PERTURBATIONS IN METABOLIC CUES: IMPLICATIONS FOR ADVERSE CARDIAC FUNCTION LEADING TO SUDDEN CARDIAC DEATH

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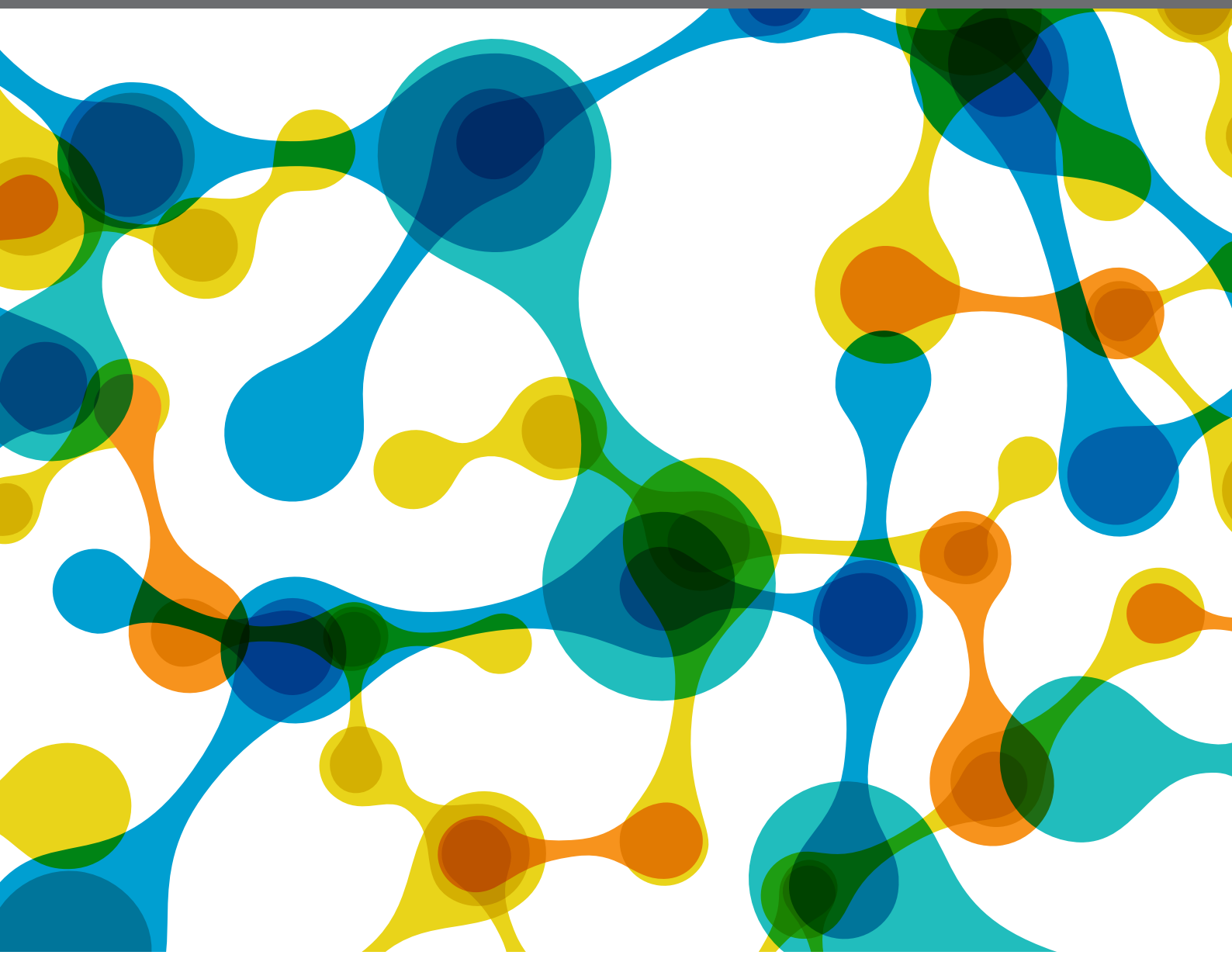

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PERTURBATIONS IN METABOLIC CUES: IMPLICATIONS FOR ADVERSE CARDIAC FUNCTION LEADING TO SUDDEN CARDIAC DEATH

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EDITORIAL
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Editorial: Perturbations in Metabolic Cues: Implications for Adverse Cardiac Function Leading to Sudden Cardiac Death

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Editorial on the Research Topic

Perturbations in Metabolic Cues: Implications for Adverse Cardiac Function Leading to Sudden Cardiac Death

Sudden cardiac death (SCD) remains a leading cause of death in the United States and accounts for ∼460,000 deaths annually (Zheng et al., 2001; Roger et al., 2012). Importantly, there is increasing evidence that metabolic syndrome, a disease process defined by a clustering of pathologies (obesity, diabetes, dyslipidemia and inflammation), is a significant contributor to the risk for SCD (Yang et al., 2015). Therefore, it is important to understand the intertwined complexity of metabolic disorders and how cardiomyocytes respond to the associated electrical and structural perturbations. In order to develop novel and effective strategies to prevent SCD in people with metabolic disorders there is a need to identify metabolic risk factors. In this Research Topic we present a series of articles from experts in various aspects of metabolic disorders and heart disease. Our hope is that the research ideas shared in these articles will create new discussions around existing gaps in knowledge and help initiate future metabolic studies in cardiac health and disease.

The Research Topic starts with a review article from van Opbergen et al. that describes an important role for pathological mitochondrial function in the development of arrhythmogenic cardiomyopathy (ACM), an electrical disease mechanism that increases the risk for SCD, particularly in young people and athletes (Corrado et al., 2017). While impaired mitochondrial function has been widely studied in the context of arrhythmogenesis, how this occurs in ACM remains unclear. The authors highlight defective mitochondrial ATP production and altered redox regulation as key mechanisms that may increase vulnerability to ACM. Thus, ACM studies that incorporate mitochondrial biology are emphasized with the expectation for novel mechanistic insights for the development of targeted therapies in people living with ACM.

A similar perspective to van Opbergen et al. is provided by Snyder et al.. In their review article, they discuss a critical role for cardiac metabolism, particularly as it relates to the electrical and structural properties that define normal cardiac sinus rhythm. Furthermore, they raise the possibility that a metabolism-based therapeutic strategy may be a promising approach in people with heart disease. The review by Xu et al. aims to emphasize the critical link between impaired cardiac metabolism and SCD. They elegantly guide the reader through the nicotinamide adenine dinucleotide (NAD^+) pathway and how its impaired function may contribute to SCD. The authors conclude with a convincing argument in favor of NAD^+-boosting therapies in people living with cardiovascular disease.

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In a continuing dialogue about ATP production, myocardial energy metabolism and cardiac contractility, the review by Greenwell et al. provides a comprehensive perspective into distinct metabolic triggers that underlie acquired or genetic cardiomyopathies. Taken together, these reviews offer an intriguing prospect of how existing, as well as emerging data, suggest that metabolic pathways are spatially and temporally intertwined at multiple levels. Therefore, future studies that consider potential outcomes due to multiple or compartmentalized pathologies are likely to be informative and shift current paradigms.

Impaired beat-to-beat Ca^{2+} dynamics predispose to fatal arrhythmias, leading to heart failure (HF) (Balke and Shorofsky, 1998; Mora et al., 2019), and two research articles on this topic provide crucial evidence for reduced expression of cardiac bridging integrator 1 (cBIN1), a membrane curvature protein that generates Ca^{2+} microdomains at the myocyte t-tubular system. First, Hitzeman et al. demonstrated a novel finding that a high cBIN1 score (or CS) is an important marker of pathologic cardiac remodeling and predicts a 1-year risk of cardiovascular events in ambulatory people who have HF with preserved ejection fraction. This conclusion is also supported by the findings of Li et al.. They revealed that, in a mouse model of pre-existing HF, exogenous cBIN1 limited HF progression, improved cardiac function, and increased survival by normalizing t-tubule Ca handling microdomains. These findings are clinically relevant and further underscore the importance of CS testing with the ability to detect smaller changes in t-tubule cBIN1 levels in pre-HF, especially those cases that are associated with metabolic disorder.

Two articles in this Research Topic focused on circadian mechanisms, arrhythmias, and risk for SCD. Bernardi et al. explores how the circadian-dependent modulation of metabolic and environmental cues, lead to altered hormonal and autonomic signaling and contribute to arrhythmias. Schroder et al. investigated how the disruption of the cardiac circadian clock by inducing the deletion of *Bmal1*, the core circadian clock transcription factor, impacts cardiac function in a knock-in mouse ($Scn5a^{+/\Delta KPQ}$) model of long QT syndrome type 3 (LQT3). The authors demonstrated that inducing the deletion of *Bmal1* in cardiomyocytes decreased the expression of several different cardiac ion channel mRNA transcripts and increased the QT interval at slow heart rates in wild-type and $Scn5a^{+/\Delta KPQ}$ mice.

The relationship between the metabolic syndrome and acquired arrhythmias was also studied by Yang et al. who investigated the effects of low magnesium (or hypomagnesemia) on electrocardiographic (ECG) parameters. Hypomagnesemia is acquired in people with metabolic disorders (Guerrero-Romero and Rodríguez-Morán, 2002), and here Yang et al. shows that people with hypomagnesemia displayed significant ECG changes, including an increase in the heart rate corrected QT interval and P wave duration, consistent with both impaired atrial and ventricular repolarization. Overall, these findings highlight how individual metabolic mechanisms may independently modify cardiac function.

The article by Espinoza et al. highlights the interplay between cardiometabolic function, parasympathetic (or vagal) motor output and the effects on cardiac function. Specifically, the authors focus their discussion on how vagal motor output may modulate metabolic cues and affect cardiac function. The authors state that vagal brainstem circuits provide a unique integrative mechanism that regulates and responds to metabolic cues to impact cardiac function.

Impaired thyroid function and the metabolic syndrome are interlinked endocrine disorders with significant morbidity and mortality in people worldwide. Additionally, thyroid hormone (TH) plays an important role in modulating cardiac electrophysiological properties. The article by Yamakawa et al. discusses the current knowledge on TH action on the cardiovascular system, relevant clinical and hemodynamic laboratory findings, and the therapeutic management for people with hyper- or hypo-thyroid heart disease. They also discuss cardiovascular medications that can disrupt normal thyroid function, including amiodarone-induced thyroid toxicity.

Overall, this Research Topic continues the discussion for how perturbations in several distinct metabolic mechanisms can have significant implications for cardiac function. Specifically, the articles published in this topic highlight how normal and impaired metabolic cues can impact electrical, structural, neural, and hormonal modulation of cardiac function. Expanding research that explores how metabolic disorders impact and contribute to heart disease will advance the field in ways that improve therapeutic options, better the quality of life for people living with heart disease and reduce the risk for SCD.

AUTHOR CONTRIBUTIONS

BD and ASA contributed significantly to this work, finalized, and approved it for publication. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Mitochondrial Dysfunction as Substrate for Arrhythmogenic Cardiomyopathy: A Search for New Disease Mechanisms

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Arrhythmogenic cardiomyopathy (ACM) is a familial heart disease, associated with ventricular arrhythmias, fibrofatty replacement of the myocardial mass and an increased risk of sudden cardiac death (SCD). Malignant ventricular arrhythmias and SCD largely occur in the pre-clinical phase of the disease, before overt structural changes occur. To prevent or interfere with ACM disease progression, more insight in mechanisms related to electrical instability are needed. Currently, numerous studies are focused on the link between cardiac arrhythmias and metabolic disease. In line with that, a potential role of mitochondrial dysfunction in ACM pathology is unclear and mitochondrial biology in the ACM heart remains understudied. In this review, we explore mitochondrial dysfunction in relation to arrhythmogenesis, and postulate a link to typical hallmarks of ACM. Mitochondrial dysfunction depletes adenosine triphosphate (ATP) production and increases levels of reactive oxygen species in the heart. Both metabolic changes affect cardiac ion channel gating, electrical conduction, intracellular calcium handling, and fibrosis formation; all well-known aspects of ACM pathophysiology. ATP-mediated structural remodeling, apoptosis, and mitochondria-related alterations have already been shown in models of PKP2 dysfunction. Yet, the limited amount of experimental evidence in ACM models makes it difficult to determine whether mitochondrial dysfunction indeed precedes and/or accompanies ACM pathogenesis. Nevertheless, current experimental ACM models can be very useful in unraveling ACM-related mitochondrial biology and in testing potential therapeutic interventions.

Keywords: arrhythmogenic cardiomyopathy, cardiac metabolism, mitochondria, ATP, oxidative stress, plakophillin-2, calcium handling, connexin 43

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INTRODUCTION

Arrhythmogenic cardiomyopathy (ACM) is a familial heart disease, associated with ventricular arrhythmias and an increased risk of sudden cardiac death (SCD). SCD often occurs as the first clinical manifestation, in particular in athletes and young adults (Basso et al., 2012). The prevalence of ACM in the general population ranges from 1:2000 to 1:5000, meaning that this disease is listed among the rare cardiovascular diseases (Basso et al., 2018). Age at time of diagnosis varies

widely, with most patients being first diagnosed between 20 and 50 years of age (Bennett et al., 2019). ACM is characterized by progressive replacement of the myocardium with fatty and fibrous tissue, ending in impairment of the ventricular systolic function and ventricular dilatation (Corrado et al., 2017a). Cardiac remodeling in ACM patients is of right ventricular predominance, though left-ventricular or bi-ventricular involvement has been recognized (Bennett et al., 2019). The fibrofatty substitution likely contributes to the development of ventricular arrhythmias by creating an anatomic substrate (Corrado et al., 2017b) though cellular mechanisms of initiation have been invoked. ACM is considered an inheritable disease, as approximately 60% of the patients carry a genetic mutation, though likely not all genetic mutations related to ACM have been identified yet (Moncayo-Arlandi and Brugada, 2017). ACM is commonly considered as a disease of the intercalated disc (ID), whereby mutations in *Plakophilin-2* (*PKP2*) associate with most cases of ACM in which a genetic cause can be found. Mutations in other genes that encode desmosomal proteins [Desmocollin2 (DSC2*)*, Desmoglein2 (DSG2*)*, Plakoglobin (PKG*)*, Desmoplakin (DSP)], have been linked to ACM as well (Basso et al., 2018). Desmosomes mediate cell–cell mechanical coupling of adjacent cardiomyocytes at the ID. ID proteins are not just considered "junctional" and "non-junctional" single function entities, but multitasking molecular complexes that jointly regulate electrical conduction and mechanical force (Leo-Macias et al., 2016a,b). Recent studies show that ID proteins are also involved in modulation of transcription pathways fundamental for homeostasis within cardiomyocytes. Specifically, the transcription of genes involved in the intracellular calcium (Ca^{2+}) homeostasis can be modified by the expression of PKP2 (Cerrone et al., 2017; Montnach et al., 2018). Besides that, PKP2 loss provokes non-transcriptional related Ca^{2+} handling dysregulation, via ID-located Cx43 hemichannels and a change in the phosphorylation state of the Ryanodine receptor (RyR2) (Kim et al., 2019). This stands along the notion that PKP2, like many others in the cardiac myocyte, is a pleiotropic gene (Cerrone et al., 2019), and it highlights the fact that ACM is not only a desmosomal disease, but aberrant activation of intracellular signaling pathways and subsequent destabilization of the intracellular homeostasis likely provokes the structural and electrical changes in the heart. Besides desmosomal mutations, ACM can also be caused by mutations in genes directly involved in cardiac Ca^{2+} dynamics, such as the ryanodine receptor (RyR2) and phospholamban (PLN). Variants in RyR2 are classically linked to the inherited arrhythmogenic disease Catecholaminergic polymorphic ventricular tachycardia (CPVT), although RyR2 missense mutations have also been found in ACM patients (Tiso et al., 2001). Moreover, the Dutch founder mutation PLN-R14del has been associated with the development of ACM (Van Der Zwaag et al., 2013).

Sudden cardiac death in ACM patients often occurs in the subclinical phase of the disease, before structural changes are present. To prevent or interfere with ACM disease progression, more insight in the mechanisms related to electrical instability in ACM hearts is needed. At present, there is widespread interest on the link between cardiac arrhythmias and metabolic disease. A common focal point is the case of Diabetes Mellitus (DM) where a changed metabolic state and altered mitochondrial balance caused by elevated blood glucose levels, glucose fluctuation, and hypoglycemia can set the stage for cardiac arrhythmias (Grisanti, 2018). The altered energy metabolism and increased oxidative stress in DM cardiomyocytes have proven to influence intracellular Ca^{2+} handling, activate Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), and cause mitochondria-induced cell death and fibrosis (El Hadi et al., 2019). Apoptosis and necrosis are important elements in structural remodeling of ACM hearts and mitochondria-related alterations have been shown in iPSC-derived cardiomyocytes (iPSC-CMs) from a patient with mutated PKP2 (Nishikawa et al., 1999; Caspi et al., 2013; Kim et al., 2013; Austin et al., 2019). Mitochondria are essential for providing adenosine triphosphate (ATP) to the cell and satisfying the energy demand for electrical activity and contraction (Yang et al., 2014). Mitochondria are also involved in the intracellular Ca^{2+} homeostasis of cardiomyocytes and ATP generated by the mitochondria fuels various ion pumps (Doenst et al., 2013; Kolwicz et al., 2013). Mitochondrial dysfunction can thereby affect the electrical stability of cardiomyocytes and favor arrhythmogenesis. In the specific case of ACM, this hypothesis has not been tested yet, though it is tempting to speculate that metabolic and mitochondrial dysfunction can serve as substrates for electrical and structural changes in ACM patients. Especially since previous studies have indicated differences in mitochondrial metabolism between the left ventricle (LV) and right ventricle (RV) in the heart and ACM is regarded a (right) chamber specific disease (Schlüter et al., 2018). In this review, we describe the mitochondrial (patho)physiology, the link between mitochondrial dysfunction and development of cardiac arrhythmias and explore a potential relation between mitochondrial dysfunction and typical hallmarks of ACM.

CARDIAC MITOCHONDRIAL (PATHO)PHYSIOLOGY

The mechanical force required for cardiac contraction consumes large amounts of energy, which needs to be replenished via the mitochondria. The heart is capable of utilizing all classes of energy substrates for ATP production, including carbohydrates, lipids, amino acids, and ketone bodies (Kolwicz et al., 2013). Lactate and glucose are the major sources for energy production during the fetal stage. After birth, the heart undergoes a metabolic adaptation, switching substrate oxidation from glucose to fatty acids (FA). β-oxidation of free FA and oxidative phosphorylation (OXPHOS) become the primary mechanisms for ATP production and produces approximately 70% of all ATP in the heart (Torrealba et al., 2017). In the healthy heart, ATP production via OXPHOS is in balance with the rate of ATP hydrolysis. This balance mediates a constant ATP level in the cell, even during intense exercise (Stanley et al., 2005). The importance of mitochondrial function in cardiomyocytes is highlighted by their high abundance in the cell. Mitochondria occupy roughly 33% of the cellular volume in each ventricular cardiomyocyte and generate more than 95% of ATP consumed

by the heart (Williams et al., 2015). ATP production is established via the mitochondrial membrane potential ($\Delta\Psi m = -180$ mV). The $\Delta\Psi m$ creates a proton motive force which provides energy necessary to phosphorylate adenosine diphosphate (ADP) to ATP (Gambardella et al., 2017). This mechanism is also one of the main sources for reactive oxygen species (ROS) in the cell (Yang et al., 2014). Under physiological conditions, $\Delta\Psi m$ is tightly controlled and ATP production is in equilibrium with the energy demands of the cell. In response to pathological stimuli, such as ischemia or structural injury, alterations in $\Delta\Psi m$ diminish the cellular ATP level and increase ROS production (Gambardella et al., 2017). If ROS production exceeds the detoxifying (scavenging) capacity of the cell, it creates oxidative stress (Gambardella et al., 2017).

The two most important factors regulating the cardiac energy production are cellular concentrations of ADP and Ca^{2+} (Granatiero et al., 2017). Cytosolic Ca^{2+} levels tightly regulate enzymes of the tricarboxylic acid cycle (TCA) cycle and excitation-contraction coupling in the heart, thereby linking myocyte contraction and energy production (Granatiero et al., 2017). The heart uses about 60–70% of all generated ATP to facilitate contraction, the remaining 30–40% is used for control of various ion pumps, such as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) (Doenst et al., 2013). About 15% of the cardiac energy is consumed via SERCA-ATPase activity, which highlights the energy cost of active Ca^{2+} signaling in the heart (Granatiero et al., 2017). The cellular Ca^{2+} homeostasis is maintained by both SR and mitochondrial Ca^{2+} cycling (Gadicherla et al., 2017). Mitochondria are defined by two structurally and functionally different membranes, the outer membrane (OMM) and the inner membrane (IMM) which surround the intermembrane space (IMS). The mitochondrial membrane contains large amounts of invaginations enclosing the mitochondrial matrix, referred to as "cristae." Cristae junctions are tubular structures, which regulate and limit the random diffusion of molecules into the IMS. Mitochondria are linked to several other intracellular structures, especially the SR, enabling Ca^{2+} and lipid transfer between these two organelles. The mitochondrial matrix $[Ca^{2+}]$ is tightly regulated via mitochondrial Ca^{2+} influx and efflux. Ca^{2+} influx requires a balanced mitochondrial membrane potential (negative charge inside) and an electrogenic Ca^{2+} uniporter (Granatiero et al., 2017). Ca^{2+} crosses the outer mitochondrial membrane (OMM) through voltage dependent anion channels (VDAC), large channels that are permanently permeable to Ca^{2+}. VDAC permits Ca^{2+} flux into the intermembrane space and the mitochondrial Ca^{2+} uniporter (MCU) allows Ca^{2+} entry from the intermembrane space into the mitochondrial matrix. The mitochondrial $[Ca^{2+}]$ plays an important role in regulation of mitochondrial ATP production (Williams et al., 2015; De Stefani et al., 2016). Ca^{2+} that enters the mitochondrial matrix can be pumped back into the intermembrane space via the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX) and via ubiquitous H^{+}/Ca^{2+} exchange. This means that the mitochondrial $[Ca^{2+}]$ is set by the dynamic balance between MCU Ca^{2+} entry and Ca^{2+} extrusion via the mNCX (Williams et al., 2015). Under physiological circumstances, the mitochondrial Ca^{2+} influx and efflux is relatively small and unlikely to affect the cytosolic Ca^{2+} concentration (Williams et al., 2015). However, a prolonged and sustained increase of the mitochondrial Ca^{2+} level opens the permeability transition pore (mPTP), collapses the membrane potential ($\Delta\Psi m$) and activates apoptotic pathways (De Stefani et al., 2016).

Under pathological conditions, the fuel metabolism of the heart shifts back from FA oxidation (FAO) to an increased reliance on glucose, like in the fetal heart (Doenst et al., 2010, 2013). In addition, the metabolic gene expression shifts back to a fetal profile and the overall oxidative metabolism and energy reserve is thus reduced (Tuomainen and Tavi, 2017). Increased glucose consumption is characterized by enhanced glucose uptake and glycolysis, without prominent adaptations in glucose oxidation. As a consequence, glucose uptake and glucose oxidation become outbalanced. In combination with decreased FAO, the mitochondrial oxidative metabolism capacity gets depleted and ultimately the cardiac energy provision shrinks (Hajri et al., 2001). Cardiac hypertrophy and heart failure also enhance ketone body oxidation and disturb amino acid metabolism, particularly the branched-chain amino acid (BCAA) catabolism (Tuomainen and Tavi, 2017). The exact metabolic consequences and functional relevance of these changes remain to be understood.

MITOCHONDRIAL-ASSOCIATED CARDIAC ARRHYTHMIAS

Cardiomyocyte excitability and electrical cell–cell coupling are critical factors for electrical activation and a synchronized contraction of the heart. Changes in ion channel composition, function, and localization increase the susceptibility toward arrhythmias (Marbán, 2002). Multiple ion channels undergo structural and functional remodeling driven by pathophysiological stimuli, such as generated by mitochondrial dysfunction (Marbán, 2002). In this chapter, we will outline the effects of mitochondrial dysfunction on parameters critical for cardiomyocyte excitability, electrical impulse propagation and contractility, likely predisposing to cardiac arrhythmias.

ATP Driven Arrhythmogenic Substrate

During mitochondrial ATP production, mitochondrial proteins, and enzymes are subjected to various post-translational modifications, such as phosphorylation and acetylation. These modifications can alter the activity of metabolic enzymes and affect downstream metabolic pathways (Parihar and Parihar, 2017). Enzymes important in FA metabolism, TCA cycle metabolism, electron transport chain (ETC) and OXPHOS can severely be dysregulated by an imbalanced ATP production (Parihar and Parihar, 2017). Dysfunction of the mitochondrial oxygen consuming capacity leads to destabilization of $\Delta\Psi m$, which causes diminished ATP production and eventually energy deficiency in the cardiomyocyte (Parihar and Parihar, 2017). The impact of mitochondria on cellular excitability is mainly mediated by energy sensing, ATP sensitive K^{+} channels on the sarcolemma (sarcKATP) (Gambardella et al., 2017). These

channels are negatively regulated via intracellular ATP levels and form a crucial link between metabolism and electrical function of the cardiomyocyte. An altered intracellular ATP/ADP ratio, as a consequence of mitochondrial dysfunction, results in opening of sarcKATP channels. This increases the K^+ efflux and shortens the action potential duration (APD) (Gambardella et al., 2017). Shortening of the APD via sarcKATP channels reduces the time for Ca^{2+} influx via L-type Ca^{2+} channels (LTCC) during the action potential plateau phase and increases the time for Ca^{2+} extrusion via NCX (Nakaya, 2014). As a consequence, less intracellular Ca^{2+} is available and excitation-contraction coupling of cardiomyocytes is impaired (Gambardella et al., 2017). Via this mechanism, insufficient ATP levels in the cardiomyocyte diminish the contractile capacity of the cell. If a large amount of sarcKATP channels open at the same time, they hold the membrane potential very close to the K^+ Nernst equilibrium potential and keep the cardiomyocytes in a non-excitable state (Brown and O'Rourke, 2010). As consequence, in regions where sarcKATP channels are open due to metabolic stress, a 'sink' is created for depolarization of the myocardium. This phenomenon is called the 'metabolic sink.' It impairs electrical conduction and is suggested to be an important substrate for cardiac arrhythmias (**Figure 1**) (Brown and O'Rourke, 2010). Blocking sarcKATP channels with HMR1883 showed to reduce the incidence of ventricular arrhythmia in rats, rabbits, pigs, and dogs (Billman et al., 1998; Wirth et al., 1999; Fischbach et al., 2004; Vajda et al., 2007). These findings have been confirmed in clinical studies where sarcKATP channel blockers reduced the incidence of ventricular arrhythmias in patients with decompensated heart failure and DM (Cacciapuoti et al., 1991; Aronson et al., 2003).

Oxidative Stress Related Arrhythmogenic Substrate

A different and important aspect of mitochondrial dysfunction is the production of ROS, which may result in oxidative stress (Gambardella et al., 2017). Oxidative stress is defined as the imbalance between the generation of ROS and the cellular antioxidant defense (El Hadi et al., 2019). ROS are unstable molecular structures such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hydroxyl radicals (OH). They can damage proteins and lipids within the cell, activate intracellular signaling cascades and induce cellular dysfunction or cell death (Tse et al., 2016; El Hadi et al., 2019). To protect cellular functions from ROS, cells have two defense mechanisms: enzymatic and non-enzymatic pathways. The enzymatic pathway includes superoxide dismutase, catalase, and glutathione peroxidase. Together they are responsible for breaking down superoxide into water and oxygen. The non-enzymatic pathways include a variety of redox-defense systems, for example antioxidant scavengers (Jeong et al., 2012). The arrhythmogenic effect of ROS depends on ROS-induced ROS release (RIRR). This is an autocatalytic process by which high levels of ROS induce further ROS release from mitochondria (Gambardella et al., 2017). Increased oxidative stress through ROS is associated with the abnormal function of intracellular organelles, including the SR and mitochondria (Tse et al., 2016).

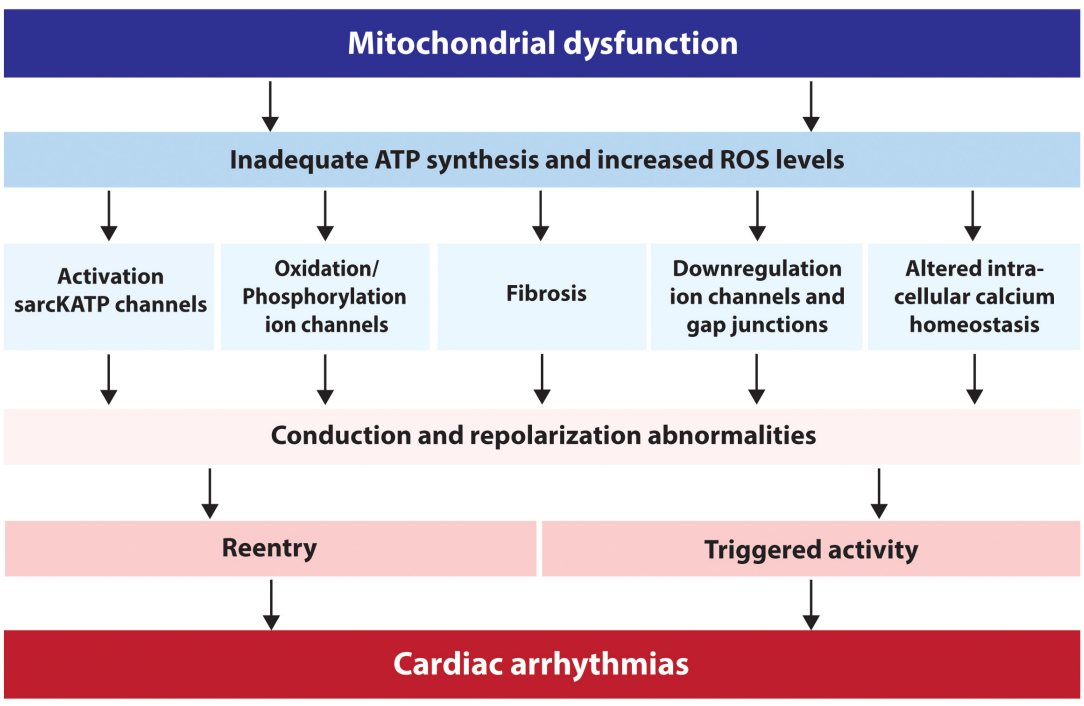

FIGURE 1 | Schematic overview of the factors via which mitochondrial dysfunction can lead to cardiac arrhythmias. ATP, adenosine triphosphate; sarcKATP, ATP sensitive potassium channels on the sarcolemma; ROS, reactive oxygen species.

Via several pathways, oxidative stress leads to repolarization- and conduction abnormalities, eventually resulting in cardiac arrhythmias (**Figure 1**) (Tse et al., 2016). For example, destabilization of $\Delta\Psi m$ can cause oxidation of ion channels via ROS, and/or indirectly through phosphorylation of ion channels via activation of protein kinases (**Figure 1**) (Foteinou et al., 2015; Yang et al., 2015). Increased ROS production can also activate the unfolded protein response (UPR) that accordingly reduces ion channel functionality and creates repolarization abnormalities (Tse et al., 2016). In addition, oxidative stress impairs gap junction conduction and electrical coupling between cardiomyocytes. Elevation of ROS levels decreases the amount of Cx43 protein in the cell and hampers gap junction conduction, resulting in cardiac arrhythmias and SCD (**Figure 1**) (Iravanian et al., 2011; Sovari et al., 2011). Moreover, ROS production alters the intracellular Ca^{2+} homeostasis (by inducing SR Ca^{2+} sparks and increasing cytosolic Ca^{2+} levels) and causes fibrosis formation (Voigt et al., 2012; Köhler et al., 2014) creating two additional substrates for conduction abnormalities, triggered activity and re-entry based arrhythmias (**Figure 1**) (Köhler et al., 2014). The relationship between ROS production, Ca^{2+} handling disturbances and structural remodeling will be explained later in this review.

Effect of Oxidative Stress on Cardiac Calcium Dynamics

Mitochondrial Calcium Overload and Oxidative Stress

The link between disturbed cytosolic Ca^{2+} levels and cardiac arrhythmias is well-established, although mitochondrial Ca^{2+} dysregulation as substrate for arrhythmias is less clear (Brown and O'Rourke, 2010). Oxidative stress can induce mitochondrial Ca^{2+} overload via the MCU. An overload of mitochondrial Ca^{2+} results in opening of mPTP, collapse of $\Delta\Psi m$, release of cytochrome c and eventually myocyte death (**Figure 2**) (Gambardella et al., 2017). It has been demonstrated that the H_2O_2-induced activation of MCU and subsequent mitochondrial Ca^{2+} overload can be decreased by overexpression of microRNA 25 (MiR-25). Overexpression of MiR-25 reduced the MCU protein levels, diminished H_2O_2 driven elevation of the mitochondrial Ca^{2+} levels and inactivated the mitochondrial apoptotic pathway (Pan et al., 2015). Prevention of mitochondrial Ca^{2+} overload, via inhibition of MCU, has also proven protective against the development of cardiac arrhythmias (Hamilton et al., 2018). Mitochondrial Ca^{2+} overload promotes excessive ROS production and oxidative stress, of which the consequences have already been discussed in the previous paragraphs.

Ryanodine Receptor Oxidation by ROS

RyR2 is a crucial component of Ca^{2+}-induced Ca^{2+}-release (CICR) and highly sensitive to oxidative stress, as it is oxidized by increased ROS levels in the cytosol (Joseph et al., 2016; Hamilton et al., 2018). Oxidation of RyR2 causes opening of the channel pore and Ca^{2+} leak out of the SR. This SR Ca^{2+} leak results in Ca^{2+} sparks, cytosolic Ca^{2+} overload and SR Ca^{2+} depletion, inducing delayed after depolarization's (DADs) and impairment of the cardiac contractile force (**Figure 2**) (Voigt et al., 2012; Santulli et al., 2015). Enhanced cytosolic Ca^{2+} levels cause mitochondrial Ca^{2+} overload via an increased MCU Ca^{2+} uptake, eventually provoking a pro-arrhythmic vicious cycle of malignant high cellular Ca^{2+} levels (**Figure 2**) (Voigt et al., 2012; Santulli et al., 2015). Elevated cytosolic Ca^{2+} levels also activate a variety of pathogenic Ca^{2+} sensitive signaling pathways in cardiomyocytes, as for example the one including Ca^{2+}/Calmodulin-dependent kinase II (CaMKII) (van Opbergen et al., 2017).

Calcium/Calmodulin-Dependent Protein Kinase II Activation by ROS

CaMKII is a multifunctional serine/threonine kinase located at the cell membrane, cytoplasm and in the nucleus of cardiomyocytes. Ca^{2+} and Calmodulin facilitate CaMKII activation under physiological conditions, though CaMKII is also a substrate for its own monomers (Erickson, 2014). Under pathological conditions ROS can directly oxidize CaMKII, leading to its persistent activity (Foteinou et al., 2015). Once activated (via ROS or elevated cytosolic Ca^{2+} levels) CaMKII can target several ion channels and transcription factors, which coordinate cardiac electrical and mechanical mechanisms (**Figure 2**) (Rokita and Anderson, 2012). CaMKII can for example hyper-phosphorylate the L-type Ca^{2+} channel ($Ca_V1.2$), $Na_V1.5$ and RyR2, thereby increasing cytosolic Ca^{2+} levels (Rokita and Anderson, 2012). This leads to contractile dysfunction and after depolarization's, as discussed before. Moreover, the increased cytosolic Ca^{2+} levels can lead to mitochondrial Ca^{2+} overload, further increasing ROS production and ultimately resulting in cell death (**Figure 2**) (Gambardella et al., 2017). It has been shown that oxidative CaMKII activation leads to after depolarization's in isolated rabbit cardiomyocytes, caused by phosphorylation of L-type Ca^{2+} (LTCC) and Na^+ channels (Yang et al., 2018). In addition, mitochondrial-targeted antioxidant treatment has shown to suppress early after depolarization's (EADs) in an *in silico* model of guinea pig cardiomyocytes (Yang et al., 2018). Phosphorylation of $Na_V1.5$ delays the I_{Na} recovery time after inactivation and enhances the persistent late Na^+ current (Wagner et al., 2006). Under pathophysiological conditions, the CaMKII induced increased Na^+ peak current further elevates diastolic Ca^{2+}, as increased cytosolic Na^+ levels stimulate the Ca^{2+} influx via the sodium- Ca^{2+} exchanger (NCX) (Yao et al., 2011). Elevated cytosolic Ca^{2+} levels enhance the phosphorylation and activation of CAMKII, ending in a positive feedback loop in which a CaMKII-dependent increase of I_{CaL}, alters the Ca^{2+} homeostasis and persistent activation of CaMKII. When during the plateau phase more Ca^{2+} and Na^+ enter the cell, EADs can develop, provoking ectopic activity in the heart (Wagner et al., 2006). Overexpression of CaMKIIδ in rabbit cardiomyocytes has proven to increase the I_{Na}, $[Na]_i$ and cause accumulation of $Ca^{2+}{}_i$ (Wagner et al., 2011). CaMKIIδ knock out in murine cardiomyocytes blunts the ROS (H_2O_2)-induced intracellular accumulation of Na^+ and Ca^{2+} and its subsequent incidence of ventricular arrhythmias, hyper-contraction, and SCD (Wagner et al., 2011). This implies that ROS-induced CaMKII activation causes cellular Na^+ overload and, as a consequence, disturbs the Ca^{2+} balance in the cell.

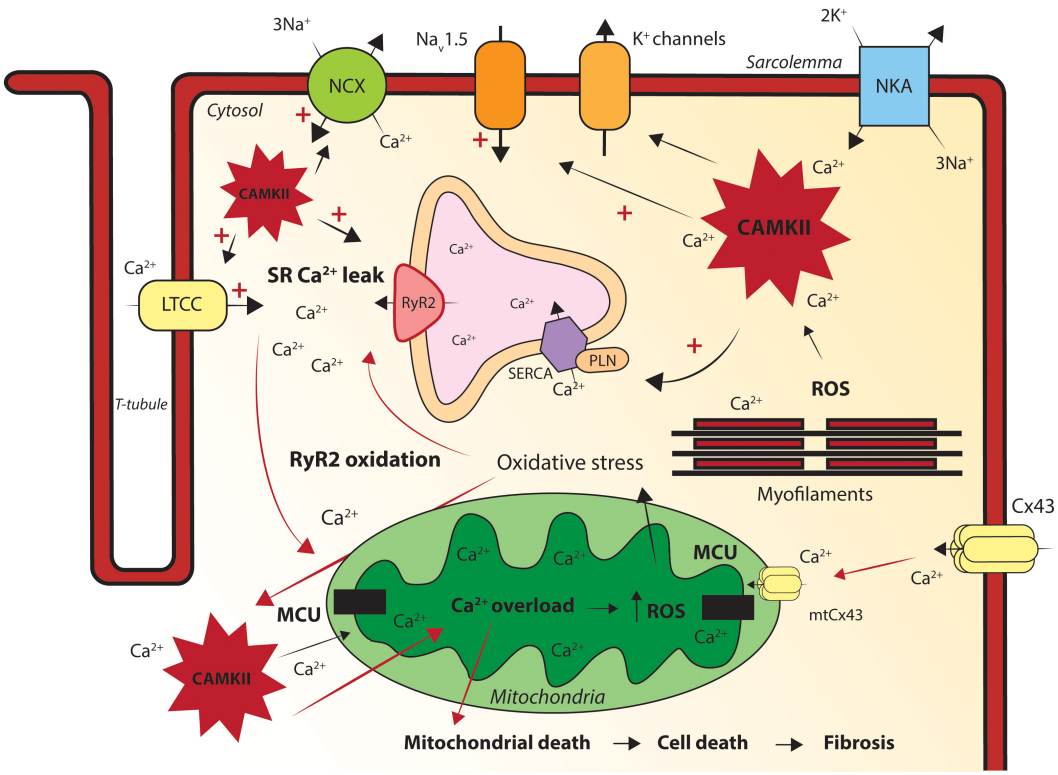

FIGURE 2 | Graphic illustration of the processes via which mitochondrial dysfunction causes intracellular calcium handling disturbances. Oxidative stress induces mitochondrial calcium (Ca^{2+}) overload via the MCU. Mitochondrial Ca^{2+} overload increases reactive oxygen species (ROS) production, oxidizes ryanodine receptors (RyR2) and will subsequently cause sarcoplasmic reticulum (SR) Ca^{2+} leakage. Excessive SR Ca^{2+} release creates elevated cytosolic Ca^{2+} levels and free Ca^{2+} ions will be transported into the mitochondria via the mitochondrial Ca^{2+} uniporter (MCU). The increased intracellular $[Ca^{2+}]$ activates the pathologic Ca^{2+} dependent protein Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). ROS and Ca^{2+} induced sustained activation of CaMKII hyper-phosphorylate the l-type Ca^{2+} channel (LTCC), sodium channel ($Na_V1.5$) and RyR2, increasing the sodium current (I_{Na}), $[Na]_i$ and $[Ca^{2+}]_i$. Connexin43 (Cx43) and mitochondrial Cx43 (mtCx43) can cause cytosolic and mitochondrial Ca^{2+} overload. Mitochondrial Ca^{2+} overload causes mitochondrial death, apoptosis and fibrosis in the heart. Disturbed intracellular Ca^{2+} dynamics and/or fibrosis formation will eventually lead to conduction and repolarization abnormalities in the heart, causing triggered activity and re-entry based cardiac arrhythmias. LTCC, l-type calcium channel; NCX, sodium calcium exchanger; NKA, sodium potassium ATPase; RyR2, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; PLN, phospholamban; Cx43, connexin43; mtCx43, mitochondrial connexin 43; MCU, mitochondrial Ca^{2+} uniporter; SR, sarcoplasmic reticulum; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; ROS, reactive oxygen species.

The Effect of ROS on the L-Type Calcium Channel and Na^+/Ca^{2+} Exchanger

The L-type Ca^{2+} channel (LTCC) is a voltage gated Ca^{2+} channel that couples electrical activation, via an action potential, to contraction of the cardiomyocyte. The effects of ROS on LTCCs are controversial (Yang et al., 2014). On one hand, it has been shown that elevated mitochondrial ROS levels increase the I_{Ca} in guinea pig ventricular cardiomyocytes (Viola et al., 2007). On the other hand, increased ROS levels decreased I_{Ca} in hamster ventricular cardiomyocytes. This could be due to energy depletion or Ca^{2+} overload, rather than oxidation of the LTCC (Hammerschmidt and Wahn, 1998). It should be taken into account that results can vary between animal species and different types of ROS. The subset of ROS components differs in reactivity and oxidation potential (Yang et al., 2014). The Na^+/Ca^{2+} exchanger (NCX) is an antiporter membrane protein which mainly works in the forward mode, pumping 3 Na^+ ions into the cardiomyocyte in exchange for 1 Ca^{2+} ion (Amin et al., 2010). Whether NCX acts in the forward or reversed mode depends on the driving force of the intracellular ion concentrations: high cytosolic $[Ca^{2+}]$ favors the forward mode, whereas high cytosolic $[Na^+]$ and a positive membrane potential favor the reverse mode (Driessen et al., 2014). The effect of oxidative stress on NCX activity is also controversial, as ROS has proven to both stimulate and decrease NCX activity (Zhang et al., 2016). Nevertheless, both RyR2 oxidation and CaMKII activation elevates the cytosolic $[Ca^{2+}]$, forcing NCX into the forward mode, inducing a depolarizing current and making the cell susceptible for DADs (Driessen et al., 2014).

To conclude, mitochondrial dysfunction can result in inadequate ATP synthesis and an increased ROS production. Insufficient supply of ATP to the cardiomyocytes results in activation of sarcKATP channels, which impair cardiomyocyte excitability, electrical impulse propagation and cardiac contractility. Furthermore, enhanced cellular ROS levels cause RIRR, aberrant opening of various ion channels, repolarization abnormalities, impaired electrical conduction and eventually provokes different types of malignant cardiac arrhythmias.

Oxidative stress resulting from mitochondrial dysfunction can induce mitochondrial Ca^{2+} overload via the MCU. Mitochondrial Ca^{2+} overload increases ROS production, oxidizes RyR2 and will subsequently cause SR Ca^{2+} leakage. Excessive SR Ca^{2+} release creates elevated cytosolic Ca^{2+} levels and free Ca^{2+} ions will be transported into the mitochondria via MCU. Mitochondrial Ca^{2+} reuptake and subsequent ROS production creates a vicious pro-arrhythmic environment in the cardiomyocyte. The increased intracellular $[Ca^{2+}]$ prolongs the APD, initiates after depolarization's, causes contractile dysfunction and importantly activates pathologic Ca^{2+} dependent signaling pathways as for example CaMKII. ROS and Ca^{2+} induced sustained activation of CaMKII will hyper-phosphorylate $Ca_V1.2$, $Na_V1.5$ and RyR2, increasing I_{Na}, $[Na]_i$ and $[Ca^{2+}]_i$. Via these mechanism, a separate vicious pro-arrhythmic cycle of altered Ca^{2+} homeostasis and CaMKII activation is initiated in the cell (Wagner et al., 2011; Bertero and Maack, 2018). Whether these mechanisms, all or in part, are present in ACM hearts, remains to be studied.

MITOCHONDRIAL DYSFUNCTION AND ACM

Arrhythmogenic cardiomyopathy is a familial heart disease, associated with ventricular arrhythmias and progressive fibrofatty replacement of the myocardium. Ventricular arrhythmias in individuals with ACM are usually exercise-related and range from frequent premature ventricular complexes (PVCs) to ventricular tachycardia (VT) and ventricular fibrillation (VF) (Benito et al., 2011). The intimate mechanism of these ventricular arrhythmias is still unclear but triggered activity and reentry-based conduction are two likely factors. These two pro-arrhythmic factors are mainly based on disturbed intercellular communication and intracellular Ca^{2+} dynamics, fibrotic infiltrates in the heart and ion channel dysfunction (Cerrone et al., 2017; van Opbergen et al., 2017; Kim et al., 2019). Examination of cardiac biopsies and post-mortem cardiac material revealed diminished expression of Cx43 and $Na_V1.5$ at the ID of ACM patients, two critical determinants of cardiomyocyte excitability and electrical cell–cell coupling (Noorman et al., 2013). In this chapter, we will explore a potential link between parameters known to be involved in ACM development and mitochondrial dysfunction.

Mitochondrial Cx43 Hemichannels and Disturbed Calcium Dynamics in ACM

Gap junctions regulate cell–cell coupling between cardiomyocytes, conducting the passage of small molecules, metabolic substrates and electrical current between the adjacent cells (Kar et al., 2012). In ACM patients, a diminished expression of Cx43 at the ID was demonstrated in a large cohort of patients (Basso et al., 2018). Under pathological conditions, Cx43 can also be redistributed as Cx43 hemichannels to the lateral side of the cardiomyocyte (Cogliati et al., 2016). Cx43 hemichannels are large non-selective conduction pores, transporting small molecules and ions via concentration gradients across the cellular membrane. By this mechanism, hemichannels can release ATP, exchange Na^+ and Ca^{2+} ions and release K^+ ions (Boengler and Schulz, 2017). Reduced ID Cx43 expression and lateral Cx43 localization contribute to abnormal electrical impulse propagation and thereby create an arrhythmogenic substrate in the ACM heart. In addition, Cx43 hemichannels in the perinexus of the cardiomyocyte (outside of the gap junctions) are suggested as important arrhythmogenic substrates in a mouse model of PKP2 deficiency (Kim et al., 2019).

Cx43 resides not only in the ID, but is also present on the mitochondrial membrane, though almost exclusively at mitochondria that are located directly beneath the sarcolemma (Boengler and Schulz, 2017). It has been suggested that ROS can increase the expression of mtCx43, via activation of the p38-MAPK pathway (Matsuyama and Kawahara, 2011). Recent studies uncovered the contribution of mtCx43 hemichannels to mitochondrial Ca^{2+} entry (Gadicherla et al., 2017). Cardiac mitochondria play an important role in the intracellular ion homeostasis of cardiomyocytes, especially for Ca^{2+} ions (Kolwicz et al., 2013). Under pathological circumstances, mitochondrial Ca^{2+} overload increases ROS production, induces oxidative stress and thereby interferes with the SR Ca^{2+} cycling and elevates cytosolic Ca^{2+} levels (Gambardella et al., 2017). Close proximity of sarcolemmal Cx43 hemichannels, mitochondria and mtCx43 likely facilitate ATP and Ca^{2+} exchange that under pathological conditions can interfere with intracellular and mitochondrial Ca^{2+} dynamics. Elevated cytosolic Ca^{2+} levels activate several pro-arrhythmic pathways and cause triggered activity in the heart (Katra and Laurita, 2005; van Opbergen et al., 2017).

Nowadays, it is more and more recognized that in ACM patients RV mechanical deterioration precedes, or occurs in parallel, with the electrical disease progression, potentially initiated via Ca^{2+} handling disturbances and/or structural remodeling (Mast et al., 2016; Cerrone et al., 2017; Taha et al., 2019). In murine PKP2cKO-RV cardiomyocytes, Cx43 hemichannels in the sarcolemma increase the membrane permeability and cause intracellular Ca^{2+} overload and ISO-induced ventricular arrhythmias (Kim et al., 2019). It is tempting to speculate that an increased mitochondrial membrane permeability, via mtCx43 hemichannels, allows excessive entry of Ca^{2+} into the mitochondria and induces mitochondrial Ca^{2+} overload upon loss of PKP2 expression (**Figure 2**). As well, previous studies have suggested that mitochondrial metabolism differs between the LV and RV. Therefore, it might be the case that mitochondrial dysfunction alters intracellular Ca^{2+} sensitive pathways and break the Ca^{2+} balance in the cell, leading to RV initiated electrical disturbances and structural remodeling in ACM patients. This could for example be mediated via reduced ATP production, enhanced ROS production, direct interference with the Ca^{2+} handling related proteins or mtCx43 hemichannels, as explained in the previous chapter (**Figure 2**).

Mitochondrial Related Fibrosis Formation in ACM

Besides ventricular arrhythmias is ACM characterized by fibrofatty replacement of the ventricular myocardium

(Bennett et al., 2019). Fibroblasts can become activated upon injury or environmental stress, triggering their differentiation into myofibroblasts, the primary collagen-producing cell (Nguyen et al., 2014). Myofibroblast differentiation further induces pro-fibrotic responses, including the production of extracellular matrix proteins, collagen and cytokines (Thannickal et al., 2003). Excessive deposition of collagen, in between or in place of the healthy myocardium, interferes with the cardiac electrophysiology. Collagen deposition in the heart acts as a physical barrier to conduction, facilitating re-entry (Nguyen et al., 2017). Molecular signatures underlying the epicardium-initiated fibrofatty replacement in ACM hearts remain largely unexplored (Corrado et al., 2017b; Sepehrkhouy et al., 2017). Previous studies have proposed that abnormal trafficking of intracellular proteins to the ID and subsequent Wnt/β-catenin and Hippo-YAP pathway activation underlie the disease pathogenesis in ACM (Pilichou et al., 2016). Involvement of mitochondrial related processes in fibrofatty infiltration of ACM hearts have not been well-studied and may be an interesting new target. Especially, since previous studies showed apoptosis in ACM-affected hearts and RV vs. LV differences in (mitochondrial) Ca^{2+} handling during the concealed stage of the disease (Nishikawa et al., 1999; Akdis et al., 2016; Austin et al., 2019; Kim et al., 2019). This suggests that alterations in mitochondrial function might serve as early component in ACM, preceding overt structural disease and apoptosis.

ATP- and ROS-Induced Collagen Production in ACM

Multiple cellular and animal models have been used to explore the role of PKP2 in pro-fibrotic processes. Dubash et al. (2016) showed that paracrine pathways might be responsible for inducing fibrosis in the setting of PKP2 deficiency. In HL-1 cells lacking PKP2, transforming growth factor (TGF)-β1 expression was increased and the p38-mitogen-activated protein kinases (MAPK) pro-fibrotic program was activated. Importantly, this study also showed that p38-MAPK was activated in neighboring, PKP2-positive, HL-1 cells (Dubash et al., 2016). These data strongly suggest cell–cell communication between PKP2 positive and negative cells and the presence of a paracrine pathway for induction of the pro-fibrotic processes. The molecular carrier of this cell–cell communication was not identified. Recently, Cerrone et al. (2018) explored adenosine as a possible pro-fibrotic molecular cell–cell messenger in the PKP2-deficient heart. Previous studies showed that binding of adenosine to the adenosine 2A receptor (A2AR) enhances collagen deposition in skin, lung, and liver tissue (Shaikh and Cronstein, 2016). A2AR activation can induce TGF-β1 expression and activate GSK3-β and Wnt-signaling pathways (Giambelluca et al., 2013; Shaikh et al., 2016; Zhang et al., 2017). In the heart, ATP can rapidly convert to adenosine, which bind to its G-coupled protein receptor (A2AR) (Peng et al., 2008; Fernández et al., 2013). In the study of Cerrone et al. (2018), we showed that ATP release was significantly higher in PKP2-deficient cells and that this effect was blunted when Cx43 was also silenced. This suggests that Cx43 hemichannels may act as a conduit for ATP release in PKP2-deficient cells (**Figure 3**). As proposed in Kim et al. (2019), reduced intercellular adhesion strength by PKP2 deficiency may disrupt the gap junction plaque integrity and create a pool of Cx43 hemichannels at the ID (perinexus). Cx43 hemichannels can passively leak intercellular solutes as ATP, via mitochondria closely resembling at the site of cell–cell contact (Leo-Macías et al., 2015). In this particular study, Cerrone et al. (2018) treated PKP2cKO mice with Istradefylline, a specific A2AR antagonist and indeed less collagen was found in both ventricles of PKP2-cKO mice after treatment.

ROS-Induced Fibroblast Differentiation in ACM

Besides Cx43 hemichannels, pannexin-1 channels are also involved in cardiac fibrosis formation, likely via release of ATP from the cardiomyocytes (Nishida et al., 2008; Kar et al., 2012; Li et al., 2015). Pannexin-1 can be activated by Ca^{2+} released from the SR and allow passage of Ca^{2+}, ATP and other small molecules across the membrane, in a manner similar to that of Cx43 hemichannels (**Figure 3**) (Li et al., 2015; Xu et al., 2018). Oxidative stress activates Pannexin-1 hemichannels and does increase ATP release of cardiomyocytes via Pannexin-1 (Zhang et al., 2008; Dolmatova et al., 2012). Pannexin-1 mediated ATP release activates G-coupled P2X and P2Y receptors, further triggering the accumulation of ROS, via their underlying pathways (**Figure 3**) (Onami et al., 2014; Díaz-Vegas et al., 2015; Xu et al., 2018). P2X and P2Y activation enhances the transcription of fibrotic genes such as TGF-β1 (Nishida et al., 2008). ATP not only directly activates P2X and P2Y receptors, but can also be converted to AMP and adenosine, activating A2AR and underlying pro-fibrotic pathways, mechanisms proven to be involved in fibrosis formation in PKP2-cKO mice (**Figure 3**) (Shaikh et al., 2016; Cerrone et al., 2018).

The fact that Pannexin-1 is activated by intracellular Ca^{2+} levels and SR Ca^{2+} release, both increased by ROS-induced RyR2 oxidation, highlights Pannexin-1 as an interesting new target in mitochondrial related pro-fibrotic processes of ACM. In addition to the indirect (Pannexin-1 mediated) ROS-induced fibrosis formation, it has been demonstrated that mitochondrial ROS also directly promotes fibroblast differentiation (Choi et al., 2014). For example, superoxide activates TGF-β1 expression, fibroblast differentiation into myofibroblasts and subsequent extracellular matrix (ECM) deposition (Cucoranu et al., 2005; Choi et al., 2014). Myofibroblasts are characterized by expression of contractile proteins, such as smooth muscle α-actin (α-SMA). Cucoranu et al. (2005) showed that TGF-β1-mediated ROS production regulates α-SMA expression in the cell and the conversion of fibroblasts into myofibroblasts. In human cardiac fibroblasts, TGF-β1 promotes the production of superoxide via stimulating NADPH oxidase 4 (Nox4) and superoxide subsequently stimulates α-SMA expression and ECM deposition. Nox4 superoxide production is regulated via Smad2/3 activation. Moreover, Smad2/3 activation leads to enhanced activation of Nox4, creating a pro-fibrotic positive-feedback loop (**Figure 3**) (Cucoranu et al., 2005).

The most direct link between mitochondrial-related processes and collagen production in ACM has been found in PKP2-cKO mice. However, there are several other ACM models were involvement of TGF-β1, GSK3-β, and Wnt-signaling pathways

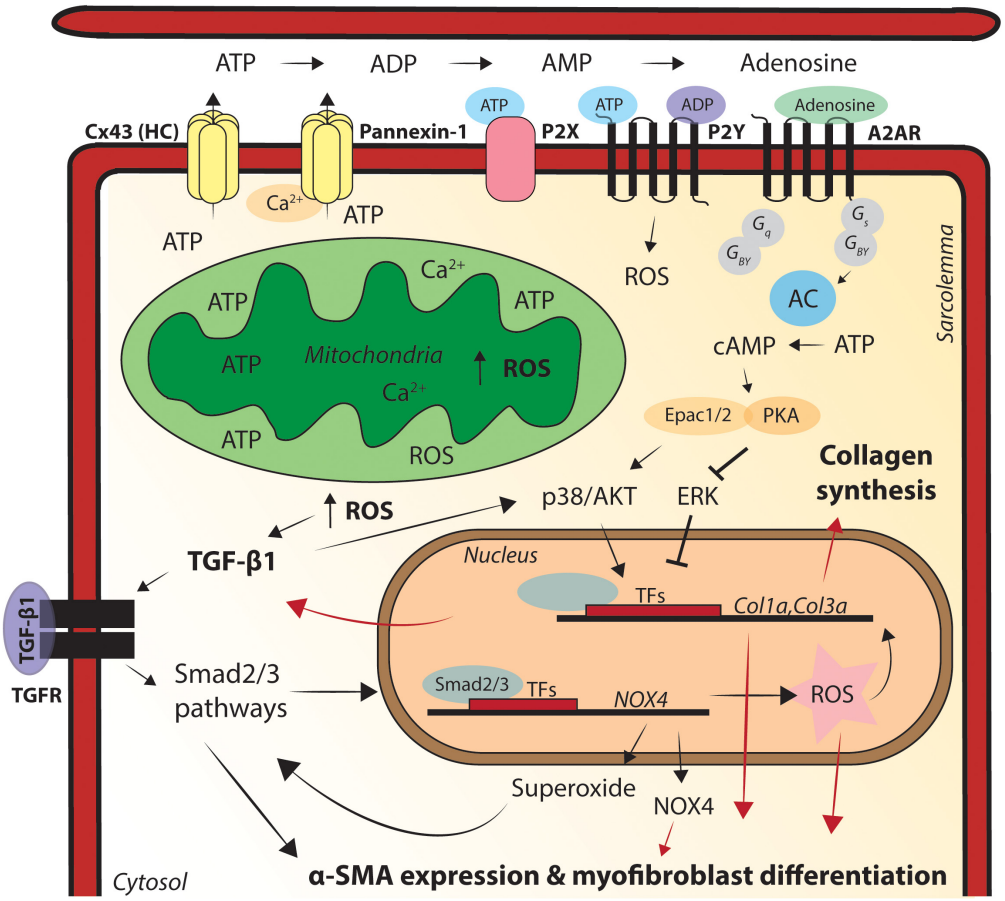

FIGURE 3 | Graphic illustration of the processes via which mitochondrial dysfunction causes pro-fibrotic remodeling in the heart. Relocation of connexin43 (Cx43) hemichannels to the lateral membrane enables release of adenosine triphosphate (ATP) into the extracellular space, via mitochondria close residing at sides of cell–cell contact. ATP can directly activate P2X/Y channels and also convert into adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine. Adenosine subsequently activates pro-fibrotic signaling pathways, via adenosine 2A receptor (A2AR) binding. Pannexin-1 is activated via enhanced cytosolic calcium (Ca^{2+}) levels and sarcoplasmic (SR) Ca^{2+} release and has the same ATP-mediated pro-fibrotic factor capacity as Cx43 hemichannels. Superoxide activates transforming growth factor (TGF)-β1 expression, fibroblast differentiation into myofibroblasts and subsequent extracellular matrix (ECM) deposition. TGF-β1-mediates reactive oxygen species (ROS) production, regulates α-smooth muscle actin (SMA) expression in the cell and the conversion of fibroblasts into myofibroblasts and TGF-β1 promotes the production of superoxide via stimulating NADPH oxidase 4 (Nox4). Nox4 superoxide production is also regulated via Smad2/3 activation, Smad2/3 activation leads to enhanced activation of Nox4, creating a pro-fibrotic positive-feedback loop in the heart. Fibrosis formation in the heart acts as a physical barrier to conduction, facilitating re-entry based cardiac arrhythmias. Cx43, connexin43; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; A2AR, adenosine 2A receptor; AC, adenylate cyclase; cAMP, cyclic AMP; PKA, protein kinase A; ROS, reactive oxygen species; TFs, transcription factors; Col1α, collagen 1α; Col3α, collagen 3α; Nox4, NADPH oxidase 4; TGF-β1, transforming growth factor beta 1; α-SMA, alpha smooth muscle actin.

in cardiac fibrosis formation has been proven (Chelko et al., 2016; Calore et al., 2019; Zheng et al., 2019). If activation of these pathways indeed is preceded by increased ROS levels, ATP release, P2X, P2Y, and/or A2AR activation would be an interesting topic for upcoming studies.

PPAR-Induced Apoptosis and Lipogenesis in ACM

Cardiac metabolic activity is tightly regulated by expression levels of metabolic enzymes and their post-translational modifiers. One important class of transcriptional regulators for FA use are nuclear Peroxisome proliferator–activated receptors (PPAR). PPARα is the predominant isoform in the heart, although PPARδ and PPARγ also regulate lipid metabolism (Kersten et al., 2000; Lopaschuk et al., 2010). PPARγ is mainly expressed in adipose tissue and contributes to adipogenesis, the differentiation of pre-adipocytes to adipocytes (Lecarpentier et al., 2010). PPARs interact with members of the PPAR-γ coactivator-1 (PGC-1) family and co-activation of these proteins increases the FA uptake capacity and FAO (Rowe et al., 2010). Recently, an interesting link was found between cardiomyocyte metabolism, apoptosis, and lipogenesis in an induced pluripotent stem cell cardiomyocyte (iPSC-CM) model of ACM. Dermal fibroblasts from patients harboring a *PKP2* mutation were reprogramed to generate iPSC-CMs and mutant PKP2 iPSC-CMs demonstrated exaggerated lipogenesis and apoptosis (Caspi et al., 2013; Kim et al., 2013). Kim et al. (2013) presented PPARα, β/δ, and γ

as key regulators in these processes, especially PPARγ seem to be activated. Experimental PPARγ over-activation in PKP2-iPS-CMs also induced exaggerated lipogenesis and pronounced apoptosis, caused by pathogenic PPARγ and PPARα co-activation (Kim et al., 2013). In addition, Kim et al. (2013) assessed the involvement of ROS in pathogenesis of PKP2 iPSC-CM and decreased ROS levels could indeed prevent cardiomyocyte death of these iPSC-CM. As discussed earlier, the heart experiences a metabolic switch after birth, changing substrate oxidation from glucose to FA (Torrealba et al., 2017). After pathogenic co-activation of PPARγ and PPARα, the mutant PKP2 iPSC-CMs also demonstrated a fuel shift from FA and glucose metabolism to pre-dominant glucose utilization (Kim et al., 2013). These data strongly suggest that pathological metabolic changes underlie disease progression in ACM patients harboring a *PKP2* mutation. The engagement of PPARs in a PKP2-related cardiomyopathy has been confirmed in the PKP2-cKO mouse model (Cerrone et al., 2017). KEGG analysis of transcriptional regulation in PKP2-cKO murine hearts presented the adipocytokine signaling pathway as one of the most upregulated pathways, with a predominant effect on PPARα (Cerrone et al., 2017). Moreover, GTEx-based analysis of human RNA abundance in the heart revealed that PPARα is a member of a PKP2-centered gene network, thus suggesting a direct regulation of PPARα levels by PKP2 expression (Montnach et al., 2018).

In conclusion, ACM is a progressive and predominantly RV disease, characterized by ventricular arrhythmias and fibrofatty infiltrates in the myocardium. Mitochondrial interference with the Ca^{2+} homeostasis in the cell, directly or indirectly via Cx43 hemichannals and mtCx43, tentatively serves as an important substrate in RV initiated electrical disturbances and structural remodeling in ACM patients. In murine PKP2cKO-RV cardiomyocytes, Cx43 hemichannels in the sarcolemma increase the membrane permeability and cause intracellular Ca^{2+} overload and ISO-induced ventricular arrhythmias. ROS can increase the expression of mtCx43 and close the proximity of sarcolemmal Cx43 hemichannels, mitochondria and mtCx43 might facilitate ATP and Ca^{2+} exchange that under pathological conditions can interfere with intracellular and mitochondrial Ca^{2+} dynamics. In addition, cellular ATP release and increased oxidative stress are important pro-fibrotic aspects in the (ACM) heart. Cx43 hemichannels in the perinexus enable release of ATP into the extracellular space, via mitochondria close residing at sides of cell–cell contact. ATP can directly activate P2X/Y channels and also convert into ADP, AMP, and adenosine. Adenosine subsequently activates pro-fibrotic signaling pathways (as TGF-β1 activation) via A2AR binding. Pannexin-1 has the same ATP-mediated pro-fibrotic factor capacity as Cx43 hemichannels. Interestingly, pannexin hemichannels are activated via enhanced cytosolic Ca^{2+} levels and SR Ca^{2+} release, which is increased by ROS-induced RyR2 oxidation. Furthermore, Nox4-derived ROS can induce fibrosis via TGF-β1 activation and fibroblast differentiation into myofibroblasts. The increased apoptosis and adipogenesis in ACM patients is likely caused by co-activation of PPARγ and PPARα, which points toward an altered metabolic state of the ACM heart.

DISCUSSION

In ACM patients, malignant ventricular arrhythmias and SCD largely occur in the pre-clinical phase of the disease, before overt structural changes. To prevent or interfere with the disease progression, more insight into the mechanisms related to electrical instability in ACM hearts is needed. At present, a large extent of research is focused on the link between cardiac arrhythmias and metabolic disease. Mitochondrial dysfunction can affect the electrical balance in cardiomyocytes and favor arrhythmogenesis. The role of mitochondrial dysfunction in ACM pathology is still unclear, and mitochondrial biology and structure in the ACM heart remains understudied. Here, we reviewed cardiac mitochondrial dysfunction, cardiac arrhythmias and the potential links to typical hallmarks of ACM.

Mitochondrial dysfunction reduces ATP production and increases ROS production in the heart. Upon insufficient ATP supply, the predominantly closed sarcKATP channels can become conductive. Open sarcKATP channels can impair electrical conduction in the heart, by reducing cardiomyocyte excitability. In addition, increased ROS production leads to abnormal opening of various ion channels and reduces the expression of gap junctions. Mitochondrial Ca^{2+} overload (via for example mtCx43) increases ROS production, oxidizes RyR2 and subsequently induces SR Ca^{2+} leakage. This results in repolarization abnormalities, ectopic activity, conduction defects and eventually (re-entry based) cardiac arrhythmias. ROS oxidation also indirectly affects ion channels, transcription factors and (pathogenic) intracellular processes, through the multifunctional protein CaMKII. Appearance of fibrosis, apoptosis, and lipogenesis is highly related to mitochondrial (dys)function. Fibrosis formation can be induced via Cx43 hemichannel-mediated ATP release, SR Ca^{2+}-driven Pannexin-1 activation and ROS-TGF-β1-Nox4 activated fibroblast differentiation. Whether some or all of these changes are present in cardiomyocytes of hearts deficient in desmosomal proteins remains to be determined. PPARγ and PPARα co-activation is a possible link between metabolic changes in PKP2-deficient patients, apoptosis and lipogenesis. Yet their relative contribution remains a subject of further investigation.

The limited amount of experimental evidence in ACM models makes it hard to determine whether mitochondrial dysfunction indeed precedes and/or accompanies ACM pathogenesis. Mutations in mitochondrial DNA can provoke mitochondrial dysfunction and activate the mechanisms we discussed. On the contrary, loss of ID integrity might also affect the intracellular ion homeostasis (e.g., Ca^{2+}) and alters mitochondrial activity and homeostasis. The cardiac stress responses upon mutations in ID-associated proteins likely activates several mechanisms related to mitochondrial dysfunction, such as inflammation and apoptosis. Postulated hypotheses are largely based upon findings in experimental PKP2 models, which are of course not representative for the entire ACM population, therefore extrapolations should be done with caution.

Nevertheless, these models can be very useful in discovering ACM-related mitochondrial biology and testing of therapeutic targets, as for example anti-oxidant treatment or genetic repair.

AUTHOR CONTRIBUTIONS

CO and LB prepared the primary manuscript. MD and TV critically revised the manuscript. CO produced the figures.

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cBIN1 Score (CS) Identifies Ambulatory HFrEF Patients and Predicts Cardiovascular Events

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Background: Cardiac Bridging Integrator 1 (cBIN1) is a membrane deformation protein that generates calcium microdomains at cardiomyocyte t-tubules, whose transcription is reduced in heart failure, and is released into blood. cBIN1 score (CS), an inverse index of plasma cBIN1, measures cellular myocardial remodeling. In patients with heart failure with preserved ejection fraction (HFpEF), CS diagnoses ambulatory heart failure and prognosticates hospitalization. The performance of CS has not been tested in patients with heart failure with reduced ejection fraction (HFrEF).

Methods and Results: CS was determined from plasma of patients recruited in a prospective study. Two comparative cohorts consisted of 158 ambulatory HFrEF patients (left ventricular ejection fraction (LVEF) $\leq$ 40%, 57 $\pm$ 10 years, 80% men) and 115 age and sex matched volunteers with no known history of HF. N-terminal pro-B-type natriuretic peptide (NT-proBNP) concentrations were also analyzed for comparison. CS follows a normal distribution with a median of 0 in the controls, which increases to a median of 1.9 ($p < 0.0001$) in HFrEF patients. CS correlates with clinically assessed New York Heart Association Class ($p = 0.007$). During 1-year follow-up, a high CS ($\geq$ 1.9) in patients predicts increased cardiovascular events (43% vs. 26%, $p = 0.01$, hazard ratio 1.9). Compared to a model with demographics, clinical risk factors, and NT-proBNP, adding CS to the model improved the overall continuous net reclassification improvement (NRI 0.64; 95% CI 0.18-1.10; $p = 0.006$). Although performance for diagnosis and prognosis was similar to CS, NT-proBNP did not prognosticate between patients whose NT-proBNP values were > 400 pg/ml.

Conclusion: CS, which is mechanistically distinct from NT-proBNP, successfully differentiates myocardial health between patients with HFrEF and matched controls. A high CS reflects advanced NYHA stage, pathologic cardiac muscle remodeling, and predicts 1-year risk of cardiovascular events in ambulatory HFrEF patients. CS is a marker of myocardial remodeling in HFrEF patients, independent of volume status.

Keywords: heart failure, cardiac muscle remodeling, ion channels, calcium handling, cBIN1 score

INTRODUCTION

Heart failure (HF) is currently one of the most burdensome global public health problems. This complex, progressive condition is caused by a cascade of pathophysiologic insults that lead to abnormally functioning myocardium, which at the myocyte level is associated with disturbed intracellular calcium homeostasis and impaired mitochondrial function and cellular metabolism. The current prevalence of HF among adults in the United States is 6.2 million and is projected to keep increasing over the next 10 years (Virani et al., 2020). Half of this population have reduced ejection fraction (HFrEF) with high morbidity and mortality mainly caused by cardiac pump failure and sudden cardiac death. Clinical management remains challenging, with a total cost of care greater than $30 billion per year. HF hospitalizations are already the highest single cost to Medicare for Americans over the age of 65, and this expense is expected to increase (Go et al., 2014). The substantial burden of this disease represents a healthcare and economic imperative to assess the health of cardiac muscle and use that information to limit future hospitalizations.

Unlike with most cancers which have advanced tissue based molecular diagnostics, our current methods at assessing prognosis in heart failure are more limited to *status quo* hemodynamics and organ function assessments (Murray et al., 2005; Chow and Senderovich, 2018; Zadikany et al., 2019). Current guidelines define HFrEF patients as having transthoracic echocardiogram obtained left ventricular ejection fraction (LVEF) of less than 40% (Yancy et al., 2013). Traditional diagnostic tools for assessing the severity of HFrEF include not only the transthoracic echocardiogram (Loeppky et al., 1984), but New York Heart Association (NYHA) assessment (Dickstein et al., 2008), cardiopulmonary exercise testing (Weber and Janicki, 1985; Corra et al., 2002), and cardiac index obtained from right heart catheterization (Ganz et al., 1971). These assessments provide valuable information regarding overall cardiac function (Ponikowski et al., 2016). However, because these assessments will vary with loading conditions, they do not necessarily correlate with intrinsic myocardial health. Furthermore, current cardiac assessment tools often require specialized equipment and highly trained medical staff, limiting accessibility for patients being seen in a general clinician's office. Clinicians could be well served with a quantitative blood-based tool that is able to effectively assess the molecular health and reserve of cardiac muscle to assist with critical clinical decision making such as the need for left ventricular assist device (LVAD) and implantable cardioverter defibrillator (ICD), as well as heart transplant priorities.

The gold standard biomarkers to assess patients with HF symptoms are the brain natriuretic peptide (BNP) family (Sun et al., 2014). Both BNP and its more stable and non-active version, N-terminal prohormone BNP (NT-proBNP) are secreted by cardiomyocytes in response to pressure and stretch. Active BNP results in a downstream natriuresis, diuresis, and vasodilatation (Lee and Daniels, 2016). As stretch-response molecules, blood available BNP biomarkers help assess fluid status and assist in evaluating patients with acute dyspnea (Januzzi et al., 2005). However, NT-proBNP is not more effective than a usual care strategy in high-risk patients with HFrEF (Felker et al., 2017). Furthermore, BNP values are affected by renal function and body mass index (BMI) (Myhre et al., 2018). Taken together, HF diagnosis and management of ambulatory patients could benefit from a quantitative assessment of the health of cardiac muscle that is insensitive to volume, BMI, or non-cardiac organ function.

Cardiac bridging integrator 1 (cBIN1) score (CS), a novel biomarker of HF, has recently been introduced as a diagnostic and prognostic test in patients with heart failure with preserved ejection fraction (HFpEF) (Nikolova et al., 2018). cBIN1 is a cardiac-specific transverse tubule (t-tubule) membrane sculpting protein, functioning to organize microdomains responsible for calcium release and excitation-contraction (EC) coupling (Hong et al., 2014), as well as efficient diastolic calcium reuptake (Liu et al., in press). In doing so, cBIN1 helps maintain intracellular calcium homeostasis particularly at functionally important microenvironment adjoining t-tubule, junctional sarcoplasmic reticulum, and mitochondria. As a result, low cardiomyocyte cBIN1, which is also measurable at the plasma level (Xu et al., 2017), is intrinsically linked to cardiac inotropy (Hong et al., 2012), lusitropy (Liu et al., in press), and arrhythmia risks (Hong et al., 2014) particularly during stress response (Fu et al., 2016). Derived from the inverse of plasma cBIN1 level (Nikolova et al., 2018), CS rises with worsening HF. In this study, we explored whether CS has the potential to diagnose HFrEF when compared with matched controls. We also explored CS's ability, within the HFrEF cohort, to accurately predict future cardiac events.

MATERIALS AND METHODS

Study Design

All human studies were approved by the institutional review board at Cedars-Sinai Medical Center. Full informed consent was obtained from all subjects prior to participation in the study. The study involved two human populations, including patients with documented HFrEF and volunteers with no known history of HF.

The HFrEF cohort, followed longitudinally in the Advanced Heart Failure clinic at Cedars-Sinai Smidt Heart Institute, consisted of those in the clinic with a known diagnosis of HFrEF (left ventricular ejection fraction (LVEF) $\leq$ 40% and history of HF). From July 2014–November 2015, 158 patients were enrolled and a blood sample was obtained from patients at the time of clinic-scheduled phlebotomy. Patients with LVEF $>$ 40% at the time of enrollment were excluded. Patient demographics, clinical information, medications, and laboratory and diagnostic test results were gathered from the hospital electronic medical records into a secure de-identified database. Subsequent clinical information was updated from chart review occurring every 3 months for 1 year.

The comparison cohort, consisting of 115 age and sex matched volunteers with no known history of HF, was obtained from the Cedars-Sinai MIRIAD IBD Consortium and Innovative Research, under similar plasma collection and preparation

as the HFrEF samples. A clinical history, including patient demographics, medical history, and current medications was obtained from each volunteer.

Sample Processing and CS Determination

Whole venous blood was drawn into EDTA lavender tubes, stored immediately at 4°C for less than 4 h, and then processed to plasma and flash frozen for storage at −80°C prior to use, as previously described (Nikolova et al., 2018). The concentration of cBIN1 was determined using a cBIN1 specific sandwich-ELISA assay provided by Sarcotein Diagnostics, as previously described (Xu et al., 2017). Findings are reported using CS, the natural log of the inverse of cBIN1 plasma concentration (Nikolova et al., 2018), which increases as cBIN1 decreases.

NT-proBNP Assay

NT-proBNP values were obtained from the plasma of control and HFrEF patients. The Cedars-Sinai Medical Center clinical laboratory referred samples to Quest Diagnostics Laboratory to perform the NT-proBNP assay using electrochemiluminescence.

Cardiac Event Defined as the Primary Outcome During Follow-Up

Cardiac events were predefined as any HF or cardiac hospitalization, left ventricular assist device (LVAD) or mechanical circulatory support (MCS) placement, orthotopic heart transplantation (OHT), or death within the 1 year after time of blood draw. All chart review and adjudication were done by a two-physician panel, who were not involved in the clinical care of the patients (YX and RZ).

Statistical Analysis

Data distributions were assessed for normality by the Kolmogorov-Smirnov test. Continuous variables with normal distributions were expressed as means and standard deviations and compared using two-sided *t*-tests. Continuous variables with non-normal distributions were analyzed with medians and interquartile ranges (IQR) and compared using Mann–Whitney U and Kruskal–Wallis tests. Categorical variables were compared using Fisher's exact or chi-square test. Receiver operating characteristic (ROC) analyses were performed to determine the sensitivity and specificity of CS and NT-proBNP to diagnose HF. Kaplan-Meier and Cox-proportional hazard analyses were used to compare the differences in event-free survival rates between patients with high and low values of CS. Model one included age, sex, BMI, NYHA class, LVEF, estimated glomular filtration rate (eGFR), and NT-proBNP. Model two included the same covariants with CS added to it. To determine the increase in discriminative value to predict 1 year outcomes following the addition of CS as a covariant in the survival model, we evaluated continuous net reclassification improvement (NRI). Two-sided *p*-values were reported and a $p < 0.05$ was considered statistically significant. Statistical analyses were conducted using SAS Version 9.3.1 software (SAS Institute, Inc.), RStudio Version 1.0.143 (RStudio, Inc.), and GraphPad Prism Version 6 (GraphPad Software).

RESULTS

Study Cohorts

158 HFrEF patients (57 ± 10 years old, 80% men) were age and sex matched to 115 volunteers with no known history of HF (54 ± 6 years old, 80% men) (**Table 1**). The HFrEF cohort consisted of 55 (35%) ischemic and 101 (64%) non-ischemic patients. Most of the non-ischemic patients have idiopathic cardiomyopathy (68%), with the remainder due to valvular disease, toxin-mediated disease, or infiltrative disease. Most patients were classified as New York Heart Association (NYHA) II or III (35 and 48%, respectively) and the prevalence of comorbidities was 45% hypertension, 37% diabetes, and 23% chronic kidney disease. The baseline median LVEF on transthoracic echocardiography was 24 ± 8%. Patients were

TABLE 1 | Baseline characteristics of HFrEF and matched controls.

Characteristics (%)	Heart failure with reduced EF (*n* = 158)	Matched controls (*n* = 115)	*p*-value
Age (SD)	57 ± 10.4	54 ± 6.1	<0.001
≤ 50 years	39 (25)	29 (25)	NS
> 50 years	119 (75)	86 (75)	
Male	126 (80)	92 (80)	NS
White	93 (59)	67 (58)	NS
BMI, kg/m2 (SD)	29 ± 6.1	29 ± 5.6	NS
Hypertension	71 (45)	13 (11)	<0.001
Diabetes	59 (37)	8 (7)	<0.001
CKD	36 (23)		
LVEF	24 ± 8.1		
Medications			
Beta-Blockers	139 (88)		
ACE-I/ARB	120 (76)		
Diuretics	125 (79)		
NYHA			
I	21 (13)		
II	55 (35)		
III	76 (48)		
IV	6 (4)		
Subtype of heart failure			
Ischemic	55 (35)		
Non-Ischemic	101 (64)		
Valvular	10 (10)		
Dilated	11 (11)		
Toxin	9 (9)		
Infiltrative	2 (2)		
Other	69 (68)		

HFrEF, heart failure with reduced ejection fraction; SD, standard deviation; BMI, body mass index; CKD, chronic kidney disease; LVEF, left ventricular ejection fraction; ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; NYHA, New York Heart Association.

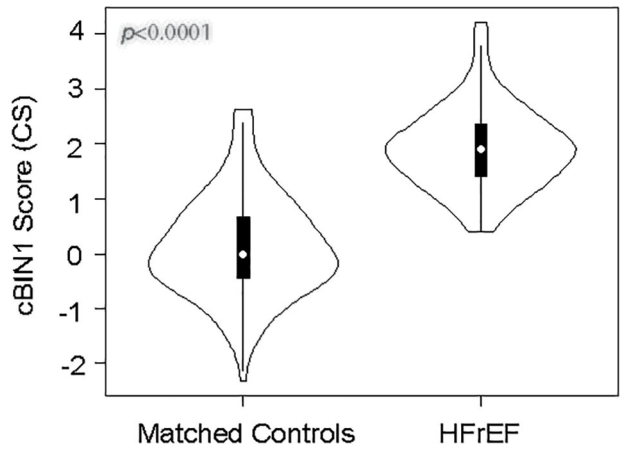

FIGURE 1 | Distribution of CS among HFrEF and matched controls. The vertical axis is the cBIN1 score (CS), and the width of each violin graph depicts the density plot at each measured value. The HFrEF cohort median CS is elevated relative to the controls, 1.9 (IQR 1.4–2.4) compared to 0 (IQR -0.5–0.7), respectively ($p < 0.0001$).

treated with guideline-directed medical therapy by the Cedars-Sinai Advanced Heart Disease group.

CS Is Elevated in Patients With HFrEF

Violin plots of CS in HFrEF and matched controls are shown in **Figure 1**. The HFrEF cohort median CS is elevated relative to the controls, 1.9 (IQR 1.4–2.4; mean ± SD 1.9 ± 0.7) compared to 0 (IQR -0.5–0.7; mean ± SD 0.1 ± 0.9), respectively ($p < 0.0001$), with an approximately normal distribution in both cohorts. The HFrEF cohort median of ln NT-proBNP level, 7.0 (IQR 6.0–8.1), is also significantly elevated compared to the median level of the control cohort, 3.3 (IQR 2.7–4.1), with $p < 0.0001$ (**Supplementary Figure S1**). NT-proBNP levels (pg/ml) have a median of 28 (IQR 15–60) in controls and a median of 1,081 (IQR 409–3,419) in HFrEF patients.

Median CS (**Table 2**) and NT-proBNP (**Supplementary Table S1**) values were analyzed among different subgroups of demographics and clinical parameters. CS and NT-proBNP do not vary with sex or age. Unlike NT-proBNP, CS does not differ among BMI categories (normal, overweight, and obese) in controls or HFrEF patients. Among HFrEF patients with normal vs. decreased estimated glomerular filtration rate (eGFR < 60 ml/min/m^2), CS does not differ (1.8 and 1.9, respectively), while patients with worsening eGFR had increased NT-proBNP levels ($p = 0.01$). CS an NT-proBNP correlate with clinically assessed New York Heart Association Class ($p = 0.007$ and $p < 0.001$, respectively). The lack of association with CS and obesity or renal function is consistent with results previously reported in patients with HFpEF and matched controls (Nikolova et al., 2018).

CS Diagnoses Failing Heart Muscle

Since CS is higher in HFrEF patients than controls, we generated receiver operating characteristic (ROC) curves for CS and NT-proBNP, adjusting for age, sex, and BMI (**Figure 2**). The area under the curve (AUC) of the ROC indicates that both CS (AUC = 0.973, red) and NT-proBNP (AUC = 0.967, blue) distinguish patients with HF from the control population. When CS and NT-proBNP were combined, the ability to determine HF from the control population, while already strong, still improved with statistical significance (AUC = 0.995, green, $p = 0.01$) suggesting that CS (muscle health) and NT-proBNP (intracardiac volume) can be complementary.

Elevated CS Prognosticates Cardiovascular Hospitalization in HFrEF Patients

Next, we explored whether, in addition to its diagnostic value, CS can serve as a prognostic marker in predicting future clinical outcomes in patients with HFrEF. During the 12-month follow-up period, we found that 66 patients (42%) had at least one cardiovascular (CV) event: 41 (26%) patients had a HF-related hospitalization, 16 (10%) patients with a hospitalization diagnosis that was cardiac, but not HF, in origin, 31 (20%) patients had OHT, 4 (3%) went onto MCS or LVAD, and 9 (6%) patients died (6 of which were due to cardiac etiology). Kaplan-Meier survival curves were generated (**Figure 3A**). The median CS level of 1.9 (drawn at the initial visit) was used as a cutoff to differentiate high verse low CS (Nikolova et al., 2018). A cutoff higher than the median of 1.9 further increases specificity of 12-month event free survival (**Figure 3B**). Using a model including age, sex, BMI, NYHA class, LVEF, eGFR, and NT-proBNP (**Table 3**), the hazard ratio (HR) of a high CS ≥ 1.9 predicting CV event rate among HFrEF patients during 1 year of follow-up was 1.9 (43 vs. 26%, $p = 0.03$). The addition of CS ≥ 1.9 significantly improved continuous net reclassification improvement (NRI 0.64; 95% CI 0.18-1.10; $p = 0.006$).

For NT-proBNP, the Kaplan-Meier survival curve using the median NT-proBNP cutoff value of 1078 pg/ml, was not quite significant in predicting 1-year CV event (40 vs. 28%, $p = 0.06$). Interestingly, the prognostic power of NT-proBNP was in the low values (<409 pg/ml in the 1st quartile) (**Supplementary Figure S2**). A low NT-proBNP predicted CV events with a HR of 3.3 (40 vs. 19%, $p = 0.03$). There was no significant prognostication in patients with NT-proBNP greater than 409 pg/ml.

DISCUSSION

The prevalence of HFrEF is increasing over time as our population continues to age, making this clinical syndrome a significant public health concern. The diagnosis of HF remains complex, involving a combination of history taking, physical examination, labs, imaging, and functional studies. Among the existing armamentarium of HF diagnostics, there is currently no invasive or non-invasive tool to measure intrinsic cardiomyocyte remodeling. CS can be used as a blood available biomarker to assist in this clinical need.

In pre-clinical animal models, pathophysiological changes in cardiomyocytes of failing hearts such as t-tubule remodeling is considered the transition point from functional compensation to

TABLE 2 | CS among subgroups in matched controls and HFrEF patients.

Characteristics	*N*	Matched controls	IQR (Q1-Q3)	*p*-value	N	HFrEF	IQR (Q1-Q3)	*p*-value
All patients	115	0.0	−0.5–0.7	–	158	1.9	1.4–2.4	–
Sex				NS				NS
Men	92	0.0	−0.5–0.8		126	1.9	1.4–2.3	
Women	23	0.0	−0.3–0.4		32	1.9	1.3–2.4	
Age (years)				NS				NS
< 55	67	−0.1	−0.5–0.7		54	1.8	1.5–2.1	
≥ 55	48	0.1	−0.4–0.7		104	2.0	1.4–2.4	
Race/Ethnicity				NS				0.03
White	67	0.1	−0.6–0.8		93	1.8	1.3–2.1	
Black	24	0.0	−0.5–0.9		26	2.1	1.8–2.6	
Hispanic	24	−0.1	−0.3–0.4		28	2.0	1.5–2.5	
Asian	0	–	–		9	1.6	1.3–2.3	
BMI (kg/m^2)				NS				NS
Normal (<25)	32	0.0	−0.5–0.7		29	1.9	1.4–2.4	
Overweight (25–29.9)	34	0.0	−0.4–0.7		65	1.9	1.4–2.4	
Obese (30–34.9)	25	−0.1	−1.0–0.4		38	2.0	1.7–2.5	
Morbid Obesity (≥35)	14	−0.2	−0.4–0.1		25	1.7	1.1–2.1	
Etiology								NS
Ischemic HFrEF					55	2.0	1.5–2.5	
Non-ischemic HFrEF					101	1.9	1.4–2.3	
eGFR ($ml/min/m^2$)								NS
<60					94	1.9	1.5–2.2	
>60					64	1.8	1.4–2.5	
NYHA								0.007
I					21	1.6	1.3–2.0	
II					55	1.8	1.5–2.3	
III					76	2.0	1.4–2.5	
IV					6	2.6	2.1–3.0	
OHT								0.0006
No					127	1.8	1.4–2.2	
Yes					31	2.3	1.7–3.0	

HFrEF, heart failure with reduced ejection fraction; IQR, interquartile range; Q1, quartile 1; Q3, quartile 3; BMI, body mass index; eGFR, estimated glomular filtration rate; NYHA, New York Heart Association; OHT, orthotopic heart transplant.

decompensated heart failure (Wei et al., 2010). cBIN1 reductions are associated with t-tubule remodeling and the progression of heart failure (Hong et al., 2012, 2014; Xu et al., 2017). With recovery of muscle, cBIN1 levels also recover (Lyon et al., 2012). Given that cBIN1 is also blood available, CS provides a blood available "liquid biopsy" of cardiac muscle.

HF progression can be reflected by different biomarkers, with robust data demonstrating the ability of natriuretic peptides (BNP and NT-proBNP) to reflect myocardial wall stress (Iwanaga et al., 2006). As a marker of acute volume overload, the diagnostic utility of BNP peptides is well-validated in detecting acute decompensated HF (Januzzi et al., 2005). However, blood BNP values are affected by obesity and renal dysfunction, in addition to requiring adjustments for age and sex (Myhre et al., 2018). Furthermore, because natriuretic peptides are reflective of volume status, it can be difficult to use BNP as a marker to distinguish between patients with severe HFrEF and patients with non-cardiac origin volume overload, such as renal failure.

There is a need for additional HF biomarkers that can help identify the intrinsic health of cardiac muscle cells independent of volume status. Because CS reflects cardiac muscle cell health, it is stable and independent of fluctuations induced by intracardiac volume, inflammatory state, or body habitus (Nikolova et al., 2018). The characteristic performance of CS observed in the current HFrEF cohort indicates that a positive CS can help accurately identify failing heart muscle and is not sensitive to comorbid conditions. Thus, CS provides an unprecedent and non-invasive tool to help with evaluation of myocyte health from EC-coupling efficiency to electrical stability, determining individual's risks of pump failure and arrhythmias. As a signature footprint from myocytes, CS is therefore particularly important at many critical clinical decision-making points for HFrEF patients, guiding medical treatment choices, clinical surveillance intervals, criteria determination for LVAD and ICD implant, and evaluation of the need and the recovery potential of a heart transplant.

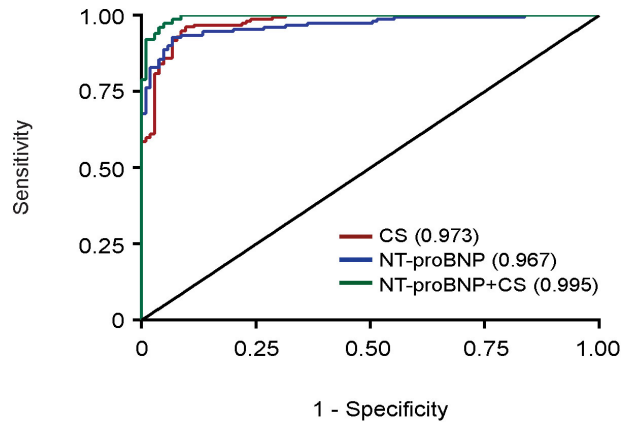

FIGURE 2 | Receiver operating characteristic (ROC) curves. ROC curve containing the various sensitivities and specificities of CS (AUC = 0.973, red), NT-proBNP (AUC = 0.967, blue), and combined NT-proBNP and CS (AUC = 0.995, green) to diagnose disease in our control and HFrEF cohorts.

TABLE 3 | Risk of CV Event in HFrEF patients with multivariate Cox regression analysis.

Variables	Model without CS			Model with CS		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age, > 55 vs. < 55 years	0.88	0.45–1.72	0.71	0.95	0.49–1.86	0.88
Sex, female vs. male	0.45	0.20–1.03	0.06	0.47	0.20–1.07	0.07
BMI, kg/m^2	1.0	0.98–1.09	0.25	1.04	0.98–1.1	0.19
NYHA class, III-IV vs. I-II	2.75	1.39–5.43	<0.01	2.59	1.33–5.06	0.01
LVEF%	1.03	0.99–1.07	0.14	1.04	1.00–1.08	0.07
eGFR, ml/min/m^2	1.00	0.98–1.01	0.53	0.99	0.98–1.01	0.36
NT-proBNP, Q4 vs. Q1	3.05	1.00–9.30	0.05	3.30	1.1–10.0	0.03
CS, >1.9 vs. <1.9	–	–	–	1.93	1.07–3.48	0.03

CV, cardiovascular; HFrEF, heart failure with reduced ejection fraction; HR, hazard ratio; CI, confidence interval; eGFR, estimated glomular filtration rate; BMI, body mass index; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction.

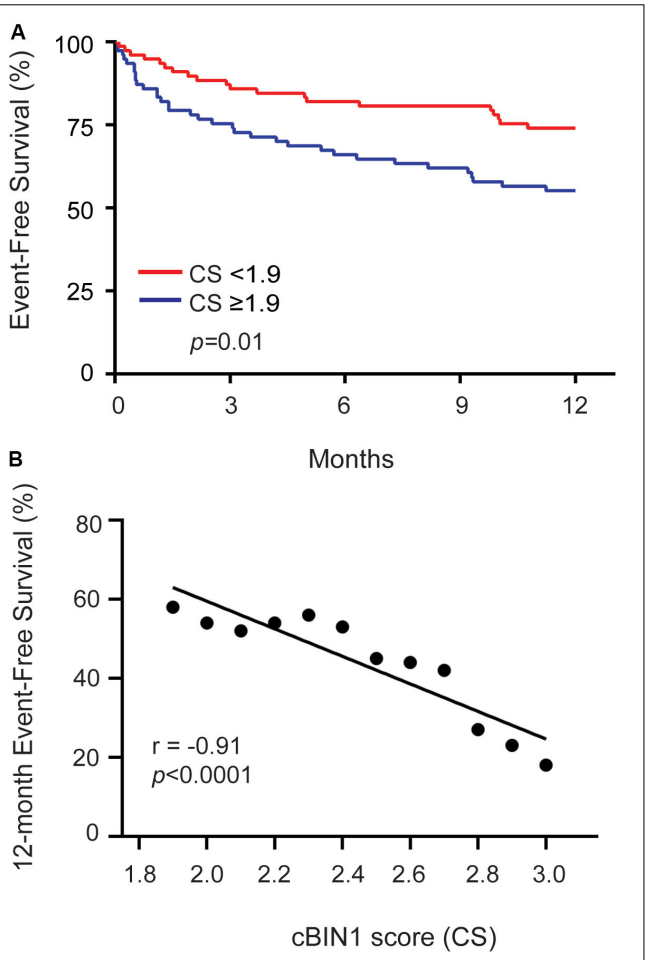

FIGURE 3 | Cardiovascular event free survival of HFrEF patients during 12-month follow-up. **(A)** Kaplain-Meier survival curve is shown here for all HFrEF patients. The red line demonstrates patients with CS < 1.9 and the blue line demonstrate patients with CS ≥ 1.9. Event free survival is defined as patients who did not have a cardiovascular (CV) event (HF-related hospitalization, cardiac hospitalization, LVAD, OHT, or death) during 12-months follow-up. A low CS (<1.9) predicted a higher event-free survival among all HFrEF patients ($p = 0.01$). **(B)** Scatterplot of 12-month event-free survival vs. CS for all CS ≥ 1.9 indicates a negative correlation (Pearsons's correlation coefficient −0.91, $p < 0.0001$).

In this report we find that within a HFrEF cohort, a CS cutoff value of greater than 1.9 accurately predicts cardiac hospitalization during a 1 year follow-up period (**Figure 3**), as it did in a HFpEF cohort (Nikolova et al., 2018). In addition, our findings indicate that the addition of CS, to a model with established risk predictors, significantly improved risk classification for CV events in HFrEF patients. In an ambulatory clinic, a high CS may provide the added prognostic information needed to help sway the pendulum of clinical care toward more aggressive surveillance or escalating care to more advanced therapies. Conversely, an advanced HFrEF patient with a low, even normal CS, may indicate an ability to postpone advanced therapies and continue monitoring with periodic clinic visits. Clinical decompensation with a high CS would indicate failing heart, whereas clinical decompensation with a low CS could suggest extracardiac factors such as renal failure or medical non-compliance are dominating the clinical decline.

Limitations

This is a real-world single center clinical patient population with no exclusion criteria and the HFrEF patients had follow up at the discretion of clinical providers. We would like to reproduce these findings in a prospective multicenter HFrEF cohort. In keeping with our prior study, we used a CS cutoff of the median (1.9). Based on the data in **Figure 3B**, investigators may choose to use a higher cutoff than the median CS, which would improve test specificity.

CONCLUSION

A protein involved in cardiomyocyte t-tubule remodeling is blood available and can function as a biomarker that helps diagnose and prognosticate ambulatory HFrEF patients. The potential advantages of CS include early pre-clinical diagnosis in asymptomatic patients, differentiating cardiac origin volume overload from extracardiac source, prognosticating outcomes in stable ambulatory patients, and evaluating myocardial recovery once on a successful therapeutic regimen. In an era of ballooning health care costs, CS is a blood-available test that substantially adds to the assessment of ambulatory HFrEF.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, RMS, upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Cedars-Sinai Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TCH, TTH, and RS contributed to conception and design of the study. TCH, YX, and RZ organized the database. TCH performed the statistical analysis. YX wrote the first draft of the manuscript. TCH, YX, RZ, AN, MK, MH, AW, TTH, and RS wrote sections of the manuscript. GM, DM, DG, DC, JM, AH, BA, LC, MK, JP, JK, and MH consented patients and collected patient samples. RB, A-MC, and SA ran ELISA assays.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00503/full#supplementary-material

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REVIEW
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Changes in Myocardial Metabolism Preceding Sudden Cardiac Death

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Heart disease is widely recognized as a major cause of death worldwide and is the leading cause of mortality in the United States. Centuries of research have focused on defining mechanistic alterations that drive cardiac pathogenesis, yet sudden cardiac death (SCD) remains a common unpredictable event that claims lives in every age group. The heart supplies blood to all tissues while maintaining a constant electrical and hormonal feedback communication with other parts of the body. As such, recent research has focused on understanding how myocardial electrical and structural properties are altered by cardiac metabolism and the various signaling pathways associated with it. The importance of cardiac metabolism in maintaining myocardial function, or lack thereof, is exemplified by shifts in cardiac substrate preference during normal development and various pathological conditions. For instance, a shift from fatty acid (FA) oxidation to oxygen-sparing glycolytic energy production has been reported in many types of cardiac pathologies. Compounded by an uncoupling of glycolysis and glucose oxidation this leads to accumulation of undesirable levels of intermediate metabolites. The resulting accumulation of intermediary metabolites impacts cardiac mitochondrial function and dysregulates metabolic pathways through several mechanisms, which will be reviewed here. Importantly, reversal of metabolic maladaptation has been shown to elicit positive therapeutic effects, limiting cardiac remodeling and at least partially restoring contractile efficiency. Therein, the underlying metabolic adaptations in an array of pathological conditions as well as recently discovered downstream effects of various substrate utilization provide guidance for future therapeutic targeting. Here, we will review recent data on alterations in substrate utilization in the healthy and diseased heart, metabolic pathways governing cardiac pathogenesis, mitochondrial function in the diseased myocardium, and potential metabolism-based therapeutic interventions in disease.

Keywords: mitochondria, heart failure, sudden cardiac death, substrate utilization, lipotoxicity, glucotoxicity

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INTRODUCTION

Cardiovascular disease is the number one cause of death in the United States and worldwide, accounting for about 17.9 million deaths globally in 2015 (Benjamin et al., 2018). As the incidence of cardiac disease increases exponentially, estimates show that by 2035, expenditures related to chronic heart disease will exceed one trillion dollars (Benjamin et al., 2018). A common terminal event for cardiovascular disease is sudden cardiac arrest (SCA) leading to sudden cardiac death

(SCD). SCA often results from ventricular tachyarrhythmias or ventricular fibrillation initiated by dysfunctional excitation contraction coupling. The genesis of the heartbeat is an intrinsic electrical phenomena of the heart, with electrical impulses driven by pacemaker cells at the sino-atrial node that propagate through the His-Purkinje bundle system, ultimately leading to synchronous ventricular contraction (Jalife et al., 2009). Ventricular contraction is a highly energetic process, as the heart is the most metabolically demanding organ in our body, contracting approximately 3 billion times during an average lifespan of 75 years. In spite of this extremely high energetic requirement, myocardial ATP stores are relatively low with a complete turnover of myocardial ATP pool every 10s (Neely and Morgan, 1974). To accomplish this highly bioenergetic coupling to mechanical contraction, the myocardium utilizes a gamut of circulating energetic substrates such as FAs, glucose, lactate, branched chain amino acids (BCAAs), and ketone bodies (**Figure 1**). These cytosolic metabolites are enzymatically processed, ultimately converging in the generation of acetyl-CoA, and/or reduced nucleotide electron carriers ($FADH_2$ and NADH), that through the electron transport chain (ETC) are used to develop the proton motive force driving ATP production.

While metabolic flexibility is an essential cardiac attribute, the use of each substrate is not only regulated but preference altered during development and in various cardiac pathologies. Under normal physiological conditions, energetic demand is primarily met by carbohydrates and FAs, with the latter being the major fuel source in the adult heart, where β-oxidation contributes to 60–80% of ATP production (Opie, 1968, 1969; Neely and Morgan, 1974). Nevertheless, in several cardiomyopathies, such as left ventricular hypertrophy, the adult heart reverts to a more fetal-like cardiometabolic state, switching from FA oxidation as the primary energy source to oxygen sparing glycolytic energy production (Taegtmeyer and Overturf, 1988; Depre et al., 1998). This review will focus on alterations in metabolism that occur as the heart progresses from healthy to diseased states (**Figure 2**). We will discuss substrate transport, the role of substrate metabolism, metabolic pathways, mitochondrial function, and ion channel modifications that precede and contribute to SCA/SCD. Lastly, we will briefly discuss potential interventions that target myocardial energy metabolism, as an approach to treat and/or prevent heart failure (HF) and SCD.

REGULATION OF GLUCOSE AND FATTY ACID ENTRY IN CARDIOMYOCYTES

In order to meet its own bioenergetic demands, the heart expresses specialized proteins that regulate substrate entry from the blood into the cytosol of cardiomyocytes. While glucose and FAs are the major cardiac metabolic substrates, the heart can also use ketone bodies and BCAAs to meet its energetic demands (**Figure 1**). Transport of substrates is specific and dependent on concentration gradients and hormonal signaling. In this section, we will discuss current knowledge of various modes for glucose and FA entry into cardiomyocytes.

Transporters of Glucose

In the adult heart, about 25 to 30% of energy used for contractile function is generated by carbohydrates (Shao and Tian, 2015). There are three known modes of glucose transport across cellular plasma membranes: (i) facilitative glucose transporters (GLUTs), which are the major form of cardiac glucose entry; (ii) sodium-glucose cotransporters (SGLTs), which use the energy of a favorable sodium gradient to transport glucose against its concentration gradient; and (iii) recently identified sugars will eventually be exported transporters (SWEETs), which promote cellular efflux of glucose (Banerjee et al., 2009; Wright, 2013). SWEETS are novel transporters of glucose that are mostly found in plants, and although understudied in humans, the human SWEET1 gene has been identified in mammary glands where it is thought to provide glucose for lactose synthesis (Chen et al., 2010). Thus far, no other major physiological function of SWEETs is known in humans.

The major path of glucose entry into the heart is via GLUTs, a family of proteins consisting of 14 members, where the main cardiac isoforms are GLUT1 and GLUT4. The expression of GLUT1 and GLUT4 is dysregulated in various cardiac diseases. In response to chronic hypoxia and ischemia, GLUT1 expression increases, leading to cardioprotection via increased glucose influx independent of insulin signaling (Brosius et al., 1997). GLUT1 is localized on the plasma membrane and plays an important role in regulating basal metabolism of cardiomyocytes. GLUT1 can be regulated by fasting and is mainly expressed in embryonic or neonatal stages of cardiac development (Kraegen et al., 1993). Genetically modified animal models have revealed an important role for GLUT1 in regulating glucose utilization for cardiac function. Murine cardiac-specific overexpression of GLUT1 enhanced cardiac glucose utilization, attenuated the development of contractile dysfunction, and improved long-term survival after transverse aortic constriction (TAC; Liao et al., 2002; Luptak et al., 2007). Moreover, acute increases in glucose uptake mediated by inducible overexpression of GLUT1 improved mitochondrial function and reduced cardiac structural remodeling but did not prevent ventricular dysfunction (Pereira et al., 2013).

Following birth, the expression of GLUT4 increases and becomes the predominant glucose transporter in adult hearts. GLUT4 is located in intracellular vesicles that translocate to the plasma membrane upon insulin stimulation or muscular contraction (Rattigan et al., 1991; Slot et al., 1991; Kraegen et al., 1993). During the development of cardiac hypertrophy, and in response to myocardial ischemia (MI), GLUT4 translocates from intracellular vesicles to the plasma membrane, increasing glucose intracellular levels (Slot et al., 1991; Young et al., 1997; Shi et al., 2016). However, GLUT4 expression is decreased in human HF with a concurrent decrease in glucose-mediated energy production (Razeghi et al., 2001; Doehner et al., 2010). Similarly, decreases in GLUT4 expression are also observed in hypertensive models showing insulin-resistance (Paternostro et al., 1995). Mouse models of GLUT4 have revealed that this transporter plays a prominent role in cardiac function. Cardiac

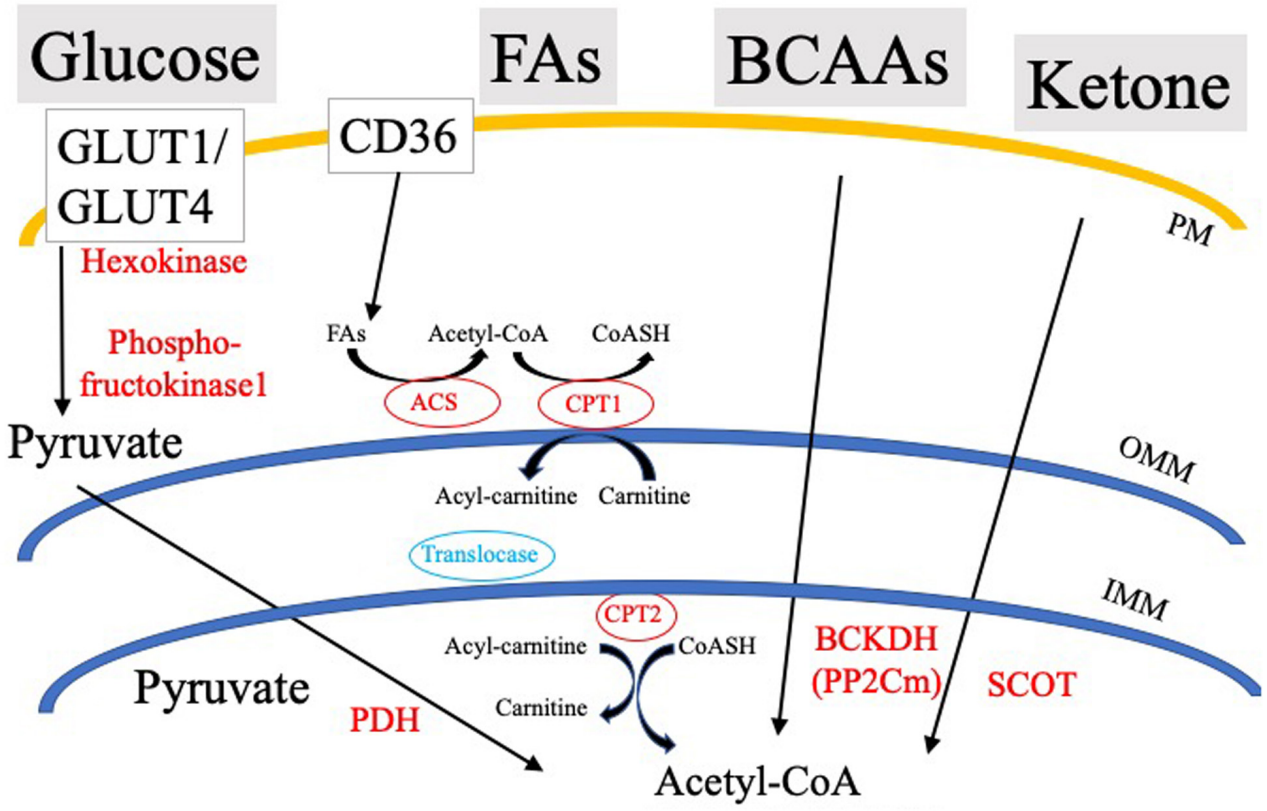

FIGURE 1 | Simplified schematic of cardiac substrate utilization and substrate-specific rate-limiting/key processes. PDH, Pyruvate dehydrogenase; SCOT, CoA Transferase; BCKDH, Branched-chain α-keto acid dehydrogenase; ACS, Acyl-CoA Synthetase; OMM, Outer mitochondrial membrane; IMM, Inner mitochondrial membrane; PM, Plasma membrane; and CPT, Carnitine palmitoyltransferase.

overexpression of GLUT4 increased glycolysis but not glucose oxidation (Belke et al., 2001), while cardiac-specific deletion of GLUT4 led to cardiac hypertrophy with preserved contractile function (Abel et al., 1999). Notably, following downregulation of GLUT4, SGLT1 may mediate glucose utilization, as it is increased 2- to 3-fold in ischemic and type 2 diabetic mellitus (T2DM) hearts (Banerjee et al., 2009) and other studies have reported that SGLT1 colocalizes with GLUT1 at the sarcolemma (Turk et al., 1991; Zhou et al., 2003). Yet, the precise functional role of SGLT1 and any compensatory role in the heart remains poorly understood.

Noteworthy is the ability of incretins to promote GLUT1 expression in cardiomyocytes. In fact, incretins like glucagon-like peptide 1 (GLP-1; produced by enteroendocrine L-cells), and glucose-dependent insulinotropic polypeptide (GIP; produced by enteroendocrine K-cells; Baggio and Drucker, 2007) can regulate appetite, glucose-mediated insulin secretion from pancreatic β-cells (Varndell et al., 1985), and increase GLUT1 expression and translocation to the sarcolemma in cardiomyopathy models (Bhashyam et al., 2010). Interestingly, the impact of incretins is not limited to diabetes-related cardiac dysfunction, as GLP-1 agonists restored cardiac function in dogs with advanced dilated cardiomyopathy (DCM; Nikolaidis et al., 2004). GIP also reduces angiotensin II-induced cardiac hypertrophy and fibrosis in mice (Hiromura et al., 2016). Nonetheless, the importance of incretin signaling in metabolism and chronic disease in humans has been underscored by the association of genetic variations in GLP-1 and GIP receptors with body weight, pancreatic islet function, and risk for T2DM (Sathananthan et al., 2010; Finan et al., 2016).

Transporters of Fatty Acids

As the healthy heart greatly depends on the oxidation of FAs for energy production, FA transport through the plasma membrane is key to myocardial metabolism (Nagoshi et al., 2011). Although FAs can passively diffuse into the plasma membrane of cells (Schaffer and Lodish, 1995), several proteins facilitate cardiomyocyte FA uptake and transport, including cluster of differentiation 36 (CD36 or FAT; Abumrad et al., 1993), plasma membrane-associated FA-binding protein or plasmalemmal FA-binding protein ($FABP_{pm}$; Stremmel et al., 1985), and FA transport proteins (FATPs; Schaffer and Lodish, 1994; Gimeno et al., 2003). In addition to these membrane bound lipid transport proteins, cardiac tissue also expresses heart-FABP (HFABP or FABP3), a cytosolic lipid binding protein that binds and transports hydrophobic lipid species through the cytoplasm (Binas et al., 1999; Storch and Thumser, 2010). In the heart, CD36 is the main transporter responsible for FA uptake.

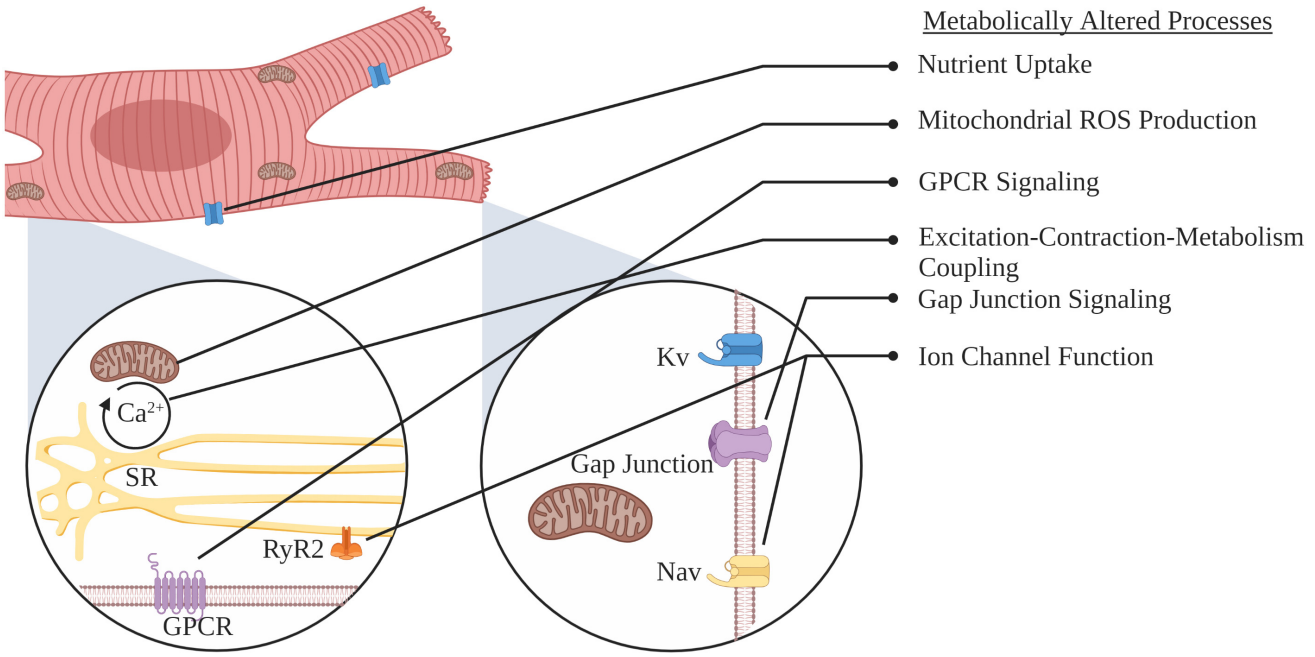

FIGURE 2 | Schematic representation of myocardial adaptations preceding SCA or SCD.

CD36 was first identified as a cellular transporter of FAs in 1993 (Abumrad et al., 1993). Several *ex vivo* and *in vivo* studies support the hypothesis that CD36 is necessary for efficient myocardial FA uptake and accumulation (Coburn et al., 2000; Bharadwaj et al., 2010). Additionally, CD36 can transduce signals that influence how cardiac FAs are utilized. For example, CD36 induces a signaling cascade that favors FA oxidation by activation of 5' adenosine monophosphate-activated protein kinase (AMPK). CD36 forms as complex with LKB1 (AMPK Kinase) and Fyn (src kinase; Samovski et al., 2015). Binding of FA to CD36 disassociates the complex, allowing for LKB1 to phosphorylate and activate AMPK (Samovski et al., 2015). Although CD36 is thought to be the main regulator of cardiac FA transport through the plasma membrane, there does not appear to be a consensus on the role of CD36 in pathological cardiac hypertrophy (Nakamura et al., 1999). For example, in mice that were subjected to TAC, cardiomyocyte-specific ablation of CD36 resulted in a more rapid progression from compensated hypertrophy to HF (Sung et al., 2017). Conversely, in diet induced obese (DIO) mice, cardiomyocyte-specific knock down of CD36 was protective and reduced signs of pathological cardiac remodeling when compared to control mice (Zhang et al., 2015). In response to hypertrophy, the expression of FATPs are significantly decreased (Vork et al., 1992), but the physiological significance of this finding remains unclear. In order for FAs to enter the mitochondria for energy production, FAs must be activated by forming fatty-acyl-CoA. The mitochondrial enzyme that mediates FA entry, carnitine palmitoyltransferase 1 (CPT1), is located on the outer mitochondrial membrane, and modulation of its activity or expression results in alterations in FA substrate utilization in the myocardium (Opie and Knuuti, 2009). CPT1 catalyzes the transesterifcation of acetyl-CoA into acylcarnitine. Subsequently, carnitine acylcarnitine translocase (CACT) transports acylcarnitine across the inner mitochondrial membrane in exchange for a free carnitine molecule. The acylcarnitine is then reconverted into acetyl-CoA via CPT2, which is located on the inner mitochondrial membrane (Houten et al., 2016). CPT1 is the rate limiting enzyme of FA entry into the mitochondria, and its regulation is integral to the control of FA oxidation. An important post-transcriptional step in the regulation of FA oxidation involves the inhibition of CPT1 by malonyl CoA, which is formed from acetyl-CoA via acetyl-CoA carboxylase (ACC; Foster, 2012). Thus, the negative feedback inhibition of CPT1 via malonyl-CoA can avert FA-driven ATP generation, and lead to the incorporation of FA into lipid droplets in the cytosol. Excess intracellular palmitate can also lead to ceramide production and protein palmitoylation of ionic channels and mediators of cardiac conduction (Chien et al., 1996; Pei et al., 2016). Relevant to HF and SCD are the studies suggesting that reversible protein S-pamitoylation plays an important role for sodium and potassium channel biosynthesis (Schmidt and Catterall, 1987). Particularly pertinent to cardiac arrhythmias is the recent study showing that palmitoylation of the major cardiac sodium channel (Nav1.5) leads to increased channel availability and late sodium current activity, promoting the generation of arrhythmogenic events (Pei et al., 2016). Although it is currently not known if palmitoylation impacts the inward rectifier potassium channel (Ik1), there is a known reciprocity between the sodium and potassium channels at the plasma membrane that impacts excitability and arrhythmia generation (Milstein et al., 2012). Thus at the very least, the inward rectifier current could be indirectly impacted by

palmitoylation of Nav1.5, this could be significant as it could also alter resting membrane potential. Palmitoylation is also known to modify the L-type calcium beta2a subunit impacting channel function (Chien et al., 1996). Thus, while it is evident that a link between cardiac metabolism and SCD exists, specific mechanisms dictating this spatiotemporal relationship remain poorly understood.

METABOLIC PATHWAYS GOVERNING CARDIAC FUNCTION AND PATHOGENESIS

Metabolic Flexibility

Metabolic flexibility is a key functional aspect of a healthy heart and it relates to the ability of a cell to adapt to its environment as determined by the utilization of nutrients, oxygen, and hormonal input. It is specifically important in the heart due to the energetic demands of continuous repetitive cardiac muscle contractions and the detrimental feedback loops initiated in its absence, to be reviewed in this section. Loss of this flexibility is thought to be one of the earliest indications of cardiac dysfunction leading to HF (Harris and Das, 1991). Indeed, the failing heart has reduced bioenergetic capacity and phosphocreatine/ATP ratio (Neubauer, 2007). Glucose and FA oxidation are the two main cardiac energy sources, but chronic overreliance or overabundance of one substrate class without the corresponding bioenergetic coupling may have deleterious consequences for myocardial function (Sharma et al., 2004; Chess and Stanley, 2008). Exemplifying this metabolic-contractile link is the potential role for glucose and FA metabolism in regulating the sarcoplasmic-reticulum calcium pump (SERCA) 2a expression via SP1 (a glucose regulated transcription factor) and peroxisome proliferation-activated receptors (PPAR; a FA regulated transcription factor), respectively (Rupp and Zarain-Herzberg, 2002). **Table 1** shows current models of cardiac pathology associated with SCD and their observed metabolic impact.

Cardiac substrate utilization is intertwined, crossing over and impacting parallel metabolic pathways and signaling integrations. For instance, cardiac-specific deletion of ACC prevented TAC-induced decreases in FA oxidation and increased reliance on glucose (Kolwicz et al., 2012). Conversely, cardiac-specific inducible pyruvate dehydrogenase α (PDHα) deletion led to decreased glucose oxidation, decreased AMPK phosphorylation, and ischemic-induced cardiac injury (Sun W. et al., 2016). These mechanistic studies are particularly relevant to pathological conditions that involve altered substrate preferences. For example, in pathological hypertrophy and ischemic heart disease, the heart heavily relies on glucose to meet its bioenergetic needs (Eberli et al., 1991). In contrast, in T2DM cardiac glucose uptake is reduced with concurrent increase in FA oxidation enzymes leading to exacerbated cardiac hypertrophy (Paternostro et al., 1999; Domenighetti et al., 2010).

Under normal physiological conditions, more than 90% of myocardial ATP production is generated via mitochondrial oxidative phosphorylation, with the remainder being produced by anaerobic glycolysis and GTP from the tricarboxylic acid (TCA) cycle (Harris and Das, 1991). Oxidative phosphorylation in the fetal heart mainly relies on glucose, while in adult stages, there is a shift in preference toward FA metabolism. Notably, in the heart, the majority of ATP produced is quickly consumed by myofilaments during contraction. Additionally, about 25% is used to fuel cardiac sarcolemmal and sarcoplasmic reticulum (SR) ion channels and transporters (Schramm et al., 1994). The link between cardiac rhythm, myocardial work, and metabolism was first explored in the early 1900s, when notable physiologists including E. H Starling developed *in situ* models of studying these relationships (Knowlton and Starling, 1912; Patterson et al., 1914). They were able to establish relationships between preload and afterload with energetic rates, oxygen consumption, and changing blood parameters (Evans, 1939). Notably, metabolic dysfunction is ubiquitously observed in chronic heart diseases that are associated with increased risk for SCD.

Lipotoxicity

In 1963, Philip Randle and colleagues hypothesized that the heart must utilize both glucose and FA to meet its high energy demands (Randle et al., 1963). However, successful oxidation of FA produces several factors that inhibit glycolysis, including NADH, ATP, citrate, and acetyl CoA. Thus, an environment where high glycolytic flux and FA oxidation simultaneously occur is rare. Flux through these pathways is influenced by feed/fast cycles and by exercise paradigms such that at different points, glucose and FA are used in different proportions and neither are over-accumulated (Randle et al., 1963). Overabundance of glucose or FA availability or dysregulation of the usage thereof may contribute to cytotoxic effects classified as lipotoxicity and glucotoxicity (Lundsgaard et al., 2019).

Lipotoxicity is induced by the abnormal accumulation of intra-myocellular fatty acids and lipid metabolites in non-adipose tissues such as the heart. Lipid storage in non-adipose sites contributes to altered cellular physiology and perhaps most notably insulin resistance. The summation of these detrimental effects of lipid accumulation is termed lipotoxicity (Sharma et al., 2004). Lipotoxicity can be mimicked via high fat diets that model western feeding habits and/or increased lipid transport into the myocardium (Chiu et al., 2001, 2005). However, the roles of various classes of lipid molecules in myocardial pathogenesis have only been recently explored. Increased levels of triacylglycerol (TG), the main dietary form of lipid, are associated with diseases such as obesity and T2DM. Yet, the role for TGs in inducing cellular lipotoxicity remains controversial (Drosatos and Schulze, 2013). Studies suggest that TG levels act as a marker of general lipid load, while an array of other lipid metabolites play a larger role in increasing or decreasing lipotoxicity. For example, while the role of TG in directly inducing lipotoxicity remains inconclusive, there is evidence that diacylglycerol (DG), a TG metabolite, is linked to lipotoxicity and induction of insulin resistance in muscle and liver (Inoguchi et al., 1992; Erion and Shulman, 2010). More recently, Law and colleagues have demonstrated that very long ceramide species (≥24 carbons) diminish mitochondrial function and increase

TABLE 1 | SCD-disease models with associated metabolic profiles and resultant toxicity.

Disease model	Change in metabolism	Metabolic toxicity	Potential therapeutic metabolic intervention	References
TAC	↓Fatty acid oxidation ↑Glycolysis ↓BCAA Catabolism ↑Ketone Body Catabolism	Glucotoxicity Lipotoxicity	Restoration of glucose Oxidation Redirection of lipid synthesis	Shao and Tian, 2015; Muthuramu et al., 2017
I/R	↓Fatty acid oxidation ↑Glycolysis	Glucotoxicity Lipotoxicity	Restoration of FAO Restoration of glucose oxidation Redirection of lipid synthesis	Bonezzi et al., 2019; Goldenberg et al., 2019
β-Adrenergic Overstimulation	↓Fatty acid oxidation ↑Glycolysis ↓BCAA Catabolism	Glucotoxicity Lipotoxicity Insulin Resistance	Restoration of glucose oxidation Restoration of insulin sensitivity	Opie and Knuuti, 2009; Shao and Tian, 2015
Diabetes	↑BCAA/BCKA Exposure ↑Ketone Body Exposure	Lipotoxicity Insulin Resistance	↑BCAA catabolism Restoration of insulin sensitivity	Lim et al., 2020

apoptosis, autophagy, and mitophagy in cardiomyocytes (Law et al., 2018). Conversely, in a model where long-chain FA synthesis was re-directed toward long-chain ceramide synthesis (20–22 carbons) a cardioprotective phenotype was demonstrated in a TAC model of left ventricular hypertrophy, demonstrating the importance of lipid saturation and chain length. This latter study used a mouse model overexpressing cardiac acyl-coenzyme A synthetase 1, and thereby affected multiple lipid metabolism parameters (Goldenberg et al., 2019). However, the lipid profile following the TAC intervention specifically deviated in ceramide species accumulation (Goldenberg et al., 2019). Additionally, saturated FAs, such as palmitic acid, have been associated with increased ceramide synthesis and cardiomyocyte apoptosis when compared to unsaturated FAs, such as oleic acid (Okere et al., 2006). In fact, restoring FA oxidation in the heart by overexpression of Long-chain-FA-CoA ligase 1 (ACSL1) in a TAC model preserved cardiac function (Goldenberg et al., 2019). Together, this emphasizes the importance of lipid diversity in the heart and how they may differently impact the development of lipotoxicity.

The main source of FAs that fuel mitochondrial oxidative metabolism are circulating free FAs (FFAs) bound to albumin and/or released from TGs that are present in very-low-density lipoproteins (VLDL) or chylomicrons (Niu et al., 2004; Bharadwaj et al., 2010). As mentioned above, extracellular FAs can enter cardiomyocytes via passive diffusion, FATPs, or CD36 (Schaffer and Lodish, 1994; Gimeno et al., 2003; Bharadwaj et al., 2010). Once FFAs are in the cytosol, they are esterified to CoA via fatty acyl-CoA synthase enzymes, forming long-chain fatty acyl-CoAs (Chiu et al., 2001, 2005). These fatty acyl-CoAs can then shuttle into mitochondria via CPT1 and CPT2. CPT1 converts the long chain fatty acyl-CoA to long chain acylcarnitine in the outer mitochondrial membrane, which is then subsequently converted back to long chain fatty acyl CoA by CPT2 in the mitochondrial inner membrane (Murthy and Pande, 1984; McGarry and Brown, 1997). As mentioned previously, a major point of regulation for fatty acyl-CoA transport into the mitochondria involves CPT1, which is robustly inhibited by the presence of malonyl CoA (Foster, 2012). The levels of malonyl CoA are determined by the balance of acetyl CoA synthesis via ACC, and degradation of malonyl CoA by malonyl CoA decarboxylase (Dyck and Lopaschuk, 2002; Ussher and Lopaschuk, 2008). For example, citrate produced from the TCA cycle can move to the cytosol and activate ACC (Lane et al., 1970; Beaty and Lane, 1983), which leads to greater production of malonyl CoA and a feedback response that reduces mitochondrial FA oxidation.

During HF, fatty acyl carnitines in the cytosol and sarcolemma are accumulated up to 10 times their normal levels, particularly in cardiac regions exposed to ischemia. The relationship between lipid accumulation and electrical abnormalities has been hypothesized in the 1980s (Corr et al., 1989). Since then multiple pathways have been established by which fatty acyl carnitines affect ion channel function and therefore arrhythmogenic events. Reactive oxygen species (ROS) generated from fatty acyl carnitines interact with redox sensitive channels, such as the ryanodine receptor, thus modulating calcium release from the SR during contraction. Palmitoyl carnitine, a fatty acyl carnitine, increases the oxidation of the ryanodine receptor 2 resulting in prolonged calcium leak following opening (Roussel et al., 2015). Direct infusion of palmitoyl carnitine at levels observed in diabetic cardiomyopathy is independently arrhythmogenic in healthy animals (Roussel et al., 2015). Palmitoyl carnitine exposure to cardiomyocytes induces the slow-inactivating sodium current, impacting current inactivation thereby augmenting intracellular sodium concentration. Subsequently, palmitoyl carnitine inhibited the sodium potassium exchanger by approximately 13%, inducing an elevation in intracellular calcium (Wu and Corr, 1995). Moreover, long-chain acyl carnitines are known to reduce gap junctional conductance by 68%, a phenomenon which has been linked to the preferential accumulation of endogenous long-chain acyl carnitines at the sarcolemma during hypoxia (Wu et al., 1993). These effects alter the incidence of early after depolarizations, delayed after depolarizations, and action potential propagation promoting the development of arrhythmias.

In addition to certain lipids that may directly induce lipotoxicity, such as ceramides and DGs, ROS is generated as a byproduct of the ETC which can induce cellular stress. ROS are generated when electrons leak from the mitochondrial ETC,

allowing free electrons to reduce molecular oxygen (Turrens, 2003). Certain pathological conditions, such as MI and HF, can increase ROS production via mitochondrial membrane leakage. Elevation of mitochondrial ROS can damage ETC complexes, further impairing ATP production in a failing heart (Murray et al., 2003). This leads to a vicious circle in which the electron leak increases ROS formation, and ROS formation alters the ETC to favor additional production of ROS. ROS can damage ETC complex proteins and can react with polyunsaturated FAs, which enhances the formation of peroxidized lipids that can dysregulate phospholipid membranes (Mylonas and Kouretas, 1999). In humans with diabetic cardiomyopathy and obesity-related cardiomyopathy, insulin resistance diminishes glucose uptake and utilization enhancing cardiac lipid accumulation (Boudina and Abel, 2007; Montaigne et al., 2014). As such, impairments in cardiac lipid metabolism that reduce intracellular TG-derived FA mobilization and oxidation promote an accumulation of intracellular TG (McGavock et al., 2007; O'Donnell et al., 2008). Interestingly, similar to citrate, insulin also inhibits FA oxidation through activation of ACC (Witters and Kemp, 1992). In the failing heart, insulin-induced inhibition of FA oxidation is impaired, but the effect of chronic dysregulation of genes associated with FA metabolism dominates such that TG and DG are still accumulated (Inoguchi et al., 1992; Zhang et al., 2013; Fukushima and Lopaschuk, 2016).

Glucotoxicity

Glucotoxicity is characterized by an intracellular accumulation of glucose metabolites and subsequent impairment in glucose-mediated oxidative phosphorylation. In multiple HF etiologies, such as left ventricular hypertrophy, the heart increases its reliance on oxygen sparing glycolytic energy production. Although glucose uptake is increased, oxidative phosphorylation is diminished, or unchanged (Mori et al., 2013; Li et al., 2017a). In HF patients, augmented glycolysis is uncoupled from oxidative phosphorylation, or mitochondrial energy production, leading to the accumulation of glycolytic byproducts such as lactate and protons (Diakos et al., 2016). These glycolytic byproducts can lead to acidosis, inducing aberrations in ATPases that regulate cytosolic Na^+ and Ca^{2+}, ultimately reducing cardiac contractility (Fiolet and Baartscheer, 2000; Jaswal et al., 2011).

High glucose alone can promote pathological cardiac development by accelerating hypertrophy, promoting ER stress, increasing ROS production, and ultimately leading to apoptosis (Ng et al., 2018; Zhang X. et al., 2018; Shi et al., 2019). When uncoupled from oxidative phosphorylation, glycolysis produces glycolytic intermediates that can accelerate flux through non-anapleurotic pathways, such as the pentose phosphate pathway (PPP), hexosamine biosynthetic pathway, or lactate shuttling (Gupte et al., 2006; Gibb et al., 2017). Specifically, increased flux through the PPP occurs in both hypertrophic and chronic HF (Meerson et al., 1967; Zimmer and Peffer, 1986). Li and colleagues recently demonstrated that restoring glucose oxidation by inhibiting pyruvate dehydrogenase kinase (PDK) 4, a rate controlling enzyme of glucose oxidation, improved cardiac function, and glucose uptake post-ischemia reperfusion injury (Li et al., 2017a). Increased glucose exposure over long periods reduces BCAA catabolism, leading to an accumulation of BCAA and BCAA metabolites (Zhang X. et al., 2018). It is well established that leucine, a BCAA, is a potent activator of the mammalian target of rapamycin (mTOR) signaling pathway (Xu and Brink, 2016). Increased mTOR signaling, a known regulator of cell growth and metabolism, promotes the progression of cardiac hypertrophy and cell death (Xu and Brink, 2016). Moreover, increased glucose exposure has been shown to alter miRNAs that control protein expression and thereby regulate gross cell metabolism. For instance, elevation of miRNA-195 upregulates multiple genes associated with hypertrophic development. Exogenous administration of this oligonucleotide can promote hypertrophy and alter cardiac mitochondrial function, while the complementary oligonucleotide provides a protective effect (Shi et al., 2019; Wang et al., 2019). Lastly, increased use of glucose has been shown to induce cell growth (Kolwicz et al., 2013; Shao et al., 2018) and alter epigenetic regulation in the nucleus (Shao et al., 2018; Lombardi et al., 2019). Even though elevation in glucose appears to be an important signaling mechanism for cell growth and differentiation, specific mechanisms detailing how chronic exposure to high levels of glucose leads to glucotoxicity remains to be further characterized.

Glucotoxicity promotes protein glycation and glycosylation, which are, respectively, non-enzymatic and enzymatic protein modifications mediated by carbohydrates. These changes impact many pathways relevant to myocardial structure-functional relationships. Increased glycation drives the formation of advanced glycation end-products, which accumulate in tissues and contribute to inflammation and subsequent structural alterations, namely fibrosis (Dziubak et al., 2018). The deposition of advanced glycation end products contributes to electrical abnormalities through alterations of the myocyte cytoskeleton, altered ion channel activity, and damage to the local autonomic nervous system that contribute to regulating cardiac rhythm (Balcıoğlu and Müderrisoğlu, 2015). Advanced glycation end products specifically inhibit voltage gated K^+ channels, Kv channels, that play a primary role in cardiac microcirculation and a secondary role in atrial repolarization, contributing to metabolic remodeling and arrhythmogenic susceptibility (Bai et al., 2015; Su et al., 2015). Relevant to atrial repolarization and His-Purkinje conduction is increased N-glycosylation of the K_{2P} potassium channel, enhancing its function and transport to the cell membrane. Interestingly, while acute or chronic high glucose exposure increase N-glycosylation of the K_{2P} channel, chronic exposure results in downregulation of its expression (Wiedmann et al., 2019). Acute hyperglycemia promotes the covalent modification of CaMKII by O-linked N-acetylglucosamine (O-GlcNAc) at Ser279, which activates CaMKII autonomously, leading to molecular memory as calcium concentration declines (Erickson et al., 2013). Increased O-GlcNAc-modified CaMKII, as observed in human diabetic hearts, enhances CaMKII-dependent activation of spontaneous SR calcium release, leading to augmented premature ventricular complexes that contribute to arrhythmogenicity (Erickson et al., 2013). Moreover, voltage gated sodium channels, the major ionic current responsible for ventricular membrane depolarization, are also heavily modulated by glucose-based and other posttranslational modifications that

can be dysregulated by metabolism. For instance, regulation of Nav1.5 glycosylation and sialyltransferase activity impacts the sodium voltage-gated activity and propensity for arrhythmias (Ufret-Vincenty et al., 2001; Ednie et al., 2013). Moreover, exposure of high glucose in CHO cells transiently overexpressing the human Nav1.5 channel led to a rightward shift in voltage dependence of conductance and steady-state fast inactivation, which was attributed to the observed increase in ROS (Fouda et al., 2020). Whether this mechanism is also pertinent to cardiac cells remains to be determined.

Ketone Body Metabolism

In HF, cardiac substrate utilization of glucose and FAs decrease with a concurrent increased reliance on ketone bodies and BCAAs (Kolwicz et al., 2013; Aubert et al., 2016; Bedi et al., 2016). In the hypertrophied and failing heart, ketone body metabolism supplements energy production following decreased FA oxidation (Aubert et al., 2016). Ketones are primarily produced within hepatocytes and form acetyl-CoA via 3-oxoacid-CoA transferase (SCOT) in extrahepatic tissue to generate energy (Laffel, 1999). The major determinant of cardiac ketone oxidation rates is circulating ketone levels (Laffel, 1999; Ikegami et al., 2017). In human HF, ketone utilization plays an important role in maintaining cardiac metabolism (Aubert et al., 2016; Bedi et al., 2016). Recently, Schugar and colleagues reported that mice subjected to TAC with a cardiomyocyte-specific ablation of SCOT (the rate limiting enzyme in ketone oxidation) displayed increased mitochondrial dysfunction, and accelerated pathological cardiac remodeling (Schugar et al., 2014). Additionally, overexpression of cardiac D-β-hydroxybutyrate dehydrogenase 1 (BDH1) decreased ROS and apoptosis in mice subjected to TAC (Uchihashi et al., 2017). Studies in isolated rat cardiomyocytes exposed to simulated hypoxia showed that β-hydroxybutyrate increases contraction and calcium in a dose-dependent manner (Klos et al., 2019). Experiments in isolated working hearts subjected to 4-weeks of TAC showed that enhancing ketone body oxidation increased energy production although it did not show significantly improvement in cardiac efficiency (Ho et al., 2019). Thus, increased myocardial ketone oxidation may be an adaptive mechanism for the failing heart. These results from animal models are supported by recent findings in humans with chronic HF that show decreased expression of lipid utilization proteins with increased expression of ketone catabolic genes (Aubert et al., 2016; Bedi et al., 2016).

Recently, the "ketogenic diet" has become popular, yet it remains unknown whether enhancing long-term ketone oxidation is adaptive or maladaptive to the human heart. There is concern about the common use of this diet, especially in obese and diabetic individuals with an increased risk for SCA/SCD. Currently, it appears that the benefit of weight loss associated with this diet may outweigh potential adverse cardiac metabolic changes (Harvey et al., 2019). In wild-type mice, ketogenic diets increase ketone oxidation genes, decrease gene profiles associated with glucose utilization, and do not alter expression of FA utilization genes (Shimizu et al., 2018). Nevertheless, long-term effects of increasing ketone bioenergetics remain to be fully detailed.

BCAA Metabolism

Branched chain amino acids can be metabolized in the cardiac muscle via the branched-chain α-ketoacid dehydrogenase (BCKDH) complex (Neinast et al., 2019). Alterations in cardiac BCAA metabolism are associated with cardiac pathologies. For example, defective BCAA catabolism disrupts glucose signaling and sensitizes the heart to ischemic-reperfusion injury (Li et al., 2017b). The expression of BCAA catabolic enzymes is decreased in HF patients, in conjunction with increased levels of BCAA and branched chain keto acids (Sun H. et al., 2016). In fact, studies in TAC-induced mice suggest that increasing BCAA metabolism preserves cardiac structure and function (Chen et al., 2019). BCAA supplementation and more generally high protein diets are common practices in order to obtain desired physiological outcomes such as in resistance training. In a large observational study, it appears that both low (<1 *g*/kg body weight) and high protein (>1.38 *g*/kg body weight) intake associate with increased risk for cardiovascular events as well as all-cause mortality even when accounting for risk factors and renal function (Halbesma et al., 2009). Specific conclusions regarding the BCAA element of these dietary classifications, however, require further studies. BCAAs only represent a small part of total protein intake, and high protein diets in observational studies will most likely differ in macronutrient and micronutrient makeup. BCAA supplementation in addition to resistance exercise training in late-stage HF patients does not improve physical and functional capacities (Pineda-Juarez et al., 2016). Nevertheless, multiple clinical trials continue to investigate a potential interaction between BCAA metabolism, BCAA supplementation, and exercise in different types of HF (Halbesma et al., 2009; Pineda-Juarez et al., 2016).

PATHOLOGICAL MITOCHONDRIAL FUNCTION

Mitochondrial Calcium Handling

Deterioration in contractile mechanics of a heart that is hypertrophied, dilated, or fibrotic correlates with altered excitation-contraction coupling and failing bioenergetics (Franz et al., 1992; Nishimura et al., 2006). Contractile force generation requires calcium signaling; while intracellular SR calcium is reviewed in detail elsewhere (Eisner et al., 2020), in this section, we will specifically provide a brief overview of mitochondrial calcium handling. Mitochondrial calcium dynamics are determined by ATP/ADP ratio, transporter expression levels, and other regulatory signals (Zhou and Tian, 2018). Calcium entry in the mitochondrial inner membrane can be facilitated by the mitochondrial calcium uniporter (MCU). Systemic MCU knockout studies did not reveal any major baseline phenotype although mitochondrial calcium handling was diminished (Pan et al., 2013). Cardiac-specific MCU deletion led to improved cardiac function post-IR (Luongo

et al., 2015), supporting the notion that MCU does not impact baseline physiology but instead modulates energetic upregulation by increasing contractility or sympathetic stress signaling. Moreover, MICU1, a regulator of the MCU (Mallilankaraman et al., 2012), was recently shown to possess an additional function where it controls cristae junction and anchoring of the MCU complex (Gottschalk et al., 2019). Mitochondrial calcium extrusion, however, is dependent on mitochondrial Na/Ca exchanger (NCLX). Murine models of inducible cardiac-specific NCLX knockout increased arrhythmogenicity and SCD, while overexpression of NCLX was cardioprotective post-IR (Luongo et al., 2017). Calcium extrusion can also be elicited via the mitochondrial permeability transition pore (MPTP), which promotes apoptotic signaling. Although the molecular identity of the MPTP is debated and unknown, recent studies have suggested that dimerization of the F1F0 ATP synthase is responsible for the pore forming MPTP (Urbani et al., 2019), with a prominent role for the c-subunit of the ATPase in MPTP formation (Neginskaya et al., 2019). Most importantly, recent studies have linked the relevance of mitochondrial calcium dynamics to human cardiac pathology. In diabetic rats, cardiac mitochondrial MCU expression is decreased, diminishing mitochondrial calcium and subsequently blunting pyruvate dehydrogenase activity, which it is the rate-limiting process in glucose oxidation (Suarez et al., 2018). In agreement are studies showing decreased levels of MCU and MICU1 in cardiac tissue from ischemic HF patients (Luongo et al., 2015). The feasibility and specificity of pharmacologically targeting these mitochondrial channels and signaling pathways remain to be determined.

Mitochondrial ROS Generation

ROS are generated when electrons leak from the mitochondrial ETC, allowing free electrons to reduce molecular oxygen (Turrens, 2003). Certain pathological conditions, such as MI and HF, increase ROS production via mitochondrial membrane leakage. Elevation of mitochondrial ROS damage ETC complexes further impairing ATP production in the failing heart (Murray et al., 2003; Redout et al., 2007; Dubouchaud et al., 2018). ROS generation is in concert with previously discussed alterations in mitochondrial calcium dynamics and reduced oxidative phosphorylation (Ide et al., 1999; Zhang H. et al., 2018). Dysfunction of complex 1, 2, and 3 of the ETC have been associated with ROS production (Redout et al., 2007; Dubouchaud et al., 2018). ROS can also trigger the reversible, transient, and minimally concerted opening of the MPTP, which is thought to have a housekeeping physiological function of releasing ROS to the cytosol. Prolonged ROS generation triggers MPTP opening, which leads to ROS-induced-ROS release promoting the destruction of the mitochondrion that may be propagated inter-mitochondrially, culminating in cell death (Zorov et al., 2000). Interestingly, deletion of cyclophilin-D, a regulator of the MPTP, led to resistance to MPTP opening and improved cell survival post-IR (Baines et al., 2005). Additionally, loss of cyclophilin-D led to greater hypertrophy and HF (Elrod et al., 2010). Mitochondrial oxygen levels can be sensed by the mitochondrially localized NADPH-oxidase 4 (Nox4). Unlike other Nox family members, Nox4 is constitutively active where 90% of the electron flux through isolated Nox4 produces H_2O_2 and 10% forms superoxide (Nisimoto et al., 2014). Cardiac-specific Nox4 overexpression is detrimental to cardiac function post-ischemia-reperfusion injury (Ago et al., 2010), re-enforcing the crucial role that Nox4 plays in regulating myocyte function. An extensive review of mitochondrial ROS is found elsewhere (Cadenas, 2018).

There is a strong link between ROS and cardiac ion channel dysfunction directly impacting cardiac electrophysiology, contractile deficiencies, and SCD. For instance, the ryanodine receptor, can be oxidized by ROS resulting in altered calcium release (Roussel et al., 2015). The carotid body is a group of neurosensory cells that sense blood oxygenation and inform ventilation and adrenergic stimulation to increase blood flow in response to ischemia. Voltage-gated potassium channels (Kv) channels are significantly impacted by redox-based reactions in the coronary vasculature and the carotid body. As a result of altered metabolism there is an increase in ROS in the coronary vasculature and carotid body, resulting in greater oxygenation and perfusion rates in an attempt to reduce hypoxic exposure (Rogers et al., 2006; Moreno-Dominguez et al., 2020). Therein ROS, plays an important direct role in the hyperemic response to increased cardiac output. Yet, long-term activation of this mechanism, either due to metabolic or structural remodeling, has adverse consequences as ROS alters a multitude of vital cardiac pathways that promote SCD. Although Kv channels are also found elsewhere in the heart, including the sino-atrial node and His-Purkinje system, how ROS alters action potential propagation in these specialized cardiac cells are currently unknown. Importantly, Kv channels have varying subunit composition in various tissues as well as modulation by other cellular redox agents such as NADH and NADPH which increase the activity of Kv channels (Dwenger et al., 2018). Kv channel activation via ROS in an acutely adaptive mechanism, such as increasing oxygenation and perfusion, contrasts Kv channel inhibition by glycation and products discussed in the section "Glucotoxicity" (Su et al., 2015; Moreno-Dominguez et al., 2020). While it is reasonable to conclude that metabolic toxicity would dysregulate this family of ion channels, further studies are required to delineate how ROS modification of various ionic channels are integrated in the resultant arrhythmic incidence of the heart.

PAST AND ON GOING THERAPEUTIC INTERVENTIONS TO IMPROVE GLUCOSE AND FA METABOLISM

Clinical Approaches to Modulate Glucose Metabolism

The interaction between insulin resistance and diabetic cardiomyopathy has been a major driver for unraveling therapies that enhance glucose metabolism. Enhancing glucose uptake has been attempted by insulin infusion called glucose–insulin–potassium (GIK) therapy, which involved exposure to high concentrations of insulin during acute ischemia (Sodi-Pallares

et al., 1962). Some studies have suggested that GIK infusion during ischemia reduced infarct size and improved post-ischemic cardiac dysfunction, but clinical trials were inconsistent and a meticulous glucose control was essential for those patients (Van den Berghe et al., 2003; Malmberg et al., 2005). GLP1 can increase glucose uptake by increasing GLUT2 expression in pancreatic islets (Villanueva-Penacarrillo et al., 2001). Exenatide is a human GLP1 receptor agonist that is currently being investigated as a potential adjunct therapy for HF. A study conducted in 2007 showed that patients treated with exenatide had decreased mortality from cardiovascular causes when compared to patients treated with placebo or standard of care (Erdmann et al., 2007). Exenatide can also contribute to vasorelaxation due to the effect on opening K_{ATP} channels and therefore preventing vessel dysfunction (Selley et al., 2014). Conversely, recent studies showed no significant benefit on cardiovascular events in T2DM patients (Mentz et al., 2018). Liraglutide is an FDA-approved drug that is currently used to treat T2DM patients. Liraglutide also activates the GLP1 receptor, and is currently being investigated as a potential treatment for HF. T2DM patients treated with liraglutide have improved mortality rates compared to placebo treated patients (Cohen and Beckey, 2016). Circulating GLP1 is quickly metabolized by dipeptidyl peptidase (DPP)-IV, and thus, a DPP-IV inhibitors can be used as adjuvant with GLP1 agonists to improve GLP1 efficacy (Deacon, 2004). One study demonstrated that an inhibitor of DPP-IV, sitagliptin, improves left ventricular function in patients with coronary artery disease (Read et al., 2010). It has been suggested that the mechanism for the cardioprotective effect of sitagliptin is via upregulation of the transient receptor potential channel (TRP), therefore increasing Ca^{2+} influx (Al-Awar et al., 2018). In contrast, a recent study revealed no benefit on reducing HF risk in patients with diabetes treated with sitagliptin (Nauck et al., 2019).

Pyruvate is a key glycolytic metabolite which has positive inotropic actions in both healthy and dysfunctional hearts, improving left ventricular function (Mentzer et al., 1989). Pyruvate can potentiate the inotropic response of β-adrenergic receptor compounds and result in increased intracellular Ca^{2+} transients via improvement of SERCA in failing human myocardium (Hermann et al., 2002). The pyruvate dehydrogenase complex (PDC) metabolizes pyruvate to acetyl-CoA, and PDK inactivates this complex via phosphorylation, leading to a buildup of glucose metabolites. Inhibiting PDK with dichloroacetate (DCA) is one approach that increases the utilization of glucose in the heart (Stacpoole et al., 1998). Clinical evidence has shown that DCA can weakly improve cardiac function through the regulation of voltage-gated K^+ channels, but unfortunately, this compound has a major toxicity risk for the liver, kidney, and nervous system (Michelakis et al., 2002). Other alternative compounds currently under development for PDK inhibition have also shown substantial side effects in multiple organs (Morrell et al., 2003; Roche and Hiromasa, 2007). Currently, another effective modulator of PDK, PS10, is being evaluated as potential drug target (Wu et al., 2018). Moreover, SGLT2s are expressed in the early proximal tubule of the kidney (Vallon et al., 2011), and while SGLT2 inhibitors have been developed with the intent to improve glucose control in diabetic patients via its impact on renal sodium-glucose re-absorptive function, it has sparked enthusiasm for the treatment of HF. The EMPA-trial revealed that T2DM patients treated with empagliflozin possessed a 38% relative risk reduction in death from cardiovascular causes (Zinman et al., 2015). Empagliflozin also significantly regulates calcium and sodium exchange, which can be a potential mechanism for the cardioprotective effect (Lee et al., 2019). While there is a consensus that improving glucose metabolism is an attractive pharmacological intervention, specific, safe, and effective compounds that provide cardiovascular benefits still need to be developed.

Therapeutic Strategies Targeting Fatty Acid Utilization

As mitochondrial FA utilization is dependent on CPT-1, this transporter is an attractive pharmacological target for HF, such that inhibiting FA oxidation can result in parallel increase of glucose utilization (Xu et al., 1995). Perhexiline maleate was developed as an antianginal drug in the 1970s (Ashrafian et al., 2007). It potentially inhibits the uptake of fatty-acyl-CoA via mitochondrial CPT-1 (Murray et al., 2005), and this inhibition of FA metabolism improves left ventricular ejection fraction (LVEF) in patients with chronic HF (Lee et al., 2005). Perhexiline also potentially protects the heart from cardiac dysfunction by inhibiting Human ether-a-go-go-related gene (HERG) channels (Walker et al., 1999). Etomoxir is an irreversible inhibitor of CPT-1 (Lopaschuk et al., 1988) that also increased SERCA2a expression, improving SR Ca^{2+} handling and cardiac function (Vetter and Rupp, 1994). It was first developed and tested in clinical trials in 1984, passing phase I and II, and becoming a promising drug for HF patients (Schmidt-Schweda and Holubarsch, 2000). Unfortunately, etomoxir treatment resulted in abnormally high liver transaminase levels and now is only used as an experimental tool for inhibiting FA oxidation (Holubarsch et al., 2007).

Trimetazidine is an inhibitor of 3-ketoacyl CoA thiolase, the terminal enzyme of FA β-oxidation (Kantor et al., 2000; Lopaschuk et al., 2003). Trimetazidine treatment reduced FA oxidation and induced a compensatory increase in glucose oxidation. Trimetazidine also reduced the accumulation of lactic acid caused by the buildup of fatty acids. Further, this drug modified calcium dynamics by inhibiting SERCA activity and modifying the density of Ca^{2+} channels in cardiomyocytes impacting left ventricular systolic and diastolic function (Kiyosue et al., 1986; Banach et al., 2006; Meng et al., 2006). In clinical trials trimetazidine significantly increased LVEF (Vitale et al., 2004), myocardial perfusion, oxidative metabolism, and work efficiency (Fragasso et al., 2006), and is being investigated as a potential therapeutic strategy for HF patients (Dezsi, 2016).

Ranolazine was FDA-approved in 2006 for the treatment of stable angina pectoris (Reddy et al., 2010). Ranolazine was first thought to inhibit FA metabolism similarly to trimetazidine, but the drug concentration required to inhibit FA β-oxidation was much higher than the recommended therapeutic dose, indicating an alternative mechanism of action (McCormack et al.,

TABLE 2 | Therapeutic interventions that may regulate glucose and FA metabolism.

	Target	Name	Clinical use	Arrhythmia events	References
Glucose metabolism	GLP-1 agonist	Exenatide	Under investigation-No significant benefit	Yes, unknown ionic alteration	Mentz et al., 2018
		Liraglutide	Under investigation	No	Cohen and Beckey, 2016
	DPP-IV inhibitor	Sitagliptin	Under investigation-No significant benefit	No	Read et al., 2010; Nauck et al., 2019
	SGLT2 inhibitor	Empaglifloxin	Glucose management-Cardiac benefit under investigation	Antiarrhythmic effect via regulates Na^{+}-Ca^{2+} exchanger	Zinman et al., 2015
	PDK inhibitor	Dichloroacetate (DCA)	Toxicity	No	Stacpoole et al., 1998
		PS10	Under investigation	Not yet determined	Wu et al., 2018
Fatty acid metabolism	CPT-1 inhibitor	Etomoxir	Adverse effects	Antiarrhythmic effect via improving Ca^{2+} handling	Vetter and Rupp, 1994; Holubarsch et al., 2007
		Perhexiline maleate	Under investigation	Antiarrhythmic effect via inhibit HERG channels	Lee et al., 2005
	β-oxidation inhibitor	Trimetazidine	Under investigation	Antiarrhythmic effect via modify Ca^{2+} channels	Fragasso et al., 2006; Dezsi, 2016
		Ranolazine	Under investigation	Antiarrhythmic effect via regulate both Na^{+} and Ca^{2+}	Sossalla et al., 2008; Maier et al., 2013
	PPARα agonist	Fenofibrate	Under investigation	Antiarrhythmic effect via K^{+} channel	Morgan et al., 2006; Elam et al., 2011, 2017

1996). Studies suggest that the primary mechanism of action for ranolazine is via inhibition of the late sodium current and diastolic Ca^{2+}overload, which benefit patients with diastolic dysfunction (Antzelevitch et al., 2004; Belardinelli et al., 2006; Fraser et al., 2006). Ranolazine has been shown in clinical studies to have therapeutic efficacy for many kinds of cardiomyopathies (Sossalla et al., 2008; Maier et al., 2013).

Lastly, PPARs are key transcriptional factors that regulate FA uptake and metabolism, and modulation of various PPAR family members may have a beneficial impact for HF. The expression of PPARα is decreased during TAC-induced HF (Kaimoto et al., 2017). However, ischemic failing rats hearts treated with the PPARα agonist fenofibrate did not show improvements in left ventricular function (Morgan et al., 2006). Additionally, fenofibrate treatment may lead to left ventricular dysfunction in mice with hypertension (Ogata et al., 2004; Brigadeau et al., 2007). Post-trial follow up from the ACCORD study confirmed the original overall neutral results of the study but revealed a beneficial impact of fenofibrate therapy in reducing cardiovascular diseases in a specific group of participants with diabetes, hypertriglyceridemia, and low high-density lipoprotein cholesterol (Elam et al., 2017). PPARγ agonists are currently used in the treatment of T2DM with suggested indirect benefits to cardiovascular disease (Elam et al., 2011). **Table 2** summarizes previous and ongoing therapeutic interventions for glucose and FA metabolism.

CONCLUSION

Atrial fibrillation, tachyarrhythmias, and dysfunction in mechanical junctions preceding cardiac structural remodeling and fibrosis may all contribute to SCD (Jalife et al., 2009; Sato et al., 2009, 2011, 2018). More recently, cardiac metabolism has been studied as a potential early causal mode that promotes and supports SCD (Wu et al., 1993; Roussel et al., 2015). Metabolic flexibility and shifts in metabolic utilization are major determinants of adaptive and maladaptive cardiac signaling. This is particularly evident in T2DM patients, where aberrant glucose control is thought to contribute to cardiac dysfunction in more than 50% of patients (Dunlay et al., 2019). While metabolic pathways are somewhat plastic, detangling specific substrate utilization pathways has been particularly challenging because substrate preference alterations include horizontal crosstalk of various metabolic pathways. Undoubtedly, intervening at early maladaptive stages is key to delay or impede the development of HF and SCD. Detailing which enzymatic reactions are critical and pharmacologically targetable is essential to developing specific compounds that intervene accurately and efficaciously in the fight against SCD events. Future studies will be paramount in unraveling key enzymatic events for the development of novel compounds and determining which population groups are more prone to be responsive to these interventions.

AUTHOR CONTRIBUTIONS

JS and RZ contributed equally to this work. JS, RZ, AL, and PS reviewed the literature, drafted the manuscript, and critically revised the manuscript.

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Exogenous Cardiac Bridging Integrator 1 Benefits Mouse Hearts With Pre-existing Pressure Overload-Induced Heart Failure

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Background: Cardiac bridging integrator 1 (cBIN1) organizes transverse tubule (t-tubule) membrane calcium handling microdomains required for normal beat-to-beat contractility. cBIN1 is transcriptionally reduced in heart failure (HF). We recently found that cBIN1 pretreatment can limit HF development in stressed mice. Here, we aim to explore whether cBIN1 replacement therapy can improve myocardial function in continuously stressed hearts with pre-existing HF.

Methods: Adult male mice were subjected to sham or transverse aortic constriction (TAC) surgery at the age of 8–10 weeks old. Adeno-associated virus 9 (AAV9) transducing cBIN1-V5 or GFP-V5 (3×10^{10} vg) was administered through retro-orbital injection at 5 weeks post-TAC. Mice were followed by echocardiography to monitor cardiac function until 20 weeks after TAC. Overall survival, heart and lung weight (LW), and HF incidence were determined. In a second set of animals in which AAV9-cBIN1 pretreatment prevents HF, we recorded cardiac pressure-volume (PV) loops and obtained myocardial immunofluorescence imaging.

Results: The overall Kaplan-Meir survival of AAV9-cBIN1 mice was 77.8%, indicating a significant partial rescue between AAV9-GFP (58.8%) and sham (100%) treated mice. In mice with ejection fraction (EF) ≥30% prior to AAV9 injection at 5 weeks post-TAC, AAV9-cBIN1 significantly increased survival to 93.3%, compared to 62.5% survival for AAV9-GFP treated mice. The effect of exogenous cBIN1 was to attenuate TAC-induced left ventricular (LV) dilation and prevent further HF development. Recovery of EF also occurs in AAV9-cBIN1-treated mice. We found that EF increases to a peak at 6–8 weeks post-viral injection. Furthermore, PV loop analysis identified that AAV9-cBIN1 increases both systolic and diastolic function of the post-TAC hearts. At the myocyte level, AAV9-cBIN1 normalizes cBIN1 expression, t-tubule membrane intensity, and intracellular distribution of Cav1.2 and ryanodine receptors (RyRs).

Conclusions: In mice with pre-existing HF, exogenous cBIN1 can normalize t-tubule calcium handling microdomains, limit HF progression, rescue cardiac function, and improve survival.

Keywords: heart failure, ion channel, cardiac bridging integrator 1, calcium handling, gene therapy

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INTRODUCTION

Heart failure (HF) is the fastest growing cardiovascular disorder affecting over 20 million people worldwide and 6.2 million Americans (Roger, 2013; Virani et al., 2020). The majority of HF-related mortality is associated with cardiac pump failure due to myocardial inotropic and lusitropic dysfunction, as well as sudden cardiac death due to increased arrhythmia burden of failing hearts. Furthermore, near 50% of HF patients are diagnosed with HF with preserved ejection fraction (HFpEF), which develops severe diastolic failure, has increased risk of arrhythmias, and lacks effective medical therapy (Virani et al., 2020). Thus, there is an urgent need to develop new therapeutic strategies that can limit and reverse HF progression.

During HF development, the pathophysiologic cellular hallmark of failing ventricular myocytes is abnormal calcium transients with impaired intracellular calcium homeostasis (Gomez et al., 1997, 2001; Litwin et al., 2000; Louch et al., 2004), which disrupts excitation-contraction (EC) coupling (Perreault et al., 1992), impairs electrical stability (Landstrom et al., 2017), and disturbs mitochondrial metabolism (Lopez-Crisosto et al., 2017). Normal beat-to-beat calcium transient relies on a sequence of intracellular events known as calcium-induced-calcium-release (CICR; Bers, 2002), where t-tubule L-type calcium channels (LTCCs)-mediated initial calcium influx will subsequently induce a massive calcium release *via* ryanodine receptors (RyRs) from the sarcoplasmic reticulum (SR) store. During relaxation, the accumulated calcium will then be removed from the cytoplasm mainly by calcium reuptake to SR *via* SR Ca^{2+}-ATPase 2a (SERCA2a) together with calcium exclusion through sodium calcium exchanger from cytosol into the extracellular space (Bers, 2008). In HF, abnormal t-tubule remodeling (Lyon et al., 2009; Louch et al., 2010; Wei et al., 2010) impairs LTCC-RyR coupling and synchronous CICR (Gomez et al., 1997; Litwin et al., 2000), resulting in diminished systolic release, EC uncoupling, and thus reduced contractility. On the other hand, HF-associated leaky RyRs (Marx et al., 2000) and abnormal SERCA2a function (Houser et al., 2000) will result in SR depletion and elevated diastolic calcium (Periasamy and Huke, 2001), resulting in severe diastolic failure and electrical instability (Erkasap, 2007). In addition, impaired calcium homeostasis triggers loss of mitochondrial membrane potential (Santulli et al., 2015) and increased permeability (Odagiri et al., 2009), which promotes the risk of mitochondrial-initiated cell death (Nakayama et al., 2007; Kinnally et al., 2011) and HF progression (Nakayama et al., 2007; Zhou and Tian, 2018). Taken together, calcium homeostasis is critical in maintaining normal cardiac pump function, electrical stability, and metabolism. Disturbed beat-to-beat calcium dynamic, as occurs in diseased hearts, will therefore lead to pump failure, lethal arrhythmias, and severe metabolic disorder.

Recently, we reported that the reorganization of intracellular calcium handling machinery could be achieved by a new approach of targeting t-tubule membrane microdomains organized by the cardiac bridging integrator 1 (cBIN1; Liu et al., 2020). We previously found that BIN1 facilitates intracellular LTCC trafficking to t-tubule microdomains (Hong et al., 2010), as well as surface clustering (Fu et al., 2016; Fu and Hong, 2016) at the t-tubule microdomains. RyRs are recruited to junctional SR (jSR) by cBIN1 for coupling with LTCCs (Fu et al., 2016). In addition to dyad organization, cBIN1 sculpted microdomains generate a protective slow diffusion zone for extracellular ions in t-tubule lumen to regulate ionic flux across t-tubule membrane (Hong et al., 2014). More recently, we found that cBIN1-microdomain is also critical in organizing the intracellular distribution of SERCA2a for diastolic calcium regulation (Liu et al., 2020). In HF, cBIN1-microdomains are disrupted due to transcriptional reduction in cBIN1 (Hong et al., 2012b; Caldwell et al., 2014; Zhou and Hong, 2017), impairing dyad formation, calcium transient regulation, and cardiac contractility. Reduced myocardial cBIN1 can be detected in human blood, a result of cBIN1-membrane turnover and microparticle release (Xu et al., 2017). In humans, plasma CS (cBIN1 score) is an index of myocyte cBIN1 level, which identifies myocardial structural remodeling, facilitating HF diagnosis and prognosis (Nikolova et al., 2018). In mouse hearts subjected to chronic stress, pretreatment with exogenous cBIN1 preserves the microdomain-organized distribution of Cav1.2 and SERCA2a, maintaining normal inotropy and lusitropy (Liu et al., 2020). These data indicate that cBIN1 replacement can be an effective HF therapy with the potential to recover myocardial function in hearts with preexisting HF.

Since increased afterload is an important primary and secondary cause of HF (Blaufarb and Sonnenblick, 1996), our current study uses a mouse model of elevated afterload induced by transverse-aortic constriction (TAC). In TAC mice, we recently reported that cBIN1 pretreatment prevents HF development (Liu et al., 2020). Here, we used adeno-associated virus 9 (AAV9)-mediated gene transfer to introduce exogenous cBIN1 in post-TAC mouse hearts with pre-existing HF. We find that *cBin1* gene therapy reduces TAC-induced pathological remodeling, limits HF progression, causes functional recovery, and ultimately reduces death and improves survival.

MATERIALS AND METHODS

Animal Model Design

All mouse procedures were reviewed and approved by the Cedars-Sinai Medical Center (CSMC) and University of Utah Institutional Animal Care and Use Committees (IACUC) and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 2011).

Adult male C57BL/6 mice (The Jackson Laboratory) were used. All mice were anesthetized at the age of 8–10 weeks and subjected to open-chest sham or TAC surgery. TAC was performed by tying a 7-0 silk suture against a 27-gauge needle between the first and second branch of the aortic arch. For sham controls, age-matched mice were subjected to open-chest mock surgery without TAC being performed. For rescue experiments, at 5 weeks post the onset of TAC, mice received retro-orbital injection of 100 μl of 3×10^{10} vector genome (vg) of AAV9 virus (Welgen, Inc.) transducing cBIN1-V5 or

GFP-V5 (Basheer et al., 2017). In the prevention experiments where cBIN1-V5 was reported to provide protection of mouse hearts against subsequent TAC-induced HF (Liu et al., 2020), mice received the same dose (3×10^{10} vg) of AAV9 virus transducing cBIN1-V5 and GFP-V5 3 weeks before TAC surgery was done.

Echocardiography

In vivo systolic and diastolic left ventricular (LV) functions were monitored by echocardiography in anesthetized mice using Vevo 3100 at baseline, pre-surgery, and every other week thereafter until the end of the experimental protocol. The trans-aortic pressure gradient was recorded using the modified Bernoulli equation [Δ Pressure gradient (mmHg) = 4 × peak velocity^2 (m/s)^2] at 2 weeks post-surgery. All surviving mice at 5 weeks post-TAC were included in the study.

Hemodynamic Measurements

In the prevention experiments, 8 weeks after TAC, mice were anesthetized with 3% isoflurane in 100% O_2 at 1.2 L/min and maintained at 1.5% isoflurane during the measurement. After intubation to facilitate breathing, mice were placed on controlled heating pads maintained at 37°C. A 1-Fr pressure-conductance microcatheter (PVR-1045; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the LV. After stabilization for 10 min, baseline P-V relations were recorded. Signals were continuously recorded using a P-V conductance system (MPVS-Ultra; Millar Instruments) connected to the PowerLab data acquisition system (AD Instruments, Colorado Springs, CO), stored, and displayed on a PC computer by the LabChart7 Software System (AD Instruments). With the use of a special P-V analysis program (PVAN; Millar Instruments), heart rate, the maximal slope of LV systolic pressure increment (dp/dt max) and diastolic pressure decrement (dp/dt min), EF, and Tau value were recorded and calculated as previously described (Pacher et al., 2008).

Immunofluorescence Labeling and Confocal Imaging

For cardiomyocyte membrane fluorescent labeling, freshly isolated ventricular cardiomyocytes from GFP-TAC and cBIN1-TAC mice were incubated with Di-8-ANNEPs for 20 min at room temperature (RT). The cells were then washed with HBSS to remove the remaining dye before live-cell imaging. For fixed-cell V5 imaging (10×), isolated cardiomyocytes were fixed in methanol at −20°C for 5 min and permeabilized and blocked with 0.5% Triton X-100 and 5% normal goat serum (NGS) in PBS for 1 h at RT. Cells were incubated with rabbit anti-V5 (Sigma) overnight at 4°C and detected by Alexa555 conjugated goat anti rabbit IgG. For tissue immunofluorescent imaging, myocardial cryo-sections were fixed with ice-cold acetone for 5 min. The primary antibodies used were mouse anti–BIN1-BAR (2F11, Rockland), mouse anti-RyR (Abcam), or rabbit anti-Cav1.2 (Alomone). Following incubation with primary antibodies and several washes with 1× PBS, cells and tissue sections were then incubated with Alexa488 or Alexa555 conjugated goat anti-mouse or rabbit secondary antibodies (Life Technologies) and mounted with DAPI containing ProLong gold.

All confocal imaging was performed on a Nikon Eclipse Ti microscope with a 100 × 1.49 numerical aperture (NA) and 60 × 1.1 or 10 × objectives. High-resolution cardiomyocyte images were obtained using a spinning-disc confocal unit (Yokogawa CSU10) with diode-pumped solid state (DPSS) lasers (486, 561, 647) generated from laser merge module 5 (Spectral applied research, CA). T-tubule membrane labeling fluorescent intensity profiles were generated by ImageJ, and peak intensity at t-tubules is quantified as previously reported (Hong et al., 2014). Power spectrum analysis was analyzed in Matlab using FFT conversion, and normalized peak power density at t-tubules was compared across groups (Hong et al., 2010; Wei et al., 2010).

Western Blotting

Tissue lysates were made from hearts flash frozen in liquid nitrogen. Frozen tissue was homogenized in radio-immunoprecipitation assay (RIPA) lysis buffer as previously described (Liu et al., 2020). Lysates were rotated head-to-toe in 4°C for 40 min, sonicated, followed by centrifugation (16,000 *g* for 25 min at 4°C) to clear cellular debris. Protein lysates were then prepared 2× sample buffer (Bio-Rad, Hercules, CA) containing 5% β-mercaptoethanol, incubated in RT for 30 min, and separated on an 8–12% gradient sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis gel. Proteins were electro-transferred to polyvinylidene difluoride (PVDF) membrane. After transfer, membranes were fixed in methanol and blocked with 5% BSA in 1× tris-buffered saline (TBS) for 1 h at RT, and incubated with primary antibody in 5% BSA in 1× TBS overnight at 4°C, followed by incubation with Alexa 647 conjugated secondary antibody (Life Technology) for 1 h at RT. Primary antibodies consisted of a custom-made polyclonal rabbit anti-BIN1 exon 13 (Anaspec) (Hong et al., 2014), mouse anti RyR (Abcam), rabbit Cav1.2 antibody (Alomone), and mouse anti-GAPDH (Millipore).

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). Normality was assessed using the Shapiro-Wilk test. Kaplan-Meier survival analysis was used to compare survival between two or three groups using the long-rank test. Continuous variables were compared using Mann-Whitney U and one-way ANOVA or Kruskal-Wallis tests. Two-way ANOVA followed by LSD *post-hoc* test was used to determine differences between two groups at two different time points. Categorical variables were analyzed using Fisher's exact or Chi-square tests. Data were analyzed using GraphPad Prism 7.0. Two-sided p values were used and $p < 0.05$ is considered statistically significant.

RESULTS

To explore whether targeting cBIN1-microdomain can be a new therapy for HF, we investigated how cardiac cBIN1 affects HF development in mice subjected to pressure overload stress.

As indicated in the experimental protocol in **Figure 1A**, mice were subjected to TAC first for 5 weeks before retro-orbital injection of AAV9 transducing cBIN1-V5 or control GFP-V5, followed by echocardiography monitoring for an additional 15 weeks after virus injection (end point at 20 weeks post-TAC). In addition to a group of mice subjected to an open-chest mock surgery (sham control, N = 12), 36 mice were subjected to TAC surgery. One mouse died before reaching Time 0 (5 weeks post-TAC), the remaining 35 surviving mice were randomized to receive AAV9-GFP (N = 17) or cBIN1 (N = 18) at 3 × 10^{10} vg. Anti-V5 labeling of cardiomyocytes isolated from mice after 15 weeks of AAV9 injection identified positive V5 signal, indicating successful transduction of exogenous protein in cardiomyocytes (**Supplementary Figure 1**). Comparable trans-aortic pressure gradient at 2 weeks post-TAC (**Figure 1B**) and myocardial dysfunction at 5 weeks post-TAC in these two groups (**Table 1**) establish a similar level of TAC-generated pressure overload and its induction of dilated cardiomyopathy.

We, then, explored the overall survival rate (non-survival is death) in all groups. As indicated in the Kaplan-Meier curves in **Figure 2**, the survival curve of AAV9-cBIN1 treated TAC mice (survival rate 77.8%, 14/18) lies significantly between the two curves of sham control (survival rate 100%, 12/12) and AAV9-GFP treated TAC mice (survival rate 58.8%, 10/17; p = 0.045 by log-rank test when comparing the three groups; **Figure 2A**), indicating partial rescue. Next, in all TAC mice not yet at end stage disease with EF ≥ 30% at Time 0 (5 weeks post-TAC and before virus injection), we further compared the survival rates between the two viral groups. The survival rate was significantly improved by AAV9-cBIN1 (p = 0.038 by log-rank test) when compared to the AAV9-GFP group (**Figure 2B**). Of the 16 AAV9-GFP mice with EF ≥ 30% at Time 0, six died within 20 weeks post-TAC with progressive EF reduction. In comparison, of the 15 AAV9-cBIN1 mice with EF ≥ 30% at Time 0, only one died within 20 weeks post-TAC. Note, all four animals from both groups with pre-AAV9 EF < 30% died during follow up, suggesting that AAV9-cBIN1 is not sufficient to improve survival in animals already at end-stage HF and with afterload still restricted. The surviving mice at 20 weeks post-TAC were then sacrificed for tissue collection for histological analysis (**Figure 3A**). Compared to the normal structure and size of the sham control hearts, AAV9-GFP hearts were visibly enlarged with an expanded left ventricle, which was reduced in AAV9-cBIN1 hearts. The ratios of heart weight (HW) and lung weight (LW) over tibial length (TL) were further evaluated. AAV9-cBIN1 mice did not have a significant increase in HW/TL and LW/TL as occurred in AAV9-GFP mice when compared to sham control mice (**Figures 3B,C**). These data indicate that *cBin1* gene therapy can block, postpone, or even reverse the worsening cycles of HF progression in hearts with pre-existing HF, resulting in functional protection and better survival.

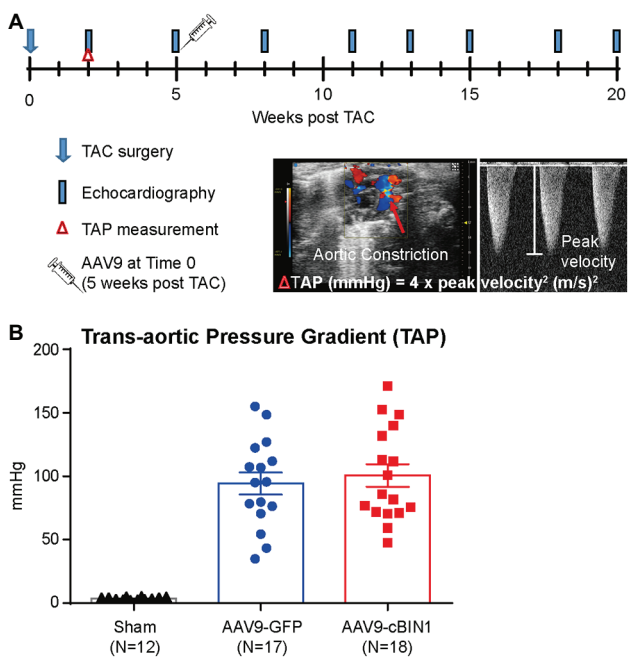

FIGURE 1 | Experimental protocol of cardiac bridging integrator 1 (cBIN1) post-treatment in mice subjected to transverse aortic constriction (TAC). **(A)** Schematic protocol: 47 mice were randomized into three groups: sham (N = 12) or TAC mice with post-treatment of AAV9-GFP (N = 17) and AAV9-cBIN1 (N = 18) administered at 5 weeks post-TAC. **(B)** Echocardiography analysis of trans-aortic pressure gradient (TAP) in the three groups.

Echocardiography-measured myocardial functional parameters were further compared across groups both before and after AAV9 treatment (**Table 1**). At 20 weeks post-TAC, AAV9-GFP mice developed significant LV contractile dysfunction (EF reduction) and chamber dilation (EDV elevation; **Figures 4A–C** and **Table 1**), which were normalized by AAV9-cBIN1 treatment. The increase in LV Mass at 20 weeks post-TAC was significantly reduced in AAV9-cBIN1 treated mice when compared to AAV9-GFP group (**Figure 4D**). In AAV9-GFP treated mice, TAC surgery resulted in a progressive increase in E/e', indicating the onset of diastolic dysfunction. AAV9-cBIN1, on the other hand, effectively blocked progressive worsening of E/e', indicating exogenous cBIN1-mediated preservation of cardiac lusitropy. As a result, E/e' values of 20 weeks post-TAC cBIN1 hearts were maintained at their pre-AAV levels, which were not significantly different from E/e' values of sham control hearts but tended to be lower than those of AAV9-GFP hearts (p = 0.071) at 20 weeks post-TAC (**Figures 4E,F**). Furthermore, the observed delta reductions in stroke volume and cardiac output in AAV9-GFP group were also abolished in AAV9-cBIN1 treated group (**Figures 4G,H**). These results indicate that *cBin1* gene therapy preserves myocardial function when administered to mice with failing hearts.

To explore the progression of systolic dysfunction in post-TAC hearts after viral injection at Time 0 (pre-AAV9, 5 weeks post-TAC), the delta EF changes (ΔEF) from pre-AAV to 3, 6, 8, 10, and 15 weeks post-AAV9 injection (corresponding to 8, 11,13, 15, and 20 weeks post-TAC) were monitored by echocardiography (**Figure 5A**). Exogenous cBIN1-induced EF recovery peaked at 6–8 weeks post-AAV9 injection with continuous improvement of EF in the following weeks, whereas progressive

TABLE 1 | Echocardiographic and physiological parameters of AAV9-GFP or AAV9-cBIN1 injected mice before and after AAV9 injection.

	Pre-AAV9 (5w-post TAC)			Post-AAV9 (20w-post TAC)		
	SHAM	**GFP**	**cBIN1**	**SHAM**	**GFP**	**cBIN1**
EF (%)	53.72 ± 2.01	41.87 ± 3.20**	44.22 ± 2.83*	48.25 ± 2.22	27.65 ± 4.19***	41.72 ± 1.72##
LVEDV (μl)	62.34 ± 3.87	82.35 ± 5.32*	78.48 ± 6.22	58.24 ± 2.52	107.64 ± 10.01***	77.96 ± 4.55##
LVESV (μl)	28.97 ± 2.46	49.44 ± 4.96*	46.43 ± 5.89*	35.29 ± 2.03	79.92 ± 10.43***	45.76 ± 3.43###
HR (bpm)	482.04 ± 14.76	516.60 ± 8.49	481.41 ± 12.40	463.77 ± 12.28	557.35 ± 20.71***	504.94 ± 8.91*, ##
SV (μl)	33.38 ± 2.17	32.91 ± 2.38	32.05 ± 1.19	32.95 ± 1.98	27.72 ± 3.28	32.20 ± 1.79
CO (ml/min)	16.00 ± 1.08	16.92 ± 1.18	15.30 ± 0.53	15.35 ± 1.17	15.37 ± 1.83	16.34 ± 1.08
LVAWs (mm)	1.40 ± 0.04	1.61 ± 0.07*	1.56 ± 0.05	1.35 ± 0.08	1.52 ± 0.08	1.61 ± 0.06*
LVAWd (mm)	0.99 ± 0.03	1.26 ± 0.06***	1.20 ± 0.03**	0.97 ± 0.06	1.29 ± 0.07***	1.28 ± 0.05***
LVPWs (mm)	1.15 ± 0.07	1.46 ± 0.07**	1.34 ± 0.06	1.10 ± 0.03	1.41 ± 0.15*	1.33 ± 0.07
LVPWd (mm)	0.79 ± 0.05	1.15 ± 0.07***	1.06 ± 0.04**	0.84 ± 0.03	1.24 ± 0.16***	1.11 ± 0.06*
LV Mass (mm)	124.39 ± 4.76	232.36 ± 15.20***	202.30 ± 8.43***	137.17 ± 9.60	300.76 ± 29.55***	223.09 ± 12.41***, ###
BW (g)	27.83 ± 0.65	29.66 ± 0.56	29.28 ± 0.40	32.41 ± 1.19	33.97 ± 0.84	34.77 ± 0.95*
HW (mg)	-	-	-	196.98 ± 7.25	288.63 ± 31.63***	234.94 ± 10.75#
LW (mg)	-	-	-	158.32 ± 5.14	245.70 ± 63.18***	165.28 ± 3.68#
HW/TL (g/m)	-	-	-	9.85 ± 0.36	14.77 ± 1.40***	11.75 ± 0.54#
LW/TL (g/m)	-	-	-	7.92 ± 0.26	12.53 ± 3.09*	8.26 ± 0.18

EF, ejection fraction; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; HR, heart rate; SV, stroke volume; CO, cardiac output; LVAWs/LVAWd, left ventricular anterior wall in systole/diastole; LVPWs/d, left ventricular posterior wall in systole/diastole; LV Mass, left ventricular mass; BW, body weight; HW, heart weight; LW, lung weight; TL, tibia length.

Data are expressed as mean ± SEM.

**Indicates $p < 0.05$.*

***Indicates $p < 0.01$.*

****Indicates $p < 0.001$ vs. sham.*

#Indicates $p < 0.05$.

##Indicates $p < 0.01$.

###Indicates $p < 0.001$ for AAV9-GFP vs. AAV9-cBIN1.

EF reduction was noted in the AAV9-GFP group. The observed recovery of EF is demonstrated on the histogram distribution of ΔEF with Gaussian fitting (**Figures 5B,C**). AAV9-cBIN1 has a right-shifted histogram distribution of ΔEF when compared to AAV9-GFP group. For instance, at 6 weeks post-AAV9, there is a medium EF (%) reduction of −15.0 in AAV9-GFP group, while a medium recovery of +6.9 in EF (%) is observed in the AAV9-cBIN1 group. These data indicate that exogenous cBIN1, when administered at 5 weeks post-TAC, can improve myocardial systolic function in hearts with TAC-induced HF.

We recently reported that, in mice receiving AAV9-cBIN1 pretreatment (3×10^{10} vg at 3 weeks prior to TAC surgery; **Figure 6A**), the incidence of TAC-induced HF is significantly reduced with a resultant better HF-free survival at 8 weeks post-TAC (Liu et al., 2020). These data are consistent with the observed myocardial protection when AAV9 was administered after TAC surgery. To further establish the cardioprotective effect of exogenous-cBIN1 in the TAC mice, intracardiac hemodynamics were obtained in AAV9-pretreated mice using invasive PV loop recording. **Figure 6B** contains representative PV loops of sham, AAV9-GFP, and AAV9-cBIN1 pretreated hearts 8 weeks after TAC surgery (**Figure 6B**). The EF and the maximal rate of pressure change during systole (dp/dt max) were decreased in AAV9-GFP group, which were normalized by exogenous cBIN1 (**Figures 6C,D**). When exploring the effects on relaxation kinetics, we found that the maximal rate of pressure decay (dp/dt min) was decreased in AAV9-GFP group but was normalized by AAV9-cBIN1 (**Figure 6E**). The increased time constant for isovolumic relaxation (Tau) in AAV9-GFP group was also rescued by *cBin1* gene transfer (**Figure 6F**), indicating improved cardiac relaxation. Together, these data indicate that exogenous cBIN1 improves cardiac inotropy and lusitropy in pressure overloaded hearts.

We previously identified that cBIN1 creates t-tubule microdomains and organizes LTCC-RyR dyads for efficient and dynamic regulation of cardiac function and EC coupling (Hong and Shaw, 2017). More recently, we found that in sympathetic overdriven mouse hearts developing diastolic dysfunction, cBIN1-microdomains are disrupted and are rescued by AAV9-cBIN1. Here, we also explored the alterations in cardiac t-tubule cBIN1-microdomains and the effect of exogenous cBIN1 in post-TAC hearts. Western blotting (**Figure 7A**) identifies that myocardial cBIN1 protein is significantly reduced in mouse hearts 8 weeks after TAC (27% less than sham controls, $p < 0.05$), which is normalized by AAV9-cBIN1 pretreatment. Along with cBIN1 rescue, membrane labeling with Di-8-ANNEPs (**Supplementary Figure 2**) identifies that compared to sham cardiomyocytes, 8 weeks post-TAC cardiomyocytes have a significantly reduced t-tubule cBIN1-microdomain intensity, which is normalized in cardiomyocytes from mice with AAV9-cBIN1 pretreatment. We, next used immunofluorescent imaging to analyze the organization of cBIN1-microdomains and dyads at the myocyte level. Power spectrum analysis of BIN1 signal identifies that the well-organized t-tubule distribution of cBIN1 in sham myocardium is disrupted in post-TAC hearts, which is preserved with AAV9-cBIN1 pretreatment (**Figure 7C**). Although total LTCC and RyR protein levels are not significantly altered

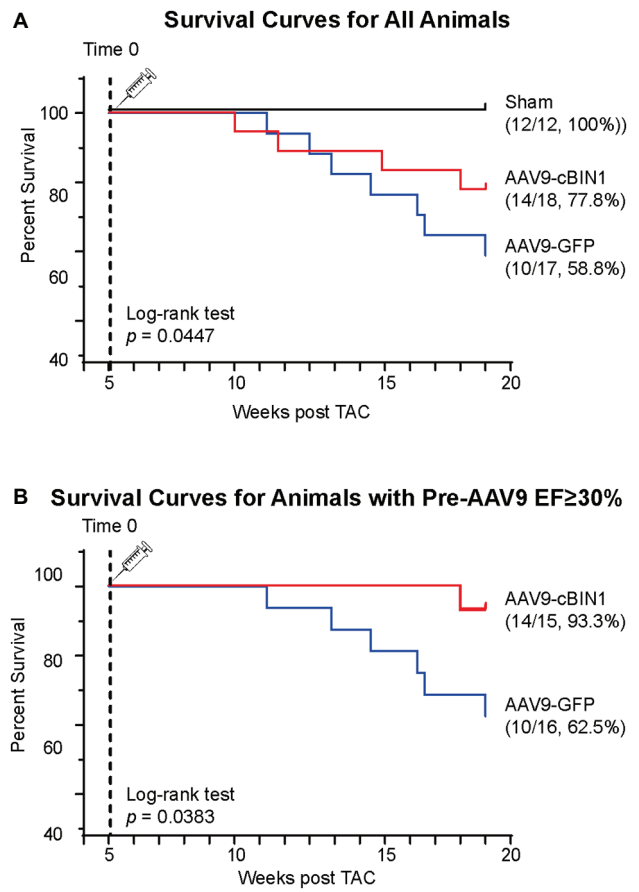

FIGURE 2 | Post-treatment with exogenous cBIN1 improves survival rates in post-TAC mice. **(A)** Kaplan-Meier survival curves for all three groups of mice: sham controls (N = 12), post-TAC mice treated with AAV9-CMV viruses transducing GFP (N = 17) or cBIN1 (N = 18). The log-rank test was used for comparison across three groups. **(B)** Kaplan-Meier survival curves for post-TAC mice not yet at end stage of disease prior to AAV9 injection (5 weeks post-TAC EF ≥ 30%) following post-treatment of AAV9-GFP (N = 16) or AAV9-cBIN1 (N = 15). The log-rank test was used for comparison of survival rates between AAV9-GFP and AAV9-cBIN1 groups.

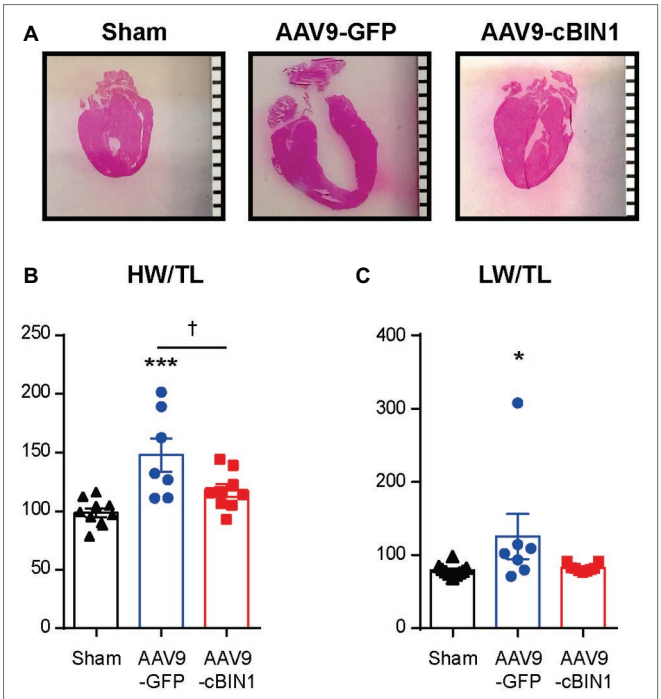

FIGURE 3 | Exogenous cBIN1 reduces TAC-induced hypertrophy and pulmonary edema. **(A)** Longitudinal heart sections with H&E staining, (scale bar, 1 mm). **(B)** The ratio of heart weight over tibia length (HW/TL) and **(C)** lung weight over tibia length (LW/TL) at 20 weeks post-TAC. Data are presented as mean ± SEM, and two-way ANOVA with Fisher's LSD test was used for statistical analysis. *,***$p < 0.05$, 0.001 vs. sham; †$p < 0.05$, comparing between GFP and cBIN1 groups.

among groups by Western blotting (**Figure 7B**), myocardial distribution of LTCCs and RyRs (**Figure 7C**) becomes disorganized in post-TAC hearts, which is also significantly improved in hearts with AAV9-cBIN1 pretreatment ($p < 0.05$). These data indicate that exogenous cBIN1 normalizes TAC-induced reduction of myocardial cBIN1, resulting in preservation of cBIN1-scultped microdomains at t-tubules. *Via* normalizing t-tubule microdomains in pressure-overloaded hearts, cBIN1 replacement therapy thus reorganizes cardiac LTCC-RyR couplons required for beat-to-beat calcium cycling and efficient EC coupling.

DISCUSSION

In this study, we report that AAV9 virus transduced-exogenous cBIN1 in myocardium, applied after a reduction in EF, can improve cardiac function and limit further development of ventricular chamber dilation and HF in mice subjected to chronic pressure overload stress. The critical role of cBIN1 in regulating cardiac function is consistent with previous findings that cBIN1-organized t-tubule microdomains are required for normal dyad formation and function (Hong et al., 2010, 2012a,b, 2014; Hong and Shaw, 2017), and that reduced cBIN1 expression contributes to weakened calcium transients and impaired cardiac contractility in failing hearts (Hong et al., 2012b; Lyon et al., 2012; Caldwell et al., 2014).

Under continuous pressure overload, myocardial remodeling starts with an adaptive hypertrophic response followed by transitioning into maladaptive cardiac dilatation, leading to worsening HF (Liu et al., 2011; Zhang et al., 2016; Zhou et al., 2016). In previous studies, we identified that the administration of AAV9-cBIN1 prior to TAC surgery preserves myocardial systolic and diastolic function, indicating the efficacy of *cBin1* gene therapy in HF prevention. In the current study, we found that exogenous cBIN1 administration after TAC-induced HF blocks further disease progression and improves the overall survival with attenuated cardiac hypertrophy and lessened pulmonary edema in mice (**Figures 2, 3**). Furthermore, exogenous cBIN1 introduced by gene transfer improves myocardial remodeling and cardiac function as measured by echocardiography (**Figures 4, 6**). Most strikingly, mice with preexisting HF exhibited recovered

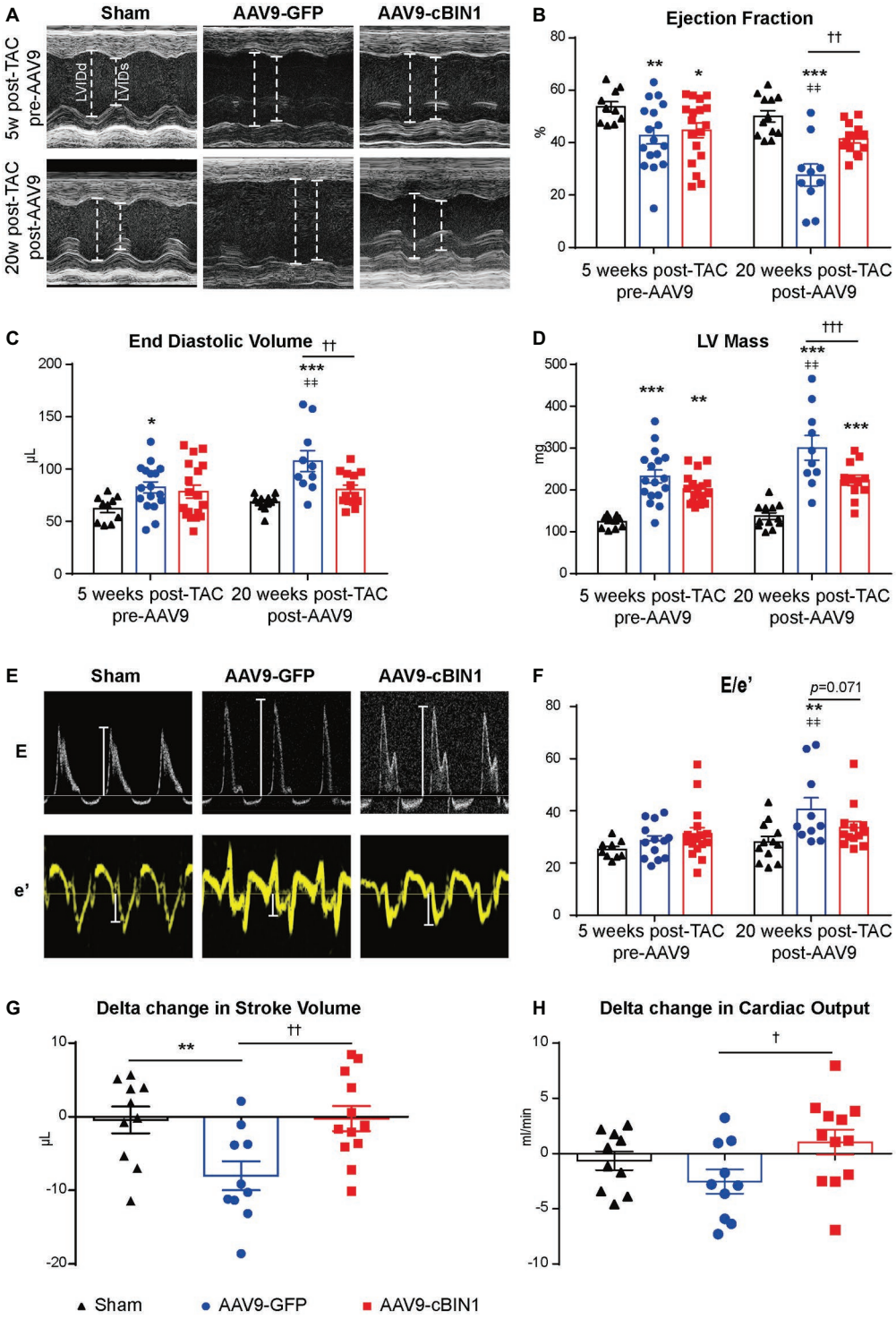

FIGURE 4 | *cBin1* gene transfer preserves myocardial systolic and diastolic function in pressure overloaded hearts. **(A)** Representative left ventricular (LV) short axis M-mode images from each group (sham, AAV9-GFP, AAV9-cBIN1) at 5 weeks post-TAC (pre-AAV9 injection) and 20 weeks post-TAC (15 weeks post-AAV9 injection). Echocardiography-measured **(B)** left ventricle ejection fraction (EF), **(C)** end-diastolic volume, and **(D)** left ventricle mass at 5 (pre-AAV9) and 20 (post-AAV9) weeks post-TAC. **(E)** Representative mitral valve inflow pulsed wave (E) Doppler images (top) and tissue Doppler images of septal mitral valve annulus (e') (bottom) at 20 weeks post TAC (15 weeks post-AAV9 injection). **(F)** Quantification of E/e' from each group at 5 (pre-AAV9) and 20 (post-AAV9) weeks post-TAC. Delta changes from 5-week to 20-week in **(G)** stroke volume (SV) and **(H)** cardiac output (CO) of each mouse ($\Delta SV = SV_{20w} – SV_{5w}$; $\Delta CO = CO_{20w} – CO_{5w}$). Data are presented as mean ± SEM, and two-way ANOVA with Fisher's LSD test was used for statistical analysis. $^{*,**,***}p < 0.05, 0.01, 0.001$ vs. sham; $^{†,††,†††}p < 0.05, 0.01, 0.001$ comparing between AAV9-GFP and -cBIN1 groups at each time point. $^{‡‡}p < 0.01$ when comparing pre- and post-AAV9 treatment in each group.

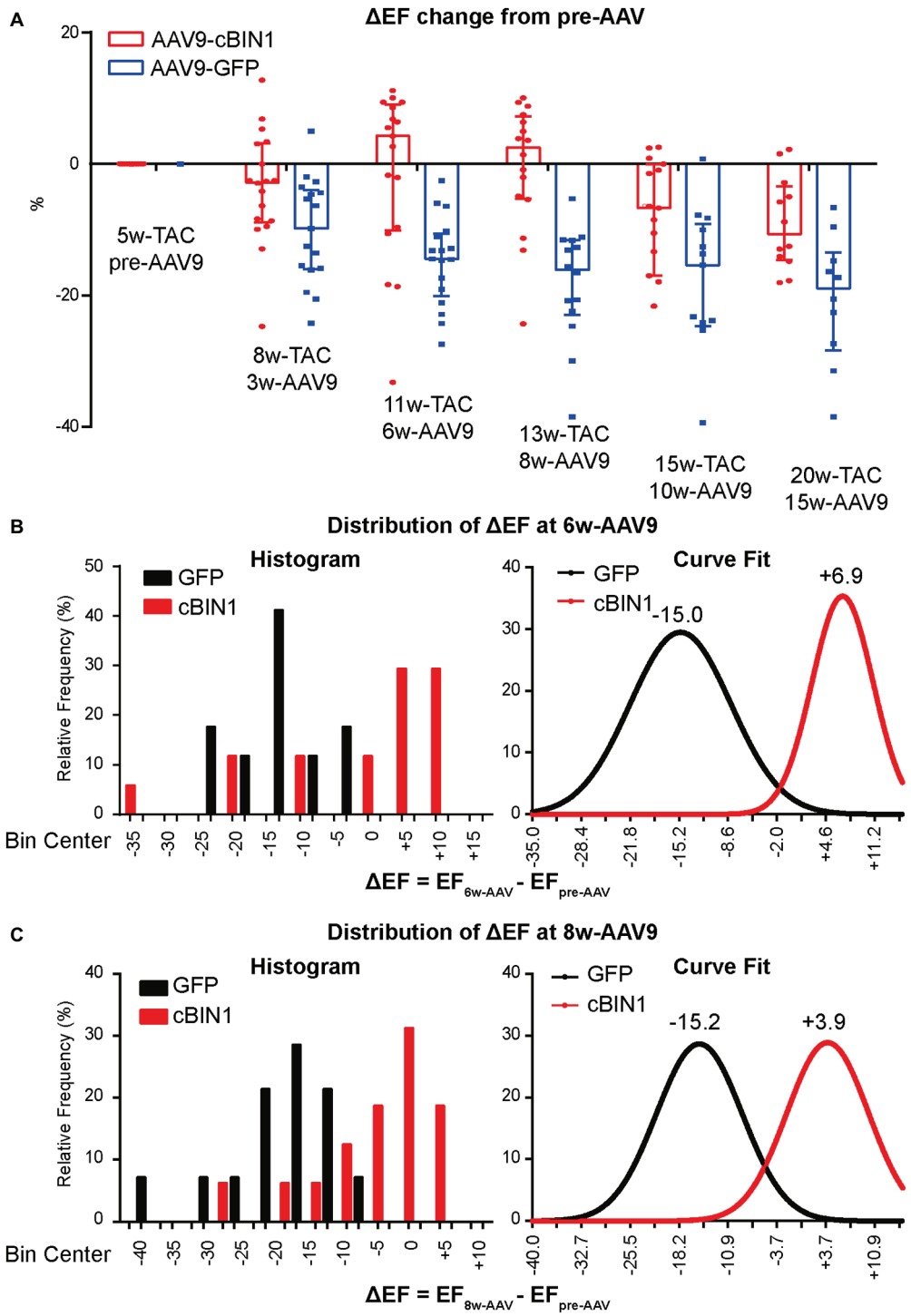

FIGURE 5 | AAV9-cBIN1 post-treatment rescues EF in post-TAC mouse hearts. **(A)** Echocardiography monitored delta EF changes (ΔEF) from pre-AAV to 3, 6, 8, 10, and 15 weeks post AAV9 injection (correspondingly 8, 11, 13, 15, and 20 weeks post-TAC) in AAV9-CMV-GFP and -cBIN1 treating groups. Histogram distribution of ΔEF at **(B)** 6-week and **(C)** 8-week post AAV9 treatment with Gaussian distribution fitting curve. Data are presented as mean ± SEM.

EF following *cBin1* gene therapy (**Figure 5**), indicating the protective effect of exogenous cBIN1 may serve as a translatable treatment for patients with diagnosed preexisting structural remodeling and HF. Furthermore, consistent with results in isoproterenol chronically stressed mouse hearts (Liu et al., 2020), gene therapy-induced cBIN1 restoration in post-TAC hearts occurs at t-tubule microdomains and is capable of inducing the reorganization of LTCC-RyR

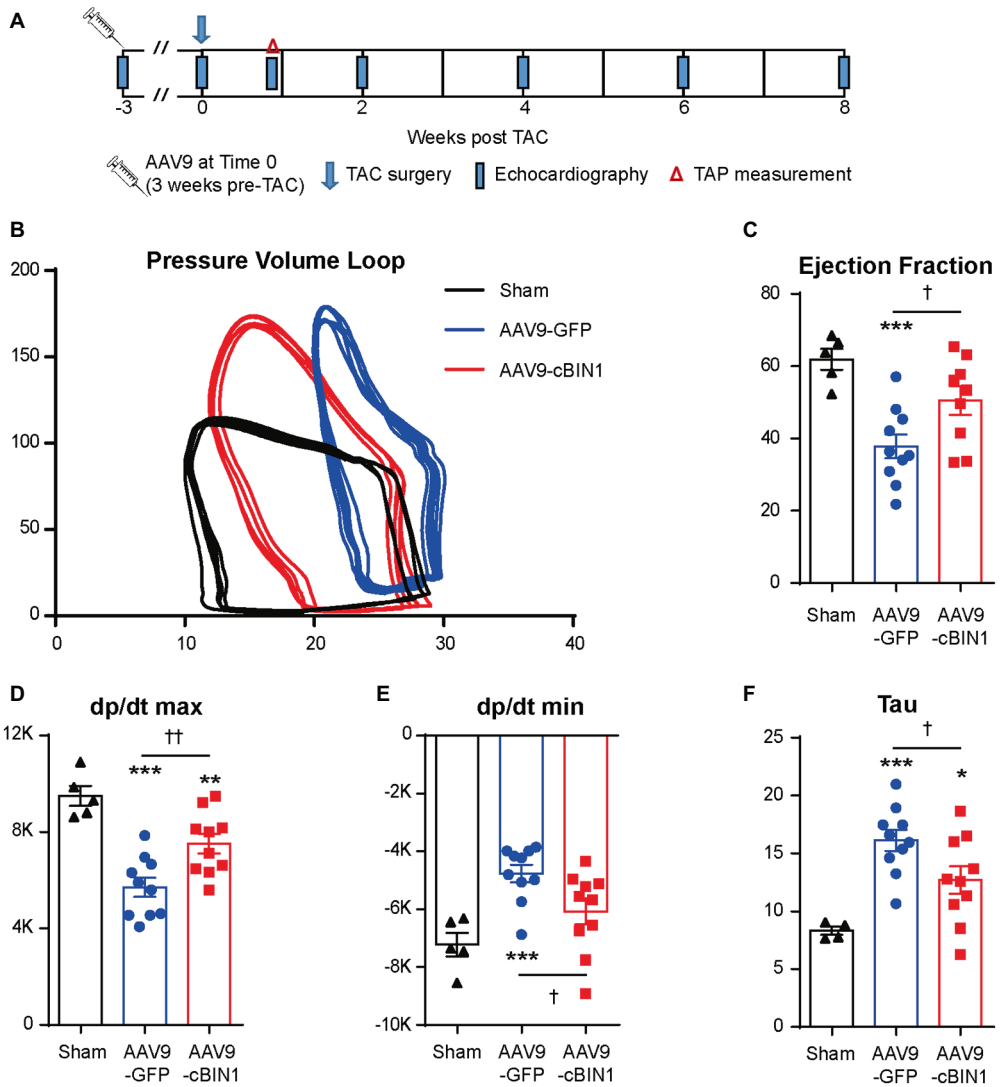

FIGURE 6 | Exogenous cBIN1 pretreatment improves myocardial pressure-volume (PV) loops. **(A)** Schematic protocol: sham ($N = 5$) or TAC with pretreatment of AAV9-GFP ($N = 10$) or cBIN1 ($N = 10$) administered 3 weeks prior to TAC. Representative PV loop **(B)**, EF **(C)**, dp/dt max **(D)**, dp/dt min **(E)** and Tau **(F)** in sham, AAV9-GFP and -cBIN1 hearts at 8 weeks post TAC surgery. Data are presented as mean ± SEM, and one-way ANOVA with Fisher's LSD test was used for statistical analysis. $^{**,***}p < 0.01$, 0.001 vs. sham; $^{\dagger,\dagger\dagger}p < 0.05$, 0.01 comparing between AAV9-GFP and AAV9-cBIN1 groups.

couplons (**Figure 7**), indicating the observed therapeutic effect is mechanistically linked to normalization of impaired calcium handling in failing cardiomyocytes.

Recently, AAV-mediated gene therapy has been shown as a promising modality for the treatment of HF (Rincon et al., 2015; Bass-Stringer et al., 2018). There are currently several completed or ongoing clinical trials of HF gene therapies targeting various pathways such as the β-adrenergic system, Ca^{2+} cycling proteins, and cell death pathways, as well as homing stem cells (Tilemann et al., 2012). We recently found that targeting the calcium regulating microdomains at t-tubules can be effectively achieved by transducing the essential microdomain-organizing protein cBIN1 (Liu et al., 2020). By stabilizing t-tubule microdomains, cBIN1 preserves cytosolic calcium homeostasis and contributes to increasing systolic calcium release, improving diastolic reuptake, limiting SR leak for electrical stability maintenance, and preserving mitochondrial function to limit mitochondrial-associated cell death. Our data indicate that this microdomain-targeting approach may serve as a new therapeutic strategy with improved efficiency in functional preservation, improving overall HF survival. Furthermore, the observed cBIN1-mediated improvement in overall survival (**Figure 2**) is a possible combined effect from improved pump function and reduced arrhythmias, both of which are regulated by cBIN1-microdomains (Hong et al., 2012b, 2014; Hong and Shaw, 2017). How cBIN1 therapy affects arrhythmia burden in failing hearts will need further analysis using *in vivo* telemetry monitoring in future studies. In addition, since TAC-induced HF is associated with mitochondrial disorder-associated

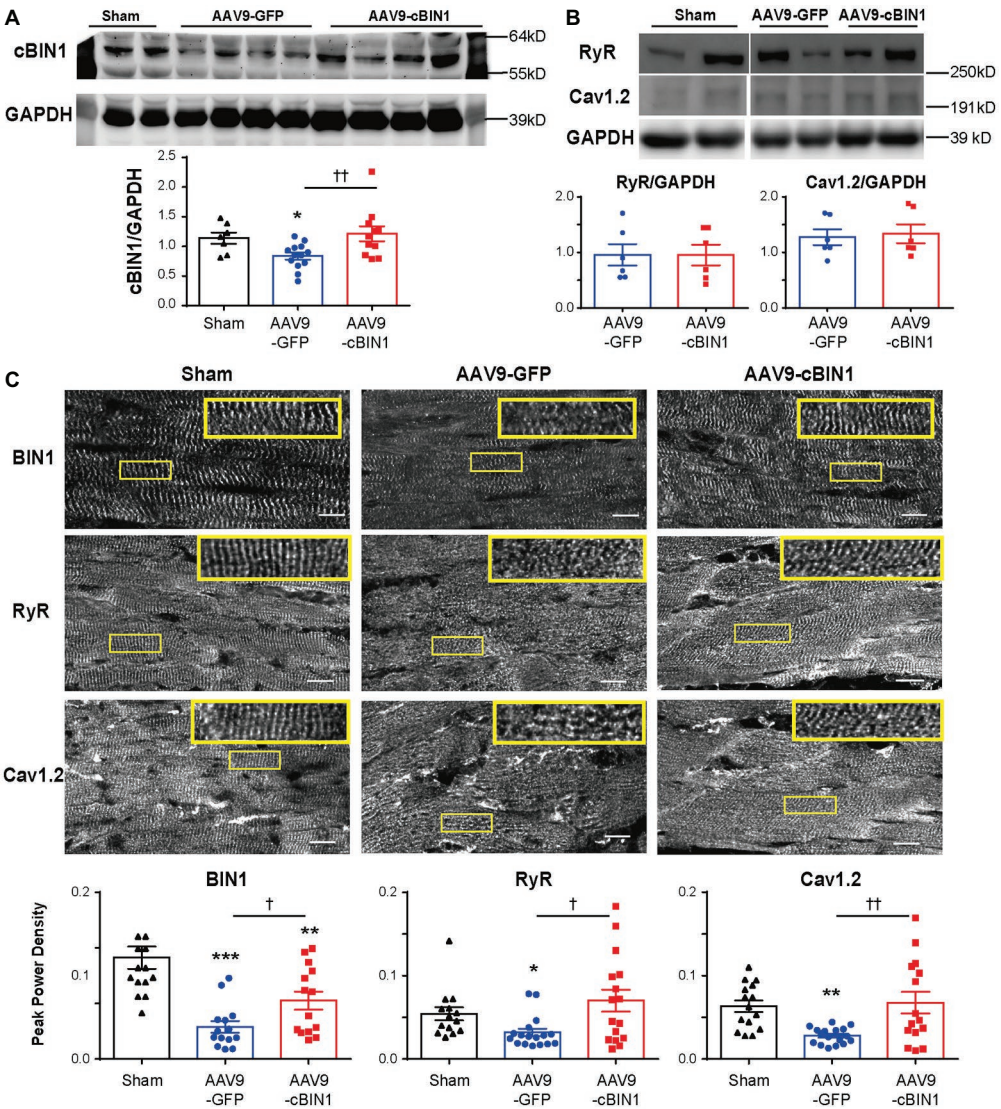

FIGURE 7 | cBIN1-microdomain reduction in post-TAC hearts can be normalized with AAV9-cBIN1 pretreatment. Western blot of **(A)** cBIN1, **(B)** ryanodine receptor (RyR), and Cav1.2 from sham, AAV9-GFP, and AAV9-cBIN1 pretreated post-TAC heart lysates. Quantitation is included in the bar graph at the bottom (n = 8 hearts per group for cBIN1, n = 6 hearts per group for cBIN1). **(C)** Representative myocardial immunofluorescent spinning-disc confocal images of BIN1 labeling (anti-BAR domain; top panel), RyR (middle panel), and Cav1.2 (bottom panel) from sham, AAV9-GFP, and AAV9-cBIN1 pretreated post-TAC hearts. The insets include enlarged images of the corresponding boxes areas. Bottom row (from left to right): Peak power density of BIN1, RyR, and Cav1.2 distribution in sham, AAV9-GFP and cBIN1-pretreated hearts at 8 weeks post-TAC surgery (n = 15–20 images from five hearts per group). Data are presented as mean ± SEM, and one-way ANOVA with Fisher's LSD test was used for statistical analysis. $^{*,**,***}p < 0.05, 0.01, 0.001$ vs. sham; $^{\dagger,\dagger\dagger}p < 0.05, 0.01$ comparing between AAV9-GFP and -cBIN1 groups.

myocyte death (Thai et al., 2018), it remains interesting in future studies to explore whether cBIN1 replacement therapy can preserve mitochondrial function and limit mitochondrial-related cell death in failing hearts.

With regard to functional recovery, although EF changes monitored from the beginning of AAV9-cBIN1 treatment shows a peak recovery at week 6 post-AAV9 followed by descending therapeutic efficiency (**Figure 5**), the positive effect is maintained at 15-week post AAV9 injection as shown in **Figure 4**. These data indicate that even a single administration of AAV9-cBIN1 at a relatively low dose (3×10^{10} vg) is effective in preserving cardiac function. Whether multiple administrations of exogenous cBIN1 with increased dosage are needed to maximize its therapeutic effect remains to be tested. Nevertheless, our current data (**Figures 4, 5**) indicate that, for patients with existing HF, *cBin1* gene therapy could potentially break the worsening cycles of HF progression and result in functional recovery of failing hearts.

In conclusion, our study reveals a protective role of exogenous cBIN1 in mouse hearts with existing HF after subjected to pressure overload. For this proof-of-concept study, we used the AAV9 vector driven by the CMV promoter

for gene delivery due to its consistent transduction efficiency and established cardiac tropism. Further experiments using *cBin1* packaged in AAV9 with a more efficient cardiac-specific promoter in mice and large mammals will be needed before clinical trials testing the efficacy and efficiency of *cBin1* gene therapy in HF patients. Future studies are also needed to explore the intracellular mechanism for cBIN1 in balancing calcium homeostasis among cytosolic microdomains at t-tubules, SR, and nearby mitochondria. Further understanding of the downstream targeting molecules and signaling pathways of cBIN1 will be needed as well for a better understanding of the interplay between *cBin1* gene therapy and HF pathophysiology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Cedars-Sinai Medical Center (CSMC) and University of Utah Institutional Animal Care and Use Committees (IACUC).

AUTHOR CONTRIBUTIONS

TH and RS contributed to conception and design the study. TH supervised the experiments. JL and SA were responsible for performing experiments and analyzing the data. KZ performed the PV loop experiments. JL and TH wrote the manuscript. TH and RS revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00708/full#supplementary-material.

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REVIEW
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NAD$^+$ Metabolism as an Emerging Therapeutic Target for Cardiovascular Diseases Associated With Sudden Cardiac Death

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In addition to its central role in mediating oxidation reduction in fuel metabolism and bioenergetics, nicotinamide adenine dinucleotide (NAD$^+$) has emerged as a vital co-substrate for a number of proteins involved in diverse cellular processes, including sirtuins, poly(ADP-ribose) polymerases and cyclic ADP-ribose synthetases. The connection with aging and age-associated diseases has led to a new wave of research in the cardiovascular field. Here, we review the basics of NAD$^+$ homeostasis, the molecular physiology and new advances in ischemic-reperfusion injury, heart failure, and arrhythmias, all of which are associated with increased risks for sudden cardiac death. Finally, we summarize the progress of NAD$^+$-boosting therapy in human cardiovascular diseases and the challenges for future studies.

Keywords: NAD$^+$, NAMPT, sirtuin, myocardium, sudden cardiac death, ischemia reperfusion, heart failure, arrhythmia

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INTRODUCTION

Sudden cardiac death (SCD) is defined as a sudden, unexpected death caused by loss of cardiac function (Zipes and Wellens, 1998; Zipes et al., 2006). SCD is responsible for 300,000 – 400,000 deaths each year in the United States with an annual incidence of 60 per 100,000 (Stecker et al., 2014). Most SCD events occur in patients who were not previously identified as being at risk for SCD (Piccini Sr et al., 2016). Despite the improving resuscitation rate and the increasing use of implantable cardioverter defibrillator, the majority who suffer a SCD will not survive. SCD remains a major international public health problem in clinical cardiology, emergency medicine, and public health (Lown, 1979; Moss, 1980).

Abbreviations: ADPR, ADP-ribose; AF, atrial fibrillation; BMAL1, brain and muscle Arnt-like protein 1; CAD, coronary artery disease; cADPR, cyclic ADPR; CLOCK, circadian locomoter output cycles protein kaput; CVD, cardiovascular diseases; DCM, dilated cardiomyopathy; eNAMPT, extracellular NAMPT; ENT, equilibrative nucleoside transporter; ETC, electron transport chain; FOX, forkhead box; GATA4, GATA binding protein 4; I/R injury, ischemia/reperfusion injury; I_{Na}, sodium current; KLF15, Kruppel-like factor 15; K_m, Michaelis constant; MAS, malate-aspartate shuttle; MI, myocardial infarction; mPTP, mitochondrial permeability transition pore; NA, nicotinic acid; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD$^+$, nicotinamide adenine dinucleotide; NADP$^+$, nicotinamide adenine dinucleotide phosphate; NAM, nicotinamide; NAMPT, NAM phosphoribosyltransferase; NMN, nicotinamide mononucleotide; NMNAT1-3, NMN adenyltransferase 1-3; NOXs, NADPH oxidases; NR, nicotinamide riboside; NRK1/2, NR kinase 1/2; PAR, poly ADP-ribose; PARP, PAR polymerases; PARylation, poly ADP-ribosylation; PLD, phospholipase D; PPP, pentose phosphate pathway; RISK, reperfusion injury salvage kinase (PI3K/AKT and ERK1/2); ROS, reactive oxygen species; SCD, sudden cardiac death; SIRT, sirtuin; SLC12A8, solute carrier family 12 member 8; TCA, tricarboxylic acid; Trp, tryptophan.

Nicotinamide adenine dinucleotide or NAD^+, is one of the most essential small molecules in mammalian cells. NAD^+ interacts with over 500 enzymes (Ansari and Raghava, 2010) and plays important roles in almost every vital aspect in cell biology and human physiology (Katsyuba et al., 2020). Dysregulation of NAD^+ homeostasis is associated with a number of diseases including cardiovascular diseases (CVD) (Hershberger et al., 2017; Matasic et al., 2018; Rajman et al., 2018; Katsyuba et al., 2020; Ralto et al., 2020). Particularly, modulation of NAD^+ metabolism has been proposed to provide beneficial effects for CVD settings that are highly associated with SCD, such as ischemia/reperfusion injury (I/R injury), heart failure and arrhythmia (Hershberger et al., 2017; Matasic et al., 2018). In this review, we will discuss how alteration in NAD^+ metabolism can lead to heart disease with the focus on I/R injury, heart failure, and arrhythmia. We will also provide a comprehensive review on animal and human studies with NAD^+ boosters, and discuss the feasibility of NAD^+-boosting therapy for SCD-associated CVD.

NAD^+ METABOLISM IN THE HEART

NAD^+ Biosynthesis

The heart, along with the kidney and the liver has the highest level of NAD^+ among all the organs (Mori et al., 2014). In mammalian cells, NAD^+ is synthesized via two distinct pathways: the *de novo* pathway and the salvage pathway (**Figure 1**; Sporty et al., 2009). The *de novo* pathway generates NAD^+ from tryptophan (Trp) through the kynurenine metabolic pathway (Badawy, 2017), or nicotinic acid (NA) through the Preiss-Handler pathway (Preiss and Handler, 1958a,b). Nevertheless, most of the extrahepatic organs including the heart, use the salvage pathway as the main route to generate NAD^+ (Mori et al., 2014; Liu et al., 2018). Mori et al. (2014) established the metabolic profiling of NAD^+ biosynthetic routes in mouse tissues by measuring the *in vitro* activity of enzymes, the levels of substrates and products, and revealed that 99.3% of NAD^+ in the heart is generated by the salvage pathway. On the other hand, enzymes involved in the *de novo* pathway are of low expression and low activity in the heart (Ikeda et al., 1965).

Salvage pathway generates NAD^+ from the NAD^+ degradation product nicotinamide (NAM) (**Figures 1**, **2**; Ralto et al., 2020). NAM is converted into an intermediate product nicotinamide mononucleotide (NMN) via NAM phosphoribosyltransferase (NAMPT) – the rate limiting enzyme in the salvage pathway. Extracellular NMN can also be transported into the cells through a NMN transporter which might be solute carrier family 12 member 8 (SLC12A8) (Grozio et al., 2019). NMN is then converted into NAD^+ by NMN adenyltransferase 1-3 (NMNAT1-3) in the final step. In addition, NMN can also be generated from another NAD^+ precursor nicotinamide riboside (NR) by NR kinase 1/2 (NRK1/2) (**Figures 1**, **2**). Cardiac expression of NRK2 is much higher than NRK1 (Ratajczak et al., 2016), suggesting that NRK2 may control the phosphorylation of NR in the heart. Moreover, NR can be generated from extracellular NAD^+ or NMN through CD73 (Grozio et al., 2013). Both NMN and NR preserve the pyridine ring, and thus do not require NAMPT to be converted back to NAD^+. Details of the NAD^+ salvage pathway is illustrated in **Figure 2**.

The Rate-Limiting Enzyme for the Salvage Pathway: NAMPT

Much attention has been drawn to NAMPT as it directly correlates with the NAD^+ levels in the heart (Revollo et al., 2004). For instance, *Nampt* expression and NAD^+ level are both reduced in the heart after I/R injury, and ectopic expression of *Nampt* restored the NAD^+ level (Hsu et al., 2009).

NAMPT expression oscillates in a circadian fashion in the heart (Um et al., 2011; Young et al., 2014; Peliciari-Garcia et al., 2016; Li et al., 2020). Previous studies have shown that *Nampt* gene expression is under the regulation of core clock machineries, circadian locomoter output cycles protein kaput (CLOCK) and brain and muscle Arnt-like protein 1 (BMAL1), as well as SIRT1, which leads to rhythmic cellular NAD^+ levels (Nakahata et al., 2009; Ramsey et al., 2009; Wijnen, 2009). CLOCK:BMAL1 transcription complex binds to E-box on the *Nampt* promoter and activates its expression. Upregulation of NAMPT increases NAD^+ production, which as a co-factor activates SIRT1 activity. In turn, SIRT1 deacetylates BMAL1 and histone H3 at the cis-acting site and silences the *Nampt* gene expression. Thus, NAD^+ connects the two feedback loops of circadian rhythmic gene expression and cellular metabolism (**Figure 3**). Furthermore, our recent study showed that Kruppel-like factor 15 (KLF15), a zinc finger transcription factor also directly regulates *Nampt* at the transcriptional level in a circadian fashion by binding to an enhancer element of *Nampt* in intron 1 (Li et al., 2020). It is interesting that KLF15 deficiency only abolishes the circadian rhythmic expression of *Nampt* and does not affect its baseline expression, while in BMAL1 knockout mice overall expression of *Nampt* is reduced (Ramsey et al., 2009). This may be explained by the observation that KLF15 and BMAL1 each binds to a different cis-regulatory element (**Figure 3**). And while KLF15 binding may be spared for basal level of *Nampt* expression during the resting phase, it is required for optimal *Nampt* expression during the active phase, when the metabolic demand is high. In addition, the oscillatory expression of *Nampt* is absent in AMPKα2 but not in AMPKα1 knockout mice, suggesting that AMPKα2 also participates in the regulation of circadian *Nampt* expression in the heart (Um et al., 2011) possibly through the core clock suppressors cryptochrome 1/2 (Lamia et al., 2009) and period 2 (Um et al., 2007).

Another intriguing feature about NAMPT is that it can also be secreted by multiple cell types, including the adipocytes (Revollo et al., 2007), hepatocytes (Garten et al., 2010), monocytes (Schilling and Hauschildt, 2012) and pancreatic β cells (Kover et al., 2013), and be present as an extracellular form (eNAMPT) in circulation. eNAMPT is secreted in extracellular vesicles (Yoshida et al., 2019), which may then be internalized by the recipient cells (Lu et al., 2019), thus elevates NAD^+ biosynthesis in adjacent tissues through paracrine effects (Lu et al., 2019; Yoshida et al., 2019), or even in remote tissues through circulation (Revollo et al., 2007; Imai, 2016; Yoshino et al., 2018). Secretion of

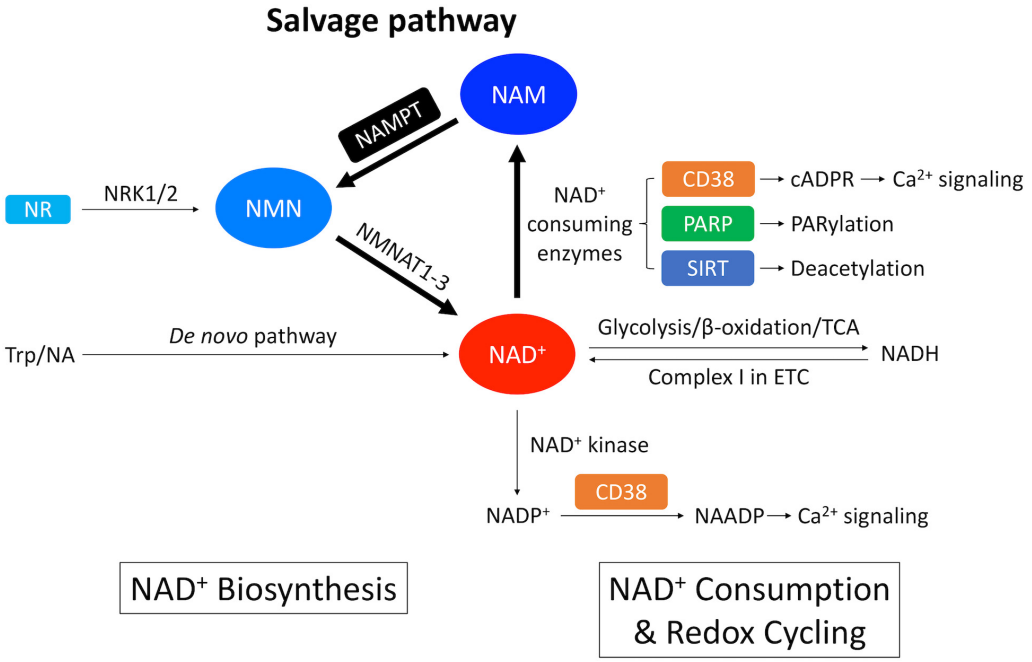

FIGURE 1 | Overview of NAD^+ metabolism in the heart. NAD^+ is synthesized through the *de novo* pathway and the salvage pathway in mammalian cells. The *de novo* pathway generates NAD^+ from Trp or NA. The salvage pathway, indicated by the thicker arrows, is the major NAD^+ biosynthesis pathway in the heart where NAM generated from NAD^+-consuming enzymes is converted into NMN through NAMPT – the rate limiting enzyme. NMN can also be generated from NR by NRK1/2. NMN is further converted into NAD^+ by NMNAT1-3. A redox potential between NAD^+ and its reduced form NADH is established by key catabolic processes including glycolysis, fatty acid β-oxidation and TCA cycle, which drives the electron transport chain (ETC). On the other hand, NAD^+ is consumed by NAD^+-consuming enzymes, including CD38 that generates cADPR, which is a Ca^{2+}-mobilizing second messenger, PARP that catalyzes protein PARylation and SIRT that catalyzes protein deacetylation. These enzymatic reactions produce NAM as a byproduct that can be recycled back into NAD^+ salvage pathway. In addition, NAD^+ can also be consumed by NAD^+ kinase which generates $NADP^+$. Through CD38, $NADP^+$ is further converted into NAADP, which is another Ca^{2+}-mobilizing second messenger.

eNAMPT can be enhanced by SIRT1- (Yoon et al., 2015) or SIRT6 (Sociali et al., 2019)-mediated deacetylation, which also increases the NAMPT enzymatic activity. On the other hand, eNAMPT has also been reported as a proinflammatory cytokine through mechanisms that are independent of its NAD^+ biosynthetic activity (Grolla et al., 2016; Carbone et al., 2017; Dalamaga et al., 2018; Yoshino et al., 2018). In the heart, cardiomyocytes (Pillai et al., 2013; Hsu et al., 2014), perivascular (Wang et al., 2009) and epicardial adipose tissues (Cheng et al., 2008) were shown to secrete eNAMPT. Whether eNAMPT is beneficial or detrimental to heart function remains controversial (Montecucco et al., 2013; Pillai et al., 2013; Yano et al., 2015).

$NAD(P)^+$-NAD(P)H Redox Cycling

NAD^+ is a hydride acceptor that can be transformed into its reduced form NADH after accepting an electron (**Figures 1**, **4**). Reduction of NAD^+ to NADH occurs during fuel catabolism. Fatty acid is the primary fuel source in the heart under physiological conditions (Grynberg and Demaison, 1996). β-oxidation of fatty acid and tricarboxylic acid (TCA) cycle reduce NAD^+ to NADH. NADH is then fed into the electron transport chain to generate ATP in the mitochondria while being oxidized back to NAD^+. NAD^+ depletion reduces ATP content in cardiomyocytes (Hsu et al., 2009). Severe NAD^+ depletion (>95%) may even disable the ability of ATP generation in cells (Del Nagro et al., 2014).

In addition, NAD^+ can be phosphorylated by NAD^+ kinase and converted into nicotinamide adenine dinucleotide phosphate ($NADP^+$) (**Figures 1**, **4**). A redox cycling is also established between $NADP^+$ and NADPH. $NADP^+$ is mostly consumed in the pentose phosphate pathway (PPP) where it is reduced into NADPH. However, the capacity of PPP is very small in the heart due to the low activity of the rate-limiting enzyme glucose-6-phosphate dehydrogenase (Zimmer, 1992). NADPH is the substrate of NADPH oxidases (NOXs). NOXs are the major reactive oxygen species (ROS)-generating enzymes in the heart and have emerged as the primary source of oxidative stress underlying a variety of CVD (Zhang et al., 2020).

NAD^+ Consumption and NAD^+-Consuming Enzymes

The homeostasis of NAD^+ relies on the balance between NAD^+ biosynthesis and NAD^+ consumption. There are three major types of enzymes that utilize NAD^+ as a co-substrate, including cyclic ADP-ribose synthetases which is mainly CD38 in the heart (Lin et al., 2017), poly ADP-ribose (PAR) polymerases (PARPs) and sirtuins (SIRTs) (**Figures 1**, **4**). These enzymatic reactions convert NAD^+ to NAM which must be recycled through salvage

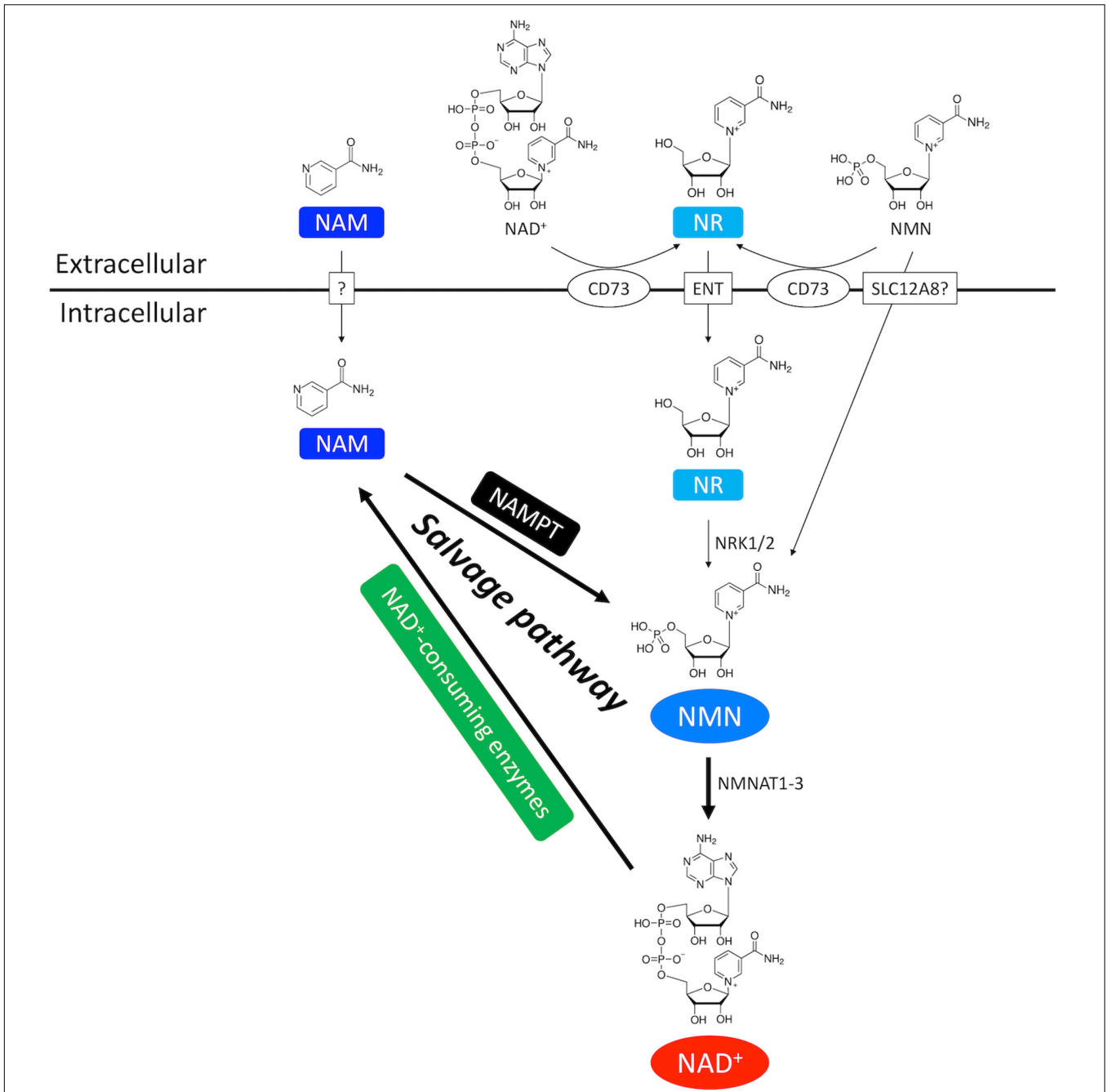

FIGURE 2 | NAD+ salvage pathway in the heart. NAM and NMN are two important intermediate NAD+ metabolites in the NAD+ salvage pathway. NAM is the byproduct of NAD+ consumption. Exogenous NAM can also enter the cytoplasm through a putative membrane transporter. NMN comes from three routes. First, NMN can be generated from NAM by the rate-limiting enzyme NAMPT. Second, extracellular NMN can be transported into cytoplasm through a membrane transporter which might be SLC12A8. Third, NMN can be generated from NR by NRK1/2. Extracellular NR, either from direct supplementation or CD73-mediated conversion from extracellular NMN and NAD+, is transported into cells through equilibrative nucleoside transporter (ENT).

pathway by rate-limiting enzyme NAMPT (**Figures 1**, **4**). It is important to note that different NAD+-consuming enzymes have different enzymatic kinetics or Michaelis constant (K_m) for NAD+. Therefore, whether activation of a particular enzyme can actually lead to rapid depletion of NAD+ would depend on the quantitative relationship between the K_m value of that particular enzyme and the physiological level of NAD+ as detailed below (Katsyuba et al., 2020).

CD38 and Ca^{2+} Signaling

CD38 is a multifunctional enzyme (Wei et al., 2014; Lin et al., 2017; Hogan et al., 2019) that metabolizes NAD+ to ADP-ribose

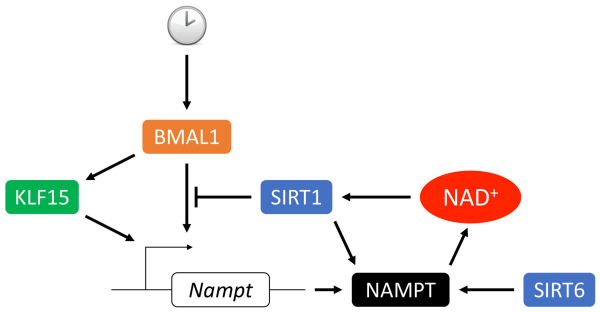

FIGURE 3 | Circadian transcriptional regulation of NAMPT. The circadian rhythmic transcription of *Nampt* in the heart depends on intricate regulation from the core clock. BMAL1 complex binds to the promoter E-box and activates *Nampt* transcription which leads to upregulation of NAMPT expression, NAD^+ biosynthesis and SIRT1 activity. In turn, SIRT1 deacetylates BMAL1 and histone H3 at the *cis*-acting site which suppresses the *Nampt* transcription. Transcription factor KLF15 integrates inputs from both the core clock and additional metabolic cues to promote *Nampt* transcription via direct binding of the enhancer region of *Nampt* in intron 1. Additionally, activity and secretion of NAMPT can be stimulated by SIRT1- or SIRT6-mediated deacetylation.

(ADPR) through its glycohydrolase activity (Kim et al., 1993; Takasawa et al., 1993) or cyclic ADP-ribose (cADPR) through its cyclase activity (Howard et al., 1993). In the presence of NA, CD38 can also mediate a base-exchange reaction in which $NADP^+$ is converted to nicotinic acid adenine dinucleotide phosphate (NAADP) (Aarhus et al., 1995). The major activity of CD38 is the hydrolysis of NAD^+ into ADPR (Dousa et al., 1996; Graeff et al., 2009). The K_m value of CD38 [16 μM (Sauve et al., 1998), 26 μM (Cakir-Kiefer et al., 2001)] for NAD^+ is far below physiological range of intracellular NAD^+ level (400–700 μM) (Hara et al., 2019), thus activation of CD38 can rapidly consume and deplete NAD^+ content. Aging and inflammation are associated with upregulated CD38 gene expression and declined NAD^+ level (Chini et al., 2018).

In the heart, CD38 expression is highest in the endothelial cells, with a relative enzymatic activity ratio of 100:20:1 in endothelium, cardiac fibroblasts and cardiomyocytes, respectively (Boslett et al., 2018). Activation of CD38 by hypoxia-reoxygenation depletes $NAD(P)^+$ in cardiac endothelial cells and impairs nitric oxide production by endothelial nitric oxide synthase that utilizes NADPH as the reducing substrate (Forstermann and Sessa, 2012), suggesting that CD38 may cause endothelial dysfunction after ischemic injury (Boslett et al., 2018). In contrast, a CD38 inhibitor luteolinidin preserved $NAD(P)^+$ level, endothelial and myocardial function in an *ex vivo* heart I/R model (Boslett et al., 2017). The cardioprotective effect of CD38 deficiency have been shown to be SIRT1- (Guan et al., 2016) or SIRT3-dependent (Wang et al., 2018) in mouse models.

Additionally, cADPR and NAADP, produced by CD38 from NAD^+ catabolism, are both important Ca^{2+}-mobilizing second messengers (Lee et al., 1989; Lee and Aarhus, 1995). cADPR activates ryanodine receptors and triggers the release of Ca^{2+} from the endoplasmic reticulum to the cytosol (Ogunbayo et al., 2011). NAADP is the most potent Ca^{2+}-mobilizing second messenger known to date with a working concentration ranging from picomolar to low nanomolar range (Galione, 2019). The receptor for NAADP remains an active pursuit but recent studies suggested a new class of Ca^{2+}-release channel, two-pore channels localized on the endolysosomes are likely to be the target for NAADP (Pitt et al., 2010; Galione, 2019). cADPR and NAADP are required for excitation-contraction coupling in the cardiomyocytes (Rakovic et al., 1996; Collins et al., 2011). Excessive production of these two messenger molecules by CD38 contributes to the pathogenesis of cardiac hypertrophy and arrhythmia (Rakovic et al., 1999; Nebel et al., 2013).

PARylation and DNA Damage

Protein poly ADP-ribosylation (PARylation) is a reversible posttranslational protein modification in response to DNA damage (Langelier et al., 2018). PARP hydrolyzes NAD^+ to build up the PAR chains, which are subsequently degraded by PAR glycohydrolase to terminate the PAR signal (Feng and Koh, 2013). Among 17 PARP family members, PARP1 accounts for over 90% of PARP catalytic activity (Alemasova and Lavrik, 2019). The activity of PARP1 can be activated to as much as 500-fold by DNA double strand breaks (D'amours et al., 1999). Similar to CD38, PARP1 has a K_m [50 μM (Ame et al., 1999), 59 μM (Mendoza-Alvarez and Alvarez-Gonzalez, 1993)] far below physiological range of NAD^+ levels. Additionally, PARylation can be very extensive as the chain length can reach over 200 units on the target proteins (D'amours et al., 1999). Therefore, excessive activation of PARP may lead to rapid depletion of intracellular NAD^+.

Increased PARP level has been found in human failing hearts (Pillai et al., 2005b). Reactive oxygen and nitrogen species generated from heart injuries induce DNA damage and activate PARP (Pacher and Szabo, 2007). In cardiomyocyte, PARP level has a linear correlation with the degree of cardiac hypertrophy induced by either swimming exercise or aortic banding (Pillai et al., 2005b). Overexpression of PARP in cardiomyocytes reduced the NAD^+ and ATP contents by as much as 60% and causes more than 50% of cell death within 48 h (Pillai et al., 2005a). The PARP-induced cardiomyocyte death is mainly mediated by SIRT1 deactivation as ectopic expression or pharmacological activation of SIRT1 prevents the PARP-induced cell death (Pillai et al., 2005a). Elevation of cardiac PARP level has also been found in patients with atrial fibrillation (AF) (Zhang et al., 2019). Either NAD^+ repletion or PARP inhibition ameliorates the contractile dysfunction in HL-1 atrial cardiomyocytes and Drosophila heart tubes after tachypacing (Zhang et al., 2019). Modulation of PARP activity by PARP inhibitors in a number of preclinical models showed improvement in the cellular energy status and cardiac function as well as attenuation in inflammation and cell death, which has been extensively reviewed elsewhere (Pacher and Szabo, 2007; Henning et al., 2018).

Sirtuins and Deacetylation

Sirtuins or SIRTs have attracted substantial attention since their life extending capability was first discovered in yeast in 1999 (Kaeberlein et al., 1999). The subsequent discovery that

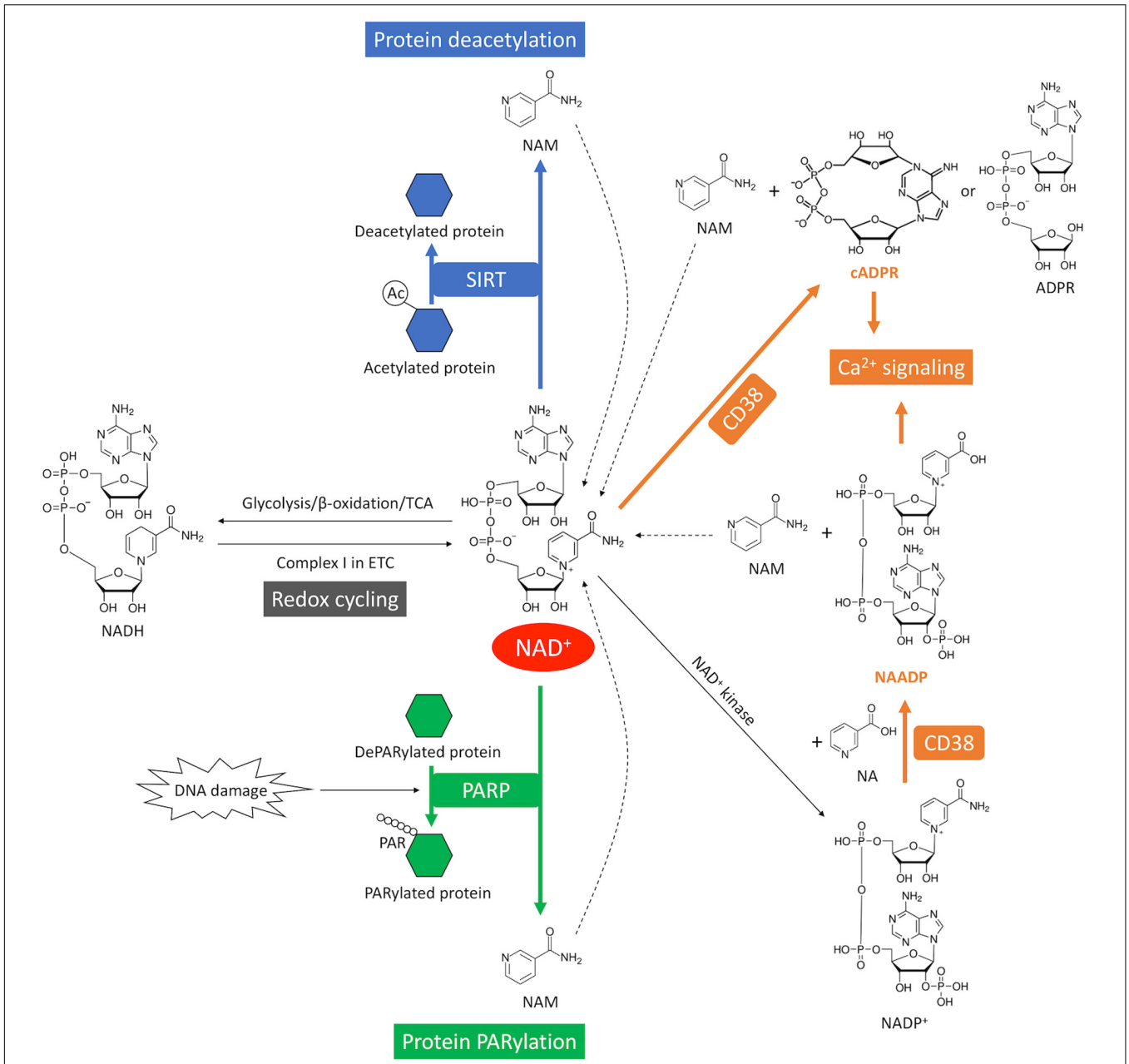

FIGURE 4 | NAD^+ consumption and redox cycling in the heart. SIRT mediates NAD^+ consumption during deacetylation, where the acetyl group is removed from the acetylated protein (blue). DNA damage activates PARP-mediated NAD^+ consumption during PARylation, where the ADP-ribose chain is added onto the target proteins (green). CD38-mediates NAD^+ consumption through cyclization or base-exchange reaction, where two important Ca^{2+}-mobilizing second messengers, cADPR or NAADP, are produced from NAD^+, respectively (orange). CD38 also generates ADPR from NAD^+ through its glycohydrolase activity. NAM is generated as a byproduct from NAD^+ consumption. The dash lines indicate recycling of NAM back into NAD^+ salvage pathway. The redox cycling between NAD^+ and NADH is indicated in gray.

SIRTs use NAD^+ as an essential substrate led to the idea that NAD^+ supplementation may be beneficial in prolonging life and healthy aging (Imai et al., 2000; Landry et al., 2000). It is now well established that SIRTs are NAD^+-dependent deacetylases, which are involved in almost every aspect of human physiology including cell metabolism, cell survival, DNA repair, transcription regulation, inflammation and circadian rhythm (Kupis et al., 2016). SIRTs catalyze the protein deacetylation by transferring the acetyl group from a target protein to its co-substrate NAD^+, which is then broken down into NAM and 2'-O-acetyl-ADPR (Sauve et al., 2001; Feldman et al., 2012). So far seven SIRT members have been discovered in mammalian cells with distinct subcellular localizations (Kupis et al., 2016). SIRT1, which is the most extensively studied SIRT, as well as SIRT6 and

SIRT7, are primarily located in the nucleus. SIRT2 resides in the cytoplasm. SIRT3, SIRT4 and SIRT5 are primarily found in the mitochondria.

Sirtuin activity and NAD^+ levels have an intricate relationship. Although SIRTs are considered NAD^+-consuming enzymes, in most physiological situations SIRTs activation does not lead to cellular NAD^+ depletion due to their enzymatic kinetics (Canto et al., 2015; Anderson et al., 2017; Katsyuba et al., 2020). The K_m of SIRT1 [94 μM (Pacholec et al., 2010), 171 μM (Smith et al., 2009), 750 μM (Madsen et al., 2016), 888 μM (Gerhart-Hines et al., 2011)], SIRT3 [280 μM (Jin et al., 2009), 880 μM (Hirschey et al., 2011)] and SIRT5 [26 μM (Roessler et al., 2015), 200 μM (Madsen et al., 2016)] lie within the physiological range of NAD^+, thus they are unlikely to cause NAD^+ depletion, however, their activities are highly dependent on the cellular NAD^+ levels. Significant disturbance on NAD^+ homeostasis can lead to abnormal SIRT1/3/5 activity which is often seen in human diseases, particularly age-related diseases such as neurodegenerative diseases, CVD and cancer (Carafa et al., 2012). In contrast, the K_m values of SIRT2 [83 μM (Borra et al., 2004)], SIRT4 [35 μM (Laurent et al., 2013)], SIRT6 [13 μM (Kugel et al., 2015)] for NAD^+ are well below the physiological range of NAD^+, so that the level of NAD^+ will not be rate-limiting for those SIRT members, instead, activation of these low-K_m SIRTs may cause a rapid depletion of cellular NAD^+ at least in theory, although supporting evidence is still lacking. Further, SIRT1 and SIRT6, directly regulate NAD^+ biosynthesis through NAMPT (Audrito et al., 2020), the rate-limiting enzyme in the NAD^+ salvage pathway (**Figure 3**). SIRT1 negatively regulates the expression of NAMPT through CLOCK:BMAL1 complex, an important mechanism that regulates the oscillatory NAD^+ levels in a circadian fashion (Nakahata et al., 2009; Ramsey et al., 2009). In addition, deacetylation of NAMPT by SIRT1 (Yoon et al., 2015) or SIRT6 (Sociali et al., 2019) enhances NAMPT enzymatic activity and eNAMPT release.

Sirtuins play a vital role in maintaining normal cardiac function and are involved in various CVD (Sundaresan et al., 2011; Bindu et al., 2016; Hershberger et al., 2017; Ianni et al., 2018). In I/R injury model, SIRT1 (Hsu et al., 2010), SIRT3 (Porter et al., 2014), SIRT5 (Boylston et al., 2015) and SIRT6 (Wang et al., 2016) have all been shown to play a cardioprotective role. For instance, Hsu et al. (2009) demonstrated that I/R injury is associated with a reduction in NAMPT expression and depletion of NAD^+ in heart, which leads to a SIRT1-dependent inhibition of autophagy and activation of apoptosis. SIRT3 also protects the heart from I/R injury by attenuating the myocardial oxidative stress and apoptosis through the SIRT3/forkhead box (FOX) O3/manganese superoxide dismutase (MnSOD) signaling pathway (Chang et al., 2019). Additionally, our recent study revealed that KLF15 deficiency caused a reduction in MnSOD activity due to hyperacetylation at $MnSOD^{K122}$, resulting in elevated oxidative stress and increased susceptibility of myocardium to I/R injury in mouse heart (Li et al., 2020). The KLF15 deficiency-induced oxidative stress in cardiomyocyte can be ameliorated by NMN, which is predominantly mediated by SIRT3 (unpublished data). Because mitochondrial NAD^+ pool is the largest subcellular NAD^+ pool in the cardiomyocytes (see section "Subcellular Compartmentalization of NAD^+"), our study suggests that SIRT3, the main deacetylase in mitochondria (Lombard et al., 2007; Hebert et al., 2013), may be more sensitive to the NAD^+ level change than any other SIRT in the myocardium.

SIRT2-7 all showed a protective effect against cardiac hypertrophy and fibrosis in mouse models (Sundaresan et al., 2012; Ryu et al., 2014; Sadhukhan et al., 2016; Luo et al., 2017; Tang et al., 2017; Yu et al., 2017) whereas the effect of SIRT1 appears to be dependent on its expression level. Alcendor et al. (2007) showed that low to moderate level expression of SIRT1 (2.5–7.5 fold) attenuated age-associated cardiac hypertrophy, fibrosis and cardiac dysfunction, while a high level expression of SIRT1 (12.5 fold) led to spontaneous cardiac pathological remodeling and heart failure. It is possible that excessively high level of SIRT1 leads to NAD^+ depletion and mitochondria dysfunction (Kawashima et al., 2011). The role of SIRTs in non-ischemic heart failure has not been extensively studied. Failing hearts from dilated cardiomyopathy (DCM) patients and mice hearts with mitochondrial complex I deficiency have increased level of mitochondrial protein acetylation (Horton et al., 2016; Lee et al., 2016), suggesting that SIRT3 may be protective against heart failure. In fact, cardiac overexpression of SIRT3 protected against angiotensin II- or doxorubicin-induced cardiac hypertrophy, dysfunction and fibrosis (Sundaresan et al., 2009; Pillai et al., 2016).

The roles of different SIRT isoforms in the context of arrhythmia remain to be further interrogated. Cardiac specific knockout of SIRT1 causes an arrhythmic phenotype resulting from $Na_v1.5$ channel hyperacetylation and dysfunction (Vikram et al., 2017). Overexpression of SIRT2 reversed the repolarization defects in check point kinase BubR1 hypomorphic mice, although the exact molecular mechanism is still unclear (North et al., 2014).

Apart from the deacetylation activity, SIRTs can also use NAD^+ as a co-substrate to catalyze protein deacylation (Sauve, 2010; Feldman et al., 2012). Each SIRT isoform has its unique substrate preference and deacylase activity (Rauh et al., 2013; Tong et al., 2017; Kumar and Lombard, 2018; Carafa et al., 2019; de Ceu Teixeira et al., 2019). For instance, SIRT5 has a strong affinity for negatively charged substrate (Rauh et al., 2013) and mediates protein desuccinylation, demalonylation, and deglutarylation (Du et al., 2011; Tan et al., 2014; Hirschey and Zhao, 2015; Kumar and Lombard, 2018). A recent study from Sadhukhan et al. (2016) revealed that succinyl-CoA is the most abundant acyl-CoA molecule in the heart. SIRT5 deletion causes an accumulation of protein lysine succinylation and leads to hypertrophic cardiomyopathy in mice. SIRT5-targeted proteins are mostly involved in metabolic pathways such as fatty acid β-oxidation, branched chain amino acid catabolism, and respiratory chain proteins (Boylston et al., 2015; Sadhukhan et al., 2016). These findings established a new paradigm of metabolic regulation by SIRT5-mediated lysine succinylation in the heart, distinct from the classic SIRT deacetylation-mediated regulatory pathways. Whether deacylation by other SIRT isoforms could also regulate the cardiac function remains to be explored in future studies.

Subcellular Compartmentalization of NAD+

Different organelles have different membrane permeability to NAD^+, and contain different NAD^+-synthetic/consuming enzymes, which results in highly subcellular compartmentalization of NAD^+ levels and NAD^+-dependent cellular functions (Stein and Imai, 2012; Canto et al., 2015; Nikiforov et al., 2015; Cohen, 2020; Katsyuba et al., 2020; Ralto et al., 2020). In general, there are two major subcellular NAD^+ pools in mammalian cells, the nucleocytoplasmic NAD^+ pool and the mitochondrial NAD^+ pool. The nuclear NAD^+ pool and the cytoplasmic NAD^+ pool are considered to be exchangeable as NAD^+ is freely interchanged through the nuclear membrane pore (Berger et al., 2005; Houtkooper et al., 2010). By using a biosensor for NAD^+, Cambronne et al. (2016) showed that in HEK293 cells, the cytoplasma (106 μM) and the nucleus (109 μM) had an almost identical level of free NAD^+ under basal condition and the NAD^+ levels decreased in a similar kinetics in response to a NAMPT inhibitor FK866. In cardiomyocytes the mitochondrial NAD^+ pool is considerably larger than the nucleocytoplasmic pool (Stein and Imai, 2012). Over 80% of NAD^+ is found in the mitochondrial NAD^+ pool in rodent cardiomyocytes (Alano et al., 2007). As the inner mitochondrial membrane is impermeable to NAD^+, the mitochondrial NAD^+ must come from the import of NAD^+ or NAD^+ precursors from cytosol via transporters or indirect exchange through the malate-aspartate shuttle (MAS) where NAD^+ is transferred out of the mitochondria in exchange for NADH into the mitochondria (Bakker et al., 2001; Nielsen et al., 2011; Satrustegui and Bak, 2015). Based on a comprehensive analysis of subcellular enzyme localizations and NAD^+ precursors, Nikiforov et al. (2015) suggested that NMN is the mitochondria precursor for NAD^+ generation. Another recent study using isotope labeling showed that NAD^+ may be directly imported into mitochondria from cytosol (Davila et al., 2018).

Modulation of compartment-specific enzymes that consume or generate NAD^+ can lead to changes in specific subcellular NAD^+ pools. For instance, PARP inhibitor Tiq-A prevented the reduction in nuclear NAD^+ level induced by inhibiting NAMPT, indicating that nuclear NAD^+ level largely depends on PARP activity (Cambronne et al., 2016). Knockdown of nuclear NMNAT1 and cytoplasmic NMNAT2 lowered the level of nucleocytoplasmic NAD^+, while knockdown of mitochondrial NMNAT3 reduced the mitochondrial NAD^+ level (Cambronne et al., 2016).

Catabolic enzymes involved in fuel metabolism as well as SIRTs are highly compartmentalized and are regulated by local NAD^+ levels. Changes in a specific subcellular NAD^+ pool can alter the activity of these enzymes and related biological processes occurred in the specific subcellular compartment. The details have been recently reviewed elsewhere (Verdin, 2015; Matasic et al., 2018; Katsyuba et al., 2020).

NAD+ IN CVD

Both reductions in NAD^+ biosynthesis and activation of NAD^+-consuming enzymes can cause NAD^+ depletion, which in turn may lead to dysregulation of numerous vital cellular functions, including fuel metabolism, SIRT-dependent regulation and CD38-mediated Ca^{2+} signaling. Chronic dysregulation of NAD^+-dependent cell functions ultimately results in the development of CVD. An increasing number of studies, particularly in rodent models, have shown that boosting NAD^+ is beneficial for CVD (Matasic et al., 2018; Yoshino et al., 2018; Hosseini et al., 2019b). Elevation of NAD^+ levels can be achieved by supplementing NAD^+, NAD^+ precursors or modulating activities of enzymes responsible for NAD^+ generation or degradation such as NAMPT, PARP and CD38 (**Figure 5**). In this review, we will focus on the *in vivo* evidence supporting the cardioprotective effects of NAD^+ restoration in etiologies and risk factors for SCD, including I/R injury, heart failure and arrhythmia.

I/R Injury

Ischemic heart disease is the main cause of SCD in the general population (El-Sherif et al., 2017). Ischemia causes ATP depletion and cardiomyocyte necrosis. Reperfusion restores the energy supply, however, paradoxically imposes oxidative stress by ROS production (Kalogeris et al., 2016). The NAD^+ levels are reduced in the myocardium after both ischemia and I/R injury (Yamamoto et al., 2014). A variety of NAD^+ precursors including NA (Trueblood et al., 2000), NAM (Sukhodub et al., 2010), NMN (Hosseini et al., 2019a), and NAD^+ itself (Zhang et al., 2016; Zhai et al., 2019), have been consistently shown to restore the NAD^+ levels, reduce myocardial infraction (MI) size and protect against I/R injury dysfunction *in vivo*.

Activation of NAD^+-biosynthetic enzymes or inhibition of NAD^+-consuming enzymes have also shown cardioprotective effects in response to I/R. Hsu et al. (2009) showed that cardiac expression of NAMPT was reduced by I/R in mice. Cardiac-specific overexpression of NAMPT increased the NAD^+ levels and reduced MI size and cardiomyocyte apoptosis in response to ischemia and I/R (Hsu et al., 2009). On the other hand, the activity of CD38 is elevated by more than 5-fold in the post-ischemic heart (Reyes et al., 2015). CD38 deficiency reduced the MI size in response to I/R injury (Guan et al., 2016). Inhibition of PARP by genetic deletion or pharmacological inhibitors have also shown therapeutic effects against I/R injury in various animal models, which has been extensively reviewed elsewhere (Zingarelli et al., 1998, 2003, 2004; Grupp et al., 1999; Pieper et al., 2000; Yang et al., 2000; Zhou et al., 2006; Pacher and Szabo, 2007).

Multiple signaling pathways are involved in mediating the cardioprotective effects from NAD^+ restoration. First, the SIRT1/FOXO1 pathway is upregulated by NAD^+ restoration in response to I/R injury (Yamamoto et al., 2014; Guan et al., 2016; Zhang et al., 2016). Elevated NAD^+ levels lead to SIRT1 activation and deacetylation of cytosolic FOXO1, which promotes its nuclear translocation (Frescas et al., 2005; Hsu et al., 2010). Nuclear FOXO1 then activates transcription of antioxidants such as *MnSOD*, which leads to increased ROS clearance and ameliorates the oxidative stress in the heart (Brunet et al., 2004; Hsu et al., 2010). FOXO1 also activates autophagic flux by transcriptional activation of *Rab7*, which preserves energy during ischemia (Hariharan et al., 2010). Second, the PI3K/AKT and ERK1/2 pro-survival kinase pathways, or the so-called

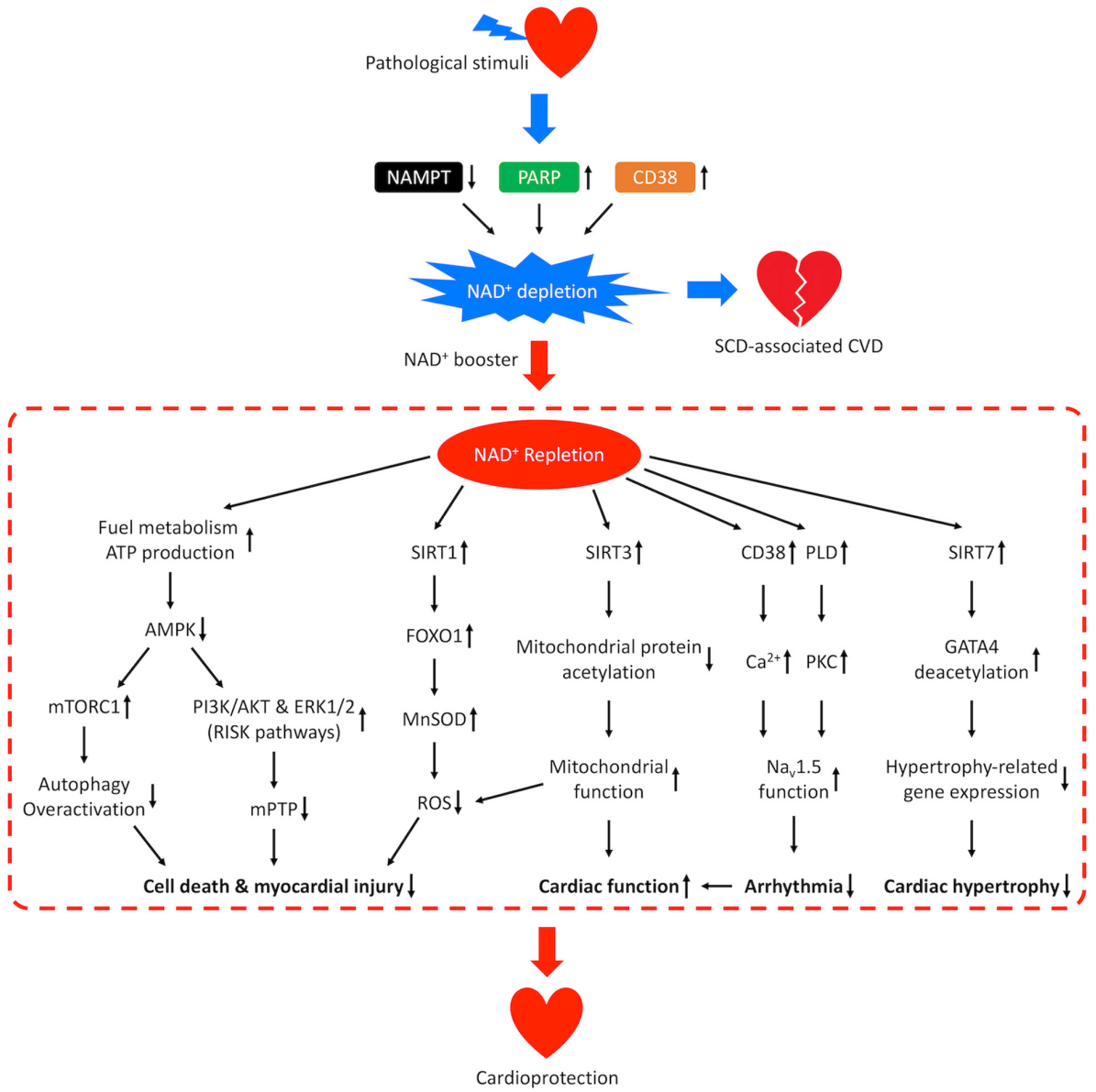

FIGURE 5 | Mechanisms of cardioprotection from NAD^+ repletion. Pathological stimuli that predispose the heart to SCD-associated CVD may result in reduced activity of NAMPT and increased activities of CD38 and PARP, which leads to NAD^+ depletion and disease progression. Repletion of NAD^+ by supplementation of NAD^+ boosters confers cardioprotective effects through multiple signaling pathways. NAD^+ repletion enhances catabolism and stimulates ATP production resulting in downregulation of AMPK, which prevents overactivation of autophagy, promotes the PI3K/AKT and ERK1/2 pro-survival kinase pathways (RISK pathways), and inhibits the mPTP-induced cell death. NAD^+ repletion also activates SIRT1/FOXO1/MnSOD pathway that increases the clearance of ROS, SIRT3-dependent mitochondrial protein deacetylation that restores mitochondrial function and SIRT7-dependent deacetylation of GATA4 which inhibits hypertrophy-related gene expression. These mechanisms lead to reduced myocardial injury and hypertrophic remodeling as well as improved cardiac function. In addition, NAD^+ repletion activates CD38-mediated Ca^{2+} signaling and PKC-dependent phosphorylation which improves the function of cardiac sodium channel $Na_V1.5$ and lowers the risk for arrhythmia.

reperfusion injury salvage kinase (RISK) pathways (Rossello and Yellon, 2018), are upregulated by NAD^+ restoration under I/R conditions (Lim et al., 2008; Sukhodub et al., 2010). NAD^+ restoration activates RISK pathways possibly through downregulation of AMPK activity. Elevation of NAD^+ level increases glycolysis and fatty acid β-oxidation which generates more ATP and decreases the AMP/ATP ratio, which reduces AMPK activity (Dyck and Lopaschuk, 2006). AMPK has been shown to suppress both PI3K/AKT signaling pathway (Tzatsos and Tsichlis, 2007) and ERK1/2 signaling pathway (Meng et al., 2011), therefore inhibition of AMPK activates the RISK pathways. RISK pathways are rapidly activated during the first few minutes of reperfusion and have been proposed as a potential therapeutic target for cardioprotective intervention

(Hausenloy and Yellon, 2004; Rossello and Yellon, 2018). Noma et al. demonstrated that activation of RISK pathways through activation of EGFR signaling confers cardioprotective effect *in vivo* (Noma et al., 2007; Pinilla-Vera et al., 2019). Mechanistically, RISK pathways reduce the opening probability of mitochondrial permeability transition pore (mPTP) and mPTP-induced cell death *in vitro* (Davidson et al., 2006). The activation of RISK pathways also upregulates the activity of a cardiac ATP-sensitive K^+ channel and leads to reduced Ca^{2+} entry, muscle contractility and energy consumption (Sukhodub et al., 2010), which potentially protects the myocardium during ischemia (Nichols, 2016).

In summary, repletion of NAD^+ levels by either direct supplementation of NAD^+ precursors or targeting the NAD^+-biosynthetic/consuming enzymes activity may be a potential therapeutic strategy to mitigate the cardiac injury from I/R. The SIRT1/FOXO1 and RISK pathways are two major players that mediate the beneficial effects through reducing ROS level, inhibiting cell death and preserving energy.

Heart Failure and Pathological Remodeling

Human failing hearts from patients with DCM showed reductions in NAD^+ level and NAD^+/NADH ratio (Horton et al., 2016; Lee et al., 2016). The cardioprotective effects of NAD^+ and its precursors (NR, NMN, and NAM) have been demonstrated in a number of small animal models for heart failure (Riehle and Bauersachs, 2019), including (1) genetic models in which the mitochondrial function is disrupted such as Frataxin (*Fxn*)-knockout model (Puccio et al., 2001; Wagner et al., 2012) [NMN (Martin et al., 2017)], NADH dehydrogenase [ubiquinone] iron-sulfur protein 4 (*Ndufs4*)-knockout model (Karamanlidis et al., 2013) [NMN (Lee et al., 2016)] and transferrin receptor (*Tfrc*)-knockout model [NR (Xu et al., 2015)]; (2) genetic models in which a cytoskeletal component is dysfunctional such as MDX mice model (Quinlan et al., 2004) [NR (Ryu et al., 2016)]; (3) genetic models in which a cardiac transcription factor is deficient such as the serum response factor (*Srf*)-knockout model (Diguet et al., 2011) [NR (Diguet et al., 2018)]; (4) non-genetic models, including pressure overload (Rockman et al., 1994) [NR (Diguet et al., 2018) and NMN (Lee et al., 2016)], volume overload (Liu et al., 1991) [NAM (Cox et al., 2002)], angiotensin II-induced hypertension (Wollert and Drexler, 1999) [NAD^+ (Pillai et al., 2010)], and isoproterenol-induced (Oudit et al., 2003) [NAD^+ (Pillai et al., 2010)] heart failure models. The cardioprotective effects of boosting NAD^+ level observed in various animal models suggest that raising the NAD^+ levels may be a promising therapeutic strategy for heart failure (Walker and Tian, 2018).

Mitochondrial dysfunction and mitochondrial protein hyperacetylation are causally linked to the development of heart failure (Rosca and Hoppel, 2013; Lee and Tian, 2015; Horton et al., 2016; Zhou and Tian, 2018). NAD^+ repletion restored SIRT3 activity and reversed mitochondrial protein hyperacetylation in the failing hearts and improved cardiac function (Lee et al., 2016). Using acetylome analysis, Lee et al. (2016) identified a subgroup of NAD^+/NADH-sensitive mitochondrial proteins, the hyperacetylation of which are highly associated with the development of heart failure, including MAS components and mPTP regulatory proteins. Apart from SIRT3, expression of SIRT1 was found to be reduced in patients with advanced heart failure (Lu et al., 2014). Similar to that in I/R injury, SIRT1/FOXO1 pathway was suppressed, which reduced the expression of antioxidants such as MnSOD and Thioredoxin1, whereas the expression of pro-apoptotic molecule BAX was increased (Lu et al., 2014). Therefore, NAD^+ repletion may reduce oxidative stress and apoptosis in failing hearts through activation of SIRT1/FOXO1 pathway. In addition, a recent study showed that NAD^+ repletion by NMN supplement activates SIRT7/GATA binding protein 4 (GATA4) pathway which confers anti-hypertrophic effects in response to pressure overload (Yamamura et al., 2020). SIRT7 directly interacts and deacetylates GATA4, which regulates its transcriptional activity and suppresses the cardiac hypertrophy-related gene expression (Yamamura et al., 2020).

The cardioprotective effects of NAD^+ repletion in heart failure may also come from inhibition of AMPK signaling pathways. AMPK is activated as a result of energy depletion and metabolic remodeling in the failing heart (Azevedo et al., 2013; Doenst et al., 2013). NAD^+ repletion can suppress the elevated AMPK activity in heart failure (Lee et al., 2016; Ryu et al., 2016; Diguet et al., 2018). As mentioned above, NAD^+ may inhibit AMPK activity indirectly through the activation of glycolysis and fatty acid β-oxidation. The effects of AMPK activation on cardiac function depends on the stage and the severity of the heart failure. In the early stage before decompensation, activation of AMPK is considered to be an adaptive mechanism which may help maintain the physiological level of autophagy (Nakamura and Sadoshima, 2018) by inhibiting mTORC1 signaling (Kim et al., 2011) and delay the transition from cardiac remodeling to heart failure (Pillai et al., 2010; Beauloye et al., 2011). Once decompensation occurs, chronic activation of AMPK can actually be detrimental to cardiac function. Overactivation of AMPK may lead to uncontrolled autophagy which triggers autophagy-dependent cell death and myocardial injury (Zhu et al., 2007; Sciarretta et al., 2018; Kaludercic et al., 2020). Further, long-term activation of AMPK suppresses the pro-survival RISK signaling pathways (Tzatsos and Tsichlis, 2007; Meng et al., 2011). Therefore, NAD^+ repletion-induced inhibition on AMPK signaling pathway may be particularly beneficial for late stage heart failure.

Intriguingly, modulation of NAMPT activity has shown contradictory effects on heart failure. Lee et al. (2016) showed that overexpression of cardiac NAMPT partially restored the cardiac function after pressure overload and completely reversed the isoproterenol-induced cardiac dysfunction in the mitochondrial complex I defective mouse model. However, Byun et al. (2019) showed that both gain and loss of NAMPT function exacerbated the pressure overload-induced heart failure. On the other hand, animal studies on PARP inhibition consistently showed significant preservation in cardiac function and promotion in survival rate in heart failure models (Booz, 2007; Pacher and Szabo, 2007; Halmosi et al., 2016; Henning et al., 2018). In

addition, knockout of CD38 was shown to be cardioprotective against angiotensin II−induced cardiac hypertrophy in mouse (Guan et al., 2017). This discrepancy may be due to the proinflammatory effect of eNAMPT (Montecucco et al., 2013; Byun et al., 2019). Further, overexpression of NAMPT may not necessarily elevate NAD^+ level if the amount of NAM is limited (Byun et al., 2019). NAMPT overexpression may also lead to excessive amount of NAD^+ and overactivation of SIRT1 which has been shown to impair mitochondrial and cardiac function (Kawashima et al., 2011).

In summary, NAD^+ repletion could provide cardioprotective effects against cardiac remodeling and heart failure through multiple mechanisms, including reduction of oxidative stress, suppression of hypertrophy-related gene expression, prevention of autophagy overactivation, restoration of mitochondrial function and activation of RISK pro-survival pathways. These findings indicate that NAD^+-boosting intervention may be a potential therapeutic strategy for cardiac remodeling and heart failure.

Arrhythmia

We have only started to explore the effects of NAD^+ on arrhythmia. Cardiac sodium current (I_{Na}) upstroke during depolarization phase is predominantly mediated by $Na_v1.5$ channel, which is the major voltage-gated sodium channel in the heart (Veerman et al., 2015). Mutations in $Na_v1.5$ are associated with a number of cardiac diseases such as long QT syndrome, AF and cardiomyopathy (Song and Shou, 2012; Shy et al., 2013; Han et al., 2018; Wilde and Amin, 2018). Matasic et al. (2020) showed that NR increased I_{Na} and reduced residual late I_{Na} *in vitro*, and more importantly administration of NR decreased QTc *in vivo*. Mechanistically, NR supplementation elevates NADH level which increases phospholipase D (PLD) activity leading to activation of PKC signaling pathway (Martin et al., 2017). PKC phosphorylation at S1503 in $Na_v1.5$ channel (Matasic et al., 2020) modulates the channel conductance and improves the I_{Na} profile in the heart (Valdivia et al., 2009; Martin et al., 2017). The anti-arrhythmic effect of NAD^+ supplementation may not be exclusively dependent on the NAD^+ levels. Liu et al. (2013) demonstrated that administration of NAD^+ restored I_{Na} from 60% to 97% in mouse with non-ischemic cardiomyopathy and improved conduction velocity in human myopathic hearts. However, the I_{Na} restoration by NAD^+ was diminished by CD38 inhibitor pelargonidin, suggesting that CD38-mediated Ca^{2+} signaling rather than the intracellular NAD^+ level plays a direct role (Liu et al., 2013).

In patients with AF and tachypaced cardiomyocytes, PARP1 was found to be hyperactive, which leads to NAD^+ depletion (Zhang et al., 2019). Excessive production of ROS observed in AF (Youn et al., 2013; Xie et al., 2015; Liang et al., 2018) may be associated with high level of DNA damage, which leads to activation of PARP1. Inhibition of PARP1 restored the NAD^+ content, reduced oxidative stress and improved cardiomyocyte contractility in rat atrial cardiomyocytes and Drosophila hearts with tachypacing (Zhang et al., 2019).

Apart from $Na_v1.5$, future study should look into other NAD^+-regulated ion transporters (Kilfoil et al., 2013) that are important for cardiac electrophysiology and their potential implications in anti-arrhythmic effects, such as Kv4.2 (Tur et al., 2016, 2017). In addition, severe NAD^+ depletion (>95%) sabotages the ability to regenerate ATP (Del Nagro et al., 2014) which may potentially impair the normal phosphorylation state and function of ion transporters in the heart (Ismailov and Benos, 1995; Grant, 2009; Bartos et al., 2015). Future study may explore whether NAD^+ repletion can restore a global level of phosphorylation on cardiac ion transporters after severe NAD^+ depletion.

In summary, there is an emerging evidence suggesting that NAD^+ replenishment plays important roles in cardiac arrhythmia.

THE EMERGING NAD+-BOOSTING THERAPY

There has been a rapidly growing attention and expectation on the clinical usage of NAD^+-boosting therapy for diseases associated with NAD^+ depletion and metabolic syndrome. Over 300 clinical trials (clinicaltrials.gov) have been conducted to test the therapeutic potential of NAD^+ precursors on Alzheimer's disease, psoriasis, obesity, diabetes, chronic kidney disease, dyslipidemia, and CVD etc (Katsyuba et al., 2020). Currently, NA (Niaspan) is the only US Food and Drug Administration (FDA)-approved NAD^+ precursor product and is used to treat dyslipidemia in the US (Villines et al., 2012; Rajman et al., 2018). However, NR, NA, NMN, and NAM are all natural products and their use does not require FDA approval. As a result, they are taken as food supplements for a broad spectrum of indications in many situations but without evaluation from well-controlled clinical studies.

Human Studies on SCD-Associated CVD

As a basis for clinical translation, the safety of NAD^+-boosting interventions in high-risk groups for CVD has been well evaluated (Dellinger et al., 2017; Martens et al., 2018). A recently published review from Katsyuba et al. (2020) summarized the human studies with NAD^+ boosters in different disease settings. Here, we will focus on human studies on SCD-associated CVD.

Since the lipid-lowering effect of NA was found in 1950s (Altschul et al., 1955; Parsons et al., 1956), most of the human studies on coronary artery disease (CAD) or ischemic heart disease have chosen NA as the NAD^+ booster of choice. Initiated in 1962, the Coronary Drug Project demonstrated that subjects treated with NA had a 10% reduction of the serum cholesterol levels (Berge and Canner, 1991). Moreover, NA was shown to reduce the levels of low-density lipoprotein while increase the level of high-density lipoprotein, and the combination therapy with colestipol (Brown et al., 1990) or statins (Guyton et al., 2008; Sang et al., 2009) offered even greater lipid lowering benefits. Consistent with the improved lipid profile, NA offers additional preventive effects on atherosclerotic progression in patients receiving colestipol (Blankenhorn et al., 1987a,b;

Brown et al., 1990) or statins (Brown et al., 2001; Taylor et al., 2004; Villines et al., 2010; Boden et al., 2011; Landray et al., 2014). Further, NA-treated subjects had a lower incidence of definite, non-fatal MI (10% vs. 14% in placebo group) (Berge and Canner, 1991). Most importantly, the NA treated group, although showing a trend toward lowering mortality at the conclusion of the trial, actually showed a significant (11%, $Z = -3.52$, $p < 0.0004$) reduction of mortality in the extended follow-up study 9 years from the conclusion of the active study. Subjects taking NA previously showed a 1.6 year extension of life (Berge and Canner, 1991). However, another two clinical trials with patients receiving statin-based therapy showed that the addition of NA failed to provide clinical benefits in reducing the risk of major vascular events or composite cardiovascular death (Boden et al., 2011; Landray et al., 2014). Because the NAD^+ level is oscillating in a circadian fashion in heart, one possible explanation for the inconsistent results is that the timing of implementing NAD^+-boosting intervention may have a significant impact on the clinical outcome. As a supportive evidence for this notion, our recent study showed that NMN administration elevated NAD^+ level and reduced MI size in mice after I/R injury at ZT2 when the NAD^+ level was at nadir, however, the improvement was not observed when NMN was supplemented at ZT14 when the NAD^+ level was at peak (Li et al., 2020). Therefore, an optimal time window may be critical for NAD^+-boosting therapy, which needs to be carefully examined in future studies. Another explanation might be the lack of a pharmacological effective dose. In fact, most of the human studies reported so far with NAD^+ boosters, including those on non-CVD, did not provide evidence for elevated NAD^+ level in experimental subjects (Katsyuba et al., 2020).

For heart failure, two small-scale pilot clinical studies, one with 5 and the other with 30 patients, aiming to assess the safety and feasibility of NR have been completed but the results have not been published yet (clinicaltrials.gov). Another pilot study on NAM is in the progress of recruiting 60 heart failure patients (clinicaltrials.gov). To date, no clinical trial has been conducted to examine the role of NAD^+-boosting strategies on arrhythmia (clinicaltrials.gov). The effects of NAD^+ boosters on patients with heart failure and arrhythmia remain to be investigated in future studies.

In summary, current human studies have shown that NAD^+-boosting therapy can reduce mortality (Carlson and Rosenhamer, 1988; Berge and Canner, 1991; Brown et al., 2001; Landray et al., 2014) and provide moderate clinical benefits for patients with CAD. However, conflicting results on critical clinical outcomes such as incidence of composite mortality and major vascular events have raised the concern that whether NAD^+-boosting therapy can ultimately become a primary treatment for CAD and other CVD. Several important aspects may help overcome these hurdles. First, it is critical to determine the effective dose of NAD^+ boosters for each individual patient. Direct measurement for NAD^+ level or NAD^+ metabolome from accessible samples [i.e., plasma (Grant et al., 2019)] should be considered. It is possible to achieve the effective therapeutic level, novel NAD^+ precursors or novel pharmaceutical formulations are required. Second, the optimal time window for NAD^+ booster supplementation remains to be established in human subjects. NAD^+-boosting therapy should coordinate with the intrinsic circadian oscillation of NAD^+ level in human body so that maximal beneficial effects can be achieved. With a more nuanced understanding of NAD^+ biology in the heart and clinical studies designed with more sophistication, we anticipate that NAD^+-boosting therapy would ultimately harness its potential for SCD-associated CVD.

AUTHOR CONTRIBUTIONS

LZ conceived the manuscript. WX, LL, and LZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Myocardial Energy Metabolism in Non-ischemic Cardiomyopathy

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As the most metabolically demanding organ in the body, the heart must generate massive amounts of energy adenosine triphosphate (ATP) from the oxidation of fatty acids, carbohydrates and other fuels (e.g., amino acids, ketone bodies), in order to sustain constant contractile function. While the healthy mature heart acts omnivorously and is highly flexible in its ability to utilize the numerous fuel sources delivered to it through its coronary circulation, the heart's ability to produce ATP from these fuel sources becomes perturbed in numerous cardiovascular disorders. This includes ischemic heart disease and myocardial infarction, as well as in various cardiomyopathies that often precede the development of overt heart failure. We herein will provide an overview of myocardial energy metabolism in the healthy heart, while describing the numerous perturbations that take place in various non-ischemic cardiomyopathies such as hypertrophic cardiomyopathy, diabetic cardiomyopathy, arrhythmogenic cardiomyopathy, and the cardiomyopathy associated with the rare genetic disease, Barth Syndrome. Based on preclinical evidence where optimizing myocardial energy metabolism has been shown to attenuate cardiac dysfunction, we will discuss the feasibility of myocardial energetics optimization as an approach to treat the cardiac pathology associated with these various non-ischemic cardiomyopathies.

Keywords: energy metabolism, cardiomyopathy, cardiac function, glucose oxidation, fatty acid oxidation

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INTRODUCTION

Relative to its size, the heart possesses a metabolic demand that far exceeds that of any other organ in the body (Lopaschuk et al., 2010; Lopaschuk and Ussher, 2016). However, despite the extraordinary energy requirements necessary to sustain constant contractile function, basal metabolic processes, and ionic homeostasis, the heart has limited energy reserves and, therefore, must continually regenerate adenosine triphosphate (ATP) in order to maintain function. Although the heart relies preferentially on fatty acid metabolism to sustain a sufficient ATP supply, the heart acts omnivorously and demonstrates a unique capability to metabolize a variety of substrates in addition to fatty acids, such as carbohydrates (glucose and lactate), ketone bodies and amino acids (Lopaschuk et al., 2010; Lopaschuk and Ussher, 2016). This flexibility of substrate utilization allows the heart to accommodate alterations in substrate availability throughout various physiological states (e.g., nutrient ingestion, fasting). Of importance, this flexibility may deteriorate in response to pathological conditions where the heart must adjust its substrate preference to accommodate

a perturbation in energy supply or demand, such as that seen during myocardial ischemia or heart failure.

With regards to the latter, heart failure is defined as a complex clinical syndrome that results from any structural or functional cardiac disorder that hinders the ability of the ventricle to fill with (diastolic dysfunction) or eject blood (systolic dysfunction) (Hunt et al., 2001). Despite the pathology of heart failure being multifactorial, it is generally accepted that the failing heart is energy-starved (Neubauer, 2007; Ussher et al., 2016). Myocardial ATP production is reduced by 30–40% in heart failure, therefore placing emphasis on the potential role of perturbed myocardial energetics in the mechanisms leading to heart failure. Chronic alterations in energy substrate utilization have been reported in heart failure, as well as in preceding cardiomyopathies (Neubauer, 2007; Taha and Lopaschuk, 2007; Lopaschuk et al., 2010). Furthermore, impaired metabolic flexibility has been postulated to contribute to the myocardial insulin resistance that characterizes the failing heart (Lopaschuk et al., 2010; Zhang et al., 2013). While heart failure may be the end diagnosis in an individual's cardiac disease, many cases of heart failure are preceded initially by different forms of cardiomyopathy (Braunwald, 2017). Depending on the classification system used, cardiomyopathies can be classified as ischemic, dilated, hypertrophic, restrictive, inflammatory, arrhythmogenic, or diabetic to list a few. Given that alterations in myocardial energy metabolism are present in numerous cardiomyopathies that lead to heart failure, optimization of energetics during the cardiomyopathy stage may represent a promising therapeutic approach to attenuate or prevent subsequent heart failure development and progression.

Accordingly, we will review the primary metabolic pathways supporting cardiac function, the alterations in intermediary energy metabolism that accompany various cardiomyopathies, and whether these alterations can be targeted to mitigate cardiomyopathy and the eventual development of overt heart failure. We will not discuss energy metabolism in ischemic cardiomyopathy or dilated cardiomyopathy, as the perturbations of energy metabolism in these cardiac disorders have been extensively characterized in numerous reviews (Taha and Lopaschuk, 2007; Jaswal et al., 2011; Heusch et al., 2014; Gibb and Hill, 2018). Rather, we will focus our efforts on detailing the perturbations of intermediary energy metabolism accompanying various non-ischemic cardiomyopathies, including hypertrophic cardiomyopathy (HCM), diabetic cardiomyopathy, the cardiomyopathy associated with the rare genetic disorder, Barth Syndrome (BTHS), and arrhythmogenic cardiomyopathy.

ENERGY METABOLISM IN THE HEALTHY HEART

The heart derives the majority (∼95%) of its ATP production from the mitochondrial oxidation of fatty acids, carbohydrates (e.g., glucose, lactate), ketone bodies (e.g., acetoacetate, β-hydroxybutyrate), and amino acids, with the remainder being produced through aerobic glycolysis (**Figure 1**; Lopaschuk et al., 2010; Lopaschuk and Ussher, 2016). In the mature heart, fatty acids account for the majority of oxidative metabolism (50–70%), with the oxidation of glucose primarily accounting for the remainder. However, in response to nutrient ingestion, carbohydrates (glucose) can become the predominant fuel in the mature, metabolically flexible heart, due to the ensuing insulin response (Lopaschuk et al., 2010; Lopaschuk and Ussher, 2016). Although the heart is capable of metabolizing ketone bodies and amino acids, the majority of studies have demonstrated minimal contribution of these substrates to myocardial ATP production (Lopaschuk and Ussher, 2016). Conversely, recent studies in isolated working mouse hearts have demonstrated that ketone bodies can become a major fuel source for the heart, particularly at circulating concentrations representative of prolonged fasting/starvation (Ho et al., 2020), and thus we will also describe the regulation of myocardial ketone body oxidation herein. In addition to supporting ATP production, it is becoming more apparent that these substrates, or various intermediates arising from the metabolism of these substrates, can also influence cardiac function via directly regulating cellular signaling pathways. This can include post-translational modifications of proteins (e.g., acetylation, *O*-N-acetyl glucosaminylation), allosteric regulation of enzymes, or via transducing G-protein coupled receptors, though these aspects will be minimally discussed herein, as they have been extensively characterized in recent reviews (D'Souza et al., 2016; Lopaschuk and Ussher, 2016; Puchalska and Crawford, 2017; Murphy and O'Neill, 2018; Collins and Chatham, 2020; Ritchie and Abel, 2020).

Fatty Acid Metabolism

The heart's coronary circulation provides it with its fatty acid supply either as free fatty acids bound to albumin, or following the release of fatty acids from the hydrolysis of triacylglycerol (TAG)-containing lipoproteins or chylomicrons, mediated predominantly by the actions of lipoprotein lipase (Teusink et al., 2003; Lopaschuk et al., 2010). The majority of extracellular fatty acid uptake into the cardiac myocyte occurs through protein-mediated transport, which involves transporters such as cluster of differentiation 36 (CD36) and fatty acid transport proteins (FATP), whereas passive diffusion is thought to contribute to a smaller extent (**Figure 1**; Luiken et al., 1999; Kuang et al., 2004; Lopaschuk et al., 2010). Once present in the cytosol, fatty acids are rapidly activated for further metabolism via esterification to coenzyme A (acyl CoA) in an ATP-dependent manner by fatty acyl CoA synthetase.

Acyl CoAs have three primary metabolic fates in the heart, where they can be stored as TAG, utilized for the biosynthesis of membranes, and most importantly, due to the heart's enormous energy demand, transported into the mitochondria for subsequent β-oxidation (Lopaschuk et al., 2010). Because the mitochondrial membrane is impermeable to long chain acyl CoAs, mitochondrial fatty acid uptake and subsequent β-oxidation is dependent upon a carnitine shuttle comprised of three enzymes. An outer mitochondrial membrane-localized carnitine palmitoyl transferase 1 (CPT-1) catalyzes the conversion of long chain acyl CoA into acylcarnitine for transport to the mitochondrial matrix by carnitine acyl translocase, following which the acyl CoA

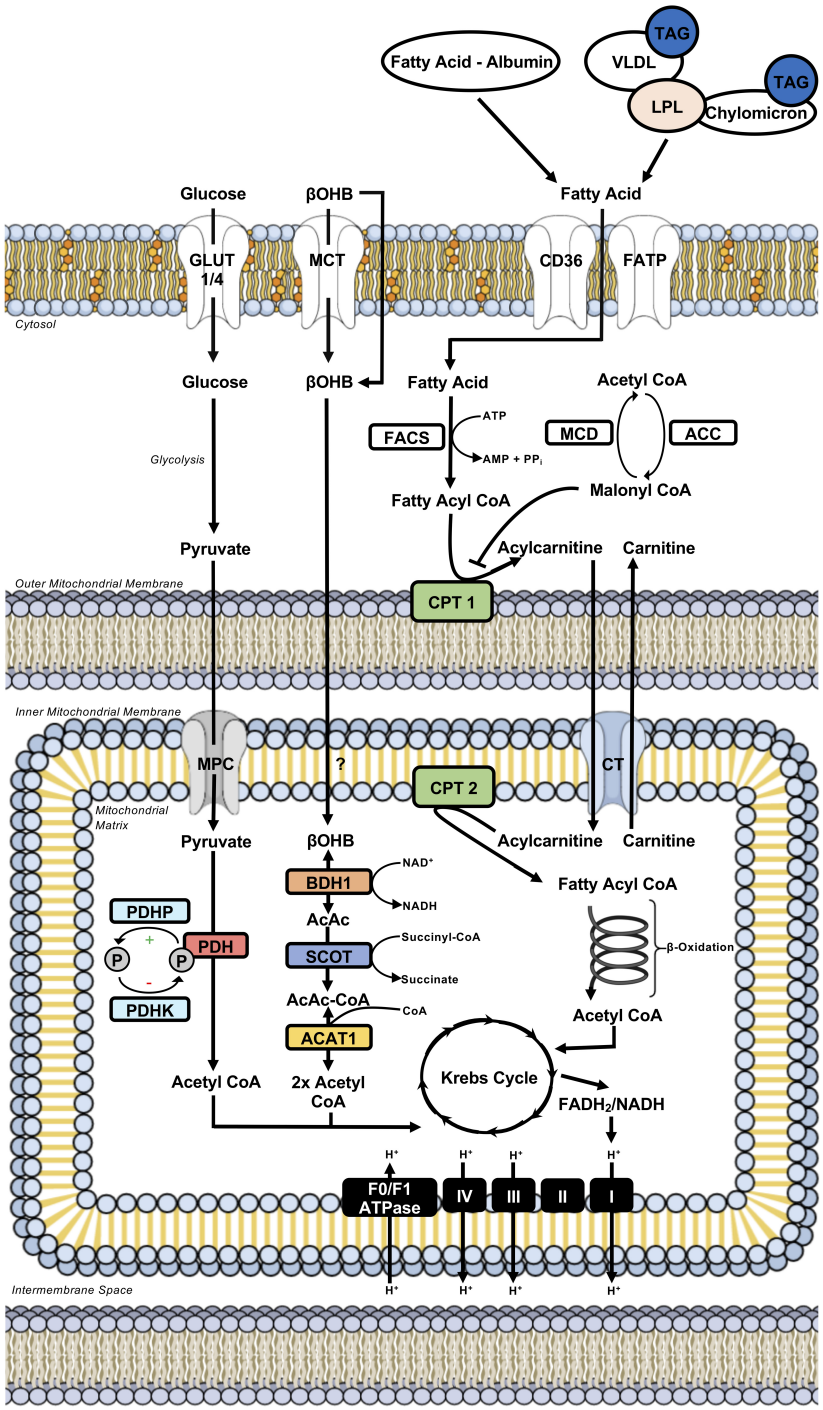

FIGURE 1 | Intermediary energy metabolism in the cardiac myocyte. Illustration depicts intermediary metabolism of carbohydrates (glucose), fatty acids, and ketone bodies (βOHB) in the myocardium. Key nodes depicting the control of uptake into the cardiac myocyte, uptake into the mitochondria, and subsequent metabolism to acetyl CoA are highlighted. Acetyl CoA from the oxidation of carbohydrates, fatty acids, and ketone bodies is subsequently metabolized further in the Krebs Cycle. This results in the formation of reducing equivalents (e.g., $FADH_2$/NADH) that donate their electrons to the complexes of the electron transport chain, driving oxidative phosphorylation and the ATP generation needed for sustaining contractile function. ACAT, acetoacetyl CoA thiolase; ACC, acetyl CoA carboxylase; BDH, β-hydroxybutyrate dehydrogenase; βOHB, β-hydroxybutyrate; CD36, cluster of differentiation 36; CPT, carnitine palmitoyl transferase; CT, carnitine translocase; FACS, fatty acyl CoA synthetase; $FADH_2$, reduced flavin adenine dinucleotide; FATP, fatty acid transport protein; GLUT, glucose transporter; LPL, lipoprotein lipase; MCD, malonyl CoA decarboxylase; MCT, monocarboxylic acid transporter; MPC, mitochondrial pyruvate carrier; NADH, reduced nicotinamide adenine dinucleotide; PDH, pyruvate dehydrogenase; PDHK, PDH kinase; PDHP, PDH phosphatase; SCOT, succinyl CoA:3-ketoacid CoA transferase; TAG, triacylglycerol; VLDL, very-low density lipoprotein.

is regenerated via CPT-2 present in the inner leaflet of the inner mitochondrial membrane (**Figure 1**; Lopaschuk et al., 2010). Of importance, this carnitine shuttle regulating mitochondrial fatty acid uptake and subsequent β-oxidation is highly sensitive to regulation via malonyl CoA, a potent endogenous inhibitor of CPT-1. Malonyl CoA is the product of acetyl CoA carboxylation via acetyl CoA carboxylase, whereas it is degraded by malonyl CoA decarboxylase. Once inside the mitochondrial matrix, the CPT-2 regenerated acyl CoA is finally subjected to repeated cycles of β-oxidation, a process involving four enzymes (acyl CoA dehydrogenase, enoyl CoA hydratase, 3-hydroxyacyl CoA dehydrogenase, and 3-ketoacyl CoA thiolase) that progressively shortens the acyl CoA via 2 carbons released as acetyl CoA with each cycle (**Figure 1**). Acetyl CoA subsequently enters the Krebs Cycle, where it joins with oxaloacetate to form citrate and undergo a series of redox reactions. The Krebs Cycle results in the formation of reducing equivalents (reduced flavin adenine dinucleotide/nicotinamide adenine dinucleotide) that donate their electrons to the complexes of the electron transport chain (ETC) for the generation of ATP during oxidative phosphorylation (**Figure 1**). The heart also contains endogenous TAG stores that can be used to support ATP production, with some studies suggesting that fatty acids taken up into the heart are first shuttled through the intracellular TAG pool prior to undergoing mitochondrial β-oxidation (Shipp et al., 1964b; Banke et al., 2010).

Glucose Metabolism

Myocardial glucose metabolism for energy production involves three major steps: glucose uptake, glycolysis, and the mitochondrial oxidation of glycolytically-derived pyruvate, a process referred to as glucose oxidation (**Figure 1**). Glucose uptake into cardiac myocytes is facilitated by glucose transporters (GLUT), of which GLUT4 and GLUT1 are responsible for insulin-dependent and insulin-independent glucose uptake, respectively (Brownsey et al., 1997; Jaswal et al., 2011). Glucose subsequently undergoes glycolysis in the cytosol, a metabolic pathway comprised of ten enzymes that result in the formation of minimal energy (2 ATP) and the three-carbon end-product, pyruvate. Glycolytically-derived pyruvate has two primary metabolic fates, where it can either be converted to lactate by lactate dehydrogenase or shuttled into the mitochondrial matrix by a monocarboxylic acid transporter [referred to as the mitochondrial pyruvate carrier (MPC)] for subsequent oxidation. In the healthy mature heart where oxygen is not limiting, the latter mechanism predominates and is regulated by the actions of pyruvate dehydrogenase (PDH), the rate-limiting enzyme of glucose oxidation (Patel and Korotchkina, 2006; Patel et al., 2014).

The PDH complex is a multienzyme complex that decarboxylates pyruvate into acetyl CoA, which has the same fate as fatty acid oxidation-derived acetyl CoA and enters the Krebs Cycle to generate reducing equivalents for supporting oxidative phosphorylation in the ETC (**Figure 1**). The PDH complex is intricately regulated by numerous post-translational modifications, including phosphorylation-mediated inactivation via four PDH kinase (PDHK) isoforms, and dephosphorylation-mediated activation via two PDH phosphatase isoforms (**Figure 1**; Patel and Korotchkina, 2006; Patel et al., 2014; Gopal et al., 2017). Recent studies have also demonstrated that PDH activity and subsequent glucose oxidation can be stimulated via sirtuin 3 mediated deacetylation (Jing et al., 2013). As many mitochondrial dehydrogenases including PDH are sensitive to calcium-mediated stimulation (Denton, 2009), it has been demonstrated that mitochondrial calcium uptake mediated by the mitochondrial calcium uniporter (MCU) can positively regulate PDH activity (Suarez et al., 2018).

The heart also contains endogenous glycogen stores from which glucose can be mobilized to support myocardial ATP production when needed. Another metabolic pathway of glucose includes the pentose phosphate pathway, which plays a major role in the production of reduced nicotinamide adenine dinucleotide phosphate needed to generate the endogenous antioxidant, reduced glutathione (Ussher et al., 2012). Although evidence suggests that perturbations in the pentose phosphate pathway may be implicated in the pathology of heart failure (Gupte et al., 2006), glucose flux through this pathway in the healthy mature heart is thought to be negligible (Shipp et al., 1964a; Ussher et al., 2012).

Ketone Body Metabolism

β-hydroxybutyrate and acetoacetate are the two primary ketone bodies that the heart utilizes to support energy production. While both are able to enter the cardiac myocyte via passive diffusion, at elevated concentrations it has also been suggested that ketone body uptake may involve monocarboxylic acid transporters (Halestrap, 1978; Puchalska and Crawford, 2017). β-hydroxybutyrate is converted into acetoacetate via the actions of β-hydroxybutyrate dehydrogenase (BDH1). Acetoacetate is subsequently activated for metabolism via esterification to CoA as acetoacetyl CoA, which is catalyzed via succinyl CoA:3-ketoacid CoA transferase (SCOT). Acetoacetyl CoA thiolase then hydrolyzes acetoacetyl CoA into two molecules of acetyl CoA, which are subject to the same metabolic fate as either glucose or fatty acid oxidation-derived acetyl CoA, thereby entering the Krebs Cycle to generate reducing equivalents for supporting oxidative phosphorylation (**Figure 1**).

Substrate Competition for Oxidative Metabolism

A significant contributor to the heart's robust metabolic flexibility to switch between fatty acids and glucose as major fuel sources during fasting and nutrient ingestion, respectively, involves substrate competition for oxidative metabolism. Joseph Shipp and colleagues were the first to demonstrate that increasing fatty acid availability to the heart leads to a marked inhibition of glucose oxidation (Shipp et al., 1961). However, credit for the reciprocal relationship by which fatty acids and glucose compete for oxidative metabolism (glucose/fatty acid cycle) is attributed to the work of Randle et al. (1963), and is thus often referred to as the "Randle Cycle." A plethora of both animal and human studies have provided strong evidence to

support substrate competition between fatty acids and glucose for oxidative metabolism in the heart (Wisneski et al., 1985; Wisneski et al., 1990; Liu et al., 2002; Buchanan et al., 2005). Conversely, recent evidence has suggested that increasing the heart's reliance on ketone bodies as an oxidative fuel source is not subject to the same reciprocal relationships that would be conjectured to lead to reduced glucose and fatty acid oxidation rates (Ho et al., 2020). Of relevance to the remainder of this review, numerous cardiac disorders including various cardiomyopathies and/or heart failure, are accompanied by metabolic alterations whereby this metabolic flexibility becomes impaired, which may directly contribute to their associated cardiac pathologies.

ENERGY METABOLISM IN HYPERTROPHIC CARDIOMYOPATHY

HCM is characterized by a thickening of the left or right ventricular wall secondary to cardiac myocyte hypertrophy and in the absence of ventricular dilation. With an estimated prevalence of 1:200, HCM is reported as the most frequent form of inherited cardiomyopathy (Semsarian et al., 2015; Braunwald, 2017; Marian and Braunwald, 2017). Our knowledge of the pathology of HCM has greatly improved through major advancements in hemodynamics and genetics, with pivotal studies by Christine and Jonathan Seidman uncovering a key role for a missense mutation in the β-myosin heavy chain gene (*MYH7*) causing familial HCM in a French Canadian family (Geisterfer-Lowrance et al., 1990). Presently, more than 1400 mutations have now been identified as being associated with a HCM phenotype, mainly in genes encoding for sarcomeric proteins (Ho et al., 2015). Numerous studies have proposed that mutation-induced changes in sarcomere function may be attributed to inefficient sarcomere contraction leading to myocardial ATP depletion in HCM (Chandra et al., 2005; Witjas-Paalberends et al., 2014). Furthermore, imaging studies have revealed that inefficient cardiac contractility may precede the development of HCM, as demonstrated by reduced myocardial external efficiency in asymptomatic mutation carriers (Timmer et al., 2011; Guclu et al., 2017). Therefore, myocardial energetics may be disturbed and potentially contribute to cardiac dysfunction in genetic/inherited HCM. However, the influence of HCM-associated mutations on myocardial energy metabolism requires further investigation as measurements of myocardial metabolic flux in humans with genetic causes of HCM is limited. On the contrary, a considerable number of studies have investigated the role of altered myocardial energy metabolism during the progression of pathological cardiac hypertrophy, and whether optimizing myocardial energy metabolism may be beneficial. Although there is much ongoing debate regarding the energy metabolism profiles that characterize the hypertrophied heart (summarized in **Table 1**), these studies may provide insight into the potential metabolic disturbances that contribute to cardiac dysfunction (primarily diastolic dysfunction) in genetic/inherited HCM.

A striking hallmark of pathological cardiac hypertrophy and subsequent heart failure is the reversion to fetal gene expression patterns, which are associated with reductions in fatty acid oxidation and a concurrent increase in glucose utilization (Sack et al., 1996; Razeghi et al., 2001; Stanley et al., 2005). Reductions in protein expression and activity of fatty acid oxidation enzymes are only observed during the decompensated heart failure stage in spontaneously hypertensive and heart failure-prone rats, though mRNA expression is already markedly reduced in these rats at the compensated hypertrophy stage (Sack et al., 1996). Moreover, in male Wistar-Kyoto rats subjected to abdominal aortic constriction (AAC) for an 8 week duration to induce compensated hypertrophy, palmitate oxidation rates are reduced during aerobic perfusion of isolated working hearts compared to sham-operated rats (Allard et al., 1994). Similarly, AAC in male C57BL/6J mice also results in a reduction in fatty acid oxidation rates during aerobic isolated working heart perfusions (Zhang et al., 2013). This result is observed at 2 weeks post-surgery in C57BL/6J mice, a time-point where normal systolic function [e.g., left ventricular ejection fraction (LVEF)] but abnormal diastolic function (e.g., reduced e′/a′ ratios) are observed, reminiscent of cardiac function profiles often seen in cases of genetic HCM (Braunwald, 2017; McKenna et al., 2017). These observations in *ex vivo* models are also observed *in vitro*, as palmitate oxidation rates are markedly reduced in neonatal rat cardiac myocytes subjected to experimental hypertrophy via treatment with the α_1-adrenergic receptor agonist, phenylephrine (100 μM) (Barger et al., 2000).

In cardiac hypertrophy, the consensus regarding elevations in glucose utilization is defined by an enhancement of glucose uptake and glycolysis, with inconsistent changes in glucose oxidation (Jaswal et al., 2011), further contributing to an altered metabolic profile recapitulating that seen in the fetal heart (Huss and Kelly, 2005). Indeed, aerobic isolated working heart perfusions performed in male Wistar-Kyoto rats subjected to AAC for an 8 week duration demonstrate significant increases in glycolysis rates with no change in glucose oxidation rates (Allard et al., 1994). Unlike the similarities previously discussed for fatty acid oxidation, studies in C57BL/6J mice subjected to AAC do not support these metabolic perturbations regarding glucose utilization (Zhang et al., 2013). Glycolysis and glucose oxidation rates were both diminished during aerobic isolated working heart perfusions in the presence of insulin concentrations (100 μU/mL) matching those used in the study in male Wistar Kyoto rats, whereas glycolysis and glucose oxidation rates were similar in the absence of insulin.

Reasons for the discrepancies in the energy metabolism profiles identified in the rat (Allard et al., 1994) and mouse (Zhang et al., 2013) studies just described remain unclear, however, they may involve species-specific differences, differences in perfusate conditions, or duration of the aortic constriction. Furthermore, while AAC in the male Wistar-Kyoto rats did produce significant cardiac hypertrophy, assessments of *in vivo* cardiac function were not performed, and thus it cannot be discerned whether both systolic and diastolic dysfunction was present in these rats. Conversely, serial assessments via ultrasound echocardiography were performed in the male C57BL/6J mice subjected to AAC. At the 2 week post-surgery time point when myocardial energy

TABLE 1 | Alterations in myocardial energy metabolism in cardiac hypertrophy.

	Experimental model	Alteration in myocardial metabolism	References
Fatty acid metabolism	Human failing heart samples and spontaneously hypertensive heart failure-prone rats	mRNA expression of fatty acid oxidation enzymes↓	Sack et al., 1996
	Human fetal, non-failing and failing adult heart samples	mRNA expression of fatty acid oxidation enzymes↓	Razeghi et al., 2001
	Male Wistar-Kyoto rats with AAC	Fatty acid oxidation↓	Allard et al., 1994
	Male C57BL/6J mice with AAC	Fatty acid oxidation↓	Zhang et al., 2013
	Male dogs with pacing-induced decompensated heart failure	Fatty acid oxidation↓	Osorio et al., 2002
	Male C57BL/6J mice with TAC	Fatty acid oxidation↓	Byrne et al., 2016
Glucose metabolism	Male Wistar-Kyoto rats with AAC	Glycolysis↑	Allard et al., 1994
	Male C57BL/6J mice with AAC	Glycolysis↓ Glucose oxidation↓	Zhang et al., 2013
	Dogs with pacing-induced decompensated heart failure	Glucose oxidation↑	Osorio et al., 2002
	Male C57BL/6J mice with TAC	Glucose oxidation↓	Byrne et al., 2016
	Male Wistar rats with AAC	Glucose uptake↑ Glycolysis↑	Tian et al., 2001; Nascimben et al., 2004
	AMPK γ2 subunit R302Q transgenic mice	Glucose uptake↑ Glycogen content↑	Folmes et al., 2009
Ketone body metabolism	Male and female C57BL/6J mice with TAC	Ketone body oxidation↑	Aubert et al., 2016; Ho et al., 2019

metabolism profiles were measured, these mice exhibited signs of diastolic dysfunction (reduced e′/a′ ratios), without systolic dysfunction (normal LVEF), consistent with cardiac function profiles observed in HCM. The degree of dysfunction is, therefore, an important aspect to consider, as the presence of systolic dysfunction contributes to a decompensated heart failure phenotype where general reductions in overall oxidative metabolism, and increases in glycolytic rates to counteract these reductions, are more likely to be observed (Osorio et al., 2002; Byrne et al., 2016).

Alterations in the transcriptional regulation of genes involved in mitochondrial oxidative metabolism may contribute to the decline in fatty acid oxidation observed in pathological cardiac hypertrophy. Myocardial mRNA expression of peroxisome proliferator-activated receptor-α (PPARα), a master regulator of fatty acid metabolism, is reduced in male Sprague-Dawley rats and C57BL/6 mice following AAC and transverse aortic constriction (TAC), respectively (Barger et al., 2000; Akki et al., 2008). This leads to reductions in the expression of numerous PPARα downstream target genes, including CPT-1 and medium chain acyl-CoA dehydrogenase. Cardiac PPARγ activity has also been linked to cardiac hypertrophy, as cardiac-specific PPARγ deficient mice exhibit a robust increase in LV size/mass (Duan et al., 2005). Conversely, cardiac-specific PPARγ overexpressing mice also demonstrate a robust cardiac hypertrophy, which is actually associated with elevations in mRNA expression of genes regulating fatty acid oxidation (e.g., CPT-1, acyl CoA oxidase) (Son et al., 2007). Reasons for these discrepancies are currently unknown, and surprisingly, deletion of PPARα alleviates the cardiac dysfunction present in cardiac-specific PPARγ overexpressing mice without impacting cardiac hypertrophy, yet actually increases myocardial fatty acid oxidation (Son et al., 2010). Reductions in myocardial carnitine content have also been observed in pathological cardiac hypertrophy in male Sprague-Dawley rats following AAC, which could contribute to decreases in fatty acid oxidation (Reibel et al., 1983).

Cardiac hypertrophy-related changes in glucose metabolism are more difficult to explain, due in part to inconsistency in the reported alterations, especially with regards to glucose oxidation. Nonetheless, despite reported differences on whether cardiac hypertrophy leads to increases, decreases, or no change in myocardial glucose oxidation rates, increases in glycolysis rates are often reported and significantly higher than glucose oxidation rates. This results in a marked uncoupling of glucose oxidation from glycolysis, thereby leading to increases in proton production and subsequent contractile inefficiency, as ATP is diverted away from supporting contractile function toward relieving intracellular acidosis (Liu et al., 2002; Jaswal et al., 2011). It has been postulated that the replenishment of Krebs Cycle intermediates via increases in anaplerosis may contribute to this uncoupling of glucose metabolism in cardiac hypertrophy (Des Rosiers et al., 2011). In male Sprague Dawley rats subjected to TAC-induced pressure overload, increases in myocardial anaplerosis are attributed to elevations of glycolytic pyruvate entering the mitochondria via malic enzyme mediated carboxylation to malate (Sorokina et al., 2007). Interestingly, cardiac hypertrophy related increases in glycolysis do not correlate with increased expression of glycolysis enzymes, however, may be due to increases in 5′AMP activated protein kinase (AMPK) activity (Tian et al., 2001; Nascimben et al., 2004), a cellular energy sensor that promotes ATP production during

low energy states (Steinberg and Kemp, 2009). Indeed, increases in myocardial AMPK activity have been observed in male Wistar rats at least 17 weeks after ascending aortic constriction, resulting in increased GLUT1 protein expression and subsequent 2-deoxyglucose uptake (Tian et al., 2001). In addition, the elevation in myocardial AMPK activity augments phosphofructokinase-2 mediated formation of fructose-2,6-bisphosphate, a potent allosteric stimulator of phosphofructokinase-1, the rate-limiting enzyme of glycolysis (Nascimben et al., 2004). It should be noted, though, that since *in vivo* cardiac function was not monitored in these rats, it is not possible to discern whether these metabolic alterations are due to potential systolic dysfunction rather than cardiac hypertrophy *per se*. Nonetheless, alterations in AMPK activity are a reasonable molecular culprit with regards to HCM, as mutations in the AMPK γ2 subunit have been shown to induce familial HCM in humans (Gollob et al., 2001; Taha and Lopaschuk, 2007; McKenna et al., 2017). One notable mutation involves a missense mutation resulting in an arginine substitution for glutamine at position 302 of the AMPK γ2 subunit (R302Q), which causes a glycogen storage HCM characterized by a familial form of Wolff-Parkinson-White syndrome. Key studies by Folmes et al. (2009) demonstrated that adenoviral-mediated overexpression of an AMPK γ2 subunit R302Q mutant increases AMPK activity, glucose uptake, glycogen synthase protein expression, and glycogen content in neonatal rat ventricular cardiac myocytes. Moreover, transgenic mice harboring a cardiac myocyte-specific AMPK γ2 subunit R302Q mutation also exhibit robust cardiac hypertrophy that is associated with massive glycogen accumulation (Folmes et al., 2009). While the described studies support a key role for increased AMPK activity and its metabolic actions in the pathology of HCM, AMPK also has contrasting anti-hypertrophic actions that need to be considered, such as inhibiting protein synthesis (Chan and Dyck, 2005).

Of interest, it has now been demonstrated in preclinical and clinical studies that ketone body oxidation is increased in both compensated hypertrophy and heart failure (Lopaschuk and Ussher, 2016; Ussher et al., 2016; Ho et al., 2019). Compensated hypertrophy in female C57BL/6J mice in response to TAC increases myocardial protein expression of BDH1, which is associated with increased ketone body oxidation as indicated by increased ^{13}C enrichment of acetyl CoA from ^{13}C-β-hydroxybutyrate (Aubert et al., 2016). The shift toward greater ketone body utilization in compensated hypertrophy and/or heart failure has been postulated to be an adaptive response to maintain oxidative metabolism in the setting of decreased fatty acid oxidation. In further support that this metabolic alteration is adaptive, mice with a cardiac-specific deficiency of SCOT exhibit blunted myocardial ketone body oxidation, which is associated with accelerated pathological ventricular remodeling and cardiac dysfunction in response to TAC-induced pressure overload (Schugar et al., 2014). However, given that cardiac-specific SCOT-deficient mouse hearts also exhibited an increase in fatty acid oxidation, it remains unclear whether the increased pathological remodeling was attributed to the increase in fatty acid oxidation or the decrease in ketone body oxidation. Furthermore, it remains to be determined whether ketone body oxidation is increased in HCM that is characterized solely by diastolic dysfunction.

Taken together, a plethora of evidence has now been documented demonstrating that cardiac hypertrophy and heart failure are characterized by pronounced metabolic alterations. Whether these perturbations in myocardial energy metabolism contribute to the pathology of HCM, or whether they are due to the cardiac hypertrophy and subsequent dysfunction itself has not been conclusively determined. As mentioned previously, various intermediates of energy metabolism can directly regulate intracellular signaling, and one must also consider whether perturbations in myocardial energy metabolism regulate cardiac hypertrophy via such signaling changes. Indeed, the accumulation of glucose-6-phosphate as a result of elevated glycolysis in cardiac hypertrophy may directly promote ventricular hypertrophy, via activation of the mammalian target of rapamycin, a key signaling mediator of cardiac myocyte protein synthesis and subsequent growth (Shioi et al., 2003; Sen et al., 2013). Despite amino acids contributing minimally to myocardial energy metabolism, it has also been demonstrated that glucose can contribute to cardiac hypertrophy by preventing Kruppel-like factor 15 regulated transcription, thereby reducing branched chain amino acid metabolism and increasing mammalian target of rapamycin activation (Shao et al., 2018). Importantly, evidence is also limited that these metabolic alterations resulting from AAC- or TAC-induced cardiac hypertrophy are present and/or contribute to the pathology of genetic/familial HCM involving mutations in sarcomeric proteins. Nonetheless, HCM due to the AMPK γ2 subunit R302Q mutation does support that this form of familial HCM shares consistency with some of the documented changes in energy metabolism in cardiac hypertrophy.

ENERGY METABOLISM IN DIABETIC CARDIOMYOPATHY

First described in diabetic patients with heart failure in the absence of myocardial ischemia, diabetic cardiomyopathy is characterized by ventricular dysfunction in the absence of underlying coronary artery disease and/or hypertension (Rubler et al., 1972). In addition, structural changes to the myocardium are present in diabetic cardiomyopathy, which include increased fibrosis and LV mass, and are often accompanied by underlying diastolic dysfunction with negligible impact on systolic function. Our understanding of diabetic cardiomyopathy has greatly improved since its original description in 1972, with numerous advancements regarding the mediators of its pathology. Some of the most extensively studied mechanisms include lipotoxicity, glucotoxicity, insulin resistance, alterations in calcium handling/signaling, fibrosis, endoplasmic reticulum stress, oxidative stress, cardiac myocyte apoptosis, and derangements in energy metabolism (Rubler et al., 1972; van de Weijer et al., 2011; Liu et al., 2012; Battiprolu et al., 2013; Ussher, 2014). With regards to the latter, myocardial energy metabolism in diabetic cardiomyopathy is substantially influenced by increased fatty acid delivery and insulin resistance, which leads to increases and decreases in fatty acid and glucose utilization, respectively. At a molecular

level, increases in cardiac myocyte lipoprotein lipase activity lead to increased fatty acid delivery (Pulinilkunnil and Rodrigues, 2006; Kim et al., 2008), whereas hyperinsulinemia-mediated increases in cardiac myocyte protein kinase Cζ activity increase sarcolemmal CD36 protein expression and subsequent fatty acid uptake (Ouwens et al., 2007; Luiken et al., 2009). As fatty acid supply in obesity/type 2 diabetes (T2D) often exceeds increases in myocardial fatty acid oxidation rates, the build-up of myocardial lipid stores (TAG) and lipid intermediates (e.g., ceramide and diacylglycerol) frequently characterizes the diabetic myocardium (Lopaschuk et al., 2007; Zlobine et al., 2016). While the accumulation of TAGs, ceramides, diacylglycerols, and other lipid intermediates may promote myocardial insulin resistance, lipotoxicity, and cardiac myocyte apoptosis in diabetic cardiomyopathy, we encourage the reader to refer to the many excellent reviews already published on this topic (van de Weijer et al., 2011; D'Souza et al., 2016; Zlobine et al., 2016). The following section will detail the perturbations in myocardial energy metabolism that characterize the hearts of individuals with T2D (summarized in **Table 2**), and how these perturbations may potentially contribute to the pathology of diabetic cardiomyopathy.

While studies in isolated working hearts from fasted obese Zucker rats have shown that fatty acid oxidation rates are impaired (Young et al., 2002), the vast majority of literature has demonstrated that obesity and/or T2D lead to marked increases in myocardial fatty acid oxidation. For example, studies in genetic models of obesity/T2D have demonstrated that fatty acid oxidation rates are increased during aerobic perfusion of isolated working hearts from either 4 or 15-week-old male *db*/*db* and *ob*/*ob* mice (Buchanan et al., 2005). Similarly, fatty acid oxidation rates are increased during aerobic isolated working heart perfusions from male C57BL/6J mice after 2 weeks of high-fat feeding (45% kcal from lard) or 3 weeks of high-fat feeding (60% kcal from lard) (Wright et al., 2009; Zhang et al., 2011). These observations also translate to models of type 1 diabetes (T1D), as aerobic isolated working heart perfusions from rodents subjected to streptozotocin mediated destruction of islet β-cells, and Akita mice harboring a genetic mutation in the insulin 2 gene, demonstrate significant increases in fatty acid oxidation rates (Sakamoto et al., 2000; How et al., 2006; Basu et al., 2009). These observations have been recapitulated in humans, as positron emission tomography (PET) imaging with [1-^{11}C]palmitate in obese women without cardiovascular disease, T1D subjects, or T2D subjects with non-ischemic cardiomyopathy, demonstrated significant increases in both myocardial fatty acid uptake and subsequent oxidation (Peterson et al., 2004; Herrero et al., 2006; Rijzewijk et al., 2009). It should be noted that many of these studies do not account for endogenous fatty acid oxidation from intracellular TAG stores, making it likely that fatty acid oxidation rates in preclinical and clinical studies of obesity/diabetes are actually being underestimated. As mentioned previously, myocardial TAG stores are increased in obesity/T2D. Furthermore, PET imaging studies with [1-^{11}C]palmitate and [^{1}H] magnetic resonance spectroscopy in obese patients without cardiovascular disease, reported that endogenous fatty acid oxidation rates are greater than exogenous plasma fatty acid oxidation rates, though a lean, healthy group for comparison was not included (Bucci et al., 2012). As such, these observations suggest that obesity/T2D increases the heart's reliance on fatty acids from all sources (exogenous and endogenous) for energy metabolism. At a molecular level, PPARα is thought to be a key mediator of these metabolic alterations, as both T1D and T2D lead to increases in myocardial PPARα expression, and cardiac-specific PPARα overexpressing mice exhibit a cardiac phenotype mimicking that seen in diabetic cardiomyopathy (Finck et al., 2002, 2003). PPARα may also potentially be a key mediator of the increased reliance on TAG-derived fatty acid oxidation in obesity/T2D, as ^{13}C nuclear magnetic resonance spectroscopy studies revealed increased TAG turnover in cardiac-specific PPARα overexpressing male mice fed a high-fat diet for 2 weeks (Banke et al., 2010). PPARα also contributes to the elevations in myocardial fatty acid uptake and subsequent lipotoxicity observed in obesity/T2D, which may be dependent on increased glycogen synthase kinase 3α (GSK3α) activity. It was demonstrated in neonatal rat cardiac myocytes that GSK3α can translocate to the nucleus and phosphorylate PPARα at serine residue 280, stimulating a biased PPARα transcriptional response that prompts increases in myocardial fatty acid uptake and lipid storage (Nakamura et al., 2019). Furthermore, mice with a cardiac-specific heterozygous GSK3α deficiency subjected to high-fat diet-induced obesity demonstrated significant improvements in diastolic function, as seen by a lower deceleration time during Tissue Doppler analysis. Conversely, increased myocardial PPARβ/δ may be protective against obesity/T2D-related cardiomyopathy, as cardiac-specific overexpression of PPARβ/δ prevents cardiac dysfunction following 8 weeks of high-fat diet supplementation (Burkart et al., 2007). This cardioprotection was attributed to PPARβ/δ increasing myocardial fatty acid oxidation but not fatty acid uptake, differing from PPARα activity, which increases both in the myocardium. Likewise, cardiac-specific deletion of PPARβ/δ in mice leads to a robust reduction in myocardial fatty acid oxidation, which results in a lipotoxic cardiomyopathy that leads to early mortality due to heart failure (Cheng et al., 2004).

With regards to impaired myocardial glucose utilization in obesity/T2D, cardiac myocytes isolated from adult *ob*/*ob* and *db*/*db* mice exhibit reduced insulin-stimulated Akt phosphorylation and 2-deoxyglucose uptake (Mazumder et al., 2004; Hafstad et al., 2006). Moreover, PET imaging with [^{18}F]fluorodeoxyglucose demonstrated marked reductions in myocardial glucose uptake during a euglycemic-hyperinsulinemic clamp in male Wistar rats fed a high-fat and high-fructose diet for 6 weeks (Menard et al., 2010). Similarly, PET imaging revealed decreased [^{18}F]fluorodeoxyglucose uptake in 12 h fasted male C57BL/6J mice fed a high-fat diet for at least 25 weeks (Battiprolu et al., 2012). On the contrary, other studies have suggested that myocardial insulin sensitivity remains intact in T2D, especially in the context of hyperinsulinemia and when plasma free fatty acid levels are matched (Lopaschuk et al., 2010). For example, 3 months of high-fat and high-cholesterol diet supplementation in male low-density lipoprotein receptor-deficient mice results in

TABLE 2 | Alterations in myocardial energy metabolism in diabetic cardiomyopathy.

	Experimental model	Alteration in myocardial metabolism	References
Fatty acid metabolism	Male Obese Zucker rats	Fatty acid oxidation↓	Young et al., 2002
	Male *db*/*db* and *ob*/*ob* mice	Fatty acid oxidation↑	Mazumder et al., 2004; Buchanan et al., 2005; Hafstad et al., 2006; How et al., 2006
	Male C57BL/6J mice fed a high-fat diet	Fatty acid oxidation↑	Wright et al., 2009; Zhang et al., 2011
	Obese women, men and women with T1D, or men with T2D and non-ischemic cardiomyopathy	Fatty acid uptake↑ Fatty acid oxidation↑	Peterson et al., 2004; Herrero et al., 2006; Rijzewijk et al., 2009
Glucose metabolism	Male *db*/*db* and *ob*/*ob* mice	Glucose uptake↓ Glucose oxidation↓	Mazumder et al., 2004; Buchanan et al., 2005; Hafstad et al., 2006; How et al., 2006
	Male Wistar rats fed a high-fat and high-fructose diet, or male C57BL/6J mice fed a high-fat diet	Glucose uptake↓	Menard et al., 2010; Battiprolu et al., 2012
	Male C57BL/6J mice fed a high-fat diet	Glucose oxidation↓	Ussher et al., 2009; Wright et al., 2009
	Male Wistar rats or male C57BL/6J mice subjected to experimental T2D (high-fat diet + low-dose streptozotocin	Glucose oxidation↓	Le Page et al., 2015; Almutairi et al., 2020
Ketone body metabolism	Men and women with T2D undergoing cardiac catheterization	Ketone body uptake↑	Mizuno et al., 2017
	Male non-obese diabetic Goto-Kakizaki rats	Ketone body oxidation↑	Abdurrachim et al., 2019

hyperinsulinemia and increased [^{18}F]fluorodeoxyglucose uptake after a 4 h fast compared to standard chow fed lean mice (Gupte et al., 2013). Despite these divergent findings regarding myocardial glucose uptake in T2D, the marked impairment of myocardial glucose oxidation in the diabetic heart has been demonstrated in numerous studies. Aerobic perfusion of isolated working hearts from *ob*/*ob* mice, *db*/*db* mice, mice subjected to experimental obesity via high-fat diet supplementation, or mice and rats subjected to experimental T2D via high-fat diet supplementation plus low-dose streptozotocin administration, demonstrate impaired basal and insulin-stimulated glucose oxidation rates (Buchanan et al., 2005; Hafstad et al., 2006; How et al., 2006; Ussher et al., 2009; Wright et al., 2009; Le Page et al., 2015). The impaired glucose oxidation in the heart in the setting of obesity/T2D is partially attributed to the previously discussed "Randle Cycle" phenomenon, whereby elevated myocardial fatty acid oxidation rates decrease glucose oxidation via substrate competition (Lopaschuk et al., 2010). Reductions in MCU activity may also be involved, as C57BL/6NHsd mice subjected to experimental T1D via 5 daily injections with streptozotocin (40 mg/kg) exhibit decreased myocardial MCU protein expression and mitochondrial calcium levels in intact-paced contracting cardiac myocytes (Suarez et al., 2018). These changes are associated with reduced myocardial PDH activity and glucose oxidation rates during isolated working heart perfusions, but are completely normalized in mice with T1D administered an adeno-associated virus expressing murine MCU mRNA via the jugular vein. In addition, molecular control once again may involve increased PPARα activity, as PDHK4 mRNA expression, a transcriptional target of PPARα and the most prominent PDHK isoform in the heart, is increased in cardiac-specific PPARα overexpressing mice (Wu et al., 2001; Finck et al., 2002). On the contrary, in the setting of obesity/T2D, increased myocardial forkhead box O1 (FoxO1) may contribute to increased PDHK4-mediated inhibition of PDH activity and subsequent glucose oxidation. Indeed, PDHK4 is also a transcriptional target of FoxO1, and pharmacological FoxO1 antagonism with the agent AS1842856 increases glucose oxidation rates in aerobically perfused isolated working hearts from male C57BL/6J mice (Gopal et al., 2017). Furthermore, cardiac-specific FoxO1 deficiency prevents chronic high-fat diet supplementation mediated increases in myocardial PDHK4 mRNA expression (Battiprolu et al., 2012). The marked reduction in myocardial glucose oxidation is thought to directly contribute to the pathology of diabetic cardiomyopathy via decreasing cardiac efficiency, whereby ATP utilization for non-contractile purposes (e.g., ionic homeostasis) is increased (Lopaschuk et al., 2007; Almutairi et al., 2020).

Although our knowledge of myocardial ketone body oxidation has grown considerably with regards to its perturbations in cardiac hypertrophy and heart failure, this is an area of limited study in the diabetic myocardium. Nonetheless, it is imperative we increase our understanding of myocardial ketone body oxidation, as it has been suggested that the marked improvement in cardiovascular outcomes in people with T2D taking sodium-glucose cotransporter 2 (SGLT2) inhibitors (e.g., empagliflozin) may be due to increased ketosis and subsequent ketone body utilization (Ferrannini et al., 2016; Lopaschuk and Verma, 2016). Studies to date suggest that ketone body utilization may be increased in T2D, as catheterization studies for blood sampling from the coronary sinus and aortic root in subjects with T2D and mild diastolic dysfunction revealed increased myocardial uptake of acetoacetate and β-hydroxybutyrate (Mizuno et al., 2017).

Furthermore, a recent study in non-obese diabetic male Goto-Kakizaki rats using hyperpolarized [3-^{13}C]acetoacetate demonstrated increased myocardial ketone body utilization, as well as increases in myocardial SCOT activity (Abdurrachim et al., 2019). However, as these rats also demonstrated cardiac hypertrophy and systolic dysfunction, whether their metabolic perturbations are due to diabetes or systolic dysfunction cannot be discerned. Of interest, the increase in myocardial SCOT activity observed in diabetic male Goto-Kakizaki rats is also seen in skeletal muscles from C57BL/6J mice after 10 weeks of high-fat diet (60% kcal from lard) supplementation (Al Batran et al., 2020), suggesting that obesity/T2D may cause an increase in cardiac and skeletal muscle ketone body oxidation. While increased myocardial ketone body oxidation is a postulated mechanism by which SGLT2 inhibitors improve cardiovascular outcomes in T2D, careful consideration should be taken with regards to systemically boosting ketone body oxidation in humans with T2D, as increases in skeletal muscle ketone body oxidation appear to promote hyperglycemia (Al Batran et al., 2020).

ENERGY METABOLISM IN BARTH SYNDROME

Despite a decreased prevalence with respect to the adult population, pediatric cardiomyopathies and heart failure are associated with a significant mortality rate and cost of care burden (Woulfe and Bruns, 2019). Many inherited cardiomyopathies affecting children involve inborn errors of metabolism that cause derangements in myocardial fatty acid oxidation, or mutations in mitochondrial DNA that impair normal oxidative phosphorylation, both of which have been reviewed extensively (Kelly and Strauss, 1994; Lee et al., 2017; Towbin and Jefferies, 2017). In this particular section, we will focus on pediatric cardiomyopathy associated with the rare genetic disorder, BTHS, which is caused by mutations in the *TAZ* gene, encoding for tafazzin, a mitochondrial transacylase critical for catalyzing the final step in cardiolipin remodeling (Jefferies, 2013; Dudek and Maack, 2017). Cardiolipin is a structurally distinct mitochondrial phospholipid with essential roles in the maintenance of mitochondrial morphology, regulation of mitochondrial protein transport and dynamics, and in maintaining the integrity and optimal activity of the ETC (Dudek and Maack, 2017; Dudek et al., 2019). Furthermore, aberrant myocardial cardiolipin content and composition, specifically reduction of the predominant tetralinoleoyl cardiolipin species, has been reported in a variety of cardiac pathologies, including idiopathic dilated cardiomyopathy (Chatfield et al., 2014), and in both human and experimental models of heart failure (Sparagna et al., 2007). As such, infantile-onset cardiomyopathy is the most common clinical feature associated with BTHS, although other phenotypic traits can include neutropenia, skeletal myopathy, exercise intolerance, 3-methylglutaconic aciduria, and pre-pubertal growth retardation (Jefferies, 2013). The cardiomyopathy associated with BTHS is primarily dilated cardiomyopathy, though HCM has also been documented, and may co-present with LV non-compaction or endocardial fibroelastosis (Spencer et al., 2006; Jefferies, 2013). In addition, the lack of a genotype-phenotype correlation suggests that physiological modifiers may exacerbate the tafazzin deficiency and contribute to the variability in clinical phenotypes of BTHS. However, due to cardiolipin's key role in maintaining the ETC, perturbations in myocardial energy metabolism may also contribute to BTHS associated cardiomyopathies, which will now be highlighted in the following section (summarized in **Table 3**).

In vitro studies have demonstrated that siRNA mediated *Taz* knockdown in neonatal rat ventricular cardiac myocytes decreases ATP levels, which was associated with increases in AMPK activity, cell surface area, and increased mRNA expression of brain natriuretic peptide, a biomarker of cardiac hypertrophy/heart failure (He, 2010). Decreases in ATP content are consistent with an impairment of oxidative phosphorylation, and cardiac mitochondria isolated from a mouse model of BTHS due to doxycycline mediated *Taz* knockdown (herein referred to as TazKD mice), display markedly reduced respiration, primarily attributed to impaired complex III activity (Kiebish et al., 2013; Powers et al., 2013). In young adults with BTHS (mean age of 26 years), a decreased myocardial energy reserve has also been observed, as indicated by a reduced phosphocreatine/ATP ratio during ^{31}P magnetic resonance spectroscopy studies (Cade et al., 2019).

Despite well-characterized myocardial energy deficiency in BTHS, the actual alterations in myocardial intermediary energy metabolism are still being delineated. Paralleling the setting of heart failure, preclinical and clinical studies suggest that BTHS is associated with reductions in myocardial fatty acid oxidation. Isolated cardiac mitochondria from 3-month-old male TazKD mice subjected to proteomics analysis demonstrated disruption of ETC supercomplexes with the fatty acid oxidation enzymes, very long-chain acyl CoA dehydrogenase and long-chain 3-hydroxyacyl CoA dehydrogenase (Huang et al., 2015). Likewise, cardiac mitochondria isolated from 2-month-old male TazKD mice demonstrated a 25% reduction in palmitoyl-L-carnitine supported fatty acid oxidation during state 3 respiration, as determined using high-resolution respirometry (Kiebish et al., 2013). Oxygen consumption rates using a Seahorse XF24 analyzer were also reduced in neonatal cardiac myocytes isolated from TazKD mice, once more suggesting that BTHS is associated with impaired myocardial fatty acid oxidation (Powers et al., 2013). Illustrating the translatability of these findings, young adults with BTHS (mean age of 26 years) exhibited reductions in myocardial fatty acid extraction as determined by PET imaging (Cade et al., 2019).

With regards to BTHS associated changes in myocardial glucose metabolism, tafazzin deficiency mediated reductions in tetralinoleoyl cardiolipin formation may influence glucose metabolism through the regulation of PDH activity. Studies in C2C12 myoblasts subjected to CRISPR/Cas9 mediated *Taz* knockout demonstrated decreased incorporation of [U-^{13}C]glucose into acetyl CoA, which was associated with an ∼50% inhibition of PDH activity (Li et al., 2019). Of interest, incubation of *Taz* knockout C2C12 myoblast mitochondria

TABLE 3 | Alterations in myocardial energy metabolism in BTHS.

	Experimental model	Alteration in myocardial metabolism	References
Fatty acid metabolism	Isolated cardiac mitochondria from male TazKD mice	Fatty acid oxidation↓ Fatty acid oxidation enzyme – ETC supercomplex interaction↓	Kiebish et al., 2013; Huang et al., 2015
	Neonatal cardiac myocytes from TazKD mice	Fatty acid oxidation↓	Powers et al., 2013
	Young adult males with BTHS	Fatty acid extraction↓	Cade et al., 2019
Glucose metabolism	Young adult males with BTHS	Glucose uptake↑ Glucose utilization↑	Cade et al., 2019
	Neonatal cardiac myocytes from TazKD mice	Glycolysis↑	Powers et al., 2013
Ketone body metabolism	Males (age range 6 months–32 years) with BTHS	Circulating β-hydroxybutyrate levels↑	Sandlers et al., 2016

with exogenous cardiolipin restored this defect, providing mechanistic support for cardiolipin having a direct regulatory role on PDH activity. While C2C12 myoblasts originate from mouse skeletal muscle, high-resolution respirometry in cardiac mitochondria isolated from 2-month-old TazKD mice revealed no impairment in pyruvate supported state 3 respiration (Kiebish et al., 2013). Likewise, significant increases in myocardial glucose extraction fraction, uptake, and utilization were observed in young adults with BTHS (mean age of 26 years) compared to healthy age-matched controls (Cade et al., 2019). While these contrasting findings in humans suggest that BTHS may be associated with increased myocardial glucose oxidation, it is important to note that glucose oxidation was not directly assessed in the aforementioned study. It remains possible that the increase in myocardial glucose uptake and extraction reflects increases in myocardial glycolysis rates that are not coupled to a proportional increase in glucose oxidation. Evidence supporting this paradigm has been observed in neonatal cardiac myocytes isolated from TazKD mice, whereby an increased rate of extracellular acidification (indicative of increased glycolysis) was reported using a Seahorse XF24 analyzer (Powers et al., 2013). Furthermore, circulating lactate levels were increased in ∼4–5-month-old male TazKD mice in response to exercise, supporting that the increase in glucose utilization was not matched by a corresponding increase in glucose oxidation (Powers et al., 2013). Similar to what we have described in HCM and diabetic cardiomyopathy, the uncoupling of glucose oxidation from glycolysis may precipitate contractile inefficiency and contribute to the pathology of BTHS-related cardiomyopathy, as ATP is diverted toward supporting non-contractile purposes.

Regarding ketone metabolism, there has been limited investigation regarding potential alterations in myocardial ketone body utilization in BTHS. However, metabolomics in plasma collected from 23 subjects with BTHS demonstrated increases in circulating β-hydroxybutyrate levels compared to plasma from 15 age-matched control subjects not known to have an inborn error of metabolism (Sandlers et al., 2016). These findings are compatible with other preclinical and clinical studies that have noted a metabolic shift toward increased myocardial ketone body utilization in both cardiac hypertrophy and heart failure (Aubert et al., 2016; BediJr., Snyder et al., 2016; Ho et al., 2019).

Although the metabolic profile characterizing the heart in BTHS remains the subject of ongoing study, it is evident that perturbations in intermediary energy metabolism are present, and it will be important to confirm whether these metabolic perturbations contribute to the pathology of BTHS-related cardiomyopathy. As a rare genetic disease, human BTHS studies of myocardial metabolism are limited in nature, and the furthering of our understanding of energy metabolism as a pathological mediator in this disorder will likely come from studies in the TazKD murine mouse model of BTHS (Acehan et al., 2011). However, cardiac phenotypes reported in TazKD mice are not consistent in the literature, with some studies demonstrating the more prevalent dilated cardiomyopathy seen in BTHS subjects (Acehan et al., 2011), whereas others have reported a HCM that is less frequently observed in BTHS subjects (Johnson et al., 2018). As such, it will be important for future studies to carefully dissect the specific cardiac phenotypes present in TazKD mice, and how they may relate to potential contrasting findings relating to perturbed myocardial energy metabolism.

ENERGY METABOLISM IN ARRHYTHMOGENIC CARDIOMYOPATHY

Arrhythmogenic cardiomyopathy is another form of inherited cardiomyopathy that is genetically heterogenous, typically caused by genetic mutations producing abnormalities in cardiac desmosomes, and it is a major risk factor for sudden cardiac death. The reader is encouraged to refer to (Corrado et al., 2017) for extensive review of the various mutations that can predispose to arrhythmogenic cardiomyopathy. Depending on geographic region the overall prevalence of arrhythmogenic cardiomyopathy can range from 1:1000 to 1:5000, though most specialists in this area are of the opinion that the real prevalence is closer to the latter (Corrado et al., 2017). Although

originally referred to as arrhythmogenic right ventricular dysplasia or arrhythmogenic right ventricular cardiomyopathy, increasing reports characterized by early and greater LV involvement have resulted in use of the broader arrhythmogenic cardiomyopathy term (Norman et al., 2005). Arrhythmogenic cardiomyopathy is characterized by ventricular arrhythmias, systolic dysfunction, and replacement of myocardium with fibrofatty tissue (Corrado et al., 2017). Few studies to date have performed any meaningful assessment of myocardial energy metabolism in arrhythmogenic cardiomyopathy and other heart rhythm disorders such as atrial fibrillation or ventricular tachycardia, though there are reports alluding to perturbations in energy metabolism.

In a rat model of stress-induced cardiac damage and arrhythmias, administration of an overdose of isoproterenol (67 mg/kg) to male Wistar rats produces a mild systolic dysfunction and notable diastolic dysfunction in a hypercontractile state 2 weeks following isoproterenol treatment (Willis et al., 2015). Moreover, isolated mitochondria from the hearts of these rats at the same time point demonstrated significant reductions in both NADH and succinate linked respiration, whereas monitoring of aconitase activity demonstrated increases in oxidative stress. In 12 to 16-week old male B6.129S mice fed a high saturated fat diet (60% kcal from palm oil) for 4 weeks that does not induce significant weight gain (∼3–4 grams) or cardiac dysfunction, *in vivo* telemetry revealed ventricular ectopy and a prolonged QT period due to an increase in NADPH oxidase 2 mediated oxidative stress (Joseph et al., 2019). Of interest, these observations were absent in mice with a whole-body deficiency of NADPH oxidase 2. Metabolic perturbations may also contribute to atrial fibrillation, as atrial expression of fatty acid binding protein 3, a key regulator of cellular fatty acid uptake and subsequent transport, is elevated in right atrial tissue biopsies from Japanese individuals undergoing heart surgery with established atrial fibrillation versus those with normal sinus rhythm (Shingu et al., 2020). Last, the cardiac ryanodine receptor (RYR2) plays a key role in excitation-contraction coupling, whereby sarcoplasmic reticulum release of calcium through the RYR2 allows for calcium to interact with cytosolic contractile proteins, and both gain and loss of function RYR2 mutations can promote arrhythmias and sudden cardiac death (Gomez and Richard, 2004). Studies in mice with a 50% reduction in cardiac RYR2 protein expression demonstrate significant reductions in heart rate assessed via *in vivo* radiotelemetry, which is associated with reductions in glucose oxidation rates assessed via isolated working heart perfusions (Bround et al., 2016).

While the above described studies lend credence to the notion that perturbations in energy metabolism may contribute to heart rhythm disturbances that are often present in people with arrhythmogenic cardiomyopathy, it should be noted that none of these studies actually assessed metabolic flux *per se*. Furthermore, whether these metabolic perturbations are causally related to heart rhythm disturbances or simply the result of arrhythmias remains to be determined, and it is also currently unknown whether such observations would translate to the genetic mutations that predispose to inherited arrhythmogenic cardiomyopathy.

ENERGY METABOLISM AS A TARGET TO ALLEVIATE CARDIOMYOPATHY

As previously discussed, numerous studies have demonstrated that during the transition from compensated hypertrophy to decompensated heart failure, myocardial fatty acid oxidation rates decline as the heart adopts a phenotype mimicking fetal metabolism. Hence, the preservation of fatty acid oxidation has been investigated as a potential therapeutic strategy to reverse maladaptive metabolic remodeling and preserve cardiac function in the setting of pathological HCM. Cardiac-specific deletion of acetyl CoA carboxylase 2 in mice, which increases myocardial fatty acid oxidation via preventing the synthesis of malonyl CoA, is associated with reductions in cardiac hypertrophy and fibrosis, as well as improved *ex vivo* and *in vivo* cardiac function at 8 weeks post-TAC (KolwiczJr., Olson et al., 2012). However, as a genetic model where myocardial fatty acid oxidation rates are chronically elevated, it remains uncertain whether stimulating myocardial fatty acid oxidation specifically at the compensated hypertrophy stage, and prior to systolic dysfunction, would yield a similar result. Although there is inconsistency regarding myocardial glucose oxidation alterations in cardiac hypertrophy, treatment of isolated working hearts from male Sprague-Dawley rats after 10 weeks of AAC with dichloroacetate (DCA, 2 mM), a PDHK inhibitor, improves *ex vivo* cardiac function as indicated by increased LV pressures (Pound et al., 2009). In addition, infusion of angiotensin II (1.5 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$), a potent vasoconstrictor and mediator of cardiac injury, for 14 days via implanted micro osmotic pumps in male C57BL/6J mice induces cardiac hypertrophy and diastolic dysfunction (Mori et al., 2012). Moreover, isolated working hearts from these mice demonstrated significant reductions in glucose oxidation rates during aerobic perfusion, concomitant with a reduction in PDH activity. Interestingly, treatment with irbesartan (50 $mg{\cdot}kg^{-1}{\cdot}day^{-1}$), an angiotensin II type 1 receptor antagonist, prevents angiotensin II infusion-induced cardiac hypertrophy and diastolic dysfunction, which is associated with increased myocardial PDH activity and subsequent glucose oxidation rates. It should be noted, though, that it remains unclear whether the increase in myocardial glucose oxidation rates is mechanistically required for how irbesartan attenuates diastolic dysfunction in response to angiotensin II infusion. Myocardial anaplerosis can also be targeted to attenuate cardiac dysfunction in response to TAC-induced pressure overload in male Sprague Dawley rats, as adenoviral delivery of a microRNA specific to malic enzyme improved contractile function during isolated Langendorff perfusions at 12 weeks post-TAC (Lahey et al., 2018). Taken together, it does appear that modifying myocardial energy metabolism can mitigate cardiac dysfunction in experimental models of cardiac hypertrophy. Nevertheless, due to inconsistencies in the models utilized, as well as whether systolic or diastolic dysfunction predominates, or whether both are present, makes it challenging to discern whether targeting

myocardial energy metabolism can mitigate the diastolic dysfunction often observed in genetic/inherited HCM.

With regards to diabetic cardiomyopathy, numerous preclinical studies have demonstrated that correcting either elevated myocardial fatty acid oxidation rates or impaired myocardial glucose oxidation rates, yields salutary actions on cardiac function. For example, treatment for 3 weeks with the antianginal agent, trimetazidine (15 mg/kg once daily), which decreases fatty acid oxidation by inhibiting 3-ketoacyl-CoA thiolase, prevented diastolic dysfunction in 26-week-old male C57BL/6J mice fed a high-fat diet for 13 weeks (Ussher et al., 2014). These findings may translate to humans with T2D, as trimetazidine treatment for 6 months (20 mg 3x daily) in subjects with T2D and idiopathic dilated cardiomyopathy ameliorated both systolic and diastolic dysfunction (Zhao et al., 2013). Moreover, mice with a whole-body deficiency of malonyl CoA decarboxylase demonstrated reductions in fatty acid oxidation due to elevated malonyl CoA levels, while also displaying marked improvements in cardiac efficiency following high-fat diet-induced obesity (Ussher et al., 2009). As the reduction in myocardial fatty acid oxidation in response to treatment with trimetazidine, or due to malonyl CoA decarboxylase deficiency, is accompanied by corresponding increases in myocardial glucose oxidation, directly stimulating glucose oxidation in the heart also yields salutary actions in diabetic cardiomyopathy. In particular, supplementation of the drinking water for 4 weeks with DCA (1 mM), augmented myocardial PDH activity and glucose oxidation rates in male Wistar rats subjected to experimental T2D via high-fat diet supplementation and low-dose streptozotocin (25 mg/kg) administration (Le Page et al., 2015). Importantly, diastolic dysfunction was also ameliorated in these rats following treatment with DCA. Similarly, liraglutide, a T2D therapy that improves cardiovascular outcomes (Al Batran et al., 2018), also increases glucose oxidation rates in male C57BL/6J mice subjected to experimental T2D via high-fat diet supplementation and low-dose streptozotocin (75 mg/kg) administration, which was once again accompanied by an alleviation of diastolic dysfunction (Almutairi et al., 2020). On the contrary, increasing myocardial fatty acid oxidation via cardiac-specific deletion of acetyl CoA carboxylase 2 alleviates both systolic and diastolic dysfunction in a mouse model of obesity-induced cardiomyopathy following 24 weeks of supplementation with a high-fat diet (Shao et al., 2020). As mentioned previously, it has been suggested that SGLT2 inhibitors (e.g., empagliflozin) may improve cardiovascular outcomes in people with T2D via increasing myocardial ketone body oxidation rates (Lopaschuk and Verma, 2016). While cardiovascular outcomes studies with SGLT2 inhibitors have not assessed diabetic cardiomyopathy *per se*, preclinical studies have demonstrated that the SGLT2 inhibitor, empagliflozin, can attenuate diastolic dysfunction in obesity-induced cardiomyopathy (Sun et al., 2020). Nonetheless, whether myocardial ketone body oxidation rates were increased was not assessed, and treatment of genetically obese *db*/*db* mice with empagliflozin did not increase ketone body oxidation rates assessed during aerobic isolated working heart perfusions (Verma et al., 2018). Therefore, future studies are necessary to further elucidate whether modulating myocardial ketone body oxidation can meaningfully impact the pathology of diabetic cardiomyopathy, and whether this may represent a potential cardioprotective mechanism of SGLT2 inhibitors.

Although there are no curative therapies for BTHS, BTHS-related cardiomyopathy is primarily managed using standard heart failure pharmacotherapy. However, in light of the documented myocardial metabolic perturbations in BTHS previously discussed, pharmacological optimization of myocardial energetics may represent a promising therapeutic approach to attenuate this form of inherited cardiomyopathy. To date, few studies have examined whether correcting defects in myocardial energy metabolism can reduce the risk for cardiomyopathy in people with BTHS. Intriguingly, treatment of 3-month-old TazKD mice with the pan-PPAR agonist (primarily PPARα), bezafibrate, via supplementation in the diet (0.05% weight/weight) for 4 months, prevented the development of dilated cardiomyopathy and systolic dysfunction (Schafer et al., 2018). Despite myocardial energy metabolism not being directly assessed, gene-ontology analysis revealed that bezafibrate treatment resulted in increased expression of genes involved in multiple intermediary energy metabolism pathways, and the actions of PPAR agonists to increase fatty acid oxidation are well documented (Lopaschuk et al., 2010). Nonetheless, Aasum et al. (2008) have shown that systemic administration of fibrates decreases myocardial fatty oxidation rates due to an elevation of hepatic fatty acid oxidation, which lowers circulating free fatty acids and TAGs, thereby decreasing fatty acid delivery to the heart. This decrease precipitated a corresponding increase in myocardial glucose oxidation and improvement in cardiac efficiency, suggesting that the potential benefit seen with bezafibrate therapy in TazKD mice may involve a similar mechanism. Furthermore, a mitochondrial targeted antioxidant that binds selectively to and stabilizes cardiolipin, elamipretide, is being investigated as a novel therapy for heart failure (Birk et al., 2013). While studies in a swine model of obesity and *ex vivo* human failing heart samples have demonstrated that elamipretide can improve mitochondrial function and integrity, it would be of interest to assess its potential utility for correcting cardiolipin defects in BTHS, and whether that is associated with a normalization of myocardial energy metabolism. Overall, future studies are still required to further delineate the specific metabolic perturbations that characterize the myocardium in BTHS, in order to better assess whether therapeutic interventions aimed at modulating myocardial energy metabolism may indeed be beneficial.

FINAL SUMMARY

Both ischemic and non-ischemic dilated cardiomyopathies often lead to overt heart failure and are characterized by perturbations in myocardial energy metabolism that may represent modifiable targets for reversing disease pathology (Taha and Lopaschuk, 2007; Jaswal et al., 2011; Heusch et al., 2014; Gibb and Hill, 2018). As our understanding of other forms of cardiomyopathies (e.g., HCM, diabetic cardiomyopathy, BTHS-related cardiomyopathy, arrhythmogenic cardiomyopathy) continues to grow, it does

appear that perturbations in myocardial energy metabolism may actually represent a shared feature of cardiomyopathy in general. Before optimization of myocardial energy metabolism should even be considered as a possible therapeutic approach to improve cardiac function in genetic/inherited HCM, it will be important to develop novel mouse models harboring the same genetic mutations responsible for the numerous inherited HCMs identified to date. Indeed, BTHS represents a form of genetic/inherited cardiomyopathy where substantial advancements in defining myocardial energy metabolism defects have been made, due to the generation of the TazKD mouse model. Likewise, as non-invasive *in vivo* imaging techniques improve for quantifying myocardial energy metabolism in humans (e.g., hyperpolarized ^{13}C magnetic resonance imaging to assess glucose oxidation), future opportunities to advance our understanding of myocardial energy metabolism in genetic/inherited HCM will likely follow. These same issues also apply to and should be considered in arrhythmogenic cardiomyopathy. If both avenues of pursuit indicate metabolic perturbations that parallel those observed in cardiac hypertrophy or cardiac arrhythmias, then identifying novel ways to feasibly reverse these defects in myocardial energy metabolism with pharmacotherapies should be explored. Finally, the perturbations in myocardial energy metabolism characterizing diabetic cardiomyopathy have been extensively studied, and appear to be a promising approach for improving cardiac function in people with T2D. Therefore, as we continue to further understand the metabolic perturbations present in various cardiomyopathies, while delineating the molecular mechanisms responsible, it should pave the way for unique targets that can be modified using a personalized medicine approach to treat human cardiomyopathy.

AUTHOR CONTRIBUTIONS

All authors researched literature, drafted and wrote the review article, and approved the submitted version.

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REVIEW
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Circadian Mechanisms: Cardiac Ion Channel Remodeling and Arrhythmias

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Circadian rhythms are involved in many physiological and pathological processes in different tissues, including the heart. Circadian rhythms play a critical role in adverse cardiac function with implications for heart failure and sudden cardiac death, highlighting a significant contribution of circadian mechanisms to normal sinus rhythm in health and disease. Cardiac arrhythmias are a leading cause of morbidity and mortality in patients with heart failure and likely cause ∼250,000 deaths annually in the United States alone; however, the molecular mechanisms are poorly understood. This suggests the need to improve our current understanding of the underlying molecular mechanisms that increase vulnerability to arrhythmias. Obesity and its associated pathologies, including diabetes, have emerged as dangerous disease conditions that predispose to adverse cardiac electrical remodeling leading to fatal arrhythmias. The increasing epidemic of obesity and diabetes suggests vulnerability to arrhythmias will remain high in patients. An important objective would be to identify novel and unappreciated cellular mechanisms or signaling pathways that modulate obesity and/or diabetes. In this review we discuss circadian rhythms control of metabolic and environmental cues, cardiac ion channels, and mechanisms that predispose to supraventricular and ventricular arrhythmias including hormonal signaling and the autonomic nervous system, and how understanding their functional interplay may help to inform the development and optimization of effective clinical and therapeutic interventions with implications for chronotherapy.

Keywords: circadian rhythm, metabolic disorders, autonomic regulation, ion channel remodeling, long QT syndrome, atrial fibrillation

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Abbreviations: AF, atrial fibrillation; ANS, autonomic nervous system; AP, action potential; APD, action potential duration; BP, blood pressure; CVD, cardiovascular disease; EAD, early afterdepolarization; ECG, electrocardiogram; ERG, ether-A-go-go-related gene; HR, heart rate; hiPSC-CM, human induced pluripotent stem cell-derived cardiomyocyte; $I_{Ca,L}$, L-type calcium current; I_f, funny current; $I_{K,ACh}$, acetylcholine-activated potassium current; I_{Kr}, rapid delayed rectifier potassium current; I_{Ks}, slow delayed rectifier potassium current; I_{Kur}, ultra-rapid delayed rectifier potassium current; I_{Na}, sodium current; I_{to}, transient outward potassium current; LQTS, long QT syndrome; SAN, sinus atrial node; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; SCD, sudden cardiac death; SCN, suprachiasmatic nuclei; TRF, time-restricted feeding; VF, ventricular fibrillation; VT, ventricular tachycardia.

INTRODUCTION

The circadian rhythm is an oscillatory physiological process that occurs within a 24-h period. This rhythmic behavior is evolutionarily conserved and has an critical role in the ability of organisms to modulate endogenous cellular and molecular activities in response to biological cues involving day/night and sleep/wake variations (Andreani et al., 2015). Adverse modulation of circadian rhythms predisposes to sleep disorders and increases risk of cardiovascular diseases and metabolic disorders with significant implications for the quality of life and longevity of patients (Bhupathiraju and Hu, 2016).

The circadian system is composed of a central clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus, consisting of over 20,000 neurons, and peripheral clocks that are present in virtually all tissues. The central clock is synchronized with the environment through external cues, particularly by light, and can entrain peripheral clocks *via* neuronal and humoral factors (Buhr and Takahashi, 2013), such as autonomic tone and glucocorticoid signaling. The rhythm of peripheral clocks can also be regulated by external stimuli, that includes light, food, temperature, physical activity, and sleep. The significance of these distinct regulatory pathways has been extensively discussed in the literature (Xie et al., 2019), and therefore not fully considered in this review. The temporal patterns of food intake have also been identified as a crucial factor that sets the timing (phase) of peripheral clocks (Damiola et al., 2000). Furthermore, the phase of central and peripheral clocks is controlled and/or regulated by different physiological cues, suggesting these phase differences can lead to pathological disease mechanisms that underlie vulnerability to heart failure or cardiovascular diseases.

The molecular machinery of the central and peripheral clocks can be defined by transcriptional/translational feedback loops consisting of the two core transcriptional factors, *CLOCK* and *BMAL1*. These transcription factors have been shown to bind to the enhancer boxes (Ebox) in the promoter region of the negative regulators *PERIOD* (*PER*) and *CRYPTOCHROME* (*CRY*; Gekakis et al., 1998). The PER and CRY proteins accumulate in the cytoplasm, which is then followed by their translocation to the nucleus, where they form a dimer complex which, in turn, suppresses the innate transcriptional activity of *CLOCK* and *BMAL1*, resulting in an oscillatory negative feedback loop mechanism (Buhr and Takahashi, 2013). This core loop is interconnected with a second loop of nuclear receptors, a transcriptional activator ROR (A/B) and a transcriptional repressor REV-ERB (A/B), both of which are activated by the heterodimer CLOCK-BMAL1, that compete for responsive elements in the regulatory sequences of the core clock genes to modulate their transcriptional activities including *BMAL1* (Buhr and Takahashi, 2013). More specifically, ROR (A/B) stimulates *BMAL1* transcription while REV-ERB (A/B) inhibits it (Guillaumond et al., 2005).

Furthermore, circadian oscillations can also modulate cellular posttranslational processes (Green, 2018), through targeted protein phosphorylation, ubiquitination (Robles et al., 2017), redox and metabolic modulatory pathways (Wang et al., 2012). Approximately 10–40% of the genes expressed in specific tissues follow a circadian pattern and these intrinsic clocks are important for the maintenance of tissue and cellular homeostatic control (Panda et al., 2002; Zhang et al., 2014). A peripheral clock is also known to be present in the heart, where it plays a pivotal role in regulating cardiac electrical excitability, metabolism, and the biophysical properties of major cardiac ionic channels (Bray and Young, 2008; Black et al., 2019). This further highlights a critical role for circadian rhythms in the modulation of cellular mechanisms that contribute to cardiac function in health and disease (**Figure 1**). In this review we discuss recent studies on circadian rhythms and the pathophysiology of cardiac ion channels. We further discuss the contribution of circadian rhythms in disease states that lead to altered cardiac electrical remodeling with implications for cardiac arrhythmias and cardiovascular disorders in general.

CIRCADIAN MODULATION OF THE AUTONOMIC NERVOUS SYSTEM AND ION CHANNEL REGULATION

The rhythmic control of cardiac events could be explained by the existence of daily oscillations in several cardiovascular parameters, including heart rate (HR; Furlan et al., 1990), heart rate variability (HRV; Bonnemeier et al., 2003a), blood pressure (BP; Millar-Craig et al., 1978), cardiac output (Cugini et al., 1993) or QT interval duration (Bonnemeier et al., 2003b), and the activity of the autonomic nervous system (ANS). Typically, ANS activity has been indirectly evaluated by measuring HRV, which is affected by HR, an index of sympathovagal balance (Bootsma et al., 1994).

Furthermore, HR, BP, and cardiac output follow diurnal patterns, defined by a morning peak (acrophase) and a nocturnal decrease (nadir) (Degaute et al., 1991; Veerman et al., 1995) and reinforces an important role for these cardiovascular parameters in defining vulnerability to arrhythmogenic events. For example, myocardial infarction, stroke, and ultimately sudden cardiac death (SCD) show a higher prevalence during morning hours.

The slowing of the HR at night, leads to a lengthening of the heart rate corrected QT interval (QT_c) (an index of ventricular repolarization) (Browne et al., 1983). This diurnal variability of repolarization is consistent with the circadian profile of catecholamine circulation (Bexton et al., 1986). In fact, the variations that occur in HR are largely regulated by the two branches of the ANS, the sympathetic and the parasympathetic nervous systems through circulating neurohumoral factors including vasoconstrictive, vasodilative, and proinflammatory cytokines. Nadir in HR diurnal oscillation is generally associated with increased parasympathetic activity at night, while the acrophase is linked with changes in the sympathetic tone during daytime (Furlan et al., 1990).

Despite a critical role for the ANS in circadian rhythms, its contribution to the diurnal variation in HR is not completely clear. The majority of studies suggest that the central clock is not involved in the control of HR by circadian rhythms, as demonstrated in transplanted hearts (Bigger et al., 1996), Langendorff-perfused hearts (Young et al., 2001), cultured

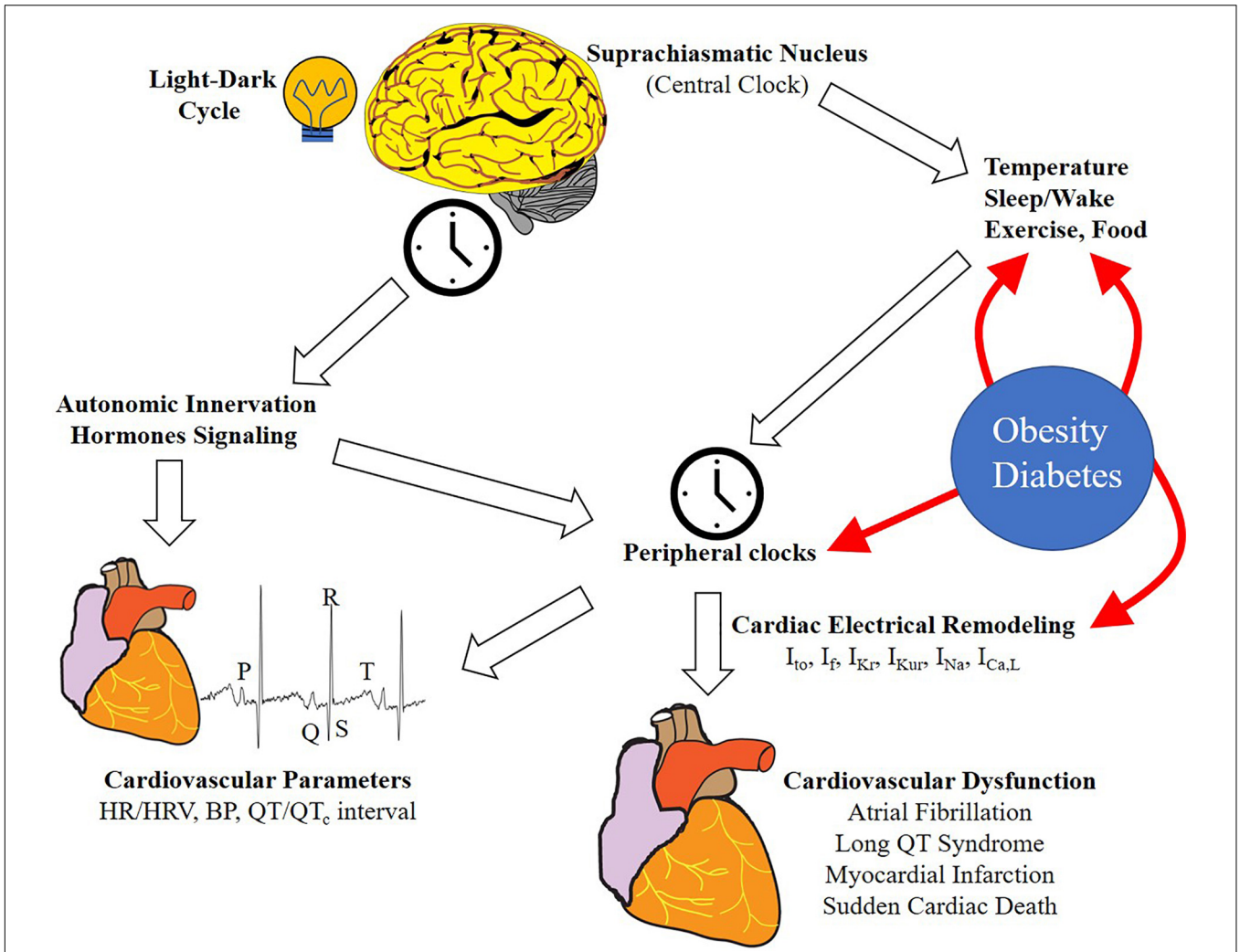

FIGURE 1 | Schematic representation of the regulation of circadian rhythms of cardiovascular function. Light/dark cycle entrains the central clock in the SCN, which in turn regulates rhythmic oscillation in peripheral tissues clocks through neurohumoral signaling. Peripheral clocks are also regulated by other stimuli including sleep/wake, food intake, exercise, and temperature. In the heart, this regulation results in the rhythmicity of different physiological (cardiovascular parameters and ion channel expression) and pathological processes (cardiovascular diseases and arrhythmia). Lifestyle can influence and alter the effect of some external cues (HFD, shiftwork etc.). Metabolic diseases (e.g., obesity and diabetes) can influence the circadian rhythms in different tissues and processes, particularly in the heart, leading to ion channel expression remodeling and increasing the risk of cardiovascular disease (CVD) and arrhythmias. HR, heart rate; HRV, heart rate variability, BP, blood pressure; I_{to}, transient outward potassium current, I_f, funny current, I_{Kr}, rapid delayed rectifier potassium current; I_{Kur}, ultra-rapid delayed rectifier potassium current; I_{Na}, sodium current; I_{CaL}, L-type calcium current.

cardiomyocyte monolayers (Durgan et al., 2005), possibly due to a lack of an intact autonomic innervation, as well as β-adrenergic receptor deficient-mice (Kim et al., 2008; Swoap et al., 2008) or models of autonomic blockade (Makino et al., 1997; Oosting et al., 1997). Moreover, in pheochromocytoma patients that show sustained and elevated levels of circulating catecholamines, the circadian mediated decrease in BP persists (Statius van Eps et al., 1993), suggesting a role for peripheral clocks in the regulation of these biological parameters. However, Tong et al. (2013) demonstrated that both SCN lesion and pharmacological ANS blockade in mice lead to a loss of circadian rhythmicity in HR, and that ANS seems to influence some cardiac ion channels gene expression.

There are other systemic rhythmic factors, including glucocorticoids (e.g., cortisol) or mineralocorticoids that may also influence the circadian rhythms of the cardiovascular system but with contrasting outcomes associated with diurnal patterns. For example, Shea et al. have demonstrated that the diurnal variation in BP is modulated or controlled by the circadian rhythms in cortisol or catecholamines (Shea et al., 2011). By contrast Imai and others showed that exogenous administration of glucocorticoids changes the rhythmic pattern of BP variations, and prevents the nocturnal-dependent decreases in BP and further suggests an important role for the hypothalamic–pituitary–adrenal axis in influencing the circadian rhythm of BP (Imai et al., 1989).

In native cardiomyocytes, mineralocorticoids, and glucocorticoids have been shown to exert their effects on cellular functions through the mineralocorticoid receptor leading to distinct functional and transcriptional outcomes (Jaisser et al., 2011; Oakley and Cidlowski, 2015). For example, glucocorticoid receptor signaling in cardiomyocytes is critical for the normal development and function of the heart. In contrast, mineralocorticoid receptor signaling in cardiomyocytes participates in the development and progression of cardiac diseases (Imai et al., 1989; Jaisser et al., 2011).

Moreover, there is a paucity of studies that have investigated the potential role of the aldosterone/cortisol-mediated mineralocorticoid receptor in the regulation of the cardiomyocyte circadian clock. However, both Tanaka et al. (2007) and Fletcher et al. (2019), have provided strong evidence for an important link between mineralocorticoid receptor and circadian clock signaling, by demonstrating that aldosterone promotes circadian rhythm dependent functional expression of clock genes (*Bmal1*, *Per1*, *Per2,* and *Rev-ErbA*) in rat cardiomyoblasts and mouse hearts. The β-adrenergic receptor agonist isoproterenol has been shown to increase the circadian rhythms of the *Per2* clock gene in ventricular mice explants (Beesley et al., 2016). This suggests that modulation of the ANS may determine the functional outcomes of cardiac ion channel expression possibly *via* synchronization of the circadian rhythms in the peripheral cardiac clock. It would also be interesting for future circadian rhythm and cardiac studies to evaluate whether mineralocorticoids and glucocorticoids can affect cardiac ion channel expression and promote arrhythmogenesis.

It is widely known that the functional expression of major cardiac ionic channels is critical for normal sinus rhythm and cardiac function. The physiological link between cardiac action potential and its ionic channels is vital for mechanistic insights into the clinical consequences that occur when there are disease-induced changes in the functional properties of these ionic channels.

A critical balance of cardiac ionic depolarizing (Na and Ca channels), and repolarizing mechanisms (K channels), is an important determinant of the duration of the cardiac action potential (AP) and refractory period (Carmeliet, 2006). This means that disease processes that either increase depolarizing currents or decrease repolarizing currents will alter this balance and predispose to reentry and/or induction of ectopic foci, that increases the likelihood of developing arrhythmogenic events (Antzelevitch and Burashnikov, 2011), and ultimately the transition to heart failure and SCD. Research efforts that are directed toward a comprehensive understanding of the link between the cardiomyocyte molecular clock and electrical instability have identified and validated novel mechanistic links associated with oscillatory ion channel expression (summarized in **Table 1**). Our hope is that these findings will trigger additional research investigations into unappreciated but significant pathways that are directly or indirectly linked to circadian molecular pathways and help to provide insights that will

TABLE 1 | Altered functional expression of ion channels by the circadian molecular clocks.

Ion Channel	Channel Subunit	Current	Localization	Circadian Rhythm	Circadian Expression	Assessment Type	Species	References
Na	$Na_v1.5$	I_{Na}	V	yes	↑ dark	mRNA	rat, mouse	Schroder et al., 2013
	HCN4	I_f	SAN	yes	↑ light	mRNA, protein, current	mouse	Wang et al., 2016
Ca	$Ca_v1.2$	$I_{Ca,L}$	V	yes	↑ light	protein, current	guinea pig	Chen et al., 2016
K	$K_v4.2$	I_{to}	A, V	yes	↑ light	mRNA, protein, current	rat, mouse	Yamashita et al., 2003; Jeyaraj et al., 2012
	KChiP2	I_{to} b subunit	A, V	yes	↑ dark	mRNA, protein	mouse	Jeyaraj et al., 2012
	$K_v3.1/3.4$	$I_{K,ACh}$	A	no		mRNA	rat	Yamashita et al., 2003
	Kv1.5	I_{Kur}	A, V	yes	↑ dark	mRNA, protein, current	rat	Yamashita et al., 2003
	ERG	I_{Kr}	A, V	yes	↑ light	mRNA	mouse, rat	Schroder et al., 2015
	$K_v7.1$	I_{Ks}	A, V	no		mRNA	mouse, rat	Yamashita et al., 2003; Schroder et al., 2015

A, atrium, V, ventricle, SAN, sinus atrial node, ↑ dark, increased expression during dark period, ↑ light, increased expression during light period. Modified from Black et al. (2019).

further advance the field of chronological modulation of cardiac function.

CIRCADIAN MODULATION OF SUPRAVENTRICULAR AND VENTRICULAR ARRHYTHMIAS

Supraventricular and ventricular arrhythmias display opposing circadian patterns. Among the supraventricular arrhythmias, atrial fibrillation (AF) is one of the most common arrhythmias in both men and women and it is characterized by increased morbidity and mortality. One major mechanism that underlies the pathogenesis of AF is rapid and disorganized atrial electrical activity that ultimately leads to loss of efficient atrial function, and altered ventricular contraction (Nattel, 2002). This means we need to have a good understanding of how major atrial ionic currents may be modulated in disease states that increase AF risk. Obesity has been shown to be an independent risk factor for AF (Vyas and Lambiase, 2019; Javed et al., 2020), suggesting that understanding how obesity-related mechanisms modulate ion channel function may inform effective pharmacological and dietary interventions in patients.

Electrical remodeling in AF includes increases in the pacemaker current, I_f (Lai et al., 1999), a strong reduction of the transient outward (I_{to}) and the ultra-rapid (I_{Kur}) K current densities (Brandt et al., 2000), and a significant reduction in the L-type Ca current, $I_{Ca,L}$ (Christ et al., 2004), which is one of the most consistent features. Furthermore, constitutive activation of the acetylcholine-activated K current ($I_{K,Ach}$), is important for the maintenance of chronic AF (Dobrev et al., 2005). AF incidence is higher during nighttime, and this has been confirmed in ICD data reported by Shusterman et al. (2012). This nocturnal prevalence is consistent with a predominance of vagal activity at night that can stimulate $I_{K,ACh}$ in atrial cardiomyocytes and inhibits I_f and $I_{Ca,L}$, thus promoting a shortening of refractory period and reentry (Chen et al., 2014). There are also more recent reports of the contribution of altered function of the rapidly (I_{Kr}) and slowly (I_{Ks}) activating components of the delayed rectifier K currents (I_K) in AF (Caballero et al., 2010; Gonzalez de la Fuente et al., 2013). We have recently shown increased current density of the delayed rectifier K current (composed of I_{Kr} and I_{Ks}) in a high-fat diet (HFD)-induced obese guinea pig model (Martinez-Mateu et al., 2019), with implications for an abbreviated atrial AP duration (APD), and propensity for AF tachycardia (Martinez-Mateu et al., 2019). There is a paucity of arrhythmia studies that investigate the modulation, by the cardiomyocyte molecular clock, of I_K function in metabolic disorders. The important role of delayed rectifier K currents in limiting cardiac repolarization in health and disease suggests that future studies that investigate their modulation by the cardiomyocyte molecular clock are likely to reveal crucial mechanistic insights that will inform targeted interventions with implications for precision medicine. Alterations in tissue properties (or impaired tissue structural integrity), and autonomic (manifested as altered sympathovagal activity) remodeling (Nattel, 2002), also predispose to AF risk.

Circadian rhythms in HR is widely attributed to variations in sympathovagal tone (Bootsma et al., 1994). Recent reports have provided evidence that HR diurnal oscillations could also be due to intrinsic circadian rhythms in the activity of the pacemaker of the heart or the sinus atrial node (SAN; Wang et al., 2016). The hyperpolarization activated cyclic nucleotide gated K channel (HCN)4 currents have been proposed to contribute to several functions including pacemaker activity in heart and brain, control of resting membrane potential, and neuronal plasticity (DiFrancesco and DiFrancesco, 2015). The hyperpolarization-activated "funny" current (or I_f), is carried by HCN channels, which exists in native cells as heterotetramers built of four HCN subunits (Novella Romanelli et al., 2016). The transcript and protein expression of HCN4 in mice SAN biopsies have been shown to exhibit circadian rhythm profiles compatible with the oscillations of HR. The density of I_f was double at the start of the awake period (higher HR) compared to the sleep period (lower HR) (Wang et al., 2016). Moreover, an *in silico* analysis of the *Hcn4* promoter has revealed the presence of conserved Ebox binding sites for the Clock-Bmal1 heterodimer (Wang et al., 2016), suggesting that its expression may be directly under the control of the cardiomyocyte molecular clock.

Gene transcripts, protein expression, and current densities of the I_{Kur} channel subunit $K_v1.5$ and I_{to} subunit $K_v4.2$ have shown significant circadian variations in rats. $K_v1.5$ is increased during the dark period, while $K_v4.2$ displayed a completely reverse pattern, with an increase during the light period (Yamashita et al., 2003). Moreover, the reversal of light stimulation for 2-weeks attenuated and reversed the circadian pattern of these channel transcripts, while β-adrenergic stimulation solely influenced oscillation in $K_v1.5$, suggesting that rhythmicity of both channels could be the result of multiple factors (internal cardiomyocyte clock, light/dark cycle, ANS activity, etc.) (Yamashita et al., 2003).

In contrast to AF, ventricular arrhythmias, including ventricular tachycardia (VT) and ventricular fibrillation (VF), are prevalent during morning hours (Siegel et al., 1992; Englund et al., 1999). One potential mechanism is possibly through increases in sympathetic activity after awakening, with β-adrenergic stimulation promoting Ca overload, afterdepolarizations, and reentry mechanisms, and therefore acting as substrates for pro-arrhythmic triggers (Gardner et al., 2016).

These observations emphasize a role for the involvement of sympathetic stimulatory pathways in the propensity and prevalence of SCD in the mornings and reinforces the importance of targeted clinical interventions that utilize β-blockers to limit the morning peaks in SCD especially after myocardial infarction (Peters et al., 1989). Furthermore, circadian variation studies (based on 24-h ECG monitoring), have also been described for distinct ventricular arrhythmias. For example, long QT Syndrome Type 1 (LQT1) and long QT Syndrome Type 2 (LQT2), display a morning prevalence, while LQT Type 3 and Brugada Syndrome, have been shown to display increased incidence at night (Stramba-Badiale et al., 2000; van den Berg et al., 2006; Takigawa et al., 2012).

Long QT Syndrome Type 2 is caused by mutations in the *KCNH2* gene leading to a loss of function of the $K_v11.1$ (hERG)

channel, and pathological decreases in the repolarizing I_{Kr} current (Curran et al., 1995; Puckerin et al., 2016). Two different variants of the ERG subunit, ERG 1a and ERG 1b, are expressed in human ventricle (Jones et al., 2004) and functional I_{Kr} is likely to consist of a combination of both variants (hERG 1a/1b) (London et al., 1997). Interestingly, compared with homomeric hERG 1a currents, hERG 1a/1b currents exhibit a twofold increase in density, rate of activation, recovery from inactivation, and deactivation (Sale et al., 2008; Aromolaran et al., 2016; Puckerin et al., 2016; Martinez-Mateu et al., 2019). It has been demonstrated that reducing hERG 1b subunit levels alters I_{Kr} kinetics and leads to cellular manifestations of pro-arrhythmia, such as APD prolongation and early afterdepolarizations (EADs), in human induced pluripotent stem cell-derived ventricular cardiomyocytes (hiPSC-CMs; Jones et al., 2014). The expression of hERG channels have been reported to follow a circadian variation, and its diurnal pattern is disrupted after cardiac-specific *Bmal1* knockout, suggesting that its control is under the cardiomyocyte molecular clock (Schroder et al., 2015). Compatible with a decrease in gene expression, I_{Kr} density in the *Bmal1* cardiac knockout was 50% smaller than in control ventricular myocytes, with no differences in gating properties (Schroder et al., 2015). In this study, the specific contribution of the distinct hERG variants to this outcome was not examined. Thus, it would be of particular interest to evaluate if the subunits are under differential circadian control, particularly considering the differences in biophysical properties of channel function, and the implication in a variety of cardiovascular disease conditions.

In a recent retrospective study in heart failure patients, an increase in QT and QT_c diurnality (QT_d and $QT_{c,d}$), representing the amplitude of their diurnal variation, has been associated with ventricular arrhythmias (Du Pre et al., 2017). The QT_d and $QT_{c,d}$ have also been shown to be increased in both congenital (LQT2) or drug-induced (Sotalol) ERG channel dysfunction (Du Pre et al., 2017), supporting the hypothesis that loss of circadian control of ion channel functional expression leads to adverse cardiovascular parameters and increased incidence of arrhythmias.

In human ventricular myocytes I_{Kr} and I_{Ks}, together with $I_{Ca,L}$, are important determinants of APD. This dynamic ion channel relationship underscores the relevance of cardiac repolarization reserve, which would be expected to limit vulnerability to arrhythmia risk by maintaining normal cardiac repolarization (Carmeliet, 2006). A novel clock-dependent oscillator, Kruppel-like factor 15 (Klf15) has been identified as a rhythmic regulator of repolarization. It has been shown to target the rhythmic expression of the α-subunit ($K_v4.2$) and the regulatory β-subunit (KChiP2), of the I_{to} current (Jeyaraj et al., 2012). Both *Klf15* deletion and overexpression in mice led to modification of I_{to} density and APD with corresponding alterations in the QT interval length, resulting in increased susceptibility to arrhythmias. This is supported by the evidence that an ECG pattern (ST-segment changes), similar to that found in Brugada syndrome, has been observed after deletion of *Klf15* in mice (Jeyaraj et al., 2012).

Expression levels of several other K channels without a circadian pattern were lower in *Bmal1* mice knockout hearts compared to control, suggesting that cardiomyocyte clock signaling might indirectly contribute to the expression of non-circadian K^+ channels genes (Schroder et al., 2015). Furthermore, in the *Bmal1* mice model, the authors demonstrated a loss of rhythmic expression of SCN5A, which encodes for the cardiac voltage-gated Na channel, with a reduction of the corresponding current I_{Na} (50%), a slowed HR and an increased incidence of arrhythmias in mice and rat ventricular myocytes (Schroder et al., 2013). It would be of particular interest to evaluate whether oscillations in Na channels are altered in LQT3 patients.

In guinea pig ventricular myocytes, Clock-Bmal1 heterodimers have been shown to regulate the circadian expression and function of L-type Ca channels, and this occurs through the PI3K-Akt signaling pathway, with corresponding oscillations in APD (Chen et al., 2016). We and others have shown that I_{Ks} and I_{Kr} contribute prominently to cardiac repolarization in guinea pig ventricular myocytes (Sanguinetti and Jurkiewicz, 1990; Bryant et al., 1998; Aromolaran et al., 2014, 2018). To our knowledge, there have been no reports of diurnal variations in I_{Ks} and I_{Kr} functional expression in guinea pig ventricular myocytes. Pathological decreases in I_{Ks} either due to congenital or inherited mutations in KCNQ1 channel subunits (Aromolaran et al., 2014; Puckerin et al., 2016), or acquired in disease states delay cardiac repolarization leading to prolongation of the QT interval (or LQT1), a disease mechanism that increases vulnerability to fatal arrhythmias such as *Torsades des Pointes* (Khan, 2002; El-Sherif and Turitto, 2003). Therefore, it is important to determine whether these ion channels may be regulated by circadian regulation. This premise is underscored by a previous report by Schroder and others (Schroder et al., 2015) showing that the molecular clock in the heart regulates the circadian expression of KCNH2 (which encodes the hERG channel) and modifies channel gene expression. The authors suggested that a disruption of cardiomyocyte circadian clock mechanisms likely unmasks the diurnal changes in ventricular action potential repolarization and predispose to an increased risk of fatal arrhythmias that underlie SCD. It will be important to determine whether similar mechanisms control cardiac KCNQ1-I_{Ks} channel functional expression.

Together, it is intriguing to speculate that modulation by circadian rhythms of ion channel functional expression and ANS activity may underlie alterations in the day/night pattern of arrhythmias and SCD.

TIME RESTRICTED FEEDING, METABOLIC DISORDERS, ION CHANNEL BIOPHYSICS AND CIRCADIAN RHYTHM PATHWAYS

Changes in the intracellular concentration of several metabolites (e.g., heme, NAD/NADH, CO, glucose, AMP/ATP, etc.) can influence the activity of the clock machinery by regulating histone modifications, DNA interactions or protein modifications (Panda, 2016). Extracellular factors, including hormones and temperature that regulate the peripheral clocks permit their

alignment with the central clock. These rhythmic patterns enable a temporal separation of distinct biochemical pathways in a more energy-efficient fashion (Panda, 2016), such that misalignment of central and peripheral clock phases may promote the development of metabolic diseases. Additionally, dietary habits associated with excessive feeding can affect circadian rhythms in distinct organs leading to a higher likelihood of developing the metabolic syndrome (Pickel and Sung, 2020; **Figure 1**).

Daily eating patterns (feeding-fasting cycle and day *vs* night meals), and time-restricted feeding (TRF) can affect peripheral circadian rhythms. For example, experiments conducted in mice fed *ad libitum* or exposed to TRF have shown how quantity, quality and timing of food intake can alter circadian rhythm physiology. Mice exposed to HFD *ad libitum* (used to induce obesity) have altered diurnal oscillations in hepatic transcriptome, compared to mice fed a standard diet (Eckel-Mahan et al., 2013). Moreover, TRF of HFD improves molecular oscillations (similar to mice fed a standard diet) (Hatori et al., 2012), and therefore suggests its potential ability to attenuate the adverse metabolic consequences of diet-induced pathologies. This suggestion is further reinforced by the demonstration that TRF is able to reduce age-dependent or HFD-dependent deterioration of cardiac function in insects (Gill et al., 2015), and that implementation of a 10-hour TRF may promote weight loss and improve sleep in humans (Gill and Panda, 2015). Moreover, changes in metabolism, as seen after TRF, can lead to an uncoupling of peripheral oscillators from the central clock, with consequent alterations of the phase of circadian gene expression in different tissues, including the heart, while not affecting the SCN clock genes (Damiola et al., 2000).

Obesity and diabetes are functionally related to alterations in circadian rhythms with an impact on cardiac function. Studies on the effect of obesity on circadian rhythmicity of cardiometabolic functions are limited, but obesity has been associated with a decrease in HRV and with a shift in its circadian pattern (Rodriguez-Colon et al., 2014). Notably, polymorphisms in the *CLOCK* gene have been associated with a differential incidence of obesity in humans, further supporting the idea that circadian rhythms have a pivotal role in the development of metabolic syndrome (Scott et al., 2008).

Diabetes leads to alterations in circadian rhythms and adversely affects cardiac function. This functional remodeling process is exemplified by circadian rhythm studies in a rat model of streptozotocin-induced diabetes (Young et al., 2002). The authors demonstrated that diabetes-induced alteration of circulating humoral factors, leads to a loss of normal synchronization of the peripheral heart clock (Young et al., 2002). This observation is further supported by pathological diurnal variations in diabetes biomarkers (including insulin, leptin, glucocorticoids, growth hormone, glucose, and circulating of free-fatty acids) (Ortiz-Caro et al., 1984; Velasco et al., 1988; Havel et al., 1998), and ANS activity (Bernardi et al., 1992). Moreover, two different *BMAL1* SNP haplotypes have been shown to be associated with type 2 diabetes and hypertension in patients, suggesting an important contribution of *BMAL1* variants to the pathogenesis of these disease mechanisms (Woon et al., 2007).

The circadian rhythm distribution of the onset of cardiovascular events is also altered in diabetes. For example, compared to non-diabetic patients the peak in acute myocardial infarction is lower in the morning, and this is followed by a second peak in the evening, with the risk of developing myocardial infarction higher during the nighttime (Hjalmarson et al., 1989). This chronological-dependent susceptibility to myocardial infarction can be explained by alterations in the circadian patterns associated with sympathovagal balance in diabetic patients that display a range of autonomic abnormalities (Bernardi et al., 1992).

There is also evidence of lower parasympathetic activity during the night, and a marked dominance in sympathetic tone in diabetic patients during both day and night (Bernardi et al., 1992). Furthermore, diabetic patients, particularly those with autonomic neuropathy, showed no decrease in BP during the night when compared to non-diabetic patients. This disruption of the circadian rhythm dependent modulation of BP is frequently associated with a poor prognosis (Felici et al., 1991). Therefore, prolonged sympathetic activity in diabetic patients may counteract the protective effect of parasympathetic tone on the cardiovascular system, which normally would then be manifested by a lower incidence of cardiac events during the nighttime (Bernardi et al., 1992). Diurnal differences in the ECG have been observed during hypoglycemia and this is generally manifested as a larger prolongation in QT_c interval throughout the daytime, suggesting a higher vulnerability to arrhythmias; while the incidence of bradycardia episodes was found to be increased during the sleep cycle (Andersen et al., 2020).

FUTURE DIRECTIONS AND CONCLUSIONS

There is increasing evidence that cardiac diseases can be influenced by circadian rhythms, and peripheral clocks can be altered in the setting of different pathologies, including diabetes, obesity, and hypertension (Maury et al., 2010). There is a lack of progress in the knowledge of arrhythmia mechanisms. However, in recent years there has been a great deal of effort to understand the molecular mechanisms of circadian rhythms that regulate cellular mechanisms in health and disease. For example, hiPSC-CMs have been widely used as disease models for arrhythmias (including LQT) (van Mil et al., 2018) and have been validated as reliable sources for drug safety studies and the assessment of a new drugs pro-arrhythmic risk with translational implications in patients. The evidence that differentiated hiPSCs acquire and exhibit circadian variation in clock genes (Umemura et al., 2019; Kaneko et al., 2020), provides the rationale for the use of these cells in circadian rhythms studies that could provide relevant mechanism-based insights that may be better predictive of disease penetrance with significant implications in patients.

The existence of circadian variations in the manifestation of cardiac events and arrhythmogenesis highlights the critical link between chronotherapy and cardiovascular disorders, particularly arrhythmias. This suggests that the timing of dietary or therapeutic interventions may be key to limiting

the incidence of disease mechanisms that impact the quality of life of patients. Several clinical trials have demonstrated a better tolerability and increased efficacy for chronotherapy compared to non-time-based treatment for different pathologies (Levi et al., 1985; Giacchetti et al., 2006; Buttgereit et al., 2008), while some other trials have failed to establish a similar and positive outcome (Levi and Okyar, 2011). This could be attributed to inter-individual circadian differences that can result from sex, age, lifestyle, genetic or disease profile. Therefore, a further understanding of the mechanisms involved in circadian regulation of biological processes is required to further improve the rigor of these approaches.

Existing molecular mechanisms of how circadian rhythms may modulate cardiovascular function are obtained in rodent models including mice and rats, that unlike humans, are nocturnal. Therefore, future studies that incorporate mechanisms in larger animal models are more likely to be rewarded with additional and/or novel mechanism-based insights that could be better translated into therapeutic and clinical significance.

There is also the added complexity of species differences associated with rational development of targeted therapeutics in patients with cardiovascular diseases. This is also because most of the current knowledge about the regulation of genes (ion channels and metabolic factors) targeted by the molecular clock, have been obtained in animal models of clock component manipulation, mainly *Bmal1* and *Clock*. Therefore, we need to exercise caution in the interpretation of outcomes in future studies due to an indirect effect of clock gene modulation, in models where these modulations may not be tissue specific. Therefore, a further analysis of clock genes and associated upstream and downstream molecular pathways could inform or potentially shift current paradigm of the circadian rhythms-dependent regulation of the cardiovascular system, and more specifically arrhythmia substrates that promote ion channel dysfunction.

AUTHOR CONTRIBUTIONS

JB and AA researched the concepts. JB wrote the first draft of the manuscript. HZ and KA edited and finalized the manuscript. AA obtained funding, conceived of, wrote, and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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The ECG Characteristics of Patients With Isolated Hypomagnesemia

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Background: Electrocardiographic (ECG) characteristics of patients with isolated hypomagnesemia are not well defined. We aimed to investigate these ECG characteristics in order to define clearly the features of isolated hypomagnesemia.

Hypothesis: Lower serum magnesium could affect ECG parameters after excluding potential confounders.

Methods: This retrospective study was of patients with low serum magnesium <0.65 mmol/L compared with the same patients after restoration to normal serum magnesium. Patients with hypokalemia, hypocalcemia and other electrolyte disturbances were excluded. ECG parameters manually determined and analyzed were P wave dispersion, PR interval, QRS duration, ST-T changes, T wave amplitude, T peak-to-end interval (Tpe), corrected Tpe (Tpec), QT, corrected QT (QTc), QT peak corrected (QTpc) and Tpe dispersion, Tpe/QT ratio.

Results: Two-hundred-and-fourteen patients with isolated hypomagnesemia were identified with 50 of them (56.9 ± 13.6 years; 25 males) being eligible for final analysis from 270,997 patients presenting April 2011–October 2017. In the period of isolated hypomagnesemia, P wave duration was found prolonged ($p \leq 0.02$); as was QTc (439 ± 27 vs. 433 ± 22, p = 0.01). Tpec (122 ± 24vs. 111 ± 22, $p = 0.000$) and Tpe/QT ratio (0.29 ± 0.05 vs. 0.27 ± 0.05, $p = 0.000$) were increased. QTpc decreased during hypomagnesemia (334 ± 28 vs. 342 ± 21, $p = 0.02$). However, no significant differences were found in PR interval, QRS duration (85 ± 12 ms vs. 86 ± 12 ms, $p = 0.122$) and ST-T segments between the patients and their own controls.

Conclusions: In patients with isolated hypomagnesemia, P wave duration, QTc, Tpec, and Tpe/QT ratio suggesting atrial depolarization and ventricular repolarization dispersion were significantly increased compared with normal magnesium levels in the same patients after restoration to normal levels.

Keywords: electrocardiogram, serum magnesium, ventricular arrhythmia, sudden cardiac death, repolarization dispersion

INTRODUCTION

Sudden cardiac death (SCD) has been widely studied because of its important public health implications (Ackerman et al., 2016); electrolyte disturbances have been thought to be related to its etiology. Magnesium is the second most abundant intra-cellular fluid cation, and is considered to be an important factor in facilitating influx of potassium ions into the myocyte, which relates to QT duration. Low serum magnesium is also regarded as a risk factor for ventricular tachycardia/fibrillation (VT/VF) and SCD (Adamopoulos et al., 2009; Del Gobbo et al., 2013; Kieboom et al., 2016). Moreover, administration of magnesium has been first line treatment for Torsades des Pointes (TdP; Neumar et al., 2010).

Studies focusing on isolated hypomagnesemia are limited. Early studies observed ST depression or T wave changes in magnesium deficient dogs (Seta et al., 1966). Other reports have suggested that low serum magnesium level is related to a higher incidence of various arrhythmias, including VT and VF and SCD (American Heart Association, 2000; Peacock et al., 2010). The possible mechanisms are thought to involve QT prolongation (Kieboom et al., 2016). However, these studies have, generally, not excluded hypokalemia, hypocalcemia and other electrolyte disturbances, or were based only on animal data. The electrocardiographic (ECG) characteristics of isolated hypomagnesemia remain not clearly defined. We, therefore, performed this study to investigate the specific changes in various ECG parameters among patients with isolated hypomagnesemia.

MATERIALS AND METHODS

Study Design

The study population comprised patients admitted to The First Affiliated Hospital of Dalian Medical University between April 2011 and October 2017. The study sample collected subjects with documented isolated low serum magnesium and those same patients when magnesium levels were returned to the normal range as their own controls to offer comparison of ECG parameters between the two states. Seven-hundred-and-sixty-seven patients with intermittent low serum magnesium levels were selected from 270,997 patients from electronic medical records; The inclusion criteria were as follows:

(1) Patients with isolated hypomagnesemia (serum magnesium levels <0.65 mmol/L) without other electrolyte disturbance according to laboratory criteria in The First Affiliated Hospital of Dalian Medical University (serum calcium levels between 2.02 and 2.60 mmol/L, serum sodium levels between 137 and 147 mmol/L, serum potassium levels between 3.50 and 5.30 mmol/L, serum chlorine levels between 99 and 110 mmol/L and serum phosphate levels between 0.87 and 1.45 mmol/L).
(2) The patients in whom both normal and low magnesium levels were available, where the separate magnesium measurements were <3 months apart.
(3) ECGs were available contemporaneously with the blood samples. Two-hundred-and-fourteen of these 767 patients were shown to have isolated hypomagnesemia and 175 patients with intermittent low serum magnesium fulfilled all the above inclusion criteria. Additionally, those patients with diagnosis of bundle branch block, fascicular block, atrial flutter/fibrillation, atrioventricular block, VT were excluded.
(4) Patients taking QT-prolonging drugs were also excluded guided by crediblemeds.[1]
(5) Further, patients with end-stage heart failure results from various etiologies.

After these exclusions, 50 patients were eligible for comparison with their own controls (**Figure 1**). Our study conforms to the Declaration of Helsinki and was approved by the Medical Ethics Committee of The First Affiliated Hospital of Dalian Medical University. Informed Patient consent was not required in our institution as this was a retrospective study according to local ethics rules.

Assessment of Serum Magnesium

Fasting blood samples were drawn from an antecubital vein and sent immediately to the laboratory in the medical center. The timing of blood drawing and ECG recording was within 24 h. Magnesium was measured in serum by the clinical laboratory in the medical center using a Hitachi 7600-210 automatic analyzer (selective electrode method). Low serum magnesium levels were defined as <0.65 mmol/L. The laboratory coefficient of variation for Mg, based on split samples sent 1 week apart blindly to our laboratory, was 2.2–3.2%, rechecked on an annual basis.

Measurements of ECGs

Standard 12-lead ECGs recorded by GE Healthcare, Chicago, IL, United States MAC5500 at a paper speed of 25 mm/s and voltage of 10 mm/mV. All ECG recordings were made with 40 Hz filtering which was carefully and correctly set in our GE machines. These devices offered some automated measurements but since we wished to record a number of measurements that were not provided, we opted to make all measurements manually. Definition of ECG parameters is shown in **Figure 2**. QT interval was manually measured as the beginning of the QRS complex to the endpoint of the T-wave. The terminal aspect of the T wave was identified as the point where the descending limb reaches the baseline. The methods of QT interval measurement have been shown in **Figure 3** and well described in our previous publications (Wang et al., 2017; Yu et al., 2017; Vink et al., 2018). According to Bazett's formula, QT-corrected (QTc) was defined as:

$$QTc = QT_{maximum}/\sqrt{RR\ interval}$$

$QT_{maximum}$ was measured from the earliest deflection of the QRS complex to the latest termination of T-wave among leads II, V2, and V5. Bazett's formula was also used for correction of other parameters including corrected Tpe (Tpec), QTpc, and

[1]https://www.crediblemeds.org/new-drug-list/

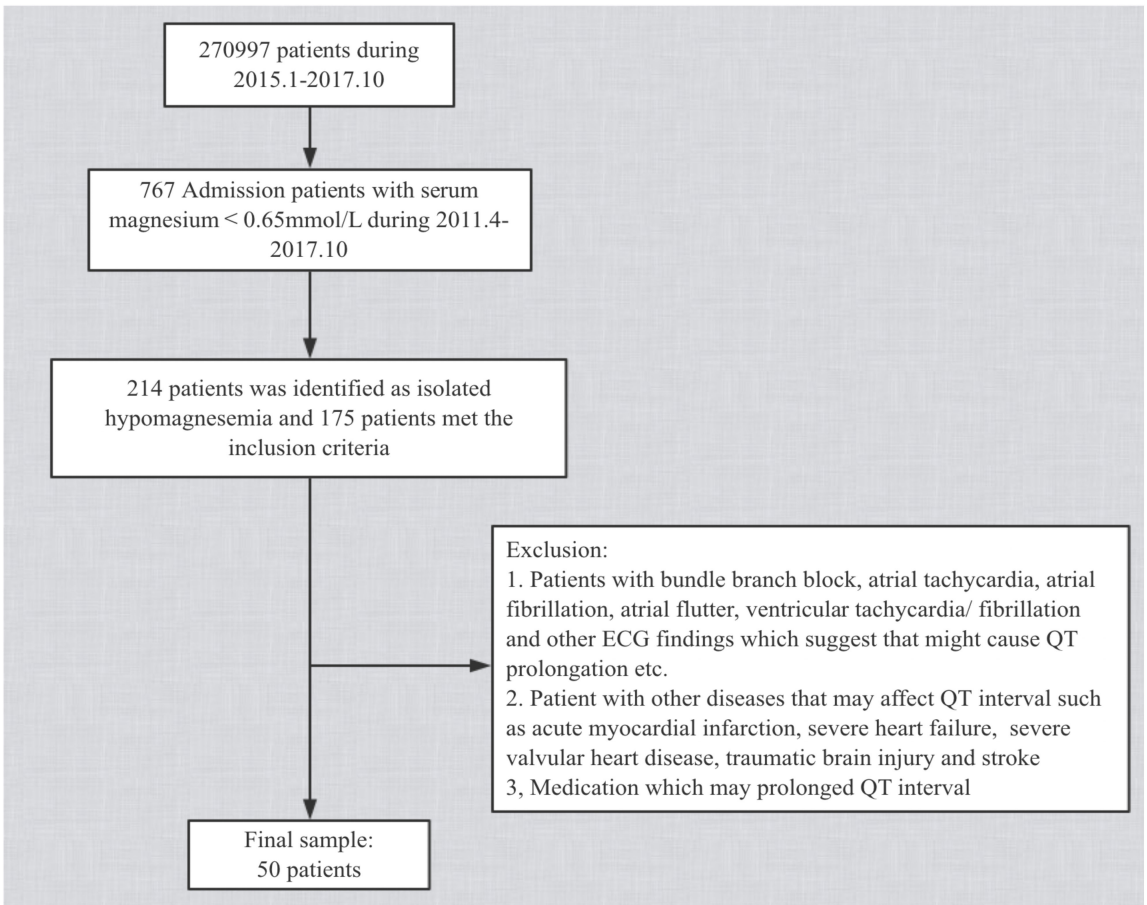

FIGURE 1 | Flow chart of baseline screening of the study population.

JT-corrected (JTc) interval. The other ECG parameters were measured as follows:

- T peak-to-end interval (Tpe) intervals were calculated in lead II, V2, V5;
- P wave duration was measured in lead II, V2 and V3;
- QT-peak (QTp) interval was measured from the beginning of the QRS complex to the peak of T-wave in lead II, V2, V5;

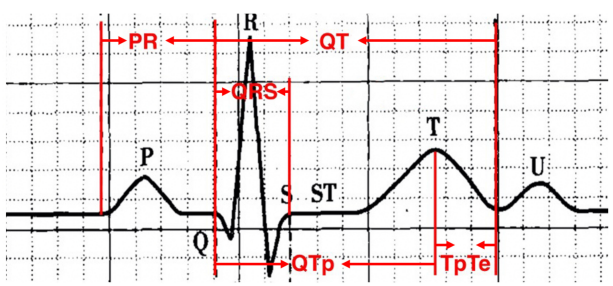

FIGURE 2 | Definition of ECG parameters.

- the peak of the T wave was defined as the highest amplitude reached by the T-wave deflection;
- the longest Tpe interval from among the leads stated above was selected as $Tpe_{maximum}$;
- the longest P wave $duration_{maximum}$ and $QTp_{maximum}$;
- JT interval was calculated by the difference between the $QT_{maximum}$ and QRS duration.

Intervals were calculated as follows;

- Tpe interval = QT interval - QTp interval; Tpec = Tpe corrected;
- Tpe/QT ratio = $Tpe_{maximum}/QT_{maximum}$;
- Tpe dispersion = $Tpe_{maximum}$ - Tpe_{minmum};
- QT dispersion = $QT_{maximum}$ - QT_{minmum};
- P wave dispersion = P wave $duration_{maximum}$ - P wave $duration_{minmum}$;
- JT interval = $QT_{maximum}$ - QRS duration;
- $QTpc = QTp_{maximum}/\sqrt{RR}$ interval;
- $Tpec = Tpe/\sqrt{RR}$ interval.

According to the recommendation of the American Heart Association (AHA), T-wave amplitude was measured from the baseline to the peak of the T wave in lead II, V2, and V3

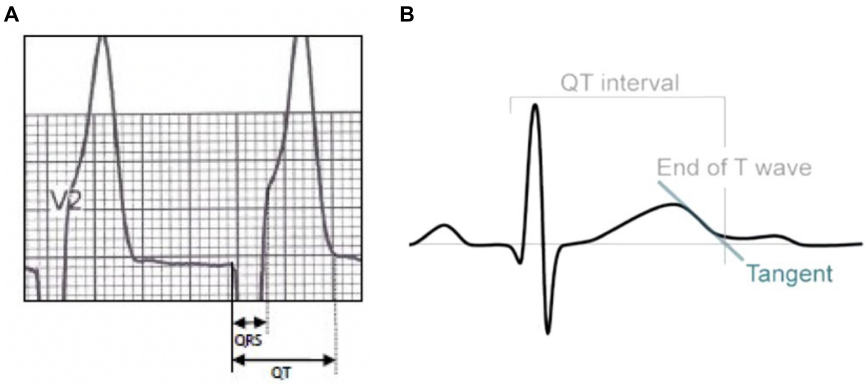

FIGURE 3 | In normal T wave morphologies, the termination of the T wave was taken to be the point of maximal change in the slope as the T wave merges with the baseline **(A)**. If there was any difficulty in determining the end of the T wave, the end point is considered as the intersection between the tangent of steepest slope of the T wave and the isoelectric line **(B)**.

(Rautaharju et al., 2009). ST depression was defined as ST segments depressed >0.1mv in any lead. All ECG measurements were made by two cardiologists from among the authors who were blind to which group each patient belonged, and verified each other's work. Measurements were performed on consecutive complexes on these leads (II, V2, and V5) whichever QT interval was the longest. We printed the ECG on A4 paper and indices were measured by calipers.

Statistical Analyses

Based on the use of patients as their own controls described above, the electrolyte levels and ECG characteristics were compared between the two measurements, abnormal (serum magnesium <0.65 mmol/L) and normal (>0.65 to <0.8 mmol/L). Continuous variables were described by mean ± (standard deviation). The Student's paired *t*-test was used to evaluate the difference for normally distributed continuous variables and Wilcoxon test for abnormally distributed variables. Kolmogorov–Smirnov test was used to test the distribution of continuous variables. Categorical variables were described in percentages and were compared using paired χ^2 tests (McNemar's test). We considered a two-sided *p*-value <0.05 to be statistically significant.

RESULTS

Patient Characteristics

Of the 767 patients with low serum magnesium levels, 159 (20.7%) also had low serum potassium and 364 (47.5%) had both low serum potassium and calcium and, thus, were excluded. **Table 1** shows the distribution of serum electrolytes stratified by serum magnesium level. No significant differences were found in other electrolytes in the included patients, neither in hypomagnesemia nor when normal.

We collected the clinical characteristics of the final studied sample, mean age 56.94 ± 13.63 years; males 50%. The average time between the two ECG recordings was 1.3 ± 0.8 (0.1–3.5) months. The common clinical diagnoses, including various cancers (38), hypertension (10), and diabetes (7), are shown in **Table 2**. Regarding the in-patient departments concerned (**Figure 4**), 66% patients (33/50) were from Oncology, the second most common department concerned was Obstetrics and Gynecology with 10% of patients, followed by Hematology (6%). Diagnoses including coronary artery disease/myocardial infarction/PCI history were not found.

TABLE 1 | Serum electrolyte characteristics of patients stratified by serum magnesium.

	Hypomagnesemia group	Normal group	*P* value
Serum magnesium (mmol/L)	0.60 ± 0.05	0.76 ± 0.09	0.000
Serum potassium (mmol/L)	4.06 ± 0.37	4.11 ± 0.34	0.39
Serum calcium (mmol/L)	2.25 ± 0.14	2.24 ± 0.13	0.70
Serum sodium (mmol/L)	139.88 ± 3.40	140.14 ± 3.51	0.63
Serum chlorine (mmol/L)	104.08 ± 4.13	103.58 ± 4.24	0.46

Study group <0.65 mmol/L, control group 0.65–0.8 mmol/L. Data are presented as mean ± standard deviation. P value derived from paired t-tests for normally distributed variables and Wilcoxon test for abnormally distributed variables.

Descriptions and Comparison of ECG Parameters

In the study group with isolated hypomagnesemia, we identified PR prolongation in 30 patients (60%). ST depression was found in 4 patients in both groups (8%). Amongst repolarization parameters, there were 24 patients (48%) with QTc prolongation, 26 (52%) patients with lower T wave amplitude in lead V2 compared with normal magnesium levels. Forty-six patients (92%) showed Tpec prolongation in lead V3 where was also the greatest increment (mean 16.9 ms). The ECG parameters of patients with low serum magnesium are presented in **Table 3**. Moreover, the inter-observer variability of QT measurement was calculated and provided in **Table 4**.

We found that patients with isolated hypomagnesemia presented significantly higher Tpe/QT ratio (0.29 ± 0.05 vs.

TABLE 2 | Baseline characteristics and clinical diagnosis of total sample.

	Study population
Demographics	
Mean age (Mean ± SD)	56.94 ± 13.63
Men (*N*, %)	25 (50%)
Diagnosis (*N*)	
Cancer	38
Hypertension	10
Diabetes	7
Anemia	3
Renal dysfunction	3
Pregnancy	4
Rheumatoid disease	4
Liver cirrhosis	3
COPD	2
Gout	2
Peptic ulcer	2
Arrhythmia	2
Stroke	1
Heart failure	1

SD, standard deviation; COPD, chronic obstructive pulmonary disease.

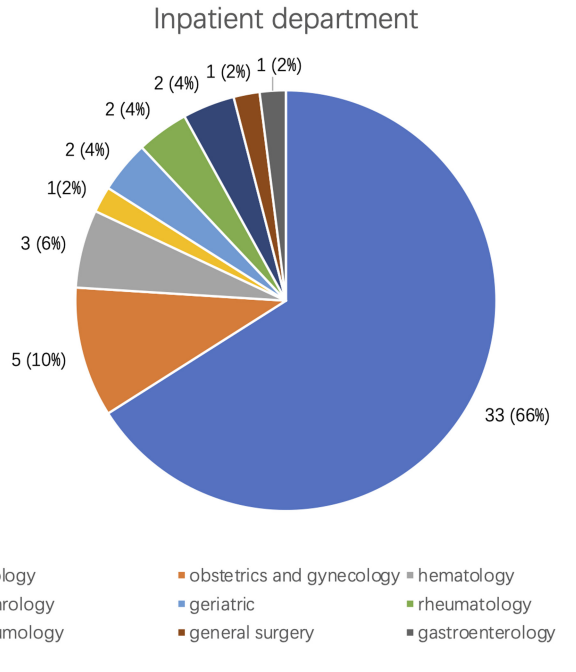

FIGURE 4 | Inpatient departments distribution of the 50 patients with isolated hypomagnesemia.

0.27 ± 0.05, $p = 0.000$), longer QTc interval (439 ± 27vs. 433 ± 22, $p = 0.01$). and JTc interval (345 ± 29 vs. 338 ± 24, $p = 0.05$). Decreased QTpc interval (338 ± 28 vs. 342 ± 21, $p = 0.02$) and Tpec interval prolongation (122 ± 24 vs. 111 ± 22, $p = 0.000$) were also observed at the time of low magnesium. These patients with low magnesium also presented longer P wave duration in all measured leads. Notably, patients with low serum magnesium

TABLE 3 | ECG characteristics of patients with isolated hypomagnesemia.

	N	Percentage (%)
QRS interval shortening	30	60.0
PR interval prolongation	22	44.0
ST depression	4	8.0
QTc prolongation	24	48.0
Tpec prolongation		
Lead II	30	60.0
Lead V2	35	70.0
Lead V3	46	92.0
Lead V4	38	76.9
Lead V5	33	66.0
Lead V6	28	53.0
T wave amplitude peaking (lead II)	14	28.0
T wave amplitude peaking (lead V2)	26	52.0
T wave amplitude peaking (lead V3)	18	26.0

QTc, QT corrected; Tpec, Tpeak to end corrected.

TABLE 4 | Inter-observer variability of QT interval measurements.

		Miss/cases	Observer 1	Observer 2	*P* value
Lead II	Study group (ms)	47/50	360 ± 38	363 ± 38	0.100
	Control group(ms)		363 ± 37	367 ± 36	0.062
Lead V2	Study group (ms)	50/50	365 ± 34	366 ± 35	0.496
	Control group (ms)		366 ± 37	369 ± 36	0.142
Lead V5	Study group (ms)	49/50	359 ± 34	362 ± 33	0.093
	Control group (ms)		365 ± 36	368 ± 36	0.171

have shorter QRS duration (85 ± 12 vs. 87 ± 12, $p = 0.122$) but not significantly so. No significant differences were found in other ECG parameters between patients at time of isolated hypomagnesemia and with normal magnesium (**Table 5**).

Difference of these ECG parameters with significant changes are shown in **Figure 5**.

DISCUSSION

Isolated hypomagnesemia has been relatively rare in our clinical practice. In this study, we analyzed the ECG parameters of patients with isolated hypomagnesemia, after excluding the concurrence of hypokalemia and/or hypocalcemia, and compared the findings with the same ECG parameters when normal serum magnesium levels were restored. We found that isolated hypomagnesemia was associated with longer P wave duration, QTc, Tpec interval and Tpe/QT ratio, indicating increased dispersion of ventricular repolarization.

Role of Serum Magnesium *in vivo*

Magnesium, the second key intracellular cation in the human body, intracellular magnesium level varies between 5 and 20 mmol/L, depending on the different tissues, with the highest concentrations in skeletal and cardiac muscle (Kamonwan and Davenport, 2018). Consequently, serum magnesium level

TABLE 5 | ECG parameter comparison between low serum magnesium and normal serum magnesium groups.

		Hypomagnesemia group	Normal group	*P* value
	P wave dispersion	15 ± 8	16 ± 7	0.46
	P wave duration (ms)			
	Lead II	101 ± 21	93 ± 19	0.02
	Lead V2	94 ± 19	86 ± 19	0.005
	Lead V3	89 ± 19	82 ± 16	0.01
	PR interval (ms)	150 ± 21	149 ± 23	0.64
	QRS duration (ms)	85 ± 12	87 ± 12	0.122
	Heart rate (beats/min)	84.84 ± 16.50	82.33 ± 16.69	0.09
	ST depression	2(4.0)	2(4.0)	1.000
	QTc (ms)	439 ± 27	433 ± 22	0.01
	QTp_max (ms)	284 ± 38	298 ± 36	0.001
	QTpc (ms)	334 ± 28	342 ± 21	0.02
	Tpe_max (ms)	103 ± 20	96 ± 18	0.000
	Tpec_max (ms)	122 ± 24	111 ± 22	0.000
	JT_max (ms)	295 ± 36	296 ± 36	0.71
	JTc (ms)	345 ± 29	338 ± 24	0.05
	Tpe/QT	0.29 ± 0.05	0.27 ± 0.05	0.000
	Tpe dispersion	38 ± 16	34 ± 15	0.199
	QT dispersion	29 ± 19	27 ± 15	0.594
Lead II	QTpc (ms)	323 ± 27	332 ± 22	0.04
	Tpec	102 ± 25	89 ± 22	0.001
Lead V2	T ampltitude (mv)	0.22 ± 0.10	0.22 ± 0.11	0.97
	QTpc (ms)	313 ± 29	319 ± 25	0.15
	Tpec (ms)	117 ± 25	105 ± 23	0.000
Lead V5	QTpc (ms)	326 ± 31	332 ± 29	0.351
	Tpec (ms)	100 ± 21	95 ± 21	0.05

Data are presented as mean ± standard deviation or number (%). P value derived from the paired t-test or Wilcoxon test (for continuous variables), and the paired χ^2 test (for categorical variables) when appropriate.
QTc, QT corrected, Tpec, Tpeak to end corrected, QTp, QTpeak interval, QTpc, QTpeak corrected.

reflects only 1% of the body magnesium content and the clinical impact of magnesium deficiency on cardiac function may be underestimated (Jeroen et al., 2015). Magnesium exerts effects on cardiac function by regulating ion channels, plays a role in activation of Na+-K+ ATPase, which participates in transportation of K+ into cells and Na+ out of cells, and is a natural calcium antagonist. These functions could be adversely affected in magnesium deficiency, which, further, may result in hypokalemia and/or hypocalcemia and alteration of ECG characteristics. Previous studies have demonstrated that patients with hypertension and coronary heart disease are likely associated with more arrhythmias such as VT/VF and SCD (Peacock et al., 2010; Reffelmann et al., 2011). The Atherosclerosis Risk in Communities (ARIC) study also observed 45% SCD risk reduction in individuals with higher serum magnesium level (>0.87 mmol/L) compared with those with serum magnesium level <0.75 mmol/L (Peacock et al., 2010). Beneficial effects of magnesium in suppression of arrhythmia secondary to myocardial ischemia or quinidine have previously been reported. The main mechanism may be that magnesium can suppress the early afterdepolarization-induced triggered activity that may be responsible for initiating episodes of Torsade de Pointes and triggered activity suppression may be related to a "stabilizing" or surface charge effect (Davidenko et al., 1989). Elevated magnesium levels shift the threshold potential to less negative values and produce a membrane stabilizing effect which is similar but less than that of calcium (Castillo and Engbaek, 1954; Frankenhaeuser and Hodgkin, 1957). In addition, magnesium also acts to suppress triggered activity by inhibition of slow inward current (Isi) and consequently shorten phase 2 of the action potential, which is reported in canine Purkinje fibers (Sebeszta et al., 1981; Davidenko et al., 1989). Moreover, other studies have indicated that serum magnesium acts by blocking the increase in intracellular calcium during myocardial ischemia (Prielipp et al., 1995; Peacock et al., 2010). Consequently, low serum magnesium contributes to the increase in intracellular calcium, and results in calcium overload during acute ischemia and reperfusion, thus, playing an important role in arrhythmogenesis. Other possible pathways may contribute to the association between lower serum magnesium levels and dyslipidemia, metabolic syndrome, endothelial dysfunction, inflammation, atherosclerosis, and vascular calcification, all of which could be mechanisms to explain the above observations (Yang et al., 2016).

However, in these early studies almost all the baseline characteristics including serum potassium levels were significantly abnormal ($P < 0.0001$), which may confound the electrophysiological effect of depletion of serum magnesium. Zhang et al. (2012) reported that individuals with magnesium deficiency were found to have associated severe ischemic heart disease and fatal arrhythmias but there were no descriptions of ECG parameters including QTc in their regression model. Therefore, our results that reveal the relationship between QTc, Tpec, and Tpe/QT ratio suggesting increased ventricular repolarization dispersion may, therefore, verify the relationship between hypomagnesemia and SCD.

P-QRS-T Complex

P wave duration was significantly prolonged with low magnesium levels. Increased P wave duration reflected slow or uncoordinated conduction in the atria. The mechanism is as yet unclear but may be a direct effect of low serum magnesium, or indirect by inhibition of K+ channels and result in a block of inward K+ current in phase 3 of the Action potential, considered as prolongation of effective refractory period. Consistent with our results in patients with hypomagnesemia, no difference was detected in PR interval in magnesium deficient beagles reported by Seta et al. (1966).

We identified a tendency to QRS shortening in 41 of 65 (63.1%) patients with low magnesium but it was not statistically significant. However, in an experimental study conducted in dogs (Syllm-Rapoport, 1962), Syllm-Rapoport (1962) found that dogs on a magnesium deficient diet had a shorter QRS duration in the resting state after several days but the observed abnormality could be attributed to diet, the resting state or other effects of the experiment. The Seta group's study (Seta et al., 1966) detected

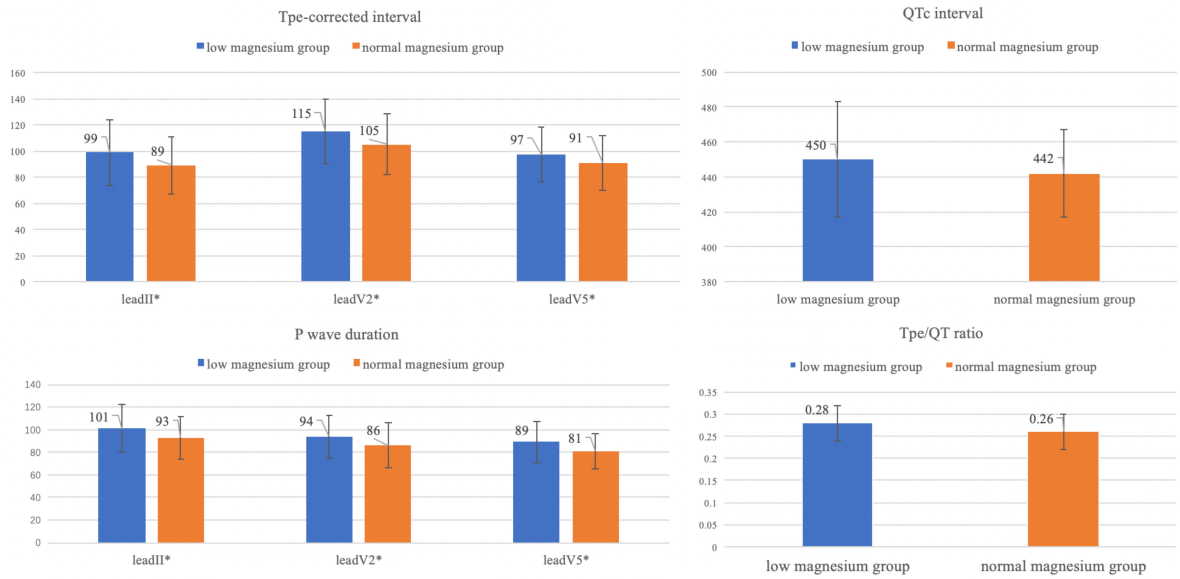

FIGURE 5 | Differences of ECG parameters with significant changes (Tpec, QTc, P wave duration, Tpe/QT ratio). *P value < 0.05.

widening of the QRS in the early stage of magnesium deficiency with peaked T waves and ST depression after 2 weeks. Thus, the most probable explanation for these differences is the fact that it is almost impossible to control all aspects of magnesium metabolism and maintain low serum magnesium levels for long durations in humans and in animals.

QTc Interval and Tpe Interval

A recent study reported that hypomagnesemia was associated with QTc prolongation and SCD from the Rotterdam cohort (Kieboom et al., 2016). However, it must be noted that they did not report the baseline data on serum potassium and other electrolytes. Possible mechanism of QTc prolongation in hypomagnesemia patients is thought to be caused, at least in part, by the effect of hypokalemia or other electrolytes disturbances. Thus, our study may add some confirmation of the possible mechanism. Similarly, another earlier study showed a significant increase in QT interval in magnesium deficient dogs but QTc was not calculated (Syllm-Rapoport, 1962). The difference in results between these two studies (Syllm-Rapoport, 1962; Kieboom et al., 2016) could be due to changes in R–R interval. Subjects with hypomagnesemia in our study had slightly higher heart rates than those with normal magnesium levels potentially increasing QTc. Moreover, reduction in Mg^{2+} ions blocks inward flow of potassium which also potentially prolongs QTc. With respect to Tpec interval, generally, the apex of the T-wave represents epicardial repolarization while the end of the T-wave represents M-cell repolarization. The global dispersion of repolarization in different regions of the ventricular wall can be represented by Tpe and Tpe/QT ratio (Xia et al., 2005; Tse et al., 2018a). Tpe also reflects the length of the ventricular vulnerable period, increase in which has been thought to be a pro-arrhythmic factor for VT/VF in patients with prolonged QTc interval (Tse et al., 2017, 2018b). Recently, our team conducted a retrospective study comparing the outcomes of 293 patients with long QT syndrome (LQTS) compared with 542 patients without LQTS. LQTS patients, as expected, had higher mortality (Yu et al., 2017). Additionally, we found, in the present study, a significant increase in Tpec interval and Tpe/QT ratio among those with QTc prolongation, suggesting that serum magnesium could directly affect electrophysiologic characteristics via prolongation of Tpec. Low serum magnesium significantly shortened the QTpc interval in our study, which raises the possibility that QTc prolongation could be the result of increased Tpec. In terms of mechanism of these changes, it is possible that Tpec prolongation was observed in the early stage of serum magnesium deficiency and, at this early stage, the effect of hypomagnesemia on Na^{+}-K^{+} ATPase is too small to have impact on serum potassium and QTc prolongation. Presently, the electrophysiologic action of low serum magnesium on cellular function is unclear but our results suggest Tpec interval prolongation may have importance in hypomagnesemic patients in clinical practice.

In this study, we draw a conclusion that atrial depolarization and ventricular repolarization dispersion were significantly increased in patients with isolated hypomagnesemia based on our results. However, we are unable to explain the reason why atrial and ventricular cardiomyocytes behave differently in electrolyte disturbances. There is currently insufficient knowledge on the influence of magnesium on the different phases of the cardiac action potential, and that it is also unknown whether there are different effects on different subtypes of cardiomyocytes or the specialized conduction system. Furthermore, we also think it should not be simply concluded that atrial and ventricular cardiomyocytes behave in opposite ways with this electrolyte disturbance, as atrial repolarization is not well reflected by the ECG, preventing us from being certain of the change in atrial

repolarization. We anticipate that further study may illuminate this area in the future.

Clinical Aspects

We identified 36 patients (72%) from Oncology and Hematology departments, the main diagnosis including lung cancer (25), leukemia (2), and lymphoma (3) that accounted for almost 80% of the total. We investigated the patients' medications and found that some chemotherapeutic drugs increase renal magnesium loss by inhibition of reabsorption. Use of cisplatin has been found to be associated with renal magnesium loss, by protein-binding adversely affecting distal tubular reabsorption of magnesium in up to 90% of cancer patients (Goren, 2003; Taguchi et al., 2005) and 19 of our total of 50 patients received intravenous cisplatin. Two patients received ifosfamide or amphotericin B, both of which may cause distal renal tubular damage, and thereby reduce serum magnesium (Klastersky, 2003; Goldman and Koren, 2004). Furthermore, leukemic cells could affect renal tubular function increasing magnesium excretion (Miltiadous et al., 2008). Five patients (10%) from the Obstetrics and Gynecology department were included in our study where magnesium metabolism may be critical; reduction of serum magnesium can enhance uterine sensitivity, thus, helping to initiate delivery in late pregnancy. Moreover, serum magnesium deficiency plays a key role in eclampsia.

Strengths and Limitations

There are limitations of our study to be acknowledged; the study has been performed in a single center, we only have a single measurement of serum magnesium without serial measurements. This methodological limitation also pertains to other studies in this field (Kieboom et al., 2016; Wannamethee et al., 2018). Further, we are unable to be certain of the temporal association between hypomagnesemia and SCD because we were unable to obtain the clinical outcome of all patients. Indeed, we may have failed to obtain all subtle changes during the fluctuation of magnesium in these patients. Preclinical studies focus on the wedge preparations, single myocardial cells may reveal more details of pathological changes in the future.

The strengths of our study are inclusion of only isolated hypomagnesemia excluding other electrolyte disturbances, patients acting as their own controls, and the availability of much of the clinical picture. Since isolated hypomagnesemia is relatively rare, monitoring of serum electrolytes including magnesium is of great value in prompting the maintenance of electrolyte balance which may reduce the risk of life-threating arrhythmia and SCD. In clinical practice, hypomagnesemia which is also likely to lead to serious clinical conditions such as SCD is not scrutinized by physicians as much as hypokalemia. More detailed descriptions of ECG characteristics of hypomagnesemia may trigger greater attention to this abnormality. Furthermore, prolongation of Tpec and Tpe/QT ratio indicating increased dispersion of ventricular repolarization can bring more understanding of the relationship between hypomagnesemia and SCD. Further clinical trials are needed in order to determine the clinical benefit and ECG characteristics of magnesium supplementation.

CONCLUSION

In patients with isolated hypomagnesemia, P wave duration, QTc, Tpec, and Tpe/QT ratio suggesting atrial depolarization and ventricular repolarization dispersion were significantly increased compared with normal magnesium levels in the same patients after restoration to normal levels.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YX designed the study in which RS also played a part. YY and XY wrote the manuscript. CC, ST, SH, RT, YW, and RZ conducted the patient selection and the ECG measurements. YY maintained the database and conducted data analysis. XY and YD provided the electronic medical record database and reviewed the flow of study. RS, LG, and YX conducted the quality assurance and reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Interplay Between Systemic Metabolic Cues and Autonomic Output: Connecting Cardiometabolic Function and Parasympathetic Circuits

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There is consensus that the heart is innervated by both the parasympathetic and sympathetic nervous system. However, the role of the parasympathetic nervous system in controlling cardiac function has received significantly less attention than the sympathetic nervous system. New neuromodulatory strategies have renewed interest in the potential of parasympathetic (or vagal) motor output to treat cardiovascular disease and poor cardiac function. This renewed interest emphasizes a critical need to better understand how vagal motor output is generated and regulated. With clear clinical links between cardiovascular and metabolic diseases, addressing this gap in knowledge is undeniably critical to our understanding of the interaction between metabolic cues and vagal motor output, notwithstanding the classical role of the parasympathetic nervous system in regulating gastrointestinal function and energy homeostasis. For this reason, this review focuses on the central, vagal circuits involved in sensing metabolic state(s) and enacting vagal motor output to influence cardiac function. It will review our current understanding of brainstem vagal circuits and their unique position to integrate metabolic signaling into cardiac activity. This will include an overview of not only how metabolic cues alter vagal brainstem circuits, but also how vagal motor output might influence overall systemic concentrations of metabolic cues known to act on the cardiac tissue. Overall, this review proposes that the vagal brainstem circuits provide an integrative network capable of regulating and responding to metabolic cues to control cardiac function.

Keywords: autonomic, metabolic, parasympathetic, cardiovascular disease, brainstem, vagus

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INTRODUCTION

While sympathoexcitation may be widely accepted as a hallmark of the pathogenesis of cardiovascular disease, decreased parasympathetic, or vagal, tone is linked to a broad spectrum of diseases, including cardiac arrhythmias, coronary heart disease, and heart failure, and is an accurate predictor of morbidity and mortality in humans and animals (Barkai and Madacsy, 1995; La Rovere et al., 1998; Nolan et al., 1998; Thayer and Lane, 2007; Franciosi et al., 2017). Experiments conducted more than 150 years ago first established the anti-arrhythmogenic effect of

vagal stimulation (Lown and Verrier, 1976). With later seminal work in dogs demonstrating that blocking muscarinic acetylcholine receptors abolished this effect of vagal nerve stimulation, there is now convincing evidence that increased vagal nerve activity can ameliorate poor cardiac function (Schwartz et al., 1984; Vanoli et al., 1991).

Significant clinical evidence also implicates metabolic diseases (i.e., obesity and diabetes) as independent risk factors for the development of cardiovascular disease (Benjamin et al., 1994; Bonow and Eckel, 2003; Baek et al., 2017; Aune et al., 2018; Rawshani et al., 2018a,b). Notably, diseases of metabolism also associate with autonomic dysfunction (Barkai and Madacsy, 1995; Vinik et al., 2013). While the mechanism(s) linking metabolic disorders with cardiac dysfunction remain heavily debated, the role of the central autonomic nervous system in the orchestration of cardiometabolic homeostasis warrants a discussion on its potential role as a neuromechanistic link between metabolic signaling and cardiovascular function. While the heart as an isolated entity is important, it is critical to understand that it represents a single part of a larger system.

Although a large scale clinical investigation suggested that chronic vagal stimulation did not improve cardiac function in patients with heart failure (Zannad et al., 2015), this study emphasizes the need for better strategies to target efferent cardiac vagal output given the known disadvantages of activating non-cardiac vagal motor neurons and vagal sensory afferent fibers (Buckley et al., 2015). Therefore, understanding the neuronal modulation of cardiac activity could provide novel mechanistic details into the pathogenesis and treatment of cardiovascular disease. This idea is further reinforced since advanced neural modulation techniques have proven effective treatments for other cardiovascular diseases, including cardiac arrhythmias (Kapa et al., 2010; Shen et al., 2011).

This review then will focus on autonomic function in the context of cardiometabolic physiology, particularly as it relates to vagal motor output. Therefore, it will aim to define basic brainstem circuits and the influence of metabolic signaling on plasticity within these circuits. It will hopefully compel more investigations into how vagal motor output is both affected by and an effector of metabolic cues.

PARASYMPATHETIC CIRCUITS AND THEIR REGULATION OF HEART RATE

Ever since the first description of cardiac innervations in the 19th century (Hirsch, 1970), significant work has been done to map cardiac autonomic networks. Therefore, it is well established that the heart is innervated by two distinct branches of the autonomic nervous system, the sympathetic and parasympathetic. Despite the well acknowledged understanding of these two divisions, the paradigm generally used in textbooks and cardiology reviews overly simplifies the regulation of cardiac function as dependent almost exclusively on sympathetic activity. Therefore, our current dogma discounts the contributions of the parasympathetic nervous system to cardiac function. All of this despite consensus that in most vertebrates, including humans, the activity of myogenic pacemaker sinoatrial (SA) nodal cells is largely regulated by the tonic, inhibitory influence of parasympathetic motor output, making vagal tonus the predominant determinant of resting heart rate (Gordan et al., 2015).

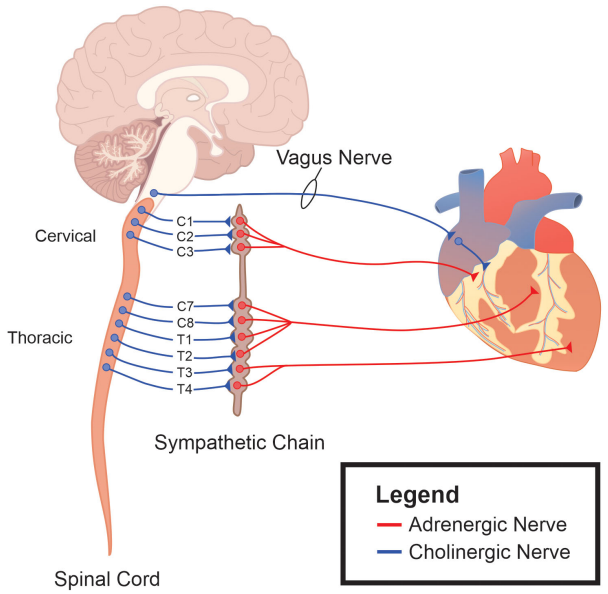

FIGURE 1 | The autonomic nervous system as an integrative control center for cardiac control. Sympathetic ganglia are located in the intermediolateral (IML) cell column of the thoracic spinal cord. The sympathetic ganglia send prominent projections to both cardiac tissue and the vascular system. Efferent parasympathetic, or vagal, originate within the brainstem and project to the epicardial fat sac in close apposition to cardiac tissue. It is these ganglia, along with their postganglionic fibers and their interconnections, that represent the final pathway for autonomic regulation of cardiac function.

Vagal motor innervation of cardiac tissue is comprised of cholinergic, preganglionic motor neurons whose cell bodies are located in the brainstem (**Figure 1**). These preganglionic neurons send their axons through the vagus nerve, and synapse onto intracardiac postganglionic motor neurons. Intracardiac vagal postganglionic neurons are also cholinergic, and traditionally thought to be subservient relay stations since the majority of these neurons lose their ability to generate spontaneous electrochemical activity when preganglionic motor neuron innervations are severed (Armour, 2008). Consequently, cardiac-related vagal efferent nerve activity is initiated at the soma of preganglionic cardiac vagal motor neurons, and alterations in their firing properties affect vagal nerve motor efferent output.

These preganglionic cardiac vagal motor neurons originate from two brainstem regions: the nucleus ambiguus (NA) and the dorsal motor nucleus of the vagus (DMV) (Standish et al., 1994; Massari et al., 1995; Cheng and Powley, 2000). The vast majority of cardiac innervation (approximately 80%) in higher mammals originates from motor neurons located in the NA. These NA cardiac vagal motor neurons uniformly exert a strong cardioinhibitory influence on heart rate (McAllen and Spyer, 1977; Geis and Wurster, 1980; Gilbey et al., 1984). Importantly, NA neurons are intrinsically silent, implicating synaptic input as a strong regulator of their overall activity (Mendelowitz, 1996). Cardiac vagal motor neurons in the NA are also critical to

cardiovascular disease development. Studies conducted in animal models of obstructive sleep apnea, for example, demonstrated that diminished vagal output activity and blunted baroreflex control of heart rate are due to changes in cardiac vagal motor neurons located in the NA, and not due to changes in intracardiac ganglia activity or innervation (Gu et al., 2007; Lin et al., 2007; Yan et al., 2009).

Little is known, however, about the innervation arising from the cardiac-projecting neurons residing within the DMV. Retrograde tracing studies indicate that cardiac motor neurons do originate in the DMV (Calaresu and Cottle, 1965; Sturrock, 1990; Standish et al., 1994), providing instrumental evidence of the existence of DMV cardiac-projecting motor neurons. However, while several studies reported cardioinhibitory activity from the DMV (Schwaber and Schneiderman, 1975; McAllen and Spyer, 1977), others suggest a lack of an effect on heart rate (Geis and Wurster, 1980). It is important to note that the majority of previous studies relied heavily on techniques with limited spatial precision and specificity. This is critical to our understanding of the DMV's contribution to heart rate since non-motor inhibitory interneurons exist within this nucleus as well (Jarvinen and Powley, 1999; Gao et al., 2009). Using techniques with improved specificity, like optogenetics, activation of DMV motor neurons increased cardiac ventricular contractility and enhanced exercise endurance in rodents (Machhada et al., 2017), protected ventricular cardiomyocytes from ischemic/reperfusion injury (Mastitskaya et al., 2012), and altered the electrical properties of cardiac tissue (Machhada et al., 2015). Importantly, these latter two results were independent of changes in heart rate, providing key experimental evidence that the DMV might be the source of vagal nerve-dependent coronary artery dilation (Reid et al., 1985; Kovach et al., 1995). However, these studies utilized a viral expression system for Phox2 cholinergic DMV neurons and could not distinguish between cardiac-projecting DMV neurons and those that project to other visceral organs. As discussed later in this review, DMV vagal motor neurons are critical in the regulation of metabolic cues, such as insulin and glucagon, and there is a possibility that these secondary humoral factors played a role. However, both anatomical and more traditional stimulation approaches do support a role for DMV activity in direct regulation of ventricular cardiomyocyte regulation (Dickerson et al., 1998; Lewis et al., 2001; Ulphani et al., 2010).

We also know relatively little in terms of the electrophysiological properties and upstream signaling network governing the activity of cardiac-projecting DMV neurons. While cardiac NA neurons have low resting membrane potentials and are silent when devoid of synaptic input, DMV neurons with other visceral organ targets exhibit a slow pace-making current (Browning et al., 1999). The presence of pace-making currents fundamentally alters the relationship of neuronal excitability and synaptic input. Therefore, if cardiac-projecting DMV neurons possess similar pace-making currents, these neurons could serve a unique role in cardiovascular autonomic regulation. In support of such a role, synaptic input to cardiac-projecting DMV neurons after heart failure undergoes unique signaling plasticity compared to cardiac-projecting NA neurons (Cauley et al., 2015). Still, given the existing controversy over the contribution of cardiac DMV motor neurons to cardiac regulation, future studies will continue to provide a more accurate depiction of cardiac parasympathetic innervation and regulation.

Central Brainstem Parasympathetic Circuits

Regardless of the location and electrophysiological properties of cardiac vagal motor neurons, upstream central brainstem signaling is critical to their final motor output. Centrally-mediated autonomic motor control of the cardiovascular system is sensitive to various sensory afferent information carried in large part by peripheral neurons located in the intrathoracic nodose ganglia, which synapse in the nucleus tractus solitarius (NTS) (**Figure 2**; Armour, 2008). First and second order NTS neurons integrate the excitatory, glutamatergic information from these peripheral afferents to influence the activity of downstream

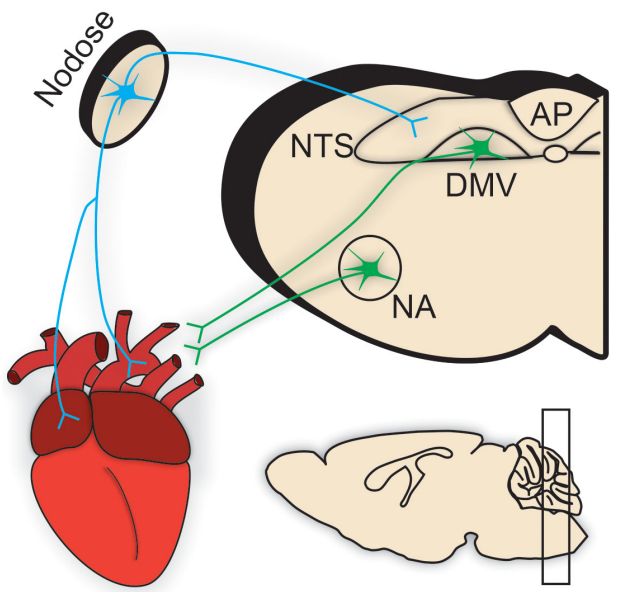

FIGURE 2 | Brainstem parasympathetic circuits. Efferent parasympathetic, or vagal, innervation (illustrated in green) to cardiac tissue originates from preganglionic brainstem motor neurons predominantly residing within the nucleus ambiguus (NA) and the dorsal motor nucleus of the vagus (DMV). These preganglionic neurons send their projections through the vagus nerve to synapse onto intracardiac parasympathetic ganglia located in close apposition to cardiac tissue [e.g., pacemaker nodal cells in the sinoatrial (SA) node].Conversely, sensory afferent information is carried by sensory neurons (shown in blue) located throughout the heart, especially in ventricular and atrial tissues, and aortic arch. Most prominent in cardiometabolic regulation are those vagal afferents in the intrathoracic nodose ganglia. Nodose ganglia afferent inputs synapse directly onto the nucleus tractus solitarius (NTS). NTS neurons [and the circumventricular organ, area postrema (AP)] integrate this sensory information from the heart (in addition to other peripheral sensory information from viscera important in the regulation of respiratory, gastrointestinal, and metabolic homeostasis). This integrated sensory information is either relayed to descending vagal motor neurons in a pathway termed "vago-vagal" reflexes or to upstream brain regions for further processing and integration. Therefore, the brainstem is a critical location for the orchestration of central motor control of cardiovascular function.

parasympathetic motor neurons. Traditionally, the caudal aspect of the NTS contains the majority of cardiovascular-related afferent synapses, following a general topographic organization (Loewy and Spyer, 1990). Important to cardiovascular regulation is the baroreceptor reflex. Increases in arterial blood pressure result in the activation of baroreceptors, which then convey this information to NTS neurons to initiate a reflexive decrease in heart rate. This is primarily achieved through increases in cardiac vagal nerve activity, and requires little to no inhibition of cardiac sympathetic nerve activity (Dun et al., 2004). Vagal motor output is also critical to other cardiovascular reflex responses including respiratory sinus arrhythmia (Dergacheva et al., 2010).

However, there is considerable overlap in visceral organ-based topography within the NTS with sensory afferent innervations from other organ systems (i.e., pulmonary stretch receptors (Katona and Jih, 1975; Taylor et al., 2014) and arterial chemoreceptors (Accorsi-Mendonca and Machado, 2013), including subdiaphragmatic organs involved in metabolic regulation (Browning et al., 1999; Browning and Travagli, 2011; Taylor et al., 2014). The NTS and its neighboring circumventricular organ, area postrema (AP), are also implicated as central metabolic sensors, including neurons that respond to insulin (Ruggeri et al., 2001; Blake and Smith, 2012), glucose (Balfour et al., 2006; Lamy et al., 2014; Boychuk et al., 2015a; Roberts et al., 2017), ghrelin (Cui et al., 2011), and leptin (Barrera et al., 2011). Importantly, the ability of these brain regions to directly sense metabolic state can influence peripheral physiology (Ritter et al., 2000; Ferreira et al., 2001; Lamy et al., 2014; Boychuk et al., 2019). For example, in terms of cardiometabolic behavior, insulin microinjections into the NTS decreased the activity of baroreceptor-sensitive NTS neurons (McKernan and Calaresu, 1996; Ruggeri et al., 2001), and despite only limited effects on resting heart rate (McKernan and Calaresu, 1996; Krowicki et al., 1998), insulin in the NTS significantly reduced the baroreflex response (McKernan and Calaresu, 1996; Ruggeri et al., 2001). Similar interactions likely occur with other metabolic signals since leptin has also been implicated in a reduction of baroreflex responses (Arnold and Diz, 2014). Therefore, information related to metabolic status is quickly and efficiently integrated into cardiovascular regulatory networks within the dorsal hindbrain. These integrated circuits are likely evolutionary mechanisms developed to allow for gross matching of cardiac output to both metabolism and respiration (Castro et al., 2003; Srivastava, 2012; Taylor et al., 2014). Therefore, the brainstem represents an integrative network of neurons responsible for sensing systemic cardiorespiratory and metabolic states and coordinating motor neuron control of such systems, resulting ultimately in the maintenance of internal homeostasis.

THE INFLUENCE OF METABOLIC DISRUPTIONS ON PARASYMPATHETIC FUNCTION

Metabolic dysregulation (i.e., obesity and diabetes) is an independent risk factor for the development of cardiovascular disease (Benjamin et al., 1994; Bonow and Eckel, 2003; Baek et al., 2017; Aune et al., 2018; Rawshani et al., 2018a,b). While metabolic disorders come with complex multisystem morbidities, longitudinal studies conducted in human patients have long indicated that vagal dysfunction is first in the autonomic dysfunction sequelae and occurs prior to overt cardiac complications (Ewing et al., 1980; Vinik et al., 2011) or induction of fasting hyperglycemia (Wu et al., 2007). Despite consensus that autonomic dysfunction is a hallmark of most cardiovascular diseases, the precise role of autonomic cardiac-related activity in the context of metabolic disruption remains debated. This section will discuss the role of two important metabolic cues, diet and inflammation, and their potential to alter vagal regulatory circuits.

High Fat Diet as a Metabolic Disruption

The nuances surrounding different diets and their relationship to cardiovascular disease are best reviewed elsewhere (Sacks et al., 2017). However, it has long been recommended that individuals wishing to reduce their risk of cardiovascular disease should reduce dietary saturated fatty acid consumption. This evidence includes randomized clinical trials reporting improvements in the incidence of poor cardiac outcomes, including sudden death, after reductions in saturated fat intake (Dayton et al., 1962; Leren, 1970). Importantly, animal models exposed to high dietary fat content mimic several characteristics of cardiovascular disease (Dobrian et al., 2000), and while the mechanisms mediating the effects of high fat diet are still up for debate, it remains possible that the effects of high fat diets on vagal circuits contribute to the development of cardiovascular disease.

In animal models, diets high in saturated fats can induce tachycardia (Van Vliet et al., 1995; Dobrian et al., 2001; Bruder-Nascimento et al., 2017), even if only a mild tachycardia (Carroll et al., 2006). Moreover, in studies that failed to identify this tachycardia, there was still significant evidence for reduced cardiac vagal tone (Williams et al., 2003; Chaar et al., 2016). This reduction in vagal drive includes an abolishment of vagal responsivity during the baroreflex (Van Vliet et al., 1995). Historically, decreased cardiac vagal tone during disease (purely cardiovascular or metabolic in nature) is attributed to vagal neuropathy (Bolinder et al., 2002; Horowitz et al., 2002). However, emerging evidence from other peripheral nerve systems demonstrates that lack of neuronal activity itself can eventually lead to neuronal degeneration and neuropathy (Gibson et al., 2014). Similar experimental evidence has not been examined for vagal motor output, but at the very least, the reduced baroreflex activity appears consistent with a reorganization of central cardiovascular circuits that results in a lack of vagal motor output early in disease progression (McCully et al., 2012).

Unfortunately, the mechanism(s) responsible for vagal circuit reorganization of the brain regions involved are still largely unknown. There are now substantial data implicating vagal afferents and the NTS as important sites in the effects of high fat diet in the context of feeding regulation (de Lartigue et al., 2011), suggesting it as a possible location for the effects of high fat diet on cardiovascular regulation. To our knowledge, only a limited number of studies examine the role of high fat

diet in the regulation of cardiac-projecting vagal motor neuron excitability and function. However, these studies do support the idea that reduced vagal drive originates from reduced vagal motor activity. High fat diet decreases c-Fos expression in the NA, suggesting a reduction in neuronal activation compared to normal chow controls (Alsuhaymi et al., 2017). Although to date there is no data on the effects of high fat diet on cardiac-projecting DMV neurons, high fat diet for 12 weeks reduces gastric-projecting DMV motor neuron excitability as measured by whole-cell patch-clamp techniques (Browning et al., 2013). Similarly, perinatal exposure to high fat diet increases GABAergic inhibitory synaptic signaling in DMV neurons (McMenamin et al., 2018; Clyburn et al., 2019). Interestingly, other conditions of metabolic dysregulation of vagal motor neurons suggest that altered synaptic signaling occurs through inappropriately low trafficking of select synaptic receptor populations out of the cellular membrane (Zsombok et al., 2011; Boychuk et al., 2015b), but this mechanism has yet to be confirmed for high fat diet or cardiac-projecting vagal motor neurons. Taken together, it remains possible that decreased neuronal activity within vagal motor neurons themselves eventually leads to reductions in vagal motor efferent drive.

The case for early inhibition of the vagal motor system can only be made through longitudinal evaluations of consumption of high fat diets (that includes earlier timepoints in the feeding paradigms). While a significant amount of research is appropriately dedicated to investigating the role of long-term high fat diet and ultimately the obesity that follows long term consumption of these diets, there is increasing evidence that consumption of high fat for a few days can affect neuronal function. Part of this reevaluation includes the emerging concept that metabolic challenges lead to neural adaptions, rendering the brain insensitive to future metabolic cues (Beutler et al., 2020; Mazzone et al., 2020). Importantly, some aspects of adaptive neural plasticity occur quickly, for example after a single bout of exercise (He et al., 2018). There are several investigations suggesting similar effects likely exist in vagal circuits. For example, patients with type 2 diabetes first show impaired glucose intolerance during the vagally-mediated component of insulin release (Gallwitz et al., 2013), and reducing dietary fat intake increases cardiac vagal activity even in non-obese patients (Pellizzer et al., 1999). In animal studies, DMV vagal motor neurons exhibit increased glutamatergic neurotransmission after short term (less than 5 days) high fat consumption (Clyburn et al., 2018). Although it has not been determined if this increase in glutamatergic signaling can drive disease pathogenesis or is compensatory in nature (for say elevated inhibitory signaling), it does confirm that high fat diet alters synaptic signaling to vagal efferent motor neurons earlier than previously considered. Reduced parasympathetic motor activity during early disease progression would not be unique to metabolic dysfunction. Reductions in parasympathetic tone occurred early and often preceded increased sympathetic activity in both animal models of and patients with heart failure (Ishise et al., 1998; Motte et al., 2005). Therefore, these types of investigations into the early influence of diets may help elucidate the neuromechanism(s) through which high fat diet contributes to the pathogenesis of cardiovascular disease (Fleming, 2002; Hartnett et al., 2015).

Inflammation as a Metabolic Disruption

Chronic inflammation is also considered a significant risk factor for the development of cardiovascular disease (de Kloet et al., 2013). Of particular importance is the role of neuroinflammation through brainstem autonomic mechanisms. As the brain's resident immune cells (Ransohoff and Brown, 2012), microglia play an important role in this signaling within the brainstem. For example, microglial activation is noted within the NTS in both experimentally-induced type-1 diabetes (Rana et al., 2014) and high fat diet animal models (Minaya et al., 2020). Additionally, vagal afferents are directly activated by proinflammatory signals (Besedovsky et al., 1986; Waise et al., 2015), suggesting that in addition to direct effects on neurons residing within the brainstem, inflammation can modulate autonomic feedback control mechanisms by modulation of vagal afferent signaling.

Another emerging modulator of central cardiovascular regulatory networks is the most abundant glial cell in the central nervous system, the astrocyte (Martinez and Kline, 2021). Originally identified for their importance in the generation of the blood brain barrier, astrocytes not only contain multiple immune receptors, but respond to a number of immune signals (Colombo and Farina, 2016). Astrocytic activity in the brainstem can influence cardiovascular function (Martinez et al., 2020). Importantly, activation of astrocytes is linked to cardiovascular dysregulation after high fat diet consumption (Worker et al., 2020), and astrocyte signaling is required for high fat diet-induced hyperphagia and obesity (Douglass et al., 2017).

Indirect pathways of neuroinflammatory activation are also implicated in cardiovascular (dys)function. The renin-angiotensin system (RAS) is classically considered an endocrine regulator of the cardiovascular system. However, it is now recognized that components of the RAS system are present within the brain, including the brainstem (Cuadra et al., 2010), and RAS signaling is elevated during metabolic disorders (Giacchetti et al., 2005). Convincing evidence now exists suggesting that central RAS signaling is critical to the development of hypertension through sympathetic activation of peripheral inflammation (de Kloet et al., 2013). However, convincing evidence now implicates the activation of vagal signaling in reducing the inflammatory response (Pavlov and Tracey, 2012), and the DMV was recently identified as the critical brain region for the generation of this response (Kressel et al., 2020). Moreover, elevated RAS signaling also increases the permeability of the blood-brain barrier (Biancardi et al., 2014). Therefore, brainstem autonomic circuits are likely exposed to other systemically circulating factors (e.g., inflammatory cytokines). Therefore, it remains possible that reduced vagal signaling promotes the inflammation typically seen during chronic metabolic conditions, like diabetes and obesity.

THE INFLUENCE OF PARASYMPATHETIC SIGNALING ON METABOLIC CUES

To fully understand the role of perturbations in metabolic cues on cardiac function, it is important to understand the role of autonomics in mediating the concentrations and sensitivities of

metabolic hormones with known cardiac modulatory abilities. It is worth mentioning that much of our understanding of the role of metabolic signaling perturbations has been insulin-centric. However, it is becoming evident that metabolic diseases come with a complex hormonal milieu. As such, there have been calls for a more diverse focus, such as on glucagon (Unger and Cherrington, 2012; Lee et al., 2014). Therefore, this section will focus on the role of the parasympathetic nervous system to regulate the concentration of and sensitivity to key metabolic cues which have established roles in cardiac regulation, namely insulin, glucagon, and GLP-1.

Insulin

First discovered more than 100 years ago, insulin is secreted from pancreatic beta cells in response to high blood glucose concentrations and its role in metabolic dysregulation is well established. Insulin receptors are widely expressed throughout the cardiovascular system, including cardiac tissue (Wang et al., 1997; Muniyappa et al., 2007; Riehle et al., 2014). Insulin resistance in cardiac tissue is suggested as a major risk factor for cardiovascular disease (Paternostro et al., 1996), and insulin resistance appears in cardiovascular diseases not explicitly related to energy homeostatic changes in metabolism, like heart failure (Mori et al., 2012; Zhang et al., 2013). Elevated sympathetic nervous system activity has been linked to the development of insulin resistance in cardiac tissue (Morisco et al., 2005; Mangmool et al., 2016), and to our knowledge similar studies have not been conducted for vagal signaling in cardiac tissue. This is a significant gap in knowledge since vagal signaling promotes insulin sensitivity in other peripheral organs, and the loss of vagal activity associates with insulin resistance (Xie et al., 1993; Xie and Lautt, 1994, 1995, 1996), leading some to conclude that vagal activity is critical to overall insulin sensitivity (Lautt, 1999).

The parasympathetic nervous system also plays an important role in the secretion of insulin. Preganglionic vagal innervation to the pancreas originates from the DMV, with little known contribution from the NA (Berthoud and Powley, 1990; Love et al., 2007; Rodriguez-Diaz et al., 2011; Chandra and Liddle, 2013). These DMV preganglionic fibers terminate on cholinergic intrapancreatic ganglia that influence pancreatic beta cells to release insulin (Thorens, 2014). However, intrapancreatic vagal ganglia also signal through non-adrenergic non-cholinergic (NANC) pathways such as nitric oxide or vasoactive intestinal polypeptide signaling (Wang et al., 1999; Love et al., 2007; Di Cairano et al., 2016) and these pathways can trigger insulin release as well (Mussa et al., 2011). While vagal motor innervation to the pancreas is relatively limited (Berthoud and Powley, 1990), there is evidence that abnormal, vagally-mediated insulin release is an early marker of diabetes (Gallwitz et al., 2013), suggesting that this may ultimately contribute to the pathogenesis of cardiovascular disease with strong metabolic connections.

Glucagon

Serving in opposition to insulin (Cherrington and Vranic, 1971), glucagon is secreted from pancreatic alpha cells in response to low blood glucose concentrations and works to increase glucose production in and secretion from the liver. In humans, a single bolus of glucagon increases heart rate (Parmley et al., 1968), which has made it a useful therapy for reversing the effects of many cardioinhibitory drugs (White, 1999). However, hyperglucagonemia is present in metabolic disorders, including type 1 and 2 diabetes (Muller et al., 1973; Ichikawa et al., 2019) and has received renewed attention in terms of its role in the hyperglycemia associated with these conditions. Therefore, the influence of vagal activity on glucagon secretion may also influence cardiac regulation.

Unlike insulin, however, our understanding of how the parasympathetic nervous system regulates glucagon secretion is more controversial. Despite evidence of parasympathetic innervation to the pancreas, very few studies have dissected out the precise innervation to alpha cells themselves (Rodriguez-Diaz et al., 2011). Using more functional approaches, there is evidence that similar to insulin, vagal stimulation increases glucagon release (Ionescu et al., 1983; Ahren and Taborsky, 1986; Berthoud et al., 1990). This has been proposed as the mechanism behind the cephalic response to food consumption when glucagon is released to prevent an insulin-induced drop in blood glucose before food is ingested (Berthoud and Powley, 1990). More recent experiments continue to link increased glucagon concentration with increased vagal nerve bundle activity through brainstem glucose sensing mechanisms (Lamy et al., 2014). However, attempts to dissect out efferent vs. afferent vagal stimulation have suggested that increases in blood glucose levels (as would occur when glucagon is released) are achieved by efferent inhibition not activation (Meyers et al., 2016). Moreover, reduced preparations such as whole-cell patch-clamp combined with *in vivo* glucagon measures also suggest that inhibition of DMV motor activity increases glucagon concentrations (Boychuk et al., 2019). Taken together, these latter data provide evidence that parasympathetic efferent tone might serve as a brake for glucagon secretion. Therefore, the renewed interest in investigations into the role of glucagon in glucose homeostasis must continue to dissect out the autonomic nervous system's contribution to its regulation since these types of studies likely have importance far beyond just glucose homeostasis.

Glucagon-Like Peptide

The small peptide hormone glucagon-like peptide-1 (GLP-1) is an incretin hormone produced from L-cells within the intestine and released during digestion of fat and carbohydrates (Drucker, 2001). Upon release, GLP-1 acts to increase gastric volume, inhibit gut motility, and increase insulin secretion (Lim and Brubaker, 2006). GLP-1 receptors are also present in cardiac tissue (Pyke et al., 2014; Baggio et al., 2018) and GLP-1 signaling in cardiac tissue results in increased heart rate *in vivo* (Hayes et al., 2008; Baggio et al., 2017) and *in vitro* (Zhao, 2013; Ang et al., 2018).

However, the action of GLP-1 in cardiac regulation may be more complex (Ussher and Drucker, 2012). There is consensus that vagal signaling positively influences the release of GLP-1 (Anini and Brubaker, 2003) since either pharmacological inhibition of vagal signaling or vagotomy reduces serum concentration of GLP-1 (Rocca and Brubaker, 1999; Anini and Brubaker, 2003). However, acute bolus of GLP-1 induces a

tachycardia through activation of GLP-1 receptors in central sympathetic regulatory networks, and not through activity at cardiac tissue itself (Hayes et al., 2008; Ghosal et al., 2013; Baggio et al., 2017). Moreover, GLP-1 receptor agonist injected into the NA not only decreased indices of cardiac vagal activity, but also depressed neurotransmission to cardiac vagal motor neurons (Griffioen et al., 2011), suggesting that NA neurons could also mediate the GLP-1-induced tachycardia. Therefore, while vagal activation likely supports the release of GLP-1, GLP-1 may negatively feedback on cardiac regulation to decrease vagal drive and induce sympathoexcitation. Adding further complexity, despite inducing a tachycardia and sympathoexcitation, GLP-1 mimetics not only improve glucose intolerance but provide cardioprotective benefits (Barnett, 2012; Zhao, 2013; Del Olmo-Garcia and Merino-Torres, 2018). These paradoxical effects of GLP-1 signaling could be the result of species-specific biology, as well as differences in approaches and outcomes tested. Since the cardioprotective nature of GLP-1 mimetics is typically examined after long term exposure, it is also possible that GLP-1 has multiple tissue specific intracellular signaling cascades based on time of exposure or concentration (Jessen et al., 2017; Tomas et al., 2020).

CONCLUSION

Given the long history of both basic science and clinical investigations into cardiac autonomic function, it can be easy to assume that we fully understand how these systems work. While we know a great deal about the anatomy of these circuits, how they process the wide variety of complex signals they receive and ultimately integrate and relay this information to peripheral organs, such as the heart, is still under active investigation. Despite the scarcity of studies investigating the effect of metabolic signaling on cardiac vagal motor neuron physiology, reports have confirmed the therapeutic potential—although varied in magnitude—of activating vagal pathways, most notably through vagal stimulation. Although these results may provide a mechanistic rationale for the importance of cardiac-related vagal tone and health, more studies investigating the plasticity within autonomic regulatory circuits related to various metabolic cues are needed to better understand the fundamental role of vagal signaling in metabolic and cardiovascular physiology.

During this discussion, it is also important to consider that the plasticity associated with a disease may not simply be an exaggeration of normal physiology. Therefore, considerable work must be done to determine the role of the brain in mediating cardiometabolic integrative homeostasis in both health and disease. These examinations into autonomic contributions to cardiometabolic function need to include time courses throughout disease progression. This will determine when important neuronal remodeling occurs, revealing important biological milestones for intervention. Continued investigation into these autonomic pathways will not only increase our understanding of these circuits, but will develop a more informed perspective that will influence current clinical treatment guidelines for patients to provide early and reliable detection markers of autonomic dysregulation, as well as a more complete management of a patient's disease and the prevention of cardiac-related morbidity and mortality.

AUTHOR CONTRIBUTIONS

LE, SF, and CB wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Cardiomyocyte Deletion of *Bmal1* Exacerbates QT- and RR-Interval Prolongation in *Scn5a*$^{+/\Delta KPQ}$ Mice

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Circadian rhythms are generated by cell autonomous circadian clocks that perform a ubiquitous cellular time-keeping function and cell type-specific functions important for normal physiology. Studies show inducing the deletion of the core circadian clock transcription factor *Bmal1* in adult mouse cardiomyocytes disrupts cardiac circadian clock function, cardiac ion channel expression, slows heart rate, and prolongs the QT-interval at slow heart rates. This study determined how inducing the deletion of *Bmal1* in adult cardiomyocytes impacted the *in vivo* electrophysiological phenotype of a knock-in mouse model for the arrhythmogenic long QT syndrome (*Scn5a*$^{+/\Delta KPQ}$). Electrocardiographic telemetry showed inducing the deletion of *Bmal1* in the cardiomyocytes of mice with or without the ΔKPQ-*Scn5a* mutation increased the QT-interval at RR-intervals that were ≥130 ms. Inducing the deletion of *Bmal1* in the cardiomyocytes of mice with or without the ΔKPQ-*Scn5a* mutation also increased the day/night rhythm-adjusted mean in the RR-interval, but it did not change the period, phase or amplitude. Compared to mice without the ΔKPQ-*Scn5a* mutation, mice with the ΔKPQ-*Scn5a* mutation had reduced heart rate variability (HRV) during the peak of the day/night rhythm in the RR-interval. Inducing the deletion of *Bmal1* in cardiomyocytes did not affect HRV in mice without the ΔKPQ-*Scn5a* mutation, but it did increase HRV in mice with the ΔKPQ-*Scn5a* mutation. The data demonstrate that deleting *Bmal1* in cardiomyocytes exacerbates QT- and RR-interval prolongation in mice with the ΔKPQ-*Scn5a* mutation.

Keywords: heart, electrophysiology, ion channel, SCN5A, long QT syndrome, *Bmal1*, circadian

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INTRODUCTION

Circadian rhythms alter physiology in anticipation of predictable changes in the daily environment. They are generated by cell autonomous circadian clocks in most of the cells in the body (Reppert and Weaver, 2002; Hastings et al., 2018; Michel and Meijer, 2020). Circadian clocks are formed by transcription-translation feedback loops that drive rhythmic changes in clock gene and protein expression with a periodicity of ∼24 h (Hastings et al., 2018; Michel and Meijer, 2020). The positive limb of the feedback loop is initiated by the transcription factors BMAL1 and CLOCK, which

heterodimerize to activate the transcription of Period (PER) and Cryptochrome (CRY). PER and CRY proteins negatively feedback on BMAL1 and CLOCK activity. In addition to functioning as ubiquitous cellular timekeepers, circadian clocks also contribute to cell- and tissue-specific changes in physiology by regulating the expression of genes outside the timekeeping network. Studies using transgenic mouse models that allow for the selective deletion of *Bmal1* in adult cardiomyocytes show the cardiomyocyte circadian clock mechanism contributes to heart rate, ventricular repolarization and the functional expression of several cardiac ion channels (Schroder et al., 2013, 2015; Delisle et al., 2020). In this study, we determined how inducing the deletion of *Bmal1* in adult cardiomyocytes impacted cardiac electrophysiology in a genetic mouse model of long QT syndrome (LQTS).

People living with congenital LQTS have a high risk for ventricular arrhythmias that can lead to syncope, seizures, and/or sudden cardiac death (SCD) (Schwartz et al., 1993; Moss, 2003). Most cases of LQTS are caused by mutations in one of three different cardiac ion channel genes (LQT1-LQT3). LQT3 is caused by mutations in the predominant voltage-gated Na+ channel expressed in the heart (*SCN5A*/Nav1.5). Some LQT3-linked mutations associate with more than one type of arrhythmia syndrome/phenotype in people, including cardiac conduction defects, atrial arrhythmias, and/or Brugada Syndrome (Remme, 2013). People who have the LQT3-linked *SCN5A* deletion mutation ΔKPQ1505-1507 can suffer LQT3 and cardiac conduction defects (Zareba et al., 2001). Heterozygous knock-in mice with the equivalent ΔKPQ-*Scn5a* mutation (*Scn5a*$^{+/\Delta KPQ}$) have abnormally long QT- and RR-intervals (Nuyens et al., 2001; Fabritz et al., 2010; Lemoine et al., 2011; Wu et al., 2012). We tested the hypothesis that inducing the deletion of *Bmal1* in *Scn5a*$^{+/\Delta KPQ}$ mice would exacerbate QT- and RR-interval prolongation using electrocardiographic (ECG) telemetry.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee at University of Kentucky. Mice for these studies were bred by crossing the floxed *Bmal1* (*Bmal1*$^{f/f}$) mouse and the cardiac-specific, *Myh6-MerCreMer* recombinase mouse (iCSΔ*Bmal1*) (Schroder et al., 2013). The *Scn5a*$^{+/\Delta KPQ}$ mice (kindly provided by Dr. Peter Carmeliet) were bred with the iCSΔ*Bmal1* mice to generate an B6Cre$^{\pm}$;B6*Bmal1*$^{f/f}$;129*Scn5a*$^{+/\Delta KPQ}$ (iCSΔ*Bmal1/Scn5a*$^{+/\Delta KPQ}$) mouse. The iCSΔ*Bmal1*$^{+/+}$ mice consisted of vehicle injected mice or iCSΔ*Bmal1* mice assessed prior to tamoxifen injection. *Cre*-recombination was activated by intraperitoneal injections of tamoxifen (2 mg/day) for 5 consecutive days to generate iCSΔ*Bmal1*$^{-/-}$ mice. The concentration and duration of the tamoxifen injections used have been shown to cause effective cardiomyocyte-specific recombination without any obvious long-term tamoxifen toxicity as assessed by changes in the structure, function, and the ECG (Eckardt et al., 2004; Andersson et al., 2009; Hall et al., 2011; Schroder et al., 2013). Mice were housed in 12 h light and 12 h dark cycles with *ad libitum* access to food and water.

Circadian Collections

mRNA collections were done as described previously (Schroder et al., 2013). Male and female iCSΔ*Bmal1* mice at 14–16 weeks of age were housed in light boxes and entrained to a 12:12-h light-dark cycle. Two weeks after the final injection of vehicle (32 mice) or tamoxifen (32 mice), mice were released into constant darkness. After 30 h in darkness (18 h after the beginning of the subjective light phase, Circadian Time or CT = 18 h), mice were euthanized under dim red light (< 5 lux) and hearts were collected every 4 h for 28 h, a total of 8 time points. RNA was prepared for quantitative PCR (qtPCR) using TaqMan (Applied Biosystems) assays to examine transcript expression. The ΔΔCT method was used for the quantification of qtPCR data. Gene expression is shown as the relative value compared with the mean vehicle value.

ECG Telemetry

In vivo ECG telemetry was performed as described previously (Schroder et al., 2013, 2014). Briefly, male mice at 14 weeks of age were anesthetized with isoflurane and transmitter units (DSI, TA11ETA-F10) were implanted in the peritoneal cavity. The two ECG leads were secured near the apex of the heart and the right acromion. Mice were housed singly and allowed to recover for 1 week before beginning recording. Telemetry data were recorded at 1000 Hz before and after the intraperitoneal injection of tamoxifen to activate deletion of *Bmal1* by *Cre*-recombination. After injection, mice were given at least 4 weeks to recover before ECG data were recorded.

Ponemah telemetry software (Data Science International) was used to quantify QT-intervals. QT-intervals were analyzed at RR-intervals ranging from 90 ± 3 ms to 140 ± 3 ms in 10 ms bins. Two independent investigators performed these analyses.

Ponemah was also used to measure RR-intervals and HRV. We plotted 3-days of averaged RR-intervals (15-min averages) and fit the individual data with the following cosine function:

$$Interval = A*cos(2\pi(t-\tau)/T) + m$$

This allowed calculation of the period (T), phase (τ), amplitude of the oscillation (A), and rhythm-adjusted mean (m).

HRV was analyzed using the time domain parameters for the standard deviation of all RR-intervals in sinus rhythm (SDNN, in ms); the root mean square differences between successive RR-intervals (RMSSD, in ms); and the percentage of normal consecutive RR-intervals differing by ≥ 6 ms similar to that previously described (Thireau et al., 2008; Fenske et al., 2016; Moghtadaei et al., 2017). SDNN is an index of total sinus rhythm HRV, whereas RMSSD is a beat to beat index of HRV and reflects abrupt changes in RR-intervals. PNN6 is thought to reflect HRV secondary to parasympathetic tone. Each parameter was measured in 15-min episodes. Individual mouse data sets

were not well described using a cosine function, so we compared averaged values that corresponded to 2–5, 8–11, 14–17, or 20–23 h after the start of the light phase (Zeitgeber time or ZT = 2–5, 8–11, 14–17, or 20–23 h, respectively).

Statistical Analysis

The data were analyzed using a two-way ANOVA to identify significant interactions (PRISM, MathWorks). We used the Šidák correction method to correct for multiple comparisons. For gene expression studies, the statistical JTK_CYCLE package was used to identify mRNA transcripts that had circadian expression profiles (Hughes et al., 2010).

RESULTS

Inducing the Deletion of *Bmal1* in Adult Cardiomyocyte Decreases the mRNA Transcript Levels for Several Cardiac Ion Channels and Transcription Factors Important for Repolarization and Conduction

Studies suggest that *Bmal1* directly and indirectly contributes to the transcription of cardiac ion channel genes *Scn5a*, *Kcnh2*, *Hcn4*, and *Kchip2* (Jeyaraj et al., 2012; Schroder et al., 2013, 2015; D'Souza et al., 2020). *Bmal1* may regulate cardiac ion channel transcription by binding to enhancer box (E-box) elements in the promoters of ion channel genes, or *Bmal1* can modify the expression of other transcription factors that regulate ion channel transcription. We wanted to determine how inducing the deletion of *Bmal1* in the adult heart impacts the expression profiles for a large number of candidate ion channel genes that encode proteins important for cardiac depolarization, repolarization, conduction, and ion channel transcription in humans and mice (London, 2001; Eckardt et al., 2004; Nerbonne and Kass, 2005; Arnolds et al., 2012; Herrmann et al., 2012; Jeyaraj et al., 2012; Mao et al., 2012; Cai et al., 2014; Nerbonne, 2014; Wahl-Schott et al., 2014; Tarradas et al., 2017).

In mice and humans, the cardiac Na^+ current (I_{Na}) generates the rapid upstroke of the action potential in the working myocardium and the funny current (I_F) initiates depolarization in the autorhythmic myocardium. *Scn5a and Hcn4* encode the major pore-forming proteins that conduct I_{Na} and I_F (Abriel, 2007; Kozasa et al., 2018; D'Souza et al., 2020). Also similar to humans, the inward rectifier K^+ current (I_{K1}) in the mouse working myocardium is responsible for stabilizing the resting membrane potential. *Kcnj2* encodes a critical pore-forming subunit that conducts I_{K1} (Nerbonne and Kass, 2005; Nerbonne, 2014). Unlike humans, the rapid and slowly activating delayed rectifier K^+ currents (I_{Ks} and I_{Kr}, respectively) are not major contributors to ventricular repolarization in mice. However, the genes that encode the pore-forming proteins for I_{Ks} and I_{Kr}, *Kcnq1,* and *Kcnh2*, are expressed in the mouse myocardium (Pond et al., 2000; Knollmann et al., 2007). Ventricular repolarization in mice is regulated by several K^+ currents, including the transient outward K^+ currents (I_{to}) and the slow K^+ currents ($I_{K,slow}$). *Kcnd2*, *Kchip2*, *Kcna5*, and *Kcnb1* encode important pore-forming and auxiliary ion channel proteins that contribute to I_{to} and $I_{K,slow}$ (Nerbonne and Kass, 2005; Nerbonne, 2014). *Gja1*, *Gja5*, and *Gjc1* encode the cardiac gap junction proteins (connexins) that mediate conduction between cardiomyocytes (Jalife et al., 1999). The *Scn4b* auxiliary Na^+ channel subunit is a genetic modifier of cardiac conduction speed in mouse models of Na^+ channelopathies (Remme et al., 2009). The transcription factor genes *Klf15*, *Tbx5*, *Gata4*, and *Foxo1* regulate the expression of certain cardiac ion channel genes, including *Kchip2* or *Scn5a*, previous studies show that these transcription factors have circadian expression profiles in the mouse heart (Arnolds et al., 2012; Jeyaraj et al., 2012; Mao et al., 2012; Pizarro et al., 2013; Cai et al., 2014; Tarradas et al., 2017).

We previously reported the *Scn5a, Kcnh2,* and *Kcnd2* mRNA transcript expression profiles in iCSΔ*Bmal1*$^{+/+}$ and iCSΔ*Bmal1*$^{-/-}$ mouse hearts (Schroder et al., 2013, 2015). We found that *Scn5a*, *Kcnh2 and Kcnd2* had circadian expression profiles in iCSΔ*Bmal1*$^{+/+}$ mouse hearts. The circadian expression profiles for *Scn5a* and *Kcnh2* (but not *Kcnd2*) were lost after inducing the deletion of *Bmal1* (iCSΔ*Bmal1*$^{-/-}$), and, compared to mRNA transcript levels in iCSΔ*Bmal1*$^{+/+}$ mouse hearts, the mRNA transcript levels for *Scn5a* and *Kcnh2* (but not *Kcnd2*) were reduced in iCSΔ*Bmal1*$^{-/-}$ mice.

In this study, *Hcn4*, *Kcnj2*, *Kcnq1*, *Kchip2*, *Kcna5*, *Kcnb1*, *Gja1*, *Gja5*, *Gjc1*, *Scn4b*, *Klf15*, *Tbx5*, *Gata4*, and *Foxo1* transcripts were assessed for both the circadian expression and overall expression level by quantitative PCR. These data demonstrate that *Hcn4, Kcnj2, Kcnq1, Scn4b*, *Tbx5*, *Gata4, Gja5,* and *Gjc1* mRNA transcript levels have circadian expression profiles in the hearts of iCSΔ*Bmal1*$^{+/+}$ mice but not in iCSΔ*Bmal1*$^{-/-}$ mice (**Figures 1A,B**). Compared to mRNA transcript levels in iCSΔ*Bmal1*$^{+/+}$ mouse hearts, the mRNA transcript levels in the hearts of iCSΔ*Bmal1*$^{-/-}$ mice for *Hcn4, Kcnj2, Kcnq1, Scn4b*, *Gata4*, and *Tbx5* (but not *Gja5* or *Gjc1*) were reduced.

Klf15, *Kchip2*, *Kcna5*, *Kcnb1*, *Gja1*, and *Foxo1* mRNA transcripts did not show a circadian expression profile in the hearts of iCSΔ*Bmal1*$^{+/+}$ or iCSΔ*Bmal1*$^{-/-}$ mice (**Figures 1C,D**). However, compared to mRNA transcript levels in the hearts of iCSΔ*Bmal1*$^{+/+}$ mice, the mRNA transcript levels for *Klf15*, *Kchip2*, *Kcna5*, and *Kcnb1* in iCSΔ*Bmal1*$^{-/-}$ mice were reduced (**Figure 1C**). The mRNA transcript levels for *Gja1* and *Foxo1* in iCSΔ*Bmal1*$^{+/+}$ and iCSΔ*Bmal1*$^{-/-}$ mice were not different (**Figure 1D**). Together, these findings demonstrate that the loss of *Bmal1* in adult cardiomyocytes causes a loss of circadian expression and/or a reduction in overall mRNA transcript levels for several cardiac ion channels and transcription factors important for cardiac depolarization, repolarization and conduction.

Inducing the Deletion of *Bmal1* Increases the QT-Interval at Slow Heart Rates

The changes in cardiac ion channel expression that occurred after inducing the deletion of *Bmal1* in adult cardiomyocytes motivated us to determine how it would impact the cardiac

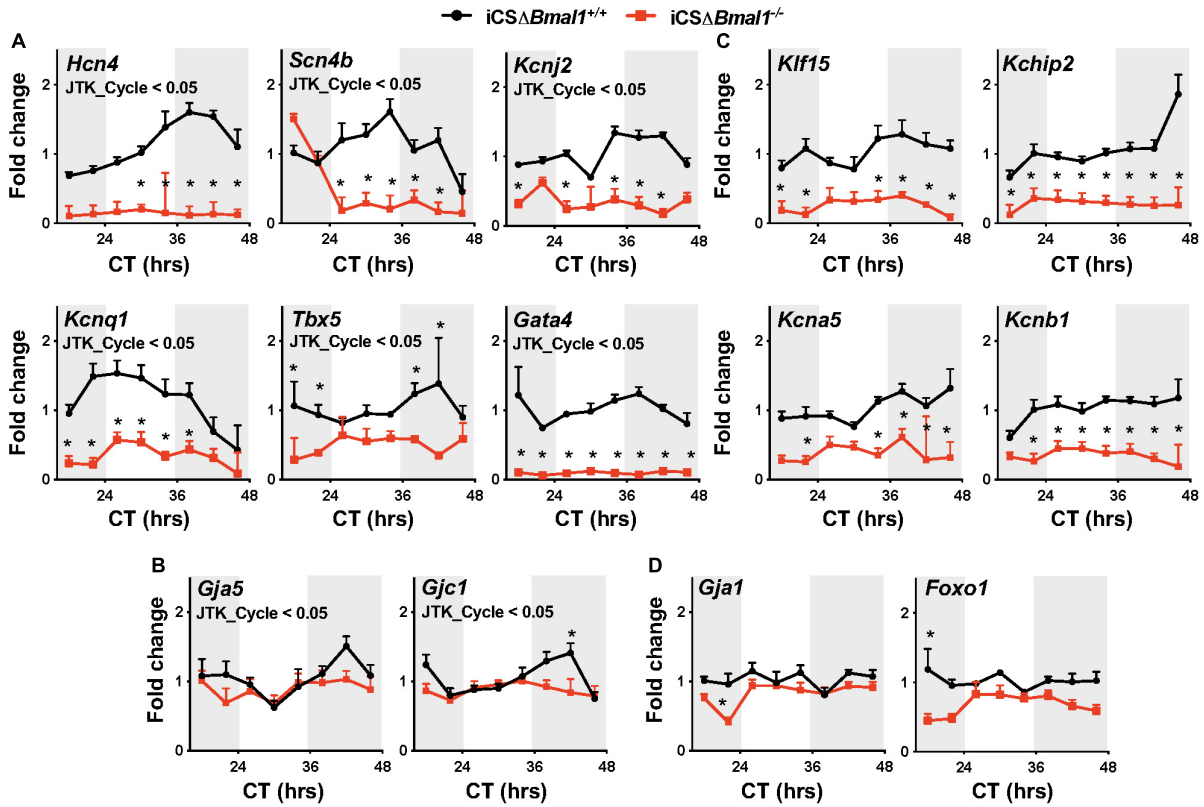

FIGURE 1 | Inducing the deletion of *Bmal1* in adult cardiomyocytes differentially impacts the temporal expression of cardiac ion channel transcripts. Shown are qPCR profiles for mRNA transcripts measured from iCSΔ*Bmal1*$^{+/+}$ (black circles) and iCSΔ*Bmal1*$^{-/-}$ (red squares) mouse hearts plotted as a function of circadian time (CT). Light and shaded regions on x-axis represent subjective light and dark cycles. **(A)** *Hcn4*, *Scn4b, Kcnj2*, *Kcnq1*, *Tbx5*, and *Gata4* mRNA transcripts had circadian expression profiles in the hearts of iCSΔ*Bmal1*$^{+/+}$ mice (JTK_Cycle < 0.05) but not iCSΔ*Bmal1*$^{-/-}$ mice. mRNA expression was lower at most time points in the hearts of iCSΔ*Bmal1*$^{-/-}$ mice when compared to iCSΔ*Bmal1*$^{+/+}$ mouse hearts (n = 3–4/time point; *p < 0.05). **(B)** *Gja5* and *Gjc1* mRNA transcripts had circadian expression profiles in iCSΔ*Bmal1*$^{+/+}$ mouse hearts (JTK_Cycle < 0.05) but not iCSΔ*Bmal1*$^{-/-}$mouse hearts. The overall transcript levels were similar in iCSΔ*Bmal1*$^{+/+}$ and iCSΔ*Bmal1*$^{-/-}$ mice at most circadian time points (n = 3-4/time point; *p < 0.05). **(C)** *Klf15*, *Kchip2*, *Kcna5*, and *Kcnb1* mRNA transcripts did not have circadian expression profiles in the hearts of iCSΔ*Bmal1*$^{+/+}$ or iCSΔ*Bmal1*$^{-/-}$ mice. (JTK_Cycle > 0.05). The overall transcript levels were lower in iCSΔ*Bmal1*$^{-/-}$ mouse hearts compared to transcript levels in iCSΔ*Bmal1*$^{+/+}$ mouse hearts at most circadian time points (n = 3–4/time point; *p < 0.05). **(D)** *Gja1* and *Foxo1* mRNA transcripts did not have circadian expression profiles in the hearts of iCSΔ*Bmal1*$^{+/+}$ or iCSΔ*Bmal1*$^{-/-}$ mice (JTK_Cycle > 0.05) and the overall transcript levels were similar at most circadian time points (n = 3–4/time point; *p < 0.05).

electrophysiological phenotype of mice with the ΔKPQ-*Scn5a* mutation. We used ECG telemetry to measure QT- and RR-intervals in conscious free moving mice housed in 12-h light and 12-h dark cycles prior to and after inducing *Bmal1* deletion in cardiomyocytes (**Figure 2A**). Previous studies show that the QT-intervals measured from wildtype C57BL/6 mice do not depend on the preceding RR-intervals (Drici et al., 1998; Speerschneider and Thomsen, 2013; Roussel et al., 2016). We confirmed these findings in the iCSΔ*Bmal1*$^{+/+}$ mice by measuring the QT-intervals at RR-intervals ranging from 90 to 140 ms in 10 ms bins (**Figure 2B**). Deletion of *Bmal1* in cardiomyocytes caused the QT-intervals to become longer at slower RR-intervals. Compared to iCSΔ*Bmal1*$^{+/+}$ mice, the iCSΔ*Bmal1*$^{-/-}$ mice had longer QT-intervals at RR-intervals that were ≥ 130 ms.

QT-intervals in mice with the ΔKPQ-*Scn5a* mutation were dependent on the duration of preceding RR-intervals (**Figure 2B**). Compared to the QT-intervals measured in the iCSΔ*Bmal1*$^{+/+}$ mice, the QT-intervals in iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice were longer at RR-intervals that were ≥ 110 ms. Compared to the iCSΔ*Bmal1*$^{-/-}$ mice, the iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ mice had longer QT-intervals at RR-intervals that were ≥ 100 ms. Inducing the deletion of *Bmal1* in mice with the ΔKPQ-*Scn5a* mutation also increased the QT-interval at RR-intervals that were ≥ 130 ms. These data demonstrate that *Bmal1* deletion in adult cardiomyocytes prolongs the QT-interval at slow heart rates in mice with or without the ΔKPQ-*Scn5a* mutation.

Inducing the Deletion of *Bmal1* Increases the Day/Night Rhythm-Adjusted Mean in *the* RR-Interval in Mice With or Without the ΔKPQ-*Scn5a* Mutation

We quantified the day/night rhythm in the RR-interval recorded from mice before and after inducing the deletion of *Bmal1*

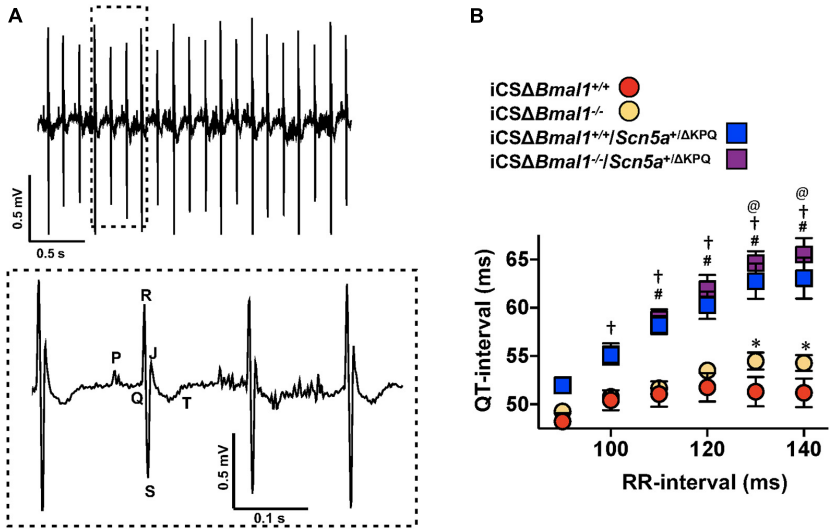

FIGURE 2 | Deletion of *Bmal1* in adult cardiomyocytes prolongs the QT-interval at longer RR-intervals. **(A)** Representative electrocardiogram trace measured from the mice used in this study. The inset shows the dashed box region on the ECG trace in more detail to highlight the P, Q, R, S, J, and T waves. **(B)** The mean QT-interval plus standard error of the mean measured between 90 and 140 ms in iCSΔ*Bmal1*$^{+/+}$ (red circles), iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ (blue squares), iCSΔ*Bmal1*$^{-/-}$ (orange circles) or iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ (purple squares) mice is shown (n = 6 and n = 8, respectively). We compared QT intervals at each RR-interval between iCSΔ*Bmal1*$^{+/+}$ and iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ ($^{\#}p < 0.05$); iCSΔ*Bmal1*$^{-/-}$ or iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ ($^{\dagger}p < 0.05$); iCSΔ*Bmal1*$^{+/+}$and iCSΔ*Bmal1*$^{-/-}$ ($^{*}p < 0.05$); and iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ and iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ ($^{@}p < 0.05$).

in adult cardiomyocytes with or without the ΔKPQ-*Scn5a* mutation. Mice were housed in 12 h light and 12 h dark cycles. The RR-intervals were averaged every 15-min and plotted as a function of ZT for 3 days (**Figure 3A**). The individual data from each mouse were fit with a cosine function to calculate the day/night period, acrophase (the ZT at which the rhythms peaked), amplitude, and rhythm adjusted mean (**Figure 3B**). The day/night rhythm in the RR-intervals had periods that were ∼24 h in mice before and after deletion of *Bmal1* in cardiomyocytes with or without the ΔKPQ-*Scn5a* mutation. The day/night rhythm in the RR-intervals peaked after the beginning of the light cycle and did not change after deletion of *Bmal1* in the cardiomyocytes of mice with or without the ΔKPQ-*Scn5a* mutation. The amplitudes in the day/night rhythm of the RR-intervals were not different before or after deletion of *Bmal1* in cardiomyocytes in both groups of mice, however, the iCSΔ*Bmal1*$^{-/-}$ mice had larger amplitudes compared to iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ mice. The biggest differences among the groups of mice were in day/night rhythm-adjusted means of the RR-intervals. Compared to iCSΔ*Bmal1*$^{+/+}$ mice, the rhythm-adjusted means for the RR-intervals were longer in iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice. Inducing the deletion of *Bmal1* in cardiomyocytes increased the rhythm-adjusted means for both groups of mice by similar amounts (**Figure 3B**). These data demonstrate that deletion of *Bmal1* in cardiomyocytes does not impact the period, phase or amplitude in the day/night rhythm in RR-intervals in mice with or without the ΔKPQ-*Scn5a* mutation. Deletion of *Bmal1* in adult cardiomyocytes prolongs the day/night rhythm-adjusted mean in the RR-intervals of mice with or without the ΔKPQ-*Scn5a* mutation.

Mice With the ΔKPQ-*Scn5a* Mutation Have Reduced HRV That Is Normalized After Inducing the Deletion of *Bmal1*

Heart rate variability measures changes in sinoatrial node function secondary to autonomic drive, autonomic sensitivity of the sinoatrial nodal cells, and/or changes in the spontaneous depolarization of SAN cells (Fenske et al., 2016). We tested the hypothesis that the HRV in the iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice was different than the iCSΔ*Bmal1*$^{+/+}$ mice. We measured the 3-day average in the SDNN, RMSSD, and pNN6 at ZT = 2-5 h, ZT = 8–11 h, ZT = 14–17 h, or ZT = 20–23 h. We found that when RR-intervals were longest (ZT = 2–5 h), the SDNN, RMSSD and pNN6 were smaller in the iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice compared to iCSΔ*Bmal1*$^{+/+}$ mice (**Figure 4**). Compared to the iCSΔ*Bmal1*$^{+/+}$ mice, the HRV did not change in the iCSΔ*Bmal1*$^{-/-}$ mice at any of the timepoints tested. Compared to iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice, the iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ mice had SDNN values that increased at all the timepoints tested; RMSSD values that increased at ZT = 2–5 h and ZT = 20–23 h; and pNN6 values that increased at ZT = 2–5 h, ZT = 8–11 h, and ZT = 20–23 h. The data demonstrate that inducing the deletion of *Bmal1* in the cardiomyocytes of mice with the ΔKPQ-*Scn5a* mutation normalize the HRV to levels similar to those seen in iCSΔ*Bmal1*$^{+/+}$ or iCSΔ*Bmal1*$^{-/-}$ mice.

DISCUSSION

This study showed that the deletion of *Bmal1* in adult cardiomyocytes disrupts the expression profiles for a number

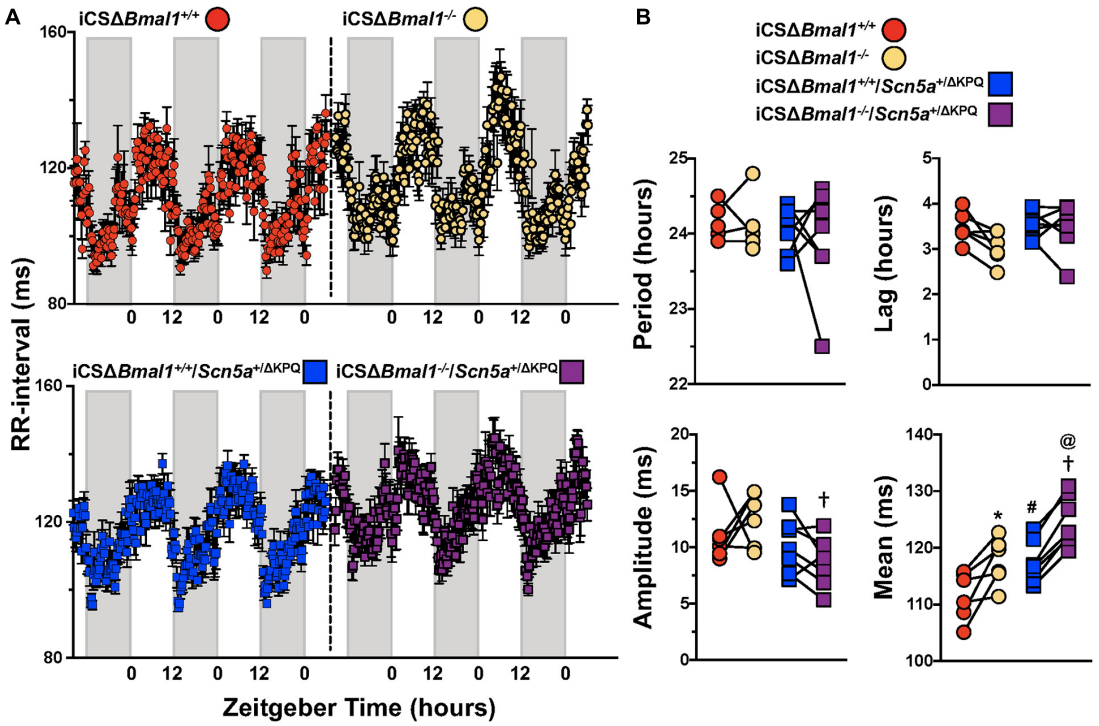

FIGURE 3 | Deletion of *Bmal1* in adult cardiomyocytes increases the rhythm adjusted mean of the RR-interval. **(A)** RR intervals were averaged in 15-min bins over 3 days for iCSΔ*Bmal1*$^{+/+}$ (red circles) and iCSΔ*Bmal1*$^{-/-}$ (orange circles) (top graph, $n = 6$) or iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ (blue circles) and iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ (purple circles) mice (bottom graph, $n = 7$). Gray shaded regions on x-axis represent lights off. **(B)** The data for each mouse were fit to cosine curves to analyze period, lag, amplitude and median. The graphical representation of the change in period, amplitude and slope for iCSΔ*Bmal1*$^{+/+}$ (red circles) and iCSΔ*Bmal1*$^{-/-}$ (orange circles) and iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ (blue squares) and iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ (purple squares) mice are shown. Analysis showed that the rhythm adjusted mean between iCSΔ*Bmal1*$^{+/+}$ and iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ ($^{\#}p < 0.05$) were different; the amplitude and rhythm adjusted mean for iCSΔ*Bmal1*$^{-/-}$ or iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ were different ($^{\dagger}p < 0.05$); the rhythm adjusted mean for iCSΔ*Bmal1*$^{+/+}$and iCSΔ*Bmal1*$^{-/-}$ were different ($^{*}p < 0.05$); and the rhythm adjusted mean for iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ and iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ were different ($^{@}p < 0.05$).

of cardiac ion channel and transcription factor transcripts important for normal cardiac repolarization, depolarization, and conduction. We tested the hypothesis that the deletion of *Bmal1* in adult cardiomyocytes would modify the cardiac electrophysiological phenotype in mice that harbor the ΔKPQ-*Scn5a* mutation *in vivo*. We found that the mice with the ΔKPQ-*Scn5a* mutation had abnormally long QT-intervals, steeper QT- and RR-interval relations, slower day/night rhythm-adjusted means in RR-intervals and decreased HRV. Inducing the deletion of *Bmal1* in the hearts of mice with the ΔKPQ-*Scn5a* mutation prolonged the QT-intervals measured at slower RR-intervals and increased the day/night rhythm-adjusted mean in RR-interval. The absolute magnitude of these changes was similar in mice without the ΔKPQ-*Scn5a* mutation, suggesting that the changes caused by deletion of *Bmal1* in cardiomyocytes were additive. The deletion of *Bmal1* in cardiomyocytes did not alter HRV in mice without the ΔKPQ-*Scn5a* mutation, but it increased the abnormally low HRV in mice with the ΔKPQ-*Scn5a* mutation. We conclude that deletion of *Bmal1* in cardiomyocytes disrupts the expression for a number of cardiac ion channel genes, prolongs the QT- and RR-intervals in mice with or without the ΔKPQ-*Scn5a* mutation, and normalizes HRV in mice with the ΔKPQ-*Scn5a* mutation.

Bmal1 Is Important for Normal Ventricular Repolarization at Slow Heart Rates

Inducing the deletion of *Bmal1* prolongs the QT-intervals at slower heart rates in mice with and without the ΔKPQ-*Scn5a* mutation (**Figure 2**). This might not have been expected in mice with the ΔKPQ-*Scn5a* mutation since previous studies show that inducing the deletion of *Bmal1* in adult cardiomyocytes decreases the functional expression of *Scn5a* by ∼30% (Schroder et al., 2013). We now show that *Bmal1* directly or indirectly contributes to the expression of many different cardiac transcripts that regulate normal depolarization, repolarization and conduction in mice (**Figure 1**; Jeyaraj et al., 2012; Schroder et al., 2015; Delisle et al., 2020). Thus, the cardiac electrophysiological properties likely do not reflect the changes in one gene but rather many different genes.

The Day/Night Rhythm in Heart Rate *in vivo* Does Not Depend on the Cardiomyocyte Circadian Clock

Inducing the deletion of *Bmal1* did not change the phase, amplitude or period in the day/night rhythm of heart rate in mice

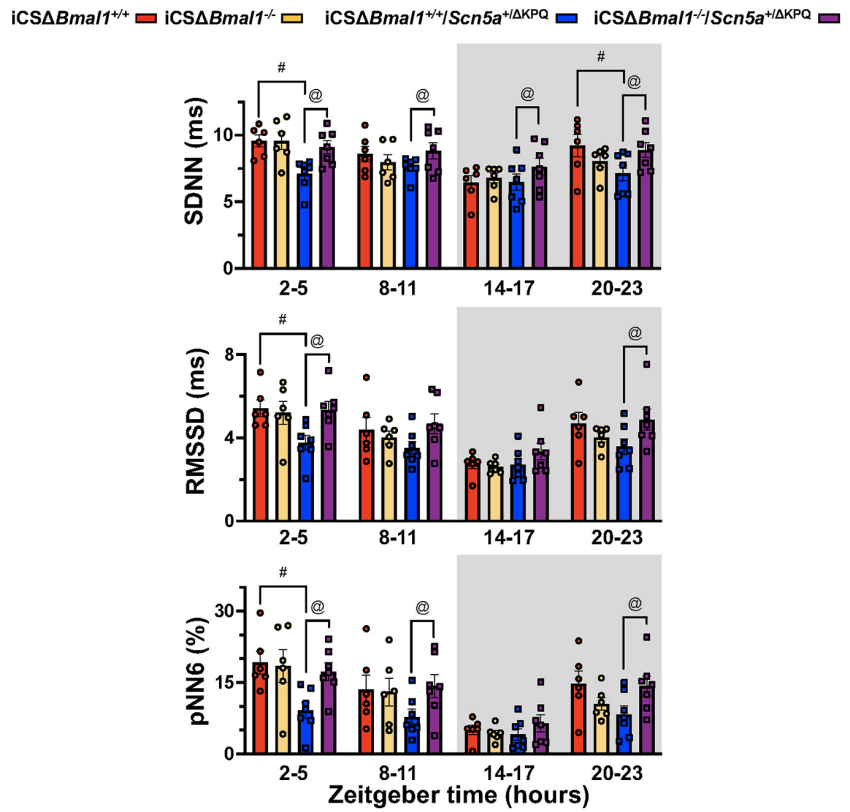

FIGURE 4 | Mice with the ΔKPQ-*Scn5a* mutation have reduced HRV and this is increased by inducing the deletion of *Bmal1* in adult cardiomyocytes. The graphical representation of the change SDNN (top graph), RMSD (middle graph) or pNN6 (bottom graph) for iCSΔ*Bmal1*$^{+/+}$ (red bars, circles), iCSΔ*Bmal1*$^{-/-}$ (orange bars, circles), or iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ (blue bars, circles), and iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ (purple bars, circles) mice measured at ZT = 2–5, 8–11, 14–17, and 20–23 h are shown (n = 6 and n = 7, respectively). Analysis detected differences in the SDNN, RMSSD and PNN6 between iCSΔ*Bmal1*$^{+/+}$ and iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice ($^{\#}p < 0.05$) at certain timepoints, as well as differences in the SDNN, RMSSD and PNN6 between iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ and iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ mice ($^{@}p < 0.05$).

with or without ΔKPQ-*Scn5a* mutation (**Figure 3**; Schroder et al., 2013). These data provide additional support that the day/night rhythm in heart rate *in vivo* does not depend on the functional circadian clock mechanism in cardiomyocytes but rather other factors including the autonomic nervous system, nocturnal behavioral patterns, core body temperature, circadian rhythms in neurohumoral signaling, etc (Sheward et al., 2010; Tong et al., 2013; Schroder et al., 2014). This study confirms previous findings that the deletion of *Bmal1* in adult cardiomyocytes slows the day/night rhythm-adjusted mean in heart rate (Schroder et al., 2013). The slowing of the mean heart rate after inducing the deletion of *Bmal1* was observed in mice with or without the ΔKPQ-*Scn5a* mutation.

The Reduction in HRV in Mice With ΔKPQ-*Scn5a* Is Not Secondary to Slower Heart Rates

Another novel finding in this study is that, compared to mice without the ΔKPQ-*Scn5a* mutation, mice with the ΔKPQ-*Scn5a* mutation had a lower HRV when heart rate was slowest. The lower HRV likely reflects sinoatrial node dysfunction, changes in the autonomic sensitivity of the sinoatrial node and/or changes in autonomic tone (Fabritz et al., 2010; Wu et al., 2012). Although studies have not shown changes in the autonomic sensitivity of the sinoatrial node in *Scn5a*$^{+/\Delta KPQ}$ mice, studies do show that, compared to WT mice, the working myocardium of *Scn5a*$^{+/\Delta KPQ}$ mice expresses fewer β-adrenergic receptors (Fabritz et al., 2010). Another possibility is that the reduction in HRV measured in the iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice reflects differences in basal heart rate compared to the iCSΔ*Bmal1*$^{+/+}$ mice. However, this seems unlikely because HRV increases as the heart rates become slower (Monfredi et al., 2014). The increase in HRV normally seen at slower heart rates might explain why HRV increased in the iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ mice (**Figures 3**, **4**). Another possible explanation for the effect on HRV is the recent observation that the loss of *Bmal1* in the heart alters the intrinsic beating rate of sinoatrial node preparations (D'Souza et al., 2020). The time of day changes in sinoatrial node intrinsic beating rate were suggested to be secondary to a reduction in the functional expression of the *Hcn4*. Consistent with these studies, we found that cardiac *Hcn4* mRNA transcripts in the hearts of iCSΔ*Bmal1*$^{+/+}$ mice had a circadian expression profile that was lost and reduced iCSΔ*Bmal1*$^{-/-}$ mice

(**Figure 1**). Whether or not the decreased expression of *Hcn4* mRNA transcript levels and/or other cardiac ion channel genes is responsible for the slower rhythm-adjusted mean in heart rate or increased HRV in mice with the ΔKPQ-*Scn5a* mutation warrants further investigation.

Study Limitations

There are several limitations to this study. Mouse models are widely employed by cardiac electrophysiologists because they are practical for determining how genetic, pharmacological and/or environmental manipulations impact arrhythmogenic triggers and/or pro-arrhythmic changes in the cardiac substrate (Dobrev and Wehrens, 2018; Clauss et al., 2019). However, there are clear species-specific differences in cardiac electrophysiology that limit extrapolation to humans (Sabir et al., 2008; Nerbonne, 2014). Mouse hearts are small and beat about 10 times faster than humans. Although human and mouse ECG waveforms have P, QRS, and T-waves, mouse hearts repolarize quickly to generate a predominant J wave and a small inverted T-wave (Mitchell et al., 1998; Kaese and Verheule, 2012; Boukens et al., 2014). As such, the underlying mechanisms for cardiac excitability and arrhythmias in mouse and human hearts are different (Sabir et al., 2008).

We did not observe any obvious spontaneous ventricular symptoms (e.g., ventricular tachyarrhythmias) in iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ or iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice during the ECG recordings (∼6-9 days). Therefore, we cannot conclude that inducing the deletion of *Bmal1* in cardiomyocytes increases arrhythmogenicity in mice with the ΔKPQ-*Scn5a* mutation. However, similar to the Nuyens et al., 2001 study (Nuyens et al., 2001), we did see that mice harboring the ΔKPQ-*Scn5a* mutation had a higher number of sinus pauses, atrioventricular block and bradyarrhythmias. The absolute number of hourly pauses/blocks during the inactive phase trended higher in the iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ mice compared to the iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ mice but was not significant (iCSΔ*Bmal1*$^{+/+}$/*Scn5a*$^{+/\Delta KPQ}$ = 6.7 ± 2.5 pauses/blocks per hour vs iCSΔ*Bmal1*$^{-/-}$/*Scn5a*$^{+/\Delta KPQ}$ = 20.3 ± 7.8 pauses/blocks per hour, n = 6 mice, p = 0.06). More invasive studies are needed to determine whether the loss of the *Bmal1* in mice with the ΔKPQ-*Scn5a* mutation have higher susceptibility to arrhythmias and/or ventricular tachyarrhythmias.

Conclusion

In summary, this study shows inducing the deletion of *Bmal1* in mouse hearts exacerbates the prolongation in the QT- and RR-intervals in mice with the ΔKPQ-*Scn5a* mutation. Specifically, it increases the abnormal prolongation in the QT-intervals at slower heart rates and causes an overall slowing of the heart rate over 24-h. These effects combine to increase the absolute number of abnormal QT-intervals during the 24-h cycle. However, due to the low absolute probability of ventricular symptoms, we cannot conclude it increases the likelihood of ventricular tachyarrhythmias. Future studies that explore the molecular mechanisms with which *Bmal1* functions to limit QT-interval prolongation at slow heart rates might lead to the identification of novel therapeutic targets.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Association for Assessment and Accreditation of Laboratory Animal Care and was approved by the Institutional Animal Care and Use Committee at University of Kentucky.

AUTHOR CONTRIBUTIONS

ES, KE, CE, and BD contributed and developed the research design. ES, JW, KS, SS, DB, and BD worked on data acquisition and analyses. ES and TS generated the animals and performed the experiments. ES, JW, KS, SS, DB, TS, CE, KE, and BD wrote, edited, and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Thyroid Hormone Plays an Important Role in Cardiac Function: From Bench to Bedside

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Thyroid hormones (THs) are synthesized in the thyroid gland, and they circulate in the blood to regulate cells, tissues, and organs in the body. In particular, they exert several effects on the cardiovascular system. It is well known that THs raise the heart rate and cardiac contractility, improve the systolic and diastolic function of the heart, and decrease systemic vascular resistance. In the past 30 years, some researchers have studied the molecular pathways that mediate the role of TH in the cardiovascular system, to better understand its mechanisms of action. Two types of mechanisms, which are genomic and non-genomic pathways, underlie the effects of THs on cardiomyocytes. In this review, we summarize the current knowledge of the action of THs in the cardiac function, the clinical manifestation and parameters of their hemodynamics, and treatment principles for patients with hyperthyroid- or hypothyroid-associated heart disease. We also describe the cardiovascular drugs that induce thyroid dysfunction and explain the mechanism underlying the thyroid toxicity of amiodarone, which is considered the most effective antiarrhythmic agent. Finally, we discuss the recent reports on the involvement of thyroid hormones in the regulation of myocardial regeneration and metabolism in the adult heart.

Keywords: thyroid hormone, hyperthyroidism, hypothyroidism, cardiovascular disease, genomic pathways, non-genomic pathways, amiodarone, cardiac regeneration

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INTRODUCTION

The thyroid gland secretes two thyroid hormones (THs), 3,5,3′-triiodothyronine (T3) and 3,5,3′,5′−tetraiodothyronine (T4 also known as thyroxine). Moreover, THs are synthesized using iodine, influence metabolism, and biosynthesize proteins in the body. These THs are regulated by thyroid stimulating hormone (TSH), which is secreted by the anterior pituitary gland. In turn, TSH is regulated by the hypothalamus via thyrotropin-releasing hormone (TRH). Thyroid hormones exhibit a variety of effects on the heart and peripheral vascular system It is well known that they raise the heart rate and cardiac contractility, improve the systolic and diastolic function of the heart, and decrease the systemic vascular resistance (SVR) in resting condition (Klein and Ojamaa, 2001).

Thyroid dysfunction, which causes hyperthyroidism and hypothyroidism, is associated with increased cardiovascular risk factors (Klein and Ojamaa, 2001; Rodondi et al., 2010; Collet et al., 2012). For example, hyperthyroidism increases the risk of atrial fibrillation (AF), cardiovascular disease (CVD), and heart failure (HF) (Biondi, 2012). Conversely, hypothyroidism is associated with hypertension and dyslipidemia and also causes CVD (Rodondi et al., 2010; Pearce, 2012). In particular, the intracellular effects of THs in cardiomyocytes occur via two types of mechanisms, genomic and non-genomic, with the genomic pathway predominating (Khan et al., 2020). The details of the mechanisms are described in Section 3.

There has been a long controversy regarding whether increased cardiovascular risk is related to thyroid dysfunction (Langén et al., 2018) and whether there is an association between thyroid disorder and the risk of sudden cardiac death (SCD) (Chaker et al., 2016). Sudden cardiac death is an unexpected death or arrest from a cardiovascular cause that occurs outside a hospital or in the emergency room (Lopshire and Zipes, 2006). The major cause of SCDs is lethal ventricular arrhythmias in patients with underlying coronary heart disease (Weisfeldt et al., 2011; Hayashi et al., 2015). Moreover, SCD also develops within 1 h. Over 50% of SCD cases are due to coronary hearts diseases (Hayashi et al., 2015), and these account for almost 20% of the total mortality.

Several groups have reported a relationship between thyroid function and SCD. Charker et al. concluded that elevated free T4 levels might increase the risk of SCD in patients with euthyroid thyroid disease, studied in a prospective population-based cohort (Chaker et al., 2016). Mitchell et al. investigated whether patients with HF with a reduced ejection fraction (HFrEF) and a thyroid functional disorder had an increased risk of SCD (Mitchell et al., 2013). They concluded that a thyroid dysfunction in patients with symptomatic HF and an ejection fraction $\leq$ 35% had a strong positive correlation with risk of death. Similar results were obtained after adjusting for known mortality predictors [Sudden Cardiac Death in Heart Failure Trial (SCD-HeFT)] (Mitchell et al., 2013). Langén et al. reported that thyroid dysfunction could be related to an increased overall mortality and risk of SCD and that large-scale randomized control trials are essential to decide whether to treat patients with mild thyroid insufficiency (Langén et al., 2018).

In this review, we summarize the effects of TH on the heart (Klein and Ojamaa, 2001) and the clinical symptoms of thyroid dysfunction from the viewpoint of the cardiology (Klein and Danzi, 2007). In addition, we discuss the changes in and the mechanisms of TH metabolism that have an influence on arrhythmias and congestive HF (Danzi and Klein, 2014). Further, we specify the cardiovascular drugs that induce thyroid dysfunction and explain the mechanism underlying the thyroid toxicity of amiodarone, which is considered the most effective antiarrhythmic agent.

EFFECTS OF THYROID HORMONES ON THE CARDIOVASCULAR SYSTEM

In the thyroid gland, two main iodinated hormones, T3 (triiodothyronine) and T4, (tetraiodothyronine; also known as thyroxine) are secreted. By binding to thyroid hormone receptors (TRs), T3 and T4 exert biological activity in responsive tissues. T3 is regarded as a biologically active hormone (Jabbar et al., 2017), and T4 has a few documented non-genomic effects but is largely regarded as a prohormone. Most T4 is deiodinated to T3 in the liver, kidneys, and skeletal muscle (Klein and Danzi, 2007). T3 is carried through blood circulation to each target tissue and organ such as the heart and peripheral blood vessels. Then, these tissues and organs are regulated by serum levels of T3 solely or preponderantly (Danzi and Klein, 2014).

Symptoms of hyperthyroidism include cardiac and hemodynamic symptoms, such as palpitations, widened pulse pressure, dyspnea on exertion, tachycardia, exercise intolerance, and AF (**Table 1**) (Dahl et al., 2008). Cardiac contractility, as well as resting heart rate, is increased by THs. Cardiac output can increase by 50–300% under hyperthyroidism compared to that in normal conditions. This enhancement of cardiac output is based on synergistic effects of a raised heart rate, increased cardiac contractility, and dilation of peripheral blood vessels (Biondi et al., 2002).

The cardiovascular symptoms of hypothyroidism are not obvious, in contrast to the distinct clinical manifestations of hyperthyroidism. Multiple characteristic phenotypes of hypothyroidism have been described, including bradycardia, diastolic hypertension, narrow pulse pressure, fatigue, myalgia, elevated cholesterol, and a feeling of puffiness (**Table 1**), but the serum TSH level is an accurate diagnostic indicator of hypothyroidism (Dahl et al., 2008). The cardiovascular effects of hypothyroidism significantly differ from those of hyperthyroidism; for example, cardiac output can be reduced by 30 to 50%, compared to that in a normal state (Danzi and Klein, 2004). However, it is significant that the treatment of hypothyroidism can normalize cardiovascular hemodynamics with a slight change in the resting heart rate (Crowley et al., 1977).

Abbreviations: AF, atrial fibrillation; AIT, amiodarone-induced thyrotoxicosis; AKT, serine/threonine-protein kinase; AMI, acute myocardial infarction; ANCA, anti-neutrophil cytoplasmic antibodies; ATD, antithyroid drugs; CBZ, carbimazole; CVD, cardiovascular disease; DUSP5, specific dual-specificity phosphatase 5; EC coupling, excitation-contraction coupling; ECG, electrocardiogram; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; ES, electrical storm; GD, Graves' disease; HF, heart failure; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; HIF-1α, Hypoxia-Inducible Factor alpha; IGF-1, insulin-like growth factor-1; IV, intravenous administration; JNK2α2, c-Jun N-terminal kinase-2α2; LT3S, low T3 syndrome; MAPK, mitogen-activated protein kinase; MMI, methimazole; MMP, matrix metalloproteinase; mH_2O_2, mitochondria-generated H_2O_2; mtRXR, mitochondrial RXR; MYH, myosin heavy chain; NCX, Na + /Ca2 + exchanger; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PLN, phospholamban; PTU, propylthiouracil; PWV, pluse wave velocity; RAI, radioactive iodine; RXR, retinoid X receptor; SCD, sudden cardiac death; SERCA2, sarcoplasmic/endoplasmic reticulum calcium ATPase 2; SVR, systemic vascular resistance; SVR, synergistic effects of reducing; TdP, torsade de pointes; TGB, thyroxine binding globulin; TH, thyroid hormone; TR, thyroid hormone receptor; TRE, thyroid hormone response element; TRH, thyrotropin-releasing hormone; VT, Ventricular tachycardia.

TABLE 1 | Cardiovascular clinical manifestations and laboratory findings associated with hyperthyroidism and hypothyroidism (Danzi and Klein, 2014).

Hyperthyroidism				
Palpitations	Anginal chest pain	Exercise intolerance	Atrial fibrillation	Cardiac hypertrophy
Systolic hypertension	Peripheral edema	Hyperdynamic precordium	Pulmonary hypertension	Heart failure
Hypothyroidism				
Fatigue	Decreased endurance	Increased serum cholesterol	Impaired cardiac contractility	Increased SVR
Bradycardia	Decreased endothelial-derived relaxation factor	Increased homocysteine	Increased C-reactive protein	

SVR, systemic vascular resistance.

MECHANISMS OF THYROID HORMONE EFFECTS AT THE CELLULAR LEVEL

Mechanisms Underlying the Intracellular Cardiac Effects of Thyroid Hormones

The effects of THs at the cardiac intracellular level are divided into genomic and non-genomic pathways (Khan et al., 2020). In the genomic pathway, THs regulate the expression of target genes by binding to nuclear receptors in cardiomyocytes. In contrast, the non-genomic pathway includes effects on ion channels of the cardiomyocytes and effects of THs on the peripheral circulation, which regulate hemodynamics and the cardiac ejection fraction (Klein and Ojamaa, 2001; Cooper and Biondi, 2012).

Genomic Response to Thyroid Hormones

Thyroid hormones (THs) regulate the expression of genes coding for cardiac proteins. T3 binds to TRs in the cardiomyocyte nucleus, and this regulates transcription by binding to thyroid hormone response elements (TREs) in regulatory regions of target genes (Kahaly and Dillmann, 2005). Thyroid hormone responses (TRs) are members of the superfamily of steroid hormone receptors. An important feature of their activity is that they bind to TREs with or without ligand, which is distinct from other steroid hormone receptors. TRs bind to TREs as retinoid X receptors (RXRa, RXRb, or RXRg) (Lazar and Chin, 1990; Giammanco et al., 2020).

Table 2 shows the regulation by TH of genes coding for cardiac proteins (Klein and Ojamaa, 2001). One effect of TH in cardiomyocytes is to control cardiac contractility and ejection fraction. THs upregulate the expression of genes encoding sodium/potassium-transporting ATPases (Na^+/K^+ ATPase), α-myosin heavy chain (myosin heavy chain 6; encoded by *MYH6*), and sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2; encoded by *ATP2A2*) and downregulate the transcription of β−myosin heavy chain (myosin heavy chain 7; encoded by *MYH7*) and phospholamban (PLN; encoded by *PLN*) (He et al., 1997; Kaasik et al., 1997; Holt et al., 1999).

α-myosin and β-myosin heavy chains are major components of the cardiomyocyte contractile structures called sarcomeres (Nadal-Ginard and Mahdavi, 1989). The upregulation of SERCA2 and the downregulation of PLN increase calcium concentrations in cardiomyocytes and enhance systolic contraction. Thyroid hormones can increase the protein expression of SERCA2 and decrease the protein expression of PLN in the sarcoplasmic reticulum, leading to improved ventricular relaxation (Kiss et al., 1994; Kranias and Hajjar, 2012). Thyroid hormones (especially T3) also have a direct inotropic effect on cardiomyocytes by upregulating the expression of the β1-adrenergic receptors (Hoit et al., 1997). On the other hand, T3 has been reported to suppress the expression of adenylate cyclase (Klein and Danzi, 2007, 2016). In another study, the authors hypothesized that T3 primarily regulates genes that control cardiac pacemaker cells, exerting a positive chronotropic effect in these cells (Klein and Danzi, 2016).

Chen et al. reported that treatment with a T4 antagonist decreases the collagen fibers in the left ventricular non-infarcted area, with increasing expression of matrix metalloproteinase-2 (MMP2) and tissue inhibitor of matrix metalloproteinases 1 to 4 (MMP1 to 4). Paradoxically, atrial fibrosis is also decreased by T4 (Chen et al., 2013; Zhang et al., 2014), and stimulation by various THs increased cardiac angiogenesis as well as cardiomyocyte growth (von Hafe et al., 2019). In addition, THs regulate several plasma membrane and ion transporters, for example Na^+/K^+-ATPase, Na^+/Ca^{2+} exchanger (NCX) and voltage-gated potassium channels, including Kv1.5, Kv4.2, and Kv4.3, at both the transcriptional and post-transcriptional levels. Consequently, THs regulate cardiomyocyte excitation-contraction coupling (EC coupling) responses (Gick et al., 1990; Ojamaa et al., 1999).

TABLE 2 | Regulation of the genes coding for cardiac proteins by THs [modified from reference (Klein and Ojamaa, 2001)].

	Upregulate	**Downregulate**
Myofilament		
	α-MHC (*MYH6*)	β-MHC (*MYH7*)
Ca handling protein		
	SERCA (*ATP2A2*)	Phosphlamban (*PLN*)
Membrane channel		
	Na^+/K^+ ATPase	Na^+/Ca^{2+} exchanger
Adrenergic receptor pathway		
	b1 Adrenergic receptor	Adenylyl cyclase type V, VI
Fibrosis		
	MMP2, TIMP1-4	
Voltage-gated potassium channels		
	Kv1.5, Kv4.2, Kv4.3	

Non-genomic Response to Thyroid Hormone

Thyroid hormones (THs) have two major non-genomic effects, on cardiomyocytes of several membrane ion channels, such as Na^+, K^+, and Ca^{2+} channels (Klein and Ojamaa, 2001; Giammanco et al., 2020). In neonatal rat cardiomyocytes, they regulate phosphatidylinositol 3-kinase (PI3K) or serine/threonine-protein kinase (AKT) signaling pathways (Kuzman et al., 2005). Thyroid hormone prevents cell death by AKT pathway in the vascular smooth muscle (Ojamaa et al., 1996).

Other than these pathways, PI3K/AKT-induced physiological hypertrophy is regulated by insulin-like growth factor-1 (IGF-1) (Fujio et al., 2000), p85α (Kenessey and Ojamaa, 2006), angiotensin-1 receptor (Diniz et al., 2009), ubiquitin proteasomes (Rajagopalan et al., 2013), epidermal growth factor receptor (Rajagopalan et al., 2008), and extracellular signal-regulated kinases (Pantos et al., 2007). THs also influence cardiac mitochondrial function (Marín-García, 2010) and regulate impaired myocardial bioenergetic status and function (Madathil et al., 2015).

In addition, plasma membrane-bound sites include integrin αvβ3, a member of a family of proteins that mediate bidirectional interactions between cells and the extracellular matrix (ECM) and regulate tissue organization and cell migration processes. The integrin αvβ3, which is a member of a family of proteins that interactions between cell and the ECM and regulate migration processes, dependent pathway is primary T4-sensitive and effective some intracellular signals, such as protein kinase B (PKB/AKT) and mitogen-activated protein kinases (MAPKs) that phosphorylate intracellular proteins. In addition, some of them can regulate the nucleus and transcription (Cayrol et al., 2019; Davis et al., 2019; Hercbergs, 2019).

The mitochondrial isoforms of other hormone receptors, including mtRXR (mitochondrial RXR), have also been identified (Casas et al., 2003). In addition of the localization of TR (thyroid receptor) in mitochondria there may be TH-mediated pathway between the nuclear and mitochondrial genome (Wirth and Meyer, 2017).

HYPERTHYROIDISM AND THE CARDIOVASCULAR SYSTEM

Overview of Hyperthyroidism

Hyperthyroidism is commonly affected by stimulation of the TSH receptors by autoantibodies [Graves' disease (GD)] or as a result of the autonomous production of THs by thyroid nodules (Cooper and Biondi, 2012). In general, the prevalence of hyperthyroidism is approximately 0.5% (Cooper and Biondi, 2012). It predominantly affects women aged 30–50 and is caused by GD in 70% of cases. Graves' disease with TSH receptor antibodies is characterized by a diffuse goiter, exophthalmos, and pretibial myxedema. Aside from GD, 20% of patients with hyperthyroidism show autonomous production of THs by a nodular goiter (Toft and Boon, 2000).

The cardiac effects in thyroid hormones upregulate resting heart rate, blood volume, and myocardial contractility compared to normal. However, as shown in **Table 1**, exacerbation of hyperthyroidism can also be detrimental to cardiac function (von Hafe et al., 2019). In patients with hyperthyroidism, exercise intolerance is caused by impaired ability to further increase the heart rate and cardiac contraction and to lower the SVR (Forfar et al., 1982). In a consecutive case series study of 24 patients, Duyff et al. reported that 16 patients showed objective signs or symptoms of neuromuscular dysfunction (Duyff et al., 2000). Cardiac output is markedly elevated in patients with hyperthyroidism. The hyperthyroidism has been implicated in a 16% increase in the risk of major cardiovascular events, as well as involvement in an increase in cardiovascular death (Selmer et al., 2014).

Hyperthyroidism and Arrhythmias

Cardiac arrhythmias or electrocardiogram (ECG) abnormalities, which include sinus tachycardia, AF, and shortened PR and QT intervals, are sometimes observed in patients with hyperthyroidism (Kahaly and Dillmann, 2005). Although it is rare, atrio-ventricular blockage might be observed in patients with GD (Mohr-Kahaly et al., 1996). In almost all patients with hyperthyroidism, the most common rhythm disturbance is sinus tachycardia (Nordyke et al., 1988; Biondi et al., 2000).

Atrial fibrillation (AF) is recognized as the most common supraventricular arrhythmia in patients with thyrotoxicosis (Nakazawa et al., 2000). In patients with hyperthyroidism, the prevalence of AF ranges between 2 and 20%, and their risk of AF is approximately six-fold higher than that of healthy people (Klein and Danzi, 2007). The primary consideration for the management of AF is to control heart rate. β-blockers are one of the widely used drugs in the treatment of AF in cases of hyperthyroidism (Klein and Danzi, 2007). These drugs can bring down the ventricular rate and stabilize the rapid symptoms, but they have little effect on converting AF to sinus rhythm or on hyperthyroidism. Therefore, treatment of hyperthyroidism is optimal for long-term AF management. This normally employs radioiodine treatment or antithyroid drugs (ATDs), which can restore sinus rhythms within a few months in the majority of hyperthyroidism patients (Nakazawa et al., 2000). One prospective study in middle-aged and elderly people in Rotterdam (Rotterdam Study) reported that the risk of AF, sudden cardiac death, and decreased life expectancy is associated with elevated free T4 levels, even if thyroid function is within normal limits (Bano et al., 2017; Razvi et al., 2018).

While the major arrythmias in patients with hyperthyroidism are atrial, ventricular arrhythmias are rare and occur about as often as in healthy people (Osman et al., 2002). Ventricular tachycardia (VT) is one of the major causes of death in patients with CVD. A cardiac electrical storm (ES) is defined as electrical instability involving hemodynamic disturbances with significant VT, occurring in at least three or more episodes within 24 h, and requiring direct current cardioversion (Dorian and Cass, 1997). This is likely in patients with hyperthyroidism, in whom VT is typically severe (Colzani et al., 2001; Jao et al., 2004). Ventricular arrhythmia is seen in thyrotoxicosis

patients undergoing antithyroid therapy, though only rarely (von Olshausen et al., 1989; Osman et al., 2002). Ventricular tachycardia usually occurs in association with underlying structural heart diseases or HF from various etiologies (Polikar et al., 1993; Marrakchi et al., 2015).

Hyperthyroidism and Heart Failure

Hypothyroidism and hyperthyroidism can both lead to HF (Schmidt-Ott and Ascheim, 2006), and the prognosis for patients with hyperthyroidism even at a mild level is poor, as these patients suffer from arrythmia, as well as HF. When these patients do not receive treatment, hyperthyroidism might lead to HF because of arrhythmias, cardiac hypertrophy, and increased blood volumes (Biondi, 2012). Furthermore, in a case-based study, patients with hyperthyroidism who did not get proper medical treatment had a higher mortality risk with CVD (Franklyn et al., 2005; Vale et al., 2019). Patients with severe hyperthyroidism can suffer from "high-output HF". Precisely, high-output HF is defined as congestive HF with increasing cardiac output (DeGroot and Leonard, 1970); resulting "tachycardia-induced cardiomyopathy" depends on the duration of high-output HF with hyperthyroidism (Cruz et al., 1990).

In young patients with hyperthyroidism, this thyrotoxicosis is not associated with underlying heart disease, and therefore, the heart is not damaged. However, symptoms of HF occur in cases of enlarged cardiac output and low SVR, and enlarged blood flow volumes caused by chronic stimulation of the renin angiotensin aldosterone system can be present. The symptoms of patients with high-output HF are breathlessness at rest, fatigability, and the accumulation of fluid with peripheral edema, pleural effusion, pulmonary hypertension, and hepatic congestion (Biondi, 2012).

Siu et al. have estimated that the risk of low-output HF is 6–15% in patients with hyperthyroidism (Siu et al., 2007). Elderly patients with hyperthyroidism might suffer from HF with a reduced ejection fraction. These low-output HF patients have low cardiac output, increasing the SVR, a reduction in ventricular contractility, and impaired left ventricular filling, without increased blood volume. The risk of HF with reduced ejection fraction is increased in patients with hyperthyroidism suffering from cardiac disorders such as ischemic heart disease, hypertensive heart, valvular disease and/or AF (Biondi, 2012). Clinically apparent hyperthyroidism with a hyperdynamic state increases the risk of AF (Cooper and Biondi, 2012). The synergistic effects of reducing SVR, increasing contractility, and increasing the heart rate augments cardiac output (Cooper and Biondi, 2012).

It has been reported that cardiovascular diseases related to thyroid function can be further improved by treating the thyroid gland (Barreto-Chaves et al., 2020). Muthukumar et al. reported that patients with cardiovascular dysfunction related to hyperthyroidism could improve their cardiac function by bringing the thyroid to normal levels with thyroid medication (Muthukumar et al., 2016). And they reported that these aforementioned patients could also have their cardiac function completely restored after total thyroidectomy (Muthukumar et al., 2016). In fact, Saad et al. have reported that treatment of the thyroid gland significantly prevented cardiac dysfunction in a mouse model of T4-induced cardiac dysfunction (Saad et al., 2017). Furthermore, reversibility of heart disease was observed after 2 weeks of T4 treatment, including cardiac hypertrophy (Saad et al., 2017).

In SardiNIA study, Delitala et al. demonstrated that serum FT4 levels are associated with carotid-femoral artery PWV (pulse wave velocity), and high levels of free T4 was is one aggravating factor of aortic stiffness. And they considered that T4 may contribute to the atherosclerosis and the aging process in the vascular system (Delitala et al., 2015; Vale et al., 2019).

Importantly, death from heart failure is the major cause of cardiovascular death in both hyperthyroidism and subclinical hyperthyroidism (Selmer et al., 2014).

Subclinical Hyperthyroidism

Subclinical hyperthyroidism is defined as a condition in which serum TSH levels are below the lower limit of normal, but the width of serum T3 and T4 concentrations is within the normal range (Surks et al., 2004; Biondi and Cooper, 2008; Bahn et al., 2011). Subclinical hyperthyroidism has two major causes. The first encompasses exogenous factors such as an excessive dosage of thyroid hormone replacement drugs, high-dose glucocorticoids, and others. The second is an endogenous factor, namely underlying thyroid disease that causes the overactivity of THs. A considerable proportion (15–20%) of patients who take levothyroxine have a low TSH serum level (Canaris et al., 2000; Vadiveloo et al., 2011; Taylor et al., 2014). In the USA, the prevalence of endogenous subclinical hyperthyroidism varies and depends on age, sex, and iodine intake. Cappola et al. reported that the prevalence of subclinical hyperthyroidism in iodine-sufficient cases is almost 2% (Cappola et al., 2006).

Several observational clinical studies have reported a relationship between subclinical hyperthyroidism and incident CVD (Parle et al., 2001; Iervasi et al., 2007), AF (Cappola et al., 2006; Collet et al., 2012), HF (Rodondi et al., 2008; Biondi et al., 2015), and cardiovascular mortality (Collet et al., 2012). It is not clear whether subclinical hyperthyroidism is associated with high-risk cardiovascular morbidity and mortality. However, the European Thyroid Association guidelines recommend that older patients with hyperthyroidism should have a serum TSH < 0.1 mU/L (Biondi et al., 2015).

Treatment of Hyperthyroidism

In this section, we review the medical history of GD, which is a major cause of hyperthyroidism. Graves' disease is treated by ATDs, which decrease TH synthesis, by radioactive iodine (RAI) therapy, or total thyroidectomy (Smith and Hegedüs, 2016; Kahaly et al., 2018). Antithyroid drugs is the major treatment in Europe, United States, and Asia (Brito et al., 2016). The main ATDs are thionamides, for example propylthiouracil (PTU), carbimazole (CBZ), and methimazole (MMI). Propylthiouracil controls the conversion from T4 to T3 by inhibiting enzymatic activity in the peripheral organs such as the liver or kidney. As a result, PTU further reduces the density of blood T3. Carbimazole acts by obstructing hormone generation in the thyroid gland, and more significantly, by affecting the hyperstimulation of the thyroid gland at a level prior to biosynthesis. Carbimazole must

be decarboxylated to produce MMI in the liver. Methimazole, as well as PTU, is absorbed immediately and accumulates at a high density in the thyroid gland, restraining the synthesis process of TH. All thionamides inhibit the coupling of iodothyronines and reduce the biosynthesis of THs (Cappola and Ladenson, 2003). As a result, these drugs inhibit the production of TH by iodide peroxidase. Specifically, iodide peroxidase oxidizes an iodide ion to iodine and iodinates a tyrosine residue of the thyroglobulin, and this process is indispensable in the production of T4. ATD has been recommended as a first choice drug for the treatment of GD, particularly for short-period GD treatment prior to thyroidectomy or RAI therapy (Bartalena, 2013; Smith and Hegedüs, 2016). Higher doses of PTU inhibit the deiodination of T4 to T3 (Cooper, 2005) and have severe side effects, such as severe hepatic disorder and anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis. However, the half-life of PTU (75 min–150 min) is much shorter than that of MMI (6 h) (Kahaly et al., 2018). Hyperthyroidism is linked to risk of enlargement in CVD and mortality in the first year following radioiodine treatment, but early diagnosis and proper treatment of hyperthyroidism along with cardiac treatment might reduce mortality (Toft and Boon, 2000).

HYPOTHYROIDISM AND THE CARDIOVASCULAR SYSTEM

Overview of Hypothyroidism

In general, hypothyroidism is diagnosed when serum TSH levels are high (usually > 10 mU/L) and serum-free T4 levels are low (< 9–10 pmol/L). Clinically apparent hypothyroidism can be seen in 0.2–2.0% of non-pregnant adults (Canaris et al., 2000). Symptomatic thyroid dysfunction is found in 1–2% of the population and is more frequent in women. Except for previous radioiodine treatment or thyroidectomy for GD, the most common cause is autoimmunity of the thyroid gland or Hashimoto's thyroiditis (Hashimoto's disease). Hashimoto's thyroiditis can often be accompanied by hard goiter, in which the thyroid gland becomes atrophied causing progressive fibrosis during the course of the disease, and resulting in diminished function of the thyroid gland. Distinct from hyperthyroidism, there is a link between low serum concentrations of HTs (T3 and T4) and a reduction in cardiac output, heart rate, stroke volume, and myocardial contractility (Toft and Boon, 2000).

One of the most important cardiac dysfunctions, especially among patients with hypothyroidism, is diastolic dysfunction. This diastolic dysfunction is observed not only at rest, but also during exercise. This exacerbates the symptoms and worsens the prognosis of potential heart failure patients in hypothyroid patients (Selmer et al., 2014; von Hafe et al., 2019). It is well known that hypothyroidism is associated with chronic heart failure. However, in rare cases, hypothyroidism may also be associated with pericardial effusion and cardiac tamponade (Grais, 2010; Patil et al., 2011).

In addition to the abovementioned clinical symptoms, remarkable changes in modifiable atherosclerotic risk factors are also observed in clinically apparent hypothyroidism, including hypercholesterolemia, diastolic hypertension, carotid intima-media thickening, and reduced production of endothelial-derived relaxation factor (nitric oxide) (Cappola and Ladenson, 2003). All of these clinical manifestations are improved by TH replacement therapy (Cappola and Ladenson, 2003).

Hypothyroidism and Arrhythmias

It is generally accepted that typical hypothyroid ECG changes include bradycardia, long-PQ segment, low voltage of the QRS complex, and flattening or T-wave inversion. However, it is important, though not well known, that hypothyroidism induces atrioventricular blockage and acquired long QT syndrome (Marrakchi et al., 2015). In the case of supraventricular arrhythmias, hypothyroidism or subclinical hypothyroidism has a certain clinical impact. For example, patients with thyroid dysfunction have a higher probability of having AF than healthy people (Sawin, 1995; Baumgartner et al., 2017). Klemperer et al. reported that perioperative T3 treatment reduces the incidence or necessity of postoperative AF in patients with normal thyroid function during cardiopulmonary bypass surgery. However, experiments on the mechanism underlying this discovery have not yet been reproduced (Klemperer et al., 1996). Kim et al. confirmed that hypothyroidism has no relationship with 10-year risk of incident AF from the famous cardiovascular cohort of the Framingham Heart Study (Kim et al., 2014).

Regarding ventricular arrhythmias, some groups have reported that patients with hypothyroidism might experience life-threatening arrhythmia, for example a torsade de pointes type ventricular tachycardia (TdP type ventricular tachycardia) and VT due to prolonged QT syndrome (Chojnowski et al., 2007). That study also reported that hypothyroidism decreases the expression of protein T3 in cardiomyocytes and that it can cause reduced cardiac contractility and heart rate, as well as delayed conduction of electrical stimulation in the heart. This might be the reason for bradycardia and elongation of the QT interval and consequent fatal arrhythmia, such as TdP-type ventricular tachycardia. In this case, the causes of long QT syndrome and shock include the decreased T3 expression and disorders of the electrolyte balance, for example hypokalemia and hypocalcemia (Chojnowski et al., 2007). For patients with hypothyroidism, it is necessary to monitor the effectiveness of amiodarone for the prevention of ventricular arrhythmic recurrence. It has been reported that lidocaine (class IB antiarrhythmic drug) or bretylium tosylate (class III antiarrhythmic drug) might be useful to prevent these paroxysmal ventricular tachycardias and endocavitary electrode stimulation, in place of amiodarone (Chess-Williams and Coker, 1989).

Hypothyroidism and Heart Failure

It is thought that TH deficiency raises the risk of developing and exacerbates HF (Rodondi et al., 2008). Basic experiments have reported that hypothyroidism suppresses myosin heavy chain 6 protein expression and enhances myosin heavy chain 7 protein expression and that hypothyroidism induces cardiac atrophy as a result. In addition, hypothyroidism is associated with the increased dilation of ventricular chambers and reduced myocardial perfusion (Liu et al., 2008; Biondi, 2012).

Congestive HF and myxedema have been recognized in patients with hypothyroidism (Schwimmer et al., 1947), and their HF and myxedema symptoms improved with treatment for hypothyroidism. More recently, it was reported that patients with hypothyroidism were among patients with reversible dilated cardiomyopathy (Khochtali et al., 2011). Recent clinical studies have reported that patients with cardiovascular disease who have reduced T3 levels have a higher risk of death from heart failure (Wang et al., 2017; Neves et al., 2019). Rezvi et al. reviewed thyroid hormone supplementation in HF (Neves et al., 2020). Studies in patients without HF as well as HFrEF suggest an effect of thyroid hormone supplementation in improving diastolic function (Pingitore et al., 2008). In the HFrEF animal model, it has been suggested that thyroid hormone supplementation in HF improves cardiac function (Vale et al., 2019).

It is interesting that the metabolism and serum levels of THs are changed by conditions of HF, myocardial infarction, and cardiac surgery. In these situations, the conversion of T4 to T3 decreases. Diseases with normal serum levels of TSH and no symptoms suggestive of hypothyroidism despite a decrease in blood thyroid hormone are called euthyroid sick syndrome or non-thyroidal illness. Among them, those with only low T3 levels are called low T3 syndrome (LT3S) (Nagayo, 2018).

Amin et al. reported that the Patients with heart failure with HFrEF (left ventricular ejection fraction $< 40\%$) and with LT3S take the oral T3 supplements (Liothyronine) for 1.5 months, and that the patients can be found to improve cardiac function considering the blood laboratory data and echocardiographic data (Liothyronine group $N = 25$, Placebo grouper $N = 25$) (Amin et al., 2015). Further, LT3S impairs cardiac dysfunction and as a result induces heart disease. Patients with advanced heart disease and LT3S have increased mortality (Pingitore et al., 2005; Gerdes and Iervasi, 2010; Mourouzis et al., 2011).

Pingitore et al. conducted a clinical study to determine whether T3 administration improves cardiac function in patients with low T3 syndrome (LT3S) who have suffered an acute myocardial infarction (AMI) (LT3S/AMI) (Pingitore et al., 2019). Pingitore et al. concluded that the patients with LTS3S/AMI had improved cardiac functions, which were assessed using cardiac MRI to evaluate various parameters (for example infarct sizes, and cardiac function), after 6 months of treatment with liothyronine (T3) therapy [The THIRST Study (Thyroid Hormone Replacement Therapy in ST elevation myocardial infarction); Phase II study] (T3 supplement group $N = 19$, Placebo grouper $N = 18$) (Pingitore et al., 2019; Lisco et al., 2020). Some researchers reported that the changes in gene expression associated with cardiac dysfunction are similar to those induced in hypothyroidism, suggesting that TH dysfunction might be one aggravating factor for HF (Kinugawa et al., 2001; Biondi, 2012).

Hyperlipidemia and Coronary Artery Disease in Hypothyroidism

THs are involved in lipid metabolism (Cappola and Ladenson, 2003). Hyperthyroidism is not an exacerbating factor for the lipid profile. For several years, hypothyroidism was thought to be linked to hyperlipidemia. In fact, many patients with clinical hypothyroidism show obvious clinical symptoms (Klein and Danzi, 2007; Jabbar et al., 2017).

There are two aspects of the association between hypothyroidism and coronary disease. First, hypothyroidism has an influence on hypertension and hypercholesterolemia in these patients. In particular, this hypertension and hypercholesterolemia due to hypothyroidism accelerate atherosclerosis. Second, it is thought that hypothyroidism reduces cardiac oxygen requirements and decreases their effective use and that this process induces CVD (Kahaly and Dillmann, 2005). It is notable that low serum T4 levels are associated with increased low-density lipoprotein-cholesterol and that hypothyroidism has also been implicated in hyper-triglyceridemia and low free fatty acid levels.

Thyroid hormone replacement is desirable in patients with hypothyroidism, even in patients with myocardial infarction. In fact, this is because thyroid hormone is said to be an important factor in regulating the structure and function of the left ventricle in the late post-myocardial infarction period (Jankauskienė et al., 2016; von Hafe et al., 2019). In molecular biology, low T3 levels can induce oxidative stress and apoptosis, which may exacerbate ventricular dysfunction (Jankauskienė et al., 2016).

Zhang et al. reported that in a rat model of cardiac reperfusion injury, the addition of T3 enhances the gene expression of transcription factor Hypoxia-Inducible Factor alpha (HIF-1α). It thereby regulates mitochondrial opening and protects cardiomyocytes (Zhang et al., 2018).

Subclinical Hypothyroidism

Subclinical hypothyroidism is defined as being associated with a serum TSH level above the normal range, but with normal TH levels. Subclinical hypothyroidism is classified into two types according to the level of TSH. Mild subclinical hypothyroidism is diagnosed by a mildly high TSH level (between 4.0–4.5 and 10.0 mU/L). Severe subclinical hypothyroidism is diagnosed by a TSH level $>$ 10.0 mU/L. However, the upper limit of normal TSH has not been clearly defined. For the diagnosis of subclinical hypothyroidism, it is necessary to consider both clinical symptoms and the level of TSH (Hamilton et al., 2008; Razvi et al., 2018). In general, the prevalence of subclinical hypothyroidism is 4–20% in adults (Biondi and Cooper, 2008; Abreu et al., 2017). There are many causes for this wide range of reported prevalence. Specifically, differences in age, sex, race, body mass index, dietary iodine intake, and serum TSH measurements at different diagnostic institutions might contribute to this. The prevalence of a high serum level of TSH is higher in Caucasians than in African-American people (Hollowell et al., 2002). It is also thought that at least 10% of older women (aged 60 and older) are diagnosed with subclinical hypothyroidism (Parle et al., 1991). This wide prevalence suggests that patients with more cardiovascular risk factors than expected are present. The cardiac function of patients with subclinical hypothyroidism shows abnormalities including extended isovolumic relaxation time and impaired ventricular filling (Monzani et al., 2001). To date, several studies of

systolic dysfunction in patients with subclinical hypothyroidism have been conducted. Ripoli et al. reported that there is an association between subclinical hypothyroidism and systolic dysfunction and that thyroxine replacement therapy improves cardiac contractility in patients with hypothyroidism (Ripoli et al., 2005). In patients with subclinical hypothyroidism during exercise, both diastolic and systolic function are impaired and as a result, exercise tolerance is reduced in these patients (Brenta et al., 2003).

It is not currently known whether there is an association between the severity of subclinical hypothyroidism and increased risk of CVD. According to meta-analyses of observational studies, patients aged < 65 years (Razvi et al., 2008) with severe hypothyroidism, defined as a TSH level > 10 mU/L, do have a high risk of CVD (Rodondi et al., 2010). According to the guidelines of the European Thyroid Association, treatment for hypothyroidism is recommended for patients with severe hypothyroid disease (serum THS > 10 mU/L), symptoms of hypothyroidism, an age < 70 years, and elevated risk of CVD (Pearce et al., 2013).

Treatment of Hypothyroidism

One favored therapy involves taking levothyroxine (L-thyroxine) with solid preparation on an empty stomach. Patients who not only have clinical manifestations of hypothyroidism, but also a diagnosis based on biochemical examinations, are recommended treatment. Levothyroxine is marketed by several pharmaceutical companies, but switching to generic levothyroxines is not recommended for patients who are stable with one regimen (Jonklaas et al., 2014; Chaker et al., 2017). In patients with clinically manifesting hypothyroidism, 1.5–1.8 mg/kg/day of levothyroxine is desirable as a daily optimal dose (Pearce et al., 2013; Jonklaas et al., 2014). Generally, in patients with CVD, the initial dose is 12.5–25.0 mg/day. Based on symptoms and the serum TSH level, it is desirable to gradually increase the dose afterward (Jonklaas et al., 2014).

Thyroid hormone replacement therapy improves diastolic function in patients with hypothyroidism and also in patients with heart failure with preserved ejection fraction (HFpEF). Thyroid hormone is said to improve cardiac diastolic function, both pathophysiologically and through gene expression. However, further preclinical and clinical studies are needed to clarify the role of thyroid hormones in the treatment of HFpEF (Neves et al., 2020). In patients with HFpEF, it is advisable to use beta-blockers in combination with thyroid hormones when they are used. The risk of cardiovascular events due to sympathetic hyperactivity in thyroid hormones can be inhibited by the combined use of beta-blockers. As a result, the combination of the two drugs can improve cardiac function while foreshadowing arrhythmias, cardiac hypertrophy, and cardiac dysfunction (Ortiz et al., 2019).

For some CVD patients with hypothyroidism, cardiac function is improved by hypothyroidism treatment. Furthermore, treatment can improve prognostic factors of thyroid function and the cardiovascular system (Crowley et al., 1977). However, it has not been clarified whether patients treated for hypothyroidism have exacerbated heart disease when the treatment is discontinued (Klein and Danzi, 2007). The clinical implications of low T3 levels in patients with normal TSH levels have also not yet been determined. Measurement of T3 does not correlate with therapeutic efficacy (Abdalla and Bianco, 2014).

DRUG-INDUCED THYROID DYSFUNCTION

Effects of Cardiovascular Drugs on Thyroid Function

The interrelationship between thyroid hormones and the heart is a very important focus area. Thus far, we have described how changes in THs cause cardiovascular dysfunction. In this section, we summarize the ways in which cardiac medications routinely used by cardiologists affect thyroid hormones.

As mentioned above, among drugs used commonly by many cardiologists, there are some that alter TH levels in patients with normal thyroid function. Several of these can alter the levels of THs in euthyroid patients. Fadel et al. summarized the effects of cardiovascular drugs on thyroid function [**Table 3**, (Fadel et al., 2000)]. When cardiovascular drugs are used, TH levels must be checked every 3–4 months. The most widely used drug that affects thyroid function is amiodarone. Approximately 50% of patients taking amiodarone for a long period have elevated serum T4 levels. However, the serum levels of T3 and TSH are within a normal range (Harjai and Licata, 1997). One of the main effects of amiodarone on thyroid function is the inhibition of the conversion of T4 to T3 by amiodarone and its major active metabolite, desethylamiodarone (Burch, 2019). Ruzieh et al. reported that the likelihood of experiencing an amiodarone-related adverse event was greater than with placebo. (relative risk about 4.44 versus placebo) (Ruzieh et al., 2019). In the following sections (6.2 to 6.4), we summarize the effect of amiodarone on thyroid function in detail.

This section summarizes the relation between some drugs in cardiology and thyroid function.

Dopamine suppressed TSH secretion in about 50% of humans. Administration of the dopamine receptor antagonists metoclopramide or domperidone increases TSH in primary hypothyroidism. Normal level of THs suppresses TSH elevation, but hypothyroidism does not respond to this suppression, resulting in an elevation response (Cooper et al., 1983). High dose fulosemide is said to attenuate the effects of thyroid hormones by binding to TGB (thyroxine binding globulin), which is one of transporter portein of THs (Fadel et al., 2000; Burch, 2019). However, there are few documents that explain the above in pharmacokinetics, and it is necessary to examine it in detail in the future. Heparin increases T3 and T4 from binding proteins indirectly by increasing free fatty acids (Mendel et al., 1987; Burch, 2019) . Propranolol in high volume inhibits the conversion of T4 to T3. As a result, it increases T4 and free T4 (Burch and Wartofsky, 2018; Burch, 2019). In hyperthyroidism, the effect of propranolol is enhanced (Shenfield, 1981), and vice versa in hypothyroidism (Burch, 2019).

TABLE 3 | Effect of cardiovascular drugs on thyroid hormone function [modified from reference (Fadel et al., 2000; Burch, 2019)].

Drugs	Medical effect	Thyroid hormone serum concentrations			Mechanism of action in the thyroid	References
		TSH	T3	T4		
Amiodarone (∼3months)	Class III antiarrhythmic drug	↑	↓	↑	Inhibition of conversion of T4 toT3 inhibition the biosynthesis and release of TH	Harjai and Licata, 1997; Martino et al., 2001; Burch, 2019
Amiodarone chronic therapy (chronic phase: 3 months ∼)	Class III antiarrhythmic drug	→	→ or ↓	↑	Inhibition of T4 to T3 conversion Inhibition the biosynthesis and release of TH	Harjai and Licata, 1997; Martino et al., 2001; Burch, 2019
Dopamine	Adrenergic drug	↓	→	→	Suppression of TSH production	Cooper et al., 1983; Fadel et al., 2000
Furosemide (high dose)	Loop diuretic drug		↓	↓, slight ↑free T4 ?	Inhibition of T3/T4 binding to TBG	Fadel et al., 2000; Burch, 2019
Heparin (IV)	Anticoagulant drug			slight ↑, slight ↑ free T4	Inhibition of T4distribution in targeted tissue	Mendel et al., 1987; Burch, 2019
Propranolol (high dose)	β1 Non-selective blocker			↑, ↑free T4	Inhibition of T4 uptake in targeted tissue	Shenfield, 1981; Burch and Wartofsky, 2018; Burch, 2019

IV; intravenous administration, TGB; thyroxine binding globulin, ↑; increase, ↓; decrease, →; unchange, ?; unknown.

Overview of Amiodarone and Thyroid Function

Amiodarone is regarded as the most effective antiarrhythmic drug but has various toxicities, and cardiologists need to exercise caution (Trohman et al., 2019). The combination of amiodarone and a beta-blocker is the preferred treatment for ES (Nademanee et al., 2000). According to the 2017 AHA/ACC/HRS Guidelines for Management of Patients with Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death, amiodarone is recommended as a Class IIb indication for the acute treatment of hemodynamically stable VT (Al-Khatib et al., 2018). Amiodarone is effective at suppressing AF, as well as ventricular arrhythmia. In particular, it is thought to be the most effective drug for maintaining sinus rhythms against paroxysmal and persistent AF for a long duration. It is also recognized as the first-choice agent for patients with AF due to HF or symptomatic AF (Singh, 2008; Al-Khatib et al., 2018).

The numerous side effects of amiodarone might involve the skin, eyes, brain, lungs, liver, and peripheral nervous system (Trohman et al., 2019). The major side effect is the production of corneal microdeposits (> 90% of treated patients). These are very similar in appearance to vortex keratopathy, which is associated with Fabry disease (D'Amico et al., 1981; Wasielica-Poslednik et al., 2011).

It is important for clinical physicians, as well as cardiologists, to keep in mind that the use of amiodarone affects thyroid function. That is, some patients using amiodarone suffer from hypothyroidism (5% to 10% of treated patients) and some suffer from hyperthyroidism (0.9–10% of treated patients) (Harjai and Licata, 1997; Trohman et al., 2019). The iodine richness of amiodarone is the recognized cause of its thyrotoxic side effects.

Changes in serum TSH, T4, and T3 levels are seen in patients receiving amiodarone treatment. The pattern of TH levels differs between the acute phase (∼ 3 months) and the chronic phase (after 3 months) after the start of amiodarone use. In both the acute and chronic phases, the serum T4 level rises and the serum T3 level decreases, though the serum reverse T3 (rT3) level rises. Interestingly, the serum TSH levels are elevated in the acute phase, but return to a normal range during the chronic phase (Martino et al., 2001). There are two reasons why serum TH levels change during these two phases, specifically (1) the unique effects of amiodarone and (2) the effects of its constituent iodine (Basaria and Cooper, 2005).

Amiodarone-Induced Hyperthyroidism

Amiodarone can cause hyperthyroidism (amiodarone-induced thyrotoxicosis: AIT), which is subdivided into two main forms. Type I AIT is usually a predisposing factor for goiter abnormalities (for example asymptomatic GD) and results from the over-synthesis and over-release of THs. The self-regulatory mechanism of THs regulates iodine metabolism in the thyroid gland according to iodine content (Harjai and Licata, 1997). Typical cases of type I AIT occur in patients with a background of non-toxic goiter or asymptomatic GD with an overdose of iodine (Trohman et al., 2019). The prevalence of type I AIT is higher in areas with low iodine intake. Treatment for AIT type I is similar to that for normal hyperthyroidism (Hamilton et al., 2020).

Type II AIT is a thyrotoxicosis cause by destructive thyroiditis and is characterized by acute or subacute destruction of the thyroid gland and massive leakage of THs in patients with no underlying thyroid disease while taking amiodarone (Tsang and Houlden, 2009). The prevalence of type II AIT is higher in areas with high iodine intake. Mild thyroiditis of type II AIT (with mild elevation of free T4 and free T3) is likely to improve spontaneously with follow-up alone. In severe type II AIT, the use of glucocorticoid is beneficial even under amiodarone administration (Daniels, 2001; Hamilton et al., 2020).

Sometimes we find the patients who have a combination of the two types of AIT. In such cases, it is advisable to treat them in both types of AIT. Specifically, type 1 should be treated with antithyroid therapy and type 2 with steroids (Osuna et al., 2017; Omidi et al., 2020).

Amiodarone-Induced Hypothyroidism

Amiodarone can also cause hypothyroidism, which inhibits the biosynthesis and release of THs. This is called amiodarone-induced hypothyroidism. This is because large amounts of iodine are released during amiodarone metabolism (Harjai and Licata, 1997). Intrathyroid iodine organization resumes with the normal synthesis of T4 and T3 (Martino et al., 1984). The development of hypothyroidism with amiodarone has been associated with previous Hashimoto's thyroiditis. However, the use of amiodarone is not contraindicated even if serum TSH levels are elevated before or during treatment, because thyroid dysfunction can be easily treated with T4 (Toft and Boon, 2000). After discontinuing amiodarone treatment, thyroid function might recover, but persistent reduced function has been observed. In particular, many patients for whom thyroid function does not improve even after the discontinuation of oral amiodarone administration are considered to have underlying autoimmune thyroid (Martino et al., 2001).

CONCLUSION

In this manuscript, we discussed how TH plays an important role in cardiovascular disease at the molecular level via genomic and non-genomic pathways. Serious cardiac complications such as arrythmia, congestive HF, and angina pectoris might arise in patients with hyperthyroidism or hypothyroidism, and their treatment requires control of the underlying TH levels. Judging from previous clinical observational studies and small-scale intervention studies, which have evaluated the association between THs and risk factors for CVD, it can be concluded that among patients who should be treated for thyroid function, those with severe CVD should be given priority thyroid treatment (Biondi and Cooper, 2008). Abnormal levels of THs might also serve as a prognostic marker (Jabbar et al., 2017).

Since the discovery of iPS cells by Takahashi and Yamanaka (2006), the study of myocardial regeneration has attracted the attention of scientists worldwide (Takahashi and Yamanaka, 2006). Several hormones have been suggested to be involved in myocardial regeneration. One of these is TH, which was recently reported to affect both the metabolic profile and the mitotic cycle in cardiomyocytes (Nakada et al., 2017; Hirose et al., 2019; Tan et al., 2019; Bogush et al., 2020).

In mouse cardiomyocytes, prolonged inhibition of TH with propylthiouracil prolongs the proliferative period of cardiomyocytes during cardiac development (Hirose et al., 2019). Transgenic mice with cardiac-specific suppression of TRα exhibited a higher number of cardiomyocytes and higher expression of cell cycle markers compared to normal mice during cardiac development. In addition, adult TRα transgenic mice demonstrated a 10-fold increase in the number of proliferating diploid cardiomyocytes, as well as improved recovery of cardiac function after ischemia-reperfusion injury (Hirose et al., 2019).

Two recent reports have shown that exogenous administration of thyroid hormone (T3) increases the number of cardiomyocytes in neonatal mouse hearts (Tan et al., 2019; Bogush et al., 2020). Tan et al. determined that T3 activates mitochondria-generated H_2O_2 (mH_2O_2), which in turn activates c-Jun N-terminal kinase-2α2 (JNK2α2) via peroxiredoxin-1 (Tan et al., 2019) to promote cardiomyocyte proliferation. Exogenous T3 stimulates proliferative ERK1/2 signaling and affects cardiomyocytes in the cardiac apex, but is suppressed in P8 by the expression of DUSP5, a nuclear phosphho-ERK1/2-specific dual-specificity phosphatase (Bogush et al., 2020).

It is important to maintain optimal levels of THs in the cardiac tissue, as well as suitable serum TH levels, to stabilize homeostasis. Further, basic studies (such as molecular biology and omics-based analysis), and clinical studies (such as cohort studies and randomized clinical trials) of THs will help us understand their effects on CVD. Moreover, we can elucidate the mechanisms of the demonstrated effects via induced arrhythmia or myocyte remodeling and dysfunction.

AUTHOR CONTRIBUTIONS

HY and YA: conceptualization and funding acquisition. HY: writing – original draft preparation. TK and YA: writing – review and editing. HY, SY, and KF: supervision. All authors contributed to the article and approved the submitted version.

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