MITOCHONDRIAL REMODELING AND DYNAMIC INTER-ORGANELLAR CONTACTS IN CARDIOVASCULAR PHYSIOPATHOLOGY

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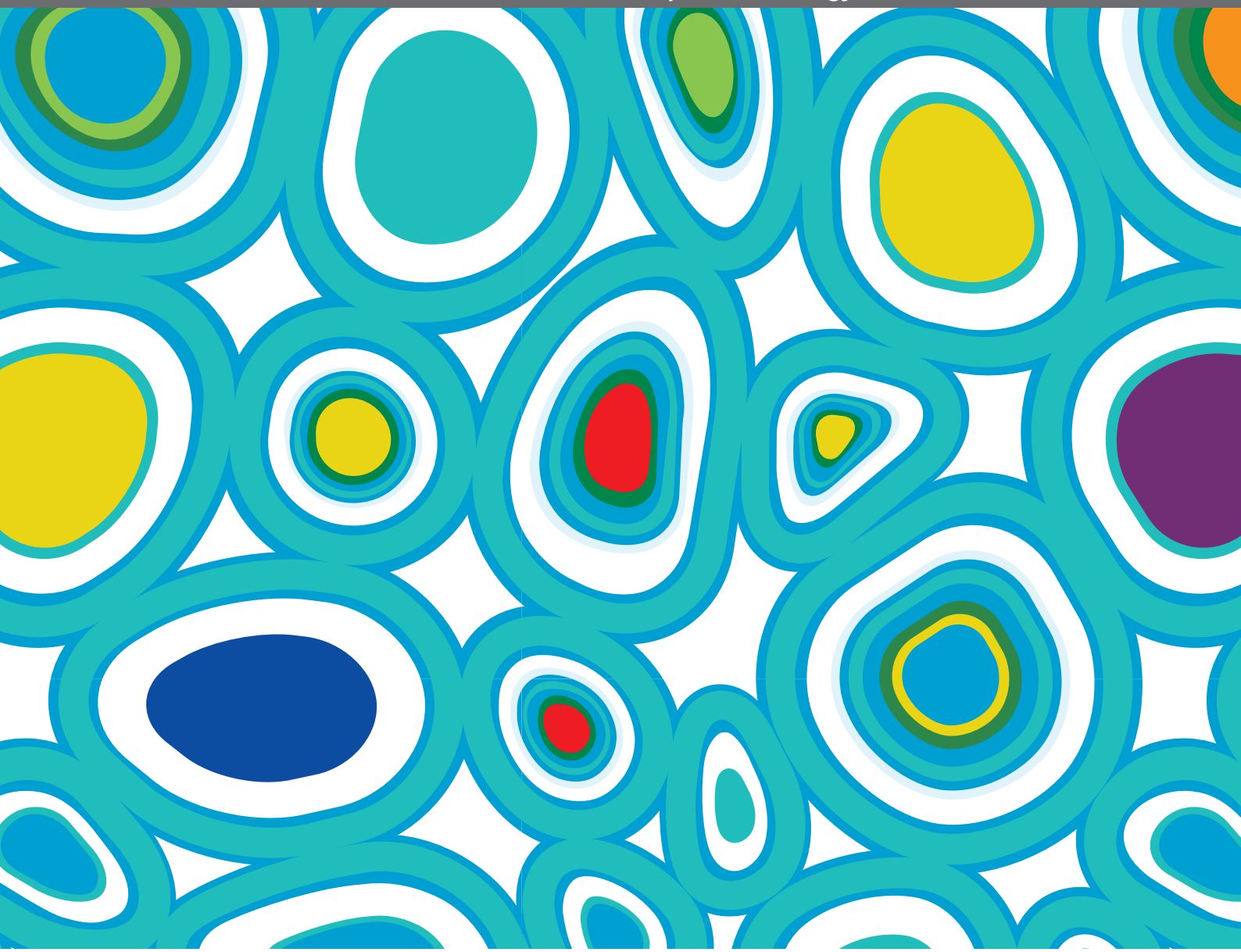

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MITOCHONDRIAL REMODELING AND DYNAMIC INTER-ORGANELLAR CONTACTS IN CARDIOVASCULAR PHYSIOPATHOLOGY

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EDITORIAL
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Editorial: Mitochondrial Remodeling and Dynamic Inter-Organellar Contacts in Cardiovascular Physiopathology

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Editorial on the Research Topic

Mitochondrial Remodeling and Dynamic Inter-Organellar Contacts in Cardiovascular Physiopathology

Emerging evidence has shown that membranes of many subcellular organelles are dynamic and engage in structural and functional communications, thereby creating new intracellular compartments by either sharing proteins or by owning a distinct pool (Rizzuto et al., 1998; Giorgi et al., 2018).

Membranes from different organelles do not fuse together, but preserve their integrity by approaching not more than a few nanometers (usually 10 nm); this distance is enough to create transient contacts which significantly impact physiological processes (e.g., lipid metabolism, material exchange) (Simmen and Tagaya, 2017; Vance, 2020) and human diseases (van Vliet and Agostinis, 2018; Simoes et al., 2020).

Growing advances in technologies, including cell fractionation (Wieckowski et al., 2009; Montesinos and Area-Gomez, 2020), confocal (Chung et al., 2015; Galmes et al., 2016), and transmission electron microscopy (Csordás et al., 2006), alongside new tools which combine biochemistry and online databases (e.g., Contact-ID) (Kwak et al., 2020), have allowed the study of contact sites in many types of living cells, in order to address new structural, functional, and modulatory properties.

Contact sites in cardiomyocytes, especially those established between sarcoplasmic reticulum (SR), and transverse tubules (TT) of the sarcolemma, and with mitochondrial membranes, are necessary for excitation-contraction coupling (ECC) efficiency (Gambardella et al., 2018) and suitable calcium signaling (Fearnley et al., 2011). The latter sustains cell survival by modulating mitochondrial ATP generation to match cardiac workload and also cell death (Jouaville et al., 1999; Traaseth et al., 2004; Bonora et al., 2019).

Among the intracellular organelles, mitochondria play an essential role in cardiomyocyte bioenergetics, because they constitute 35% of the total cell volume to satisfy the high-energy demand of heart (Elfering et al., 2004; Benard et al., 2007). As such, it is not surprising that mitochondrial dysfunction underlies several defects observed during heart development and differentiation, participating actively in the pathogenesis of a number of cardiovascular diseases (Santulli et al., 2015; Bravo-Sagua et al., 2020). Hence, maintaining a healthy mitochondrial population is an essential homeostatic requirement that the cell retains by controlling multiple checkpoints including a balanced ratio between mitophagy and biogenesis, including mitochondrial fission and fusion (Morciano et al., 2020).

The present collection includes 11 reports subdivided in the following categories: basic mechanisms, human diseases, and therapies.

BASIC MECHANISMS

Five out of 11 reports belong to this category and are authored by Rossini and Filadi, Lin et al., Gilkerson et al., Lynch et al., and Piquereau et al.. The authors highlighted the importance of the cytoarchitecture, especially SR-mitochondria contact sites and spatio-temporal mitochondrial remodeling, in some molecular pathways essential for cardiomyocyte function. These include calcium signaling, one of the main players in mitochondrial bioenergetics and cardiac contractility; in this context, organelles and proteins involved in intracellular calcium fluxes have been analyzed both *in vitro* and *in vivo*. Moreover, new insights have been provided about reactive oxygen species (ROS) production, mitochondrial dynamics, and quality control in the adaptation of the heart to multiple stress conditions. Lastly, there is a report highlighting the ability of sex hormones as factors able to influence metabolism via mitochondrial remodeling.

DISEASES

Four manuscripts authored by Gao et al., Salazar-Ramírez et al., Ramaccini et al., and Kumar et al. report compelling evidence of how mitochondrial dysfunction and alterations in organelle communication can impact cellular homeostasis in cardiovascular diseases. Indeed, the rewiring of calcium signaling at SR-mitochondria interface (but also at the sarcolemma), the imbalance in mitophagy, defects in fusion-fission machinery, lipid biosynthesis, ATP and ROS production are analyzed in a wide range of pathologies including dilated cardiomyopathy (DCM), heart failure, ischemia-reperfusion injury, and cardiac arrythmia.

THERAPIES

In CVD, the altered mitochondrial remodeling and impaired inter-organellar communications of cardiomyocytes may be amenable to therapeutic interventions, especially considering the dynamic and reversible nature of these interactions (Ferrandi et al., 2013; Sabbah, 2016; Siasos et al., 2018; Kerkhofs et al., 2019). In this sense, the last 2 reports authored by Elorza et al. and Angebault et al. summarize the currently available therapies targeting mitochondrial fitness (e.g., maintaining the correct balance of biogenesis and the control of mitochondrial heteroplasmy to prevent age-related diseases) and report the beneficial effects of metformin in mice affected by Duchenne muscular dystrophy (DMD)-associated cardiomyopathy. In this preclinical model, metformin was able to normalize SR-mitochondria interactions, and restore the function of the electron transport chain (ETC) Complex I and the expression of mitochondrial calcium-handling protein complexes.

AUTHOR CONTRIBUTIONS

GS and GMor conceived, wrote, and finalized the Editorial. GMon and VP wrote the Editorial.

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MINI REVIEW
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Sarcoplasmic Reticulum-Mitochondria Kissing in Cardiomyocytes: Ca^{2+}, ATP, and Undisclosed Secrets

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In cardiomyocytes, to carry out cell contraction, the distribution, morphology, and dynamic interaction of different cellular organelles are tightly regulated. For instance, the repetitive close apposition between junctional sarcoplasmic reticulum (jSR) and specialized sarcolemma invaginations, called transverse-tubules (TTs), is essential for an efficient excitation-contraction coupling (ECC). Upon an action potential, Ca^{2+} microdomains, generated in synchrony at the interface between TTs and jSR, underlie the prompt increase in cytosolic Ca^{2+} concentration, ultimately responsible for cell contraction during systole. This process requires a considerable amount of energy and the active participation of mitochondria, which encompass ∼30% of the cell volume and represent the major source of ATP in the heart. Importantly, in adult cardiomyocytes, mitochondria are distributed in a highly orderly fashion and strategically juxtaposed with SR. By taking advantage of the vicinity to Ca^{2+} releasing sites, they take up Ca^{2+} and modulate ATP synthesis according to the specific cardiac workload. Interestingly, with respect to SR, a biased, polarized positioning of mitochondrial Ca^{2+} uptake/efflux machineries has been reported, hinting the importance of a strictly regulated mitochondrial Ca^{2+} handling for heart activity. This notion, however, has been questioned by the observation that, in some mouse models, the deficiency of specific molecules, modulating mitochondrial Ca^{2+} dynamics, triggers non-obvious cardiac phenotypes. This review will briefly summarize the physiological significance of SR-mitochondria apposition in cardiomyocytes, as well as the pathological consequences of an altered organelle communication, focusing on Ca^{2+} signaling. We will discuss ongoing debates and propose future research directions.

Keywords: sarcoplasmic reticulum, mitochondria, cardiomyocyte, heart, Ca^{2+}, ATP, organelle contacts, MCU

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INTRODUCTION

In muscle cells, the sarcoplasmic reticulum (SR) is a specialized, differentiated domain of the endoplasmic reticulum (ER), generated from a reorganization of the ER membranes during myogenesis (Michalak and Opas, 2009). SR consists of a network of membranes, closely associated with the myofibrils, specialized in the regulation of Ca^{2+} transport and thus the control of

excitation-contraction coupling (ECC). Though ER and SR membranes/lumen are continuous, protein distribution is polarized, with completely different kinetics of Ca^{2+} uptake and release between the two compartments. As an example, ryanodine receptors (RyRs) and calsequestrin are enriched in the SR, whereas inositol-trisphosphate receptors (IP3Rs) and calreticulin in the ER (Michalak and Opas, 2009). Importantly, SR can be divided into two distinct domains: the longitudinal SR (formed by interconnected tubules surrounding myofibrils) and junctional SR (jSR) (formed by the terminal cisternae of the longitudinal SR juxtaposed with TTs) (**Figure 1**).

As to mitochondria, in adult cardiomyocytes, they are densely packed between jSR and parallel tubules of longitudinal SR, tethered to both SR domains. Specifically, seminal ultrastructural studies revealed, by electron microscopy (EM), a highly orderly organelle juxtaposition between SR and mitochondria, maintained by electron-dense filamentous tethers, whose length is approximately 10 nm (Ramesh et al., 1998; Sharma et al., 2000; Boncompagni et al., 2009; Hayashi et al., 2009). The molecular identity of these tethers is not completely clear (see also below). However, ER-mitochondria "bridges" in non-cardiac cells have been reported to be composed by proteins, to extend for 6–15 nm and to frequently occur in clusters, where they are spaced 13–22 nm apart (Csordás et al., 2006). Importantly, in mice, SR-mitochondria association is generated after birth in a developmentally regulated process, in which mitochondria progressively migrate from a random, predominantly sub-sarcolemma distribution, to their definitive positioning between the myofibrils. In this process, which parallels the maturation of both the primitive TT system and of SR, mitochondria gradually tether to jSR (Franzini-Armstrong, 2007; Boncompagni et al., 2009), with a progressive increase in the occurrence of tethers and organelle contacts.

In the early postnatal days, SR-mitochondria contacts are scarcely observed, possibly suggesting a non-essential role for cell viability; yet, their developmentally regulated appearance hints a structural and/or functional advantage. However, while the ultrastructural features of this coupling have been well defined in the heart, on the other hand (and in contrast with other tissues) its functional significance is still debated. Below, we briefly review these aspects.

STRUCTURAL ORGANIZATION OF SR-MITOCHONDRIA COUPLING

Within SR, the specific distribution of the Ca^{2+} releasing channels (RyR2 in cardiomyocytes) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) argues against a significant contribution of mitochondrial localization to the process of mitochondrial Ca^{2+} uptake (see below). Conversely, some experimental evidence suggests this latter could take advantage of the strategic organelle positioning. Indeed, RyRs do not homogenously distribute in the SR membrane, but are specifically recruited on the jSR subdomain facing the TTs (**Figure 1**). Here, they are closely associated with the TT-located L-type voltage-gated Ca^{2+} channels (VOCCs), originating the "Ca^{2+} releasing unit" (CRU). In these interface regions (dyadic clefts), TTs and jSR juxtapose with a gap of ∼10–15 nm, where electron-dense structures, representing RyR "feet," are observed (Eisner et al., 2017). Upon an action potential (AP), TTs guarantee an almost simultaneous spreading of sarcolemma depolarization, inducing Ca^{2+} entry through VOCCs. This Ca^{2+} influx generates local Ca^{2+} microdomains, intense enough to stimulate the Ca^{2+}-mediated opening of RyRs, a process called "Ca^{2+}-induced-Ca^{2+}-release" (CICR). In turn, RyR opening triggers a massive Ca^{2+} release from the SR into the dyadic cleft, promptly spreading in the whole cell and ultimately responsible for cell contraction. Of note, mitochondria are excluded from the dyadic clefts, being tethered to jSR on the opposite side (Sharma et al., 2000). This implies that SR lumen separates RyRs from mitochondria (**Figure 1**). Therefore, before reaching the outer mitochondrial membrane (OMM), Ca^{2+} must diffuse around the jSR cisternae, dissipating part of the steep concentration gradient generated in the nearest CRU proximity. The distance between RyR feet located at the edge of jSR and the OMM of the closest mitochondria is ∼35–40 nm, whereas Ca^{2+}, released in the middle of the dyadic cleft, must cover an additional distance to firstly reach the edge of the junction (∼230 nm), and then the OMM (Sharma et al., 2000). This range of distances is considerably higher than that commonly observed in different, non-muscular cell types, where ER-mitochondria Ca^{2+} tunneling is guaranteed by the very close apposition between the two organelles (∼10–25 nm; Filadi et al., 2017c) and by the strategic interaction between IP3Rs on the ER and voltage-dependent-anion-channels (VDACs) on the OMM (Szabadkai et al., 2006).

Sarco/endoplasmic reticulum Ca^{2+}-ATPase is also excluded from the dyadic cleft (Fearnley et al., 2011), but is abundant in the longitudinal SR (which surround mitochondria; Greenstein and Winslow, 2011), promptly taking up Ca^{2+} that reaches these regions and further hindering mitochondrial Ca^{2+} uptake. Of note, the overall cytosolic Ca^{2+} peak reached during an AP is ∼1–3 μM (Fearnley et al., 2011). All these considerations argue against the possibility that, during physiological ECC, mitochondria are exposed to sufficiently high Ca^{2+} concentrations [Ca^{2+}] to match the relatively low Ca^{2+} affinity of the mitochondrial Ca^{2+} uniporter complex (MCUC). This latter is endowed with a Kd for Ca^{2+} of ∼15 μM and is composed by the pore-forming subunits (MCU and/or the dominant negative isoform MCUb), by the essential component EMRE (a 10 kDa protein spanning the inner mitochondrial membrane, IMM) and by additional regulatory subunits residing in the mitochondrial intermembrane space (MICU1, MICU2 and, in certain tissues, MICU3; reviewed in Mammucari et al., 2018). However, the peculiar ultrastructure of cardiac organelle juxtaposition may overcome these apparent odds. Indeed, at the dyadic cleft: (a) the very close jSR-TT apposition and the small volume of the cleft (Hayashi et al., 2009), strongly favor lateral Ca^{2+} diffusion along the junction, limiting its radial dispersion; (b) several RyR feet fuel CICR and amplify Ca^{2+} microdomain, up to 100 μM (Fearnley et al., 2011); (c) the absence of SERCA (and, possibly, the local depletion of cytosolic Ca^{2+} buffers) enhance Ca^{2+} spreading; (d) longitudinal SR elements, frequently connecting the two jSR cisternae located

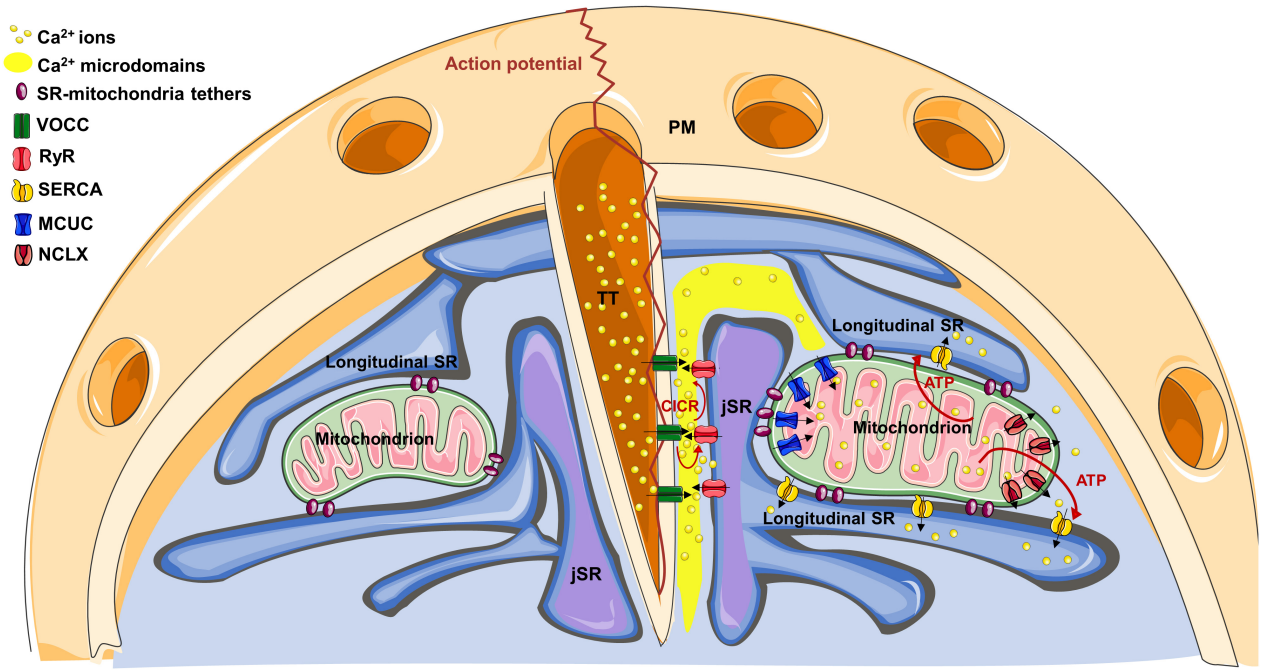

FIGURE 1 | The cartoon represents the structural organization of TT-SR-mitochondria association within a cardiomyocyte. On the right, note how Ca^{2+} microdomains, generated at TT-JSR interface, can reach the edges of juxtaposed mitochondria, favoring mitochondrial Ca^{2+} uptake through the MCUC. See the text for details on the specific Ca^{2+} channel/pump distribution.

at the opposite sides of a TT (Boncompagni et al., 2009), may limit Ca^{2+} diffusion outside of the junction (**Figure 1**). Thus, the specific arrangement of juxtaposed membranes and Ca^{2+} handling proteins may create a "railway" enhancing Ca^{2+} diffusion over relatively long distances, from the junction to the nearby mitochondria, before further spreading in the whole cell. This would imply an heterogeneous Ca^{2+} uptake among mitochondrial population (Pacher et al., 2002; Lu et al., 2013), with those organelles located in closer apposition with the CRU experiencing the highest Ca^{2+} rises.

Interestingly, along the IMM of cardiac mitochondria, the distribution of both the MCUC and the Na^{+}/Ca^{2+} exchanger (NCLX, the major mitochondrial Ca^{2+} efflux mechanism in excitable cells; Palty et al., 2010) is not homogenous. Intriguingly, a strategic positioning of MCUC toward areas juxtaposed with jSR has been reported (De La Fuente et al., 2016), whereas NCLX is actively excluded from these regions, but abundantly expressed in the sub-mitochondrial fraction free of contacts (De La Fuente et al., 2018). This biased positioning of the uptake and efflux machineries has been suggested to enhance the efficiency of the excitation-energetics coupling. Indeed, it favors the generation of mitochondrial Ca^{2+} signals capable of sustaining mitochondrial metabolism (see below), while minimizing the energetic cost (in terms of transient mitochondrial depolarization) inevitably associated with mitochondrial Ca^{2+} uptake (De La Fuente et al., 2018). The mechanisms underlying this sub-mitochondrial protein distribution are unknown. However, in rat heart, it has been observed that the OMM-IMM contact points are aligned with those between jSR and mitochondria (Garcia-Perez et al., 2011). This possibly suggests that the OMM-IMM contacts may work as a platform for the anchorage of specific structures (such as the tethers connecting the OMM with SR, composed by proteins possibly anchored to particular lipids) and the recruitment/exclusion of specific protein complexes (such as MCUC or NCLX).

Overall, the tightly regulated organization of the machineries that control mitochondrial Ca^{2+} dynamics, specifically observed in cardiomyocytes, argues in favor of the key importance of mitochondria-jSR apposition and, more in general, of mitochondrial Ca^{2+} signaling, for heart physiology.

SR-MITOCHONDRIA TETHERS

While the composition of the protein complexes mediating ER-mitochondria physical/functional tethering has been intensively studied in non-muscular cells (Filadi et al., 2017c), the molecular identity of SR-mitochondria tethers has been poorly investigated in the heart. Most studies focused on mitofusin-2 (MFN2), an OMM protein (in part present also at the ER membranes) involved in mitochondrial fusion. The role of this protein as a putative ER-mitochondria tether has been a matter of intense debate, suggesting either a pro-tethering (de Brito and Scorrano, 2008; Naon et al., 2016) or an anti-tethering (Cosson et al., 2012; Filadi et al., 2015, 2017b) function. Similarly, in the heart, contradictory results have been obtained. The cardiomyocyte-specific deletion of the *Mfn2* gene after birth has been observed to

reduce the length of SR-mitochondria contacts by ∼30%, but the distance between jSR and the OMM was not significantly altered (Chen et al., 2012). In isolated cardiomyocytes from this mouse model, upon isoproterenol exposure, higher cytosolic and lower mitochondrial Ca^{2+} rises, associated with a reduced stimulation of the Krebs cycle, were observed. On the other hand, in a different mouse model in which *Mfn2* was specifically deleted in the embryonic heart, jSR-mitochondria apposition was found to be maintained, without significant differences in Ca^{2+} dynamics (Papanicolaou et al., 2011). Importantly, profound alterations of mitochondrial morphology were observed in both models. However, to the best of our knowledge, *Mfn2* ablation has never been reported to completely disrupt SR-mitochondria contacts, nor to decrease the presence of electron-dense filamentous bridges between the two organelles, possibly suggesting that MFN2 is not essential for their formation. Likely, multiple tethers may exist and exert redundant activities.

For instance, recently, the cardiac-specific downregulation of protein-tyrosine-phosphatase-interacting-protein 51 (PTPIP51) has been demonstrated to reduce the extension of SR-mitochondria contacts, while increasing organelle distance, reducing caffeine-induced SR to mitochondria Ca^{2+} transfer and protecting from ischemia-reperfusion damage (Qiao et al., 2017). The opposite effects have been observed upon PTPIP51 overexpression, suggesting that it may participate in contact stabilization, as reported for ER-mitochondria tethering (Stoica et al., 2014). Similarly, FUN14-domain-containing 1 (FUNDC1) on the OMM has been shown to bind IP3R2 on ER membranes, maintaining structural organelle coupling, favoring IP3-linked ER-mitochondria Ca^{2+} shuttling and sustaining cardiac functionality (Wu et al., 2017). However, whether FUNDC1 activity is limited to ER-mitochondria association, or extend also to the SR-mitochondria one, is not completely clear. Indeed, whether specialized SR-mitochondria tethers exist, or they are completely redundant with those mediating ER-mitochondria apposition, remains an unanswered question.

FUNCTIONAL EVIDENCE OF SR-MITOCHONDRIA Ca^{2+} COUPLING

As far as SR-mitochondria Ca^{2+} transfer in the heart is concerned, in the microdomain that bathes the ends of the mitochondria closer to the CRUs, mathematical models calculated a $[Ca^{2+}]$ of ∼10-20 μM (Williams et al., 2011, 2013), lasting for ∼10 ms (Cheng and Lederer, 2008). In rat neonatal cardiomyocytes, the existence of Ca^{2+} hot-spots during spontaneous Ca^{2+} oscillations has been demonstrated by an OMM-targeted FRET-based Ca^{2+} probe (Drago et al., 2012). Upon caffeine-induced RyR stimulation, a prompt mitochondrial Ca^{2+} uptake was observed in permeabilized rat ventricular cardiomyocytes, with a marginal reduction when the fast Ca^{2+} buffer BAPTA was included in the experimental buffer (Sharma et al., 2000). Similarly, in permeabilized cardiac H9c2 cells stimulated with caffeine, it has been calculated that ∼25% of the Ca^{2+} released from SR is taken up by mitochondria (Pacher et al., 2000), further suggesting that they are exposed to sustained Ca^{2+} microdomains. It is worth noting, however, that caffeine stimulation triggers a higher and slower cytosolic Ca^{2+} rise, compared with the physiological Ca^{2+} oscillations observed during ECC, thereby differently impacting on the machinery that mediate mitochondrial Ca^{2+} uptake.

Whether and how mitochondria decode cytosolic Ca^{2+} transients during ECC is debated. Contrasting data have been reported, suggesting either smoothened, slow changes in steady-state mitochondrial Ca^{2+} levels (Miyata et al., 1991; Griffiths et al., 1997; Sedova et al., 2006), or rapid beat-to-beat oscillations, closely following those in the cytosol (Chacon et al., 1996; Robert et al., 2001; Bell et al., 2006; Maack et al., 2006; Garcia-Perez et al., 2008; Drago et al., 2012). The controversy may depend on both the specific Ca^{2+} probes (chemical dyes or genetically encoded indicators) and types of stimulations (electrical, β-adrenergic or spontaneous contraction) used in the different studies. This topic has been extensively reviewed and the interested readers are referred to a comprehensive contribution (De la Fuente and Sheu, 2019). Recently, however, by using the FRET-based Cameleon Ca^{2+} probe 4mtD3cpv expressed in cultured rat cardiomyocytes, it has been demonstrated that, upon a low frequency electrical stimulation (0.1 Hz), mitochondria promptly take up Ca^{2+} (rise time ∼49 ms), whereas their Ca^{2+} efflux is relatively slow (decay half time ∼1.17 s) (Wüst et al., 2017). At higher frequency stimulation, this asymmetry results in a progressive rise in mitochondrial $[Ca^{2+}]$, until a new balance between uptake and efflux is reached, yet maintaining a beat-to-beat oscillatory pattern (Wüst et al., 2017). These results confirmed those previously observed in rabbit ventricular myocytes, where the repetitive rise in mitochondrial $[Ca^{2+}]$ was observed to be higher and to occur earlier in the pool of mitochondria closer to jSR (Lu et al., 2013). Importantly, the observation that the efflux kinetics are much slower than the uptake ones implies that beat-to-beat mitochondrial Ca^{2+} oscillations might be more easily observed (and could have a larger physiological relevance) in organisms endowed with lower heart rates, such as humans [heart rate ∼1–1.5 Hz; note the match with the reported half time of mitochondrial Ca^{2+} efflux, ∼1.17 s (Wüst et al., 2017)].

PHYSIOLOGICAL SIGNIFICANCE OF MITOCHONDRIAL Ca^{2+} UPTAKE IN CARDIOMYOCYTES

As to the possible significance of mitochondrial Ca^{2+} transients in the heart, they have been consistently suggested to stimulate ATP synthesis, thus matching the increased energy demand upon cell contraction (Yaniv et al., 2010; Dorn and Maack, 2013). Indeed, in the mitochondrial matrix, Ca^{2+} stimulates Krebs cycle, thereby enhancing the synthesis of NADH and $FADH_2$, in turn fuelling the electron transport chain (ETC) and, ultimately, ATP synthase (reviewed in Rossi et al., 2019). Moreover, in the mitochondrial-intermembrane-space, Ca^{2+} stimulates the activity of different metabolite transporters, further enhancing mitochondrial metabolism (Rossi et al., 2019). Last, but not least, the Ca^{2+}-mediated stimulation of the Krebs cycle increases the

mitochondrial antioxidative capacity by enhancing the synthesis of NADPH, in turn essential for the activity of key H_2O_2-eliminating enzymes, such as glutathione peroxidase (Kohlhaas et al., 2017). Importantly, reactive oxygen species (ROS, which, in mitochondria, are mainly generated as by-products of oxidative phosphorylation) can act as signaling molecules and regulate the activity of different Ca^{2+} handling protein, including SERCA and RyR2 (Feno et al., 2019). Therefore, a complex crosstalk exists between Ca^{2+} and ROS signaling, in which SR-mitochondria juxtaposition likely plays a key role, as observed for ER-mitochondria interface and IP3R-mediated Ca^{2+} transfer (Booth et al., 2016).

An increase in the heart workload is associated with an increase in the frequency and amplitude of cytosolic Ca^{2+} transients, enhancing myofilament contraction. In turn, this triggers ATP consumption, to allow both muscle relaxation and recovery of basal cytosolic Ca^{2+} levels. Therefore, the capacity of mitochondria to decipher transient cytosolic Ca^{2+} oscillations would represent an elegant mechanism to match, in real-time, the acute ATP demand of the beating heart. On the other hand, slower and smoothened mitochondrial Ca^{2+} dynamics would not provide mitochondria with a sufficient temporal resolution to face acute fluctuations of cell energy need. Interestingly, while the close juxtaposition of mitochondria with jSR favors mitochondrial Ca^{2+} uptake (see above), the organelle coupling with longitudinal SR may provide a structural advantage to fuel SERCA (which is abundant in this SR domain) with both mitochondria-derived ATP (Wilding et al., 2006) and Ca^{2+} released through NCLX after mitochondrial Ca^{2+} uptake (as suggested in different cell models; Malli et al., 2005; Poburko et al., 2009). Overall, evidence has been provided suggesting that mitochondrial Ca^{2+} signals are important for ATP synthesis in cardiomyocytes (Maack et al., 2006; Garcia-Perez et al., 2008; Liu and O'Rourke, 2009; Chen et al., 2012; Bertero and Maack, 2018; Wescott et al., 2019), but whether this regulation is carried out on a beat-to-beat basis is not clear. A defective SR-mitochondria coupling, reducing organelle Ca^{2+} exchange and maximal mitochondrial respiration, while increasing mitochondrial ROS, has been reported to associate with heart aging (Fernandez-Sanz et al., 2014). Recently, a critical role for IP3R-mediated (but not for RyR-mediated) SR-mitochondria Ca^{2+} transfer on mitochondrial ATP synthesis has been reported in mouse ventricular myocytes (Seidlmayer et al., 2016), suggesting that different Ca^{2+} signaling pathways may differently impact on mitochondrial metabolism.

In addition to the role played in the modulation of cardiomyocyte bioenergetics, mitochondrial Ca^{2+} uptake has been suggested to buffer part of the Ca^{2+} released from SR during ECC, thereby shaping cytosolic Ca^{2+} oscillations and modulating cell contraction. In rat neonatal cardiomyocytes, MCU downregulation by siRNA (decreasing mitochondrial Ca^{2+} uptake) increases cytosolic Ca^{2+} peaks and contraction during spontaneous Ca^{2+} pacing, whereas MCU overexpression triggers the opposite effects (Drago et al., 2012). Similarly, in adult cardiomyocytes from an MCU-cKO mouse model, isoproterenol-induced cytosolic Ca^{2+} transients were higher compared with WT animals (Luongo et al., 2015). Finally, upon field stimulation, higher diastolic and systolic cytosolic [Ca^{2+}], associated with lower ATP levels, were observed in ventricular myocytes from transgenic mice selectively expressing in myocardium a dominant-negative form of MCU (Rasmussen et al., 2015). However, contradictory results are present in literature. Indeed, earlier studies failed to detect a significant impact of mitochondrial Ca^{2+} uptake on cytosolic Ca^{2+} transients in cardiomyocytes (Andrienko et al., 2009; Williams et al., 2013; Boyman et al., 2014). As discussed above, differences between species or experimental setups may underlie part of the discrepancies. Importantly, it is worth noting that cytosolic Ca^{2+} dynamics, among other determinants, are deeply affected by SERCA activity, which controls both the amount of Ca^{2+} stored within SR and the speed/amplitude of Ca^{2+} re-uptake after release. SERCA activity, however, is quite sensitive to ATP availability (Tian and Ingwall, 1996). Therefore, the process of mitochondrial Ca^{2+} uptake may affect cytosolic Ca^{2+} transients not only directly (by buffering the cation), but also indirectly, by modulating ATP availability.

Above, we discussed the importance of mitochondrial Ca^{2+} signaling in cardiomyocytes. However, recent experimental evidence cast doubts on its overall significance. In particular, three different mouse models, in which whole organism or cardiac mitochondrial Ca^{2+} accumulation was severely compromised by manipulating MCUC composition (Pan et al., 2013; Holmström et al., 2015; Kwong et al., 2015; Luongo et al., 2015; Rasmussen et al., 2015; Wu et al., 2015), displayed an almost normal heart functionality at rest. On the other hand, upon β-adrenergic stimulation, a reduced contractile responsiveness, associated with a decreased cardiac performance, was observed (Kwong et al., 2015; Luongo et al., 2015; Rasmussen et al., 2015). These results may suggest that MCU-mediated mitochondrial Ca^{2+} uptake is dispensable for basal cardiac activity, but is necessary upon acute increases of heart workload. The lack of gross alterations in the absence of MCU might be surprising. Importantly, however, the impact of mitochondrial Ca^{2+} uptake on heart performance could be species-specific, depending on the different heart rates and thus on the amplitude of the beat-to-beat Ca^{2+} oscillations in the mitochondrial matrix (see above). Moreover, it should be noted that, although the process of rapid mitochondrial Ca^{2+} uptake is severely (if not completely) compromised in these models, yet the resting mitochondrial Ca^{2+} levels are not deeply affected (Kwong et al., 2015). This may suggest the possible existence of additional (though less efficient) mitochondrial Ca^{2+} uptake mechanisms. For instance, a mitochondria-specific RyR1 (mRyR1), mediating Ca^{2+} uptake, has been reported to be expressed in the IMM of heart organelles and activated at low cytosolic [Ca^{2+}] (Beutner et al., 2001, 2005). Though the existence and the physiological importance of mRyR1 is still controversial, it has been recently suggested to enhance ATP synthesis, by transferring Ca^{2+} from SR to mitochondria upon IP3-dependent cardiomyocyte stimulation (Seidlmayer et al., 2016).

Overall, the importance of a strictly regulated mitochondrial Ca^{2+} signaling is outlined by a series of evidences. NCLX deletion in adult mouse heart is lethal, inducing mitochondrial Ca^{2+} overload, activation of the mitochondrial permeability transition

pore, necrotic cell death, and sudden heart failure (Luongo et al., 2017). Importantly, NCLX ablation is well tolerated when performed shortly after birth, hinting unknown compensatory adaptations in the maturing heart, and NCLX overexpression in mice was observed to protect from ischemia-reperfusion injury and reduce infarct size (Luongo et al., 2017). On the same line, in some (but not all) mouse models, MCU deletion has been demonstrated to protect from cardiac ischemia-reperfusion damage, by preventing mitochondrial Ca^{2+} overload (Kwong et al., 2015; Luongo et al., 2015). Moreover, the expression of the dominant negative MCU subunit (MCUb) is particularly abundant in the heart (Raffaello et al., 2013) and MCUC density and activity is very low (Fieni et al., 2012). In particular, MCU translation, regulated by microRNA-1 levels, decreases during postnatal cardiac growth, both in mice and humans, whereas MCUb expression increases in mice (Zaglia et al., 2017). The reason why, during cardiomyocyte maturation, the increase in SR-mitochondria association (Boncompagni et al., 2009) is paralleled by a decreased MCU expression (Zaglia et al., 2017) remains an outstanding question. It is tempting to speculate that, in mature heart cells, mitochondria develop specific toolkits to decipher low-level, basal Ca^{2+} fluctuations, while becoming particularly sensitive to Ca^{2+} overload. Interestingly, in physiologic and pathologic cardiac hypertrophy, MCU protein levels increase, a process prevented by β-blocker treatment (Zaglia et al., 2017). Furthermore, the expression of the MCUC regulatory subunit MICU2 is altered in patients with ventricular hypertrophy, and MICU2-KO mice display diastolic dysfunction, possibly associated with delayed cytosolic Ca^{2+} re-uptake and decreased cardiomyocyte relaxation (Bick et al., 2017).

Finally, in cardiomyocytes, cyclic AMP (cAMP) and Ca^{2+} signaling are well known to intersect and modulate with each other (reviewed in Filadi et al., 2017a). Interestingly, recently, in neonatal rat ventricular cardiomyocytes, the presence of a compartmentalized cAMP-PKA signal at the OMM has been demonstrated (Burdyga et al., 2018). Whether the narrow gap between SR and OMM might further shape this localized pathway will require further investigations.

CONCLUSION

In the last decade, the rapid growth in our knowledge of the mechanisms regulating the complex balance between mitochondrial Ca^{2+} signal and cell metabolism has tremendously increased our understanding of cardiomyocyte physiopathology, yet prompting additional and unpredicted questions. The intimate relationship between SR and mitochondria appears critical to regulate mitochondrial Ca^{2+} uptake. Nevertheless, whether mitochondria take advantage of their strategic positioning to efficiently detect Ca^{2+} microdomains on a beat-to-beat basis is still matter of debate. Species-specific differences, partly due to diverse heart rates, may dramatically impact on the complex balance between Ca^{2+} uptake/efflux dynamics, resulting in substantially different outcomes. Moreover, the lack of severe phenotypes in mice lacking key MCUC components suggests that additional routes, guaranteeing a sufficient, albeit minimal, Ca^{2+} signal in the mitochondrial matrix might exist. Alternatively, unknown compensatory mechanisms may occur (see for example, the dramatically different effect of NCLX deletion in adult vs newborn mouse heart; Luongo et al., 2017).

In addition to Ca^{2+} transfer, lipid metabolism is known to be modulated by ER-mitochondria tethering; yet, as far as SR-mitochondria juxtaposition is concerned, this aspect has been largely neglected in literature. Similarly, the impact of SR-mitochondria contacts on autophagy, as well as on the regulation of mitochondrial dynamics and morphology, has not been sufficiently explored in the heart. Clearly, an in-depth investigation of the molecules regulating SR-mitochondria coupling appears urgent to study these issues. The development of novel techniques, enabling to simultaneously measure with sufficient spatial and temporal resolution different parameters, will offer the opportunity to further raise the bar, allowing to precisely evaluate the impact of these pathways in different cardiac pathologies.

AUTHOR CONTRIBUTIONS

MR and RF revised the literature, wrote and discussed the manuscript. All authors contributed to the article and approved the submitted version.

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Energetic Interactions Between Subcellular Organelles in Striated Muscles

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Adult striated muscle cells present highly organized structure with densely packed intracellular organelles and a very sparse cytosol accounting for only few percent of cell volume. These cells have a high and fluctuating energy demand that, in continuously working oxidative muscles, is fulfilled mainly by oxidative metabolism. ATP produced by mitochondria should be directed to the main energy consumers, ATPases of the excitation-contraction system; at the same time, ADP near ATPases should rapidly be eliminated. This is achieved by phosphotransfer kinases, the most important being creatine kinase (CK). Specific CK isoenzymes are located in mitochondria and in close proximity to ATPases, forming efficient energy shuttle between these structures. In addition to phosphotransfer kinases, ATP/ADP can be directly channeled between mitochondria co-localized with ATPases in a process called "direct adenine nucleotide channeling, DANC." This process is highly plastic so that inactivation of the CK system increases the participation of DANC to energy supply owing to the rearrangement of cell structure. The machinery for DANC is built during postnatal development in parallel with the increase in mitochondrial mass, organization, and complexification of the cell structure. Disorganization of cell architecture remodels the mitochondrial network and decreases the efficacy of DANC, showing that this process is intimately linked to cardiomyocyte structure. Accordingly, in heart failure, disorganization of the cell structure along with decrease in mitochondrial mass reduces the efficacy of DANC and together with alteration of the CK shuttle participates in energetic deficiency contributing to contractile failure.

Keywords: sarcoplasmic reticulum, creatine kinase, organelle interaction, energy metabolism, mitochondria, skeletal muscle cells, cardiac muscle

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INTRODUCTION

In mammals, evolutionary and ontological changes have progressed toward a high degree of specialization and complexification of all cell types. Striated muscle cells are exquisitely modeled and adapted to the specificity of each kind of movement: slow or fast, repetitive or sustained, phasic or tonic. This is true whether morphological, histological, biochemical, or energetic points

of view are considered. The muscle-specificity of the cytoarchitecture reflects the functional role of each muscle type. Myofilaments are forming the contractile apparatus to induce cell contraction; sarcoplasmic reticulum maintains calcium release and reuptake, glycolytic complexes and oxidative abilities within mitochondria, are necessary for energy provision. These main cell components vary in morphologic, quantitative and qualitative biochemical composition and organization. Muscle cells are the main energy consumers of the organism. Energy yield, transport and regeneration are adequately adapted to each contractile pattern. Striated muscle cells have thus developed specialized energy transport systems and organelle interactions.

ENERGETIC COMPARTMENTATION IN MUSCLE CELLS

Striated muscles can be divided in two main types: the fast skeletal muscle involved in fast running and escape for short periods of time, and the slow muscles designed to maintain continuous contraction for posture or cyclic contractions for the cardiac pump. In both types, ATP is provided either by glycolytic complexes or mitochondria but in different proportions. Mitochondria produce a high amount of energy-rich phosphates mostly from carbohydrates and fatty acids but at a relatively slow rate, while glycolysis and energy reserve [phosphocreatine (PCr), and ATP] can quickly provide energy for fast contraction but in limited amount.

The heart, being slow muscle, has to function permanently and cyclically, thus it mainly relies on oxidative metabolism allowing provision of enough energy on a "pay as you go" manner, achieved by high mitochondria volume (30–40% cell volume) densely packed and organized in rows between myofibrils (40% cell volume) (**Figure 1A**). On the other hand, fast skeletal muscle has low mitochondrial volume (5–10%) mainly arranged around T tubules near Z-lines and high content of contractile apparatus (70%) (**Figure 1B**). It relies mostly on quickly mobilizable energy reserves in the form of ATP and PCr, to sustain fast and intense contractile activity on a "twitch now, pay later" manner (Katz, 2001).

Because muscle cell is densely packed and contains only a few percent of free cytosol, it cannot be considered as a well-mixed bag. Some intracellular organelles are in close contact; others need specialized energy transport systems to interact. Indeed, it is now evident that cellular energy metabolism occurs in many specialized "microcompartments," where energy is transferred preferentially from generating modules directly to consuming ones (Zala et al., 2017).

Efficient transfer systems allow production, transfer and utilization of energy between the different cell compartments (Ventura-Clapier et al., 1998). The main one is the creatine kinase (CK) system (**Figure 2**). CK catalyzes the reversible transfer of a phosphate moiety between ATP and creatine (ATP + creatine $\Leftrightarrow$ ADP + PCr + H^+). An important property of the CK system is that its total activity, its isoform distribution, and the concentration of guanidino substrates are highly variable among muscles. In striated muscles, specific CK isoenzymes are bound to intracellular compartments, and are functionally coupled to enzymes and transport systems involved in ATP production and utilization. The dimeric MM-CK is bound to myosin and sarcoplasmic reticulum in close vicinity to myosin ATPase and Ca^{2+}-ATPase of the SR (SERCA). This bound-CK is functionally coupled to these ATPases so that ADP produced is immediately and locally re-phosphorylated at the expenses of PCr. This kinetically and thermodynamically favors ATPase activity by increasing concentration of its substrate (ATP) and decreasing accumulation of the reaction product, ADP. A specific dimeric or octameric isoenzyme of CK is present in the intermembrane space of mitochondria (mi-CK) where it transfers the phosphate moiety from ATP to creatine, thus allowing ADP to be immediately rephosphorylated by mitochondria and PCr to be transferred to cytosol and channeled by cytosolic MM-CK to sites of utilization. MM-CK is also bound to glycolytic complexes in the cytosol. Additional energy transfer systems are represented by adenylate kinase for example (Dzeja and Terzic, 2003). The importance of such energy transfer systems has been largely documented by different groups and well-described in books and reviews (Saks and Ventura-Clapier, 1994; Saks et al., 1998, 2006; Joubert et al., 2001; Dzeja and Terzic, 2009; Guzun et al., 2015). CK compartmentation determines high cellular efficiency and fine specialization of differentiated muscle cells (Ventura-Clapier et al., 1998). Efficiency of phosphotransfer circuits declines significantly during aging, participating in vulnerability of the aging myocardium (Nemutlu et al., 2015; Tepp et al., 2017).

A structural energy transfer system coexists with the enzymatic ones. In cardiomyocytes, due to the close proximity between mitochondria and sarcoplasmic reticulum or myofibrils, a direct adenine nucleotide channeling (DANC) of ATP from mitochondria and ADP from contractile proteins or SR exists that is more efficient that bulk ATP diffusion to fulfill energy requirements. Therefore, ATP produced by mitochondria is able to sustain calcium uptake and contractile speed (Kaasik et al., 2001) better than cytosolic ATP (**Figure 2**).

Importance and plasticity of the different energy transfer systems are exemplified in transgenic animals. In cardiomyocytes deficient in MM and mi-CK, effectiveness of DANC between mitochondria and SR or myofibrils is largely increased and is accompanied by marked cytoarchitectural modifications (Kaasik et al., 2001). More numerous mitochondria are reorganized within myofilaments, providing decreased diffusion distances for adenine nucleotides (**Figure 1C**). CK deficiency also induces a redirecting of phosphotransfer flux through alternative adenylate kinase, glycolytic and guanine nucleotide systems (Dzeja et al., 1999, 2011). Such energetic re-wiring, together with increased mitochondrial and glycolytic capacities, ultrastructural rearrangements and increased DANC, represent an adaptive mechanism to CK deficiency.

The remodeling of mutant CK-deficient fast skeletal muscle is even more impressive (**Figure 1D**). In normal fast skeletal muscle, mitochondrial content is low and DANC is absent. CK-deficiency induces increased mitochondrial content, marked ultrastructural rearrangement and building of DANC between mitochondria and myofilaments or SR showing that spatial

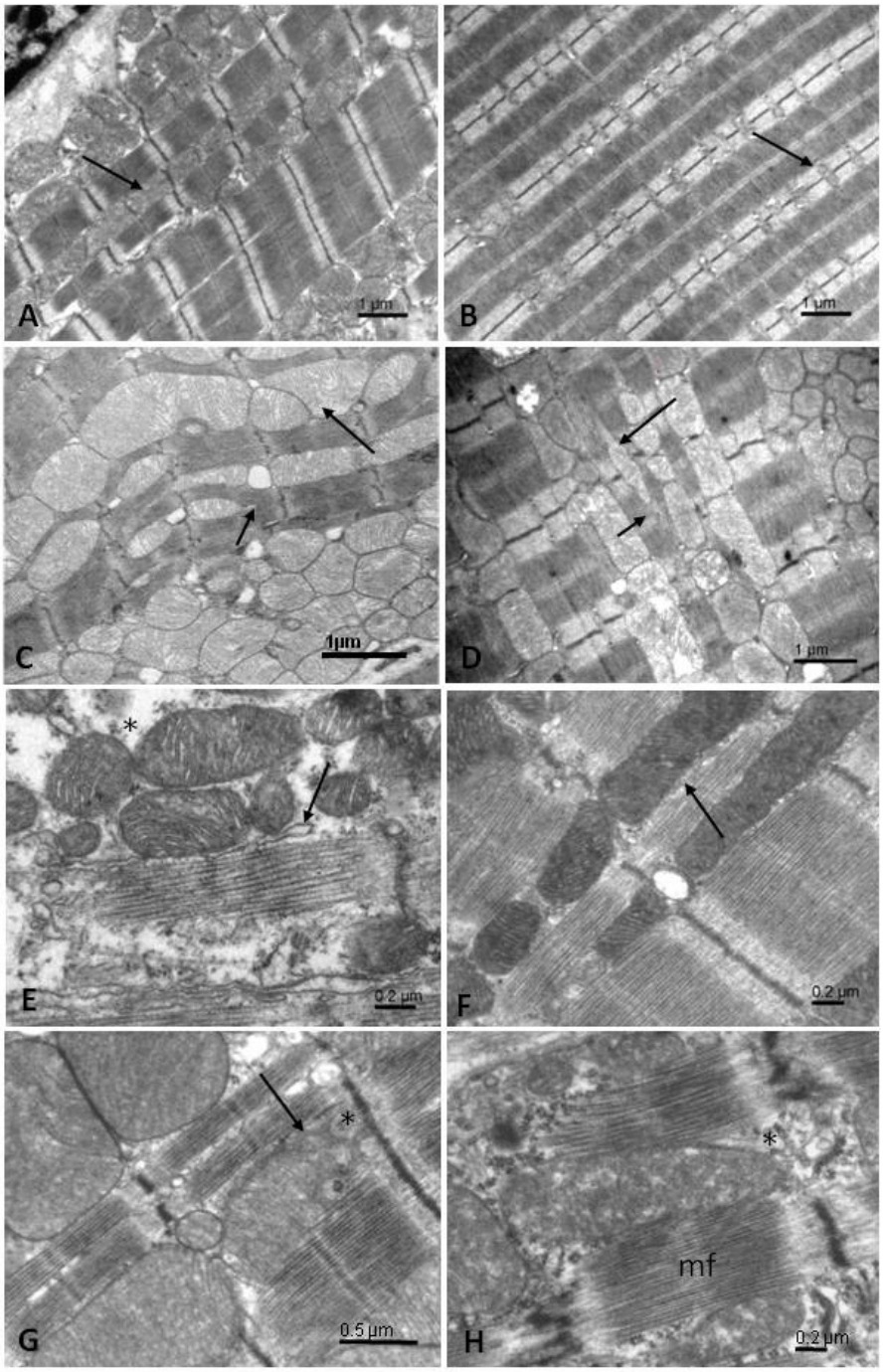

FIGURE 1 | Electron microscopic pictures of mitochondrial organization in mouse muscle cells. **(A)** Cardiomyocyte with regularly arranged mitochondrial rows between myofibrils. **(B)** Muscle fiber from white *gastocnemius* with small mitochondria occurring near the Z-lines. **(C)** Mitochondrial clusters and branching of myofibrils in CK-deficient cardiomyocyte. **(D)** increased mitochondrial content in CK-deficient *gastrocnemius muscle.* Long arrows: mitochondria; short arrows—branching of myofibrils. **(E)** Mitochondrial environment in 3-days old cardiomyocyte. **(F)** Occurrence of mitochondria in 21-days old cardiomyocyte. **(G)** Environment of mitochondria in control adult cardiomyocyte. **(H)** Mitochondrial environment in cardiomyocyte of adult failing heart. Long arrow: sarcoplasmic reticulum; *cytosol; mf, myofibrils.

relations among organelles of muscle cells undergo adaptation in response to non-structural stimuli like metabolic deficiency (Kaasik et al., 2003; Novotova et al., 2006). However, these adaptive mechanisms are still limited as CK-deficient mice exhibit muscle atrophy and decreased exercise capacities (Momken et al., 2005).

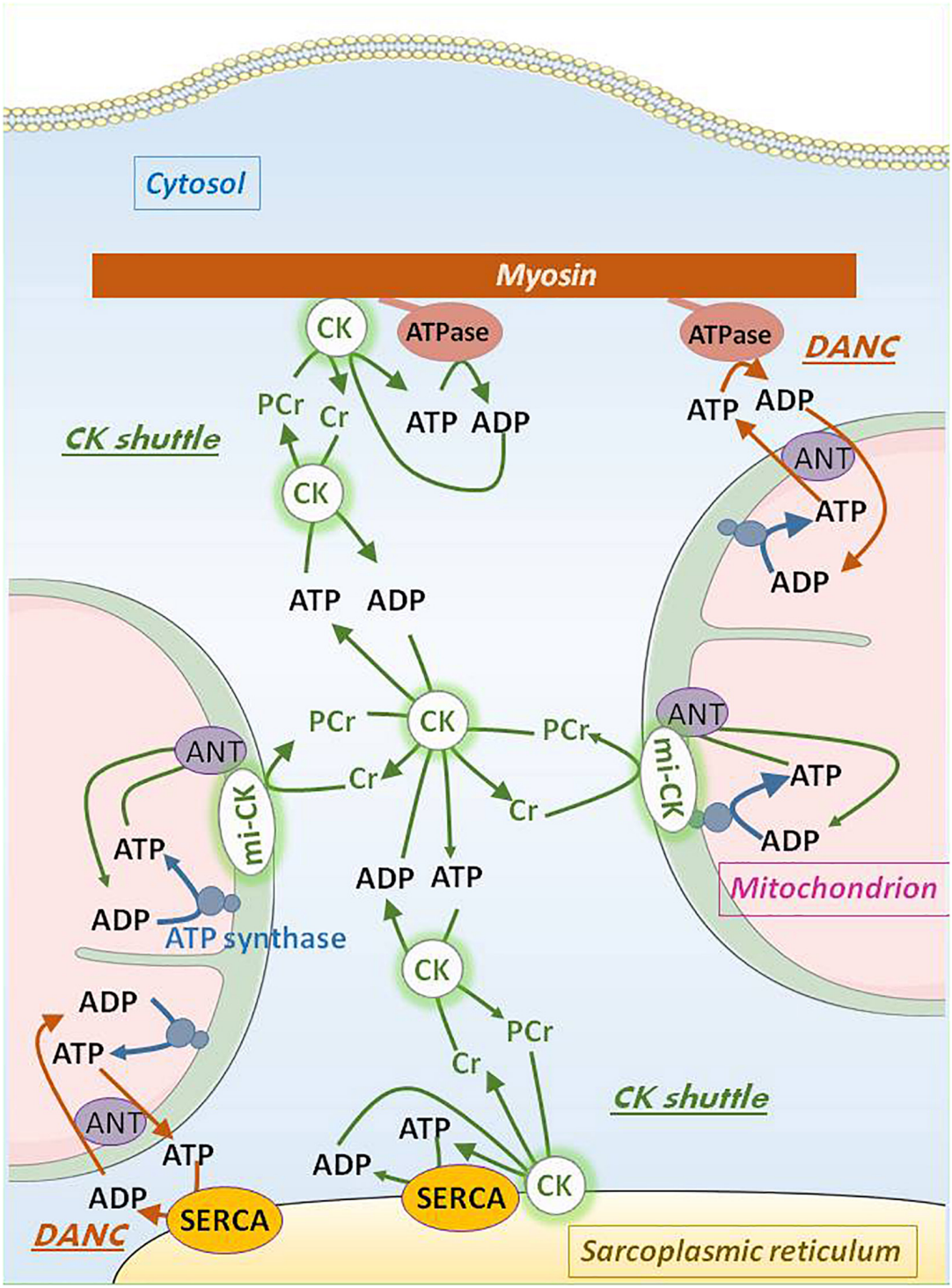

FIGURE 2 | Intracellular adenine nucleotide transport by DANC and CK shuttle in oxidative muscle cells. ATP generated in the mitochondrial matrix by ATP synthase is transported by adenine nucleotide translocase (ANT) across the inner mitochondrial membrane to the intermembrane space whereas ADP is transported by ANT in the opposite direction. Co-localization of the mitochondria with cell ATPase (like myosin ATPase or sarcoplasmic reticulum Ca^{2+}-ATPase, SERCA) allows for bi-directional direct adenine nucleotide channeling (DANC). Functional mitochondrial creatine kinase (mi-CK) coupled with ANT uses ATP and creatine to produce phosphocreatine (PCr) which diffuses in the cytosol, and ADP which is taken back in the matrix by ANT. MM-CK bound to different organelles near ATPases traps cytosolic PCr to locally rephosphorylate ADP thus replenishing local ATP concentration near ATPases. Creatine liberated by this reaction can be reused by mi-CK. Cytosolic soluble MM-CK maintains the equilibrium between all chemical actors of the CK shuttle (ATP, ADP, PCr, and creatine). Relatively high cytosolic concentrations of PCr and creatine make efficient the bi-directional flux of these two substances.

ENERGETIC COMPARTMENTATION DURING DEVELOPMENT

During development, the heart faces an increase in the contractile demand owing to the growth and the increase in activity level of the fetus/newborn as well as the relative diminution of the cardiac mass. Consequently, this period is marked by development of higher capacities and increased efficiency of ATP production involving a progressive increase in mitochondrial mass (Piquereau et al., 2010). Mitochondria quickly become the main energy source of the growing cardiomyocyte (Piquereau and Ventura-Clapier, 2018), and the cellular machinery thus becomes more and more dependent on energy transfer from these organelles to ATPases. In parallel, development is a period during which cardiomyocyte is subjected to a profound reorganization of intracellular structure which gets denser and denser because of a significant augmentation in the amount of myofilaments, sarcoplasmic reticulum and mitochondria (Porter et al., 2011). Whereas this complexification of cytoarchitecture allows cardiomyocyte to contract more efficiently, it also makes adenine nucleotide diffusion within cytosol less easy owing to densification (Saks et al., 2008; Piquereau et al., 2010). Simple ATP/ADP diffusion becomes less and less efficient as the cardiomyocyte grows and the early period of postnatal development is the scene of the establishment of the energy transfer systems (CK shuttle and DANC) required for a suitable energy input in particular for SERCA and ATPase of myosin myofilament (Piquereau et al., 2010).

The establishment of an efficient energy transfer from mitochondria to energy consumers is a progressive process which occurs during the first weeks of postnatal development in rodent (Piquereau et al., 2010). In fetal heart, the cardiomyocyte exhibits a loose cytoarchitecture (Hirschy et al., 2006; Lozyk et al., 2006) and energy is mainly produced by supramolecular complexes of glycolytic enzymes locally maintaining ATP/ADP ratio in the vicinity of the ATPases (Brooks and Storey, 1991; Ventura-Clapier et al., 2011). These specificities of the prenatal cardiomyocyte combined to the relatively low energy fluxes at this stage probably explain the fact that CK shuttle and DANC do not seem to be required in fetal heart which can operate without these optimized energy transfer mechanisms (Hoerter et al., 1991, 1994; Tiivel et al., 2000). The increase in cardiac workload around birth and the progressive densification of intracellular structure during the first weeks of *ex utero* life trigger rapid energetic adaptations. Thus, whereas CK activity in the fetal heart is very low and does not allow energy transfer through the aforementioned phosphotransfer system (Hoerter et al., 1991, 1994; Fischer et al., 2010), the important increase in CK activity, creatine transporter protein amount and creatine content in the cardiac muscle cell just before or/and after birth leads to the establishment of a fully functional CK shuttle in only a few weeks (depending on species) which is then rapidly able to support ATPases activity and ensure positive feedback on production of energy by mitochondria (Hoerter et al., 1991, 1994; Fischer et al., 2010; Piquereau et al., 2010; Anmann et al., 2014). For instance, CK bound to sarcoplasmic reticulum and myofilament is able to efficiently provide energy to SERCA and myosin-ATPase only 3 weeks after birth in mouse (Piquereau et al., 2010). Although the modulations of energy metabolism in the early development are often explained by the profound changes occurring at birth, like blood concentration of energy substrates (Lopaschuk and Jaswal, 2010), the maturation of the phosphotransfer shuttle could rather be dependent on the maturity state of the cardiomyocyte. While functional CK compartmentation appears in the first 3 weeks after birth in mouse and rabbit, it is established before birth in guinea pig which exhibits a heart at a more advanced maturation state at birth (Hoerter et al., 1994).

Unlike CK shuttle, the efficiency of which depends on the expression of CK isoforms in striated muscle, functional DANC does not require the presence of any specific energy transfer enzyme. It only needs a precise spatial arrangement of the organelles within the cytosol which is ensured by the highly organized cytoarchitecture of the cardiomyocyte (Kaasik et al., 2003; Wilding et al., 2006; Piquereau et al., 2010). The establishment of energy microdomains, in which ATP and ADP are directly channeled between mitochondria and ATPases occurs at the early postnatal development during which important architectural maturation profoundly modulates the spatial organization of the intracellular components of the cardiomyocyte (Piquereau et al., 2010; **Figures 1E,F**). In mouse, effective DANC appears at the end of the first week after birth (Piquereau et al., 2010) which corresponds to the hyperplasic phase of cardiac growth (Alkass et al., 2015). This is a period during which cardiomyocyte acquires a mature intracellular organization; mitochondria and SERCA/myosin-ATPases become closer and closer, thereby allowing direct channeling of compounds between these entities and the emergence of energy microdomains in particular. This quick maturation of subcellular structures in cardiomyocyte during perinatal period has also been recently shown in rat (Lipsett et al., 2019). Although this study especially focused on calcium microdomains that have largely been investigated in the heart (De la Fuente and Sheu, 2019), it confirms that efficiency of interorganelle communications largely depend on a specific subcellular organization that is precociously established during development.

ENERGETIC COMPARTMENTATION IN CARDIOVASCULAR PHYSIOPATHOLOGY

In various types of cardiovascular pathology, adenine nucleotide transfer between mitochondria and ATPases could be impaired either due to CK functional activity lowering or cell remodeling leading to inefficient DANC. Indeed, the CK activity in cardiomyocytes was shown to be rather sensitive to various pathologies. Major alterations of the CK system (decreases in the activities of the mi-, MM-, and MB-CK isoenzymes) in the failing heart are well-established in a wide variety of models and species, including humans (see Lygate et al., 2007 and references therein). Our group demonstrated that heart failure impaired the CK-dependent phosphotransfer systems not only in myocardium but also in skeletal muscle

(De Sousa et al., 1999, 2000, 2001). Voluntary exercise is able to normalize the CK system lowered in slow skeletal muscle under conditions of heart failure (De Sousa et al., 2002). A severe impairment of the CK-driven energy transport system was shown in doxorubicin-induced cardiotoxicity (for review see Tokarska-Schlattner et al., 2006). Chronic doxorubicin-induced damage leads not only to inactivation, which was observed with all CK isoforms, but also to further specific injury of the mi-CK isoform, namely dissociation of octamers into dimers and inhibition of mi-CK binding to mitochondrial membranes, in particular to cardiolipin.

Creatine kinase system is known to be altered also in acute models of pathology. Increased phosphate concentration in the cytosol due to high energy phosphate degradation under short-term ischemic conditions solubilizes mi-CK and decreases the functional coupling between this enzyme and mitochondrial adenine nucleotide translocase (Veksler and Ventura-Clapier, 1994). This effect lowers the efficiency of the CK system in spite of the fact that mi-CK is still present in the mitochondrial compartment. Ischemia followed by reperfusion leads to well-known down-regulation of the CK system (for review see Cao et al., 2018). Mitochondrial CK is highly susceptible to oxidative modifications (for example by increased local reactive oxygen species production), leading to enzymatic inactivation and octamer dissociation, as well as formation of crystalline mitochondrial inclusion bodies, all resulting in loss of mi-CK function (Schlattner et al., 2006). Elevated nitric oxide production is also able to inhibit mi-CK thus lowering the CK system efficiency (Kaasik et al., 1999).

Interestingly, mi-CK overexpression in mice diminished ischemic contracture and improved functional recovery after ischemia (Whittington et al., 2018). Similarly, MM-CK overexpression also improves contractile function and ATP kinetics in post-ischemic myocardium (Akki et al., 2012). Theoretically, efficiency of CK shuttle could be increased by elevation of creatine content in the cytosol of cardiomyocytes. Indeed, moderately elevating myocardial creatine levels by over-expression of the creatine transporter improved energetic and functional post-ischemic recovery and reduced myocardial injury (Lygate et al., 2012).

Various pathologies associated with muscle cell remodeling are also able to alter the intracellular adenine nucleotide transfer. Importance of cell architecture for DANC was demonstrated in studies where spatial interactions between energy-utilizing and producing sites are perturbed by mutations in various cytoskeletal proteins resulting in cytoarchitectural disorganization (Wilding et al., 2006). Among them, muscle LIM protein (MLP) is known to be a key regulator of myogenesis, promoting myogenic differentiation. MLP-null mouse hearts have disorganized myofibrils with widened Z-lines; intermyofibrillar mitochondria are irregularly dispersed and not arranged in longitudinal columns. Mitochondria, myofibrils, and SR are less tightly packed, with more cytosol visible, and increased content of subsarcolemmal mitochondria. Nevertheless, this remodeling does not change maximal respiration rate, mitochondrial content or total CK activity. However, such modified mitochondrial network having normal oxidative activity give significantly weaker energy support to the SR calcium loading obviously due to impaired DANC. Another model of cytoarchitectural perturbation is myocardium lacking desmin, a muscle-specific intermediate filament protein linking the mitochondria to the cytoskeleton. Like for MLP-KO myocardium, mitochondrial support for SR calcium uptake is specifically decreased in the desmin-null hearts, despite normal oxidative capacity thus suggesting less efficient DANC (Wilding et al., 2006). These experiments clearly demonstrated that cytoarchitectural perturbation could promote energetic dysfunction via DANC impairment.

Failing myocardium is known to exhibit reduced mitochondrial content. However, lowered CK activity and cytoarchitectural disorganization, which may compromise mitochondria SR and myofilament interactions, are able to perturb intracellular energy transfer thus aggravating the pathology. Experimental heart failure is associated with misalignment of mitochondria and myofibrils, heterogeneity of mitochondrial shape and size and mitochondrial degradation so that zig-zagging Z lines appears with fewer contacts between mitochondria and myofibrils (Joubert et al., 2008; **Figures 1G,H**). These changes are the basis for the decreased energy transfer between mitochondria and myosin-ATPase in addition to the decrease in energy production. Indeed, while in healthy rats both CK and mitochondria contribute equally to SR and myofibrillar functions, in failing heart, mitochondria are less efficient than CK in maintaining an adequate energy supply for contraction. Such data suggest that energetic remodeling occurs in heart failure, which alters the ratio of efficacy between the two main energy sources. Due to the thick structure of myofibrils, the core of myofilaments may be more dependent on mitochondrial form, mass and architectural arrangement than SR, which is in closer physical interaction with mitochondria. On the other hand, because CK is closely bound to myofilaments and SR, CK efficacy seems to depend more on the amount of bound CK than on architectural design. Thus, in heart failure mitochondria could be more limiting than CK for the regulation of ATP/ADP ratio in the vicinity of myofibrils as compared to SR. Maintaining the close interaction between mitochondria and myofibrils appears to be crucial for optimal energetic regulation (Joubert et al., 2008).

Impaired adenine nucleotide channeling has also been demonstrated in overloaded myocardium of spontaneously hypertensive rats (SHRs) (Power et al., 2016). This myocardium shows a significantly longer distance between the centers of myofibril to mitochondria in the SHR hearts, which increases transverse metabolite diffusion distances. As a result, ADP channeling toward mitochondria is weakened, thus lowering the stimulation of mitochondrial respiration by ADP. Along with reduced CK functional activity, this mechanism appears to contribute to the energy state impairment in overloaded myocardium. Interestingly, experimental pulmonary artery hypertension leading to right ventricle hypertrophy also induces an increase in diffusion distances between the myofilaments and mitochondria (Power et al., 2019) thus impairing the

adenine nucleotide channeling. Such a remodeling seems to be a common feature of myocardial hypertrophy induced by an elevated afterload.

CONCLUSION

In muscle cells, cytoarchitecture, energy yield, and biochemical composition are all intimately linked to determine specific muscle functions. Highly structured cytoarchitecture involving direct organelle interaction, compartmentalized phosphotransfer kinases, and bound glycolytic enzymes allows high efficiency and fine-tuning of energy transduction system. Consequently, it has to be considered that genetic modifications, ontologic development and pathologies induce joint adaptation and remodeling of this complex network in order to match specialized muscle cell functions.

AUTHOR CONTRIBUTIONS

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REVIEW
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Mitochondria-Associated Endoplasmic Reticulum Membranes in Cardiovascular Diseases

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The endoplasmic reticulum (ER) and mitochondria are physically connected to form dedicated structural domains known as mitochondria-associated ER membranes (MAMs), which participate in fundamental biological processes, including lipid and calcium (Ca^{2+}) homeostasis, mitochondrial dynamics and other related cellular behaviors such as autophagy, ER stress, inflammation and apoptosis. Many studies have proved the importance of MAMs in maintaining the normal function of both organelles, and the abnormal amount, structure or function of MAMs is related to the occurrence of cardiovascular diseases. Here, we review the knowledge regarding the components of MAMs according to their different functions and the specific roles of MAMs in cardiovascular physiology and pathophysiology, focusing on some highly prevalent cardiovascular diseases, including ischemia-reperfusion, diabetic cardiomyopathy, heart failure, pulmonary arterial hypertension and systemic vascular diseases. Finally, we summarize the possible mechanisms of MAM in cardiovascular diseases and put forward some obstacles in the understanding of MAM function we may encounter.

Keywords: mitochondria-associated ER membrane, cardiovascular diseases, SR-mitochondrial contact, mitochondrial bioenergetics, metabolic transition

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Abbreviations: Ac-CoA, acetyl-coenzyme A; AKT, serine/threonine kinase; AMPK, adenosine 5′-monophosphate (AMP)-activated protein kinase; ASC, apoptosis-associated speck-like protein; ATF6, activating transcription factor 6; ATG14L, autophagy-related 14-like; BCL-XL, B cell lymphoma extra-large; Ca^{2+}, calcium; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; cFLIPL, FADD-like apoptosis regulators; CypD, peptidyl-prolyl *cis–trans* isomerase D; DRP1, dynamin-related protein 1; EC, endothelial cell; ER, endoplasmic reticulum; FACL4, fatty acid CoA ligase 4; Fis1, fission 1 protein; FUNDC1, FUN14 domain-containing protein 1; GLUT4, glucose transporter type 4; GRP75, chaperone 75 kDa glucose-regulated protein; GSK3β, glycogen synthase kinase 3β; HF, heart failure; IMM, inner mitochondrial membrane; IL-1β, interleukin 1β; IP3R, inositol-1,4,5-triphosphate receptor; I/R, ischemia-reperfusion; IRE1, inositol-requiring enzyme 1; MAM, mitochondria-associated ER membrane; MCU, mitochondrial calcium uniporter; MFN2, mitofusin 2; Mff, mitochondrion fission factor; mPTP, mitochondrial permeability transition pore; mTORC2, mammalian target of rapamycin complex 2; NE, norepinephrine; NCLX, Na^{+}/Ca^{2+} exchanger 1; NO, nitric oxide; NOGO B, neurite outgrowth inhibitor B; NLRP3, pyrin domain-containing 3 protein; OMM, outer mitochondrial membrane; OPA1, optic atrophy protein 1; ORP5, oxysterol-binding protein-related protein 5; ox-LDL, oxidative low-density lipoprotein; PACS2, phosphofurin acidic cluster sorting protein 2; PAH, pulmonary arterial hypertension; PASMC, pulmonary artery smooth muscle cell; PDGF, platelet-derived growth factor; PDH, pyruvate dehydrogenase complex; PDK, PDH kinase; PE, phosphatidylethanolamine; PEMT2, phosphatidyl ethanolamine methyltransferase 2; PERK, protein kinase-like ER kinase; PML, promyelocytic leukemia protein; PS, phosphatidylserine; PSS, PS synthase; PTEN, phosphatase and tensin homolog; RMDB3, regulator of microtubule dynamics 3; ROS, reactive oxygen species; RYR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TAC, thoracic aortic constriction; TCA, tricarboxylic acid; TEM, transmission electron microscopy; TNF-α, tumor necrosis factor-α; TRPM8, transient receptor potential melastatine 8; TXNIP, thioredoxin interacting protein; T2D, type 2 diabetes mellitus; UCP2, uncoupling protein 2; UPR, unfolded protein reaction; VAMP, vesicle associated membrane protein; VAPB, VAMP-associated protein B; VDAC1, voltage-dependent anion-selective channel protein 1; VSMC, vascular smooth muscle cell; YME1L1, YME1 Like 1 ATPase.

INTRODUCTION

As the main energy-producing organelles in eukaryotic cells, mitochondria are very important in maintaining the metabolism and function of cells, especially those with high energy demand, such as continuously contracting cardiomyocytes. The main energy substrate, fatty acids, or other substrates (mainly glucose, lactic acid, ketone body, etc.), require mitochondrial oxidative phosphorylation to generate enough ATP for maintaining contraction (Bertero and Maack, 2018). Mitochondrial disorders, accompanied by repressed mitochondrial oxidative activity, are detrimental to cardiac function, leading to cardiac hypertrophy and eventually HF (Buchwald et al., 1990; Bertero and Maack, 2018). On the other hand, as the main function of blood vessels is to transport oxygen to other tissues, the basal oxygen consumption of vascular ECs and smooth muscle cells is very low and the energy required is mainly from glycolysis, with concomitant lower mitochondrial content (Paul, 1983; Culic et al., 1997). However, impaired vascular mitochondrial function has been associated with metabolic transition of vascular cells and increased ROS production, resulting in the loss of EC barrier function and the increase of VSMC *trans*-differentiation and proliferation, hallmarks of arterial stiffness, lipid deposition, vascular remodeling and other vascular pathological changes (Dromparis and Michelakis, 2013). Taken together, abnormal mitochondrial metabolism plays a critical role in the development of cardiovascular diseases.

Similar as other membrane organelles, mitochondria are highly dynamic structures, and their mass, morphology, location and composition are constantly changing according to cellular requirements (Pernas and Scorrano, 2016). In addition, mounting evidence have proved the existence of physical and functional communication between mitochondria and other organelles, such as plasma membrane, nucleus and ER (Lackner, 2019). ER-mitochondrial coupling is the first and best described type of organelle interaction. The first evidence of physical interaction between these membranes comes from electron microscopy studies more than 60 years ago (Copeland and Dalton, 1959). About 30 years later, ER-mitochondrial contact was isolated by subcellular isolation using Percoll density gradient and lead to the production of the term MAMs (Vance, 1990). This contact is considered to be critically important for mitochondrial metabolism as at these sites calcium (Ca^{2+}) is directly transferred from the ER storage area to the mitochondria, and increases the activities of Krebs cycle and electron transport chain, thus stimulating ATP synthesis in the mitochondrial matrix (Cardenas et al., 2010). However, overactivation of electron transport chain by excessive mitochondrial Ca^{2+}, which is evident during ischemia–reperfusion, is directly linked to enhanced ROS production (Brookes et al., 2004), and mitochondrial Ca^{2+} overload also induces apoptosis by opening the mPTP on the mitochondrial outer membrane, leading to rapid collapse of the membrane potential and swelling of mitochondria (Kwong and Molkentin, 2015). Therefore, the aberrant formation of MAMs is a main step of mitochondrial dysfunction.

In addition to acting as a regulator of mitochondrial energy production, MAMs are also important in regulating the contractile function of arteries and hearts by modulating transient intracellular Ca^{2+} concentration (Herring et al., 2006; Orchard and Brette, 2008). The disorder of mitochondrial Ca^{2+} buffering mediated by MAMs often leads to the abnormal increase of Ca^{2+} in the cytoplasm, thus activating the calcium signaling pathway related to cardiac hypertrophy and HF (Frey et al., 2000). Therefore, in this review, we will first introduce the molecular components of MAMs and the evidence of ER-mitochondria communication in cardiovascular system according to their different roles in regulating mitochondrial morphology and function. We then discuss the role of MAMs in cardiac and vascular function, and how dysfunction of MAMs is associated with highly prevalent cardiovascular diseases such as HF, myocardial hypertrophy and hypertension.

THE COMPONENTS AND FUNCTION OF MAMS

Lipid Synthesis and Transfer

Phospholipid transport and synthesis is the first recognized function of the ER mitochondrial interface (Rusinol et al., 1994). In fact, the first proteins found on the MAMs, PEMT2 and phosphatidylserine synthase 1 and 2 (PSS1/2) (Cui et al., 1993; Stone and Vance, 2000), are related to lipid metabolism, and FACL4, which is involved in the synthesis of triacylglycerol, is currently regarded as one of the most reliable MAM markers (Rusinol et al., 1994). The enrichment of cholesterol and sphingolipid in MAM is related to the accumulation of caveolin-1 (Sala-Vila et al., 2016). After synthesis, caveolin-1 is inserted into the ER, not only participates in cholesterol transport to the plasma membrane, but also regulates ER-mitochondrial cholesterol transfer (Quest et al., 2008). In addition, the enrichment of synthetic enzymes at MAM promote the local generation of main structural component of biological membranes, phosphatidylcholine, PE, and phosphatidylserine (Vance, 1990). Phosphatidylserine synthesized in ER requires mitochondrial specific phospholipase to produce PE, which is then converted to phosphatidylcholine in the ER (Shiao et al., 1995). This transferring process is carried out by ORP5 and ORP8, two proteins known to be involved in the phosphatidylserine transfer from the ER to plasma membrane or MAM (Galmes et al., 2016). In addition, phospholipid acids are synthesized in ER and must be transferred to mitochondria for modification to produce mitochondrial cardiolipin that exerts cardioprotective function (Dennis and Kennedy, 1972; Osman et al., 2011). Cardiolipin interacts strongly with, and is required for the stability and activity of many integral membrane proteins of the IMM (Musatov and Sedlak, 2017), including the mitochondrial Ca^{2+} uniporter (MCU) that mediates Ca^{2+} uptake in mitochondrial matrix (Ghosh et al., 2020). Levels of individual species of cholesterol esters, PEs, and triacylglycerols are associated with cardiovascular diseases (Stegemann et al., 2014).

Ca^{2+} Transfer

The energy released by electron transport from the mitochondrial oxidative respiratory chain is used to form a proton gradient across the inner membrane of mitochondria, which drives ATP synthesis and also creates a driving force for Ca^{2+} absorption (Giorgi et al., 2018). However, the entry of Ca^{2+} into mitochondrial matrix is a process that exhausts mitochondrial potential and competes with ATP generation, thus requires precise regulation (Giorgi et al., 2018). Importantly, mitochondria must be exposed to high concentration of Ca^{2+} in order to take up Ca^{2+} due to the limitation of MCU on IMM (Rizzuto et al., 1998). Thus, mitochondrial Ca^{2+} uptake is most likely to occur near the Ca^{2+} releasing stores, such as ER (Giorgi et al., 2018). Through MAMs, Ca^{2+} is transferred directly from the ER to mitochondria and controls key mitochondrial functions, such as apoptosis and energy generation (Giorgi et al., 2018). This local and rapid uptake of mitochondrial Ca^{2+} can prevent excessive increase of cytosolic Ca^{2+} and control the Ca^{2+} signals to occur locally (Laude and Simpson, 2009).

The effective transfer of Ca^{2+} between the ER and mitochondria is mediated by a complex of multiple proteins. The main channel of Ca^{2+} release of the ER, Inositol-1,4,5-triphosphate receptor type 1 (IP3R1), is responsible for forming a high Ca^{2+} domain in the ER vicinity. The VDAC1 acts as a Ca^{2+} uptake channel in the OMM. The third component of the complex is mitochondrial stress 70 protein, also known as GRP75, which connects two channels through their cytosolic portions to form VDAC1/GRP75/IP3R1 channel complex (Szabadkai et al., 2006). The overexpression of GRP75 does not increase the ER-mitochondrial contact, so GRP75 may play a role in the established contact points (Szabadkai et al., 2006). In this way, Ca^{2+} is transferred directly from ER to cytosol and across the OMM, then Ca^{2+} is transported into mitochondrial matrix via MCU (Baughman et al., 2011; De Stefani et al., 2011). In excitable cell types, such as cardiomyocytes and VSMCs, the RYR on SR is also present in MAMs and plays a key role in organelle Ca^{2+} transfer in these cells (Eisner et al., 2013).

Recently, a new family of TRPM8 channel isoforms as functional ER Ca^{2+} release channels expressed in MAMs has been identified (Bidaux et al., 2018). We also confirmed that SR-resident TRPM8 participated in the regulation of cellular and mitochondrial Ca^{2+} homeostasis in the VSMCs. TRPM8 activation by menthol antagonized angiotensin II (AngII)-induced mitochondrial respiratory dysfunction and excess ROS generation by preserving mitochondrial Ca^{2+}-dependent PDH activity, thus lowered blood pressure in cold or in AngII-induced hypertensive mice (Xiong et al., 2017). Meanwhile, restoration of ER-mitochondrial Ca^{2+} transfer by activation of TRPM8 seems to be beneficial for restricting cytosolic Ca^{2+} signaling that accounts for vascular constriction (Sun et al., 2014). However, whether there are other TRP channels located in MAM and involved in the Ca^{2+} transfer from ER to mitochondria remains to be further investigated.

Mitochondrial Dynamics

Mitochondria are dynamic organelles continuously undergoing fusion and fission. A proper balance between these two opposing processes is essential for cell survival and for maintaining the shape, the size and the number of mitochondria (Hoppins and Nunnari, 2012; Youle and van der Bliek, 2012). The main mitochondrial dynamic protein accounting for fission is DRP1. It is a cytosolic GTPase, which is recruited from the cytoplasm to form a contractile ring on the mitochondria, thus driving the cleavage process. Mitochondrial fission often occurs at positions where ER tubules contact and constrict mitochondria and facilitates the recruitment of DRP1 (Friedman et al., 2011). On the other hand, the process of mitochondrial fusion is controlled by GTPases mitofusin 1 (MFN1), mitofusin 2 (MFN2) and mitochondrial dynamic-like 120 kDa protein [also known as optic atrophy protein 1 (OPA1)]. As GTPases, MFN2 on the ER surface can form dimers with either MFN1 or MFN2 located on the mitochondria, and drives the fusion of outer membrane (OMM) of mitochondria (Chen et al., 2003; de Brito and Scorrano, 2008). This interaction not only determines the distance between organelles, but also enable coordinated regulation of ER and mitochondria dynamics (Chen et al., 2003; de Brito and Scorrano, 2008). However, this widely accepted model has been challenged by results from quantitative EM analysis, which demonstrate that MFN2 ablation or silencing increases the close contacts between the two organelles and facilitates Ca^{2+} transfer from the ER to mitochondria (Cosson et al., 2012; Filadi et al., 2015). Thus, MFN2 is more like a tethered antagonist, which prevents excessive proximity between the two organelles. The exact role of MFN2 in ER-mitochondria contact remains debated. OPA1 exists in the mitochondrial inner membrane (IMM) and the intramitochondrial space, which is responsible for maintaining cristae structure and mediating IMM fusion (Frezza et al., 2006; Meeusen et al., 2006).

Autophagy, ER Stress, Inflammation and Apoptosis

In addition to regulating the morphology and function of mitochondria, MAMs are also involved in many important cellular behaviors, such as autophagy, ER stress, inflammation and apoptosis. MAMs not only provide an appropriate space for the occurrence of cell pathways, but also recruit some key regulatory factors responsible for these behaviors.

Autophagy is an evolutionarily conserved self-digestion process of intracellular material turnover in eukaryotes, which involves the formation of double-membrane vesicles called autophagosomes. The formation of autophagosome is initiated by the recruitment of pre-autophagosome marker ATG14L at the MAMs (Hamasaki et al., 2013). At rest, syntaxin-17 binds to DRP1, but in the absence of nutrients, DRP1 is replaced by ATG14L, which promotes the enrichment of different proteins involved in autophagy in MAMs (Arasaki et al., 2015). mTORC2, a key inducer of autophagy, is located in MAMs and regulates its integrity (Colombi et al., 2011). It is also required for normal cardiac physiology and ensures cardiomyocyte survival in response to pressure overload (Sciarretta et al., 2018). Growth

factors stimulate the activation of mTORC2 and AKT, which are then translocated to MAMs to phosphorylate some key components of MAM maintaining mitochondrial potential, ATP production and Ca^{2+} uptake (Betz et al., 2013).

The changes of ER oxidation can lead to the aberrant formation of disulfide bonds and the accumulation of peptides, thus activating a series of intracellular reactions called UPR (Zeeshan et al., 2016). UPR as an acute response has been found in many types of cardiovascular disease (Zhang et al., 2019). The stimulation of UPR leads to three main response mechanisms: inositol-requiring enzyme 1α (IRE1α), PERK and ATF6, which regulate the protein folding ability of ER (Song et al., 2018). In the early stage of UPR, the increase of ER mitochondrial contact sites is beneficial (Bravo et al., 2011). Verfaillie et al. (2012) reported that without PERK, endogenous apoptosis induced by ER stress was weakened due to the reduction of MAM formation and hampered ROS signal transmission to adjacent mitochondria. The presence of IRE1 in MAMs determines the effectiveness of IP3R, which is conducive to the transfer of Ca^{2+} to mitochondria (Carreras-Sureda et al., 2019). These mechanisms connect ER stress and mitochondrial function, thus affecting the fate of cells. MAMs also provide a place for ROS generation. Oxidative condition activated PKCβ induces ser36 phosphorylation of p66Shc, resulting in p66Shc transfer to mitochondria or MAMs, where ROS could be produced (Giorgio et al., 2005; Pinton et al., 2007). ROS generation from p66Shc might facilitate short-term repair response (Akhmedov et al., 2015), but contribute to the development of many types of cardiovascular disease in a long term (Boengler et al., 2019).

A class of nucleotide oligomerization domain-like receptors (NLRs) sense abnormal cytosolic changes, such as microbial invasion, tissue damage and cell stress, and form multiprotein complexes called "inflammasome," which are linked to the pathogenesis of several cardiovascular diseases (Liu et al., 2018). The NLRP3 inflammasome initiates proteolysis of pro-inflammatory cytokine interleukin 1β (IL-1β) (Gross et al., 2011). In resting state, NLRP3 localizes in cytoplasm and ER. Upon stimulation, NLRP3 inflammasome could be recruited to the MAM sites accompanied with its adaptor ASC, suggesting that NLRP3 strategically accumulates at mitochondria to sense mitochondrial damage (Zhou et al., 2011). Thus, MAMs play a critical role in initiating inflammation by acting as an inflammatory platform.

Ca^{2+} transfer from ER to mitochondria is a key factor in a series of events leading to apoptosis, and there are many proteins that control death and survival in MAMs. For example, BCL-2 protein family includes anti apoptotic and pro-apoptotic members, which control the sensitivity of cells to apoptosis signals. BCL-XL (also known as Bcl-2-like protein 1), a member of the anti-apoptotic family, partially localizes to MAM, increases Ca^{2+} transfer from ER to mitochondria as an adaptive response to increase mitochondrial bioenergetics and prevent intracellular Ca^{2+} overload after thapsigargin stimulation (Williams et al., 2016). A typical tumor suppressor PTEN has also been shown to be present in MAM. It antagonizes AKT signal, interacts with IP3R1, and enhances the transfer of Ca^{2+} from ER to mitochondria, which makes cells more sensitive to apoptosis (Bononi et al., 2013). In the process of apoptosis, extracellular signals trigger cell death by activating caspase 8, which then activates downstream caspases to decompose cells (Wu et al., 2014). A caspase 8 inhibitor located at MAM, cFLIPL, prevents caspase 8-mediated NOGO B cleavage, thus maintaining the integrity of ER morphology and ER-mitochondrial contacts (Marini et al., 2015). In conclusion, these findings suggest that apoptosis is strictly regulated in MAMs.

The above-mentioned functions of MAMs in cell physiology are depicted in **Figure 1**.

THE ROLE OF MAMS IN THE REGULATION OF CARDIOVASCULAR PHYSIOLOGICAL FUNCTION

Although MAMs were first observed more than 60 years ago, their potential role in the cardiovascular system has not been the focus of debate until the last 20 years. Significant differences in SR-mitochondrial communication were observed in cardiac and vascular tissues, which were mainly based on the cellular function regulated by this coupling. However, Ca^{2+} transfer between organelles seems to play an important role in both the heart and the vascular system.

Heart

SR is a membrane-bound structure existing in muscle cells (myocardium and skeletal muscle), similar to ER in other cells. The main function of SR is to store Ca^{2+} (Eisner et al., 2013). In the early 1990s, different research groups provided evidence that mitochondrial Ca^{2+} uptake was involved in myocardial contractile regulation. SR releases Ca^{2+} in response to electrical stimulation or pharmacological activation of RYR and increases mitochondrial Ca^{2+} level (Bassani et al., 1992; Negretti et al., 1993; Szalai et al., 2000). When the mitochondrial membrane potential was partially inhibited, the kinetics of cytosolic Ca^{2+} during contraction induced by electricity or caffeine was slightly changed, but the shortening degree of cardiomyocytes was reduced by more than twice, suggesting that the transfer of Ca^{2+} from SR to mitochondria was involved in cardiac contraction (Bassani et al., 1992; Negretti et al., 1993). Subsequently, studies showed that the mitochondrial Ca^{2+} oscillates synchronously with cytosolic Ca^{2+} in cardiomyocytes (Robert et al., 2001). In fact, the basal Ca^{2+} concentration in mitochondria fluctuated between 145 and 175 nmol/l in response to SR-derived Ca^{2+} release during electrical stimulation (Lu X. et al., 2013). Consistent with these observations, an increase or decrease in the level of MCU results in a lower or higher amplitude of the spontaneous cytoplasmic Ca^{2+} peak, respectively, indicating a two-way communication between these intercellular compartments (Drago et al., 2012). It is worth noting that the specific effect of mitochondrial Ca^{2+} treatment on the cytosolic Ca^{2+} level of cardiomyocytes is still controversial, which is mainly due to the moderate Ca^{2+} uptake capacity of mitochondria compared with other Ca^{2+}-scavenging entities. Comparative studies showed that, despite the same biophysical properties as other tissues, MCU-derived current densities were

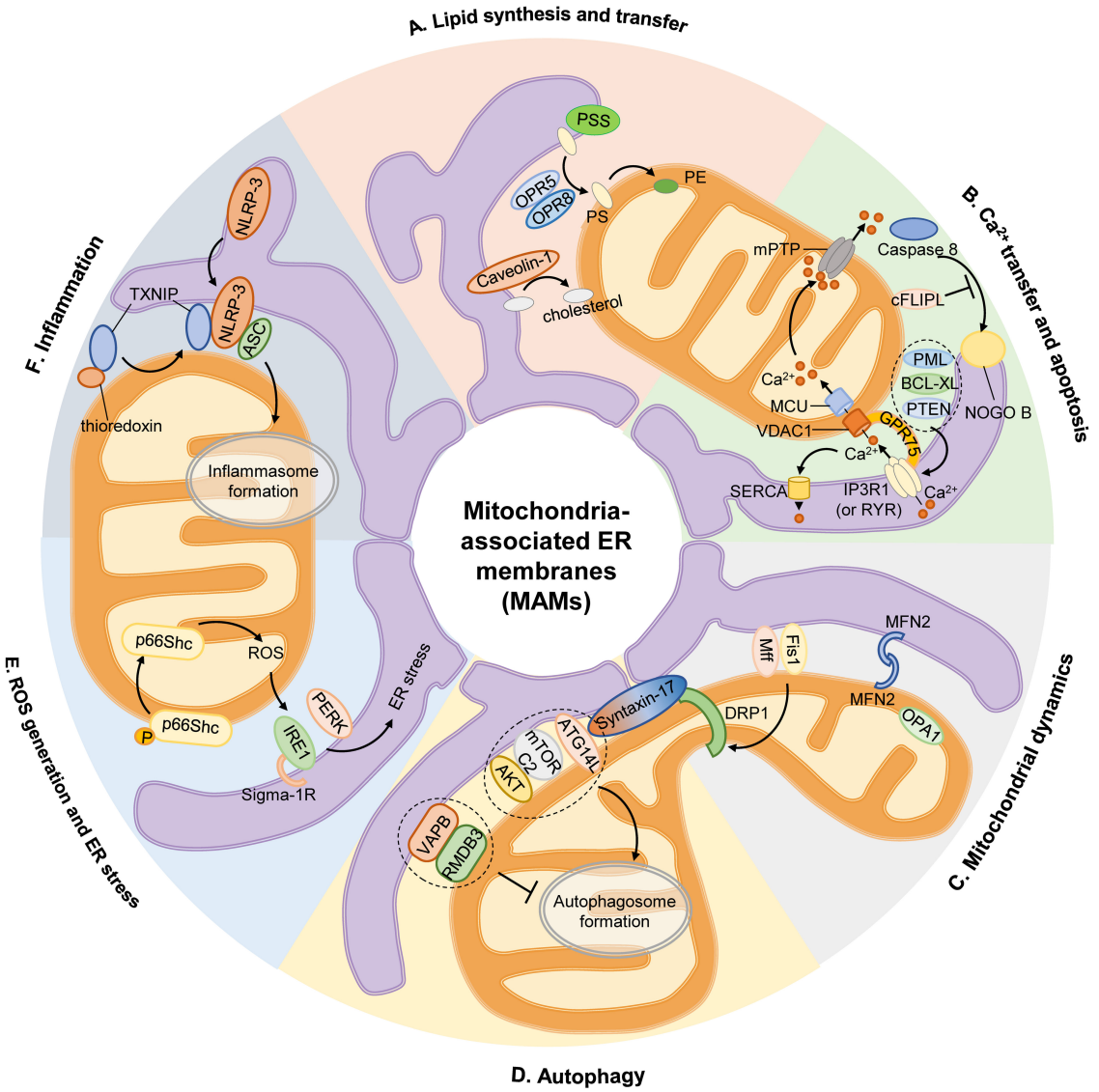

FIGURE 1 | Mitochondria-associated ER membranes and cell physiology. (A) Lipid synthesis and transfer. The MAMs account for PS generation through PSS, and PS synthesized in ER is then transferred to mitochondria by ORP5 and ORP8 for further conversion to PE. In addition, caveolin-1 inserted into the ER participates in ER-mitochondrial cholesterol transfer. (B) Ca^{2+} transfer and apoptosis. Ca^{2+} transfer from ER to mitochondria is mediated by a protein complex consisting IP3R1 in ER or RYR in SR, GRP75, and VDAC1 in OMM, then Ca^{2+} is transported into mitochondrial matrix via MCU. Excessive mitochondrial Ca^{2+} uptake triggers opening of mPTP to initiate apoptosis. Activation of caspase 8 activates downstream caspases and induces NOGO B cleavage, which is inhibited by cFLIPL. Some proteins are located on MAM govern the apoptotic pathway by preserving intraorganellar Ca^{2+} transfer, such as PML, BCL-XL, or PTEN. (C) Mitochondrial dynamics. The recruitment of main mitochondrial fission protein DRP1 to MAM is regulated by Mff, Fis1 and Syntaxin-17. And mitochondrial fusion is controlled by MFN2 on OMM and OPA1 on IMM. (D) Autophagy. At rest, Syntaxin-17 binds to DRP1, but in the absence of nutrients, DRP1 is replaced by pre-autophagosome marker ATG14L, which promotes the enrichment of mTORC2 and AKT to initiate the formation of autophagosome. The anchor sets formed by VAPB in ER and RMDB3 on OMM regulate autophagy by maintaining MAMs. (E) ROS generation and ER stress. Oxidative condition induces ser36 phosphorylation of p66Shc, resulting in p66Shc transfer to MAMs and produce ROS, which stimulates ER stress via IRE1 and PERK. Sigma-1R located on MAMs could stabilize IRE1. (F) Inflammation. Upon stimulation, NLRP3 is transferred from ER to MAMs where it interacts with its adaptor ASC and TXNIP to initiate inflammasome formation. AKT, serine/threonine kinase; ASC, apoptosis-associated speck-like protein; ATG14L, autophagy-related 14-like; BCL-XL, B cell lymphoma extra-large; cFLIPL, FADD-like apoptosis regulators; DRP1, dynamin-related protein 1; ER, endoplasmic reticulum; Fis1, fission 1 protein; GRP75, chaperone 75 kDa glucose-regulated protein; IP3R1, inositol-1,4,5-triphosphate receptor type 1; IMM, inner mitochondrial membrane; IRE1, inositol-requiring enzyme 1; MAM, mitochondria-associated ER membrane; Mff, mitochondrion fission factor; MCU, mitochondrial calcium uniporter; MFN2, mitofusin 2; mTORC2, mammalian target of rapamycin complex 2; NOGO B, neurite outgrowth inhibitor B; NLRP3, pyrin domain-containing 3 protein; OPA1, optic atrophy protein 1; ORP5, oxysterol-binding protein-related protein 5; OMM, outer mitochondrial membrane; PE, phosphatidylethanolamine; PERK, protein kinase-like ER kinase; PML, promyelocytic leukemia protein; PS, phosphatidylserine; PSS, PS synthase; PTEN, phosphatase and tensin homolog; RMDB3, regulator of microtubule dynamics 3; ROS, reactive oxygen species; RYR, ryanodine receptor; TXNIP, thioredoxin interacting protein; VAMP, vesicle associated membrane protein; VAPB, VAMP-associated protein B; VDAC1, voltage-dependent anion-selective channel protein 1.

unexpectedly lower in mouse cardiac mitochondria (Fieni et al., 2012). In addition, it is estimated that 1–15% of Ca^{2+} in the cytosol is removed by mitochondria during heart beating, while the remaining percentage is mobilized by SERCA and NCLX (Drago et al., 2012; Fieni et al., 2012). Mitochondria transiently take up Ca^{2+} and can contribute to buffering cytosolic Ca^{2+} rises, but in most cases, mitochondrial Ca^{2+} is released quite quickly through the efflux machinery, such as NCLX in cardiomyocytes. Deletion of NCLX in adult mouse hearts causes sudden death with severe myocardial dysfunction and fulminant HF due to mitochondrial Ca^{2+} overload, whereas overexpression of NCLX in the mouse heart displays potent beneficial effect of augmenting mitochondrial Ca^{2+} clearance, and protecting against ischemia-induced cardiomyocyte necrosis and HF (Luongo et al., 2017).

The Ca^{2+} transfer from SR to mitochondria is mainly restricted in the MAM regions, where local high Ca^{2+} domains are formed. The distance between SR, RYR and OMM is nearly 40 nm and the concentration of Ca^{2+} in mitochondria decreases rapidly, as the distance between organelles and Z-line and transverse tubule in cardiac sarcomeres increases (Sharma et al., 2000; Lu X. et al., 2013). SR-mitochondrial Ca^{2+} transfer seems to occur through direct physical contact in cardiomyocytes. Purified microsomes isolated from rat cardiomyocytes have been shown to be responsive to caffeine stimulation, which is due to the presence of RYR2-containing SR vesicles in these microsomes (Garcia-Perez et al., 2008). This delegate structure allows mitochondria produce ATP closely coupled to RYR2-mediated Ca^{2+} release and the energy demand of the cardiomyocytes (De la Fuente and Sheu, 2019). However, the activity of RYR2 could be enhanced by mitochondrial ROS, leading to aberrant Ca^{2+} leak from the SR and diminished systolic Ca^{2+} transients, contributing to a blunted response to sympathetic stimulation (Cooper et al., 2013). In addition, enhancement of RYR2 activity promotes mitochondrial ROS production in a mitochondrial Ca^{2+}-dependent manner, forming a positive feedback process that is detrimental to intracellular Ca^{2+} handling in cardiomyocytes (Hamilton et al., 2020).

MFN2 is the most likely protein involved in the tether between SR and cardiac mitochondria. Under basal conditions, cardiac-specific MFN2 knockout mice show cardiac hypertrophy and moderate diastolic dysfunction, but no systolic dysfunction (Papanicolaou et al., 2011). In contrast, these mice show significant systolic dysfunction under a β-adrenergic stress condition (Papanicolaou et al., 2011). TEM showed that mitochondria were abnormally large and elongated, and the contact between SR and mitochondria was reduced without affecting inter-organelle distance in MFN2 but not MFN1 knockout hearts (Chen et al., 2012). In addition, compared with the control group, MFN2 deficient cardiomyocytes displayed abnormal mitochondrial spatial distribution, low mitochondrial membrane potential and reduced Ca^{2+} uptake (Chen et al., 2012).

In addition, FUN14 domain-containing protein 1 (FUNDC1) is a mitochondrial outer membrane protein involved in maintaining MAM formation by binding with IP3R2. According to the results of TEM, the deletion of FUNDC1 resulted in an 80% reduction in ER and mitochondrial contact (Wu et al., 2017), while MFN2 deletion only resulted in a 30% reduction (Chen et al., 2012). The mutation of FUNDC1 makes it unable to interact with IP3R2, which significantly reduces the contact between ER and mitochondria, similar to the situation after FUNDC1 ablation (Wu et al., 2017). Further studies showed that FUNDC1 inhibited the ubiquitination and degradation of IP3R2 by direct interaction, and improved the stability of IP3R2 (Wu et al., 2017). Mitochondria in the hearts of FUNDC1 knockout mice are larger and more elongated and these mice had significantly lower early and late ventricular filling velocity ratio, decreased ejection fraction, shortening fraction and cardiac output, showing diastolic and systolic dysfunction (Wu et al., 2017).

Mitochondrial dynamics in myocardial tissue is also important for cardiac function. The changes in mitochondrial dynamics can be observed in neonatal rat cardiomyocytes (Kuzmicic et al., 2014), and the fission-fusion cycle is estimated to take nearly 2 weeks in adult cardiomyocytes (Chen et al., 2011). Although the heart appearance of DRP1 knockout mice was normal, the beating rate of isolated cardiomyocytes was lower than that of wild-type mice (Wakabayashi et al., 2009). A recent study demonstrated that decreased DRP-1 expression by mdivi-1 treatment or siRNA knockdown proteolytically cleaved OPA1, and altered the expression of mitochondrial oxidative phosphorylation complex proteins, resulting in defects in mitochondrial respiration, suppressed autophagy and increased mitochondrial ROS production (Aishwarya et al., 2020). Thus, normal mitochondrial fission might be crucial for energy supply in cardiomyocytes. Accordingly, cardiac-specific ablation of DRP1 in mice is lethal, and controlled deletion during adult life results in diminished survival, cardiac hypertrophy, fibrosis and reduced systolic function (Ikeda et al., 2015).

Vasculature

Compared to cardiac muscle, there are fewer studies on SR-mitochondrial communication in vasculature. Rather than providing energy, mitochondria in ECs are more likely to act as mediators of signal transduction as signaling organelles that control cytosolic Ca^{2+} signaling or modify ROS, as they are reported to be glycolytic and to minimally rely on mitochondria for ATP generation (Wilson et al., 2019). As angiogenesis requires ECs to be in acidic and hypoxic surroundings, a reliance on anaerobic metabolism may enable ECs to form new vessels (Eelen et al., 2018). In ECs, spontaneous ER Ca^{2+} release events and subsequent Ca^{2+} signaling usually occur preferentially at sites of contact between ECs and VSMCs, and this process is maintained and tightly controlled by mitochondrial ATP synthesis (Wilson et al., 2019). MAM formation is increased under hypoxia, which directly induce endothelial mitochondrial damage, leading to elevated ROS production and mitophagy. Disruption of MAMs in ECs attenuates mitochondrial impairment, cell apoptosis, and inflammatory response and increases NO release (Yang et al., 2019). Similarly, increased MAM formation also participates in oxidized low-density lipoprotein (ox-LDL)-induced EC apoptosis, the initial step of atherogenesis. Silencing a tethering protein in MAM, PACS2, inhibits ox-LDL-induced cell apoptosis, as well as concomitant mitochondrial Ca^{2+}

elevation, ROS production, and cytochrome c release (Yu et al., 2019). These findings confirm the importance of MAM in regulating endothelial function and participating in related vascular diseases.

In PASMCs, mechanical stress triggers a calcium release by subplasmalemmal RYR1 and is then buffered by mitochondria (Gilbert et al., 2014). The ability of mitochondria to regulate SR-derived Ca^{2+} signal is related to the control of SR luminal Ca^{2+} level, because pharmacological depolarization or MCU inhibition precludes the activation of store-operated Ca^{2+} entry channels, resulting in the decreased VSMC proliferation (Munoz et al., 2011). Also, Ca^{2+}/calmodulin-dependent kinase II (CaMKII) in the mitochondrial matrix of VSMCs promotes mitochondrial Ca^{2+} entry by phosphorylating MCU at S92. In a transgenic model of selective mitochondrial CaMKII inhibition in VSMCs, neointimal hyperplasia was significantly reduced after vascular injury (Nguyen et al., 2018). The change of VSMC phenotype is the main cause of many diseases, including hypertension and atherosclerosis, and mitochondrial metabolic regulation is closely related to VSMC differentiation (Owens et al., 2004). SR-mitochondrial communication regulates the oxidative metabolism of VSMCs, because increased Ca^{2+} flow between these organelles is associated with increased mitochondrial activity and more efficient use of glucose (Morales et al., 2014).

Also, little is known about the role of mitochondrial dynamics in ECs and VSMCs compared with that in cardiomyocytes. Some studies have observed changes in mitochondrial morphology that result in the formation of a longer or fragmented network, at least *in vitro*, within a few minutes (Wang et al., 2015; Torres et al., 2016). In most cases, these changes are related to the regulation of cell proliferation. For example, DRP1 activity and mitochondrion fission are required to induce proliferation by mitogenic factors, such as PDGF (Salabei and Hill, 2013; Wang et al., 2015). MFN2 levels are down-regulated when VSMCs switch to proliferative state (Salabei and Hill, 2013). Therefore, the disruption of mitochondrial network seems to be a key step in VSMC proliferation. Recently, TNF-α, a potent pro-inflammatory cytokine, has been discovered to induce mitochondrial fission in ECs via DRP1 (Forrester et al., 2020). Silencing of endothelial DRP1 prevents leukocyte adhesion and proinflammatory proteome induction, suggesting a potential cross-communication between the canonical inflammation pathway and mitochondrial fission (Forrester et al., 2020).

THE ROLE OF MAMS IN THE DEVELOPMENT OF CARDIOVASCULAR DISEASES

Compared with the above-mentioned important role of MAM in cardiovascular system physiology, there are few studies on the mechanism of MAM in the occurrence of cardiovascular diseases. So far, studies have shown that MAM proteins are involved in the occurrence of cardiovascular disease, which indirectly reflects the critical role of MAM.

Ischemia-Reperfusion

The harmful effects of coronary artery disease on myocardium are usually attributed to I/R injury. The subsequent pathological manifestations, from cardiomyocyte death to myocardial injury and cardiac dysfunction, are progressive factors of HF and death (Yellon and Hausenloy, 2007). An important mediator of myocardial I/R injury is mPTP, which is the main driver of cell death (Hausenloy and Yellon, 2003). The abnormal increase of mitochondrial Ca^{2+} leads to the dissipation of mitochondrial membrane potential, mitochondrial swelling and the release of proapoptotic factors, such as cytochrome c, into the cytoplasm, resulting in subsequent pore opening (Ong et al., 2015). On the basis of the results of hypoxia-reoxygenation and I/R, SR-mitochondrial Ca^{2+} transfer is considered to be harmful to cardiac injury. During hypoxia-reoxygenation, the interaction between CypD, a regulatory component of mPTP, and the Ca^{2+} channeling complex (VDAC1-GRP75-IP3R1) increases, resulting in increased mitochondrial Ca^{2+} load and cardiomyocyte death (Paillard et al., 2013). Reduction of CypD-IP3R1 interaction by inhibition of either, using their respective inhibitors NIM811 or 2-aminoethoxydiphenylborate, prevents mitochondrial Ca^{2+} overload and cell death in adult mouse cardiomyocytes (Paillard et al., 2013). Downregulation of CypD or IP3R1 also leads to similar results. In addition, downregulation of MFN2 also prevents mitochondrial Ca^{2+} overload and lethal cell damage by reducing the interaction between CypD and VDAC1-IP3R1 (Paillard et al., 2013). Thus, reducing SR-mitochondrial contact protects against I/R injury by reducing mitochondrial Ca^{2+} overload. Interestingly, ischemic or hypoxic preconditioning, a procedure that protects the myocardium from I/R injury, requires CypD participation to prevent I/R induced cell death, as hypoxic preconditioning reduced cell death in wild-type adult cardiomyocytes, but not in CypD deficient cardiomyocytes exposed to simulated I/R injury (Hausenloy et al., 2010). Similarly, inhibition of CypD by cyclosporin A reduces the beneficial effect of hypoxic preconditioning on I/R (Hausenloy et al., 2010). In addition, treatment of cyclosporin A in adult cardiomyocytes decreased mitochondrial ROS and AKT, the most important mediators of hypoxic preconditioning, and extracellular signal-regulated kinases 1/2 pro-survival activation is reduced in CypD-deficient hearts (Hausenloy et al., 2010). These observations highlight the importance of MAM controlling mPTP and Ca^{2+} channels.

Interacting with the IP3R1-GRP75-VDAC1 complex at the MAM sites, GSK3β is an important regulator of organelle Ca^{2+} transfer in cardiomyocytes (Gomez et al., 2016). Inhibition of GSK3β by SB21 reduces the interactions between the components of this complex and the transfer of Ca^{2+} from SR to mitochondria (Gomez et al., 2016). Similarly, rabbits and mice treated with GSK3β inhibitors (MLS2776 and MLS2778) had smaller infarct size compared with control (Nikolaou et al., 2019). Also, during hypoxia-reoxygenation, SB21 prevented the increase of IP3R1 activity by reducing GSK3β-mediated phosphorylation of IP3R1, and prevented mitochondrial Ca^{2+} overload (Gomez et al., 2016). Consistent with these observations, the use of small interfering RNA targeting VDAC1 can reduce the translocation of GSK3β to mitochondria and prevent the opening of mPTP in

response to cellular stress (Tanno et al., 2014). These observations suggest that GSK3β plays an important role in controlling Ca^{2+} flow from SR to mitochondria during I/R. However, loss of GSK-3 in adult cardiac myocytes resulted in induction of mitotic catastrophe, with increased DNA content and multinucleation, leading to apoptosis and severe fatal dilated cardiomyopathy (Zhou et al., 2016).

Cardiomyocyte metabolism is seriously affected by ischemia and hypoxia (Bertero and Maack, 2018). As the main regulator of cell metabolism, mitochondrial dynamics also plays an important role in I/R by regulating the opening of mPTP. As expected, the mitochondrial network is fragmented during ischemia, which is associated with increased cell death (Ong et al., 2010). However, the main function of fission is to produce more mitochondria to meet the energy requirements of myocardial cells during I/R. Inhibition of mitochondrial fission by inactive DRP1 (DRP1K38A) can reduce the sensitivity of HL-1 cells to mPTP opening and reduce cell death induced by I/R (Ong et al., 2010). In addition, inhibitor of mitochondrial fission by mdivi-1 in HL-1 cells and isolated adult cardiomyocytes promotes mitochondrial elongation and decreased cell death after I/R (Ong et al., 2010). Mdivi-1 treatment also reduces the infarct size of the heart in mice with myocardial infarction (Ong et al., 2010). In addition, inhibition of mitochondrial fission by blocking DRP1 function reduces oxygen dependence and increases leak-associated oxygen consumption in cardiomyocytes (Zepeda et al., 2014). This inhibition can produce a protective mitochondrial uncoupling effect and reduce heart injury after I/R (Aldakkak et al., 2008). Similarly, the increased DRP1 activity due to enhanced interaction of filamin A with the GTPase domain of DRP1 also exists in peri-infarct regions characterized by mitochondrial hyperfission (Nishimura et al., 2018). In addition, compared with wild-type mice, mice lacking MFN1 and MFN2 were resistant to the opening of mPTP, which could protect them from acute myocardial I/R injury and reduce the infarct size (Hall et al., 2016). Ischemia also reduces the level of IMM fusion protein OPA1. Although OPA1 overexpression increases mitochondrial fusion in H9c2 myoblasts, it fails to prevent ischemia-induced apoptosis (Chen et al., 2009).

Diabetic Cardiomyopathy

Diabetic cardiomyopathy is a special form of heart disease, characterized by lipid accumulation in myocardial cells and left ventricular hypertrophy, which leads to systolic dysfunction (Tan et al., 2020). The role of MAMs in diabetes has been studied in detail. For example, in a mouse model of type 2 diabetes mellitus (T2D), disruption of ER-mitochondrial interaction is an early event prior to mitochondrial dysfunction and insulin resistance (Tubbs et al., 2018). Compared with the control group, the number of IP3R-VDAC1 complexes determined by *in situ* proximity ligation assay was significantly reduced in T2D subjects (Thivolet et al., 2017). However, the formation of MAM is increased in diabetic cardiomyopathy. As shown by the quantitative analysis of TEM images, the association between SR and mitochondria increased significantly in the hearts of diabetic Akita mice that carry a single nucleotide substitution in the insulin 2 gene (Wu et al., 2019). Confocal imaging and Pearson correlation coefficient analysis showed that the formation of MAM increased in high glucose (HG) treated neonatal mouse cardiomyocytes (Wu et al., 2019). Similarly, HG significantly increased the expression of FUNDC1 and IP3R2 in neonatal mouse cardiomyocytes (Wu et al., 2019). Compared with non-diabetic donors, FUNDC1 level in heart tissue of diabetic patients was significantly increased (Wu et al., 2019). Moreover, the deletion of FUNDC1 significantly reduced MAM formation in Akita heart (Wu et al., 2019). These results suggest that FUNDC1 is required for diabetic induced cardiac MAM formation. Cardiac-specific FUNDC1 deletion almost completely prevented STZ-induced cardiac abnormalities in diabetic mice, keeping cardiac function at normal values (Wu et al., 2019). It can be speculated that the increase of MAM in high glucose environment may cause mitochondrial calcium overload and damage mitochondrial function, which is the key determinant of HF. Metformin, a famous antidiabetic drug, activates AMP-activated protein kinase (AMPK), improves cardiac function by restoring mitochondrial and cardiac ultrastructure in mice (Xie et al., 2011), and reduces the incidence of myocardial infarction in diabetic patients (Griffin et al., 2017). Studies have shown that the inactivation of AMPK leads to diabetic cardiomyopathy by increasing the MAMs associated with FUNDC1 (Wu et al., 2019). In contrast, we recently observed a negative role of AMPK on MAM formation by reducing the expression of FUNDC1 (Wei et al., 2020). Also, under energy stress, considerable amounts of AMPK translocate from cytosol to the MAM and the mitochondrion as mitochondrial fission occurs, where they interact directly with MFN2 to initiate autophagy (Hu Y. et al., 2020). These findings suggest that the cardiovascular benefits of metformin may depend on its regulation of MAM formation.

Heart Failure (HF)

Coronary artery disease, as well as other cardiovascular diseases, initially leads to compensatory myocardial hypertrophy, which, if worsened, can lead to HF. In the process of cardiac hypertrophy and its transition to HF, changes in SR- mitochondrial contact have been observed (Gutierrez et al., 2014; Santulli et al., 2015). In the norepinephrine-induced hypertrophic environment, the distance between SR and mitochondria in cardiomyocytes is increased, which reduces the Ca^{2+} re-uptake of mitochondria (Gutierrez et al., 2014). This may be a compensatory or adaptive mechanism to buffer the increased SR Ca^{2+} leakage during HF. However, it results in the decrease of mitochondrial oxidative activity, which forced the metabolism of cardiomyocytes into glycolysis, thus promoting the occurrence of hypertrophy (Bertero and Maack, 2018). In addition, SR-mitochondrial communication deficiency and low efficiency of Ca^{2+} exchange may be the prerequisite for pathological myocardial hypertrophy in aged mice (Fernandez-Sanz et al., 2014). Similarly, blocking SR-mitochondrial Ca^{2+} transfer by cardiac-specific knockout of RYR2 results in spontaneous myocardial hypertrophy and fibrous hyperplasia in mice (Bround et al., 2013). Therefore, mitochondrial Ca^{2+} maladjustment seems to be an obvious feature of HF. However, there is no conclusive data on the changes of mitochondrial Ca^{2+} level. Some reports have shown that significant perturbations of

cytosolic cation levels have been observed in HF, including an increase in Na^+ levels (Shimizu and Minamino, 2016), which contributes to the outflow of mitochondrial Ca^{2+} through the mitochondrial NCLX and reduces the mitochondrial bioenergetic responses (Maack et al., 2006). In the guinea pig HF model, the increase of Na^+ in cytoplasm promoted the production of mitochondrial H_2O_2 (Kohlhaas et al., 2010). Interestingly, a compound blocking NCLX (CGP-37157) enhanced mitochondrial Ca^{2+} accumulation, reduced H_2O_2 production, and restored mitochondrial energy supply (Liu and O'Rourke, 2008; Kohlhaas et al., 2010). Contrary to the above results, mitochondrial Ca^{2+} overload caused by SR Ca^{2+} leakage through RYR2 channel was detected in the mouse model of posy-myocardial infarction, leading to changes in normal mitochondrial function and a harmful increase in mitochondrial Ca^{2+} levels (Santulli et al., 2015). The researchers suggested that there was a positive feedback loop between SR Ca^{2+} leakage and mitochondrial ROS production, which leads to RYR2 leakage and intracellular Ca^{2+} increase (Santulli et al., 2015). In line with this view, blocking CaSR reduces the intercellular Ca^{2+} transfer, mitochondrial Ca^{2+} overload and apoptosis (Lu F. H. et al., 2013).

Inadaptable cardiac hypertrophy, which leads to HF, can produce a variety of functional disorders, including changes in mitochondrial dynamics. Decreased MFN2 levels were observed in both *in vitro* and *in vivo* models, such as spontaneously hypertensive rats and hypertrophy caused by pressure overload induced by transverse aortic contraction (Fang et al., 2007). In addition, decreased OPA1 levels were associated with mitochondrial network fragmentation in rat and human HF models (Chen et al., 2009), and decreased MFN1 and MFN2 levels associated with mitochondrial network changes were detected in guinea pig HF models (Goh et al., 2016). Norepinephrine can induce cardiomyocyte hypertrophy and mitochondrial fission by regulating DRP1 function (Pennanen et al., 2014). DRP1 acetylation increases its activity and mitochondrial translocation, resulting in cardiomyocyte hypertrophy and dysfunction in response to excessive lipid supply (Hu Q. et al., 2020). Overexpression of inactive DRP1 (DRP1K38A) in cultured neonatal rat cardiomyocytes prevented mitochondrial network damage and noradrenaline-induced hypertrophy (Pennanen et al., 2014). It should be noted that in the absence of other external stimuli, reducing MFN2 levels is sufficient to induce hypertrophic cardiomyocyte growth (Pennanen et al., 2014). Consistent with these results, moderate myocardial hypertrophy and mild functional deterioration were observed in cardiac-specific MFN2 deficient mice (Papanicolaou et al., 2011). On the other hand, down-regulation of OPA1 increases the response of myocardium to mechanical stress. Higher levels of cardiac hypertrophy associated with changes in ventricular function were detected in $OPA1^{+/-}$ mice exposed to transverse aortic contraction compared to wild-type mice (Piquereau et al., 2012). Cardiac-specific deletion of ATP-dependent zinc metalloproteinase YME1L1 impairs mitochondrial morphology and leads to progressive dilated cardiomyopathy by increasing OPA1 degradation (Wai et al., 2015). Therefore, maintaining the balance between mitochondrial fusion and fission seems to be the key to maintaining normal cardiac function. Treatments that regulate mitochondrial dynamics can be used to prevent cardiac hypertrophy and HF. For example, in a mouse model of pressure overload induced by transverse aortic contraction, mdivi-1 treatment can reduce cardiac fibrosis and left ventricular dysfunction (Givvimani et al., 2012). However, a 2016 report showed that DRP1-dependent mitophagy has a protective effect on HF induced by pressure overload (Shirakabe et al., 2016), so mdivi-1 may have a harmful effect on the later stage of HF.

Endoplasmic reticulum stress involving PERK and eIF2α-ATF4-CHOP signaling has recently been considered as a critical step for development of cardiac hypertrophy and HF. As a member of ER reticulon family that define the tubular morphology of the ER, NOGO B acts as a negative regulator of ER–mitochondria contacts (Sutendra et al., 2011). Inhibition of NOGO B promotes cardiomyocyte hypertrophy and cardiac fibroblast activation by activating the PERK/ATF4 signaling pathway and ATF6 branches of ER stress pathways (Li et al., 2018).

Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) is a progressive and fatal disease characterized by the gradual increase of pulmonary vascular resistance and pulmonary arterial pressure, which eventually leads to right ventricular dysfunction and death (Galie et al., 2016). The long-term survival rate of PAH is depressing. The survival rate of patients with PAH decreases significantly after symptoms appear, and lung transplantation is the only choice for patients with advanced disease (Galie et al., 2016). PAH is characterized by vascular remodeling caused by phenotypic changes in PASMCs. In PAH, PASMCs with quiescent contractile phenotype transit to a highly proliferative phenotype and resistance to apoptosis, resulting in occlusion of small pulmonary vessels (Galie et al., 2016). Although the etiology of PAH is diverse and often multifactorial, phenotypic transformation, proliferation, hypertrophy and other cytological behaviors of PASMC are related to the changes of metabolic patterns (Paulin and Michelakis, 2014; Sutendra and Michelakis, 2014). Specifically, PASMC converts from ATP production from mitochondrial oxidative substrates to relying primarily on cytoplasmic glycolysis for energy, similar to the Warburg effect described in cancer cells (Cottrill and Chan, 2013; Sutendra and Michelakis, 2014). At the same time, the inhibition of mitochondrial oxidation leads to the decrease of mitochondrial membrane hyperpolarization and ROS, which increases the threshold of mPTP opening and apoptosis in PASMC, and the metabolites previously oxidized in mitochondria can now be used as precursors to synthesize macromolecules needed for cell growth and proliferation (Cottrill and Chan, 2013; Sutendra and Michelakis, 2014). The inhibition of mitochondrial oxidation in PAH may be the consequence of altered functional contact between SR and mitochondria, which leads to the decrease of mitochondrial Ca^{2+} concentration and Ca^{2+}-dependent dehydrogenase activity. As the gatekeeper of complete oxidation of glucose in mitochondria, PDH complex converts pyruvate derived from glycolysis into mitochondrial Ac-CoA.

Therefore, PDH is a key factor of metabolic transfer observed in PASMCs, and its activity is inhibited by phosphorylation (Sutendra and Michelakis, 2014). Mitochondrial Ca^{2+} regulates the phosphorylation of PDH by activating PDH phosphatase (Denton et al., 1972) and inhibiting PDH kinase (PDK) (Cooper et al., 1974), thus enhancing PDH activity and facilitating glucose complete oxidation. Activation of PDH with dichloroacetic acid (PDK inhibitor) can prevent hypoxia-induced metabolic and phenotypic changes in PASMCs (McMurtry et al., 2004; Sutendra and Michelakis, 2014). On the other hand, mitochondrial UCP2 acts as a selective modulator of MCU-dependent mitochondrial Ca^{2+} inward current (Bondarenko et al., 2015), while UCP2 knockout in PASMCs reduces mitochondrial Ca^{2+} level and the activity of Ca^{2+}-dependent enzymes, simulating the effect of hypoxia (Dromparis et al., 2013b). In addition, UCP2-deficient mice produce spontaneous PAH, which highlights the relevance of this mechanism in the development of the disease (Dromparis et al., 2013b). Similarly, we also noticed that UCP2 deficiency was associated with increased mitochondrial ROS generation and reduced NO production in the endothelium (Xiong et al., 2016), which might also play a role in vascular dysfunction.

The common characteristics of several PAH related diseases, such as hypoxia, viral infection and inflammation, are the causes of SR stress (Sutendra and Michelakis, 2014). The activation of ATF6α axis, a cAMP-dependent transcription factor of SR stress response, leads to the increase of NOGO B expression in SR (Sutendra et al., 2011), which leads to the structural change of SR, and increases the distance between the organelle and mitochondria, leading to the damage of mitochondrial function (Sutendra et al., 2011; Sutendra and Michelakis, 2014). According to the mechanism of PAH, mice lacking NOGO B are resistant to PAH induced by chronic hypoxia (Sutendra et al., 2011). In addition, the use of small-molecular chemical chaperones prevents and reverses the established PAH and its related cell phenotypes in two rodent disease models by reducing SR stress (Dromparis et al., 2013a).

Another aspect of mitochondrial function associated with phenotypic changes observed in PASMCs is mitochondrial dynamics. As reported in other cell types, the increased proliferation of PASMCs in the pathogenesis of PAH is coordinated with the disruption of mitochondrial network in M phase, so that the distribution of mitochondria in daughter cells is equal (Taguchi et al., 2007; Ryan et al., 2015). In fact, PASMCs isolated from PAH patients showed that mitochondrial fragmentation was associated with decreased expression of the fusion protein MFN2 and increased levels of the fission protein DRP1 (Taguchi et al., 2007; Ryan et al., 2015). MiR-34a-3p-mediated epigenetic upregulation of DRP1 adapter proteins MiD49 and MiD51 increases mitotic fission, which drives pathological proliferation and apoptosis resistance in PAH (Chen et al., 2018). It should be noted that the phenotypic changes of PASMCs can be prevented by controlling mitochondrial dynamics by mdivi-1 treatment or MFN2 overexpression. These strategies produce cell cycle arrest in PASMCs of PAH patients *in vitro*, reverse the established PAH *in vivo*, restore pulmonary artery remodeling, reduce pulmonary vascular resistance and right ventricular hypertrophy, and improve the motor ability of rodent models (Marsboom et al., 2012; Ryan et al., 2013). In addition, MFN2 not only plays a role in mitochondrial fusion, but also participates in the binding and communication between SR and mitochondria. In PAH patients and mouse models, the decrease of MFN2 level may also be related to the increased distance between the two organelles (Ryan et al., 2013).

Systemic Cardiovascular Diseases

In addition to the respiratory system, the phenotypic changes of VSMCs also play a role in the development of different diseases, such as hypertension and atherosclerosis (Chiong et al., 2013). Although there is indirect evidence to support the role of SR-mitochondrial communication in these diseases, some studies have shown that this communication plays a role in the pathogenesis of these diseases. Using high-resolution confocal microscopy and proximity ligation assays, Moulis et al. (2019) found an increase in MAM contacts in VSMCs upon stimulation with atherogenic lipids. Unlike what has been previously described in ECs, the disruption of MAM contacts by PACS-2 knockdown facilitated VSMC apoptosis, an initial step for atherogenesis and plaque rupture, by inhibiting mitophagosome formation and mitophagy (Moulis et al., 2019). Most studies have focused on the role of MFN2 in the transition of VSMCs from contractile and resting phenotypes to hyperproliferative and migratory phenotypes. In this regard, VSMCs from spontaneously hypertensive rats or balloon-injured arteries showed higher proliferation rate and lower level of MFN2 (Wang et al., 2015; Torres et al., 2016). On the contrary, MFN2 overexpression inhibited the proliferation of these cells, neointima formation and carotid restenosis induced by balloon injury in rat carotid arteries (Chen et al., 2004; Guo X. et al., 2007; Guo Y. H. et al., 2007). In addition, in apolipoprotein E (ApoE)-deficient mice, the progression of carotid atherosclerosis is accompanied by a decrease in MFN2 levels (Chen et al., 2004), and overexpression of MFN2 inhibits oxLDL-induced VSMC proliferation during atherogenesis (Guo Y. H. et al., 2007). Although these findings do not indicate the specific role of SR-mitochondrial contact in vascular pathology, the correlation between MFN2 level, VSMC proliferation and SR-mitochondrial contact has been observed in view of the other functions of MFN2 besides organelle tethering. In rat aortic VSMCs, MFN2 level increased in G_0/G_1 phase, mitochondrial elongation and MAMs increased (Li et al., 2015). Similarly, the overexpression of MFN2 was associated with G_0/G_1 phase arrest and increased number of renal tubular mitochondria and SR-mitochondrial contact sites. On the contrary, MFN2 gene knockout was associated with the increase of S phase cells, the disruption of mitochondrial network and the decrease of SR-mitochondrial contact sites (Li et al., 2015).

Although there is no direct evidence for the role of SR-mitochondrial contact in the pathogenesis of systemic vascular diseases, there is more data on the role of mitochondrial dynamics in this area. During the phenotypic changes of VSMCs, the decrease of MFN2 expression may be related to the mitochondrial network disruption observed in proliferative cells. In this regard, PDGF, a known inducer of VSMC phenotype changes, has been shown to increase mitochondrial fission and

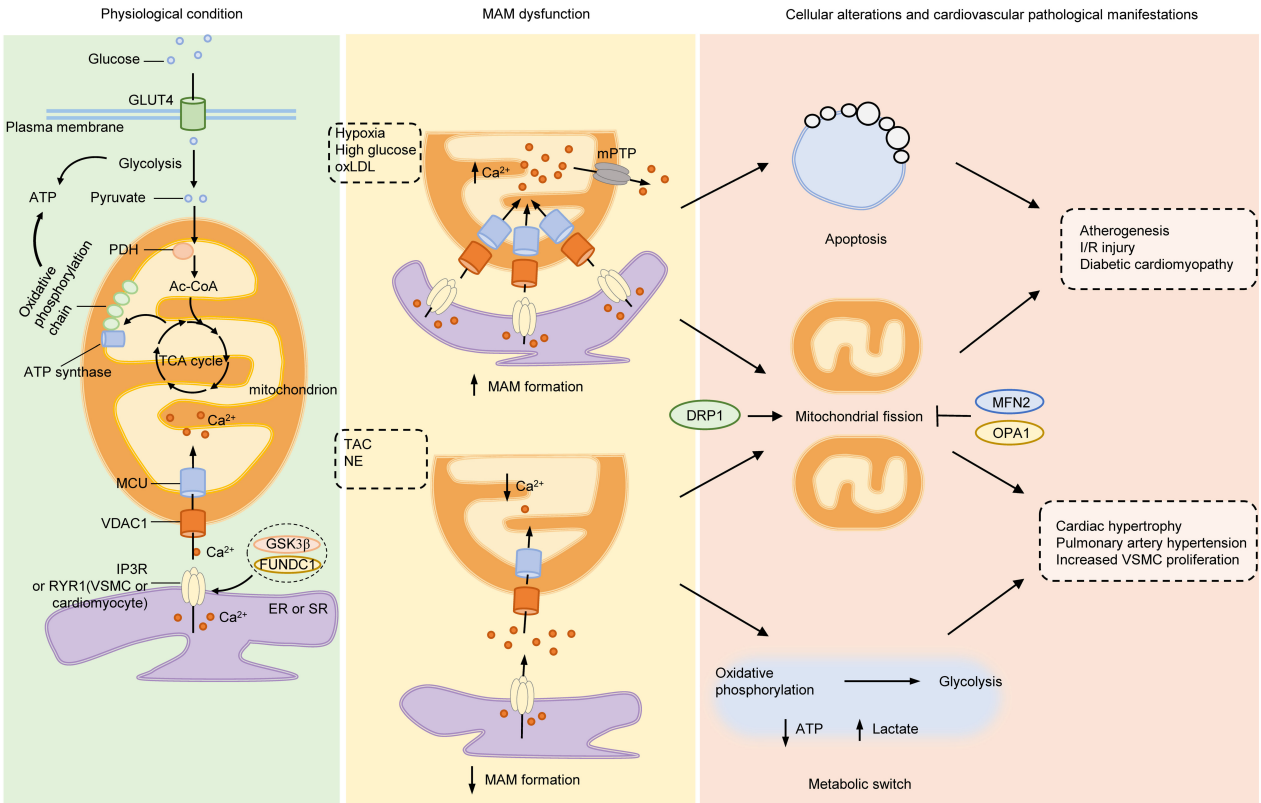

FIGURE 2 | Mitochondria-associated ER membranes and pathogenesis of cardiovascular diseases. Under normal conditions, glucose is taken up into cytoplasm via GLUT4, where it is divided into two molecules of pyruvate through glycolysis. PDH converts pyruvate derived from glycolysis into mitochondrial Ac-CoA, which enters into TCA cycle to generate substrates required for electron transfer of oxidative phosphorylation chain located on IMM. Both glycolysis and mitochondrial oxidative phosphorylation produce ATP but the efficiency of mitochondria is much higher than glycolysis. Ca^{2+} transferred from ER to mitochondria via IP3R (or RYR in VSMCs and cardiomyocytes)/VDAC1 complex acts as stimulants of main enzymes in TCA cycle to enhance the ability of mitochondrial bioenergetics. Under pathological stimulants, such as hypoxia, high glucose or ox-LDL, the formation of MAMs increases and lead to excessive mitochondrial Ca^{2+} uptake, which opens the mPTP to initiate apoptosis and mitochondrial fragmentation, the early steps of atherogenesis, I/R injury and diabetic cardiomyocyte damage. On the other hand, under TAC or NE stimulation, the formation of MAMs is reduced, with increased gap between ER and mitochondria, resulting in lowered mitochondrial Ca^{2+} level and elevated cytosolic Ca^{2+}. The insufficient mitochondrial Ca^{2+} lowers the activity of oxidative phosphorylation, initiates metabolic switch to glycolysis to generate ATP, resulting in increased lactate production and mitochondrial fission. Inhibition of mitochondrial energy production evokes phenotype switch of VSMCs or cardiomyocytes from contractile to synthetic, a major step for developing hypertrophy-associated diseases, such as HF, PAH, and systemic vascular diseases. Mitochondrial dynamics participates in both processes as either inhibition of DRP1 or activation of MFN2 or OPA1 exerts beneficial effects on cardiovascular diseases. Ac-CoA, acetyl-coenzyme A; DRP1 dynamin-related protein 1; ER, endoplasmic reticulum; FUNDC1, FUN14 domain-containing protein 1; GLUT4, glucose transporter type 4; GSK3β, glycogen synthase kinase 3β; IMM, inner mitochondrial membrane; IP3R, inositol-1,4,5-triphosphate receptor; I/R, ischemia-reperfusion; MAMs, mitochondria-associated ER membranes; MCU, mitochondrial calcium uniporter; MFN2, mitofusin 2; mPTP, mitochondrial permeability transition pore; NE, norepinephrine; OPA1, optic atrophy protein 1; ox-LDL, oxidative low-density lipoprotein; PDH, pyruvate dehydrogenase complex; RYR, ryanodine receptor; TAC, thoracic aortic constriction; TCA, tricarboxylic acid; VDAC1, voltage-dependent anion-selective channel protein 1; VSMC, vascular smooth muscle cell.

VSMC proliferation and migration (Salabei and Hill, 2013; Wang et al., 2015). High glucose concentration in the culture media, is an experimental condition that mimics the high glucose levels present in the blood of people affected by diabetes, can also induce high proliferation phenotype of VSMCs cultured *in vitro*, indicating the development of diabetic vascular complications (Maimaitijiang et al., 2016). DRP1 has been found enriched in calcified regions of human carotid arteries, and mice heterozygous for DRP1 deletion are resisted to vascular calcification in an atherosclerosis model (Rogers et al., 2017). It should be noted that inhibition of mitochondrial fission by mdivi-1 or by expression of DRP1K38A can prevent VSMCs from over proliferation at high glucose level (Maimaitijiang et al., 2016), AngII infusion (Cooper et al., 2020) or PDGF stimulation (Salabei and Hill, 2013; Wang et al., 2015). In addition, DRP1K38A transgene in mice displayed a protective effect on intimal vascular proliferation after vascular injury (Wang et al., 2015).

CONCLUSION

As MAM connects two important organelles, ER and mitochondria, the formation, structure and function of MAM display significant regulatory roles in cellular behaviors

associated with ER and mitochondria. This review summarizes the function and mechanism of MAM in regulating cell behavior, and its importance in cardiovascular physiology and pathophysiology from three aspects. First, the Ca^{2+} transfer from ER to mitochondria mediated by MAM affects the energy metabolic patterns of cardiovascular cells, which are related to cardiomyocyte hypertrophy, VSMC phenotype transition and proliferation. Second, the Ca^{2+} transfer of MAM not only affects the Ca^{2+} levels of mitochondria and ER itself, but also affects the local cytosolic Ca^{2+} concentration which might induce subsequent pro-hypertrophic Ca^{2+} signaling and contractile response. Third, MAM not only triggers the cellular behaviors related to ER and mitochondria by modulation lipid and Ca^{2+} transfer, such as ER stress and apoptosis caused by mitochondrial Ca^{2+} overload, but also acts as a platform where inflammasome and mitochondrial fission occurs by recruiting key signal molecules and effector proteins. Thus, the interaction between these organelles is an important factor in the pathophysiology of cardiovascular system (**Figure 2**). However, the role of ER-mitochondrial communication in cardiovascular diseases has not been paid enough attention, especially in the blood vessels with less mitochondrial content. Although many molecules involved in the regulation of MAM function are summarized in this paper, their functions in cardiovascular system have not been fully studied.

At present, there are two major obstacles in the way of identifying the role of MAM in the cardiovascular system. On the one hand, cardiomyocytes mainly rely on mitochondria for energy supply, whereas glycolysis is predominant in vascular ECs, and VSMCs stand between the two. Obviously, the effects of MAM formation in these cell types are not identical, sometimes even opposite. Therefore, it is very important to explore the role of MAM in a cell-specific manner, which also raises a question for us that how to regulate the formation and function of MAM according to different cell types, so as to develop new treatment methods for cardiovascular diseases. Regulating communication between these organelles, on the other hand, is a double-edged sword. The increase of ER-mitochondrial proximity increases mitochondrial Ca^{2+} uptake, thus activated ATP synthesis, while mitochondrial Ca^{2+} overload triggered the opening of mPTP, resulting in mitochondrial swelling and pro-apoptotic factors released to the cytoplasm. Thus, the concentration of mitochondrial Ca^{2+} needs to be appropriately controlled. Therefore, the precise regulation of MAM formation and function might be a promising way to develop more specific treatment strategies and selectively restrain the progression of cardiovascular diseases.

AUTHOR CONTRIBUTIONS

PG, ZY, and ZZ designed the scope of the review. PG performed the document searching and wrote the manuscript. PG prepared the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mitochondrial Membrane Intracellular Communication in Healthy and Diseased Myocardium

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Research efforts in the twenty-first century have been paramount to the discovery and development of novel pharmacological treatments in a variety of diseases resulting in improved life expectancy. Yet, cardiac disease remains a leading cause of morbidity and mortality worldwide. Over time, there has been an expansion in conditions such as atrial fibrillation (AF) and heart failure (HF). Although past research has elucidated specific pathways that participate in the development of distinct cardiac pathologies, the exact mechanisms of action leading to disease remain to be fully characterized. Protein turnover and cellular bioenergetics are integral components of cardiac diseases, highlighting the importance of mitochondria and endoplasmic reticulum (ER) in driving cellular homeostasis. More specifically, the interactions between mitochondria and ER are crucial to calcium signaling, apoptosis induction, autophagy, and lipid biosynthesis. Here, we summarize mitochondrial and ER functions and physical interactions in healthy physiological states. We then transition to perturbations that occur in response to pathophysiological challenges and how this alters mitochondrial–ER and other intracellular organelle interactions. Finally, we discuss lifestyle interventions and innovative therapeutic targets that may be used to restore beneficial mitochondrial and ER interactions, thereby improving cardiac function.

Keywords: mitochondria, endoplasmic reticulum stress, heart, mitochondria-ER communication, membrane communication mechanism

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INTRODUCTION

Mitochondria are paramount for energy production in all tissues, especially in highly energetic organs such as the heart. In the myocardium, mitochondria compose around 30% of the total cell volume and synthesize 6 kg of ATP per day via oxidative phosphorylation (Vásquez-Trincado et al., 2016). Mitochondria also have extensive interactive networks with many organelles within the cell, most notably the endoplasmic reticulum (ER). Through these interactions, the mitochondrion also influence Ca^{2+} signaling, reactive oxygen species (ROS), and cell death (Szymański et al., 2017).

Although cardiac disease death rate since the 1950s has substantially declined, it is still the leading cause of mortality among American adults (National Center for Health Statistics, 2018). According to the 2018 National Health Interview Survey, 11.2% of adults in the United States are

affected by cardiac diseases, with heart failure (HF) being the most common diagnosis (National Center for Health Statistics, 2018). In 2014, there were 1.1 million emergency department visits with HF as the primary cause and 4.1 million visits with HF as comorbidity. The direct medical costs of HF in 2012 were estimated to be $30.7 billion, with a projected increase to $69.7 billion by 2030 (Jackson et al., 2018). Globally, in 2017, cardiovascular disease was indicated as the cause of nearly 18 million deaths contributing to approximately 330 million years of life lost. These deaths represent a 21.1% increase over the previous decade (Roth et al., 2018).

The expansion of cardiac disease burden is partly due to increased life expectancy and a partial understanding of contributing factors that promote cardiac pathology. Despite the significant amount of research being performed in this area, treatment strategies for many types of cardiac diseases, including HF, remain limited. This review will discuss mitochondrial dynamics, mitochondria and ER roles in cardiac physiology, and therapeutic interventions that focus on mitochondrial and ER stress.

IMPORTANCE OF MITOCHONDRIAL DYNAMICS, ULTRASTRUCTURE, AND MORPHOLOGY IN CARDIAC PHYSIOLOGY

The mitochondrion is a double membrane organelle composed of an outer membrane, intermembrane space, inner membrane, and matrix. The inner mitochondrial membrane exhibits complex folding to increase functional surface area confined within the outer mitochondrial membrane. The electron transport chain, ATP synthase, and many other mitochondrial membrane proteins are localized within the inner mitochondrial membrane (Zick et al., 2009). In yeast and certain mammalian cells, ER tubules constrict the mitochondria prior to the recruitment of proteins involved in mitochondria dynamics (Friedman et al., 2011). Unlike non-cardiac mitochondria, mitochondria in the adult heart are partially immobile, possessing restricted capacity to move and distribute in cytoplasmic tubular networks (Ong et al., 2017). Indeed, continuous mitochondrial networks can span the length of the cell both in the transverse and longitudinal directions. These networks function to exchange cristae components and substrates (Franzini-Armstrong, 2007; Picard et al., 2015). The presence of mitochondrial nanotunnels in cardiomyocytes, but not in skeletal muscle, suggests a direct mode of communication between cardiac mitochondria across distances up to several microns. These nanotunnels contain an outer membrane as well as cristae and therefore allow the sharing of matrix components (Huang et al., 2013; Eisner et al., 2017; Lavorato et al., 2017). Due to limited mitochondrial movement in the myocardium, this mode of communication has been implicated in ensuring content sharing in the absence of proximal direct contact. These structures have been visualized both by live-cell confocal imaging (Huang et al., 2013) and electron microscopy (Lavorato et al., 2017). Indeed, cardiac nanotunnels appear to have distinct spatiotemporal dynamics compared to other mitochondrial contacts in terms of formation, transport, and structures formed (Huang et al., 2013). The role of mitochondria nanotunnels in cardiac pathologies remain to be fully characterized.

Mitochondria in adult cardiomyocytes are divided into three groups based on their location and function: interfibrillar, subsarcolemmal, and perinuclear (Palmer et al., 1977). Interfibrillar mitochondria comprise most of the mitochondrial population and are found between myofibrils. They have tubular cristae that are involved in ATP production for cardiomyocyte contraction and Ca^{2+} signaling. Subsarcolemmal mitochondria are located under the sarcolemmal membrane and have lamellar cristae used for energy generation for ion channels and cell signaling (Riva et al., 2005; Ong et al., 2017). Perinuclear mitochondria are clustered around the sides of the nucleus and are postulated to aid in transcriptional regulation (Santel and Fuller, 2001; Ong et al., 2017). These different types of mitochondria are all functionally linked, thus participating in electrical conduction coupling among cardiac cells (Amchenkova et al., 1988).

Mitochondrial ultrastructure and dynamics are integral for cardiac tissue maintenance and homeostasis. Cardiac pathophysiological conditions have been linked to alterations in these processes and include swelling, loss or reorientation of cristae, distortion of configuration, or vacuoles found in the inner or outer compartments (Hoppel et al., 2009). Of note, cardiac pathologies have been linked to the presence of giant mitochondria. The clearance of dysfunctional mitochondria is a significant component in mitochondrial network health and involves a process known as mitophagy (Galluzzi et al., 2017). Giant mitochondria can be formed by either fusion of two mitochondria via increased expression of mitofusins (Mfn) or by the growth of a single mitochondrion (Kraus and Cain, 1980; Arbustini et al., 1998; Santel and Fuller, 2001). However, the mechanism of how giant mitochondria directly participate in cardiac dysfunction remains largely understudied. Mitochondrial morphology is regulated by fission and fusion (**Figure 1**), a balanced process required for a healthy cell.

Mitochondrial fission is used in repair, cell division, and mitophagy, while fusion is used to elongate and exchange matrix components. Dysregulation of these processes is observed in various cardiovascular pathologies and linked to disease progression (Vásquez-Trincado et al., 2016). Fission is regulated by GTPases mitochondrial fission 1 protein (Fis1), dynamin-related protein 1 (Drp1), mitochondrial fission factor (Mff), and mitochondrial dynamic proteins of 49 and 51 kDa (MiD49/51). These outer mitochondrial membrane proteins act as receptors for Drp1 and work to recruit Drp1 to the mitochondria as Drp1 lacks a mitochondrial targeting sequence (Gandre-Babbe and Van Der Bliek, 2008; Losón et al., 2013). They are potentially redundant as each receptor can interact with Drp1 independently; nevertheless, it remains unclear whether these receptors compete or work in consortium in the overall process (Yu et al., 2020). It is reported that in 293T cells, MiD49/51 and Mff may compete with each other to interact with Drp1 (Yu et al., 2017). The importance of Fis1 in translocating Drp1 to the mitochondria remains controversial. Studies have reported that knockout of human Fis1 (hFis1) in HeLa and HCT116 cells does

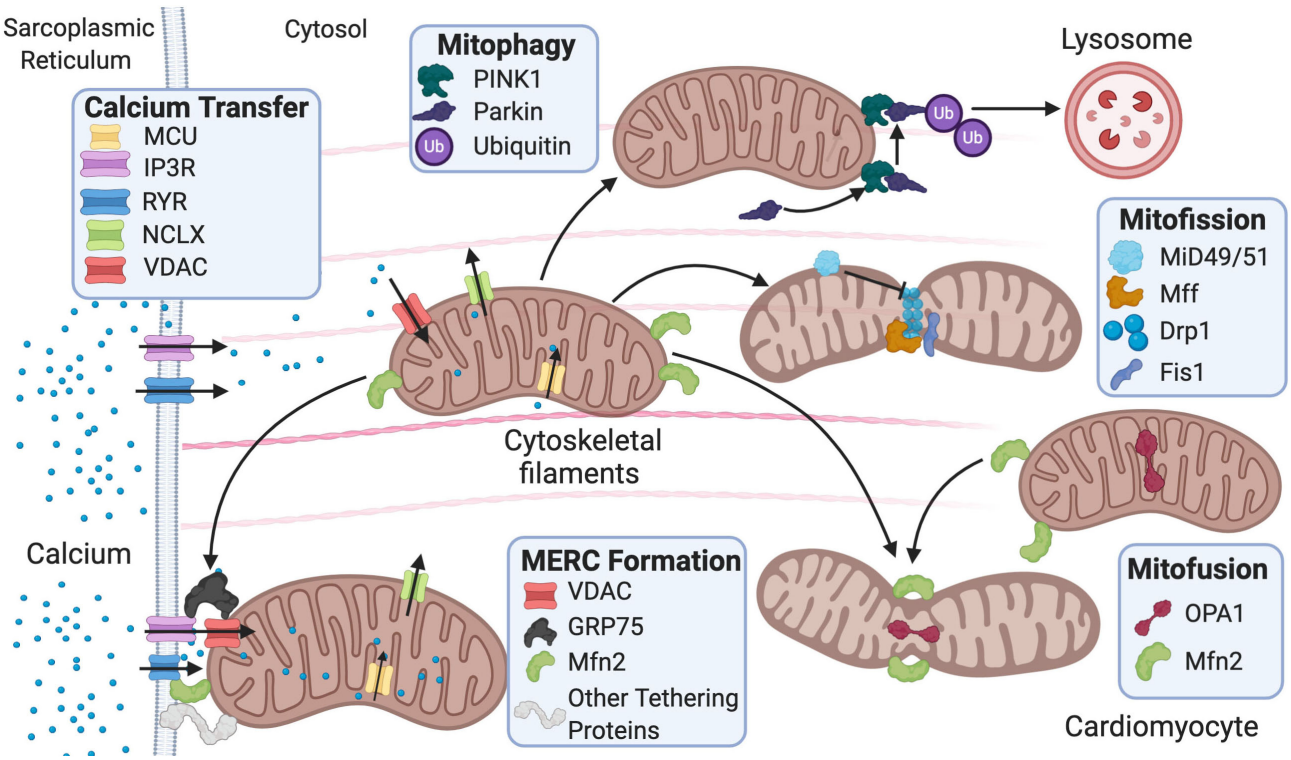

FIGURE 1 | Fusion and fission protein dynamics and how sarcoplasmic reticulum (SR) and mitochondrial communication participate in these processes. Fusion and fission are both regulated by GTPases. Mitofusion occurs through tethering of both mitochondrial membranes and then fusion via Mfn1/2 and OPA1. Mitofission occurs via Drp1-mediated constriction of the mitochondria, which is inhibited by MiD49/51. Fis1 acts as a recruiting molecule for Drp1, while Mff is the receptor for Drp1. MERC formation is mainly regulated by Mfn2 but also involves the IP_3R-GRP75–VDAC complex and other numerous proteins. Mitophagy is initiated by PINK1 phosphorylation of Parkin, leading to targeting of the mitochondria for degradation by the lysosome. MERC, mitochondrial-ER contact; IP_3R, inositol 1,4,5-trisphosphate receptor; GRP75, glucose-related protein 75; VDAC, voltage-dependent anion channel; RyR, ryanodine receptor; NCLX, Na^+/Ca^{2+} exchanger, Mfn, mitofusin; Opa1, optic atrophy 1; Fis1, mitochondrial fission 1 protein; Drp1, dynamin-related protein 1 Mff, mitochondrial fission factor; MiD49/51, mitochondrial dynamic proteins 49/51; PINK1, PTEN-induced kinase 1.

not affect Drp1 binding to the mitochondria (Lee et al., 2004; Otera et al., 2010), while another study using mouse embryonic stem cells reported that Fis1 is heavily involved in recruiting Drp1 to the mitochondria—potentially more than Mff (Seo et al., 2020).

The importance of mitochondrial fission has been highlighted by studying how the downregulation of Drp1 causes mitochondrial elongation, hindrance of mitophagy, increased probability for mitochondrial permeability transition pore (mPTP) opening, and altered stress responses. Genetic mouse models of Drp1 downregulation developed cardiac dysfunction and possessed increased susceptibility for ischemia/reperfusion (I/R) injury (Ikeda et al., 2015). Moreover, mitochondrial fission helps cell repair by extruding damaged components, which is then targeted for removal and degradation via mitophagy. Drp-1-mediated fission has been shown to act as an adaptive mechanism during cellular stress, although its role in exercise is debatable. One study found Drp1-mediated fission in mice to amplify cardiac and mitochondrial function during exercise (Coronado et al., 2018). A more recent study also performed in mice found fission mediated by Mcl-1—a potential receptor for Drp1—to reduce nutrient deprivation-induced cell death but, on the other hand, reduce mitochondrial ability to adapt to a sudden increase in energy demand and workload (Moyzis et al., 2020). As a balanced regulation of fission is necessary for healthy cells, overexpression of Drp1 also induced mitochondrial dysfunction and excessive cell death in the cardiomyocytes via apoptosis (Ikeda et al., 2015).

Drp1 is extensively regulated by many posttranslational mechanisms, including phosphorylation of residues Ser-637 and Ser-616. Phosphorylation of Ser-616 activates Drp1, promoting cytosolic to mitochondrial translocation, and inducing fission (Marsboom et al., 2012); on the contrary, phosphorylation of Ser-637 retains Drp1 in the cytoplasm (Chang and Blackstone, 2007). Studies have reported that Drp1 is phosphorylated at Ser-637 and dephosphorylated at Ser-616 in starvation conditions (Rambold et al., 2011), while the opposite is true during hyperglycemic conditions (Gawlowski et al., 2012). The inhibition of Drp1 during starvation would lead to fusion and increase mitochondrial respiration (Rambold et al., 2011), supporting the notion that mitochondrial dynamics are linked to the metabolic state of cells (Seo et al., 2020).

Fusion is regulated by GTPases mitofusin 1/2 (MFN1/2) and optic atrophy 1 (OPA1) (**Figure 1**). Fusion occurs stepwise with mitochondria tethering together first via MFN1/2. Then,

the outer mitochondrial membranes fuse as a result of MFN activity (Chen and Chan, 2005). Last, the inner mitochondrial membranes are fused via OPA1 (Cipolat et al., 2004). Studies have reported that MFN1/2 can act redundantly during fusion, and ablation of both results in mitochondrial structural abnormalities and dilated cardiomyopathy in the postnatal heart (Papanicolaou et al., 2012). Postnatally, the heart undergoes rapid proliferation of mitochondria and an increase in respiratory function. Thus, the ablation of both MFNs is lethal in murine models (Papanicolaou et al., 2012) as is the case with homozygous OPA1 mutants (Piquereau et al., 2012). Interestingly, no alteration in mitochondrial respiration was found in mice with a heterozygous OPA1 deletion, but there was mitochondrial network remodeling and increased sensitivity to hemodynamic stress (Piquereau et al., 2012). Ubiquitination of MFNs promotes degradation, which allows for unopposed mitochondrial fission during the removal of damaged mitochondria via mitophagy (Gegg et al., 2010). For mitophagy to occur, mitochondria need to undergo fission, thus inhibition of fusion aids this process. Drp1, on the other hand, was not found to be ubiquitinated during mitophagy as expected (Gegg et al., 2010). The homeostatic balance between fusion and fission is vital for the maintenance of healthy mitochondria in the heart. Similar to "yin and yang," these opposite and contrary forces are complementary and interconnected, such that dysregulation in either of these processes leads to mitochondrial dysfunction related to cardiac pathologies.

Interestingly, in addition to ER connections, the role of mitochondria has extended to other subcellular membranes. Recent studies have shown that mitochondria can shed mitochondrial-derived vesicles (MDVs) with specific cargoes to the peroxisome or the late-endosome (Neuspiel et al., 2008; Braschi et al., 2010; Soubannier et al., 2012). While these MDVs are distinct from mitophagy-related mechanisms, MDVs have been linked to alterations in cellular physiology and metabolism, although precise mechanisms of action remain largely unknown requiring further investigation.

CALCIUM SIGNALING, APOPTOSIS, AND FERROPTOSIS

Calcium entry in the mitochondria plays an important role in metabolic regulation and cell death mechanisms. A prominent role for mitochondrial calcium entry has been attributed to the mitochondrial calcium uniporter (MCU) complex. Surprisingly, global ablation of MCU has no overt baseline phenotype as these animals had an apparent normal lifespan. While acute mitochondrial calcium entry in the MCU knockout was absent, mitochondrial calcium levels were reduced but existent in mitochondria derived from MCU knockout mice, suggesting the existence of other forms of mitochondrial calcium entry (Pan et al., 2013). Although perhaps moderately speculative, this could be partially attributed to connexin 43 (Cx43), as calcium is permissive through these channels, and Cx43 has been reported to be localized in the mitochondria (Gadicherla et al., 2017). Moreover, the viability of MCU knockout mice depended on the genetic background, as MCU KO was embryonically lethal in C57/BL6 but not in CD1 background (Murphy et al., 2014). Interestingly, cardiac-specific ablation of MCU led to cardioprotection post-I/R (Luongo et al., 2015). Additionally, a variety of proteins have been reported to regulate the MCU complex, among which are spastic paraplegia 7 (SPG7), mitochondrial calcium uptake 1 (MICU1), mitochondrial calcium uniporter 1 (MCUR1), and essential MCU regulator (EMRE) (Mallilankaraman et al., 2012a,b; Shanmughapriya et al., 2015; Vais et al., 2016). Studies over decades have shown that mitochondrial calcium overload can lead to the opening of the mitochondrial permeability transition pore (mPTP) and subsequent cell death. Although mPTP pore function is widely accepted to be vital for cell death mechanisms, the molecular identity of the pore-forming channel remain largely debated. Novel evidence has pointed to an additional function for the F-ATPase, particularly for the c-subunit of the ATPase as the pore-forming channel (Alavian et al., 2015; Urbani et al., 2019). This knowledge has been counter argued by John Walker's group (Nobel Laureate 1997), where persistent permeability transition pore is observed in human mitochondria devoid of assembled ATP synthase (Carroll et al., 2019). For a more extensive review of mitochondrial calcium and its regulators, we defer to another recent review (Garbincius et al., 2020).

Ca^{2+} signaling can occur via a physical connection between the mitochondria and ER, which is thought to be mediated by MFN2 tethering of both organelles (**Figure 1**) (De Brito and Scorrano, 2008). Nevertheless, there are several controversies surrounding the functional role of MFN2. For instance, reports have shown opposing effects of Mfn2 deficiency in the susceptibility to I/R injury. One study found decreased susceptibility to I/R injury due to delayed mPTP opening from decreased Ca^{2+} intake (Papanicolaou et al., 2011). Another study found increased susceptibility to I/R injury as a result of dysfunctional autophagy and disturbed mitochondrial ultrastructure (Zhao et al., 2012). It is thought that this discrepancy may be due to differences in timing and location as both studies used murine models (Zhao et al., 2012). Moreover, there have been opposing reports on the impact of ablation of Mfn2 on mitochondria–ER junctional distance. One study found the distance between the two organelles to increase by approximately 30% (Chen et al., 2012), while another found no difference in distance (Papanicolaou et al., 2012). It is possible that the discrepancy is due to timing of the Mfn2 gene deletion as the former study ablated Mfn2 during embryonic stages in mice, and the latter ablated Mfn2 after birth in mice (De la Fuente and Sheu, 2019). This is intriguing as it remains unclear how the mitochondria respond to rapid cytosolic Ca^{2+} transients from the ER in contracting cardiac myocytes (Andrienko et al., 2009). Two models describe how cardiac mitochondria interpret Ca^{2+} transients released by the sarcoplasmic reticulum (SR). One model states that mitochondria imports and releases Ca^{2+} with each heartbeat, while the other proposes that mitochondria gradually take up Ca^{2+} until a steady-state environment is reached (Sedova et al., 2006). It is hypothesized that the former model is used in larger animal models relative to mice, while the latter is found mainly in smaller animal models such as mice (Griffiths, 1999). Studies have shown that in addition to Ca^{2+}

cycling, cardiac mitochondria are also involved in cellular Ca^{2+} buffering. It was found that in neonatal cardiomyocytes, Ca^{2+} is taken up by the mitochondria in systole and released back into the cytosol during diastole. Between 1% and 15% of cytosolic Ca^{2+} is thought to be buffered by cardiac mitochondria (Drago et al., 2012). Mitochondrial fusion frequency is also coupled with the frequency of Ca^{2+} transient oscillations, rather than sustained transients, and cardiomyocyte contractions (Eisner et al., 2017). In cases where a ryanodine receptor (RyR2) channel mutation is present in murine models, pathologies arise due to altered Ca^{2+} oscillations and inability of cardiomyocytes to contract (Lavorato et al., 2017) linked to fewer fusion events (Eisner et al., 2017). Further studies are needed to expand our understanding on the role of MFN2, ER–mitochondrial Ca^{2+} communication, and susceptibility to cardiac injury.

Mitochondrial Ca^{2+} is an important regulator of cell death. In fact, apoptosis is a major form of cell death in cardiomyocytes that occurs in response to ischemia or metabolic stress linked to decreased ATP levels, dysfunctional electron transport chain (ETC), or excessive oxidative stress. It involves mitochondrial fragmentation, increased mitochondrial permeability caused by Ca^{2+} influx, proapoptotic protein assembly, and cytochrome C release to the cytosol. Pro-apoptotic factors BCL2-interacting protein 3 (BNip3) and BNIP3-like (Nix) form heterodimers with antiapoptotic factors such as B-cell lymphoma—extra large (BclxL) and B-cell lymphoma 2 (Bcl2), which then permit activation of proapoptotic proteins BCL2-associated X (BAX) and BCL2 antagonist killer (BAK). BAX and BAK are pore-forming proteins and increase mitochondrial outer membrane permeability, allowing for the release of cytochrome c into the cytosol and initiation of apoptosis (Gálvez et al., 2006; Dorn, 2010). Apoptosis signaling can be initiated through a secondary pathway when Fis1 on the mitochondrial outer membrane binds to BAP31 on the ER membrane (Iwasawa et al., 2011). Drp1 is not only involved in mitochondrial fission, as it can also promote mitochondrial fragmentation and subsequent apoptotic signaling (Frank et al., 2001). Mitochondrial fragmentation is a component of apoptosis that occurs through mitochondrial fission (Oettinghaus et al., 2016). Nevertheless, Drp1 is not essential for cell death as other proapoptotic proteins can be released in the cytosol to induce apoptosis in the absence of Drp1. Contrarily, Drp1 may induce apoptosis on its own without other proapoptotic proteins (Oettinghaus et al., 2016).

The Bcl-2 family of proteins are paramount regulators of cellular apoptosis. Evidence suggests that these proteins work by decreasing Ca^{2+} release from the ER, increasing IP_3R channel leakage, dampening Ca^{2+} overload in the mitochondria, and mPTP opening probability in response to stress (Bittremieux et al., 2016). mPTP opening dissipates mitochondrial membrane potential, causing swelling and release of proapoptotic factors into the cytoplasm (De Giorgi et al., 2002). Thus, increased mitochondrial Ca^{2+} influx promotes mPTP opening, followed by ATP depletion and cell death (Dorn, 2010).

Ferroptosis is a newly defined form of apoptosis that is dependent on iron. It results from the accumulation of lipid ROS driven by the deficiency of the scavenging antioxidant, glutathione. Iron is used as a catalyst in lipid peroxide-generating reactions; thus, deficiency of ferritin or iron concentration will drive ROS production. Ferroptosis is associated with changes in mitochondrial morphology, such as cristae enlargement and mitochondrial fragmentation. A study using human fibrosarcoma HT1080 cells and mouse embryonic fibroblasts showed that mitochondrial tricarboxylic acid (TCA) cycle and ETC function are essential for potent ferroptosis (Gao et al., 2019). The TCA cycle is required as glutamine, and downstream TCA cycle metabolites are used in the initiation of ferroptosis, while the ETC is necessary to ensure ROS production. Studies were performed in HT1080 cells depleted of mitochondria via Parkin-mediated mitophagy, which caused them to become resistant to ferroptosis (Gao et al., 2019).

Inhibition of ferroptosis has been reported to reduce I/R-related cardiac pathology, protect from cardiomyopathy, and reduce HF incidence (Fang et al., 2019). Heme oxygenase-1 (Hmox1) catalyzes heme degradation, which releases free iron, leading to ferroptosis and HF. Hmox1 is considered to play paradoxical roles on its status as a cardioprotective protein. One study found that Hmox1 can serve a cardioprotective role as overexpression in mice can protect against I/R injury and permanent coronary ligation-induced HF (Wang et al., 2010). On the other hand, another study found that inhibition of Hmox1 in mice is cardioprotective, in a manner that is similar to the effects of iron chelation (Fang et al., 2019). Similarly, a third study showed that overexpression of Hmox1 in murine models leads to spontaneous HF by 1 year of age despite being protected against isoproterenol-induced cardiomyopathy (Allwood et al., 2014). Further studies are required to delineate precise mechanisms of Hmox1 and iron-related cardiac pathologies.

MEMBRANE CONTACTS AND COMMUNICATION BETWEEN ENDOPLASMIC RETICULUM AND MITOCHONDRIA

The mitochondrion has numerous contacts with other organelles such as peroxisomes and the plasma membrane, but the most well-characterized interactions involve ER membranes—an organelle involved in lipid and protein synthesis (Szymański et al., 2017). The peroxisome is an organelle used for oxidation and lipid synthesis, and the plasma membrane is a semipermeable membrane that surrounds the cell. The ultrastructural organization of the contact sites between the mitochondria and ER is termed the mitochondrial–ER contact (MERC), while the collection of proteins and lipids that form the MERC is called the mitochondrial-associated ER membrane (MAM) (**Figure 1**) (Giacomello and Pellegrini, 2016). An early study using HeLa cells showed that up to 20% of the mitochondrial surface is near ER membranes (Rizzuto et al., 1998). Later on, another study using RBL-2H3 and H9c2 cells proposed that all mitochondria are in contact with the ER (Csordás et al., 2010). While the percentage of ER-mitochondrial membrane contacts remains debatable, it is well-established that the outer membrane of the mitochondria, MAMs, and ER

membranes are well-connected, and even the inner membrane of the mitochondria continues to influence pattern, structure, and physiology of adjacent mitochondria (Vincent et al., 2017). Specific proteins have been reported to play a significant role in tethering the ER and mitochondria (Lee and Min, 2018). While some proteins are common to various cellular membranes, others are specific to MAMs.

Although the MERC tethering system has been extensively investigated in yeast and mammalian cells, the exact composition and potential interactions have not been fully characterized (Lee and Min, 2018). In yeast cells, a tethering complex called the ER–mitochondria encounter structure (ERMES) connects the ER to the mitochondrial outer membrane. It is comprised of maintenance of mitochondrial morphology protein 1 (MMM1), mitochondrial disruption and morphology protein 10 (MDM10), mitochondrial disruption and morphology protein 12 (MDM12), and mitochondrial disruption and morphology protein 34 (MDM34) (Kornmann et al., 2009). Specific protein localization via confocal fluorescence microscopy was determined in wild-type and mutant cells that expressed each protein independently. MMM1 was found to be on the ER membrane, MDM10/34 was observed on the mitochondrial outer membrane, and MDM12 was found to be the linker molecule between the two subcellular components (Kornmann et al., 2009).

Mammalian cells lack an ERMES counterpart and are thought to have a more complicated protein interface than yeast cells (Lee and Min, 2018). In mammals, MFN2 is a tethering protein (De Brito and Scorrano, 2008), independent of its role in mitochondrial fusion. Although it is unclear whether MFN2 increases the mito–ER junctional distance, as stated before, cardiac-specific MFN2 knockout studies have led to decreased mitochondrial sensitivity to the SR Ca^{2+} release (Chen et al., 2012; Papanicolaou et al., 2012). The amount of SR-associated RyRs in cardiomyocyte MAMs without MFN2 was significantly lower than wild-type cells despite the overall unchanged cardiac content of RyRs. These effects are either less pronounced or not observed in cells where MFN1 is knocked out (Chen et al., 2012). Nonetheless, in cardiac cells with dual MFN1 and MFN2 ablation, mitochondrial fragmentation, and rapidly lethal dilated cardiomyopathy was observed (Chen et al., 2012).

Other major proteins involved in tethering include vesicle-associated membrane protein-associated protein-B (VAPB), protein tyrosine phosphatase-interacting protein-51 (PTPIP51), glucose-related protein 75 (GRP75), inositol 1,4,5-trisphosphate receptor (IP_3R), voltage-dependent anion channel (VDAC), B-cell receptor-associated protein 31 (BAP31), FIS1, and PDZ domain-containing 8 (PDZD8) (Lee and Min, 2018). In addition to tethering, these proteins play a part in regulating Ca^{2+} buffering, lipid processing, mitochondrial fusion, and autophagy. The abundance, localization, and/or interactions of these proteins often lead to different outcomes, further masking our understanding of this complex process (Lee and Min, 2018). For instance, VAPB is an ER membrane protein, while PTPIP51 is an outer mitochondrial membrane protein. Together, they participate in Ca^{2+} regulation and autophagy. Overexpression of both proteins impairs autophagy and increases mitochondrial Ca^{2+} uptake, while deficiency of both has the opposite effect (Gomez-Suaga et al., 2017). IP_3Rs are located on the ER membrane and facilitate Ca^{2+} efflux toward the mitochondria, while VDAC is situated on the outer mitochondrial membrane and allows for Ca^{2+} influx into the mitochondria. GRP75 is a cytosolic linker that bridges IP_3R and VDAC and promotes delivery of Ca^{2+} into the mitochondria. Cardiac mitochondrial Ca^{2+} uptake is then mediated via the MCU complex in conjunction with VDAC (Szabadkai et al., 2006; Xu et al., 2018). A study analyzing the downregulation of GRP75 in neuronal cells showed reduced ER–mitochondrial coupling and attenuation of Ca^{2+} transfer from the ER to the mitochondria (Honrath et al., 2017). This allowed for mitochondrial resistance to Ca^{2+} overload during oxidative stress. Nevertheless, it remains unclear whether GRP75 inhibition holds potential as a pharmacological target against oxidative stress in cardiomyocytes (Honrath et al., 2017). BAP31 is located on the ER membrane, and FIS1 can be found on the mitochondrial outer membrane. Together, they regulate a secondary pathway for initiation of apoptosis. FIS1 elicits apoptosis signaling via an early and specific caspase-like protease activity that facilitates the cleavage of BAP31 into its proapoptotic form (Iwasawa et al., 2011). This cleavage allows for the recruitment and cleavage of procaspase-8 into caspase-8, a powerful activator of apoptosis. The BAP31–FIS1 complex releases Ca^{2+} downstream from the ER that further amplifies cell death via a positive feedback loop (Iwasawa et al., 2011). PDZD8 is another crucial tethering protein that participates in Ca^{2+} dynamics between the ER and mitochondria. PDZD8 knockdown in neuronal cells significantly decreased mitochondrial Ca^{2+} import despite no change in the Ca^{2+} import machinery, thus implicating the protein in ER–mitochondrial tethering (Hirabayashi et al., 2017). In this latter study, PDZD8 was identified as an ortholog to MMM1 in the yeast ERMES complex, although another study using phylogenetic analyses showed it to be a paralog instead (Wideman et al., 2018).

The ER–mitochondrial tether additionally plays a role in lipid trafficking (Rusiñol et al., 1994). Specific proteins and enzymes involved in lipid synthesis and transfer are enriched in the MAM subdomain of the ER. Examples of these proteins include acyl-coenzyme A cholesterol acyltransferase (ACAT1), phosphatidylserine (PS) synthase, phosphatidylethanolamine N-methyltransferase 2 (PEMT2), fatty acid coenzyme A (CoA) ligase 4 (ACS4), and diacylglycerol O-acyltransferase 2 (DGAT2) (Szymański et al., 2017). ACS4 is used to synthesize acyl CoA used as a precursor for triacylglycerols (TAGs), and DGAT2 catalyzes the final step in TAG synthesis. In a study that examined DGAT2, a known mitochondrial targeting sequence was found in the N-terminus (Stone et al., 2009). MAMs are crucial for the movement of phospholipids between the ER and mitochondria (Hernández-Alvarez et al., 2019). A study using human models of non-alcoholic steatohepatitis and mouse models of non-alcoholic liver disease found decreased levels of Mfn2. In the same study, liver-specific Mfn2 knockout in mouse models resulted in disturbances of ER–mitochondrial PS transfer, revealing a novel mechanism in the development of liver disease (Hernández-Alvarez et al., 2019). Mitochondria import PS synthesized in the ER via PS synthase, which can be decarboxylated into phosphatidylethanolamine

(PE). Synthesized PE is able to be exported back to the ER, where it can be methylated via PEMT2 into phosphatidylcholine (PC) (Ridgway and Vance, 1987). ACAT1 catalyzes the synthesis of cholesterol esters, which allows for control of the equilibrium between cytosolic and membrane-bound cholesterol (Puglielli et al., 2001). Cholesterol transfer to the mitochondria from the ER provides material for steroid synthesis. Cholesterol transfer, along with membrane organization and stability, is regulated by caveolin 1 (CAV1), which is abundantly present in MERCs, although its role is controversial. One study using a genetic knockout model found CAV1 to be essential for MERC recruitment and regulation (Sala-Vila et al., 2016), while another study using an inducible CAV1 expression system found CAV1 to cause impairment of MERC communication and remodeling (Bravo-Sagua et al., 2019).

Mitochondrial dynamics are also influenced by MERC tethering, where phospholipid composition of membranes regulates mitochondrial fusion and fission. The main two phospholipids involved are phosphatidic acid (PA) and cardiolipin (CL) (Yu et al., 2020). The *de novo* mitochondrial synthesis of CL is followed by cycles of CL deacylation and reacylation, which can result in the generation of an array of CL species. Increased exposure of CLs occurs on the outer mitochondrial membrane during mitochondrial stress, where they can serve as binding sites for signaling proteins (Schlame and Haldar, 1993; Osman et al., 2011). CL works in the outer mitochondrial membrane to stimulate pro-fission GTPase Drp1, while PA interacts with Drp1 to inhibit it and promote fusion (Bustillo-Zabalbeitia et al., 2014; Adachi et al., 2016). CL can also promote fusion by interacting with OPA1 on the inner membrane, while PA can stimulate fusion by participating in it with MFN (Choi et al., 2006; Ban et al., 2017).

ROLE OF MITOCHONDRIA IN CARDIAC HEALTH

Mitochondria are the main energy source in cells and are especially important in energy-intensive organs such as the heart. Energy is produced in the form of ATP via oxidative phosphorylation. A balance in the concentration of ATP, adenosine diphosphate (ADP), creatine phosphate (CrP), and inorganic phosphate (Pi) are essential for a healthy heart. In the heart, changes in ATP, ADP, and CrP levels are detected to ensure that transient changes in their levels can cause large heart rate alterations (Balaban et al., 1986). The levels of ATP are thought to be low due to fast turnover and intensive energetic demand (Chistiakov et al., 2018). The enzyme creatine kinase produces phosphocreatine, which is involved in ATP buffering in the myocardium. When there are conditions of high ATP turnover, phosphocreatine concentration is altered to increase oxidative phosphorylation (Bark, 1980). Creatine kinase exists in an octameric form, which is very reactive, and a dimeric form, which is slower. In heart disease, creatine kinase is found predominantly in the slow dimeric form due to disassociation of the octamer. This causes dysfunctional oxidative phosphorylation and less ATP production due to impaired phosphocreatine hydrolysis (Soboll et al., 1999).

The heart obtains over half of its energy from fatty acid oxidation, though it can preferentially utilize glucose instead of lipids depending on energy demands (Ljubkovic et al., 2019). The level of malonyl CoA determines this switch, as increased malonyl CoA levels are associated with increased glucose oxidation and decreased fatty acid oxidation. In the heart, high activity of malonyl CoA decarboxylase (MCD), which converts malonyl CoA to acetyl CoA, maintains cardiac fatty acid substrate preference (Dyck et al., 2004). Studies have proposed that a switch in substrate availability can be altered by insulin signaling (Ljubkovic et al., 2019). In pathologic conditions such as HF or dilated cardiomyopathy, cardiac metabolism shifts toward glucose oxidation under resting conditions. However, under stress, this switch becomes detrimental due to disrupted insulin signaling and inefficient glucose uptake (Neglia et al., 2007).

An efficient mitochondrial ETC uses 98% of the electrons for ATP production (Chistiakov et al., 2018). The small percentage of electrons that escape the ETC and generate superoxide radicals are normally quickly broken down by superoxide dismutase (Boveris et al., 1976). Although the exact mechanism is not fully characterized, a small amount of ROS production can be cardioprotective by triggering protective mechanisms before and after I/R injury (Murry et al., 1986; Zhao et al., 2003). These processes are referred to as preconditioning and postconditioning, respectively. Uncoupling of the ETC results in ROS overproduction and inefficient ATP synthesis. In addition to cellular death, excessive ROS generation can induce atherogenesis through increasing vessel inflammation, oxidized low-density lipoprotein attachment to the vessel wall, endothelial dysfunction, and plaque-induced rupturing (Förstermann et al., 2017).

A healthy heart depends on balanced maintenance of contractile function and constant energy production. Regulation of cellular protein integrity in the heart is termed proteostasis, which involves heat shock response chaperones, autophagy, the ubiquitin–proteasome system, and the unfolded protein response (UPR) (**Figure 2**) (Arrieta et al., 2020). These processes all act when the mitochondria are under cellular stress. The mitochondrial protein folding environment is complex and the mitochondrial UPR (UPR^{mt}) is not as well-researched as its counterpart, the ER UPR (UPR^{ER}) (Li et al., 2019). UPR^{ER} will be discussed in the next section.

At normal physiological conditions, when UPR^{mt} is not activated, mitochondrial precursors must be transcribed and translated in the cytoplasm where they are maintained in an unfolded state guided by cytosolic heat shock protein (HSP) chaperones prior to being imported into the mitochondrion, where proteins are then folded (Priesnitz and Becker, 2018). Import stress such as reduced efficiency due to mitochondrial membrane depolarization or mismatch in production of respiratory complex subunits from mtDNA vs. nuclear DNA, leads to the buildup of misfolded or unfolded proteins (**Figure 2**) (Quirós et al., 2015; Rolland et al., 2019). These unfolded proteins activate the UPR^{mt}, resulting in the transcription and

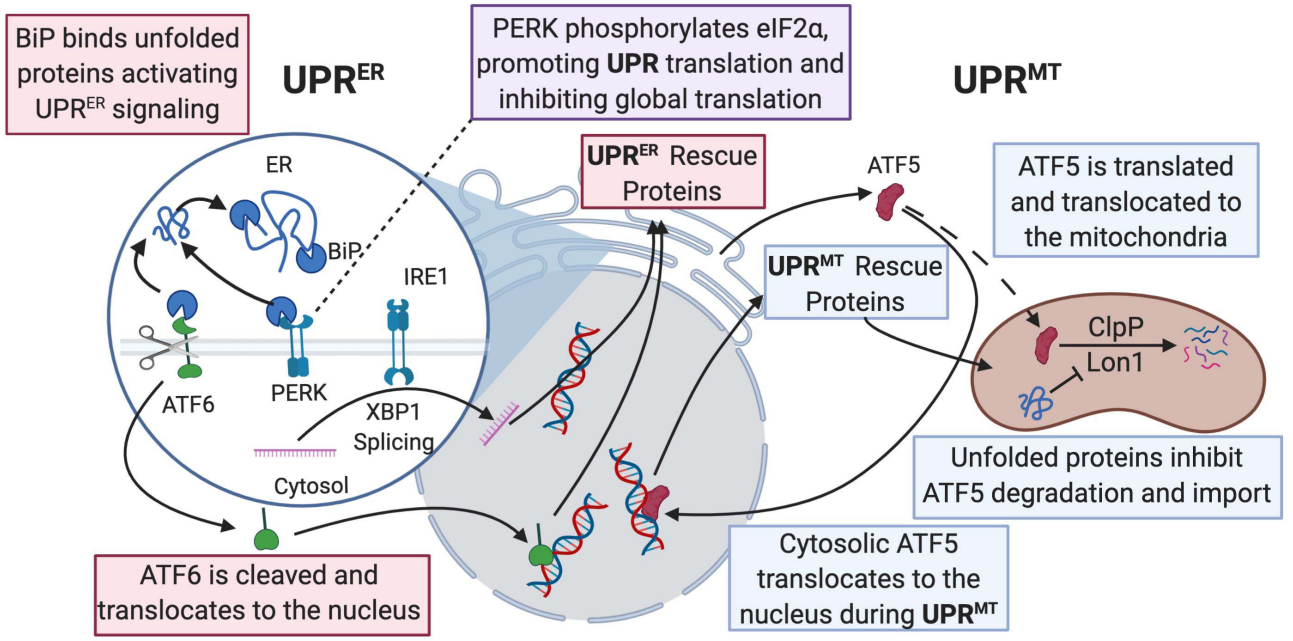

FIGURE 2 | UPR^{ER} and UPR^{mt} mechanistic commonalities and differences are described in this figure. BiP binds to misfolded proteins and promotes UPR^{ER} signaling, allowing for activation of the ATF6, PERK, and IRE1 pathways. ATF6 acts as a transcription factor and activates production of protein-folding machinery. PERK phosphorylates eIF2α, promoting translation of both UPR^{ER} and UPR^{mt} protein-folding machinery and downregulating global translation. IRE1 splices Xbp1 and allows the spliced product to transcribe protein chaperones in the nucleus. In the ATF5 pathway of UPR^{mt}, ClpP, and Lon1 diverge from degradation of ATF5 to degradation of misfolded proteins and the accumulation of ATF5 results in transcription of UPR-associated proteins in the nucleus. Dashed line is ATF5 movement in normal physiological conditions; solid line is enhanced when UPRmt is activated. UPR, unfolded protein response; BiP, binding immunoglobulin protein; ATF, activating transcription factor; PERK, protein kinase RNA-like endoplasmic reticulum kinase; ClpP, ATP-dependent Clp protease proteolytic subunit; Lon1, Lon protease homolog 1; IRE1, inositol-requiring kinase 1; Xbp1, X-box binding protein 1; eIF2α; eukaryotic translation initiation factor 2A.

translation of HSP10/60, and proteases, ATP-dependent Clp protease proteolytic subunit (ClpP) and lon protease homolog 1 (Lon1), to fold the proteins or repair damaged proteins (Martinus et al., 1996; Zhao et al., 2002; Haynes et al., 2007). The initiation of the UPR^{mt} can occur through two separate pathways. One pathway involves activating transcription factor 5 (ATF5), a transcription factor imported into the mitochondria, where Lon1 and ClpP degrade ATF5 under normal conditions. However, under cellular stress, the accumulation of misfolded proteins in the mitochondria prevents mitochondrial import of ATF5. Decreased mitochondrial import of ATF results in increased Lon1- and ClpP-induced degradation of the misfolded proteins in place of ATF5. The reduced mitochondrial import of ATF5 leads to increased trafficking of ATF5 to the nucleus, resulting in the transcription of UPR-associated proteins, promoting cellular defenses against stress signaling (Nargund et al., 2012; Fiorese et al., 2016). In addition to directly influencing protein translation, Lon1 has been shown to influence transcription indirectly. During activation of UPR^{mt}, Lon1 degradation of mitochondrial ribonuclease P catalytic subunit (MRPP3) increases MRPP3 turnover, leading to an accumulation of Mrpp3 RNA precursors resulting in impairment of mitochondrial translation (Münch and Harper, 2016). Knockdown of ClpP caused alteration of mitochondrial morphology, excessive ROS production, and a breakdown of oxidative phosphorylation even under non-stressed conditions (Deepa et al., 2016). This study suggested a more global role of ClpP for mitochondrial maintenance than impacting the UPR^{mt}. The second pathway is initiated by protein kinase RNA-like endoplasmic reticulum kinase (PERK) phosphorylating eukaryotic translation initiation factor 2A (eIF2α), which inhibits protein translation and activates ATF4/5 and C/EBP homologous protein (CHOP) translation. C-Jun N-terminal kinase 2 (JNK2) also binds to the transcription factor C-Jun, which results in the transcription of CHOP. ATF4/5 and CHOP all assist in transcribing other UPR^{mt} proteins (Aldridge et al., 2007; Baker et al., 2012; Verfaillie et al., 2012). The cumulative effects of both UPR^{mt} pathways result in reduced overall mitochondrial protein translation and increased translation of specific proteins that assist with refolding or degrading unfolded or misfolded proteins.

Mitophagy is another process that plays a role in proteostasis. Unlike the UPR^{mt}, which occurs when mitochondria are still salvageable, mitophagy occurs when mitochondria are irreparably damaged (Pickles et al., 2018). Mitophagy is initiated by phosphorylation and mitochondrial recruitment of ubiquitin ligase Parkin by PTEN-induced kinase 1 (PINK1) during stress (Kane et al., 2014). Phosphorylated Parkin recruits autophagy receptors by placing ubiquitin chains on mitochondrial outer membrane proteins (Lazarou et al., 2015). p53-induced inhibition of Parkin exacerbated cardiac aging and

dysfunction, suggesting that activation of mitophagy could be a potential target for therapy in diseases related to aging or mitochondrial dysfunction (Hoshino et al., 2013; Pires Da Silva et al., 2020). Interestingly, Drp1—a key protein involved in mitochondrial fission—is also essential for mitophagy. Deficiency of Drp1 resulted in loss of mitophagy, promotion of cardiac dysfunction, and increased susceptibility to I/R injury (Ikeda et al., 2015). Mitophagy is a particularly fascinating topic of research as it is a reparative protective mechanism that may become pathological when uncontrolled.

Alterations in epigenetic regulation are known to promote various pathologies. Recent studies have shown the importance of metabolite diversion from bioenergetic pathways to nuclear epigenetic regulation. Mitochondrial calcium exchange has been shown to participate in myofibroblast activation by promoting shuttling of α-ketoglutarate from the mitochondria to the nucleus where it activates histone demethylases enhancing chromatin accessibility (Lombardi et al., 2019). The concept of retrograde signaling is strongly supported by oncometabolites such as 2-hydroxyglutarate, which are enhanced in gain-of-function mutations in isocitrate dehydrogenases, shown to participate in epigenetic modification of various tumors (Shim et al., 2014). How mitochondrial dynamics may alter retrograde signaling and/or be involved in cellular differentiation as it pertains to cardiac pathologies remain to be fully characterized.

ROLE OF ENDOPLASMIC RETICULUM (SARCOPLASMIC RETICULUM) IN CARDIAC HEALTH

The ER is the bridge between many different types of membranes and organelles. Important membrane junctions between the ER and other organelles include the ER–endosome (Friedman et al., 2013), ER–peroxisome (Knoblach et al., 2013), ER–Golgi (Peretti et al., 2008), and ER–mitochondria junctions (Shore and Tata, 1977). Along with its extensive contacts, the ER continuity allows other organelles to communicate rapidly via the ER network (Wozniak et al., 2009). A specialized form of the ER exists in cardiomyocytes called the sarcoplasmic reticulum (SR) which acts as a reservoir for Ca^{2+} to facilitate intracellular Ca^{2+} release and storage. Upon depolarization, Ca^{2+} enters the cell and binds to RyRs, which induces calcium-induced Ca^{2+} release (CICR) (Otsu et al., 1990). CICR in the SR is crucial in muscle contraction and relaxation (Terracciano and MacLeod, 1997). Other proteins found in the SR involved in Ca^{2+} signaling include Ca^{2+}-ATPases (SERCAs), which work to import Ca^{2+} into the ER and IP_3Rs, which release Ca^{2+} into the cytoplasm along with RyRs (**Figure 1**) (Nixon et al., 1994). The SR is also the location that controls excitation–contraction coupling in cardiac myocytes (Li et al., 2019). When Ca^{2+} homeostasis becomes dysfunctional, toxic misfolded proteins can accumulate and cause ER stress. Ca^{2+} overload has been proposed to cause protein unfolding and activation of the ER stress response (Wiersma et al., 2017). ER stress has been directly implicated in the progression of atrial fibrillation (AF) (Wiersma et al., 2017).

The ER function comprises protein synthesis, protein folding, Ca^{2+} signaling, and proteostasis during conditions of stress. The ER is the site where proteins imported from cytosolic ribosomes are folded and modified. Proteins that are processed in the ER make up one-third of all synthesized proteins, and they are vital for cardiomyocyte function (Blackwood et al., 2019). A crucial aspect of protein folding is the formation of disulfide bonds, which is catalyzed by combining the protein disulfide isomerase and ER oxidoreductin-1 (PDI-Ero1) complex and an oxidized folding environment (Tu and Weissman, 2002; Araki et al., 2013). ROS is generated as a byproduct of disulfide bond formation during oxidative protein folding, which must be eliminated to maintain ER proteostasis. Any disturbance in redox homeostasis results in ER stress and activation of the UPR^{ER} response (**Figure 2**) (Zhang et al., 2019).

The UPR^{ER}, like the UPR^{mt}, works in an attempt to mitigate ER stress through three ER membrane-embedded sensors: protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring kinase 1 (IRE1) (Senft and Ronai, 2015). The activation of UPR^{ER} is regulated by the protein BiP, which inactivates the three sensors by binding to them. Binding immunoglobulin protein or heat shock protein family A member 5 (BiP/HSPA5) has a high affinity for misfolded proteins and, in response to their accumulation, will dissociate from PERK, ATF6, and IRE1 (Bertolotti et al., 2000). BiP will then act as a chaperone for the misfolded proteins, while the sensors can activate the three prongs of the ER stress response (Bettigole and Glimcher, 2015). Once ER stress is alleviated, BiP rapidly binds to the sensors again (Bertolotti et al., 2000).

ATF6 acts as a transcription factor via its N-terminus once activated through sites 1 and 2 proteolysis in the Golgi apparatus (**Figure 2**) (Haze et al., 1999). It works to induce genes in the protein-folding machinery that were previously not thought to play a role in protein folding (Jin et al., 2017). For instance, one study showed that ATF6 had antioxidant properties as it induced catalase activity during I/R conditions. Catalase can neutralize ROS generated as a byproduct of protein folding and protect the heart from I/R injury (Jin et al., 2017). ATF6 has been proposed as a pharmacological therapy since studies showed that selective activation of ATF6 can reduce reperfusion damage and preserve cardiac function (Blackwood et al., 2019). IRE1 activates its ER stress response by splicing X-box-binding protein 1 (Xbp1) mRNA, allowing Xbp1 to become an active transcription factor in the nucleus (Yoshida et al., 2001). Xbp1 contributes to mitigating ER stress by transcribing chaperones and proteins that assist with protein degradation (Lee et al., 2005). It also has transcriptional targets that encompass lipid metabolism (Lee et al., 2005), cellular differentiation (Acosta-Alvear et al., 2007), and elongation of the secretory protein apparatus (Shaffer et al., 2004). In a mouse model of HF with preserved ejection fraction, there was deficiency of IRE1 and Xbp1. However, restoration of Xbp1 ameliorated the phenotype in a disorder with no effective clinical therapies (Schiattarella et al., 2019). PERK acts similarly to its function in the UPR^{mt} by phosphorylating translation factor eIF2α, which causes massive downregulation of protein translation (Harding et al., 2000). This results in preferential UPR^{ER}-related protein translation

with a global reduction in overall protein translation. ATF4 is a protein that is preferentially translated via this pathway, where it acts as a potent activator of proapoptotic factor CHOP (Dey et al., 2010; Teske et al., 2013). CHOP may be beneficial in mild cases of cardiac ER stress, but during conditions of prolonged ER stress, it is likely harmful (Fu et al., 2010). CHOP-deficient mice had attenuated hypertrophy signs and cardiac dysfunction compared to wild-type mice, suggesting that CHOP could be implicated in the development of cardiac pathology (Fu et al., 2010). Moreover, PERK was cardioprotective in conditions of pressure overload-induced congestive HF (Liu et al., 2014). These studies indicate the complexity of the UPR^{ER} and showcases that there are instances where it is adaptive, and other cases where it is maladaptive (Arrieta et al., 2020). Hence, mild and short-lived cardiac ER stress may be beneficial to clear unfolded, misfolded, or aged proteins to maintain proper cardiac function. Prolonged ER stress that cannot be quelled, on the other hand, may be detrimental as it can result in apoptosis of cardiac cells. Since cardiomyocytes are terminally differentiated and cannot be largely replenished, excessive apoptosis will lead to cardiac dysfunction resulting in HF (Fang et al., 2019).

MITOCHONDRIA AND SARCOPLASMIC RETICULUM INTERACTIONS IN CARDIAC PHYSIOLOGY

The mitochondrion and SR have numerous interactions with each other, which are necessary for healthy cardiac function. Mitochondria are spatially and functionally organized in close contact with the SR. They are tightly associated in areas that support lipid and protein transfer but are more detached in areas of Ca^{2+} delivery (Csordás et al., 2010). This spatial organization contributes to the mitochondrial uptake of Ca^{2+} release from the SR via IP_3Rs, which is essential for mitochondrial ATP production (**Figure 1**) (Nixon et al., 1994). Ca^{2+} transfer between these two organelles is regulated by MFN2 (De Brito and Scorrano, 2008). Dysfunction of MFN2 and other proteins found in the MAM can alter Ca^{2+} signaling, leading to aberrant signaling that promotes cardiovascular pathogenesis (Chen et al., 2012).

Ca^{2+} is also imperative to excitation–contraction coupling in cardiomyocytes, and research has attempted to elucidate whether mitochondria contribute to cytosolic Ca^{2+} regulation in contracting cardiac cells (Affolter et al., 1976). Recent evidence indicates that mitochondria participate in cardiomyocyte calcium dynamics by interacting with the SR to regulate beat-to-beat phasic calcium cycling. It is known that with every cardiac cycle, Ca^{2+} influx and efflux in the mitochondria occurs, respectively, via the MCU and the Na/Ca^{2+} exchanger (NCLX); nevertheless, the involvement of these processes in regulating excitation–contraction coupling remain largely debated. Recently, the ablation of the mitochondrial NCLX has supported the notion that indeed mitochondrial calcium regulation is vital and participates in cytosolic calcium levels and arrhythmia generation (Luongo et al., 2017). In the following sections, we will briefly describe the role of mito–SR interactions in cardiac hypertrophy, myocardial I/R injury, AF, and diabetic and inherited cardiomyopathy.

Cardiac Hypertrophy and Heart Failure

SR–mitochondria Ca^{2+} dysregulation is thought to be a significant driver in the pathogenesis of cardiac hypertrophy and HF. This could be due to both mitochondrial Ca^{2+} overload and Ca^{2+} deficiency. Mitochondrial Ca^{2+} overload has been shown to promote mPTP opening and ROS generation. In conditions of augmented Ca^{2+} intake, increased ETC activity results in electron leakage and superoxide formation (Zoccarato et al., 2004). The increase in ROS production can lead to posttranslational modification of RyR2, causing the channel to become leaky (Santulli et al., 2015). This results in upregulation of calcium uptake by the mitochondria causing excessive mitochondrial fragmentation, reduction in size, increased permeability of the mPTP pore, and apoptosis initiation. This positive feedback loop of Ca^{2+} influx causes severe mitochondrial loss of function and HF (Santulli et al., 2015). On the other hand, mitochondrial Ca^{2+} deficiency can occur due to increased cytosolic levels of Na^{+}. Increased Na^{+} levels cause increased activity of NCLX, resulting in Ca^{2+} efflux from the mitochondria. Reduced Ca^{2+} levels are thought to inhibit TCA cycle activity and increase H_2O_2 levels due to impaired antioxidant capabilities via a reduction in nicotinamide adenine dinucleotide phosphate (NADPH) from slower TCA cycle rates. Furthermore, H_2O_2 can increase Na^{+} current and influx into the mitochondria, amplifying this detrimental feedback loop (Kohlhaas et al., 2010). Inhibition of the NCLX restored Ca^{2+} levels, maintaining mitochondrial redox potential, and replenishing energy supply (Liu and Rourke, 2008). This mechanism is promising in the development of novel pharmacological strategies to treat cardiac hypertrophy and HF.

Excessive adrenergic stimulation can increase the distance between SR–mitochondrial contacts leading to mitochondrial fragmentation, impaired function, and cardiac hypertrophy (Gutiérrez et al., 2014). Norepinephrine increases the distance between the SR and mitochondria in cardiomyocytes, causing inefficient Ca^{2+} transfer (Gutiérrez et al., 2014). The mechanism by which fragmentation occurs is through an increase in Ca^{2+} and calcineurin activation. Calcineurin promotes DRP1-dependent constriction of the mitochondria, whereas the downregulation of calcineurin was shown to inhibit hypertrophy (Pennanen et al., 2014). In addition to alteration in calcineurin signaling, these hypertrophic hearts were also found to have reduced levels of MFN2, resulting in less tethering between the SR and mitochondria and mitochondrial fission (Pennanen et al., 2014). In summary, there is evidence that dysregulation of Ca^{2+} signaling and impairment of mitochondrial dynamics participate in the development of hypertrophy and HF and may be used in the future to develop novel treatment strategies.

Myocardial Infarction and Ischemia/Reperfusion Injury

An MI is defined as the cardiomyocyte death that occurs as a result of prolonged ischemia, while I/R injury describes the tissue damage when perfusion is restored after an ischemic

event. Dysfunction in Ca^{2+} transfer and contacts between mitochondria and SR can contribute to cardiac I/R injury. The upregulation of VDAC1, GRP75, and IP_3R channel complex during I/R can lead to Ca^{2+} overload, thus triggering the opening of the mPTP. Once the mPTP pore opens, oxidative stress, mitochondrial swelling, and release of cytochrome c into the cytoplasm ensue leading to cell death (Zhu et al., 2018). Independent downregulation of VDAC1, GRP75, IP_3R, Mfn2, and cyclophilin D (CypD) (mPTP regulator) decreased Ca^{2+} transfer and reduced cell death after I/R injury (Paillard et al., 2013). During I/R, GSK3β activates IP_3R channels via phosphorylation. SB21 inhibition of GSK3β reduces IP_3R channel opening and subsequent mPTP-mediated cell death (Gomez et al., 2016). Administration of sulodexide, a glycosaminoglycan that inhibits ER stress, reduces I/R-induced cellular apoptosis, leading to cardioprotection in murine models (Shen et al., 2019). Activation of the phosphoinositide 3-kinase/protein kinase B (PI_3K/Akt) pathway causes downstream increase in Bcl-2, an antiapoptotic factor, and a decrease in Bax, a proapoptotic factor, thus reducing apoptosis (Shen et al., 2019). It is paramount to fully understand the role of mito–SR communication in ischemic cardiac injury as it may provide novel and unexplored mechanisms that can be explored for new pharmacological interventions.

Atrial Fibrillation

ER stress-induced cardiac remodeling, Ca^{2+} overload, and excessive autophagy are the main drivers of AF. ER stress can occur through deficiency of chaperone proteins, such as HSPA5, whereas alleviation of ER stress can be achieved with the addition of 4PBA, a chemical chaperone. ER stress has also been found to activate mitochondrial apoptosis via the mitogen-activated protein kinase (MAPK) pathway (Shi et al., 2015). Ca^{2+} overload in models of AF has been found to occur through multiple mechanisms. One study using murine models found that the oxidation of RyR2 channels increased intracellular Ca^{2+} release to excessive levels (Xie et al., 2015). Another study using HL-1 atrial cardiomyocytes found that MCU-mediated Ca^{2+} influx enhanced tachypacing-induced mitochondrial dysfunction (Wiersma et al., 2019). Inhibition of this channel via treatment with Ru360 prevented devastating mitochondrial changes. The same study found aberrant ATP levels in models of AF and overexpression of the chaperone HSP60, mitochondrial fragmentation, cardiac remodeling, and contractile dysfunction (Wiersma et al., 2019). Mitochondrial dysfunction is pivotal in the development of AF as it is thought that structural mitochondrial changes exist before AF onset, but AF exacerbates mitochondrial functional impairment, thus promoting a positive feedback loop (Wiersma et al., 2019).

Diabetic and Inherited Cardiomyopathy

ER stress and alterations in the interactions between the ER and mitochondria are crucial in the development of both diabetic and inherited cardiomyopathy. The pathogenesis of diabetic cardiomyopathy stems from impaired insulin signaling resulting in the metabolic switch from glucose metabolism to fatty acid oxidation. There is also decreased activity and presence of β-oxidation enzymes (Ljubkovic et al., 2019). This results in the development of metabolic stress and impaired functional cardiomyocyte efficiency (Ljubkovic et al., 2019). Increased mPTP Ca^{2+} sensitivity and intrinsic caspase-9 signaling are observed in human diabetic cardiomyocytes, presumably due to long-term metabolic stress, leading to an overproduction of mitochondrial ROS (Anderson et al., 2011). In human diabetic heart tissue, there were elevated levels of UPR^{ER} proteins CHOP and GRP78, linking cardiac diabetes to ER stress processes (Ljubkovic et al., 2019). Compromised cardiac contractility is another feature of diabetic cardiomyopathy, which has been linked to defective Ca^{2+} signaling linked to erratic RyR2 channel behavior (Yaras et al., 2005). ER stress-mediated apoptosis and ROS production in conditions of hyperglycemia have been reported in rats with diabetic cardiomyopathy (Yang et al., 2017). Administration of exogenous H_2S inhibited apoptosis and ER stress via the suppression of hyperglycemia and MFN2-induced oxidative stress (Yang et al., 2017). There is limited evidence linking MERCs and pathogenesis of diabetic CM, but one study found that FUNDC1—an outer mitochondrial membrane protein—levels are elevated in diabetic patients compared to those in nondiabetic patients. It was concluded in the study that diabetes facilitates Fundc1-mediated MERC formation, and inhibition of Fundc1 could be a potential therapy for diabetic CM (Wu et al., 2019).

Inherited cardiomyopathy comprises around 50% of all cases of cardiomyopathy, with the most prevalent forms being dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) (Towbin, 2014; Sacchetto et al., 2019). The most common disease-causing mutation in HCM comes from genes encoding sarcomeric proteins (Singh et al., 2017). The Mybpc3 gene encodes for cardiac myosin-binding protein C (cMyBP-C). A mutation of the Mybpc3 gene in human tissue, and mouse knock-in models supported the notion that autophagy is impaired, whereas activation of autophagy resulted in amelioration of cardiomyopathy (Singh et al., 2017). In DCM patients with S143P mutation in the lamin A/C gene, there was activation of the UPR^{ER} system as observed by increased ER stress markers (GRP78, IRE1, and ATF6) (Ortega et al., 2014; West et al., 2016). As intraorganellar membrane communication plays a role in many of the processes mentioned above, further understanding of these mechanisms may aid in the development of new pharmacological targets that will, at the very least, delay pathological outcomes.

THERAPEUTIC TARGETS FOR MITOCHONDRIAL–ENDOPLASMIC RETICULUM INTERACTIONS

Lifestyle Interventions

Participation in secondary prevention lifestyle changes such as exercise training and caloric restriction has been shown to protect against cardiac risk factors and improve the quality of life of individuals (Clark et al., 2005). Swimming exercise in aged mice suppressed ER stress responses delaying ROS-mediated cell damage by enhancing antioxidation mechanisms

via increased superoxide dismutase (Chang et al., 2020). Resistance exercise and aerobic exercise significantly decreased the expression of ER stress markers (CHOP, eIF2α, and PERK) (Kim et al., 2018). Moreover, exercise training was further potentiated when performed concurrently with a lower caloric intake (Kim et al., 2017). This agrees with results showing that caloric restriction delays proteostasis collapse that occurs with cardiac pathogenesis by maintaining robust UPR^{ER} activity (Matai et al., 2019).

Pharmacological Interventions

Pharmacologic interventions are essential in cases where lifestyle modifications are unable to slow the progression of cardiac disease. They work to restore the balance of the ER–mitochondrial interactions and reduce ER and mitochondrial maladaptive outcomes. The use of 4BPA, a chemical chaperone, which relieves ER stress by reducing misfolded protein aggregation, has been contemplated for the use in cardiac pathologies. 4BPA is available to treat urea cycle disorders and is undergoing human clinical trials for neurological protein misfolding disorders. Thus, this strategy is also under consideration for cardiac diseases, particularly for AF, where autophagosome formation appears to be a hallmark (Wiersma et al., 2017). Another option for intervention is SIRT1, a deacetylase, which has been found to increase beneficial autophagy and decrease ER stress-induced cell death in cardiomyocytes. It promotes autophagy via indirect activation of the eukaryotic elongation factor 2 kinase (eEFK2/eEF2) pathway, possibly through regulation of the acetylation state of eIF2α (Pires Da Silva et al., 2020). The role of beta-blockers in alleviating ER stress in conditions of hypertrophy and HF are also being studied. Beta blockade inhibits beta-adrenergic hyperactivation and drastically reduces ER-mediated apoptosis in cardiomyocytes of hypertrophic and failing hearts (Ni et al., 2011). Last, the administration of taurine, a conditionally essential amino acid, has been implicated in the downregulation of mitochondrial and UPR-dependent cell apoptosis as well as ER stress markers (Yang et al., 2013).

CONCLUSIONS

Significant scientific achievements in the twenty-first century have promoted novel pharmacological interventions to maintain cardiac function, yet cardiac disease remains a top cause of death worldwide. Interactions between mitochondria and ER are essential for a healthy myocardium as these processes participate in energy production, apoptosis, ROS management, protein folding, and Ca^{2+} signaling. Disruption in ER or mitochondrial function can play a role in development in hypertrophy, heart failure, myocardial I/R injury, AF, HCM, DCM, and diabetic cardiomyopathy. Several of these pathologies stem from common MERC alterations, but it is unknown why these changes result in different pathologies. Studies further delineating precise mechanisms that regulate intra-organellar membrane communication hold promise to unravel novel therapies that conserve cardiac function by maintaining the ER and mitochondria homeostatic balance and overall cell health.

AUTHOR CONTRIBUTIONS

VK, AL, and PS reviewed the literature, drafted, and edited the manuscript. VK and JS edited the manuscript and prepared illustrations. AR, SK, AD, and PS reviewed the article and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mitochondrial Function and Dysfunction in Dilated Cardiomyopathy

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Cardiac tissue requires a persistent production of energy in order to exert its pumping function. Therefore, the maintenance of this function relies on mitochondria that represent the "powerhouse" of all cardiac activities. Mitochondria being one of the key players for the proper functioning of the mammalian heart suggests continual regulation and organization. Mitochondria adapt to cellular energy demands via fusion-fission events and, as a proof-reading ability, undergo mitophagy in cases of abnormalities. Ca^{2+} fluxes play a pivotal role in regulating all mitochondrial functions, including ATP production, metabolism, oxidative stress balance and apoptosis. Communication between mitochondria and others organelles, especially the sarcoplasmic reticulum is required for optimal function. Consequently, abnormal mitochondrial activity results in decreased energy production leading to pathological conditions. In this review, we will describe how mitochondrial function or dysfunction impacts cardiac activities and the development of dilated cardiomyopathy.

Keywords: mitochondria, cardiomyocytes, cardiomyopathies, organoids model, sarcoplasmic reticulum, Ca ATPase (SERCA) 2+, calcium, heart function

INTRODUCTION

Mitochondria are highly dynamic organelles, universally recognized as the "powerhouse" of eukaryotic cells, especially in those that require high-energy demand such as cardiomyocytes (Nan et al., 2017). In these cells mitochondria occupy 30% of the total volume of the cell and supply, through oxidative phosphorylation (OXPHOS), approximately 6 kg of adenosine triphosphate (ATP) per day that is required to sustain cardiac function (Cao and Zheng, 2019). In addition to their pivotal role in energy production, mitochondria are the central hub of cellular metabolism providing metabolites for biosynthesis and also producing reactive oxygen species

(ROS). Under physiological conditions ROS act as second messengers that are maintained at low concentrations by the scavenging system present in the cell. However, ROS are hyper-produced in many cardiovascular diseases (CVDs), which impairs heart function (Murphy et al., 2016).

It is well established that mitochondrial calcium (Ca^{2+}) fluxes are a key regulator of cardiac function, controlling not only ATP production and mitochondrial metabolism, but also playing a pivotal role in the modulation of muscle contraction (Walsh et al., 2009). In cardiomyocytes mitochondria are well organized and in close proximity to the sarcoplasmatic reticulum (SR), where most cellular Ca^{2+} is stored (Frederick and Shaw, 2007). Therefore, mitochondria are highly sensitive to Ca^{2+} oscillations. The release of Ca^{2+} from SR to mitochondria ensures a balanced activation of SR ATPase and mitochondrial ATP synthesis; all of which contribute to controlling the energy metabolism within a cell (Balaban et al., 2003). Hence, the maintenance of Ca^{2+} homeostasis is a fundamental requirement for optimal mitochondrial function as mitochondria are a key checkpoint regulating cell survival and cell death.

It is thus not surprising that the maintenance of efficient inter-organelle-communication as well as a conserved "mitochondrial quality control" system (MQC), are fundamental for sustaining mitochondrial bioenergetics demand and metabolic functions (Campos et al., 2016). The term MQC refers to mitochondrial fusion and fission machinery (also called mitochondrial dynamics) and autophagy (called mitophagy when pertaining to mitochondria) (Fan et al., 2020). As we will explain in detail in this review, mitochondrial fusion has the ability to respond to high-energy demand conditions by recovering mitochondria that have been damaged and creating elongated interconnected mitochondrial networks. Fission, however, is the process by which dysfunctional mitochondria are separated and segregated away from healthy ones. These dysfunctional mitochondria may be subsequently either recovered or eliminated through mitophagy (Murphy et al., 2016; Fan et al., 2020; Forte et al., 2020; Oh et al., 2020). These complex processes provide the balance for maintaining proper mitochondrial dynamics through regulation of mitochondrial size, shape and number (Youle and Karbowski, 2005; Piquereau et al., 2013).

An increasing number of studies on cardiac mitochondria have determined that dysfunction in their structure and function contributes to the pathogenesis of CVD including dysrhythmias, ischemia-reperfusion injury (IRI) and cardiomyopathies (CMPs); all of which culminate in end-stage heart failure (HF) (Brown and O'Rourke, 2010; Cadenas et al., 2010; Rosca and Hoppel, 2010; Verdejo et al., 2012).

In this review, we will provide an overview of the main functions of mitochondria within cardiac tissue. Furthermore, we will discuss the involvement of mitochondrial impairment in CVD, focusing our attention on dilated cardiomyopathy (DCM) leading to heart failure. Dilated cardiomyopathy is associated with decreased mitochondrial biogenesis and we will examine DCM subtypes and how mitochondria are dysregulated in these conditions. We will highlight the paucity of targeted treatments for DCM and the necessity for understanding the molecular mechanisms involved in DCM onset and progression. Finally, we will the need for new methods to tease out the complexities of dilated cardiomyopathy, such as the potential use of cardiac organoids to investigate the underlying molecular mechanisms of cardiac function and to develop new targeted therapies for dilated cardiomyopathy.

MITOCHONDRIAL FUNCTIONS IN THE HEART

Bioenergetics, Ca^{2+} Homeostasis, Cell Death

In heart mitochondria, the primary source of carbons for ATP production relies on fatty acid oxidation (FAO) (**Figure 1**). Products of beta-oxidation are directed into the tricarboxylic acid cycle (TCA): the starting compound acetyl-CoA enters the cycle and undergoes a series of reactions where electrons are extracted from TCA intermediates in the form of the reducing equivalents NADH and $FADH_2$ and in turn fueling the electron transport chain (ETC) for ATP synthesis (Murphy et al., 2016; Martínez-Reyes and Chandel, 2020). The ETC creates an electrochemical gradient ($\Delta\Psi m$ is -180 mV) along the intermitochondrial membrane (IMM) interface which acts as a driving force for mitochondrial Ca^{2+} uptake (Giorgi et al., 2012, 2018b; **Figure 1**). Mitochondria are calcium-buffering organelles in which under resting conditions mitochondrial Ca^{2+} concentrations are kept low, but after a stimulus Ca^{2+} is transferred from the SR into the mitochondria that transiently and rapidly takes up large quantities of Ca^{2+} (Giorgi et al., 2018a,b). Lastly, Ca^{2+} is extruded from mitochondria by the Na^{+}/ Ca^{2+} antiporter (NCLX) (Giorgi et al., 2018a; **Figure 1**).

However, under pathological conditions, a cytosolic Ca^{2+} overload initiates a large and persistent Ca^{2+} uptake by mitochondria, which triggers the opening of the mitochondrial permeability transition pore (mPTP; a nonspecific pore) (**Figure 1**; Giorgi et al., 2012; Morganti et al., 2018). mPTP allows for free passage of small molecules and ions (<1.5 kDa) across the IMM, leading to membrane potential dissipation, and consequent imbalance in ATP production, mitochondrial swelling and mitochondrial outer membrane (OMM) rupture all of which cause regulated cell death (RCD) through either apoptosis or necrosis (**Figure 1**; Bonora et al., 2015). The activation of one of these cell death pathways depends upon the severity of the damage and the kinetics of the pore opening (Javadov and Karmazyn, 2007). mPTP is also involved in the pathogenesis of Ischemia/reperfusion injury (IRI) (Morciano et al., 2015). For example, a moderate injury, which occurs in the case of a short ischemic period, may lead to a short pore opening time thereby triggering apoptosis. On the other hand, a more severe and persistent insult such as a longer hypoxic event may lead to persistent pore opening inducing cell death through necrosis. Myocardial infarction exhibits a necrotic area at the core of the ischemic zone that is surrounded by apoptotic markers, indicating decreased cardiomyocyte survival upon an ischemic event (Javadov and Karmazyn, 2007; Morciano et al., 2015). The mPTP structure remains an area of

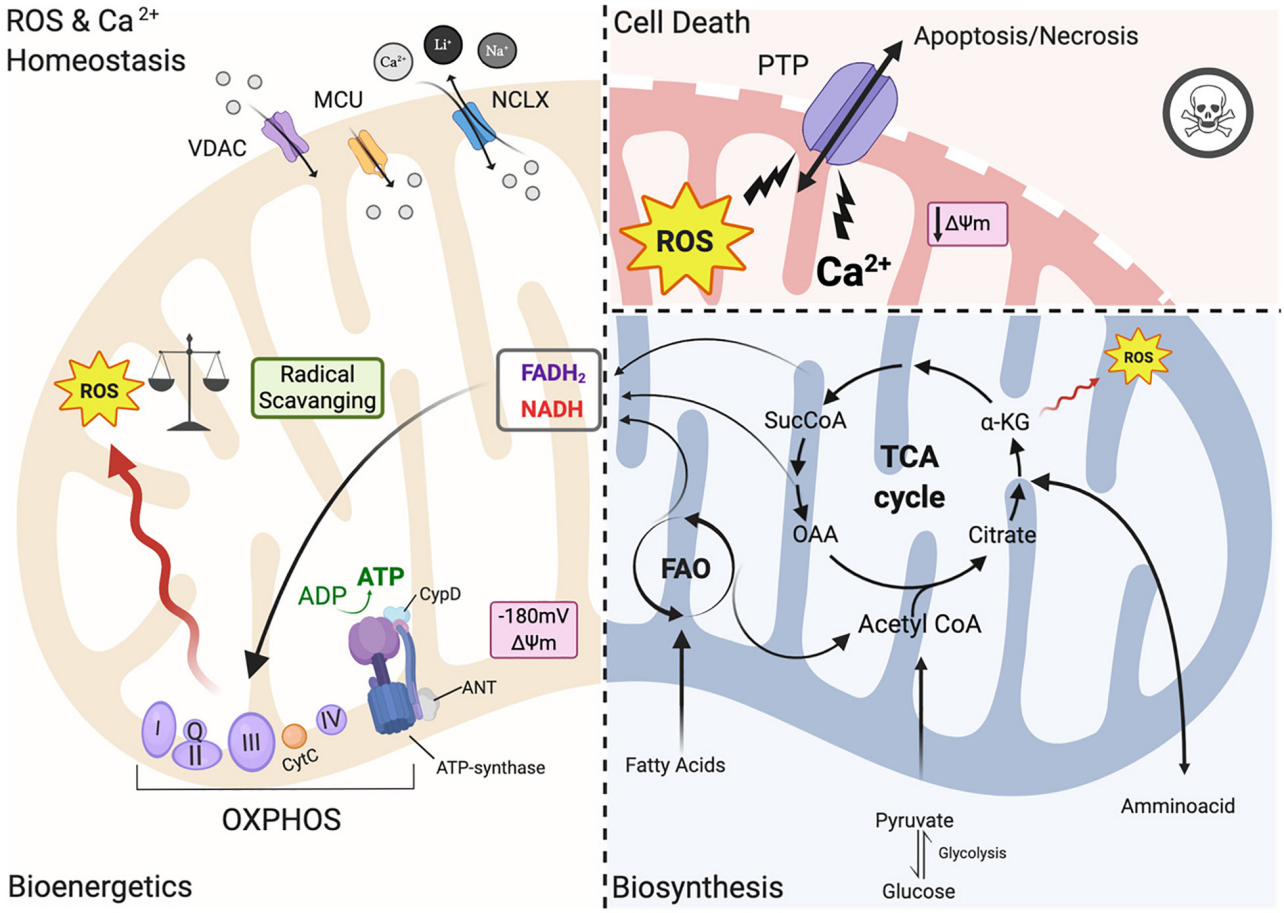

FIGURE 1 | Mitochondrial functions. ***Left panel:*** under physiological conditions, mitochondria functions are the core of bioenergetics activities, providing ATP throughout the OXPHOS, which is also an important source of ROS. Basal ROS levels are maintained by the radical scavenging network. Additionally, mitochondria are calcium-buffering organelles. Ca^{2+} homeostasis is finely controlled by its uptake through voltage-dependent anion-selective channel proteins (VDACs) and the mitochondrial Ca^{2+} uniporter (MCU) complex, Ca^{2+} efflux is controlled by NCLX. ***Right top panel*:** pathological conditions, ROS burst and mitochondrial Ca^{2+} overload activate regulated cell death (RCD) inducing either apoptosis or necrosis pathway through the PTPC opening. ***Right-bottom panel*:** Ca^{2+} uptake activates mitochondrial metabolism. Fatty acids are metabolized via FAO toward the TCA cycle providing energy as $FADH_2$ and NADPH are building blocks for biosynthesis. Voltage-dependent anion-selective channel proteins (VDAC), Mitochondrial Calcium Uniporter Complex (MCUC), Mitochondrial Na^+/Ca^{2+} exchanger (NCLX), oxidative phosphorylation (OXPHOS), Permeability transition pore complex (PTPC), ADP/ATP translocase (ANT) and peptidyl-prolyl *cis-trans* isomerase Cyclophilin D (CypD), cytochrome C (cyt C), adenosine triphosphate (ATP), reactive oxygen species (ROS), tricarboxylic acid cycle (TCA), fatty acid oxidation (FAO), α -ketoglutarate dehydrogenase (α-KG), oxaloacetate (OAA), Acetyl coenzyme A (Acetyl CoA) mitochondrial membrane potential ($\Delta\psi_m$) (Created with BioRender.com).

intense study; the latest findings have been reviewed recently by Bonora et al. (2020).

In the past few years, the mitochondrial F1/F0 ATP Synthase (ATP synthase) has been recognized as a key component of pore formation along with ADP/ATP translocase (ANT) and peptidyl-prolyl *cis-trans* isomerase Cyclophilin D (CypD) (**Figure 1**; Bonora et al., 2017; Morciano et al., 2017; Bonora and Pinton, 2019) that together regulate the opening of the permeability transition pore complex (PTPC) (Bonora et al., 2020). It has been demonstrated that dissociation of ATP synthase dimers upon mitochondrial permeability transition (MPT) induction, in particular the C subunit of the F0 part (in its c-ring form), is a key component of the pore (Bonora et al., 2013, 2015, 2017). Mitochondria isolated from *Ppif*-null mice strongly validates the role of CypD as a pore regulator (Basso et al., 2005) because these mitochondria are unresponsive to mitochondrial Ca^{2+} overload. Moreover, upon knocking out all three ANT isoforms simultaneously MPT is inhibited (Karch et al., 2019). It remains controversial whether ANT represents a key pore regulator or is a part of the pore, and futher studies are needed to understand its role in PTPC opening (Bonora and Pinton, 2019).

Thus, in order to avoid mitochondrial Ca^{2+} overload and consequent activation of regulated cell death (RCD), Ca^{2+} uptake has to be finely controlled. Ca^{2+} released by the ER rapidly enters the mitochondrial intermembrane space (IMS) by Voltage-dependent anion channels (VDACs), which are localized at the OMM (**Figure 1**; Giorgi et al., 2018b). The channel exists in three isoforms (VDAC1, VDAC2, VDAC3) expressed almost ubiquitously among tissues with different sub-mitochondrial ratios (Messina et al., 2012). It exhibits two conformations: the

open pore conformation with a low transmembrane potential, showing high-conductance and weak anionselective; whereas increasing potential leads to a closed state conformation characterized by cation selectivity and impermeable to nucleotide passage (Giorgi et al., 2018b). In recent years, the mitochondrial Ca^{2+} uniporter (MCU) was identified as a major player in the regulation of mitochondrial Ca^{2+} homeostasis (**Figure 1**; Kirichok et al., 2004). MCU is a multiprotein complex (MCUC) situated at the IMM, which has a low affinity for Ca^{2+} ions but is highly selective and regulated by auxiliar proteins that make up part of the MCUC (Marchi and Pinton, 2014). MCUC involvement in cardioprotection has been widely studied in recent years: several mouse models with MCU deletions have been developed including a cardiac-specific dominant-negative MCU mouse that is expressed in neonates (Rasmussen et al., 2015), a cardiac conditional MCU-KO mouse (Luongo et al., 2015), and a tamoxifen-inducible cardiac-specific loss of MCU in adult mice (Kwong et al., 2015). Mitochondria isolated from the hearts of these mice are characterized by reduced mitochondrial Ca^{2+} influx with subsequent reduced susceptibility to mPTP opening and loss of mPTP-related cardioprotection (Kwong et al., 2015; Luongo et al., 2015). On the contrary, deletion of NCLX, a key component of Ca^{2+} release, is lethal to cells because it induces mitochondrial Ca^{2+} overload and consequent PTP opening (Luongo et al., 2015).

Mitochondria and Biosynthesis

Mitochondria contribute to cell metabolism by providing building blocks for the synthesis of macromolecules necessary for the maintenance of cellular homeostasis and cell growth. As mentioned above, the TCA cycle represents a metabolic engine in mitochondria where these catabolic and anabolic reactions intersect (Martínez-Reyes and Chandel, 2020). As the cycle runs, metabolic intermediates may be utilized for different biosynthetic reactions (**Figure 1**; Spinelli and Haigis, 2018). These biosynthetic reactions not only consume the TCA cycle intermediates and direct them away from ATP production but also require substantial energy input (Ritterhoff et al., 2020). Thus, whether the intermediates will be used for synthetic purposes is dependent on the energy state of the cell. Energy requirements for sustained cardiac contractile function are high and most of the cardiac metabolism is directed toward the production of ATP (Doenst et al., 2013; Ritterhoff and Tian, 2017; **Figure 1**). Conversely, biosynthetic demands in non-proliferative cardiomyocytes of the adult heart are rather low, especially compared to highly proliferative cells such as cancer cells (Karlstaedt et al., 2018). However, biosynthesis increases considerably during cardiac hypertrophy (Karlstaedt et al., 2018; Ritterhoff et al., 2020).

Whenever metabolic intermediates are removed from the TCA cycle for biosynthetic reactions, they need to be restored to ensure the cycle's continued running (Martínez-Reyes and Chandel, 2020). This replenishment of the intermediate pool is named anaplerosis. Increased anaplerotic flux through carboxylation of glycolysis-derived pyruvate to malate was previously reported in hypertrophied rat hearts and paralleled by elevated expression of malic enzyme, which catalyzes this reaction (Sorokina et al., 2007; Pound et al., 2009). During cardiac hypertrophy, there is an energy source switch from FAO to increased glucose utilization, with a general reduction in oxidative metabolism (Doenst et al., 2013; Ritterhoff and Tian, 2017; Ritterhoff et al., 2020). Taken together, increasing the use of pyruvate for anaplerosis reduces its accessibility for oxidation and may lead to energy inefficiency of the TCA cycle (Sorokina et al., 2007; Pound et al., 2009), which contributes to contractile dysfunction and subsequent heart failure. A better understanding of how these mechanisms are regulated is needed for potential targeted treatments of cardiac dysfunction.

ROS Generation and Regulation

Mitochondria are one of the important sources of ROS production within most mammalian cells, including cardiomyocytes (**Figure 1**; Chen and Zweier, 2014). Moreover, interspecies comparisons performed in recent years show that ROS regulatory systems are dependent on organism, type of tissue, physiological state, age and pathological conditions to finely tune the underlying responses (Barja, 1999). The primary ROS generated in cardiac mitochondria is superoxide radical anion ($O_2^{\cdot -}$), which can be reduced through dismutation to hydrogen peroxide (H_2O_2). Hydroxyl radicals ($OH^{\cdot}$) are also generated from the decomposition of hydroperoxides, or by the reaction of excited atomic oxygen with water. The mitochondrial respiratory chain is a powerful endogenous source of $O_2^{\cdot -}$, which is a toxic by-product of oxidative phosphorylation (**Figure 1**). Electrons from NADH and $FADH_2$ flow through the electron chain to reduce oxygen to form H_2O (**Figure 1**). Large amounts of $O_2^{\cdot -}$ are generated when oxygen is incompletely reduced due to electron leaking at complexes I and III (Kussmaul and Hirst, 2006; Bleier and Dröse, 2013; Vinogradov and Grivennikova, 2016). Apart from the sites of ROS production within the mitochondrial respiratory chain there are other mitochondrial enzymes that generate either $O_2^{\cdot -}$ or H_2O_2. For example, NADPH oxidase 4 (Nox4) is an important source of ROS in heart mitochondria. Nox4 expression is upregulated in failing cardiomyocytes and contributes to the increase of mitochondrial $O_2^{\cdot -}$ levels that drives oxidative stress (Kuroda et al., 2010). α-ketoglutarate dehydrogenase (α-KGDH) is one of the TCA enzymes that is the most vulnerable to environmental changes (**Figure 1**; Tretter, 2004). α-KGDH generates $O_2^{\cdot -}$ during its catalytic function upon excessive NADH levels (Tretter and Adam-Vizi, 2005), making α-KGDH an important mitochondrial site for ROS production.

The ROS scavenging network coordinately works to maintain proper basal ROS levels and redox signaling in cells to control mitochondrial oxidative stress (**Figure 1**). The mitochondrial antioxidant defense system includes endogenous antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and the peroxiredoxin/thioredoxin system (discussed in greater detail in Peoples et al., 2019). ROS are not only byproducts of mitochondrial metabolism (**Figure 1**), but are commonly involved as second messengers in cellular signaling impacting both adaptive and maladaptive cardiomyocytes responses (Cave et al., 2006). The redox-sensitive cellular processes are involved

in cardiac development and differentiation, angiogenesis, cardiac regeneration and cardiomyocyte apoptosis (Tretter, 2004). Indeed, basal levels of ROS are required for human embryonic stem cells (ESCs) to differentiate into cardiomyocytes (Ji et al., 2010). In particular, accumulating evidence points to mitochondrial-mediated ROS generation as having a key role in cardiomyocyte differentiation. Nox4-dependent mitochondrial oxidative stress is one of the major pathways activated in undifferentiated ESCs, driving their differentiation into cardiomyocytes (Murray et al., 2013). Accordingly, Nox4 depletion in ESCs impairs cardiomyocyte differentiation (Li et al., 2006). Another study demonstrated Nox4 expression was significantly reduced in differentiated cardiomyocytes (Crespo et al., 2010).

Oxidative stress signaling also orchestrates angiogenesis in cardiomyocytes through Nox4 regulation. Nox4 is involved in the stimulation of angiogenesis, protecting against contractile dysfunction and hypertrophy under situations of chronic load-induced stress (Zhang et al., 2010). Furthermore, functional alterations of mitochondria and the subsequent increase of ROS production are critical in cardiac repair and regeneration. Postnatal heart maturation is associated with the transition from glycolytic to oxidative metabolism, which drives an increase in ROS production derived from the ETC and reduces cardiomyocyte regeneration capacity (Puente et al., 2014). In general, ROS levels are increased in response to heart damage including ischemic injury (Chouchani et al., 2014; Puente et al., 2014). Blocking ROS production and activation of scavenger systems promotes heart regeneration after cardiac injury (Tao et al., 2016). Conversely, mitochondrial dysfunction and the subsequent increase of mitochondrial ROS cause cardiomyocytes cell cycle arrest and activates apoptotic responses resulting in lethal dilated cardiomyopathy (Yang et al., 2018; Zhang et al., 2018). Overall, further insight into cellular mechanisms by which mitochondrial redox signaling disturbs physiological oxidative stress may uncover novel CVD therapeutic targets.

PHYSICAL AND FUNCTIONAL COMMUNICATION

In adult cardiomyocytes mitochondria mobility is limited with mitochondria moving along microtubule networks (Frederick and Shaw, 2007). In most mammalian cells mitochondria generally cluster around the nucleus (Yoon, 2004), but mitochondria can be at different cytoplasmic locations leading to mitochondrial heterogeneity within different cell types (Kuznetsov et al., 2009; Piquereau et al., 2013). In cardiomyocytes, this heterogenous population can be divided up into three separate populations, characterized by their location within the cardiomyofibers: subsarcolemmal mitochondria (SSM), intermyofibrillar mitochondria (IFM) or perinuclear mitochondria (PNM) (Shimada et al., 1984). Electron microscopy and transmission electron microscopy show these distinct populations of mitochondria as the morphology is unique for both their location and function (Shimada et al., 1984; Manneschi and Federico, 1995; Vendelin et al., 2005; Wikstrom et al., 2009). SSM are located just under the surface sarcolemma and possess closely packed cristae. Holmuhamedov and colleagues characterized and assessed SSM, finding that SSM have a high sensitivity to Ca^{2+} overload-mediated inhibition of ATP synthesis (Holmuhamedov et al., 2012). PNM are clustered at nuclear pores between and around the two nuclei commonly found in cardiomyocytes. Due to their well-developed curved cristae, PNM have little matrix area that allows for higher ATP production (Hackenbrock, 1966). Lu and coworkers provide us with one of the few studies on PNM and found that PNM morphology is more spherical than IFM and SSM, where the lack of myofibrillar constraints allows for the PNM spherical shape and its high mobility. This group also determined that PNM are physically closer to protein synthesis sites for perinuclear mitochondrial biogenesis, indicating that PNM are involved in transcription and translation processes (Piquereau et al., 2013; Lu et al., 2019). Lastly, IFM are very well organized, as they lay closely parallel to contractile myofilaments. This highly organized structure may cause IFMs to be restricted in their position and mobility; however it also provides bioenergetic support needed for contraction and mitochondrial interaction with the cytoskeleton and the SR (Wilding et al., 2006). IFM form an interface with the SR, which allows molecules to be transported between the SR and mitochondria for effective signal transduction (Eisner et al., 2013).

The SR is of extreme importance in cardiomyocytes, as it critically regulates excitation-contraction coupling by releasing its stored Ca^{2+} via the type 2 ryanodine receptor (RyR2) (Fu et al., 2006; **Figure 2**). To understand the release of Ca^{2+} by the SR, we will discuss the SR compartments. Structurally, the SR is a diverse organelle, consisting of junctional, corbular, and network SR. These components of the SR form a complex tubular network where the network SR is formed by a series of interconnected tubules that are located in the region between the transverse-tubules (T-tubules) (**Figure 2**). The junctional SR is the domain where specialized junctions are formed with the sarcolemma T-tubules, allowing the SR to bring its ryanodine sensitive Ca^{2+} channels (RyRs) in close range with the sarcolemma voltage-gated L-type Ca^{2+} channels (Franzini-Armstrong and Protasi, 1997; Franzini-Armstrong et al., 1999; **Figure 2**). The corbular SR expresses RyRs as well, however it does not form junctions with the sarcolemma (Vega et al., 2011). The membrane depolarization, as a result of excitation-contraction (EC), causes the L-type Ca^{2+} channels to open up close to the junctional SR (jSR). This results in a small amount of Ca^{2+} entering the limited cytosolic space that separates the SR and the T-tubule sarcolemma. This increase in Ca^{2+} concentration exceeds the threshold for the RyR2 to be activated through a mechanism of Ca^{2+}-induced Ca^{2+} release (CICR) (**Figure 2**; Fabiato, 1983). This activation of a small number of clustered RyR2 affects the concentration of intracellular calcium, inducing "calcium sparks" (Cheng et al., 1993). When multiple "sparks" are activated by the EC, a rise of intracellular calcium can be detected in the dyadic cleft (Sharma et al., 2000), thereby initiating myocardial contractions.

Interestingly, it has been reported that L-type Ca^{2+} channels regulate mitochondrial functions through actin filaments

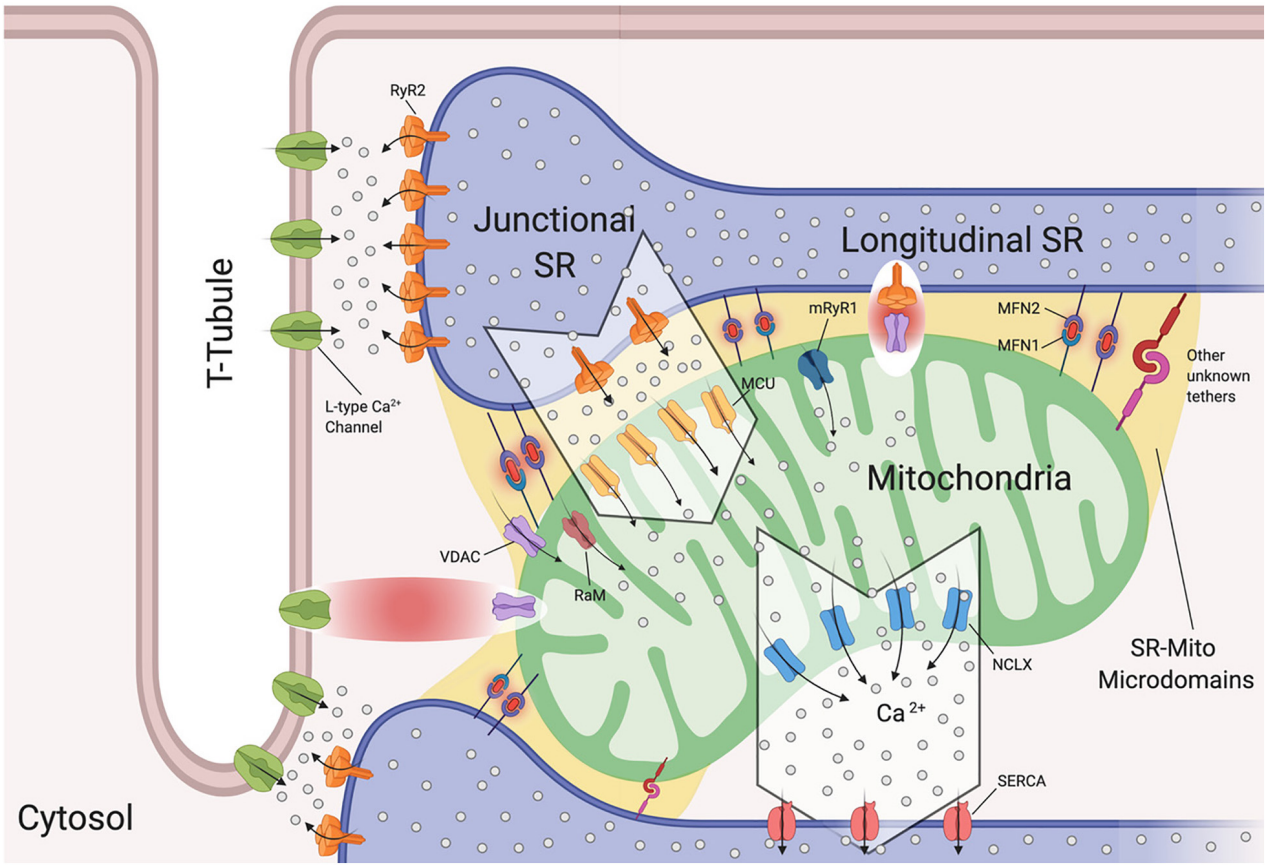

FIGURE 2 | Sarcoplasmic reticulum-mitochondria communications. Sarcoplasmic reticulum (SR) is in close proximity to T-tubules and mitochondria. Depicted are the channels involved in Ca^{2+} flux by which SR regulates excitation-contraction coupling and Ca^{2+} signaling with mitochondria. Microdomains where Ca^{2+} exchanges occur are shown. Ryanodine Receptor 2 (RyR2) and L-Type Ca^{2+} Channels located at the t-tubule-SR interface; voltage-dependent anion-selective channel proteins (VDAC) colocalize with RyR2 and also with L-Type Ca^{2+} channels, Mitochondrial Calcium Uniporter Complex (MCUC) is mainly located at the SR-Mito interface, Rapid modes of Ca^{2+} uptake (RaM), ryanodyne receptor type 1 (mRyR1), Mitochondrial Na^{+}/Ca^{2+} exchanger (NCLX) is located at the opposite side of MCUC near the sarcoplasmic/endoplasmic reticulum Ca2+ ATPases (SERCA). Mitofusin1/2 (MFN1/2) function as SR-mitochondrial tethers (Created with BioRender.com).

(Viola and Hool, 2010). Viola and colleagues demonstrated that Ca^{2+} influx through this channel increases superoxide production, NADH levels, metabolism and also mitochondrial membrane potential in a calcium independent pathway (Viola et al., 2009). The cytoplasmic β-subunit of the L-type Ca^{2+} channel is anchored to the actin cytoskeleton. Disruption of this tether decreases Ca^{2+} flux, leading to poor oxygen consumption and ATP production by the mitochondria (Viola et al., 2009). Moreover, this group found that cardiomyocytes isolated from *mdx* mice (a mouse model of Duschennes muscular dystrophy that in patients causes dilated cardiomyopathy) had impaired communication between L-type Ca^{2+} and mitochondria through an alteration in the cytoskeletal network that led to a decrease in metabolic functions (Viola et al., 2014). They were the first to show a physical and functional association between L-type Ca^{2+} and VDAC through F-actin (Viola et al., 2013, 2014; **Figure 2**). They further reported that *mdx* cardiomyocytes maintain a higher level of resting calcium and L-type Ca^{2+} channel activation plays a role in the observed mitochondrial calcium changes all of which may promote DCM (Viola et al., 2013).

Interactions between the SR and mitochondria play a key role in cardiomyocyte contraction and multiple studies have provided us with evidence of mitochondrial Ca^{2+} uptake, and thus increased mitochondrial Ca^{2+} levels, in response to SR-mediated Ca^{2+} release (Bassani et al., 1992; Negretti et al., 1993; Szalai et al., 2000). As mentioned previously, cardiac mitochondria uptake cytosolic Ca^{2+} through MCUC. However, this channel requires at least a concentration of 2–5 μM of free Ca^{2+} in the SR's bulk in order to be activated (Kirichok et al., 2004). This Ca^{2+} concentration is reached only within specific microdomains at the SR-mitochondria interface (**Figure 2**). Specifically, in such extremely structured cells, MCUC is expressed more in areas in close contact with the jSR, which contain Ca^{2+}-releasing RyR2 channels (**Figure 2**; De La Fuente et al., 2016). Moreover, Ca^{2+} fluxes must be tightly regulated. De la Fuente and colleagues demonstrated that MCUC and NCLX are spatially excluded in cardiac mitochondria (**Figure 2**) in order to optimize Ca^{2+} signals and sustain mitochondrial metabolism required for cardiomyocyte contraction, while also reducing the energy required for mitochondrial membrane

potential depolarization (De La Fuente et al., 2016, 2018). It remains controversial whether mitochondrial Ca^{2+} uptake occurs quickly and synchronously with the cytosolic Ca^{2+} fluctuations in a beat to beat model (García-Pérez et al., 2008) or if it increases slowly (Griffiths et al., 1997). The differences between these two models rely on the experimental approach used in terms of probes, stimulation and species (De la Fuente and Sheu, 2019).

It should be noted that at the microdomain level, the molecular bridge permitting Ca^{2+} exchange between SR-Mitochondria is formed by RyR2 and VDAC2 channels (**Figure 2**; Min et al., 2012). Moreover, in aged cardiomyoctes the physical interaction between RyR2 and VDAC is significantly reduced, leading to lower mitochondrial Ca^{2+} uptake, thereby promoting oxidative stress and energy impairment (Fernandez-Sanz et al., 2014). However, this event is independent of RyR2 and VDAC expression levels and does not correlate with MFN2 levels (Fernandez-Sanz et al., 2014).

It has become widely accepted that mitochondrial dysfunction is associated with heart disease (Bonora et al., 2019). Pathological SR-dependent Ca^{2+} leak through RyR2 channels is involved in excessive mitochondrial Ca^{2+} uptake (Santulli et al., 2015; Ruiz-Meana et al., 2019). Santulli et al. were the first to show using a murine model that a feedback loop exists between SR and mitochondria where Ca^{2+} leak through RyR2 channels causes mitochondrial Ca^{2+} overload and ROS burst that enhances Ca^{2+} leak and thereby worsening mitochondrial dysfunction (Santulli et al., 2015). Moreover, in human and murine senescent cardiomyocytes, the SR-mitochondria Ca^{2+} exchange is significantly impaired due to RyR2 glycation (Ruiz-Meana et al., 2019). These aged cardiomyocytes display a deficient dicarbonyl detoxification pathway initiating Ca^{2+} leak through RyR2 channels and further increasing mitochondrial Ca^{2+} uptake. Taken together, this mechanism is involved in the transition from a healthy cardiomyocyte to a failing cardiomyocyte, as it may induce bioenergetic deficit through mitochondrial damage leading to mitochondrial dysfunction (Ruiz-Meana et al., 2019).

It is important to mention that MCUC is not the only manner in which mitochondrial Ca^{2+} uptake occurs in cardiomyocytes. Three different approaches of *MCU*-knockout mice (global constitutive, cardiac-specific, dominant negative overexpression) have been developed (Pan et al., 2013; Kwong et al., 2015; Luongo et al., 2015). These models demonstrate that MCUC is dispensable for heart function in basal cardiac activity, while under "fight-or-flight" conditions MCU deletion shows inhibition of acute mitchondrial Ca^{2+} uptake. Moreover, these mice are highly protected from mPTP opening during ischemia-reperfusion injury. Therefore, in basal resting conditions, mitochondrial Ca^{2+} influx for maintaining ATP production and cardiac metabolism occurs through other channels such as rapid modes of Ca^{2+} uptake (RaM) (Buntinas et al., 2001) and ryanodyne receptor type 1 (mRyR1) (Beutner et al., 2001, 2005; **Figure 2**) both of which are located in the IMM. RaM displays a faster Ca^{2+} uptake compared to MCUC (Buntinas et al., 2001), while mRyR1 opens at lower cytosolic Ca^{2+} concentrations (Beutner et al., 2001, 2005).

As discussed above, in cardiomyocytes SR-mitochondria Ca^{2+} transfer occurs mainly in areas of direct physical contact. However, the proteins involved in the tethering have been poorly investigated in the heart. Currently, mitofusin 2 (MFN2) is the protein that has been suggested to tether this physical interaction (**Figure 2**; Papanicolaou et al., 2011; Chen et al., 2012). It remains a matter of discussion whether MFN2 acts as a tether or a spacer at the ER/SR-mito interface. Two different *MFN2*-KO mouse models have been generated showing opposite results. When the gene deletion is made after birth the distance of SR-mitochondria increased leading to a rise of Ca^{2+} concentration in the SR (Chen et al., 2012). Moreover, isopronenterol stimulation of cardiomyocytes, display an increase in cytosolic Ca^{2+} concentrations and lower mitochondrial Ca^{2} uptake. Of note, this model does not show mitochondrial bioenergetic impairment (Chen et al., 2012). On the other hand, if the gene is deleted at the embryonic stage, there are no differences in the SR-mitochondria distance and therefore, no alterations in Ca^{2+} fluxes. This result may be due to compensatory remodeling (Papanicolaou et al., 2012). Each of these mouse models demonstrates mitochondrial morphology alterations, contractile depression and cardiac hypertrophy in the adults. However, more studies are needed to investigate the role of MFN2 in tethering SR-mitochondria and also to determine whether other proteins may be involved in this tethering (**Figure 2**).

MITOCHONDRIA DYNAMICS

Fusion and Fission

Mitochondrial health is tightly correlated to the ability of these dynamic organelles to move and change their morphology in response to the surrounding environment (Ong et al., 2017). The term "mitochondrial dynamics" refers to fusion and fission processes of mitochondrial structures within a living cell. Mitochondria constantly shape themselves through fusion and fission in response to changes in energy requirements (Hu et al., 2019). The balance between mitochondrial fusion and fission determines the number (biogenesis), morphology and activity of these multifunctional organelles (Marín-García and Akhmedov, 2016). In the heart, rapid responses to body demands depend on this balance of fusion and fission that modulate multiple mitochondrial functions such as energy production, ROS generation, Ca^{2+} homeostasis and cell death (Marín-García and Akhmedov, 2016).

Several GTPases are involved in the fission and fusion processes, which utilize GTP energy to guide conformational changes (Urbani and Babu, 2019). Mitochondrial fission is driven by dynamin-related protein-1 (Drp1) that is recruited to mitochondria by human fission protein-1 (hFis1), mitochondrial fission factor (Mff) and mitochondrial dynamics proteins 49 and 51 (MiD49 and 51) (**Figure 3**; Ong et al., 2017). This process, characterized by the fragmentation of mitochondria into more restricted and rounded organelles (Sharp and Archer, 2015; Sciarretta et al., 2018), is essential for mitosis and is required for the specific clearance of injured mitochondria through mitophagy (Ong et al., 2017). Specifically, in the heart,

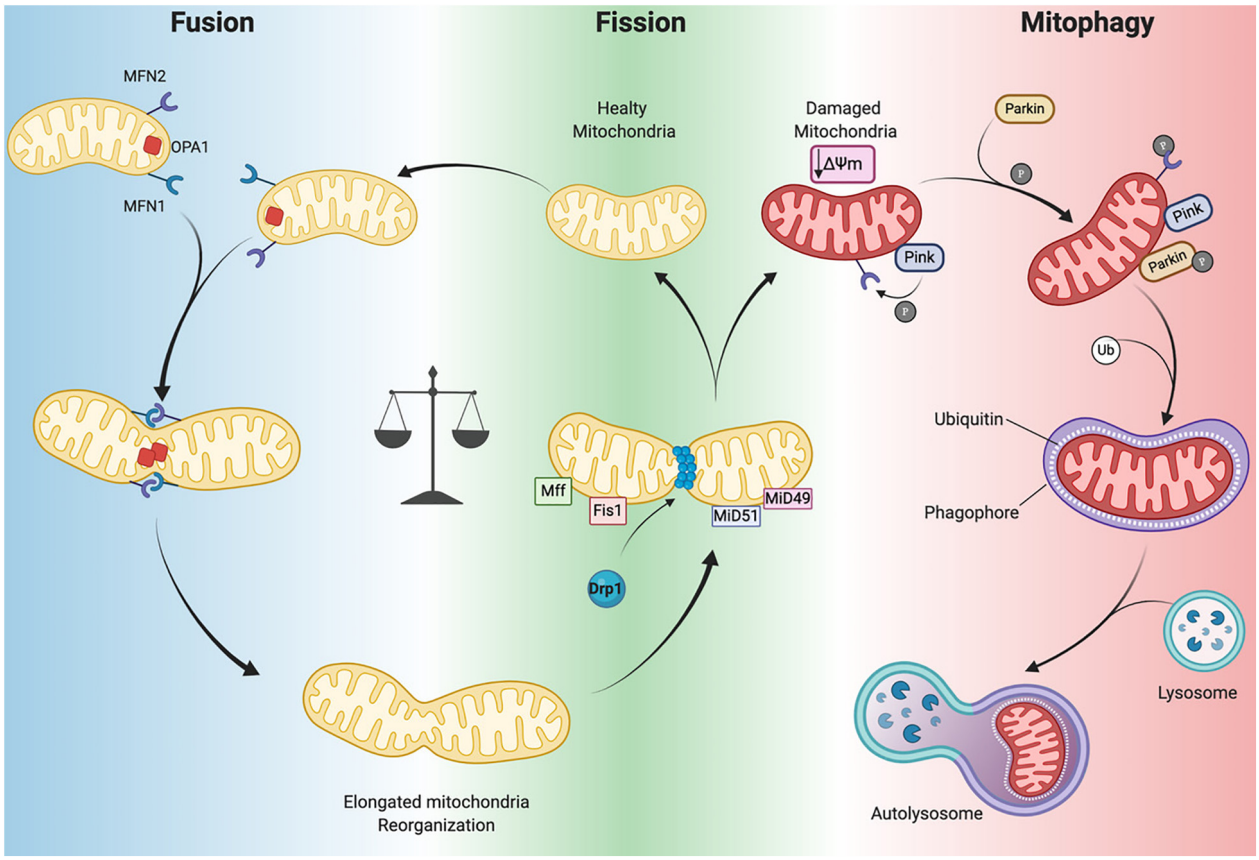

FIGURE 3 | Mitochondrial dynamics. Mitochondrial neworks are mantained by the balance betwen fusion (***left panel***) and fission (***central panel***). Damaged mitochondria are cleared by mitophagy pathway (***right panel***). Dynamin-related protein-1 (Drp1), fission protein-1 (Fis1), mitochondrial fission factor (Mff), mitochondrial dynamics proteins 49 and 51 (MiD49 and MiD51), mitofusin 1/2 (MFN1 and MFN2) optic atrophy 1(Opa1), PTEN-induced kinase 1 (PINK1) ubiquitin ligase Parkin, phosphorylation (P), Ubiquitin (Ub), mitochondrial membrane potential ($\Delta\psi_m$) (Created with BioRender.com).

mitophagy is used to preserve a healthy pool of mitochondria under stress conditions (Nan et al., 2017). Mitochondrial outer membrane fusion is directed by mitofusin 1 (MFN1) and MFN2 and mitochondrial inner membrane fusion is executed by optic atrophy 1(Opa1), leading to the formation of functional elongated organelles (**Figure 3**; Ong et al., 2017). This process is fundamental for mitochondrial DNA (mtDNA) maintenance and inheritance, membrane potential transmission and Ca^{2+} signaling within the mitochondrial machinery (Westermann, 2010; Archer, 2013; Elgass et al., 2013; Hoppins, 2014). Of note, in stress-free conditions neonatal and adult cardiomyocytes demonstrate differences in the rate of mitochondrial dynamics. Indeed, in neonatal cardiomyocytes fusion and fission processes are more frequent and rapid compared to the levels observed in adult cardiomyocytes (Forte et al., 2020). Mitochondria in neonatal cardiomyocytes are highly mobile and distributed throughout the cytoplasm within a filamentous network, while adult cardiomyocyte mitochondria are more static and spatially arranged into three subpopulations, as described above, which constrains their movements (Vásquez-Trincado et al., 2016). The homeostasis of mitochondrial dynamism is ensured by the interaction and cooperation of the aforementioned proteins. Moreover, alterations in the balance of mitochondrial dynamics are correlated to cardiac disorders (Marín-García and Akhmedov, 2016; Hu et al., 2019) leading to aberrant mitochondrial network morphology.

Mitophagy

Mitophagy is a specific form of autophagy exploited by the cellular machinery to digest dysfunctional and senescent mitochondria through autophagosomes, under basal and stress conditions (Shires and Gustafsson, 2015). This process is tightly regulated by the mitochondrial PTEN-induced kinase 1 (PINK1) and the cytosolic ubiquitin ligase Parkin. In Parkin-mediated mitophagy, upon loss of $\Delta\Psi$m, PINK1 accumulates in the OMM where it recruits Parkin (**Figure 3**). This may occur directly by PINK1-mediated phosphorylation of Parkin (Koyano et al., 2014) or indirectly by phosphorylation of MFN2 (**Figure 3**; Chen and Dorn, 2013). Indeed, Matsua and colleagues reported that upon a $\Delta\Psi$m decrease, cytosolic Parkin is recruited to mitochondria by PINK1 through Parkin's phosphorylation at Ser^{65} within its ubiquitin-like domain. This phosphorylation event is necessary for the efficient translocation of Parkin to mitochondria (an initial step of mitophagy). Upon activation

parkin initiates ubiquitination of mitochondrial proteins to promote phagasome recruitment and subsequent degradation of mitochondrial proteins by the lysosome (**Figure 3**; Kondapalli et al., 2012; Shiba-Fukushima et al., 2012; Koyano et al., 2014).

It has also been observed that the phosphorylation of MFN2 by PINK1 is essential for Parkin recruitment to damaged mitochondria, thus suggesting a connection between mitochondrial dynamics and mitophagy (**Figure 3**; Chen and Dorn, 2013). A detailed discussion of mitophagy is reviewed elsewhere (Shires and Gustafsson, 2015; Sciarretta et al., 2018). Mitochondrial autophagy plays a critical cardioprotective role; although when impaired it is detrimental to the heart (Saito and Sadoshima, 2015; Morciano et al., 2020). In mouse hearts lacking *Mfn2* expression there is a reduction in Parkin-mediated mitophagy and contractility and increased hypertrophy leading to heart failure by 30 weeks of age (Song et al., 2014). As mentioned previously, during ischemia/reperfusion (I/R) mitophagy appears to protect the heart. Indeed, in a cardiac-specific conditional *Drp1* knockout mouse the inhibition of the mitophagic flux causes accumulation of injured and dysfunctional mitochondria, leading to cardiomyocyte death during reperfusion (Ikeda et al., 2015). In addition, ablation of *Drp1* in adult mouse cardiomyocytes dampens mitochondrial fission and significantly upregulates Parkin, which leads to mitophagy and lethal cardiomyopathy (Song et al., 2015). Notably, loss of Parkin in adult mouse hearts did not affect function. However, in neonates a lethal cardiomyopathy due to defective mitophagy clearance of fetal mitochondria was observed in cardiomyocyte-specific *Parkin* ablation within three weeks after birth (Gong et al., 2015). Moreover, *PINK1* deficiency in mice leads to cardiac mitochondrial dysfunction and excessive oxidative stress (Billia et al., 2011). These findings highlight that PINK1/Parkin and the mitophagy machinery are crucial for cardiac homeostasis. Moreover, mitophagy impairment results in defective cellular homeostasis, leading to cardiomyopathy and ultimately heart failure (Nan et al., 2017; Bonora et al., 2019).

DILATED CARDIOMYOPATHY and MITOCHONDRIAL DYSFUNCTION

Cardiomyopathies are a heterogeneous condition, which effect myocardial structure and function culminating in heart failure. Due to the complexity of this disease its classification continues to evolve. However, the scenario is not simple, since any attempt at classification is limited by the criterion choice of classification itself (phenotype, etiology, clinical, morphological, functional). Additionally, the heterogeneity and all the overlapping forms of cardiomyopathy make this work more complex (Report of the WHO/ISFC task force on the definition and classification of cardiomyopathies; WHO/ISFC, 1980; Maron et al., 2006; Thiene et al., 2007; Arbustini et al., 2014).

Among all cardiomyopathies, dilated cardiomyopathy (DCM) continues to lack proper characterization and understanding (Merlo et al., 2019). It is presented with a mixed etiology and high incidence of genetic mutations. Patients with dilated cardiomyopathy may present with ventricular arrhythmias in the absence of signs of heart failure. An arrhythmogenic indication may be found in arrhythmogenic right ventricular (RV) cardiomyopathy (ARVC) and left-dominant arrhythmogenic cardiomyopathy, hypertrophic cardiomyopathy and LV noncompaction all of which are associated with increased risk of sudden cardiac death. Furthermore, DCM may also occur in patients with mitochondrial cardiomyopathy and metabolic disorders (Thiene et al., 2007; Merlo et al., 2019). Moreover, new genetic mutations continue to be identified for DC, its related peripartum cardiomyopathy and those of the arrhythmogenic cardiomyopathies, including ARVC, left-dominant arrhythmogenic cardiomyopathy, and channelopathies (Sen-Chowdhry et al., 2008, 2010; Spezzacatene et al., 2015).

In this section we will focus on how changes in mitochondrial health are associated with dilated cardiomyopathy onset and progression (**Figure 4**). Dilated cardiomyopathy characteristics resulting from gene mutations have been identified in patients with peripartum cardiomyopathy, metabolic disorders, mitochondrial dynamics, OXPHOS dysfunction, Fatty acid and cardiolipin metabolism (Barth's syndrome), all of which we will touch on here (**Figure 4**). In each of these syndromes echocardiographic data points to dilated cardiomyopathy presenting with left ventricular systolic dysfunction (left ventricular ejection fraction of <45% or fractional shortening of <30%) and cardiac hypertrophy. A number of mitochondrial diseases including OXPHOS disorders (Marin-Garcia et al., 2000; Alston et al., 2012, 2015; Jain-Ghai et al., 2013) and Barth syndrome (Hoch, 1992; Spencer et al., 2006; Gebert et al., 2009; Wang H.-Y.J. et al., 2014) correlate with the incidence of DCM, suggesting mitochondrial function as a key prognostic in the pathogenesis of DCM.

Dilated Cardiomyopathy Types and Mitochondrial Dynamics

Dilated cardiomyopathy (DCM) is a leading cause of heart failure worldwide with a higher incidence in underdeveloped countries, however its prevalence varies due to geographic and socioeconomic conditions (Bozkurt et al., 2016). It is a form of heart disease that presents with dilation of the left or both ventricles and leads to systolic dysfunction and subsequent heart failure (**Figure 4**; Dellefave and McNally, 2010; De Paris et al., 2019). DCM is also considered idiopathic if no other vascular conditions such as hypertension are detected (Mestroni et al., 1999). Between 20–35% of idiopathic DCM cases may be linked to a family history with an inherited gene defect (Hershberger et al., 2010). Primarily mutations in genes that are involved in sarcomere structure and contractility, cytoskeletal arrangement, electrolytes balance and mitochondrial function (De Paris et al., 2019) are linked to DCM. In DCM, the cardiac muscle becomes thin and weakened causing the open area of the chamber to become enlarged. As a result, the heart is unable to pump blood efficiently (NIH, 2020). In order to maintain cardiac output, ventricular volumes increase and sarcomere contractility is reduced thereby, producing the thin-walled dilated appearance that characterizes DCM (De Paris et al., 2019). This ventricular

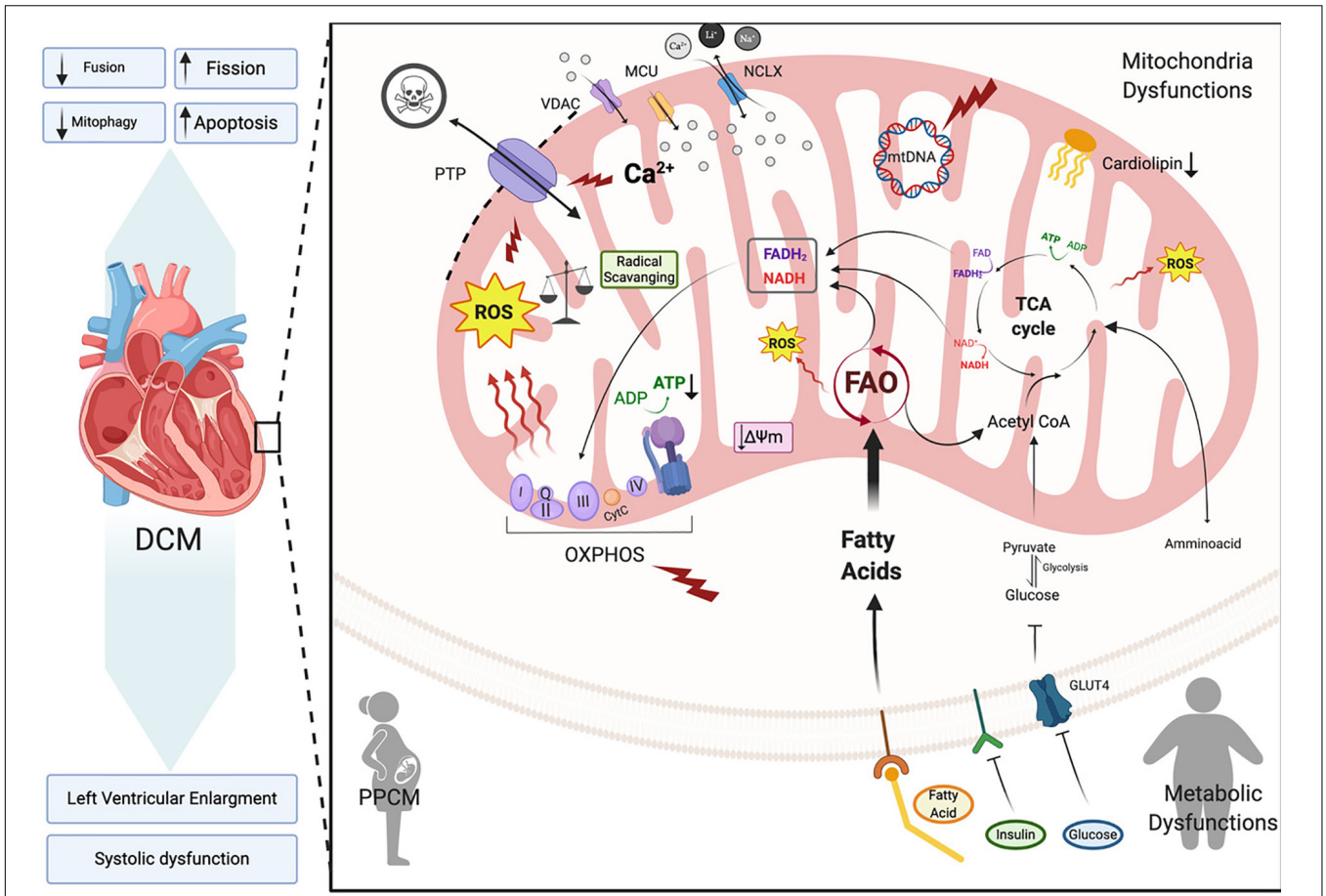

FIGURE 4 | Dilated cardiomyopathy. Schematic representation of the primary pathway of cardiomyocyte mitochondrial dysfunction, which induces dilated cardiomyopathy (DCM). Metabolic imbalance, ROS overproduction and dysregulation of Ca^{2+} homeostasis are key changes inducing dilated cardiomyopathy. These overall changes cause increased mitochondrial fission events and activation of cardiomyocyte apoptosis. Voltage-dependent anion-selective channel proteins (VDAC), Mitochondrial Calcium Uniporter Complex (MCUC), Mitochondrial Na^{+}/Ca^{2+} exchanger (NCLX), oxidative phosphorylation (OXPHOS), adenosine triphosphate (ATP), reactive oxigen species (ROS), tricarboxylic acid cycle (TCA), fatty acid oxidation (FAO), Glucose transporter type 4 (GLUT-4), mitochondrial membrane potential ($\Delta\psi_m$), cytochrome C (cyt C) (Created with BioRender.com).

remodeling is driven by irregular cardiomyocyte pathophysiology encompassing cardiomyocyte hypertrophy, impaired calcium cycling, apoptosis and fibrosis. A decrease in cardiac efficiency, as measured by myocardial oxygen consumption, leads to a progressive weakening of energy-starved cardiac myocytes pushing the heart toward heart failure (De Paris et al., 2019; Chen, 2020).

Because the heart is an organ requiring a high energy demand, regulation of mitochondrial metabolism plays an important role in the pathogenesis of this and other CVDs (Rosca and Hoppel, 2010). During the first stages of DCM, increased mitochondrial number (**Figure 4**) acts as a compensating mechanism for maintaining energy supply (Zak et al., 1980). However, the number of mitochondria declines during DCM progression leading to a reduction in ATP, decreased contractility and increased ROS all of which results in diastolic dysfunction and heart failure (Flarsheim et al., 1996; Goffart et al., 2004; **Figure 4**). Oxidative stress that presents as a consequence of increased ROS production is a key part of the pathogenesis of DCM. *In vitro* studies on isolated rat cardiomyocytes determined that physiologic stretch induces a Ca^{2+} spike through activation of NADPH Oxidase 2 (NOX2) and subsequently the ryanodine receptors in the SR. The authors found that in healthy cardiomyocytes, NOX regulation of ROS production plays a beneficial role through oxidation of the RyR2 channel, which mediates cardiac Ca^{2+}-induced Ca^{2+} release (**Figure 2**). However, in muscular dystrophy *mdx* diseased cardiomyocytes these Ca^{2+} sparks induce arrhythmogenic Ca^{2+} waves (Prosser et al., 2011). Moreover, in an *mdx* mouse model of Duchenne muscular dystrophy Ca^{2+} release led to hyperactive ROS and subsequent cardiomyopathy. Whether this occurs in DCM remains to be determined. However, in patients with DCM, NADPH-upregulation of NOX increases ROS production through elevated rac1-GTPase activity (Maack et al., 2003). Furthermore, isolated ventricular cardiomyocytes from a rabbit heart failure model display decreased Ca^{2+} uptake resulting in reduced mitochondrial NADPH availability (Despa et al., 2002). Decreased NADPH subsequently increases

ROS (Kohlhaas et al., 2010; **Figure 4**). Taken together, these findings point to Ca^{2+} controlled uptake at the mitochondria as a key regulator of ROS and an imbalance in NADPH-ROS causes disturbances in excitation-contraction coupling leading to cardiac dysfunction (Sag et al., 2013) and heart failure. ROS overproduction may also induce myocardial fibrosis, which is a common factor in DCM patients presenting with diastolic and systolic dysfunction (Assomull et al., 2006; Herpel et al., 2006). Cardiac stress, in general, plays a key role in initiating intrinsic apoptotic mechanisms in cardiomyocytes through mitochondrial dysfunction (Green and Reed, 1998). As detailed in previous sections dysfunctional mitochondria are efficiently removed by mitophagy in cardiomyocytes for cell maintenance and survival (**Figure 3**; Vásquez-Trincado et al., 2016). However, during cardiac stress, autophagy flux is reduced and damaged mitochondria accumulate resulting in enhanced oxidative stress and cardiomyocyte apoptosis (**Figure 4**; Campos et al., 2016). Uncontrolled autophagy is a component of the pathogenesis of DCM, cardiac hypertrophy and ischemic heart disease (Chistiakov et al., 2018).

Mitochondrial Fusion and Fission Alterations in Dilated Cardiomyopathy

The role of mitochondrial morphological alterations in the physiopathology of cardiomyopathies have become more apparent in recent years with changes in mitochondrial fission and fusion being at the forefront. Mitochondrial fission and fusion proteins are essential for normal cardiac remodeling and homeostasis (Chen et al., 2011). Impairments in mitochondrial morphology and function due to genetic deletion of fission and fusion proteins and their interacting partners may lead to dilated cardiomyopathy and heart failure (Nan et al., 2017; Ong et al., 2017; Hernandez-Resendiz et al., 2020). Dynamin-related protein 1 (DRP1) is involved in controlling mitochondrial fission. Genetic ablation of the *Drp1* gene is embryonically lethal at day E12.5 (Manczak et al., 2012), however cardiac-specific deletion of *Drp1* in the murine adult heart triggers mitochondrial elongation and mitophagy suppression leading to a higher susceptibility to ischemia/reperfusion and cardiomyopathy (Ikeda et al., 2015). Further studies demonstrated that Python mutant mice with a *Drp1* gene point mutation (C425F) develop mitochondrial defects and DCM, as a result of diminished mitochondrial fission and mitophagy (Cahill et al., 2015). This C452F mutation is within the M domain, which is highly conserved and involved in protein-protein interactions (Cahill et al., 2015). Moreover, mice deficient in the mitochondrial fission regulator *Mff* also develop DCM leading to heart failure and death. Interestingly, the simultaneous deletion of mitochondrial fission and fusion regulator genes *MFF (mitofission)* and *MFN (*mitofusin) rescued *Mff* knockout mice, with improved cardiac function, enhanced mitochondrial oxidative capacity and increased survival (Chen et al., 2015).

In regards to fusion, cardiac-specific ablation of mitofusins *Mfn1* and *Mfn2* in mouse embryos causes death at days E9.5–10.5 (Chen et al., 2011). At late embryonic stage, the genetic inactivation of both these mitofusins promotes mitochondrial dysfunction leading to the development of a lethal cardiomyopathy; possibly due to biogenesis alterations, diminished mtDNA and enhanced mitochondrial fragmentation (Papanicolaou et al., 2012). Furthermore, conditional cardiac *Mfn1/Mfn2* gene deletions in adult mouse hearts present with mitochondrial fragmentation, mitochondrial respiratory chain deterioration and develop a lethal DCM. Interestingly, loss of the *Mfn1* gene alone is well tolerated in mice (Papanicolaou et al., 2011; Chen et al., 2012), whereas *Mfn2*-null mice display mitochondrial enlargement (Chen and Dorn, 2013), increased ROS production (Song et al., 2014) and cardiac hypertrophy (Papanicolaou et al., 2011). Proteolytic processing of fusion protein Opa1 also plays a critical role in the regulation of mitochondrial fusion. Opa1 proteolysis by stress-activated OMA1 peptidase induces mitochondrial fragmentation and DCM onset in a cardiac-specific *Yme1l* peptidase-null mouse (Wai et al., 2015). Taken together, alterations in mitochondrial fusion and fission machinery in the heart promotes mitochondrial metabolic impairments that induce dilated cardiomyopathy.

Dilated Cardiomyopathy: Subtypes and Syndromes

Peripartum Cardiomyopathy

Peripartum cardiomyopathy (PPCM) is a rare form of DCM that develops in the last month of pregnancy or within five months postpartum. It presents with left ventricular systolic dysfunction (left ventricular ejection fraction of $< 45\%$ or fractional shortening of $<30\%$) and cardiac hypertrophy (Satpathy et al., 2008). PPCM occurs in the absence of any identifiable cause and is exclusive to patients with no prior history of heart disease (Crane, 1976; Hibbard, 1999). PPCM occurs worldwide with an incidence of 1:1,000 births (Sliwa et al., 2017; Cunningham et al., 2019) with its incidence varying geographically being the highest in the United States, South Africa, Nigeria, and Haiti. In the US, its incidence is 1:2,230 live births (Mielniczuk et al., 2006; Brar et al., 2007; Gunderson et al., 2011; Kolte et al., 2014; Cunningham et al., 2019). Mortality also varies depending location with an estimate of 3–40% of patients diagnosed with PPCM Although it is a condition of unknown etiology, it also occurs in women with a previous history of pre-eclampsia or as a result of multiple pregnancies (Kolte et al., 2014; Arany and Elkayam, 2016).

PPCM shares many similarities with DCM including clinical symptoms such as ventricular dilation and systolic dysfunction (**Figure 4**; Pearson et al., 2000; Elliott et al., 2007). However, both diseases differ in progression and outcome and there are molecular differences. Similar to DCM, oxidative stress plays a key role in the pathogenesis of PPCM in patients and in mouse models in which ROS levels are highly increased compared to controls. According to Hilfiker-Kleiner and colleagues (Hilfiker-Kleiner et al., 2007), one possible reason for this increase is due to a cardiomyocyte-specific deletion of signal transducer and activator of transcription 3 (STAT3) as studied in a mouse model of PPCM. In these mice downregulation of the antioxidant Manganese superoxide dismutase (MnSOD)

increases ROS production within mitochondria, which activates Cathepsin D that cleaves prolactin (PRL, 23 kDa) into a smaller 16 kDa piece. This negatively affects cardiomyocyte microvasculature and metabolism in these mice (Hilfiker-Kleiner et al., 2007, 2012; Hilfiker-Kleiner and Sliwa, 2014). STAT3 levels are downregulated in PPCM patient hearts suggesting that its expression may be cardioprotective during pregnancy (Ricke-Hoch et al., 2013). Importantly, PLR levels increase and remain high toward the end of pregnancy and after delivery which is in accord with the development of PPCM (Grattan et al., 2008). PPCM patients present with changes in PRL as well (Hilfiker-Kleiner et al., 2007; Haghikia et al., 2013) suggesting an oxidative stress plays a key role in PPCM.

A different mouse model of PPCM containing a cardiac specific deletion of PPARγ coactivator-1α (PGC-1α) showed that MnSOD was also reduced in the heart thus increasing ROS production and resulting in disturbed mitochondrial metabolism (Patten et al., 2012). Although research in cardiomyopathy-related genes have begun to elucidate the pathogenesis of PPCM, the molecular mechanisms underlying the development and progression of PPCM and development of targeted therapies have yet to be elucidated.

Early case studies in cardiomyopathy-related genes identified a clinical overlap between PPCM and DCM, however the extent of this interconnection remains unknown (Lee and Judge, 2017). More recent studies have reported that mutations in cardiac sarcomere proteins are a pathogenic cause of PPCM. These mutations include deleterious truncations in the titin encoding *TTN* gene that is a clinical feature shared with idiopathic DCM (van Spaendonck-Zwarts et al., 2014; Ware et al., 2016; Ballard, 2019). Titin is part of the structural organization and assembly of the sarcomere from the Z-disc to the M-line along with other developmental, regulatory and mechanical functions in cardiac and skeletal muscle (Lee and Judge, 2017). Mutations in many sarcomeric proteins located at the Z-disc may also lead to cardiomyopathies (Knöll et al., 2002; Frank et al., 2006; Sheikh et al., 2007). For example, mutations in the myosin heavy chain 7 (*MYH7*) gene that encodes the sarcomeric protein β-Myosin Heavy Chain (β-MHC) cause PPCM (Walsh et al., 2010; Fiorillo et al., 2016; Bollen and van der Velden, 2017). β-MHC forms the heavy chain structure of type II myosin in sarcomeres, which in a sliding mechanism with actin filaments, generates the mechanical forces needed for muscle contraction. Mutations in the *STAT3* gene have also been found to contribute to PPCM (Ballard, 2019; Harhous et al., 2019). Other genes in which mutations have been reported to be associated with PPCM onset and progression include truncations in *DMD* (dystrophin that causes Duchenne's Muscular Dystrophy) (Cheng and Prior, 2013; Ahmed et al., 2016), *DSP* (desmoplakin) (Ware et al., 2016), *TPM1* (α-tropomyosin)(Ware et al., 2016), and missense mutations in *MYBPC3* (cardiac myosin binding protein C) (Morales et al., 2010), *TNNC1* (cardiac troponin C) (Mestroni et al., 1994), *TNNT2* (cardiac troponin T) (Morales et al., 2010), and *LAMP2* (lysosome-associated membrane protein) (Ware et al., 2016). This list of gene mutations causing PPCM continues to grow as more genes are identified (Lee and Judge, 2017; Ballard, 2019). Additional evidence supporting an involvement of gene mutations in PPCM includes familial incidence, genome-wide association studies and variability of occurrence of PPCM among women from different regions and ethnicities (Lee and Judge, 2017).

Metabolic Disorders and Dilated Cardiomyopathy

Metabolic cardiomyopathy is a heart muscle disorder that primarily develops in the presence of chronic metabolic conditions, such as type 2 diabetes, obesity, and insulin resistance (**Figure 4**; Nishida and Otsu, 2017). These conditions are frequently overlapping, resulting in similar metabolic-related structural and functional cardiac alterations, independent of hypertension or coronary artery disease and are collectively referred to as diabetic cardiomyopathy (Nakamura and Sadoshima, 2020). During the early stage of this disorder, metabolic disturbances do not cause significant structural changes in the heart, but result in other cellular abnormalities (e.g., impaired mitochondrial function, oxidative and ER stress and altered Ca^{2+} handling) all of which contribute to changes in diastolic function (**Figure 4**; Nishida and Otsu, 2017). However, as the disease progresses, these abnormalities accumulate, culminating in cardiomyocyte death, hypertrophy, fibrosis, and diastolic and systolic dysfunction (Riehle and Bauersachs, 2019; Tan et al., 2020; **Figure 4**). The etiology of metabolic cardiomyopathy is multifactorial and has been previously reviewed (Nishida and Otsu, 2017; Riehle and Bauersachs, 2019; Nakamura and Sadoshima, 2020; Tan et al., 2020).

In the presence of obesity, type 2 diabetes, or insulin resistance, the heart functions with a dysregulated energy metabolism. More specifically, impaired insulin-receptor signaling leads to reduced translocation of glucose transporter 4 to the cell membrane resulting in reduced glucose uptake and availability for oxidation (**Figure 4**; Cook et al., 2010; Tan et al., 2020). On the other hand, increased fatty acid uptake (**Figure 4**) and utilization occur due to increased membrane localization of fatty acid translocase (FAT/CD36) and higher PPAR-α activity (Coort et al., 2004; Nakamura et al., 2019; Riehle and Bauersachs, 2019), which induces the expression of genes involved in fatty acid uptake and oxidation and further prevents glucose oxidation through stimulation of pyruvate dehydrogenase kinase 4 expression (Nakamura and Sadoshima, 2020). Consequently, high rates of FAO and loss of glucose availability increase oxygen consumption, impair cardiac efficiency, and induce mitochondrial ROS production (**Figure 4**; Boudina et al., 2007; Borghetti et al., 2018). This imbalance between fatty acid uptake and oxidation leads to excessive accumulation of lipids and lipotoxic intermediates (e.g., ceramides) in cardiomyocytes, which has been associated with increased ROS levels, ER stress, mitochondrial membrane remodeling, and cardiomyocyte apoptosis (**Figure 4**; Bikman and Summers, 2011; Wende et al., 2012; Riehle and Bauersachs, 2019). Under hyperglycemic conditions, toxic glucose intermediates may contribute to the generation of advanced-glycosylation end products (AGEs) that trigger enhanced proinflammatory and profibrotic signaling in the heart (Singh et al., 2001;

Nakamura and Sadoshima, 2020). These pathways promote increased extracellular matrix (ECM) protein production and reduced activity of ECM-degrading enzymes, both of which contribute to cardiac fibrosis and contractile dysfunction (Westermann et al., 2007; D'Souza et al., 2011; Borghetti et al., 2018). Additionally, activation of the renin-angiotensin-aldosterone system increases angiotensin II that stimulates cardiac fibrosis and hypertrophy (Kumar et al., 2012).

Despite the significant body of research reporting various possible mechanisms contributing to metabolic cardiomyopathy, the pathology of this disorder is still not entirely understood. Several studies have proposed that diabetes-induced changes in mitochondrial function lead to cardiomyopathy. A detailed review by Schilling (2015) discusses the hypothesis that diabetes promotes mitochondrial dynamic dysregulation, which is a trigger in the development of diabetic-induced cardiomyopathy progression and heart failure. However, more mechanistic studies are needed to confirm this hypothesis and understand the underlying pathobiology to advance treatment with targeted therapeutics. It should be noted that in a broader context, metabolic cardiomyopathy can also develop as a consequence of different inherited metabolic storage disorders that manifest during childhood (Albakri, 2019). However, their pathology differs from systemic disease-related cardiomyopathy and involves altered energy production due to deficiencies in certain enzymes regulating glycogen, glycolipid, and glycosaminoglycan metabolism (Guertl et al., 2001; Albakri, 2019).

Mitochondrial-Associated Dilated Cardiomyopathy

Approximately 50% of patients suffering with mitochondrial diseases also present with cardiomyopathy (Florian et al., 2015). In many cases mitochondrial cardiomyopathies have an underlying genetic component resulting in malfunction of mitochondrial respiratory chain, FAO or cardiolipin synthesis and alterations of mitochondrial dynamics (El-Hattab and Scaglia, 2016a; **Figure 4**).

Dilated Cardiomyopathy Associated With OXPHOS Dysfunction

Taking into account that cardiac muscles are one of the high energy tissues in the body, it is not surprising that mitochondrial disorders associated with OXPHOS dysfunction manifest as cardiomyopathy (**Figure 4**; Thorburn et al., 2004). Moreover, mitochondrial disorder-related cardiomyopathies may be associated with defects in the synthesis of coenzyme Q10 (Potgieter et al., 2013), synthesis of the OXPHOS Fe–S clusters, transport of adenine nucleotides across IMM maintenance of mtDNA, transfer of mitochondrial RNAs, ribosomal proteins, ribosomal RNAs and translation factors (El-Hattab and Scaglia, 2016b). Mutations in the genes encoding mitochondrial proteins often lead to aberrant OXPHOS machinery resulting in not only an ATP deficiency but also increased ROS production and/or alterations in the antioxidant defense system, nitric oxide (NO) deficiency and dysregulation of Ca^{2+} homeostasis (El-Hattab and Scaglia, 2016a,b).

Dilated Cardiomyopathies Associated With Fatty Acid Oxidation Alterations

The energy substrates primarily used by the heart include fatty acids and carbohydrates; however fatty acids are the main energy substrate for the heart and they provide the majority of cofactors crucial for mitochondrial oxidative phosphorylation. All things considered, it is not surprising that alterations in the mitochondrial FAO pathway lead to the development of heart failure. Fatty acid and glucose metabolism are interconnected to regulate each other in a process referred to as the glucose/fatty acid cycle often called Randle Cycle (Randle et al., 1963; Arslanian and Kalhan, 1994). Interestingly, in the heart an increased rate of FAO decreases glucose oxidation and conversly an increased rate of glucose oxidation inhibits FAO (**Figure 4**). Alterations in key enzymes involved in FAO lead to mitochondrial cardiomyopathy. These enzymes include very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD); trifunctional protein (TFP); carnitine-acylcarnitine translocase (CACT); carnitine palmitoyltransferase type 2 (CPT2); carnitine transporter (CT) and multiple acyl-CoA dehydrogenase (MAD) (Merritt et al., 2018). In the case of diabetic cardiomyopathy (where an oversupply of fatty acids is responsible for the observed cardiac lipotoxicity) excess fatty acids promote accumulation of lipid intermediates and surprisingly, in the case of diabetes, it is accompanied by increased FAO (**Figure 4**; Fillmore et al., 2014).

Dilated Cardiomyopathy Associated With Cardiolipin Synthesis Dysregulation

Cardiolipin is an essential constituent of IMM and contributes up to 20% of total IMM lipids (Schlame and Greenberg, 2017; Tatsuta and Langer, 2017). Due to the fact that IMM is comprised of approximately 75% protein and 25% lipid, alterations in the cardiolipin content and/or its composition have a direct impact on the structure and properties of the IMM and influence many mitochondrial processes including oxidative phosphorylation and protein translocation to mitochondria. Cardiolipin is essential (other phospholipids cannot substitute for it) for optimal function of the mitochondrial respiratory chain complexes I, III, and IV and is required for the structural integrity and formation of respiratory chain supercomplexes (**Figure 4**; Pfeiffer et al., 2003; Dudek et al., 2013; Letts et al., 2016). Moreover, changes in cardiolipin content or its' species composition contribute to higher ROS production (Paradies et al., 2004; Nickel et al., 2014). Interestingly, mutations in the *DNAJC19* gene encoding a component of the mitochondrial protein import machinery in the IMM also induces cardiolipin accumulation with altered acyl chain. This is most likely due to DNAJC19's interaction with prohibitins (PBH) to regulate cardiolipin remodeling (Richter-Dennerlein et al., 2014).

Barth Syndrome (BTHS) is a rare X-linked recessive mitochondrial cardiomyopathy caused by an altered cardiolipin metabolism. BTHS pathology includes changes in mitochondrial membrane phospholipids, lactic acidosis, organic aciduria and skeletal muscle weakness. BTHS is linked to gene mutations in the phospholipid transacylase localized to mitochondria, *taffazzin* (*TAZ)* (Dudek and Maack, 2017) and is involved in

cardiolipin acyl chain remodeling. In physiological conditions up to 90% of cardiolipin in cardiac mitochondria exist as tetralinoleoylcardiolipin. Decreases in tafazzin activity reduce cardiolipin abundance and increase monolysocardiolipin levels. There are several detailed reviews on BTHS and cardiolipin metabolism in cardiomyopathy (Ikon and Ryan, 2017; see Dudek, 2017).

TARGETING MITOCHONDRIA TO ATTENUATE DILATED CARDIOMYOPATHY

Overall patients presenting with dilated cardiomyopathy continue to progress to end stage heart failure (1:3 patients) with disease maintenance occurring in 1:4 patients. Currently, a heart transplant is the best treatment option because there are no targeted therapies available due to the high complexity of dilated cardiomyopathy (Begic et al., 2018). Targeted therapies need to be developed that treat this complex disease potentially by targeting the underlying molecular pathways that are dysregulated. For example, targeting the OXPHOS pathway and subsequent mitochondrial dysfunction may be a way to attenuate cardiomyocyte death and maintain cardiac function (Brown et al., 2017). Advances in genetic analysis and molecular pathway alterations due to these gene mutations has paved the way for early detection and focused research on the development of potential preventative therapies (Verdonschot et al., 2019). Currently, mitochondrial-specific targeting therapies for dilated cardiomyopathy have not yet entered the clinic as they are at the basic research level. As research continues it will help determine whether restoring mitochondrial function may be way an efficacious way to treat dilated cardiomyopathies.

One research area that has emerged recently is the development of cardiac-specific organoids to study the complexities of cardiovascular disease. The term organoid is used to describe *in vitro* 3D multicellular tissues generated from pluripotent and adult stem cells that recapitulate some of the key structural and functional features of their *in vivo* counterparts (de Souza, 2018). Advances in efficient differentiation of induced-pluripotent cells (iPSC) into several cardiac cell types provide a novel unlimited cell source for developing cardiac organoids, especially due to the difficulties of adult stem cell isolation and ethical concerns regarding embryonic cells (Moretti et al., 2013; Zamani et al., 2018). Unlike traditional 2D cell cultures, cardiac organoids provide more accurate representations of the complexity of cell-cell and cell-extracellular matrix interactions. Moreover, being derived from human stem cells, they reflect cardiac (patho)physiology more appropriately compared with animal models thereby helping to overcome substantial functional between-species differences and animal to human translation issues (Moretti et al., 2013).

To date, iPSC cells and derived 3D organoids are used to model several cardiac disorders caused by cardiomyocyte gene mutations including dilated cardiomyopathy, hypertrophic cardiomyopathy, Barth's syndrome and glycogen-storage cardiomyopathy (Wang G. et al., 2014; Hinson et al., 2015, 2016; Cashman et al., 2016; Nugraha et al., 2019). In addition to these monogenic diseases, cardiac organoids may provide great promise for modeling complex, lifestyle-related heart pathologies including acute myocardial infarction (Richards et al., 2020) and cardiomyopathy. For instance, relevant *in vitro* models of metabolic cardiomyopathy should embody all of the key features of the diabetic heart, including metabolic shift, lipotoxicity, insulin resistance and altered functionality (contractility and changes in mitochondrial function). Most of these characteristics have been previously recapitulated in 2D *in vitro* models that use iPSC-derived cardiomyocytes exposed to a diabetic-like environment (Drawnel et al., 2014). However, iPSC-derived cardiomyocytes are somewhat immature, with a fetal-like profile, which relies primarily on glucose utilization, compared to adult cardiomyocytes where metabolism depends on FAO. Ideally, a model of metabolic cardiomyopathy would provide evidence of this metabolic switch, which may be challenging to achieve using iPSC-derived cardiomyocytes (Jiang et al., 2018; Granéli et al., 2019). Nevertheless, considerable advances have been made in overcoming this issue of immaturity, especially using 3D culture, mechanical, or electrical conditioning techniques that provide a more adult-like cardiomyocyte phenotype in terms of oxidative metabolism, gene expression and calcium handling (Ronaldson-Bouchard et al., 2018; Golforoush and Schneider, 2020). Moreover, combining different cardiac cell types into 3D tissue-like organoids promotes the maturity and function of iPSCs-derived cardiomyocytes (Giacomelli et al., 2017; Golforoush and Schneider, 2020). Recently, cardiac organoids containing four different cell types (cardiomyocytes, epicardial, endothelial cells, and cardiac fibroblast) derived from the same iPSC have been created (Helms et al., 2019). Furthermore, Lee and colleagues developed a 3D heart organoid comprised of cardiomyocytes, conducting tissues, endothelial and smooth muscle cells, with atrial and ventricular parts, myocardial contraction, and gene expression profiles resembling their *in vivo* counterparts (Lee et al., 2020). Further research is necessary to determine how these organoids may be used to accurately model the complex pathology of dilated cardiomyopathy for a better understanding of underlying molecular mechanisms, mitochondrial function and identification of potential therapeutic targets.

CONCLUSION

Mitochondria are the central hub of the cell, controlling and regulating several different functions such as bioenergetics, biosynthesis, ROS production and cell death. Considering the high-energy demand of the heart, much research has focused on how mitochondria play a central role in maintaining myocardial homeostasis. Morphological and functional changes of mitochondria are associated with CVD. Therefore, a finely tuned healthy mitochondrial network is required in order to prevent CVDs. Mechanisms of surveillance are also required to conserve mitochondrial fitness and alterations of this quality control system, which requires a delicate balance between fusion-fission machinery and mitophagy and loss of this balance,

may lead to the accumulation of damaged and dysfunctional mitochondria. Loss of healthy mitochondria in the heart is linked directly with a decline of energy supply, increased ROS and culminates with cardiomyopathy onset and heart failure.

To date, current therapies focus on reducing heart energy demand and preventing further worsening of cardiac muscle function (Acquatella, 2000; Verdonschot et al., 2019). Continued research into the molecular mechanisms of CVD is needed to develop targeted therapies. Indeed, the continuing development of novel approaches such as organoids to study the aberrant pathology of the heart will further our understanding of how mitochondria are cardioprotective and identify new therapeutic targets for treating and preventing cardiomyopathies.

AUTHOR CONTRIBUTIONS

DR: conceptualization, writing—original draft, writing—review and editing. VM-U, FA, LM, YP, MW, IK, and MG: writing—original draft. PP: review and editing. CG: conceptualization, review, and editing. MM: conceptualization, funding acquisition, review, and editing. All authors have read and agreed to the published version of the manuscript.

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Metformin Reverses the Enhanced Myocardial SR/ER–Mitochondria Interaction and Impaired Complex I-Driven Respiration in Dystrophin-Deficient Mice

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Besides skeletal muscle dysfunction, Duchenne muscular dystrophy (DMD) exhibits a progressive cardiomyopathy characterized by an impaired calcium (Ca^{2+}) homeostasis and a mitochondrial dysfunction. Here we aimed to determine whether sarco-endoplasmic reticulum (SR/ER)–mitochondria interactions and mitochondrial function were impaired in dystrophic heart at the early stage of the pathology. For this purpose, ventricular cardiomyocytes and mitochondria were isolated from 3-month-old dystrophin-deficient mice (*mdx* mice). The number of contacts points between the SR/ER Ca^{2+} release channels (IP3R1) and the porine of the outer membrane of the mitochondria, VDAC1, measured using *in situ* proximity ligation assay, was greater in *mdx* cardiomyocytes. Expression levels of IP3R1 as well as the mitochondrial Ca^{2+} uniporter (MCU) and its regulated subunit, MICU1, were also increased in *mdx* heart. MICU2 expression was however unchanged. Furthermore, the mitochondrial Ca^{2+} uptake kinetics and the mitochondrial Ca^{2+} content were significantly increased. Meanwhile, the Ca^{2+}-dependent pyruvate dehydrogenase phosphorylation was reduced, and its activity significantly increased. In Ca^{2+}-free conditions, pyruvate-driven complex I respiration was decreased whereas in the presence of Ca^{2+}, complex I-mediated respiration was boosted. Further, impaired complex I-mediated respiration was independent of its intrinsic activity or expression, which remains unchanged but is accompanied by an increase in mitochondrial reactive oxygen species production. Finally, *mdx* mice were treated with the complex I modulator metformin for 1 month. Metformin normalized the SR/ER-mitochondria interaction, decreased MICU1 expression and mitochondrial Ca^{2+} content, and enhanced complex I-driven respiration. In summary, before any sign of dilated cardiomyopathy, the DMD heart displays an aberrant SR/ER-mitochondria coupling with an increase mitochondrial Ca^{2+} homeostasis and a complex I dysfunction. Such remodeling could be reversed by metformin providing a novel therapeutic perspective in DMD.

Keywords: Duchenne muscular dystrophy cardiomyopathy, mitochondria-associated ER membrane, mitochondrial calcium uniporter, MICU1, calcium

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common X-linked disorder (1/3,500 newborn male affected) caused by non-sense mutations in dystrophin gene and resulting in the absence of the large protein dystrophin (427 kDa) (Hoffman, 2020). Dystrophin links the cytoskeleton to a complex of proteins at the cell surface, which, in turn, interacts with the extracellular matrix. Dystrophin deficiency causes progressive muscle weakness and cardiac failure. Cardiac involvement is inevitable and progresses with age toward a dilated cardiomyopathy (DCM) with an increased frequency of ventricular arrhythmia and sudden cardiac death. Among DMD patients, the cardiac phenotype varies with age from no discernible cardiac remodeling or dysfunction to early onset of DCM with heart failure (Sasaki et al., 1998; Amedro et al., 2019; Segawa et al., 2020). Whereas more than 90% of DMD patients display echocardiographic features of left ventricular remodeling and contractile dysfunction by the age of 18 years 11/26/20 5:34:00 PM, in the murine model of DMD, the *mdx* mice, similar defects are evident at 42 weeks of age (Quinlan et al., 2004).

At the cellular level, altered calcium (Ca^{2+}) homeostasis is one of the first pathophysiological features associated with dystrophin deficiency. Before any sign of DCM and cardiomyopathy, the calcium channels of the sarcoplasmic reticulum (SR), the type 2 ryanodine receptors (RyR2), are leaky due to posttranslational remodeling (Fauconnier et al., 2010). Associated with an increased Ca^{2+} influx due to sarcolemmal damages and overactivation of stretch-activated channels and/or L-type Ca^{2+} current, these defects increase the diastolic Ca^{2+} level and promote fatal cardiac arrhythmias (Williams and Allen, 2007a; Jung et al., 2008; Ullrich et al., 2009; Fauconnier et al., 2010; Prosser et al., 2011). In parallel, DMD is associated with a progressive deterioration of the mitochondrial ultrastructure and function (Kyrychenko et al., 2015). Mitochondrial defects were mainly characterized in skeletal muscle where a significant uncoupling of the oxidative phosphorylation, defects in complex I, and a reduction of ATP synthesis were observed (Sperl et al., 1997; Kuznetsov et al., 1998; Percival et al., 2013; Rybalka et al., 2014; Moore et al., 2020). In heart muscle, a metabolic shift from fatty acid oxidation to carbohydrate oxidation has also been observed prior to the onset of DCM and heart failure (Khairallah et al., 2007, 2008; Burelle et al., 2010). Furthermore, mitochondrial Ca^{2+} uptake as well as mitochondrial reactive oxygen species (ROS) production and oxidative damages are increased in the DMD *mdx* mouse model and contribute to the pathogenesis of heart failure in DMD (Williams and Allen, 2007b; Dubinin et al., 2020; Hughes et al., 2020). Importantly, the first signs of mitochondrial and bioenergetic deficiencies precede the decline in myocardial function. Therefore, targeting mitochondrial function and/or metabolism in DMD has become a therapeutic issue over the last decade. Among the therapeutic candidates, the antidiabetic drug metformin has recently been evaluated in DMD both in preclinical studies and in clinical trials (Ljubicic and Jasmin, 2015; Hafner et al., 2016, 2019; Mantuano et al., 2018; Vitiello et al., 2019). This N,N-dimethylbiguanide interferes with the activity of mitochondrial complex I and activates AMP-activated protein kinase (AMPK), a critical hub for metabolic-mediated signaling pathways (for a review, see Foretz et al., 2014). Although the variability of the severity profile and the different stages of the pathology will require larger-scale clinical trials, treatment with metformin in combination with nitric oxide precursors has shown encouraging positive effects, particularly on motor function (Hafner et al., 2019). In *mdx* mice, long-term treatment with metformin alone also improves diaphragm function and limits muscle damage and the development of fibrosis due to exercise (Mantuano et al., 2018). To date, the only evidence of a beneficial effect of metformin in the development of cardiomyopathy associated with DMD is a decrease in the mass index of the heart (Mantuano et al., 2018).

The tight connection between the sarco-endoplasmic reticulum (SR/ER) and the mitochondria, also called mitochondria-associated ER membrane (MAM), is essential for the maintenance of energy metabolism, Ca^{2+} homeostasis, and cell fate (Rossini and Filadi, 2020). The tethering is maintained by mitofusin-2 (MFN2) as well as by a macromolecular complex comprising the SR/ER Ca^{2+} channel IP3 receptor 1 (IP3R1) and the porine of the outer membrane of the mitochondria (OMM), VDAC1 (voltage-dependent anion channel 1). These two channels are linked by a chaperone, GRP75, forming a direct Ca^{2+} channeling between the SR/ER and the mitochondria (Lee and Min, 2018). To cross the inner mitochondrial membrane (IMM), Ca^{2+} passes through the mitochondrial Ca^{2+} uniporter (MCU) complex creating an IP3R1–GRP75–VDAC–MCU Ca^{2+} transfer axis (Lee and Min, 2018). MCU is a highly selective Ca^{2+} channel which comprises a membrane-spanning 40-kDa protein that forms a low-conductance Ca^{2+}-selective pore. MCU exists as a large protein complex (∼480 kDa) comprising MICU1 and MICU2 which give it its Ca^{2+} sensitivity (Tarasova et al., 2019). This SR/ER–mitochondria interaction and contact points have never been studied in DMD hearts. However, at the early stage of the pathology, we have recently demonstrated an impaired SR/ER–mitochondria interaction in *mdx* skeletal muscles associated with an alteration of Ca^{2+} homeostasis, and increase in the unfolding protein response (UPR or ER stress) and muscle weakness (Pauly et al., 2017).

The aim of this study was thus to establish whether, in *mdx* mice that show no signs of DCM, the interconnection between SR/ER and mitochondria is modified and whether it may interfere with myocardial mitochondrial function. Our results demonstrate that increased SR/ER–mitochondria interaction is associated with increased mitochondrial Ca^{2+} content and a disrupted mitochondrial function. One-month metformin treatment reversed these defects providing a novel therapeutic perspective in DMD.

METHODS

Animal and Cell Isolation

All experiments were conformed to the institutional ethics committee for animal experiments and received the agreement from the national *Ministère de l'enseignement supérieur et de la recherche* (N° #16473-2018082016141320). Male *mdx* or WT (wild-type, C57bl/10ScSn) mice (CNRS, IGMM, France) were

used at 3 months of age. According to the animal-to-human translation factor for drug treatments and as previously shown, a daily dose of metformin of 200 mg/kg/day was given for 1 month in 50 ml of drinking water (Reagan-Shaw et al., 2008; Mantuano et al., 2018). Mice receiving metformin treatment were housed in a single cage to ensure homogeneity between groups. Left ventricular myocytes were enzymatically dissociated as previously described (Fauconnier et al., 2005). In brief, after removal, the heart was retrogradely perfused at 37°C with a modified enzyme solution [0.1 mg.mL^{-1} of Liberase™ (Roche)] containing 113 mM NaCl, 4.7 mM KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4, 12 mM NaHCO3, 10 mM KHCO3, 10 mM Hepes, 30 mM taurine (pH 7.4). Isolated myocytes were then transferred to the same enzyme-free solution containing 1 mM $CaCl_2$ prior experiments.

Western Blot

Twenty to thirty micrograms of total or mitochondrial protein was separated on SDS-polyacrylamide gel and electro-blotted onto a nitrocellulose membrane (Bio-Rad). Membranes were saturated with blocking buffer for 1 h at room temperature and incubated overnight at 4°C with monoclonal mouse anti-VDAC (1:1000, Abcam), anti-OXPHOS (1:5000, Abcam), anti-IP3R1 (1:1000, Santa Cruz), anti-NDUFA13 (1:1000, Abcam), anti-GAPDH (1:10 000, Abcam) or with polyclonal rabbit anti-MCU (1:500, Abcam), anti-MICU1 (1:500, Thermo Fisher), anti-MICU2 (1:500, Sigma Aldrich), anti-PDH-E1α (1:1000, Abcam), anti-PDHE1α phosphor Ser 293 (1:1000, Abcam), anti-PDHE1α phosphor Ser 300 (1:1000, Millipore), anti-PDHE1α phosphor Ser 232 (1:1000, Calbiochem), anti-PDK4 (1:1000 Novus Biotech), anti-GRP75 (1:1000, Santa Cruz), anti-SIGMA1R (1:1000, Cell Signaling), anti-MFN2 (1:1000, Abcam), and anti-Hsp60 (1:1000, Abcam). Hsp60 and GAPDH were used as loading controls. All immunoblots were developed and quantified using the Odyssey infrared imaging system (LICOR Biosystems) and infrared-labeled secondary antibodies. Band intensities were quantified with ImageJ.

Quantitative Real-Time RT-PCR

Isolated cardiomyocyte RNA was extracted with the TRIzol Reagent (Life Technologies). The level of target mRNAs was measured by reverse transcription (Superscript II, Invitrogen, 1 μg total RNA) followed by quantitative real-time PCR using a RotorGene (Corbett Research). TATA-binding protein (TBP) mRNA was used as a housekeeping gene *Tbp* (forward 5′-TGG TGTGCACAGGAGCCAAG-3′, reverse 5′-TTCACATCACA GCTCCCCAC-3′). The primer sequences of ER stress-related genes are *Hspa5* forward 5′-CCACCTCCAATATCAACTTG-3′, *Hspa5* reverse 5′-ACGATCAGGGCAACCGCATCA-3′; *Ddit3* forward 5′-CTGGAAGCCTGGTATGAGGA-3′, *Ddit3* reverse 5′-CTCTGACTGGAATCTGGAGA-3′; total *Xbp1* forward 5′-GTGCAGGCCCAGTTGTCACC-3′, *Xbp1* reverse 5′-TCTG GGTAGACCTCTGGGAG-3′; U-*Xbp1* forward 5′-CAGACTA TGTGCACCTCTGC-3′, U *Xbp1* reverse 5′-TCTGGGTAGAC CTCTGGGAG-3′; *Atf3* forward 5′-CCAGGTCTCTGCCTCA GAAG-3′, *Atf3* reverse 5′-CATCTCCCAGGGGTCTGTTGT-3′; *Atf4* forward 5′-TCGATGCTCTGTTTCGAATG-3′, *Atf4* reverse 5′-AGAATGTAAAGGGGGCAACC-3′; *Atf6* forward 5′-TCGA GGCTGGGTTCATAGAC-3′, *Atf6* reverse 5′-CTGTGTACTG GACAGCCATC-3′.

Proximity Ligation Assay

The Proximity Ligation Assay (PLA) was performed as previously described (Paillard et al., 2013; Pauly et al., 2017). Cells were fixed with paraformaldehyde 4% and permeabilized at RT with 0.1% Triton-X100. After washing, they were incubated with blocking buffer for 30 min at 37°C. The blocking solution was removed before incubation of primary antibodies (anti-VDAC Abcam, ab1734, 1:200, and anti-IP3R1, Santa Cruz, sc-28614, 1:200, or anti-GRP75, Santa Cruz, sc-13967) overnight at 4°C. The cells were washed two times using PBS with 0.01% Tween. The two PLA probes 1:5 were prepared in antibody diluent 20 min before incubation for 1 h at 37°C. Next, cells were incubated with mix containing 5× ligation stock (diluted 1:5 in water) and 1× ligation solution (diluted 1:40) for 30 min at 37°C. Next, the cells were incubated with mix containing 5× amplification stock (diluted 1:5 in water) and polymerase (diluted 1:80) for 100 min at 37°C. Finally, the cells were washed with Dapi (diluted 1:500) in wash buffer B 1× and mounted using Dako fluorescent mounting medium (S3023) and analyzed using a fluorescence microscope (excitation: 594 nm, emission: 624 nm, magnification: 40×).

Mitochondrial Isolation and Endogenous Mitochondrial Ca^{2+} Content

Mitochondrial isolation protocols were performed and adapted from Frezza et al. (2007). Briefly, left ventricles (LV) or isolated cardiomyocytes were homogenized with Teflon pestle in cold isolation buffer (pH 7.4, 225 mM Mannitol, 75 mM Sucrose, and 30 mM Tris with anti-protease 1×) and homogenized by 10 strokes with a motorized Potter Elvejhem (1500 rpm). Nuclei and cellular debris were pelleted at 800 g for 10 min at 4°C. Supernatants containing mitochondria were centrifuged twice at 9000 g for 10 min to pellet mitochondria. Mitochondria were resuspended in isolation buffer, and protein concentration was determined by a bicinchoninic assay kit. Isolated mitochondria pellets were diluted in HCl 0.6 N, homogenized, and sonicated. After incubation during 30 min in boiling water, mitochondria were centrifuged for 5 min at 10 000 g, and the supernatant was recovered. Ca^{2+} content in supernatant was determined spectrophotometrically (Tecan) using an o-cresolphthalein complexone assay according to the manufacturer's instructions (TECO Diagnostics). Results are expressed in mM of Ca^{2+} per μg of protein.

Mitochondrial Respiration and Mitochondrial Ca^{2+} Uptake

Respiration was measured on digitonin-permeabilized cardiomyocytes (15 μg per million cells) in the respiratory buffer containing 10 mM KH_2PO_4, 3 mM $MgCl_2{\cdot}6H_2O$, 0.5 mM EGTA, 20 mM taurine, 60 mM K-lactobionate, 20 mM HEPES, 110 mM sucrose, and 1 g/L BSA, pH 7.1. The respiratory rates of 10 000 cardiomyocytes were recorded at 37°C using a high-resolution Oxygraph respirometer (Oroboros, Innsbruck, Austria) after

addition of different substrates. Briefly, the aerobic glycolysis pathway was stimulated by addition of pyruvate and malate. Pyruvate, the last substrate generated by glycolysis, and malate activate the Krebs cycle after their conversion in acetyl coenzyme A and oxaloacetic acid, respectively. Under these conditions, the complex I of the mitochondrial respiratory chain was activated by NADH produced. Complex II-driven respiration was obtained by addition of succinate and rotenone (to inhibit the complex I), and complex IV-driven respiration was permitted by addition of antimycin A (to inhibit the complex III), ascorbate and N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) to reduce cytochrome c. In some experiments, the free Ca^{2+} was set to 400 nM in the respiratory buffer using the Maxchelator program (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/). The respiratory coupling ratio (RCR) was measured in all conditions, and no differences between groups were observed (**Supplementary Figure 1**). Mitochondrial Ca^{2+} uptake was measured with Calcium Green (Invitrogen) with the O2k-Fluorescence LED2-Module on isolated mitochondria in the presence of thaspigargin (10 μM). The decay time constant of the calcium green fluorescence was measured after application of 25 μM extramitochondrial Ca^{2+} and reflects mitochondrial Ca^{2+} uptake.

Enzymology

Activity of the pyruvate dehydrogenase (PDH) was obtained by using Pyruvate Dehydrogenase Enzyme Activity Microplate Assay (Abcam ab109902) and 100 μg of mitochondria isolated from heart homogenates according to the manufacturer's instruction. Mitochondrial enzymatic activities of complexes I and IV and citrate synthase were measured on mitochondria isolated from heart homogenates at 37°C using a SAFAS spectrophotometer and standard method (Angebault et al., 2018).

ROS Measurement

Mitochondrial anion superoxide production was monitored using the fluorescent indicator MitoSOX Red (Invitrogen) and confocal microscopy as described previously (Fauconnier et al., 2007). Briefly, cardiomyocytes were loaded with MitoSOX Red (5 μM) for 30 min at room temperature, followed by washout. Confocal images were obtained after 5 min of 1-Hz stimulation in standard Tyrode solution and subsequently after addition of antimycin A (50 μM) to estimate the maximal complex I superoxide production capacity. MitoSOX Red fluorescence was measured in the same region of the cell at each time point. The signal from each cell was normalized to that immediately before pacing.

Statistics

All data are presented as mean ± SEM. Normality of distribution, controlled by Agostino–Person omnibus normality test, not being respected; the Mann–Whitney test was used for all experiments. For multiple comparisons, the non-parametric Kruskal–Wallis test was applied. The significance level was set at $\alpha = 0.05$.

RESULTS

SR/ER–Mitochondria Interaction and Mitochondrial Ca^{2+} Uptake in *mdx* Cardiomyocytes

We first determined whether SR/ER–mitochondria interactions are altered in mdx mouse cardiomyocytes as previously reported in skeletal muscle, namely, a decrease in SR/ER–mitochondria interaction (Pauly et al., 2017). For this purpose, we first measured the expression level of proteins involved in the SR/ER-mitochondria tethering. Although the MFN2 expression as well as VDAC1 and GRP75 levels were similar in both groups, IP3R1 and its regulatory subunits SIGMA-1R (Sig-1R) were significantly increased in *mdx* cardiomyocytes (**Figures 1A,B**). The expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase type 2a (SERCA2a) and its regulatory protein, phospholamban (PLB), and its phosphorylation were also unchanged, although the Ca^{2+} load of the SR was reduced (**Supplementary Figure 2**). We next evaluated the contact points between the two organelles using the *in situ* PLA assay. The IP3R1–VDAC1 interaction was significantly enhanced as well as the GRP75–VDAC1 connections, indicating that the physical SR/ER–mitochondria interconnection *via* IP3R1 and VDAC is enhanced in *mdx* cardiomyocytes (**Figure 1C**). However, the immunoprecipitation of IP3R1 did not show a difference in the VDAC/IP3R1 and GRP75/IP3R1 ratio meaning that the increase is at least in part related to the increase in the expression of IP3R1 (**Supplementary Figure 3**). In addition, except *Atf3* gene expression which increases, mRNA levels of ER stress markers were unchanged in *mdx* heart (**Supplementary Figure 4**). The increased IP3R1–GRP75–VDAC1 interaction is supposed to enhance the direct channeling for Ca^{2+} from the SR/ER to the mitochondria. This is reinforced by an increase in both MCU and MICU1 expression level as reported recently (Dubinin et al., 2020). Importantly, the MICU2 expression remained unchanged (**Figures 2A,B**). This structural remodeling was associated with a faster mitochondrial Ca^{2+} uptake indicating functional changing of the MCU complex (**Figure 2C**). Similarly, mitochondrial Ca^{2+} content was also significantly elevated compared to WT mitochondria (**Figure 2D**). Unlike skeletal muscle, these data indicate an increase in SR/ER–mitochondria interactions and mitochondrial Ca^{2+} uptake in the *mdx* heart.

PDH Activity in *mdx* Cardiomyocytes

Once in the mitochondrial matrix, Ca^{2+} regulates several processes that interfere with the mitochondrial function and the metabolic flux. Among them, Ca^{2+} controls the activity of the PDH, which is the entry point for the glycolytic product pyruvate into the oxidative metabolism. The PDH phosphorylation by the PDH kinase (PDK) inhibits its activity whereas its dephosphorylation by the Ca^{2+}-dependent PDH phosphatase (PDP) increases its activity. We thus measured the PDH phosphorylation in *mdx* LV mitochondria. The total PDH content and the PDK4 expression were comparable in both groups; however, the phosphorylation levels of the three phosphorylable serines (pPDH-ser232, pPDH-ser293, and pPDH-ser 300) were

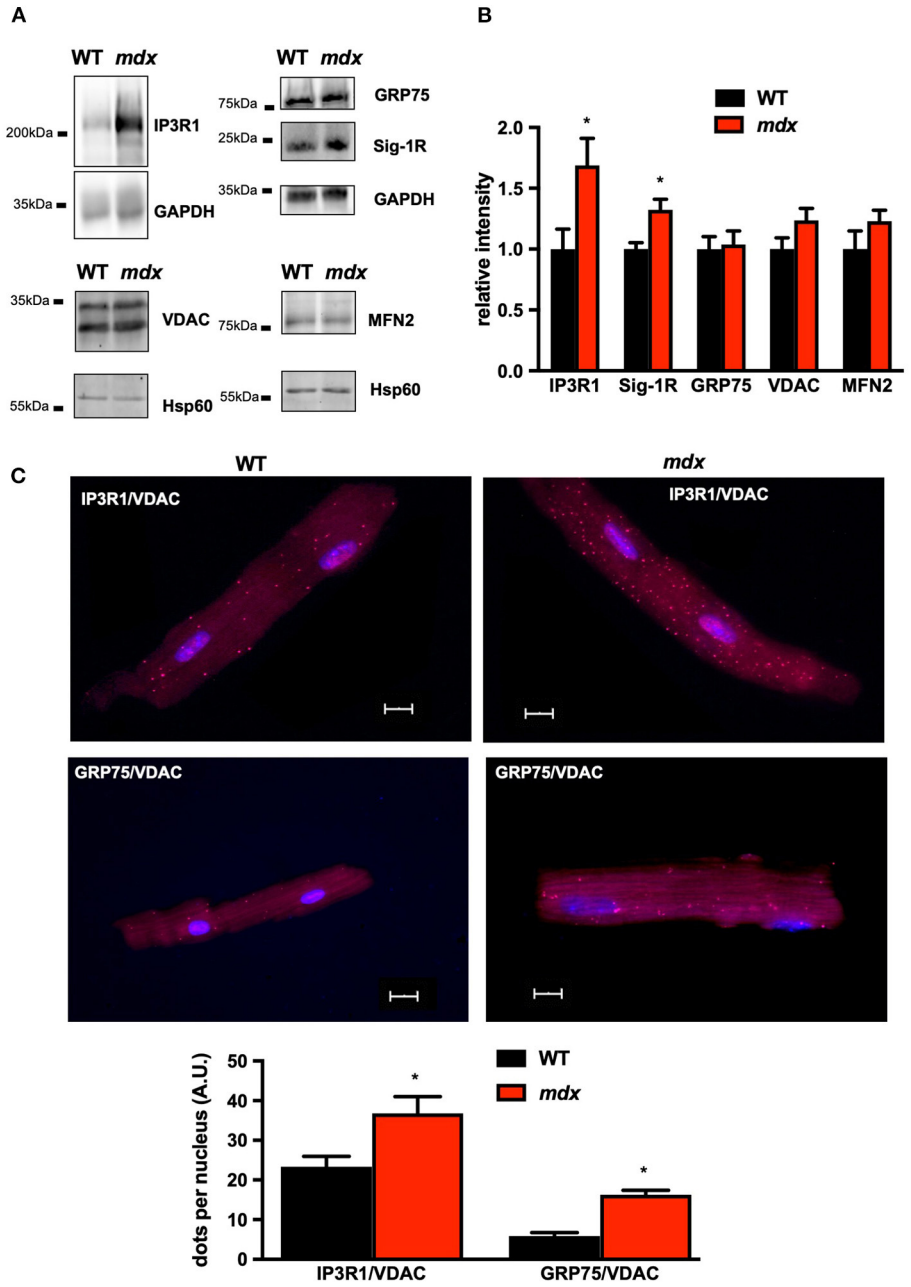

FIGURE 1 | Increase in interconnection between SR/ER and mitochondria in *mdx* cardiomyocytes. All quantifications of proteins were normalized either to GAPDH when heart homogenates were used or to Hsp60 when Western blot were performed on isolated mitochondria and expressed relative to WT. **(A)** Representative immunoblots and quantification **(B)** of IP3R1, GRP75, Sig-1R, and VDAC. Quantification of IP3R1, GRP75, MFN2, and Sig-1R was carried out from $N = 6$ *mdx* and WT hearts and from $N = 5$ *mdx* and WT hearts for VDAC. Data are mean ± SEM. For IP3R1 $^*p = 0.0317$ and for Sig-1R $^*p = 0.0043$ *mdx* vs. WT. **(C)** Representative images and quantitative analysis of IP3R1-VDAC and GRP75-VDAC1 interaction measured by *in situ* PLA on isolated cardiomyoctytes from *mdx* ($N = 3$, $n > 49$) and WT ($N = 3$; $n > 44$). The numbers of dots were normalized to the number of nucleus per cells. Data are mean ± SEM. $^*p = 0.0057$ *mdx* vs. WT for IP3R1-VDAC and $p < 0.0001$ *mdx* vs. WT for GRP75-VDAC1.

significantly reduced (**Figures 3A,B**; **Supplementary Figure 5**). Furthermore, the PDH activity was significantly increased in LV *mdx* mitochondria consistent with the decrease in PDH phosphorylation (**Figures 3B,C**).

Ca^{2+} Regulates Oxidative Phosphorylation in *mdx* Cardiomyocytes

Increased PDH activity may impact carbohydrate metabolic flux and pyruvate-mediated oxidative phosphorylation. We

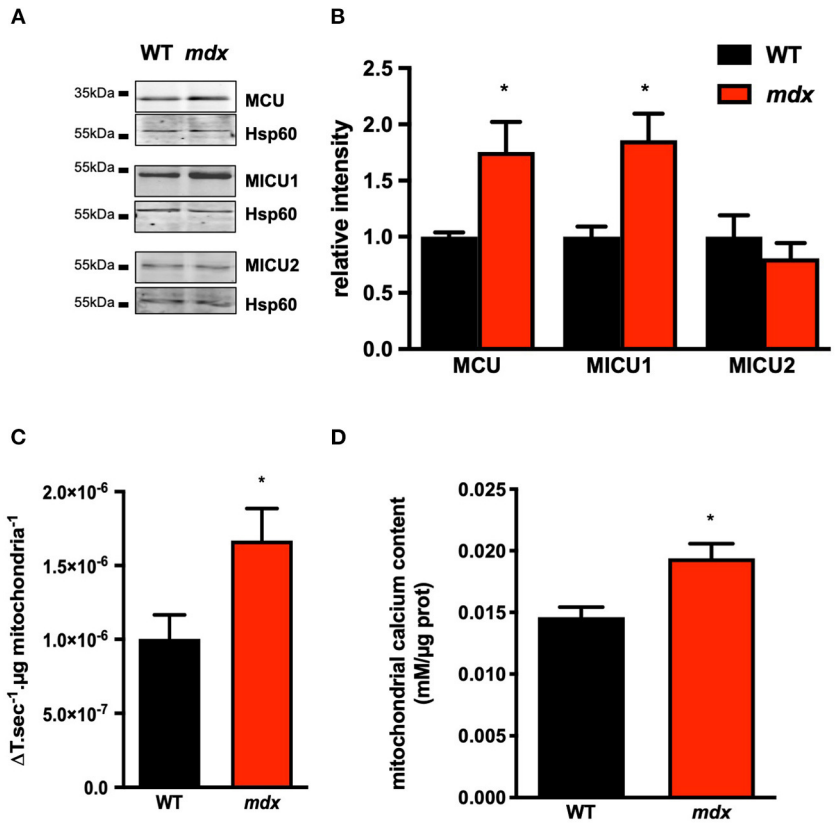

FIGURE 2 | Increase MCU/MICU1 expression and mitochondrial Ca^{2+} in *mdx* cardiomyocytes. All Western blots were performed on isolated mitochondria and quantifications of proteins were normalized to Hsp60 and expressed relative to WT. **(A)** Representative immunoblots and quantification **(B)** of MCU, MICU1, and MICU2. Data are mean ± SEM, $^*p = 0.0043$ and $^*p = 0.0022$ for MCU and MICU1, respectively, *mdx* ($N = 6$) vs. WT ($N = 6$). **(C)** The mean rate of mitochondrial Ca^{2+} uptake measured as the decay time constant of calcium green fluorescence in presence of 25 µM of Ca^{2+}. Data are mean ± SEM, $^*p = 0.0476$ *mdx* ($N = 6$) vs. WT ($N = 5$). **(D)** Mean of the absolute mitochondrial Ca^{2+} content in *mdx* ($N = 6$) and WT ($N = 6$) hearts. Data are mean ± SEM, $^*p = 0.0152$ *mdx* vs. WT.

next measured maximal mitochondrial respiration rates in a Ca^{2+}-free conditions using pyruvate/malate as carbohydrate substrates to feed the complex I. In the presence of ADP, the complex I-mediated oxygen consumption was significantly reduced whereas complex II- and complex IV-related respiration and the RCR were comparable in both groups (**Figure 4A**; **Supplementary Figure 1**). These results indicate that only the complex I-driven oxygen consumption is altered in *mdx* mice. In order to determine whether the decrease in the respiration mediated by complex I is related to Ca^{2+}, we measured complex I-driven oxygen consumption in the presence of 400 nM Ca^{2+}. In this condition, the complex I-mediated respiration rate was significantly enhanced in *mdx* mitochondria compared to WT (**Figure 4B**), while the expression of complex I subunits is unchanged (**Figures 4C,D**). It is noteworthy that the expression of the other complexes is also unchanged compared to WT (**Supplementary Figure 6**). Another parameter which could explain the decrease in complex I-driven oxygen consumption is the capacity of the complex I to transfer electron. We performed quantification of enzymatic activity, but we did not observe any modification in enzymatic activities of complexes I and IV nor of the activity of citrate synthase in *mdx* mice (**Figure 4E**). A preserved complex I activity and a reduced complex I-mediated respiration could indicate an electron leak at the complex I level and superoxide anion ($O_2.-$) production. We have indeed observed an increase in Mitosox red fluorescence in paced *mdx* cardiomyocytes but also in the presence of the complex III inhibitor antimycin A, demonstrating an increase in the production of $O_2.-$ at the level of complex I compared to WT (**Figure 4F**). Altogether, these data indicate an impaired carbohydrate-mediated mitochondrial respiration at the level of complex I which is partly compensated by the increase in mitochondrial Ca^{2+}.

Metformin Improves SR/ER-Mitochondrial Interaction and Mitochondrial Function

We next investigated the effects of a treatment with metformin, the antidiabetic drugs targeting mitochondrial complex I. After 1 month in drinking water, the level of phosphorylation of AMPK and acetyl-CoA carboxylase (ACC), one of the downstream targets of AMPK, was significantly increased validating the efficacy treatment with metformin (**Supplementary Figure 7**). We next evaluated the SR/ER–mitochondria interaction using

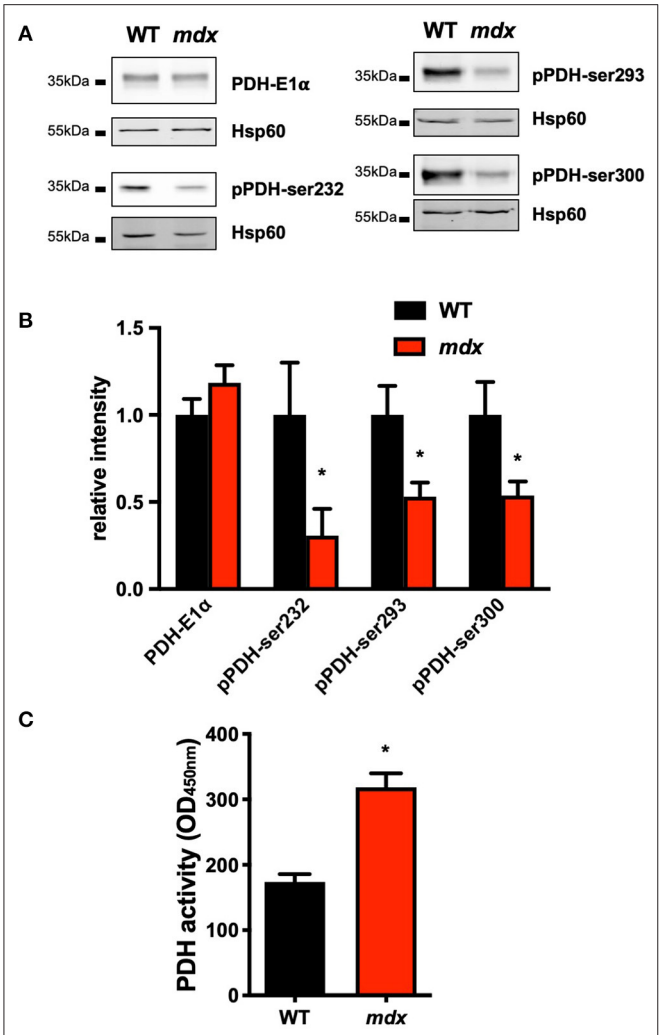

FIGURE 3 | Increase PDH activity in *mdx* hearts. All Western blots were performed on isolated mitochondria and quantifications of proteins were normalized to Hsp60 and expressed relative to WT. **(A)** Representative immunoblots and **(B)** quantification of total pyruvate dehydrogenase subunit E1α (PDH-E1α), PDH phosphorylated on Serine 232 (*$p = 0.0262$), 293 (*$p = 0.0499$), and 300 (*$p = 0.0281$). Data are mean ± SEM, *mdx* ($N = 8$) vs. WT ($N = 8$). **(C)** Mean PDH activity measured on isolated mitochondria. Data are mean ± SEM, *$p = 0.0043$ *mdx* ($N = 6$) vs. WT ($N = 6$).

in situ PLA assay. As shown in **Figure 5**, metformin treatment decreased IP3R1/VDAC contacts (**Figures 5A,B**) whereas the expression levels of IP3R1, VDAC, GRP75, or Sig-1R remained similar (**Supplementary Figure 7**). Although MCU expression is also unchanged, MICU1 level is decreased and this is accompanied by a decrease in the mitochondrial Ca^{2+} content (**Figures 5C,D**). The decrease in mitochondrial Ca^{2+} content is accompanied by an increase of the phosphorylation level of the PDH but only on the Ser232 site (**Figures 6A,B**). However, this is not sufficient to affect the PDH activity which remained comparable to untreated *mdx* mice (**Figure 6C**). Finally, the complex I-mediated oxygen consumption under pyruvate/malate substrate is significantly enhanced (**Figure 6D**), indicating that metformin treatment in addition to restoration of SR/ER-mitochondrial interactions optimizes mitochondrial function in *mdx* ventricular cardiomyocytes.

DISCUSSION

Dystrophin deficiency causes profound striated skeletal muscle lesions which lead to major muscle weakness at the early stage of the pathology and ultimately to fatal respiratory failure. Secondary to peripheral muscle deficiencies, a progressive cardiomyopathy develops with left ventricle dilatation, fibrosis, and arrhythmias (Sasaki et al., 1998; Fauconnier et al., 2010; Amedro et al., 2019; Segawa et al., 2020). Over the last decade, due to the improvement of the management of patients' respiratory failure, cardiac failure has become a major cause of death. Although cardiac and skeletal muscles share similar pathophysiological mechanisms, which are more or less shifted in time, some of these processes appear to be regulated differently. Indeed, despite an increase of IP3R1 expression in both tissues, here we demonstrated an increase in SR/ER–mitochondria interactions, characterized by an increase in IP3R1–GRP75–VDAC contact points, whereas in skeletal fibers such interactions were decreased (Pauly et al., 2017). Interestingly, the IP3R1/GRP75 or IP3R1/VDAC1 protein–protein binding remained unchanged, indicating that the elevation in PLA signals is most likely related to an increase in IP3R1 expression. Although the expression of MFN2 is unchanged, electron microscopy would be useful to establish whether the SR/ER–mitochondria tethering, and the physical distances between the two organelles, is disturbed in mdx hearts prior to the development of DCM. IP3Rs are involved in many cellular processes like metabolism, secretion, gene transcription, cell fate, and ER stress (Kiviluoto et al., 2013), but compared to RyR2 which is 50–100 times more expressed in left ventricles, the contribution of the IP3Rs to the cardiac beat-to-beat Ca^{2+} homeostasis and SR Ca^{2+} leak is unlikely (Kockskämper et al., 2008).

Increase of IP3R1 expression is accompanied by an elevation in Sig-1R, a transmembrane chaperone located in MAMs interacting with IP3Rs and ER stress sensors (for review Delprat et al., 2020). Sig-1R has been shown to (i) stabilize IP3R1 in MAMs contributing to the strengthening of ER–mitochondria contact and ER–mitochondria Ca^{2+} transfer and (ii) bind the protein chaperone BiP in the ER lumen stabilizing the ER stress response proteins (Hayashi and Su, 2007). Although a prolonged disrupted IP3R1–GRP75–VDAC interaction promotes ER stress (Rieusset et al., 2016; Pauly et al., 2017), an increase in contact points and Sig-1R expression has been reported in the early stage of the UPR response to sustain cell homeostasis and bioenergetics and alleviate ER stress (Hayashi and Su, 2007; Bravo et al., 2011; Delprat et al., 2020). Consistently, in contrast to skeletal muscle and with the exception of *Atf3*, UPR-inducible genes and ER sensor expression remained unchanged in the present study. Of note, the absence of ER stress response in the *mdx* heart does not exclude a mitochondrial stress response *per se*.

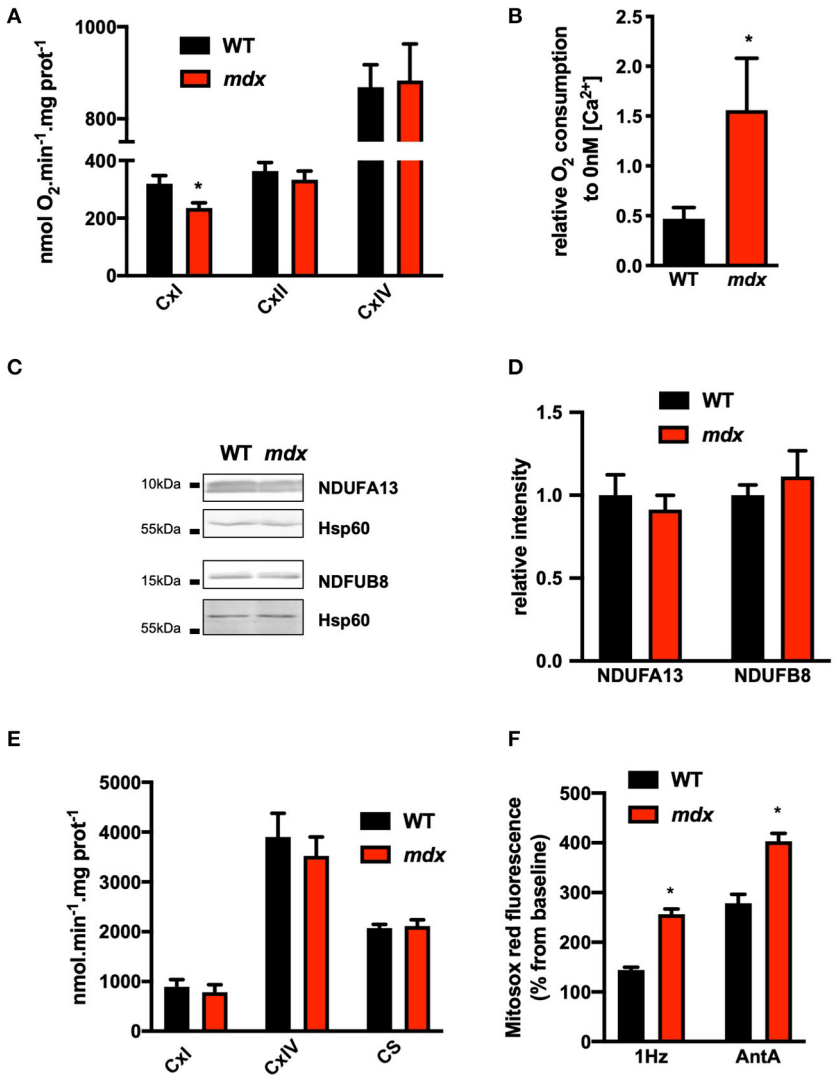

FIGURE 4 | Impaired complex I-mediated mitochondrial respiration in *mdx* hearts. **(A)** Respiration rates under glycolysis protocols in permeabilized isolated ventricular cardiomyocytes from WT and *mdx* mice ($N = 5$–7). Complex I-dependent respiration State 3 (EIII) is determined in presence of malate and pyruvate (CxI). Complex II and IV-dependent respiration State 3 (EIII) is obtained by addition of succinate and rotenone (CxII) and ascorbate/TMPD (CxIV), respectively. Data are mean ± SEM, $^*p = 0.0379$ *mdx* vs. WT. **(B)** Mean complex I-dependent respiration in the presence of 400 nM extramitochondrial Ca^{2+} relative to 0 nM Ca^{2+}. Data are mean ± SEM, $^*p = 0.0260$ *mdx* ($N = 6$) vs. WT ($N = 6$). **(C)** Representative immunoblots and **(D)** quantification of two subunits of complex I (NDUFA13: CxI 13 and NDUFB8: CxI 20). Isolated mitochondria from $N = 6$ hearts were tested in *mdx* and WT mice, and Hsp60 was used as loading control and expressed relative to WT. Data are mean ± SEM. **(E)** Enzymatic activities of complexes I and IV and citrate synthase measured on isolated mitochondria from Data are means ± SEM, $p > 0.05$ *mdx* ($N = 5$) vs. WT ($N = 5$). **(F)** Mitochondrial ROS production is evaluated under confocal microscopy with MitoSOX red and expressed as percentage of baseline after electric stimulation at 1 Hz ($^*p = 0.0071$) or Antimycin A ($^*p = 0.0435$) addition. Data are mean ± SEM, *mdx* ($N = 10$) vs. WT ($N = 9$).

As indicated, the mitigation of ER stress might be related to an enhancement of Ca^{2+} transfer and mitochondrial function (Bravo et al., 2011). Here, the increase in the IP3R1–GRP75–VDAC1 juxtaposition was associated with an increase in the MCU–MICU1 complex suggesting a reinforcement of the IP3R1–GRP75–VDAC–MCU Ca^{2+} transfer axis. As shown recently by Dubinin et al. (2020), we also observed an increase expression of MICU1. MICU1/2 subunits form heterodimers located in the mitochondrial intermembrane space regulating the Ca^{2+}-dependent gating and threshold properties of the MCU. At low Ca^{2+} concentrations, the MICU1/MICU2 dimer keeps MCU in the close state, and at higher Ca^{2+} level, MICU2-dependent inhibition is released and MICU1 activates MCU allosterically (Payne et al., 2017; Tarasova et al., 2019). An increase in the MICU1/MICU2 ratio reduces the Ca^{2+} threshold for MCU activation and increases the Ca^{2+} sensitivity of the MCU complex (Payne et al., 2017). Here, increased expression of MCU and MICU1 is not associated with a change in MICU2, which may in addition to decreased expression of the dominant negative isoform of MCU (MCUb) enhanced the mitochondrial Ca^{2+}

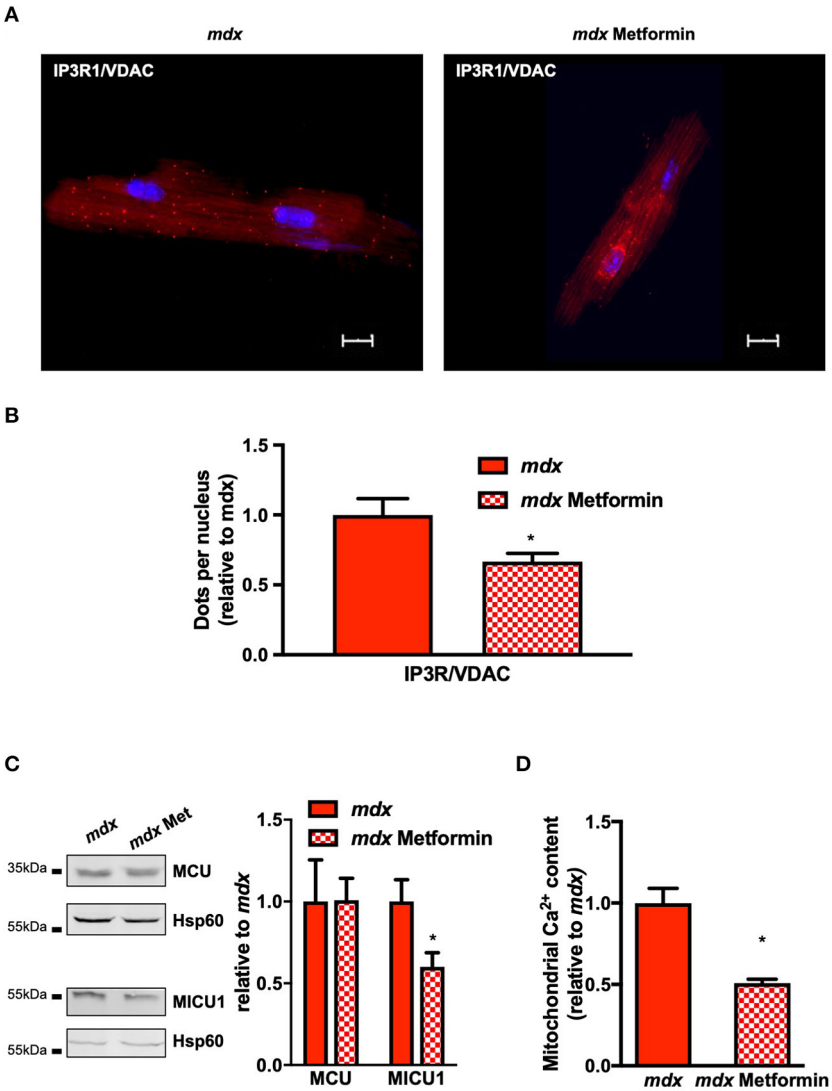

FIGURE 5 | Chronic metformin treatment decreases SR/ER and mitochondria interaction and mitochondrial Ca^{2+} content in *mdx* cardiomyocytes. All Western blots were performed on isolated mitochondria, and quantifications of proteins were normalized to Hsp60 and expressed relative to *mdx*. **(A, B)** Representative images and quantitative analysis of IP3R1–VDAC interaction measured by *in situ* PLA on isolated cardiomyocytes from *mdx* ($N = 3$, $n = 49$) and *mdx* + metformin ($N = 2$; $n = 33$). The numbers of dots were normalized to the number of nucleus per cells and expressed relative to *mdx* mean value. Mean ± SEM, $^*p = 0.046$ *mdx* vs. *mdx* + metformin. **(C)** Representative immunoblots and quantification of MCU and MICU1. Data are mean ± SEM, $^*p = 0.0303$ *mdx* ($N = 6$) vs. *mdx* + metformin ($N = 6$). **(D)** Mean of the absolute mitochondrial Ca^{2+} content in *mdx* ($N = 4$) and *mdx* + metformin ($N = 6$) hearts. Mean ± SEM, $^*p = 0.0095$ mdx vs. *mdx* + metformin.

uptake and content as shown in *mdx* ventricular mitochondria [**Figure 2**; (Dubinin et al., 2020)]. This change in MICU1 expression may affect the pharmacology of MCU and should be considered for future therapeutic strategies aimed at directly targeting mitochondrial Ca^{2+} uptake (Kon et al., 2017; Márta et al., 2020). The elevation of mitochondrial Ca^{2+} content is therefore the result of a remodeling of the MCU complex associated with the increase in the leakage of Ca^{2+} from the SR, the elevation of diastolic Ca^{2+}, and the strengthening of the contact points between the two organelles.

More generally, the enhancement of mitochondrial Ca^{2+} uptake and content has several functional consequences. It increases the metabolic and respiration rate by stimulating the respiratory chain and the activities of several enzymes involved in the metabolic flux and the Krebs cycle. Among them, the PDH is the entry point for the glycolytic product pyruvate into the oxidative metabolism. In the mitochondrial matrix, Ca^{2+} activates the PDH phosphatase 1 that dephosphorylates PDH to increase its activity, and thus the use of carbohydrates for energy production. Here PDH phosphorylation at three serine residues (pSer232, pSer293, pSer300 in the mouse) on the alpha chain of the E1 subunit is significantly decreased in conjunction with an increase in PDH activity. This increases in PDH activity may account for the previously described elevation

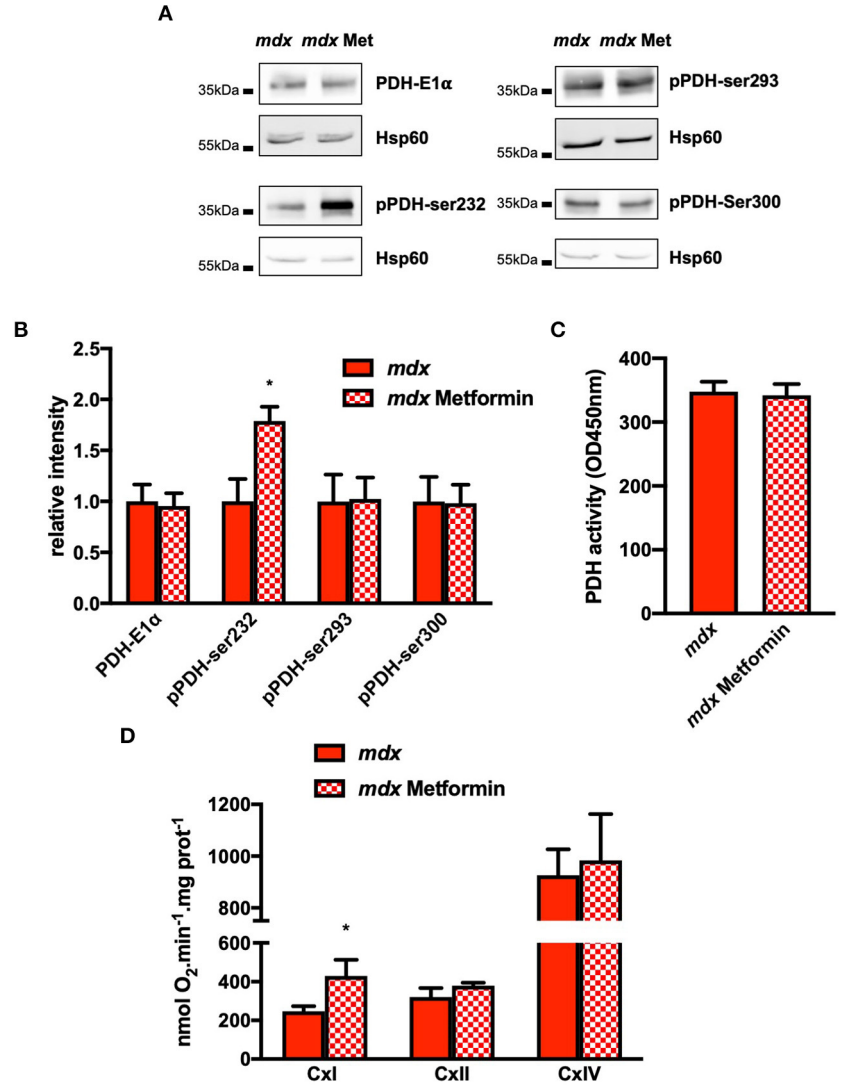

FIGURE 6 | Chronic metformin treatment increases PDH Ser232 phosphorylation and complex I-mediated respiration in *mdx* hearts. All Western blots were performed on isolated mitochondria and quantifications of proteins were normalized to Hsp60 and expressed relative to *mdx*. **(A)** Representative immunoblots and **(B)** quantification of total pyruvate dehydrogenase subunit E1α (PDH-E1α), PDH phosphorylated on Serine 232, 293, and 300. Data are mean ± SEM, $^*p = 0.0317$ *mdx* ($N = 4$–6) vs. *mdx* + metformin ($N = 4$–6). **(C)** Mean PDH activity measured on isolated mitochondria. Data are mean ± SEM, $p > 0.05$ *mdx* ($N = 6$) vs. *mdx* + metformin ($N = 7$). **(D)** Complex I-dependent respiration State 3 (EIII) is determined in the presence of malate and pyruvate (CxI). Complex II- and IV-dependent respiration State 3 (EIII) is obtained by addition of succinate and rotenone (CxII) and ascorbate/TMPD (CxIV), respectively. Data are mean ± SEM, $^*p = 0.036$ *mdx* ($N = 6$) vs. *mdx* + metformin ($N = 9$).

in the pyruvate decarboxylation and the shift from fatty acid to carbohydrate oxidation in the heart of both *mdx* and DMD patients (Perloff et al., 1984; Quinlivan et al., 1996; Momose et al., 2001; Naruse et al., 2004; Khairallah et al., 2007). PDH catalyzes the irreversible step of oxidative decarboxylation of pyruvate to produce acetyl-CoA and fuel the tricarboxylic acid cycle and electron transport chain. The increased PDH activity thus enhances the glycolytic flux, and pyruvate becomes the privileged substrate for the oxidative phosphorylation (Sun et al., 2015). However, in the absence of Ca^{2+} and as recently reported, the pyruvate-driven complex I respiration is reduced independently of any change in complex I expression level (Hughes et al., 2020). Such impairment might be related to posttranslational modifications; however, intrinsic complex I activity remained unchanged. It is noteworthy that mitochondrial respiration analyzed with substrates other than pyruvate, such as glutamate, does not appear to show any difference in *mdx* heart (Ascah et al., 2011; Viola et al., 2013). Remarkably, in the *mdx* diaphragm, the mitochondrial rate of ATP production was partly improved by directly stimulating Complex II, suggesting that Kreb's-driven NADH-dependent complex I function is defective (Rybalka et al., 2014). In addition, in the presence of Ca^{2+}, complex I-mediated mitochondrial respiration is boosted, suggesting adaptive mechanisms to sustain the energy demand. On the

one hand, it can increase the rate of pyruvate consumption to improve NADH production but, on the other hand, enhance electron leakage and mitochondrial ROS production (Williams and Allen, 2007b; Viola et al., 2013). Although an increase in mitochondrial ROS production is commonly observed in the heart of *mdx* (Williams and Allen, 2007b; Viola et al., 2013; Kuno et al., 2018; Hughes et al., 2020), the exact mechanisms linking complex I to ROS production remain to be established. It would be interesting to determine whether ROS are produced in the forward direction or in the reverse direction of electron transfer (Hirst and Roessler, 2016). Although we have not explored these mechanisms in detail, electron leakage from complex II is somewhat unlikely because the succinate-induced respiration is comparable in *mdx* and WT hearts. In the forward mode hypothesis, targeting the function of complex I may improve mitochondrial activity and metabolism.

We here tested metformin, an antidiabetic drug with pleiotropic properties that are related to its mitochondrial effects (for review see Foretz et al., 2014; Vial et al., 2019). Long-term treatment with metformin has already demonstrated some beneficial effects on motor function in stable DMD patients with encouraging evidence regarding muscle degeneration and histopathology (Hafner et al., 2016, 2019). The exact mechanisms of action of metformin have yet to be elucidated; however, at high doses, metformin inhibits the oxidation of NADH at the complex I level. Consequently, the ADP:ATP or AMP:ATP ratios increase which is thought to activate AMPK, a hub for major metabolic and energy sensing pathways (Horman et al., 2012; Foretz et al., 2014; He and Wondisford, 2015). In addition, at low doses, metformin has also been shown to activate AMPK independent of direct inhibition of complex I (He and Wondisford, 2015). In all cases, activation of AMPK demonstrated significant beneficial effects on the dystrophic phenotype in skeletal and cardiac muscles. Importantly, it improves mechanical signaling, muscle strength, and force; limits muscle necrosis, fibrosis, and inflammation; and stimulates the oxidative phenotype, mitochondrial function, and autophagy processes (Ljubicic et al., 2011, 2012; Pauly et al., 2012; Garbincius and Michele, 2015; Juban et al., 2018). Metformin has also been shown to increase PGC1-α expression in *mdx* muscle fibers, a central transcriptional coactivator regulating a wide range of biological processes such as mitochondrial biogenesis, oxidative phosphorylation, and muscle regeneration (Scarpulla, 2011; Ljubicic and Jasmin, 2015; Suntar et al., 2020). In addition, increased activation of AMPK stimulates autophagic clearance of defective mitochondria and may thus improve mitochondrial function (De Palma et al., 2012; Pauly et al., 2012). More generally, the maintenance of cell fate and cell proteostasis are emerging therapeutic targets in DMD (De Palma et al., 2012), but depending on the phase, severity, and inflammatory state of the pathology, the therapeutic window is critical for such emerging strategies (Farini et al., 2019). Here, in line with other studies, treatment with metformin also improves pyruvate-mediated mitochondrial respiration (Wang et al., 2019) and increasing the level of ACC phosphorylation would also facilitate fatty acid oxidation (Saddik et al., 1993). In parallel, MICU1 expression decreased but MCU did not, which is consistent with a recent report demonstrating a causal link between the pyruvate fluxe and consumption and MICU1 expression, suggesting that MICU1 could serve as a metabolic sensor (Nemani et al., 2020). Moreover, the IP3R1–VDAC1 interactions also decrease, indicating a reduction in the SR/ER–mitochondria contact points. Although the mechanism remains to be established, changes in SR/ER–mitochondria interaction are causally related to energy metabolism. AMPK activation has recently been shown to reduce the formation of cardiac MAM in hyperglycemia and decrease IP3-induced Ca^{2+} release (Arias-del-Val et al., 2019; Wu et al., 2019). Remodeling of the IP3R1–VDAC1–MCU axis under metformin treatment also reduced mitochondrial Ca^{2+} content and enhanced the phosphorylation of the Ser232 site of the PDH-E1α subunit, which was not sufficient to reduce PDH activity in *mdx* cardiomyocytes. All three phosphorylation sites can restrain enzyme activity; however, Ser293 phosphorylation has a higher inhibitory impact than Ser300 and even more than Ser232 phosphorylation (Korotchkina and Patel, 1995; Gray et al., 2014). Finally, metformin can also impact ROS production in a different way, first of all as a modulator of complex I activity and then by its effect on mitochondrial Ca^{2+} content. AMPK activation has also been reported to decrease mitochondrial production of ROS (Foretz et al., 2014).

Myocardial metabolic and mitochondrial impairments as well as mitochondrial and cellular Ca^{2+} handling have been extensively studied in DMD during the last decade. All the studies agree that these disorders, which precede the onset of structural remodeling and deterioration of myocardial macroscopic function, play a central role in the progression of the pathology to heart failure (for review: Esposito and Carsana, 2019). Here, for the first time, our data suggests that the enhancement of SR/ER–mitochondria contact points and increasing mitochondrial Ca^{2+} uptake and content would enhance glycolytic flux and complex I respiration. However, the downside of this scheme is that increasing mitochondrial Ca^{2+} would increase ROS production, sensitize mPTP, further impair metabolic flexibility, and alter ATP production and Ca^{2+} handling creating an amplification loop with all the ingredients toward contractile dysfunction, arrhythmia, fibrosis, and finally heart failure. Accordingly, long-term metformin treatment has already proved to have some beneficial effects on the development of DCM linked to mutations in the dystrophin–glycoprotein complex (Mantuano et al., 2018; Kanamori et al., 2019). Although more studies are needed to understand and characterize the beneficial effects of chronic treatment with metformin on the development of the cardiomyopathy associated with DMD, in the present study, metformin already demonstrates beneficial effects on the aberrant SR/ER-mitochondria interaction and increased mitochondrial Ca^{2+} as well as mitochondrial function. It will therefore be very important in the near future to determine whether treatment with metformin can improve dystrophic cardiomyopathy in the advanced stage of the pathology. To conclude, our data support the concept that metformin alone or in combination with other drugs might be a potential therapeutic strategy to ameliorate the dystrophinopathies (Casteels et al., 2010; Ljubicic and Jasmin,

2015; Hafner et al., 2016, 2019; Mantuano et al., 2018; Vitiello et al., 2019).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité d'éthique régional en expérimentation animale languedoc Roussillon–Ministère de l'enseignement supérieur de la recherche et de l'innovation (N° #16473-2018082016141320).

AUTHOR CONTRIBUTIONS

CA and MP performed and analyzed the experiments. ML and JR performed the experiments. AL designed the experiments, discussed the data, and read the manuscript. JF designed the experiments, collected and discussed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.609493/full#supplementary-material

Supplementary Figure 1 | Respiratory control ratio (RCR) was measured in **(A)** WT ($N = 7$), *mdx* ($N = 13$) and *mdx* + met ($N = 9$) hearts and **(B)** in WT ($N = 6$) and *mdx* ($N = 6$) in presence of in presence of 0 nM or 400 nM extramitochondrial Ca^{2+}. Data are mean ± SEM, $p > 0.05$ *mdx* vs. WT.

Supplementary Figure 2 | SERCA2a, PLB expression, and SR Ca^{2+} load. **(A)** Full length immunoblots and quantification of SERCA2a, phospholamban (PLB), and Phospho-Phospholamban (Ser16/Thr17) (pPLB-Ser16; pPLB-Thr17) were normalized to GAPDH. Data are mean ± SEM, $p > 0.05$ *mdx* ($N = 4$-6) *vs.* WT ($N = 4$-6). **(B)** Mean values of the amplitude of caffeine-induced SR Ca^{2+} release, estimating the SR Ca^{2+} load. $^{*}p = 0.0182$ WT ($N = 3$; $N = 9$) vs. *mdx* ($N = 3$; $N = 10$).

Supplementary Figure 3 | IP3R1 immunoprecipitation. Anti-IP3R1 antibody (1:200) was used to immunoprecipitate IP3R1 from heart homogenate. Samples were incubated with an anti-IP3R1 antibody in 0.5 ml of a modified RIPA buffer (10 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1% Triton; 5 mM NaF and protease inhibitor cocktail) for 2 h at 4°C. The immune complex was incubated with protein A/G magnetic beads (Pierce 88802) at 4°C for 2 h, after which the beads were washed out three times with RIPA buffer. Proteins were separated on SDS/PAGE gels and transferred onto nitrocellulose membranes for 1 h at 100 V. The immunoblots were prepared using antibodies against IP3R1 (1:1,000), anti-GRP75 antibody (1:1,000, Santa Cruz) and anti-VDAC (1:300). All immunoblots were developed and quantified using the Odyssey infrared imaging system (LICOR Biosystems) and infrared-labeled secondary antibodies. After immunoprecipitation (IP) of IP3R1 from heart muscle of WT or mdx mice, immunoblots were used to detect IP3R1, Grp75, and VDAC. Data are mean ± SEM, $p > 0.05$ *mdx* ($N = 3$) vs. WT ($N = 3$).

Supplementary Figure 4 | (A) Transcript levels of UPR response genes quantified by 30 reverse transcription quantitative polymerase chain reaction (RT-qPCR) in WT ($N = 4$) and *mdx* ($N = 4$) hearts. The mRNA levels were normalized to the reference gene TBP. Data are means ± SEM, $^{*}p = 0.0286$ *mdx* vs. WT. **(B)** PLA technical negative control obtained in absence of primary antibody.

Supplementary Figure 5 | Pyruvate dehydrogenase kinase 4 (PDK4) expression. Full length immunoblots and quantification of PDK4 were normalized to Hsp60. Data are mean ± SEM, $p > 0.05$ *mdx* ($N = 5$) vs. WT ($N = 5$).

Supplementary Figure 6 | Protein expression of mitochondrial respiratory chain complexes. All western blots were performed on isolated mitochondria and quantifications of proteins are normalized to Hsp60. **(A)** Representative immunoblots and **(B)** quantification of complex I (CxI), complex 40 II(CxII), complex III (CxIII), complex IV (CxIV), complex V (Cx V). Data are mean ± SEM, *mdx* ($N = 6$) vs. WT ($N = 6$).

Supplementary Figure 7 | Phosphorylation level of AMPK and ACC, and proteins expression of SR/ER and mitochondria contacts points. All quantifications of proteins are normalized either to GAPDH or Actin when heart homogenates were used or to Hsp60 when western blot were performed on isolated mitochondria. **(A)** Representative immunoblots and quantification of IP3R1, GRP75, Sig-1R, and VDAC. Quantification of IP3R1, GRP75, MFN2, and Sig-1R was carried out from $N = 6$ *mdx* and *mdx* +metformin hearts. Data are mean ± SEM. **(B)** Mean value of pAMPK/AMPK and pACC/ACC ratio. Ratio were established after proteins normalization to Actin from $N = 6$ WT, *mdx* and *mdx* +metformin hearts. Full lengths gels are in supplemental information. WT vs. *mdx*+ metformin $^{\#}p = 0.0101$ and *mdx* vs. *mdx*+metformin $^{*}p = 0.0419$.

Supplementary Information | All full lengths gel for all protein probed.

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Mitochondrial and Sarcoplasmic Reticulum Interconnection in Cardiac Arrhythmia

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Ca^{2+} plays a pivotal role in mitochondrial energy production, contraction, and apoptosis. Mitochondrial Ca^{2+}-targeted fluorescent probes have demonstrated that mitochondria Ca^{2+} transients are synchronized with Ca^{2+} fluxes occurring in the sarcoplasmic reticulum (SR). The presence of specialized proteins tethering SR to mitochondria ensures the local Ca^{2+} flux between these organelles. Furthermore, communication between SR and mitochondria impacts their functionality in a bidirectional manner. Mitochondrial Ca^{2+} uptake through the mitochondrial Ca^{2+} uniplex is essential for ATP production and controlled reactive oxygen species levels for proper cellular signaling. Conversely, mitochondrial ATP ensures the proper functioning of SR Ca^{2+}-handling proteins, which ensures that mitochondria receive an adequate supply of Ca^{2+}. Recent evidence suggests that altered SR Ca^{2+} proteins, such as ryanodine receptors and the sarco/endoplasmic reticulum Ca^{2+} ATPase pump, play an important role in maintaining proper cardiac membrane excitability, which may be initiated and potentiated when mitochondria are dysfunctional. This recognized mitochondrial role offers the opportunity to develop new therapeutic approaches aimed at preventing cardiac arrhythmias in cardiac disease.

Keywords: mitochondria, sarcoplasmic reticulum, arrhythmia, calcium, heart failure, ischemia/reperfusion injury

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INTRODUCTION

Arrhythmias can be defined as any disturbance in the normal electrical sequence of the heart. These disturbances may cause the electrical impulse to travel slowly, rapidly, or in an erratic manner. However, few studies have calculated the overall burden of arrhythmias. Incidence has been reported to be about 2.35% in the United Kingdom's general population (Khurshid et al., 2018). In Mexico, only data for atrial fibrillation (AF) exist, and it is estimated to affect 2% of the general population (Lara-Vaca et al., 2014). These data are consistent with the estimated global rates of approximately 1–4% (Zulkifly et al., 2018). Although it may seem like a low percentage, electrical abnormalities appear in up to 39% of patients with cardiopathies (Vazquez Ruiz de Castroviejo et al., 2005). Within this population, sudden cardiac death constitutes a significant cause of mortality. Sudden cardiac death is defined as when the death of a patient occurs, most commonly by a fatal ventricular arrhythmia, within 1 h of the onset of symptoms when there is a witness or within 24 h of last

being seen alive when no witness is available (Adabag et al., 2010). Arrhythmia susceptibility is especially concerning in high-risk populations, such as heart failure (HF) patients, in which the incidence of sudden cardiac death reaches approximately 15% per year; this population continues to grow, as it is the outcome of almost all cardiovascular pathologies (Lee et al., 2011; Srinivasan and Schilling, 2018). Sudden cardiac death has been documented to be responsible for 20–30% of all cardiac deaths worldwide and about 7–18% in the United States (Rodríguez-Reyes et al., 2015). Analyzing the process of arrhythmogenesis at the cellular level is, therefore, of vital importance to better understand the underlying mechanisms that lead to its development and elucidate new potential therapeutic targets to prevent it.

Focal activity and re-entry are proposed as the main mechanisms of cardiac arrhythmia. Re-entry is associated with conduction abnormalities and occurs when a propagating impulse fails to extinguish after normal activation of the heart tissue and re-excites the heart after completion of the refractory period. A reduction in the cardiac impulse's wavelength, which is a product of the conduction time and refractory period (Smeets et al., 1986; Wijffels et al., 1995), is a determinant of re-entry. Changes in the expression and function of membrane ion channels (termed electrical remodeling) affect the cardiac conduction properties and refractory period, facilitating functional re-entry (Papadatos et al., 2002). The alteration of gap junction-specialized structures that couple myocytes (Kirchhoff et al., 1998; Jansen et al., 2010), the deposition of extracellular matrix components (Verheule et al., 2004; Krul et al., 2015), and cardiac enlargement (Eijsbouts et al., 2003; Vranka et al., 2007) are considered part of the structural remodeling that increases the chances of arrhythmia via anatomical re-entry mechanisms. Triggered activity refers to the impulse initiation resulting from depolarization of the membrane potential after the upstroke phase of the action potential (AP). Afterdepolarizations that occur during the repolarization phase of the AP are called early-afterdepolarizations (EADs), whereas those occurring after completion of the membrane repolarization are known as delayed-afterdepolarizations (DADs). The modulation of membrane ion channels that prolong the AP facilitates the appearance of EADs, whereas the appearance of DADs is commonly associated with altered intracellular Ca^{2+} homeostasis. Both increased Ca^{2+} overload conditions and Ryanodine receptor (RyR2) dysfunction (leakiness) promote spontaneous Ca^{2+} releases from the sarcoplasmic reticulum (SR) and activate the Na^{+}/Ca^{2+} exchanger (NCX) in its forward mode. In this operating mode, a net inward Na^{+} current (I_{ti}) is present, which depolarizes the membrane and causes DADs. If these DADs are large enough, they can reach the threshold activation of the Na^{+} current and generate a full arrhythmogenic AP. Both EADs and DADs are known as trigged activity and play an important role in initiating cardiac arrhythmias. There is compelling evidence that mitochondria play an important role in the generation of Ca^{2+}-triggered arrhythmia, a process that requires an understanding of cellular Ca^{2+} fluxes.

The SR is the organelle in charge of storing and releasing Ca^{2+} into the cytosol. RyR2 opening occurs after a small initial amount of Ca^{2+} passes through L-type Ca^{2+} channels (LTCCs) in the sarcolemma in response to membrane depolarization during the AP (Bers, 2002). The sudden increase in cytosolic Ca^{2+} levels activates the myofibrils in sarcomeres, and contraction occurs. During the diastolic phase, Ca^{2+} is removed from the cytosol, and around 70% of total cytosolic Ca^{2+} is pumped back into the SR (Bassani et al., 1994) by the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). Ca^{2+} is extruded into the extracellular space by NCX activation, which counterbalances the entry of Ca^{2+} through LTCCs. Only a discreet quantity of cytosolic Ca^{2+} is removed by mitochondria (Bers, 2002), which occurs through a Ca^{2+} channel in the inner mitochondrial membrane (IMM) known as mitochondrial Ca^{2+} uniplex (mCU) (previously called the mitochondrial Ca^{2+} uniporter). Mitochondrial Ca^{2+} extrusion is carried out by the mitochondrial NCX, which possesses slow kinetics compared to the mCU, allowing the accumulation of this ion in the mitochondria. The local SR-Mitochondria Ca^{2+} flux is facilitated by anchoring proteins that anchor both organelles (Kohlhaas and Maack, 2013). The dependence on Ca^{2+} import in mitochondrial reactive oxygen species (ROS) production and energetics highlights the importance of coordinated regulation between SR Ca^{2+} fluxes and mitochondrial homeostasis. In this review, we focus on describing the cross signals that occur between these two organelles and how dysregulation of this intertwined signaling may be involved in altered cardiac rhythms.

THE MITOCHONDRIA-SR INTERCONNECTION: PROXIMITY ENABLES CROSSTALK

The structure of the cardiomyocyte has been extensively reviewed (Eisner et al., 2017), and emphasis has been placed on the proximity of T-tubules, where LTCCs reside, with the terminal cisternae of the SR, where RyR2s are more concentrated. The approximate distance between both structures has been calculated to be only about 15 nm (Scriven et al., 2013). This distance is what enables the two structures to react to each other's activation. This unit, comprised of a T-tubule with its corresponding terminal cisternae of the SR, is called a dyad; it is considered the functional unit of the heart and is in charge of excitation-contraction-coupling (ECC). Nonetheless, because of its proximity, mitochondria could also be regarded as part of this functional unit, as they also play a role in responding to stimulation and producing a proper contractile response. Fluorescence (Friedman et al., 2011) and electron microscopy (Csordás et al., 2006) techniques have been used to reveal physical interactions between these organelles, with protein-like structures linking both membranes. Protein structures between the mitochondria and the SR have been described elsewhere (Lopez-Crisosto et al., 2017; Martinvalet, 2018; Giorgi et al., 2018). Several structures have been found to create bridges between the organelles, securing proximity (**Figure 1**). The complexity of SR-mitochondria bridging proteins is high, so the removal of individual structures could be compensated for by other components. Describing the interactions of each element found to link both organelles in detail is out of the scope of

this review, but a brief description will be provided. The first of such structures is the Ca^{2+} channel inositol-3-phosphate receptor (IP3R), joined to the mitochondrial voltage-dependent anion channel (VDAC) through protein GRP75 (Szabadkai et al., 2006). This communication enables the rapid movement of Ca^{2+} ions from the SR into the mitochondrial intermembrane space when IP3 is released through the protein kinase C (PKC) pathway. Similarly, IP3R2 has been described as binding with the FUN14 domain containing 1 (FUNDC1) to modulate SR Ca^{2+} release (Wu et al., 2017). The VDAC has also been described as having physical interactions with the RyR2, which, coupled with mCU co-localization with the RyR2, helps explain how mitochondrial Ca^{2+} transport is possible (Kohlhaas and Maack, 2013). Other structures involved in maintaining a connection are the SR vesicle-associated membrane protein-associated protein B/C (VAPB), whose function is not fully understood, although it has been shown to regulate Ca^{2+} transport between both organelles (De Vos et al., 2012). The mitochondrial protein tyrosine phosphatase-interacting protein 51 (PTPIP51) seems to have more structural functions (Stoica et al., 2014). The ER-mitochondria encounter structure (ERMES) is a protein complex characterized in yeast that bridges both organelles and has diverse biological functions. Although no homolog structure has been described in mammals, an ortholog of one of its components, PDZ domain-containing protein 8 (PDZD8), was described recently (Giorgi et al., 2018). Other structures, such as Mitofusin2, a protein involved in mitochondrial dynamics, have also been described as being able to form dimers that bridge both organelles (de Brito and Scorrano, 2008), presumably to organize mitochondrial dynamics. Similarly, SR protein inverted formin 2 (INF2) serves as an anchor for actin filaments to reach the mitochondria, thereby providing scaffolding for mitochondrial constriction in mitochondrial dynamics (Korobova et al., 2013; Manor et al., 2015). These connections help maintain the close gap between mitochondria and the SR. Intermyofibrillar mitochondria have been measured as close as 33 nm to the RyR2 in the SR and as far away as 188 nm (Ramesh et al., 1998). This proximity enables the existence of a microdomain where secure communication can take place. For instance, cellular Ca^{2+} concentrations vary from about 100–500 nM globally between resting and peak concentrations in the AP (Bers, 2002). Furthermore, within the dyadic cleft, Ca^{2+} levels can get as high as 100 μM at the periphery when the RyR2 releases Ca^{2+} from the SR, stimulated by LTCCs (Langer and Peskoff, 1996). These high levels of Ca^{2+} are maintained for about 10 ms, affecting a region of about 2 μm, although not in the same concentrations, before descending as Ca^{2+} diffuses to other cellular regions (Cheng and Lederer, 2008). This localization places the mitochondria well within reach of accessing high local Ca^{2+} concentrations. Although 10 ms may not seem very long, this is enough time for Ca^{2+} to be transported through the IMM by the mCU into the mitochondrial matrix. Two models of the mitochondrial response to changes in cytosolic Ca^{2+} levels have been described (O'Rourke and Blatter, 2009). The first one, originally proposed by Crompton (Crompton, 1990), indicates that the mitochondrial Ca^{2+} concentration increases slowly and gradually with a faster AP firing rate until influx and efflux are balanced entirely, and a new steady state is achieved. However, slow changes in the mitochondrial Ca^{2+} concentration may not be able to stimulate ATP production fast enough to meet immediate metabolic needs. Subsequently, beat-to-beat changes in mitochondrial Ca^{2+} are slight, and the energetic requirements of integrated mitochondrial Ca^{2+} transport are marginal. The other model describes mitochondria as having the ability to comprehensively sense rapid cytosolic Ca^{2+} changes, presenting with oscillations on a beat-to-beat basis (O'Rourke and Blatter, 2009). This process would imply that mitochondria can not only rapidly internalize but also extrude Ca^{2+} ions. In consolidating both models, a mitochondrial Ca^{2+} transient was described by Lu et al. (2013), which has some key differences when compared to its cytosolic counterpart. As the firing rate of the AP increases, a decrease in amplitude maintains a more stable concentration throughout the whole AP by slowly increasing diastolic Ca^{2+} concentrations, although a faster decline was also noted compared to baseline after stimulating with a catecholamine analog (Lu et al., 2013). It is estimated that this happens because mitochondrial structures pump Ca^{2+} into the cytosol more slowly, primarily the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX), as opposed to the combined pumping force of SERCA in the SR and the sarcolemmal NCX. While it has been calculated that the percentage of cellular Ca^{2+} taken up by mitochondria is modest (Bassani et al., 1994), it can have significant effects on excitation-contraction-energetics coupling (ECEC). The main reason is that by increasing mitochondrial Ca^{2+} concentration, dehydrogenases from the Krebs cycle change to a more active form. In turn, these dehydrogenases produce more high-energy products ($NADH^{+}$ and $FADH_2$) for the electron transport chain (ETC) to use as substrates and generate the mitochondrial membrane potential ($\Delta\Psi m$) and subsequent ATP synthesis under energy-demanding states, such as when adrenergic stimulation takes place (Fernandez-Sada et al., 2014; Kwong et al., 2015). This ATP is then transported to the cytosol, where it is used by the sarcomere to relax its myofibrils and, equally necessary, used by a wide range of pumps to maintain ion balance. One of these pumps is SERCA, which returns most Ca^{2+} ions, about 70% (Bassani et al., 1994), into the SR after the AP finishes and reestablishes the basal Ca^{2+} concentration. This is just one example of how mitochondria can communicate with the SR under demanding and stressful conditions, according to ECEC.

MITOCHONDRIAL CA^{2+} SIGNALING AND CARDIAC ARRHYTHMIA

High cytosolic Ca^{2+} concentrations trigger mitochondrial Ca^{2+} transport by the mCU. Under homeostatic conditions, this process is finite, and Ca^{2+} can slowly be transported back into the cytosol by the mNCX. However, if Ca^{2+} cannot be extruded out of the mitochondria before more Ca^{2+} enters the mCU, then Ca^{2+} overload ensues. High mitochondrial Ca^{2+} concentrations cause higher ROS production, loss of mitochondrial membrane integrity with subsequent $\Delta\Psi m$ loss, and mPTP opening (Kazak et al., 2017). This last event is an initial step in the signaling

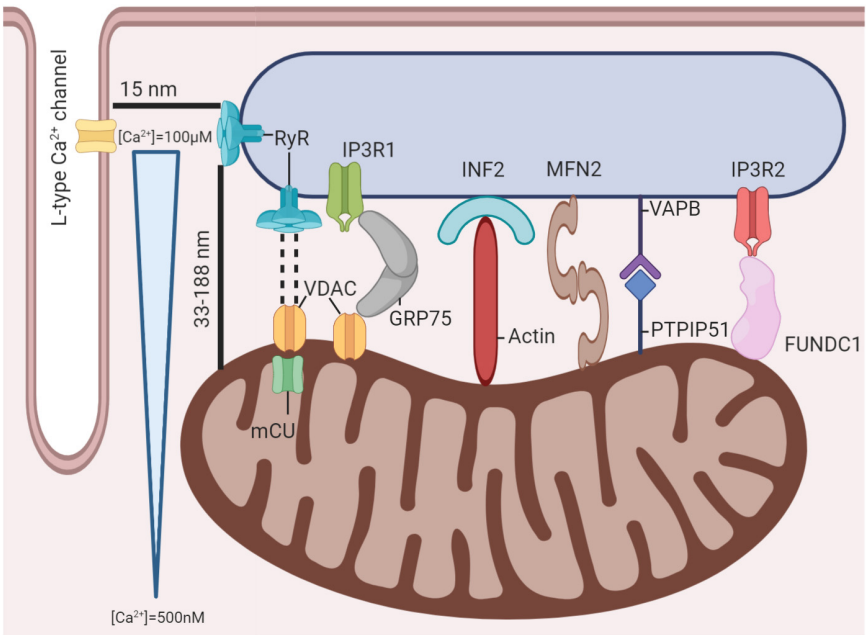

FIGURE 1 | Mitochondria-SR-T tubule microdomain. Several protein structures have been demonstrated to exist between mitochondria and the SR. These structures maintain proximity between the organelles and enable mitochondria to experience significant Ca^{2+} fluctuations with each AP despite the global cardiomyocyte's Ca^{2+} concentration remaining relatively unchanged.

cascade for the mitochondrial-mediated apoptosis pathway, causing further alterations in heart rhythmicity and organization at a cellular level. Whether SR or mitochondrial dysfunction causes the initial Ca^{2+} overload is debatable. Nonetheless, it is a safe bet to argue that this process may be self-maintained. Based on murine models of HF and AF associated with RyR2, as well as atrial samples from patients with chronic AF, the Marks group has reported that increased RyR2-mediated leakage is a potential mechanism for augmented mitochondrial Ca^{2+} content and ROS production. Post-translational analysis has revealed increased RyR2 oxidation and dissociation of the RyR2 inhibitory protein, calstabin2 or FKBP, which may further exacerbate RyR2 leakage. Thus, a feedback loop mechanism in Ca^{2+} signaling between the SR and mitochondria promotes intracellular Ca^{2+} mishandling. The SR-mitochondria interconnection in Ca^{2+} fluxes may offer the possibility of targeting mitochondrial Ca^{2+} overload to ameliorate RyR2-mediated leakage (Santulli et al., 2015; Xie et al., 2015). The importance of mitochondria in this vicious cycle is exemplified in a recent study conducted by Hamilton et al. (2020), who demonstrated that mitochondrial ROS targeting ameliorates RyR2 leakiness in a mouse model of catecholaminergic polymorphic ventricular tachycardia (CPVT). As part of the Ca^{2+}-handling proteins, mitochondrial membrane components *per se* are susceptible to redox modulation, which contributes to altered Ca^{2+} homeostasis in the myocyte. This is the case for the mCU, whose oxidation at cysteine 97 activates the channel, leading to mitochondrial Ca^{2+} overload and ROS production (Dong et al., 2017). The impact of altered mitochondrial Ca^{2+} in membrane excitability and arrhythmia has also been investigated. Myocytes from non-ischemic HF showed increased mitochondrial Ca^{2+} transients, enhanced LTCC currents, AP prolongation, and the appearance of EADs (Xie et al., 2018). Cardiac activity monitored by telemetry has demonstrated QT prolongation and a high incidence of ventricular arrhythmia following the application of isoproterenol (ISO), a catecholamine analog.

A potential causal role of mitochondrial Ca^{2+} signaling in arrhythmia is illustrated by genetically and pharmacologically blocking the mCU. We previously reported that selective mCU inhibition has the therapeutic potential to prevent catecholamine-induced toxicity, as observed in HF (Fernandez-Sada et al., 2014). In this regard, using mCU transgenic mice, two independent groups confirmed our findings of the mCU's critical role in matching the metabolic output during the adrenergic response (Kwong et al., 2015; Wu et al., 2015). Blocking the mCU with ruthenium red (RuR) or Ru 360 min before the induction of ischemia-reperfusion (I/R) injury prevents the appearance of ventricular arrhythmia in rat models (Garcia-Rivas Gde et al., 2006; Menezes-Rodrigues et al., 2020). In contrast, other studies have reported that enhancing Ca^{2+} fluxes between the SR and mitochondria using mitochondrial Ca^{2+} uptake enhancers is beneficial in preventing the appearance of ventricular arrhythmia associated with RyR2 dysfunction (Schweitzer et al., 2017). In atrial myocytes, the Blatter group has reported that inhibiting the mCU with Ru360 aggravates the Ca^{2+} alternans ratio and decreases the alternans' pacing threshold. These arrhythmogenic effects are restored when myocytes are treated with spermine, suggesting that mitochondrial Ca^{2+} uptake could be a potential candidate for AF treatment (Oropeza-Almazan and Blatter, 2020). Thus, the

effect of blocking mitochondrial Ca^{2+} uptake, either protecting from or promoting arrhythmia, may be dependent on the cardiac condition associated with Ca^{2+} overload. For example, in I/R injury, where a massive and acute Ca^{2+} imbalance occurs, closing the mCU prevents mitochondrial damage, whereas stimulated Ca^{2+} transport in AF could ameliorate the emerging Ca^{2+}-triggered arrhythmia by dissipating Ca^{2+} waves and lowering diastolic Ca^{2+} levels.

MITOCHONDRIAL ENERGETIC DEBACLE IN CARDIAC ARRHYTHMIA

About 90% of the heart's ATP is produced through oxidative phosphorylation in mitochondria (Harris and Das, 1991). Given that the heart beats continuously, cardiac muscle contraction is an extremely ATP-demanding process. Furthermore, mitochondria constitute about 25% of the volume of human cardiomyocytes, even more so in rats and mice (∼30%) (Schaper et al., 1985), which reflects the organelle's relevance in cardiac tissue. Rising cytosolic Ca^{2+} levels during the AP stimulates Ca^{2+} transport into the mitochondrial matrix. This rising intramitochondrial Ca^{2+} concentration activates dehydrogenases involved in the Krebs cycle, which, in turn, supplies the ETC with $NADH^{+}$ and $FADH_2$ to meet the cell's energetic demand (McCormack and Denton, 1990; Glancy and Balaban, 2012; Fernandez-Sada et al., 2014). In this sense, in cardiac cells, the Ca^{2+} signal is coupled with the energy demand through a process called ECEC. This process links Ca^{2+} handling and the contraction of the cardiomyocyte with relaxation and ATP production in mitochondria (Maack and O'Rourke, 2007). Energetic depletion can affect highly dependent ion channels and transporters that participate in proper intracellular Ca^{2+} handling. A prominent example of such a protein is SERCA, which needs ATP to transport Ca^{2+} into the SR during myocardium relaxation (Bers, 2002). Impairing SERCA's functioning reduces SR Ca^{2+} content and increases the cytosol's diastolic Ca^{2+} concentration. Another example of an ATP-driven protein function is sarcKATP, a potassium ion channel in the sarcoplasmic membrane that opens upon a low ATP/ADP ratio. High ATP/ADP ratios usually inhibit this channel, which limits the reactivation of inward currents by prolonging the refractory period and avoiding functional re-entry. The O'Rourke lab recently proposed that sarcKATP channels are the means by which changes in the $\Delta\Psi m$ cause altered cellular membrane excitability. In their work, metabolic dysfunction induced by the mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) increased IKATP and shortened the AP and refractory period, leading to re-entry into myocyte monolayers. The proarrhythmogenic effects of FCCP are attenuated by the IKATP inhibitor glibenclamide (Zhou et al., 2014). Depleting mitochondrial ATP production may impact membrane excitability by promoting T-wave alternans. In the electrocardiogram (ECG), T-wave alternans are linked to ventricular arrhythmia and constitute a prominent arrhythmogenic mechanism in the settings of myocardial infarction and ischemia, conditions characterized by the depletion of mitochondrial ATP production. T-wave alternans reflect beat-to-beat variation in the action potential duration (APD) at a cellular level, which are known as APD alternans, and numerous studies have reported that they are coupled to intracellular Ca^{2+} release variations (Ca^{2+} transient alternans). In an isolated rabbit heart, FCCP perfusion caused a higher incidence of APD and Ca^{2+} transient alternans, increased interventricular heterogeneity, impaired cardiac conduction velocity, and promoted ventricular arrhythmias in optical mapping studies. In this study, APD and Ca^{2+} alternans were also induced under ischemic conditions in rabbit heart preparation, validating the clinical relevance of blunted mitochondria energetics in cardiac arrhythmogenesis (Smith et al., 2013). In contrast, several other studies have reported that mitochondrial uncoupling proteins (UCPs) confer protection against cardiac arrhythmias in conditions of I/R. Subjecting cardiac UCP3 knockout ($UCP3^{-/-}$) mice to I/R injury produced twofold larger infarcts, a high propensity for ventricular tachycardia, and excessive ROS generation when compared to WT mice. Pretreatment with FCCP prior to I/R injury relieved cardiac stress in $UCP3^{-/-}$ hearts and improved ventricular functioning (Ozcan et al., 2013). Thus, the balance between mitochondrial energetics preservation should be a target for better cardiac arrhythmia protection in cardiac ischemia. Mitophagy, the degradation of mitochondria by autophagy, is considered a protective mechanism against the damage that mitochondria experience during I/R injury. Adenoviral expression of the uncoupling protein 2 (UCP2) in the heart promoted mitochondria mitophagy in the settings of I/R injury and offered cardiac output preservation comparable with ischemic preconditioning (Wu et al., 2019). The significance in cardiac arrhythmia was not addressed in this study, but it may be the case since another study reported that the genetic ablation of $UCP2^{-/-}$ in mice resulted in AP and QT shortening, leading to a high propensity for ventricular arrhythmias (Larbig et al., 2017). In the setting of AF, high AP firing imposes a high demand for ATP in the atrial chambers, causing mitochondria remodeling, altered Ca^{2+} handling, and impaired atrial contraction. In atrial-like myocytes (HL-1 cells), 6–8 h of tachypacing caused dissipation of the $\Delta\Psi m$ and mitochondrial network, as well as the loss of mitochondrial Ca^{2+} transients. Interestingly, treatment with SS31, a compound that accelerates the formation of ATP from ADP, protected against mitochondria remodeling, impaired respiration, and Ca^{2+} homeostasis (Wiersma et al., 2019). The AMP-activated protein kinase (AMPK) is a cellular energy sensor that activates when the AMP/ATP ratio increases. Expression of a mutant (T172D) of the AMPK α1-subunit in rat ventricular myocytes resulted in the impaired open-state inactivation of Nav1.5, which led to AP prolongation and a high incidence of EADs at the cellular level. Whether this arrhythmogenic mechanism operates in conditions of high metabolic stress, as in ischemic HF and AF genetic syndrome, has not been yet determined (Harada et al., 2012). Similarly, impaired cardiac metabolism has been associated with several other cellular changes that increase the risk of arrhythmia generation. ATP, while mainly used by pumps and contractile machinery, is also used as a second messenger. ATP by itself can

bind with RyR2 or Mg^{2+}, a RyR2 inhibitor, and lower the Ca^{2+} threshold needed to elicit a Ca^{2+}-induced-Ca^{2+} release (Fill and Copello, 2002; Meissner, 2004). Low ATP would mean RyR2 inhibition with a subsequent deficient Ca^{2+} release. Impaired metabolism also increases phosphate levels, decrease ATP levels which, inhibit sarcolemmal Na^+/K^+ ATPase and activate the glycolytic pathway with the conversion of pyruvate to lactic acid, lowering the cell's pH. The low pH activates the sarcolemmal Na^+/H^+ exchanger, increasing the cytosolic Na^+ concentration. Na^+ accumulation is further aggravated by less active Na^+/K^+ ATPase and the translocation of connexin hemichannels to the non-junctional membrane, which turn into non-selective cation channels during ischemia or metabolic inhibition. High cytosolic Na^+ levels thus stimulate Ca^{2+} extrusion from the mitochondrial matrix through the mNCX, further inhibiting ATP synthesis and increasing cytosolic Ca^{2+} levels by inhibiting the sarcolemmal NCX (Yang et al., 2015).

Translocating connexin hemichannels to a non-junctional membrane, transforming them into non-selective channels, can also initiate other signaling pathways that promote arrhythmias (Kim et al., 2019). Because subsarcolemmal mitochondria also express connexin 43 in their IMM (Boengler et al., 2009), their proximity to the sarcolemma could enable the transport of messengers, such as ATP and Ca^{2+}, between compartments, which could increase the risk of cellular and mitochondrial Ca^{2+} overload and arrhythmias. Another channel with properties similar to connexin is pannexin 1 (Panx1). This channel is activated by cytoplasmic Ca^{2+} (Locovei et al., 2006) and ROS (Zhang et al., 2008) and has been reported to release ATP into the extracellular matrix upon activation in fibrotic processes (Dolmatova et al., 2012). ATP released into the medium by these channels activates P2X and P2Y receptors, or it can be rapidly converted to adenosine in the extracellular matrix, binding to its G-coupled receptor, A2AR, and inducing TGF-β1 expression (Shaikh et al., 2016; Zhang J. et al., 2017). This cytokine then stimulates collagen deposition and fibrosis of the cardiac tissue (Cerrone et al., 2018), which is known to act as a physical barrier to electrical signaling conduction, facilitating re-entry and arrhythmia propagation.

MITOCHONDRIAL REDOX IMBALANCE AND CARDIAC ARRHYTHMIA

In mitochondria, ROS is produced when electrons flowing through the ETC react prematurely with oxygen before reaching complex IV to form radical superoxide. These unstable molecules can then transform into hydrogen peroxide by mitochondrial superoxide dismutase or into hydroxyl radicals if they encounter a metal with which to react (Alfadda and Sallam, 2012). These byproducts of the ETC, while having a relatively short half-life, can diffuse to nearby cellular compartments and have various effects on a wide range of proteins. In the mitochondria-SR microdomain, proteins, such as the RyR2 and SERCA, are possible sites of protein oxidation, as well as other proteins implicated in the AP propagation that also reside within or near the dyadic cleft, all of which can affect cellular Ca^{2+} handling.

The RyR2 contains at least 21 free cysteine residues per monomer deemed to be susceptible to oxidation (Nikolaienko et al., 2018). Anzai et al. (1998) reported that hydroxyl radicals increased the opening probability of RyR2 channels reconstituted in lipid bilayers, an effect that was reverted by the SH-reducing agent dithiothreitol (Boraso and Williams, 1994; Anzai et al., 1998). At the cellular level, exposing cardiac myocytes to ROS increased the RyR2-mediated leak in permeabilized isolated ventricular myocytes, further supporting an up-regulatory effect of ROS (Terentyev et al., 2008). In another study, the β-adrenergic stimulation of myocytes promoted ROS production, oxidized RyR2, and, along with the RyR2 phosphorylation, enhanced the appearance of Ca^{2+} waves. MitoTEMPO treatment ameliorated the incidence of Ca^{2+} waves, indicating that mitochondria play a role in the production of oxidative stress (Bovo et al., 2012).

The proper functioning of the RyR2 is assisted by associated proteins, including calstabin and the FKBP12.5 factor. This factor exerts intrinsic negative regulation of the RyR2 and disrupts the RyR2-FKBP interaction, resulting in SR Ca^{2+} leakage and atrial and ventricular arrhythmias (Wehrens et al., 2003; Sood et al., 2008). The RyR2-FKBP association is redox-regulated (Zissimopoulos et al., 2007). As mentioned previously, highly oxidized levels of RyR2 were associated with increased SR Ca^{2+} leakage and an increased propensity for AF in a mouse model of CPVT. RyR2 oxidation was correlated with FKBP depletion from the RyR2, providing the molecular basis for atrial arrhythmia. Notably, the pharmacological stabilization of RyR2-FKBP binding prevented diastolic spontaneous Ca^{2+} releases in isolated atrial myocytes and decreased the propensity for AF *in vivo* (Shan et al., 2012). Disruption of the RyR2-FKBP interaction by oxidative stress has also been identified as the mechanism by which palmitoyl-carnitine (PC) promotes RyR2 leakage and, subsequently, cardiac arrhythmia (Roussel et al., 2015). In this study, the measurement of ROS production by MitoSox showed increased mitochondrial superoxide amounts in myocytes pre-exposed to PC, suggesting that mitochondrial ROS reduces RyR2-FKBP stability.

Oxidative stress also affects the RyR2 channel's activity by activating Ca^{2+} calmodulin kinase II (CaMKII) (Erickson et al., 2008). The clinical significance of CaMKII oxidation in atrial arrhythmogenesis was first explored by Mark E. Anderson's group. They reported an increase in oxidized CaMKII levels in atria from patients with AF, and the causality of AF was provided in a mouse model infused with the pro-oxidant hormone angiotensin II. After 3 weeks of treatment, angiotensin caused CaMKII oxidation, promoted SR Ca^{2+} leakage in isolated atrial myocytes and increased *in vivo* susceptibility to AF. The genetic inhibition of CaMKII oxidation reduced the propensity for AF, suggesting that CaMKII acts as a coupler between oxidative stress and atrial arrhythmia (Purohit et al., 2013). In line with these findings, Zhang et al. (2014) reported that the overexpression of NADPH oxidase 4 (NOX4) in zebrafish embryos resulted in increased superoxide production, CaMKII activation, and cardiac arrhythmia. Interestingly, MitoTEMPO treatment prevented NOX4-induced H_2O_2 production, indicating that mitochondrial oxidative stress plays a role in cardiac arrhythmia.

The SERCA pump plays a critical role in myocyte relaxation by promoting the recapture of Ca^{2+} by the SR. This pump is also subjected to oxidative regulation. Cys674 is identified as a critical target for oxidative modification, including oxidation (Qin et al., 2013) and sulfonylation (Hobai et al., 2013). The exposure of an SR membrane preparation to H_2O_2 demonstrated that ROS slows down the recapture of Ca^{2+}, which is consistent with an inhibitory effect (Qin et al., 2013). Loss of SERCA function caused by oxidative stress is, in part, responsible for diastolic dysfunction in aging (Herraiz-Martínez et al., 2015), inherited (Robinson et al., 2018) or acquired (Beuckelmann and Erdmann, 1992) cardiac conditions, and metabolic dysregulation (Bugger and Abel, 2014). Suppressed SERCA pump activity prolongs the Ca^{2+} spark duration life (O'Neill et al., 2004), increasing the chances of reaching the NCX and inducing triggered activity. The role of SERCA oxidation in cardiac arrhythmia in the setting of mitochondrial dysfunction deserves further exploration. As part of the SR Ca^{2+}-handling proteins, ROS is implicated in the development of cardiac substrates and triggers by causing the direct oxidation of membrane ion channels and structural elements or by indirectly targeting signaling pathways that modulate their function and expression. These topics have been extensively reviewed, and we would like to refer readers to excellent reviews in the area (Zima and Blatter, 2006; Takimoto and Kass, 2007; Jeong et al., 2012; Rutledge and Dudley, 2013).

Aging is a complex multi-factorial process characterized by a progressive decline in the organ function. Mitochondrial dysfunction also increases with aging and the resulting accumulation of ROS may contribute to the aging process by gradually damaging cellular components, a theory known as "the free radical theory of aging" proposed by Harman (1956). Aging is a primary risk factor for AF, the most common arrhythmia in the elderly population. AF may be the result of the accumulation of damaged biomolecules by mitochondrial oxidative stress over the years being the mitochondrial DNA (mtDNA) one of the exposed targets. Analysis of the mtDNA integrity in atrial samples from patients with persistent AF revealed deletion of the 4977 bp, as well as a higher mitochondrial content of 8-OHdG compared with control patients, suggesting DNA damage by oxidative stress (Lin et al., 2003). A connection between oxidative stress and the pathophysiology of persistent AF may also have a genetic basis which is exemplified in the study conducted by Kim et al. (2003) who compared the transcriptional profile between control patients and patients with chronic AF using cDNA microarrays. Among those upregulated genes in atrial samples from AF patients, five were encoding enzymatic systems associated with ROS production.

Pulmonary vein (PV) dysregulation is involved in the initiation of AF and a study demonstrated that the reduction of the atrial arrhythmia burden in patients undergoing isolation of PVs correlated with significantly lower plasma levels of oxidative markers (advanced glycation end-products and thiobarbituric-acid reacting substances) 9 months after the intervention (Böhm et al., 2016). From the experimental side, Wongcharoen et al. (2007) reported that PVs isolated from aged rabbits showed more depolarized resting membrane potentials and larger amplitude of EADs compared young PV preparations. Whether the high cellular arrhythmogenicity in aged PVs is linked to oxidative stress remains to be elucidated. Atrial remodeling, which contributes to the progression of permanent AF, also increases with aging and animal studies support a causal role for oxidative stress. In this regard, pioglitazone, an activator of the peroxisome-activated receptor gamma (PPAR-γ), decreased atrial fibrosis, NADPH oxidase subunits p22phox and gp91phox expression and the duration of pacing-induced AF in aged rats (Xu et al., 2012).

Aging regulates mitochondrial Ca^{2+} dependent processes including apoptosis and energetics by directly affecting mitochondrial components or their spatial relationship to the SR. For instance, the pharmacological inhibition of mPTP is reduced in hearts from senescent rats compared to younger animals when subjected to ischemia/reperfusion injury (Zhu et al., 2010). On the other hand, decreased mitochondrial Ca^{2+} uptake occurs in old isolated cardiomyocytes which correlated with lower mitochondrial NAD(P)H/NAD(P^+) ratio in response to electrical pacing. Interestingly, immunolabelling of permeabilized myocytes with anti-RyR and anti-VDAC revealed an age-dependent reduction of the fraction of the RyR2 overlapping with VDAC, whereas quantitative redox proteomics showed augmented oxidation of VDAC proteins. Thus, oxidation of mitochondrial membrane components may contribute to the disruption of the spatial proximity between mitochondria and the SR, which may cause defective Ca^{2+}-dependent mitochondrial energetics (Fernandez-Sanz et al., 2014). Which in turn others have appointed this increase in mitochondrial ROS to dysfunctional mitochondria which oxidize the RyR2, developing an increased risk of arrhythmia (Cooper et al., 2013). This increased arrhythmia risk was reduced by MitoTEMPO (Murphy et al., 2019).

MITOPHAGY IN CARDIAC ARRHYTHMIA

Since proper mitochondrial functionality is needed to prevent potentially damaging environments that predispose cardiac tissue to arrhythmias, defective remodeling of these organelles is also a potential mechanism for mitochondrial dysfunction with subsequent ROS generation and Ca^{2+} mishandling. Such is the case in the aging heart, were defective mitophagy has been described to ensue. This has been shown to cause accumulation of dysfunctional mitochondria and spontaneous Ca^{2+} release under adrenergic stimulation, which were prevented upon treatment with Torin1, an autophagy enhancer (Murphy et al., 2019). Another example is the SPRED2 deficiency murine model, an intracellular inhibitor of the ERK-MAPK pathway. SPRED2$^{-/-}$ mice have been described to develop cardiomyocyte hypertrophy, fibrosis, impaired excitability, and arrhythmias, which were associated with impaired mitophagy and the accumulation of defective mitochondria, along with increased ROS production, caspase 3 activity, and apoptosis, as this pathway seems to inhibit normal autophagy signaling. Selumetinib, a MAPK signaling inhibitor, restored autophagic flux *in vivo,* although no effects on arrhythmias were tested (Ullrich et al., 2019). Nonetheless, this proves the importance of proper mitochondrial remodeling to prevent damage accumulation and provides yet

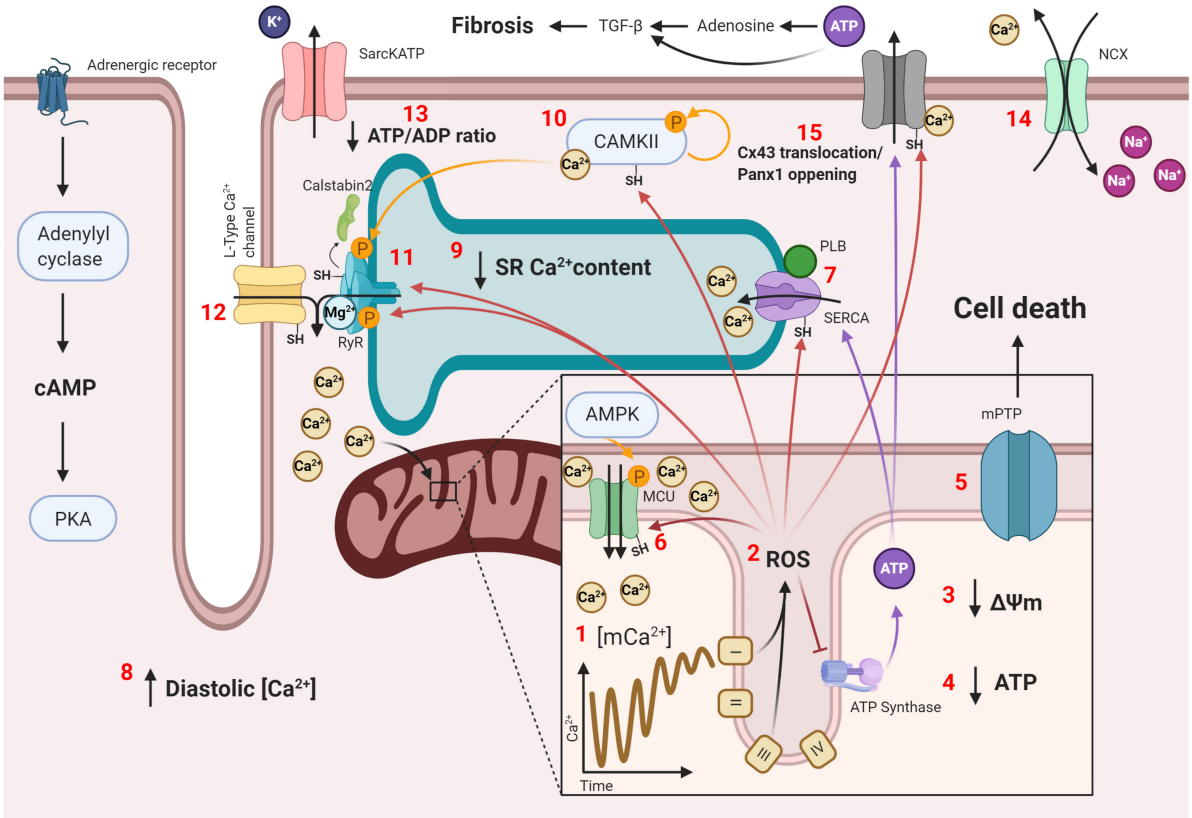

FIGURE 2 | Mitochondria-SR-T tubule communication in arrhythmogenesis. Conditions that favor Ca^{2+} overload, such as chronic adrenergic stimulation, cause the following: **(1)** increased Ca^{2+} transport through the mCU to the mitochondrial matrix, shortening mitochondrial Ca^{2+} transients' amplitude and stabilizing the mitochondrial Ca^{2+} concentration at a high level, which causes mitochondrial Ca^{2+} overload; **(2)** increased ROS production by mitochondrial structures, such as the ETC, with subsequent **(3)** loss of $\Delta\Psi m$ and **(4)** decreased ATP production; **(5)** opening of the mPTP, which initializes the cell death signal; **(6)** increased mCU activity by oxidation and, possibly, AMPK phosphorylation following a decline in the ATP concentration, which increases Ca^{2+} transport and ROS production; **(7)** decreased SERCA activity by oxidation and decreased PLB phosphorylation, which **(8)** increases the diastolic Ca^{2+} level and **(9)** decreases SR Ca^{2+} content, reducing contractility; **(10)** increased CAMKII activity by Ca^{2+} activation, oxidation, and autophosphorylation; **(11)** sensibilization of the RyR2 to luminal Ca^{2+} content with a reduced refractory state by oxidation and CAMKII hyperphosphorylation, with a subsequent unbinding of its modulating protein, calstabin2, and lower sensitivity to cytosolic Ca^{2+} by Mg^{2+} binding; **(12)** oxidation of LTCCs, which increases the amount of Ca^{2+} entering the cardiomyocyte upon activation; **(13)** opening of sarcKATP channels due to a reduced ATP/ADP ratio, which decreases the cardiomyocyte's bathmotropism and dromotropism; **(14)** increased activity of the NCX, which slowly depolarizes the cardiomyocyte and increases the risk of an unsolicited AP; **(15)** connexin 43 (Cx43) translocation in certain cardiac pathologies or pannexin 1 (Panx1) opening with Ca^{2+} or ROS transports ATP into the extracellular matrix, where it is transformed into adenosine, and both ATP and adenosine cause TGF-β1 expression and fibrosis. All these changes combined produce, at the cellular level, Ca^{2+} mishandling with subsequent low contractility and spontaneous contraction that can propagate to neighboring cardiomyocytes and, at the tissular level, patches of slow conduction by reduced dromotropism or collagen deposition caused by cardiomyocyte cell death and fibrosis, which enable possible re-entry zones for sustained arrhythmias.

another possible therapeutic target, mitochondrial dynamics. The term mitochondrial dynamics refers to the changes by which individual mitochondria go through the process of fusion and fission within the cell to isolate damaged mitochondria or to better couple the generation of $\Delta\Psi m$ to ATP synthesis, respectively. This is important in certain cardiac pathologies, such as HF. Pressure-overload HF murine models have been described to have an mitophagic blockade, which causes damaged mitochondria to accumulate and mitochondrial dysfunction to ensue (Yu et al., 2018). Regarding the different states of the mitochondrial network, fused mitochondria predomination has been described to be beneficial for cardiac function (Wai et al., 2015; Qin et al., 2020), as they are better at ATP synthesis and have lower ROS generation. Following this train of thought, inhibiting fission, promoting fusion, or maintaining an adequate mitophagy profile could potentially protect cardiac electrical signaling and organization. A summary with the presented mechanisms of arrhythmias are shown in **Figure 2**.

NOVEL TARGETS IN SR-MITOCHONDRIA INTERCONNECTION AS POTENTIAL ANTI-ARRHYTHMIC DRUGS

The development of new therapeutics could break the vicious cycle of Ca^{2+} overload, mitochondrial dysfunction, and ROS

TABLE 1 | Summary of therapeutic strategies used to prevent arrhythmogenic events and their main findings.

Therapeutic target	Compound	Mechanism of action	Models used	Main findings	References
mCU	Ru_{360}	Prevents mitochondrial Ca^{2+} overload by inhibiting mitochondrial Ca^{2+} transport	Pacing-induced VF model; I/R murine model; NI-HF murine model; pressure overload HF model	Reverted VF into VT; prevented arrhythmias upon reperfusion; prevented DADs in isolated cardiomyocytes; and VF when challenged with ISO; better mitophagy profile	Kimura et al., 2005; Garcia-Rivas Gde et al., 2006; Hamilton et al., 2018; Xie et al., 2018; Yu et al., 2018
	Efsevin	VDAC2 enhancer, increases Ca^{2+} transport into mitochondria	Zebra fish cardiac tissue	Prevented spark propagation into Ca^{2+} waves	Shimizu et al., 2015; Schweitzer et al., 2017
	Kaempferol	mCU enhancer, increases Ca^{2+} transport into mitochondrial matrix	CPVT murine model and iPSC-derived human cardiomyocytes	Prevented diastolic Ca^{2+} waves and spontaneous AP in both models as well as VT *in vivo*	Schweitzer et al., 2017
	Spermine	mCU enhancer, increases Ca^{2+} transport into mitochondrial matrix	Tachypacing protocol in atrial rabbit cardiomyocytes	Lowered Ca^{2+} transient alternans	Oropeza-Almazan and Blatter, 2020
	CGP-37157	mNCX inhibitor, reduces Ca^{2+} transport out of the mitochondria	Porcine model of I/R	Suppressed ventricular arrhythmias, lowered J point depression and expedited ST segment resolution	Sventzouri et al., 2018
	Cariporide	N^{+}/H^{+} exchanger inhibitor, potential regulator of mitochondria uptake	I/R model; isolated cardiomyocytes with ouabain poisoning; cardiomyocytes with angiotensin II and endothelin 1 administration	Prevented reperfusion arrhythmia, preserved metabolic status; prevented Ca^{2+} overload, delayed mPTP opening and hyper contracture; reduced ROS production and Ca^{2+} induced swelling	Sugiyama et al., 1999; Toda et al., 2007; Garciarena et al., 2008
mROS	Glutathione	ROS scavenger to prevent ROS damage	I/R in isolated rat hearts	Reduced reperfusion arrhythmias	Woodward and Zakaria, 1985
	Ascorbic acid	ROS scavenger to prevent ROS damage	I/R in isolated rat hearts	Reduced reperfusion arrhythmias; reduced lipid peroxidation, preserved mitochondria respiratory function and improved survival	Woodward and Zakaria, 1985; Tsai et al., 2011
	N- acetylcysteine	Glutathione precursor	I/R in isolated rat and dog hearts; murine model of fatty acid accumulation	Reduced reperfusion arrhythmias, reduced infarct size, improved mechanical function recovery; Preserved $\Delta\Psi m$ and Ca2+ transient amplitude, reduced Ca2+ sparks and prevented VT	Qiu et al., 1990; Sochman et al., 1990; Roussel et al., 2015
	TEMPOL	ROS scavenger to prevent ROS damage	I/R in rat model	Reduced arrhythmias, lipid peroxidation, preserved mitochondria respiratory function and improved survival	Tsai et al., 2011
	MitoTEMPO	ROS scavenger to prevent ROS damage	ISO-exposed isolated rabbit cardiomyocytes; CPVT murine model; HF guinea pig model; human end-stage HF cardiomyocytes	Partially prevented SR Ca2+ leak, Ca2+ waves, and ROS production; reduced ROS generation with subsequent RyR2 oxidation and leakage; prevented sudden cardiac death, ventricular arrhythmias and remodeling; reduced RyR2 hyperactivity and Ca2+ waves	Bovo et al., 2012; Ni et al., 2016; Dey et al., 2018; Dries et al., 2018; Hamilton et al., 2020
	SS-31 and SS-20	Most likely ROS scavenger and ROS production inhibitor	I/R model	Reduced infarct size, severity, and duration of arrhythmias and lipid peroxidation	Cho et al., 2007
	Quercetin / quercetin-filled liposomes	ROS scavenger to prevent ROS damage	I/R murine and dog model	Reduced arrhythmias and platelet aggregation; prevented peroxynitrite-induced arrhythmias	Xiao et al., 1993; Soloviev et al., 2002; Lozano et al., 2019

(Continued)

TABLE 1 | Continued

Therapeutic target	Compound	Mechanism of action	Models used	Main findings	References
	Resveratrol	ROS scavenger, sirtuin activator	I/R murine model; cardiomyocytes from human-induced pluripotent stem cells from patients with very long-chain acyl-CoA dehydrogenase deficiency	Reduced reperfusion arrhythmias and mortality, decreased LDH and increased NO levels; reduced DADs and cytosolic Ca2+ levels as well as normalizing APD by decreasing fatty acid oxidation intermediates	Hung et al., 2000; Knottnerus et al., 2020
	DTT	Restores reduced thiol groups in oxidized proteins	Isolated atrial cardiomyocytes exposed to TNF-α	Decreased transient amplitude, spark frequency and duration, and ROS production	Zuo et al., 2018
mPTP	CsA	Inhibits mPTP opening	I/R murine model; chronic AV block canine model; VF murine model; rabbit I/R model	Reduced arrhythmias, cardiac edema and cardiomyocyte death; reduced hypertrophy and arrhythmia susceptibility, no changes in electrical remodeling; no protection found; CsA offered no protection	Arteaga et al., 1992; Schreiner et al., 2004; Brown et al., 2008; Ayoub et al., 2017
	Bongkrekic acid	Inhibits ANT, reducing Ca2+ overload and ROS production	Murine model of fatty acid accumulation	Preserved $\Delta\Psi$m and Ca2+ transient amplitude, reduced ROS production, RyR2 oxidation, Ca2+ sparks and prevented VT	Roussel et al., 2015
	Octylguanidine	Inhibits mPTP opening	I/R murine model; hyperthyroid I/R murine model	Prevented arrhythmias and edema, preserved blood pressure, and mitochondrial function; prevented arrhythmias and preserved hemodynamic parameters, mitochondrial function, and reduced inflammatory cytokines	Parra et al., 2005; Pavón et al., 2009
	Citicoline	Inhibits mPTP opening	I/R	Prevented arrhythmia and pressure drop during reperfusion, maintained $\Delta\Psi$m and reduced ROS damage	Hernández-Esquivel et al., 2014
	4-ClDzp	Ligand to TPSO, a mPTP regulator	Rabbit I/R model; guinea pig I/R	Preserved cardiac function; preserved $\Delta\Psi$m, reduced AP shortening and prevented reperfusion arrhythmias	Akar et al., 2005; Brown et al., 2008
CAMKII	KN-93	CAMKII inhibitor	Genetic murine models: Gain-of-function RyR2, CAMKII overexpression, constitutively active CAN, CPVT Diabetic hyperglycemia murine model Human cardiomyocytes from diabetic HF patients Cardiomyocytes derived from induced pluripotent stem cells from CPVT patients Trabeculae from HF patients	Prevented AF after fast pacing; prevented arrhythmias after ISO administration; decreased premature contractions and sparks; improved contractile function	Khoo et al., 2006; Chelu et al., 2009; Sag et al., 2009; Sossalla et al., 2010; Liu et al., 2011; Di et al., 2013; Erickson et al., 2013
	AIP	CAMKII inhibitor	CAMKII overexpressing murine model; trabeculae from HF patients; impaired glucose tolerance murine model	Prevented afterdepolarization in isolated cardiomyocyte; improved contractile function; prevented arrhythmias and arrhythmogenic cellular events	Sag et al., 2009; Sossalla et al., 2010; Federico et al., 2019

(Continued)

TABLE 1 | Continued

Therapeutic target	Compound	Mechanism of action	Models used	Main findings	References
RyR2	Dantrolene	RyR2 stabilizer	CPVT KI murine model *Ex vivo* rabbit heart Long QT syndrome model Atrial infarct sheep model Cardiac arrest pig model Isolated cardiomyocytes from AF or endstage HF patients Cardiomyocytes from induced pluripotent stem cells from CPVT patients Clinical trial with CPVT patients	Prevented VT and decreased spark frequency; reduction of EADs and VT frequency; prevented AF; same efficacy as amiodarone to regain spontaneous circulation and hemodynamic stability in cardiac arrest; reduced spark frequency, Ca2+ waves, Ca2+ leak and spontaneous transients; reduced premature ventricular beats	Kobayashi et al., 2010; Penttinen et al., 2015; Hartmann et al., 2017; Wiesmann et al., 2017; Avula et al., 2018; Frommeyer et al., 2018
Calstabin2	JTV519	Increases affinity of calstabin2 to the RyR2	HF dog model KD calstabin2 murine model HEK293 with CPVT Ry mutations *in vitro* model 3D engineered heart tissue with optical activated ion channel model	Abolished abnormal RyR2 gating; prevented VT and sudden cardiac death	Kohno et al., 2003; Lehnart et al., 2004a; Wehrens et al., 2004; Lemme et al., 2019
	S107	Increases affinity of calstabin2 to the RyR2	CPVT murine models DMD murine model Cardiomyocytes derived from induced pluripotent stem cells from CPVT patients CPVT analogue nematode model	Prevented arrhythmias and seizures; reduced Ca2+ leak; prevented DADs	Lehnart et al., 2008; Fauconnier et al., 2010; Shan et al., 2012; Sasaki et al., 2016; Fischer et al., 2017
Mitochondrial dynamics	Mdivi-1	Mitochondrial fission inhibitor	I/R murine model	Reduced arrhythmias and preserved hemodynamic parameters	Maneechote et al., 2018
	M1	Mitochondrial fusion promoter	I/R murine model	Reduced arrhythmias and preserved hemodynamic parameters	Maneechote et al., 2019
mitoKATP	4CPI	H2S pro-drug (a mitoKATP agonist)	I/R model	Reduced tissue injury, ROS production, prevent reperfusion arrhythmias and improved recovery	Testai et al., 2016
	Compound A	mitoKATP agonist	I/R model	Reduce reperfusion arrhythmias, better results than diazoxide, no added effect with sarcKATP blocker	Gonca et al., 2016

generation to substantially reduce the incidence of sudden cardiac death and disease progression, especially in high-risk patients, such as those with HF. Fortunately, there are a few candidates that may prove useful in doing so. A summary of such compounds can be reviewed in **Table 1**.

Regulation of Mitochondrial Ca^{2+} Transport

Considering that the mCU seems to be the central piece in the positive feedback cycle, mCU inhibition may be efficient in stopping the cycle and maintaining mitochondrial integrity. Ru_{360} is a potent mCU inhibitor. It is an oxygen-bridged small molecule with a dinuclear ruthenium center complexed with amine groups. This molecule was first fully described in 1998 and synthesized from a familiar component, RuR. RuR was first used as the mCU inhibitor to study mitochondrial Ca^{2+} dynamics but was found to be non-specific for the mCU, as it has various effects on other cellular components. Ru_{360} was then described as being more potent (IC50 of 0.184 vs. 6.85 nM for RuR) and having a much greater affinity for the mCU, with a Kd of about 0.34 nM. Doses of up to 10 μM had no effect on SERCA, the RyR2, NCX, LTCCs, or myofibrils (Matlib et al., 1998; García-Rivas et al., 2005), which demonstrates its high specificity for the mCU and safety. In this sense, Ru_{360} treatment partially inhibited the mCU, maintaining proper mechanical performance of the heart and mitochondrial matrix free-Ca^{2+} concentrations at basal levels, despite high concentrations of cytosolic Ca^{2+} after ischemia/reperfusion (I/R) injury (García-Rivas et al., 2005). In an open-chest rat model of I/R, a bolus of Ru_{360} given before ischemia significantly prevented the occurrence of ventricular fibrillation (VF) and hemodynamic dysfunction. Ru_{360} administration was associated with protecting mitochondria from depolarization by mPTP opening and thus preserved mitochondrial functioning (Garcia-Rivas Gde et al., 2006). Ru_{360} infusion also reverted the

progression of pacing-induced sustained VF into ventricular tachycardia, a less severe form of arrhythmia, in an isolated heart model. This was associated with a reduction in the APD, refractory period, and slope of the APD restitution curves (Kimura et al., 2005).

Since then, Ru_{360} has been used to research the effects of inhibiting mitochondrial Ca^{2+} transport on various models of cardiac injury. It has already proved its efficacy in preventing EADs in ventricular myocytes in a non-ischemic HF model (Xie et al., 2018). It has also been shown to reduce VF in mice with thoracic aorta banding when presented with a catecholamine challenge. It is worth mentioning that inhibiting mitochondrial Ca^{2+} transport was related to less oxidized RyR2 in isolated cardiomyocytes, as opposed to other drugs that increase mitochondrial Ca^{2+} content, such as kaempferol, the mCU agonist, and CGP-37157, the mNCX inhibitor, which enhances RyR2 oxidation. Isolated cardiomyocytes also presented with improved Ca^{2+} homeostasis and less spontaneous Ca^{2+} waves (Hamilton et al., 2018). This further proves that mitochondrial Ca^{2+} overload and ROS generation can affect SR components and should be taken into consideration more fully. Finally, mitochondrial Ca^{2+} transport inhibition was described as useful in maintaining proper ECG signaling in a murine pressure-overload HF model by enhancing mitochondrial autophagy (Yu et al., 2018). It has been previously described that mitochondrial dynamics (i.e., fusion, fission, and mitophagy) are impaired in a wide range of cardiovascular diseases and could be another potential therapeutic target (Vásquez-Trincado et al., 2016). Unsurprisingly, mitophagy was found to be impaired in the HF model and inhibiting mitochondrial Ca^{2+} transport seemed to prevent the accumulation of dysfunctional mitochondria, although no clear explanation was given for why this happened. It appears that there is much more to be described regarding the affected mitochondrial signaling mechanisms when mitochondrial Ca^{2+} overload ensues.

Inhibiting mitochondrial Ca^{2+} transport seems to be a plausible therapeutic strategy to prevent arrhythmias. Nonetheless, there is evidence indicating that the opposite approach would be more effective. It has been observed that efsevin, a VDAC2 enhancer, allows better Ca^{2+} transport from the SR to mitochondria by interacting directly inside the channel pore (Wilting et al., 2020). This enhanced Ca^{2+} transport was observed to abolish Ca^{2+} spark propagation into Ca^{2+} waves and erratic contractions by limiting the Ca^{2+} spark's temporal and spatial boundaries, effectively acting as a "Ca^{2+} sponge" (Shimizu et al., 2015). Similarly, in a CPVT murine model, diastolic Ca^{2+} waves and spontaneous APs in isolated cardiomyocytes were prevented by enhancing mitochondrial Ca^{2+} transport via either efsevin or the mCU enhancer, kaempferol. Ventricular tachycardia was prevented *in vivo*, and similar results were found in iPSC-derived human cardiomyocytes (Schweitzer et al., 2017). Furthermore, enhancing mitochondrial Ca^{2+} content using the mNCX inhibitor CGP-37157 suppressed ventricular arrhythmias, lowered the depression of the J point during ischemia, and expedited ST-segment resolution after reperfusion in a porcine model of I/R (Sventzouri et al., 2018). Likewise, enhancing mitochondrial Ca^{2+} transport using the mCU activator spermine in isolated rabbit atrial cardiomyocytes undergoing a tachypacing protocol to induce AF demonstrated a protective effect against Ca^{2+} transient alternans. However, inhibiting mCU Ca^{2+} transport with Ru_{360} lowered the threshold needed to achieve alternans and enhanced the severity of the alternans (Oropeza-Almazan and Blatter, 2020).

Similar to the approach of using a specific mCU inhibitor, using gene therapy to downregulate mCU expression is another feasible alternative. With the development of better protocols to direct gene therapy to specific tissues, it may be possible to knockout (KO) mCU expression using siRNA (Oropeza-Almazan et al., 2017). Transgenic mice with mCU KO in cardiac tissue were shown to have less mPTP opening than their WT counterparts and appeared to have no problems when resting and upon being put on a treadmill. These mCU KO mice could even sprint for as long as their WT counterparts, provided that they were given a 30-min "warm-up" beforehand, as they were unable to do so acutely (Kwong et al., 2015). This finding correlates with the finding of similar mitochondrial basal Ca^{2+} levels between groups, while the KO group needed more time to accumulate Ca^{2+} after stress stimulation. Cardiomyocytes from the KO group also showed less mPTP opening compared to the WT group, and the KO hearts had a smaller affected area after I/R injury. Another study using mice with mCU knockdown in cardiac tissue showed that upon being submitted to a non-ischemic HF model, they presented with reduced Na^+/Ca^{2+} exchange currents, decreased APD, no observed EADs, and a reduced incidence of VF compared to the WT group (Xie et al., 2018). Combining these findings with the recent development of nanometric particles used for drug delivery places this type of therapy in the possible near future, as it has been recently proven that siRNA can be packaged and protected from degradation while maintaining its biological effects *in vitro* studies. Furthermore, cariporide, an N^+/H^+ exchange inhibitor, has proven useful in not only preventing arrhythmias in an I/R model but also preserving the metabolic status of the heart (Sugiyama et al., 1999). At the mitochondrial level, cariporide has also been shown to have mitochondrial protective properties. In isolated ventricular myocytes, cariporide prevented mitochondrial Ca^{2+} overload upon ouabain administration. No effect was seen in preventing $\Delta\Psi m$ reduction, although mPTP opening was delayed significantly. This translated the ouabain taking longer to cause hyper contracture, which would indicate global ATP depletion and, thus, more preserved mitochondrial functioning (Toda et al., 2007). Notably, cariporide decreased Ca^{2+}-induced mitochondrial swelling, comparable to the effects of Cyclosporine A (CsA) (Garciarena et al., 2008). N^+/H^+ exchange inhibitors seem to be a promising therapeutic approach for cardioprotection, although they require further studies to elucidate their precise mechanism of action in regulating mitochondrial Ca^{2+} handling to better design a therapeutic strategy.

Modulation of Redox Homeostasis

Cellular ROS Scavengers

Since increased mitochondrial ROS generation seems to be the initial insult that starts the positive feedback loop, antioxidants

could be useful in boosting the myocardium's ROS defense pool. Since their first trials in animal models, ROS scavengers have had beneficial effects in preventing arrhythmias during I/R. Superoxide dismutase, glutathione, and ascorbic acid significantly reduced reperfusion arrhythmias in isolated rat hearts (Woodward and Zakaria, 1985). N-acetylcysteine, a precursor for glutathione synthesis, also demonstrated protection against reperfusion arrhythmias in isolated rat heart (Qiu et al., 1990) and dog models (Sochman et al., 1990) of I/R, reducing infarct size, preventing reperfusion arrhythmias, and improving the recovery of mechanical functioning during reperfusion. Another dog model of I/R was infused with superoxide dismutase. High-dose infusion was correlated with a reduction in ventricular extrasystoles, improved regional contractile force, and increased blood flow during reperfusion (Kónya et al., 1992). Other non-specific antioxidants, such as ascorbic acid and tempol, have also proven useful in preventing necrosis and dysfunction after VF and cardiopulmonary resuscitation (CPR). VF was induced in rats, and after 5 min, CPR and electrical shocks were used to induce a return to spontaneous circulation. Administering ascorbic acid or tempol at the start of CPR in a murine model of I/R was correlated with reduced myocardial necrosis and lipid peroxidation, preserved mitochondrial respiratory function, and a higher and faster rate of return of spontaneous circulation, along with better survival rates at 72 h post-CPR (Tsai et al., 2011).

Since the endoplasmic reticulum (ER) is yet another site where redox signaling is integrated, it is a potential ROS control organelle. Selenium (Se) is essential for the function of at least 25 selenoproteins, including the antioxidant glutathione peroxidase (GPx). In patients undergoing primary coronary artery bypass grafts, preoperative reduced levels of Se, GPx, and malondialdehyde correlated with high incidence of postoperative AF (McDonald et al., 2016). Moreover, low Se concentration is inversely associated with coronary heart diseases in observational studies (Flores-Mateo et al., 2006). In animal studies, administration of selenoprotein T (PSELT5) prevented the increase of cardiac ROS levels and infarct size area following I/R injury (Rocca et al., 2018). In primary culture of cardiomyocytes, low concentration of Se impaired mitochondrial function and reduced expression of Kv1.2 channel (Zhang C. et al., 2017). Although no electrophysiologic consequences were provided in this study, a reduced I_{K1} would predict AP prolongation and the facilitation of EADs. Recently, it has been described that ATF6, an ER transcription factor, is responsible for inducing antioxidative stress programs, such as catalase and ROS scavenging selenoproteins, in a setting with a high level of accumulation of misfolded proteins, such as in I/R (Jin et al., 2017). Selenoproteins were also shown to recover contractility and reduce infarct size in an isolated rat heart I/R model (Rocca et al., 2018) and treatment with dexmedetomidine, a sedative and analgesic with antioxidant properties, along with Na_2SeO_3, a Se supply for selenoproteins synthesis, was shown to confer cytoprotection against cellular damage in a cellular model of oxygen and glucose deprivation/reoxygenation by reducing apoptosis, LDH release, and ROS levels (Wang et al., 2020). However, the clinical application of Se should be taken with caution since it was demonstrated that the administration of this element in a patient population with acute myocardial infarction associated with HF did not prevent the appearance of ventricular arrhythmia (Tränkmann et al., 1999). Thus, it is possible that supplementation of selenium may be more beneficial as a preventive therapy. Nonetheless, this places the possibility of targeting the ER antioxidant resources as a mean to keep ROS levels down in a pathological setting with a high risk of arrhythmia.

Other antioxidants, such as quercetin and resveratrol, have been useful in preventing cellular damage and preserving mitochondrial functioning upon challenge with insults *in vitro* models, such as I/R (Lozano et al., 2019), and in reducing right-heart remodeling and dysfunction in a murine model of pulmonary arterial hypertension (Vázquez-Garza et al., 2020). Regarding arrhythmias, treatment with quercetin has proven useful in preventing platelet aggregation and arrhythmias in an I/R murine model. The protective effect is thought to be partially caused by inhibiting platelet aggregation and thus preventing microthrombi during reperfusion, which would cause heterogenous circulation when blood flow is restored, predisposing the cardiac tissue to arrhythmias (Xiao et al., 1993). A more recent study using treatment with quercetin-filled phosphatidylcholine liposomes was able to prevent peroxynitrite-induced arrhythmias in isolated murine papillary muscle and in an I/R dog model (Soloviev et al., 2002).

Resveratrol was shown to reduce lactate dehydrogenase levels, an indicator of cellular damage, as well as increase NO levels in carotid blood during I/R arrhythmias, although there was no effect on ischemia-induced arrhythmias and mortality (Hung et al., 2000). It was also observed that cardiomyocytes from human-induced pluripotent stem cells from patients with very long-chain acyl-CoA dehydrogenase deficiency presented with a shorter APD, as well as a greater incidence of DADs and higher cytosolic Ca^{2+} concentration. Resveratrol treatment reportedly abolished all these changes in one of the two patients enrolled in the study, presumably by increasing the amount of defective mitochondrial proteins, which had residual activity; however, the other patient had a more severe form of the disease and no residual activity (Knottnerus et al., 2020). This decreased the accumulation of fatty acid oxidation intermediates, which seem to have caused the changes described and is a possible explanation for the higher risk of arrhythmias in patients with this genetic disease.

Another potential molecule worth mentioning is DL-dithiothreitol (DTT), which restores thiol groups in oxidized proteins to their reduced state, somewhat inhibiting ROS effects. Treatment with DTT reversed Ca^{2+}-handling disorders observed in isolated atrial cardiomyocytes after exposure to TNF-α, such as a decreased transient amplitude, increased spark frequency and duration, and increased mitochondrial ROS production (Zuo et al., 2018), suggesting that antioxidant therapy is a promising anti-arrhythmic therapy. Finally, it has also been demonstrated that bongkrekic acid, an adenine nucleotide translocase (ANT) inhibitor, has potential as an antiarrhythmic drug. In a murine model of fatty acid accumulation, fatty acids caused a partial inhibition of ANT, which was associated with

increased ROS production, decreased $\Delta\Psi$m, RyR2 oxidation, decreased Ca^{2+} transient amplitude, increased Ca^{2+} sparks and Ca^{2+} wave incidence, and an increased propensity to develop non-sustained ventricular tachycardia. Adding bongkrekic acid or N-acetylcysteine decreased ROS production, RyR2 oxidation, and Ca^{2+} spark frequency, as well as preserved $\Delta\Psi$m and the Ca^{2+} transient amplitude and prevented the development of non-sustained ventricular tachycardia (Roussel et al., 2015).

It was previously thought that antioxidants like quercetin and resveratrol, due to their low bioavailability and non-specificity to any tissue, would make them poor candidates for new therapies (Formica and Regelson, 1995). This is backed by the fact that only limited benefits have been found when using such therapies (Askari et al., 2012; Wilson et al., 2016; Vázquez-Garza et al., 2020). Nonetheless, similar to the gene therapy described previously, the development of nanomaterials capable of packaging the components, protecting them from degradation, and directing them to a specific tissue places these often-dismissed compounds back on the table. This new way of delivering compounds has opened a wide spectrum of possible components that were disregarded in the past due to having low bioavailability or a small therapeutic window or because they were unable to pass through the cellular membrane. Furthermore, nanomaterials make it possible for two or more different compounds to be placed in the same particle, increasing the number of possibilities exponentially. A more in-depth review of nanoparticles is available (Lozano et al., 2018). Even drugs that are being used today may be packaged to provide the same or a better outcome while reducing adverse effects. It is only a matter of time before these compounds demonstrate their real potential in preserving mitochondrial integrity in a pathologic setting, such as arrhythmias. Previous clinical trials targeting ROS scavenging as an antiarrhythmic therapy have had mixed results (El-Hamamsy et al., 2007; Hicks et al., 2007; Negi et al., 2011; Rodrigo et al., 2013; Martínez-González et al., 2014; Stanger et al., 2014), some of which may be debated to be due to other effects, not only ROS scavenging (Martínez-González et al., 2014). Furthermore, a meta-analysis of clinical studies with commonly used antioxidants concluded that the studies with positive results were flawed by the small sample size and the lack of evidence of a real antioxidant effect in these patients (Violi et al., 2014). Nonetheless, there have been no clinical trials targeting mitochondrial ROS specifically, and thus the possibility of this being a new therapeutic target should not be dismissed.

Specific Targeting of Mitochondrial ROS Production

MitoTEMPO, a mitochondria-specific molecule, is composed of a combination of the antioxidant piperidine nitroxide tempo and the lipophilic cation triphenylphosphonium. The lipophilic cation section of the molecule enables it to pass through bilipid layers and accumulate predominantly where there is an accumulation of negative charges, which means that it is drawn toward $\Delta\Psi$m. Mitochondrial concentrations of MitoTEMPO are several 100-fold when compared to cytosol. This makes it a suitable candidate to prevent excessive mitochondrial ROS accumulation and has already been tested in a murine model of diabetic cardiomyopathy. Daily dosing reduced superoxide generation in cardiomyocytes, reduced mitochondrial ROS generation, decreased apoptosis, and reduced myocardial hypertrophy, which were increased in the diabetic group without MitoTEMPO. The treatment group was also found to have a preserved ejection fraction and fractional shortening when measured by echocardiography, both of which were reduced in the diabetic group without MitoTEMPO (Ni et al., 2016). Similarly, in isolated rabbit cardiomyocytes, ISO exposure was associated with increased Ca^{2+} leakage, Ca^{2+} waves, and ROS production, as evidenced by fewer free thiols in the RyR2. These effects were partially prevented using MitoTEMPO or other ROS scavengers (Bovo et al., 2012) and were corroborated in a murine model of CPVT (Hamilton et al., 2020). MitoTEMPO has also been described as being able to prevent sudden cardiac death and ventricular arrhythmias in an HF guinea pig model, as well as prevent remodeling (Dey et al., 2018). Other mitochondria-specific molecules have also produced similar results. In an I/R model, peptides SS-31 and SS-20 reduced infarct size and severity, as well as the duration of reperfusion arrhythmias and lipid peroxidation, most likely by scavenging and reducing ROS production (Cho et al., 2007). Observations of human ventricular myocytes from patients with end-stage HF further support this idea. In this condition, it has been shown that RyR2s become uncoupled from nearby T-tubules and prone to spontaneous activation. Treatment with mitochondrial ROS scavengers reduces the receptor's hyperactivity and spontaneous Ca^{2+} wave formation under adrenergic stimulation (Dries et al., 2018). These compounds are, therefore, another viable treatment option to reduce mitochondrial ROS accumulation and subsequent mitochondrial dysfunction.

Modulation of mPTP Opening

Despite its elusive composition, the mPTP is strongly regulated by cyclophilin D (CypD), the VDAC, ANT, the phosphate carrier, ATP synthase, and the translocator protein (TSPO), formerly known as the peripheral benzodiazepine receptor (Alves-Figueiredo et al., 2021). A causative relationship between ROS-induced mPTP opening and $\Delta\Psi_m$ depolarization has also been demonstrated in numerous studies. In particular, there is some evidence that the mPTP may also be regulated by TSPO through interaction with the other components (Zulian et al., 2011). Using TSPO ligands has been shown to inhibit $\Delta\Psi_m$ depolarization after excessive ROS exposure (Leducq et al., 2003). TSPO became known as a potential therapeutic target when it was shown to activate a channel in the IMM, the inner membrane anion channel (IMAC). This channel is described as being able to depolarize $\Delta\Psi_m$ in an mPTP-independent way, causing oscillations in $\Delta\Psi_m$ and intermittent sarcKATP channel activation (Aon et al., 2003). These oscillations cause variation in the APD, which promotes a reentry pathway to the electrical stimuli. Inhibiting TSPO, therefore, has the potential to cover two different pathways to mitochondrial dysfunction, IMAC-mediated and mPTP-mediated (Gambardella et al., 2017), making this protein a potential therapeutic target. Although studies have shown that TSPO ligands can reduce ROS levels and abolish metabolic and electrophysiological changes, they have yet to be tested in a clinical setting (Akar et al., 2005). Protection

against mitochondrial depolarization has been observed to translate into reduced APD shortening and inexcitability in a dose-dependent manner. However, the use of FGIN-1-27, an IMAC activator, promoted faster shortening of the APD and resulted in an earlier loss of conduction during reperfusion (Akar et al., 2005). Hearts treated with an IMAC activator prior to ischemia are more responsive to it. High-resolution optical mapping demonstrated small areas of conduction blockage during early ischemia that persisted throughout the whole recording, making it more suitable for reentry, which caused VF. IMAC inhibition stabilized $\Delta\Psi m$ *in vitro*, suppressed reperfusion arrhythmias, and promoted faster AP recovery (Akar et al., 2005). The protective effect on AP recovery was also demonstrated in a rabbit model of I/R (Brown et al., 2008). Notably, the antiarrhythmic effect afforded by TSPO ligands was not evident in hearts treated with the mPTP desensitizer CsA. This finding indicates that IMAC and mPTP opening are primary mitochondrial mediators of acute postischemic arrhythmias. Finally, mPTP opening, which can be viewed as an irreversible loss of mitochondrial function, leads to cell death. Over time, cardiomyocytes are replaced with fibrotic tissue, which generates a physical barrier that prevents proper stimulation of the myocardium in an orderly manner. This pathological remodeling creates possible paths for re-entry arrhythmias.

As mentioned previously, mPTP opening is the process by which mitochondrial membrane integrity is permanently lost, and content from the mitochondrial matrix flows toward the cytosol. Thus, the mitochondria are no longer able to maintain their $\Delta\Psi m$, ATP is unable to be synthesized, and there is a subsequent loss of ionic balance. Between the leaked components that are translocated to the cytosol, cytochrome C, which normally takes part in the ETC, starts a signaling cascade for cell apoptosis. Cardiomyocyte death may be the most challenging arrhythmogenic process to deal with, as cardiomyocytes do not regenerate and are replaced with fibrotic tissue. This fibrotic tissue not only reduces the myocardium's pool of available contractile units to create effective blood pumping but also creates zones in which electrical signals do not travel at the proper speed, paving the way for disorganized contraction. CsA, a well-known immunosuppressor, was found to be a potent mPTP inhibitor many years ago (Crompton et al., 1988). Later, it was found that the compound's inhibitory effect on the mPTP is achieved by inhibiting CypD, a component required for mPTP opening (Davidson and Halestrap, 1990; Griffiths and Halestrap, 1991). It was discovered that inhibiting mPTP with CsA reduces I/R-induced arrhythmias by preserving cardiac tissue from suffering edema and reducing cardiomyocyte death, presumably by protecting mitochondria from Ca^{2+}-induced damage (Arteaga et al., 1992). mPTP inhibition was then corroborated as a potential strategy when another inhibitor, octylguanidine, was observed to protect against arrhythmias and edema caused by I/R, as well as maintain blood pressure and mitochondrial function (Parra et al., 2005). The effect was later confirmed in a hyperthyroid murine model undergoing I/R. Octylguanidine protects against arrhythmias by maintaining mitochondrial functioning and cardiac hemodynamic parameters and reducing inflammatory cytokines (Pavón et al., 2009). Another mPTP inhibitor, citicoline, was also useful in preventing arrhythmia and blood pressure drop during I/R. Mitochondria maintained adequate $\Delta\Psi m$, high respiratory control, cis-aconitase enzyme activity, and mDNA integrity (Hernández-Esquivel et al., 2014). It was then shown that mPTP inhibition by CsA administration was related to reduced infarct size in a murine model of I/R (Xie and Yu, 2007). CsA was also shown to reduce hypertrophy and arrhythmia susceptibility in a canine model of chronic atrioventricular blockage. CsA administration was not associated with reducing the electrical remodeling observed in the model, as indicated by a prolonged effective refractory period similar to the no-treatment group, although it did reduce the thickness of free and septal ventricular walls. Nonetheless, the CsA group showed no susceptibility to drug-induced polymorphic ventricular tachycardia (Schreiner et al., 2004). This led to the conclusion that electrical remodeling is only partially responsible for the susceptibility to arrhythmias seen in the model and that hypertrophy is also a necessary substrate for arrhythmogenesis. However, more recent studies found no protective effect when using CsA and measuring mPTP opening *in vivo* in a VF murine model. There were no differences in troponin I, cytochrome c, or NAD^+ levels when compared to the group treated with just the vehicle, as well as in hemodynamic and left ventricular (LV) function parameters (Ayoub et al., 2017). Although it could be argued that CsA was administered at half the dosing concentration used in previous studies and was administered for only 5 min before inducing VF or before starting the resuscitation protocol. Similar results were found when comparing 4′-chlorodiazepam (4-ClDzp), a ligand for TSPO, to CsA in a rabbit I/R model. CsA offered no protection against arrhythmias, in contrast to 4-ClDzp, which preserved normal cardiac functioning when given as an infusion throughout the I/R or as a single bolus before reperfusion (Brown et al., 2008). Another study utilized guinea pig hearts in an I/R protocol. 4-ClDzp preserved $\Delta\Psi m$, reduced AP shortening, and prevented reperfusion arrhythmias. Recovery was remarkably higher than in the group treated with CsA (Akar et al., 2005). This evidence raises the possibility of yet another potential target for new anti-arrhythmic therapies, and some authors have even proposed that the mediator of post-ischemic arrhythmias is the IMAC, which can be regulated via TSPO, rather than the mPTP (Motloch et al., 2015). However, a CypD KO murine model proved that the mPTP does, indeed, play a role in arrhythmogenesis. At the cellular level, cardiomyocytes from CypD KO mice were much less likely to undergo mPTP opening upon the addition of FCCP. Likewise, the incidence of Ca^{2+} waves and Ca^{2+} alternans was much lower than in their WT counterparts. Similar results were also obtained when CsA was added to WT cardiomyocytes. At the *ex vivo* heart level, ECGs showed ST-T wave alternans in the WT group, but the alternans were absent in the CypD KO group and only observed in one heart in the CsA group. Arrhythmia scores were consistently much lower upon FCCP addition or I/R (Gordan et al., 2016). Using the same CypD KO model, arrhythmia susceptibility was tested under an iron overload protocol. Again, WT cardiomyocytes presented with increased ROS production, diminished $\Delta\Psi m$, and frequent Ca^{2+} waves, while *ex vivo*

hearts presented with arrhythmias upon stimulation. Although the CypD KO group presented with similar ROS production and decreased $\Delta\Psi m$, its Ca^{2+} waves were significantly less frequent, and arrhythmia scores were lower. Similar results were found when the WT group was treated with CsA (Gordan et al., 2020). Although there may be contradictory evidence, CsA is still thought to be a molecule with therapeutic potential.

These findings have led to the use of CsA as a positive control for mitochondrial protection against mPTP, with which other treatments can be compared. However, when CsA was used as a treatment for out-of-hospital cardiac arrest in the CYRUS trial, there was no difference in organ failure, survival at hospital admission, survival at 24 h, survival at hospital discharge, or favorable neurologic outcomes when compared to the control group (Argaud et al., 2016). This finding led to the reasoning that in clinical practice, patients who are susceptible to cardiac arrest or have a baseline cardiopathy have already undergone adaptations in their cardiac tissue that make them less susceptible to mPTP protection with CsA, unlike murine models of ischemia-reperfusion that use healthy and young animals in both groups. After integrating the information presented here, it is possible that mPTP inhibition delays irreversible mitochondrial damage, giving the cardiomyocyte time to fix the underlying cause. It may be helpful in a young, healthy animal model in which the insult is temporal and where the cardiac tissue can deal with the ROS generated after reperfusion, given the extra time CsA provides before mPTP opening. However, in the context of a patient with severe atherosclerosis or HF, which is more commonly the case in patients with a high risk of arrhythmias, there is already increased ROS production and Ca^{2+} overload, which decreased the cell's resources considerably before ischemia. Delaying mPTP opening would have little effect in preserving cardiac tissue since the cardiomyocyte is unable to re-establish homeostatic conditions, even if given more time with CsA. Nonetheless, there is still the possibility that CsA, used in combination with another therapy, could synergize by delaying mPTP opening, and other therapies could take effect in cardiomyocytes, which would not have had enough time for recovery otherwise.

Regulation of Mitochondrial Quality Control

Excessive mitochondrial fission has been observed to promote cell death in I/R models. Mdivi-1, a fission inhibitor that has been shown to prevent apoptosis, and M1, a cell-permeable phenylhydrazone fusion promoter, were administered in an I/R murine model (Maneechote et al., 2018, 2019). Both treatments significantly reduced arrhythmias after reperfusion when given prior to ischemia, as well as reduced the decline in hemodynamic parameters. Preventing excessive fission with the subsequent reduction in $\Delta\Psi m$ and ATP synthesis and increasing ROS generation is yet another potential therapeutic strategy to prevent arrhythmia generation in high-risk patients. As stated previously, a pressure-overload HF murine model was shown to have a blockade in the cell's mitophagic function, leading to damaged mitochondria accumulation. When given the mCU inhibitor RuR or Ru_{360}, the mitophagic profile improved to reveal no blockade, along with improvement in cardiac function, reduction in ventricular asynchrony, and preserved mitochondrial integrity (Yu et al., 2018). This finding indicates that some therapies, such as mCU modulation, have effects at different levels and signaling pathways. Further research is needed to elucidate the mechanisms by which this happens to better understand the effects that such strategies have at the mitochondrial and cellular levels.

Maintaining Energetic Balance With High-Energy Phosphate Analogs

A central point of Ca^{2+} dysregulation is the energetic deficiency that ensues given the lower ATP production by dysfunctional mitochondria, accompanied by higher energetic demand from a less efficient Ca^{2+} removal system. Thus, replenishing the energetic reserve with exogenous molecules is another possible approach. In this regard, cyclocreatine, a creatine analog that is permeable to membranes, has been used mainly as a therapy for I/R injury. Prior treatment with cyclocreatine in the diet has been shown to preserve ATP levels longer during ischemia in chicken hearts (Turner and Walker, 1985) when measured by conventional techniques, as well as in rat hearts when measured via nuclear magnetic resonance imaging (Osbakken et al., 1992). Cyclocreatine has also been demonstrated to have anti-inflammatory effects in isolated heart (Elgebaly et al., 1990) and *in vivo* (Elgebaly et al., 1993) models of ischemia. Recovery of cardiac function after cold storage has also been improved when hearts were previously treated with cyclocreatine phosphate (Elgebaly et al., 1994), as well as in models of cardiopulmonary bypass (Houser et al., 1995). Previous studies have only been conducted in the context of ischemia and in clinical trials using other high-energy phosphate analogs without favorable results in HF or myocardial infarction patients (Horjus et al., 2011). However, it may still be worthwhile to research the effects of compounds like cyclocreatine in arrhythmia models, as it has been stated that the failure of these substances to preserve ATP levels and, thus, cardiac function may be due to kinetic differences in their ability to become a substrate for creatine kinase and their phosphoryl group transfer potential. Although monotherapy with high-energy phosphate analogs may not be effective in preventing arrhythmias, it can still potentiate the effects of other therapies by increasing and preserving the heart energy pool.

MitoK_{ATP} Channel Modulators

MitoK_{ATP} channels are structures within the IMM that, upon opening, partially dissipate $\Delta\Psi m$. This partial depolarization diminishes the driving force for Ca^{2+} entry and thus prevents Ca^{2+} overload and generates ROS, which have been associated with the protection conferred by ischemic preconditioning (Pain et al., 2000). Agonists of this channel have been assessed as a possible therapeutic approach. 4-carboxy phenyl-isothiocyanate (4CPI), a hydrogen sulfide (H2S) pro-drug and mitoK_{ATP} agonist, reduced tissue injury and ROS production, prevented arrhythmias, and improved recovery upon reperfusion in murine models of I/R; these effects were lost when 5-hydroxydecanoic acid, a mitoK_{ATP} blocker, was added (Testai et al., 2016).

Compound A, another mitoK$_{ATP}$ agonist, has also been proven to reduce reperfusion arrhythmias in I/R, with effects even more pronounced than diazoxide, a more commonly used mitoK$_{ATP}$ agonist. Unfortunately, these effects were not more effective when used in combination with a sarcolemmal K$_{ATP}$ blocker (Gonca et al., 2016). Even sevoflurane, a commonly used anesthetic, has been demonstrated to have protective effects dependent on mitoK$_{ATP}$ opening. Preconditioning isolated rat hearts with sevoflurane was demonstrated to have protective effects upon I/R, as evidenced by smaller infarct size, reduced cardiac troponin I levels, upregulated PKC, and downregulated caspase 8. This protection was lost when hearts were pretreated with 5-hydroxydecanoic acid, suggesting the role of mitoK$_{ATP}$ in the protective effects of sevoflurane (Wang et al., 2015). While these results seem promising, more studies are needed, mainly in other models susceptible to generating arrhythmias, to obtain more solid evidence that mitoK$_{ATP}$ is a potential antiarrhythmic therapy.

In the last few years, there has been considerable advancement in understanding the molecular mechanisms of cardiac disease. This has laid a path to the development of better therapeutic strategies that were first ignored or disregarded as potentially ineffective. It has also led to the discontinuation of practices that were once thought to be helpful but then proved to be either neutral or damaging. It may be time to expand our viewpoint and explore the crosstalk that takes place between organelles and seems to orchestrate Ca^{2+} homeostasis rather than focus on a single cellular component for a new therapy to prevent sudden cardiac death. This may be the right way to prevent arrhythmogenesis, by tackling the central mechanism that leads to different cellular malfunctions.

Mitochondrial-SR Interaction

CAMKII Modulation to Prevent SR Ca^{2+} Leaks Into Mitochondria

Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) is an enzyme normally activated by Ca^{2+}-calmodulin and phosphorylates a wide range of proteins, including RyR2, PLB, and LTCCs. Increased CAMKII activity has been observed in pathological processes, such as cardiac hypertrophy and HF (Kirchhefer et al., 1999; Zhang et al., 2003). Whether a higher phosphorylated state of the RyR2 leads to a subsequently higher opening probability in diastolic Ca^{2+} concentrations, the generation of Ca^{2+} sparks, lower SR Ca^{2+} content, and a negative force-frequency relationship (Ai et al., 2005; MacDonnell et al., 2008; Kushnir et al., 2010) remains controversial. Unsurprisingly, CAMKII has been a potential target in the prevention of hypertrophy, arrhythmias, and HF. Modulating its activity has led to interesting results regarding cardiac tissue protection. KN-93, the most well-known CAMKII inhibitor, has been shown to prevent AF in mice with a gain-of-function mutation in the RyR2 after fast atrial pacing (Chelu et al., 2009). AIP, another CAMKII inhibitor, has also been shown to prevent afterdepolarization events in isolated cardiomyocytes in a murine model overexpressing CaMKIIδ_C. Additionally, KN-93 administration *in vivo* prevented ISO-induced arrhythmias (Sag et al., 2009). In a murine model of diabetic hyperglycemia, KN-93 administration reduced premature ventricular contractions, as well as Ca^{2+} sparks, and was further supported with similar findings from diabetic failing heart donors (Erickson et al., 2013). This treatment strategy has also proved to be effective in a murine model expressing constitutively active Ca^{2+}-dependent phosphatase calcineurin (CAN), in which KN-93 administration reduced arrhythmia susceptibility, as well as improved ventricular cardiomyocyte contractile functioning (Khoo et al., 2006). Similarly, an impaired glucose tolerance murine model was found to have arrhythmias and arrhythmogenic events related to hyperphosphorylation of Ca^{2+} handling proteins in isolated ventricular cardiomyocytes, which were prevented when a transgenic model that expressed AIP was submitted to the same impaired glucose tolerance protocol (Federico et al., 2019). Furthermore, CaMKII also regulates mitochondrial functioning by controlling fatty acid oxidation through palmitoyl-CoA transferase I (Sharma et al., 2010) and Ca^{2+}-dependent dehydrogenases via the mCU (Joiner et al., 2012); however, some studies do not support this conclusion (Nickel et al., 2020). CaMKII might enhance mCU activity by phosphorylating the mCU's N-terminals and concomitantly lead to mPTP opening by mitochondrial Ca^{2+} overload and, ultimately, cell death upon I/R injury or dilated cardiomyopathy (Joiner et al., 2012). Chronic CaMKII activation has also been shown to be involved in mitochondrial gene reprogramming, leading to mitochondrial dysfunction and increased oxidative stress in cardiac hypertrophy (Joiner and Koval, 2014). CaMKII also mediates Drp1 phosphorylation, which has been shown to increase the frequency of mPTP opening upon chronic β-adrenergic stimulation (Xu et al., 2016). Although the use of a CaMKII inhibitor has not been demonstrated in mitochondrial physiology, KN-93 administration has also been used in a murine model of CPVT. Arrhythmias were abolished when treated with KN-93, and the spark frequency was reduced in isolated cardiomyocytes when treated with ISO (Liu et al., 2011). These findings are further supported by cardiomyocytes derived from induced pluripotent stem cells of CPVT patients presenting with similar findings. All this evidence suggests that CAMKII activation plays a major role not only in arrhythmogenesis but also in other cardiac pathologies, such as HF progression. Further studies regarding the development of a more specific component with a much higher affinity could bring us closer to the development of a new therapeutic regime.

RyR2 Stabilizers to Reduce Mitochondrial Ca^{2+} Overload

Dantrolene is a muscle relaxant known widely for its use in treating malignant hyperthermia. This condition arises when a patient with a mutation in the RyR2 is exposed to inhaled halogenated anesthetics. This mutation makes the RyR2 susceptible to massive Ca^{2+} release, which is associated with muscle breakdown, elevated serum creatinine kinase, hypotension, hyperthermia, tachycardia, and intraoperative death. Its mechanism of action was unknown until a few years ago, when it was found to bind the RyR2 and stabilize domains within the receptor (Paul-Pletzer et al., 2002;

Kobayashi et al., 2005). Its mechanism of action seems suitable for preventing stochastic Ca^{2+} release during diastole and has been studied in various scenarios. It has shown therapeutic potential in a murine model of CPVT, in which mice were subjected to a knock-in procedure of a known mutation that causes the pathology. Transgenic mice presented with ventricular tachycardia when stimulated with epinephrine or by running on a treadmill. Pretreatment with dantrolene for 7–10 days inhibited VT. In isolated cardiomyocytes, the spark frequency was also diminished (Kobayashi et al., 2010). In an *ex vivo* model of long-QT syndrome, rabbit hearts were infused with erythromycin or veratridine to mimic the condition. When the potassium concentration was lowered, the hearts presented with early EADs and polymorphic ventricular tachycardia. Infusion with dantrolene resulted in a significant reduction of EADs and polymorphic VT (Frommeyer et al., 2018). Dantrolene also regulates mitochondrial-SR interconnection in cardiomyocytes from a model of HF with increased stochastic RyR2 activity. The author identified that spontaneous Ca^{2+} waves, which can subsequently propagate and trigger organ-wide arrhythmia, were increased in the presence of the mCU agonist (Hamilton et al., 2018). Under these conditions, kaempferol-treated ventricular myocytes showed a significant increase in the percentage of cells presenting with spontaneous Ca^{2+} waves. Interestingly, stabilization of the RyR2 with dantrolene reduced the proarrhythmic effects of kaempferol on Ca^{2+} handling (Hamilton et al., 2018). In this context, some authors (Beutner et al., 2001) have identified a RyR2-like protein in the IMM. This elusive Ca^{2+} transporter demonstrates that it might be possible to modulate dantrolene-sensitive mitochondrial functions, such as Ca^{2+}-dependent O_2 consumption, Ca^{2+}-dependent mPTP and swelling, and cytochrome c release (Beutner et al., 2001), with a direct effect on mitochondrial RyR2.

Dantrolene has also been used to treat AF in a sheep model of atrial infarction. Left atrial myocardial infarction was induced by ligating the atrial branch of the left anterior descending coronary artery. After the procedure, numerous episodes of AF appeared. It was noted that these episodes were produced by spontaneous focal discharges occurring in the zone between the infarcted and normal tissue and were inducible with ISO. Dantrolene administration after the procedure prevented the focal discharges and AF, presumably by maintaining a proper RyR2 response to calmodulin (Avula et al., 2018). It has also been compared to amiodarone in VF treatment using a pig model of cardiac arrest. Under anesthesia, VF was induced by an electrode in the right ventricle and left untreated for 8 min. Afterward, chest compressions and ventilation were started, along with the administration of either amiodarone, dantrolene, or saline. After 4 min of resuscitation, defibrillation was attempted. The rates of return to spontaneous circulation and hemodynamic stability were not statistically different in the dantrolene and amiodarone groups (Wiesmann et al., 2017). This finding implies that dantrolene is as effective as other anti-arrhythmic drugs currently in use. In a more clinical setting, dantrolene has shown efficacy in reducing spark frequency, diastolic SR Ca^{2+} leakage, Ca^{2+} waves, and spontaneous Ca^{2+} transients in isolated human cardiomyocytes from patients with AF or end-stage HF (Hartmann et al., 2017). Similarly, a small group of patients with CPVT underwent an exercise stress test, and ECG was recorded before and after dantrolene administration. Premature ventricular complexes were reduced in patients enrolled in the study. Similarly, cardiomyocytes derived from induced pluripotent stem cells from fibroblasts from these patients were stimulated with epinephrine, and Ca^{2+} transients were recorded. Dantrolene, again, abolished most of the abnormalities registered in patients (Penttinen et al., 2015). Notably, some patients who responded to treatment had mutations in the N-terminal or central cytosolic region of the RyR2 protein, while the non-responders had their mutations in or near the transmembrane domain. This brings attention to the fact that, at least in the case of CPVT, the baseline pathology needs to be checked to ensure pharmaceutical effectiveness.

However, dissociation of this protein with the RyR2 macromolecular complex has been described in a variety of cardiac pathologies, such as HF, hereditary forms of exercise-induced arrhythmias, and sudden cardiac death. Dissociation of this protein causes the RyR2 to become more sensitized to Ca^{2+}-dependent activation, increasing diastolic Ca^{2+} leakage, impairing Ca^{2+} cycling, and decreasing contractility (Lehnart et al., 2004b). Increasing the affinity of calstabin2 to the RyR2 macromolecular complex with the 1,4-benzothiazepine derivative molecule JTV519 or S107 has been useful in preventing arrhythmias and sudden cardiac death. In one of the first experiments, JTV519 was found to correct abnormal RyR2 channel gating in dogs with induced HF (Kohno et al., 2003). In another study, calstabin2$^{\pm}$ mice presented with ventricular tachycardia and sudden cardiac death when put under strenuous exercise. However, these events were prevented entirely when treated with JTV519 (Wehrens et al., 2004). Treatment with JTV519 was associated with a decreased opening probability of individual RyR2 channels when subjected to diastolic Ca^{2+} concentrations compared to the untreated group, which had abnormally high opening probabilities. Calstabin2$^{-/-}$ mice, however, exhibited no protection from JTV519, demonstrating that calstabin2 must be present for JTV519 to prevent stochastic RyR2 channel opening. This molecule has also been shown to stabilize RyR2 opening in the context of mutations found in CPVT patients. Treatment with JTV519 was found to increase the association between calstabin2 and the RyR2 and normalize the channel's functioning (Lehnart et al., 2004a). This was recently demonstrated using 3D-engineered heart tissue transfected to express channelrhodopsin-2, an ion channel activated by light. Under a chronic optical tachypacing protocol, the tissue remodeling showed a propensity for tachycardic episodes when submitted to a burst pacing protocol. This propensity was abolished when JTV519 was administered (Lemme et al., 2019). In addition, in guinea-pig ventricular muscles subjected to no-flow ischemia followed by reperfusion, JTV519 administration significantly improved post-ischemic contractile recovery. However, the potential benefit of JTV519 administration in models of I/R injury was blocked with 5-HD, thereby suggesting that the activation of mitochondrial K_{ATP} channels mediates the cardioprotective effects of JTV519 (Ito et al., 2000).

Given the non-specific effects that JTV519 could have on other ion channels, a new molecule, S107, was initially developed as a treatment to increase skeletal muscle exercise capacity (Bellinger et al., 2008). However, this new molecule prevented arrhythmias and seizures in mice harboring a CPVT mutation in the RyR2 (Lehnart et al., 2008). The protective effect was also observed in a Duchenne muscular dystrophy (DMD) murine model. DMD mice were found to have structural remodeling in their cardiac tissue. This remodeling predisposed the model to ventricular arrhythmias, which were prevented completely with S107 administration (Fauconnier et al., 2010). S107 was also able to suppress AF in different murine models with *knock-in* mutations known to cause CPVT in humans (Shan et al., 2012). In another study using cardiomyocytes derived from induced pluripotent stem cells from a human patient with CPVT, pre-incubation with S107 prior to ISO exposure dramatically reduced the percentage of cells that developed DADs (Sasaki et al., 2016). Finally, a new optogenetic animal model using nematodes with mutations in proteins analogous to mutations in the RyR2 or calsequestrin in CPVT patients displayed similar muscular dysfunction findings upon a stress challenge with a faster pacing rate; muscular dysfunction was prevented with the use of S107 (Fischer et al., 2017).

SERCA Modulation to Improve ECEC

Numerous studies have shown that SERCA's functionality is reduced in common cardiac pathologies and conditions prone to developing arrhythmias. Such is the case of AF, in which reduced SERCA expression has been described in the peripheral blood cells of patients with AF, and these levels of expression can be used to predict the clinical response to treatment, such as epicardial thoracoscopic PV isolation (Sardu et al., 2020). Unsurprisingly, increasing SERCA's capacity to pump Ca^{2+} into the SR to increase SR Ca^{2+} content, which, in turn, increases the amount of Ca^{2+} released during CICR and translates into generating a greater contractile force, along with a lower probability of stochastic RyR2 opening, by decreasing cytosolic Ca^{2+} concentrations during diastole. This is one of the most studied approaches to treating cardiac dysfunction and has been assessed from different viewpoints. A small group of patients with advanced HF received an intracoronary infusion of an adeno-associated viral vector (AAV) containing genetic material that coded for SER-CA2a. These patients presented with improvements in symptomatic, functional, biomarker, and LV functional parameters, along with no significant adverse effects in other organs (Jaski et al., 2009). Notably, two patients who did not experience clinical improvement despite the therapy already had circulating antibodies against the viral vector before transfusion. In a phase 2a trial, 39 patients received an intracoronary infusion of the AAV. The same parameters were assessed. Again, the treatment group presented with improvement, an increased time from therapy to the occurrence of clinical events related to HF, and a decreased frequency of cardiovascular events in the following 12 months (Jessup et al., 2011). However, when the phase 2b trial was conducted, unfortunately, after a median follow-up of 17.5 months, the treatment group showed no improvement in time to recurrent events or mortality when compared to placebo (Greenberg et al., 2016). The reason these results were so different from previous studies remains unclear. It is hypothesized that the previous studies' results were affected by chance, considering the small number of patients, or the formulation used in the CUPID 2 trial was not effective enough. Although the outcome was unexpected, it at least proved gene therapy is a viable option, as there were no major adverse effects when compared to placebo. Furthermore, the trial provides insight into what needs further development before a second attempt to implement gene therapy is performed.

Another strategy used to enhance SERCA's Ca^{2+} pumping capacity is to functionally reduce its inhibitor protein, PLB. This strategy has been studied with a PLB KO murine model. This model presented with a similar magnitude in the LTCC current when compared to wild-type, although with a faster decay. Regarding differences, a larger AP that decayed faster had greater SR Ca^{2+} content, better ECC (measured as Δ cytosolic Ca^{2+} concentration/LTCC current), and more frequent and greater Ca^{2+} sparks were noted, although the decay times were similar (Santana et al., 1997). This model also presented with better diastolic functioning in Doppler and color M-mode echocardiography (Schmidt et al., 2002). Furthermore, combining a SERCA overexpression model with a PLB KO model resulted in an even more enhanced cardiac state. Mice with both genetic modifications presented with higher maximal rates of contraction and relaxation and lower Ca^{2+} transient decay times when compared to groups with either single mutation. No histological or pathological changes were found in the double transgenic model (Zhao et al., 2003). This demonstrates the synergy achieved when enhancing SERCA's functionality from two different approaches, making it possible to simultaneously address SERCA's dysfunction with different treatments. Interestingly, these transgenic mice exhibited increased oxygen consumption to meet the demand for increased ATP consumption. Higher levels of mitochondrial oxygen consumption were associated with increased Ca^{2+}-dependent pyruvate dehydrogenase activity. These findings suggest that the ablation of PLB requires metabolic adaptations to establish proper ECEC (Chu et al., 1996).

Concerns about enhancing SERCA in a dysfunctional setting have been expressed. Because the RyR2 is commonly dysfunctional in most settings where SERCA is less efficient in removing diastolic Ca^{2+}, it is implied that increasing SR Ca^{2+} without addressing the increased opening probability of the RyR2 could result in an enhanced spark frequency and higher Ca^{2+} wave propagation, caused by RyR2 opening due to increased luminal Ca^{2+} sensibility, with subsequent arrhythmia development. Nonetheless, studies involving the enhancement of SERCA's functioning in such settings, including HF models and patients with advanced HF, have not shown such adverse effects. This may be due to SERCA's enhanced efficiency being more beneficial by reducing diastolic Ca^{2+}, as it seems that the RyR2 still needs cytosolic Ca^{2+} for stochastic opening, even under conditions of increased sensitivity to luminal Ca^{2+} content, since reaching a certain SR Ca^{2+} threshold seems insufficient for diastolic RyR2 opening (Belevych et al., 2012).

FINAL REMARKS

The SR and mitochondria engage in constant, intimate communication to properly respond to workload and metabolic needs. However, their interconnectedness leaves both organelles vulnerable to malfunction if one should have its safety mechanisms overrun. In this case, conditions that favor Ca^{2+} overload, such as chronic adrenergic stimulation, could elevate the diastolic mitochondrial Ca^{2+} concentration to the point at which Ca^{2+} overload ensues. This overload increases ROS production and, once the antioxidant system is overrun, causes $\Delta\Psi m$ loss (by mPTP and IMAC-mediated mechanisms), decreased ATP production, and mPTP opening. Furthermore, increased ROS oxidates the mCU, along with possible phosphorylation from AMPK, secondary to low ATP synthesis, which further increases Ca^{2+} transport into the matrix, creating a positive feedback loop for more ROS production. ROS can also affect nearby structures, such as SERCA, which decreases its effectiveness in pumping Ca^{2+} back into the SR. This translates into reduced SR content and higher cytoplasmic Ca^{2+} concentrations, reducing contractility. Ca^{2+} can then activate CAMKII, which can also be activated by mitochondrial ROS. CAMKII can then autophosphorylate itself to stay in a permanent active form. RyR2 is activated by CAMKII phosphorylation and ROS, which causes its regulator protein, calstabin, to detach from the RyR2 complex. This increased sensibilization promotes stochastic opening with subsequent Ca^{2+} leaks and Ca^{2+} waves, further increasing mitochondrial Ca^{2+} transport. On the sarcolemma, the oxidation of LTCCs increase the amount of Ca^{2+} that enters the cardiomyocyte upon activation. A reduction in the ATP concentration caused by mitochondrial dysfunction opens sarcKATP channels, reducing the cardiomyocyte's bathmotropism and dromotropism. The constantly high Ca^{2+} concentration also activates NCX, slowly depolarizing the cell and promoting unsolicited depolarizations and APs. Finally, connexin 43 translocation or Panx1 opening due to increased Ca^{2+} and ROS translocates ATP and adenine into the extracellular matrix, where they activate signaling pathways for TGF-β1 expression, with subsequent fibrosis. This represents two pathways to cardiac tissue fibrosis: cell death-dependent and TGF-β1-dependent. Both have the same outcomes, creating patches of slow conduction and promoting reentry. All these effects make a suitable environment for conduction dysfunction and arrhythmia generation. Up until now, classic antiarrhythmic drugs' main mechanism known to prevent arrhythmias does not include the mitochondrial-SR interconnection. However, they have effects on either of these organelles (Sugiyama et al., 1985; Sano et al., 1990; Deng and Zhang, 1993; Tsutsumi et al., 2001; Afanas'ev et al., 2002; Ugdyzhekova et al., 2005; Wang et al., 2007; Bannister et al., 2015; Kryshtal et al., 2020). Flecainide can inhibit RyR2 opening, although its relevance in preventing arrhythmias is still controversial (Bannister et al., 2015; Kryshtal et al., 2020). Lidocaine might inhibit mitoK_{ATP} channels, as shown in isolated cardiomyocytes utilizing a mitochondrial redox state reporter as a surrogate for mitoK_{ATP} opening (Tsutsumi et al., 2001), and prevents mitochondrial Ca^{2+} overload in a model of closed-chest VF and resuscitation (Wang et al., 2007), but the widely reported mechanism of action of Lidocaine is prolonging the inactivation of the fast voltage-gated Na^{+} channels, inhibiting spontaneous depolarization (Sheu and Lederer, 1985). Amiodarone was found to preserve mitochondrial respiration after ischemia in a model of ischemia-induced ventricular arrhythmias (Sano et al., 1990) and was shown to potentiate the SR's ability to accumulate Ca^{2+} in either rat or isolated myocardial strips from coronary heart disease patients (Afanas'ev et al., 2002; Ugdyzhekova et al., 2005). However, its reported action is by inhibiting the I_{Kr} current, prolonging phase 3 of the AP. Finally, Verapamil blocks voltage-dependent Ca^{2+} channels, decreasing impulse conduction through the AV node, but has been reported that indirectly prevents mitochondrial Ca^{2+} accumulation (Sugiyama et al., 1985) and reduce mitochondrial oxidative stress in the context of ischemia-reperfusion by a lower MDA content in mitochondria (Deng and Zhang, 1993). These effects could be additional antiarrhythmic mechanisms that should be further addressed to fully understand and assess their physiological relevance for future antiarrhythmic therapy developing.

CONCLUSION

In the last few years, there have been considerable advancements in understanding the molecular mechanisms of cardiac disease, especially arrhythmogenesis. These advancements laid the path for developing better therapeutic strategies that were first ignored or disregarded as potentially ineffective. Gaining a better understanding of the molecular mechanisms involved in arrhythmia generation provides insights that could lead to new therapeutic strategies. It may be time to expand our viewpoint and explore the crosstalk taking place between mitochondria-SR interconnection that seem to orchestrate Ca^{2+} homeostasis instead of focusing on a single cellular component. This may be the most effective way to prevent arrhythmogenesis, by tackling the central mechanism that leads to different cellular malfunctions.

AUTHOR CONTRIBUTIONS

GG-R conception and designed the review. FS-R, RR-M, and GG-R analyzed the literature and drafted the manuscript. GG-R and RR-M contributed to critial review of the manuscript. This work was submitted in partial fulfillment of the requirements for the Ph.D. degree (FS-R) for the Doctorate in Biomedical Sciences. Tecnologico de Monterrey. All authors contributed to the article and approved the submitted version.

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Sex Hormone Regulation of Proteins Modulating Mitochondrial Metabolism, Dynamics and Inter-Organellar Cross Talk in Cardiovascular Disease

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Cardiovascular disease (CVD) is the leading cause of death in the U.S. and worldwide. Sex-related disparities have been identified in the presentation and incidence rate of CVD. Mitochondrial dysfunction plays a role in both the etiology and pathology of CVD. Recent work has suggested that the sex hormones play a role in regulating mitochondrial dynamics, metabolism, and cross talk with other organelles. Specifically, the female sex hormone, estrogen, has both a direct and an indirect role in regulating mitochondrial biogenesis via PGC-1α, dynamics through Opa1, Mfn1, Mfn2, and Drp1, as well as metabolism and redox signaling through the antioxidant response element. Furthermore, data suggests that testosterone is cardioprotective in males and may regulate mitochondrial biogenesis through PGC-1α and dynamics via Mfn1 and Drp1. These cell-signaling hubs are essential in maintaining mitochondrial integrity and cell viability, ultimately impacting CVD survival. PGC-1α also plays a crucial role in inter-organellar cross talk between the mitochondria and other organelles such as the peroxisome. This inter-organellar signaling is an avenue for ameliorating rampant ROS produced by dysregulated mitochondria and for regulating intrinsic apoptosis by modulating intracellular Ca^{2+} levels through interactions with the endoplasmic reticulum. There is a need for future research on the regulatory role of the sex hormones, particularly testosterone, and their cardioprotective effects. This review hopes to highlight the regulatory role of sex hormones on mitochondrial signaling and their function in the underlying disparities between men and women in CVD.

Keywords: sexual dimorphism, cardiovascular disease, estrogen, testosterone, mitochondria

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CARDIOVASCULAR DISEASE AND SEX STEROID SIGNALING

Cardiovascular disease (CVD) is modulated by mitochondrial dysfunction, calcium handling, aging, etc., which are reviewed in detail in the corresponding reviews (Khoury et al., 1992; Sandstede et al., 2000; Berridge, 2003; Lou et al., 2012; Keller and Howlett, 2016; Ventura-Clapier et al., 2017; Virani et al., 2020). Sex disparities in the cardiovascular system including heart size, body

size, adipose deposition, etc. have been linked to variations in CVD risk and rates. In this review, we will focus on sex hormone driven differences in CVD. In general, women express higher levels of estrogen and estrogen receptors (ERs) than men, while men express higher levels of testosterone and androgen receptors (ARs) than women; as both sexes age, there is a decrease in the predominant sex hormone. While the primary sex hormone decreases, there is a concurrent increase in estrogen in men and an increased ratio of testosterone to estrogen in women (Araujo and Wittert, 2011; Bowling et al., 2014; Zhao et al., 2018).

Currently, there are conflicting results on the role of testosterone in CVD, but it is documented that the decrease in testosterone in men with age and the higher ratio of testosterone to estrogen in post-menopausal women may be linked to increased CVD incidence (Freeman et al., 2010; Elagizi et al., 2018). Human studies have shown confounding results regarding the cardioprotective effects of menopausal hormone therapy to increase estrogen levels in women during the post-menopausal period (Stampfer et al., 1991; Rossouw et al., 2002; Hodis and Mack, 2014). The stark decrease in estrogen levels in the post-menopausal period have also been linked to obesity and metabolic syndrome incidence (Araujo and Wittert, 2011; Ziaei and Mohseni, 2013; Bowling et al., 2014; Moore et al., 2017; Zhao et al., 2018; Terrazas et al., 2019). The sex hormones also regulate differences in metabolism, specifically in regards to fat accumulation and body shape between men and women, which are known modulators of CVD risk (Bjorntorp, 1997; Fui et al., 2014; Mercado et al., 2015; Van Pelt et al., 2015; Leeners et al., 2017; Terrazas et al., 2019). The metabolic differences associated with changes in hormone status—which are influenced by a plethora of factors including sex chromosomes, gene expression and regulation, and epigenetics—are key to understanding CVD disparities between men and women (Ventura-Clapier et al., 2019). This review will focus on sex hormone signaling and its potential cardioprotective effects, discuss controversial findings regarding sex hormone signaling, and highlight the need for further research to create efficacious and sex-specific CVD treatments.

To elucidate the roles of estrogen and testosterone in CVD, it is essential to understand the roles of their associated receptors, including estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), G protein coupled estrogen receptor (GPER/GCPR30), and ARs. The regulation of estrogen and androgen receptor expression is challenging to study, as they are sex-, age-, cell type-, and organelle-specific (Lizotte et al., 2009; Dart et al., 2013; Bowling et al., 2014; Hutson et al., 2019). At the cellular level, it has been shown that varying cell types express different levels of sex hormone receptors, highlighting that each organ system may have differential sexual dimorphic regulation (Erlandsson et al., 2001; Deroo and Korach, 2006; Levin, 2009; Dart et al., 2013; Mahmoodzadeh and Dworatzek, 2019; Ventura-Clapier et al., 2019). Within the cell, sex hormone receptors can be found in a variety of locations including the cell membrane, nucleus, mitochondria, and endoplasmic reticulum, although, again, these locations vary depending upon the cell type (Levin, 2009; Lizotte et al., 2009; Luo and Kim, 2016; Pedernera et al., 2017). In cardiomyocytes, the hormone receptors are expressed at different locations on various organelles; for example, both ERα and ERβ have been found to be localized to the mitochondria, while GPER has been localized to both the cell membrane and the endoplasmic reticulum (Lizotte et al., 2009; Luo and Kim, 2016; Zimmerman et al., 2016; Pedernera et al., 2017; Gourdy et al., 2018; Ventura-Clapier et al., 2019). While receptor expression within the cardiovascular system is known, further studies understanding how the ERs and ARs change based on age and hormone status is needed.

The mitochondrion plays an integral role in the production of the steroid hormones, as it is the site wherein the first step of sex hormone synthesis occurs (Miller, 2013). These same sex hormones are implicated in regulating mitochondrial dynamics and function. Cholesterol is the building block for the steroid hormones, specifically the C27-steroid cholesterol, which enters the mitochondria through the steroidogenic acute regulatory protein where the cytochrome P450 enzyme, CYP11A1, produces pregnenolone; pregnenolone can subsequently be transported back into the cytosol and converted, through a series of enzymatic steps, into either estrogen (estradiol) or testosterone (Hu et al., 2010; Miller and Bose, 2011; Samavat and Kurzer, 2015). The enzyme aromatase converts testosterone into estradiol, and recent studies have found elevated aromatase correlates with metabolic dysfunction in women (Araujo and Wittert, 2011; Iyengar et al., 2017). It has also been shown that adipose tissue is the primary producer of estrogen in post-menopausal women, adding to the complexity of estrogen signaling (Cleland et al., 1985; Chen and Madak-Erdogan, 2018). The differences in serum estrogen and testosterone levels can greatly influence cellular processes and adipose deposition, which can modulate CVD risk.

Testosterone and dihydrotestosterone activate the nuclear AR, which regulates transcription of genes located near androgen response elements or via a DNA binding independent pathway to activate ERK, Akt, and MAPK pathways (Benten et al., 1999b; Davey and Grossmann, 2016). Plasma membrane associated ARs play an important role in calcium (Ca^{2+}) signaling, in addition to influencing endoplasmic reticulum signaling and apoptosis (Benten et al., 1999a,b; Segawa et al., 2002; Davey and Grossmann, 2016; Azhary et al., 2018). AR and androgen hormones are also essential for the development and normal physiology of the cardiovascular system (Ikeda et al., 2005). In comparison, the classical pathway of ERα activation involves its association with heat shock proteins within the cytosol; once estrogen binds ERα and/or ERβ, they can dimerize and translocate to the nucleus to activate transcription via the estrogen response element (Levin, 2009). ERα has also been shown to induce signaling cascades —including Akt, PKA and ERK1/2, and eNOS— through a membrane-initiated sequence whereby a post-translationally modified pool of ERα is localized near the plasma membrane due to an interaction with caveolin 1 (Levin, 2009; Yasar et al., 2017). After estrogen binds the receptor, it induces additional signaling pathways. ERα and ERβ are found on the plasma membrane, as both homo- and heterodimers, and expression is differential based on cell type, as previously mentioned (Li L. et al., 2003; Levin, 2009; Bowling et al., 2014). ERβ has been shown to localize to the mitochondria in cardiomyocytes of both humans and rodents, and has been

proposed to play a role in mitochondrial integrity (Yang et al., 2004). GPER, a membrane-bound estrogen receptor, induces cAMP, IP3, Ca^{2+}, and the MAPK/ERK pathways (Aronica et al., 1994; Improta-Brears et al., 1999; Filardo et al., 2000). Unlike ERα and ERβ expression, which are strictly regulated by estrogen levels and decrease in the post-menopausal period, GPER levels appear to be unaffected by circulating estrogen levels induced by menopause, but may fluctuate with estrous cycle (Cheng et al., 2014; Zimmerman et al., 2016). Data also suggests that GPER activation is protective after a vascular injury in ERα and ERβ KO mice, and can regulate mitochondrial function and biogenesis in ovariectomized mice (Bowling et al., 2014; Sbert-Roig et al., 2016; Mahmoodzadeh and Dworatzek, 2019). Therefore, more research is needed to determine the expression and role of GPER in preventing CVD injury.

CELLULAR METABOLISM

Mitochondria play a crucial role in many molecular pathways and cellular bioenergetics. Mitochondria comprise about 35% of the entire cell volume in cardiomyocytes, making their function even more crucial to proper cardiovascular function (Dedkova and Blatter, 2012; Consolini et al., 2017). The mitochondrion contains its own small genome, encoding 37 mitochondrial proteins which are translated in the mitochondria, while the remaining proteins and RNAs are encoded by the nuclear genome (Lee and Han, 2017). Since the majority of mitochondrial proteins arise from nuclear transcription, cross talk between the mitochondria and the nucleus is imperative for effective metabolism and function. Mitochondrial proteins and their precursors are transported from cytosolic ribosomes and the endoplasmic reticulum into the mitochondria and then integrated with mitochondrially-derived proteins via sorting, assembly, and importing machinery (Pfanner and Meijer, 1997; Ellenrieder et al., 2015, 2017; Doan et al., 2020). This, in conjunction with the formation of phospholipid precursors by the endoplasmic reticulum for the mitochondria, such as cardiolipin, shows the importance of cross communication for proper mitochondrial function, as well as modulation by hormones (Ellenrieder et al., 2017; Pozdniakova et al., 2018; Acaz-Fonseca et al., 2020).

Mitochondria utilize a variety of energy sources including glucose, fatty acids, and amino acids to produce reducing equivalents for mitochondrial respiration and ATP production, which is essential in cardiac tissue. Developing cardiomyocytes prefer glucose as their energy source, whereas adult cardiomyocytes prefer fatty acids; recent studies suggest that alteration of energy sources in cardiomyocyte metabolism can contribute to CVD progression (Piquereau et al., 2010, 2012; Krzywanski et al., 2011; Martin et al., 2014; Siasos et al., 2018). Mitochondrial metabolism produces reactive oxygen species (ROS), which are increased in mitochondrial dysfunction and prevalent in CVD (Wang and Zou, 2018). To reduce ROS, antioxidant proteins, including superoxide dismutase 1 (SOD1), SOD2, and glutathione peroxidase (GPx) can be upregulated (**Table 1**). In the heart, estrogen and testosterone have been shown to increase these antioxidant enzymes, and may act in a cardioprotective manner (Barp et al., 2002; Strehlow et al., 2003; Zhang et al., 2011; Lee et al., 2012; Liu H. et al., 2014; Liu Z. et al., 2014; Redmann et al., 2014; Pozdniakova et al., 2018; Mahmoodzadeh and Dworatzek, 2019; Ventura-Clapier et al., 2019; Asmis and Giordano-Mooga, 2020; Casin and Kohr, 2020). In the vasculature, GPER has been shown to modulate ROS through downregulation of oxidative stress proteins such as NADPH oxidase 4 (NOX4), Prostaglandin-endoperoxide synthase 2 (PTGS2), and GPx1 in addition to upregulating antioxidant proteins such as SIRT3 and GSTK1 (Wang H. et al., 2018; Ogola et al., 2019). Additionally, it has been shown that GPER downregulates an essential autophagy protein light chain 3—LC3I/LC3II—via upregulation of PTEN-induced putative kinase 1 (PINK1), and a concurrent decrease in mitochondrial parkin localization indicating a decrease in mitophagy (Feng et al., 2017). GPER activation, through mitochondrial and lysosomal cross talk mechanisms, is an important mitigator of ROS in CVD (Lee et al., 2012; Feng et al., 2017). These same antioxidant proteins have been associated with peroxisomal function and are key players in the peroxisome's regulation of intracellular ROS levels (Schrader and Fahimi, 2006; Wang Q. et al., 2015). Because ROS regulation is critical in metabolic homeostasis and mitochondrial dynamics, perturbation of these processes often leads to increased risk of pathological outcomes, such as CVD (Krzywanski et al., 2011; Siasos et al., 2018). Hence, understanding how mitochondrial dynamics and cross talk are impacted by ROS and modulated by sex hormones is critical in elucidating the mechanisms underlying CVD.

MITOCHONDRIAL BIOGENESIS AND DYNAMICS

Under normal conditions, mitochondrial turnover in the adult heart occurs every 2 weeks (Dorn et al., 2015). Mitochondrial dynamics is the process of mitochondrial growth and division designed to maintain homeostasis by utilizing the fission, fusion, mitophagy, and recycling processes during growth and development as well as under environmental stressors such as ischemia, hypoxia, or oxidative stress (Dorn et al., 2015; Dorn, 2016). Recent studies have shown that regulating mitochondrial homeostasis is crucial in mitigating CVD disruption of these processes which has been previously to worse pathological outcomes or increased death (Jornayvaz and Shulman, 2010; Sun et al., 2013; Redmann et al., 2014). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) is a transcriptional coactivator protein crucial for maintaining homeostasis of this organelle by targeting genes involved in electron transport chain (ETC) and apoptotic signaling (Liang and Ward, 2006; Lai et al., 2008; Wang F. et al., 2015). AMP-activated protein kinase (AMPK) is an upstream regulator of PGC-1α involved in energy homeostasis and mitochondrial biogenesis and has been shown to be regulated through the sex hormones (Jornayvaz and Shulman, 2010; Varanita et al., 2015; Wang F. et al., 2015; Park et al., 2017; Hevener et al., 2020). In cardiomyocytes, activated estrogen receptors found on the cell membrane can upregulate PGC-1α activity, thereby

TABLE 1 | Impact of sex hormones on cardiac cell protein expression.

Protein/channel	Model	Testosterone (including DHT) regulation	Estrogen (including estradiol, estrone, and estrogen) regulation
ERα	(Lizotte et al., 2009) CD1 mice		Timing specific regulation
	(Park et al., 2017) Human skeletal muscle		Upregulated
	(Bowling et al., 2014) ERα KO and WT mice		Regulates expression
ERβ	(Lizotte et al., 2009) CD1 mice		No regulation
	(Park et al., 2017) Human skeletal muscle		Upregulated
	(Bowling et al., 2014) ERβ KO and WT mice		Regulates expression
GPER	(Wang H. et al., 2018) GPER-KO mice		
AR	(Lizotte et al., 2009; Pedernera et al., 2017) CD1 mice		
	(Kerkhofs et al., 2009; Huang et al., 2016) ARKO and ARKI mice		
	(Hanke et al., 2001) Rabbit aorta	Upregulation	
	(Dart et al., 2013) ARE-Luc knock-in mice	Upregulation	No regulation
	(Marsh James et al., 1998) Rat cardiomyocytes	Upregulation	No regulation
Unspecified SOD	(Zhang et al., 2011) Tfm mice	Upregulated	Upregulated
	(Barp et al., 2002) Wistar rats		Upregulated
	(Cruz-Topete et al., 2020) Mice cardiomyocytes	Upregulated	
SOD1	(Strehlow et al., 2003) Rat VSMCs		Upregulated
SOD2	(Strehlow et al., 2003) Rat VSMCs		Upregulated
	(Lone et al., 2017) MCF-7		Upregulated
	(Liu Z. et al., 2014) HAECs		Upregulated
	(Liu H. et al., 2014) Rat cardiomyocytes		Increased activity
GPx	(Zhang et al., 2011) Tfm mice	Upregulated	
	(Cruz-Topete et al., 2020) Mouse cardiomyocytes	Upregulated	
nNOS	(Casin and Kohr, 2020) Mouse cardiomyocytes		Upregulated
eNOS	(Casin and Kohr, 2020) Mouse cardiomyocytes		Upregulated
NOX4	(Wang H. et al., 2018) GPER-KO mice		Downregulated
	(Cruz-Topete et al., 2020) Human sex studies	Inconclusive	Inconclusive
	(Ogola et al., 2019) VSMCs GPER-KO mice		Downregulated
PTGS2	(Wang H. et al., 2018) GPER-KO mice		Downregulated
SIRT3	(Lone et al., 2017) MCF-7		Upregulated
	(Wang H. et al., 2018) GPER-KO mice		Upregulated
GSTK1	(Wang H. et al., 2018) GPER-KO mice		Upregulated
K^+ATP Channel	(Sakamoto and Kurokawa, 2019) Rat cardiomyocytes	Opens channels during reperfusion, cardioprotective	Opens channels during reperfusion, cardioprotective
	(Gao et al., 2014) SUR_2KO mice		Regulated, cardioprotective
	(Er et al., 2004) Sprague-Dawley rats cardiomyocytes	Activated, cardioprotective	
	(Ballantyne et al., 2013) H9c2 cells	Upregulates expression	Upregulates expression
SERCA	(Witayavanitkul et al., 2013) ORX mice cardiomyocytes	Increases activation	
			(Hill and Muldrew, 2014) Upregulates expression
PGC-1α	(Witt et al., 2008) AC16 cell line		Upregulation
	(Klinge, 2008) MCF-7 and H1793 cell lines		Upregulation via NRF-1
	(Klinge, 2008) Mouse cardiac tissue		Upregulation
	(Park et al., 2017) Human skeletal muscle		Upregulation
	(Wang F. et al., 2015) Wistar rat cardiomyocytes	Upregulates via AMPK	
Drp1	(Martin et al., 2014) PGC-1α and PGC-1β DKO mice		Upregulated*
	(Sastre-Serra et al., 2013) MDA-MB-231 cells		Upregulated
	(Sastre-Serra et al., 2013) T47D cells		Upregulated
	(Sastre-Serra et al., 2013) MCF-7 cells		No Effect
	(Capllonch-Amer et al., 2014) 3T3-L1 adipocytes	Downregulated	Upregulated
	(Lee et al., 2020) jLNCaP Cells	Upregulated	

(Continued)

TABLE 1 | Continued

Protein/channel	Model	Testosterone (including DHT) regulation	Estrogen (including estradiol, estrone, and estrogen) regulation
Mfn1	(Martin et al., 2014) PGC-1α and PGC-1β DKO mice		Upregulated*
	(Papanicolaou et al., 2012) Mouse cardiomyocytes		Upregulated*
	(Sastre-Serra et al., 2013) MDA-MB-231 cells		Upregulated
	(Sastre-Serra et al., 2013) T47D cells		Upregulated
	(Sastre-Serra et al., 2013) MCF-7 cells		Upregulated
	(Capllonch-Amer et al., 2014)3T3-L1 adipocytes	Upregulated	No Effect
	(Lee et al., 2020) LNCaP cells	No Effect	
Mfn2	(Martin et al., 2014)PGC-1α and PGC-1β DKO mice		Upregulated*
	(Papanicolaou et al., 2012) Mouse cardiomyocytes		Upregulated*
	(Sastre-Serra et al., 2013) MDA-MB-231 cells		Upregulated
	(Sastre-Serra et al., 2013) T47D cells		Upregulated
	(Sastre-Serra et al., 2013) MCF-7 cells		Upregulated
	(Lee et al., 2020) LNCaP cells	No Effect	
Opa1	(Martin et al., 2014) PGC-1α and PGC-1β DKO mice		Upregulated*
	(Sastre-Serra et al., 2013) MDA-MB-231 cells		Upregulated
	(Sastre-Serra et al., 2013) T47D cells		Upregulated
	(Sastre-Serra et al., 2013) MCF-7 cells		Upregulated
	(Capllonch-Amer et al., 2014) 3T3-L1 adipocytes	No Effect	Upregulated
	(Lee et al., 2020) LNCaP cells	No Effect	
Fis1	(Martin et al., 2014) PGC-1α and PGC-1β DKO mice		Upregulated*
	(Sastre-Serra et al., 2013) MDA-MB-231 cells		No effect
	(Sastre-Serra et al., 2013) T47D cells		No effect
	(Sastre-Serra et al., 2013)MCF-7 cells		Downregulated
	(Capllonch-Amer et al., 2014) 3T3-L1 adipocytes	Downregulated	No effect
	(Lee et al., 2020) LNCaP cells	No effect	
PINK1	(Feng et al., 2017) Sprague–Dawley rats		Upregulated
LC3I/LC3II	(Feng et al., 2017) Sprague–Dawley rats		Downregulated
	(Papanicolaou et al., 2012) Mouse cardiomyocytes		Downregulated*

Summary of estrogen and testosterone regulation of antioxidant proteins, mitochondrial dynamics, intracellular Ca^{2+}, and inter-organellar cross talk, both directly and indirectly.
**Proteins indirectly regulated by the sex hormones via PGC-1α.*

regulating ATP synthesis, substrate oxidation, and phosphate transfer (Klinge, 2008; Witt et al., 2008). PGC-1α can also target genes that regulate metabolism in the heart, such as estrogen-related receptor alpha (ERRα), which protects against stressors of CVD (Huss et al., 2004; Martin et al., 2014; Li H. et al., 2015). While estrogen does not directly bind to ERRα, it activates genes regulated to mitochondrial biogenesis, and further studies are needed to understand the sexually dimorphic regulation (Horard and Vanacker, 2003). These data indicate a crucial signaling role for estrogen in the maintenance of mitochondria.

Estrogen has also been shown to activate peroxisome proliferator-activated receptor α (PPARα), a partner of PGC-1α, which functions to transcriptionally regulate fatty acid metabolism in the heart. Activation of PPARα induces the expression of Pex genes leading to peroxisomal biogenesis, while simultaneously inducing the expression of mitochondrial fusion and fission proteins mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), dynamin-related protein-1 (Drp1), and fission protein 1 (Fis1) (**Table 1**; Bagattin et al., 2010; Papanicolaou et al., 2012; Schrader et al., 2012; Martin et al., 2014; Varanita et al., 2015). Increased number of peroxisomes in conjunction with sustained mitochondrial integrity increases β-oxidation of long chain fatty acids and fatty acid-induced cellular respiration. It has been further suggested that these tissues upregulate PGC-1α in response to increased lipid intake, acting as a compensatory mechanism for high fat diets and metabolic dysregulation. This co-regulation of peroxisomal and mitochondrial biogenesis has been established in brown adipose tissue, liver and skeletal muscle (Huang et al., 2017, 2019; Hevener et al., 2020). Work has yet to be done showing the proliferation of peroxisomes in cardiac tissue, but findings in other tissues is highly suggestive of the need for future research in this area.

PPARα KO mice have severely impaired cardiac function due to lipid accumulation and hypoglycemia which causes death in all males but only 25% of females; however, pretreatment of β-estradiol in males with ablated PPARα survived, implicating estrogen signaling as a crucial mechanism for cardiac metabolism (Nöhammer et al., 2003). Estrogen plays a crucial role in

cardiac lipid metabolism for both males and females *in vivo* (Djouadi et al., 1998). This data, again, highlights the importance of estrogen signaling in cardiac metabolism.

Many cardiovascular pathologies have notable alterations in mitochondrial network morphology. Mitochondrial fission is a process by which mitochondria alter their physical structure; symmetrical division for replication or asymmetrical division to remove damaged organelle components (Shirihai et al., 2015). Asymmetrical fission acts as quality control for damaged mitochondria resulting in fragmentation, which can be utilized for selective mitophagy (Ong et al., 2010; Shirihai et al., 2015). While both processes serve as protective mechanisms for cellular damage and apoptosis through the mitochondria, the mechanisms of activation via other cellular organelles are different. In mitochondrial fusion, the mitochondria fuse with other organelles to repair and regenerate, as opposed to mitochondrial fission, where DNA replication is upregulated in response to mitochondrial damage, inhibiting cytochrome c release and corresponding apoptosis (Chen et al., 2012; Varanita et al., 2015; Dorn, 2016; Hevener et al., 2020). Major proteins associated with fission and fusion include Drp1, Fis1, Mfn1 and Mfn2, and the optic atrophy-1 protein (Opa1). Drp1 is recruited to the outer mitochondrial matrix (OMM) and has been shown to interact with the endoplasmic reticulum, highlighting the importance of inter-organellar cross talk during mitochondrial fission events (Ishihara et al., 2009). Mfn1 and 2 are responsible for fusing OMMs and tethering the mitochondria to the SR for Ca^{2+} signaling, making mitofusin proteins indispensable to inter-molecular and inter-organellar interactions (Chen et al., 2012). These proteins also have an important role in mitochondrial quality control by mediating fusion, guiding protein folding, and preventing ROS-induced mitophagy (Shirihai et al., 2015; Song et al., 2015). Opa1 mediates inner mitochondrial membrane fusion and maintains cristae structure, which ensures proper ETC function (Varanita et al., 2015). This increase in cristae integrity can reduce ROS and prevent cytochrome c release, preventing and reducing mitochondrial dysfunction and apoptosis in highly-metabolic tissues, like the heart and brain (Ong et al., 2010; Varanita et al., 2015).

Abnormal fission and fusion leading to reduced cristae integrity and less functionally efficient morphology—overtly spherical or elongated—are known contributors to heart failure due to their effects on metabolism and apoptosis (Ong et al., 2010; Papanicolaou et al., 2012; Dorn, 2016). Mitochondrial fission opens the mitochondrial permeability transition pore (MPTP) which can result in cell necrosis or mitophagy if not properly managed, as seen in ischemic conditions (Parra et al., 2008; Shirihai et al., 2015; Song et al., 2015). Activation of GPER and ERα has been shown to preserve mitochondrial function and decrease mitophagy after ischemia reperfusion injury via MPTP signaling through MEK/ERK activation, thereby decreasing apoptosis (Feng et al., 2017; Mahmoodzadeh and Dworatzek, 2019). These data, again, suggest the estrogen has cardioprotective effects by preserving mitochondrial integrity and inhibiting apoptosis. Testosterone has also been shown to protect against myocardial infarction through the AMPK pathway, elevating PGC-1α and preserving mitochondrial integrity leading to decreased cardiomyocyte apoptosis, as demonstrated by rodent models (Witt et al., 2008; Wang F. et al., 2015). The ability of both estrogen and testosterone to activate PGC-1α in cardiac tissue has been extensively studied, and it is well-established that PGC-1α regulates the transcription of Drp1, Fis1, Mfn1, Mfn2, Opa1, and other important mitochondrial dynamic proteins (**Table 1**; Witt et al., 2008; Papanicolaou et al., 2012; Martin et al., 2014; Wang F. et al., 2015; Park et al., 2017). This therefore implies a potentially shared pathway for cardioprotection by estrogen and testosterone, but direct evidence has remained elusive. Adding to the complexity, direct regulation of mitochondrial dynamics by the sex hormones has been well established in brain, breast cancer, prostate cancer, and adipocyte models but more research is needed to better characterize the direct effects of estrogen and testosterone in modulating signaling in cardiac tissue as well as inter-organellar cross talk between the mitochondria and other cellular organelles (Sastre-Serra et al., 2013; Capllonch-Amer et al., 2014; Lejri et al., 2018; Lee et al., 2020).

SARCOPLASMIC RETICULUM AND MITOCHONDRIAL CROSS TALK

The K_{ATP} channel, found on both the mitochondria and the sarcoplasmic reticulum (SR), alters the electrochemical gradient through an influx of K^+ into each organelle (Ranki et al., 2002; Er et al., 2004; Ballantyne et al., 2013; Gao et al., 2014; Bayat et al., 2016; Sakamoto and Kurokawa, 2019). In the mitochondrion, this change in K^+ concentration causes an increase in K^+/H^+ antiporter activity, inducing an efflux of H^+ ions from the mitochondrial intermembranous space. The resulting decreased membrane potential impairs organelle efficiency and reduces mitochondrial production of ATP. The activation of the K_{ATP} channel by both estrogen and testosterone has been shown to be cardioprotective in models of ischemia reperfusion injury (Er et al., 2004; Ballantyne et al., 2013; Gao et al., 2014; Sakamoto and Kurokawa, 2019). Estrogen, but not testosterone, can also regulate the SR K_{ATP} channel, which has also been shown to preserve cardiac function after ischemia reperfusion injury (Ranki et al., 2002). Evidence further suggests that testosterone may have a down-regulation effect on SR K_{ATP} channels in exercise models, suggesting a potentially antithetical effect from estrogen (Bayat et al., 2016). GPER activation has also been indicated as a possible mitigator of cell death during reperfusion injury through modulation of mitochondrial integrity, further supporting estrogen's cardioprotective properties (Lee et al., 2012; Feng et al., 2017; Groban et al., 2019). Taken together, these data suggest potential mechanisms of cardioprotection via sex hormone regulation of K_{ATP} channels on mitochondria and sarcoplasmic reticula.

Another important ion to consider in this interorganellar crosstalk is calcium (Ca^{2+}). Ca^{2+} is a divalent ion, and an essential mineral vital for cellular signaling, which has been a critical area of study for several decades (Berridge, 2003; Brookes

et al., 2004; Clapham, 2007). Calcium signaling has also been extensively studied in the mitochondrion and plays important roles in the regulation of many enzymes in the Krebs cycle, electron transport complexes such as ATP synthase, as well as many other enzymes (Das and Harris, 1990; McCormack et al., 1990; Consolini et al., 2017). In cardiomyocytes, an essential mechanism of cardiac function is the maintenance of high levels of ATP in order to properly activate SERCA—a Ca^{2+} pump on the surface of the sarcoplasmic reticulum—and induce the reloading of Ca^{2+} in action potentials (Rosano et al., 1999; Piquereau et al., 2010; Witayavanitkul et al., 2013). Modulation of SERCA levels or activity and Ca^{2+} burden by testosterone is suggested to be cardioprotective during ischemic events (Witayavanitkul et al., 2013). Additionally, it has been shown that estrogen can increase SERCA protein expression, particularly SERCA2b, which causes a decrease in intracellular Ca^{2+}, thus increasing cell survival in coronary arteries (Witayavanitkul et al., 2013; Hill and Muldrew, 2014; Groban et al., 2019).

In cardiomycocytes, move phrase to after (CICR), the primary driver of Ca^{2+} release is through Ca^{2+} induced Ca^{2+} release (CICR). One example of a channel which exists in the inner mitochondrial membranes and the SR are the ryanodine receptors (RyR/mRyR), which are responsible for releasing intracellular stores of Ca^{2+} ions through CICR (Beutner et al., 2005; Altschafl et al., 2007; Gambardella et al., 2018). Leaky RyR on the SR has been directly implicated in heart failure, as excess cytosolic calcium is absorbed by mitochondria resulting in dysregulation and further RyR leak from oxidative damage generated by mitochondrial ROS (Santulli et al., 2015). In contrast, when Ca^{2+} is released from inositol-triphosphate receptors (IP_3R)—receptors responsible for Ca^{2+} release and a mechanism for triggering CICR—and absorbed by mitochondria, whose function, mitochondria or IP3R and ATP production increases; however, when mitochondria number is compromised—such as in heart failure, the Ca^{2+} release can induce arrhythmias (Hohendanner et al., 2015; Seidlmayer et al., 2016). The effect of estrogen on RyR expression and function is mixed and poorly understood, whereas growing evidence is implicating testosterone in increasing expression and function (Tsang et al., 2009; Hsu et al., 2015; Evanson et al., 2018; Groban et al., 2019; Mahmoodzadeh and Dworatzek, 2019; Jiao et al., 2020). For IP_3R, the role of estrogens is even more poorly understood, with some work implicating estrogen's ability to activate IP_3 production in liver and smooth muscle cells, whereas testosterone has been shown to directly trigger IP_3 production and IP_3R activation in cardiomyocytes (Marino et al., 1998; Vicencio et al., 2006). In summary, these data suggest that the sex hormones play an important role in regulating intracellular Ca^{2+}, itself a regulator of cellular apoptosis through the mitochondrion, thereby highlighting an additional cardioprotective role of the sex hormones (Pinton et al., 2008; Groban et al., 2019).

CELL DEATH

There is conflicting information regarding the role of the sex hormones in regulating cell death in cardiac tissue, and therefore,

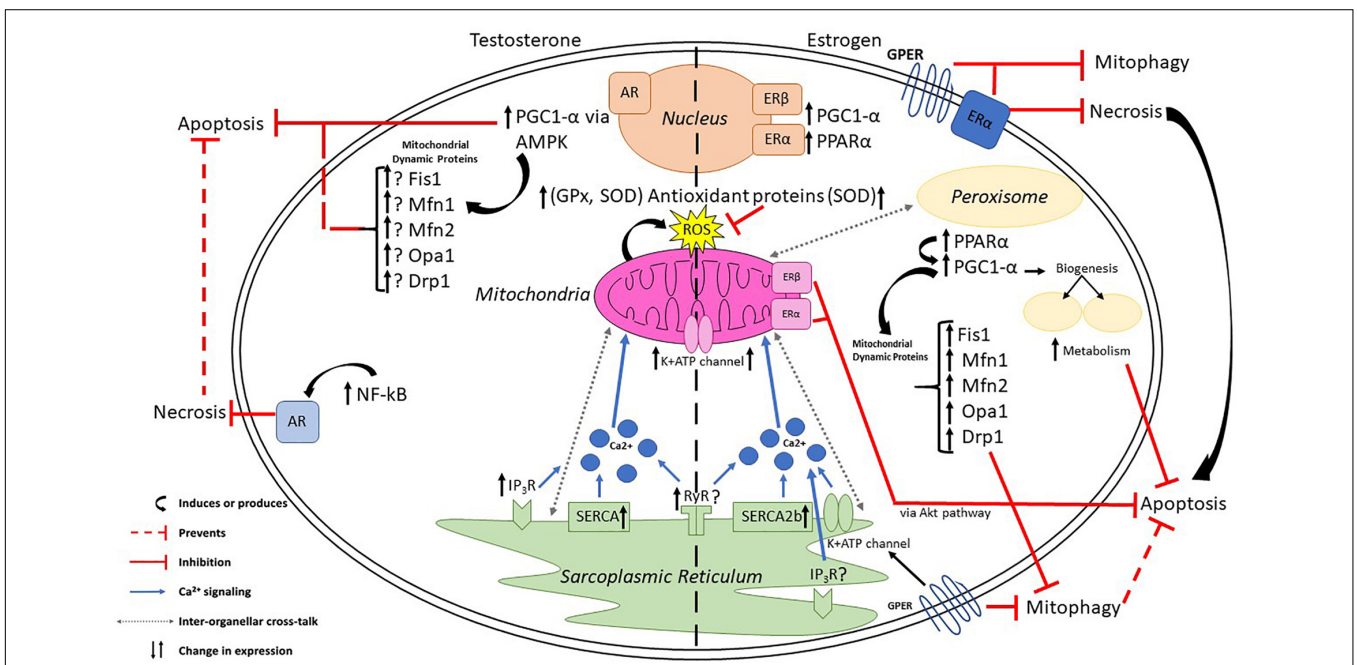

FIGURE 1 | Estrogen and testosterone influence mitochondrial dynamics and cross talk between organelles. Estrogen, via PPARα, upregulates PGC-1α to initiate transcription of mitochondrial dynamic proteins and induce peroxisomal biogenesis. Testosterone upregulates PGC-1α, via AMPK, to influence mitochondrial dynamics. Both sex hormones increase antioxidant proteins to mitigate damaging reactive oxygen species, regulate Ca^{2+} signaling via K^+ATP channels on the sarcoplasmic reticulum, and prevent various forms of cell death in CVD including apoptosis, necrosis, and mitophagy.

indicated a need for further research in this area (Djouadi et al., 1998; Morris and Channer, 2012; Hsieh et al., 2015; Wang F. et al., 2015; Gagliano-Juca and Basaria, 2018; Jones and Kelly, 2018). Cell death pathways, including apoptosis, autophagy, necrosis, and pyroptosis, have been implicated in inducing cell death in CVD among various cell types within the heart including cardiomyocytes, endothelial cells, and monocytes/macrophages (Subramanian and Shaha, 2007; Wang F. et al., 2010; Chen et al., 2014; Feng et al., 2017; Amgalan et al., 2020; Di Florio et al., 2020). In macrophages, estrogen has been shown to regulate intracellular Ca^{2+} levels, which modulates Bcl-2 activity, and decreases Bax translocation to the mitochondria, thereby preserving cell viability through inhibition of intrinsic apoptosis (Subramanian and Shaha, 2007). Preliminary data in cardiomyocytes has shown that estrogen regulates Akt through ERα which attenuates ROS-induced intrinsic apoptosis in female mice, but not males (Wang F. et al., 2010; Hevener et al., 2020). While the previous study determined ERβ does not play an anti-apoptotic role in response to ROS, estrogen signaling through ERβ has been shown to decrease cardiac apoptosis by increasing mitochondrial Complex IV in rodent trauma-hemorrhage models (Hsieh et al., 2006). However, more recent studies have indicated that ERβ is not highly expressed on cardiomyocytes, and therefore may not play a major cardioprotective role in CVD (Pugach et al., 2016; Groban et al., 2019).

Testosterone has been shown to indirectly regulate necrosis of cardiomyocytes through NF-κB apoptotic signaling pathways, however, more studies on the regulation of cell death by testosterone are essential to better understand its role in CVD (Xiao et al., 2015). Estrogen and testosterone's roles in apoptosis may be altered according to receptor expression and cell type (Pugach et al., 2016). Therefore, further studies are necessary to better understand how signaling via both estrogen and testosterone influence cardiac apoptosis in CVD. Necrosis and pyroptosis are inflammatory cell death pathways regulated via caspase enzyme activity, which is induced when irreversible damage occurs in the tissues (Bergsbaken et al., 2009; Gao et al., 2019; Zhaolin et al., 2019). These pathways, more specifically, are initiated when the MPTP is damaged or uncontrolled in mitochondrial fission, as seen in CVD (Song et al., 2015; Amgalan et al., 2020). Estrogen treatment has been shown to inhibit necrosis and induce apoptosis in both sexes with lupus nephritis (Jog and Caricchio, 2013). In general, research on pyroptosis is limited in cardiac tissue and work has yet to indicate a direct role of the sex hormones.

CONCLUSION

There are clinical disparities in CVD risk and incidence, which could be caused by known sexually dimorphic differences in cardiac cells and tissues. These differences are driven by the sex hormones—estrogen and testosterone—and the presence of their receptors ERα, ERβ, GPER and AR, which are expressed differentially in varying organ systems and cell types. Studies have shown that both estrogen and testosterone can directly regulate mitochondrial biogenesis, ROS production, inter-organellar interactions between the mitochondria and the endoplasmic reticulum, in addition to preserving cell viability (**Figure 1**). Nevertheless, further studies are needed to better understand the exact mechanism of each sex hormone in regulating mitochondrial dynamics, specifically the regulation of mitochondrial fission and fusion proteins, so to establish differential function of each and elucidating the cause of CVD disparities between the sexes. Research on the cardioprotective effects of sex hormones has predominantly focused on estrogen, leaving much to be studied regarding testosterone's regulatory function in CVD. This review hopes to inspire others to begin focusing on the regulatory role of sex hormones in mitochondrial function and dynamics, as well as inter-organellar cross talk.

AUTHOR CONTRIBUTIONS

SL wrote the mitochondrial dynamics section and metabolism, edited the manuscript, and created the **Figure 1**. JB wrote the section on cell death and Ca^{2+} regulation, created the **Table 1**, and reviewed the manuscript. MS compiled the sources for metabolism and dynamics, reviewed the manuscript, table, and figure. SG-M wrote the introduction and conclusion, edited the text, figures and table, and reviewed the final manuscript. All authors contributed to the article and approved the submitted version. MRS Co-wrote metabolism and Ca^{2+} sections, edited text, and reviewed the manuscript, table, and figure.

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REVIEW
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mtDNA Heteroplasmy at the Core of Aging-Associated Heart Failure. An Integrative View of OXPHOS and Mitochondrial Life Cycle in Cardiac Mitochondrial Physiology

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The most common aging-associated diseases are cardiovascular diseases which affect 40% of elderly people. Elderly people are prone to suffer aging-associated diseases which are not only related to health and medical cost but also to labor, household productivity and mortality cost. Aging is becoming a world problem and it is estimated that 21.8% of global population will be older than 65 years old in 2050; and for the first time in human history, there will be more elderly people than children. It is well accepted that the origin of aging-associated cardiovascular diseases is mitochondrial dysfunction. Mitochondria have their own genome (mtDNA) that is circular, double-stranded, and 16,569 bp long in humans. There are between 500 to 6000 mtDNA copies per cell which are tissue-specific. As a by-product of ATP production, reactive oxygen species (ROS) are generated which damage proteins, lipids, and mtDNA. ROS-mutated mtDNA co-existing with wild type mtDNA is called mtDNA heteroplasmy. The progressive increase in mtDNA heteroplasmy causes progressive mitochondrial dysfunction leading to a loss in their bioenergetic capacity, disruption in the balance of mitochondrial fusion and fission events (mitochondrial dynamics, MtDy) and decreased mitophagy. This failure in mitochondrial physiology leads to the accumulation of depolarized and ROS-generating mitochondria. Thus, besides attenuated ATP production, dysfunctional mitochondria interfere with proper cellular metabolism and signaling pathways in cardiac cells, contributing to the development of aging-associated cardiovascular diseases. In this context, there is a growing interest to enhance mitochondrial function by decreasing mtDNA heteroplasmy. Reduction in mtDNA heteroplasmy is associated with increased mitophagy, proper MtDy balance and mitochondrial biogenesis; and those processes can delay the onset or progression of cardiovascular diseases. This has led to the development of mitochondrial therapies based on the application of nutritional, pharmacological and genetic treatments. Those seeking to have a positive impact on mtDNA integrity, mitochondrial biogenesis, dynamics and mitophagy in old and sick

hearts. This review covers the current knowledge of mitochondrial physiopathology in aging, how disruption of OXPHOS or mitochondrial life cycle alter mtDNA and cardiac cell function; and novel mitochondrial therapies to protect and rescue our heart from cardiovascular diseases.

Keywords: cardiac, OXPHOS, ROS, mtDNA heteroplasmy, aging, heart failure, mitophagy, biogenesis

INTRODUCTION

The world is aging at a very high speed and with it, the aging-associated diseases are going up. According to Lutz et al. (2008), the proportion of the global population of 60 + years was 10% in 2000 but is projected to be 21.8% in 2050 and then to 32.2% in 2100. Current projections on aging show that in 2050 there will be more elderly people (65+) than children (0-14 years old) for the first time in human history. This is enormous pressure for the health care system and for the economy of any country because older adults are affected by several and diverse chronic diseases - aging-associated diseases- that contribute to disability, diminish the quality of life and increase health- and long term- care costs. The cost of aging-associated diseases is not only related to medical care but also to labor and household productivity losses and mortality costs. Furthermore, old people in many cases are affected by a combination of diseases which increases the cost even more. Older people are more susceptible to get ill when they work or live under environmental risk factors such as air pollution, tobacco smoke, particulate matter and ozone and/or under low quality of life risk factors such as sedentarism and bad food quality, leading to obesity. This negative environmental exposure may cause or exacerbate respiratory and cardiovascular diseases (Van Houtven et al., 2008). Cardiovascular diseases are the leading cause of death among older people reaching up to 40% of deaths in people over 65 years old (Van Houtven et al., 2008; Ginneken, 2017). Therefore, new heart failure treatments are of high relevance to improve the life quality and lifespan of older people and also to decrease the pressure and costs on the public health system. Working on new methodologies and technologies will make a contribution to expediting and advancing drug discovery and genetic therapies in this area.

Our heart is a wonderful organ built at the beginning of our existence, during embryonic development, and never ever stops working until we die. It is a perfect machine that pumps, day and night, blood into all tissues, resting only between beats. Furthermore, if we exercise or have an increase in oxygen demand, our heart beats even harder to satisfy tissue demands. It has been estimated that our heart beats 36 million times per year and to support such a high load of work, our heart must be able to produce an enormous amount of ATP that has been estimated in about 30-40 Kg per day (Ferrari et al., 2003) which is about one-third of total ATP produced by our body (a normal individual produces 90-100 Kg of ATP daily). Cardiac cells are contractile striated cells with an average render cell volume of 30.5 pL (picoLiters) and 140 μm $\times$ 32 μm $\times$ 13 μm (LxWxD) average dimension in mammals. The cardiac cell capacitance, which is proportional to the amount of membrane and membrane invaginations, varies among different mammal species between 138 and 300 pF (picoFaraday). The amount of membrane invaginations is explained by the presence of T-tubules (50% membrane), endoplasmic reticulum and mitochondria which are all involved in cardiac cell contraction (Satoh et al., 1996). The ultrastructural analysis of cardiac cells also reveals that the volume density of mitochondria is over 25.3%, the myofibrils 52.3% and the cytoplasm 22.3%. One-third of the cardiac volume is filled with mitochondria (Barth et al., 1992); although Huang et al. (2013) estimate mitochondrial volume to be up to 40%.

KEEPING HEALTHY AND HAPPY MITOCHONDRIA: OXPHOS AND MLC

Mitochondria are cellular organelles that are well known as the powerhouse of the cell in charge of satisfying ATP demands. However, today, mitochondria are also acknowledged as a metabolic hub that integrates intracellular signaling to elaborate and execute a cellular response to adapt cell metabolism to external or internal environmental changes, taking primary action in cell fate determination. Fit and healthy mitochondria will be able to satisfy the heart's energy demands, but dysfunctional mitochondria won't, therefore a cardiac failure is expected.

In general, for all of our cells (excluding erythrocytes that do not have mitochondria) two convoluted mitochondrial systems work collaboratively to maintain a healthy mitochondrial population. The Oxidative Phosphorylation (OXPHOS) system and the Mitochondrial Quality Control System also known as the Mitochondrial Life Cycle (MLC). Together they complement each other to keep mitochondrial function i.e., energy production (ATP), reactive oxygen species (ROS) detoxification, proper metabolism and retrograde signaling (**Figure 1**).

OXPHOS is made of the Electron Transport Chain (ETC) to generate the proton motive force (mitochondrial membrane potential) and the ATP synthase that utilizes the proton motive force to generate ATP. The ETC or respiratory chain is formed by 4 respiratory complexes plus the ubiquinone and the cytochrome c, located in the inner mitochondrial membrane that forms cristae. OXPHOS is fed by NADH at the level of complex I and FADH2 at the level of complex II. Both reductant equivalents are mostly generated by the Krebs (TCA) cycle which in turn is dependent on calcium and respiratory substrates such as carbohydrates and lipids. As a byproduct of the respiratory chain, the superoxide radical (a ROS molecule) is generated, which has

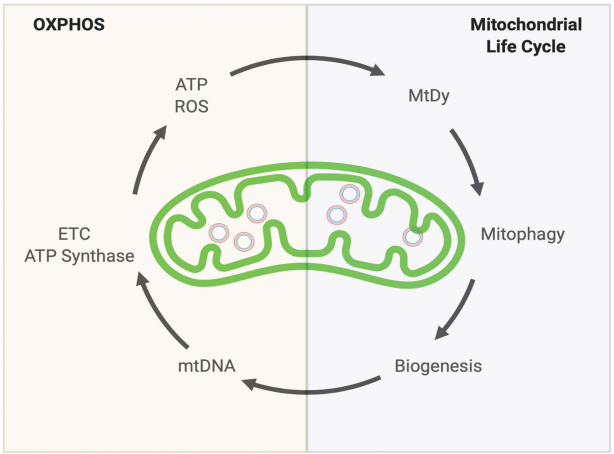

FIGURE 1 | Keeping a healthy mitochondrial population. Mitochondria's health depends on two complementary systems. The first system is the OXPHOS. This will provide energy (ATP) and reactive oxygen species (ROS) which are both necessary to drive the second system: The Mitochondrial Life cycle. Through mitochondrial fusion and fission events (Mitochondrial dynamics, MtDy), mitochondria are segregated to be eliminated by mitophagy. The induction of mitophagy is coupled with mitochondrial biogenesis which involves mitochondrial DNA (mtDNA) replication. mtDNA encodes for 11 respiratory complex subunits (7 subunits for complex I, 1 for complex III, 3 for complex IV) plus 2 subunits of the ATP synthase that will allow OXPHOS assembly to keep going the virtuous circle. Any disruption or delay in this running circle will end up in the loss of energy, mutations in mtDNA, decrease in mtDNA copy number and increased ROS leading to mitochondrial dysfunction, cell death, aging and aging-associated diseases.

been involved in aging and aging-associated diseases. In this way, a very simplistic OXPHOS equation is:

$$\text{Respiratory Substrates} + \text{Calcium} =$$
$$\text{NADH} + \text{FADH2} = \text{ATP} + \text{ROS} + \text{Heat}$$

The OXPHOS system is encoded by two independent genetic material: nuclear DNA and mitochondrial DNA (mtDNA). The mtDNA is a plasmid DNA of 16 kb (circular, closed), doubled stranded and codes for 37 genes (13 OXPHOS protein-encoding mRNA, 2 rRNA and 22 tRNA). It is composed of a heavy strand and a light strand based on the proportion of heavier nucleotides (adenine and guanine). It also has a large non-coding region (around 1 kb) that contains the regulatory elements for the initiation and termination of the transcription of both strands. The D-Loop (Displacement Loop) or control region, is within this non-coding zone and contains the origin of replication of the heavy strand and high-variability zones called HVRI, HVR II and HVR III where most of the polymorphisms associated with mtDNA occur. Each molecule of mtDNA is packaged in structures called nucleoids made of proteins involved in their replication and transcription, such as TFAM. Nucleoids are located in the mitochondrial matrix associated with the internal membrane and the oxidative phosphorylation system (Brown et al., 2011).

Mitochondria have their own genome is maternally inherited and this configures each individual with their own maternal surname - called a haplogroup. All mitochondrial haplogroups derive from a common ancestor, from a black African woman, called "The Mitochondrial Eve" from 200,000 years ago (Cann et al., 1987). At present, a total of 20 haplogroups are recognized, each with its respective subdivisions, which were established by the acquisition of mutations and natural selection, according to the patterns of migration and human settlement, and fixed in each of the different ethnic groups. This is how the haplogroup J, D4a, D4b2b, D5 have been associated with longevity; the H5 and United Kingdom, at risk of suffering from Alzheimer's disease; the U, with a predisposition to psychosis in bipolar disorders; and others associated with resistance to altitude, with athletic performance, predisposition to diabetes, cancer or cardiovascular diseases (Hiona, 2008; Lakatos et al., 2010; Nishigaki et al., 2010; Picard et al., 2016; Chocron et al., 2019). Knowing that the haplogroup confers adaptive advantages and/or disease propensity and, therefore, their identification can also become a tool of preventive diagnosis that can be applied since the birth of a new being.

mtDNA is prone to oxidative or replicative damage. Each mitochondrion has 3 to 10 mtDNA copies which undergo mutations by either the reactive oxygen species (ROS) generated as a byproduct of electron transfer chain due to electron leak mainly from respiratory complex I and III (Brand et al., 2013) or alterations in the mtDNA replication or repair. A higher mutation load in mtDNA means lower OXPHOS functionality giving rise to more ROS and more mtDNA mutations in a vicious cycle. The coexistence of wild type mtDNA and mutated mtDNA in the same mitochondrion is known as heteroplasmy. As the percentage of mutant mtDNA rises above certain thresholds (60-70%), cellular homeostatic mechanisms are disrupted, leading to mitochondrial dysfunction, cellular atrophy, and death, contributing to age-related diseases. Heteroplasmy also leads to broad changes in gene expression that can shift abruptly as the percentage of mutant mtDNA increases (Lakshmanan et al., 2018). To date, more than 400 mtDNA mutations have been associated with human diseases (Li et al., 2010).

The mitochondrial life cycle involves three processes: mitochondrial dynamics, mitochondrial selective autophagy (mitophagy) and mitochondrial biogenesis. Through MtDy, mitochondria share or dilute components and also segregate dysfunctional mitochondrial units for degradation by mitophagy (Legros et al., 2002; Liesa et al., 2009). While mitochondria are being degraded, mitochondrial biogenesis is turned on to maintain mitochondrial population and homeostasis (Palikaras et al., 2015a; Sin et al., 2016). In addition, mitochondrial biogenesis responds to energy demands and environmental factors such as cold and hypoxia (Tohme et al., 2017; Gureev, 2019).

Mitochondrial Dynamics (MtDy) refers to the ability of two mitochondria to fuse each other (fusion) or one mitochondrion to divide into two daughter mitochondria (fission). Mitochondrial morphology and size are dependent on mitochondrial fusion and fission events. Importantly, changes in MtDy regulate bioenergetics outputs including respiratory rate,

energy expenditure and ATP synthesis as well as apoptosis and the segregation and elimination of dysfunctional mitochondrial units by mitophagy (Bénard et al., 2007; Sheridan and Martin, 2010; Westermann, 2012). Mitochondrial fission is dependent on the protein DRP1 that has been recognized as a mitochondrial fission promoter (Smirnova et al., 2001). It is located in the cytosol, but translocate to mitochondria through its binding to the mitochondrial receptor proteins FIS1, MFF, MiD49, and MiD51 (Losón et al., 2013), which are all located in the mitochondrial outer membrane (MOM). Mitochondrial fusion is dependent on MFN1 and MFN2, located in the MOM, and OPA1, which is located in the mitochondrial inner membrane (Liesa et al., 2009). A fusion event is often followed by a fission event generating one hyperpolarized and one depolarized daughter mitochondrion. The depolarized unit loses OPA1, MFN1, and MFN2 and thus its ability to fuse, becoming a target for degradation by mitophagy; while the hyperpolarized unit remains in the cytosol and can fuse again. In this way, cells keep a more active, healthier and functional mitochondrial web (Twig et al., 2008).

Mitochondrial Selective Autophagy or Mitophagy is a type of macroautophagy that delivers mitochondria to lysosomes for degradation. It is essential for the recycling and protective process of mitochondria to maintain cellular homeostasis. Mitophagy initiates with the formation of a double-membrane phagophore which elongates and closes to generate a mature, double-membrane autophagosome that engulfs mitochondria. Then, autophagosomes are fused to lysosomes for content degradation (Youle and Narendra, 2011). Mitophagy allows the removal of dysfunctional, depolarized and/or damaged mitochondria for mitochondrial turnover, ROS management, and programmed mitochondrial clearance, as seen in erythropoiesis (Schweers et al., 2007), lens differentiation (Bassnett, 2002), and mature T-lymphocytes (Pua et al., 2009). Mitophagy is also essential for embryonic development by removal of paternal sperm mitochondria from the fertilized eggs, leaving only the maternal ones (Rojansky et al., 2016; Pickles et al., 2018). In this regard, cells display basal mitophagy, stress-induced mitophagy and programmed mitophagy, being the cardiac muscle one of the tissues with enhanced basal mitophagy (Palikaras et al., 2018).

The process of mitophagy requires two systems: The autophagic core machinery, which is common to all types of autophagy; and the mitochondrial receptors and adaptors, a specific set of mitochondrial and/or cytosolic proteins needed for the assembly of mitochondria with the autophagic core machinery. The Autophagic Core Machinery is composed of ATG proteins that are found from yeast to mammals, indicating that autophagy is an evolutionarily conserved process. Phagophore initiation and formation is dependent on the ATG16L1 complex, made of ATG16L1, ATG5, and ATG12 which localize to the isolation membrane and dissociates from it upon the completion of autophagosome formation; and ATG8/LC3, which upon the formation of autophagosome, remains in on both sides—inside and outside of the double-membrane structure. While in yeast there is a single ATG8 protein, in mammals there are seven orthologs of ATG8 named LC3A, LC3B, LC3C, GABARAP, GABARAPL1, GABARAPL2, GABARAPL3 (thereafter they will be called LC3) suggesting a complex diversification of their function. LC3/ATG8 can be found in two isoforms: LC3-I and LC3-II. LC3-I is the inactive form that is constitutively expressed and found in the cytosol. Upon induction of autophagy, LC3-I is converted to LC3-II by site-specific proteolysis and lipidation near to the C-terminus to generate the autophagosome. LC3 is considered a reliable marker for on-going autophagy (Ashrafi and Schwarz, 2013; Lee and Lee, 2016).

The Mitochondrial Receptors interact directly or indirectly through adaptors, with LC3 via the LC3-interacting Region (LIR), a tetrapeptide sequence W/YXXL/I. Independent and non-redundant mitophagy pathways have been defined according to the mitochondrial receptors and adaptors that are involved in it. Those are (i) Ubiquitin-Dependent Mitophagy, with the PINK1-PARKIN protein axis. PINK1 (PTEN-induced putative protein kinase 1) is a mitochondrial protein whose import is dependent on mitochondrial membrane potential. Once imported, PINK1 is degraded by mitochondrial proteases. However, if mitochondria become depolarized, PINK1 is no longer imported and accumulates on the mitochondrial surface to phosphorylate ubiquitin and Mfn2, allowing the recruitment of the E3 ubiquitin ligase PARKIN from the cytosol. PARKIN mediates a hyper-ubiquitination of the mitochondrial outer membrane proteins, which are recognized by ubiquitin-binding adaptors such as P62/SQSTM1, OPTN, TAX1BP1, NDP52, NBR1, TOLLIP and HDAC6. These adaptor proteins interact and bind LC3 by means of their LIR domain. Of note, P62/SQSTM1 has been also used as a reliable indicator of the mitophagic flux, because under mitophagic defects it accumulates in response to stress stimuli (Xu et al., 2015; Yamaguchi et al., 2016). This PINK/PARKIN mitophagy is the canonical pathway for mitochondrial turnover and mitochondrial quality control. (ii) Ubiquitin-Independent Mitophagy is dependent on mitochondrial receptors interacting directly with LC3 through the LIR domain. Several and diverse mitochondrial LC3 receptors have been discovered, which also account for the complexity of mitophagy. NIX, BNIP3 (Zhang and Ney, 2009), FUNDC (Liu et al., 2012), FKBP8 (Bhujabal et al., 2017; Yoo et al., 2020), NLRX1 (Zhang et al., 2019) are located in the MOM; Prohibitins (Wei et al., 2017), in the MIM and, MsrB2 in the mitochondrial matrix (Lee et al., 2019). Not only proteins may bind LC3, but also the membrane phospholipid cardiolipin (Chu et al., 2013) mainly found in the MIM. NIX protein is highly expressed in erythroid precursors and is needed for erythropoiesis (Novak et al., 2009). Also, NIX, BNIP3 and FUNDC trigger mitophagy in response to hypoxia (Sowter et al., 2001; Bellot et al., 2009; Liu et al., 2012), Prohibitins, MsrB2 and cardiolipin could be accessible to cytosolic proteins if MOM or MIM are disrupted.

Mitochondrial dynamics and the mitophagy pathways with their mitochondrial receptors, adaptors and regulator proteins that localize to mitochondria, seem to have not redundant functions and might act cooperatively, providing multiple mechanisms to clear out damaged mitochondria under different conditions. In fact, the mitophagy protein FUNDC1 has been shown to interact with the MtDy proteins DRP1 or OPA1 to coordinate mitochondrial fission or fusion and mitophagy. Upon mitochondrial stress, FUNDC1-OPA1 interaction is dismissed,

promoting DRP1 translocation to mitochondria for fission and a new association FUNDC1-DRP1 for mitophagy. This reveals the complexity and importance that MtDy and Mitophagy play for cellular physiology.

Mitochondrial Biogenesis: When mitochondria are eliminated by mitophagy or when more mitochondria are needed to meet energy demands, the mitochondrial biogenesis program is started. Interestingly, mitochondrial biogenesis involves four independent processes: mtDNA replication, mtDNA transcription, protein translation and mitochondrial membrane biogenesis, which are not necessarily synchronized adding more complexity and regulation capabilities to mitochondria. Unfortunately, the mechanisms controlling these three processes are not fully understood or discovered. In fish acclimated to cold, it was shown that mitochondria are able to remodel differentially according to ATP demands, oxygen demands, or both. Under oxygen demands, membrane biogenesis is upregulated increasing the mitochondrial volume/mass but not OXPHOS protein densities. Having an extra membrane improves oxygen diffusion since oxygen diffuses easily and faster in lipidic than in an aqueous environment. Under ATP demand, mitochondria increase the OXPHOS densities without increasing their volume. In the case of ATP and oxygen demands, both protein density and membrane are increased (O'Brien, 2010). On the other hand, it has been reported that mtDNA replication is controlled by oxidative damage in the D-loop region which favors TFAM (a mitochondrial transcription factor) binding to mtDNA for replication. This oxidative damage may be caused by hypoxia, linking the lack of oxygen to mtDNA replication (Pastukh et al., 2016).

MITOCHONDRIA IN CARDIAC CELLS

The aforementioned mitochondrial features for OXPHOS and mitochondrial life cycle are general for all mitochondria. But what is distinctive for heart mitochondria? (**Table 1**). Heart striated cells are special and different from other cell types. They have a particular shape and contract throughout our lives from early development until we die. Heart cells demand an enormous amount of energy and then mitochondria must do the work. Mitochondria in cardiac cells have been subdivided into interfibrillar, subsarcolemmal and perinuclear mitochondria, according to their distribution in the cell; and are tightly packed between myofibrils with almost no chance of free or flexible movement. However, Hendgen-Cotta et al. (2018) claimed that all cardiac mitochondria in mice are interconnected and, therefore, they are not different populations. Cardiac mitochondria seen under transmission electron microscopy look well organized to each other and actually mitochondrial cristae seem to be aligned between two adjacent mitochondria. Three-dimensional reconstruction of murine heart mitochondria has shown that they have an oval but irregular shape. Changes in mitochondrial morphology have been associated with metabolic and bioenergetic reprogramming. The heart is not the exception since most of the cardiopathies are associated with alteration in mitochondrial morphology. Fragmented, swollen, reduced mitochondrial number, reduced cristae densities are the main morphological features of human heart failure which are nicely tabulated in Daghistani et al. (2018). Interestingly, different cardiac pathologies have shown distinct morphological mitochondria and cristae patterns. Heart mitochondrial cristae are very long, mostly going all throughout the mitochondrial matrix. In comparison, cristae length in non-cardiac mitochondria is about 1/3-1/4 of mitochondrial width. Cristae hold the OXPHOS system and considering the length of cristae and the electrodense nature of mitochondrial matrix seen by TEM (in accordance with condense mitochondrial state and opposed to orthodox one), it is possible to suggest that cardiac mitochondria are fully packed with OXPHOS having a highly oxidative metabolism. In fact, nice work from Williams et al. (2018) analyzed the mitochondrial proteome from 5 different mouse tissues and showed that in heart tissues half of the total proteome (55%) correspond to mitochondrial proteins, which was higher than brain, liver, skeletal muscle and brown adipose tissue. When looking at OXPHOS protein expression, cardiac mitochondria displayed the highest OXPHOS protein density for both all ETC respiratory complexes and the ATP synthase as compared with other tissues.

The super oxidative metabolism of cardiac cells is explained by the use of triacylglycerols and fatty acids as the main oxidative fuels, followed by glucose and other carbohydrates, which enter to the TCA cycle-OXPHOS to produce 80-90% of total ATP. The relative contribution of fat and carbohydrate to energy provision for the heart is 70% and 30%, respectively (Stoll et al., 2018) (Taegtmeyer et al., 2016). Furthermore, OXPHOS in cardiac mitochondria is equipped with specific subunit isoforms for the cytochrome c oxidase (complex IV) to make the ETC more efficient (Sinkler et al., 2017). The assembly of respiratory complexes in supercomplexes or respirosomes, reported for bovine, ovine and porcine hearts (Letts et al., 2016; Sousa et al., 2016) plus post-translational modifications in respiratory complexes such as O-GlcNAcylation, the ability to increase calcium uptake, and de-sensitize the opening of the mPTP can also improve OXPHOS efficiency (Ma et al., 2015; Stoll et al., 2018). OXPHOS efficiency is not exclusively dependent on OXPHOS proteins but also on other proteins involved in the bioenergetic capacity of cardiac cells. This is the case of the Adenine Nucleotide Transporter (ANT) protein, which is nuclear-encoded and allows the exchange of ATP by ADP through the inner mitochondrial membrane. ANT KO mouse display impaired activity of complex I, increase in ROS and oxidative damage. They also display a reduction in OPA1 leading to a fragmented mitochondrial web and sensitization to the opening of mPTP which has been associated with cardiopathies (Strauss et al., 2013).

Heart mitochondria have an active mitochondrial life cycle. Albeit to be highly packed with almost no freedom for movement, mitochondrial dynamics, mitophagy and biogenesis are critical for heart function (Eisner et al., 2017; Wu et al., 2019). Heart and skeletal muscle mitochondria feature specialized structures called nanotunnels that allow component mixing between mitochondria which resemble a fusion event (Huang et al., 2013). Disruption of MtDy has drastic consequences for cell

TABLE 1 | Summary of main characteristics found in mitochondria from non-cardiac cells, cardiomyocytes and aged cardiomyocytes.

	Young Standard Mitochondria*	Young Cardiac Mitochondria	Aged-Cardiac Mitochondria
mtDNA			
Mutation levels	– Low	– Low	– High
Heteroplasmy levels	– Low (<60%)	– Low (<60%)	– High (>60%)
Copy number	– High	– High	– Low
Biomass and Morphology			
Cell Occupancy (% of total cell Volume)	3-8%	30-40%	<30-40%
Subpopulations	– None	– Interfibrillar, subsarcolemmal and perinuclear	– Interfibrillar, subsarcolemmal and perinuclear
Mitochondrial Network	– Mostly tubular shape and branched mitochondria	– Mostly oval with irregular shape. – Highly packed between myofibrils.	– Fragmented, Shrunken or Swollen
Cristae Dimensions	– Length: 1/3 of mitochondrial width. (12 to 40 nm)	– Length: All throughout mitochondrial width (30-40 nm) – Aligned between adjacent mitochondria	– Altered cristae structure.
Morphological state seen under TEM	– Orthodox state	– Condensed state	– Orthodox, Condense and Swollen states
Bioenergetics			
OXPHOS protein Density	– Middle	– High	– Middle-Low
Special OXPHOS subunit isoforms	– Most tissues have ubiquitous COX subunit isoform expression	– Special Heart (H) Subunit Isoforms COX VIa H COX VIIa H COX VIII H	– Unknown
OXPHOS post-translational modifications	– Low levels of O-GlcNAcylated proteins	– High levels of O-GlcNAcylated proteins	– Unknown
Main Respiratory Substrate	– Glucose	– Fatty Acids – Triacylglycerides	– Glucose
ATP production	20-50%	80%	<80%
Calcium management	100nM	100-800nM	– Disrupted Calcium Homeostasis
ROS production	– Low	– Low-Middle	– High
mPTP status	– Physiological Flickering	– Desensitized to deal with increased Ca^{++} uptake and ROS	– Sensitized. Pathological Flickering and Opening
Mitoflashes	– Low (mPTP dependent?)	– Low (mPTP dependent?)	– High (ANT dependent)
Mitochondrial Life Cycle			
Predominant control of mitochondrial biomass	– PGC-1α – NFE2L2 (Activators of Mito Biogenesis)	– PGC-1α – NFE2L2 (Activators of Mito Biogenesis)	– NcoRI – SMRT? – RIP140? (Repressors of Mito Biogenesis)
Mitochondrial fusion and fission events	– Frequent events between adjacent mitochondria	– Frequent events between adjacent mitochondria. – Nanotunnel generation for long distance fusion events	– Decreased fusion and fission events. – Decreased nanotunnel generation?
Predominant basal mitophagy pathway	– PINK/PARKIN/P62	– PINK/PARKIN/P62. – Autophagy-dependent exospheres release	– Decreased PINK/PARKIN/P62 pathway. – Low Autophagy-dependent exospheres release. – Increased receptor mediated mitophagy gene expression. – Increased MUL1 expression?

** General features found in mitochondria from different types of cells. Variations may exist.*
? Expected to occur but not demonstrated yet. TEM, Transmission Electron Microscopy.

physiology which are mainly associated with the accumulation of dysfunctional and ROS-generating mitochondria and heart failure. In humans, mutations in MFN2 and OPA1 cause Charcot–Marie–Tooth disease type 2A and dominant optic atrophy respectively. Also, complete loss of mitochondrial fusion results in a dramatic decrease in mtDNA content,

loss of membrane potential and reduced respiratory chain function in both cultured cells and tissues. After conditionally knocking out Mfn1 and Mfn2 genes in adult rats, the mitochondrial fission in cardiomyocytes increased leading to abnormal cellular respiration, eventually leading to progressive dilated cardiomyopathy. Excessive mitochondrial fission has been associated with decreased mitochondrial function and increased ROS (Jheng et al., 2011; Haileselassie et al., 2019). FIS1 up-regulation decreased cellular ATP levels in anoxic cardiomyocytes (Wang et al., 2012). On the other hand, an excessive fusion is also deleterious for cardiac cells. MFF mutant mice died at 13 weeks due to heart failure caused by severe dilated cardiomyopathy. Mutant tissues showed decreased mitochondrial density and respiratory chain activity, and increased mitochondria size (García-Palmer, 2008; Zhou et al., 2017; Zhou and Tian, 2018). A thoughtful review on mitochondrial dynamics and cardiac biology is found in Vasquez-Trincado et al. (2016).

Although the role and the importance of the proper balance of MtDy for cell physiology are well accepted, there is no clarity about the signaling mechanisms that might be associated with heart failure. In our laboratory studying the role of MtDy in erythropoiesis, it was discovered a link between the mPTP and MtDy to control cell differentiation in erythropoiesis (Gonzalez-Ibanez et al., 2020). Interestingly, the role of the mPTP in cellular differentiation has been reported in the past for early embryonic cardiomyocytes, where a frequent opening of the mPTP is needed to maintain an immature mitochondrial morphology with low oxidative capacity. On the other hand, the closure of the pore was required for proper differentiation into cardiac muscle cells (Hom et al., 2011; Folmes et al., 2012). One of the triggers of mPTP opening is an elevated Ca++ concentration in the mitochondrial matrix (Bernardi and Di Lisa, 2015) and the up-regulation of FIS-1 by means of its interaction with the ER protein BAP31 might favor calcium entrance. In fact, mitochondrial calcium overload is a key determinant in heart failure (Iwasawa et al., 2011; Santulli et al., 2015).

Mitophagy is the cellular process to dismiss dysfunctional mitochondria and secure ATP demand for heart cells. As it was discussed before, many pathways and proteins are implicated in mitophagy which responds to different stimuli like ROS, hypoxia and mitochondrial depolarization. The great variability and diversity of proteins and pathways controlling mitochondria elimination reflect the importance and complexity of mitophagy for cell function that we are still far to fully understand. In cardiac cells, it is clear that mitophagy plays a major role in keeping healthy mitochondria and many or all known mitophagy pathways have been shown to play a critical role. In fact, it was recently published a novel and astonishing mechanism in cardiac cells to get rid of dysfunctional mitochondria, especially under cardiac stress. Dysfunctional mitochondria are expelled out from cardiomyocytes in special vesicles called exophers which are made by means of the cardiac mitophagic machinery. These expelled mitochondria are engulfed by macrophages which are resident in the cardiac tissue. Blocking the mitophagy machinery, reduction of macrophages or ablation of the specific exopher receptor caused and accumulation of damaged mitochondria and dysfunction in the heart (Nicolás-Ávila et al., 2020). In general, disruption of mitophagy is associated with heart failure and cardiopathies Deep details on mitophagy and cardiovascular diseases can be found in Morciano et al. (2020). On the other hand, induction of mitophagy, by any means (exercise, food, specific drugs or hypothermia), has shown a protective effect on many cardiovascular diseases.

CARDIAC MITOCHONDRIA IN AGING

Much of the cardiac literature describes a relationship between different cardiopathies and mitochondrial dysfunction. Daghistani et al. (2018), Chaanine (2019) showed that mitochondria changed their morphology from elongated to fragmented in heart failures. Lin and Kerkelä (2020), that there is a reduction in ETC and bioenergetic capacity in cardiomyopathies; and Vasquez-Trincado et al. (2016), Bravo-San Pedro et al. (2017) that mitochondrial dynamics and mitophagy are altered or reduced in cardiovascular diseases. In the end, most of those pathologies are related to an exacerbated amount of ROS, dysregulation of calcium homeostasis and the aperture of the mPTP. Mitochondria are constantly adapting bioenergetics, metabolism and signaling to allow cell function and survival. Thus, aged mitochondria (**Table 1**), with altered ROS and calcium homeostasis and dysregulation of the mPTP leading to ATP crisis, are one of the main causes of heart failure (Brand et al., 2013). Recently, the ANT protein was shown to increase mitochondrial proton leak - mitoflashes - in aged cardiomyocytes. This pathological proton leak is associated with inefficient ATP synthesis and increased electron flux and oxygen consumption, increasing ROS production and mPTP sensitivity. Interestingly, the treatment with the drug SS-31, a tetrapeptide that binds to cardiolipin-containing membranes and improves membrane stability and cristae curvatures (Birk et al., 2014), arrested proton leak, restored mitochondrial functionality, reduced ROS production and more surprisingly rejuvenated old cardiomyocytes (Zhang et al., 2020). Cardiac aging is characterized by a decrease in the number of mitochondria in the heart and a reduction in the area of the inner mitochondrial membrane implicating OXPHOS at the center of cardiac mitochondrial metabolism deficiency during aging. Respiratory complex III and IV deficiency in aging caused a decreased efficiency of OXPHOS, affecting also the number and mass of interfibrillar myofibrils (Stoll et al., 2018; Lin and Kerkelä, 2020).

The main driver of mitochondrial dysfunction in aging is the mtDNA, which progressively accumulates mutations and reduces its copy number as humans are getting old. In fact, mtDNA mutations cause aging, heart failure and many other aging-associated diseases. In 2004, it was published in Nature journal the "mutator mouse" whose mitochondrial DNA polymerase (POLG) had a dysfunctional proofreading domain which introduced random point mutations in the mtDNA, increasing mtDNA heteroplasmy (Trifunovic et al., 2004). The "mutator mouse" at the early age of 6 months, presented all the symptoms of old age such as blindness, deafness, alopecia, muscular atrophy, anemia, hump development and

heart hypertrophy. The creation of this mutator mouse is an irrefutable proof that mtDNA mutations are a cause of aging and cardiovascular diseases. mtDNA mutations lead to OXPHOS inefficiency and then energy shortages especially in the tissues with the highest energy demands such as the heart, muscle and brain, which are the most vulnerable. By 40 weeks of age, mutator mice presented an enlarged volume of the left ventricle along with an increase in weight in relation to body weight. Histochemical analysis of the mutator mouse's heart revealed a mosaic pattern with cytochrome c oxidase deficiency in some cardiomyocytes, which also occurs in the aged human hearts. TEM analysis of mitochondria ultrastructure confirmed the accumulation of fragmented, abnormal mitochondria. In addition, a decreased ATP production rate was noted (Trifunovic et al., 2004).

Another form of mtDNA damage during aging in humans is large mtDNA deletions. Mitochondrial diseases are usually associated with 5kb deletions of mtDNA which have been shown to accumulate in the human's brain, muscle and heart (Chocron et al., 2019). Mutant mtDNA co-exists with wild type mtDNA giving rise to mtDNA heteroplasmy affecting the proper function of OXPHOS in a vicious cycle. Some pathogenic mtDNA mutations and a reduction in their copy number in cardiac cells have been specifically associated with impaired cardiac function and cardiac diseases and then, mtDNA mutations and copy number can be used as potential biomarkers of heart diseases (Ashar et al., 2017; Bray and Ballinger, 2017; Elosua, 2018; Galera-Monge et al., 2019). In fact, Elosua (2018) is emphatic when claiming that "mtDNA has been largely forgotten in cardiovascular research."

The increase in mtDNA heteroplasmy not only affects mitochondrial function but also affects and influences nuclear genome mutations and the severity of cardiovascular diseases. This is also proof of mitochondria acting as a powerful signaling platform regulating both transcriptional and epigenetic nuclear gene expression. This mitochondria-nucleus retrograde communication acts in a synergistic way to produce cardiomyopathies or increase their severity. McManus et al. (2019) showed that ablation of ANT (the mitochondrial Adenine Nucleotide Transporter) in nuclear DNA is associated with cardiopathies where OXPHOS, MtDy and mitochondrial morphology are affected. However, in the presence of mtDNA mutations, cardiopathies are much more severe. mtDNA-related mitochondrial genetic diseases are associated with the development of cardiomyopathies in 40% approximately (Bates et al., 2012). Along with mtDNA mutations, the mtDNA haplogroups are also predictors of both lifespan and risk of several age diseases. Dr. Wallace's group showed the type of mitochondrial haplogroups influences the severity of cardiovascular diseases with haplogroups U and H being the most affected (Strauss et al., 2013). On the other hand, the haplogroups J and T are associated with a reduced risk of cardiovascular disease (Veronese et al., 2019).

Reduction in heteroplasmy and restoration of mtDNA copy number is achieved by the coupling of OXPHOS with the Mitochondrial Life Cycle. It has been reported damaged DNA invokes mitophagy (Dan et al., 2020) and that adult post-mitotic tissue can eliminate mitochondria carrying damaged mtDNA by mitophagy which help to preserve cellular and mitochondrial function, suggesting that removal of the mutant mtDNA is protective for cells (Kandul et al., 2016). Mitochondrial turnover decreases with age, so there is growing interest in enhancing the pathways of mitophagy by discovering new pharmacological targets or cell mechanisms to counteract mitochondrial heteroplasmy (Diot et al., 2016). The aggravation of cardiac aging together with the accumulation of damaged mitochondria in cells can be caused by impaired and deficient mitophagy that results in increased heteroplasmy and altered mitochondrial metabolism (Quan et al., 2020). In fact, many of the pathways that improve health and extend longevity in various organisms all converge on mitophagy which is dependent on mitochondrial biogenesis and dynamics. A decrease in mitochondrial fission, functional mitochondria and NIX and FUNDC-1 receptor-mediated mitophagy may result from mtDNA mutations associated with aging (Lampert et al., 2019; Wu et al., 2019). Furthermore, Woodall et al. (2019) examined the role and expression levels of Parkin on accelerated cardiac failure in the mtDNA POLG mutator mice. It was observed that Parkin decreases in the mutator mouse's heart with age. However, the restoration of Parkin level by means of its overexpression did not rescue the cardiac hypertrophy in the POLG mouse. Besides, deletion of parkin did not worsen heart disease. The authors claimed that mitochondria were not altered in their bioenergetics and that their function did not decline with age. This author's interpretation is controversial since they observed a clear and significant decrease in complex II and IV protein levels, and TEM images showed altered cristae morphology in mutant mice with age, which might be indicative of mitochondrial dysfunction. An interesting discovery in the POLG mutant mouse's heart is the upregulation of the receptor-mediated mitophagy genes and the appearance of larger mitochondria, which is in agreement with the upregulation of NIX protein myocardial hypertrophy (Yussman et al., 2002). This larger mitochondrial phenotype might be due, among other causes, to a decreased production and expulsion of exospheres that are dependent on the autophagic machinery (Nicolás-Ávila et al., 2020). However, the reduction of parkin in aging may be compensated by the upregulation of MUL1, a ubiquitin E3 ligase that is involved in regulating mitochondrial dynamics by promoting MFN2 degradation and leading to mitochondrial fragmentation and Parkin independent-mitophagy (Yun et al., 2014; Cai and Jeong, 2020). In cardiac cells, upregulation of MUL1 has been shown to fragment mitochondria and alter cristae structure, reduce mitochondrial membrane potential, promote cytochrome c release and sensitize mPTP, making it a potential mechanism of cell death and heart failure in aging (Yang et al., 2016; Wang et al., 2020).

Stressed mitochondria have been shown to release mtDNA into cytosol which is recognized as a Damage-Associated Mitochondrial Patterns (DAMPs), that trigger an inflammatory response. This pro-inflammatory environment may activate fibroblast proliferation and excessive production of extracellular matrix proteins which correlates with cardiomyopathies (West et al., 2011; Lin and Kerkelä, 2020). These alterations at molecular levels are associated with cellular changes such as

hypertrophy, fibrosis, accumulation of misfolded proteins, loss of cardiac cells, extracellular matrix remodeling and amyloid deposition and structural changes in the myocardium like left ventricle hypertrophy and left auricle hypertrophy, which causes diastolic dysfunction (Steenman and Lande, 2017; Liang and Gustafsson, 2020). It has also been reported that mtDNA and whole mitochondria are released into bloodstream which offers a new biomarker of mitochondrial function and stress. mtDNA may travel naked (circulating cell-free mtDNA) which is recognized by immune cells to initiate the immune response, or they can go inside extracellular vesicles (EVs). Circulating EVs have been described as a novel mechanism of intercellular communication. In aging, Lazo et al. (2020) showed that mtDNA in EVs is diminished as compared with young people and even more, EVs containing old and damaged mtDNA affects mitochondrial bioenergetic in other cells and tissues. Picca et al. (2019) pointed out that EVs allow the elimination of dysfunctional or damage mitochondrial components; and Freeman et al. (2018) proved that EV secretion is dependent on autophagy. All these antecedents support the hypothesis that a decline in mtDNA containing EVs is due to a defect in mitophagy with aging.

LINKING OXPHOS, MITOCHONDRIAL LIFE CYCLE AND mtDNA: SIGNALING PATHWAYS

The master regulator of mitochondrial biogenesis is the protein PGC-1α which is a transcriptional co-activator that induces nuclear- and mitochondrial- encoded gene transcription coordinating both genomes. PGC-1α can be stimulated/induced by physical exercise, cold and hypoxia. The signaling pathways to activate PGC-1α are diverse and well described (see Gureev, 2019; Oka et al., 2020). In mammals, an increase in the AMP/ATP ratio given by an overwhelming ATP demand or reduction in ATP synthesis activates AMPK (AMP protein kinase) which in turn will activate PGC-1α and inhibit mTOR (mechanistic target of rapamycin kinase). In this way, AMPK activation stimulates mitochondrial biogenesis and mitophagy (Kupr and Handschin, 2015). As a transcriptional co-activator, PGC-1α does not bind directly to DNA promoters but interacts and activates the transcription factors Nuclear Respiratory Factor 1 and 2 (NRF1 and NRF2), Estrogen-Related Receptor (ERR) and Peroxisome Proliferator Activated Receptor (PPAR). In general, NRF2 induces nuclear-encoded OXPHOS protein expression and NRF1 induces the expression of mitochondrial transcription factors TFAM and TFB to initiate mtDNA replication and transcription. ERR and PPAR are involved in mitochondrial biogenesis and dynamics, Krebs' cycle and mitochondrial fatty acid oxidation expression genes. Furthermore, the increase in NAD + /NADH ratio as a result of the electron transport chain induces SIRT1 that also stimulates PGC-1α.

An essential component in mitochondrial signaling is ROS. In addition, ROS regulates mitochondrial dynamics by promoting mitochondrial fission and disrupting mitochondrial fusion, decreasing the OPA1 active isoform and degrading MFN1/2; and mitophagy. In cardiac myocytes, increased ROS induces a non-canonical function of the human Telomerase Reverse Transcriptase (hTERT), which reversibly translocate from the nucleus to the mitochondria to bind and protect mtDNA, reduce ROS production and increase OXPHOS efficiency (Haendeler et al., 2009; Ait-Aissa et al., 2019). Besides, the up-regulation of TERT is dependent on PGC-1α (Zhang et al., 2018) and positively regulates PINK1 function and stabilizes its mitochondrial localization to promote mitophagy (Shin and Chung, 2020). PINK1 other than phosphorylating ubiquitin and Parkin to initiate mitophagy, phosphorylates PARIS and leads to its Parkin ubiquitination and elimination. Therefore, when the PINK/Parkin axis is inhibited, PARIS accumulates and represses PGC-1α (Lee et al., 2017); and the knockdown of PARIS with CRISPR/Cas9 regains mitochondrial biogenesis (Kumar et al., 2020).

There is another parallel and independent pathway in controlling mitochondrial biogenesis given by the transcription factor Nuclear Erythroid-Related Factor 2 (NFE2L2. *It is also named NRF2, but in this work will be called NFE2L2 to avoid confusion). It has been described that NFE2L2 can translocate to the nucleus under an increase in ROS, specifically H2O2 and binds to the antioxidant response elements (ARE) in the promotor of NRF1 to induce TFAM. In nematodes, it was found that the orthologous protein of human NIX/BNIP3, named DCT-1 that works together with PINK and PRD (Parkin orthologous) is controlled by SKN-1, which is also linked to mtDNA replication. SKN-1 deficient nematodes display reduced mitophagy and reduced mitochondrial biogenesis. Even stimulation of mitophagy does not work in SKN-1 KO cells (Palikaras et al., 2015b). SKN-1 is a sensor of oxidative stress generated by defective mitochondria and is required for the expression of several genes related to mitochondrial biogenesis. SKN-1 is the orthologous of human NFE2L2 (Palikaras et al., 2015b). In mammals, Ivankovic et al. (2016) established the relationship among mitochondrial damage, NFE2L2 induction, P62, mitochondrial biogenesis (mtDNA replication) and protection against oxidative stress, in addition to lysosomal biogenesis to support the formation and degradation of autolysosomes. Increased autophagy involves an interaction between the autophagy adaptor p62/SQSTM1 and KEAP1, the cytosolic inhibitor of NFE2L2 allowing the accumulation of NFE2L2 in the nucleus, and then increased expression of nuclear-encoded mitochondrial genes needed for mitochondrial biogenesis and antioxidant response (Ichimura et al., 2013; Katsuragi et al., 2016).

In aging, it has been widely reported a decreased mitophagy (Wu et al., 2019; Bakula and Scheibye-Knudsen, 2020) commanded by a reduction of Parkin protein expression and the concomitant upregulation of the Nuclear Receptor Corepressor 1 (NCoR1) and its homologous protein SMRT, which counteracts the transcriptional control commanded by the SIRT1-AMPK/PGC1-alpha axis. In addition, NCoR1 will downregulate lipogenic and antioxidant genes favoring the metabolic switch from lipid oxidation to glucose oxidation and diminishing the antioxidant capacity of cells. NCoR1 KO animal models have greater mitochondrial biogenesis, mitophagy and

lifespan (Perez-Schindler et al., 2012; Fan et al., 2013; Liu et al., 2013; Ou-Yang et al., 2018). The stabilization of NCoR1 in aging is because this corepressor is normally bound to LC3 family protein and degraded by mitophagy. Arrested mitophagy will cause its accumulation and downstream effects (Saito et al., 2019). Despite these antecedents, the role of NCoR1 in cardiac aging is still controversial and needs more research. Li et al. (2019a) suggested that NCoR1 has a protective cardiac effect by suppressing cardiac hypertrophy and Wang et al. (2018) have shown that NCoR1 is downregulated in old mouse hearts. In this regard, NCoR1 is not the only transcriptional co-repressor involved in aging but also there is SMRT and RIP140 that might play a relevant role (Fan et al., 2013; Liu et al., 2013).

PROPOSED MODEL OF CARDIAC AGING

The core of our model and the main driver of aging is mtDNA heteroplasmy (**Figure 2**). In young people with a healthy heart and cardiomyocytes, OXPHOS efficiency is high and the mtDNA mutation level, is low (mutated mtDNA is represented with one or more "X" on the double-stranded circular DNA). Under this scenario operates the mitochondrial life cycle to dilute mutated copies of mtDNA by means of MtDy and mitophagy. This is what it is called in our model The Virtuous Cycle. One of the main controllers of mitophagy is the PINK/PARKIN/P62 axis. When active, two repressors of mitochondrial biogenesis, PARIS and KEAP1 are inhibited and degraded, unleashing the transcriptional coactivator PGC-1α and the transcription factor NFE2L2 which are also activated by AMPK -via increased AMP/ATP ratio- and ROS respectively. AMPK will also inhibit the mTOR pathway to induce mitophagy. In addition, increased NAD + /NADH activates SIRT1 which also induces PGC-1α for mitochondrial biogenesis. Once the autophagosomes are formed, they are eliminated via their fusion with lysosomes or expelled out of the cell as exospheres, to be engulfed by macrophages. The latter is a new mechanism described only for cardiomyocytes.

As mtDNA heteroplasmy exceeds 60%, mtDNA replication and transcription will be affected, reducing mtDNA copy number and proteins needed for respiratory chain assembly, leading to mitochondrial dysfunction and disruption of mitophagy. As a result, dysfunctional mitochondria will accumulate within cells, and cellular bioenergetics needs will not be satisfied causing an ATP-crisis, cell atrophy or death, ultimately leading to aging and aging-associated diseases. In fact, aging-associated diseases such as cancer, diabetes, heart disease, muscle weakness or atrophy, Alzheimer's disease and Parkinson's disease among others, are mainly due to a loss of mtDNA integrity and a failure in the mitochondrial life cycle (biogenesis, MtDy, mitophagy). To date, more than 400 mtDNA mutations, as well as a reduction in mtDNA copy number and a decrease in mitophagy, have been associated with human diseases (Tuppen et al., 2010; Li et al., 2019b). mtDNA mutations and heteroplasmy will affect the performance of the OXPHOS system reducing its efficiency due to the progressive loss of respiratory complex assembly and coupling, generating an abnormal amount of ROS. This ROS will produce more mtDNA mutations, increasing progressively the level of mtDNA heteroplasmy. This process corresponds to the Vicious Cycle No1 in our model. ROS not only affect mtDNA but also proteins and lipids. Thus, the mtDNA replication machinery, as well as membrane proteins and membrane phospholipids, including cardiolipin will be damaged. In this regard, ANT protein has been associated with increased mitoflash generation (transient mitochondrial depolarization), which uncouples ATP synthesis from the electron transport chain and ROS generation. Under these conditions, the mPTP is prone to open, collapse mitochondrial function, release mtDNA and other DAMPs, and trigger an inflammatory response and cell death. In parallel, an excessive amount of ROS inhibits the PINK/PARKIN/P62 axis meaning that PARIS and KEAP1 will be active and then NFE2L2 and PGC-1α inactive. Then, mitochondrial biogenesis will be reduced. In addition, inhibition of the PINK/PARKIN/P62 axis will activate NCoR1 which in turn blocks PGC-1α and mitochondrial biogenesis. This is called the vicious cycle No2. As mitophagy gets decreased, it is expected that exosphere release is also affected, accumulating in the cytosol many dysfunctional mitochondria prone to release more DAMPs and to induce cell death. Another important feature, in aging, is the metabolic switch from fatty acid oxidation to glucose oxidation, given the inhibition of NFE2L2 and PGC-1α which upregulates the fatty acid oxidation and antioxidant genes; and the activation of NCoR1 which down-regulates the lipogenic and ketogenic genes, and also the antioxidant genes. All these metabolic changes occurring in aging find their onset in the heteroplasmic mtDNA; and the final consequence of this, is having dysfunctional and hypertrophic cardiomyocytes leading to heart failure.

MITOCHONDRIAL THERAPIES FOR A HEALTHY HEART

Decreased heteroplasmy, increased mtDNA copy number, increased mitochondrial biogenesis and mitophagy have been shown to cause rejuvenation. Old mice that had a substantial loss of mtDNA copy number and reduced mtDNA gene expression showed improved memory performance after mtDNA replication and transcription were stimulated. The transgenic mouse called "mtDNA depleter" evidenced skin wrinkles and hair loss, associated with reduced copies of mtDNA. However, by restoring mtDNA copies, the same animal rejuvenated and returned to normal. In transgenic mice expressing PGC-1α under the MKC promoter, overexpression of PGC-1α promoted mitochondrial biogenesis in skeletal and cardiac muscle from fetal life onward, ameliorated aging phenotypes and prevented aging-associated cardiomyopathies in adults (Yuan et al., 2016). Regarding mitophagy, pharmaceutic compounds, calorie restriction or genetic manipulation have been proven to augment lifespan in different organisms via increased mitophagy. Upregulation of systemic ATG5 expression, a protein involved in autophagosome formation, in transgenic mice have increased mitophagy resulting in improved health, longer life spans and less weight gain with aging (Pyo et al., 2019). Besides, enhanced mitophagy and less fibrosis during aging were found in those

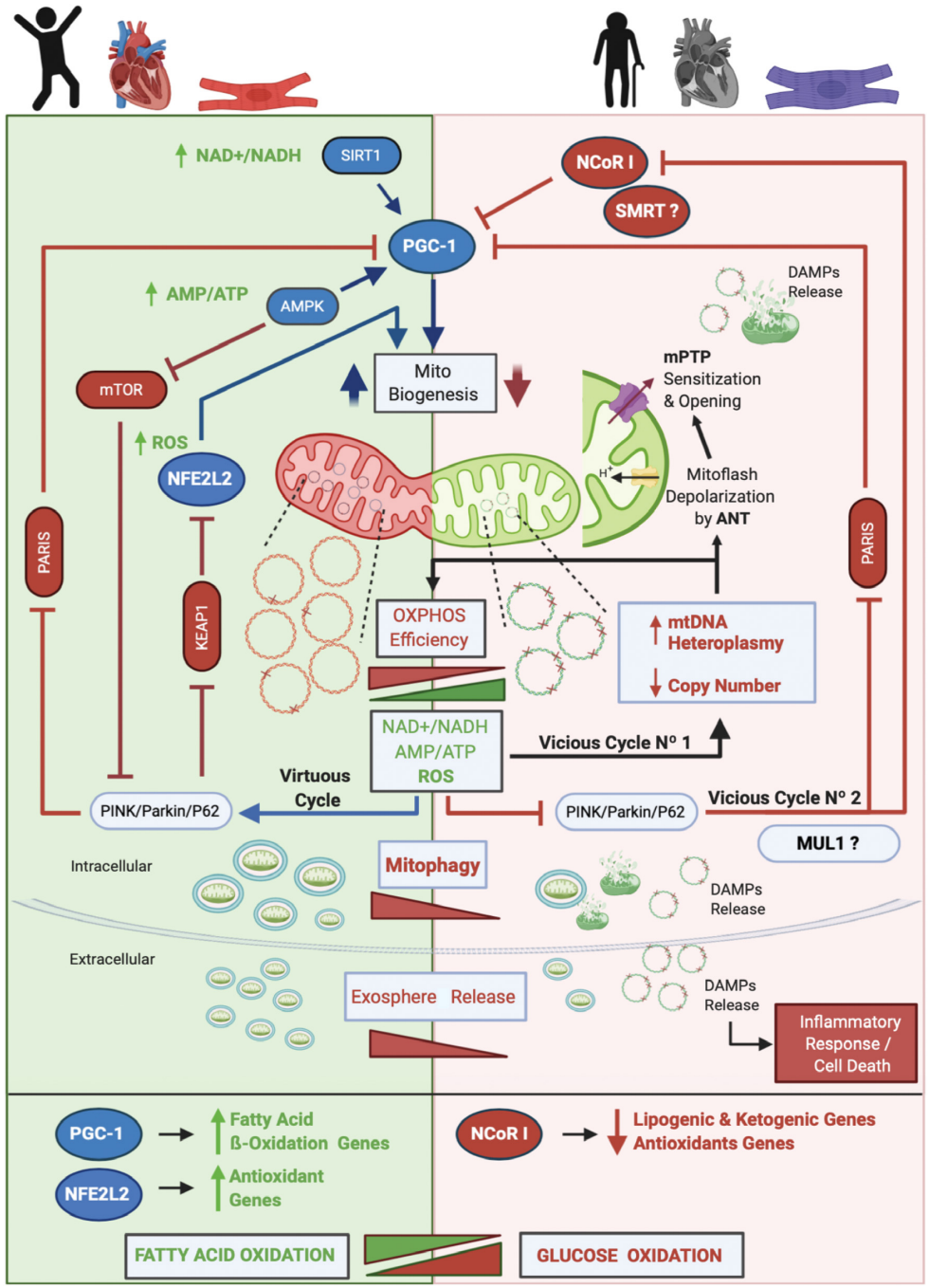

FIGURE 2 | Proposed Model of Cardiac Aging. The core of our model and the main driver of aging is mtDNA heteroplasmy. In aging, two vicious cycles were defined. Vicious cycle No1 will progressively increase the level of mtDNA heteroplasmy and decrease OXPHOS efficiency at the point to affect mitophagy and set the onset of Vicious cycle No2 that will progressively inhibit mitochondrial biogenesis and antioxidant response due to inhibition of PGC-1α and stimulation and upregulation of NCoR1. As a result, dysfunctional mitochondria will accumulate, increasing the number of mitoflashes and pathological ROS to finally trigger the mPTP opening and the release of DAMPs to initiate an inflammatory cell response and an apoptotic/necrotic stimulus. Furthermore, a metabolic switch from fatty acid oxidation to glucose oxidation will occur. Altogether, these events lead to cardiovascular diseases and cardiac failure. On the other hand, in young people, a low level of mtDNA heteroplasmy will keep the efficiency of the OXPHOS system which will maintain low NAD + /NADH and AMP/ATP ratios and a physiological amount of ROS. These conditions allow the Virtuous Cycle where MtDy will segregate dysfunctional mitochondrial units for degradation by mitophagy and will activate PGC-1α and NFE2L2 for mitochondrial biogenesis. Of note, cardiac cells are able to expel out mitochondria in vesicles called exospheres that are degraded by surrounding macrophages. In this model, we hypothesized that exosphere release, which is dependent on the autophagic machinery, is decreased in aging. Also, it is not clear yet whether in cardiac aging the protein MUL1 is involved in mitophagy, but it has been shown to be upregulated under cardiac damage. Along with NCoR1, another transcriptional corepressor like SMRT has been shown to be involved in aging.

mouse hearts. This was also established by the Levine group and others that constitutive activation of the autophagic gene Beclin1 enhanced autophagy resulting in remarkable health improvement and a longer lifespan in mice. Additionally, those mutant mice when aged, have less interstitial fibrosis and cardiac hypertrophy, meaning that maintenance of autophagy in the heart postpones or avoids cardiac aging and protects the heart during sepsis (Fernández et al., 2018; Sun et al., 2018). Even in injured cardiac tissue, mitophagy stimulation improves cellular function via a decrease in ROS and apoptosis, and mitochondrial function improvement (Torrealba et al., 2017). After ischemia/reperfusion injured heart, hypothermia has been shown to protect the cardiac tissues by increasing autophagy, autophagy flux and mitochondrial content in farm pigs (Marek-Iannucci et al., 2019). On the other hand, autophagy and autophagic flux impairment in the heart caused its accelerated aging, remodeling it toward heart hypertrophy and dysfunctional mitochondria, which are associated with heart failure and cardiomyocyte degeneration (Takemura et al., 2018; Liang and Gustafsson, 2020).

In the biomedical field, there is a growing interest in enhancing mitochondrial function to combat aging-associated diseases and improve lifespan. This has led to the development of nutraceutical therapies based on the application of antioxidants, vitamins or respiratory substrates and the discovery of new drugs, which have shown a positive antiaging effect. Some of the well-known compounds are resveratrol, an antioxidant found in red fruits and red wines; and rapamycin, a macrolide compound that is a blocker of the mTOR pathway, and then a powerful inducer of mitophagy, prolonging life in mice and other model organisms and it prevents age-associated symptoms in mammals including humans. The ketogenic diets (low glucose, high ketone bodies), albeit their mechanism of action is not well understood, have been shown to inhibit mTOR, stimulate mitophagy, mitochondrial biogenesis and mtDNA replication, and reduce oxidative stress (Santra et al., 2004; Wallace et al., 2010; Gano et al., 2014). The novel discoveries, drugs or technologies include the catechinic acid, a polyphenol widely present in tea and fruits, that is able to induce mitophagy, improve fitness and extend lifespan in aged nematodes (*C. elegans*) (Wu et al., 2020); the catalpol, an iridoid glucoside widely abundant in the root of *Rehmannia glutinosa*, that has a powerful antioxidant effect, decreases DNA damage and stimulates the PGC-1α TERT axis (Zhang et al., 2018). The Telomerase Activator 65 (TA-65), a compound extracted from *Astragalus membranaceus* has been also shown to extend lifespan by increasing TERT expression and reducing markers of cardiovascular disease and inflammation (Fernandez et al., 2018). The tetrapeptide SS-31 binds to cardiolipin from the inner mitochondrial membrane, stabilizing membranes and the integral membrane mitochondrial proteins, in particular, ANT protein to prevent and even revert aging-associated mitochondrial dysfunction in cardiomyocytes (Szeto, 2006; Birk et al., 2014; Zhang et al., 2020). Finally, the thiazolidinedione pioglitazone has been shown to be a potent inductor of the PGC-1α, mitochondrial biogenesis and antioxidant mitochondrial defense (Bogacka et al., 2005; Butterick et al., 2016).

The correlation between mtDNA integrity loss and aging has allowed the development of experimental strategies that seek to eliminate mutated mtDNA or increase wild type mtDNA to restore mitochondrial function and reverse the aging phenotype. The rationale of these strategies is that, if the ratio between the wild type mtDNA and the mutated mtDNA can be adjusted, the presence of wild type molecules will mitigate the effect of the mutants and therefore the defect will be corrected. Four different strategies have been developed (Taylor and Turnbull, 2005; Patananan et al., 2016; Aravintha Siva et al., 2019) (i) Transfer of isolated xenogeneic mitochondria, which are deposited in the cell culture medium and enter the cells by macropinocytosis (Kitani et al., 2014). It has recently been shown that mitochondria therapy is capable of promoting the regeneration of damaged hippocampal neurons (Katrangi et al., 2007; Chien et al., 2018; Kim et al., 2018). Other mitochondria transfer technique is by centrifugation and also by the generation of mitocytoplasts (Yang and Koob, 2012). (ii) Transfer of exogenous wild type mtDNA, by direct microinjection of mtDNA into the host cell or by the use of "carriers" such as the mitochondrial transduction domain (MTD) conjugated to the mitochondrial transcription factor A (MTD-TFAM), MITO Porters, DQAsomes, and nanoagents (Niazi et al., 2013; Zhang and Zhang, 2016; Aravintha Siva et al., 2019). Exogenous mtDNA has also been transferred into muscle cells via hydrodynamic limb venous injection (Yasuzaki et al., 2013). These methodologies have shown promising and surprising results in the rescue of mitochondrial and cellular function. (iii) Elimination of heteroplasmy. These techniques are named the antigenomic mtDNA Therapy; the mitochondrial-targeted restriction endonucleases; zinc-finger nucleases type; and the effector nucleases of the transcription activator type (Transcription Activator-like effector Endonucleases), are all based on the delivery of endonucleases into mitochondria, which will specifically recognize and degrade the mutated mtDNA. An attempt has also been made to carry out the CRISPR/Cas9 technology in mitochondria, but the results have not been reproducible (Jo et al., 2015). (iv) Mixed mtDNA transfer system. This technology proposes direct electroporation of isolated exogenous mitochondria with wild type mtDNA. Next, mitochondria must then be transferred to the zygotes or cultured cells through microinjections (Collombet et al., 1997; Yoon et al., 2010). Recently, it was published in Nature journal a new technology to edit mitochondrial DNA which combines mitotalen proteins, Cas9 and the Uracyl glycosidase inhibitor with a bacterial cytidine deaminase toxin. This technology acts as a repair/edit system on mutant forms of mtDNA. It does not eliminate mtDNA copies, but rather, restore them (Mok et al., 2020). This revolutionary system was tested in HEK293T cells and it remains open to the question if it will also be efficient in other cellular models like cardiac cells.

In summary, heart mitochondria must satisfy an enormous amount of energy to keep the heart beating from conception to death. To do that, mitochondria are equipped with two complex systems that work collaboratively, the OXPHOS system and the Mitochondrial Life Cycle to keep safe and healthy

the mtDNA and then mitochondria. Any disruption of these processes is associated with heart failure. On the other hand, the potentiation of these is beneficial to improve heart health. In aging, mtDNA is prone to undergo mutations leading to heteroplasmy which is associated with increased ROS affecting negatively both OXPHOS and the mitochondrial life cycle. Mitochondrial therapies have been developed to deal with mtDNA heteroplasmy. Many of them focus on Mitophagy stimulation which decreases mtDNA heteroplasmy and has a protective effect in cardiac cells. The development of new therapies aims to decrease mtDNA heteroplasmy in cells either by the transfer of mutation-free wild type mtDNA or by the direct elimination of mutant mtDNA. The results of these new technologies are promising in restoring mitochondrial and cellular function *in vitro* and open a new era in the field of mitochondrial medicine to combat aging-associated heart failure.

AUTHOR CONTRIBUTIONS

JS helped with the writing and discussion of the manuscript. AE conceived, wrote, and funded the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Quality Matters? The Involvement of Mitochondrial Quality Control in Cardiovascular Disease

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Cardiovascular diseases are one of the leading causes of death and global health problems worldwide. Multiple factors are known to affect the cardiovascular system from lifestyles, genes, underlying comorbidities, and age. Requiring high workload, metabolism of the heart is largely dependent on continuous power supply via mitochondria through effective oxidative respiration. Mitochondria not only serve as cellular power plants, but are also involved in many critical cellular processes, including the generation of intracellular reactive oxygen species (ROS) and regulating cellular survival. To cope with environmental stress, mitochondrial function has been suggested to be essential during bioenergetics adaptation resulting in cardiac pathological remodeling. Thus, mitochondrial dysfunction has been advocated in various aspects of cardiovascular pathology including the response to ischemia/reperfusion (I/R) injury, hypertension (HTN), and cardiovascular complications related to type 2 diabetes mellitus (DM). Therefore, mitochondrial homeostasis through mitochondrial dynamics and quality control is pivotal in the maintenance of cardiac health. Impairment of the segregation of damaged components and degradation of unhealthy mitochondria through autophagic mechanisms may play a crucial role in the pathogenesis of various cardiac disorders. This article provides in-depth understanding of the current literature regarding mitochondrial remodeling and dynamics in cardiovascular diseases.

Keywords: mitochondria, mitophagy, cardiovascular disease, nucleus, hypertension, ischemic heart, diabetic cardiomyopathy, mitochondrial haplogroup

INTRODUCTION

Cardiovascular diseases are one of the leading causes of death worldwide causing global health problems (Yusuf et al., 2001). Multiple factors are known to affect the cardiovascular system from lifestyles, genes, epidemiological transition, and age (Gluckman et al., 2009). Biological aging has long been known as an inevitable risk factor of cardiovascular diseases, and it has been suggested

that pathogenesis of cardiovascular dysfunction may be related to inflammation, oxidative stress, DNA damage, telomere shortening, lipotoxicity, and mitochondrial damage (Gluckman et al., 2009; Kubben and Misteli, 2017; Wu et al., 2019). With the overwhelming workload and energy required, metabolism of the heart is largely dependent on mitochondria, the major and most efficient producers of cellular adenosine triphosphate (ATP) (Wu et al., 2019). In addition to being major cellular power plants through oxidative phosphorylation (OXPHOS), mitochondria also serve as sites for β-oxidation, the Krebs cycle, calcium reservoirs, the initiators of the intrinsic apoptotic pathway, and the regulators of necrosis (Spinelli and Haigis, 2018). Thus, these highly dynamic and complex organelles have gained huge attention among researchers and accumulating data have advocated the critical roles of mitochondrial function in various aspects of cardiovascular pathology including: hypertension (HTN), atherosclerosis, ischemia/reperfusion (IR) injury, and type 2 diabetes mellitus (T2DM) (Fearon and Faux, 2009; Ong and Hausenloy, 2010; Vasquez-Trincado et al., 2016; **Figure 1**).

Mitochondria are essential for the functioning of cardiomyocytes where they occupy around 30% of the total cell volume and generate up to 30 kg of ATP per day (Lin K.J. et al., 2019). A double membrane organelle, the mitochondrion is composed of a mitochondrial outer membrane (MOM), an intermembrane space (IMS), and the convoluted mitochondrial inner membrane (MIM) surrounding the central mitochondrial matrix. It is on the MIM that the OXPHOS pathway takes place and where most of the ATP production occurs, while the tricarcoxylic acid (TCA) cycle in the mitochondrial matrix supports the fuel for this machinery (Hall et al., 2014). The OXPHOS pathway produces more than 95% of cellular energy via the electron transport chain (ETC) and involves five protein complexes assembled on the MIM. During the OXPHOS process, membrane potential is generated across the MIM. Abnormal membrane potential may signal the cell to undergo various stress responses and even mitochondrial-mediated apoptosis. Reactive oxygen species (ROS) are generated during OXPHOS when electrons leak out during electron transportation from the ETC to oxygen. Generated as metabolic byproducts, ROS are highly reactive chemical molecules formed due to the electron acceptability of O_2 and perform a dual role as harmful, protective, or signaling factors according to the balance of ROS production and disposal (Droge, 2002). Highly reactive with unpaired electrons over their molecular outer layer, at unbalanced conditions, these ROS can cause DNA and RNA damage, lipid peroxidation, protein carbonylation, imbalance cellular redox and irreversible impairment of the mitochondria, and eventually cellular death and cardiovascular pathology (Zhao et al., 2019).

Since mitochondrial function is crucial for normal cellular activity, strict mitochondrial quality control through coordination of various processes such as proteostasis, morphology regulation, and autophagy to ensure cellular homeostasis is key to exploiting the pathogenesis of cardiovascular diseases (Hammerling and Gustafsson, 2014; Anzell et al., 2018; Picca et al., 2018; Tahrir et al., 2019). A dynamic organelle, the mitochondrion forms networks to maintain its integrity and interchange mitochondrial material in response to cellular stress through the process of fusion. On the other hand, damaged mitochondria can be dispatched through fission where damaged parts could be degraded. Hence, mitochondria undergo continuous dynamic and morphological alterations in response to a stressed cellular environment (Eisner et al., 2018). Dysfunctional mitochondria can then be efficiently degraded through the process of mitochondrial autophagy, mitophagy.

As healthy mitochondria may be essential for maintaining normal cardiovascular function, further treatments targeting mitochondrial quality control may act as a preventive strategy for these notorious clinical conditions. Thus in this review, we will focus on mitochondrial quality control concerning the pathophysiology of cardiovascular diseases.

MITOCHONDRIAL BIOLOGY

Mitochondria have long been the subject of intense investigation due to their multiple roles in the cellular survival of eukaryocytes (Duchen, 2004; Van Remmen and Jones, 2009; Wang and Youle, 2016). In the following section, we will describe this organelle in different aspects including mitochondrial genetics, the OXPHOS system, and mitochondria-related programmed cell death.

Mitochondrial Genetics

According to endosymbiotic theories, mitochondria were once parasitic bacteria, later becoming the semi isolated entities in the cell. Like their prokaryote ancestors, mitochondria contain their own hereditary material, the double-stranded and circular mitochondrial DNA (mtDNA). Highly compact with only two regulatory regions and exons without introns, mtDNA contains 37 genes encoding 22 transfer RNAs, two ribosomal RNAs, and 13 polypeptides that are crucial subunits belonging to the enzyme complexes of the OXPHOS system (Wiedemann and Pfanner, 2017). Following a maternal pattern of inheritance, the mitochondria genome resides outside of the nucleus and relies immensely on products encoded in the nuclear DNA (Taylor and Turnbull, 2005; Jang and Lim, 2018). Mitochondria are major sites of ROS production under physiological conditions, and the structure of the mtDNA molecule is similar to bacteria with only limited self-repairing systems; therefore, mtDNA are prone to mutation generation under consistent attacks of free radicals produced *in situ* compared to the nuclear genome (Chinnery et al., 1999). With around 2–10 copies of mtDNA present in each mitochondrion and upward of 100s and 1,000s of mitochondria in each cell, a heterogenous mixture of mutations in mtDNA-encoded genes with wild-types can be present, known as heteroplasmy (Stewart and Chinnery, 2015). The vast majority of these mitochondrial genetic alterations are not directly tied to pathogenesis of diseases. Through human evolution, some mtDNA variants are preferentially passed down the maternal line (Stewart and Chinnery, 2021) and different

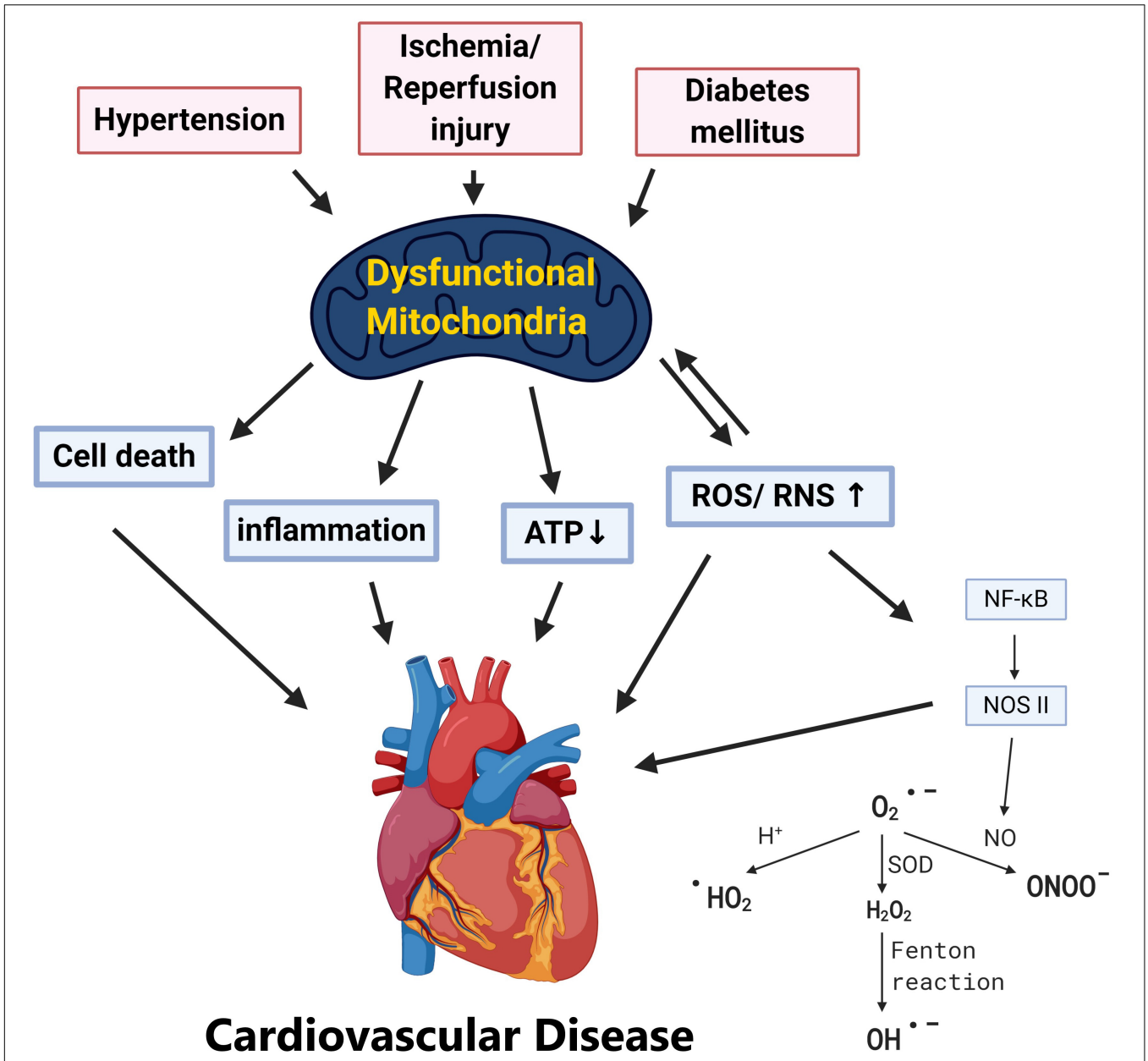

FIGURE 1 | Pathological stress including hypertension, ischemia/reperfusion, and diabetes mellitus results in dysfunctional mitochondria. Various cardiovascular risk factors including hypertension, ischemia/reperfusion injury, and diabetes leads to mitochondrial dysfunction. Without adequate quality control of the damaged mitochondria, this may result in (1) ATP depletion, (2) overproduction of ROS/RNS, (3) mitochondria-dependent cell death (apoptosis), and (4) systemic inflammation which provokes cardiovascular pathogenesis.

combinations of single nuclear polymorphisms (SNPs) in mtDNA inherited from a common ancestor can be defined into mitochondrial haplogroups, which can be used to represent genetic populations on the mitochondrial phylogenetic tree (Kazuno et al., 2006). mtDNA variants have attracted special attention due to their potential for affecting redox homeostasis and causing alteration of mitochondrial function. Mitochondrial haplogroups, considered to represent genetic populations on the mitochondrial phylogenetic tree can also be utilized as biomarkers for disease association. Recently, Liou et al. (2016) reported that the mitochondrial haplogroup B5 is resistant to the development of PD in Taiwanese people of ethnic Chinese background (Liou et al., 2016). In their cellular studies, the B5 cybrid, containing G8584A/A10398G variants, showed more resistance to the complex I inhibitor, rotenone, with lower ROS generation and apoptosis rate than the B4 cybrid which does not harbor these variants (Liou et al., 2016). These results further provide direct genetic and

functional evidence that mtDNA variations and consequential mitochondrial function alternation can potentially influence the risks of clinical diseases. Fetterman et al. (2013) also demonstrated in a mitochondrial-nuclear exchange mouse model of mtDNA polymorphisms that mtDNA genetic background significantly modulated mitochondrial bioenergetics, cellular ROS production, and susceptibility to cardiac volume overload, independent of nuclear background.

Mitochondrial Oxidative Phosphorylation and ROS Production

A structurally two membraned organelle, the mitochondrion harbors two compartments, the central matrix and the IMS with the MIM in between and the MOM as the outermost layer. The highly folded MIM forms the cristae housing the OXPHOS machinery, which is comprised of the electron transport chain (ETC), complex I (CI) to complex IV (CIV), and F1F0-ATP synthase (complex V). Using NADH as a substrate for CI and succinic acid for CII, the ETC complexes transfer electrons from electron donors to electron acceptors via redox reactions which generates energy and drives protons from the matrix side of CI, CIII, and CIV across the MIM into the IMS side. Therefore, an electro-chemical gradient is generated across the MIM with accumulation of protons pumped into the IMS and generates mitochondrial membrane potential ($\Delta\Psi$). This electro-chemical gradient driven by substrate oxidation is then coupled to the synthesis of ATP with phosphorylation of ADP at complex V, the process is called OXPHOS (Schumacker et al., 2014; Zhao et al., 2019).

In most circumstances, the oxygen molecule acts as the final acceptor of electrons at complex IV to form harmless water. However, both as a consequence of normal electron transport or during mitochondrial dysfunction, electrons can leak from the ETC, especially at complexes I and III, to oxygen to generate superoxide anions ($O_2^{\bullet-}$) (Gorrini et al., 2013; Lieburg-Apers et al., 2015). $O_2^{\bullet-}$ can then be converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD), and later to hydroxyl radicals (HO) via the Fenton reaction (Thomas et al., 2009; Zorov et al., 2014). Interaction of $O_2^{\bullet-}$ with protons can also generate hydroperoxyl radicals (HOO). ROS are highly reactive and cause oxidative damage to macromolecules (Lin et al., 2018, Lin K.J. et al., 2019). Since unbalanced high ROS concentrations are toxic, mitochondria possess powerful antioxidant systems to prevent excess oxidative stress induction (Zorov et al., 2014). Mitochondrial antioxidant defenses include antioxidative enzymes such as glutathione reductase, glutathione peroxidase, SOD (the Mn-dependent isoform (Mn SOD, SOD2) in the mitochondrial matrix and the Cu,Zn-dependent isoform (Cu,Zn SOD, SOD1) in the IMS and cytosol), thioredoxin, peroxiredoxins, and catalase (Mailloux, 2018). Under physiological conditions, the balance between ROS generation and ROS scavenging is highly regulated to provide efficient detoxification. However, oxidative stress can occur when ROS production overwhelms these defense systems leading to organelle damage (Richter, 1995). Therefore, mitochondria are more susceptible to the accumulation of damaged mtDNA and vulnerable to oxidative damage, as they are not only constantly surrounded and attacked by the ROS produced in situ, but also have an insufficient DNA repair system due to limited organelle genomic capacity (Druzhyna et al., 2008).

Mitochondria-Dependent Apoptosis

Mitochondria also play a central role in the regulation of apoptotic cell death. Apoptosis can be initiated through extrinsic or intrinsic mitochondrial pathways (Lopez and Tait, 2015). Under excessive cellular stress, activation of intrinsic pathways causes mitochondrial outer membrane permeabilization (MOMP) which then causes the release of the apoptotic signal cytochrome *c* from the intermembrane space into the cytosol. Once released, cytochrome *c* binds to apoptotic protease activating factor-1 (APAF -1) forming the structure apoptosome. Apoptosome in turn recruits and activates pro-caspase-9, which then cleaves and activates caspase-3 and caspase-7, the major executors of apoptosis. The activation of caspase proteases subsequently cleaves up to hundreds of proteins of the cell causing programmed cell death (Taylor et al., 2008; Tait and Green, 2010). Other pro-apoptotic proteins released from the mitochondrial IMS into the cytoplasm includes the second mitochondria-derived activator of caspases (SMAC) and Omi which antagonize endogenous inhibitors of caspase function called the X-linked inhibitor of apoptosis protein (XIAP) (Suzuki et al., 2004; Hui et al., 2011; Vasudevan and Ryoo, 2015; Singh et al., 2019; Tang et al., 2019). In the intrinsic mitochondrial apoptosis pathway, the B-cell lymphoma 2 (BCL-2) family consists of both apoptotic and anti-apoptotic proteins and regulates MOMP (Kalkavan and Green, 2018; Singh et al., 2019). BCL-2 effector proteins modulate the activation of the pro-apoptotic process including BCL-2-associated X protein (BAX), BCL-2 antagonist or killer (BAK), and BH3 interacting domain death agonist (Bid) and antiapoptotic signals including BCL-2, B-cell lymphoma-extra large (BCL-xL), and myeloid cell leukemia 1 (MCL-1) (Kalkavan and Green, 2018; Malina et al., 2018). The MOMP executioner, BAX is translocated to the MOM and BAK must be disengaged from antiapoptotic BCL-2 proteins (MCL-1 and BCL-xL) to activate cellular apoptosis. After activation of either or both BAX or BAK results in the proteins forming pores on the MOM and cause MOMP (Singh et al., 2019). These BCL-2 family proteins also take part in the extrinsic pathway which is typically activated through the binding of a subset of the TNF receptor superfamily such as tumor necrosis factor 1 (TNFR1), TNF-related apoptosis-inducing ligand (TRAIL) receptors (DR4 and DR5), and Fas (Apo-1; CD95) (Ping et al., 2015). A cross-talk between extrinsic and intrinsic pathways is needed to ensure the process of the extrinsic death pathway. With the binding of death ligands on these receptors, caspase-8 is activated and then further engagement of caspase-3 and caspase-7 occurs. Yet in some cells such as hepatocytes and pancreatic cells, pathways lacking MOMP is not enough to bring about cell death. In such occasions caspase-8 will cleave Bid, a BCL-2 family protein, to truncated Bid (tBid), which in turn activates BAX and BAK proteins to activate MOMP and eventually cell death (Wang and Youle, 2009; Singh et al., 2019). Under extreme environments, damaged

mitochondria can behave like cellular sensors to activate intrinsic death signals leading to the point of no return with mitochondrial quality control being crucial for cellular survival.

MITOCHONDRIAL QUALITY CONTROL

Dysfunction of mitochondria caused by oxidative stress has severe cellular consequences linked to human diseases. Several surveillance strategies have evolved to limit mitochondrial damage and ensure cellular survival. As previously mentioned, controlling the abundances of ROS production is the first line of defense. However, when damage has occurred, a secondary set of repair pathways take place to ensure the normal functionality of molecules. For example, the methionine sulfide reductase (Msr) system that consists of MsrA and MsrB reduces a subset of oxidized proteins, namely oxidized methionine moieties, back to their reduced methionine. Also, chaperones contribute through refolding the damaged misfolded proteins back to their native three dimensional structures (Fischer et al., 2012; Penna et al., 2018). In spite of all these protective and repair mechanisms, the vast majority of damaged proteins cannot be efficiently repaired. The accumulation of irreversibly damaged components fosters further acceleration of mitochondrial dysfunction and subsequent cell death; therefore, the removal of these denatured proteins is essential. Proteolysis and removal of these dysfunctional proteins through the cytosolic ubiquitin /26S proteasome system (UPS) has been shown to be a crucial part in the quality control of mitochondrial proteins (Tatsuta and Langer, 2008). Apart from cytosolic UPS, mitochondria quality is largely regulated by mitochondrial dynamics and mitophagy. The processes of mitochondrial fusion facilitate the redistribution of mitochondrial components while fission (division) allows for damaged mitochondria to be degraded through fragmentation. The process of mitophagy is responsible for the degradation and recycling of damaged mitochondria (**Figure 2**). Recently, these mitochondrial quality control mechanisms have been shown to be heavily involved in various cardiovascular pathological conditions (Anzell et al., 2018).

Mitochondrial Fusion and Fission

Mitochondria form highly dynamic and intricate networks, balancing on the opposing processes of morphological changes: mitochondrial fission and fusion (Parra et al., 2011; Lin K.J. et al., 2019). The mitochondria fusion process is primary regulated by three dynamin-related guanosine triphosphatases (GTPases) called mitofusin 1, mitofusin 2 (MFN1 and MFN2), and optic atrophy protein 1 (OPA1). In addition to the regulation of mitochondrial fusion, outer membrane MFN1 and MFN2 have been revealed to undertake ER-associated degradation (ERAD) through polyubiquitination by the E3 ubiquitin ligase parkin and thus stimulate damaged mitochondrial protein degradation through UPS (Picca et al., 2018). On the other hand, mitochondrial fission 1 protein (FIS1) and dynamin-related protein 1 (DRP1) regulates mitochondrial fission (Ishihara et al., 2004; Lin et al., 2018). Mitochondria fusion describes the fusion of two neighboring mitochondria to allow the sharing of metabolites, enzymes, and genetic materials. This process involves the integration of MFN1 with MFN 2 (heterodimers) or MFN2 with MFN2 (homodimers) to achieve fusion of the MOM (Ishihara et al., 2004; Chen and Chan, 2009; Westermann, 2012; Hall et al., 2014), while Opa1 mediates the fusion of the MIM as well as maintaining the normal inner membrane cristae structure (Malka et al., 2005; Song et al., 2007). Such an act preserves mitochondria membrane integrity and membrane permeability, improving mitochondrial resistance to injury from oxidative damage. Meanwhile, damaged mitochondria can be removed through mitochondrial fission, the division or fragmentation from one mitochondria to two (Chen and Chan, 2009; Ong et al., 2010; Elgass et al., 2013; Otera et al., 2013). This process is regulated by GTPase DRP1, mitochondrial FIS1, and mitochondrial fission factor (MFF). FIS1 acts as an inhibition factor of mitochondrial fusion and also as a receptor for the recruitment of fission-related proteins such as DRP1 to the outer mitochondrial membrane, where it initiates cleavage of the mitochondria by interaction with FIS1, MFF, and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49, MiD51) (Palmer et al., 2011; Onoue et al., 2013; Otera et al., 2013; Hall et al., 2014; Atkins et al., 2016). Oxidative damage of this organelle may cause loss of $\Delta\Psi$, which leads to phosphatase and tensin homolog (PTEN)-induced putative kinase (PINK) stabilization on the MOM which recruits the E3 ubiquitin ligase parkin to ubiquitinate mitochondrial proteins and induce mitochondrial fragmentation. Excessive fragmentation of mitochondria can induce the release of cytochrome c into the cytoplasm and trigger apoptotic cell death. Balance between fusion and fission of the mitochondria has been shown to be of great importance in a wide range of cardiovascular diseases. Quality control through degradation of damaged mitochondria via mitochondrial autophagy, mitophagy, is also closely related to mitochondrial fission as these damaged/fragmented organelles can be engulfed and degraded by lysosomes through the formation of autophagolysosomes (Zaffagnini and Martens, 2016).

Mitochondrial Turnover Through Autophagy-Mitophagy

All cells need a way to eliminate unwanted or damaged parts. The sophisticated system autophagy is crucial to prevent accumulation of toxic waste, make room for the incorporation of new elements, or reuse old building blocks (Klionsky et al., 2011; Morales et al., 2020). Three known types of autophagy have been reported, namely macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. Macroautophagy relies on the formation of autophagosomes to sequester and transport cargo to the lysosome. CMA transports unfolded proteins directly across the lysosomal membrane. Microautophagy involves the direct uptake of smaller cellular waste through invagination of the lysosomal membrane (Morales et al., 2020). Macroautophagy typically involves the degradation of large cellular components, even organelles and has been the most well-described pathway for mitochondrial turnover, called mitophagy, therefore we will be referring to macroautophagy as autophagy

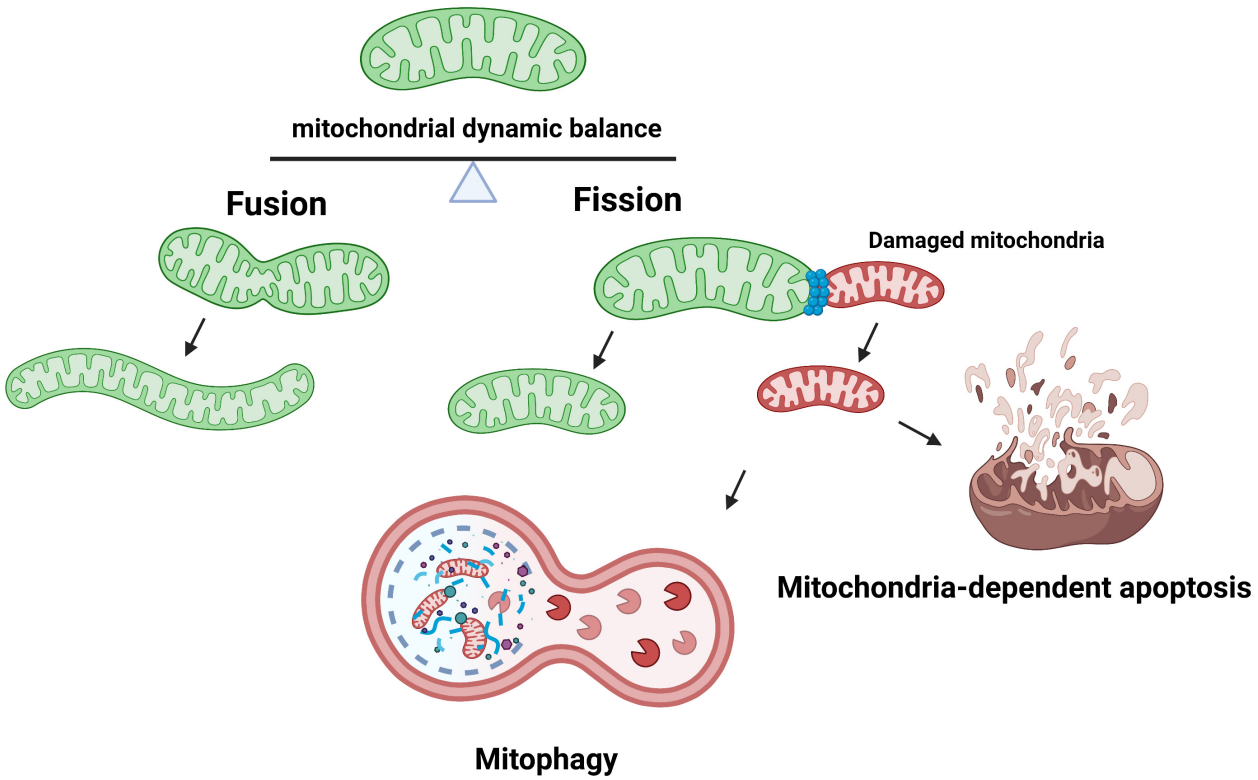

FIGURE 2 | Mitochondrial dynamics and quality control. Mitochondria fuse to merge intra-organelle contents including the mitochondrial DNA and prevent permanent loss of essential components. The fusion process is mediated by the GTPase proteins mitofusins 1 and 2 (MFN1/2) on the mitochondrial outer membrane, and optic atrophy type 1 (OPA1) on the mitochondrial inner membrane. Mitochondrial fission, on the other hand, can create new mitochondria or enable quality control by segregating damaged mitochondria for subsequent degradation via mitophagy. The fission process is primarily carried out by dynamin-related protein 1 (DRP1) and mitochondrial outer membrane mitochondrial fission 1 protein (FIS1). If mitochondria quality control is dysfunctional, the mitochondria may induce mitochondria-dependent apoptosis.

from now on. While non-selective autophagy is activated in responses to reduced nutrient availability or certain cellular stresses and comprise mainly the engulfment and degradation of bulk cytosolic material, selective autophagy labels specific molecules and structures destined for degradation (Parzych and Klionsky, 2013). As mentioned previously, this type of autophagy involves ubiquitin, ubiquitin-like conjugation, and activation systems. These molecular tags act as cargo recognition which ultimately expands to generate the autophagosome which then fuses with the late endosome or lysosome (Hansen et al., 2018). Both types of autophagy processes undergo the stages of (1) initiation, (2) phagophore formation, (3) elongation/expansion, (4) autophagosome-lysosome fusion, and (5) degradation (**Figure 3**). More than 30 autophagy-related gene (ATG) family proteins act as important mediators and orchestrate this catabolic process of damaged organelles (Parzych and Klionsky, 2013; Zaffagnini and Martens, 2016). During the initiation process, the ATG family proteins are regulated through the inhibition of the mammalian target of rapamycin (mTOR)/activation of the AMP-activated protein kinase (AMPK) pathway (Yamada and Singh, 2012). Under stress/starvation, reduction of mTOR activity releases its inhibition on the UNC-51–like kinase (ULK1) family interacting protein of 200 kD (FIP200)-ATG13 complex and thus autophagy is induced (Hara et al., 2008). Meanwhile, AMPK drives autophagy through reducing mTOR complex 1 (mTORC1) activity, while simultaneously directs the ULK1 complex to the site of autophagosome formation (Hansen et al., 2018). At the same time, with the downregulation of mTORC1, fine tune regulatory pathways such as the activation of death-associated protein 1 (DAP1), a negative regulator of autophagy, prevent the uncontrolled upregulation of autophagy (Hansen et al., 2018). The ULK1 complex regulates the recuitment of the vacuolar protein sorting 34 (VPS34) complex (Mijaljica et al., 2012). However, to form the VPS34 complex and the initiation of autophagy, *de novo* formation of the nucleation complex via the dissociation of Beclin 1 from BCL-2 is also needed. The dissociated Beclin 1 then forms the transient VPS34 complex with ATG 14-like (ATG14L), VPS15, and the lipid kinase VPS34 to generate the functional class III phosphatidylinositol 3-kinase (PI3K) complex which is responsible for the catalyzation of phospholipid phosphatidylinositol phosphate (PIP) to phospholipid phosphatidylinositol 3-phosphate (PI3P) (Zachari and Ganley, 2017). PI3P then signals PI3P-binding proteins like WD repeat domain phosphoinositide-interacting protein

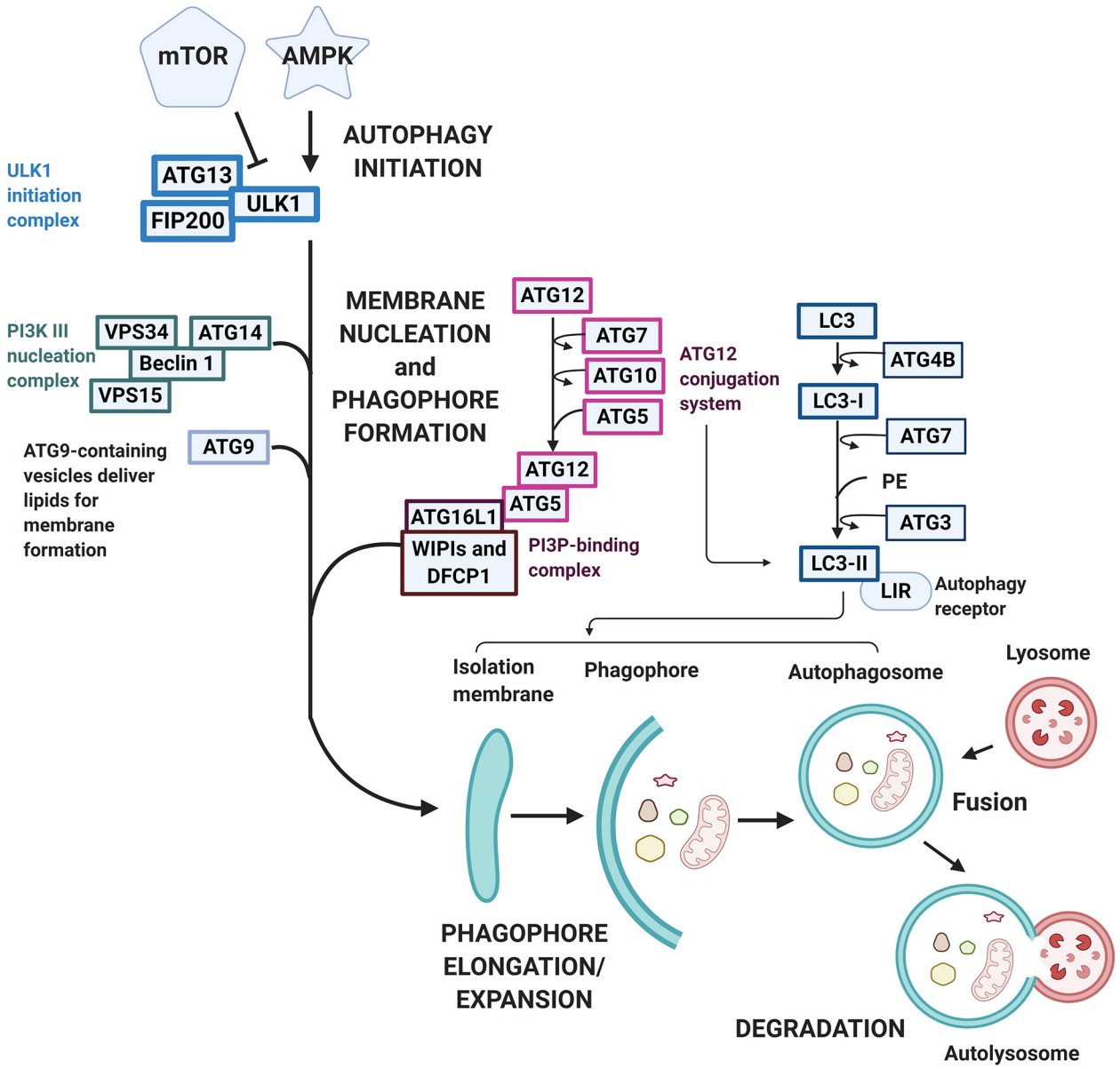

FIGURE 3 | Macroautophagy pathway and signaling. Macroautophagy is activated following various stimulation under cellular stress conditions. The autophagic pathway typically includes several steps including (1) initiation, (2) phagophore formation, (3) elongation/expansion, (4) autophagosome-lysosome fusion, and (5) degradation. mTOR and AMPK regulates the initiation process with AMPK acting as an activator while mTOR acts as an inhibitor. Under cellular stress, mTOR reduces its inhibition on the ULK1-FIP200-ATG13 complex, whereas AMPK reduces mTORC activity and directs ULK-1 to the site of autophagy. The ULK-1 complex regulates the nucleation and phagophore formation process through the recruitment of VPS34 and the formation of PI3P. PI3P then contributes to the phagophore formation through the signaling of WIPI2B and DFCP to drive the phagophore formation. A double-membrane vesicle (phagophore) begins to form and elongate into an autophagosome through two ubiquitin-like conjugation cascades, the ATG5-ATG12 and the LC3 systems. The LC3 is first processed to LC3-I by ATG4B and then activated by ATG7. LC3-I is later transformed into LC3-II via ATG3 and incorporated into the limiting membrane which harbors the LIRs. The sealed degradation components are then further degraded in an acidic environment containing hydrolytic enzymes through the fusion of the matured autophagosome with lysosome to form an autolysosome.

2 (WIPI2B) and double FYVE containing protein 1 (DFCP1) to the phagophore site and drives phagophore formation and eventually the formation of autophagosomes (Xie et al., 2008). For the elongation/expansion of phagophores into autophagosomes, PI3P also targets other ATG molecules, such as the recruitment of membranes through the shuttling of ATG9 and the activation of two ubiquitin-like conjugation cascades, the ATG5-ATG12 and light chain-3 (LC3) systems (Dall'Armi et al., 2013; Nascimbeni et al., 2017). ATG7 is required for catalyzing the activation and binding of ATG12 with ATG5, which

subsequently interacts with ATG16 and is recruited to the site of autophagosomes. Concurrently LC3 is processed by ATG4B to expose a COOH-terminal glycine and generate LC3-1. LC3-1 is transferred to ATG3 after activation by ATG7, and transformed to the active form LC3-II with lipidation through adding phosphatidylethanolamine (PE) to its C-terminal glycine via reactions requiring the ATG5-ATG12-ATG16 complex (Dooley et al., 2014). LC3-II is then incorporated into the limiting membrane /expanding vesicle and is considered an important marker of autophagy activation. The limiting membrane eventually seals forming the autophagosome and delivers the cargo to the lysosome to be degraded. Besides the bulk autophagy process, LC3 also plays a role in the finer selective autophagy where damaged or misfolded molecules can be selectively labeled and processed. For this more efficient process to occur, recognition of the cargo and the tethering of the cargo to the autophagosome is needed (Zaffagnini and Martens, 2016). Sequestosome 1 (SQSTM1) or P62 protein in this case acts as the recognition receptor for ubiquitinated proteins and organelles. These P62 proteins contains one LC3-interacting region (LIR) motif otherwise known as the ATG8-interacting motif (AIM), which localizes to sites of autophagosome formation and binds with LC3 or other ATG8 proteins (Pankiv et al., 2007; Ichimura and Komatsu, 2010). With the tagging and recognition systems, selective cargo removal and degradation through autophagy can be achieved (Anzell et al., 2018). As the heart undergoes stressful circumstances, elimination of damaged organelles such as mitochondria plays a critical role in the balance or remodeling of the heart under pathological environments.

Age and Mitochondria Quality Control

Aging is an irreversible biological process that typically involves the gradual deterioration of cardiac function and provokes various cardiovascular diseases through telomere shortening, genomic instability, oxidative stress, lipotoxicity, metabolic dysregulation, apoptosis, and mitophagy (Ren and Zhang, 2018; Wang S. et al., 2019; Ajoolabady et al., 2020; Onishi et al., 2021). Among these pathological etiologies, recent evidence has drawn light on and proposed that quality control through mitophagy plays a hand in the regulation of longevity and cardiovascular function in aging (Ren et al., 2017; Fernández et al., 2018; Nakamura and Yoshimori, 2018). Thai et al. (2019) reported increased parkin protein expression in aged rabbits and maximal expression in HF hearts where it was barely detectable in young animals. Associated reduction of MFN2 and DRP1 by almost 50% in such aged HF rabbit models was also observed suggesting rescuing mitochondrial fusion may preserve aging-related mitophagy defects. Additionally, β-hydroxybutyrate (β-OHB), which is one of the most abundant ketone bodies in human circulation, is shown to enhance mitophagy in young and aging myocytes, but not in HF. This could be improved, however, with the enhancement of mitochondria fusion with the TAT-MP1 glycine peptide (Thai et al., 2019). Liang et al. (2020) reported increased parkin levels with reduced DRP1−mediated mitochondrial fission and reduced autophagosome formation in the heart of aged mice further suggesting the involvement of dysregulated mitophagy in aging-related cardiovascular pathologies. Double knockout of Akt2 and AMPK enhanced age-associated suppression in mitophagy, including decreased mitophagy regulators Atg5, Atg7, Beclin 1, LC3BII-to-LC3BI ratio, PTEN-induced putative kinase 1 (Pink1), parkin, Bnip3, FundC1, and the mitochondrial biogenesis cofactor PGC-1α (Wang S. et al., 2019). These observations indicate the role of compromised autophagy and mitophagy in aging cardiovascular pathology and possible benefits of targeting proper mitochondria quality control in cardiac aging.

MITOCHONDRIAL INTER-TALK WITH NUCLEUS

Mitochondria are essential for mammalian cells, yet they do not function as individual entities. Heavily reliant on cellular support and vice versa, the mitochondria actively signal and interact with other subcellular compartments. This inter-organelle coordination with highly organized intracellular localization is essential for cellular function and survival. To fulfill their tasks, mitochondria are in constant communication with the nucleus, endoplasmic reticulum (ER), Golgi apparatus, and other vesicular organelles (Shin et al., 2017, Shai et al., 2018; Wong et al., 2018; Lin et al., 2020). This diverse array of interplay enables multiple independent interactions without interfering with other cellular processes through compartmentalization and thus enables mitochondria to undergo multi-sided subcellular processes and metabolic cues. Inter-organelle communication between the mitochondria and other organelles especially the nucleus has been suggested to play a role in the pathophysiology of various chronic diseases.

Out of the 1,200 mitochondrial proteins required in mitochondrial biogenesis, most of the mitochondrial proteins are encoded by nuclear genes, translated and imported into the mitochondria through mitochondrial membrane translocase complexes (Pfanner et al., 2019). The biogenesis of the mitochondrial OXPHOS system is also under dual genetic control of nuclear and mitochondrial genes. Therefore, a delicate balance between nuclear and mitochondrial-encoded mitochondrial proteins is orchestrated and monitored for organelle health (Eisenberg-Bord and Schuldiner, 2017). The communication between mitochondria and the nucleus is essential for malfunctioned mitochondria to trigger compensatory nucleus responses in order to survive [64]. The nucleus controls mitochondrial gene expression and posttranslational modifications, so-called anterograde signaling; mitochondria modulate nuclear gene expression and cellular protein activity through signal transport originating from the mitochondria, termed retrograde signaling.

Anterograde Signaling From the Nucleus to Mitochondria

A set of nuclear-encoded factors coordinately regulate mitochondrial function through anterograde signals in

response to cellular environmental alteration. The process involves the expression of nuclear transcription factors and co-regulators that regulate the expression of nuclear-encoded mitochondrial proteomes, and the production of nuclear-encoded mitochondrial factors that control mtDNA gene expression (Quirós et al., 2016). All of the mitochondrial factors that activate mitochondrial transcription and translation are encoded in the nucleus, mostly nuclear respiratory factors (NRFs). There are two nuclear transcription factors, NRF1 and NRF2α also known as GA-binding protein-α (GABPα) (Miglio et al., 2009). These transcription factors, mainly NRF1, modulate the production of various nuclear-encoded mitochondrial proteins including cytochrome c, the vast majority of subunits for OXPHOS assembly, components of the mitochondrial protein import machinery, as well as proteins involved in mtDNA replication, transcription, and translation (Blesa et al., 2008). Additionally, NRF1 integrates mtDNA gene expression through direct control of the expression of important mitochondrial transcription machinery: the mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor A (TFAM), transcription specificity factors (TFB1M and TFB2M), and transcription termination factor (mTERF) (Bruni et al., 2010). During initiation of mtDNA transcription, TFAM binds to sequence specific regions of mtDNA, recruiting POLRMT to TFAM–mtDNA sequences then recruits TFB2M to accomplish promoter melting and separation of the two strands of the mtDNA thus initiating mtRNA synthesis (Kuhl et al., 2016). POLRMT also contributes to mitochondrial gene expression by regulating mtDNA replication via generation of RNA primers and interaction with TFB1M to mediate mitochondrial ribosome subunit assembly (Surovtseva and Shadel, 2013). In addition to the regulation of mtDNA transcription, TFAM is involved in many functions including mtDNA maintenance, replication, and likely also mtDNA repair (Gaspari et al., 2004; Kuhl et al., 2016). There is strong experimental evidence that the amount of TFAM directly regulates the mtDNA copy number and that mtDNA levels also reciprocally affect TFAM levels (Picca and Lezza, 2015). Additionally, there are nuclear receptors, such as peroxisome proliferator-activated receptors (PPARs) and estrogen-related receptors (ERRs), that activate the expression of nuclear-encoded mitochondrial proteins. PPARδ stimulates enzyme expressions involved in mitochondrial fatty acid oxidation while ERRs modulate the expression of nuclear-encoded mitochondrial protein in the TCA cycle, OXPHOS, and the fatty acid oxidation process (Xia et al., 2019). Estradiol acts centrally and systemically to regulate energy balance and metabolism. Sex differences in cardiovascular diseases suggest a protective role for estrogens in some diseases and the sexually dimorphic differences have been reviewed and discussed recently by Klinge (2020).

The nuclear transcription factors regulating mitochondrial protein expression require fine tuning by co-activators and co-repressors. Co-activators for stimulating mitochondrial biogenesis include the PPAR γ co-activator (PGC) family containing PGC-1α, PGC-1β, and PGC-1-related co-activator (PRC) (Gleyzer and Scarpulla, 2011). The PGCs are master regulators of mitochondrial biogenesis and play central roles in coordination and driving energy metabolism, fatty acid oxidation, gluconeogenesis, peroxisomal remodeling, and oxidative phosphorylation (Scarpulla et al., 2012). Among them, PGC-1α integrates and coordinates the activity of multiple transcription factors, including NRFs, ERRs, and PPARs and mitochondrial TFAM, which are all involved in mitochondrial biogenesis (Corona and Duchen, 2016). Other metabolism-involved transcription factors targeted by PGC-1α include Forkhead box protein O1 (FOXO1), Sterol regulatory element-binding proteins (SREBPs), Forkhead box protein A2 (FOXA2), and SRY-box transcription factor 9 (Sox9) (Gureev et al., 2019). A known co-repressor, receptor interacting protein 140 (RIP140), maintains the balance with co-activators by inhibiting mitochondrial biogenesis (Morrish and Hockenbery, 2014).

These nuclear factors and co-activators are further regulated by upstream sensors in response to changes in cellular conditions such as PGC-1α activation in cellular mitochondrial homeostasis (Fernandez-Marcos and Auwerx, 2011). In the cell, energy shortage with low ATP/AMP sensed by AMPK elevates cellular NAD^+ levels and activates sirtuin 1 (SIRT1) to upregulate PGC-1α which leads to consequential mitochondrial biogenesis (Joo et al., 2016). Similarly, Ca^{2+} release from mitochondria during exercising and cold stress can also activates PGC-1α through promoting the mitochondrial biogenesis activation of protein kinase A (PKA) and *cAMP-response element binding* protein (CREB) (Chen et al., 2010; Gill and La Merrill, 2017; Gill et al., 2019).

Retrograde Signaling From Mitochondria to the Nucleus

Mitochondria are a self-monitoring network, and organelle dysfunction activates retrograde signaling to the nucleus for activation of nuclear genes involved in metabolic reprogramming and stress response in restoring mitochondrial function (Liu and Butow, 2006).

The Mitochondrial Unfolded Protein Response (UPR^{mt})

A retrograde signaling pathway, the UPR^{mt}, in which signals are activated upon impaired mitochondrial protein import, transduct to the nucleus, and induce transcription factors to regulate repair of the mitochondrial network and prevent cell death (Semenza, 2011; Shpilka and Haynes, 2018; Cavalcante et al., 2020). UPR^{mt} promotes a rewiring of cellular metabolism that includes the suppression of TCA and OXPHOS encoding genes potentially to relieve mitochondrial stress and enhance genes expression in glycolysis and amino acid catabolism simultaneously as alternative energy sources to promote cellular survival (Anzell et al., 2018; Pfanner et al., 2019). In addition to cellular metabolism alterations, UPR^{mt} increases expression of mitochondrial localized chaperones, proteases, protein import machinery in proteostasis, as well as the expression of anti-oxidative proteins in redox homeostasis in order to repair dysfunctional mitochondria. Therefore, mitochondrial working burden is decreased, damaged mitochondrial proteins are

efficiently cleaved and processed, mitochondrial oxidative stress is contained, and the cellular back-up energy is maintained by cellular glycolysis to augment mitochondrial recovery (Quirós et al., 2016). Various conditions of mitochondrial dysfunction could affect mitochondrial protein import through the MOM including OXPHOS impairment, mtDNA defects, perturbation in mitochondrial translation and protein synthesis, overexpression of irreversibly misfolded mitochondrial proteins, mitochondrial proteostasis imbalance, excess ROS generation, and amino acid depletion (Qureshi et al., 2017). In *Caenorhabditis elegans*, a main messenger for the initiation of UPR^{mt}, is the activating transcription factor associated with stress-1 (ATFS-1) which has two targeting sequences: the nuclear localization sequence (NLS) and the mitochondrial-targeting sequence (MTS). In a healthy mitochondrial network, the MTS prevails and ATFS-1 is imported into the mitochondria where it is degraded by the matrix-localized protease Lon. In the event of mitochondria defect, mitochondrial protein import is impaired and ATFS-1 is directed to the nucleus via NLS and activates UPR^{mt} (Wu et al., 2018). In the nucleus, ATFS-1 downregulates OXPHOS gene transcription, upregulates the expression of mitochondrial chaperones and proteases for proteostasis, and imports machineries to restore mitochondrial homeostasis (Wu et al., 2018). In mammals, the regulation of UPR^{mt} is not yet completely elucidated. However, evidence has shown that transcription factors C/EBP homologous protein (CHOP), *activating transcription factor 4* (ATF4), and ATF5 are involved in mammalian UPR^{mt} (Shpilka and Haynes, 2018). Their activation is reliant upon the eukaryotic translation initiation factor 2 subunit 1 (eIF2α) which is catalyzed by four kinases including general control non-derepressible-2 kinase (GCN2), protein kinase RNA (PKR), PKR-like endoplasmic reticulum kinase (PERK), and heme-regulated inhibitor kinase (HRI) in response to diverse cellular stresses (Teske et al., 2013; van Leeuwen and Rabouille, 2019). The activation of eIF2α results in reduced global protein synthesis and preferential translation of the transcription factors CHOP, ATF4, and ATF5 (Qureshi et al., 2017). ATF5 is thought to be orthologous to the C. elegans' ATFS-1 and is regulated via mitochondrial protein import efficiency and other diverse forms of mitochondrial stress (Melber and Haynes, 2018). In mammals, CHOP, ATF4, and ATF5 increase the expression of mitochondrial chaperones and proteases, metabolic remodeling, the mitochondrial-protein-import-related complex, mitochondrial biogenesis related components, and anti-oxidative enzymes (SOD, glutathione synthesis machinery, and ubiquinone synthesis genes) (Qureshi et al., 2017). When perturbations occur within the mitochondrial intermembrane space, the nuclear hormone receptor estrogen receptor α (ERα) is activated in addition to CHOP, ATF4, and ATF5 to increase intermembrane space protease high-temperature requirement A2 (HTRA2) expression and also NRF1 for mitochondrial biogenesis promotion. UPR^{mt} also mediates global gene silencing in response to eIF2α activation probably due to the need for cutting energy expenses in anabolism under decreased TCA-OXPHOS-ATP production. The global gene silencing in UPR^{mt} is mediated through chromatin remodeling due to cytosolic protein LIN-65 translocation to the nucleus to induce chromatin compaction. Since the mitochondrial stress response genes need to be kept transcriptionally competent under global gene silencing, UPR^{mt} activates the histone lysine demethylases Jumonji C domain-containing protein -1.2 (JMJD-1.2) and JMJD-3.1 and the homeo box transcription factor defective proventriculus -1 (DVE-1) to promote an open chromatin state (Merkwirth et al., 2016; Livezey et al., 2018).

Other Retrograde Signaling Pathways

Besides UPR^{mt}, there are other retrograde signals induced by mitochondrial damage. The low ATP/AMP caused by mitochondrial dysfunction activates AMPK and inhibits the mTORC1, which stimulates mitochondrial biogenesis, autophagy, and lysosomal degradation (da Cunha et al., 2015; Herzig and Shaw, 2018). The mTOR kinase pathway integrates a multitude of extracellular and intracellular cues to drive growth and proliferation. Under stress and starvation, inhibition of mTORC1 coordinates energy consumption by the mRNA translation machinery and mitochondrial energy production by stimulating synthesis of nucleus-encoded mitochondria-related proteins including TFAM, mitochondrial ribosomal proteins, and components of complexes I and V (Morita et al., 2015). Mitochondrial oxidative stress also signals the nucleus to upregulate antioxidative enzymes in nDNA through activation of transcription factors such as nuclear factor erythroid 2 related like 2 (NFE2L2) (Merry and Ristow, 2016; Hu et al., 2018). Excessive mitochondrial ROS under a hypoxic environment activates nucleus transcription factor hypoxia inducible factor 1 (HIF-1) which switches gene expression from oxidative to glycolytic metabolism. HIF-1 can also trigger mitophagy by activating the gene encoding proapoptotic BNIP3 (Zhang and Ney, 2009; Semenza, 2011). Ca^{2+} retrograde signaling occurs in the event of mitochondrial stress when the mitochondria lose their membrane potential with consequent release of Ca^{2+} into the cytoplasm. Elevated free cytosolic Ca^{2+} activates phosphatase calcineurin, which activates the transcription factors nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NFATC). The two translocate to the nucleus to promote synthesis of proteins involved in Ca^{2+} transport and storage (Liu et al., 2017). Elevated cytosolic Ca^{2+} also activates other Ca^{2+}-regulated kinases, such as calcium/calmodulin-dependent protein kinase IV (CAMKIV), Ca^{2+}-dependent protein kinase C, c-Jun N-terminal kinases (JNK), and p38 MAPK; which stimulate different transcription factors such as CREB, early growth response protein 1 (EGR1), cAMP-dependent transcription factor ATF2, CCAAT/enhancer-binding protein δ (CEBPδ), and CHOP to mediate mitochondrial adaptation, Ca^{2+} metabolism, glucose metabolism, and cell proliferation (Giorgi et al., 2018). Another important type of retrograde signaling is the mitochondrial involvement of the intrinsic apoptosis pathway. With the release of mitochondrial pro-apoptotic proteins into the cytosol, downstream CASP 3 executes the cleavage and destruction of subcellular structures, including the nucleus (Prokhorova et al., 2018; Tang et al., 2019).

ASSOCIATION OF CARDIOVASCULAR DISEASES AND MITOCHONDRIAL DYSFUNCTION AND FUTURE TREATMENT STRATEGIES

Mitochondria with their multitude of functions are essential in high-energy demand cardiac tissues. Here we discuss three common cardiovascular diseases (HTN, Ischemic heart disease, and diabetic cardiomyopathy) and their relationship to mitochondrial dysfunction. Apart from these diseases, mitochondrial genetic haplotypes are also associated with different pathological changes in cardiovascular diseases. Since the discovery of these mitochondrial-related pathologies, mitochondrial-targeting therapeutic treatments have emerged.

Hypertension

HTN has been considered crucial in the pathogenesis of many cardiovascular diseases. Both inflammatory damage and endothelial dysfunction of vascular structure as well as activation of the sympathetic nervous system have been alluded to have a hand in the pathogenesis of HTN (Lahera et al., 2017; Morales et al., 2020). Additionally, mitochondria dysfunction has been suggested to play a role in the pathophysiology of HTN (Barki-Harrington et al., 2004). Angiotensin (Ang) II is an important stimulus for HTN and its effects on mitochondrial function have been shown to be through the elevation of mitochondrial oxidative damage, decreases in endothelial nitric oxide ($NO^{\cdot}$) bioavailability, and the induction of vascular oxidative stress. Ang II causes mitochondria dysfunction through a protein kinase C dependent pathway leading to NADPH oxidase (NOX) activation and the formation of excess ROS such as $O_2^{\bullet -}$ and H_2O_2 after a reaction of SOD and peroxynitrite ($ONOO^-$) in the presence of NO, respectively (Iuchi et al., 2003; Doughan et al., 2008). Doughan et al. (2008) reported that the deleterious effect that Ang II has on mitochondria in bovine aortic endothelial cells leads to the generation of a virtuous cycle involving the increase of mitochondrial H_2O_2 production, the activation of cellular NOX, the increase of intracellular $O_2^{\bullet -}$ production, and diminishing $NO^{\cdot}$ bioavailability which eventually contributes to endothelial dysfunction and activation of apoptotic signaling (Davidson, 2010). Recently, mitochondria dynamics and quality control have been shown to be involved in the process of endothelial dysfunction. Lugus et al. (2011) have shown that with the downregulation of MFN1, the major mitochondrial fusion regulator lowers angiogenic responses to vascular endothelia growth factor (VEGF) and the activation of endothelial nitric oxide synthase (eNOS) in cultured endothelial cells. Knockout of either MFN1 or MFN2 resulted in the disarray of the mitochondrial homeostasis and the decrease of mitochondria $\Delta\Psi$ through the blunting of VEGF signaling pathways associated with oxidative metabolism (Lugus et al., 2011). Both N^G-nitro-L-arginine methyl ester (L-NAME) treated endothelial cellular and Sprague Dawley rat models have suggested that under eNOS dysfunction, mitochondria tend to shift away from fusion and that excessive fission may be an underlying cause of endothelial cell dysfunction in postischemic hearts (Giedt et al., 2012; Miller et al., 2013). The finding that genetic variation in the PARK2 gene, which encodes the important mitophagy regulator parkin, is significantly associated with HTN in both Nigerian and Korean populations further supports the relationship of mitochondrial quality control and susceptibility to HTN (Tayo Bamidele et al., 2009; Jin et al., 2011). The protective effect of mitophagy on the development of HTN has been shown, as overexpression of cathepsin S and enhanced ATG5-mediated mitophagy contributes to the decrease of both ROS production and NF-κB-related inflammatory responses using an Ang II-induced HTN mice model (Pan et al., 2012; Zhao et al., 2014). At the same time, ER stress influences mitochondria through specialized complexes through the ER-associated mitochondria membrane and the exchanges of Ca^{2+}, lipids, metabolites, and signaling molecules (Young, 2017). Inter-organelle actions of the ER leading to activation of cytosolic UPR can also cause the increase in inflammation, ROS production, and apoptosis (Sharkey et al., 2009; Santos et al., 2013). Adipocyte-related ER stress in obese subjects shows an increase of the ER chaperone glucose-regulated protein 78 (GRP78), while activating UPR signals resulting in the turning on of transcription factor 4, CHOP, and Bax in an obese ovine-related HTN model induced by increased food intake and reduced activity (Sharkey et al., 2009; Santos et al., 2013). CNS activation of UPR might also be a mediator of HTN via changes in synaptic transmission, neuronal signaling, and autonomic overaction (Young, 2017). Indeed, when the ER stress inducer thapsigargin, which causes UPR activation by perturbing cellular Ca^{2+}, is applied to the brain lateral cerebral ventricle in mice it results in elevation in arterial blood pressure (Bravo et al., 2012; Young et al., 2012).

In additional to endothelial damage, HTN also causes structural alterations to the mitochondria of the cardiomyocyte, including decreased mitochondria mass and density, mitochondria swelling, and cristae remodeling (Ritz and Berrut, 2005; Eirin et al., 2014). Decrease in mitochondria size and density combined with osmotic swelling of the mitochondria lead to a gradual compromise of oxidative capacity (Ritz and Berrut, 2005). HTN was found to be associated with loss of cardiolipin, a phospholipid required for proper cristae formation, which can further threaten the stability of the ETC complexes (Cogliati et al., 2013). Lack of cardiolipin may also result in dysfunctional mitochondria dynamics, the opening of mPTP, the releasing of cytochrome C, and triggering apoptosis (Osman et al., 2011). Inhibition of mitochondria fission with the DRP1 inhibitor prevents cardiomyocytes apoptosis in spontaneous hypertensive rat models (Qi et al., 2018). The same group also found that Ang II treatment of cultured neonatal rat cardiomyocytes elevated DRP1 expression and enhanced mitochondrial fission as well as cellular apoptosis. This Ang II-induced hypertensive cardiomyopathy was suggested to be sirtuin 1(Sirt1)/P53/DRP1-dependent (Qi et al., 2018). HTN enhanced excessive ROS production can damage DNA and activate the poly (ADP-ribose) polymerase (PARP) enzyme (Dizdaroglu and Jaruga, 2012; Alemasova and Lavrik, 2019). Activated PARP enzyme binds to the damaged DNA region via cleaving NAD^+, thus depleting NAD^+ reserves and resulting in programmed cell death (Ordog et al., 2021). Ordog et al.

(2021) that showed PARP-inhibition prevented DRP1 expression and increased fusion proteins while preserving mitochondria structure and function in cardiomyocytes. These recent findings provide valuable clues to the fact that multi-functional pathways may aid in the development of HTN-related cardiovascular complications where mitochondria quality control through mitophagy and inter-organelle inter-talk between mitochondria and ER plays a pivotal role.

Ischemic Heart Disease

Pathological cardiac conditions resulting from ischemic cardiac injuries and occlusion of a coronary vessel induces a cascade of tissue hypoxia and cellular ATP depletion (Peoples et al., 2019). A series of events may contribute to this process of ischemic and reperfusion damage to cardiac tissues with impairment of mitochondrial integrity being central to this process, including the generation of ROS, opening of the mitochondrial permeability transition pore (mPTP), and activation of intrinsic apoptosis (Kulek et al., 2020). Under these drastic changes in nutrient and oxygen availabilities, a decrease in oxidative phosphorylation results in a decrease in cellular ATP, and a loss of mitochondrial membrane potential (Murphy and Steenbergen, 2008). With the I/R injuries and cytosolic changes of the cardiomyocytes, lowering of intracellular pH due to lactate accumulation results in cytosolic Ca^{2+} overload (Murphy and Steenbergen, 2008). High cytosolic Ca^{2+} in turn leads to an overload of mitochondrial Ca^{2+}, which combined with unregulated ROS production from IRI, brings about the opening of the mPTP (Halestrap and Pasdois, 2009). The act of mPTP opening gives rise to the permeabilization of the MIM, thus causing mitochondrial depolarization, loss of membrane potential, swelling, rupture, and ultimately cell death (Halestrap and Pasdois, 2009; Kwong and Molkentin, 2015). Therapeutic strategies targeting to prevent the opening of mPTP through immunosuppressant cyclosporin-A (CsA) and sanglifehrin-A (SFA) have been shown to protect the myocardium from IRI in preclinical studies (Hausenloy et al., 2003; Hausenloy and Yellon, 2003). Hausenloy et al. (2002, 2003) have shown that CsA and SFA significantly decreases myocardial infarct size using isolated rat heart models subjected to 35 min of ischemia and 120 min of reperfusion. However, Cung et al. (2015) found that intravenous cyclosporine did not improve clinical outcomes in patients with anterior STEMI who had been referred for primary percutaneous coronary intervention, more than those with placebo in the Does Cyclosporine Improve Clinical Outcome in ST Elevation Myocardial Infarction Patients (CIRCUS) trial. Other inhibitors of mPTP such as 3,5-Seco-4-nor-cholestan-5-one oxime-3-ol (TRO40303) and elamipretide (MTP-131) also did not show any effect on limiting ischemic injury as can be seen in the MITOCARE and EMBRACE STEMI studies (Atar et al., 2015; Gibson et al., 2016). Facing difficulties in direct inhibition of mPTP, halting mPTP through targeting activators such as ROS may be a novel therapeutic strategy. Adlam et al. (2005) and Ribeiro Junior et al. (2018) revealed promising results in rat I/R models with mitochondria-targeted antioxidants such as mitoQ. As mentioned previously, mitochondrial quality control through mitophagy is crucial for proper maintenance mitochondrial function in cells under stress. In most mammalian tissues, mitochondria adapts to stress through a series mitochondrial dynamic processes including fission and fusion (Kulek et al., 2020). However, it has been reported that in adult cardiomyocytes, mitochondria dynamics are scarce with slow mobility and little morphology change (Song and Dorn, 2015). Thus, the sequestration and mitophagic removal of this damaged organelle is paramount for maintaining mitochondria homeostasis and relieving IRI damage (Song and Dorn, 2015). Therefore, quality control of mitochondria via mitophagy under I/R stress can be on a knife's edge, as excessive activation of mitophagy may result in disastrous loss of mitochondria leading to cellular apoptosis (Kulek et al., 2020). In the meanwhile, insufficient mitophagic activation in response to cellular stress may give rise to the accumulation of dysfunctional mitochondria (Kulek et al., 2020). Factors known for inducing autophagy like Beclin 1, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), and FUN14 domain containing 1 (FUNDC1), and anti-autophagic factors like BCL2 have been proposed to play roles during IRI (Georgakopoulos et al., 2017; Zhang et al., 2017). Matsui et al. (2007) found that for *Becn1*$^{+/-}$ mice cardiomyocytes, autophagy is protective during cardiac ischemia, but is detrimental during reperfusion. However, a protective effect through inhibition of *Bnip3*-related autophagy has been demonstrated in studies by Diwan et al. (2007), which knocked out this pro-mitophagic regulator *Bnip3* and preserved left ventricle systolic performance and diminished left ventricle dilation in *Bnip3*$^{-/-}$ mice under I/R-induced myocardial damage. Though this myocardial salvage effect may be due to inhibition of apoptosis via caspase-dependent apoptosis and mPTP opening as is later supported by Matsui et al. (2007) and Bravo-San Pedro et al. (2017). Ischemia can also cause nuclear receptor subfamily 4 group A member 1 (NR4A1) expression, which in turn activates serine/threonine kinase casein kinase 2α (CK2α) and promotes CK2-related phosphorylation of MFF and FUNDC1 (Zhou et al., 2018a). Phosphorylated activation of MFF leads to fatal mitochondrial fission in addition to the inactivation of FUNDC1-associated protective mitophagy (Zhou et al., 2018a). Zhou et al. (2018a) found that in NR4A1 knockout mice, the loss of CK2 restores FUNDC1-mediated mitophagy further providing a survival advantage to cardiac myocytes in response to I/R stress. Zhang et al. also showed that this mitophagy receptor FUNDC1 interacts with LC3 to mediate mitophagy in response to hypoxia in cultured cardiomyocytes. Using a *Fundc1*−/− mouse model, these authors also provide evidence that FUNDC1-mediated mitophagy regulates mitochondrial quality control in vivo and plays a crucial role in maintaining functional integrity in platelet activation and reduces I/R-induced heart injury (Zhang et al., 2016, 2017). Therefore, how to rightly facilitate and balance mitophagy to protect the heart under I/R stress remains to be elucidated by therapeutic strategies, and is therefore a new topic for investigation.

Diabetic Cardiomyopathy

Patients with T2DM are vulnerable to vascular disease, and heart failure is the main cause of death in the diabetic

population (Missiroli et al., 2018). Additionally, prevalence of T2DM is reaching pandemic proportions (Mozaffarian et al., 2015), and mortality and morbidity related to its macrovascular and microvascular complications have steadily been on the rise (Kearney, 2015; Kochanek et al., 2016). These vascular complications originate from insulin-resistance–related endothelial dysfunction, leading to higher risks of cardiovascular disease-related mortality in DM patients (Ting et al., 1996; Pieper et al., 1997). Furthermore, clinical outcomes associated with heart failure are considerably worse with an increase of mortality for patients with DM than for those without (Jia et al., 2018). A recent term "diabetic cardiomyopathy" is used to define the existence of abnormal myocardial structure and performance in the absence of other cardiac risk factors, such as coronary artery disease, HTN, and significant valvular disease, in individuals with DM (Yancy et al., 2013; Jia et al., 2018). Dysfunctional mitochondria have been proposed as a common pathologic mechanism of diabetic cardiac dysfunction (Schilling, 2015). The cardiomyocyte utilizes different substrates simultaneously for energy production with up to 60–70% ATP production from the mitochondrial oxidation of fatty acids and a lesser extent of 30–40% ATP from glucose, lactate, and other substrates (Bugger and Abel, 2010). However, in a diabetic heart, glucose utilization is greatly diminished due to insulin resistance, impaired pyruvate dehydrogenase activity, and reduced glucose transporter translocation (Heather and Clarke, 2011; Heather et al., 2013). Therefore, diabetic cardiomyocytes almost exclusively rely on mitochondrial fatty acid oxidation for ATP generation (Wright et al., 2009). This produces greater levels of oxidative stress and potentially provokes mitochondrial dysfunction, the release of pro-apoptotic factors such as cytochrome *C*, apoptosis inducing factor, and Smac/DIABLO, and altered mitochondrial Ca^{2+} handling which eventually lead to the death of cardiomyocytes (Brown et al., 2017). Mitochondria are important regulators in the cellular fuel choice of fatty acid or glucose metabolism under the changing metabolic environment. For example, fatty acid uptake is enhanced in diabetic cardiomyocytes which cause the activation of PPAR-α, an activator of genes involved in fatty acid uptake and β-oxidation. PPAR-α, on the other hand, suppresses the expressions of genes associated with the TCA and mitochondrial OXPHOS (Lee et al., 2017). Supporting this, Finck et al. reported that the activation of cardiac PPAR-α overexpression results in reciprocal repression enzymes involved in glucose uptake and utilization pathways (Finck et al., 2002). Ferreira et al. (2013) also showed that mitochondria from the heart of an STZ-induced type 1 DM mouse model presented lower OXPHOS activity and downregulation of mitochondrial transcription factor TFAM (Jiang et al., 2014; Khuchua et al., 2018). The increase of fatty acid β-oxidation augments delivery of electrons to the mitochondrial electron transport chain, causing elevated mitochondrial inner membrane potential, which stimulates mitochondrial ROS production (Liesa and Shirihai, 2013). Excessive fatty acid uptake in diabetic hearts reduce the expressions of genes involved in mitochondrial OXPHOS (Lee, 2020). Supporting these, Petersen et al. (2004) have also reported that insulin resistance in the skeletal muscle of insulin-resistant offspring of patients with T2DM is associated with the dysregulation of intra-myocellular fatty acid metabolism, possibly because of an inherited defect in mitochondrial OXPHOS. In a normal functioning cardiomyocyte, mitochondria oxygen consumption is generally tightly coupled to ATP synthesis via the ETC chain. However, in a diabetic heart there is increased proton bypass of the final ATPase to reenter the mitochondrial matrix known as mitochondrial uncoupling which causes the decrease in ATP production efficiency (Krauss et al., 2005; Demine et al., 2019). This uncoupling causes oxidative stress and is mostly noted at the mitochondrial complex I and III. Moreover, mitochondrial OXPHOS complexes were noted to be suppressed in a diabetic heart associated with increased ROS and nitrogen free radical production leading to cell death in human myocardial samples and a streptozotocin (STZ)-induced type 1 DM model (Sivitz and Yorek, 2010; Raza et al., 2011). Of note, abnormal metabolism developed in diabetic pathogenesis-induced altered mitochondrial morphology through imbalanced mitochondrial dynamic regulators such as MFN1, MFN2, OPA1, and DRP1 in various tissues (Williams and Caino, 2018). Particularly, T2DM is associated with reduced MFN2 expression, impaired mitochondrial fusion, fragmented mitochondria networks which are related to depressed OXPHOS, and glucose intolerance (Mootha et al., 2003). Montaigne et al. (2014) also demonstrated that MFN1 was decreased with mitochondrial fragmentation in relation to myocardium contractile dysfunction in human T2DM subjects (Liesa and Shirihai, 2013). Lack of fusion machinery has been noted in diabetic cardiomyocytes and Parra et al. (2014) demonstrated in rat cardiomyocytes that the fusion machinery OPA1 could be stimulated by insulin administration and promote mitochondrial fusion through a mechanism involving the Akt-mTOR-NFκB signaling pathway.

It is generally supported that alteration of mitochondrial architecture activates mitochondrial quality control mechanisms such as autophagy. Autophagy in the heart is intricately regulated and both protective or detrimental results have been reported. It is generally suggested that autophagy is suppressed in type 1 and induced in T2DM (Mellor et al., 2011). Using both transgenic and STZ-induced type 1 DM mouse models, Xu et al. (2013) reported markedly decreased autophagy core components including LC3, ATG5, and ATG12 which demonstrate inhibited autophagic flux in the cardiomyocytes of type 1 DM animals. This study also demonstrated that restoration of autophagy to transgenic mice further exacerbated diabetic cardiac injury; while cardiac damage in WT diabetic mice was substantially attenuated in Beclin 1 or ATG16L1-deficient (beclin $1^{+/-}$ or ATG16L1-HM) mice. Improved glucose blood levels, reduced free fatty acids, and triglycerides were observed with the suppression of autophagy regulators Beclin 1 or ATG16L1 in STZ and OVE26 diabetic mice (Xu et al., 2013). Also noted in the study is that reduced autophagy was associated with increased expression and lysosomal localization of Rab9, which is involved in the Rab9-dependent alternative autophagic pathway (Nishida et al., 2009). Together, this study suggests that the inhibition of autophagy was an adaptive response to limit cardiac dysfunction

in type 1 DM, with upregulation of alternative autophagy and improved mitophagy (Xu et al., 2013). On the other hand, Kanamori et al. (2015) demonstrated in STZ-induced type 1 DM mice that induced autophagic activity was significantly associated with impaired diastolic function as documented with increasing LC3-II, SQSTM1/p62, cathepsin D, and an abundance of autophagic vacuoles and lysosomes detected via electron-microscopy.

A distinct bioenergetic impairment of heart mitochondrial subpopulations in diabetic cardiomyopathy is associated with obesity (Lai et al., 2020). Reduced cardiac efficiency, decreased mitochondrial energetics, enhanced oxidative damage, increased fatty acid oxidation, and change of heart substrate usage is found in both obese and diabetic patients (Kasper et al., 1992; Boudina et al., 2007; Boden, 2008; Peterson and Gropler, 2010). Toledo et al. (2006) showed in a study with 11 obese insulin-resistant human participants that mitochondria morphological alterations of skeletal muscles are associated with improvements in insulin resistance and weight loss. Tong et al. (2019) recently reported that mitophagy was associated with obesity in an Atg7-dependent manner. This group demonstrated the elevation of LC3-II localization to mitochondria after 3 weeks of high fat feeding. Thomas et al. (2019) also showed a decrease in cardiac parkin, a crucial protein of mitophagy in obese mice which suggests parkin-dependent mitophagy may contribute to obesity-related cardiovascular risk. All of the above suggest that both DM and obesity cardiomyopathy are pathologically associated with mitochondrial function maintenance.

The Association of Mitochondrial Genetic Variations With Cardiovascular Disease

As have been described in the mitochondria biology section, the mitochondrion plays a pivotal role in cell physiology not only in supplying energy to the cells but is also involved in various important cellular functions. Variations of mtDNA can potentially alter mitochondrial function and thus possibly links to clinical disease (Ma et al., 2014). The association between mtDNA variations and cardiovascular risks have been well-described. For example, the pathological mtDNA A3243G mutation causes mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes syndrome (MELAS), and maternal inherited diabetes mellitus (Liou et al., 2000, 2003, 2004; Chen et al., 2004). Both the coding and control regions of mtDNA play roles in the generation of diabetes. An mtDNA variant commonly found in the general population, the T-to-C transition at np 16189, has been shown to be positively correlated with blood fasting insulin and T2DM in a population-based case-control study in Cambridgeshire, United Kingdom (Poulton et al., 2002). Based on previous studies, this mtDNA T16189C variant is associated with increased oxidative damage, altered antioxidative status in T2DM patients, metabolic syndrome, higher fasting insulin concentration, insulin resistance index, and lacunar cerebral infarction in the Asian population (Lin et al., 2005; Liou et al., 2007, 2010; Tiao et al., 2009; Wang et al., 2009). Tanaka et al. (2007) studied genotypes for 25 polymorphisms in the coding region of the mitochondrial genome and revealed that haplogroup N9a is significantly associated with resistance to the metabolic syndrome in women. Our group has reported that the haplotype B4 (carrying the T16189C variant) is associated with T2DM (odds ratio [OR], 1.54 [95% CI 1.18–2.02]; $P < 0.001$) in the Chinese population, whereas subjects harboring haplogroup D4 have borderline resistance against DM generation (0.68 [0.49–0.94]; $P = 0.02$) (Liou et al., 2010). Recently, a patient study with 830 Taiwanese ischemic stroke patients and 966 normal controls revealed an association between mitochondrial haplogroup F1 and risk of ischemic stroke (OR 1.72:1.27–2.34, $p = 0.001$) (Tsai et al., 2020). In the same study, with the usage of a hybrid technique, Tsai et al. (2020) demonstrated that mitochondrial haplogroup F1 cybrids were associated with decreased oxygen consumption, higher mitochondrial ROS production, and lower mitochondrial membrane potential. These cybrids were also noted to be prone to inflammation, with increased expression of several inflammatory cytokines. Recently Wei et al. (2021) analyzed 996 mtDNA components in the peripheral blood of patients with cardiovascular disease and detected strong associations between the patients' clinical characteristics and both mtDNA copy number and rare mtDNA variants. All these data support the involvement of mitochondrial genetic variation in the pathogenesis of T2DM and associated vascular diseases in both clinical and functional studies.

Therapeutic Strategies Targeting Mitochondria Protection in Cardiovascular Diseases

Mitochondrial dysfunction may lead to the pathology of many common disorders, including I/R injury (Marin et al., 2021), heart failure, metabolic disease (for example HTN, insulin resistance, and obesity) (Jha et al., 2017; Tavallaie et al., 2020), and neurodegeneration (Reeve et al., 2016; Riley and Tait, 2020). Several strategies aimed at therapeutically restoring mitochondrial function are emerging, including behavioral interventions of diet or exercise (Ascensao et al., 2007; Miller et al., 2020), exposure to hypoxia, and stem cell therapies (Lin T.K. et al., 2019). Gene therapies to correct a defective gene and degrade mutated mtDNA are also developing for traditional mitochondria diseases caused by pathologic mutation in mtDNA (Slone and Huang, 2020). Agents have recently been developed specifically for the treatment of mitochondrial dysfunction including antioxidants such as coenzyme Q10 (CoQ10), protective compounds targeting the mitochondria such as MitoQ, agents to replenish NAD^+ pools such as nicotinamide mononucleotide (NMN) and Bendavia (SS31), and the inhibitor of the mitochondrial permeability transition pore (mPTP) cyclosporin A (CsA) (Scatena et al., 2007; Murphy and Hartley, 2018). Apart from these strategies, some specific medications already proved and used widely for clinical conditions have been suggested to provide an additional mitochondrial protection effect and their clinical use can potentially be expanded for the purpose of prevention and treatment of cardiovascular diseases.

Repurposing Drugs to Target Mitochondrial-Related Cardiovascular Diseases

Given the high failure rate in clinical trial and costly and slow pace of new drug development, repurposing "old" drugs to treat both common and rare diseases is increasingly becoming an attractive concept because it involves the use of safety-proven compounds, with potentially lower overall development costs and shorter development timelines (Pushpakom et al., 2019). Experimental approaches have also identified repurposable candidate drugs targeting the mitochondria for common pathologies (Yoshida, 2017; Bell et al., 2018; Pellattiero and Scorrano, 2018). For example, metolazone, a diuretic primarily used to treat congestive heart failure and high blood pressure, was found to upregulate protective cellular chaperone, heat shock protein-6, and induce UPR^{mt}, a mitochondrial stress response shown to promote longevity in model organisms (Ito et al., 2021). Ito et al. (2021) also demonstrated that metolazone extended the lifespan of Caenorhabditis elegans and specifically induced the expression of mitochondrial chaperones for UPR^{mt} in the HeLa cell line.

Numerous prescribed anti-diabetic agents worldwide have been found to possess cardiovascular benefits through their original glucose lowering effect. Beside, some anti-diabetic drugs have recently been proposed to be associated with mitochondrial function regulation (Foretz et al., 2014). For example, metformin, the most commonly prescribed anti-diabetic agent was shown to have the advantage of counteracting cardiovascular complications associated with diabetes in the large cohort United Kingdom Prospective Diabetic Study (UKPDS) (Bailey and Grant, 1998). Studies further show metformin to improve mitochondrial respiratory activity through paradoxical mechanisms, through reducing cellular oxygen consumption via inhibiting mitochondrial complex 1 activity at high levels of metformin or by activation of AMPK (Wang Y. et al., 2019). Another T2DM drug that have been shown to possess mitochondrial regulatory mechanisms are the sodium glucose cotransporter 2 (SGLT2) inhibitors. In three cardiovascular outcome trials: the EMPA-REG OUTCOME trial (7064 participants) (Zinman et al., 2015; Fitchett et al., 2019), CANVAS Program (4330 participants) (Neal et al., 2017), and DECLARE TIMI 58 trial (17,190 participants) (Wiviott et al., 2019) SGLT2 inhibitors have been shown to reduce cardiovascular events including mortality and hospitalization for heart failure, in patients with T2DM. The recent DAPA-HF trial additionally demonstrated SGLT2 inhibitor to reduce major outcomes in patients with established heart failure with a reduced ejection fraction (HfrEF), regardless of the presence of diabetes (McMurray et al., 2019). Two other trials currently evaluating the effects of SGLT2 inhibitors in patients with established heart failure with a preserved ejection fraction (HFpEF) regardless of the presence of diabetes are the EMPEROR-Preserved trial [NCT03057951], and the DELIVER trial [NCT03619213] (Anker et al., 2019). Salutary mechanisms of SGLT2 inhibitors in cardiomyocytes through mitochondrial function-mediated beneficial effects have been demonstrated by Takagi et al. (2018), who reported SGLT 2 inhibitors to alleviate mitochondrial dysfunction via restoration of dynamic proteins to normal values in OPA1, Mfn1, Mfn2, Fis1, and Drp1 in high-fat diet rat models. Another mechanisms of SGLT 2 inhibitor on mitochondria was through activating AMPK and suppressing mitochondrial fission (Zhou et al., 2018b). The other T2DM drug is the glucagon-like peptide-1 receptors agonists (GLP-1RA). GLP-1 receptors are found on human cardiac tissue (Anagnostis et al., 2011), and are found to have cytoprotective actions in the heart (Baggio et al., 2018). In the LEADER trial and SUSTAIN-6 trial, GLP-1RA also showed effects of reducing the rates of myocardial infarction, cardiovascular deaths, and stroke (Marso et al., 2016; Aroda et al., 2017; Nauck et al., 2017). GLP-1 RA additionally improved vasodilation and significantly reduced systolic blood pressure (Plutzky, 2011). Tomas et al. (2011) show GLP-1-derived nonapeptide GLP-1(28–36) amide targets to mitochondria and suppresses the OXPHOS process and oxidative stress (Ussher and Drucker, 2012). Chang et al. (2018) demonstrated that GLP-1 analog decreases mitochondrial morphological abnormalities, reduces oxidative stress, enhances ATP synthesis, mitochondrial ATPase activity and $\Delta\Psi$, decreases mitochondrial calcium overload and inhibits the opening of mPTP in an cellular model of I/R injury. Therefore, demonstrating GLP-1 analogue cardioprotective effects. In myocardial infarction mice, circulating GLP−1 concentrations were markedly elevated with association of increased AMPK activity which stimulated the mitochondrial respiratory capacity of non−infarcted tissue areas considered as a compensatory protective mechanism (Diebold et al., 2018). The above support the potential of drug repurposing targeting mitochondrial function modulation and implicate the dual effects of mitochondrial-protective benefits additional to their original pharmacological mechanisms for the treatment of cardiovascular disease.

PERSPECTIVE

Proper mitochondria functioning is fundamental for cellular health and of extreme importance in high-energy demand tissues. The quality control of the organelle is crucial for the removal of damaged mitochondria and maintaining homeostasis to preserve cardiac function under pathological conditions. As has been shown above, in the pathogenesis of cardiovascular diseases such as HTN, ischemic heart disease, and T2DM, mitochondria dysfunction plays a potential role in not only cellular injuries but also the progression of these diseases. Thus, providing and sustaining a healthy mitochondrial network through mitochondrial quality control can be pivotal for the outcome of the cardiac injury under various pathologic stresses. Recent development of mitochondria targeting agents have provided pharmacological means of alleviating these mitochondrial functional impairments. Old drugs have also been approached to search for potential underlying mitochondrial targeting mechanisms. These therapeutic measures present alternative or coadministration pharmacological choices and bring innovation, challenge, and hope to future remedies of common pathological pathways of cardiovascular disease.

Rising evidence has given us an insight on how reducing oxidative stress, facilitating mitophagy, and even inter-organelle interaction can provide cardioprotective effects. Targeting and modulating these protective pathways may throw new light on the treatment of cardiovascular disease in the future.

AUTHOR CONTRIBUTIONS

K-LL contributed to the concept generation, data interpretation, drafting of the manuscript, and graphic drawing. S-DC contributed to the concept generation, data interpretation, and drafting of the manuscript. K-JL contributed to the concept generation, graphic drawing, and drafting of the manuscript. C-WL, Y-CC, P-WW, and J-HC contributed to the concept generation, data interpretation, and approval of the article. T-KL contributed to concept generation, data interpretation, graphic drawing, drafting of the manuscript, and approval of the article. All authors contributed to the article and approved the submitted version.

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GLOSSARY

ATP	Adenosine triphosphate	MiD49	Mitochondrial dynamics protein of 49
AIM	ATG8-interacting motif	MiD51	Mitochondrial dynamics protein of 51
AMPK	AMP-activated protein kinase	MIM	Mitochondrial inner membrane
Ang	Angiotensin	MOM	Mitochondrial outer membrane
APAF -1	Apoptotic protease activating factor-1	MOMP	Mitochondrial outer membrane permeabilization
ATFS-1	Activating transcription factor associated with stress-1	mPTP	Mitochondrial permeability transition pore
ATF	*Activating transcription factor*	Msr	Methionine sulfide reductase
ATG	Autophagy related gene	mtDNA	Mitochondrial DNA
ATG14L	Autophagy related gene-like	mTERF	Mitochondria transcription termination factor
BAK	BCL-2 antagonist or killer	mTOR	Mammalian target of rapamycin
BAX	BCL-2-associated X protein	mTORC1	mTOR complex 1
BCL-2	B-cell lymphoma 2	MTS	Mitochondrial-targeting sequence
BCL-xL	B-cell lymphoma-extra large	NFE2L2	Nuclear factor erythroid 2 related like 2
Becn1	Beclin 1	NFATC	Nuclear factor of activated T cells
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3	NF-κB	nuclear factor-κB
Ca^{2+}	Calcium	NLS	Nuclear localization sequence
CAMKIV	Calcium/calmodulin-dependent protein kinase IV	NOX	NADPH oxidase
C/EBP	CCAAT/enhancer-binding protein	NRF	Nuclear respiratory factor
CHOP	C/EBP homologous protein	NR4A1	Nuclear receptor subfamily 4 group A member 1
CK2	Casein kinase 2	$ONOO^-$	Peroxynitrite
CMA	Chaperone-mediated autophagy	OPA1	Optic atrophy protein 1
CREB	*cAMP-response element binding* protein	OXPHOS	Oxidative-phosphorylation
CsA	Cyclosporin-A	$O_2{\cdot}^-$	Superoxide
DAP 1	Death-associated protein 1	PE	Phosphatidylethanolamine
DFCP-1	Double FYVE containing protein 1	PRC	PGC-1-related co-activator
DM	Diabetes mellitus	PINK	Phosphatase and tensin homolog (PTEN)-induced putative kinase
DRP1	Dynamin-related protein	PIP	Phospholipid phosphatidylinositol phosphate
DVE-1	Defective proventriculus -1	PI3K	Phosphatidylinositol 3-kinase
EGR1	Early growth response protein 1	PI3P	Phosphatidylinositol 3-phosphate
eNOS	Endothelial nitric oxide synthase	PKA	Protein kinase A
ER	Endoplasmic reticulum	POLRMT	Mitochondrial RNA polymerase
ERAD	ER-associated degradation	PPARs	Peroxisome proliferator-activated receptors
ERR	Estrogen-related receptors	PGC	PPAR γ coactivator
ERα	Estrogen receptor α	RIP140	Receptor interacting protein 140
ETC	Electron transport chain	ROS	Reactive oxygen species
GABPα	GA-binding protein-α	SFA	Sanglifehrin-A
GLP-1RA	Glucagon-like peptide-1 receptors agonists	SGLT2	Sodium glucose cotransporter 2
FIS1	Fission 1	SIRT1	Sirtuin 1
FOXA-2	Forkhead box protein A2	SMAC	Second mitochondria-derived activator of caspases
FOXO1	Forkhead box protein O1	Sox9	SRY-Box Transcription Factor 9
FUNDC1	FUN14 domain containing 1	SQSTM1	Sequestosome 1
GTPases	Guanosine triphosphatase	SREBPs	Sterol regulatory element-binding proteins
GRP78	Glucose-regulated protein 78	TCA	Tricarboxylic acid cycle
HIF-1	Hypoxia inducible factor 1	TFAM	Transcription factor A, mitochondrial
HTN	Hypertension	TFB1M	Transcription Factor B1, Mitochondria
H_2O_2	Hydrogen peroxide	TFB2M	Transcription Factor B2, Mitochondria
IMS	Intermembrane space	TNF	Tumor necrosis factor
I/R	Ischemia/reperfusion	TNFR1	Tumor necrosis factor 1

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ATP	Adenosine triphosphate	MiD49	Mitochondrial dynamics protein of 49
IRI	Ischemia/reperfusion injury	Trail	TNF-related apoptosis-inducing ligand
JMJD	Jumonji C domain-containing protein	UPS	Ubiquitin /26S proteasome system
JNK	c-Jun N-terminal kinases	ULK-1	UNC-51–like kinase
LC3	Light chain-3	VPS34	Vacuolar protein sorting 34
LIR	LC3-interacting region	VEGF	Vascular endothelia growth factor
L-NAME	N^G-nitro-L-arginine methyl ester	WIPI2B	WD repeat domain phosphoinositide-interacting protein 2
MCL-1	Myeloid cell leukemia 1	XIAP	X-linked inhibitor of apoptosis protein
MFF	Mitochondrial fission factor	$\Delta\Psi$	Mitochondrial membrane potential
MFN1	Mitofusin 1		
MFN2	Mitofusin 2		

frontiers in Cell and Developmental Biology

REVIEW
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Mitochondrial OMA1 and OPA1 as Gatekeepers of Organellar Structure/Function and Cellular Stress Response

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Mammalian mitochondria are emerging as a critical stress-responsive contributor to cellular life/death and developmental outcomes. Maintained as an organellar network distributed throughout the cell, mitochondria respond to cellular stimuli and stresses through highly sensitive structural dynamics, particularly in energetically demanding cell settings such as cardiac and muscle tissues. Fusion allows individual mitochondria to form an interconnected reticular network, while fission divides the network into a collection of vesicular organelles. Crucially, optic atrophy-1 (OPA1) directly links mitochondrial structure and bioenergetic function: when the transmembrane potential across the inner membrane ($\Delta\Psi_m$) is intact, long L-OPA1 isoforms carry out fusion of the mitochondrial inner membrane. When $\Delta\Psi_m$ is lost, L-OPA1 is cleaved to short, fusion-inactive S-OPA1 isoforms by the stress-sensitive OMA1 metalloprotease, causing the mitochondrial network to collapse to a fragmented population of organelles. This proteolytic mechanism provides sensitive regulation of organellar structure/function but also engages directly with apoptotic factors as a major mechanism of mitochondrial participation in cellular stress response. Furthermore, emerging evidence suggests that this proteolytic mechanism may have critical importance for cell developmental programs, particularly in cardiac, neuronal, and stem cell settings. OMA1's role as a key mitochondrial stress-sensitive protease motivates exciting new questions regarding its mechanistic regulation and interactions, as well as its broader importance through involvement in apoptotic, stress response, and developmental pathways.

Keywords: mitochondria, apoptosis, OPA1, OMA1, development

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INTRODUCTION

The mitochondria of mammalian cells are increasingly understood to be a highly dynamic organellar network, using opposing fission and fusion pathways to homeostatically balance mitochondrial organization and bioenergetic function. Fusion of the inner membrane, mediated by optic atrophy-1 (OPA1), is a stress-sensitive mechanism of mitochondrial dynamic homeostasis, controlled by the OMA1 metalloprotease. Loss of OPA1 fusion causes the collapse of the mitochondrial network and promotes apoptosis. Our current understanding of OMA1's crucial role in mitochondrial dynamics demonstrates that this proteolytic mechanism has broad importance

to cell stress response, raising exciting new questions regarding OMA1's mechanistic regulation, participation in apoptosis, and novel roles in differentiation and development.

MITOCHONDRIAL DYNAMICS AND BIOENERGETIC FUNCTION

From their earliest descriptions as "thread-like granules" giving rise to their designation as mitochondria, these organelles have undergone a profound reappraisal to our current understanding of mitochondrial structure/function as a highly responsive, dynamic network. Early work using light microscopy allowed investigators to appreciate the filamentous nature of the mitochondrial network (Ernster and Schatz, 1981). The advent of thin-section transmission electron microscopy, including seminal works by Palade (1952) and Sjostrand (1953), advanced the understanding of the multimembrane organization of the organelle, in which the outer membrane envelopes the organelle, while the inner membrane opposes the outer membrane at the periphery of the organelle but also is organized into tubules or folds (cristae) that extend through the interior matrix compartment of the organelle (Frey and Mannella, 2000). This internal organization provides a maximized surface/area ratio as the site of oxidative phosphorylation (OXPHOS). The multisubunit electron transport Complexes I–IV utilize NADH and $FADH_2$ to establish a proton-motive transmembrane potential ($\Delta\Psi_m$). This electrochemical gradient then powers the F_1F_0 ATP synthase, which uses $\Delta\Psi_m$ to drive synthesis of ATP from ADP and Pi (DiMauro and Schon, 2003). As such, the mitochondrial inner membrane is highly specialized for bioenergetics, with structural adaptations to maximize metabolic function. Thin-section TEM images, while highly informative, also gave rise to the somewhat erroneous canonical textbook view of mitochondria as static, bean-shaped "batteries," with one or two of these organelles tucked away at the back of the cell. Advances in fluorescence and imaging technology led to a reappraisal of mitochondrial ultrastructure, revealing the pleiomorphic, dynamic nature of the mitochondrial structure as a highly interconnected reticular network, a population of isolated vesicular organelles, or a balance of the two states (Amchenkova et al., 1988; Rizzuto et al., 1998). At the same time, the specific factors governing these elegant organellar dynamics emerged, demonstrating a set of sensitive, responsive factors that govern mitochondrial structural dynamics by balancing both organellar fission and fusion events. Mitochondria undergo fission by the recruitment of the cytosolic dynamin-related protein-1 (DRP1) to the mitochondrial outer membrane, which forms a multimeric collar around the mitochondrial tubule and constricts for membrane scission (Smirnova et al., 2001). DRP1 is bound at the outer membrane by an array of interacting partners, including mitochondrial fission protein-1 (Fis1), mitochondrial fission factor (Mff) (Gandre-Babbe and van der Bliek, 2008), and mitochondrial dynamic factors of 49 kDa (MiD49) and 51 kDa (MiD51) (Loson et al., 2013; Palmer et al., 2013). A variety of cellular stimuli, including $\Delta\Psi_m$ uncouplers, low serum, or pro-apoptotic stimuli such as staurosporine and etoposide (Loson et al., 2013), cause phosphorylation-sensitive activation of DRP1's mitochondrial recruitment, leading to fission of the mitochondrial network (Dickey and Strack, 2011; Ji et al., 2015). **Figure 1** intriguing mechanistic questions remain for the mitochondrial fission machinery; for example, Voeltz and coworkers found that dynamin-2 (Dyn2) plays a role in completing separation of the two organelles during fission (Lee et al., 2016), while Raimundo's group found that DRP1 was sufficient to complete fission without Dyn2 and additional dynamin partners (Fonseca et al., 2019). Fission is balanced by a separate set of factors controlling mitochondrial fusion. Mitofusins 1 and 2 maintain fusion of the mitochondrial outer membrane, independent of bioenergetic function (Santel and Fuller, 2001; Chen et al., 2003). Fusion of the mitochondrial inner membrane, conversely, requires an intact $\Delta\Psi_m$ and is mediated by OPA1.

OPA1 AND OMA1: STRESS-SENSITIVE MITOCHONDRIAL FUSION

Optic atrophy-1 is an essential GTPase responsible for fusion of the mitochondrial inner membrane. There are a total of eight mRNA splice variants of *OPA1*, processed for tissue-dependent expression (Ishihara et al., 2006). Following translation, mitochondrial importation, and insertion into the inner membrane, the high concentration of cardiolipin (CL) allows for CL-OPA1 tethering or OPA1:OPA1 homotypic association, followed by GTP-dependent membrane fusion (Ban et al., 2017; **Figure 1**). Recent crystallographic and cryo-EM analyses of OPA1's yeast homolog Mgm1, provide new insights into how OPA1 remodels the inner membrane to mediate fusion (Faelber et al., 2019; Yan et al., 2020). In addition to facilitating inner-membrane fusion, OPA1 promotes dimerization of ATP synthase (Patten et al., 2014) and interacts with the multisubunit Mitochondrial contact site and Cristae Organizing System (MICOS) to help mediate cristae organization in addition to remodeling of the inner membrane (Hu et al., 2020; Stephan et al., 2020). Western blot analysis shows five distinct protein isoforms of OPA1: two long (L-OPA1) isoforms that mediate inner-membrane fusion and three short (S-OPA1) fusion-inactive isoforms. This pattern results from cleavage at OPA1's S1 and S2 sites, which release S-OPA1 into the intermembrane space (Griparic et al., 2007; Guillery et al., 2008). Basal levels of S-OPA1 are produced by constitutive cleavage of OPA1 at the S2 site (Griparic et al., 2004), producing a steady-state balance of long and short OPA1 isoforms. Intriguingly, treatment of cells with some pharmacological inhibitors of mitochondrial OXPHOS, such as valinomycin, oligomycin, or carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), but not others (rotenone, cycloheximide, antimycin A), causes inducible cleavage of L-OPA1 (Griparic et al., 2007; Guillery et al., 2008), demonstrating that L-OPA1 is specifically processed in response to loss of $\Delta\Psi_m$. This loss of fusion causes unopposed mitochondrial fission and fragmentation of the mitochondrial network (**Figure 2**). Moreover, the two distinct pathways may impact each other mechanistically: fission-active Fis1 binds

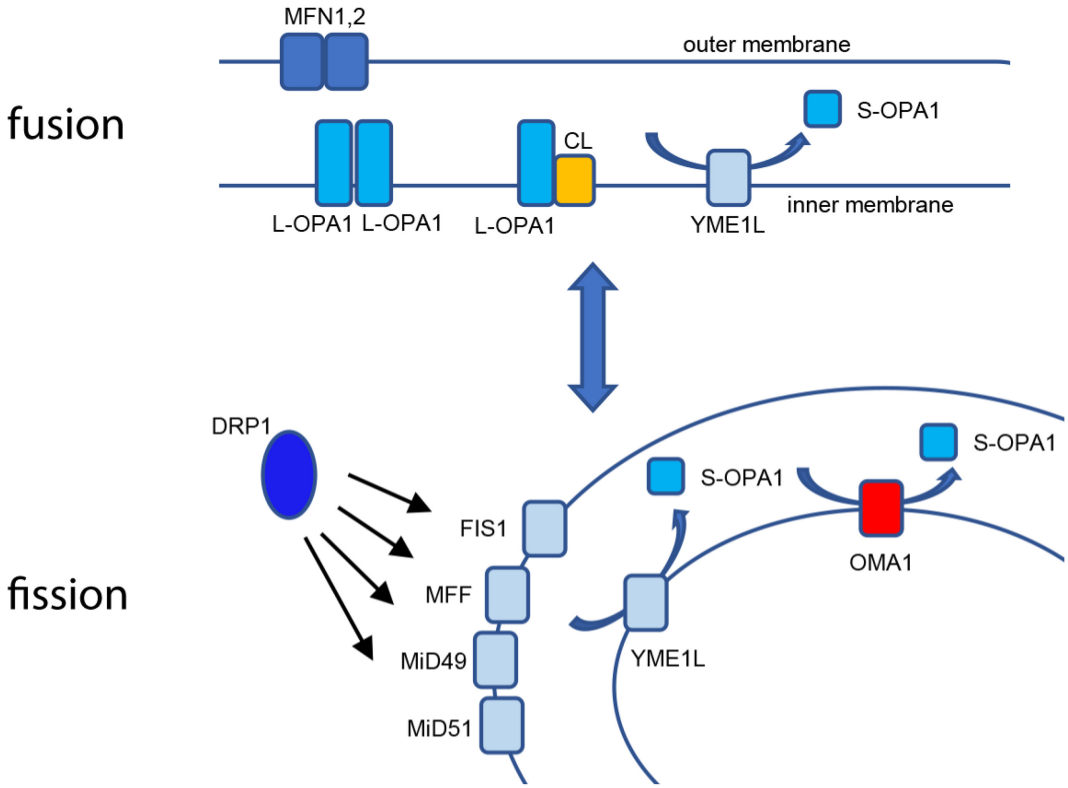

FIGURE 1 | Mitochondrial fusion and fission. Fusion of the mitochondrial outer membrane is carried out at MFN1 and 2, while L-OPA1 maintains continuity of the inner membrane, either by homotypic interaction or by binding cardiolipin (CL). YME1L constitutively cleaves L-OPA1, resulting in basal S-OPA1. Fission is mediated by recruitment of cytosolic DRP1 to the outer membrane using actin-dependent dynamics, where it is bound by mitochondrial binding partners FIS1, MFF, MiD49, and MiD51. When activated, OMA1 cleaves L-OPA1 to S-OPA1 in cooperation with YME1L for accumulation of fusion-inactive S-OPA1.

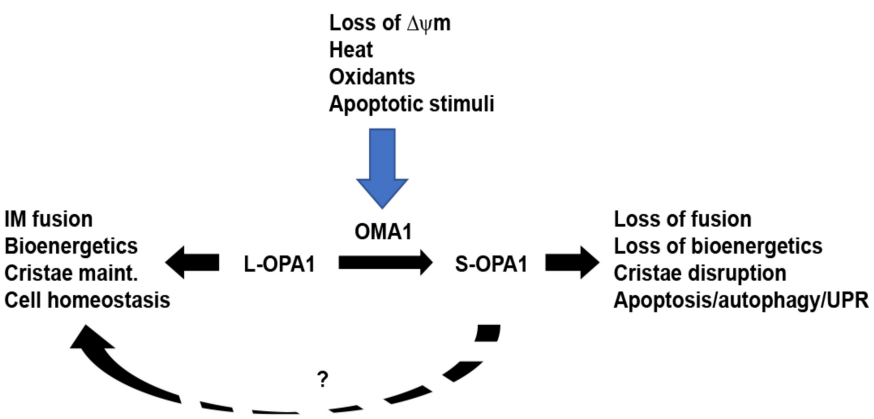

FIGURE 2 | OMA1 controls stress-sensitive cleavage of long OPA1 isoforms. Under steady-state conditions, mitochondria maintain a balance of long, fusion-active L-OPA1 and short, fusion-inactive S-OPA1 isoforms. While YME1L (not shown) causes constitutive cleavage of L-OPA1 to produce steady-state S-OPA1, the OMA1 metalloprotease is activated by a range of stress stimuli. Upon activation, OMA1 cleaves L-OPA1, causing accumulation of S-OPA1, mitochondrial fragmentation, and loss of mitochondrial bioenergetics. This, in turn, primes the cell for increased stress response via mechanisms including apoptosis, autophagy, and unfolded protein response. While S-OPA1 isoforms cannot mediate mitochondrial inner membrane fusion, they may contribute to maintaining mitochondrial homeostasis (dashed line, ?).

to MFN1 and 2, as well as OPA1 (Yu et al., 2019). These findings suggest that the interactions of the two distinct organelle remodeling pathways, both with each other and with bioenergetic function, are more complex than previously appreciated. Consistent with this, fission and fusion events both occur at sites of mitochondria-endoplasmic reticulum (ER)

contact (Friedman et al., 2011; Abrisch et al., 2020), indicating a higher-order spatial coordination of fission and fusion pathways.

The overlapping with m-AAA protease (OMA1) metalloprotease was concurrently identified as the stress-sensitive protease responsible for L-OPA1 cleavage upon dissipation of $\Delta\Psi_m$: Langer's group, having previously identified OMA1 in yeast as a mitochondrial metallopeptidase (Kaser et al., 2003), found that knockdown of OMA1 in mammalian cells prevents CCCP-induced OPA1 processing (Ehses et al., 2009), while van der Bliek's group similarly found that OMA1 is an inner-membrane-localized protease that mediates CCCP-inducible L-OPA1 cleavage (Head et al., 2009). OMA1 has since emerged as a major mitochondrial factor for sensing and responding to cellular stress. Subsequently, a range of stimuli have been shown to activate OMA1-mediated OPA1 processing, including oligomycin, ATP depletion (Rainbolt et al., 2016), oxidants (Rainbolt et al., 2015; Garcia et al., 2018), valinomycin, and heat (Baker et al., 2014). OMA1 acts in close cooperation with the *i*-AAA protease YME1L, with YME1L constitutively cleaving L-OPA1 for a steady-state balance of long and short OPA1 isoforms, while OMA1 is stress-activated to complete L-OPA1 cleavage. L-OPA1 is inducibly cleaved by OMA1 at the S1 site, while YME1L cleaves OPA1 at the S2 site (Anand et al., 2014). Cells lacking both YME1L and OMA1 show only L-OPA1 isoforms (Anand et al., 2014), while OMA1 becomes degraded itself following activation by CCCP (Zhang et al., 2014) in a YME1L-dependent manner (Rainbolt et al., 2016). More recently, a YME1L-dependent third OPA1 cleavage site (S3) has been identified (Wang et al., 2020). Mechanistically, OMA1 is localized to the mitochondrial inner membrane (Ehses et al., 2009; Head et al., 2009). The C-terminal M48 domain of OMA1, oriented toward the intermembrane space, is responsible for carrying out OMA1's proteolytic activity, while the matrix-oriented N-terminal domain appears to play an important role in sensing changes in $\Delta\Psi_m$: OMA1 variants lacking the positively charged N-terminal domain are unable to cleave L-OPA1 in response to loss of $\Delta\Psi_m$ (Baker et al., 2014). Recent work shows that localized fluctuations in $\Delta\Psi_m$, or "flickering," cause OMA1 activation events as a protective stress response against mitochondrial hyperfusion (Murata et al., 2020), demonstrating a highly sensitive, responsive mode of action. In addition to interacting with YME1L, OMA1 also appears to interact with other inner-membrane factors as part its emerging roles in apoptosis and other cellular stress response pathways.

OMA1, OPA1, AND CELLULAR APOPTOSIS

As elegant as the stress-sensitive mechanisms of OMA1-mediated OPA1 proteolysis are, their greater importance to the cell at large is becoming more broadly evident, given their mechanistic involvement in cell-wide stress responses including apoptosis, autophagy, and integrated stress response. A range of cell and organismal studies demonstrate that OPA1 homeostasis directly contributes to apoptosis and other cellular stress pathways, demonstrating a broader impact for mitochondrial dynamics on cellular life and death. Moreover, a growing literature supports developmental roles for OPA1 in cellular differentiation, particularly in energetically demanding contexts such as myocardial and neuronal cell settings.

The arrival of mitochondria as a mechanistic component of apoptosis significantly broadened the organelle's importance beyond bioenergetics. A variety of apoptotic stimuli activate the release of cytochrome c from the mitochondria to the cytosol, where it activates caspases for apoptotic cell death (Bossy-Wetzel et al., 1998). Strikingly, both loss and proteolytic processing of OPA1 are associated with apoptotic induction. Knockdown of OPA1 causes mitochondrial fragmentation, followed by cytochrome c release and apoptotic induction in HeLa cells (Olichon et al., 2002). Induction of apoptosis via Bim/tBid causes cleavage of L-OPA1 as part of Bax/Bak-mediated apoptosis (Jiang et al., 2014). OPA1's role in maintaining the cristae formation of the inner membrane allows it to play a role in remodeling the cristae to allow cytochrome c release upon induction of apoptosis (Cipolat et al., 2006), with evidence that this role is functionally distinct from OPA1's role in inner-membrane fusion (Frezza et al., 2006). OPA1-mediated cristae reorganization is indeed required for Bax-mediated cytochrome c release and apoptosis (Yamaguchi et al., 2008). Consistent with this, a range of findings demonstrate that OMA1 plays a key role in regulating apoptosis (**Figure 2**). In identifying OMA1 as the stress-responsive protease that cleaves L-OPA1, van der Bliek's group found that knockdown of OMA1 blunts staurosporine-induced apoptosis (Head et al., 2009). Similarly, pro-apoptotic Bax and Bak, which are recruited to the mitochondria upon apoptotic induction, activate OMA1, while OMA1 knockdown or knockout dramatically attenuates Bim/tBid-induced apoptosis (Jiang et al., 2014).

These cell-based findings are in agreement with organismal findings, in which genetic modification of OMA1/OPA1 homeostasis dramatically impacts physiology. Activation of OMA1 in mouse heart leads to mitochondrial fragmentation and disrupted metabolism, causing dilated cardiomyopathy and heart failure (Wai et al., 2015), while genetic ablation of OMA1 prevents OPA1 cleavage, delaying neuronal apoptosis in a murine neurodegeneration model (Korwitz et al., 2016), as well as mouse models of heart failure (Acin-Perez et al., 2018). Consistent with this, OMA1 silencing confers increased cell proliferation and migration in patient-derived metastatic cancer cells (Daverey et al., 2019), while adenoviral delivery of OPA1 rescues mitochondrial dysfunction in *in vitro* models (Maloney et al., 2020). Taken together, these findings suggest that OMA1's role in controlling stress-sensitive OPA1 cleavage has crucial importance to cellular fate through modulation of apoptosis.

NEW QUESTIONS: MOLECULAR INTERACTIONS, APOPTOTIC PRIMING, AND DEVELOPMENTAL ROLES

The emerging role of OMA1 as a critical stress-sensitive protease responsible for mitochondrial homeostasis, as well as broader cellular stress response, motivates a range of intriguing questions regarding's interactions and regulation at the inner membrane,

as well as the mechanistic contributions of OMA1 to cellular apoptosis. These underlying mechanisms may also have key developmental roles for cells in a variety of lineages, as a small but growing literature indicates that OMA1 and OPA1 are important for differentiation and development.

OMA1's proteolytic activation and regulatory interactions with other factors represent key areas of mechanistic inquiry for the field. OMA1 interacts with, and is likely to be regulated by, factors including YME1L (Anand et al., 2014; Rainbolt et al., 2016), P32 (Noh et al., 2020), prohibitin (Anderson et al., 2019), and AFG3L2, indicating that the activation of OMA1 within the inner membrane is likely controlled by a complex set of events and interactions within the mitochondrial interior. Within the inner membrane, OMA1 appears to associate as a hexameric oligomer (Levytskyy et al., 2017). While this multimeric OMA1 interacts with the multiple interacting partners described above, it is unclear how this occurs within the inner membrane. Moreover, the N-terminal $\Delta\Psi_m$ sensor domain is oriented on the matrix side of the inner membrane and is required for stress-sensitive proteolytic activity (Baker et al., 2014), but exactly how this positively charged, loosely structured domain senses changes in $\Delta\Psi_m$ and activates OMA1's proteolytic activity is unclear. To further our working understanding of OMA1 regulation, these fundamental mechanisms, as well as the regulatory effects of the interacting factors, require clarification.

Moreover, the precise molecular mechanisms behind OMA1's role in "priming" apoptosis remain to be determined: loss of $\Delta\Psi_m$ precedes translocation of Bax to the mitochondria but is not sufficient in and of itself to induce apoptosis (Bossy-Wetzel et al., 1998; Smaili et al., 2001). Furthermore, the precise functional roles of the long and short OPA1 isoforms remain unclear. While L-OPA1 isoforms are clearly required for inner-membrane fusion, recent findings indicate that the cleaved short S-OPA1 isoforms, often thought to be non-functional due to their inability to mediate membrane fusion, actually play roles in maintaining mitochondrial bioenergetics and cristae structure (Lee et al., 2017) and may confer protection against oxidative necrotic cell death (Lee et al., 2020). These findings raise new questions regarding whether the loss of L-OPA1 or the accumulation of S-OPA1 is mechanistically responsible for the apoptotic priming associated with OMA1 activation. This illustrates the range of questions remaining to be resolved regarding the mechanistic impacts of OMA1 and OPA1 on apoptosis in mammalian cells. Similarly, OMA1 is directly involved in activating the cell-wide integrated stress response (ISR). OMA1 cleaves mitochondrially localized DELE1, releasing it to the cytosol, where it interacts with HRI to activate EIF2a, initiating integrated stress response (Fessler et al., 2020; Guo et al., 2020). Loss of OPA1 activates unfolded protein response, associated with age-related muscle loss and inflammation (Tezze et al., 2017), while increased L-OPA1 suppresses mitochondrial autophagy (Lang et al., 2017). Collectively, these findings reveal a central role for OMA1 and OPA1 as mitochondrial stress sensors, with crucial impacts on cell-wide stress response mechanisms.

Given the importance of stress-sensitive OPA1 balance to mitochondrial structure/function balance, as well as cellular apoptosis and stress-response pathways, it is a short leap of logic to envision a role for OPA1 in cellular development. Consistent with this, a modest but growing literature is emerging to reveal key roles for OPA1 in neuronal and cardiac differentiation. Recently, OPA1 was shown to be required for development of GABAergic neurons from embryonic stem cells (Caglayan et al., 2020), while haploinsufficient OPA1 iPSCs demonstrate degeneration of dopaminergic neurons (Jonikas et al., 2018). Gene trapping of OPA1 in murine ESCs causes impaired cardiac differentiation and development (Kasahara et al., 2013). These findings provide intriguing clues to novel OPA1 developmental roles for OPA1 homeostasis, providing a new direction for OPA1's impact beyond organellar dynamics and apoptosis.

Taken together, these findings reveal that OMA1 and OPA1 control a highly sensitive mechanism for mitochondrial structure/function homeostasis but also play outsized roles in crucial cell-wide signaling pathways including apoptosis and development. A range of intriguing mechanistic questions remain to be answered in characterizing the mechanisms and broader impacts of this mitochondrial "gatekeeper" mechanism. The interactions of OMA1 and OPA1 with an increasing number of inner-membrane proteins and lipids suggest that delineating the higher-order organization of proteases, scaffolding proteins, and interacting lipids within the inner membrane will be critical to effectively understanding the mechanisms of mitochondrial structure/function homeostasis and apoptotic stress response.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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