CARDIAC REMODELING: NEW INSIGHTS IN PHYSIOLOGICAL AND PATHOLOGICAL ADAPTATIONS

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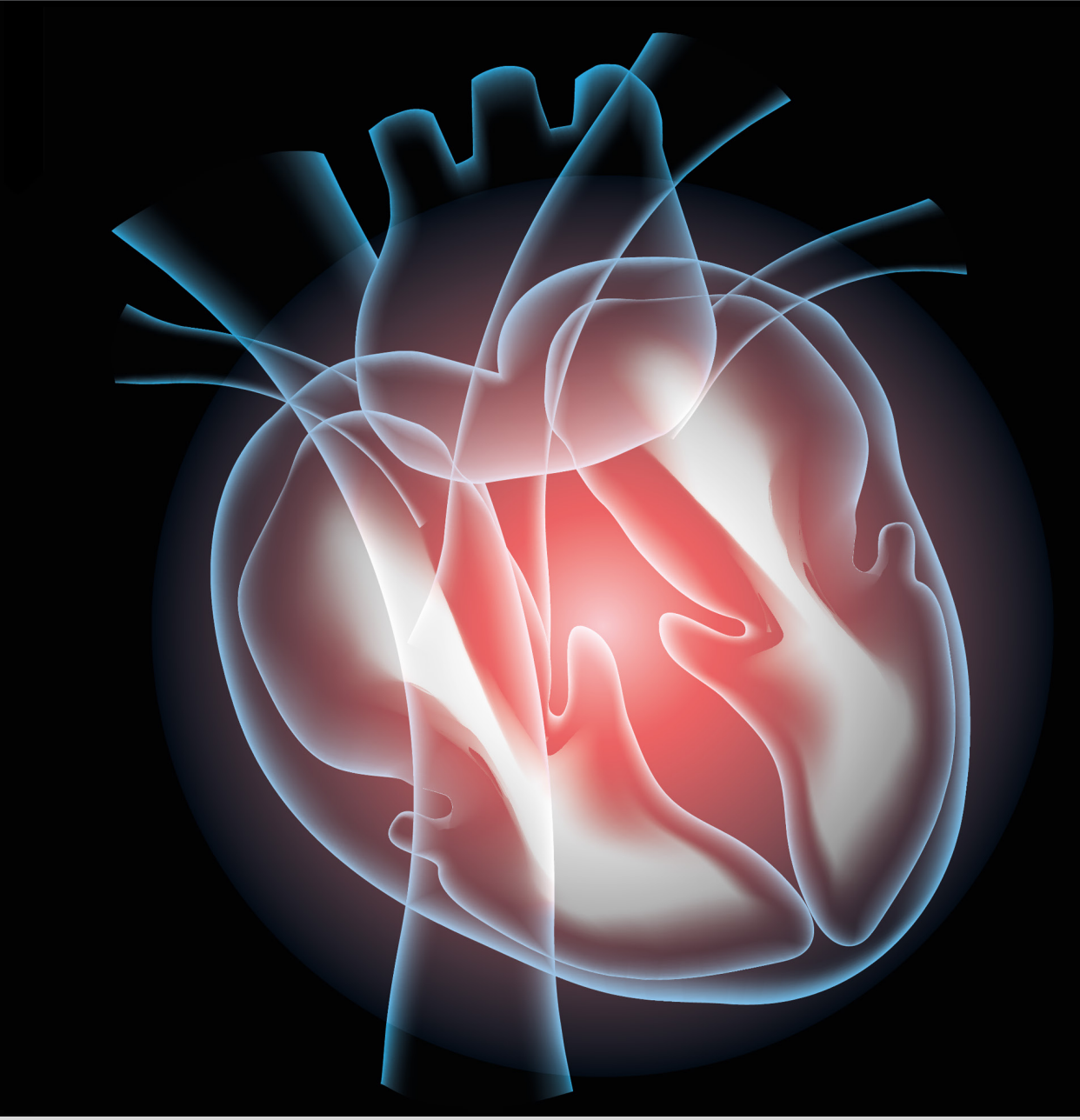

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CARDIAC REMODELING: NEW INSIGHTS IN PHYSIOLOGICAL AND PATHOLOGICAL ADAPTATIONS

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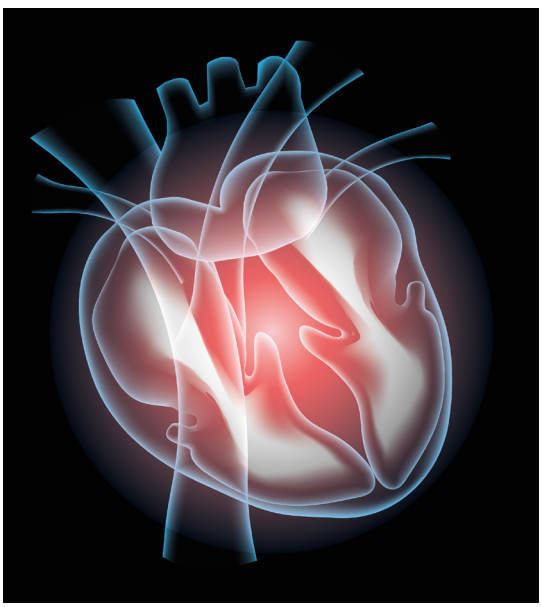

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The effective management of Cardiac remodeling(CR), remains a major challenge. Heart failure remains the leading cause of death in industrialized countries. Yet, despite the enormity of the problem, effective therapeutic interventions remain elusive. In fact, several initially promising agents were found to decrease mortality in patients recovering from myocardial infarction. Cardiac remodeling is defined as molecular and interstitial changes, manifested clinically by changes in size, mass , geometry and function of the heart in response to certain aggression. Initially, ventricular remodeling aims to maintain stable cardiac function in situations of aggression.

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Table of Contents

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EDITORIAL
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Editorial: Cardiac Remodeling: New Insights in Physiological and Pathological Adaptations

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Keywords: cardiac remodeling, physiology, heart failure, fibrosis, inflammation

Editorial on the Research Topic

Cardiac Remodeling: New Insights in Physiological and Pathological Adaptations

INTRODUCTION

Cardiac remodeling (CR) is a complex process involving cardiac myocyte growth and death, vascular rarefaction, fibrosis, inflammation, and electrophysiological alterations (Burchfield et al., 2013; Xie et al., 2013). CR can be conceptualized as a set of cellular, cardiac and interstitial changes in the heart, manifested clinically by changes in the size, mass (hypertrophy and atrophy), geometry (wall thickness and heart shape) and function, in response to a given stimulus—it is one of the major responses of the heart to biomechanical stresses and pathological stimuli (Zornoff et al., 2009; Gajarsa and Kloner, 2011; Braunwald, 2013; Heusch et al., 2014; Sekaran et al., 2017). We can also detect the presence of scarred areas, fibrosis and inflammatory infiltrate.

The most common methods used to detect these CR are echocardiography, ventriculography, tomography, magnetic resonance imaging, and most recently PET cardiac scans (Anand et al., 2002; Presotto et al., 2016). Biomarkers have been also used as indicators of CR, among them we can mention myosin heavy chain alterations (decrease of alpha and increase of beta-myosin), increase of caveolin, increase in neuronal nitric oxide synthase expression and increase of angiotensin converting enzyme, increase of alpha-actin, increase of galectin-3, increase of natriuretic peptides, decrease of GLUT-4 and increase Of GLUT-1, decrease of SERCA2a. Another feature is the substitution of the energetic preferential substrate of fatty acids for glucose (Swynghedauw, 2006; Eschalier et al., 2014; Liquori et al., 2014).

The aim of the present research topic was therefore to bring together key experiments, advances and new findings related to several aspects of CR.

Cardiac dysfunction is the main implication of the process of remodeling. It begins with genetic alterations in response to an insult to the heart, which is consequently manifested by cellular and molecular cardiac alterations, which result in progressive loss of ventricular function, initially asymptomatic and later with signs and symptoms characteristic of heart failure (Cohn et al., 2000; Azevedo et al., 2016). These stem from consequence of an excess of stimulation of the sympathetic system and the renin-angiotensin-aldosterone system which stimulates intracellular signaling pathways with a consequent increase in protein synthesis in myocytes and fibroblasts causing cellular hypertrophy and fibrosis, activation of growth factors, activation of metalloproteinases, hemodynamic overload by vasoconstriction and water retention, increase of oxidative stress and direct cytotoxic effect, leading to cell death by necrosis or apoptosis (Florea and Cohn, 2014; Sayer and Bhat, 2014). Another factor that is potentially responsible for altered cardiac function in the remodeling heart is the energy deficit, which results from the imbalance between supply and consumption of oxygen, including also the decrease in use of free fatty acids and increased

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use of glucose as a consequence of the reduction of β-oxidation, and abnormal accumulation of triglycerides and lipotoxicity may occur (Doenst et al., 2013; Santos et al., 2014; Nishida and Otsu, 2016).

Autophagy is an intracellular process in which defective or unnecessary cytoplasmic components are degraded by lysosomes which can result in accumulation of defective proteins, and in a process known as proteotoxicity (Tarone, 2014; Wang and Wang, 2015).

CR is also associated with oxidative stress due to an increase in the production of reactive species with the reduction of antioxidant defenses; this phenomenon can lead to several conditions, such as lipid peroxidation, changes in proteins responsible for calcium transit, activation of signaling pathways for hypertrophy, protein oxidation, DNA damage, cellular dysfunction, proliferation of fibroblasts, activation of metalloproteinases, stimulation of apoptosis, among others (Münzel et al., 2015).

Recent studies uncovered miR-22 as an important regulator for CR. miR-22 modulates the expression and function of genes involved in the hypertrophic response, sarcomere reorganization, and metabolic program shift during CR (Huang and Wang, 2014; Liu et al., 2015).

In CR there is evidence of changes in the calcium transport system, such as decrease of the L-channels and ryanodine receptors, as well as a decrease in the activity of calsequestrin and calmodulin kinase, and a reductionin in phospholamban phosphorylation. These alterations result in a decreased of supply of calcium during systole and increased calcium in diastole. These changes in the proteins in calcium transit may contribute to cardiac dysfunction (Luo and Anderson, 2013; Feridooni et al., 2015).

In addition, the collagen content plays a critical role in the maintenance of the architecture and cardiac function. In the CR process, there may be an imbalance between the synthesis and degradation of collagen with several deleterious effects, such as increased myocardial stiffness, diastolic dysfunction, worsening in coronary flow and the occurrence of malignant arrhythmias (Spinale et al., 2013; Deb and Ubil, 2014; Leask, 2015; López et al., 2015).

The present research topic also provides readers with fundamental advancement in our understanding of the regulation of CR. Indeed, in an elegant study, Gibb et al. examined nocturnal/diurnal behavior, treadmill exercise compliance, and systemic as well as cardiac-specific exercise adaptations in two commonly used mouse strains C57BL/6J, and FVB/NJ mice. FVB/NJ strain is a useful and robust mouse model for examining cardiac adaptations to treadmill exercise and that treadmill training during daytime hours does not negatively affect exercise compliance or capacity. Finally, they provide new insights to demonstrate that FVB/NJ mice are a suitable and robust model for understanding the mechanisms underlying cardiac adaptations to exercise (Gibb et al.).

In other article, Ruiz-Hurtado et al. investigated whether mild (20% food intake reduction) and short-term (2-weeks) caloric restriction prevented the obese cardiomyopathy phenotype and improved the metabolic profile of young (14 weeks of age) genetically obese Zucker fa/fa rats. The results suggest that with mild and short-term caloric restriction prevented an obesity-induced cardiomyopathy phenotype in young obese fa/fa rats independently of the cardiac metabolic profile.

Bai et al. contributed a very interesting review paper focused on the mechanisms underlying the emergence of post-acidosis arrhythmia at the tissue level, altered source-sink interactions and electrical heterogeneity due to acidosis-induced cellular electrophysiological alterations which may increase susceptibility to post-acidosis ventricular arrhythmias.

Due to the diversity and high quality of the articles compiled here, we hope that this can offer new ideas and research projects for new advances in the field.

AUTHOR CONTRIBUTIONS

LR: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; Drafting the work or revising it critically for important intellectual content; Final approval of the version to be published. AP: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; Drafting the work or revising it critically for important intellectual content; Final approval of the version to be published.

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ORIGINAL RESEARCH
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Mechanisms Underlying the Emergence of Post-acidosis Arrhythmia at the Tissue Level: A Theoretical Study

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Acidosis has complex electrophysiological effects, which are associated with a high recurrence of ventricular arrhythmias. Through multi-scale cardiac computer modeling, this study investigated the mechanisms underlying the emergence of post-acidosis arrhythmia at the tissue level. In simulations, ten Tusscher-Panfilov ventricular model was modified to incorporate various data on acidosis-induced alterations of cellular electrophysiology and intercellular electrical coupling. The single cell models were incorporated into multicellular one-dimensional (1D) fiber and 2D sheet tissue models. Electrophysiological effects were quantified as changes of action potential profile, sink-source interactions of fiber tissue, and the vulnerability of tissue to the genesis of unidirectional conduction that led to initiation of re-entry. It was shown that acidosis-induced sarcoplasmic reticulum (SR) calcium load contributed to delayed afterdepolarizations (DADs) in single cells. These DADs may be synchronized to overcome the source-sink mismatch arising from intercellular electrotonic coupling, and produce a premature ventricular complex (PVC) at the tissue level. The PVC conduction can be unidirectionally blocked in the transmural ventricular wall with altered electrical heterogeneity, resulting in the genesis of re-entry. In conclusion, altered source-sink interactions and electrical heterogeneity due to acidosis-induced cellular electrophysiological alterations may increase susceptibility to post-acidosis ventricular arrhythmias.

Keywords: post acidosis arrhythmias, ventricular tachycardia, premature ventricular complexes, delayed afterdepolarization, transmural dispersion of repolarization, sink-source mismatch

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INTRODUCTION

Ischaemic heart disease is the leading cause of sudden cardiac death and reperfusion is the treatment to reduce the size of a myocardial infarction in patients (Carden and Granger, 2000). The myocardial injury paradoxically occurs with the acute reperfusion of ischaemic myocardium (Brooks et al., 1995). It has been suggested that reperfusion-induced arrhythmias, one form of myocardial reperfusion injury, are mainly due to the acute acidosis recovery (Nagai et al., 2010; Niwano and Tojo, 2010). In fact, gradual reperfusion induced by ischaemic postconditioning, has been shown to delay restoration of intracellular pH and prevent reperfusion-induced ventricular

tachycardia (VT) and ventricular fibrillation (VF) (Avkiran et al., 1996). Therefore, identifying the mechanisms underlying the development of VT/VF in acidotic settings is important for clinical practices to restore intracellular pH during reperfusion (Vandenberg et al., 1993; Kapur et al., 2009; Nagai et al., 2010). However, it is difficult to identify such mechanisms due to fast change of substrates involved in ventricular arrhythmias during ischaemia-reperfusion injury (IRI) in patients. Although previous animal studies have suggested that VT might arise from focal and/or reentrant activities (Boineau and Cox, 1973; Orchard and Cingolani, 1994; Pogwizd et al., 1998; Jie et al., 2010), the precise mechanisms leading to increased arrhythmic risk in the IRI remain incompletely understood.

A marked acidosis occurs during myocardial ischaemia (Park et al., 1999), which may play a crucial role in the arrhythmogenesis during IRI. Experimental studies have suggested that post-acidosis arrhythmias during acute reperfusion are mainly triggered by delayed afterdepolarizations (DADs) at the cellular level (Said et al., 2008; Lascano et al., 2013). DADs are membrane depolarization of cardiac myocytes that appear following the repolarization of the action potential (AP), and are possibly related to the high frequency of spontaneous calcium sparks produced by overloaded calcium in the sarcoplasmic reticulum (SR) (Mészáros et al., 2001). Although the calcium overloaded SR was observed during acidosis, the DADs occurred after acidosis (Said et al., 2008). The explanation for this phenomenon may be given by the experimental evidence showing the phosphorylation of the Thr^{17} site of phospholamban (PLN) due to calcium/calmodulin-dependent protein kinase II (CaMKII) activation (Said et al., 2008) and the inhibitory effects of acidosis on ryanodine receptor 2 (RyR2) (Balnave and Vaughan-Jones, 2000) and sodium-calcium exchanger (NCX) (Terracciano and MacLeod, 1994). In particular, the enhanced effects of CaMKII activation may offset the direct inhibitory effect of acidosis on calcium-ATPase 2a (SERCA2a) and therefore the calcium overloaded SR during acidosis (Lascano et al., 2013). Further studies have showed that gradual reperfusion instead of acute reperfusion can delay recovery of acidosis and protect against ventricular arrhythmogenesis (Avkiran et al., 1996; Kin et al., 2004; Inserte et al., 2009). From these results it is reasonable to expect that the abrupt pH restoration may produce spontaneous SR calcium release and the consequent DADs, thus providing a potential mechanistic link between IRI and ventricular arrhythmogenesis. However, the DADs observed in isolated cells cannot be extrapolated directly to focal arrhythmias in the intact heart. This connection cannot be made because the capability of DADs to evolve into focal arrhythmias depends on their ability to spread into surrounding neighbor cells. In this case, cells with DADs act as a "source" of excitation to drive the surrounding quiescent myocardium, and the surrounding myocardium acts as a "sink" that would suppress the "excitation source" because of intercellular electrotonic interaction. Therefore, for localized DADs to propagate, it requires two conditions: (1) each cell in the DAD region (acting as a "source") must reach its activation threshold and initiate an action potential (AP), and (2) cells in the surrounding tissue (acting as a "sink") must receive sufficient stimulus current flowing from the "source" through intercellular gap junctional coupling to be excited (Spector, 2013). Thus, important questions need to be answered, such as the extent to which the SR calcium load is required to depolarize the cell in the "source" region to trigger an AP; and the number of DAD cells required to overcome the source-sink mismatch (i.e., insufficient "source" to drive the "sink") between an acidotic zone and the surrounding regions to produce a PVC in cardiac tissues.

In addition to DADs in single myocytes, life-threatening VT/VF in intact hearts of patients during IRI also was observed (Tsujita et al., 2004). When VT/VF is initiated, reentry of the propagation occurs and forms a spiral wave in tissues. Despite its importance in clinical settings, a clear comprehension of the mechanisms underlying re-entrant arrhythmia in post acidosis at tissue level is lacking. Although it is known that changes in AP and cytoplasmic calcium concentration induced by acidosis may constitute arrhythmogenic substrates for reentry, the evolution of reentry resulted from acidosis-induced electrophysiological changes has not yet been fully characterized. Multiple experimental studies demonstrated that increased cytoplasmic calcium concentration may increase gap junction resistance (Noma and Tsuboi, 1987; Peracchia, 2004), and down-regulation of connexin proteins as well as the presence of severe fibrosis were also observed in ischaemic tissues (de Groot et al., 2001; de Groot and Coronel, 2004; Sánchez et al., 2011; Saffitz and Kleber, 2012). Cell-to-cell uncoupling would be expected to lead to slow propagation of excitation waves (Saffitz and Kleber, 2012). Moreover, different laboratories have shown that acidosis causes heterogeneous changes (prolongation /abbreviation) of APs (Levites et al., 1975; Bethell et al., 1998; Komukai et al., 2002; Kazusa et al., 2014), resulting in altered electrical heterogeneity in tissues and therefore dispersion of repolarization (TDR), increasing susceptibility to re-entrant arrhythmias (Kuo et al., 1983; Laurita and Rosenbaum, 2000; Bernus et al., 2005; Qu et al., 2006; Jie et al., 2008; Jie and Trayanova, 2010). Whereas the development of reentry has been demonstrated to be associated with slow conduction and TDR (Boineau and Cox, 1973; Kuo et al., 1983), this association is not clear for the gradual evolution from DADs at the cellular level to PVCs and reentry at the tissue level in post acidosis arrhythmias.

Modeling studies have shed light on the mechanisms of post-acidosis arrhythmias. Based on experimental data on acid-sensing ion channels, computational models provided physiological insights into the relationship between acidosis-induced changes in electrophysiological properties and ventricular arrhythmogenesis (Crampin and Smith, 2006; Crampin et al., 2006; Roberts and Christini, 2011, 2012; Lascano et al., 2013). In single-cell simulations, the pro-arrhythmic role of CaMKII activation (Lascano et al., 2013) and sodium-potassium pump (Roberts and Christini, 2011) have been demonstrated, respectively, as they contribute to an increase in cytoplasmic calcium concentration and a rise in intracellular sodium concentration, which may contribute to the genesis of DADs after acidosis. The emergence of arrhythmias arising from DADs induced by reperfusion remains lacking. Characterizing this evolution, through the use of multi-scale models from subcellular, cellular, tissue, organ to system levels, has the

potential to help understand better the mechanisms underlying the reperfusion induced arrhythmias in IRI.

The main objective of the present work is to explore the mechanisms underlying the emergence of post-acidosis arrhythmia. For this purpose, single cell, 1D tissue strand and 2D tissue sheet simulations were performed to investigate the functional impacts of acidotic conditions on the electrical activity, with a particular focus on the genesis of spiral waves in the ventricular transmural wall. These models were used to study: (1) the quantitative relationship between the SR calcium content and the DAD amplitude; (2) the DAD amplitude required to reach the threshold for triggering an AP; (3) the recovery time of intracellular acidosis that influences the protection from the occurrence of DADs; (4) the individual contribution of the SR calcium load and the gap junction uncoupling to the enhanced source-sink interactions for producing a PVC; and (5) the effect of electrical heterogeneity on the vulnerability of tissue to unidirectional conduction block that facilitates the initiation of re-entry. These results may provide insights into the evolution of reperfusion-induced VT.

MATERIALS AND METHODS

Model of DADs in Single Cardiac Myocytes

To model the electrical excitation behavior of cardiac myocytes, well-characterized AP models developed by ten Tusscher et al. (TP06 model) were used (ten Tusscher and Panfilov, 2006). These models are chosen because they are not only based on available human ventricular data but also reproduce the ion channel kinetics and membrane potentials of human ventricular cells. Most importantly, these models incorporated transmural heterogeneity in ventricular electrophysiology to reproduce APs of human epicardial (Epi), mid-myocardial (Mid) and endocardial (Endo) cells. These models have been suggested to be suited for the study of spiral wave dynamics in human ventricular tissues (ten Tusscher et al., 2009).

To reproduce cardiac electrical behavior under acidotic and post acidotic conditions, some modifications to the TP06 models were included. Specifically, the calcium release from the SR (Irel) was modeled as a single flow, which combined the actions of calcium-induced-calcium release from the SR and calcium leak through RyR2 (Lascano et al., 2013). Intracellular pH regulation (Crampin and Smith, 2006) and CaMKII activation (Decker et al., 2009) were also introduced into TP06 cell models. Details can be found in Supplementary Information.

The protocol of pH changes (PPC) similar to that in a previous study (Lascano et al., 2013) was used to perform single cell simulations. PPC consisted of a 1-min-long control period, a 6-min-long acidosis period and a 5-min-long post acidosis period. Intracellular pH was set to 6.7 during the acidosis period and was set to 7.15 during the control period and post acidosis period. Single cells were paced with a constant pacing frequency of 70 beats/min. This PPC was used to predict the triggering of DADs. Changes in fraction of activated CaMKII ($CaMK_{active}$), maximum cytoplasmic calcium concentration ($[Ca^{2+}]_i(max)$), maximum SR calcium concentration ($[Ca^{2+}]_{SR}(max)$), maximum intracellular sodium concentration ($[Na^+]_i(max)$) and the NCX current (INCX) in the PPC were used to study impairment of calcium handling. The time courses of voltage waveforms, underlying INCX, Irel, cytoplasmic calcium concentration ($[Ca^{2+}]_i$) as well as SR calcium concentration ($[Ca^{2+}]_{SR}$) were analyzed to investigate the effect of post acidosis in genesis of DADs. The occurrence time of DADs in the single cells was calculated as the time interval between the beginning time of the last stimulus during acidosis and the time when the first DAD occurred in the PPC. We also assumed that the occurrence time of the PVC in tissues was the same as that of DADs in single cells. In addition, action potential duration (APD) was recorded as the time interval between the time of stimulus onset and 90% repolarization of the AP. Suprathreshold DAD was also defined as the ectopic beats, which can reach to the depolarization threshold required to trigger an AP.

Model of Intracellular pH Restoration

Previous studies have indicated that the period of pH recovery might range from tens of seconds to a few minutes (Avkiran et al., 1996; Park et al., 1999; Inserte et al., 2009). Indirect data also has suggested that a delayed recovery of intracellular pH during reperfusion is involved in postconditioning protection (Maruki et al., 1993; Avkiran et al., 1996; Kin et al., 2004; Fujita et al., 2007; Inserte et al., 2008, 2009). Moreover, the change of intracellular pH during reperfusion was close to a linear variation (Inserte et al., 2009).To simulate a delayed pH recovery process, a linear function with different rates of the pH change was used to model pH restoration. Specifically, three different recovery processes were considered: (i) the fast recovery period of 0.1 min; (ii) the slow recovery period of 0.5 min; and (iii) the gradual recovery period of 4 min. In addition, multiple pH restoration protocols (P_X) with a recovery time of X min were modeled to evaluate the effect of pH restoration time on the probability of generating DADs. These protocols included P_0, P_0.2, P_0.5, P_1, P_2, P_3 and P_4. In each case, the number of DADs and the maximal DAD amplitude during the recovery period were quantified.

Model of PVCs in a One-Dimensional (1D) Homogeneous Cable

To investigate the cellular level conditions required for DADs to trigger a PVC at the multicellular tissue level, a 15-mm-long epicardial strand model consisting of 100 myocytes was constructed. The middle of the strand contained an acidotic region in which myocytes were set as DAD generating cells. Varying numbers of myocytes in the acidotic region (i.e., varying sizes of the acidotic region) were also considered. To evaluate the AP inducibility in the model, the SR calcium content (peak $[Ca^{2+}]_{SR}$) and the amplitude of cytoplasmic calcium transient (peak $[Ca^{2+}]_i$) associated with the DADs generating were measured. The DAD amplitude (DADA) was calculated as the difference between the resting potential and the maximum voltage of DADs. The voltage threshold of DADs was computed as the minimum DADA required to trigger an AP. The minimum number of cells (either generating subthreshold or suprathreshold DADs) required to develop a PVC in the

strand was calculated. The effects of the SR calcium load and gap junction uncoupling on the minimal number of acidotic cells required to initiate a PVC were investigated to evaluate the sink-source relationship of cardiac tissues. As the SR calcium content gradually decreased after acidosis, peak $[Ca^{2+}]_{SR}$ was varied from the maximum $[Ca^{2+}]_{SR}$ at the end of acidosis to that at the end of post acidosis. In addition, increasing $[Ca^{2+}]_i$ may increase gap junction resistance (Noma and Tsuboi, 1987; Peracchia, 2004) and gap junctional uncoupling was also observed in ischaemic tissues. The extent of gap junctional uncoupling was modeled by decreasing the diffusion coefficient (D) (see Equation 1 in Numerical methods) from 100% (0.154 mm^2/ms) to 80, 60, 40, and 20%.

Model of Unidirectional Conduction Block in a 1D Transmural Ventricular Fiber

To assess the role of repolarization dispersion in making tissue susceptible to unidirectional conduction block, a 15-mm-long transmural fiber model was developed. The fiber consisted of a 3.75-mm-long Endo region, a 5.25-mm-long Mid region and a 6-mm-long Epi region (APs for Endo cells, Mid cells and Epi cells under the basal condition are shown in **Figure 1**). The total length of the transmural cable was within the width range (∼8–15 mm) of human ventricular wall (Drouin et al., 1995; Yan et al., 1998) and the proportions of each subdomain used in this study were consistent with those used in other studies (Zhang et al., 2008; Adeniran et al., 2012). The cellular uncoupling between the midmyocardium and epicardium was modeled with a 5-fold decrease in the diffusion coefficient at the epicardium-midmyocardium border, as previously suggested by Gima and Rudy (2002). To examine the electrical heterogeneity of the tissue, an excitation wave propagating from the endocardium to the epicardium was initiated by a stimulus (with an amplitude of $-40\ \mu A/cm^2$ and a duration of 1 ms), and the fiber repolarization time was measured as the latest repolarization time of cells in the fiber. TDR was quantified by computing the difference (the time interval between the earliest repolarization time and the latest repolarization time) in repolarization time along the fiber. The inducibility of unidirectional conduction block for a TDR value was quantified by computing the vulnerable window (VW), during which a PVC may evoke a unidirectional conduction wave. The unidirectional conduction block was created by applying a premature stimulus (for simulating an ectopic beat) to an epicardial region with a time delay after the previous stimulus. The increase in TDR (Levites et al., 1975) and the decrease in the current density of fast delayed rectifier potassium currents (IKr) (Jiang et al., 2000) were observed in ischaemic tissues. Moreover, the TDR may be mainly modulated by the midmyocardium, because APD prolongation in the midmyocardium is much greater than that in the endocardium or epicardium (Ueda et al., 2004). Therefore, the extent of TDR was varied by decreasing the conductance of IKr from 100% (Normal) to 80% (#1), 60% (#2), 40% (#3) and 20% (#4) in the midmyocardium to examine whether increased TDR promoted inducibility of unidirectional conduction block. Repolarization gradient was also calculated as repolarization time change rate per millimeter along the strand.

Model of Reentry in a 2D Transmural Ventricular Sheet

To determine whether the DADs caused by the calcium overloaded SR at the cellular level can evolve into reentrant excitation waves at the tissue level, and to determine the effects of TDR and gap junctional uncoupling on the genesis of reentry, computer simulations were performed in a transmural ventricular tissue sheet (consisting of 100 × 500 grid points, with each point representing a cell). The 2D model was constructed by expanding the 1D model of a 15-mm-long transmural fiber into a 75-mm-wide sheet. In the model, the size of the local acidotic region was chosen to be $6 \times 45\ mm^2$ (corresponding to 40 × 300 DAD generating cells) such that it was large enough to trigger a PVC in the tissue. In simulations, a planar excitation wave, which was elicited by a stimulus applied to the end of the endocardium side, propagated toward the epicardial region. After the conditioning wave, a PVC was produced in the acidotic epicardial region. When the PVC occurred within VW of cardiac tissues, unidirectional conduction of the PVC-evoked excitation wave was observed, leading to the formation of a spiral wave. Dynamics of excitation waves under normal, increased TDR (#2) and the #2 TDR combined with slow conduction conditions were investigated.

Numerical Methods

The monodomain model in cardiac electrophysiology was used to describe the reaction-diffusion system in simulating cardiac dynamics (ten Tusscher and Panfilov, 2006). The governing equation is

$$\frac{\partial E_m}{\partial t} = \frac{I}{C_m} + D\left\{\frac{\partial^2 E_m}{\partial x^2} + \frac{\partial^2 E_m}{\partial y^2}\right\} \quad (1)$$

where $C_m = 1\ \mu F/cm^2$ is the capacitance, D denotes the diffusion coefficient, I denotes the total transmembrane current and V_m is the membrane voltage. D was set to a constant value of 0.154 mm^2/ms that gave a conduction velocity (CV) of 74.2 cm/s, which was similar to the CV of excitation waves in human heart (Taggart et al., 2000). Time step (Δt) is 0.02 ms and space step ($\Delta x = \Delta y$) is 0.15 mm, which is close to the length of ventricular myocytes. The partial differential equations were solved by an explicit forward Euler approximation. Simulations were carried out on a 64 G memory with Intel core i703930K 64-bit CPU system. Efficient parallelization was implemented using GPU acceleration.

RESULTS

Acidosis-Induced Electrophysiological Changes at the Subcellular Level

Figure 2A illustrates altered calcium handling in the PPC during control, acidosis and post acidosis, and Supplementary Figure 4 is an enlarged view of changes in membrane potential (Em), $[Na^+]_i$, $[Ca^{2+}]_i$, $[Ca^{2+}]_{SR}$ and INCX at different times during the PPC. An arrhythmic pattern of phase-4 depolarization upon returning to normal pH was predicted (**Figure 2B**). The DAD was produced by the acidosis-induced increase in

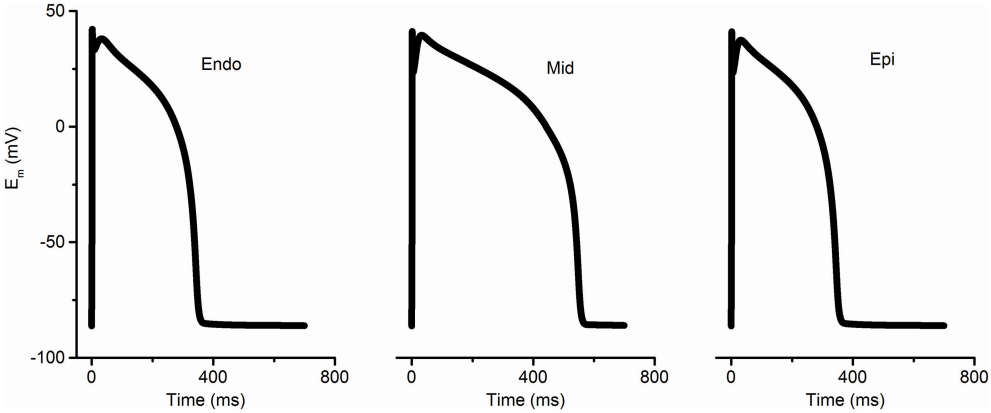

FIGURE 1 | Simulated membrane potentials (E_m) with the modified TP 06 model (ten Tusscher and Panfilov, 2006) for endocardium (Endo), mid-myocardium (Mid), and epicardium (Epi) under normal conditions.

$[Ca^{2+}]_{SR}$, which, consequently, enhanced the SR calcium leak, accompanied by an inward INCX that depolarized the cell. Increased INCX contributed to the inward current responsible for membrane depolarization amplification, which may lead to suprathreshold DADs (Jie and Trayanova, 2010). As pH reached its normal value, $[Ca^{2+}]_{SR}$ decreased, leading to a gradual reduction in the INCX, which caused a gradual decrease in membrane depolarization, resulting in subthreshold DADs. These ectopic beats were characterized by membrane depolarization associated with abnormalities in INCX, Irel and $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SR}$(**Figure 2B**).

To analyze altered calcium handling related to DADs generating, changes in $CaMK_{active}$, INCX, $[Ca^{2+}]_i$(max), $[Ca^{2+}]_{SR}$(max) and $[Na^+]_i$(max) due to pH alterations were examined for the normal, acidosis and post-acidosis conditions. At the beginning of acidosis, there was a sharp decrease in $[Ca^{2+}]_i$(max), $[Ca^{2+}]_{SR}$(max) and INCX, then acidosis produced a gradual increase in $[Na^+]_i$(max), $[Ca^{2+}]_i$(max), $[Ca^{2+}]_{SR}$(max) and INCX, and restoration of the intracellular pH caused abnormal $[Ca^{2+}]_i$(max), $[Ca^{2+}]_{SR}$(max), INCX, leading to membrane depolarization (**Figure 2A**). In detail, acidosis increased $[Ca^{2+}]_{SR}$, which reached to a maximum value of 4.86 mM at the end of the acidosis period. After acidosis, the calcium overload SR augmented Irel, which led to the first suprathreshold DADs with an occurrence time of 420 ms. Then, $[Ca^{2+}]_{SR}$ decreased, consequently, resulting in a gradually decrease in the membrane depolarization, generating subthreshold DADs with an occurrence time of 1,315 ms (**Figure 2B**).

Protection Effects of Delayed Recovery of Intracellular pH on the Development of Triggered Activity at the Cellular Level

To investigate the effects of the recovery time of intracellular pH on development of triggered activity in myocytes, three pH restoration protocols were used to examine the inducibility of triggered activity. With the fast pH recovery of 0.1 min, $[Ca^{2+}]_{SR}$ decreased suddenly, and the spontaneous SR calcium release increased quickly, leading to a suprathreshold DAD (**Figure 3A**). With the slow pH recovery of 0.5 min, $[Ca^{2+}]_{SR}$ decreased gradually, and the spontaneous SR calcium release was limited. As the pH restoration proceeded, subthreshold DAD was triggered (**Figure 3B**). With the gradual pH restoration of 4 min, delayed recovery of the intracellular pH attenuated the SR calcium load and suppressed the occurrence of DADs (**Figure 3C**).

Furthermore, multiple pH restoration protocols (P_X) with an X-min-long recovery time were used to assess the probability of generating DADs (**Figure 3D**). In simulations, no DAD was observed for slow pH recovery processes ($t \geq 4$ min). However, when the pH recovered quickly ($0.15 \leq t < 4$ min), the number of post-acidosis DADs increased, and subthreshold DADs were triggered. As the pH restoration became faster ($0 \leq t < 0.15$ min), suprathreshold DADs were obtained (**Figure 3E**). In addition, the number of ectopic beats gradually decreased with the increase of pH restoration time (**Figure 3F**). With a time course of 0, 0.1, 0.2, 0.5, 1, 2, 3, and 4 min of pH recovery, the numbers of DADs during post acidosis were 26, 25, 23, 20, 17, 9, 4, and 0, respectively. Compared with the control condition (P_0), the incidences of DADs in P_0.1, P_0.2, P_0.5, P_1, P_2, P_3, and P_4 decreased by 4, 11, 23, 34, 65, 84, and 100%, respectively. In the simulation, at least 4 min of pH restoration was required to achieve sustained protection against DADs.

Requirement for the PVCs Development Arising from DAD Generating Cells

The size of the acidotic region required to produce a PVC was examined in a 1D homogeneous strand. For the subthreshold DADs, although cells in the cable exhibited subthreshold DADs, the acidotic region failed to produce a PVC (**Figure 4A**). Similarly to subthreshold DADs, the acidotic region with 24 suprathreshold DAD cells could not overcome the sink-source mismatch to produce a propagating excitation wave (**Figure 4B**), while this region with 25 cells exhibiting suprathreshold DAD could do so (**Figure 4C**). Therefore, cells exhibiting

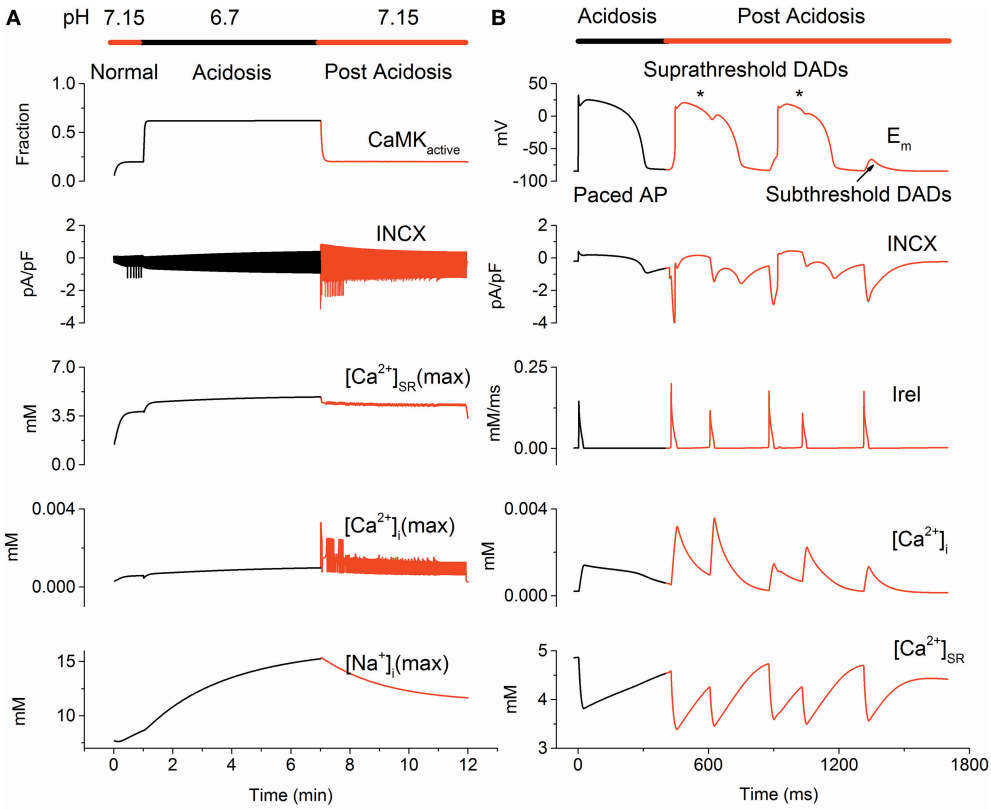

FIGURE 2 | The protocol of pH changes (PPC) predicted delayed afterdepolarizations (DADs) on returning to normal pH. (A) Changes of $CaMK_{active}$, INCX, $[Ca^{2+}]_{SR}$(max), $[Ca^{2+}]_i$(max) and $[Na^+]_i$(max) due to pH alterations in the PPC. **(B)** Time course of membrane potential (E_m), INCX, Irel, $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SR}$ during acidosis (Black) and post acidosis (Red). Suprathreshold DADs marked by an asterisk and subthreshold DADs marked with an arrow are shown. $CaMK_{active}$, fraction of activated calcium/calmodulin-dependent protein kinase II; $[Ca^{2+}]_i$(max), maximum cytoplasmic calcium concentration; $[Ca^{2+}]_{SR}$(max), maximum sarcoplasmic reticulum calcium concentration; $[Na^+]_i$(max), maximum intracellular sodium concentration; INCX, the sodium-calcium exchanger current and Irel, sarcoplasmic reticulum calcium release flux.

suprathreshold DADs that act as a "source" of excitation could initiate excitation wave propagating in the surrounding normal cells ("sink"), generating a PVC at the fiber tissue level.

Analysis of subcellular SR function has shown that the DADA is generally $[Ca^{2+}]_{SR}$-dependent (Schlotthauer and Bers, 2000; Katra and Laurita, 2005). SR calcium overload increased the spontaneous calcium release, promoting membrane depolarization toward the threshold to trigger an AP, the precursor of PVC. The minimal value of peak $[Ca^{2+}]_{SR}$ contributing to the genesis of a suprathreshold DAD was 4.45 mM during post acidosis. The voltage threshold for inducing suprathreshold DADs was ~18.15 mV (from −84.15 to −66 mV) (**Figures 5A,B**). For the suprathreshold DAD case, the length of the acidotic region required to trigger a propagating excitation wave was 14.4 mm, corresponding to 96 myocytes. If the peak $[Ca^{2+}]_{SR}$ was augmented to increase the DADA further above the voltage threshold, the number of cells in the acidotic region decreased significantly (**Figure 5C**). The maximum $[Ca^{2+}]_{SR}$ during acidosis was 4.86 mM, and the corresponding DADA at this point was 109.1 mV; consequently, the minimal number of DAD generating cells required to elicit a propagating AP was 25 (3.75 mm). Acidosis-induced SR calcium overload may cause membrane depolarization after acidosis and consequently enhance the "source" of excitation required for the generation of PVCs.

To investigate the effect of gap junctional uncoupling on the development of PVCs, we performed simulations with the diffusion coefficient decreasing from 100 to 80, 60, 40, and 20%, which reduced CV from 74.2 to 65.8, 56.3, 45, and 30.1 cm/s, respectively. When the peak $[Ca^{2+}]_{SR}$ was 4.86 mM, the size of the acidotic region was significantly reduced with the decrease in diffusion coefficient. In detail, the length of the acidotic region required to trigger a PVC was reduced from 3.75 to 3.15, 2.85, 2.25, and 1.5 mm when the diffusion coefficient was decreased by 20, 40, 60, and 80%, respectively (**Figure 5D**). In the simulation, gap junctional uncoupling may increase tissue resistance, which could decrease the "sink" of the surrounding normal tissue, facilitating the PVCs generation.

Taken together, these simulations showed that acidosis-induced electrophysiological changes may increase the "source" of excitation and decrease the "sink" of cardiac tissues, resulting in increased susceptibility to PVCs during post acidosis.

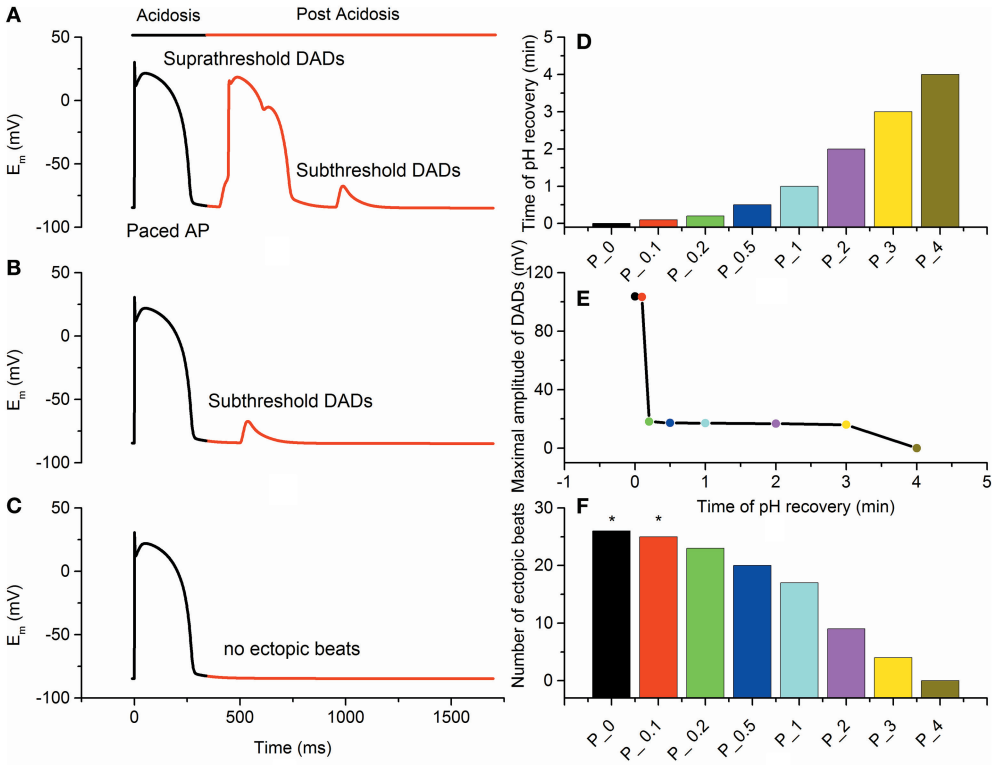

FIGURE 3 | Epicardial membrane potentials (E_m) with different pH recovery protocols after acidosis. (A) The fast pH recovery of 0.1 min triggered suprathreshold delayed afterdepolarizations (DADs). **(B)** The slow pH recovery of 0.5 min triggered subthreshold DADs. **(C)** The gradual pH recovery of 4 min suppressed the occurrence of DADs. **(D)** pH recovery time for different simulation protocols. Each bar represents the time of pH recovery for each protocol. **(E)** The maximal amplitude of DADs after acidosis gradually decreased with the recovery time of pH. **(F)** The number of ectopic beats measured under each condition. Suprathreshold DADs (marked by an asterisk) were triggered in P_0 and P_0.1.

Pro-arrhythmic Effects of Altered Electrical Heterogeneity on Inducibility of Unidirectional Conduction Block at the Fiber Tissue Level

Excitation wave propagation in regionally ischaemic tissues indicated that the onset of reentry was associated with repolarization dispersion (Nash et al., 2003). To examine the effects of TDR on the initiation of reentry and determine the degree of TDR required to induce unidirectional conduction block in response to a PVC, a 1D transmural fiber containing an acidotic region large enough to produce a PVC was used to perform simulations. A stimulus was applied to the first 3 cells at the endocardial (Endo) end, and the ability of the initiated wave to propagate to the epicardial (Epi) region was studied under five conditions (including Normal, #1, #2, #3, and #4). The repolarization time increased from 420 ms (Normal) to 423.5 ms (#1), 427.3 ms (#2), 431.2 ms (#3), and 435.4 ms (#4) (**Figure 6A**). The TDR due to the electrophysiological heterogeneity of cell types across the ventricular wall was also augmented from 40.52 ms (Normal) to 43.93 ms (#1), 47.52 ms (#2), 51.3 ms(#3), and 55.34 ms (#4), respectively (**Figure 6C**). Most importantly, cellular uncoupling between the subepicardium and midmyocardium resulted in an abrupt increase in the repolarization gradient from 14.6 ms/mm (Normal) to 16.0 ms/mm (#1), 17.5 ms/mm (#2), 19.0 ms/mm (#3), and 20.6 ms/mm (#4), respectively (**Figure 6B**). The augmented TDR increased the VW (**Figure 6D**) and produced steep spatial gradients of repolarization that may be responsible for unidirectional conduction block (**Figure 6F**). In detail, for the normal TDR, the repolarization gradient between the subepicardium and midmyocardium in response to the local PVC failed to cause a unidirectional conduction (**Figure 6E**), because the upper limit of the VW in the normal settings was below the occurrence time of the PVCs induced in the post-acidosis period (420 ms, threshold of unidirectional conduction block) (**Figure 6D**). If the TDR was progressively increased, as might occur physiologically during acidosis (Jie et al., 2010), unidirectional conduction block generated when the TDR was above the critical threshold (>43.93 ms). In the #2 case, the tissue surrounding the local acidotic region partly recovered for re-excitation, and an excitation wave induced by the post-acidosis PVC could propagate to the recovered tissue (the subepicardium) and be blocked by the unrecovered tissue (the midmyocardium), so unidirectional conduction was obtained (**Figure 6F**). The findings implied that increased TDR in response to a PVC which can arising from an acidotic region may lead to unidirectional conduction block favoring the development of reentry.

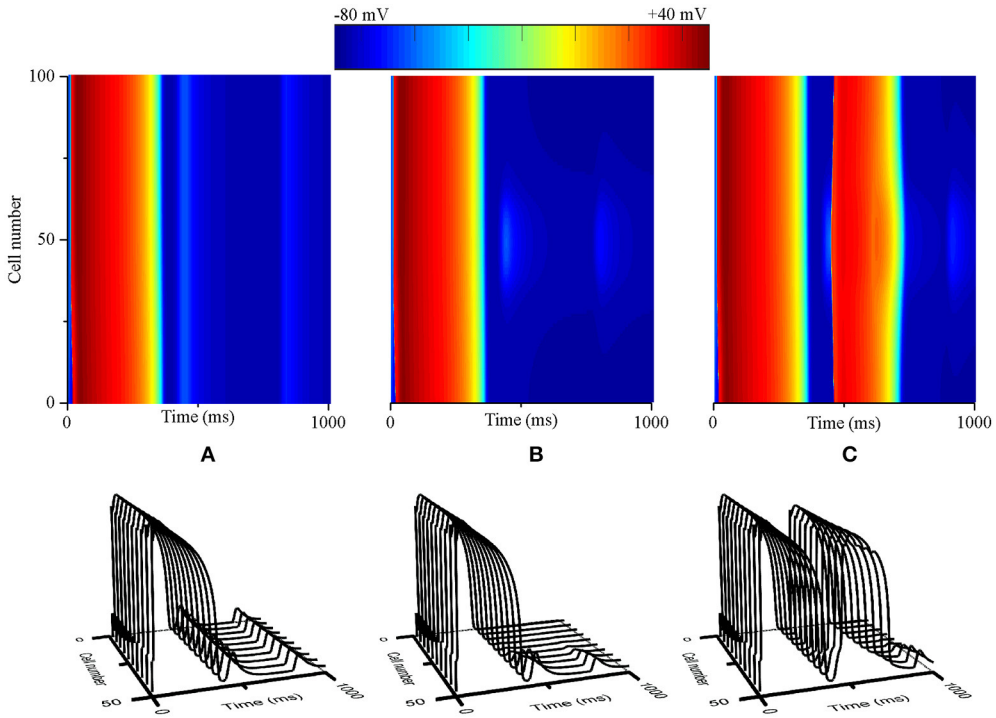

FIGURE 4 | Requirements for delayed afterdepolarizations (DADs) to produce a premature ventricular complex (PVC) in the 1D cable in which a central region of contiguous myocytes exhibiting DADs was surrounded by normal cells. (A) No PVC produced due to subthreshold DADs in the central region. **(B)** No PVC generated, though cells in the central region exhibiting suprathreshold DAD but the length of the region is under a critical value (e.g., 24 cells). **(C)** A PVC generated as the length of the region exhibiting suprathreshold DAD is over a critical value (e.g., 25 cells).

The Reentry Evaluation from PVCs at the Tissue Sheet Level

To determine whether interactions between triggered activity and local repolarization dispersion produce reentry, further simulations using a 2D transmural tissue model with an acidotic region were conducted to examine the initiation of reentry caused by PVCs and altered electrical heterogeneity. For the normal condition, a planar conditioning wave was initiated by a conditioning stimulus that propagated from the endocardium to the epicardium (**Figure 7A**, Time = 10 ms), and then the acidotic region generated a PVC which occurred at 420 ms and propagated to the surrounding tissue (**Figure 7A**, Time = 421 ms). However, the PVC produced bidirectional conduction (**Figure 7A**, Time = 510 ms) and disappeared at 840 ms, because the occurrence time of the PVC was out of VW. When the TDR was increased, the PVC occurred at 420 ms which is within VW under cases #2, #3, and #4 (**Figure 6D**). Thus, in the #2 case, the 2D tissue generated unidirectional conduction block in response to the PVC and promoted the initiation of reentry (**Figure 7B**, Time = 590 ms), but the spiral wave spontaneously terminated within 930 ms. The reason for this result may be that the tissue size could not support the spiral wave due to the large wavelength (WL) (WL = APD × CV). If the WL was shortened by slowing the conduction, i.e., as observed in ischaemic patients with gap junctional uncoupling, the spiral wave was self-maintained throughout the period of simulation in the #2 case with the reduction in D from 0.154 mm^2/ms to 0.0616 mm^2/ms, which corresponded to a decrease in CV from 74.2 to 45 cm/s (**Figure 7C**). The persistent reentrant waves can be explained by the short WL, resulting in a reduction in the size of the substrate required to facilitate and maintain reentry. Video files showing re-entry in 2D models are included in Supplementary Video 1.

The re-entry initiation is not only facilitated by the transmural heterogeneity, but also promoted by the tissue anisotropy. An anisotropic 2D ventricular tissue sheet with an acidotic island (Supplementary Figure 2) was also modeled to explain the re-entry initiation. The effect of pH restoration time on the inducibility of reentrant arrhythmias was investigated. Dynamics of excitation waves under the suprathreshold DADs (Supplementary Figure 3A), subthreshold DADs (Supplementary Figure 3B) and normal (Supplementary Figure 3C) conditions were investigated. Details can be found in Supplementary Material. The suprathreshold DADs evolved into a PVC, and finally produced a figure-of-eight reentry in the anisotropic tissue (Supplementary Figure 3A).

DISCUSSION

Summary of Major Findings

The present study was an attempt to provide mechanistic insight into the complex electrophysiological effect of acute

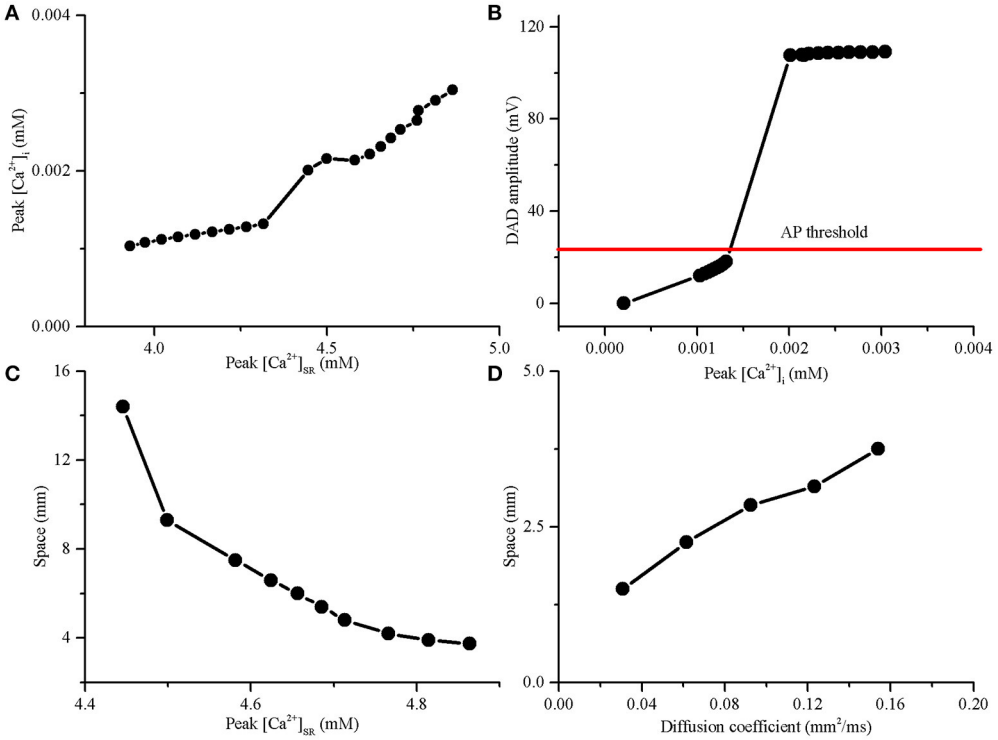

FIGURE 5 | The delayed afterdepolarization (DAD) amplitude required to trigger an action potential (AP) and the length of DAD generating region in a 15-mm-long 1D cable required to trigger a premature ventricular complex (PVC). (A) Amplitude of cytoplasmic calcium transient (peak $[Ca^{2+}]_i$) increased with SR calcium content (peak $[Ca^{2+}]_{SR}$). **(B)** The DAD amplitude increased with the increase of peak $[Ca^{2+}]_i$ in a single myocyte. **(C)** The SR calcium overload significantly decreased the length of DAD generating region required to trigger a PVC. **(D)** Slow conduction by decreasing the diffusion coefficient can decrease the length of the DAD generating region for producing a PVC.

acidosis on the evolution of reentrant arrhythmia at the tissue level. It was a comprehensive framework for understanding the mechanisms responsible for postacidotic arrhythmias originated from microscopic electrophysiological alterations. In this work, acidosis was assumed to affect calcium handling at the subcellular level, kinetic properties of ion channels at the cellular level, and electrical heterogeneity and gap junctional coupling at the tissue level. Under these circumstances, simulation results indicated that reentry can be spontaneously initiated in cardiac tissues during post acidosis and that the initiation of reentry was affected by recovery time of intracellular pH, the extent of SR calcium load, the size of the acidotic zone, the extent of repolarization dispersion and the degree of gap junction uncoupling. To unravel the mechanisms underlying arrhythmogenesis after acidosis, effect of pH restoration time on the inducibility of DADs, effect of SR calcium overload and gap junction uncoupling on the source-sink relationship required to the PVCs producing and effect of altered repolarization dispersion on the development of unidirectional conduction block necessary to the genesis of reentry were investigated. It was shown that acute pH restoration during post acidosis modified the balance of ion flow inside and outside the ventricular cell, resulting in DADs. These DADs may increase the "source" of excitation, while gap junction uncoupling can decrease the "sink" of cardiac tissue, both of which mediated the source-sink balance that can constitute the requirements for PVC generation. Synchronized DADs can overcome the source-sink mismatch and produce a PVC in ventricular tissues. The conduction of a PVC can be unidirectionally blocked and form a spiral wave if the PVC occurs within the VW of the tissue. And acidotic tissue may increase electrophysiological heterogeneity and consequently alter repolarization dispersion and elevate the upper limit of VW, increasing the susceptibility to re-entrant arrhythmias. These mechanisms are discussed in detail in the sections to follow.

Mechanistic Insights

SR calcium overload has been linked to arrhythmias after acidosis. Experimental and computational studies have shown that ectopic activity occur after acidosis when the SR becomes calcium-overloaded for an increase in CaMKII phosphorylation of PLN (Mattiazzi et al., 2007; Said et al., 2008; Pedersen et al., 2009; Lascano et al., 2013). DAD-induced triggered activity was prevented by decreasing the SR calcium content upon application of a CaMKII inhibitor. The DADA, which depended on the extent of the SR calcium load, may determine the inducibility of arrhythmias. Since suprathreshold DADs can initiate APs for propagation to adjacent cells, the results support the notion of a major role of SR calcium overload in the onset of ectopic

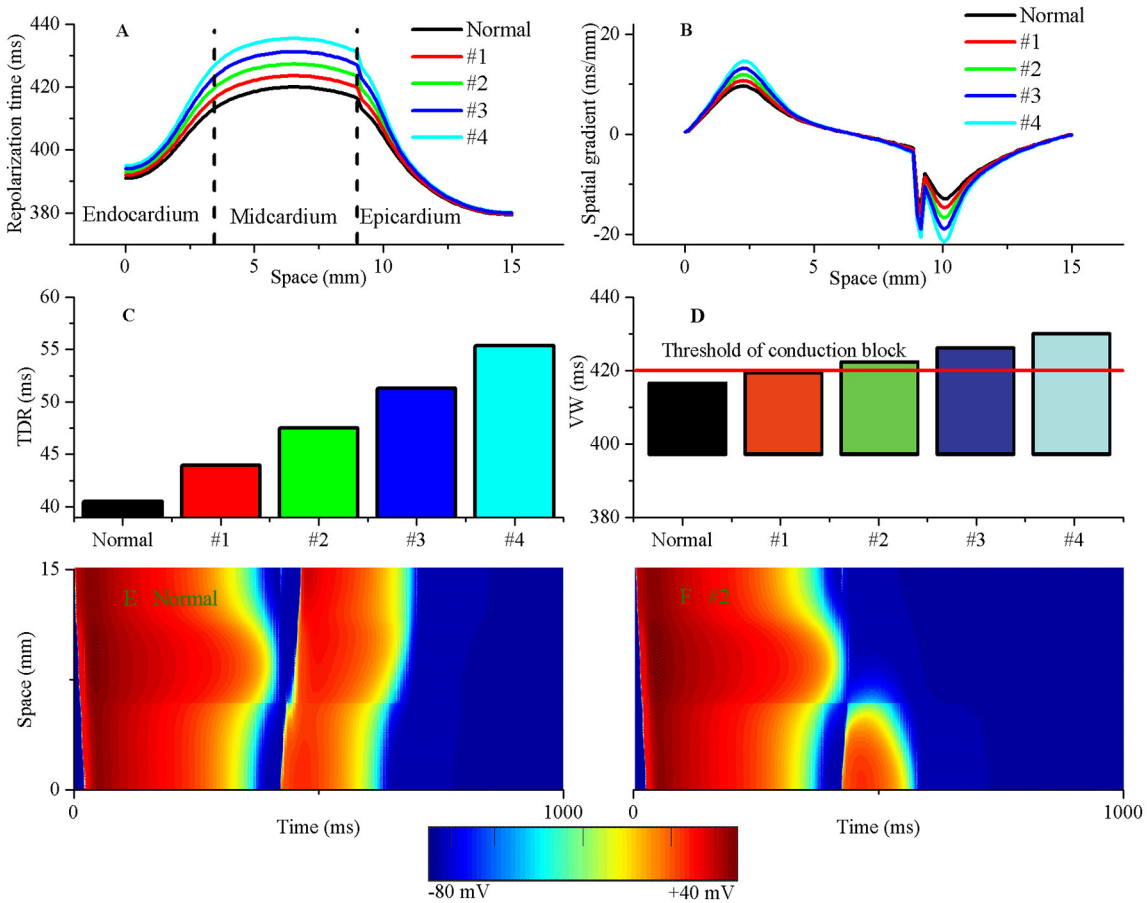

FIGURE 6 | The augmented transmural dispersion of repolarization (TDR) can increase tissue vulnerability to unidirectional conduction block. The repolarization time **(A)**, spatial gradients of repolarization time **(B)**, TDR **(C)** and vulnerable window (VW) **(D)** are shown under normal, #1, #2, #3, and #4 conditions. The VW progressively grew when TDR was augmented. The threshold of unidirectional conduction block in post acidosis was equal to 420 ms when suprathreshold DADs occurred in single cells. **(E)** A premature ventricular complex (PVC) occurred without tissue's VW under the normal condition and produced a bidirectional conduction, whereas a unidirectional conduction formed when a PVC occurred within tissue's VW under the #2 condition **(F)**.

activity. Recent experimental data indicated that a rabbit heart with focal arrhythmia showed a consistent increase in SR calcium content (Schlotthauer and Bers, 2000) and suprathreshold DADs after acidosis were observed in rat-isolated epicardial myocytes (Said et al., 2008). In agreement with previous findings, the simulation results showed that $[Na^+]_i$, $[Ca^{2+}]_{SR}$, and $[Ca^{2+}]_i$ gradually accumulated during acidosis. An increase in diastolic $[Ca^{2+}]_i$ caused by SR calcium leak was also predicted during post acidosis. The SR calcium leak due to the SR calcium load greatly augmented the incidence of calcium-induced transient depolariztheation after acidosis. Importantly, the DADA can reach the threshold to trigger an AP, thus increasing the likelihood of having PVCs.

It is more likely to produce PVCs during post acidosis at the beginning than at the end. The simulation results support the common perception that the SR calcium load resulting from acidosis is pro-arrhythmic. Synchronized SR calcium release due to SR calcium load can generate a PVC after acidosis. The propensity for PVCs to occur is consistent with experimental observations (Fujiwara et al., 2008). Local β-adrenergic receptor stimulation caused spatiotemporal synchronization of the SR calcium overload and DADs, and these synchronized DADs overcame the sink impedance to trigger focal arrhythmia in the rabbit heart (Myles et al., 2012). Although PVC inducibility was related to area of the DAD generating region, the difference in the same area of acidotic regions did not account for the high arrhythmia propensity at beginning of the post-acidosis period. The mechanisms underlying the increased arrhythmogenic potentials upon returning to normal pH were not fully characterized. The simulation results suggest a mechanism by which SR calcium load after acidosis may increase the incidence of arrhythmias. It is well established that the area of DAD generating region is proportional to the amount of calcium in the SR (Xie et al., 2010; Myles et al., 2012). A larger amount of SR calcium might lead to an increase in the DADA, resulting in a "source" of excitation, thus significantly decreasing the area of acidotic regions required to induce a PVC. Indeed, it was shown that peak $[Ca^{2+}]_{SR}$ reached the maximum at the end of

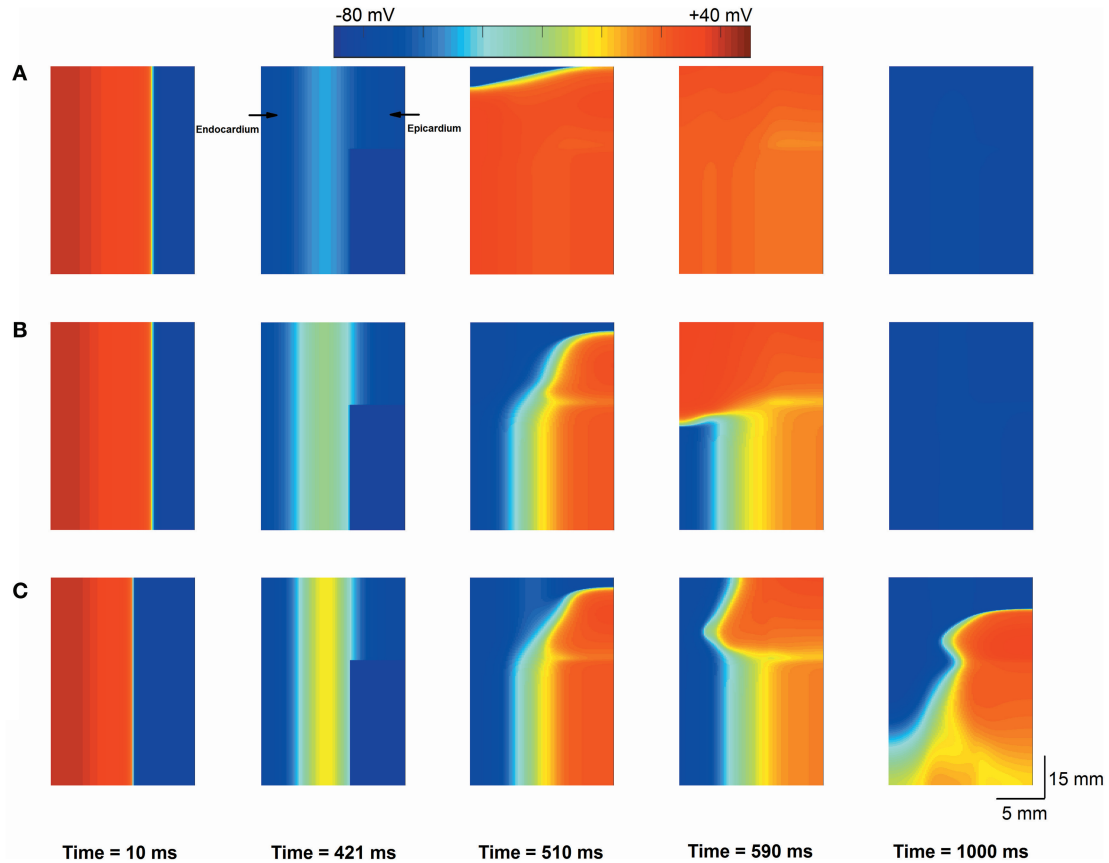

FIGURE 7 | Snapshots of excitation waves in a 2D transmural ventricular tissue. Under the normal transmural dispersion of repolarization (TDR) **(A)**, #2 TDR **(B)** and #2 TDR combined with slow conduction **(C)** conditions, a planar conditioning wave generated at the end of the endocardium and propagated to the epicardium (Time = 10 ms), and then a premature ventricular complex (PVC) due to the acidotic region occurred at Time = 420 ms. Bidirectional conduction **(A)** and unidirectional conduction block **(B,C)** were observed (Time = 590 ms), but a spiral wave self-terminated under the #2 TDR condition **(B)** and maintained in #2 TDR combined with slow conduction settings **(C)** (Time = 1,000 ms).

acidosis. The maximum $[Ca^{2+}]_{SR}$ caused the SR calcium release, leading to the maximum DADA. Thus, the number of DAD generating myocytes required to initiate a PVC was the minimum upon returning to normal pH, and consequently the maximal propensity to PVCs.

Delayed recovery of intracellular acidosis during post-acidosis can prevent the triggering of ventricular arrhythmias (Avkiran et al., 1996). It was shown that the prolongation of intracellular acidosis could suppress the occurrence of DADs. The results further suggested that postconditioning protection may depend on the prolongation of pH recovery during reperfusion and at least 4 min of pH restoration was required to achieve sustained protection against DADs. The minimum time for pH restoration required for this protection is within the time range of rat hearts (2–4 min) (Avkiran et al., 1996; Fujita et al., 2007). Previous studies have shown that, acidic reperfusion can significantly suppress the incidence of ventricular fibrillation in regionally ischaemic isolated rat hearts and that the protective mechanism may involve enhanced recovery of sodium-potassium-ATPase activity as well as inhibition of sodium influx (Avkiran et al., 1996); this assertion was confirmed in a previous simulation study that suggested increased intracellular sodium was a critical determinant of SR reloading and hence DAD maintenance (Lascano et al., 2013). In addition, postconditioning protection may contribute to prolongation of intracellular acidosis during reperfusion. At least 2 min of acidic reperfusion was required to achieve sustained protection against VF (Avkiran et al., 1996), and a further delay in pH recovery (>3.5) could improve functional recovery and reduce infarct size (Inserte et al., 2009). Hence, the results may be used to explain the postconditioning protection during reperfusion.

Repolarization dispersion provided a key connection between PVCs and VT in cardiac tissues. It was shown that an increase in tissue electrophysiological heterogeneity promoted the onset of reentry in the transmural ventricular tissue with an acidotic region; the acidotic region can generate a PVC. The results support the previous notion that an increase in repolarization dispersion plays an important role in the genesis of reentry (Wu and Zipes, 2001, 2002). It is possible that unidirectional conduction block occurs in the presence of a PVC, since the

repolarization dispersion may increase the tissue VW and a PVC arising from an acidotic region is easily triggered within the VW]the first PVC occurred at 420 ms, which is more than, but fairly close to, the maximum time in the rabbit heart (from 300 to 400 ms)] (Myles et al., 2012). Previous studies have shown that PVCs mainly occur in the epicardium and that the TDR is significantly increased during acidosis (Said et al., 2008). In addition, unidirectional conduction block due to cellular uncoupling between midmyocardium and epicardium was also observed (de Groot et al., 2001). Hence, the transmural ventricular tissue with acidotic regions likely increased the incidence of unidirectional conduction block, thus facilitating the initiation of reentry, which may be used to explain the occurrence of VT observed in patients. These findings were supported by experimental studies that showed the decline in TDR prevented the generation of VT (Dhalla et al., 2009).

We also found that slow conduction, which may occur under conditions of gap junctional uncoupling, increased susceptibility to post-acidosis arrhythmias. These findings were supported by a previous study which has suggested an extreme reduction in the number of gap junction channels is arrhythmogenic during ischaemia-reperfusion (Sánchez et al., 2011). On the one hand, the number of contiguous myocytes exhibiting suprathreshold DADs for producing a propagating PVC was significantly reduced in gap junctional uncoupling settings. This effect can be attributed to a reduction in the sink size of the surrounding tissue, so the minimal source of excitation required to trigger a PVC decreased. On the other hand, the size of transmural ventricular tissues necessary to produce persistent spiral waves was greatly decreased in gap junctional uncoupling settings. Slow conduction is thought to lead to a decrease in WL. The results showed that spiral waves were self-maintained under slow conduction conditions. Therefore, our findings support the assertion that gap junctional uncoupling may increase the risk for arrhythmias.

Relevance to Previous Simulation Studies

Crampin et al. developed a dynamical model of pH regulation and used the Luo-Rudy-dynamic (LRd) model to investigate possible roles of acidosis on key ionic species. Calcium and sodium loading were predicted in their simulations. They suggested that the most significant effects of acidosis were elevated $[Na^+]_i$, inhibition of NCX, and the direct interaction of protons with the contractile machinery (Crampin and Smith, 2006).

Crampin et al. also carried out a mathematical study on acidosis to determine the effects of changes in pH on the membrane potential and calcium handling. They observed increased $[Ca^{2+}]_i$ at both peak and resting levels and AP shortening. They suggested that the rise in $[Na^+]_i$ mediated changes in $[Ca^{2+}]_i$ (Crampin et al., 2006).

Lascano et al. modified a human myocyte model consisting of CaMKII effects on ion flows and contractile constants, and investigated the molecular mechanisms underlying the triggered. They observed SR calcium loading and post-acidotic DADs upon returning to normal pH. They concluded that DADs in single cells depended on CaMKII effect on L-type calcium channel and SERCA2a (Lascano et al., 2013).

We have developed a human ventricular acidotic model consisting of pH and CaMKII regulations, and analyzed the functional influence of acidosis on cardiac electrical activity and ECGs. We observed heterogeneous APD abbreviation and a PVC in the simulated ECG waveform (Liu et al., 2016).

Our simulation results are in agreement with and extend the findings of these previous studies, adding to the possibilities that acidosis-induced electrical alterations may mediate tissue's source-sink interactions and consequently underlie the evolution from acidosis-induced DADs at the cellular level to PVCs at the tissue level, that acidosis-induced electrical heterogeneity can increase spatial gradients of repolarization and facilitate the evolution from PVCs into re-entry at the sheet tissue level, and that prolonged transient acidosis during the early reperfusion phase may underlie the protective mechanism of ventricular arrhythmogenesis.

Significance of the Study

It is well known that the heart becomes acid in a number of pathological conditions, most dramatically during ischaemia-reperfusion. It has been suggested that the development of arrhythmias during reperfusion is due to the associated acidosis (Orchard et al., 1987; Orchard and Cingolani, 1994; Avkiran et al., 1996; Said et al., 2008; Kapur et al., 2009; Nagai et al., 2010; Niwano and Tojo, 2010; Lascano et al., 2013). However, it is difficult to identify mechanisms underlying these effects of acidosis because the substrates of ventricular arrhythmias change too rapidly to be observed during IRI in patients. Further studies have also shown that electrical alterations, which provide a potential mechanistic link between acidosis and ventricular arrhythmias, occur during ischaemia-reperfusion (Pinto and Boyden, 1999). Electrical changes (e.g., calcium handling and afterdepolarizations) of ventricular myocyctes have been observed in a number of animal models of ischaemia-reperfusion (Orchard et al., 1987; Avkiran et al., 1996; Wu and Zipes, 2001; Said et al., 2008; Dhalla et al., 2009; Kapur et al., 2009), as well as in human ischaemia-reperfusion (Adams and Pelter, 2002). Structural alterations (including gap junctional uncoupling) of ventricular tissues were also observed in isolated mouse hearts of ischemia-reperfusion and in patients with persistent VF (Luqman et al., 2007). Thus, this study may provide insight into the mechanisms underlying the ischaemia-reperfusion induced ventricular arrhythmias.

Clinical Implications

The prolonged transient acidosis has important clinical implications. The heart was protected against acute myocardial infarction by interrupting myocardial reperfusion with several short-lived episodes of myocardial ischaemia, a phenomenon termed "ischaemic postconditioning (IPost)." IPost was found to confer a myriad of protective effects, including reduced levels of myocardial oedema, oxidative stress, and polymorphonuclear neutrophil accumulation, as well as preserved endothelial function (Hausenloy and Yellon, 2016). In addition, IPost is associated with the protection against ventricular arrhythmogenesis (Avkiran et al., 1996), perhaps

related to low inducibility of triggered activity via prolonged transient acidosis during the early reperfusion phase.

Limitations

The limitations (e.g., the model lacks the ability to simulate calcium waves and contractility) of the original TP06 model have been discussed elsewhere (ten Tusscher and Panfilov, 2006; Zhang et al., 2008; ten Tusscher et al., 2009). Here we explain several limitations specific to the work. Firstly, the geometry structure of tissue sheet used in simulations is idealized rather than realistic. Although the model geometry does not represent that of the realistic ventricular slice, the use of an idealized geometry was necessary to enable elucidation of the mechanism of arrhythmogenesis which was caused by electrical heterogeneity rather than geometrical structure. Secondly, SR calcium release randomly occurred in single cells and the exact time of PVCs in ventricular tissues was not directly measured, so this study assumed that the concurrence time of PVCs was the same as that of DADs in single cell. It may affect reentry initiation, however, the mechanisms uncovered here will remain valid. Thirdly, our model does not account for the spatial gradient and shape of the local acidotic region, which would cause repolarization dispersion in the acidotic region. However, the study focused on the effect of electrical intrinsic heterogeneity on arrhythmogenesis in post acidosis and this design can support the uncovered mechanisms by eliminating the influence of the spatial gradient from the acidotic region. Fourthly, our model simulated the increase in TDR by prolonging repolarization of the midmyocardium with decreased the conductance of IKr,but many ion currents changed in ischaemic tissues. Special attention should be paid while trying to conduct data analysis. Fifthly, due to the lack of a precise measurement of gap junction uncoupling, the relative efficacy among the decrease in the diffusion coefficient might be model specific and further refinement of these models is required. Sixthly, the kinetics of pH recovery were modeled with a linear function which was not realistic, but protection mechanisms upon prolongation of pH recovery will remain valid. Seventhly, even though acidosis is likely to occur during ischemia/reperfusion, electrical alterations considered in our study may result from other effects of ischemia and special caution should be paid in using these simulated results. Eighthly, early afterdepolarizations (EADs) were also observed, but the ionic mechanism underlying EADs during reperfusion is complex and role of EADs in post-acidosis arrhythmias remains incompletely understood, which warrants further study in future. Despite these limitations, the study may provide detailed mechanisms underlying the evolution of VT from acute regional acidosis.

CONCLUSION

The multi-scale cardiac modeling provides a framework to explain the evolution of postacidotic arrhythmias. Through multi-scale acidotic models, the relationship between acidosis-induced electrophysiological changes and ventricular arrhythmogenesis was built. The simulation results suggest that although SR calcium overload is a well-known ionic mechanism of triggered activity, source-sink interactions and electrical heterogeneity are critical determinants of the emergence of post-acidosis arrhythmias.

AUTHOR CONTRIBUTIONS

JB and KW conceived and designed the experiments; JB and RY performed the simulations, prepared figures and analyzed the results; JB, RY, KW, and HZ drafted and edited the manuscript. All authors reviewed the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Mild and Short-Term Caloric Restriction Prevents Obesity-Induced Cardiomyopathy in Young Zucker Rats without Changing in Metabolites and Fatty Acids Cardiac Profile

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Caloric restriction (CR) ameliorates cardiac dysfunction associated with obesity. However, most of the studies have been performed under severe CR (30–65% caloric intake decrease) for several months or even years in aged animals. Here, we investigated whether mild (20% food intake reduction) and short-term (2-weeks) CR prevented the obese cardiomyopathy phenotype and improved the metabolic profile of young (14 weeks of age) genetically obese Zucker *fa/fa* rats. Heart weight (HW) and HW/tibia length ratio was significantly lower in *fa/fa* rats after 2 weeks of CR than in counterparts fed *ad libitum*. Invasive pressure measurements showed that systolic blood pressure, maximal rate of positive left ventricle (LV) pressure, LV systolic pressure and LV end-diastolic pressure were all significantly higher in obese *fa/fa* rats than in *lean* counterparts, which were prevented by CR. Magnetic resonance imaging revealed that the increase in LV end-systolic volume, stroke volume and LV wall thickness observed in *fa/fa* rats was significantly lower in animals on CR diet. Histological analysis also revealed that CR blocked the significant increase in cardiomyocyte diameter in obese *fa/fa* rats. High resolution magic angle spinning magnetic resonance spectroscopy analysis of the LV revealed a global decrease in metabolites such as taurine, creatine and phosphocreatine, glutamate, glutamine and glutathione, in obese *fa/fa* rats, whereas

lactate concentration was increased. By contrast, fatty acid concentrations in LV tissue were significantly elevated in obese *fa/fa* rats. CR failed to restore the LV metabolomic profile of obese *fa/fa* rats. In conclusion, mild and short-term CR prevented an obesity-induced cardiomyopathy phenotype in young obese *fa/fa* rats independently of the cardiac metabolic profile.

Keywords: caloric restriction, hypertrophy, hypertension, nuclear magnetic resonance, metabolite profile, obesity-induced cardiomyopathy

INTRODUCTION

Obesity is a strong independent risk factor for cardiovascular (CV) disease and is associated with an increased CV mortality (Adams et al., 2006; Adabag et al., 2015). The prevalence of obesity in childhood and adolescence is reaching epidemic proportions. Moreover, it has been recently shown that increased body mass index in late adolescence, even within a clinically accepted normal range, is strongly associated with higher CV mortality in young adulthood or midlife (Twig et al., 2016). Unless this clinical situation is addressed at an early stage in life, it is likely that adverse CV outcomes will occur as a consequence of cardiac damage resulting from overweight and obesity.

Despite the well-established link between obesity and CV risk, the pathogenesis of obesity-induced cardiomyopathy is not fully understood. Several factors as deleterious metabolic and structural remodeling contribute significantly to the subsequent functional cardiac damage. In this sense, in the obese-induced cardiomyopathy the heart undergoes a metabolic remodeling in response to the high energy demand characterized by a reduction of the energy reserve compounds as phosphocreatine and by a lipid overload, both conditions impairing cardiac efficiency (Kolwicz et al., 2013). These metabolic changes observed in hearts with obese-induce cardiomyopathy occur early and often precede later functional changes as ventricular dysfunction (Peterson et al., 2004). Thus, the most common structural and physiological changes of the heart in obesity include cardiac hypertrophy and diastolic dysfunction (Alpert, 2001; Rider et al., 2014), and both are associated with all-cause mortality (Levy et al., 1988; Bhatia et al., 2006).

Several strategies are available to protect the heart from obesity-induced cardiomyopathy, with caloric restriction (CR) as one of the most common, cost-effective non-pharmacological and non-surgical interventions used to reduce body weight (BW) and CV risk. CR is defined as a state in which energy intake is reduced below normal *ad libitum* (AL) intake without malnutrition (García-Prieto and Fernández-Alfonso, 2016). CR increases longevity and improves the outcome of obesity-associated diseases, including CV disease (Fontana et al., 2010). Clinical and experimental studies have shown that CR ameliorates cardiac dysfunction associated with obesity, although the majority of these studies were carried out under one or more of the following conditions: (1) high to severe CR regimen (30–65% reduction of food intake; Zheng et al., 2009; Niemann et al., 2010; Shinmura et al., 2011; Takatsu et al., 2013; Melo et al., 2016); (2) long-term CR (several months) (Meyer et al., 2006; Hammer et al., 2008; Zheng et al., 2009; Niemann et al., 2010; Melo et al., 2016); and (3) adult or elderly individuals (Meyer et al., 2006; Hammer et al., 2008; Niemann et al., 2010; Shinmura et al., 2011). The potential benefits of a milder shorter-term CR regimen on hearts with obese-induced cardiomyopathy in a younger population are much less known. Testing a mild CR regimen and in young animals would help to elucidate the relative contribution that changes in the metabolic, structural and functional remodeling have on obese cardiomyopathy in at early stage of development. Thus, the purpose of the present study was to investigate the impact of mild (20% food intake reduction) and short-term (2 weeks) CR on the cardiac phenotype in young genetically obese Zucker rats through the parallel analysis of cardiac function, cardiomyocyte structure and metabolic profile.

MATERIALS AND METHODS

Animals and Caloric Restriction Protocol

Eight-week old male obese Zucker rats (*fa/fa)* and non-obese control (*lean*) were housed under specific controlled dark-light cycles (12/12h, lights on at 8:00 a.m.) and temperature (22°C), with standard chow and water *ad libitum* (AL). To control BW and food intake, animals were housed individually and daily monitored for 4 weeks. Then, animals were randomly divided into two groups and assigned either to an AL or to a CR diet (80% of AL) for two additional weeks. A scheme of the protocol is shown in **Figure 1A**. Adjustment of 20% CR was done individually based on the previous food intake values. Rats were weighed before sacrifice. All animal procedures were conducted in accordance with the recommendations of the Spanish Animal Care and Use Committee according to the guidelines for ethical care of experimental animals of the European Union (2010/63/EU). The study was approved by the Ethical Committee of Universidad Complutense and Comunidad de Madrid (reference: PROEX 413/15).

Hemodynamic Parameters

Rats were anesthetized with ketamine (Imalgene 1000, Merial; 80 mg/kg i.p.) and xylazine (Rompun 2%, Bayer; 8 mg/kg i.p.), and a 0.58/0.97 mm inner- and outer-diameter, respectively, catheter was inserted in the left ventricle (LV) through the right carotid artery. The catheter was connected to a data acquisition system (PowerLab 4/30, ADInstruments, UK) and signals were digitally stored for analysis using the LabChart 7.0 Pro software. Systolic (SBP) and diastolic (DBP) blood pressure, LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), the first derivative of LV pressure rise over time (dP/dt_{max}), and the first derivative of LV pressure decline over time (dP/dt_{min})

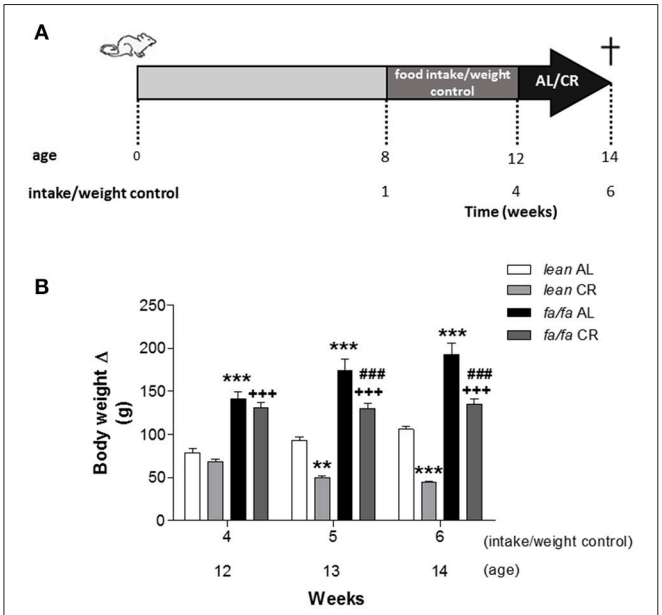

FIGURE 1 | Scheme of experimental protocol (A) and body weight gain **(B)** of *lean* and *fa/fa* rats with and without caloric restriction. Half of the animals of each experimental group were fed with 80% of their daily food intake (CR) and the other half were maintained on AL diet. Data are mean±SEM ($n = 8$ animals per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. *lean* AL group; $^{+++}P < 0.001$ vs. *lean* CR group; $^{\#\#\#}P < 0.001$ vs. *fa/fa* AL group.

were analyzed. After hemodynamic measurements, animals were sacrificed and hearts were removed for study. Hearts were weighed and heart weight (HW)/tibia length ratio was used as an index of cardiac hypertrophy.

Magnetic Resonance Imaging Acquisition and Analysis

Magnetic resonance imaging (MRI) was performed with a Biospec BMT 47/40 spectrometer (Bruker, Ettlingen, Germany) operating at 4.7-Teslas, equipped with a 12-cm gradient system. Rats were anesthetized with an isoflurane and oxygen mixture (3% in oxygen at 1.5 L/min for induction and 1.0–1.5% at 1.0 L/min during experiments). Body temperature was monitored and maintained at 36°C. Heart rate (HR) and respiration were monitored and used to trigger image acquisition with 1025 SAM monitoring and gating system (SA Instruments, Inc., USA). Several gradient echo images with different orientations were acquired to localize the short axis planes. Images were cardiac and respiratory triggered and up to 7 slices were acquired in a cardiac cycle. Repetition time (RT) was variable depending on the animal's heart and respiration rate. Other imaging parameters were as follows: echo time (ET) = 2.7 ms; flip angle (θ) = 80°; field of view (FOV) = 6 × 6 cm^2; slice thickness = 2.0 mm; matrix size = 128 × 128; number of averaged images = 2. Once the short axis was set, a multislice white blood CINE sequence was used to image the rat's entire heart. For these experiments, a cardiac and respiratory-triggered FLASH sequence was used. Ten images per cardiac cycle were acquired to completely cover the cardiac cycle. Other imaging parameters were as follows: ET = 2 ms; $\theta = 80°$; FOV = 5.12 × 5.12 cm^2; slice thickness = 1.5 mm; matrix size = 128 × 128; number of averaged images = 8. A total of 8–10 slices were acquired to cover the whole heart. The data were transferred to a PC for analysis, which was carried out with ImageJ 1.49v (NIH, USA). For each slice of the CINE sequence, the LV was segmented in the images corresponding to diastole and systole. The LV volume for each slice was summed to obtain the total left ventricular (LV) volume at the end of diastole and systole (EDV and ESV, respectively). The heart rate for each rat was calculated as the mean value of the heart rate during the entire CINE experiment. Ejection fraction (EF), stroke volume (SV), and cardiac output (CO) were obtained from these data applying these formula: EF = [EDV-ESV/LVEDV]∗100; SV = EDV-ESV; CO = SV ∗ HR

Histological Analysis

Hearts were fixed in 4% buffered formaldehyde solution for 48 h. Hearts were sectioned transversally including both ventricles. Cardiac sections were embedded in paraffin. Histological sections were obtained at 5 μm and stained with periodic acid solution and Schiff's reagent. Cardiomyocytes of the left ventricular wall were photographed in three high power fields (×40 objective) for each animal. The minor diameter of cardiomyocytes was measured. We only measured those cardiomyocytes with a recognizable nucleus. Diameter lower than 13 μm were rejected since they were considered as a dichotomy branch of cardiomyocyte; and diameters higher than 30 μm were rejected because borders between cardiomyocyte were not well delimited. The cardiomyocyte diameter was obtained using the morphometric ImagenJ software.

Real-Time PCR for mRNA Collagen Type I Analysis

Total RNA was extracted from frozen LV tissue with TRIzol reagent (Invitrogen) and 1 μg was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit for RT-PCR (Roche). Real-time PCR was performed on a MyiQ Real-Time PCR System (Bio-Rad) using Taqman Gene Expression Assays (Applied Biosystem). PCR thermocycling parameters were 50°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression of type I collagen was measured using the Quanti SYBR Green RT protocol (Roche). Melting curve data were collected to check the PCR specificity. Each cDNA was amplified in triplicate and the corresponding sample without reverse transcriptase (no-RT sample) was included as a negative control. Gene expression levels were normalized to that of 36B4 mRNA. Replicates were then averaged, and fold induction was determined using a $^{\Delta\Delta}$Ct-based fold-change calculation. The primers used were type I collagen (forward primer 5′-AAT GGC ACG GCT GTG TGC GA-3′, reverse primer 5′- AGC ACT CGC CCT CCC GTC TT-3′) and 36B4 (forward primer 5′-AGA TGC AGC AGA TCC GCA T-3′), (reverse primer 5′-GTT CTT GCC CAT CAG CAC C-3′).

Metabolites and Fatty Acids Quantified by ^{1}H HR-MAS NMR

High-resolution magic angle spinning (^{1}H HR-MAS) NMR spectroscopy was performed at 500.13 MHz using a Bruker AMX500 spectrometer 11.7 T (Bruker Rheinstetten, Germany) on frozen LV samples placed within a 50 µl zirconium oxide rotor with cylindrical insert and spun at 4000 Hz spinning rate. Standard solvent suppressed spectra were acquired into 16 k data points, averaged over 256 acquisitions, total acquisition ∼14 min, using a standard Bruker sequence (noesypr1d) with a relaxation delay of 2 s and a mixing time of 150 ms. A spectral with of 8333.33 Hz was used. All spectra were processed using TOPSPIN software, version 1.3 (Bruker). Prior to Fourier transformation, the FIDs were multiplied by an exponential weight function corresponding to a line broadening of 0.3 Hz. Spectra were phased, baseline-corrected and referenced to the sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate singlet at δ 0ppm. Several ^{1}H, ^{1}H 2D experiments were performed to carry out the component assignments. Gradient selected COSY90 was acquired under the following conditions: water presaturation during relaxation delay, spectral with of 8333 Hz in both dimensions, 2 k data points in f_2 and 384 increments in f_1. An unshifted sinusoidal window function was applied in both dimensions and zero filling in f_1 dimension. ^{1}H, ^{1}H TOCSY was registered in TPPI phase-sensitive mode, with water presaturation during 2 s relaxation delay, a spectral with of 8333 Hz in both dimensions, a 70 ms mixing time, 2 k data points in f_2 and 384 increments in f_1. Zero filling in f_1 and unshifted squared sinusoidal window function in both dimensions were applied before Fourier transformation.

Statistical Analysis

Multivariate statistical algorithms were used to classify ^{1}H HR-MAS NMR spectra of LV samples and identify distinct metabolic profiles for the different tissues. For pattern recognition analysis, ^{1}H NMR spectra were data reduced using the software program AMIX (Analysis of MIXtures version 3.6.8, Bruker) by subdivision into integral regions of 0.04 ppm between δ 0.4 and 9 ppm (excluding the water region from 5.2 to 4.7 ppm). Individual integral regions were normalized to the total sum of integral region following exclusion of the water resonance. Principal component analysis (PCA) was applied to the data. Loadings plots from the PCA were used to identify the peaks mainly responsible for the significant differences. Peaks were therefore associated with specific metabolites based upon existing literature. Results were expressed as mean ± SEM. All comparisons were carried out using Student's *t*-test or one-way analysis of variance with a Neuman-Keuls *post hoc* test to compare between groups. Statistical significance was accepted at $P < 0.05$. Analysis was performed with GraphPad Prism 5.0 software.

RESULTS

Caloric Restriction and Macroscopic Parameters

Before CR, both *lean* AL and *fa/fa* AL animals gained weight, but BW increase was significantly higher in *fa/fa* rats compared to *lean* ones at week 12 (**Figure 1B**), related to cumulative food intake was also significantly higher in *fa/fa* AL rats compared to *lean* AL ones (Supplemental Figure 1; $P < 0.001$). During CR, BW remained stable in *fa/fa* CR rats, whereas it was significantly reduced in *lean* CR animals ($P < 0.001$). At the end of the study (14 week-old animals, data summarize in Supplemental Table 1), BW was significantly higher in *fa/fa* AL rats compared to lean AL ones, and significantly decreased in both groups after CR. However, tibia length (TL) was similar between all groups, suggesting that CR reduces adipose tissue amount but does not affect rat growth.

Assessment of Cardiac Function

Invasive pressure measurements showed that SBP was significantly higher in *fa/fa* AL than in *lean* AL rats (152.1 vs. 124.2 mmHg, $P < 0.01$; **Figure 2A**), and this increase was prevented in *fa/fa* CR rats (133.4 mmHg; $P < 0.05$ compared with *fa/fa* AL rats). No differences were found for DBP among groups (**Figure 2B**). Both dP/dt_{max} and dP/dt_{min} were significantly higher in *fa/fa* AL than in *lean* AL rats ($P < 0.05$; **Figure 2C**). CR normalized the magnitude of dP/dt_{max} and dP/dt_{min} in *fa/fa* CR rats to the level seen in the *lean* CR rats, being the values of both parameters lower in *fa/fa* CR rats ($P = 0.059$ and $P < 0.05$, respectively; **Figure 2C**). LVSP (**Figure 2D**) and LVEDP (**Figure 2E**) were significantly higher in *fa/fa* AL than in *lean* AL rats ($P < 0.01$ and $P < 0.05$, respectively), and both were normalized in *fa/fa* CR rats ($P < 0.05$).

The cardiac MRI images of midventricular slides throughout cardiac cycle are shown in **Figure 3**. No differences were found in heart rate (HR) among groups during MRI analysis (**Table 1**). LVEDV but not LVESV, was higher in *fa/fa* AL than in *lean* AL rats ($P < 0.05$; **Table 1**), and as a consequence, SV and CO were also significantly higher in *fa/fa* AL rats ($P < 0.05$; **Table 1**). CR normalized the magnitude of LVEDV in *fa/fa* CR rats to the level seen in the *lean* CR rats ($P < 0.05$), and SV significantly decreased in *fa/fa* CR rats ($P < 0.05$). No differences were found in EF between *lean* and *fa/fa* groups of rats independently of food intake applied (**Table 1**).

Cardiac Structure and Cardiomyocyte Size

HW (**Figure 4A**) and HW/tibia length ratio (**Figure 4B**) were significantly higher in *fa/fa* AL than in *lean* AL rats ($P < 0.01$). After CR, HW was significantly lower in both *fa/fa* CR and *lean* CR groups, although HW/tibia length ratio was significantly lower only in *fa/fa* CR compared with *lean* CR rats ($P < 0.001$; **Figures 4A,B**).

LV wall thickness was also greater in *fa/fa* AL than in *lean* AL rats ($P < 0.01$; **Figures 4C,D**), and this parameter was significantly lower in *fa/fa* CR rats ($P < 0.05$).

Histological examination of heart tissue (**Figure 4E**) illustrated the extent of cardiac hypertrophy in *fa/fa* AL rats, which was characterized by an increase in the diameter of cardiomyocytes in *fa/fa* AL rats relative to *lean* AL counterparts ($P < 0.005$; **Figure 4F**). Cardiomyocyte size was lower both in *fa/fa* CR and *lean* CR rats ($P < 0.005$; **Figure 4F**). To assess whether cardiac hypertrophy was associated with myocardial fibrosis, collagen deposition and collagen I gene levels were analyzed in *lean* AL and *fa/fa* AL rats. There was no evidence of

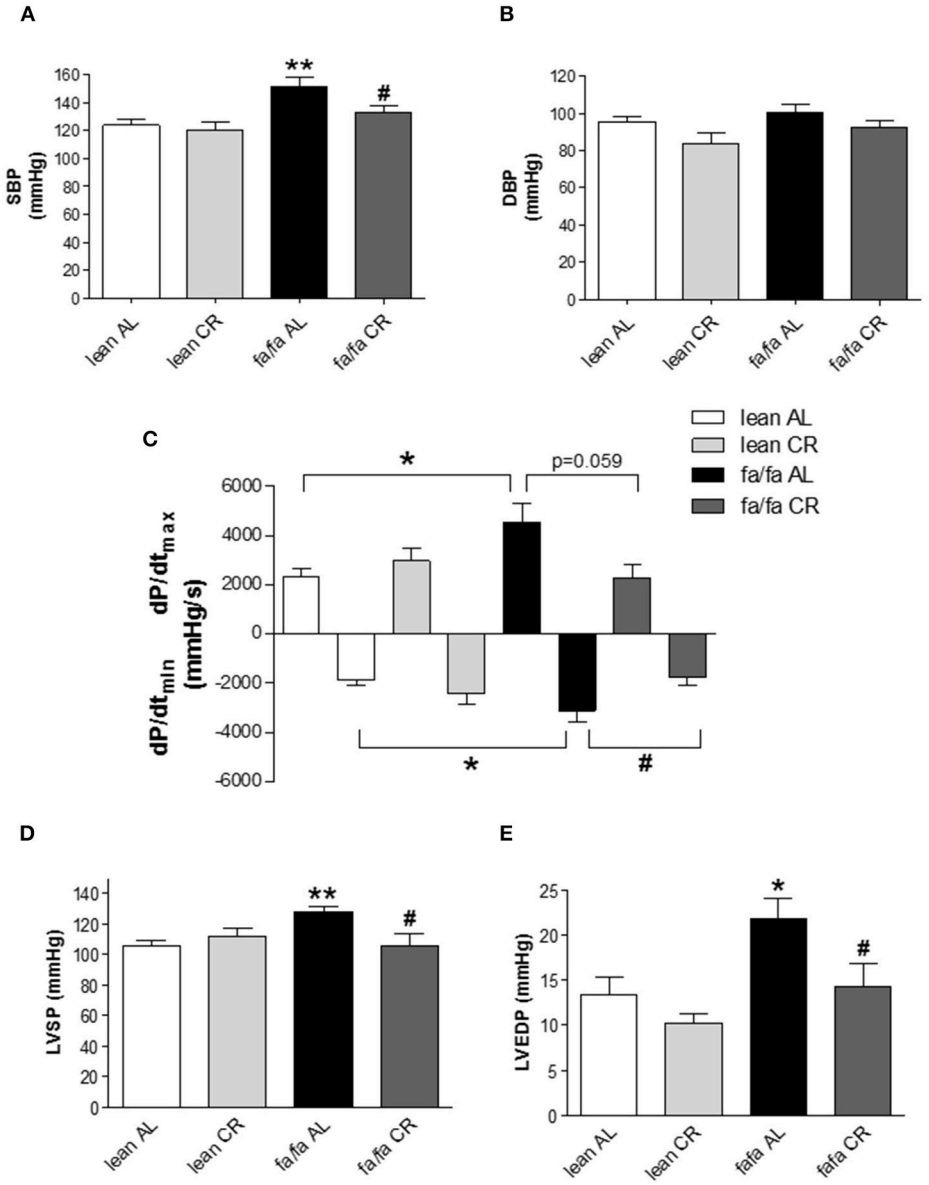

FIGURE 2 | Left ventricular pressure characteristics of *lean* and obese *fa/fa* rats with and without caloric restriction. (A) Systolic blood pressure (SBP) and **(B)** diastolic blood pressure (DBP) in *lean* and *fa/fa* rats on AL or CR diet. **(C)** Maximal rate of pressure increase during systole (dP/dt_{max}) and maximal rate of pressure decay during diastole (dP/dt_{min}) in *lean* and *fa/fa* rats on AL and CR diet. **(D)** Peak left ventricular systolic pressure (LVSP) and **(E)** Left ventricular end-diastolic pressure (LVEDP) in *lean* and *fa/fa* rats on AL or CR diet. Data are mean±SEM ($n = 7$–12 animals per group). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. *lean* AL rats; $^{\#}P < 0.05$ vs. *fa/fa* AL rats.

pathological fibrosis in Masson's trichrome-stained sections of LV (data not shown). Moreover, no differences were detected in the relative expression of collagen type I mRNA between *lean* AL and *fa/fa* AL rats as measured by quantitative PCR (19.53 ± 0.49 vs. 21.35 ± 0.92 arbitrary units, respectively, $P = 0.117$).

Myocardial Metabolites and Fatty Acids Profile

^{1}H HRMAS NMR spectra showed characteristic signals from many low molecular weight metabolites, including taurine, creatine plus phosphocreatine, glutamate, glutamine, glutathione, alanine and lactate, in LV extracts of each group (**Table 2**), as well as fatty acids including n-3 acyl chains, unsaturated and poly-unsaturated fatty acids (**Table 3**). ^{1}H HRMAS NMR analysis of the cardiac metabolites profile revealed a significant decrease in the concentration of taurine ($P < 0.01$), creatine plus phosphocreatine ($P < 0.001$), glutamate ($P < 0.01$), glutamine ($P < 0.001$), and glutathione ($P < 0.01$) in *fa/fa* AL rats relative to *lean* AL counterparts. By contrast, lactate concentration was significantly higher in *fa/fa* AL rats than

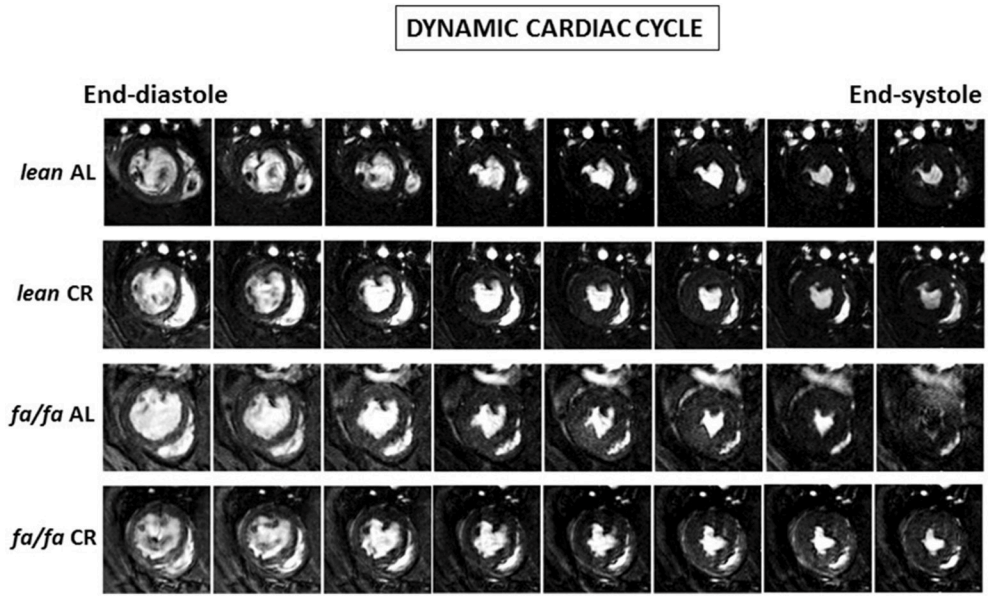

FIGURE 3 | Cardiac cycle MRI images. Representative transverse mid-papillary MR sections along the cardiac cycle obtained from *lean* and obese *fa/fa* rats with and without short and moderate caloric restriction.

TABLE 1 | Cardiac functional parameters determined by MRI.

Parameters	*lean* AL	*lean* CR	*fa/fa* AL	*fa/fa* CR
HR (bpm)	275.8 ± 15.5	307.7 ± 17.3	284.7 ± 18.4	293.2 ± 15.1
LVEDV (μL)	374.5 ± 36.4	387.9 ± 12.7	494.3 ± 23.8*	399 ± 33.2#
LVESV (μL)	127.3 ± 8.6	116.0 ± 6.3	113.5 ± 3.9	108.4 ± 3.1
EF (%)	68.8 ± 2.7	70.1 ± 1.5	76.9 ± 1.2	72.4 ± 2.0
SV (μL)	256.9 ± 26.0	271.9 ± 11.5	380.8 ± 22.7*	290.7 ± 31.8#
CO (mL/s)	1.25 ± 0.16	1.40 ± 0.12	1.77 ± 0.07*	1.55 ± 0.23

*Data are mean ± SEM (N = 4–5 for each group). HR, heart rate; LV, left ventricular; LVEDV, LV end-diastolic volume; LVESD, LV end-systolic volume; EF, ejection fraction; SV, stroke volume; CO, cardiac output. *P < 0.05 vs. lean AL; #P < 0.05 vs. fa/fa AL.*

in *lean* AL counterparts ($P < 0.001$; **Table 2**). With respect to unsaturated fatty acids, in particular n-3 acyl chains, unsaturated and poly-unsaturated fatty acids, were all significantly higher in *fa/fa* rats than in *lean* counterparts ($P < 0.01$ and $P < 0.001$ **Table 3**). All these changes observed in both metabolites and fatty acids cardiac contect in *fa/fa* AL were unchanged in *fa/fa* CR rats. Therefore, mild and short-term CR does not change the cardiac metabolic profile of genetically obese *fa/fa* rats. In fact, the nonsupervised PCA showed a clear clustering of two groups, one for all *lean* animals (AL and CR; see dark and light blue dots into the blue circle of **Figure 5**), and another for all *fa/fa* animals, irrespective of whether that they were on AL or CR diets (**Figure 5**; see dark and light red dots into the red circle).

DISCUSSION

The major findings of the present study can be summarized as follows: (1) short-term (2 weeks) and mild (20%) CR is sufficient to prevent cardiac dysfunction in young obese *fa/fa* rats; and (2) cardiac improvement associated with mild CR occurs even in the presence of a cardiometabolic profile characteristic of obesity.

Several factors including metabolic, structural and functional remodeling are involved in the development of obese-induced cardiomyopathy. Hemodynamic analysis and cardiac MRI were used to assess *in vivo* cardiac function. Whereas no changes in HR were detected between groups, LV cavity, LV wall thickness, LVEDV, SV and CO were all significantly higher in young obese *fa/fa* AL rats than in *lean* AL counterparts. However, this occurred despite of unchanged LVESV and EF, indicating that compensatory hypertrophy occurred in young obese *fa/fa* AL rats in response to increased overload by higher SBP. Moreover, both dP/dt_{max} and dP/dt_{min} were increased in *fa/fa* AL rats, which indicates an enhancement of LV contractility and distensibility as a compensatory mechanism of cardiac function. Collectively, these changes in cardiac function are characteristic of early stage obese cardiomyopathy. Our results are in concordance with the majority of studies on obese populations in which no effect on global measures of systolic function, such as LVESV or EF are observed (Powell et al., 2006; Rider et al., 2014). Prevention at this early stage is very important in terms of avoiding advanced obese cardiomyopathy characterized by LV systolic dysfunction. In the present study, we show that compensatory cardiac hypertrophy and related functional changes in *fa/fa* rats under AL diet can be prevented by CR diet, likely due to a lower hemodynamic overload normalizing SBP, LVSP and LVEDP values, and consequently parameters of cardiac function including LVEDV and SV. Therefore, our results point out that the prevention of an increase in systolic BP in fa/fa CR rats was the most probably candidate for avoiding the subsequent cardiac alterations. In addition to compensatory functional

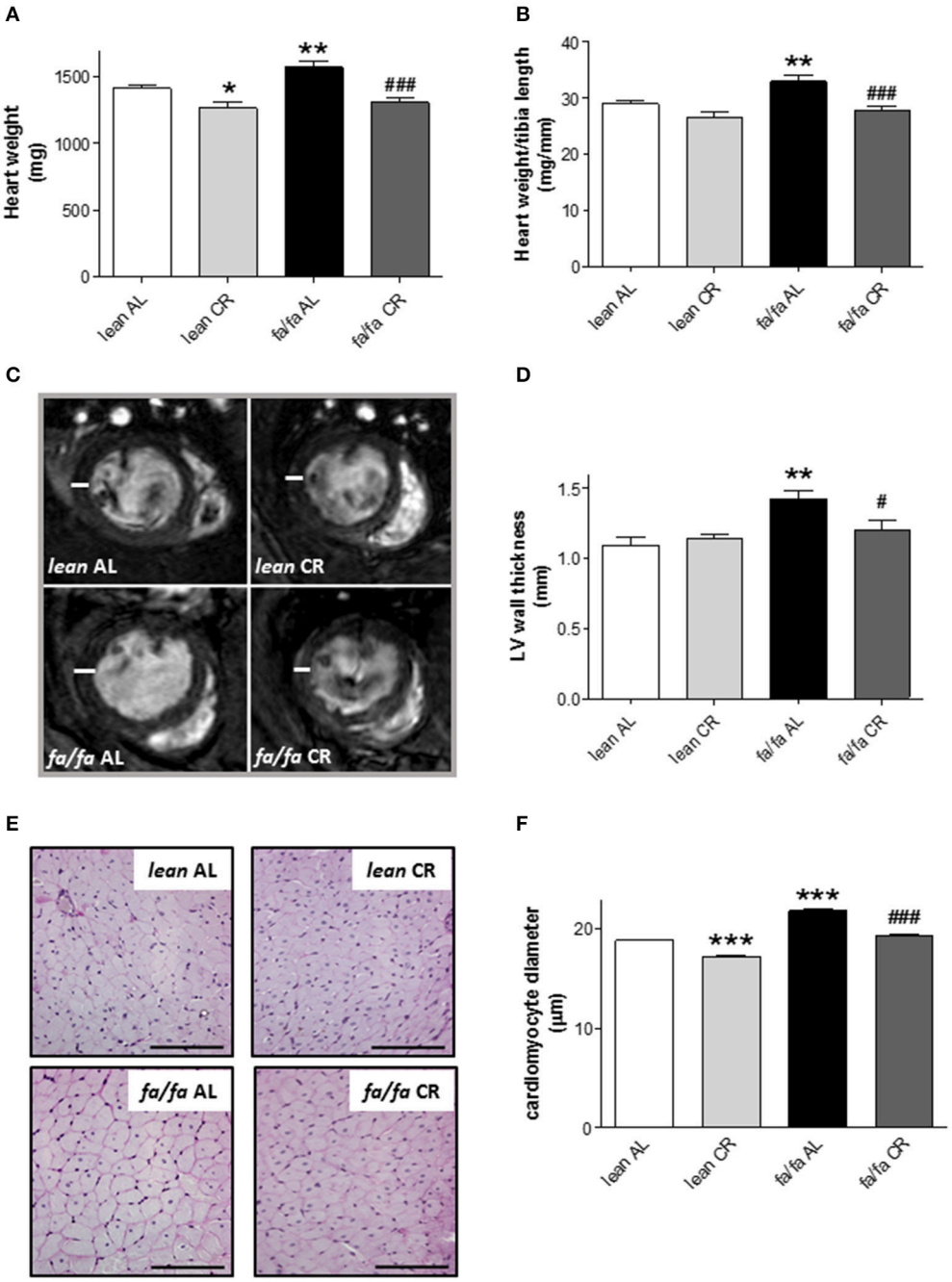

FIGURE 4 | Heart dimensions and cardiomyocyte diameter of *lean* and obese *fa/fa* rats with and without short and moderate caloric restriction. (A,B) Heart weight (HW) **(A)** and HW to tibia length (TL) ratio (HW/TL) **(B)** of each group of animals. **(C)** Representative transverse mid-papillary MR sections during end diastole from *lean* and obese *fa/fa* rats with and without short and moderate caloric restriction. **(D)** Quantitative analysis of LV free wall thickness of each group of animals. Data are mean±SEM ($n = 5$ animals per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. *lean* AL rats; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. *fa/fa* AL rats. **(E)** Representative images of cardiomyocyte cross-surfaces obtained from *lean* and *fa/fa* rats with and without short and moderate caloric restriction (bar: 100 µm). Micrographs of cardiomyocyte sections were stained with PAS reagent. **(F)** Quantitative analysis of cardiomyocyte diameter measured in µm of each group of animals. Data are mean±SEM ($n = 5$ animals per group). $^{***}P < 0.005$ vs. *lean* AL rats; $^{\#\#\#}P < 0.005$ vs. *fa/fa* AL rats.

changes in *fa/fa* rats, structural changes also occurred in the whole heart and at the level of cardiomyocytes. Thus, HW/tibia length ratio, LV wall thickness and cardiomyocyte diameter were all significantly increased in *fa/fa* AL than in *lean* AL rats, and all were prevented by mild short-term CR. Clearly, the lack of deleterious fibrosis in *fa/fa* rats probably favored the

TABLE 2 | Metabolites identified by ^{1}H HRMAS NMR from the Principal Component Analysis (PCA).

	lean AL	*lean* CR	*fa/fa* AL	*fa/fa* CR
Taurine (3.42)	44.3 ± 1.42	45.9 ± 2.92	31.84 ± 1.62**	32.45 ± 2.96^{++}
Creatine+Phosphocreatine (3.03)	64.3 ± 1.67	61.7 ± 4.03	42.2 ± 1.28***	43.1 ± 3.14^{+++}
Glutamate (2.33)	3.6 ± 0.22	3.1 ± 0.18	2.5 ± 0.16**	2.3 ± 0.11^{+}
Glutamine (3.78)	14.8 ± 0.44	13.9 ± 0.51	9.6 ± 0.56***	10.2 ± 0.79^{+++}
Glutamine/Glutamate	4.2 ± 0.32	4.5 ± 0.30	3.9 ± 0.14	4.4 ± 0.34
Glutathione (4.58)	2.1 ± 0.10	1.9 ± 0.21	1.4 ± 0.07**	1.2 ± 0.05^{++}
Alanine (1.47)	4.5 ± 0.17	4.4 ± 0.25	3.8 ± 0.24	4.0 ± 0.38
Lactate (1.33)	61.5 ± 2.64	66.0 ± 2.54	82.2 ± 4.22***	86.0 ± 2.94^{++}

*Data are mean ± SEM (n = 5 for each group). **P < 0.01, ***P < 0.001 vs. lean AL; $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. lean CR. Number between brackets correspond to the middle point of the bucket.*

TABLE 3 | Fatty acids identified by ^{1}H HRMAS NMR from the Principal Component Analysis (PCA).

	lean AL	*lean* CR	*fa/fa* AL	*fa/fa* CR
n-3 acyl chains	36.5 ± 1.68	43.5 ± 4.86	60.7 ± 2.31**	63.2 ± 5.82^{++}
Unsaturated fatty acids	2.5 ± 0.33	2.7 ± 0.37	5.7 ± 0.38***	5.2 ± 0.59^{+++}
Poly-unsaturated fatty acids	1.7 ± 0.05	1.7 ± 0.17	2.3 ± 0.11**	2.1 ± 0.03^{+}

*Data are mean ± SEM (n = 5 for each group). **P < 0.01, ***P < 0.001 vs. lean AL; $^{+}$P < 0.05, $^{++}$P < 0.01, $^{+++}$P < 0.001 vs. lean CR.*

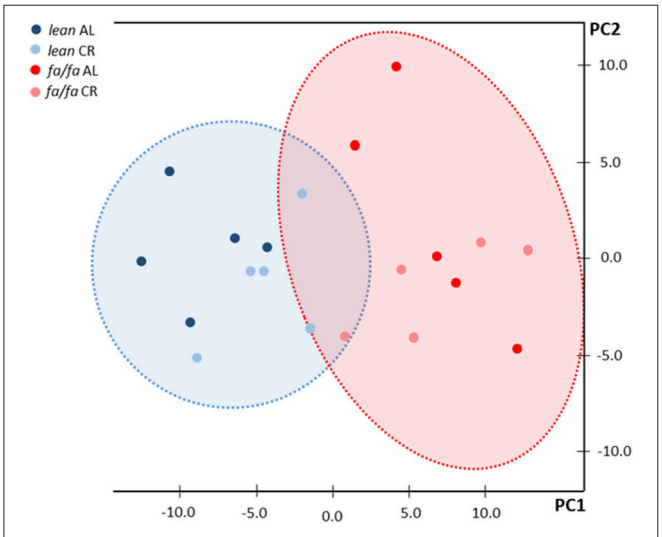

FIGURE 5 | Principal component analysis (PCA) of NMR data. Blue circle corresponds to *lean* rats and red circle corresponds to *fa/fa* rats. Dark blue dots: *lean* AL rats; light blue dots: *lean* CR rats; dark red dots: *fa/fa* AL rats; light red dots: *fa/fa* CR rats.

rapid normalization of hemodynamic parameters and cardiac function.

With respect to metabolites profile, it has been proposed in recent years that impairment in myocardial phosphate energetics, such as creatine and phosphocreatine depletion, can occur in cardiac hypertrophy associated with obesity (Rider et al., 2012, 2014). A decline in the total creatine pool together with elevated fatty acid levels induce an important increase in mitochondrial uncoupling, which can lead to diastolic dysfunction (Diamant et al., 2003; Faller et al., 2013). This scenario is evident in young obese *fa/fa* rats. We observed a general decrease in the cardiac concentration of taurine, creatine and phosphocreatine, glutamate, glutamine, and glutathione linked to the obesity background of the animals. It has been previously described that a general drop in the concentration of these metabolites might be explained by the increase in the extracellular mass due to fibrotic tissue accumulation and excessive collagen deposition (Roncalli et al., 2007). However, collagen type I expression was at controls levels in hearts of *fa/fa* AL rats and no areas of interstitial fibrosis were detected. Supporting these results, *fa/fa* AL rats had a similar glutamine to glutamate ratio to *lean* AL rats. A decrease in this ratio would be expected in cardiac fibrosis because transforming growth factor beta, a key regulator of collagen production, upregulates phosphate-dependent glutaminase (Roncalli et al., 2007). Therefore, our results support the notion that hearts of obese young *fa/fa* AL rats are no more fibrotic than their *lean* AL counterparts, and the alterations in metabolite profile probably occur only at the level of the cardiomyocyte. The exception to the general drop in metabolites was for lactate, whose concentration was significantly higher in *fa/fa* rats than in *lean* rats. This increase in cardiac lactate together with a significant decrease in glutamate implies a shift to a glycolytic phenotype in *fa/fa* rats. Moreover, the accumulation of lactate derived from pyruvate oxidation has been related to an elevated energy demand as for example during increased muscle exercise (Goodwin and Taegtmeyer, 2000), a situation closely associated with the compensatory cardiac hypertrophy observed in young obese *fa/fa* rats. On the other hand, fatty acids are also a predominant substrate used in the adult myocardium (Kolwicz et al., 2013). In our study n-3 fatty acyl changes, unsaturated and poly-unsaturated, significantly accumulated in obese young *fa/fa* rats. Therefore, both conditions, i.e. a decrease in the creatine pool and an accumulation of fatty acids, might be directly responsible for cardiac dysfunction, especially at the diastolic level observed in *fa/fa* rats. Remarkably, we observed that the

alteration in metabolites and fatty acids were maintained in *fa/fa* rats despite normalization of cardiac structure and function by mild short-term CR. Another possibility to explain these results might be the fa/fa rats phenotype. This genetic obesity model develops insulin resistance, which could be also present during CR, explaining the presence of similar cardiac metabolic profile observed in fa/fa AL and CR rats. In addition, results from non-supervised PCA indicated that the main change in the metabolic profile was not attributable to differences related to cardiac hypertrophy, hypertension or cardiac diastolic dysfunction, but rather to obesity background. All these results indicate that the recovery of myocardial energetic balance is not necessary to re-establish an adequate cardiac structure and function, suggesting that the beneficial CV effects of a short and mild CR are independent from metabolic remodeling, at least in an early stage of obesity. Therefore, the cellular hypertrophic response at the early stage of obese cardiomyopathy in young *fa/fa* rats is probably secondary to increased wall stress forced by cavity dilatation and increased filling pressures, and not to metabolic remodeling.

It is important to consider that, in contrast to lean rats CR avoided the weight gain in fa/fa rats. This maintenance of BW during the 2 weeks of CR was associated with only a moderate loss of BW in fa/fa CR relative to fa/fa AL rats at the end of the study. However, this absence of weight gain in fa/fa CR rats probably favored that these rats did not develop hypertension and the subsequent cardiac alterations associated with a compensated hypertrophy. Therefore, mild CR provides clinically valuable cardioprotective effects in terms of structure and function, and can prevent obese cardiomyopathy without the need for extreme weight loss. In contrast to our approach, the majority of published studies using experimental models where cardiac benefits are linked to CR were carried out under more severe CR regimens (30–65%), during long-term (several months), and/or on aging-animals (Dolinsky et al., 2010; Niemann et al., 2010; Shinmura et al., 2011; Finckenberg et al., 2012; Takatsu et al., 2013; Kobara et al., 2015; Melo et al., 2016). Along the same line, human obese cardiomyopathy in adulthood complicated by the presence of hypertension is associated with alterations in cardiac structure and function (Meyer et al., 2006; Hammer et al., 2008). In this setting, long-term CR during several months (Hammer et al., 2008) or even years (Meyer et al., 2006) as well as after bariatric surgery (Ikonomidis et al., 2007) has been demonstrated to improve myocardial function especially at the diastolic level. However, CR regimes for prolonged periods and/or with an appreciable reduction in food intake are difficult to implement and sustain in the obese population.

Finally, it is important to note that alterations in cardiac function and morphology present in obese adults are also apparent in the younger obese population. Thus, obesity, hypertension and concentric cardiac hypertrophy are independent predictors of diastolic dysfunction in obese children and adolescents (Dhuper et al., 2011). In this sense, the present study together with recent results from our group that demonstrate a restoration in impaired endothelial dysfunction in obese Zucker rats (García-Prieto et al., 2015), offer some insight into the effects that mild and short-term CR have in terms of CV prevention in obesity at an early stage. Our results support data from other studies that affirm that the positive benefits of CR are greater when sooner CR starts (Han and Ren, 2010; Melo et al., 2016) and, as demonstrated in the present study, CV benefits can occur without changes in the cardiac metabolic profile. One limitation of this study is not having cardiac pre-CR values in fa/fa animals in order to demonstrate whether the mild and short CR applied here was able to prevent or revert cardiac damage. Nonetheless, it is very likely that fa/fa AL rats did not have an established obesity-induced cardiomyopathy just before starting CR at 12 weeks of age, supporting the assumption that the mild and short-term CR applied here would be able to prevent the development of cardiac damage in these animal.

In summary, our results indicate that mild and short-term CR protects hearts from obesity-related cardiomyopathy even when the cardiometabolic profile remains altered. Therefore, early dietary intervention with a moderate CR of easy compliance when obese cardiomyopathy is first detected might reverse key cardiac alterations linked to obesity.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: GR-H, BS and MSF-A; performed experiments: GR-H, CG-P, HP-O, J-VM, PV-V, MEF-V, MF-V; analyzed the data: GR-H, JR, MF-V, CG-P, HP-O, PV-V, BS, MSF-A; contributed reagents/materials/analysis tools: GR-H, LB, BS, JR, MSF-A; wrote the paper: GR-H and MSF-A.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00042/full#supplementary-material

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frontiers in Physiology

ORIGINAL RESEARCH
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FVB/NJ Mice Are a Useful Model for Examining Cardiac Adaptations to Treadmill Exercise

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Mice are commonly used to examine the mechanisms by which exercise improves cardiometabolic health; however, exercise compliance and adaptations are often strain-dependent or are variable due to inconsistency in exercise training protocols. In this study, we examined nocturnal/diurnal behavior, treadmill exercise compliance, and systemic as well as cardiac-specific exercise adaptations in two commonly used mouse strains, C57BL/6J, and FVB/NJ mice. Metabolic cage analysis indicated a strong nocturnal nature of C57BL/6J mice, whereas FVB/NJ mice showed no circadian element to activity, food or water intake, VO_2, or VCO_2. Initial exercise capacity tests revealed that, compared with C57BL/6J mice, FVB/NJ mice are capable of achieving nearly 2-fold higher workloads prior to exhaustion. FVB/NJ mice tested during the day were capable of achieving significantly more work compared with their night-tested counterparts. Following 4 weeks of training, FVB/NJ mice showed significant increases in exercise capacity as well as physiologic cardiac growth characterized by enlarged myocytes and higher mitochondrial DNA content. C57BL/6J mice showed no increases in exercise capacity or cardiac growth regardless of whether they exercised during the day or the night. This lack of adaptation in C57BL/6J mice was attributable, at least in part, to their progressive loss of compliance to the treadmill training protocol. We conclude that the FVB/NJ strain is a useful and robust mouse model for examining cardiac adaptations to treadmill exercise and that treadmill training during daytime hours does not negatively affect exercise compliance or capacity.

Keywords: cardiac hypertrophy, physical activity, exercise, mouse strain, mitochondria, circadian, compliance, metabolism

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INTRODUCTION

Regular exercise improves cardiovascular health (Blair et al., 1996; Mora et al., 2007; Joyner and Green, 2009), augments musculoskeletal function (Egan and Zierath, 2013), and increases both healthspan (Mercken et al., 2012; Egan and Zierath, 2013; de Cabo et al., 2014) and lifespan (Paffenbarger et al., 1986; Blair et al., 1989, 1996; Myers et al., 2002). Nevertheless, the molecular mechanisms by which exercise promotes health are poorly understood (Neufer et al., 2015). Exercise studies commonly use murine models, which are valuable for identifying critical gene programs that contribute to exercise adaptation, primarily, because they offer the benefit

of relatively rapid and controlled genetic modification (e.g., Niebauer et al., 1999; Bernstein, 2003; Kemi et al., 2007; Riehle et al., 2014). Nevertheless, sources of variability in such studies could confound our understanding of how exercise mitigates disease or increases overall health. Minimization of confounding factors is an important consideration for designing exercise studies as well as for interpreting the results obtained.

Although mouse models cannot perfectly recapitulate the complex physiological changes occurring in humans with physical activity, they can phenocopy particular aspects of physiological adaptation. For this purpose, three models of mouse exercise are used frequently: treadmill training, forced swimming, and voluntary wheel running. While each of these models have advantages and limitations (Bernstein, 2003; Wang et al., 2010), treadmill training provides the investigative advantage of being able to control the amount of work performed in each training session. This is critical for understanding dependency of (patho) physiological adaptations on exercise workload and becomes increasingly important in studies of mice having different masses (e.g., obesity studies). Unlike the treadmill modality, work cannot be calculated easily in swimming or voluntary wheel exercise. For these reasons, treadmill training is a reliable, well-controlled, and often superior model of exercise for research studies. Nevertheless, several factors contribute to treadmill exercise non-compliance and to exercise-induced adaptations. These include, but are not limited to, mouse strain, exercise environment, acclimatization, motivation, and assessments of exhaustion (Perrino et al., 2011; Platt et al., 2015).

Of these, mouse strain is of principal importance. Inbred strains of mice and rats have pronounced differences in their ability to exercise, or their choice to do so (Ebihara et al., 1978; Barbato et al., 1998; Lightfoot et al., 2001; Lerman et al., 2002; Massett and Berk, 2005). A preponderance of genetic mouse models are on the C57BL/6J or the FVB/NJ background (Taketo et al., 1991; Battey et al., 1999); however, these strains show strikingly different preferences and capacities for exercise. For instance, C57BL/6J mice appear to be poor treadmill runners, yet display superior capacity on voluntary exercise wheels, while the opposite is true for FVB/NJ mice (Lerman et al., 2002; Massett and Berk, 2005). The reason(s) for these differences in training modality preference remain unclear. One reason could relate to the fact that laboratory mice are primarily nocturnal animals, demonstrating the highest activity and food consumption during the night cycle (Kohsaka et al., 2007; Laposky et al., 2008; Arble et al., 2009); however, to our knowledge, the impact of time of exercise (day or night) on treadmill exercise capacity and compliance has not been investigated. The goals of this study were: (1) to test for differences in treadmill exercise capacity and compliance between C57BL/6J and FVB/NJ mice; (2) to delineate whether diurnal or nocturnal training influences exercise compliance and adaptive responses to training; and (3) to assess systemic and cardiac-specific exercise adaptations in mice compliant with treadmill exercise protocols. Our study demonstrates that FVB/NJ mice are a superior strain for treadmill exercise and that the timing of treadmill exercise, at least in this strain, does not influence compliance or exercise-induced adaptation.

MATERIALS AND METHODS

Animals

All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. C57BL/6J and FVB/NJ mice were ordered from Jackson Laboratory (Bay Harbor, ME) at 12 weeks of age and allowed to acclimate at the University of Louisville animal facility for 3 weeks. At 15 weeks of age, male mice were assigned randomly by strain to sedentary (SED) or exercise (EXE-Day or EXE-Night) groups. Food and water were provided *ad libitum,* and the mice were maintained on a 12:12-h light-dark schedule. Because the majority of published studies regarding exercise in mice have utilized male mice, we chose this gender for our study, which enables comparison with the literature. At the conclusion of the study and 24 h after the last exercise session, the mice were fully anesthetized with sodium pentobarbital (40 mg/kg, i.p.), followed by euthanasia via excision of the heart. These procedures are consistent with the AVMA *Guidelines on Euthanasia*.

Metabolic Phenotyping

To assess for differences in basal metabolism and diurnal/nocturnal behavior, metabolic cage analyses were performed in naïve 15-week-old C57BL/6J and FVB/NJ mice, essentially as described (Sansbury et al., 2012; Cummins et al., 2014). Body weight was recorded prior to the initial and final exercise capacity tests to assess for changes in total body mass. Oxygen consumption rates, carbon dioxide production rates, respiratory exchange ratios, food consumption, water consumption and activity (sum of ambulatory and fine movements) were measured using a physiological/metabolic cage system (TSE PhenoMaster System, Bad Homberg, Germany) as described previously (Sansbury et al., 2012; Cummins et al., 2014).

Exercise Capacity Testing

We performed exercise familiarization and capacity testing in a manner similar to that outlined previously (Massett and Berk, 2005), with minor modifications (**Figures 1A,B**). Mice were familiarized to the motorized rodent treadmill (Columbus Instruments, Columbus OH) on the Wednesday and Thursday before the first week of training. Familiarization consisted of an initial 10 min period where the treadmill speed and incline were set to zero with shock grid settings of 25 V, 0.34 mA, and 2 Hz. The treadmill speed was then increased steadily to 10 m/min (Wed) and 12 m/min (Thurs) for an additional 10 min.

On the Friday immediately following familiarization to the treadmill, we subjected mice to an exercise capacity test (**Figure 1B**). For this, the mice were acclimated to the treadmill for 10 min, with the speed and incline set initially to zero. The treadmill speed was then increased to 8.5 m/min with an angle of inclination set to 0° for 9 min. Next, the treadmill speed and incline were increased to 10 m/min and 5°, respectively, for 3 min. The speed was then increased by 2.5 m/min every 3 min

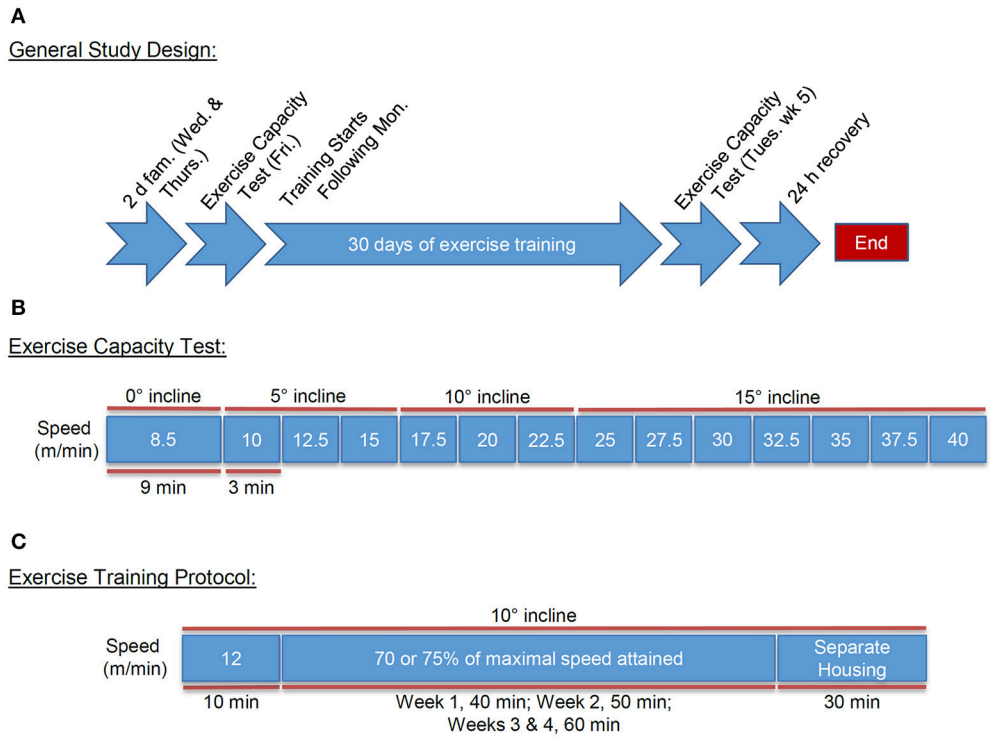

FIGURE 1 | Exercise testing and training design. Schematic of the familiarization, testing, and treadmill training design: **(A)** General treadmill training design including: familiarization, pre- and post-exercise capacity testing, and exercise training regimen; **(B)** Exercise capacity testing protocol; and **(C)** Treadmill training regimen including: warm-up and exercise training intensity and durations, and post-training housing.

to a maximum speed of 40 m/min, while inclination increased by 5° every 9 min until a maximum incline of 15° was achieved.

We developed strict *a priori* criteria for exercise-induced exhaustion. These criteria were: (1) 10 consecutive seconds on the electric grid; (2) spending more than 50% of time on the grid; and/or (3) lack of motivation to manual prodding. Each mouse was removed immediately from their respective lane once one or more of these criteria was reached. Following the protocol, the mice were housed separately for 30 min to avoid noticeable aggressive behavior following exercise.

Following 4 weeks of training, we repeated this testing protocol to assess changes in exercise capacity. Exercise capacity was measured using the parameters of distance run (meters achieved prior to exhaustion) and work accomplished [calculated as the product of body weight (kg) and vertical distance (m); vertical distance = (distance run) ($\sin\theta$), where θ = the angle of inclination of the treadmill from 0° to 15°] as outlined previously (Massett and Berk, 2005).

Exercise Training

Mice assigned to exercise training groups were subjected to a 4-week protocol of forced treadmill running. The training protocol commenced the Monday after the initial exercise capacity testing with mice exercising 5 days/week (Mon-Fri) at 70 or 75% of the maximal speed achieved during the initial exercise capacity test and an inclination appropriate to the speed (**Figure 1C**). Prior to each training bout, we provided mice with a "warm-up" period of 10 min at 0 m/min and 10 min at 12 m/min to promote exercise protocol compliance and to minimize risk of injury. For comparison between the groups, training intensity was set at 20.8 m/min at a 10° incline for FVB/NJ mice and 16.4 m/min at a 5° incline for C57BL/6J mice, which corresponded to 70% of the maximal speed and the appropriate incline at the calculated speed for each strain during the initial exercise capacity test. In subsequent studies of FVB/NJ mice, mice were exercised only during the day, and in these groups, we implemented a more intensive training protocol to further examine systemic and cardiac adaptations to treadmill running. For this, training intensity was set at 75% of the maximal initial exercise capacity, which corresponded to 22.3 m/min at a 10° incline. In all groups, we progressively increased the workload of the mice, such that they trained for 40 min during week 1, 50 min during week 2, and 60 min during weeks 3 and 4. We chose this progressive intensity protocol to prevent training plateau and to stimulate exercise-induced adaptations (Dudley et al., 1982; Hildebrandt et al., 2003; De Angelis et al., 2004; Massett and Berk, 2005).

Assessment of Protocol Compliance

To prevent injury and record protocol compliance, we monitored the mice carefully during each exercise session. Upon meeting pre-established indicators of exhaustion, mice were removed from the treadmill, and the time run was recorded (See

Supplemental Table 1). We used the percentage of total sessions and total minutes completed throughout the 4-week training program as a measure of compliance. Before and after the initial exercise capacity test, we measured blood lactate levels, which provided a biochemical indicator of exercise-induced exhaustion at or near maximal oxygen consumption (VO_{2max}) (McConnell, 1988; Pederson et al., 2005; Ferreira et al., 2007; Hakimi et al., 2007). We recorded lactate concentration in 0.7 μl of blood from a small tail clip (Lactate Plus meter; Nova Biomedical) prior to the protocol and upon meeting the exhaustion criteria defined above. High lactate levels increase confidence in a successful exercise capacity test by ensuring that failure to continue is due to exhaustion at or near VO_2max and not a failure to comply with the protocol (Von Wittke et al., 1994; Gladden, 2004; Billat et al., 2005).

Histology

Following euthanasia, tissue was excised and rapidly fixed for immunohistochemical analysis or immediately snap frozen in liquid nitrogen and stored at −80°C. Tissue was fixed in 10% formalin, paraffin embedded, and sectioned at 4 μm. Heart cross-sections were stained with 4′6-diamidino-2-phenylindole (DAPI; Invitrogen) and wheat germ agglutinin (WGA; ThermoFisher) for quantification of cardiomyocyte cross-sectional area. Quantitative measurements were determined using Nikon Elements software.

Relative Mitochondrial DNA Measurements

Mitochondrial abundance in heart tissue was estimated by measuring mitochondrial DNA (mtDNA) abundance relative to nuclear DNA (nDNA), similar to our previous studies (Cummins et al., 2014; Salabei et al., 2016). Briefly, total DNA was isolated using a QIAamp DNA Mini Kit (Qiagen). A 25-mg aliquot of the tissue was homogenized, followed by overnight digestion in proteinase K at 55°C. Following isolation, relative amounts of mtDNA and nDNA were compared using quantitative real-time PCR, using 2 ng of the isolated DNA. Primers for cytochrome *b* (mtDNA) and β-actin (nDNA) were used. The sequences are: cytochrome *b*, 5′-TTGGGTTGTTTGATC CTGTTTCG-3′ and 5′-CTTCGCTTTCCACTTCATCTTACC-3′; and β-actin, 5′-CAGGATGCCTCTCTTGCTCT-3′ and 5′-CGTCTTCCCCTCCATCGT-3′.

Statistical Analysis

Unpaired or paired Student's *t*-test was used for direct comparisons; multiple groups were compared by one-way and two-way ANOVA followed by Bonferroni or Sidak Multiple Comparison test, as appropriate. For ratio-based statistical comparisons, the data were log-transformed, and unpaired Student's *t*-test was applied for assessing statistical significance between groups; we used a one-sample *t*-test for intragroup differences from a ratio of 1 (used to determine significant chronobiological differences in metabolic cage endpoints, i.e., food and water intake, physical movement, VO_2, VCO_2, RER). A $p \leq 0.05$ was considered statistically significant.

RESULTS

FVB/NJ Mice Are Not Nocturnal

Laboratory mice, in general, are a nocturnal species (Kohsaka et al., 2007; Laposky et al., 2008; Arble et al., 2009), and they typically choose to participate in voluntary exercise at night (Verwey et al., 2013). Thus, the time at which treadmill protocols are executed could be critical for ensuring exercise regimen compliance and adaptation. Therefore, we first examined the circadian characteristics of the mouse strains by measuring their food and water intake, locomotion, VO_2, VCO_2 and RER by placing untrained (naïve) mice in metabolic chambers. To assess differences between day and night behavior, a ratio (night values:day values) was calculated for each parameter. Although, overall food consumption was not different between the two strains, the C57BL/6J mice consumed, on average, 3-fold more food at night. In contrast, the FVB/NJ mice showed no difference in food consumption in the night compared with the day (**Figure 2A**). Cumulative water intake was not different between the strains; however, C57BL/6J mice showed significantly higher water intake at night (**Figure 2B**). Similarly, C57BL/6J mice were more active at night compared with FVB/NJ mice (**Figure 2C**). In general, FVB/NJ mice had fewer total beam breaks per hour (C57BL/6J = 2583 ± 711; FVB/NJ = 1736 ± 582; $p < 0.01$), indicating that cage behavioral activity is lower in this strain.

Metabolic analysis yielded analogous results. In C57BL/6J mice, oxygen consumption (VO_2; **Figure 2D**), carbon dioxide production (VCO_2; **Figure 2E**), and respiratory exchange ratios (RER; **Figure 2F**) were higher at night compared with the day. Conversely, the FVB/NJ mice did not show metabolic differences in the day vs. the night. Thus, the behavioral and metabolic qualities of C57BL/6J mice are supportive of their known nocturnal nature and are in stark contrast to FVB/NJ mice which demonstrate a relative lack of features characteristic of the nocturnal phenotype. Additionally, FVB/NJ mice had higher rates of oxygen consumption (C57BL/6J = 3713 ± 327 ml/h/kg; FVB/NJ = 4058 ± 346 ml/h/kg; $p < 0.05$) which contributed to lower respiratory exchange ratios (C57BL/6J = 0.93 ± 0.02; FVB/NJ = 0.86 ± 0.02; $p < 0.0001$).

Effect of Strain and Time of Treadmill Running on Initial Exercise Capacity

To determine the effects of mouse genetic background on initial exercise capacity, we subjected 15-week-old C57BL/6J and FVB/NJ mice to exercise capacity testing. The mice were exercised either during the day (i.e., between 9 a.m. and 12 p.m.) under normal laboratory lighting or at night (i.e., between 7:00 p.m. and 10:00 p.m.) under dark room conditions (safelight red lamp). We chose the latter time based on metabolic cage activity data, which showed increased voluntary locomotor activity starting at 6 p.m. Compared with C57BL/6J mice, the FVB/NJ strain ran ∼1.5-fold farther (**Figure 3A**) and demonstrated ∼2-fold greater initial capacity for treadmill work (**Figure 3B**), regardless of when the mice were tested. Despite an apparent trend toward lower initial exercise capacity at night, we found no statistically significant differences between C57BL/6J mice exercised during the day or the night (**Figures 3A,B**);

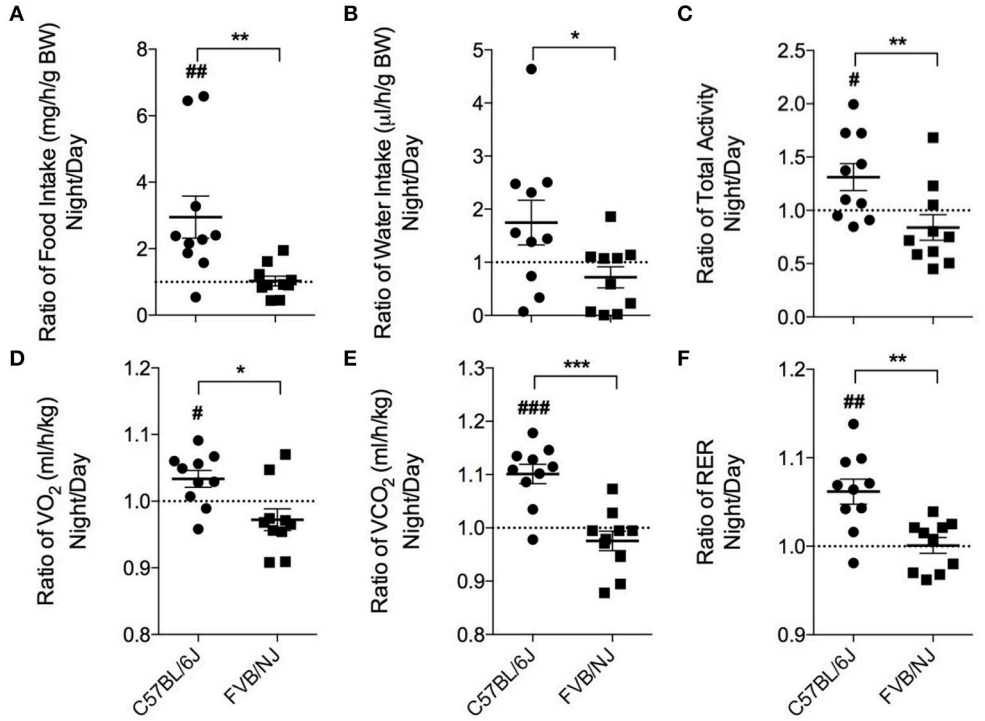

FIGURE 2 | Chronobiological characteristics of C57BL/6J and FVB/NJ mice. Ratios of the average night and day values from metabolic cage analysis: **(A)** Food intake, **(B)** Water intake, **(C)** Total activity, **(D)** VO_2, **(E)** VCO_2, and **(F)** Respiratory exchange ratio (RER). $n = 10$ per group. *significance between C57BL/6 and FVB/NJ, #significance from a ratio of 1; $^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***,\#\#\#}p < 0.001$.

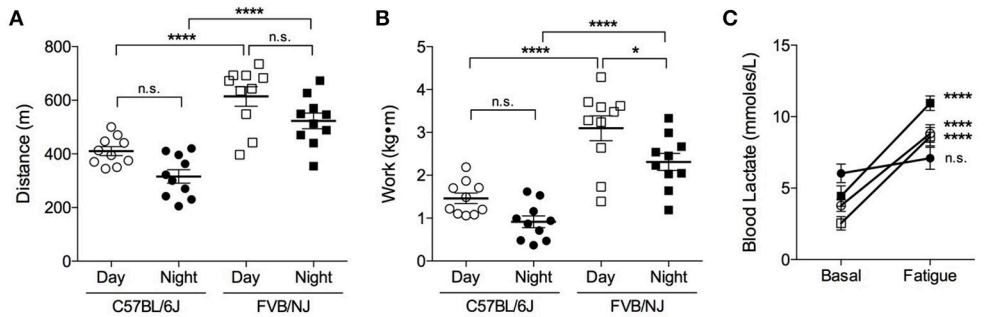

FIGURE 3 | FVB/NJ mice display a higher initial exercise capacity than C57BL/6J mice. Measurements of exercise capacity and fatigue in 15-week old C57BL/6J and FVB/NJ mice tested during the day or at night: **(A)** Comparison of distance achieved; **(B)** Comparison of work accomplished; **(C)** Blood lactate levels in C57BL/6J and FVB/NJ mice basally and following fatigue from exercise testing. $n = 10$ mice per group, $^{*}p < 0.05$, $^{****}p < 0.0001$.

however, FVB/NJ mice showed significantly lower levels of work performed at night (**Figure 3B**).

In preliminary assessments, we observed that C57BL/6J mice received more shocks than FVB/NJ mice during treadmill exercise. Therefore, to confirm that reliable exercise capacity values were obtained and to rule out non-compliance to the testing protocol, we recorded blood lactate levels prior to and immediately following the initial exercise capacity test. Resting blood lactate values were similar in C57BL/6J and FVB/NJ mice (**Figure 3C**). Although, it should be noted that the night C57BL/6J group had a slightly higher resting lactate level, in general, blood lactate increased by 2–4-fold in both strains upon meeting criteria for exhaustion (**Figure 3C**); both resting and maximal lactate abundances are within the ranges previously published for resting mice and mice exercising at or near their VO_{2max}, respectively (Pederson et al., 2005; Ferreira et al., 2007; Hakimi et al., 2007). Collectively, these results suggest that FVB/NJ mice demonstrate a superior ability to perform treadmill work compared with C57BL/6J mice, and they have higher initial exercise capacities when tested during the day.

Effect of Strain and Time of Training on Exercise Compliance and Adaptation

To determine if mouse strain or the time of training (i.e., day vs. night) influences exercise protocol compliance and adaptive responses to exercise, C57BL/6J and FVB/NJ mice were subjected to a 4-week training program, with one group from each strain training at night, and one group training during the day. Each day, we recorded compliance to the training program for individual mice (**Supplemental Table 1**). The C57BL/6J mice completed only 50–60% of their training sessions (**Figures 4A,C**), resulting in less time exercising in general (**Figure 4D**). Interestingly, compliance in C57BL/6J mice diminished progressively with duration of the training protocol (**Figure 4A**, **Supplemental Table 1**). While FVB/NJ mice were generally compliant when exercised during the day or the night, 100% of the day FVB/NJ group complied with the protocol, whereas FVB/NJ mice exercising at night appeared to show modestly compromised compliance (**Figures 4B,D**); however, this did not achieve statistical significance.

Pre-training and post-training exercise capacity tests showed that, while both FVB/NJ exercise groups significantly increased exercise capacity after 4 weeks of training, the C57BL/6J mice showed either no improvement or a decrease in the distance run or the work accomplished following training (**Figures 5A,B**). This lack of response to exercise in the C57BL/6J mice is likely due to poor compliance to the protocol.

To determine if compliance of C57BL/6J mice to the protocol correlated with improvements in exercise capacity, we plotted the percent improvement in distance and work against the percent time completed during the exercise regimen. As shown in **Figures 5C,D**, there is a significant, albeit weak, correlation between the percent time completed and exercise capacity in this strain. Collectively, these results show that, unlike FVB/NJ mice, C57BL/6J mice are not compliant with this extended treadmill exercise protocol, and that the time at which FVB/NJ mice exercise does not markedly influence their exercise capacity.

Physiological cardiac growth is a common endpoint used to verify cardiometabolic adaptation to exercise (Maillet et al., 2013). Exercise training increased heart weight to tibia length (HW/TL) in both FVB/NJ exercise groups, whereas the C57BL/6J mice showed no changes in heart size (**Table 1**). We did not find a significant correlation between exercise time completed and HW/TL in the C57BL/6J strain (data not shown). These results indicate that exercise causes cardiac growth in FVB/NJ mice and that C57BL/6J mice, likely due to lack of compliance to this treadmill protocol, fail to demonstrate physiological cardiac growth.

FVB/NJ Mice Display Robust Adaptations Following Exercise Training

It is known that, compared with the C57BL/6J strain, FVB/NJ mice can achieve higher critical running speeds on the treadmill (Billat et al., 2005), which indicates that their training regimen could be intensified further to evoke more robust adaptations to exercise. To test this, we trained an independent group of FVB/NJ mice at a slightly higher intensity (75% of their initial maximum exercise capacity) for 4 weeks. Compared with pre-training exercise capacity values, this more intensive protocol yielded a ∼1.7-fold improvement in distance run and a ∼2.4-fold increase in work in the mice (**Figures 6A,B**). As expected, cardiac size was significantly higher in exercised mice (**Figures 6C,D**). In exercise-adapted hearts, myocyte cross sectional area was found to be 22% higher than that found in hearts of sedentary mice, and the myocyte area distribution curves were shifted to the right (**Figures 6E–G**), indicating that the exercise-induced cardiac growth in this strain is due primarily to an increase in cardiomyocyte size. Furthermore, mitochondrial biogenesis, estimated using the ratio of relative mtDNA to nDNA content, was 53% higher in the exercise-adapted mice (**Figure 6H**). Collectively, these results indicate that treadmill exercise in FVB/NJ mice elicits robust systemic and cardiac muscle adaptations.

DISCUSSION

The goals of this study were to examine treadmill exercise capacity, compliance, and adaptation in two commonly used strains of laboratory mice. We found that C57BL/6J mice have significantly lower treadmill exercise capacity compared with FVB/NJ mice. C57BL/6J mice, in our hands, became progressively non-compliant with the exercise protocol, which is a likely reason underlying their lack of adaptation. Conversely, FVB/NJ mice demonstrated near perfect compliance and showed robust increases in exercise capacity as well as cardiac adaptations. Because laboratory mice are typically nocturnal, we also compared night and day behavior in the strains. Our metabolic cage data suggest that, contrary to the C57BL/6J strain, FVB/NJ mice have no proclivity for activity and feeding at night, and that their metabolic phenotype lacks circadian variation. Moreover, despite the nocturnal nature of C57BL/6J mice, exercising them at night did not improve compliance or exercise capacity. Collectively, these results indicate that FVB/NJ mice are a superior model for examining systemic and tissue-specific adaptation to treadmill exercise and that diurnal training appears to promote higher exercise compliance and capacity.

The increase in exercise capacity and cardiac adaptations in FVB/NJ mice are likely a consequence of their remarkable compliance as well as their superior ability to perform treadmill work. Indeed, other studies show that the FVB/NJ strain has a higher critical running speed (Billat et al., 2005) and is able to run farther than C57BL/6J mice (Lerman et al., 2002; Massett and Berk, 2005). To compare exercise capacity and adaptations between the strains in a controlled manner, we used equivalent relative intensities of training in the strains, which equated to 70% of the speed achieved during initial exercise capacity tests. These experiments showed that FVB/NJ mice could improve their exercise capacity and that exercise in this strain causes physiologic cardiac growth. Training FVB/NJ mice at a higher workload (initiated at 75% of their initial maximum speed) appeared to evoke superior improvements in distance run and work and provoked physiologic cardiac growth. These results

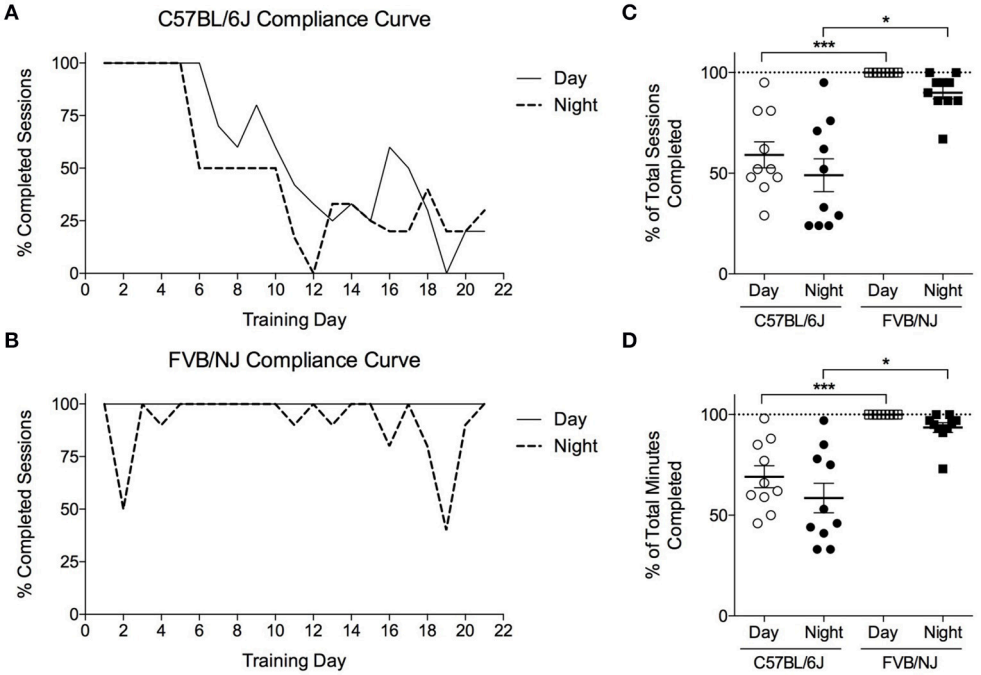

FIGURE 4 | Compliance of FVB/NJ and C57BL/6J to the treadmill training protocol. Compliance to a 4-weeks treadmill training protocol in mice: **(A,B)** Compliance curves indicating the percent of mice that completed each training session; **(C)** Compliance measured as the percent of total sessions; or **(D)** Total minutes completed by mice throughout the exercise training program. $n = 10$ per group. $^{*}p < 0.05$, $^{***}p < 0.001$.

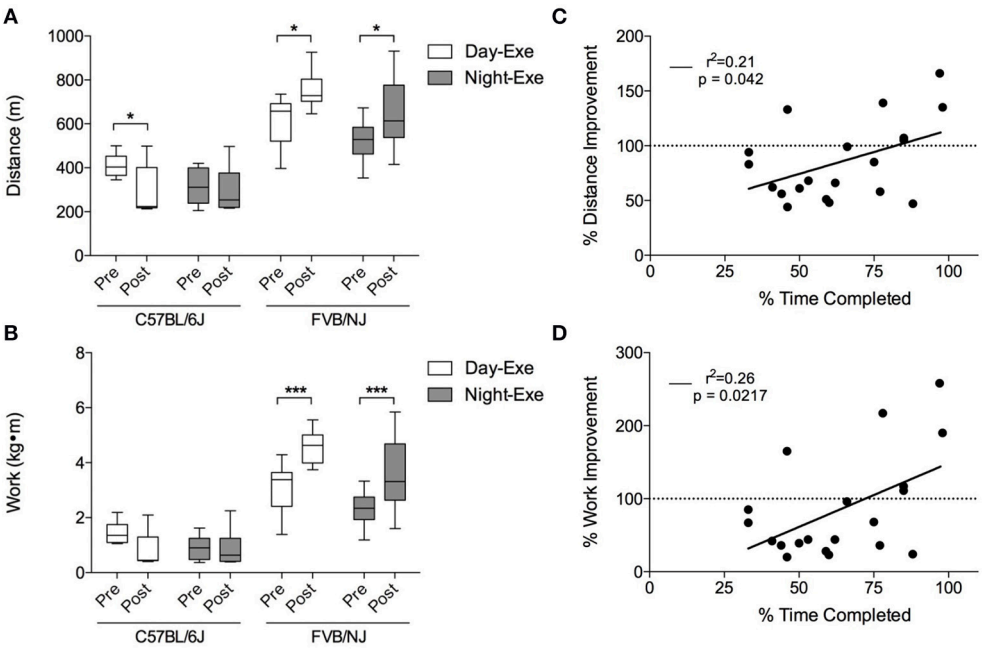

FIGURE 5 | Treadmill training-induced improvements in exercise capacity in C57BL/6J and FVB/NJ mice. Changes in exercise capacity in the mouse strains: **(A)** Distance run and **(B)** work accomplished during initial and final exercise testing following 4 weeks of treadmill training. $n = 10$ per group. $^{*}p < 0.05$, $^{***}p < 0.001$. Panels C and D show correlations of compliance and **(C)** percent distance and **(D)** percent work improved (in the C57BL/6J strain). $n = 20$ mice (includes the C57BL/6J day and the C57BL/6J night groups).

TABLE 1 | Measurements of exercise-induced cardiac growth.

	C57BL/6J	FVB/NJ
BODY MASS, g		
SED	28.2 ± 2.3	30.5 ± 2.6
EXE-Day	27.3 ± 1.4	29.9 ± 1.7
EXE-Night	26.4 ± 1.8	28.5 ± 1.0
HEART MASS, mg		
SED	123.7 ± 15.0	115.4 ± 6.1
EXE-Day	118.7 ± 10.1	135.8 ± 5.5***
EXE-Night	118.0 ± 13.0	130.5 ± 9.1*
HW/BW, mg/g		
SED	4.4 ± 0.4	3.8 ± 0.3
EXE-Day	4.3 ± 0.2	4.6 ± 0.2****
EXE-Night	4.5 ± 0.4	4.6 ± 0.2****
HW/TL, mg/mm		
SED	6.9 ± 0.9	6.3 ± 0.3
EXE-Day	6.6 ± 0.5	7.6 ± 0.3***
EXE-Night	6.6 ± 0.7	7.3 ± 0.4*

*Mice were trained for 4 weeks at 70% of their initial exercise capacity. Values are means ± SD; n = 10/group. HW/BW, heart weight-to-body weight ratio; HW/TL, heart weight-to-tibia length ratio. Statistical comparisons are between SED and EXE-Day and SED and EXE-Night. n = 10 per group. *p < 0.05, ***p < 0.001, ****p < 0.0001.*

indicate that the FVB strain is useful for examining intensity-dependent adaptations to exercise.

The usefulness of the treadmill model lies in its ability to control the level of exercise-associated work and to relate biochemical and physiological adaptations to workload. This is, of course, dependent on compliance to the treadmill protocol. We show that, while C57BL/6J mice completed the protocol during the first week of training, they become increasingly unwilling to run thereafter. It is unlikely that this decrease in compliance is due to overtraining because it occurred upon only the second week of training (and after a 2-day rest period), and the mice did not resume their ability to exercise, even after long periods of refusing to comply with the protocol. Furthermore, the mice did not lose weight during the protocol, which can be an indicator of overtraining (Kadaja et al., 2010). Although, it remains unclear why the C57BL/6J mice choose to run less after the first week, it could be that their tendency to receive more shocks is a negative stimulus for running, or that they associate the shocks with a positive outcome, i.e., removal from the treadmill. Interestingly, C57BL/6J mice are prone to footshock-induced analgesia (Moskowitz et al., 1985; Pavone et al., 1986), and they show progressively decreased shock avoidance behavior compared with other strains (Stavnes and Sprott, 1975). It is also conceivable that this strain is more susceptible to negative stress in the brain caused by forced running (Moraska et al., 2000), as opposed to voluntary running. Thus, it is possible that this strain acclimates to the repeated treadmill shocks that they receive during training.

Escape behavior is another potential contributing factor to the lack of compliance of C57BL/6J mice. A study by Mori and Makino showed that C57BL/6J mice escape and avoid shock by moving to an adjacent compartment in an L-type movement pattern rather than an R-type (rearing and jumping forward) pattern (Mori and Makino, 1994). This is of particular importance because the R-type pattern must be invoked to escape shock in our treadmill apparatus. Regardless, it would appear that the lack of both physiologic cardiac growth and improvements in exercise capacity in C57BL/6J mice is a result of non-compliance with the treadmill protocol.

We also show that FVB/NJ mice lack biological rhythmicity compared with C57BL/6J mice and that the time of day does not appear to affect compliance to the treadmill protocol. Lack of circadian behavior in FVB/NJ mice is a likely consequence of expression of the *retinal degenerative* (*rd*) mutation, which renders them blind to visual images and appears to underlie aberrant circadian wheel running behavior and spatial awareness (Pugh et al., 2004). Conversely, C57BL/6J mice appear to see well: they demonstrate entrainment to a 12:12 h light:dark cycle, and they re-entrain to phase advances (Pugh et al., 2004). Of note, while FVB/NJ mice are blind, C57BL/6J mice become deaf, progressively losing their hearing during the first year of life (Mikaelian, 1979; Henry and Chole, 1980; Willott, 1986). Nevertheless, despite the impaired senses of these strains, the lack of circadian variation in the FVB/NJ strain, and the presence of a nocturnal phenotype in C57BL/6J mice, the timing at which the mice were tested and trained did not appear to have a strong impact. However, we do show that, for FVB/NJ mice, running during the day is associated with a higher initial exercise capacity. Because calculations for the 4-week training regimen are based on this initial capacity, it is possible that testing initial exercise capacities of mice during the day could result in a higher training workload, which could equate to increased physiological adaptations.

Although it is unclear whether the mutations that underlie loss of sight or hearing in FVB/NJ or C57BL/6J mice contribute to their compliance to exercise protocols, it is clear that C57BL/6J and FVB/NJ mice have different exercise capabilities and preferences. Consistent with previous studies (Lerman et al., 2002; Massett and Berk, 2005), we found that C57BL/6J mice have lower treadmill exercise capacity, which did not improve even when they were tested at night. Nevertheless, several studies show that C57BL/6J mice are quite adept at voluntary wheel exercise (e.g., Katzeff et al., 1988; Carter et al., 1995; Lerman et al., 2002; Massett and Berk, 2005; Werner et al., 2008, 2009; Falls et al., 2010; Konhilas et al., 2015), even load bearing wheels which simulate resistance training (Konhilas et al., 2005); and, consistent with their nocturnal behavior, wheel activity in C57BL/6J mice peaks during the night (Pugh et al., 2004). This suggests that the C57BL/6J strain is more amenable to the wheel running modality than treadmill exercise. While several investigators have exercised C57BL/6J mice using a treadmill protocol successfully (reviewed in Perrino et al., 2011), only a few studies document robust increases in exercise capacity or physiological adaptations to extended treadmill training in this strain (Kemi et al., 2002; Ferreira et al., 2010; Sturgeon et al., 2015). Most studies show adaptations that appear relatively minimal compared with other strains (e.g., Massett and Berk, 2005; Ericsson et al., 2010; Miyagi et al.,

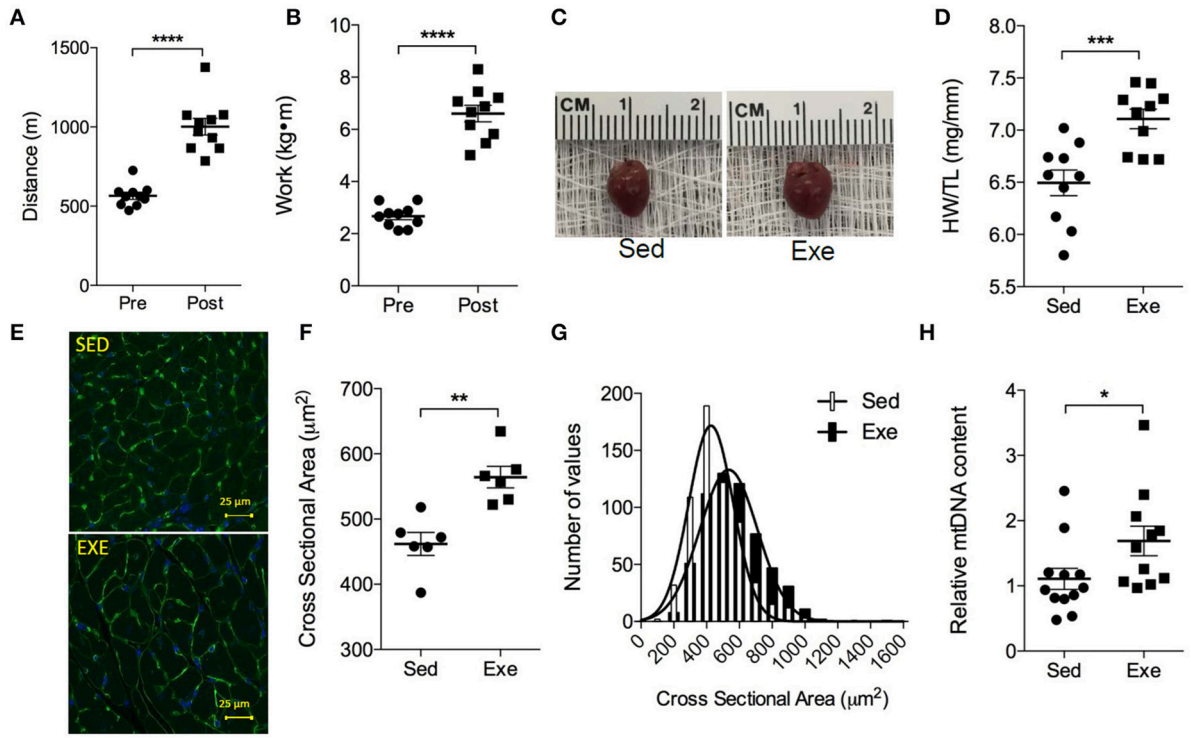

FIGURE 6 | FVB/NJ mice display robust cardiometabolic adaptations to treadmill exercise training. Adaptations to a 4-week treadmill training program in FVB/NJ mice, where the training regimen was set based on 75% of their initial exercise capacity: **(A)** Distance run and **(B)** work accomplished during exercise testing; **(C)** Representative images of hearts from Sed and Exe mice; **(D)** HW/TL; **(E)** Representative cross sections of hearts stained with WGA and DAPI; **(F,G)** quantification of myocyte cross sectional area in heart sections; and **(H)** Mitochondrial biogenesis, as indicated by relative mitochondrial (mt) DNA content. $n = 6$–10 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

2014) or demonstrate that additional exercise mimetics (e.g., AMPK or PPARδ agonists) may be required to promote robust physiological adaptations to treadmill exercise in C57BL/6J mice (Narkar et al., 2008).

Several limitations of our study deserve mention. We did not measure VO_{2max} or identify anaerobic thresholds, which can delineate metabolic crossover points (Petrosino et al., 2016); however, we used stringent criteria for determining fatigue (i.e., behavioral criteria decided upon *a priori*) as well as blood lactate measurements to ensure that mice meeting these criteria were fatigued and not simply incompliant with the exercise capacity protocol. Although we show that FVB/NJ mice are proficient treadmill runners, their lack of circadian behavior suggests that they may not be suitable for investigating chronobiological changes associated with exercise (e.g., exercise-induced changes in *clock* genes). In addition, we did not test whether lower speeds in C57BL/6J mice or different motivating stimuli would increase compliance to the protocol. Some studies in rats (Wisløff et al., 2001), as well as C57BL/6J mice (Kemi et al., 2002), use chocolate as a reward-based incentive to comply with the protocol. We did not test the use of reward in our study, nor did we examine whether alternative aversive stimuli (e.g., air puffs instead of shocks) would improve exercise compliance in the C57 strain. Thus, we do not rule out the possibility that C57BL/6J mice could be coaxed to run with an intensity and compliance similar to that of FVB/NJ mice; however, it is clear to us that improved treadmill compliance in C57BL/6J mice would appear to require a different protocol than that used here, or a reward that encourages continual compliance.

In summary, our findings indicate that FVB/NJ mice are a useful strain for testing treadmill exercise-mediated adaptations. This strain complies well with forced treadmill training and shows a robust capacity for cardiac exercise adaptation. Unlike C57BL/6J mice, FVB/NJ mice do not have a strong nocturnal nature, and they appear to show higher initial exercise capacities and comply better when trained during daytime hours. We also found that, in our hands, C57BL/6J mice show poor compliance to the treadmill exercise regimen, regardless of when they exercise. These findings demonstrate that FVB/NJ mice are a suitable and robust model for understanding the mechanisms underlying cardiac adaptations to exercise.

AUTHOR CONTRIBUTIONS

AG: Design and execution of experiments, analysis of data, manuscript preparation and writing, and financial support; LM: Design and execution of experiments, analysis of data; DR: Statistical analysis; DC: Data analysis and presentation;

AB: Experimental design and financial support; and BH: Experimental design, data analysis, manuscript preparation, writing, and financial support.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00636/full#supplementary-material

Supplemental Table 1 | Exercise compliance chart.

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MINI REVIEW
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Mitochondrial Quality Control in Cardiac Diseases

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Disruption of mitochondrial homeostasis is a hallmark of cardiac diseases. Therefore, maintenance of mitochondrial integrity through different surveillance mechanisms is critical for cardiomyocyte survival. In this review, we discuss the most recent findings on the central role of mitochondrial quality control processes including regulation of mitochondrial redox balance, aldehyde metabolism, proteostasis, dynamics, and clearance in cardiac diseases, highlighting their potential as therapeutic targets.

Keywords: bioenergetics, oxidative stress, mitochondrial unfolded protein response, fusion-fission balance, mitophagy, aldehyde dehydrogenase 2, 4-hydroxynonenal

INTRODUCTION

Cardiac disease remains the leading cause of morbidity and mortality worldwide (Bayeva et al., 2013). Despite the fact that the mechanisms underlying the pathophysiology of heart disease are multiple and complex, recent research provides evidence that loss of mitochondrial homeostasis is a hallmark of cardiomyocyte dysfunction and death (Disatnik et al., 2013; Yogalingam et al., 2013).

Mitochondria are critical organelles for the maintenance of cardiac physiology since cardiomyocytes have a high demand for ATP synthesis and increased oxygen uptake rate. These mitochondrial features also make them the major source of reactive oxygen species (ROS) under pathological circumstances (Figueira et al., 2013). Indeed, disruption of mitochondrial electrochemical and redox properties is tightly associated with the onset and progression of cardiac pathophysiology (Palaniyandi et al., 2010; Gomes et al., 2015). Despite of their main role in ensuring that cardiac energy demands are met by energy supply, mitochondria have been lately highlighted as strategic intracellular nodes/transducers where signals converge. Mitochondria also serve as multi effector players in a wide range of intracellular signaling pathways that ultimately regulates nuclear gene expression, ions homeostasis, and apoptosis (Campos et al., 2013).

Considering the pivotal role of mitochondria in regulating intracellular homeostasis, eukaryotic cells have developed several mechanisms of surveillance capable of maintaining mitochondrial integrity and functionality upon stress (Baker et al., 2011; Kotiadis et al., 2014). These quality control machineries can be divided into three levels of surveillance. The first level of defense involves a multilayer network of detoxifying systems capable of fighting oxygen- and aldehyde-mediated mitochondrial toxicity. The second line of defense relies to mitochondrial proteases and chaperones responsible for the maintenance of mitochondrial proteostasis. The third level of defense involves the control of mitochondrial morphology and number through two interconnected processes: mitochondrial dynamics (fusion and fission) and mitophagy (mitochondrial clearance). These three levels of defense are well-conserved quality control mechanisms capable of regulating mitochondrial redox state, content, size and number due to changes in mitochondrial bioenergetics, biochemical, or electrochemical properties (Twig et al., 2008).

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In this review, we will approach the aforementioned mechanisms of mitochondrial surveillance and defense in health and cardiac diseases; highlighting their critical role in the maintenance of cardiac homeostasis.

MITOCHONDRIAL DETOXIFYING SYSTEMS (FIRST LEVEL OF DEFENSE)

Detoxifying systems are critical mitochondrial quality control mechanisms that provide protection from biogenic and xenogeneic molecules such as ROS and aldehydes.

The term oxidative stress describes conditions that result from the imbalance between free radical generation and its detoxification. Aerobic organisms have developed a series of antioxidant defenses, including antioxidant compounds and enzymes that directly react with and neutralize oxidizing agents; therefore minimizing the oxidative stress-induced cellular damage (Kornfeld et al., 2015).

Mitochondria are the main source of cellular ROS as well as have the highest antioxidant capacity (Figueira et al., 2013). Accumulation of mitochondrial ROS is critical to cardiac ischemia-reperfusion injury and heart failure (Campos et al., 2012, 2013; Chouchani et al., 2014). The use of experimental animal models has contributed to the understanding of the role of antioxidant system as a first line of defense against the pathogenesis of cardiac diseases. SOD deficiency has been linked to excessive ROS accumulation and cardiac mitochondrial dysfunction, thus contributing to the establishment of pathological ventricular hypertrophy and heart failure in mice (Strassburger et al., 2005; Morten et al., 2006; Lu et al., 2008). Moreover, cardiac overexpression of either MnSOD or Cu-ZnSOD isoforms attenuates cardiac ischemia-reperfusion injury and heart failure in mice (Chen et al., 1998; Miller et al., 2010).

In general, enhanced activity of enzymatic antioxidants (i.e., catalase, glutathione peroxidase, thioredoxin, and peroxiredoxin) or increased levels of non-enzymatic antioxidants (i.e., glutathione, N-acetylcysteine, ubiquinol, α-tocopherol, ascorbic acid, and lipoic acid) reduce cardiac oxidative stress in rodents by maintaining ROS at nanomolar levels (Goszcz et al., 2015). However, these findings have not been reproduced in randomized controlled trial (Kritharides and Stocker, 2002; Ye et al., 2013). One possible explanation for the failure in clinical trials relies to the inability of these antioxidants to properly counteract mitochondrial oxidative stress. Recent studies have demonstrated that the development of antioxidants that are target to and selectively accumulates within mitochondria attenuate mitochondrial-specific oxidative damage (Subramanian et al., 2010; Oyewole and Birch-Machin, 2015; Ni et al., 2016). Finally, antioxidant in excess should have detrimental effects on intracellular redox signaling, a ROS-mediated process that positively regulates a vast array of signaling pathways (Ristow et al., 2009).

Recent findings revealed that not only elevated ROS are critical to cardiac diseases, but accumulation of endogenous toxic aldehydes also plays detrimental role in the cardiovascular system (Chen et al., 2010; Chen and Zweier, 2014). 4-hydroxynonenal (4-HNE), a major end-product of peroxidative degradation of mitochondrial phospholipids, is a highly reactive aldehyde that readily forms protein adducts via the Michael addition (Roede and Jones, 2010). Accumulation of 4-HNE causes cardiac mitochondrial dysfunction in a dose-dependent manner and contributes to cardiac pathophysiology in rodents (Campos et al., 2012; Gomes et al., 2014, 2015). Excessive 4-HNE adducts formation has also been reported in human failing hearts (Nakamura et al., 2002; Ferreira et al., 2012) and was associated with impaired cardiac bioenergetics, contractility properties and proteostasis (Campos et al., 2012; Ferreira et al., 2012). Therefore, accumulation of 4-HNE appears to be a hallmark of cardiac diseases (**Figure 1A**).

Aldehyde dehydrogenase 2 (ALDH2), a member of the aldehyde dehydrogenase family (Marchitti et al., 2008; Vasiliou et al., 2013), is a mitochondrial tetrameric enzyme responsible for the conversion of toxic aldehydes such as 4-HNE into inactive acids (Perez-Miller et al., 2010; Sobreira et al., 2011; Ferreira and Mochly-Rosen, 2012; Josan et al., 2013). ALDH2 has emerged as key enzyme in the protection of cardiac myocytes and neurons against aldehydic overload (Sun et al., 2011; Ferreira and Mochly-Rosen, 2012; Zambelli et al., 2014). Pharmacological ALDH2 activation using a small molecule (Alda-1) protects the heart against ischemia-reperfusion injury (Chen et al., 2008, 2014; Gross et al., 2015). We have recently reported that reducing cardiac aldehydic load via selective activation of mitochondrial ALDH2 is sufficient to improve ventricular function in heart failure through a better cardiac mitochondrial bioenergetics and reduced ROS generation (Gomes et al., 2014, 2015). These findings point out ALDH2 as a critical player in the maintenance of mitochondrial quality control, and highlight the potential therapeutic value of ALDH2 activators in counteracting the detrimental role of aldehydes in cardiac disease (**Figure 1A**).

MITOCHONDRIAL PROTEOSTASIS SURVEILLANCE (SECOND LEVEL OF DEFENSE)

The endosymbiotic theory proposes that mitochondria (a former free-living proteobacterium) were engulfed by a precursor eukaryotic cell about 1.5 billion years ago (Gray et al., 1999). During the evolutionary process most of the mitochondrial genome was transferred to nuclear genome. Approximately 1100 mitochondrial proteins are encoded by the nucleus, translated in the cytosol and imported to mitochondria (Pagliarini et al., 2008; Baker et al., 2011). These proteins work in synchrony with the 13 mitochondrial-encoded proteins (all subunits of the electron transport chain) to maintain ATP production (Jovaisaite and Auwerx, 2015). Changes in the stoichiometric balance between nuclear- and mitochondrial-encoded proteins due to improper mitochondrial protein import and folding, as well as mutations in mitochondrial DNA, affect organelle proteostasis, which is sufficient to disrupt the integrity and functionality of mitochondria. Therefore, a network of mitochondrial chaperones

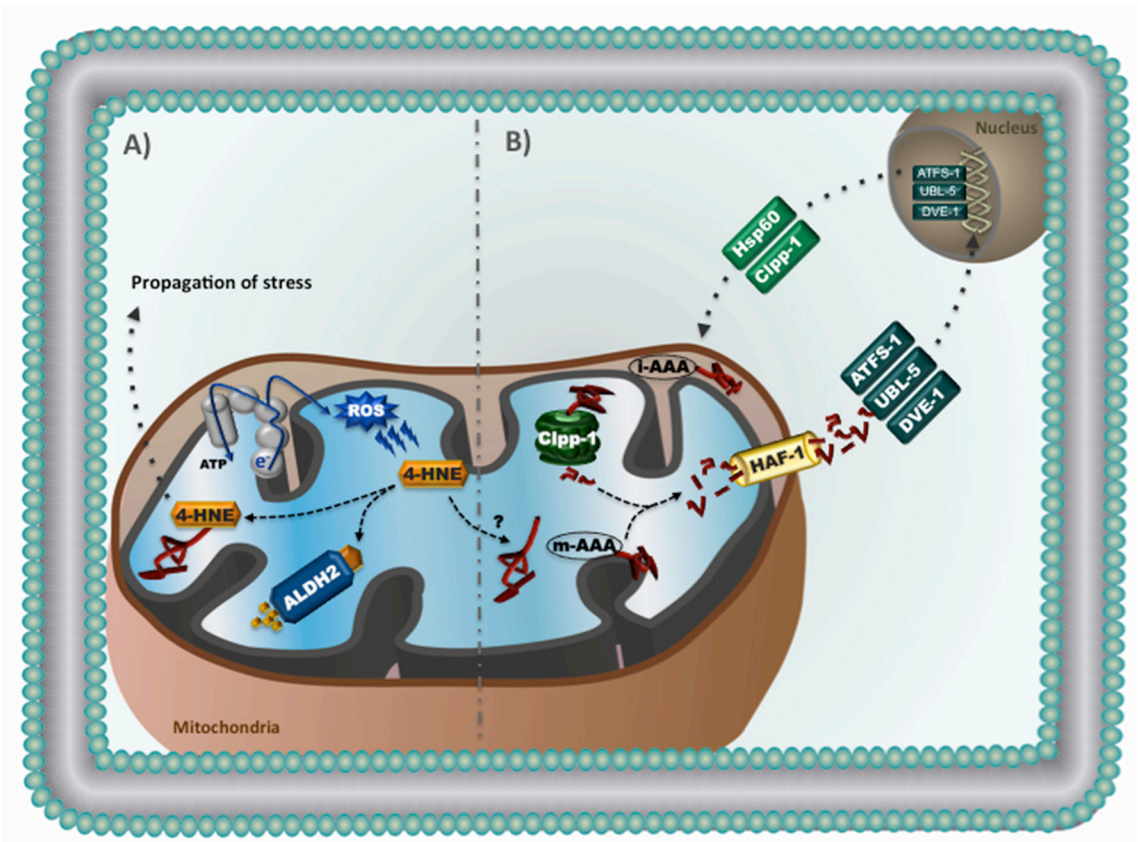

FIGURE 1 | (A) Reactive aldehydes in mitochondria: excessive reactive oxygen species causes lipid peroxidation and consequent generation of 4-hydroxynonenal (4-HNE), a highly reactive aldehyde that readily forms protein adducts. Accumulation of 4-HNE adducts causes mitochondrial dysfunction and contributes to the propagation of stress related to cardiac pathophysiology. Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial enzyme responsible for the conversion of 4-HNE into inactive acids. **(B)** Mitochondrial unfolded protein response (UPR^{mt}): Accumulation of mitochondrial unfolded proteins results in increased proteolysis and release of mitochondrial-generated peptides to the cytosol. It is suggested that these peptides activate cytosolic transcription factors (i.e., ATFS-1, UBL-5, and DVE-1). Once inside the nucleus, these transcription factors promote upregulation of genes involved in mitochondrial proteostasis (i.e., chaperones and proteases), thus relieving stress and re-establishing mitochondrial homeostasis. The UPR^{mt} has been characterized in the nematode *Caenorhabditis elegans*.

and proteases are crucial for maintenance of organelle protein homeostasis.

Mitochondrial chaperones and chaperonins including mtHsp70, Hsp60, and Hsp10 are part of mitochondrial quality control system. These proteins assist mitochondrial protein import machinery, protein folding, electron transport chain assembly, and prevention of aggregation of unfolded proteins (Baker et al., 2011; Pellegrino et al., 2013). Loss of function of mitochondrial chaperones has been implicated in several pathological processes including cardiovascular diseases (MacKenzie and Payne, 2007). Elevated Hsp60 protein levels have been reported in human failing hearts, which might suggest a compensatory response due to disrupted mitochondrial proteostasis (Knowlton et al., 1998). Indeed, combined or individual overexpression of Hsp60 and Hsp10 protects mitochondrial function and prevents cardiomyocyte death induced by either hypoxia-reoxygenation injury or doxorubicin (Lin et al., 2001; Hollander et al., 2003; Shan et al., 2003).

Another critical component of mitochondrial quality control relies to proteolytic systems located at different mitochondrial compartments. Mitochondrial proteases are required to degrade misfolded, damaged, and unfolded proteins no longer capable of being refolded by chaperones. Lon and ClpXP are typical proteases located in the matrix. Lon degrades denatured and oxidized proteins while the substrates of ClpXP still have to be identified. Another class of proteases is located at mitochondrial inner membrane. These AAA metalloproteases (ATPases associated with a number of cellular activities) expose their catalytic sites either to matrix (m-AAA protease) or to intermembrane space (i-AAA protease) and are involved in processing and degradation of proteins (Griparic et al., 2007; Song et al., 2007; Bonn et al., 2011; Gerdes et al., 2012). Both i-AAA and m-AAA proteases mainly degrade unassembled mitochondrial intermembrane proteins (Baker et al., 2011). Disruption of i-AAA and m-AAA proteases affects mitochondria morphology, proteostasis, and bioenergetics (Nolden et al., 2005; Griparic et al., 2007). Recently,

Wai et al. demonstrated that cardiac-specific ablation of the m-AAA protease YME1L alters mitochondrial morphology and metabolism and causes heart failure in mice (Wai et al., 2015).

Another important mitochondrial quality control mechanism is characterized by an inter-organelle communication between mitochondria and nucleus (known as mitochondrial retrograde signaling), which regulates mitochondrial proteostasis by upregulating nuclear-encoded mitochondrial genes (**Figure 1B**; Haynes et al., 2013). This process has been characterized and well-explored in the nematode *Caenorhabditis elegans*, where disruption of mitochondrial unfolded protein response (UPR^{mt}) perturbs mitochondrial function and negatively affects longevity (Haynes et al., 2010). Briefly, loss of mitochondrial proteostasis triggers UPR^{mt}, which may results in accumulation of mitochondrial-derived peptides in the cytosol and translocation of the transcriptional factor ATFS-1 to the nucleus. In association with others transcriptional factors (UBL-5 and DVE-1), ATFS-1 promotes upregulation of genes involved in mitochondrial proteostasis (i.e., chaperones and proteases), glycolytic metabolism, detoxification, and innate immune response, thus relieving stress and re-establishing mitochondrial homeostasis (Benedetti et al., 2006; Jovaisaite et al., 2014; Nargund et al., 2015).

Fiorese et al. recently described how UPR^{mt} is regulated in mammals (Fiorese et al., 2016). They suggest that ATF5, a mammalian transcriptional factor, has a protective role during mitochondrial dysfunction by regulating UPR^{mt} signaling, similar to ATFS-1 in worms. The role of UPR^{mt} signaling in cardiovascular diseases is unknown. It is expected that UPR^{mt} signaling might be activated in failing hearts or during ischemia-reperfusion injury to compensate impaired mitochondrial proteostasis. In fact, UPR^{mt} activation protects *C. elegans* against ischemic injury (Durieux et al., 2011; Kaufman and Crowder, 2015).

MITOCHONDRIAL DYNAMICS AND MITOPHAGY (THIRD LEVEL OF DEFENSE)

Mitochondria usually form a highly dynamic network that plays crucial role in cardiac bioenergetics. The maintenance of mitochondrial size, shape, number and location relies to well-conserved quality control mechanisms including mitochondrial fusion-fission machinery, and mitophagy (**Figure 2**; Liesa et al., 2009; Dorn and Kitsis, 2015; Shirihai et al., 2015). Disruption of these quality control processes result in accumulation of abnormal mitochondria, which is likely associated to cardiovascular diseases (Dorn, 2013; Andres et al., 2015).

Fusion Machinery

Mitochondrial fusion is important for the exchange of DNA, proteins and metabolites between two neighboring mitochondria, therefore helping the maintenance of oxidative phosphorylation, membrane potential and DNA replication/repair (Liesa et al., 2009). Mitochondrial fusion requires both the outer and the inner membranes of two mitochondria to fuse (Disatnik et al., 2015). Two members of the large GTPase dynamin family orchestrate the fusion of the outer mitochondrial membrane (mitofusins 1 and 2—Mfn1 and Mfn2) while OPA1 (optic atrophy factor 1) mediates inner membrane fusion (**Figure 2**). OPA1 is subjected to proteolytic processing where accumulation of short forms is accompanied by fragmentation of mitochondria network and loss of cristae structures (Griparic et al., 2007; van der Bliek et al., 2013). Despite of its role in regulating mitochondrial dynamics, Mfn2 also links mitochondria to the endoplasmic reticulum, a critical process for controlling calcium handling and cell death in cardiomyocytes (Dorn et al., 2015).

Mitochondrial fusion is critical for heart physiology. Combined Mfn1 and Mfn2 ablation of mouse hearts results in accumulation of fragmented mitochondria and leads to heart failure (Chen et al., 2011). Of interest, Hall et al. recently demonstrated that double Mfn1/Mfn2 knockout animals are protected from acute ischemia-reperfusion due to impaired mitochondria/sarcoplasmic reticulum tethering (Hall et al., 2016). These findings suggest that Mfn1 and Mfn2 may play either protective or detrimental role according to stress condition. Disruption of mitochondrial dynamics through deletion of OPA1 or imbalanced OPA1 processing also causes mitochondrial fragmentation, bioenergetics deficit and heart failure in mice (Chen et al., 2012; Wai et al., 2015). Overall, enzymes that control mitochondrial fusion are critical for the maintenance of bioenergetics, cellular viability, and cardiac function. Therefore, pharmacological and non-pharmacological interventions that modulate Mfn1, Mfn2 and OPA1 may have cardioprotective effects against acute and chronic pathological conditions.

Fission Machinery

Fission is critical for segregating dysfunctional mitochondria, where the impaired daughter organelle can be either recovered by fusing with healthy mitochondria or eliminated through autophagy (Gottlieb and Bernstein, 2016; **Figure 2**). Mitochondrial fission is triggered by dynamin-related protein 1 (Drp1). Upon activation, Drp1 translocates from the cytosol to the outer mitochondria membrane, binds to adaptor proteins and constrict both mitochondrial outer and inner membranes (Suzuki et al., 2005; Otera et al., 2010). Impaired mitochondrial fission due to a missense mutation in Dnm1 (Drp1) causes progressive energy deficient and dilated cardiomyopathy in rodents (Ashrafian et al., 2010).

Excessive mitochondrial fission has a negative impact on heart during ischemia-reperfusion injury (Disatnik et al., 2015). Inhibition of mitochondrial fission using a dominant-negative mutant form of Drp1 protects cultured cardiomyocytes against hypoxia-reoxygenation stress (Ong et al., 2010). More recently, we demonstrated that the use of a selective inhibitor of the fission machinery (p110 peptide; Qi et al., 2013), which inhibits the interaction of fission proteins Fis1/Drp1, decreases mitochondrial fission and improves bioenergetics in different models of ischemia-reperfusion injury, including primary cardiomyocytes, *ex vivo* heart model, and an *in vivo*

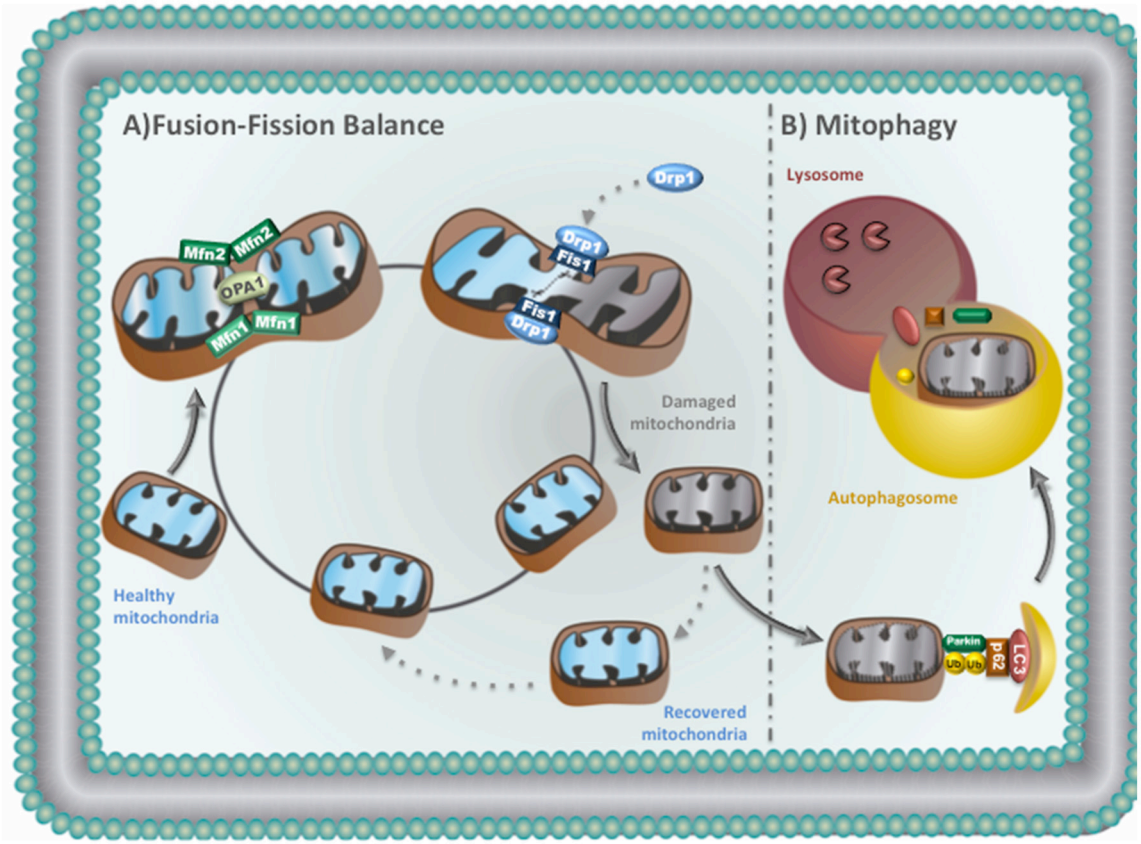

FIGURE 2 | (A) Mitochondrial fusion-fission balance: Mitochondrial fission: Drp1 translocates to mitochondria and binds to Fis1 to promote fission. Daughter mitochondria can be recovered or removed. Recovered mitochondria re-enter in the life cycle to fuse with healthy mitochondria. Mitochondrial fusion: outer membrane fusion is regulated by Mfn1 and Mfn2, while OPA1 is responsible for inner mitochondrial membrane fusion. **(B)** Mitophagy: damaged mitochondria can be sequestered by autophagosomes. The autophagosomes then fuse with lysosomes to degrade sequestered mitochondria.

myocardial infarction model (Disatnik et al., 2013). Of interest, a single dose of p110 peptide at reperfusion after transient coronary artery occlusion is sufficient to inhibit excessive mitochondrial fragmentation, increase mitochondrial oxygen consumption, and improve cardiac function in the long-term (Disatnik et al., 2013). Together, these studies provide evidence that blocking excessive mitochondrial fission protects the heart against acute ischemia-reperfusion injury in rodents. Therefore, therapies capable of reducing excessive mitochondrial fragmentation may become a valuable tool against myocardial infarction.

Mitophagy

Accumulation of dysfunctional mitochondria is suggested to play a key role in cardiac pathophysiology. Therefore, turnover of mitochondria is necessary to maintain cardiac homeostasis. Impaired mitochondria can be selectively targeted and eliminated through a process termed mitochondrial autophagy (mitophagy; **Figure 2B**). Briefly, in healthy mitochondria PINK1 (PTEN-induced putative kinase 1) is targeted to the mitochondrial inner membrane where it is degraded. During stress, dissipation of mitochondrial membrane potential results in accumulation of PINK1 on the mitochondrial surface, which phosphorylates/activates the E3 ubiquitin ligase Parkin (Kane et al., 2014). Parkin ubiquitinates key proteins that can be degraded by the proteasome or recognized by autophagy adaptor proteins. These adaptor proteins are able to act as cargo receptors, which recruit them to autophagosomes (Lazarou et al., 2015). The autophagosomes then fuse with lysosomes to degrade sequestered mitochondria (**Figure 2B**; Youle and Narendra, 2011). This process is known as macroautophagy. Mitochondria can also be directly engulfed through membrane invagination by lysosome, a process called microautophagy (Yogalingam et al., 2013; Hwang et al., 2015). Both macroautophagy and microautophagy are critical to remove damaged mitochondria during cardiac stress (Disatnik et al., 2015).

Markers of autophagy are upregulated in cardiac diseases (Nakai et al., 2007; Zhu et al., 2007) However, accumulation of these proteins may reflect either increased or insufficient autophagy flux (Klionsky et al., 2016). Studies using genetically modified animals provide evidence that autophagy

is critical for the maintenance of cardiac homeostasis (Kubli et al., 2013; Gong et al., 2015). Tissue-specific knockout of autophagy-related genes causes accumulation of disorganized mitochondria and oxidative stress (Tanaka et al., 2000; Wu et al., 2009) whereas overexpression of these genes ameliorates cardiomyopathy (Bhuiyan et al., 2013).

Disruption of mitophagy in Parkin-deficient hearts is sufficient to cause accumulation of dysfunctional mitochondria, oxidative stress, apoptosis, left ventricular dysfunction, and pathological cardiac hypertrophy (Gong et al., 2015). Moreover, defective mitophagy exacerbates cardiac damage induced by myocardial infarction (Narendra et al., 2008). These findings suggest that proper elimination of damaged mitochondria during stress conditions (i.e., myocardial infarction or heart failure) protect against oxidative stress and apoptosis; therefore contributing to the maintenance of cardiac physiology. Of interest, mitophagy markers are reduced in end-stage human heart failure (Billia et al., 2011). Saito et al. recently demonstrated that reduced autophagic vacuoles in cardiomyocytes are associated with poor heart failure prognosis in humans (Saito et al., 2016).

Finally, mitophagy seems to play a critical role during acute cardiac ischemia-reperfusion injury, a process characterized by accumulation of damaged mitochondria and severe oxidative stress. Pharmacological upregulation of autophagy prevents the onset of cell death following ischemia-reperfusion injury (Hamacher-Brady et al., 2006; Sala-Mercado et al., 2010; Yogalingam et al., 2013). However, future studies are required to test whether this cardioprotective effect occurs through enhanced clearance of damaged mitochondria. Of interest, depletion of either Parkin or p62/SQTSTM1 is sufficient to abolish the cardioprotective effects of both ischemic preconditioning and simvastatin treatment in rodents (Huang et al., 2011; Andres et al., 2014).

SUMMARY AND PERSPECTIVES

Mitochondria are essential organelles for the maintenance of myocardial homeostasis. They play critical role in bioenergetics, redox balance, ion homeostasis, and cell death. The importance of functional mitochondrial to the heart has been highlighted by the fact that situations that lead to mitochondrial dysfunction are often associated with cardiac diseases. Many of these diseases manifest later in life, where mitochondria seems to be less functional. Therefore, different levels of mechanisms of surveillance and quality control capable of detecting and fixing defects that affect mitochondrial performance are critical for the maintenance of long-lived cells with high energy demand such as cardiomyocytes. The central role of mitochondrial quality control in the health of myocardium has been recently reported. As described above, the machinery regulating mitochondrial quality control including mitochondrial redox balance, aldehyde metabolism, proteostasis, dynamics, and clearance are potential novel therapeutic targets for cardiac diseases. However, future research focusing on the critical molecular events involved in mitochondrial quality control is needed to develop better pharmacological interventions.

AUTHOR CONTRIBUTIONS

JCC, LHMB, LRGB, and VL wrote the manuscript. JCBF designed, supervised, and wrote the manuscript.

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Mitochondrial and Metabolic Gene Expression in the Aged Rat Heart

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Aging is associated with a decline in cardiac function. Exercise intervention has been suggested as a way to improve this decrement. Age-related decline in cardiac function is associated with decreases in fatty acid oxidation, mitochondrial function, and AMP-activated protein kinase (AMPK) activity. The molecular mechanisms involved with age-related changes in mitochondrial function and substrate metabolism are poorly understood. We determined gene expression differences in hearts of Young (6 mo), Old (33 mo), and old exercise trained (Old + EXE) (34 mo) FBN rats, using Qiagen PCR arrays for Glucose, Fatty acid, and Mitochondrial metabolism. Old rats demonstrated decreased ($p < 0.05$) expression for key genes in fatty acid oxidation, mitochondrial function, and AMPK signaling. There were no differences in the expression of genes involved in glucose metabolism with age. These gene expression changes occurred prior to altered protein translation as we found no differences in the protein content of peroxisome proliferator activated receptor gamma, coactivators 1 alpha (PGC-1α), peroxisome proliferator activated receptor alpha (PPARα), and AMPKα_2 between young and old hearts. Four months of exercise training did not attenuate the decline in the gene expression in aged hearts. Despite this lack of change in gene expression, exercise-trained rats demonstrated increased exercise capacity compared to their sedentary counterparts. Taken together, our results show that differential expression of genes associated with fatty acid metabolism, AMPK signaling and mitochondrial function decrease in the aging heart which may play a role in age-related declines in fatty acid oxidation, AMPK activity, and mitochondrial function in the heart.

Keywords: mitochondrial biogenesis, gene expression, exercise training, fatty acid metabolism, glucose metabolism

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INTRODUCTION

Aging is associated with a decline in cardiac function in humans as well as rodents (Lakatta and Sollott, 2002). The decline in cardiac function with age is associated with changes in mitochondrial function and energy metabolism (Abu-Erreish et al., 1977; McMillin et al., 1993; Fannin et al., 1999; Kates et al., 2003; Bhashyam et al., 2007; Chakravarti et al., 2008). Since the myocardium derives nearly all of its energetic needs from the oxidation of pyruvate and fatty acids within the mitochondria, mitochondrial changes have great potential to contribute to cardiac dysfunction with age. Indeed, mitochondrial energetic deficiency with aging has been well-documented (McMillin et al., 1993; Fannin et al., 1999; Phaneuf and Leeuwenburgh, 2002; Chakravarti et al., 2008; Ventura-Clapier et al., 2008). The mechanisms of this mitochondrial dysfunction may include biogenesis

that is inadequate to match the increasing demand (Goffart et al., 2004), as well as increased mitochondrial uncoupling and decreased substrate availability (Murray et al., 2004). Several studies have documented age-dependent impairment in the mitochondrial respiratory capacity (Kumaran et al., 2005; Navarro and Boveris, 2007).

Concomitant with mitochondria functional changes there is a change in cardiac substrate utilization during the aging process. At rest, fatty acids are the primary substrate for ATP supply in the myocardium and glucose uptake and oxidation supplies the remainder of the carbon substrates (Wisneski et al., 1985). There is some evidence that this substrate utilization changes with age (Abu-Erreish et al., 1977; McMillin et al., 1993; Sample et al., 2006), with most evidence indicating an age-related reduction in fatty acid oxidation, with the implication of increased reliance on glucose, although this is not universally seen (Sample et al., 2006). Interestingly, it has been determined that glucose utilization itself does not increase (Abu-Erreish et al., 1977; McMillin et al., 1993; Kates et al., 2003).

The molecular mechanisms underlying age-related changes in mitochondrial function or substrate energy metabolism is largely unknown. Decreases in mRNA levels of peroxisome proliferator-activated receptor (PPARα), and some of its downstream targets (i.e., CPT-1, β-hydroxyacyl dehydrogenase) have been observed with age (Iemitsu et al., 2002). In addition, peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) protein content is reduced in the aging left ventricle (Turdi et al., 2010). PPARα is a transcription factor for genes involved with fatty acid transport and β-oxidation (Huss and Kelly, 2004). PGC-1α, a co-transcription factor is known to stimulate mitochondrial DNA replication and the coding for genes involved with oxidative phosphorylation (Huss and Kelly, 2004). Another mechanism for age-related changes in substrate metabolism may be related to AMP-activated protein kinase (AMPK) activity. Dually activated by AMP and by upstream phosphorylation, this kinase promotes fatty acid oxidation, glucose uptake, and glycogenolysis while it inhibits anabolic processes such as fatty acid synthesis (Munday et al., 1988; Coven et al., 2003; Hawley et al., 2003, 2005; Scott et al., 2004). There is evidence that AMPK activity declines with age (Gonzalez et al., 2004; Turdi et al., 2010). Changes in the expression, protein content, or activity of PPARα, PGC-1α, and AMPK can help explain some of the known changes in substrate metabolism and mitochondrial function with age. However, these processes are the result of complex pathways that require the coordinated expression and function of a large number of genes and proteins, but there are few studies that have examined the effects of aging on the expression of the comprehensive array of genes that are associated with substrate metabolism and mitochondrial function.

Exercise training is known to improve indices of cardiac function in humans (Fortney et al., 1992; Seals et al., 1994) and rodents (Spurgeon et al., 1983; Iemitsu et al., 2002). Specifically, these functional improvements include, increased contractile function (Spurgeon et al., 1983; Fortney et al., 1992; Seals et al., 1994; Iemitsu et al., 2002) and increased maximal oxygen uptake (VO_{2max}) (Ogawa et al., 1992; Seals et al., 1994; Stratton et al., 1994). There is some evidence that exercise can alter the metabolic phenotype of the aging heart. Exercise training in aging Wistar rats increased AMPK activity, PPARα mRNA and protein content, and proteins involved in fatty acid oxidation in the young and aged heart (Iemitsu et al., 2002; Rimbaud et al., 2009; Dobrzyn et al., 2013). Also, lifelong voluntary wheel running in mice increased electron transport chain (ETC) complex IV gene expression (Bronikowski et al., 2003). There is molecular evidence that exercise training can increase the mRNA and protein levels of important proteins involved with fatty acid oxidation and oxidative phosphorylation, these markers suggest that these metabolic pathways may be improved with exercise training (Iemitsu et al., 2002; Bronikowski et al., 2003; Rimbaud et al., 2009; Dobrzyn et al., 2013). However, substrate metabolism and mitochondrial oxidative phosphorylation in the heart are regulated by the transcription, translation, and activity of many genes in order to optimally function.

Therefore, the aims of this study was to: (1) determine the effects of age on the expression of a large number of genes related to the pathways of glucose and fatty acid metabolism, and mitochondrial function; and (2) determine whether exercise training could mitigate age-related changes in the expression of metabolic and mitochondrial genes in the aging rat heart. We hypothesize that expression of genes associated with the pathways of fatty acid metabolism, AMPK signaling, and mitochondrial function will decrease with age and that the addition of exercise training in these aged rats will mitigate this decrease in gene expression.

MATERIALS AND METHODS

Animals

Male Fischer 344 x Brown Norway hybrid rats (FBN), were obtained from the National Institute on Aging colony at Harlan Industries (Indianapolis, IN). The FBN hybrid rat is a long-lived strain with a median life-span of 33 months and a maximum life-span of 40 months. The FBN rat is considered a "healthy aging model" widely used and highly recommended for gerontological research. All rats were confined to standard size rodent cages and housed 2 rats per cage. Rats had access to food and water *ad libitum* and were acclimated to reverse daylight (12 h dark, 12 h light). Body weights and average food intake were monitored through the course of the study. Rats were randomly assigned to one of three groups: Young (6 month), Old (33–34 month), and Old + Exercise (Old+EXE) (33–34 month). Animal housing and handling was carried out under the guidelines of the University of Wisconsin-Madison Institutional Animal Care and Use Committees and conducted in pathogen-free facilities that are accredited by the American Association of Accreditation of Laboratory Animal Care.

Tissue Collection

Old and Old + EXE hearts were removed and flash frozen in liquid nitrogen 72 h from the last maximal exercise test in order to control for transient gene expression changes due to acute exercise (Neufer and Dohm, 1993; Pilegaard et al., 2000). From the Old ($n = 9$) and Old + EXE ($n = 9$) rats, 5 hearts

were randomly selected for qRT-PCR, Western blot, and citrate synthase experiments, while Young ($n = 5$) hearts were used for molecular analysis.

Maximal Exercise Testing

Assessment of peak exercise capacity occurred each month beginning at 30 months until 34 months of age. The exercise test started with a treadmill speed at 5 m/min and progressively increased by 3 m/min at each exercise stage. Animals ran at each new treadmill speed for 3 min to assure steady-state values. To encourage the rats to run, the treadmill was equipped with an electric shock grid at the rear of the treadmill. The shock grid was set to deliver a 0.2 mA current, which gives an uncomfortable shock but does not physically harm or injure the rat. The test was terminated when animals were no longer able to maintain position on the treadmill, and the highest speed was recorded as peak exercise capacity. Blood lactate levels were measured during the graded exercise tests. Blood lactate levels were used to quantify relative exercise intensity. At the end of each 3 min interval, animals were briefly removed from the treadmill, immobilized with the tail extended, the lateral tail vein pierced, and a drop ($\sim$25 μL) of blood was collected on a lactate strip inserted into a lactate meter (Lactate Plus Meter, Nova Biomedical). Blood lactate values were recorded at each exercise intensity level during the exercise test.

Training Protocol

Trained animals were exercised on a motor driven treadmill during their 12 h dark cycle. Before training began, animals were treadmill acclimatized for 2 weeks at 5 m/min for 5 min during the first week and at 5 m/min for 10 min the second week. After acclimatization, maximal exercise tests were performed and blood lactate measurements were taken at each successive speed on the treadmill during the test. The endurance trained rats began exercise training at 30 months of age with a training program that following 2 weeks of treadmill acclimation consisted of 30 min/day, 5 days per week at a speed that corresponded to each animal's lactate threshold. Thus, the training speed was adjusted each month based on the results of the maximal exercise tests (above). All exercise training sessions included a 3 min warm-up period at 5 m/min. No negative stimuli (electric shock) were used during the daily exercise training of the animals to minimize stress involved in exercise for these aging animals.

RNA Isolation and cDNA Preparation

Rats were terminated using isoflurane/pneumothorax euthanasia and hearts were removed and flash frozen in liquid nitrogen. Total RNA from each rat was isolated from the left ventricular free wall using RNeasy® Microarray Tissue Mini Kit (Qiagen) according to the manufacturer instruction. Immediately after elution RNA concentration and purity was measured spectrophotometrically (Beckman-Coulter). For each sample 700 ng of total RNA was reversed transcribed using the RT^2 First Strand Kit (Qiagen). The reaction was performed at 42°C for 15 min followed by a termination step at 95°C for 5 min. cDNA was stored at −20°C until qRT-PCR.

qRT-PCR Data Analysis

A total of 251 genes of interest across three PCR arrays from Qiagen were assayed for this study (Glucose Metabolism RT^2 Profiler PCR Array, Fatty Acid Metabolism RT^2 Profiler PCR Array and RT^2 Custom PCR Array for mitochondrial function). A list of all of the genes contained in each array can be found in **Tables 1–3**. PCR was performed on Step-One Plus PCR system (Applied Biosystems), according to the manufacturer's instructions. For data analysis, the $\Delta\Delta$Ct method was used with the aid of a Microsoft Excel spreadsheet containing algorithms and a student's *t*-test was used to analyze differences in fold-changes in gene expression provided by the manufacturer (Hassmann-Poznańska et al., 2013; Wu et al., 2013; Okada et al., 2014). The $\Delta\Delta$Ct method for calculating differences in gene expression are as follows: (ΔCt = Ct^{GOI} − Ct$^{AVG\ HKG}$, where GOI is the gene of interest and HKG is the housekeeping gene selected. $\Delta\Delta$Ct = (ΔCt (Experimental group) −ΔCt (Control)). The housekeeping gene selected for data analysis was Ribosomal protein, large P1 (Rplp1). Fold-changes were then calculated and expressed as log-normalized ratios of values from Old/Young, Old + EXE/Young and Old + EXE/Old heart tissues.

Western Blotting

Whole cell lysate from isolated left ventricle was prepared using CelLytic™ MT Cell Lysis Reagent and 1:100 dilution of Protease Inhibitor Cocktail (Sigma, St. Louis, MO). The protein concentration was determined using Bio-Rad Protein Assay (BioRad, Hercules, CA). Thirty micrograms of whole cellular protein per lane was separated by SDS-PAGE with a 4–12% Bis-Tris Criterion™ XT gel (XT MOPS running buffer) and blotted onto a nitrocellulose membrane. The membrane was incubated with blocking buffer (5% non-fat dry milk/TBS/0.1% Tween 20) at room temperature for 1 h. The membrane was then probed with the primary antibodies diluted in blocking buffer overnight at 4°C. Subsequently, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody diluted 1:2000 in blocking buffer. Blots were developed with Clarity™ Western ECL substrate (BioRad, Hercules, CA) and imaged with GE ImageQuant LAS-4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA). The image of the blots was uploaded and densitometry analysis was done with Image Studio Lite (LI-COR Biosciences, Lincoln, NE). Protein content was measured from the densitometry units from PGC-1α, AMPKα_2, PPARα, and normalized to vinculin.

Antibodies

Rabbit anti-PGC-1α, AMPKα_2, and Vinculin antibodies were acquired from Cell Signaling Technology, Inc., Beverly, MA. Rabbit anti-PPARα antibody was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

Citrate Synthase Activity

Maximal citrate synthase (CS) activity was determined in left ventricular homogenates using (Citrate Synthase Assay Kit, Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol with absorbance kinetically measured at

TABLE 1 | Gene list Glucose metabolism array.

Symbol	Description
Acly	ATP citrate lyase
Aco1	Aconitase 1, soluble
Aco2	Aconitase 2, mitochondrial
Agl	Amylo-1,6-glucosidase, 4-alpha-glucanotransferase
Aldoa	Aldolase A, fructose-bisphosphate
Aldob	Aldolase B, fructose-bisphosphate
Aldoc	Aldolase C, fructose-bisphosphate
Bpgm	2,3-bisphosphoglycerate mutase
Cs	Citrate synthase
Dlat	Dihydrolipoamide S-acetyltransferase
Dld	Dihydrolipoamide dehydrogenase
Dlst	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)
Eno1	Enolase 1, (alpha)
Eno2	Enolase 2, gamma, neuronal
Eno3	Enolase 3, beta, muscle
Fbp1	Fructose-1,6-bisphosphatase 1
Fbp2	Fructose-1,6-bisphosphatase 2
Fh	Fumarate hydratase 1
G6pc	Glucose-6-phosphatase, catalytic subunit
G6pc3	Glucose 6 phosphatase, catalytic, 3
G6pd	Glucose-6-phosphate dehydrogenase
Galm	Galactose mutarotase (aldose 1-epimerase)
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
Gapdhs	Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic
Gck	Glucokinase
Gpi	Glucose phosphate isomerase
Gsk3a	Glycogen synthase kinase 3 alpha
Gsk3b	Glycogen synthase kinase 3 beta
Gys1	Glycogen synthase 1, muscle
Gys2	Glycogen synthase 2
H6pd	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
Hk2	Hexokinase 2
Hk3	Hexokinase 3 (white cell)
Idh1	Isocitrate dehydrogenase 1 (NADP+), soluble
Idh2	Isocitrate dehydrogenase 2 (NADP+), mitochondrial
Idh3a	Isocitrate dehydrogenase 3 (NAD+) alpha
Idh3b	Isocitrate dehydrogenase 3 (NAD+) beta
Idh3g	Isocitrate dehydrogenase 3 (NAD), gamma
Mdh1	Malate dehydrogenase 1, NAD (soluble)
Mdh1b	Malate dehydrogenase 1B, NAD (soluble)
Mdh2	Malate dehydrogenase 2, NAD (mitochondrial)
Ogdhl	Oxoglutarate dehydrogenase-like
Pc	Pyruvate carboxylase
Pck1	Phosphoenolpyruvate carboxykinase 1 (soluble)
Pck2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
Pdha2	Pyruvate dehydrogenase (lipoamide) alpha 2
Pdhb	Pyruvate dehydrogenase (lipoamide) beta
Pdhx	Pyruvate dehydrogenase complex, component X
Pdk1	Pyruvate dehydrogenase kinase, isozyme 1
Pdk2	Pyruvate dehydrogenase kinase, isozyme 2
Pdk3	Pyruvate dehydrogenase kinase, isozyme 3
Pdk4	Pyruvate dehydrogenase kinase, isozyme 4
Pdp2	Pyruvate dehyrogenase phosphatase catalytic subunit 2
Pdpr	Pyruvate dehydrogenase phosphatase regulatory subunit
Pfkl	Phosphofructokinase, liver
Pgam2	Phosphoglycerate mutase 2 (muscle)
Pgk1	Phosphoglycerate kinase 1
Pgk2	Phosphoglycerate kinase 2
Pgls	6-phosphogluconolactonase
Pgm1	Phosphoglucomutase 1
Pgm2	Phosphoglucomutase 2
Pgm3	Phosphoglucomutase 3
Phka1	Phosphorylase kinase, alpha 1
Phkb	Phosphorylase kinase, beta
Phkg1	Phosphorylase kinase, gamma 1
Phkg2	Phosphorylase kinase, gamma 2 (testis)
Pklr	Pyruvate kinase, liver, and RBC
Prps1	Phosphoribosyl pyrophosphate synthetase 1
Prps1l1	Phosphoribosyl pyrophosphate synthetase 1-like 1
Pygl	Phosphorylase, glycogen, liver
Pygm	Phosphorylase, glycogen, muscle
Rbks	Ribokinase
Rpia	Ribose 5-phosphate isomerase A
Sdha	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
Sdhb	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)
Sdhc	Succinate dehydrogenase complex, subunit C, integral membrane protein
Sdhd	Succinate dehydrogenase complex, subunit D, integral membrane protein
Sucla2	Succinate-CoA ligase, ADP-forming, beta subunit
Suclg1	Succinate-CoA ligase, alpha subunit
Suclg2	Succinate-CoA ligase, GDP-forming, beta subunit
Taldo1	Transaldolase 1
Tkt	Transketolase
Tpi1	Triosephosphate isomerase 1
Ugp2	UDP-glucose pyrophosphorylase 2

412 nm at baseline and after addition of oxaloacetate (Sigma-Aldrich). CS activity was normalized to protein content with tissue protein determined using the Bio-Rad protein assay.

Statistical Analysis

A two-tailed Student's *t*-test was used for analyzing differences in gene expression and exercise performance. A one-way ANOVA was utilized to analyze differences in protein content and citrate synthase activity. *Post-hoc* analysis was performed when the one-way ANOVA was significant using Tukey's LSD to assess between group differences. Significance was determined at $p < 0.05$.

RESULTS

Effects of Exercise Training on Exercise Performance

The effect of 4 months of exercise training on exercise performance in 34 month old trained (Old + EXE) FBN rats compared to sedentary (Old SED) rats was significant

TABLE 2 | Gene list for Fatty Acid metabolism array.

Symbol	Description
Acaa1a	Acetyl-Coenzyme A acyltransferase 1A
Acaa2	Acetyl-Coenzyme A acyltransferase 2
Acad10	Acyl-Coenzyme A dehydrogenase family, member 10
Acad11	Acyl-Coenzyme A dehydrogenase family, member 11
Acad9	Acyl-Coenzyme A dehydrogenase family, member 9
Acadl	Acyl-Coenzyme A dehydrogenase, long-chain
Acadm	Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain
Acads	Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain
Acadsb	Acyl-Coenzyme A dehydrogenase, short/branched chain
Acadvl	Acyl-Coenzyme A dehydrogenase, very long chain
Acat1	Acetyl-coenzyme A acetyltransferase 1
Acat2	Acetyl-Coenzyme A acetyltransferase 3
Acot12	Acyl-CoA thioesterase 12
Acot2	Acyl-CoA thioesterase 2
Acot3	Acyl-CoA thioesterase 3
Acot7	Acyl-CoA thioesterase 7
Acot8	Acyl-CoA thioesterase 8
Acot9	Acyl-CoA thioesterase 9
Acox1	Acyl-Coenzyme A oxidase 1, palmitoyl
Acox2	Acyl-Coenzyme A oxidase 2, branched chain
Acox3	Acyl-Coenzyme A oxidase 3, pristanoyl
Acsbg1	Acyl-CoA synthetase bubblegum family member 1
Acsbg2	Acyl-CoA synthetase bubblegum family member 2
Acsl1	Acyl-CoA synthetase long-chain family member 1
Acsl3	Acyl-CoA synthetase long-chain family member 3
Acsl4	Acyl-CoA synthetase long-chain family member 4
Acsl5	Acyl-CoA synthetase long-chain family member 5
Acsl6	Acyl-CoA synthetase long-chain family member 6
Acsm2a	Acyl-CoA synthetase medium-chain family member 2
Acsm3	Acyl-CoA synthetase medium-chain family member 3
Acsm4	Acyl-CoA synthetase medium-chain family member 4
Acsm5	Acyl-CoA synthetase medium-chain family member 5
Aldh2	Aldehyde dehydrogenase 2 family (mitochondrial)
Bdh1	3-hydroxybutyrate dehydrogenase, type 1
Bdh2	3-hydroxybutyrate dehydrogenase, type 2
Cpt1a	Carnitine palmitoyltransferase 1a, liver
Cpt1b	Carnitine palmitoyltransferase 1b, muscle
Cpt1c	Carnitine palmitoyltransferase 1c
Cpt2	Carnitine palmitoyltransferase 2
Crat	Carnitine acetyltransferase
Crot	Carnitine O-octanoyltransferase
Decr1	2,4-dienoyl CoA reductase 1, mitochondrial
Decr2	2,4-dienoyl CoA reductase 2, peroxisomal
Echs1	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial
Eci2	Enoyl-Coenzyme A delta isomerase 2
Ehhadh	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
Fabp1	Fatty acid binding protein 1, liver
Fabp2	Fatty acid binding protein 2, intestinal
Fabp3	Fatty acid binding protein 3, muscle, and heart
Fabp4	Fatty acid binding protein 4, adipocyte
Fabp5	Fatty acid binding protein 5, epidermal
Fabp6	Fatty acid binding protein 6, ileal
Fabp7	Fatty acid binding protein 7, brain
Gcdh	Glutaryl-Coenzyme A dehydrogenase
Gk	Glycerol kinase
Gk2	Glycerol kinase 2
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)
Gpd2	Glycerol-3-phosphate dehydrogenase 2, mitochondrial
Hadha	Hydroxyacyl-Coenzyme A dehydrogenase[a]
Hmgcl	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)
Lipe	Lipase, hormone sensitive
Lpl	Lipoprotein lipase
Mcee	Methylmalonyl CoA epimerase
Mut	Methylmalonyl-Coenzyme A mutase
Oxct2a	3-oxoacid CoA transferase 2A
Pecr	Peroxisomal trans-2-enoyl-CoA reductase
Ppa1	Pyrophosphatase (inorganic) 1
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit
Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit
Prkab1	Protein kinase, AMP-activated, beta 1 non-catalytic subunit
Prkab2	Protein kinase, AMP-activated, beta 2 non-catalytic subunit
Prkaca	Protein kinase, cAMP-dependent, catalytic, alpha
Prkacb	Protein kinase, cAMP dependent, catalytic, beta
Prkag1	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit
Prkag2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit
Prkag3	Protein kinase, AMP-activated, gamma 3 non-catalytic subunit
Slc27a1	Solute carrier family 27 (fatty acid transporter), member 1
Slc27a2	Solute carrier family 27 (fatty acid transporter), member 2
Slc27a3	Solute carrier family 27 (fatty acid transporter), member 3
Slc27a4	Solute carrier family 27 (fatty acid transporter), member 4
Slc27a5	Solute carrier family 27 (fatty acid transporter), member 5
Slc27a6	Solute carrier family 27 (fatty acid transporter), member 6

[a] *Hydroxyacyl-Coenzyme A dehydrogenase 3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit.*

(**Figure 1**). We measured exercise capacity in two different ways; (1) treadmill speed at lactate threshold (LT) and (2) peak exercise capacity (the final treadmill speed achieved during the maximal exercise test). Exercise training significantly improved lactate threshold ($p < 0.01$) in the Old + EXE rats compared to Old SED rats (**Figure 1A**). Peak exercise capacity was also greater ($p = 0.01$) in the Old + EXE rats compared to Old SED rats (**Figure 1B**).

Gene Expression Changes with Age

To determine the effects of age on the expression of genes involved in cardiac glucose and fatty acid metabolism as well as mitochondrial function we performed qRT-PCR using three different PCR arrays. **Table 4** shows all genes in the energetic

TABLE 3 | Gene list for the Custom array.

Symbol	Description
Ppargc1	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
Nrf1	Nuclear respiratory factor 1
Tfam	Transcription factor A, mitochondrial
Sir2	Sirtuin (Silent mating type information regulation 2 homolog) 2
Esrra	Estrogen related receptor, alpha
Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit
CREB1	cAMP response element binding protein 1
CaMK4	Calcium/calmodulin-dependent protein kinase IV
SOD2	Superoxide dismutase 2, mitochondrial
UCP2	Uncoupling protein 2 (mitochondrial proton carrier)
UCP3	Uncoupling protein 3 (mitochondrial proton carrier)
Bax	Bcl2–associated X protein
Bcl2	B-cell CLL/lymphoma 2
Casp3	Caspase 3
Gabpa	GA binding protein transcription factor (alpha subunit) (i.e., NRF-2a)
UCP1	Uncoupling protein 1 (mitochondrial proton carrier)
Ppara	Peroxisome proliferator activated receptor alpha
Slc2a4	Solute carrier family 2 (facilitated glucose transporter) member 4
CD36	CD 36 molecule (thrombospondin receptor)
Acacb	Acetyl-Coenzyme A carboxylase beta
Mlycd	Malonyl-CoA decarboxylase
Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1
Ndufa10	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10
Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 11
Ndufa2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2
Ndufa5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5
Ndufa6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6
Ndufa7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7
Ndufa8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8
Ndufa9	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 9
Ndufab1	NADH dehydrogenase (ubiquinone) 1 alpha/beta subcomplex, 1
Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2
Ndufb3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3
Ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5
Ndufb6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6
Ndufb7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7
Ndufb8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8
Ndufb9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9
Ndufc2	NADH dehydrogenase (ubiquinone) 1 subcomplex unknown, 2
Ndufs1	NADH dehyrogenase (ubiquinone) Fe-S protein 1
Ndufs2	NADH dehyrogenase (ubiquinone) Fe-S protein 2
Ndufs3	NADH dehyrogenase (ubiquinone) Fe-S protein 3
Ndufs4	NADH dehyrogenase (ubiquinone) Fe-S protein 4
Ndufs6	NADH dehyrogenase (ubiquinone) Fe-S protein 6
Ndufs7	NADH dehyrogenase (ubiquinone) Fe-S protein 7
Ndufs8	NADH dehyrogenase (ubiquinone) Fe-S protein 8
Ndufv1	NADH dehydrogenase (ubiquinone) flavoprotein 1
Ndufv2	NADH dehyrogenase (ubiquinone) flavoprotein 2
Bcs1l	BCS1-like yeast
Cyc1	Cytochrome c-1
Uqcrb	Ubiquinol-cytochrome c reductase binding protein
Uqcrc1	Ubiquinol-cytochrome c reductase core protein 1
Uqcrc2	Ubiquinol-cytochrome c reductase core protein 2
Uqcrfs1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
Uqcrh	Ubiquinol-cytochrome c reductase hinge protein
Uqcrq	Ubiquinol-cytochrome c reductase, complex III subunit VII
Cox15	COX 15 homolog, cytochrome c oxidase assembly protein (yeast)
Cox17	COX 17 cytochrome c oxidase assembly homolog (*S. cerevisiae*)
Cox4i1	Cytochrome c oxidase subunit IV isoform 1
Cox4i2	Cytochrome c oxidase subunit IV isoform 2
Cox5a	Cytochrome c oxidase subunit Va
Cox5b	Cytochrome c oxidase subunit Vb
Cox6a1	Cytochrome c oxidase subunit VIa polypeptide 1
Cox6a2	Cytochrome c oxidase subunit VIa polypeptide 2
Cox6c	Cytochrome c oxidase subunit VIc
Cox7a2	Cytochrome c oxidase subunit VIIa polypeptide 2
Cox7a2l	Cytochrome c oxidase subunit VIIa polypeptide 2 like
Cox7b	Cytochrome c oxidase subunit VIIb polypeptide
Cox8a	Cytochrome c oxidase subunit VIIIa
Cox8c	Cytochrome c oxidase subunit VIIIc
mfn1	Mitofusin 1
mfn2	Mitofusin 2
fis1	Fission 1 (mitochondrial outer membrane) homolog (*S. cerevisiae*)
lonp1	Lon protease
Aifm2	Apoptosis-inducing factor, mitochondrial-associated 2
Bcl2l1	Bcl2-like 1
Clpb	ClpB caseinolytic peptidase B homolog (*E. coli*) (i.e. HSP 78)
pnpt1	Polyribonucleotide nucleotidyltransferase 1
Me1	Malic enzyme 1, NADP(+)-dependent, cytosolic
Foxo3	Forkhead box O3
Camkk2	Calcium/calmodulin-dependent protein kinase kinase 2,beta
Stk11	Serine/threonine kinase 11 (i.e., LKB1)
Ppargc1b	Peroxisome proliferator-activated receptor gamma, coactivator 1 beta
Tp53	Tumor protein p53

pathways for which expression was significantly different between Old and Young rats. Between the three arrays there were a total of 44 genes that were differentially expressed ($p < 0.05$) in Old compared to Young. Of the 44 genes differentially expressed, 42 of these genes had decreased expression in Old compared to Young. Notably, the expression profile of three important pathways of energy production were altered with age; fatty acid oxidation (FAO), mitochondrial biogenesis, and AMPK signaling. There were no significant changes in glucose metabolism gene expression with age. **Figure 2** shows changes in expression with age in key genes involved in the pathways of FAO, mitochondrial biogenesis, and AMPK signaling.

Effects of Exercise Training on Gene Expression in Aged Hearts

Differences in gene expression were observed between Old and Old + EXE rat hearts (**Table 5**). 70 genes associated with glucose metabolism, FA metabolism and mitochondrial function were altered with exercise training in cardiac tissue of

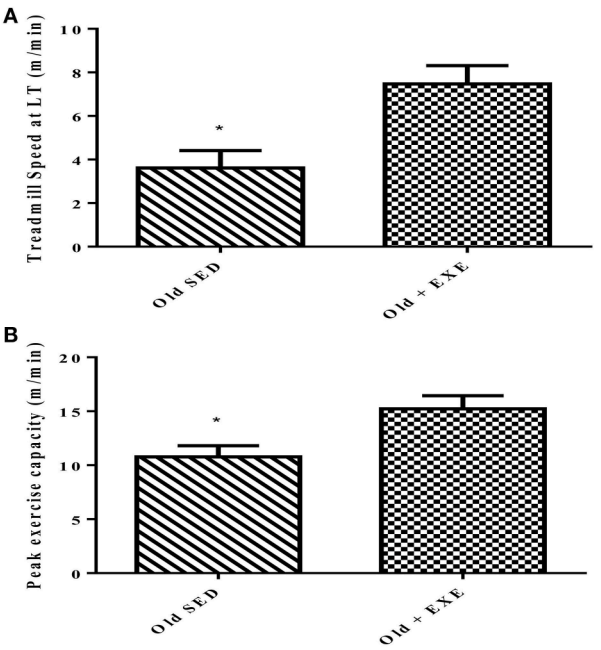

FIGURE 1 | Effects of exercise training on peak exercise capacity in 34 month old FBN rats. Values are the Means ± S.E.M of treadmill speed and peak exercise capacity of trained and sedentary rats. **(A)** We measured exercise capacity as a function of treadmill speed at lactate threshold (LT) **(B)** Peak exercise capacity was measured as the maximal speed at the end of the maximal exercise testing. Old SED ($n = 9$), Old + EXE ($n = 9$). *$P < 0.05$.

old rats compared to that of old sedentary rats. Of these 70 genes, only three were upregulated (*Prkaa1, Slc27a1, Slc27a4*), while 67 genes were downregulated with exercise training and involved genes from the Kreb's cycle, glucose transport (Glut4), fatty acid oxidation, and the mitochondrial electron transport chain.

Western Blot Analysis

In order to determine if the gene expression changes with age and exercise training were associated with altered protein content, we selected one protein from fatty acid oxidation (PPARα), AMPK signaling ($AMPK\alpha_2$), and mitochondrial biogenesis/function (PGC-1α) categories shown in **Figure 2**. Each of these genes had decreased mRNA expression and decreased protein content between Young and Old, although not significantly for PPARα (**Figure 3**). PGC-1α and $AMPK\alpha_2$ protein content was decreased in the Old + EXE compared to Young and not different than Old, but PPARα protein content was greater in the Old + EXE rat hearts compared to Old rat hearts.

Citrate Synthase Activity

To determine mitochondrial function and volume we assayed citrate synthase activity in left ventricular homogenates. We found that Old rat hearts had increased citrate synthase activity compared to Young hearts, and the Old + EXE hearts demonstrated no differences in citrate synthase activity compared to Young or Old hearts (**Figure 4**).

DISCUSSION

To our knowledge this is the first study to determine the effects of age on the expression of a comprehensive group of genes related to cardiac substrate metabolism and mitochondrial function. The other primary aim of this study was to determine whether exercise training in aged rats could alter the age-related gene expression phenotype. We hypothesized that genes associated with fatty acid oxidation, AMPK signaling, and mitochondrial biogenesis/function would be decreased with age and that exercise training would mitigate these changes in aged rat hearts. We found that aging results in the decreased expression of many genes involved with energy metabolism and mitochondrial function but found that exercise training did not improve the downregulation of these genes. In fact, exercise training in aged rats resulted in the downregulation of 67 genes associated with energy metabolism and mitochondrial function compared to aged sedentary rat hearts. Genes associated with glucose metabolism were unaffected by age. The declines in metabolic gene expression were profound, as we observed decreased expression in 42 genes involved with fatty acid metabolism and mitochondrial function in aged hearts compared to young hearts. In aged sedentary hearts altered protein content of PGC-1α and $AMPK\alpha_2$ corresponded to declines in gene expression compared to young hearts. We observed that cardiac muscle citrate synthase activity; a common measure of mitochondrial volume in skeletal muscle (Larsen et al., 2012) was increased in the aged heart compared to the young heart, while exercise training in aged hearts showed no differences between young and old sedentary rat hearts. Overall, these results suggest a significant change in the expression of genes associated with cardiac metabolic pathways with age that is not improved with exercise training.

We determined that a large number of genes involved with fatty acid metabolism and mitochondrial energy metabolism/biogenesis were downregulated with age, consistent with previous reports suggesting age-related reductions in fatty acid oxidation (Abu-Erreish et al., 1977; McMillin et al., 1993; Kates et al., 2003), AMPK activity (Gonzalez et al., 2004; Turdi et al., 2010; Zhao et al., 2014), and mitochondrial function (Fannin et al., 1999; Wanagat et al., 2002; Kumaran et al., 2005; Bhashyam et al., 2007; Preston et al., 2008; Jian et al., 2011). Specifically, we found genes associated with FAO (i.e., CPT-2, HADHA), AMPK signaling (i.e., $AMPK\alpha_2$, CaMKK2, LKB1), and mitochondrial biogenesis (PGC-1α, PGC-1β) with age-related decrements in expression in the myocardium. Cardiac PGC-1α expression has previously been shown to decline with age (Preston et al., 2008; Turdi et al., 2010), although others have observed no age-related change in cardiac PGC-1α gene expression (LeMoine et al., 2006). Our results demonstrated that the genes associated with fatty acid oxidation that were downregulated with age were primarily PPARα regulated transcripts. As in previous studies, we found that expression of FAO genes downstream of PPARα were decreased (Iemitsu et al., 2002; LeMoine et al., 2006). However, our finding of a decline in PPARα regulated genes associated with FAO in aged hearts occurred despite no change in expression of the PPARα gene itself between young and old hearts. Although, this decline in

TABLE 4 | Age-related gene expression changes in the left ventricle.

Gene name	Gene symbol	Fold Regulation	*p*-value
FA METABOLISM			
Acyl-CoA thioesterase 12	Acot12	2.88	0.026
Acyl-CoA thioesterase 7	Acot7	−1.86	0.017
Acyl-CoA thioesterase 8	Acot8	−1.44	0.02
Acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	−2.1	0.032
Acyl-CoA synthetase bubblegum family member 1	Acsbg1	2.09	0.034
Acyl-CoA synthetase long-chain family member 6	Acsl6	−1.98	0.029
3-hydroxybutyrate dehydrogenase, type 1	Bdh1	−2.55	0.036
Carnitine palmitoyltransferase 1c	Cpt1c	−1.49	0.027
Carnitine palmitoyltransferase 2	Cpt2	−2.02	0.001
Carnitine acetyltransferase	Crat	−2.69	0.011
2,4-dienoyl CoA reductase 1, mitochondrial	Decr1	−2.05	0.03
Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	Echs1	−2.39	0.004
Enoyl-Coenzyme A delta isomerase 2	Eci2	−1.79	0.049
Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	Ehhadh	−3.29	0.034
Fatty acid binding protein 3, muscle and heart	Fabp3	−2.84	0.017
Glutaryl-Coenzyme A dehydrogenase	Gcdh	−1.97	0.013
Glycerol-3-phosphate dehydrogenase 2, mitochondrial	Gpd2	−2.64	0.028
Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	Hadha	−2.23	0.035
3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase	Hmgcl	−1.51	0.03
Lipase, hormone sensitive	Lipe	−2.14	0.03
Lipoprotein lipase	Lpl	−2.31	0.025
Peroxisomal trans-2-enoyl-CoA reductase	Pecr	−1.67	0.013
Pyrophosphatase (inorganic) 1	Ppa1	−1.78	0.03
Protein kinase, AMP-activated, alpha 2 catalytic subunit	Prkaa2	−2.09	0.034
Protein kinase, AMP-activated, beta 1 non-catalytic subunit	Prkab1	−3.96	0.041
Protein kinase, AMP-activated, beta 2 non-catalytic subunit	Prkab2	−1.7	0.041
Protein kinase, cAMP-dependent, catalytic, alpha	Prkaca	−2.03	0.005
Protein kinase, cAMP dependent, catalytic, beta	Prkacb	−1.65	0.031
Protein kinase, AMP-activated, gamma 1 non-catalytic subunit	Prkag1	−1.77	0.002
Solute carrier family 27 (fatty acid transporter), member 1	Slc27a1	−2.02	0.03
MITOCHONDRIAL FUNCTION			
Peroxisome proliferator—activated receptor gamma, coactivator 1 alpha	Ppargc1	−2.14	0.03
NADH dehydrogenase (ubiquinone) Fe-S protein 7	Ndufs7	−2.24	0.015
Ubiquinol - cytochrome c reductase, complex III subunit VII	Uqcrq	−1.48	0.05
COX17 cytochrome c oxidase assembly homolog (*S. cerevisiae*)	Cox17	−1.68	0.011
Cytochrome c oxidase subunit IV isoform 1	Cox4i1	−1.72	0.02
Cytochrome c oxidase subunit VIIa polypeptide 2 like	Cox7a2l	−1.35	0.049
Mitofusin 1	mfn1	−2.41	0.01
Mitofusin 2	mfn2	−3.21	0.028
Clpb caseinolytic peptidase B homolog (*E. coli*) (i.e. HSP78)	clpb	−2.3	0.039
Calcium/calmodulin-dependent protein kinase kinase 2, beta	Camkk2	−1.77	0.017
Serine/threonine kinase 11 (i.e. LKB1)	Stk11	−1.86	0.029
Peroxisome proliferator - activated receptor gamma, coactivator 1 beta	Ppargc1b	−2.69	0.012
Tumor protein p53	Tp53	−2.19	0.025

genes associated with FAO is likely explained by reduced PPARα protein content in old hearts compared to young. This finding suggests that in the aged heart, changes in PPARα is likely to occur downstream of gene expression. Lastly, we demonstrated that the gene expression of AMPKα_2 decreased with age, which is the major catalytic subunit in the heart and responsible for the phosphorylation of downstream proteins that confer AMPK's effect on energy metabolism in the heart (Dolinsky and Dyck, 2006). Also, we found decreased gene expression in the aging heart of two protein kinases (CaMKK2 & LKB1)

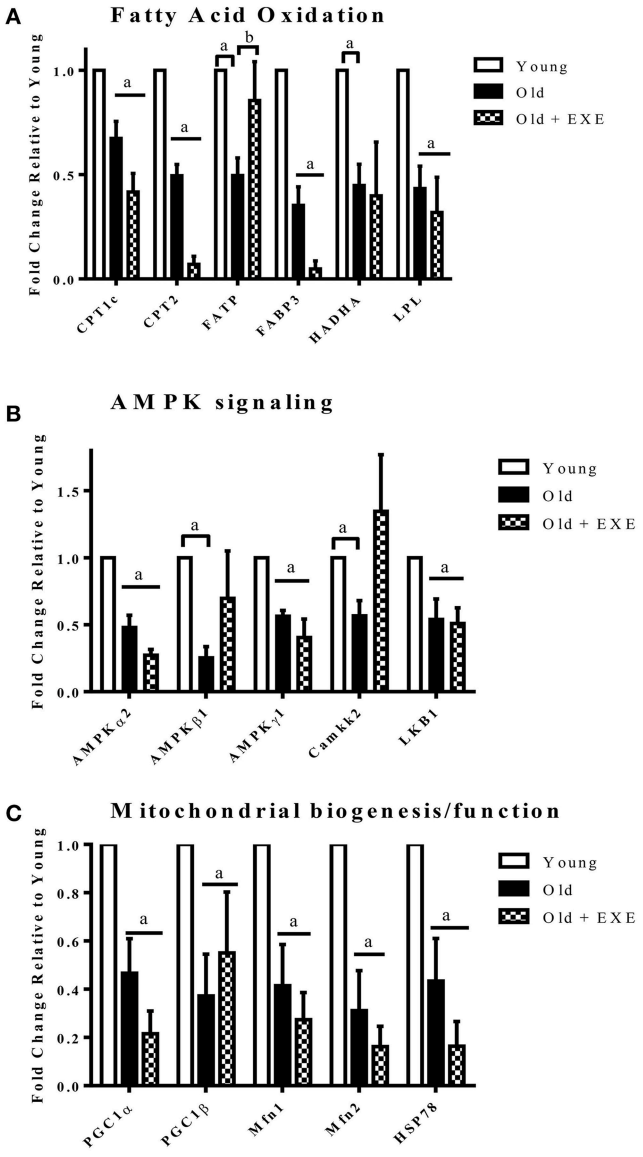

FIGURE 2 | Fold changes in gene expression Old and Old + EXE hearts relative to young hearts (Values are the Means ± S.E.M, $n = 5$ per group). (A) Genes involved in fatty acid transport and oxidation decline with age and this attenuation is not mitigated with exercise. (CPT1c, Carnitine-palmitoyl transferase 1c; CPT2, Carnitine palmitoyl transferase 2; FATP, Fatty acid transporter; FABP3, Fatty acid binding protein 3; HADHA, Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-CoA hydratase (trifunctional protein); LPL, Lipoprotein lipase). **(B)** AMPK signaling decrements with age involve changes in the gene expression of AMPK subunits and upstream kinases. (AMPKα2, Protein kinase; AMP, activated alpha 2 catalytic subunit; AMPKβ1, Protein kinase; AMP, activated beta 1 non-catalytic subunit; AMPKγ1, Protein kinase; AMP, activated gamma 1 non-catalytic sununit; Camkk2, Calcium/calmodulin-dependent protein kinase kinase 2 beta; LKB1, Liver kinase B 1). **(C)** Mitochondrial biogenesis and maintenance of mitochondrial function decline with age and exercise does not appear to improve the attenuation. (PGC1α, Peroxisome proliferator—activated receptor gamma, coactivator 1 alpha; PGC1β, Peroxisome proliferator—activator receptor gamma, coactivator 1 beta; Mfn1, Mitofusin 1; Mfn2, Mitofusin 2; HSP78, Heat shock protein 78). $^{a}P < 0.05$ vs. Young, $^{b}P < 0.05$ vs. Old + EXE.

that have been found to phosphorylate AMPK. These findings suggest that declining transcripts of AMPK and two upstream activators (CaMKK2 & LKB1), may play a role in the reduced AMPK activity that has been previously observed in aged hearts (Gonzalez et al., 2004; Turdi et al., 2010).

There is little information with regard to gene expression and protein content changes in exercise-trained aged rat hearts with regard to fatty acid oxidation, AMPK signaling, or mitochondrial function, but functional studies led us to hypothesize that exercise training would improve the age-related declines in metabolic gene expression. Surprisingly, we found that exercise training did not attenuate the age-related downregulation in the expression of genes involving fatty acid oxidation, AMPK signaling, and mitochondrial function. Specifically, in old exercise-trained hearts compared to old hearts we found a significant decline in the expression of the PPARα gene. PPARα regulated genes that were downregulated by exercise were Acyl CoA dehydrogenases (Acad), CD36, CPT1b, and CPT2. There were no differences in AMPK signaling genes (**Figure 2B**) between Old + EXE and Old rats; however, there was an upregulation of $AMPK\alpha_1$ in the exercise trained rats. $AMPK\alpha_1$ is ubiquitously expressed in cells and has lower levels of expression in the myocardium compared to $AMPK\alpha_2$ (Dolinsky and Dyck, 2006). We also found that compared to old rat hearts, exercise trained rat hearts demonstrated further downregulation of many genes involved with glucose transport (Glut4), Kreb's cycle and mitochondrial function (complex I and III in the electron transport chain). This downregulation of genes we observed in exercise trained aged hearts will require further work in order to better understand how exercise training induced downregulation of these genes affects cardiac function.

In order to determine if changes in gene expression with age were associated with altered protein content we measured the protein content of PGC-1α, PPARα, and $AMPK\alpha_2$. We found that the protein levels of PGC-1α and $AMPK\alpha_2$ were decreased in Old hearts compared to Young hearts and PPARα protein content trended toward a significant decline which was similar to the mRNA expression for these genes. In a previous report, mitochondrial oxygen consumption and expression of genes associated with mitogenesis and mitochondrial energy metabolism were both decreased, but mitochondrial number was increased and the mitochondrial marker, citrate synthase was not different in the senescent hearts compared to young (Preston et al., 2008). We observed a similar trend, of an increase in citrate synthase activity in Old hearts compared to Young hearts despite a decrease in gene expression of a large number of genes associated with mitochondrial biogenesis.

Western blot analysis determined that in the old-exercise trained rats PGC-1α and $AMPK\alpha_2$ protein content decreased compared to young hearts, similar to our findings of decreased gene expression of these two genes. However, we found that PGC-1α mRNA expression was reduced in Old + EXE hearts compared to Old hearts, yet the protein content of PGC-1α was similar. Also, we found that the PPARα mRNA expression was significantly reduced in Old + EXE hearts compared to Young and Old hearts, yet PPARα protein content was significantly greater in Old + EXE hearts compared to Old hearts and similar

TABLE 5 | Exercise-related changes in gene expression in Old FBN rats.

Gene name	Gene symbol	Fold Regulation	*p*-value
GLUCOSE METABOLISM			
Amylo-1,6-glucosidase, 4-alpha-glucanotransferase	Agl	−6.15	0.024
Aldolase A, fructose-bisphosphate	Aldoa	−5.28	0.036
Enolase 1, (alpha)	Eno1	−4.1	0.042
Enolase 2, gamma, neuronal	Eno2	−3.27	0.019
Glucose phosphate isomerase	Gpi	−5.59	0.005
Glycogen synthase kinase 3 alpha	Gsk3a	−3.17	0.046
Isocitrate dehydrogenase 3 (NAD+) beta	Idh3b	−7.7	0.04
Isocitrate dehydrogenase 3 (NAD), gamma	Idh3g	−5.41	0.027
Malate dehydrogenase 2, NAD (mitochondrial)	Mdh2	−6.31	0.009
Pyruvate dehydrogenase kinase, isozyme 1	Pdk1	−9.29	0.004
Pyruvate dehydrogenase kinase, isozyme 2	Pdk2	−6.48	0.006
Phosphoglucomutase 3	Pgm3	−1.7	0.037
Phosphorylase kinase, gamma 1	Phkg1	−2.78	0.011
Ribose 5-phosphate isomerase A	Rpia	−2.38	0.018
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	Sdha	−3.93	0.038
Succinate-CoA ligase, ADP-forming, beta subunit	Sucla2	−5.64	0.021
Solute carrier family 2 (facilitated glucose transporter), member 4	Slc2a4	−10.45	0.025
FA METABOLISM			
Acetyl-Coenzyme A acyltransferase 1A	Acaa1a	−9.79	0.001
Acetyl-Coenzyme A acyltransferase 2	Acaa2	−11.7	0.029
Acyl-Coenzyme A dehydrogenase family, member 10	Acad10	−7.02	0.002
Acyl-Coenzyme A dehydrogenase family, member 11	Acad11	−14.38	0.002
Acyl-Coenzyme A dehydrogenase family, member 9	Acad9	−8.81	0.008
Acyl-Coenzyme A dehydrogenase, long-chain	Acadl	−21.18	0.002
Acyl-Coenzyme A dehydrogenase, C-4 to C-12 straight chain	Acadm	−15.17	0.009
Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	Acads	−17.09	0.001
Acyl-Coenzyme A dehydrogenase, short/branched chain	Acadsb	−7.75	0.007
Acyl-Coenzyme A dehydrogenase, very long chain	Acadvl	−9.49	0.002
Acetyl-coenzyme A acetyltransferase 1	Acat1	−14.79	0.004
Acetyl-Coenzyme A acetyltransferase 3	Acat2	−6.25	0.003
Acyl-CoA thioesterase 2	Acot2	−18.92	< 0.001
Acyl-CoA thioesterase 3	Acot3	−3.52	0.024
Acyl-CoA thioesterase 7	Acot7	−4.43	0.027
Acyl-CoA thioesterase 8	Acot8	−2.68	0.002
Acyl-CoA thioesterase 9	Acot9	−5.41	0.001
Acyl-Coenzyme A oxidase 1, palmitoyl	Acox1	−5.16	0.007
Acyl-Coenzyme A oxidase 3, pristanoyl	Acox3	−4.47	0.015
Acyl-CoA synthetase long-chain family member 1	Acsl1	−18.06	< 0.001
Acyl-CoA synthetase long-chain family member 3	Acsl3	−3.6	0.008
Acyl-CoA synthetase long-chain family member 4	Acsl4	−2.46	0.011
Acyl-CoA synthetase long-chain family member 5	Acsl5	−2.23	0.001
Acyl-CoA synthetase long-chain family member 6	Acsl6	−5.54	0.01
3-hydroxybutyrate dehydrogenase, type 1	Bdh1	−10.48	0.04
3-hydroxybutyrate dehydrogenase, type 2	Bdh2	−5.03	0.008
Carnitine palmitoyltransferase 1a, liver	Cpt1a	−3.04	0.033
Carnitine palmitoyltransferase 1b, muscle	Cpt1b	−7.93	0.003
Carnitine palmitoyltransferase 2	Cpt2	−7.02	0.004
Carnitine acetyltransferase	Crat	−4.27	0.018
Fatty acid binding protein 3, muscle and heart	Fabp3	−7.31	0.034
Protein kinase, AMP-activated, alpha 1 catalytic subunit	Prkaa1	1.83	0.042

(Continued)

TABLE 5 | Continued

Gene name	Gene symbol	Fold Regulation	*p*-value
Protein kinase, cAMP-dependent, catalytic, alpha	Prkaca	−2.89	0.041
Protein kinase, cAMP dependent, catalytic, beta	Prkacb	−1.43	0.047
Solute carrier family 27 (fatty acid transporter), member 1	Slc27a1	1.73	0.033
Solute carrier family 27 (fatty acid transporter), member 4	Slc27a4	3.15	0.002
CD36 molecule (thrombospondin receptor)	CD36	−2.97	0.001
Peroxisome proliferator activated receptor alpha	Ppara	−9.28	0.029
MITOCHONDRIAL FUNCTION			
Sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*)	Sirt1	−3.27	0.036
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 1	Ndufa1	−2.24	0.04
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 6 (B14)	Ndufa6	−4.7	0.041
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 7	Ndufa7	−5.67	0.033
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 8	Ndufa8	−10.41	0.005
NADH dehyrogenase (ubiquinone) 1 alpha subcomplex, 9	Ndufa9	−5.71	0.018
NADH dehyrogenase (ubiquinone) 1, alpha/beta subcomplex, 1	Ndufab1	−5.67	0.001
NADH dehyrogenase (ubiquinone) 1 beta subcomplex, 2	Ndufb2	−4.49	0.006
NADH dehyrogenase (ubiquinone) 1 beta subcomplex, 5	Ndufb5	−4.72	0.032
NADH dehyrogenase (ubiquinone) 1 beta subcomplex, 8	Ndufb8	−4.92	0.01
NADH dehyrogenase (ubiquinone) Fe-S protein 6	Ndufs6	−2.94	0.047
Cytochrome c-1	Cyc1	−5.47	0.033
Ubiquinol-cytochrome c reductase core protein 2	Uqcrc2	−5.38	0.016
Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	Uqcrfs1	−5.11	0.045

compared to Young hearts. These results are somewhat conflicted to a report demonstrating that exercise training attenuated the age-related decrement in PPARα mRNA expression and protein content in aged rat myocardium (Iemitsu et al., 2002). This study indicated that decreased mRNA expression did result in similar declines in protein content. However, functional changes do not always coincide with coordinate changes in gene expression or protein content (Burelle et al., 2004; Zhu et al., 2013; Lai et al., 2014). Changes in gene expression due to exercise training in aged hearts may or may not coincide with changes in that gene's protein product. Specifically, one study demonstrated that exercise training elicited adaptations that lead to an increase in exercise capacity along with augmented glucose and fatty acid oxidation in the myocardium despite no changes in the protein content of several proteins involved with glucose and fatty acid metabolism (Burelle et al., 2004). Our results demonstrated despite many genes involved with FAO in the exercise trained aged heart was reduced to aged sedentary hearts, PPARα protein content was greater in exercise trained aged hearts compared to aged sedentary hearts. Future studies looking at the protein content of major proteins involved with β-oxidation and fatty acid transport would enable us to see if exercise training in aged hearts tends to uncouple gene expression from protein content.

Citrate synthase is a common marker of mitochondrial volume used to indicate endurance exercise adaptations in skeletal muscle (Larsen et al., 2012). We examined citrate synthase activity in cardiac muscle and found, surprisingly, that aging resulted in an increase in citrate synthase activity and exercise training diminished this effect. Citrate synthase activity was not different in old-exercise trained hearts compared to young or old hearts. This is consistent with previous studies demonstrating that exercise training in rats did not elicit increases in cardiac citrate synthase activity (Oscai et al., 1971; Murakami et al., 1995; Zonderland et al., 1999; Siu et al., 2003; Rimbaud et al., 2009). We found that exercise training improved functional exercise capacity (**Figure 4**) despite either no changes in gene expression compared to Old hearts or in some cases, a further reduction in the expression of genes associated with energy metabolism and mitochondrial function in the heart. These data suggest that exercise training may impact myocardial energy metabolism and mitochondrial function downstream of gene expression. Also, exercise is known to induce adaptations in skeletal muscle (Hall et al., 1994; Bengtsson et al., 2001; Betik et al., 2008; Kang et al., 2013), which may have been responsible for the increased exercise capacity in our old exercise-trained rats.

LIMITATIONS

One limitation to this study is that we did not determine whether exercise training in young rats leads to a similar downregulation of the expression of these cardiac genes that we found in the exercise trained aged hearts. Cardiac gene expression changes due to exercise training in young rats have been well-studied. These studies showed that mitochondrial or metabolic gene expression in the young rat heart to either increase (Hall et al., 1994; Rimbaud et al., 2009; Dobrzyn et al., 2013; Wadley et al., 2016) or not change (Murakami et al., 1995; Iemitsu et al., 2003; Alessio et al., 2014) with exercise training compared to young sedentary rats. Specifically, young hearts respond to exercise training by increasing the expression of genes associated with

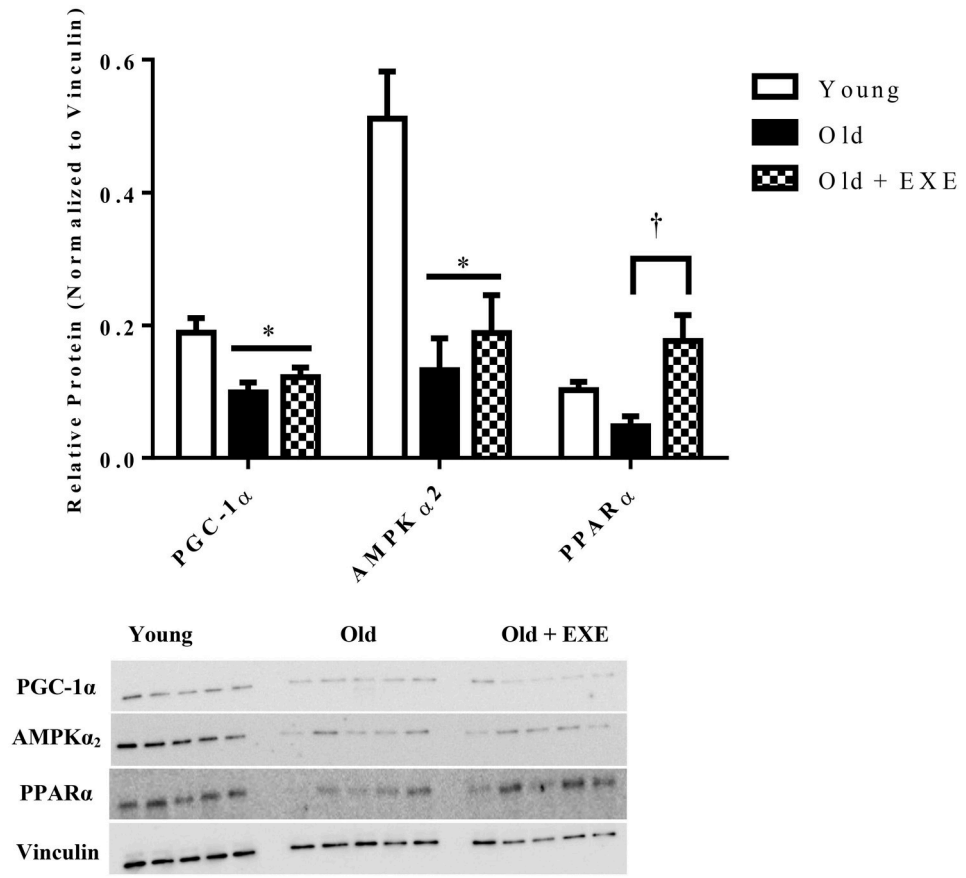

FIGURE 3 | Relative protein content in Young, Old, and Old + EXE groups ($n = 5$ per group). Values represent Means ± S.E.M. Old + EXE demonstrate increases in PGC-1α but decreased PPARα and AMPKα_2 protein content compared to Young and Old, respectively. *$P < 0.05$ vs. Young, $^{\dagger}P < 0.05$ vs. Old + EXE.

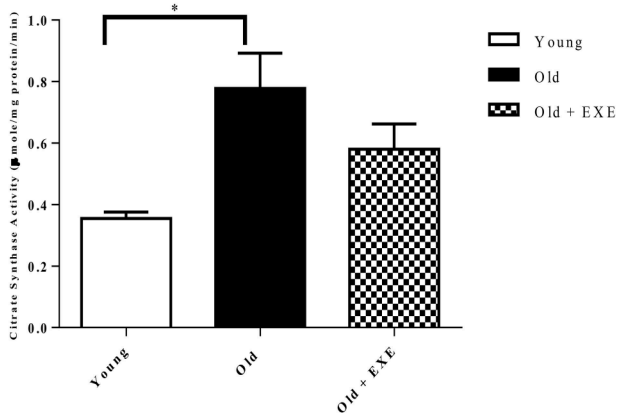

FIGURE 4 | Citrate synthase activity in left ventricular homogenates in young, old, and old exercise-trained hearts ($n = 5$ per group). Values indicate Means ± S.E.M. *$P < 0.05$.

glucose transport (Hall et al., 1994; Rimbaud et al., 2009), fatty acid oxidation (Rimbaud et al., 2009; Dobrzyn et al., 2013), and mitochondrial biogenesis (i.e., PGC-1α and Cox4il; Rinaldi et al., 2013; Wadley et al., 2016). The results of our study (further decreases in gene expression with exercise training compared to sedentary aging) compared to these previous studies suggest that gene expression changes due to exercise training may be different in the hearts of aged exercise-trained rats compared to young hearts. Future works looking at post-translational modifications and protein activity in genes associated with fatty acid oxidation and mitochondrial function may elucidate molecular mechanisms involved in potential differential exercise training responses between young and old rat hearts.

Another limitation to this study is that our primary endpoint measure was the expression of genes associated with metabolic signaling pathways, substrate energy metabolism and mitochondrial function. Along with our data, previous reports have indicated that changes in tissue mRNA levels are not necessarily predictive of changes in protein levels (Hall et al., 1994; Zhu et al., 2013; Lai et al., 2014). Interestingly, other studies have reported that roughly two-thirds of the variance in protein content may be explained by mRNA concentration (Lu et al., 2007; Shankavaram et al., 2007). However, due to an increasing appreciation of miRNA interactions, post-translational modifications effecting protein activity, and the unpredictable protein response to stimuli (i.e., exercise training),

mRNA is not always a good predictor of protein abundance (Burelle et al., 2004; Lu et al., 2007; Shankavaram et al., 2007). Future studies looking at expression changes in cardiac metabolic and mitochondrial genes, may need to include miRNA expression changes and post-translational protein modifications which will provide a more thorough understanding of metabolic and mitochondrial function in the aged and exercise trained rat heart.

CONCLUSION

This study was the first to utilize a comprehensive approach in the study of age and exercise effects in aged hearts on substrate metabolism and mitochondrial function by an integration of gene expression, protein content, and protein activity. We found that cardiac aging results in the downregulation of a large number of genes associated with fatty acid metabolism, AMPK signaling and mitochondrial function, and exercise in aged-rats did not attenuate these changes and resulted in a further downregulation of expression in many metabolic and mitochondrial genes compared to aged sedentary rat hearts. We also found that gene expression changes may or may not coincide with protein expression. Taken together, our results demonstrated extensive age-related molecular changes in fatty acid metabolism, AMPK signaling and mitochondrial function. These molecular changes are far-reaching and cannot be described by changes in a single gene or a given gene's protein product.

AUTHOR CONTRIBUTIONS

GB, GD, and JA designed the study. GB, SM, JS, and GD performed the experiments. GB, GD, JS, and SM analyzed the data. GB and GD drafted the manuscript and guarantors of the paper.

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REVIEW
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Ongoing controversies surrounding cardiac remodeling: is it black and white—or rather fifty shades of gray?

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Cardiac remodeling describes the heart's multimodal response to a myriad of external or intrinsic stimuli and stressors most of which are probably only incompletely elucidated to date. Over many years the signaling molecules involved in these remodeling processes have been dichotomized according to a classic antagonistic view of black and white, i.e., attributed either a solely maladaptive or entirely beneficial character. By dissecting controversies, recent developments and shifts in perspective surrounding the three major cardiac signaling molecules calcineurin (Cn), protein kinase A (PKA) and calcium/calmodulin-dependent kinase II (CaMKII), this review challenges this dualistic view and advocates the nature and dignity of each of these key mediators of cardiac remodeling as a multilayered, highly context-sensitive and sophisticated continuum that can be markedly swayed and influenced by a multitude of environmental factors and crosstalk mechanisms. Furthermore this review delineates the importance and essential contributions of degradation and proteolysis to cardiac plasticity and homeostasis and finally aims to integrate the various aspects of protein synthesis and turnover into a comprehensive picture.

Keywords: cardiac remodeling, plasticity, calcineurin, PKA, CaMKII, signaling, protein turnover

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Introduction

Heart failure is the leading cause of death in industrialized countries (Nichols et al., 2014). It is generally preceded by remodeling processes to adapt to alterations in wall tension in the myocardium or stress by external (hormonal) stimuli. In this regard, cardiac remodeling describes the heart's fascinating capability to respond and adapt to various stimuli. The mechanisms of cardiac plasticity, i.e., the potential of the heart to shrink or grow, are tremendous as the dynamic growth range of the myocardium exceeds 100% (Hill and Olson, 2008). Successful adaption to and adequate reduction of increased wall tension are ultimate goals of cardiac remodeling and facilitating preservation or even augmentation of cardiac pump

Abbreviations: AKT, Protein kinase B (PKB); ANF, atrial natriuretic factor; βAR, beta-adrenergic receptor; BNP, brain natriuretic peptide; CaMKII, Calcium/Calmodulin-dependent Kinase II; CnA, Calcineurin A; CnB, Calcineurin B; Cn, Calcineurin; cAMP, Cyclic adenosine monophosphate; DCM, Dilated Cardiomyopathy; DRM, Desmin-Related Cardiomyopathy; ERK, extracellular-signal-regulated kinases; Fbxl22, F-box and leucine-rich repeat protein 22; HCM, Hypertrophic Cardiomyopathy; HDAC, Histone Deacytelase; MEF-2, myocyte enhancer factor-2; MFM, Myofribrillar Myopathy; MuRF, Muscle Ring Finger Protein; NFAT, nuclear factor of activated T cells; PKA, Protein Kinase A; PKC, Protein Kinase C; RCAN, regulator of calcineurin; ROS, Reactive Oxygen Species; UPS, Ubiquitin Proteasome System.

function (Frey and Olson, 2003; Hill and Olson, 2008). The multifaceted mechanisms of cardiac remodeling and plasticity have traditionally been divided into beneficial (or "physiological") or maladaptive (or "pathological") hypertrophic remodeling of the heart on the one hand and cardiac atrophy on the other hand.

While cardiac growth during maturation ("postnatal hypertrophy"), maternal cardiac growth during pregnancy and exercise-induced cardiac hypertrophy are all considered physiological entities of hypertrophy, pathological hypertrophic responses are observed upon sustained neurohumoral activation and abnormal mechanical stretch of the myocardium as common findings of various cardiac disease entities. These include but are not limited to ischemic events (myocardial infarction), pressure overload conditions such as arterial hypertension and aortic valve stenosis, genetic disorders due to alterations in key sarcomeric or metabolic proteins as well as infectious and toxic triggers (Hill and Olson, 2008; Kehat and Molkentin, 2010; van Berlo et al., 2013; Tham et al., 2015). Atrophic remodeling is generally observed in patients with protracted bed rest and ventricular unloading as well as malignant disease (Hill and Olson, 2008; Cosper and Leinwand, 2011; Springer et al., 2014).

Physiological hypertrophy of the heart—as it is observed in postnatal growth as well as in response to pregnancy and exercise—is characterized by a fine-tuned and orchestrated process of beneficial adaptations. These modulations result in decreased cardiac wall stress, augmentation of pumping performance and improvements of vascularization, while maladaptive effects of the increased workload that lead to hypertrophic remodeling are countered and kept at bay. Various signaling molecules and pathways have been shown to take part in these adaptive processes and several excellent reviews have summarized our present knowledge on this physiological aspect of cardiac remodeling (Hill and Olson, 2008; Maillet et al., 2013). Generally considered as beneficial, these pathways are triggered by endocrine mediators or mechanosensors governing a number of adaptive intracellular signaling cascades. Ligands that result in physiological downstream signaling include vascular endothelial growth factor B (VEGF-B), insulin, growth hormone (GH) and insulin-like growth factor (IGF1) as well as the thyroid hormone triiodothyronine (T3) (Yoshida et al., 2010; Maillet et al., 2013; Tham et al., 2015). Via their respective receptors, these stimuli converge on downstream pathways controlling the induction of adaptive gene programs and protein synthesis, and direct cellular metabolism and energy production. Well-characterized cascades and pathways regulating these homeostatic mechanisms comprise phosphoinositide 3 kinase (PI3K)/AKT, mTOR complex 1 (mTORC1), ERK1/2 and AMP-activated protein kinase (AMPK) (Baker et al., 1993; Liu et al., 1993; McMullen et al., 2003; Seth et al., 2009; Bostrom et al., 2010; Zhang et al., 2010). Translating stretch-stimuli to downstream signaling, the mechanosensing apparatus is controlled by transient receptor potential (TRP) channels, integrins and various z-disc associated proteins such as muscle LIM protein (MLP), actinin-associated LIM protein (ALP) and Nebulette (NEB) as well as the sarcomere-spanning protein titin (Linke, 2008; Seth et al., 2009; Frank and Frey, 2011; Luedde et al., 2011; Hamdani et al., 2013; Maillet et al., 2013). While the notion of (these) mediators as the "good guys" in cardiac remodeling seems very appealing and is founded on robust scientific work(s), a note of caution seems advisable not to fall for a false dichotomy bearing in mind what Paracelsus postulated about 500 years ago: "Dosis sola venenum facit" (Shiojima et al., 2005; Hill and Olson, 2008; Tham et al., 2015).

Pathological hypertrophic remodeling on the other hand—while initially compensatory by reduction of ventricular wall stress and temporal preservation of cardiac pump function—eventually evolves into a devastating spiral of maladaptive alterations culminating in heart failure and death (Hill and Olson, 2008; Kehat and Molkentin, 2010; Burchfield et al., 2013; Molkentin, 2013; Tham et al., 2015). In this regard, pathological hypertrophic remodeling is characterized by multiple facets of ultimately maladaptive mechanisms such as altered calcium handling, changes in metabolic patterns and gene expression, affection of individual cell fate and survival as well as modifications of the extracellular environment by fibrosis. Cardiac stress by increased neurohumoral activation (mainly endothelin 1, angiotensin II and catecholamines) and abnormal mechanical stretch converge on a number of sensors (mostly G-proteins and strain-sensitive cellular elements) which translate these stimuli into respective stress response pathways (Hill and Olson, 2008; Kehat and Molkentin, 2010; Burchfield et al., 2013; Molkentin, 2013; Tham et al., 2015). Key second messengers that were suggested in this regard include calcium, phospholipase C, guanylyl, and adenylyl cyclases. Downstream kinases, phosphatases and other enzymes with critical roles in pathologic remodeling comprise calcineurin, protein kinase A (PKA) and C (PKC), cGMP-dependent protein kinases (PKG) calcium-calmodulin-dependent kinase II (CaMKII) and mitogen-activated protein kinases (MAPKs) with their respective downstream components—to name but a few (Benovic et al., 1986; Molkentin et al., 1998; Allen and Leinwand, 2002; Backs et al., 2006, 2009; Casey et al., 2010; Kehat and Molkentin, 2010; Burchfield et al., 2013; van Berlo et al., 2013; Kreusser et al., 2014; Tham et al., 2015).

Several outstanding reviews have been written in recent years dissecting details of these mediators and pathways integral to maladaptive/pathological remodeling processes (Kehat and Molkentin, 2010; Burchfield et al., 2013; van Berlo et al., 2013; Bisping et al., 2014; Tham et al., 2015). Therefore this review will focus on a very limited number of crucial effectors highlighting new aspects and shifts in perspective that might challenge long-standing dogmas.

Notably, RNA-based signaling pathways and mediators as well as epigenetic and other posttranslational modifiers are currently evolving as further crucial contributors to cardiac remodeling. However, elaborations on these developments would transcend the scope of this review. Furthermore they have extensively been covered in recent publications and reviews that focused on each of the above issues individually (Papait et al., 2013; Lehmann et al., 2014; Liu et al., 2015; Gillette and Hill, 2015; Philippen et al., 2015; Piccoli et al., 2015; Thum and Condorelli, 2015; Uchida and Dimmeler, 2015).

Allegory: Cardiac Remodeling as Tuning of a Machine

Allegorically, cardiac plasticity could be referred to as the maintenance and adjustment of a machine subjected to heavy and undulating workload. The machine (*heart*) can be optimized (*remodeled*) by technicians (*key mediators*) to perform according to the present demand by employing different fuel (*metabolic*), spare parts and logistics (*structural and functional alterations*). These remodeling efforts can either be construed as long-lasting, sustainable and deliberate optimizations (as it is generally observed in physiological hypertrophy), or endeavors can be targeted at drastic, short-term improvements which will not be sustainable for a longer period of time but provide immediate and powerful responses to increased demands (as observed in many aspects of pathological hypertrophy). Furthermore, it appears crucial whether the demand will have to be fulfilled infinitely (analogous to many pathological stress models) or whether periods exist when the machine can (temporarily) cool down and reduce its output (as in exercise-related hypertrophy).

Atrophy on the other hand could be seen as dismantling of a machine and its appendices in response to reduced demand. This dismantling process can be achieved by a multitude of different ways depending on focus and technique of involved technicians and parties.

Finalizing this allegorical approach, the technicians (*key mediators and signaling molecules*) driving these changes of the machine may be similar or even the same in various aspects of remodeling. However, their intentions, perspectives and approaches may be different depending on a vast number of environmental, extrinsic and intrinsic factors.

Translating this simile to cardiac pathophysiology, it is our opinion that key protagonists in cardiac plasticity may not as easily be characterized as "good" or "bad," beneficial or maladaptive. To our minds, these essential mediators may exert different functions and adaptations depending on timing, duration and dosage of respective stimuli as well as crosstalk with other crucial protagonists that are simultaneously activated leading to numerous distinct posttranslational modifications.

For instance, the phosphatase calcineurin and its role in cardiac remodeling have been studied for almost two decades now (Molkentin et al., 1998; Molkentin, 2013). While it was initially described and for a long time considered only as key mediator of pathological hypertrophy and remodeling, evidence has been accumulating in recent years that calcineurin might also play an important role in physiological cardiac growth (Heineke et al., 2010a; Felkin et al., 2011; Chung et al., 2013; Hudson and Price, 2013; Molkentin, 2013; Kreusser et al., 2014; Mattiazzi and Kranias, 2014). The resulting scientific discussion whether calcineurin is the "bad guy" in cardiac growth nicely illustrates how complex cardiac remodeling actually is: it raises the question, whether the sophisticated means of cardiac hypertrophy and remodeling can simply be broken down into bad or good, black or white, or whether we should not consider the multifaceted and finely tuned cardiac response mechanisms as a continuum (of shades of gray) (**Figure 1**).

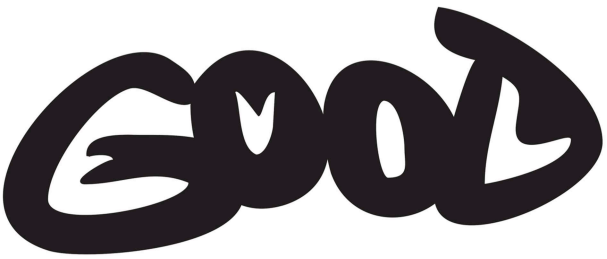

FIGURE 1 | Illustration of *good* and *evil* as an optical illusion/ambigram. This illustration of the words *good* and *evil* in the context of an optical illusion is implemented to emphasize two things: 1. Expectations drive our perception and may therefore create a bias, i.e., if we are looking for something positive, we will probably recognize the word *good* first. If negative expectations prevail, the word *evil* will most likely be seen first. In this regard, we would like to emphasize that research efforts are prone to hold to the same pattern and this bias has to be mindfully dealt with and kept in mind. 2. The idiomatic phrase of "two sides to every coin" is reflected in the concomitant depiction of *good* and *evil* serving as a simile that key mediators of cardiac remodeling processes cannot be dichotomized in solely good or bad protagonists as the nature and dignity of their signaling will range a context-sensitive continuum with positive *(good)* and negative *(evil)* results. *Good-Evil Ambigram designed by and courtesy of Punya Mishra (punyamishra.com)*.

To our minds, future research should therefore concentrate on elucidating the myriad of crosstalk mechanisms between the currently identified main pathways and key protagonists. This will enable us to meticulously modulate the major signaling pathways and molecules by augmenting those sidetracks that are desirable in response to a given stressor, while repressing those that perturb or prohibit adequate cardiac homeostasis.

While there is broad consensus on the fact that hypertrophy and atrophy can be either beneficial or maladaptive, the exact triggers, mediators and pathways leading to these distinct entities are under constant debate. In this regard, several mediators that had been classified as maladaptive have been rehabilitated over the years, whereas seemingly benign players have been attributed potential "dark sides" (Heineke et al., 2010a; Peng et al., 2010; Felkin et al., 2011; Chung et al., 2013; Zhang et al., 2013; Kreusser et al., 2014; Li et al., 2014; Mattiazzi and Kranias, 2014; Tham et al., 2015).

As cardiac remodeling mechanisms involve a myriad of different protagonists and interactions, this review will focus on ongoing controversies and current developments surrounding adaptive vs. maladaptive signaling in cardiac remodeling (Calcineurin, PKA, CaMKII) as well as shifts in perspective in terms of relative importance of select mechanisms in cardiac remodeling (namely proteasomal/degradation mechanisms).

Calcineurin-Signaling

Calcineurin (Cn), one of the heart's most intensively studied enzymes, is a calcium-dependent serine/threonine phosphatase controlling key functions and processes in response to a wide range of stress stimuli. Calcineurin is composed as a heterodimer of catalytic (CnA) and smaller regulatory subunits (CnB) with CnA-alpha and CnA-beta as well as CnB1 constituting the relevant myocardial isoforms (Taigen et al., 2000; Bueno et al.,

2002). Generally activated by calcium (or to a lesser extent calpain-cleavage), calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) as its major downstream target (Molkentin et al., 1998; Hogan et al., 2003; Wilkins et al., 2004). Upon dephosphorylation, NFAT in turn translocates to the nucleus where it orchestrates the re-activation of fetal genes such as atrial natriuretic factor (ANF), brain natriuretic peptide (BNP) and skeletal actin (SA) as part of the prototypical cardiac stress response (Molkentin et al., 1998; Hogan et al., 2003; Frey et al., 2004; Wilkins et al., 2004; Frank et al., 2007). While it is important to note that this activation of the fetal gene program principally constitutes adaptive efforts to cope with the increased cardiac stress, it is still generally perceived as an epiphenomenon and utilized as a marker of unresolved and (therefore in the long run) pathological stress.

Almost two decades ago, Molkentin and Olson—in a very elegant study—first described calcineurin as a key mediator of pathologic cardiac hypertrophy and consecutive progression to heart failure (Molkentin et al., 1998). In the following, numerous studies elaborated on the role of calcineurin in cardiac hypertrophy and remodeling as well as heart failure. These well conducted studies provided overwhelming evidence that calcineurin is indeed at the center of cardiac hypertrophy and remodeling (Zou et al., 2001; Hill et al., 2002; Wilkins et al., 2004; Molkentin, 2013; van Berlo et al., 2013). In this regard, loss of function models by either targeting calcineurin directly (Zou et al., 2001; Bueno et al., 2002) or one of its modulators such as regulator of calcineurin (RCAN), AKAP-79 or Cabin-1 (De Windt et al., 2001; Rothermel et al., 2001; Hill et al., 2002) established beyond doubt that downregulation of calcineurin prevented cardiac hypertrophy.

Moreover, data from most experiments on pharmacological inhibition of calcineurin fortified this notion (Sussman et al., 1998; Burchfield et al., 2013; Molkentin, 2013). Consequently, overexpression studies with either activated calcineurin (Molkentin et al., 1998) or knockdown of its negative modulators (Frey et al., 2004) provided the complimentary picture of increased susceptibility to heart failure in the wake of exaggerated (pathological) hypertrophy (Molkentin, 2013).

Therefore, the vast majority of experiments led to the general opinion that calcineurin is the "bad guy" in cardiac remodeling, following the classical black-and-white dualism of attributing either solely "pathological" or "physiological" qualities and functions to a given cellular component or signaling pathway. In recent years, this (false) dichotomy has evolved into a more sophisticated view of calcineurin in particular and cardiac remodeling in general (Heineke et al., 2010a; Felkin et al., 2011; Heineke and Ritter, 2012; Chung et al., 2013; Molkentin, 2013; Seto et al., 2013; Kreusser et al., 2014). In this regard, the entirely black (-and-white) picture has evolved as several shades of gray have been elucidated in calcineurin's role.

Although robust scientific evidence demonstrates the maladaptive nature of many pathways on which calcineurin has a major impact, a number of studies on protective aspects of calcineurin-mediated signaling have accumulated over the past decade. Furthermore, one pitfall from studies on regulators of calcineurin, that deserves mentioning, concerns the notion that these regulators of calcineurin may not only or primarily target calcineurin, but may very well also affect other important signaling mediators.

While first evidence of protective aspects of calcineurin signaling was already observed in terms of anti-apoptotic properties by De Windt et al. (2000), a number of studies in the years to follow fortified the notion that calcineurin may indeed provide beneficial signaling under special circumstances and should not be reduced to the maladaptive and destructive culprit (Bueno et al., 2004; Frey et al., 2008; Heineke et al., 2010a; Chung et al., 2013; Kreusser et al., 2014). These studies rather served as a warning against demonizing calcineurin in the wake of compelling data on maladaptive aspects of excessive calcineurin activity.

Consistent with this notion of a context-sensitive dignity of calcineurin signaling, a knock-out study of calsarcin-2, a negative modifier of calcineurin activity in skeletal muscle, even demonstrated enhanced exercise performance in absence of a myopathic phenotype (Frey et al., 2008), while deletion of calsarcin-1, the cardiac-enriched isoform repressing calcineurin activity, resulted in accelerated cardiomyopathy and heart failure due to exaggerated calcineurin signaling (Frey et al., 2000, 2004, 2008). These exciting observations hinted at multiple layers of calcineurin signaling with physiological facets.

Intriguingly, Leinwand and colleagues published data on the importance of calcineurin in regard to cardiac hypertrophy during pregnancy, another classical prototype of physiological cardiac remodeling (Chung et al., 2013). In particular, they show calcineurin to be upregulated in pregnant mice as part of physiological cardiac remodeling during early pregnancy. Their observation of biphasic regulation of calcineurin during pregnancy supports the idea that timing and intensity of a stimulus and its responsive signaling cascades might be more important than the individual effectors themselves in terms of long-term consequences of the respective stimuli (Chung et al., 2013). Furthermore, their observations strengthen the notion that no single mediator, but crosstalk between signaling cascades (specifically calcineurin, ERK and Akt as well as hormonal balances) constitutes the paramount principle governing cardiac response mechanisms to the physiologic remodeling stimuli induced by pregnancy. Therefore, context and duration of calcineurin activation may be the key determinants whether calcineurin exerts its predominantly negative effects on cardiac remodeling pathways or whether it contributes to adaptive and beneficial signaling cascades in response to stressors (Chung et al., 2013).

Insight on regulation of the nature of calcineurin signaling has also been provided by Kreußer et al., illustrating calcineurin as a key contributor to various entities of hypertrophy, but CaMKII as a major mediator in conducting maladaptive stimuli (Kreusser et al., 2014). Indeed, calcineurin appears to be (co-)regulated by CaMKII. While co-activation during pathological stress results in significant impairment of cardiac function in the wake of cardiac remodeling, deletion of the two cardiac CaMKII genes δ and γ in this setting is sufficient to generate a cardiac phenotype that resembles a more physiological nature (calcineurin-dependent cardiac hypertrophy without significant systolic dysfunction).

This observation provides evidence for the conclusion that calcineurin—while contributing to hypertrophic remodeling—may confer beneficial changes in a context-dependent response to external stressors (Kreusser et al., 2014).

Observations from studies by Lara-Pezzi's group add another layer of complexity, as they show induction of a splicing variant of calcineurin (CnAβ1) to be protective after myocardial infarction (Felkin et al., 2011). These findings hint at intricate and sophisticated switching mechanisms in response to cellular stressors emphasizing that no single pathway, but the interactions of multiple cascades may determine remodeling to be adaptive or maladaptive.

In an interesting review, Heineke and Ritter elaborate on the concept of compartment/subdomain-specific modification of calcineurin activity. This would allow for context-dependent physiological or pathological means of remodeling (Heineke and Ritter, 2012). Work on the calcium and integrin-binding-protein-1 (CIB1) exemplarily illustrate how differential binding and translocation of important cellular signaling mediators (in this case calcineurin) affect the nature of downstream effects and course of remodeling by compartmentalization (Heineke et al., 2010). It is still unclear to what degree this concept of "fishing for calcium" by different mediators of stress signals can unravel the mysteries of the diverse characters of cardiac remodeling.

Looking further downstream at possible modifiers of calcineurin signaling that may sway the dignity of a stimulus, interactions with other transcription factors (such as MEF2, AP-1, GATA4) as effectors of multiple upstream cascades modify NFAT-dependent DNA-interaction and integrate various pathways into meticulously tuned stress responses (Hogan et al., 2003; Heineke et al., 2010; Heineke and Ritter, 2012).

Taken together, it is well established that calcineurin is at the center of cardiac hypertrophy. However, evidence is accumulating that calcineurin's role may very well be part of a continuum of (fifty or more) shades of gray in cardiac remodeling rather than confined to the black-and-white dualism that dominated the last decades of research in the field of cardiac remodeling.

Protein Kinase A—Signaling in Cardiac Remodeling

Protein kinase A or cyclic AMP-dependent Kinase (PKA) constitutes the canonical downstream effector of the β-adrenergic cascade in myocardial signaling. Activation of the beta-adrenergic receptor (βAR), perhaps the heart's most crucial subclass of G-protein coupled receptor (GPCR), by catecholamines primarily elicits an increase in cellular cAMP via induction of cytosolic adenylyl cyclase (Benovic et al., 1986; Xie et al., 2013; van Berlo et al., 2013; Tham et al., 2015). Consequently, a number of critical signaling chains and downstream effectors are activated, contributing greatly to the immediate cardiac stress response ("fight or flight" concept) by boosting myocyte inotropy, chronotropy, and lusitropy. Mechanistically, elevation of cAMP predominantly results in activation of PKA. Activated PKA in turn rules over numerous critical downstream pathways by phosphorylating pivotal calcium-handling proteins and crucial parts of the myocytes' contractile apparatus targeted at optimization of cardiac contractility and (augmented) function. In this regard, PKA is at the heart of the myocytes' signaling pathways which integrate stimuli and stressors into orchestrated cascades that enhance cardiac remodeling and therefore ensure short- and long-term homeostasis and integrity of cellular functions (Benovic et al., 1986; Backs et al., 2011; Burchfield et al., 2013; Xie et al., 2013; van Berlo et al., 2013; Tham et al., 2015). As tremendous efforts in PKA research have elucidated and meticulously characterized a myriad of aspects of PKA signaling, this review can only highlight some recent frontiers, controversies and shifts in perspective. It aims to pick up on the notion that—with increasing knowledge—simplified models might have to make room for concepts of multi-layered and meticulously tuned processes, since key players themselves and associated cellular pathways prove even more complex than initially perceived.

Therefore, this review will focus on the following aspects of cardiac remodeling as far as PKA is concerned: importance of duration and alternate means of PKA stimulation/activation, and (in the following section) crosstalk of PKA with CaMKII, perhaps the most intricately intertwined protagonists and pathways of myocyte remodeling.

The (erratic) viewpoint of PKA as a detrimental player in cardiac remodeling and heart failure was significantly stimulated by the adverse cardiac phenotype observed in a transgenic mouse model overexpressing constitutively active PKA (Antos et al., 2001). In the years to follow, a more sophisticated picture of the role of PKA in the myocyte's stress response and remodeling processes emerged as the significance of dosage, time dependency and hierarchic crosstalk with other mediators such as CaMKII became apparent. In this regard, it became clear that PKA elicits protective effects upon immediate β-adrenergic stimulation, e.g., by phosphorylation of sarcomeric structures such as titin, while transgression to heart failure was actually found to be associated with decreased phosphorylation of PKA dependent targets. These findings fortified the idea that PKA might have a more pronounced role in early remodeling as opposed to chronic stress (Yamasaki et al., 2002; Kruger and Linke, 2006; Grimm and Brown, 2010; Backs et al., 2011; Fischer et al., 2013a).

In line with these observations, short-term stimulation of the β-adrenergic cascade confers a number of protective effects and mediates essential "fight or flight" responses. Chronic stimuli cause the remodeling processes to turn into a detrimental spiral of beta-receptor desensitization and pathologic hypertrophy with subsequent development of heart failure. Long-term β-adrenergic stimulation leads to negative modulation of the respective receptors as well as downstream effectors and targets by a number of key regulators, but the exact mechanisms of this duration-dependent switch in effects of beta-adrenergic stimulation and stress are incompletely understood (Engelhardt et al., 1999; Bristow, 2000; Hill and Olson, 2008; Zhang et al., 2013; van Berlo et al., 2013). However, it is widely accepted that GPCR receptor kinases (GRKs) constitute a pivotal class of

enzymes which restrict the dynamic range of βAR-function by phosphorylation of the receptors themselves and by modification of beta-arrestin signaling, a side-track of the β-cascade which is crucial for protective downstream effects (Strulovici et al., 1984; Benovic et al., 1986). Targeting of these duration-dependent negative regulators offers a promising approach to take advantage of PKA-mediated increases in cardiac contractility while repressing those intermediates and modulators in the PKA-cascade that lead to detrimental remodeling and subsequent heart failure (van Berlo et al., 2013). For instance, blocking GRK2 interaction with the GPCR alleviates pathologic remodeling, whereas amplified contractility is still observed (Casey et al., 2010).

The second aspect of PKA that we would like to zero in on in this review is the means by which PKA gets activated, as this seems to play a critical role in downstream effects. Furthermore, it illustrates that various environmental stimuli may divergently modify the character and dignity of remodeling and therefore induce distinctly different phenotypes, although they employ one and the same kinase. The above depicted β-adrenergic cascade serves as the predominant extracellular means to activate PKA. However, a number of alternate mechanisms have been proposed with reactive oxygen species (ROS) as the most intriguing elicitor (Brennan et al., 2006; Sag et al., 2013; van Berlo et al., 2013; Wagner et al., 2014; Tham et al., 2015).

Reactive oxygen species (ROS) and respective sensors and counterparts are integral parts of (sub)cellular signaling in many compartments of each cell. They contribute greatly to the myocyte's stress response and remodeling efforts (Burgoyne et al., 2013; Sag et al., 2013; Wagner et al., 2014; Tham et al., 2015). In this regard, ROS can either directly affect target structures by oxidation or trigger a number of enzymes to modify downstream effectors leading to altered calcium signaling and contractility, gating of ion channels as well as differential regulation of homeostatic mechanisms concerned with trophy, metabolism and cell survival (Burgoyne et al., 2013; Sag et al., 2013; Wagner et al., 2014; Tham et al., 2015).

Therefore, amongst others a publication from Phil Eaton's group deserves special attention. In multiple well-conducted experiments they demonstrate dose-dependent activation of PKA by dimerization of its regulatory subunit and consecutive compartmentalized modification of respective PKA targets. Notably, this activation of PKA by H2O2 was independent of cAMP levels, the mediator of βAR-induced PKA activation (Brennan et al., 2006). Interestingly, the study provides evidence that the postulated targets of oxidant-induced PKA are at least in part congruent with those phosphorylated upon classic beta-adrenergic activation of PKA. While many of the mechanistic details of this alternate pathway of PKA activation remain elusive, it has become evident that oxidant-induced activation confers a means of targeting PKA to its subcellular compartments and inducing its kinase activity in these select environments. Furthermore, these findings imply that the effects of PKA may be dependent on and vary with multiple environmental factors (β-adrenergic stimulation, ROS, intracellular calcium, etc.) whose crosstalk and relative contributions may determine PKA's quintessential role in any given situation. For instance, ROS-induced activation of PKA—similarly to beta-adrenoreceptor-mediated induction of PKA—results in an increase of cardiac contractility. However, the degree, dosage and duration of ROS stress appear to strongly influence these remodeling mechanisms as strong and long-lasting ROS stimuli seem to reverse the aforementioned pro-inotropic effects (Goldhaber and Liu, 1994; Yao et al., 2003). Suggesting another regulatory principle compatible to the previously described differential effects of different isoforms in calcineurin signaling, Brennan and colleagues also speculate on the possibility that redox-sensitivity applies mainly for type I PKA, while the type II entity may be more prone to β-adrenergic signaling (Brennan et al., 2006; Felkin et al., 2011).

Therefore this example of differential activation of PKA by ROS (as well as the regulatory mechanisms in terms of stability and degradation touched upon later in this review) may serve as another illustration of the complexity of cardiomyocyte signaling and remodeling mechanisms.

CaMKII-Signaling in Cardiac Remodeling

Calcium-Calmodulin-dependent Kinase II (CaMKII) is at the center of multiple cardiac signaling cascades and modulates the heart's response to various extrinsic and intrinsic stimuli (Anderson et al., 2011; van Berlo et al., 2013; Grandi et al., 2014; Kreusser et al., 2014; Kreusser and Backs, 2014; Mattiazzi and Kranias, 2014; Weinreuter et al., 2014; Tham et al., 2015). The main cardiac isoforms of CaMKII, gamma and delta, have been studied in depth in terms of their role in cardiac remodeling processes. As several excellent reviews (Anderson et al., 2011; Maier, 2012; Fischer et al., 2013b; Herren et al., 2013) and a very comprehensive topic series on CaMKII (synopsis in Grandi et al., 2014) have extensively covered the spectrum of CaMKII in cardiac physiology and pathology only very recently, the scope of this review will limit its attention to interactions and crosstalk with the aforementioned protagonists of cardiac signaling, calcineurin and PKA, as well as emerging twists and desiderata of CaMKII research.

In regard to the latter, CaMKII activation requires calcium and calmodulin to dissolve the autoinhibitory conformation of CaMKII, thereby exposing both the regulatory and catalytic domain of the molecule. In the wake of prolonged activation, autophosphorylation at T287 occurs as a major posttranslational mechanism to stabilize CaMKII in its activated form (Meyer et al., 1992; Erickson, 2014). In recent years, a number of studies have uncovered multiple alternate posttranslational modifications of CaMKII (especially of the regulatory domain and mainly activating in nature) offering a new conundrum as to the contributions and interdependency of these various regulatory mechanisms (Erickson, 2014). Select principles of this divergent activation of CaMK include oxidation, glycosylation and nitrosylation as well as interactions with intracellular proteins (such as alpha-actinin or the NMDA receptor in the brain) (Jalan-Sakrikar et al., 2012; Burgoyne et al., 2013; Erickson et al., 2013; Sag et al., 2013; Coultrap and Bayer, 2014; Erickson, 2014).

As with calcium, the previously mentioned activation of CaMKII via ROS seems especially intriguing as it parallels the induction of PKA by ROS. This parallelism construes another enigma in terms of the hierarchical, spatial and temporal patterns and regulatory mechanisms that determine which of the two kinases is preferentially targeted and therefore supreme in its effects at any given time. Furthermore, this ROS/oxidant-dependent co-induction of CaMKII and PKA is especially enthralling, as both kinases do not only share a great number of binding partners and downstream effectors such as sarcomeric proteins (e.g., cMyBPC, actinin), calcium handling proteins (RyR, PLN) as well as various calcium and sodium channels (e.g., NaV1.5), but sometimes even phosphorylate a protein at the exact same amino acid position (Faul et al., 2007; Anderson et al., 2011; Herren et al., 2013; Sag et al., 2013; Dobrev and Wehrens, 2014; Mattiazzi and Kranias, 2014; Wagner et al., 2014).

In some instances, this mystery of multiplicity, redundancy or even competition of PKA and CaMKII has been partially unraveled, as PKA was demonstrated to regulate histone deacetylase 4 (HDAC4) by inducing proteolysis, thereby selectively repressing myocyte enhancer factor-2 (MEF2) activity and shutting out CaMKII signaling tracks (Backs et al., 2011). Interestingly, this modulation of HDAC4 confers a switch toward a more protective cascade by favoring SRF over MEF2 as HDAC4-NT's primary target structure. In this way it serves as a gateway disrupting sidetracks that would cause the nature of cardiac remodeling to take a maladaptive direction (Backs et al., 2011).

A second example of progress in this area refers to the relative importance of the key signaling mediators calcineurin and CaMKII in regard to their roles in (maladaptive) cardiac remodeling. As previously delineated both signaling pathways (CaMKII and calcineurin) critically contribute to the pressure overload-induced phenotype of adverse remodeling, but knockout of both cardiac CaMKII genes demonstrates the supremacy of CaMKII for the maladaptive nature of remodeling in this setting, while calcineurin—when isolated from CaMKII crosstalk and interdependency—also seems to trigger beneficial processes in terms of a more physiological remodeling phenotype (Kreusser et al., 2014).

However, passing the buck to CaMKII and thereby advocating it as the black sheep in terms of cardiac remodeling does not seem justified since experimental evidence points at more intricate and multi-layered regulatory mechanisms. For instance, the isoform or subtype dependent variances in downstream signaling described for CaN and PKA have also been proposed for CaMKII and may significantly complicate and impede simplified explanations following a dualistic projection (Peng et al., 2010; Mishra et al., 2011; Gray and Heller Brown, 2014).

To further elaborate on the enigma of multiplicity, redundancy or even competition, the multi-adaptor z-disc protein myopodin has not only been shown to be phosphorylated by both PKA and CaMKII (both resulting in nuclear import of myopodin), but has been shown to be dephosphorylated by calcineurin, the third pivotal mediator of cardiac remodeling covered in this review (Faul et al., 2007; Linnemann et al., 2010). Similar data in terms of unresolved redundancy and/or multiplicity exists in regard to calcium or sodium handling proteins such as the RyR or the NaV1.5 channel (Camors and Valdivia, 2014; Dobrev and Wehrens, 2014; Grandi and Herren, 2014). While most of the biological implications and functions of these tantalizing observations remain elusive so far, these findings are nevertheless illustrative of the concept that key players in cardiomyocyte signaling significantly intertwine with their intermediates and downstream effectors so that dichotomizing protagonists in dualistic cascades may not always be as helpful as initially thought.

Deciphering crosstalk of CaMKII and PKA as two of the major signaling kinases in the heart has intrigued scientists for years and led to elucidation of various intermediates which constitute indirect signaling loops and pathways that integrate signals from one of these cascades into the other and modulate the response of one kinase depending on the other's signaling.

Exemplarily, recent works from Mika and colleagues demonstrate a feedback loop in which CaMKII phosphorylates PDE4D, which in turn leads to downregulation of cytosolic cAMP levels and consequent inactivation of PKA (Mika et al., 2015). Interestingly, although both kinases show this variety of identical interaction partners and targets, direct interaction and modulation of one of the kinases by the other has never been demonstrated to date.

In summary, there is broad consensus in the scientific community that factors like duration, intensity and environmental setting of extrinsic and intrinsic stimuli are most crucial for the downstream mediation of cardiac remodeling. In this regard, the realization of signaling pathways and single effectors integrating into a myriad of complex interactions and crosstalk-dependent downstream effects have led to the belief that these mechanisms of fine-tuning are paramount for our understanding of how the heart construes various stimuli, incorporates and orchestrates these into signaling cascades and alters gene expression to adapt to these stimuli. Modulation of interactions and crosstalk between cellular effectors, downstream targets in regard to gene expression as well as modification of the perception of stressors may therefore hold promise for future therapeutic approaches rather than aiming for the intermediates in signaling themselves—in reminiscence of the Greek philosopher Sophocles who postulated: "Do not kill the messenger!"

Protein Maintenance and Turnover as Critical Setscrews of Remodeling

Growing Tired (?)—Get a Shrink Before Burn-Out!

As mentioned above cardiac plasticity covers a wide and dynamic range, which allows for distinct and impressive adaptations to altered hemodynamics and demands. Key determinants of cardiac homeostasis in this regard include mechanisms which govern the dynamics of protein synthesis and degradation, thereby creating an adequate equilibrium suitable for any given situation. Over the past decades, research has mainly focused on protein synthesis with its respective appendices, adaptations, modulations and control mechanisms in an effort to elucidate

therapeutic potential of modifications of key mediators in these signaling pathways. In recent years, accumulating experimental evidence has led to a gradual shift in perspective (Hill and Olson, 2008; Portbury et al., 2011; Willis et al., 2014). While modes of protein synthesis and their modulation have remained as critical pillars in cardiac remodeling research, interest in mechanisms of degradation and atrophy has steadily grown (Hill and Olson, 2008; Portbury et al., 2012; Lavandero et al., 2013, 2015; Willis and Patterson, 2013; Willis et al., 2014). This is based on the notion that protein synthesis and degradation—while seemingly at opposing poles of cellular regulatory mechanisms—are in reality closely associated and hugely intertwined balancing mechanisms that guarantee adequate cellular responses and homeostasis (Willis and Patterson, 2013).

Therefore, the second part of our review will focus on this shift in perspective and the essential contributions of degradation and proteolysis to cardiac plasticity and homeostasis.

The cell is in dire need of tools which ensure adequate removal of misfolded or no longer-needed proteins as well as mechanisms that allow for well-controlled and regulated conversion and remodeling of its structural and functional units in response to intrinsic and extrinsic stimuli (Willis and Patterson, 2013). This deserves special emphasis in regard to the heart, more specifically cardiomyocytes, as these cells are terminally differentiated and characterized by a very limited regenerative potential (Willis and Patterson, 2013). Therefore, cardiomyocyte homeostasis, health and survival rely heavily on maintenance of protein quality control as well as critical balancing of synthesis, folding and turnover of proteins (Willis et al., 2009a). Cellular mechanisms to achieve this equilibrium include molecular chaperones and folding enzymes (nicely reviewed in Hartl et al., 2011; Christians et al., 2014 and therefore only briefly touched upon here) and coordinated systems for degradation and proteolysis, mainly autophagy and the ubiquitin-proteasome system (UPS) (Willis and Patterson, 2013; Willis et al., 2014; Lavandero et al., 2015)—elaborated on below. Only briefly mentioned here, but nevertheless very interestingly, SUMOylation has emerged as another important factor in regard to modification of protein turnover and homeostasis (Gupta et al., 2014).

Initially coined by Belgian biochemist Christian de Duve, the term "autophagy" describes the basic cellular approach to degrade and recycle dysfunctional or unnecessary components by lysosomal digestion (Blobel, 2013; Ohsumi, 2014). In this process, target structures are segregated into autophagosomes, double-membrane vesicles, which are then amalgamated to lysosomes. Consequently, lysosomal hydrolases mediate the breakdown and recycling of target structures resulting in cellular clearance (Lavandero et al., 2013, 2015; Ohsumi, 2014).

The Ubiquitin-Proteasome System is the second main effector of cellular degradation (Willis and Patterson, 2013; Willis et al., 2014; Drews and Taegtmeyer, 2014). While autophagy generally tackles (larger) aggregates, fractions or whole organelles, the UPS functions as a highly specific and meticulously tuned machinery by targeting single molecules that need to be degraded (Willis et al., 2014). Upon canonical labeling of lysine residues with ubiquitin chains, dispensable proteins are recognized and subsequently degraded by the 26S proteasome, a ubiquitous protease, marking the catalytic destination of the UPS pathway. Substrate-specificity of UPS-mediated degradation is achieved by a myriad of different ubiquitin ligases, which exhibit idiosyncratic binding sites tailored to their individual targets. Interestingly, downregulation of CSN (COP9 signalosome) confers a general reduction of F-box proteins, an important group of ubiquitin ligases, resulting in cardiac hypertrophy and severe heart failure (Su et al., 2011). This observation nicely depicts the homeostatic role of ubiquitin ligases in cardiac remodeling.

Adding another layer of complexity, various deubiquitinating enzymes may modify or even counteract the action of E3 ubiquitin ligases (Bosch-Comas et al., 2006; Bernardi et al., 2013).

There is significant crosstalk, cooperation and interdependency between the two basic mechanisms of cardiac proteolysis. In this regard, overload of and impairments in the UPS lead to compensatory upregulation of autophagy as an emergency solution or bypass to cope with the overflow. Yet, if both major systems are overwhelmed and failing (hypertrophic) remodeling accelerates and cardiac function declines (Tannous et al., 2008a,b; Su et al., 2011; Willis and Patterson, 2013).

Substrate-specific recognition by E3-ligases and subsequent degradation not only allow for the disposal of misfolded, damaged or no longer necessary proteins but offer a finely tuned and orchestrated means to direct cellular signaling. The cardiac sarcomere deserves special emphasis in this regard, as it serves as a pivotal node of signaling integrating stretch-responsive messages originating from the cell membrane, calcium-derived mechanisms of excitation-contraction (EC) coupling as well as multiple other cellular pathways which modulate the heart's quintessential function: force-generation for contraction of blood to supply the body with oxygen (Frank and Frey, 2011; Knoll et al., 2011; Portbury et al., 2011; van Berlo et al., 2013).

Illustrating this notion, most of the cardiac ubiquitin ligases identified to date target key sarcomeric proteins or molecules entangled with its associated network of signal effectors. For instance, the muscle-specific E3-ubiquitin ligase atrogin has been shown to ubiquitinate and thus to facilitate degradation of calcineurin (Li et al., 2004) which—as detailed above—represents one of the crucial enzymes in mediation of cardiac remodeling. Moreover, atrogin may exert its signaling effects in regard to hypertrophic remodeling by inducing the FoxO-transcription factor through non-canonical K63-ubiquitination (Li et al., 2007). FoxO in turn may induce atrogin (Sandri et al., 2004), while evidence suggests that at least in skeletal muscle both of the aforementioned (FoxO and atrogin) are additionally regulated by HDACs (Potthoff et al., 2007; Moresi et al., 2010). This may serve as a beautiful illustration of the complex crosstalk and interdependency of the UPS and key signaling elements of the cell.

Furthermore, the cardiac muscle-RING finger proteins (MuRFs) target parts of the contractile apparatus such as troponin I (MuRF-1) and myosin heavy chain (MuRF-1 and −3). In congruence, MuRFs (especially MuRF-1) have been shown as essential modulators of the hypertrophic remodeling process (Willis et al., 2007, 2009a,b, 2014; Fielitz et al., 2007a,b; Portbury et al., 2011; Willis and Patterson, 2013). Of note, the classic pathological vs. physiological dichotomy observed in research on

other key players of myocardial remodeling is not commonly attributed in UPS research, where seemingly contradictive or at least multi-layered data have created the picture of gradual continua of context- and stimulus-specific rather than ligase-specific reactions and pathways. This complex nature of UPS-mediated reactions to cardiac stress is marvelously illustrated by contrasting works from Willis and colleagues: on the one hand transgenic overexpression of MuRF-1 mediates cardioprotective effects in models of ischemia, on the other hand MuRF-1 transgenic mice demonstrate progressive decline in cardiac function as well as increased susceptibility to TAC-induced heart failure (Willis et al., 2007, 2009b). Additionally, studies by Fielitz et al. show a loss of MuRF-3 to be detrimental as this deficiency renders the heart more prone to rupturing after myocardial infarction (Fielitz et al., 2007b). Furthermore, these mice deficient of MuRF-1 and MuRF-3 show increased susceptibility for adverse hypertrophic remodeling along with findings of storage and aggregate myopathies (Fielitz et al., 2007a). The high redundancy of E3 ligases in regard to their respective targets as well as the critical involvement of the UPS in terms of metabolism add even more layers of complexity (Adams et al., 2008; Witt et al., 2008; Willis et al., 2014). Intriguingly, further downstream proteasome modification itself can also contribute to or even sway the nature of cardiac remodeling processes (Drews et al., 2010).

A second example of the absence of a black-and-white dualism can be derived from data on atrogin in settings of myocardial remodeling:

While the observation that atrogin-transgenic mice are protected from TAC-induced pathologic cardiac hypertrophy is promising, it is remarkable that the very same mice are still prone to TAC-mediated deterioration of cardiac function on the other hand (Li et al., 2004; Portbury et al., 2012). Furthermore, atrogin-knockout mice exhibit both protection from pathologic hypertrophic remodeling and resistance to pressure-overload induced cardiac dysfunction (Usui et al., 2011).

These observations offer a conundrum that clearly opposes the classic partition into solely "good" or "bad" players that has been used in regard to other mediators of cardiac remodeling in the past.

Myofibrillar myopathies (MFM)/desmin-related myopathies (DRM), generally clustered as a subgroup of the heterogeneous group of dilated cardiomyopathies (DCM), also highlight the critical implications of the UPS for remodeling research. They prove beyond doubt that loss of degradation capacities—which is generally due to mutations in sarcomeric proteins, that render these mutated proteins less stable in the constant wear and tear, or even cause them to be misfolded from the beginning—is indeed causative and crucial for the development of hypertrophic remodeling and deterioration of cardiac function in its wake (Kley et al., 2013; Willis et al., 2014; Willis and Patterson, 2013; Schlossarek et al., 2014a). Desminopathy and filaminopathy are the best described representatives of these MFMs/DCMs. For the group of desminopathies, mutations in either desmin or its molecular chaperone (CryAB) have been shown to be associated with impaired autosomal degradation of the respective aggregates. Identification of mechanisms to enhance UPS- and autophagy-mediated elimination of these devastating aggregates of misfolded proteins appears paramount to counter adverse remodeling and consequent heart failure (Willis and Patterson, 2013; Willis et al., 2014; Schlossarek et al., 2014a). An exploratory study by Cohen and colleagues who analyzed filament composition and breakdown in atrophying muscle upon fasting suggests involvement of Trim32 in desmin ubiquitylation and degradation (Cohen et al., 2012). It nicely illustrates the need for further comprehensive *in vivo* studies to delineate the roles of Trim32 and other ubiquitin ligases as suitable catalysts for degradation of WT and mutant desmin and its appendices as the prototypical proteins of DCM/DRM.

Filaminopathies deriving from mutated filamin entities may also be promising objectives in UPS research, as two cardiac ubiquitin ligases, MuRF-3 and fbxl22, have so far been identified to target filamin C for proteasomal degradation and upregulation of these E3 ligases may therefore offer great therapeutic potential in this disease (Fielitz et al., 2007b; Spaich et al., 2012; Kley et al., 2013; Willis et al., 2014).

Much the same applies for other types of cardiomyopathies that derive from mutated sarcomeric proteins. For instance, numerous studies demonstrate that mutations in cardiac myosin binding protein C (cMyBPC) lead to hypertrophic cardiomyopathy (HCM) associated with impairment of the UPS, establishing cMyBPC as the paradigm of HCM-studies (Schlossarek et al., 2014a,b). Conversely and illustrative of the complexity of achieving homeostasis in regard to protein turnover and maintenance, proteasomal inhibition appears to ameliorate the decline in cardiac function in a hypertrophic cardiomyopathy model of mutated cMyBPC (Schlossarek et al., 2014b). While MuRF-1 has been shown to reduce cMyBPC levels, this occurs independent of its ubiquitin ligase activity and is likely due to indirect effects (Mearini et al., 2010). Furthermore, promising observations from *in vitro* studies demonstrate atrogin to be involved in degradation of mutated cMyBPC. On the downside, atrogin fails to target WT cMyBPC—potentially due to divergent localization of mutant cMyBPC (Mearini et al., 2010). These findings underscore that the underlying mechanisms and their therapeutic potential remain largely elusive and should be targeted by future research, along with intensive efforts to identify further E3 ligases involved in cMyBPC degradation.

The vast field of cardiomyopathies has seen significant advances in identification of causative gene mutations along with respective phenotyping. However, in many cases the implications of these mutations in regard to the UPS have not been understood yet and are far from utilization of the therapeutic potential which a distinct mapping of culprits and mutated target genes to respective ubiquitin ligases would harbor (Willis et al., 2014; Drews and Taegtmeyer, 2014; Schlossarek et al., 2014a). For instance, alpha-actinin, a key sarcomeric protein in terms of structure and signaling, has repeatedly been implicated in cardiac remodeling and disease development (DCM) but so far only *in vitro* data exists on its mode of degradation (Spaich et al., 2012). While the observation that the cardiac E3 ligase fbxl22 facilitates the degradation of alpha-actinin is auspicious, it plainly exemplifies and advocates, yet again, the need for future remodeling research to shift in perspective

from solely analyzing the side of protein synthesis to minutely identifying and characterizing how each and every cardiac protein is regulated in terms of its homeostatic equilibrium and turnover.

Taken together this exemplary but to no end comprehensive presentation of autophagy- and UPS-related cardiac disease entities and mechanisms is supposed to reflect the multifaceted involvement and key roles of the degradation machinery in cardiac remodeling and disease development.

Conclusion and Future Perspective

Finally, to integrate both parts of our review we will provide two examples that nicely illustrate and thus emphasize the intertwining character of cardiac remodeling with its complexity and diverse crosstalk mechanisms:

The first part of our review detailed evolving controversies on major cardiac signaling pathways and their key protagonists—namely calcineurin, PKA and CaMKII—while the second part

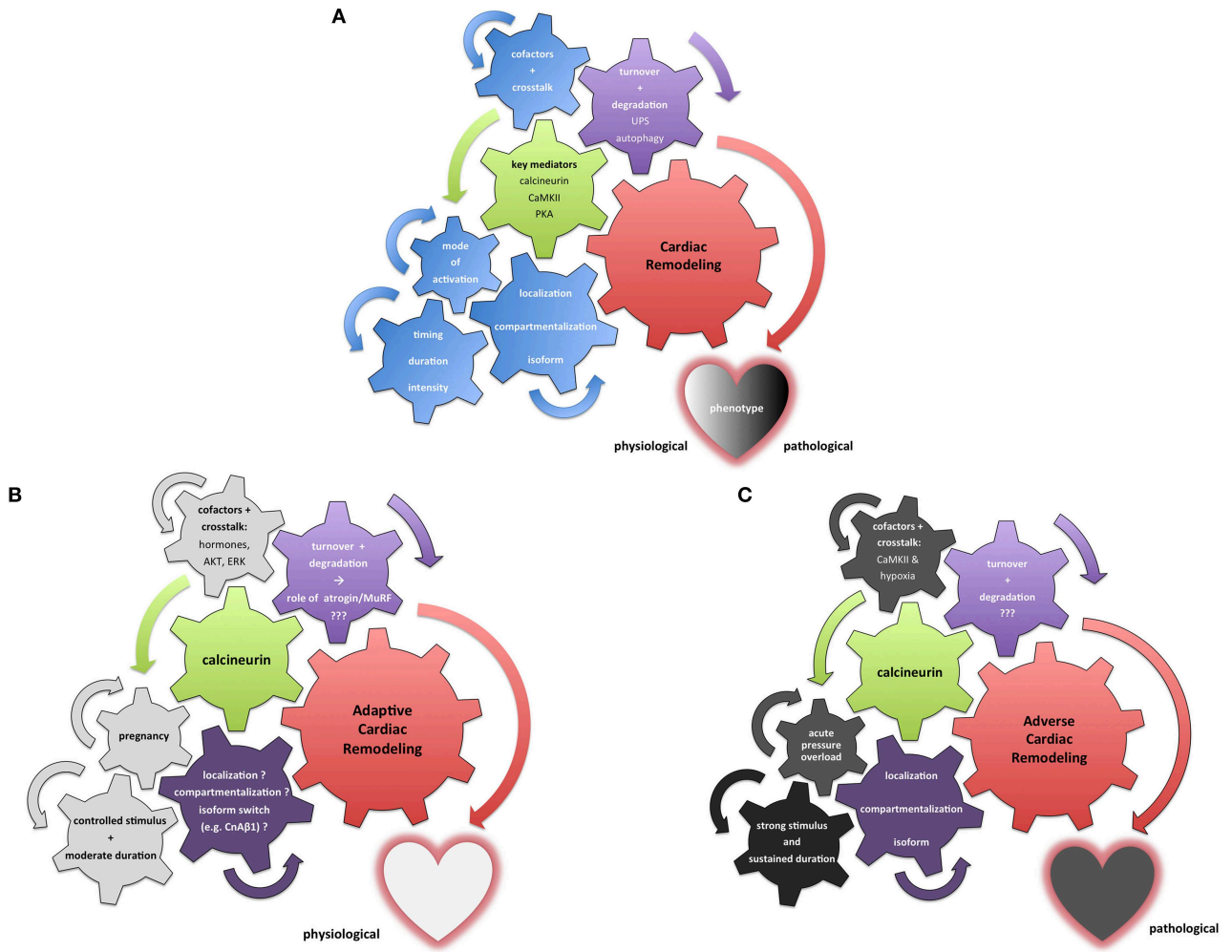

FIGURE 2 | Integrative illustration of the complexity and context-sensitivity of cardiac remodeling. (A) Neutral illustration how various setscrews determine the result of cardiac remodeling processes. Multiple pivotal molecules such as calcineurin, PKA and CaMKII have been identified as key mediators of myocardial signaling (green) in the remodeling process (red). A dualistic perspective of "*bad*" and "*good*" mediators fails to integrate the myriad of context-dependent stimuli and crosstalk mechanisms (depicted as the blue setscrews in this illustration). Furthermore, protein maintenance and degradation (purple) contribute to the dignity and nature of cardiac remodeling processes. **(B)** Exemplary illustration of calcineurin's role in adaptive cardiac remodeling processes that result in a beneficial phenotype. While a number of stimuli and setscrews remain only incompletely understood or even elusive so far (purple), multiple relevant extrinsic and intrinsic factors potentially modulate calcineurin signaling toward a more beneficial response (depicted in light gray). Pregnancy-associated cardiac remodeling has been shown to be beneficial in nature; hormonal and temporal factors depicted here appear to contribute significantly to this adaptive dignity of calcineurin signaling. **(C)** Contrasting illustration of calcineurin's role in a maladaptive setting promoting cardiac remodeling processes that result in an adverse phenotype. While multiple factors—especially in regard to protein turnover—remain only incompletely understood or even elusive so far (purple), numerous extrinsic and intrinsic stimuli and stressors drive calcineurin signaling toward pathological effects with an adverse cardiac phenotype (depicted in dark gray). The pressure overload phenotype has been intensively studied; evidence suggests that calcineurin's signaling is swayed toward a maladaptive course not only by sudden onset, pronounced duration and intensity of the remodeling stimulus but also by cofactors such as activated CaMKII.

focused on the idea that not only protein synthesis but also protein turnover and degradation are paramount for cellular homeostasis. Despite huge efforts in both fields of research to enhance our understanding how cardiac remodeling processes are governed, it seems surprising that at least for two of the formerly mentioned pivotal mediators of cardiac remodeling, PKA and CaMKII, knowledge in regard to the regulation of their turnover and degradation remains very scarce. To the best of our minds, no ubiquitin ligase has yet been identified and proven as a critical regulator of CaMKII. In terms of PKA ubiquitylation and degradation, only the ubiquitin ligase Praja2 has so far been shown to associate with and ubiquitinate the regulatory subunit of PKA in specific compartments (Lignitto et al., 2011). Furthermore, with atrogin and MuRF-1 only two ubiquitin ligases have been validated to perform E3 ligase activity upon calcineurin thereby targeting it for proteasomal degradation (Li et al., 2004; Maejima et al., 2014; Schlossarek et al., 2014a).

In light of the multiplicity and redundancy seen for other key cellular proteins (p53 is targeted by at least 10 different ubiquitin ligases, proteasomal degradation of c-Jun has been shown to be conferred by at least 4 different E3 ligases), it appears very likely that a number of ubiquitin ligases targeting PKA, CaMKII and CnA are still unidentified and may be very important for a comprehensive picture of the mechanisms that control cardiac remodeling (Portbury et al., 2012; Willis et al., 2014; Maejima et al., 2014).

The second interesting notion highlighting the integrative and highly complex mechanisms that govern cardiac plasticity and remodeling concerns the observation that exercise, therefore physiologic hypertrophic stimuli, may alleviate myofibrillar myopathies and proteotoxicity in the wake of UPS impairments and dysfunction (pathological cardiac remodeling) or lead to improvement of UPS function itself (Maloyan et al., 2007; Willis and Patterson, 2013; de Andrade et al., 2015). This intriguing interdependency demands for further clarification of the underlying crosstalk to enhance our understanding how induction of certain mediators by exercise may provide tools for renewal of cellular homeostasis in failing myocardium.

In any case, this observation underscores the concept that seemingly distant stimuli and protagonists of myocardial signaling (exercise on the one hand, the UPS on the other hand) intricately affect each other and prove the cardiac remodeling processes to be comprehensive and minutely tuned programs that integrate all cellular pathways into a highly plastic and flexible machinery (**Figure 2**).

In summary, this review provides some thought-provoking impulses on emerging or ongoing controversies and shifts in perspective surrounding myocardial remodeling rather than trying to comprehensively cover the myriad of elaborate mechanisms of cardiac signaling.

Therefore the two key messages are:

1. Protagonists of cardiac remodeling demonstrate multifaceted crosstalk and significant interdependency in a highly context-sensitive nature. Therefore it is our opinion that they cannot be easily classified in terms of a black and white dualism but appear to roam a gradual continuum of "fifty (or more) shades of gray."
2. While protein synthesis and its regulation remain paramount for our understanding of cardiac adaptations, a shift in perspective has occurred in recent years as the mechanisms of maintenance and turnover of proteins have increasingly been recognized as essential aspects of sustained homeostasis—or to put it in other words: "Growing tired (?)—get a shrink before burn-out!"

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Air pollution and adverse cardiac remodeling: clinical effects and basic mechanisms

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Exposure to air pollution has long been known to trigger cardiovascular events, primarily through activation of local and systemic inflammatory pathways that affect the vasculature. Detrimental effects of air pollution exposure on heart failure and cardiac remodeling have also been described in human populations. Recent studies in both human subjects and animal models have provided insights into the basic physiological, cellular and molecular mechanisms that play a role in adverse cardiac remodeling. This review will give a brief overview of the relationship between air pollution and cardiovascular disease, describe the clinical effects of air pollution exposure on cardiac remodeling, describe the basic mechanisms that affect remodeling as described in human and animal systems and will discuss future areas of investigation.

Keywords: air pollution, diesel exhaust particulates, cardiac remodeling, cardiac hypertrophy, heart failure

Introduction

Exposure to various forms of air pollution has been linked to cardiovascular disease in multiple human and animal studies (reviewed in Chin, 2015). Air pollution consists of many gaseous and particulate components and can vary significantly in chemical composition depending on local environmental conditions. Particulate matter air pollution, comprised primarily of fine particulates derived from combustion, is a major component of ambient air pollution and exhibits the most cardiotoxic effects (Brook et al., 2004, 2010; Miller et al., 2007; Chin, 2015). Ambient particulates are generally categorized on the basis of size, and most toxicity studies have focused on particulates less than or equal to 10 μm in diameter, referred to as PM_{10} (reviewed in (Brook et al., 2004)), or a subset of PM_{10}, particulates less than or equal to 2.5 μm in diameter, referred to as $PM_{2.5}$. Particles in this size range are especially toxic because of their ability to reach the alveoli, where they activate multiple pathophysiological mechanisms (Brook et al., 2010; Chin, 2015). Both acute and chronic $PM_{2.5}$ exposure have been associated with exacerbation of ischemic heart disease (Xie et al., 2015), heart failure (Shah et al., 2013), cerebrovascular disease (Stafoggia et al., 2014), thrombosis (Baccarelli et al., 2008), hypertension (Bellavia et al., 2013), and arrhythmias (Bartell et al., 2013). This review will focus specifically on physiological mechanisms that are activated by PM exposure to promote adverse cardiac remodeling (Atkinson et al., 2013).

The basic physiological mechanisms by which $PM_{2.5}$ promotes cardiovascular disease have been studied extensively in both humans and animal models, though many questions remain (Brook et al., 2010; Chin, 2015). The most commonly accepted mechanism is that inhalation into the lungs promotes a local inflammatory response that then "spills over" into the circulation, where soluble

and cellular mediators can then promote systemic vascular oxidative stress and inflammation that affect the heart and vasculature. This systemic effect can also be amplified by effects on adipose and liver tissue, promoting the release of adipokines and acute phase reactants, which can alter vascular tone, resulting in insulin resistance, dyslipidemia and hypercoagulability (reviewed in Brook et al., 2010). Another mechanism involves activation of autonomic reflex arcs in the lung, mediated by TRP receptors, which results in elevated sympathetic nervous system activity and reduced parasympathetic nervous system activity that then promotes vasoconstriction, endothelial dysfunction, platelet aggregation, hypertension and arrhythmogenesis. A third mechanism involves exit of ultrafine particulates or soluble components associated with these particulates from the lungs into the circulation where they directly promote vasoconstriction, endothelial dysfunction, vascular oxidative stress, hypertension and possibly atherosclerosis and platelet aggregation. A fourth mechanism involves the epigenetic modification of DNA in circulating cells and target tissues, resulting in altered gene expression patterns that promote conditions such as hypertension (Bellavia et al., 2013). These mechanisms and their downstream effects on cardiac remodeling are shown in **Table 1**. The specific mechanisms by which air pollution exposure affects cardiac remodeling can be indirect and in conjunction with other processes such as ischemia or hypertension or direct and mediated by systemic inflammatory mediators and/or epigenetic reprogramming.

Epidemiological Associations between Particulate Matter Exposure and Abnormal Cardiac Remodeling

In one study, the relationship between traffic-related air pollution exposure, cardiac hypertrophy and left ventricular function was specifically analyzed, using an estimation of exposure based on proximity to major roadways and magnetic resonance imaging in a multi-ethnic patient population known to be free of coronary artery disease. Participants living within 50 m of a major roadway were found to have increased left ventricular mass index but no change in ejection fraction (Van Hee et al., 2009). A follow up study identified associations between single nucleotide polymorphisms in the genes encoding the type I angiotensin II receptor (AGTR1) and arachidonate 15-lipoxygenase (ALOX15) and increased left ventricular mass associated with living in proximity to major roadways (Van Hee et al., 2010). These genes are important in vascular function, inflammation and oxidative stress, implying that the observed increase in LV mass may arise through these mechanisms. Interestingly, exposure to PM_{10} but not $PM_{2.5}$ is associated with development of heart failure in an English cohort (Atkinson et al., 2013).

Immunological Effects of PM Exposure

Systemic inflammation is a common mechanism that causes both direct and indirect effects on cardiac remodeling (**Table 1**). Exposure can trigger both the adaptive and innate immune responses. In mice, $PM_{2.5}$ exposure releases oxidized phospholipids in the lungs that then activate Toll-like Receptor 4 (TLR4)/NADPH oxidase-dependent mechanisms to promote systemic inflammation (Kampfrath et al., 2011). In primary mouse macrophages from Toll-like Receptor 2 (TLR2) knockout mice, the Tumor Necrosis Factor-α (TNF-α) and Interleukin-6 (IL-6) response to $PM_{2.5}$ exposure is blunted (Shoenfelt et al., 2009). Upregulation of IL-6 in mice in response to PM_{10} intratracheal instillation has been associated with an increase in coagulation (Mutlu et al., 2007), demonstrating how inflammation may provoke vascular occlusion and ischemia that can influence cardiac remodeling.

A study examining the response of transformed human bronchial epithelial cells to diesel exhaust particulate exposure showed an induction of CYP1A1 and downstream increased expression of IL-6 and IL-8 (Totlandsdal et al., 2010). In humans, short term PM_{10} exposure is associated with increased levels of circulating IL-1β, IL-6 and TNF-α (Tsai et al., 2012). Controlled short-term exposure of human subjects to $PM_{2.5}$ also leads to elevations in circulating IL-6 (Urch et al., 2010). Transcriptional profiling of peripheral blood mononuclear cells from human subjects exposed to $PM_{2.5}$ reveals changes in expression of genes associated with inflammation, oxidative stress and coagulation (Peretz et al., 2007; Pettit et al., 2012), suggesting that these mechanisms are clinically relevant. Chronic human exposure to $PM_{2.5}$ leads to hypermethylation of the TLR2 promoter in leukocytes and promotes autonomic dysfunction (Zhong

TABLE 1 | Pathophysiological effects of particulate matter air pollution and their relevance to cardiac remodeling.

Pathophysiological mechanisms	Downstream effects relevant to cardiac remodeling
Local pulmonary inflammatory response with systemic spread (Mutlu et al., 2007; Urch et al., 2010; Kampfrath et al., 2011; Tsai et al., 2012)	- ↑Systemic inflammation - Vascular oxidative stress - Release of acute phase reactants - ↑Coagulation - Insulin resistance
Activation of autonomic reflex arcs in lungs (Rhoden et al., 2005; Ghelfi et al., 2008; Zhong et al., 2015)	- ↑ Sympathetic nervous system activity - ↓ Parasympathetic nervous system activity - ↑ Vasoconstriction - Endothelial dysfunction - Platelet aggregation - Hypertension - Arrhythmogenesis
Release of ultrafine particles or particulate components into the bloodstream (Nemmar et al., 2002, 2003a,b)	- ↑Systemic inflammation - Vascular oxidative stress - Endothelial dysfunction - Hypertension - Platelet aggregation - Atherosclerosis (?)
Exposure triggers changes in epigenetic regulation (Yauk et al., 2008; Bellavia et al., 2013; Zhong et al., 2015)	- Changes in leukocyte gene expression, affecting immune response

et al., 2015). Other human studies have not shown changes in circulating inflammatory mediators, however, indicating that these studies are not always consistent (Mills et al., 2008). The reason for these variable inflammatory reactions may be context dependent, as the time of exposure and chemical composition of PM used in the studies may alter the response.

Particulate Matter Air Pollution, Ischemic Injury and Adverse Cardiac Remodeling

Ischemic injury to myocardium is well known to lead to adverse cardiac remodeling. Occlusion of coronary artery blood flow to myocardium leads to infarction and myocyte necrosis, followed by inflammation, scarring and remodeling of the heart to compensate for reduced contractile function. Occlusion can occur as the result of progressive atherosclerosis, acute vascular thrombosis, or a combination of the two, which is most likely. Endothelial dysfunction resulting in vasoconstriction can also play a role. $PM_{2.5}$ exposure has been shown to exacerbate ischemic injury to myocardium in many studies, through effects on atherosclerosis, thrombosis and vasoconstriction (Brook et al., 2010; Chin, 2015).

An early study demonstrated that exposure of dogs to concentrated ambient particles leads to increased ST segment elevation after 5 min coronary artery occlusion (Wellenius et al., 2003). The combination of a high fat diet and chronic $PM_{2.5}$ exposure has been shown to increase vascular oxidative stress and promote progression of atherosclerosis (Sun et al., 2005). Ultrafine particles ($PM_{0.1}$) have been shown to promote oxidative stress and early atherosclerosis (Araujo et al., 2008). Studies on experimental atherosclerosis in two different mouse models have shown that long term exposure to $PM_{2.5}$ in the form of inhaled concentrated ambient particulates (CAPs) promotes increased CD36 expression in plaque macrophages that facilitates greater uptake and accumulation of an oxidized form of cholesterol in atherosclerotic lesions (Rao et al., 2014). Chronic exposure to CAPs can also promote inflammatory monocyte egress from bone marrow, production of reactive oxygen species and subsequent vascular dysfunction as measured by isolated aortic ring contraction through TLR4 activation of NADPH oxidase in mice (Kampfrath et al., 2011).

Acute ischemic events that lead to myocardial infarction and adverse cardiac remodeling in patients are generally mediated by atherosclerotic plaque rupture and occluding thrombus formation on the surface of the ruptured plaque. In a hamster model, intratracheal administration of diesel exhaust particulates (DEPs) has been shown to enhance experimental thrombus formation (Nemmar et al., 2003a). Follow up studies indicated that the enhanced thrombosis is mediated by production of histamine in the lungs (Nemmar et al., 2003b) and that stabilization of mast cells reduces this effect (Nemmar et al., 2004). In mice, intratracheal administration of PM_{10} has been shown to increase lung production of IL-6, reduce bleeding time, prothrombin time and activated partial thromboplastin time, increase plasma fibrinogen and increase activities of coagulation factors II, VIII and X (Mutlu et al., 2007). In hamsters, $PM_{2.5}$-induced thrombosis is also reportedly further enhanced by angiotensin II-induced hypertension (Nemmar et al., 2011). In mice, either inhalation or intratracheal instillation of CAPs leads to increases in lung and adipose tissue PAI-1, implying that reduction in fibrinolysis may also play a role (Budinger et al., 2011). A more recent study in mice showed that inhaled CAPs promotes activation of the sympathetic nervous system and systemic catecholamine release, which in turn activates β2-adrenergic receptor-dependent release of IL-6 from alveolar macrophages to promote a prothrombotic state as measured by $FeCl_3$-induced carotid artery time to loss of blood flow (Chiarella et al., 2014). Acute exposure of healthy human volunteers to DEPs promotes an increase in thrombus formation, platelet-neutrophil and platelet-monocyte aggregates as measured *ex vivo* in a Badimon chamber, implying an increase in platelet activation (Lucking et al., 2008).

Particulate Matter Air Pollution, Hypertension and Adverse Cardiac Remodeling

Hypertension is well known to promote adverse cardiac remodeling, by inducing concentric hypertrophy, eccentric hypertrophy, systolic and diastolic dysfunction (Santos and Shah, 2014). Reported effects of PM exposure on blood pressure vary, which may reflect differences in experimental methodology but may also reflect a need for interaction with other factors (Brook et al., 2010). Short-term (10 weeks) exposure to CAPs reportedly promotes vascular oxidative stress, increases hypertension in response to angiotensin II and activates Rho/ROCK in rats (Sun et al., 2008). Acute exposure of rats to CAPs (4 days) increases expression of endothelin receptor-A in rat hearts, which correlates with an increase in blood pressure, implying a role for endothelin in $PM_{2.5}$-mediated hypertension (Ito et al., 2008). Exposure of spontaneously hypertensive rats to ultrafine particles for 24 h leads to increases in blood pressure 1–3 days after exposure that are associated with increased expression of endothelin-1 messenger RNA in lungs and increased circulating levels of plasma renin and angiotensins I and II (Upadhyay et al., 2008). Short-term exposure (12 weeks) of mice to CAPs confirms the potentiating effect of $PM_{2.5}$ exposure on angiotensin II-induced hypertension in rats, through a similar Rho/ROCK mechanism, but also promotes increased cardiac hypertrophy and collagen deposition (Ying et al., 2009). Long-term (6 months) exposure to CAPs leads to activation of the sympathetic nervous system, hypothalamic inflammation and systemic hypertension in mice (Ying et al., 2014). The source of air pollution is likely to affect toxicity, however, as some experiments using DEPs have not shown any effect of either acute (7 days), short term (1 month) or chronic exposure (6 months) on cardiac remodeling in mice in both an angiotensin II infusion model and a transverse aortic constriction (TAC)

model. This discrepancy between DEPs and CAPs likely reflects additional toxins that associate with CAPs in the atmosphere that are not initially associated with DEPs (Liu et al., 2013). Controlled exposure studies in human subjects have shown that acute exposure to CAPs in conjunction with ozone leads to transient diastolic hypertension, possibly due to autonomic imbalance (Urch et al., 2005) and that short-term, controlled human exposure to $PM_{2.5}$ CAPs resulted in an increase in systolic blood pressure that correlates with reduced DNA methylation in Alu repetitive elements in circulating leukocytes (Bellavia et al., 2013).

Diffuse Myocardial Damage, Cardiac Fibrosis and Abnormal Cardiac Remodeling

Abnormal cardiac remodeling due to cardiac fibrosis can develop in response to myocardial injury, oxidative stress, mechanical stress and/or the influence of circulating mediators

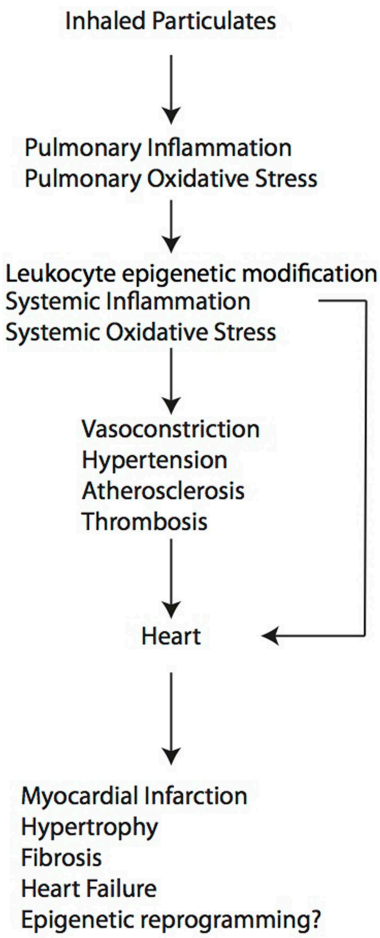

FIGURE 1 | A working model of how air pollution exposure promotes adverse cardiac remodeling.

and is associated with changes in DNA methylation (reviewed in Tao et al., 2014). A hallmark of this process is activation of cardiac fibroblasts to generate excessive amounts of extracellular matrix proteins. Cardiac fibrosis can then lead to both systolic and diastolic dysfunction. In Wistar–Kyoto rats, protracted, repeated inhalation exposure to oil combustion-derived particulate matter leads to the development of multifocal, inflammatory, degenerative and fibrotic lesions in the myocardium (Kodavanti et al., 2003). A follow up study indicated that PM-associated zinc appears to be an important contributor to focal subepicardial inflammation, myocardial degeneration, fibrosis and mitochondrial DNA damage after 16 weeks of intratracheal instillation. There were also measurable effects on mRNA expression for genes involved in signaling, ion channel function, oxidative stress, mitochondrial fatty acid metabolism and cell cycle regulation (Kodavanti et al., 2008). A 3-month exposure of mice to CAPs has been shown to exacerbate angiotensin II-induced cardiac hypertrophy and fibrosis in a Rho kinase dependent manner (Ying et al., 2009). A 9-month exposure of mice to CAPs results in increased ventricular size along with systolic and diastolic dysfunction at the organ level, attributable to myocardial fibrosis at the tissue level and associated with decreased antioxidant activity as well as reduced contractile reserve. At the cellular level, isolated myocytes showed reduced fractional shortening, decreased shortening velocity and increased relaxation time. At the molecular level, expression of hypertrophic and profibrotic markers were increased (Wold et al., 2012). Exposure of rats to diluted motorcycle exhaust leads to increased heart weight, wall thickness and histological evidence for focal cardiac degeneration and necrosis, mononuclear cell infiltration and fibrosis. Cardiac antioxidant enzyme activities for glutathione S-transferase, superoxidase dismutase and glutathione peroxidase are also reduced. Analysis of mRNA expression in these hearts reveals increased levels of interleukin-β, atrial natriuretic peptide, collagen type I, collagen type III, connective tissue growth factor and transforming growth factor β1 transcripts, suggesting that exposure to motorcycle exhaust promotes hypertrophy and fibrosis through mechanisms involving oxidative stress and inflammation (Chen et al., 2013), although transcript levels do not necessarily reflect amount of protein expressed.

Abnormal Cardiac Remodeling in Adults after Early Life Diesel Exhaust Exposure

Early life exposure to air pollution is known to cause low birth weight (Dadvand et al., 2013) and abnormal lung development in human populations (Gauderman et al., 2004, 2007). A recent study from the Netherlands found that children that are chronically exposed to $PM_{2.5}$ have increased diastolic blood pressure (Bilenko et al., 2015), and a Boston newborn study found that third trimester maternal exposure to $PM_{2.5}$ is correlated with increased newborn systolic blood pressure (van Rossem et al., 2015). A study of mouse exposure to DEPs during several

discrete developmental windows demonstrated an increased susceptibility to pressure overload-induced heart failure in mice that were exposed *in utero* and up to the age of weaning. There was no change in basal cardiac function prior to TAC surgery. The magnitude of the effect was similar to that seen in animals exposed throughout life from conception through adulthood. Exposure during the adult period from weaning through the age of 12 weeks had no effect on susceptibility to heart failure. The hearts of animals exposed early in life showed increased heart weight to body weight ratios and interstitial fibrosis but no significant change in myocyte cross sectional area when compared to those from control mice exposed to filtered air. Interestingly, the lungs of mice exposed early in life showed differential induction of interleukin-6 expression after TAC surgery (Weldy et al., 2013). A follow up study in which exposure to DEPs was limited solely to gestation demonstrated that *in utero* exposure alone is sufficient to convey a long lasting susceptibility to myocardial fibrosis and heart failure when *in utero*-exposed adult mice were subjected to TAC surgery, suggesting that mediators in the maternal circulation could cross the placenta and promote long lasting susceptibility in the developing embryo hearts. Further findings include a baseline increased body weight to tibia length ratio and paradoxically lower baseline blood pressure in adult male animals. Histological examination of hearts from exposed animals showed increased interstitial fibrosis but no significant difference in myocyte cross sectional area. Examination of placental and fetal tissue from exposed dams showed a higher rate of fetal reabsorption, a lower average placental weight, increased incidence of placental hemorrhage, an increase in leukocyte infiltration and an increase in vascular oxidative stress (Weldy et al., 2014).

Future Challenges in Understanding the Role of Air Pollution in Adverse Cardiac Remodeling

As described above, particulate air pollution can cause adverse cardiac remodeling through multiple indirect mechanisms, by exacerbating processes known to promote injury to myocardium such as atherosclerosis, vasoconstriction, thrombosis and hypertension (**Figure 1**). At present, however, little is known about effects on cardiomyocytes and cardiac fibroblasts at the cellular and molecular level, especially during development. For example, it is not known whether the cardiac fibrosis that develops in some models is due to a primary activation of cardiac fibroblasts or whether it reflects a primary injury to cardiomyocytes, resulting in cell death and replacement fibrosis. In the case of early life exposure, the molecular events that occur in the developing heart that confer later susceptibility to heart failure induced by pressure overload in the adulthood are completely unknown. Since air pollution exposure is also known to cause epigenetic modification in circulating leukocytes (Tarantini et al., 2009; Bellavia et al., 2013) and sperm (Yauk et al., 2008), and dynamic DNA methylation changes orchestrate cardiomyocyte development, maturation and disease (Gilsbach et al., 2014), future studies directed toward epigenetic regulation in cardiomyocytes may elucidate further the pathways and events that are responsible for pathological cardiac remodeling.

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Multipotent stem cells of the heart—do they have therapeutic promise?

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The last decade has brought a comprehensive change in our view of cardiac remodeling processes under both physiological and pathological conditions, and cardiac stem cells have become important new players in the general mainframe of cardiac homeostasis. Different types of cardiac stem cells show different capacities for differentiation into the three major cardiac lineages: myocytes, endothelial cells and smooth muscle cells. Physiologically, cardiac stem cells contribute to cardiac homeostasis through continual cellular turnover. Pathologically, these cells exhibit a high level of proliferative activity in an apparent attempt to repair acute cardiac injury, indicating that these cells possess (albeit limited) regenerative potential. In addition to cardiac stem cells, mesenchymal stem cells represent another multipotent cell population in the heart; these cells are located in regions near pericytes and exhibit regenerative, angiogenic, antiapoptotic, and immunosuppressive properties. The discovery of these resident cardiac stem cells was followed by a number of experimental studies in animal models of cardiomyopathies, in which cardiac stem cells were tested as a therapeutic option to overcome the limited transdifferentiating potential of hematopoietic or mesenchymal stem cells derived from bone marrow. The promising results of these studies prompted clinical studies of the role of these cells, which have demonstrated the safety and practicability of cellular therapies for the treatment of heart disease. However, questions remain regarding this new therapeutic approach. Thus, the aim of the present review was to discuss the multitude of different cardiac stem cells that have been identified, their possible functional roles in the cardiac regenerative process, and their potential therapeutic uses in treating cardiac diseases.

Keywords: cardiac stem cells, cardiac remodeling, cell therapy, regenerative process, cardiac homeostasis

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The increase in the average human lifespan has been accompanied by an increased incidence of chronic diseases (Torella et al., 2007). These chronic non-communicable diseases are the result of a number of risk factors and are characterized by a prolonged natural course and the potential for developmental disabilities. These diseases are not only prevalent but also aggressive, and they dominate death statistics. According to the World Health Organization (WHO), cardiovascular diseases (including heart attack and stroke) represent the largest number of deaths from non-communicable diseases, 17.5 million people annually, followed by cancer (8.2 million) and respiratory diseases (4 million)[1].

[1] Available at: http://www.who.int/mediacentre/factsheets/fs355/en/ Acessed January 10, 2015.

Heart failure is a particularly lethal, disabling, and expensive-to-treat disorder that has reached epidemic levels in industrialized nations (Bolli et al., 2011). Even with significant advances in the treatment of coronary artery disease and the management of acute myocardial infarction in humans, this pathological event remains the leading cause of mortality in developed countries (Gnecchi et al., 2008; Wang et al., 2014) and is highly associated with morbidity because a large population of infarct survivors will develop cardiac failure (Hellermann et al., 2002). In addition to a significant level of morbidity, and direct cost of treatments by physicians and other professionals, hospital services, medications, home health care, and other medical durables, the health-related expenditures associated with cardiovascular diseases include indirect costs, such as lost productivity resulting from morbidity and premature mortality (Go et al., 2013). If all forms of major cardiovascular disease were eliminated, human life expectancy might increase by approximately 7 years (Go et al., 2014).

Given the striking impact of these diseases on mortality rates, most prognostic models of heart failure focus on mortality, which is easily determined and highly relevant; however, other clinical outcomes also rank high in importance to individual patients (Allen et al., 2012). Because a large fraction of patients with advanced heart failure report poor quality of life and significant emotional distress due to debilitating symptoms such as breathlessness, fatigue, and sleeping difficulties (Hallas et al., 2011), quality of life is a significant outcome to be considered. When advanced heart failure patients discuss their goals, quality of life is an important issue because how well they will live is as important as how long they will live (Allen et al., 2012).

Depending on the specific condition(s), therapeutic options for patients living with heart diseases include lifestyle changes, drugs to prevent disease advancement, angioplasty, bypass surgery, pacemakers, left ventricular assist devices and, finally, heart transplantation (Smith et al., 2008a). This final treatment is unavailable to many patients because of a lack of donor organs or the presence of comorbidities that make the procedure inviable.

Given the deleterious consequences of heart disease, new therapeutic approaches are needed because the progression of heart disease involves loss of the myocardium, scar formation and remodeling of remaining cardiac tissue (Smith et al., 2008a). Invasive treatments and optimized medical therapies are increasingly successful in addressing the acute manifestations of coronary artery diseases. However, although these treatment strategies often extend the lives of patients, they may not return to healthy lives (Torella et al., 2007). As the incidence of heart failure increases exponentially, novel therapies using cellular treatments or regenerative strategies are being sought (Chong et al., 2011). In particular, patients have high expectations for cellular therapy (Lovell and Mathur, 2011).

Cardiac Physiological Homeostasis—the Heart as a Dynamic Organ

Fortunately, the last decade has witnessed the dawn of a new era in myocardial biology (Ellison et al., 2012), with the initiation of many promising studies of treatment strategies based on cellular therapies for heart patients. Until recently, the heart was considered a static and post-mitotic organ lacking regenerative capacity (Beltrami et al., 2003; Chan et al., 2009; Lovell and Mathur, 2011; Ellison et al., 2012), i.e., the number of cardiomyocytes in an individual was established at birth (Barile et al., 2007; Kajstura et al., 2010b), and cardiomyocyte hypertrophy was considered the only cellular adaptive response of the heart (Lovell and Mathur, 2011). In this context, it was assumed that the age of a cardiomyocyte would correspond to the age of the organism (Torella et al., 2007; Kajstura et al., 2010a) and that a cardiomyocyte, lacking the ability to divide, could only increase its size or die, and the process of cellular survival was ensured by the continual replacement of intracellular organelles.

Careful, reproducible, and widely reported experiments have overthrown this static-organ theory. Convincing evidence suggests that cardiomyocytes are renewed throughout life, with continual cellular turnover in the mammalian heart, which also has an intrinsic regenerative capacity (Barile et al., 2007). However, the precise rate of cardiomyocyte turnover remains unclear (Ellison et al., 2013). This rate is difficult to study in humans because, in most cases, the necessary methodology involves the use of analogous nucleotide markers (Bergmann et al., 2010). Nuclear bomb testing during the Cold War resulted in integration of ^{14}C into cellular DNA, making it possible to determine the age of cardiomyocytes in humans based on analysis of the natural radioactive isotype of carbon to estimate their turnover rate (Bergmann et al., 2010). These studies suggested an annual turnover rate of 0.2–2%; however, a clear negative correlation between the cellular renewal rate and age was observed (Bergmann et al., 2010).

In addition, the source of cardiomyocyte renewal throughout life is unknown (Chong et al., 2011). At least two different origins of these cells must be considered when examining the stimuli that regulate the renewal of cardiomyocytes. As early as, Anversa and Kajstura (1998) proposed that preexisting cardiac cells can divide both under physiological conditions and in response to cardiac overload. Subsequent detailed *in vitro* studies, supported by video microscopy, confirmed the mitotic capacity of cardiomyocytes, particularly mononucleated cardiac myocytes, despite their complex organization (Bersell et al., 2009). At baseline, the mitotic capacity is quite limited, but a considerable proportion of mitotic cardiomyocytes are observed in ischemic hearts and, compared to normal hearts, infarcted hearts have 70 times as many myocytes undergoing mitosis within the border zone (Beltrami et al., 2001).

The second source of mitotic cardiac cells considers the role of cardiac stem cells (CSCs). In 2003, the heart was shown to be regulated by its own pool of stem cells (Beltrami et al., 2003), which established the role of these multipotent cells in regulating the rate of cellular turnover and preserving organ homeostasis.

Cardiac Stem Cells

CSCs were first isolated by Beltrami et al. (2003) and characterized as a population of cells that were positive for the c-kit surface receptor (Di Felice et al., 2009). In addition to the presence of this receptor, CSCs exhibit clonogenic and self-renewal capacities and

multipotentiality, allowing them to differentiate along the three main cardiac lineages: myocytes, endothelial cells and smooth muscle cells (Di Felice et al., 2009) (**Figure 1**).

In addition to c-kit, other specific phenotypic markers define other "types" of CSCs, although some of these markers may be co-expressed by some cells. CSCs of particular interest include (i) c-kit$^+$; (ii) side population cells; (iii) Sca-1$^+$; (iv) Isl1$^+$; and (v) CSCs derived from cardiospheres (Chan et al., 2009). These CSCs all exhibit properties consistent with "real stem cells," including the following: (i) a lack of complete differentiation; (ii) the ability to divide without limitation; (iii) symmetrical division to generate two daughter stem cells to expand the stem cell compartment of the heart, i.e., self-renewal, or even asymmetrical to generate one daughter stem cell and a cell bound to a specific cellular lineage (Urbanek et al., 2006; Kajstura et al., 2010b) that subsequently undergoes terminal cellular differentiation (Raff, 2003; Leri et al., 2005).

c-Kit$^+$ Cardiac Stem Cells

c-Kit$^+$ CSCs are undifferentiated cells whose *in vitro* and *in vivo* properties are essentially identical and indistinguishable between species (Ferreira-Martins et al., 2012). c-Kit is a transmembrane receptor for a tyrosine kinase factor, and its ligand–stem cell factor (SCF)–is an early hematopoietic growth factor (Chen et al., 2013). c-Kit$^+$ cells are the most widely studied CSCs. These cells are one-tenth the size of cardiomyocytes and may express cardiac-specific-lineage transcription factors such as Nkx2.5, GATA4, and Mef2 (Beltrami et al., 2003; Barile et al., 2007). Their transcriptional profile indicates that c-Kit$^+$ cells are the most primitive population present in the heart and may play a role in early mesodermal development and stem-cell signaling pathways (Dey et al., 2013). Because the c-Kit receptor is also expressed by various differentiated adult cells, such as mast cells (Fang et al., 2012), in addition to being positive for c-kit, CSCs must also be negative for various cell-specific lineage markers (e.g., c-Kit$^+$Lin$^-$).

c-Kit$^+$Lin$^-$ CSCs are found in small clusters in the interstices between well-differentiated myocytes, in which it is possible to observe cells at several stages of early cardiac myogenic differentiation based on their expression of the characteristic transcription factors, allowing one to infer that these clusters represent myogenic precursors and progenitor cells derived from the activation of more primitive stem cells (Beltrami et al., 2003).

Human cardiac homeostasis is closely related to β-adrenergic signaling throughout life, and an important study demonstrated the existence of a direct relationship between the β-adrenergic receptor system and c-Kit$^+$ CSCs (Khan et al., 2013). c-Kit$^+$ CSCs exhibit cardiac β2-adrenergic receptors at their membranes; and the function assigned to them is to stimulate multipotent CSCs to proliferate via the ERK-Akt pathway (Khan et al., 2013).

When isolated c-Kit$^+$ cells are grown in suspension, they form spheres containing hundreds of cells similar to the pseudo-embryoid bodies formed by neural stem cells (neurospheres). By

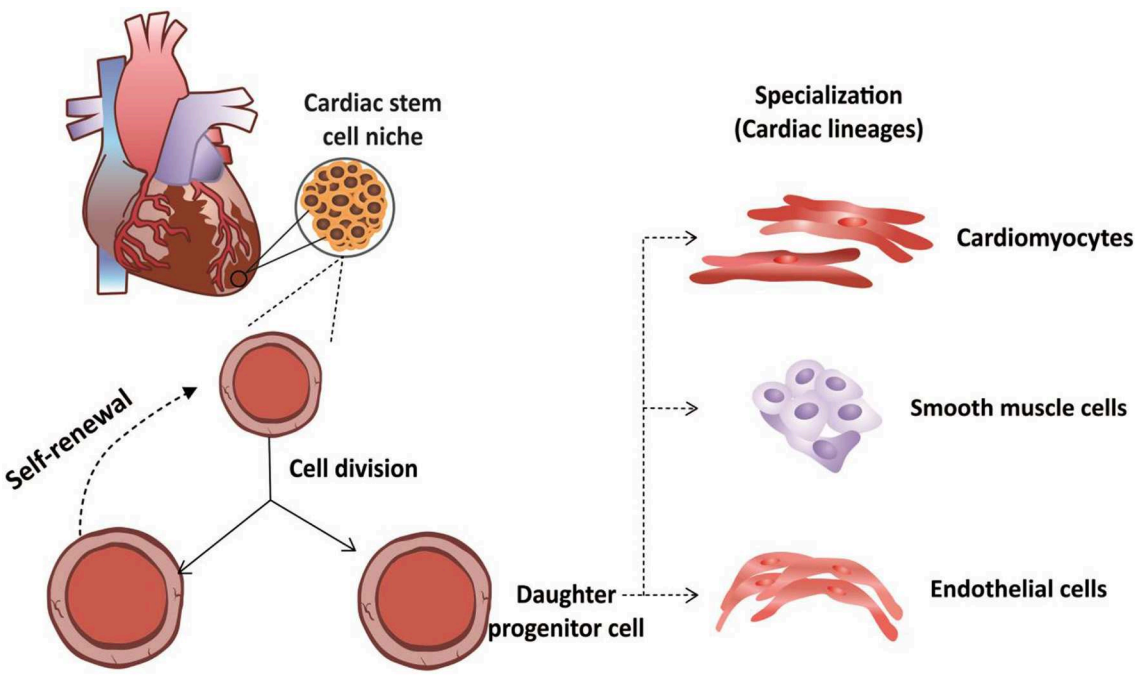

FIGURE 1 | Functional properties of cardiac stem cells. Cardiac stem cells are not differentiated cells and can divide without limitation. During cellular division, these cells can divide through symmetrical division to increase their numbers. Alternatively, these stem cells can undergo asymmetrical cellular division to produce both a daughter stem cell and a progenitor cell, the latter of which can differentiates along the three major cardiac lineages: cardiomyocytes, endothelial cells or smooth muscle cells.

analogy, these spheres were called cardiospheres and represent a population of multipotent cells with distinct characteristics (Torella et al., 2007).

Cardiosphere-derived Stem Cells

Initially isolated by Messina et al. (2004), cardiosphere-derived cells (CDCs) can differentiate into myocytes, endothelial cells and smooth muscle cells (Li et al., 2010). CDCs have been characterized as clonogenic cells, and they express markers of stem cells and endothelial progenitor cells (Messina et al., 2004). Stem cells derived from cardiospheres in a three-dimensional configuration exhibit a higher rate of c-Kit expression and increased expression of Nanog and SOX2, two important stem-cell transcription factors (Li et al., 2010).

A recent report investigating stem cell surface markers showed that CDCs are $CD105^+$ stromal cells of intrinsic cardiac origin that include a variable fraction of CD90 (Thy-1)-positive cells and a small minority of c-kit^+ cells, which have been argued to represent cardiac progenitors (Cheng et al., 2014). A clinical trial in which CDCs were transplanted into infarcted areas revealed that the c-kit^+ cells in cardiospheres are not an important determinant of the therapeutic efficacy of CDCs, whereas $CD90^+$ cells appear to undermine the overall benefit of CDC therapy (Cheng et al., 2014). Thus, c-kit expression on CDCs may be irrelevant to the therapeutic efficacy of this stem cell type. Moreover, the dissociation of cardiospheres into single cells appears to decrease their expression of extracellular matrix and adhesion molecules and affect their resistance to oxidative stress, negating the improved cellular engraftment and functional benefit of CDCs *in vivo* (Li et al., 2010).

Sca-1^+ Cardiac Stem Cells

Sca-1^+ cells, which express the surface marker stem cell antigen 1, were initially isolated from mouse hearts by Oh et al. (2003) and Matsuura et al. (2004), who demonstrated that these cells can differentiate into distinct cell lines *in vitro* (Di Felice et al., 2009). This differentiation ability is retained even after long-term propagation *in vitro* (Wang et al., 2014). Sca-1 is a convenient marker for stem-cell studies because it is expressed by stem/progenitor cells and its expression is upregulated in a variety of murine cancer stem cells. Recent studies have identified Sca-1^+ in various tissues, such as the mammary gland (Welm et al., 2002), prostate (Xin et al., 2005), dermis (Toma et al., 2005), skeletal muscle (Gussoni et al., 1999), heart (Rosenblatt-velin et al., 2005), and liver (Wulf et al., 2003). However, in all cases, it is unclear if the Sca-1^+ populations are tissue-specific precursor/stem cells or are the hematopoietic, mesenchymal, or endothelial precursor/stem cells associated with these tissues (Holmes and Stanford, 2007). The Sca-1 marker is used to characterize CSCs only in association with other specific markers to exclude differentiated cells and cells of other lineages.

Cardiac Sca-1^+ cells are capable of cardiac repair and have high levels of telomerase. These cells have the ability to migrate and home to areas of injured myocardium (Oh et al., 2003). *In vitro*, they can be propagated for a long period without any significant changes in marker expression (Wang et al., 2014). Molecular identification studies have indicated that Sca-1^+ cells are more strongly correlated with cardiomyocytes compared to c-Kit^+ cells (Dey et al., 2013). Moreover, Sca-1^+ cells obtained from adult hearts express GATA4 and Csx/Nkx-2.5, suggesting that they are committed to the cardiomyocyte lineage to some degree (Matsuura et al., 2004).

Some Sca-1^+ cells are committed to the cardiomyogenic lineage before birth; subsequently, Sca1-derived cells play a significant role by contributing continuously to cardiomyogenesis (Uchida et al., 2013). However, it appears that $Sca1^+$ cells are more responsive to inductive cues that promote differentiation into cardiomyocytes in healthy compared to diseased myocardium (Uchida et al., 2013), which might explain the limited but important contribution of Sca-1^+ CSCs to the adult myocardium. Recent findings partly corroborate this theory, demonstrating the relevant role of endothelial cells in generating cardiomyocyte progeny in the adult heart, with direct involvement of cardiac Sca-1^+ cells in this process (Fioret et al., 2014). Lineage tracing experiments have revealed endothelial progeny in perivascular areas, including Sca-1^+ cardiac progenitor cells, suggesting that a significant fraction of $Sca1^+$ CSCs are descendants of endothelial cells (Fioret et al., 2014). These Sca-1^+ cells proliferate, leave the coronary niche, and differentiate into cardiomyocytes, which indicates that the coronary vessels serve as an Sca-1 cell niche and further suggest that a negative impact on this niche environment evoked by any type of insult to coronary vessels could affect the proliferation and differentiation of CSCs (Fioret et al., 2014).

Side-population Stem Cells

Another phenotypic class of CSCs is side population cells and was initially discovered by Hierlihy et al. (2002) based on their identification of cells sensitive to verapamil in the adult human myocardium. These cells are characterized by their ability to promote the efflux of metabolic markers, such as Hoechst dye, due to high expression of membrane pumps and genes conferring resistance to multiple drugs (Hierlihy et al., 2002; Martin et al., 2004). This efflux ability is due to their expression of the Abcg2 protein, which belongs to the family of transporters linked to the ATP cassette (ABC transporters), early during cardiac development, which endows these cells with their characteristic phenotype (Di Felice et al., 2009). Under pathological conditions, human Abcg2 mRNA levels increase, suggesting the possibility of the activation and proliferation of side population cells in response to cardiac diseases in an attempt to promote the reparative potential (Smith et al., 2008b). Increased numbers of cells expressing Abcg2 have also been detected in the marginal zone of acute infarcted myocardium (Barile et al., 2007).

When co-cultured with cardiomyocytes, side population cells express markers of terminal differentiation and exhibit the electrophysiological characteristics of differentiated cardiac myocytes, including their response to β-adrenergic agonists (Barile et al., 2007). Under physiological conditions, side population cells can be isolated only from the hearts of very young mammals,

and the number of these cells decreases rapidly during the first weeks of life (Barile et al., 2007). Approximately 93% of side population cells are also positive for Sca-1 (Oh et al., 2003).

Isl1$^+$ Stem Cells

The characteristic marker of Isl1$^+$ cells is the LIM-homeodomain transcription factor Islet-1 (Laugwitz et al., 2005). Some of the distribution features of these cells suggest that Isl1$^+$ cells are organ-specific progenitor cells remaining from the fetal progenitor population (Laugwitz et al., 2005). Murine or human Isl1$^+$ cells derived from embryonic stem cells have been shown to give rise to cardiomyocytes, which exhibit appropriate electrophysiological properties, such as action potentials and Ca^{2+} transients (Cagavi et al., 2014), as well as smooth muscle cells and endothelial cells (Lui et al., 2013). Contrary to previous assumptions, Isl1$^+$ cells are not exclusively in an embryonic state because there is a subpopulation of endogenous CSCs that persist throughout life and co-express both c-kit and isl1 (Fuentes et al., 2013). Because all progenitor cells expressing Isl1 also express c-kit but not all c-kit$^+$ cells express Isl1, Isl1$^+$c-kit$^+$ progenitor cells may be a subpopulation of c-kit$^+$ progenitors (Fuentes et al., 2013).

Comparisons of embryonic and adult Isl1$^+$ cells have revealed that their significantly different levels of proliferative and migratory abilities are a consequence of age (Fuentes et al., 2013). Cardiac progenitor cells derived from embryonic stem cells may be a valid source for cell-based cardiac-repair therapies because their use would address the age-related decline in cardiac progenitor cell function; furthermore, the potential risk of teratoma formation and genomic alterations attributed to embryonic cells is minimal for Isl1$^+$ cells because they are highly committed cardiovascular cells (Cagavi et al., 2014).

Cardiac Mesenchymal Stem Cells

Another class of multipotent cells present in the heart are cardiac mesenchymal stem cells (cMSCs), which have been referred to in the literature as cardiac mesenchymal stem-like cells (Ryzhov et al., 2014) and cardiac mesenchymal-like stromal cells (Vecellio et al., 2012). MSCs were initially derived from the plastic-adherent fraction of components of mononuclear cells isolated by density-gradient centrifugation of bone marrow cells and culturing on an adherent surface (Friedenstein et al., 1974). MSCs have since been derived from many organs other than bone marrow (da Silva Meirelles et al., 2006) and were recently, identified in the cardiac stroma (Chong et al., 2011).

The phenotypic characterization of this cell type is complex, and there is not a specific marker or combination of markers to identify MSCs (Javazon et al., 2004). In culture, the phenotype of MSCs is altered; the surface markers of freshly isolated mesenchymal cells differ from those maintained in culture for a long period (Eggenhofer et al., 2014). In addition to the absence of a "phenotypic marker," the characteristics of MSCs differ depending on the species of origin (Javazon et al., 2004). Therefore, MSCs are usually defined based on a combination of physical, morphological, phenotypic, and functional properties (Javazon et al., 2004; Le Blanc and Ringdén, 2005). The following minimal criteria have been established for the identification of MSCs: (i) adherence to plastic; (ii) adipogenic, chondrogenic, and osteogenic differentiation capacities; (iii) expression of CD73, CD90 and CD105 and the absence of surface markers such as CD45, CD34, CD11b and CD14, CD79α or CD19 and HLA-DR.; and (iv) ability to generate colony-forming unit fibroblasts (CFU-Fs) (Dominici et al., 2006; Gambini et al., 2011).

In contrast to other stem cells present in the heart, MSCs lose their multipotentiality with passage in culture and enter senescence (Javazon et al., 2004), which means that the growth potential of these cells is limited. Bulk cultures of freshly isolated cardiac CFU-Fs grow exponentially for approximately 40 passages before entering senescence (Chong et al., 2011). Despite this limitation, these cells have important regenerative, angiogenic, antiapoptotic, and immunosuppressive properties (Nora et al., 2012).

MSCs can suppress monocyte differentiation into dendritic cells and down-regulate the expression of costimulatory molecules by mature dendritic cells, thereby decreasing their secretion of IL-12 and suppressing T-cell activation and proliferation (Jiang et al., 2005). In addition, MSCs regulate the function of macrophages by polarizing these cells preferentially to the M2 anti-inflammatory phenotype and consequently creating an environment favorable for accommodating therapeutic MSCs (Cho et al., 2014).

In the heart, cardiac MSCs are negative for c-Kit and positive for both pericyte (CD146$^+$) and fibroblast markers (vimentin and human fibroblast surface antigen) and exhibit features similar to those of syngeneic MSCs extracted from bone marrow, such as comparable morphology and the expression of mesenchymal antigens (CD105, CD73, CD29, and CD44) (Vecellio et al., 2012). However, comparison of bone marrow and cardiac CFU-Fs revealed distinct lineage signatures, indicating that they arise from different progenitor beds during development (Chong et al., 2011). Thus, a preferable differentiation capacity appears to develop depending on the source of the MSCs (Kern et al., 2006).

Thus, given the criteria of each CSC "type" and the variety of primitive cells existing in the heart, it is unlikely that these CSCs perform the same biological functions (Dey et al., 2013). It remains to be elucidated whether phenotypically different CSCs are different types of undifferentiated cells originating from different precursors (Wang et al., 2014) or only represent different developmental stages of the same generic CSC (Torella et al., 2007; Ellison et al., 2013), with only modest variations in phenotype (Ferreira-Martins et al., 2012).

Organization and Distribution of Resident Stem Cells in the Heart

In the heart, the CSCs are organized in niches that are preferentially allocated in the atrium and ventricular apex, areas that are protected because they are exposed to low levels of hemodynamic stress (Leri, 2009). These niches are connected by supporting cells, such as fibroblasts and myocytes, highlighting the importance of connexins and cadherins, which play roles in

the formation of gap junctions and adherens junctions at the interfaces of these different cell types (Barile et al., 2007; Ferreira-Martins et al., 2012). These cardiac niches create the microenvironment necessary for the long-term residence, survival, and growth of CSCs (Urbanek et al., 2006), providing nutrition and enabling them to maintain tissue homeostasis (Barile et al., 2007). The homeostasis of these niches is mediated by symmetric and asymmetric mitosis, with a predominance of asymmetric mitosis, which ensures the protection of the pool of stem cells and the production of lineage-committed cells (Urbanek et al., 2006; Barile et al., 2007).

On the other hand, cardiac MSCs are located near pericytes and are widely distributed in different organs in association with vessel walls (da Silva Meirelles et al., 2006). In the heart, MSCs occupy a perivascular, adventitial niche and may represent a population of progenitor cells that are able to maintain the integrity of the matrix, stroma, and vessels of the heart and contribute to the parenchyma, particularly in cases of injury and disease (Chong et al., 2011).

Extracardiac Stem Cells in the Heart

Stem cells other than resident CSCs are found in the heart. Evidence for the presence of mobile stem cells in the heart was provided by human heart transplantation experiments in which a significant number of Y-chromosome positive myocytes and coronary vessels were observed in the transplanted heart when the donor was female and the recipient was male (Quaini et al., 2002). In this case, the male cells in the female heart suggested the existence of mobile stem cells that were capable of differentiating into cardiomyocytes, endothelial cells or vascular smooth muscle cells (Beltrami et al., 2003). When the origin and population dynamics of cardiac progenitor cells were investigated using a heterotopic mouse heart transplantation model, cells that migrated to the heart after transplantation gradually ceased expressing the hematopoietic markers CD45 and CD34 and began to express cardio-specific transcription factors such as GATA-4 and Nkx2.5, hence acquiring a cardiac-cell phenotype (Li et al., 2007). Conditions other than organ transplantation that have the potential to attract circulating stem cells and the potential sources of these cells are being investigated.

Hematopoietic stem cells (HSCs) reside predominantly in the bone marrow but can be found in the peripheral blood in low numbers (Wright et al., 2001) and in the spleen, which is considered an active hematopoietic organ because it serves as a reservoir for hematopoietic stem cells for relatively immediate use (Morita et al., 2011). Little is known about the physiological role of these HSCs in normal adult tissue homeostasis (Zhang et al., 2006), although it has been described that HSCs have the ability to acquire a cardiomyocyte phenotype (Orlic et al., 2001; Rota et al., 2007).

The chemokine SDF-1α (stromal-derived factor-1) has a well-described role in the subsequent migration and homing of stem cells. SDF-1α belongs to the subfamily of chemokines that can facilitate the transmigration of hematopoietic cells through the endothelial cell barrier (Chen et al., 2013). In an infarction model, the attraction exercised by SDF-1 is not homogeneous for the different types of stem cells because more c-kit$^+$ than Sca-1$^+$ cells were recruited, for example (Zhang et al., 2007). This chemokine also participates in the recruitment of resident CSCs in specific situations by binding to the CXCR4 molecules of these cells (Zakharova et al., 2010; Wang et al., 2012; Yan et al., 2012; Chen et al., 2013; Ellison et al., 2013). The up-regulation of CXCR4 expression increases the vascularization of the injured myocardium, and the SDF-1/CXCR4 axis appears to be particularly important in the chemotaxis, homing, engraftment and attachment of progenitor cells to the injured myocardium (Wang et al., 2006b).

Hypoxic preconditioning increases the expression of CXCR4 in c-Kit$^+$ cells, which indicates that this response is related to the promotion of cellular survival achieved by activating the SDF1-α/CXCR4 axis (Yan et al., 2012). Furthermore, the rapid increase in chemokines/cytokines such as SDF-1α contributes to the upregulation of MMP-9 (matrix-metalloproteinase 9) expression, which is involved in the recruitment of hematopoietic stem cells (Heissig et al., 2002). Matrix metalloproteinases are a family of endopeptidases that act as physiological regulators of the extracellular matrix (Shah et al., 2011). For example, upon cardiac injury, the activation of c-kit$^+$ cells via an MMP-9 dependent pathway is required to mobilize progenitor cells from bone marrow to home to the heart (Fazel et al., 2008). In an acute myocardial infarction model, an increase in MMP-9 levels was observed, which may represent an endogenous mechanism for the recruitment of undifferentiated stem cells to the area of myocardial injury (Shah et al., 2011).

It is not clear if the cells attracted to the injured heart due to chemokine stimulus or stimulation by other factors released by the remaining heart cells or, in the absence of injury, due to the growth factors released by the heart are able to acquire a differentiated cardiac-cell phenotype. Using parabiotic pairs of animals, Wagers et al. (2002) observed that despite high levels of cross hematopoietic grafting, under physiological and stable conditions, multiple tissues showed no evidence of grafting of the circulating hematopoietic stem cells on non-hematopoietic tissue (Wagers et al., 2002). These findings suggested that hematopoietic cells do not develop into non-hematopoietic cells; however, the authors could not rule out atypical properties arising in response to severe injury or selective pressure (Wagers et al., 2002). Their theory was that at steady state, tissue regeneration appears to derive predominantly from progenitor cells resident in the tissue rather than from circulating cells (Wagers et al., 2002). Subsequently, to investigate cellular migration in response to pathological stimulus, Balsam et al. (2004) performed a left anterior descending coronary-artery ligation immediately after parabiosis surgery in mice. After 8 weeks, large numbers of GFP$^+$ cells were observed surrounding the scar tissue; however, these cells co-expressed CD45, some co-expressed cell marker B (B220), a few co-expressed a T-cell marker (CD3), and none expressed cardiac or smooth muscle markers (Balsam et al., 2004). These results suggest that even after cardiac injury, circulating cells do not regenerate myocardium. However, the descending artery was ligated immediately after the animals were joined, and circulatory chimerism was not yet established, which may have led to an underestimation

of the cells that migrated to the lesion foci (Aicher et al., 2007).

In another model used to investigate stem-cell migration, mice were subjected to irradiation, and bone marrow cells were replaced by eGFP$^+$ bone marrow cells. After cardiac injury, several types of histological analysis revealed that no cardiac cells with fluorescent-protein expression were present in the heart, indicating that stem cells derived from the bone marrow do not directly contribute to cardiomyocyte formation in injured or uninjured hearts (Ellison et al., 2013). Thus, the currently available evidence indicates that the resident or organ-specific stem cells contribute to the maintenance of tissue homeostasis, whereas the non-resident circulating stem cells that are attracted to organs minimally contribute to this physiological process, highlighting the restorative activities of these cells when attracted to an organ by certain stimuli (**Figure 2**), which was first demonstrated in 2002 via chimerism in a post-transplantation organ (Laflamme, 2002; Quaini et al., 2002; Angelini et al., 2007).

Cardiac Stem Cells under Homeostatic or Unstable Conditions

In addition to contributing to the maintenance of tissue homeostasis (Ferreira-Martins et al., 2012; Waring et al., 2014), CSCs participate in reparative processes under pathological conditions (Bailey et al., 2009; Ellison et al., 2013). However, extensive damage cannot be completely reversed, i.e., the regenerative potential of stem cells in damaged tissues is limited (Bailey et al., 2009; Chan et al., 2009; Hatzistergos et al., 2010). Therefore, massive degenerative events such as myocardial infarction cannot be counteracted by resident CSCs (Smith et al., 2008b).

Under physiological conditions, the pool of resident c-kit$^+$ CSCs present during early cardiac development were suggested to be responsible for expanding the myocyte mass during embryonic, fetal, and immediate postnatal development (Ferreira-Martins et al., 2012). However, the results of a recent study overturned this assumption, by demonstrating, using a very elegant model and analyzing different stages of cardiac development as well as the postnatal period, that the c-kit$^+$ CSCs contributed minimally to cardiomyocyte formation (van Berlo et al., 2014). By contrast, these cells play a significant role in the origin of endothelial cells (van Berlo et al., 2014). Furthermore, the physiological role of c-Kit$^+$ CSCs includes paracrine activity. *In vitro* experiments revealed an increased rate of cardiomyocyte survival when c-Kit$^+$ CSCs were co-cultured with adult rat cardiomyocytes, and the secretion of growth factors, such as IGF-1 (insulin-like growth factor 1) and VEGF (vascular endothelial growth factor), appeared to be responsible for the beneficial effect (Miyamoto et al., 2010). In addition, with respect to cardiac repair after heart injury, c-Kit$^+$ CSCs can acquire a cardiomyocyte phenotype (Ellison et al., 2013), highlighting the different activities of these multipotent cells under physiological and pathological conditions.

Certain conditions can lead to functional impairment of CSCs. In the pathological heart, the homeostatic control of stem cell growth is defective, resulting in cellular senescence (Chimenti et al., 2003; Urbanek et al., 2005) and apoptosis (Urbanek et al., 2005). Thus, although the number of CSCs increases after an ischemic cardiac event, the level of regeneration is not in the expected range, which is at least partially attributable to the acquisition of a senescent phenotype by many CSCs in infarcted hearts. These cells thus lose their regenerative capacity and become non-cycling, non-differentiating cells (Urbanek et al., 2005).

Functional problems also occur in CSCs in chronic systemic disease, and these changes have implications for the heart. Diabetes leads to decompensated myopathy, an etiology that is poorly understood. Because the heart constantly renews itself, an imbalance between cellular death and regeneration may occur in diabetes, and this imbalance may be mediated by defects in the growth and survival of c-Kit$^+$ CSCs, which are massively reduced in number, as well as reductions in telomere length (Rota et al., 2006). The impairment in cardiac progenitor cell function as a consequence of the enhanced oxidative stress that occurs with this disease affects cellular turnover, resulting in an excessive number of old, dying, and poorly contracting myocytes and, ultimately, ventricular failure, which in turns may result in the pathological manifestations of diabetic myopathy (Rota et al., 2006). When diabetes is treated with antioxidants, the numbers and functional abilities of both progenitor cardiac cells and mature cardiac cells are preserved, and a marked recovery of ventricular function is observed (Delucchi et al., 2012), supporting the theory that oxidative stress in diabetes leads to the loss of CSCs.

The same damaging effects of oxidative stress appear to be involved in the cardiac injury observed in subjects treated with anti-tumor therapies. Anthracyclines are effective drugs for the treatment of some neoplastic diseases (De Angelis et al., 2010), but they have profound effects on the structure and function of the heart, which over time causes cardiomyopathy that evolves to congestive heart failure (Takemura and Fujiwara, 2007). The cardiotoxicity of the anthracyclines is dose dependent, limiting their aggressive use (De Angelis et al., 2010). *In vitro* studies have demonstrated that anthracyclines promote oxidative stress and the activation of p53, a protein that regulates the cell cycle, thereby affecting the growth and survival of c-Kit$^+$ CSCs. This effect supports the theory that defects in progenitor cell function may condition the development of cardiac myopathy *in vivo* (De Angelis et al., 2010; Prezioso et al., 2010). Thus, deleterious cardiac effects with different causes may be due to targeting of CSCs, emphasizing that injury to these cells that contribute to the maintenance of cardiac homeostasis will have inevitable cardiac consequences.

Chronological age is a major predictor of the presence of biomarkers of human CSC senescence such as telomere shortening, attenuated telomerase activity, telomere dysfunction, and the expression of $p21^{Cip1}$ and $p16^{INK4a}$, both of which are senescence-associated proteins (Cesselli et al., 2011), raising questions about the functional capacity of cells taken from aging hearts and hearts in chronic failure. Comparative analyses of CSCs isolated from transplanted hearts (both donor and explanted hearts) have revealed that the clonogenic capacity of CSCs in the heart in heart failure is three times lower than that of cells from healthy hearts. By contrast, the differentiation

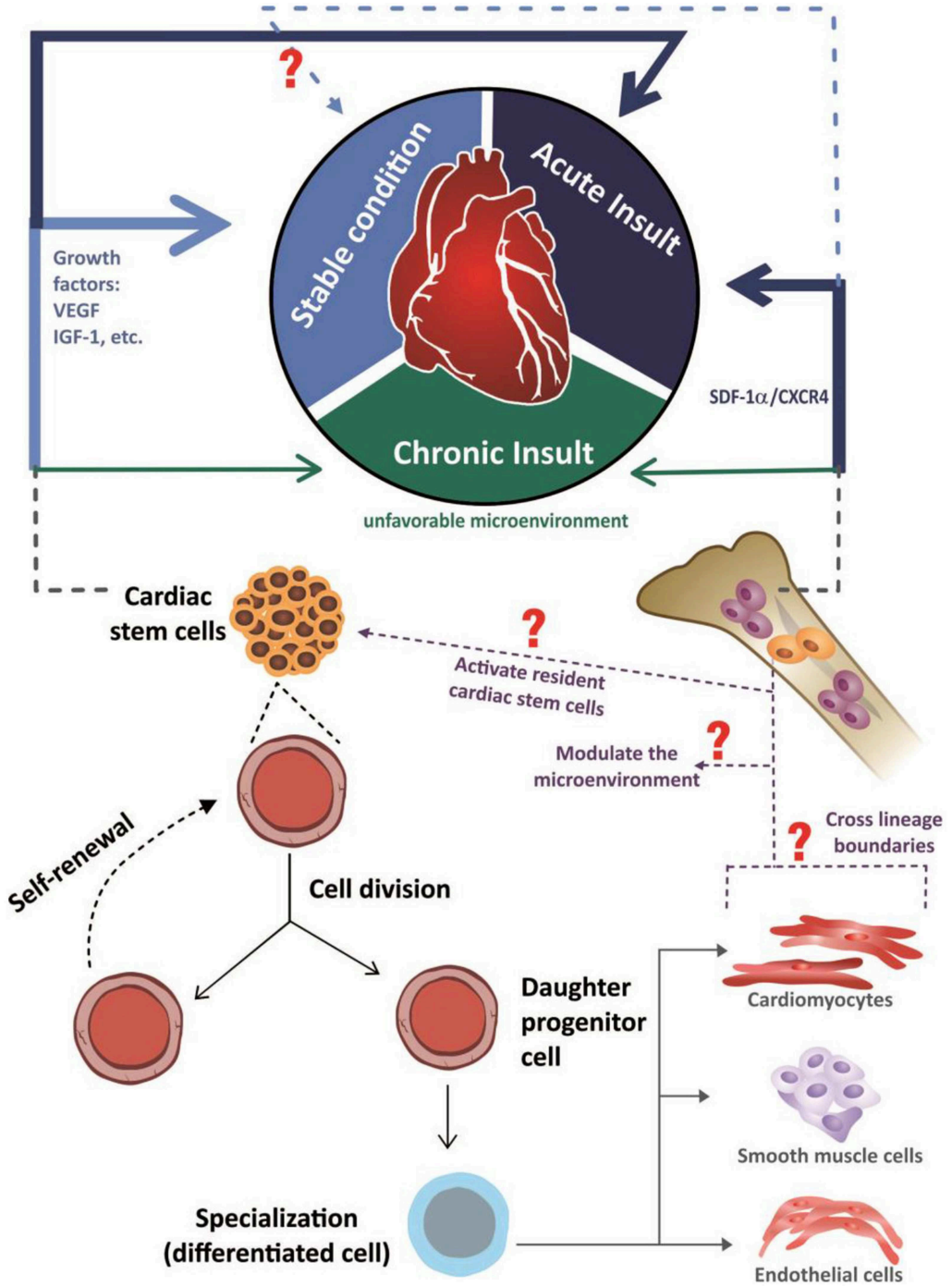

FIGURE 2 | Schematic representation of the mechanisms through which stem cells are involved in both cardiac homeostasis and in the heart repair. In response to acute insult, the damaged myocardium elicits the activation of cardiac and also extracardiac stem cells (represented by the continuous dark blue arrows). The cardiac stem cells are activated in response to growth factors, like VEGF (vascular endothelial growth factor) and IGF-1(insulin-like growth factor 1), while extracardiac stem cells (schematically represented by hematopoietic bone marrow cells) are chemo- attracted into the injured sites through the axis SDF-1α/CXCR4 (stromal-derived factor-1/receptor CXCR4). With the insult chronicity, the participation of cardiac and extracardiac stem cells in the damaged heart is reduced, possibly due to an unfavorable microenvironment to the action of these cells (represented by the continuous dark green arrows). Considering the uninjured heart, in a stable condition, the available information indicates that cardiac homeostasis is ensured by an active role of cardiac stem cells (represented by the continuous light blue arrow), with an unclear role of extracardiac stem cells (represented by light blue dashed arrow). Seeing both acute, chronic and stable conditions, in response to environmental stimuli, cardiac stem cells differentiate into cardiomyocytes, endothelial cells or smooth muscle cells and also, possibly, release soluble autocrine/paracrine factors that play roles in both stem cells self-renewal and myocardial protection/neovascularization respectively, while extra cardiac stem cells are involved in the beneficial modulation of the microenvironment, since the capacity of them to acquire a differentiated phenotype, crossing lineage boundaries, is questionable.

potentials of CSCs from healthy and infarcted hearts were comparable and yielded similar proportions of myocytes, smooth muscle cells, and endothelial cells, indicating the possibility of autologous transplantation in individuals with heart failure (Cesselli et al., 2011). Thus, even in decompensated hearts, there is a compartment of functionally competent CSCs that, despite lower growth kinetics, retain the ability to expand and differentiate. These characteristics are promising for the clinical application of these cells (Cesselli et al., 2011).

MSCs are affected by aging and exhibit diminished expression of Nanog, a transcription factor that retains MSCs in a primitive state, as well as increased adipocytic potential (Cieslik et al., 2011). Furthermore, fibroblasts derived from aged MSCs exhibit reduced expression of transforming growth factor-β (TGF- β) receptors, attenuated contractility and migratory ability (Cieslik et al., 2011), and decreased expression of α-SMA, an indicator of poor maturation into myofibroblasts (Cieslik et al., 2013). In addition, the fibroblasts derived from elderly MSCs exhibit increased expression of type I collagen due to the elevation of circulating plasma insulin levels with aging (Cieslik et al., 2013). Future *in vivo* studies using pathological models treated with cardiac MSCs isolated from animals of different ages could be able to reveal possible differences in the functional abilities of aged cells.

Cellular Therapy with Stem Cells as a Treatment Option

A massive number of cardiomyocytes die after a heart attack, and the basic concept for cellular therapy following myocardial infarction must consider this large-scale cellular loss resulting in heart failure (Lovell and Mathur, 2011). Therefore, treatments based on stem cell transplantation aim to avoid the loss or failure of cardiovascular function (Sánchez et al., 2010) and to rescue the numbers of cardiomyocytes to ensure good cardiac performance (Noseda and Schneider, 2010). It is important to repair not only the cardiomyocytes but also the endothelial and smooth muscle cells, which are crucial for developing a new network of arteries to bring nutrients and oxygen to the cardiomyocytes and restore organ function after heart damage. In the acute phase of a myocardial infarction (MI), the goals of cellular therapy are to prevent cardiomyocyte death, promote local neoangiogenesis, improve myocardial perfusion, and reduce local inflammatory responses (Takashima et al., 2013). Another major objective of an ideal clinical intervention is to avoid scar formation or replace scar tissue with functioning cardiac muscle tissue (Curtis and Russell, 2010).

Orlic et al. (2001) demonstrated that c-Kit^{+}Lin^{-} bone-marrow cells could regenerate the infarcted myocardium by demonstrating that the newly formed myocardium occupied 68% of the infarcted portion of the ventricle 9 days after transplanting the bone marrow cells. However, these results were not reproduced by other researchers, who consistently demonstrated that hematopoietic stem cells did not readily acquire a cardiac phenotype (Balsam et al., 2004; Murry et al., 2004). This discrepancy was attributed to the assays used to detect cardiomyogenic differentiation, which was exclusively based on immunofluorescence staining and is thus difficult to use in tissues with high levels of non-specific autofluorescence, as is typically encountered in the infarcted heart (Murry et al., 2004).

However, despite the absence or extremely low rate of acquisition of a cardiac phenotype, experimental studies and, to a lesser extent, clinical studies (Schächinger et al., 2006; Tendera et al., 2009; Hare et al., 2012) have investigated the use of stem cells derived from bone marrow in cases of cardiac injury. Optimistic results have been reported, such as an acceleration of left ventricular ejection fraction (LVEF) recovery after acute myocardial infarction, although this was observed without a long-term benefit on left ventricular systolic function measured at 18 months after bone marrow cells transfer (Meyer et al., 2006). However, a trend in favor of this cell therapy is defended, particularly considering patients with severely impaired LVEF at baseline (Tendera et al., 2009). The favorable results arising from the transplantation of bone marrow cells have been attributed to humoral (Cho et al., 2007) and paracrine effects rather than the direct regeneration of various cardiac cells through transdifferentiation (Loffredo et al., 2011).

Importantly, bone marrow-derived stem cells are easy to obtain in sufficient numbers for transplantation, which hastens the transplantation process because it is not necessary to seed these cells in culture to expand their number. However, because the ability of this cell type to differentiate to replace dysfunctional cardiac tissue is limited, other stem cells with this capability have been sought. Thus, criteria for the ideal cell type for the promotion of consistent improvements in the damaged myocardium with a real potential to replace lost cells have been established. These cells should be capable of differentiating into functional cardiomyocytes and of forming new vessels (Sánchez et al., 2010), indicating cardiac commitment, and they should be able to integrate with the target tissue by developing gap junctions with host cells without inducing immune reactions. Lastly, from a practical standpoint, these cells should preferably exhibit some degree of resistance to ischemia to avoid massive apoptosis during cellular transfer (Gaetani et al., 2009).

Thus, among the types of stem cells studied to date, cardiac progenitor/stem cells appear to represent an attractive option for use in clinical trials because they are intrinsically more likely to possess all of the characteristics required to repair the damaged heart (Lovell and Mathur, 2011) and improve cardiac function after myocardial injury (Torella et al., 2007).

Cellular Therapy with Cardiac Stem Cells–Experimental Models

Initial studies of CSCs considered the potential for tropism to the site of cardiac injury but demonstrated that all regenerating cells were located in the necrotic area and not the spared myocardium, even though the cells were injected at the border between the ischemic and healthy myocardium (Beltrami et al., 2003). The injured myocardium provides a *milieu* that supports the homing, nesting, survival, and differentiation of CSCs (Ellison et al., 2013). Furthermore, cardiac tropism is

CXCR4-SDF-1 axis-dependent, and the enhanced expression of SDF-1 in the surviving myocardium serves as a positive chemotactic agent (Ellison et al., 2013), activating the ligands of the CSCs themselves through auto/paracrine feedback (Torella et al., 2007).

Therefore, the potential of CSCs as a convenient source for autologous stem-cell therapy has been considered for clinical trials of myocardial regeneration (Gaetani et al., 2009). One of the main advantages of autologous transplantation is the elimination of the potential for a potent immunological response against the allograft in the host. However, CSCs are significantly more difficult to harvest and isolate than bone marrow-derived mononuclear cells (Lovell and Mathur, 2011). Furthermore, the number of cells that can be obtained from an individual is quite small; however, these cells can be expanded in culture (Gaetani et al., 2009). Thus, it is feasible that cells isolated from very small fragments of human myocardium and expanded many-fold *in vitro* could reach numbers appropriate for *in vivo* transplantation in patients while retaining their differentiation potential (Messina et al., 2004).

Among the different stem cells types already described, c-kit$^+$ CSCs as well as cells derived from cardiospheres have been more frequently utilized in experimental and clinical studies because they are cell populations with well-defined characteristics and extraction and expansion methods. Moreover, compared to the other adult stem cells in the heart, they appear to be in the most primitive stage of differentiation.

Experimental models of myocardial infarction have demonstrated satisfactory responses after the administration of CSCs, such as an improvement of myocardial remodeling following injury via a combination of a reduction in the cell death rate, induction of endogenous repair mechanisms, and the contribution of new cells to the injured myocardium (Bailey et al., 2009). The results suggested that c-Kit$^+$ CSCs promote myocardial regeneration, which contributes to the recovery of the structure and function of the damaged heart (De Angelis et al., 2010).

Studies employing a model of diffuse myocardial damage causing acute heart failure revealed that the extent of myocardial injury is even more pronounced when endogenous c-Kit$^+$ CSCs are ablated from the adult myocardium (Ellison et al., 2013). Following the administration of c-Kit$^+$ CSCs, cardiac function was restored through the regeneration of the lost cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts and even through the maintenance of the stemness of the stem cells (Ellison et al., 2013).

When cells derived from cardiospheres were administrated to transgenic animals with dilated cardiomyopathy, anti-apoptotic, anti-fibrotic and cardioproliferative effects were observed, as well as a decrease in mortality (Aminzadeh et al., 2015). However, the beneficial effects of administering these cells to treat pathological processes were most pronounced when the cells were supplied in the form of cardiospheres, thereby ensuring that a niche similar to that self-developed through these stem-cell structures was maintained (Li et al., 2010).

In an *in vivo* study in which Sca-1$^+$ CSCs were transplanted in a mouse cardiac-infarction model, significant angiogenesis was observed in the peri-infarction regions, although with minimal differentiation of the Sca-1$^+$ cells into endothelial cells, contrary to the substantial *in vitro* endothelial-cell differentiation capacity of these cells (Wang et al., 2006a). These cells transdifferentiated into cardiomyocytes more frequently, albeit the absolute number of newly differentiated cardiomyocytes was low and most likely insufficient to make substantial direct contributions to left ventricular structure, function, or bioenergetic characteristics (Wang et al., 2006a). However, despite the low rate of transdifferentiation of these cells, transplantation of Sca-1$^+$ CSCs attenuated the development of adverse structural, functional, and energetic abnormalities associated with left ventricular remodeling post-myocardial infarction, likely due to paracrine effects (Wang et al., 2006a).

When the effects of bone marrow-derived mesenchymal stem cells were compared to those of mesenchymal stem cells derived from the human heart following direct injection into the myocardium of chronically infarcted rats, the cMSCs survived longer in the tissue, better promoted angiogenesis via paracrine mechanisms, and more efficiently differentiated into cardiomyocytes than the MSCs derived from the bone marrow (Rossini et al., 2011). In addition, combining these cells with other types of stem cells appears to be important. In an animal model, combining stromal cells derived from heart tissue with stem cells derived from cardiospheres resulted in increased survival of this cell group upon transplantation in the infarcted areas, and the cardiac contractile function and extent of replacement of the injured myocardium were optimized because this combination of cells promoted vascularization and cardiomyogenesis (Zakharova et al., 2010).

Furthermore, because the paracrine action exerted by MSCs can be attributed, at least in part, to the extracellular vesicles released by MSCs (previously referred to as microvesicles or exosomes) (Bian et al., 2014), which have the capacity to change the phenotype of injured cells through the horizontal transfer of mRNA, microRNA and proteins, it appears that these cells have the potential to be exploited as an alternative to stem cell-based therapy in novel therapeutic approaches to repair damaged tissues (Biancone et al., 2012). *In vivo,* the extracellular microvesicles derived from bone marrow-derived MSCs elicited neoangiogenesis in ischemic hearts and improved functional recovery in an rat cardiac-infarct model (Bian et al., 2014). Extracts of extracellular vesicles directly isolated from supernatants of cardiac MSCs might be used to treat cardiac injuries with even more promising results, although this topic awaits investigation.

Based on the encouraging results of experimental studies, a rapid transition from promising preclinical experiments to early clinical trials occurred (Lovell and Mathur, 2011), although numerous unresolved questions remain with respect to the underlying molecular mechanisms involved in cell-based therapies (Hoshino et al., 2007). Thus, cellular therapy has advanced rapidly as a treatment option, with some concern within the scientific community that this may have been occurring too quickly and uncritically (Smith et al., 2008a). The nature of this rapid transition meant that early studies considered the safety and feasibility of cellular therapy and were of sufficient power to assess its efficacy; however, these studies were sufficient

to provoke early optimism in the medical community, even it was based on the results of small studies (Lovell and Mathur, 2011).

Cellular Therapy with Cardiac Stem Cells–Clinical Studies

The large body of preclinical evidence motivated human clinical trials of CSCs (Bolli et al., 2011; Chugh et al., 2012). With the primary goal of investigating the safety and feasibility of using autologous CSCs for the treatment of heart failure resulting from ischemic heart disease, the SCIPIO (Stem Cell Infusion in Patients with Ischemic cardiOmyopathy) study, the first study of CSC treatment in humans, received approval to conduct a phase 1 clinical trial using CSCs (Chugh et al., 2012). Methodologically, the investigators used autologous intracoronary infusion of c-Kit$^+$/Lin$^-$ CSCs derived from auricles that were surgically harvested during coronary artery bypass graft (CABG) in patients with ischemic cardiomyopathy. The LVEF was improved by 8% at 4 months after stem-cell administration, in contrast to no change in the control patients. However, the phase I SCIPIO study was randomized and performed at a single center, and autologous CSCs were administered to patients with severe heart failure secondary to ischemic heart disease (Bolli et al., 2011). In addition, the increase in ejection fraction (EF) was even higher 1 year after the administration of CSCs, suggesting that the CSCs continued to improve left ventricular (LV) function over time, whereas the control group did not exhibit improvement in the EF over time, ruling out any spontaneous temporal benefit (Bolli et al., 2011). Furthermore, this improvement in LV function was coupled with a concomitant decrease in infarct size, which was consistently observed using three different methods of cardiac magnetic resonance and was accompanied by an increase in the LV viable mass, implying the robust regeneration of myocardial tissue (Chugh et al., 2012). The NYHA functional classification also improved in the group that received autologous CSC transplantation, as did their quality of life (Bolli et al., 2011). Thus, the purpose of the SCIPIO study, to investigate the safety of cellular administration, was achieved, and no adverse effects attributable to CSC administration were noted (Bolli et al., 2011), permitting the conclusion that the functional benefits of CSCs were inversely related to the baseline functional status of the myocardial region. That is, the lower the baseline function, the greater the improvement afforded by CSC infusion (Chugh et al., 2012). In addition to the safety of the procedure, this study also demonstrated the feasibility of isolating and expanding CSCs obtained from cardiac tissue during CABG surgery (Chugh et al., 2012).

The CADUCEUS (CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction) clinical trial was another prospective randomized trial of intracoronary infusions of autologous CDCs in patients with recent myocardial infarction and residual systolic dysfunction. The phase 1 trial revealed the feasibility and safety of this therapy, with no major adverse cardiovascular events or cardiac tumor development (Makkar et al., 2012). More importantly, CDCs treatment reduced the extent of the scar mass and increased the level of regional contractility observed at the 6-month follow-up but did not improve the end-diastolic volume, end-systolic volume or the LVEF (Makkar et al., 2012). The final results at the 1-year endpoint did not raise significant safety concerns and proved that, despite improvements in the scar size and regional functionality based on magnetic resonance imaging (MRI)-based analysis of myocardial regeneration and clinical correlates of the regenerative efficacy, no improvements in global function, such as improvements in the NYHA functional class, the peak VO_2, the distance walked in 6 min or the quality of life after therapy using CDCs, were detected (Malliaras et al., 2014). Covariate analysis revealed that a higher baseline scar size was strongly associated with a greater reduction in scar size 1 year after cellular infusion (Malliaras et al., 2014). Importantly, a patient who underwent late treatment and received the infused cells at 14 months post-MI responded similarly to patients who were infused earlier (at 1.5–3 months post-MI), indicating that CDCs administration might confer benefits to chronic ischemic cardiomyopathic patients similar to those observed in convalescent MI patients (Malliaras et al., 2014).

Another randomized, double-blind, placebo-controlled clinical trial (phase I/II) that is still in progress is using, in contrast to the CADUCEUS trial, allogeneic CDCs instead of autologous cells to achieve myocardial regeneration. The purpose of the ALLSTAR study (ALLogeneic heart STem cells to achieve myocArdial Regeneration) is to investigate the safety and efficacy of the treatment in decreasing infarct size, with the primary efficacy endpoint being the relative percentage improvement in infarct size as assessed using magnetic resonance imaging at 6 and 12 months post-infusion (Allogeneic Heart Stem Cells to Achieve Myocardial Regeneration (ALLSTAR) (NCT01458405)[2]. More recently, a phase I/II randomized, double-blind, placebo controlled clinical trial was proposed by a European study group initiative (CARE-MI) to investigate the therapeutic role of allogeneic CSCs in patients with congestive heart failure due to myocardial infarction (Crisostomo et al., 2015).

Despite the favorable results observed in some individuals treated with stem cells, certain clinical expectations were not achieved because the levels of improvement in cardiac tissue structure or functionality were less than those previously observed in experimental and pre-clinical trials. Although several challenges remain, such as optimization of the therapeutic protocols and technological improvement of stem cell delivery devices, it is believed that the current phase III studies will provide new insights and accelerate the further development and acceptance of cardiovascular regenerative medicine, particularly in relation to reduced mortality following stem cell therapy and the optimal sub selection of stem cells for clinical applications (Takashima et al., 2013; Cheng et al., 2014).

Although clinical studies using stem cells obtained from different sources for cardiac-repair purposes have generally demonstrated that CSC therapy is safe, it remains unclear why these trials have failed to translate preclinical expectations in humans

[2]Available at: https://clinicaltrials.gov/ct2/show/study/NCT01458405 Accessed January 10, 2015.

(Lovell and Mathur, 2011). Various explanations for this discrepancy have been suggested, such as the injection of an insufficient number of cells or the wrong cell type, the use of an inappropriate delivery method, inappropriate timing relative to the age of the infarct, and differences between animal models and humans, including the absence of comorbidities in the animal model (Lovell and Mathur, 2011) (**Figure 3**).

The results of both clinical and bench studies on this topic demonstrate that there is much that remains to be investigated in this field, particularly regarding the biodistribution of injected cells, the optimal method and route for stem cell delivery, the extent of cellular differentiation of these cells within the target area (Hoshino et al., 2007) the precise paracrine signals that drive the cell fate decision of multipotent progenitors, and the development of novel approaches to deliver these signals *in vivo* (Lui et al., 2013).

Cellular Therapy with Cardiac Stem Cells–Future Directions

It may be relevant to investigate the effects of administering combinations of stem cells derived from different sources as alternative therapies and to determine the ideal number of these cells to be used, the best timing of injection after damage has occurred, the optimal frequency of injections, and the mechanisms of action of the administered cells, as well as the extents of their homing/grafting and survival and their preferential homing routes (Sánchez et al., 2010). However, many clinical trials have demonstrated small improvements in FE that are sufficient for substantial clinical improvement following stem cell administration, indicating the relevance of this strategy (Lovell and Mathur, 2011). Based on these results, various alternatives are being formulated (Smith et al., 2008a).

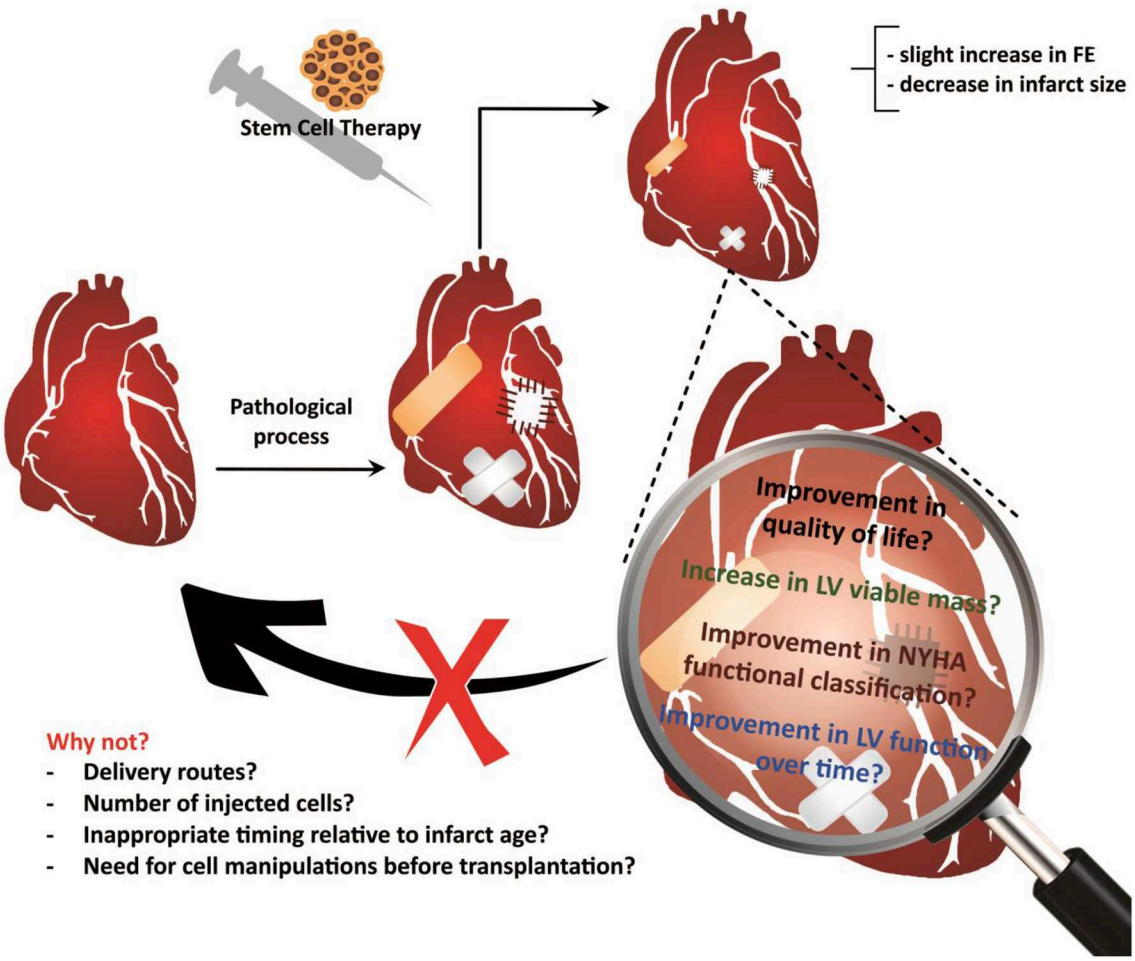

FIGURE 3 | Treatment using cardiac stem cells and associated outcomes. Results support the feasibility and safety of the cardiac stem cell delivery procedures, although the reports of outcomes have been limited and primarily concerned to functional improvements or reductions in the infarct size. Future studies will allow investigate other equally important outcomes, such as improvements in the quality of life, maintaining the improvement in ventricular function over time, and decreasing the mortality rate, among others. However, there is still much to be explored in basic studies, which may reveal the reasons why therapy using cardiac stem cells is not able to heal the injured heart.

In vitro studies have indicated the possibility of manipulating stem cells prior to transplantation to optimize their application. For example, when c-kit$^+$ CSCs exhibit an increased level of expression of the transcription factor GATA4, their cardiosphere-forming ability is reduced, indicating that their clonogenicity and multipotentiality are also decreased, whereas, their ability to differentiate into cardiomyocytes is increased (Miyamoto et al., 2010). *In vivo* assays are required to confirm these conclusions.

Thus, uncertainties remain about the next steps to advance the promising strategy of cellular therapy (**Figure 3**). Some have suggested a return to the bench, whereas others support the continuation of clinical practice because there are many examples of new treatments that have been implemented with only a provisional understanding of the underlying mechanisms, followed by elucidation of the mechanisms with clinical experience (Lovell and Mathur, 2011). Supporters of the continuity of clinical studies base their opinions on the fact that, to date, the follow-up period of subjects treated using CSCs is still short, and the vast majority of outcomes investigated were cardiac functional parameters or infarct size. Future studies may provide information about the impact of cellular therapy on the long-term quality of life of treated patients, the possible functional improvements that are maintained over time and the reduction of the rate of or lack of progression of heart failure or of the increase in the late mortality rate following cardiac injury.

The electrophysiological properties of the *in vivo* CSC-derived cardiomyocytes also remain to be investigated (Smith et al., 2008a) because they were demonstrated to be as important in tissue repopulation as the coupling of these newly formed cells to the residual myocyte populations in the heart. The functional integration of these cells was satisfactorily demonstrated in a preclinical study that used an *ex vivo* preparation and two-photon microscopic analysis (Bearzi et al., 2007), but the functional integration of these cells remains to be demonstrated in clinical studies. Likewise, understanding the physiology of the resident CSCs may lead to a better understanding of how to expand and induce these cells to differentiate into functional cardiomyocytes (Di Felice et al., 2009) as well as vascular and endothelial cells within the heart.

To summarize, the perspectives for future studies appear challenging yet encouraging; in particular, the interplay between basic and clinical research will be critical for the development of the field and for achievement of the final goal, which is a safe and efficient therapy utilizing cardiac stem cells to regenerate lost myocardium.

Conclusions

The currently available data encourage further investigation into the potential application of cardiac multipotent cells to treat the damaged heart. Although CSCs hold therapeutic promise for clinical applicability, much remains to be explored.

Author Contributions

CL drafted the study plan, designed the research plans and wrote the review. TA and CL contributed equally to the scientific research materials and the critical discussion of relevant issues. VS revised the manuscript critically for important intellectual content and coordinated the performance of this study.

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REVIEW
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Myocardial infarction: a critical role of macrophages in cardiac remodeling

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Ischemic heart disease is a common condition and a leading cause of mortality and morbidity. Macrophages, besides their role in host defense and tissue homeostasis, are critical players in the pathophysiological processes induced by myocardial infarction. In this article we will summarize the current understanding of the role of monocytes and macrophages in myocardial damage and cardiac remodeling in relation to their origin and developmental paths. Furthermore, we describe their potential implications in therapeutic strategies to modulate myocardial healing and regeneration.

Keywords: heart, development, myocardial infarction, macrophages, yolk sac, hematopoiesis, inflammation

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Introduction

Cardiac diseases, especially acute myocardial infarction and congestive heart failure, are among the most frequent causes of death in the western world and are on the uprise in developing countries. Even though the survival after myocardial infarction has significantly improved by early revascularization therapy and drug treatment, a significant number of patients develop heart failure. A broad spectrum of local and systemic mechanisms are initiated in myocardial infarction and contribute to cardiac remodeling. If uncontrolled, they may lead to negative changes in the geometry, structure and function of the ventricle and thus may have deleterious effects on cardiac function in the long term. Therefore, great interest lies in the discovery of new therapeutic strategies to treat the conditions, which precede heart failure.

In myocardial infarction, occlusion of a coronary vessel (i.e., after coronary artery plaque rupture) results in acutely diminished oxygen supply to the myocardium, which leads to myocyte necrosis and acute inflammation. As the human heart has only limited regeneration capacity (Bergmann et al., 2009), infarct healing is–in addition to restoration of oxygen supply–very much dependent on the inflammatory response. Macrophages are an integral part of the innate immune response. They are equipped with an array of pathogen recognition receptors which can activate phagocytosis of pathogens and the secretion of cytokines and chemokines. They present antigens on their cell surface by constitutively expressed major histocompatibility complex II (MHC II) and thereby interact closely with the adaptive immune system.

In vertebrates, macrophages are found in all organs, where they adopt distinct phenotypes and functions that are shaped by the environment of the organ of residence (Gosselin et al., 2014; Lavin et al., 2014). Macrophages are also found in larger numbers in the mammalian heart (Jung et al., 2000; Epelman et al., 2014a; Heidt et al., 2014; Molawi et al., 2014).

Macrophages have long been thought to be of importance in cardiac remodeling after myocardial infarction in humans (Mallory et al., 1939). In autopsy reports macrophage numbers associated closely with different stages of myocardial infarction (Chang et al., 2013). Likewise, macrophages

play a critical role in experimental infarction models including rodents (Yang et al., 2002) and swine (Vilahur et al., 2011). In this review we will summarize the functions of monocytes and macrophages in myocardial infarction and outline potential therapeutic strategies to improve infarct healing and outcome by manipulating distinct subsets of mononuclear phagocytes.

Recruitment of Monocytes Into the Ischemic Heart

The immune response to myocardial ischemia can be divided into distinct but overlapping inflammatory phases, in a simplified view an early (3–4 days) and a late (7–10 days) phase. The kinetics are dependent on the pathological conditions and respective disease models (e.g., species analyzed, chronic ligation vs. ischemia and reperfusion model). Within 30 min after induction of infarction through ligation of the left anterior descending artery (LAD), monocytes are recruited to the site of injury as shown by *in vivo* microscopy (Jung et al., 2013; Nahrendorf and Swirski, 2013). However, the applied reporter mice did not permit distinguishing between the monocyte subsets involved. In mice, blood monocytes are divided into two principal subsets: Ly6C^{hi} CCR2^{+} CX3CR1^{lo} "inflammatory" monocytes (CD14^{+} in humans) and a less frequent subset of Ly6C^{lo} CCR2^{-} CX3CR1^{hi} monocytes (CD14^{dim} CD16^{+} in humans). Ly6C^{lo} monocyte "patrol" the luminal side of the endothelium of small blood vessels under homeostatic and inflammatory conditions (Auffray et al., 2007). Initial work suggested that the early phase after infarction is dominated by inflammatory (Ly6C^{hi}) monocytes (Nahrendorf et al., 2007). They phagocytose toxic molecules and cell debris, have proteolytic activity and produce inflammatory cytokines like interleukin (IL)-1α, IL-6 and TNFα (Nahrendorf et al., 2007; Troidl et al., 2009; Woollard and Geissmann, 2010; Leuschner et al., 2012). Thus, by exercising inflammatory functions they are potentially harmful in this early phase and promote myocardial damage (Kaikita et al., 2004). In contrast to Ly6C^{hi} monocytes, non-classical Ly6C^{lo} monocytes were thought to play a role in the later stages after infarction (Nahrendorf et al., 2007). Through secretion of anti-inflammatory cytokines and growth factors, like VEGF and TGF-ß, they could promote angiogenesis, extracellular matrix synthesis and myocardial healing (Nahrendorf et al., 2007; Troidl et al., 2009). However, the clear categorization of monocyte subsets into distinct phases and functions after infarction has recently been challenged. Ly6C^{lo} monocytes are critically dependent on the transcription factor Nr4a1 for their development and survival (Hanna et al., 2011). Interestingly, absence of Ly6C^{lo} monocytes in Nr4a1-deficient animals does not abrogate the bi-phasic inflammatory response. These findings might be explained by the concept that Ly6C^{lo} cells derive from Ly6C^{hi} monocytes, which display high plasticity and develop into either proinflammatory or anti-inflammatory monocytes/macrophages (Hilgendorf et al., 2014; Ismahil et al., 2014). The transcription factor Nr4a1 thereby seems to serve as a master switch that controls the polarity of monocytes and could represent a potential target to modulate the local inflammatory response, which drives adverse cardiac remodeling. The findings also indicate that inflammatory Ly6C^{hi} monocytes are the predominant monocyte subset to be recruited to the infarct area not only in the first days of infarction but throughout the course of post-infarct remodeling (Nahrendorf et al., 2010; Hilgendorf et al., 2014; Ismahil et al., 2014). This is supported by data in humans demonstrating that peak levels of the corresponding subset of inflammatory (CD14^{+}) monocytes correlate negatively with the recovery of left ventricular function after acute myocardial infarction (Tsujioka et al., 2009). In mice, inhibition of Ly6C^{hi} inflammatory monocyte recruitment into the infarcted myocardium by genetic ablation of C-C chemokine receptor type 2 (CCR2) reduced adverse cardiac remodeling (Kaikita et al., 2004).

Future studies are needed to clearly define the role and impact of monocyte subsets in the course of infarction. In any case, preclinical (Panizzi et al., 2010) and clinical data (Tsujioka et al., 2009; Aoki et al., 2011) indicate that monocyte/macrophage recruitment needs to be tightly controlled as dysregulated infiltration results in infarct expansion, left ventricular dilation, and heart failure. In detail, monocytosis disturbs resolution of inflammation in murine infarcts and consequently enhances left ventricular remodeling (Panizzi et al., 2010). This is associated with the extent of myocardial salvage and the recovery of left ventricular function after acute infarction (Tsujioka et al., 2009).

As a side note, neutrophils accumulate in larger numbers during the early phase of ischemic injury, but disappear within approximately 24 h whereas the monocyte/macrophage response is sustained (Jung et al., 2013; Nahrendorf and Swirski, 2013). In addition to the recruitment of blood derived monocytes, tissue-resident macrophages in the heart represent another source of innate immune cells, which likely contribute to a local inflammatory process in the ischemic myocardium (Epelman et al., 2014a).

Origin of Resident Cardiac Macrophages

The formerly believed rational, that blood-derived monocytes are the only source of macrophages in the heart, has been challenged by recent work establishing the yolk sac as a common source of macrophages in adult tissues (Ginhoux et al., 2010; Schulz et al., 2012; Gomez Perdiguero et al., 2015). In line with these findings, also cardiac macrophages have their origin, at least to some extent, in the yolk sac (YS). Tamoxifen-induced pulse-labeling of Csf1r^{+} YS macrophages between embryonic days E8.5 and E9.5, when definitive hematopoiesis is not yet present, labeled a proportion of resident macrophages in the adult mouse heart (Epelman et al., 2014a; Molawi et al., 2014) (**Figure 1A**). These initial fate-mapping strategies were useful to assess the qualitative contribution of YS progenitors to adult tissue macrophages (Schulz et al., 2012; Epelman et al., 2014b). More recent work assessed the quantitative potential of YS progenitors for tissue resident macrophages *in vivo,* and established the YS as the key source of macrophages in adult tissues (Gomez Perdiguero et al., 2015). While YS-derived macrophages initially display a common signature (Schulz et al., 2012), their distinct phenotype and function is later shaped by local environmental cues specific for the respective tissue of residence (Gosselin et al., 2014; Lavin et al., 2014). Tissue macrophages perform specific duties, such as homeostatic

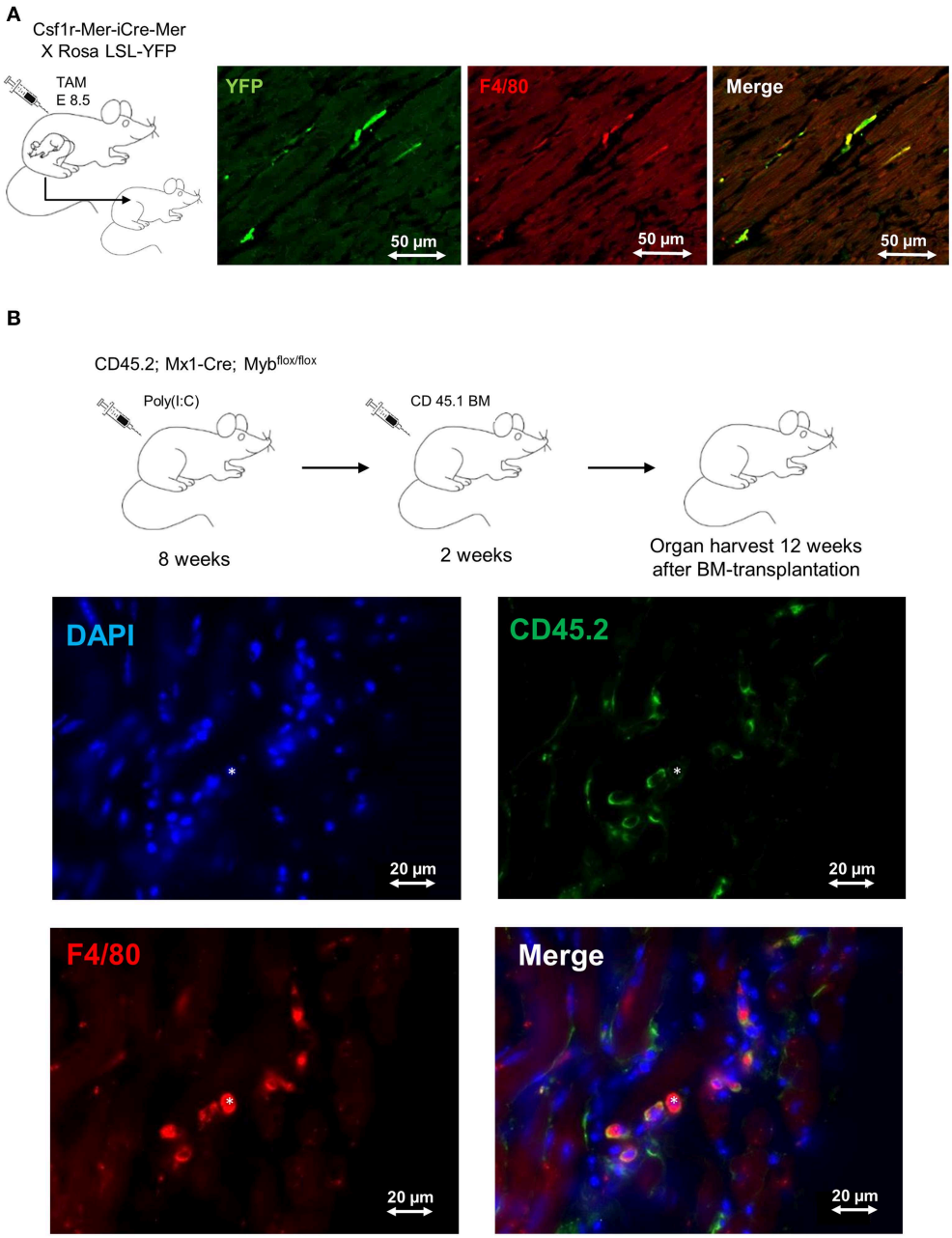

FIGURE 1 | Fate mapping analysis of cardiac macrophages. **(A)** Frozen tissue sections of 1 year old mouse. FVB strain Csf1r^{MeriCreMer} females mated with BL6 ROSA $^{LSL-YFP/LSL-YFP}$ male were pulse labeled by injection of 75µg/g hydroxytamoxifen on embryonic day 8.5 (E8.5). This labeling regime allowed the specific labeling of Csf1r^{+} cells derived from the yolk sac, as definitive hematopoiesis is not present at that time point. 1 year old mice were stained for F4/80 (red) and YFP (green); double-positive cells represent yolk-sac derived macrophages. Bars 50 µm (Schulz et al., 2012; Epelman et al., 2014a). **(B)** Frozen tissue sections of 6 month old *Myb*-deficient BM chimera (CD45.1/CD45.2). BM ablation was obtained through deletion of *Myb* by injection of poly(I:C) into CD45.2; Mx1-Cre; Myb$^{flox/flox}$ mice. Whole BM (10^7 cells) from CD 45.1 mice was transplanted after BM-ablation as described earlier (Schulz et al., 2012). The heart was examined 12 weeks after BM transplantation and sections were stained with DAPI (blue), F4/80 (red) and CD45.2 (green); double-positive (F4/80 positive, CD45.2 positive) cells represent host-derived macrophages; (∗) represents a donor-derived macrophage (F4/80 positive, CD45.2 negative). Bars 20 µm.

functions and immune surveillance in other tissues (Davies et al., 2013). Nevertheless, their role in inflammatory processes in the heart needs to be determined.

It remains unknown to date, for how long YS-derived macrophages reside in adult tissues. In many organs, such as the brain and the liver, these cells are not replaced at steady

state (Gomez Perdiguero et al., 2015) and can persist independently of HSCs (Schulz et al., 2012) (**Figure 1B**). However, a specific environment (e.g., intestine) or bacterial infections may result in replacement of tissue macrophages by bone marrow-derived monocytes (Bain et al., 2014; Bleriot et al., 2015). In the intestine, YS-derived macrophages are replaced by bone marrow monocytes within the first weeks of life due to substantial changes of the environment. Interestingly, the number of YS-derived resident macrophages in the mouse heart also seems to be impermanent and declines with age. As mice grow older the proliferation rate of cardiac macrophages decreases and is probably insufficient to maintain the resident macrophage pool (Molawi et al., 2014). Likewise, resident macrophages in the heart are lost as a consequence of myocardial infarction. They need to be substituted either through monocyte intermediates, which are recruited from the circulation, or by local proliferation and expansion of resident cells. Further experimental evidence is needed to define the quantitative contribution of circulation monocytes to the cardiac macrophage pool during aging and in the setting of myocardial injury (Heidt et al., 2014; Lavine et al., 2014; Molawi et al., 2014). Finally, it is still unknown to what extent monocyte-derived macrophages are distinct from resident macrophages with respect to their effector functions in tissues (Schulz and Massberg, 2014). Undoubtedly, the findings will have implications for our understanding of cardiac homeostasis and disease.

Macrophage Subsets in the Heart

Recently, it has become clear that cardiac macrophages consist of several different subpopulations (Epelman et al., 2014a; Heidt et al., 2014; Lavine et al., 2014; Molawi et al., 2014). Elaborate multicolor flow cytometry studies allowed a comprehensive characterization of different macrophage subsets by cell surface markers including CD11b, F4/80, Cx3Cr1, major histocompatibility complex II (MHC II), CCR2 and Ly6C.

Accumulating evidence suggests that the composition of macrophage subsets in the myocardium undergoes dynamic changes in the course of life. This needs to be kept in mind when discussing cardiac macrophage populations in the heart, especially as the macrophage subsets have different regenerative and inflammatory potential. In newborn mice cardiac macrophages display a MHC II^{lo} phenotype and have predominantly originated from the yolk sac. This subset of macrophages seems to have distinct, probably beneficial, effects on the myocardium under stress. As mice age, the macrophage phenotype diversifies into a MHC II^{lo} and a MHC II^{hi} phenotype. Fate mapping analysis in adult mice using pulse labeling of macrophages indicated that the contribution of embryo-derived cardiac macrophages to the MHC II^{lo} subset was higher than to MHC II^{hi} macrophages. Between birth and 30 weeks of age resident macrophages become increasingly replaced by monocyte-derived macrophages from the bone marrow (Lavine et al., 2014; Molawi et al., 2014).

Another surface marker to describe macrophage subsets is CCR2, the receptor for chemokine monocyte chemoattractant protein-1 (MCP-1/CCL2), which facilitates monocyte chemotaxis. This marker helps to identity macrophages, which recently derived from circulating monocytes (Epelman et al., 2014a; Lavine et al., 2014). Using CCR2 and MHC II, cardiac monocyte, and macrophage subsets of the neonatal and adult heart could be defined. In a nutshell, at least two subsets exist in the neonatal heart at steady state, namely a $CCR2^{-}$/MHC II^{lo} subset derived predominantly from the yolk sac and a $CCR2^{+}$/MHC II^{lo} subset derived from definitive hematopoiesis. In contrast, in the adult heart (6 weeks of age), the monocyte/macrophage population could be divided into 4 subsets. The $CCR2^{-}$ population had a diversified phenotype with MHC II^{lo} and MHC II^{hi} macrophage subsets and a mixed developmental origin. In contrast, the $CCR2^{+}$ population derived exclusively from definitive hematopoiesis and could be subdivided into MHC II^{hi} and MHC II^{lo} populations (Lavine et al., 2014).

Age-Dependent Response of Macrophages to Myocardial Stress

Besides the unknown influence of the origin of macrophages on myocardial repair, it has become clear that macrophages exhibit a distinct potential to facilitate regeneration during different stages of life. Subjecting neonatal mice to myocardial infarction on postnatal day 1 (P1), the ischemic myocardium has been shown to completely regenerate by proliferation of preexisting cardiomyocytes. This regeneration capacity is already lost during the first week after birth. Induction of myocardial infarction 14 days postpartum (P14) showed left ventricular scarring and cardiomyocyte hypertrophy comparable to the pathophysiological changes seen in adult animals after myocardial infarction (Porrello et al., 2013). Macrophages differed in number, localization, and gene expression profile in mice subjected to myocardial infarction on P1 and P14 after birth. Even more strikingly, the regenerative capacity of the neonatal heart after myocardial infarction was lost when macrophages were depleted using clodronate liposomes. The depletion of macrophages in neonates was accompanied by a loss of neovascularization (Aurora et al., 2014). These results stress the importance of macrophages in the regeneration potential of neonatal mice.

In fact, neonatal mice react to myocardial stress (e.g., after *in vivo* cardiomyocyte cell ablation) with an expansion of the MHC II^{lo} macrophage subpopulation. It could be shown that these macrophages possess proangiogenic and reparative properties which, at least partially, contribute to the regenerative capacity of the neonatal heart. Fate mapping analysis revealed that this macrophage subset is of yolk sac origin. In contrast, adult mice respond to myocardial injury with the recruitment of inflammatory MHC II^{hi} $CCR2^{+}$ macrophages from the blood circulation and loss of potentially beneficial $CCR2^{-}$ (resident) macrophages. These mice developed pathological left ventricular remodeling including cardiomyocyte hypertrophy, fibrosis, and LV dilation resulting in a decreased LV function. Blocking the recruitment of $CCR2^{+}$ monocytes into the injured myocardium preserved cardiac MHC II^{lo} macrophages in the adult heart and showed beneficial effects on coronary angiogenesis and attenuated inflammation (Lavine et al., 2014). This

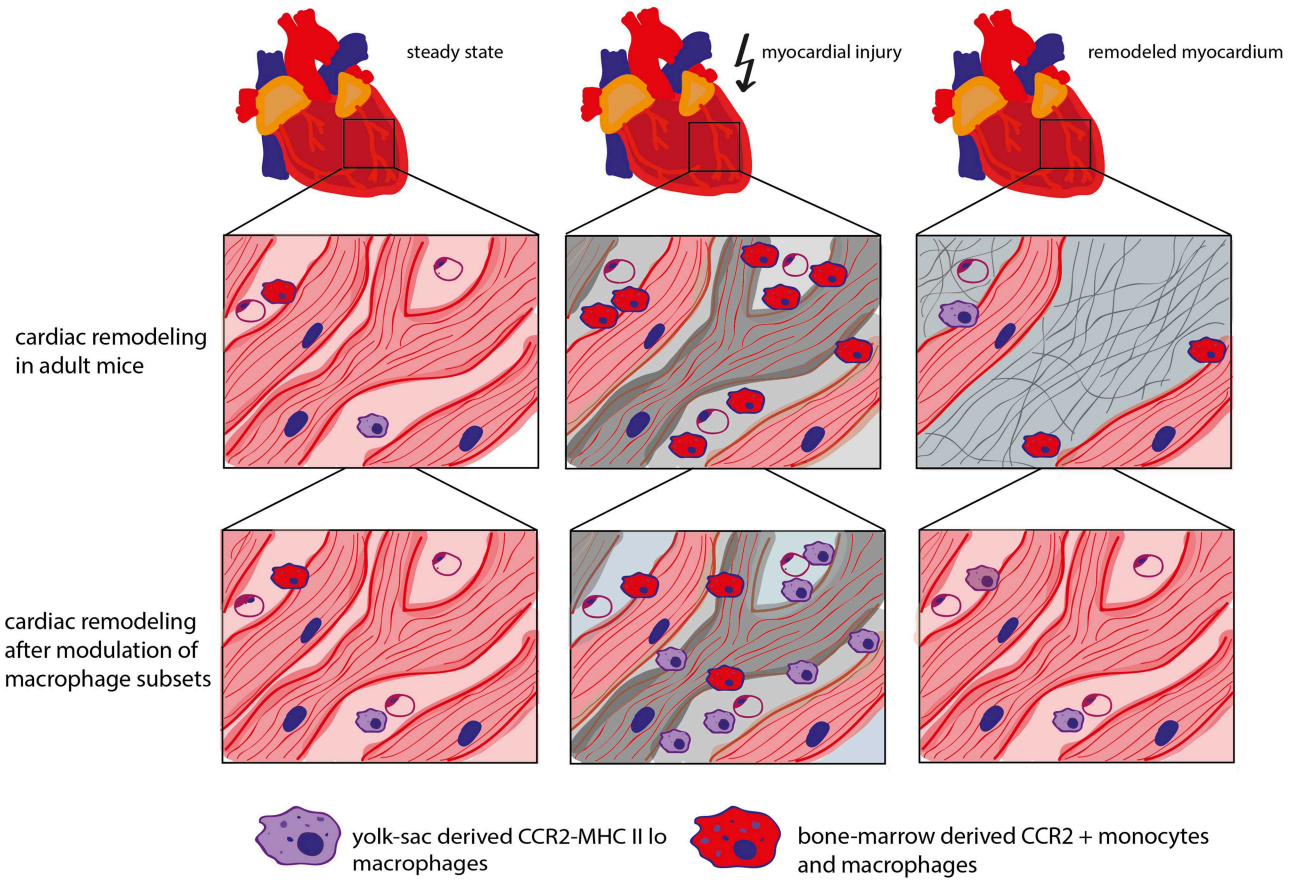

FIGURE 2 | Role of different macrophage subtypes in cardiac remodeling. In steady state, the adult mouse heart contains various types of macrophage populations, which are of yolk sac (YS) or bone marrow (BM) origin dependent on the mouse age. CCR2$^-$ macrophages are of mixed origin but predominately derive from YS/embryonic precursors. CCR2$^+$ monocytes and macrophages are derived from the bone marrow. In adult mice, myocardial stress triggers the depletion of resident cardiac macrophages followed by the recruitment of "inflammatory" CCR2$^+$ monocytes. Inhibition of CCR2 leads to a decreased recruitment of CCR2$^+$monocytes into the myocardium and protects resident cardiac macrophages which has a beneficial impact on coronary angiogenesis, inflammation and cardiac remodeling (Kaikita et al., 2004; Lavine et al., 2014).

underscores a beneficial role of resident cardiac macrophages (**Figure 2**).

It is currently unknown whether the effects of the different macrophage populations on cardiac remodeling after *in vivo* cardiomyocyte cell ablation also apply to the myocardial infarction model. Intriguingly, myocardial infarction in adult mice is accompanied by the loss of resident cardiac macrophages. Consequently, blood-derived monocytes are recruited to the ischemic myocardium and repopulate the cardiac resident macrophage population (Heidt et al., 2014). Further studies are required to unravel the role of the different macrophage populations in infarct healing.

Macrophages as Therapeutic Targets for Myocardial Repair

Monocytes and macrophages play a detrimental role in the pathophysiological processes triggered by infarction and therefore represent a potential therapeutic target to promote myocardial repair and functional regeneration. Yet we have only begun to understand the spatiotemporal relationships and functions of the different macrophage subsets in the course of infarct healing. It is likely that cell-type specific therapies as well as adequate timing of respective therapies will be able to achieve a more beneficial outcome in cardiovascular remodeling. In the following, we will highlight differential strategies targeting monocytes and macrophages, which have the potential to reduce the inflammatory burden in the post-infarct myocardium.

One approach that has been investigated for some time is blocking the recruitment of inflammatory monocytes. This can be achieved for example by interfering with chemokine gradients resulting in a reduction of circulating inflammatory monocytes (e.g., ablation of CCR2), or by inhibition of their recruitment into the ischemic myocardium (e.g., C-X-C chemokine receptor type 6 (CXCR6), macrophage migration inhibitory factor MIF). Reduction of inflammatory CCR2$^+$ monocytes either using a knockout model of CCR2 or an antibody-mediated approach

attenuated the inflammatory response after myocardial infarction and had beneficial effects on cardiac remodeling (Kaikita et al., 2004; Lavine et al., 2014). Likewise, disruption of the CXCL16-CXCR6 axis in an ischemia/reperfusion model, led to a decreased number of $CD11b^+$ cells in the infarcted tissue resulting in improved cardiac function and attenuated cardiac remodeling through a diminished autophagic response (Zhao et al., 2013). Inhibition of MIF influences apoptotic pathways and other signaling cascades. Importantly, plasma MIF levels are associated with infarct size and extent of cardiac remodeling in humans. However, blockade of MIF is rather unspecific, and among other effects, also reduces the influx of neutrophils (Gao et al., 2011; Chan et al., 2013).

Another approach to modulate macrophage function is to modify their environment. As shown only recently, macrophage phenotypes and functions are shaped by the microenvironment of the organ of residence (Gosselin et al., 2014; Lavin et al., 2014). For example, transplantation of differentiated peritoneal macrophages into the lung environment induced reprogramming of the transcriptional landscape of these cells and their acquirement of new tissue specific functions. It is tempting to speculate that the same is true for the cardiac microenvironment.

Further, resident macrophages also interact with surrounding immune cells, including those of the adaptive immune system. Regulatory T cells have recently been shown to modulate monocyte/macrophage differentiation in the setting of myocardial infarction with a positive impact on wound healing and remodeling (Weirather et al., 2014). Whether enhancement of regulatory T-cells is beneficial for infarct repair is currently unknown. In addition, B lymphocytes interact with monocytes in the course of cardiac repair after myocardial infarction. In this setting, B cells facilitate mobilization and recruitment of $Ly6C^{hi}$ monocytes into the ischemic myocardium by secretion of CCL7. Genetic as well as antibody-induced depletion of B cells reduced circulatory monocytes and inflammatory $Ly6C^{hi}$ monocytes in the myocardium which improved cardiac function and attenuated myocardial injury (Zouggari et al., 2013). However, the precise mechanisms of the crosstalk between the adaptive immune system and monocytes/macrophages and its therapeutic potential in cardiac remodeling will require further experimental data.

Depletion of macrophages could represent another approach to modulate myocardial inflammation. However, this approach is likely to represent a double edged sword. Depletion of by injection of clodronate liposomes during the first week after myocardial injury led to reduced removal of necrotic cells, impaired neovascularization and increased scar formation. This resulted in a higher mortality of macrophage-depleted mice (van Amerongen et al., 2007; Ben-Mordechai et al., 2013; Frantz et al., 2013). Further, clodronate liposomes deplete most phagocytic cells, including dendritic cells (Leenen et al., 1998) and are therefore not specific. Still, phagocytic properties of mononuclear phagocytes may be harnessed to deliver drugs to their site of action, as reviewed elsewhere (Ben-Mordechai et al., 2015).

Finally, resident cardiac macrophages might be targeted through the alteration of intracellular signals, which for example induce proliferation or apoptosis. IL-4 has been shown to directly signal to tissue-resident macrophages and induce their proliferation above homeostatic levels (Jenkins et al., 2013). Targeting expression of the transcription factor MafB may inhibit macrophage apoptosis in tissues during inflammatory conditions (Hamada et al., 2014). Small-interfering RNA could thereby help achieve robust gene silencing. Delivery of respective particles in an encapsuled form revealed an efficient uptake by phagocytes (Aouadi et al., 2009). However, strategies targeting function of resident macrophages will have to account for their physiological role in tissue homeostasis (e.g., Nr4a1) in organs, including the heart (Schulz and Massberg, 2014).

In summary, modulation of macrophage functions represents an interesting therapeutic strategy. Its success, however, will depend on a deeper understanding of the heterogeneity of macrophage subsets and their developmental origin and molecular regulation. Further, the regulatory signals derived from the local microenvironment in the heart at steady state and in the course of infarction, which modulate the phenotype and functional specification of macrophages, require further investigation.

Concluding Remarks

Despite their abundance in the myocardium, the function of the different cardiac macrophage populations at steady state and in response to stress is largely unknown. There is no doubt that future studies will reveal the precise contribution of monocyte/macrophage populations to myocardial remodeling in pathological conditions thereby integrating findings on their developmental origin and regulation. Of note, recent data in mouse models of infarction suggest that "time" may matter in various ways. First, monocyte/macrophage populations may have a distinct role at different stages of myocardial remodeling and thus, targeting these immune cells at specific time points could further improve wound healing and functional outcome. Second, it may also play a role at which age experimental models (e.g., myocardial infarction) are performed, as macrophage populations in the heart are –at least in mice– replaced over time (Molawi et al., 2014) and also the recovery potential abates with age (Lavine et al., 2014). These recent studies on the heterogeneity and kinetics of monocytes and macrophage subsets in the mammalian heart have paved the way for new therapeutical strategies to target infarct repair after myocardial infarction. Despite the recent advancement on our understanding of the pathomechanisms, specifically the kinetics and regulation of inflammatory processes and wound healing in the diseased heart, early reperfusion therapy for patients who have myocardial infarction remains the essential basis to preserve myocardial function and reduce mortality.

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Syncope: epidemiology, etiology, and prognosis

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Syncope is a common medical problem, with a frequency between 15% and 39%. In the general population, the annual number episodes are 18.1–39.7 per 1000 patients, with similar incidence between genders. The first report of the incidence of syncope is 6.2 per 1000 person-years. However, there is a significant increase in the incidence of syncope after 70 years of age with rate annual 19.5 per thousand individuals after 80 years. It presents a recurrence rate of 35% and 29% of physical injury. Among the causes of syncope, the mediated neural reflex, known as neurocardiogenic or vasovagal syncope, is the most frequent. The others are of cardiac origin, orthostatic hypotension, carotid sinus hypersensitivity, neurological and endocrinological causes and psychiatric disorders. The diagnosis of syncope can be made by clinical method associated with the electrocardiogram in up 50% of patients. Its prognosis is determined by the underlying etiology specifically the presence and severity of cardiac disease. The annual mortality can reach between 18 and 33% if cardiac cause, and between 0 and 12% if the non-cardiac cause. Thus, it is imperative to identify its cause and risk stratification for positive impact in reducing morbidity and mortality.

Keywords: vasovagal syncope, cardiac arrhythmias, orthostatic intolerance, epidemiology, prognosis

DEFINITION AND EPIDEMIOLOGY

Syncope is the sudden loss of consciousness, associated with inability to maintain postural tone, with immediate and spontaneous recovery without requiring electrical or chemical cardioversion. This framework is secondary to cerebral hypoperfusion, with short duration (average 12 seconds). It has a prevalence of 42%, considering a life time of 70 years and an annual incidence of 6% (Moya et al., 2009). Its frequency varies from 15% (below 18 years of age) (Lewis and Dhala, 1999) to 39% (among medical students) (Serletes et al., 2006), reaching 23% among the elderly (Lipsitz et al., 1985). In the general population, the annual number episodes are 18.1–39.7 per 1000 patients, with similar incidence between genders, and with high prevalence between 10 and 30 years of age, mainly of vasovagal syncope (Moya et al., 2009). The first report of the incidence of syncope is 6.2 per 1000 person-years. However, there is a significant increase in the incidence of syncope after 70 years of age, with 5.7 episodes/1000 individuals per year between 60 and 69 years old and with 11.1 episodes/1000 individuals per year between 70 and 79 years age. After 80 years, the annual incidence may reach 19.5 per 1000 individuals (Soteriades et al., 2002; Colman et al., 2004).

This framework is responsible for 3–5% of emergency department visits, with a hospitalization rate in about 40% of cases, with an average stay of 5.5 days. Beyond this morbidity, there is a recurrence rate of approximately 35% and 29% of physical injury, and major trauma in 4.7% of patients. In elderly patients with syncope due to carotid sinus hypersensitivity, the prevalence of trauma may reach 43% (Brignole et al., 2006; Moya et al., 2009). Besides the social impact with worsening quality of life, there is also the economic impact, with higher costs attributed to hospitalization with an estimated $2.4 billion annual cost (Sun, 2013).

ETIOLOGY

Among the causes of syncope, the mediated neural reflex, known as neurocardiogenic or vasovagal syncope, is the most frequent, accounting for one third of the causes and reaching 66% of cases in emergency units (Alboni et al., 2001; Brignole et al., 2006). The diagnosis of syncope can be made by clinical method associated with the electrocardiogram in up 50% of patients (Moya et al., 2009). The clinical history should be performed with proficiency, and each episode of syncope should be well characterized about presence of prodrome, precipitating factors, conditions in which it occurred, position of the patient, and other associated signs such as nausea, pallor, diaphoresis, muscle twitching, confusion, physical injury, palpitations, dyspnea, chest pain, and cyanosis. The personal medical history and family history are also important to identify the cause of syncope. The Calgary Score is one of the diagnostic tools, including seven clinical issues and allow diagnosis vasovagal syncope with 89% sensitivity and 91% specificity (Sheldon et al., 2006). The physical examination should be comprehensive, including measurement of blood pressure in the supine position and within 3 min in the orthostatic position, the blood pressure measurements in both arms (to detect subclavian steal), and the exam of the cardiovascular system, which can identify signs of disease and/or arrhythmias, and other systems.

The main causes of syncope are summarized in **Table 1** and will be discussed below.

Table 1 | Common causes of syncope.

Pathophysiological origin	Causes
Reflex syncope	Vasovagal, situational syncope, orthostatic hypotension, carotid sinus hypersensitivity
Cardiac syncope	Structural heart disease, bradyarrhythmias, tachyarrhythmias
Neurological causes	Cerebrovascular disease, autonomic dysfunction, subclavian steal syndrome
Others causes	Endocrinological causes, psychiatric disorders

VASOVAGAL SYNCOPE

This term was first used by William Gowers in 1907 and described the mechanism of vasovagal syncope by Thomas Lewis in 1932 (Lewis, 1932). It shows bimodal distribution, but is most common in young people. Precipitating factors are prolonged sitting position or standing position, emotional stress, pain, heat, venous puncture, alcohol use, dehydration, use of diuretics and vasodilators. Prodromes of nausea, vomiting, abdominal pain, diaphoresis, pallor, palpitations, and dizziness may occur and are more common in young people. After loss of consciousness, tonic-clonic contractions, if they occur, are of short duration (<15 s). There are no aura, headache, sleep, sphincter release, mental confusion, and duration of loss of consciousness greater than 5 min, such as occurs in epilepsy (Kapoor, 2002; Moya et al., 2009). The neuromediated syncope is called situational syncope when it occurs in conditions that trigger the Valsalva maneuver, such as urination, defecation, coughing, visceral pain, carry weight.

The mechanism of vasovagal syncope is explained by the Bezold–Jarisch reflex, which is triggered due to decreased venous return resulting in inadequate ventricular filling and vigorous cardiac contraction. It occurs by the action of mechanoreceptors (C fibers) preferentially located in the inferolateral wall of the left ventricle, but also in the atria and in the pulmonary artery, and manifests with hypotension and paradoxical bradycardia due to increased activity of inhibitory receptors and consequent parasympathetic hyperactivity (Medow et al., 2008; Moya et al., 2009).

Initial therapy of vasovagal syncope is non-pharmacological. The recommendations are to identify and avoid the precipitating factors, assuming the supine position during the period of prodromes, and nutritional guidelines. These include hydration with fluid intake 2–2.5 liters per day and sodium supplementation. Aerobic and isometric exercises of the upper and lower limbs reduce the recurrence of vasovagal syncope. Tilt training compared with conventional treatment shows limited efficacy in controlled studies. The use of compression stockings in the lower limbs may be recommended, however it has limited efficacy and poor adherence. If failure of initial recommendations, the next step is pharmacological treatment with the use of fludrocortisone if vasodepressor response. Beta blockers are contraindicated. Implantation of a pacemaker is class IIa indication for patients over 40 years of age and cardioinhibitory response with asystole (Moya et al., 2009; Raj and Coffin, 2013).

CARDIAC CAUSES

The causes are structural heart diseases or conditions that result in decreased cardiac output such as aortic stenosis, hypertrophic cardiomyopathy, ischemic heart disease, heart failure, aortic dissection, cardiac tamponade, prosthetic valve thrombosis, cardiac tumors, pulmonary hypertension, pulmonary embolism etc. Both bradyarrhythmias and tachyarrhythmias are frequent causes of cardiac syncope due to impaired cardiac output secondary to the frequency of the arrhythmia, its origin, to systolic ventricular dysfunction. A family history of sudden cardiac death is an important data for the hypothesis channelopathy (Moya et al., 2009; Rosanio et al., 2013). Treatment depends on the cause cardiac syncope, with treatment directed to the structural heart disease, pacemaker implantation, if bradyarrhythmias, or use of antiarrhythmic, catheter ablation or implantable cardioverter-defibrillator, if tachyarrhythmias (Moya et al., 2009).

ORTHOSTATIC HYPOTENSION

Orthostatic or postural hypotension (OH) presents as falls, dizziness, or syncope, resulting in functional impairment, with head injury, bone fractures and hospitalization. It is more common in the elderly, with frequency rates of up to 55% in those that residing in institutions, and with prevalence of 12%. Its classic definition is a drop of at least 20 mmHg in systolic and/or 10 mmHg in diastolic blood pressure within 3 min to take an orthostatic position. There are other presentations such as initial OH, when there is a greater than 40 mmHg drop in blood pressure, with less than 30 s in duration, and progressive OH, when the fall of blood pressure levels are gradual, between 3 and 30 min after taking standing position, without bradycardia (Goldstein and Sharabi, 2009; Moya et al., 2009).

Beyond the control of the precipitating factor, hydration and salt intake, treatment of syncope postural hypotension can be done with fludrocortisone, midodrine. Other measures are abdominal compression and elevation of the head of the bed (Moya et al., 2009; Raj and Coffin, 2013).

Postprandial hypotension is a common cause of syncope in the elderly, with a prevalence that can reach 67%, especially in the elderly who live in institutions. It is defined as the drop of at least 20 mmHg in systolic blood pressure or absolute value of the systolic pressure lower than 90 mmHg (those with systolic blood pressure of at least 100 mmHg), within 2 h after meals. The pathophysiology is due to sympathetic dysfunction, with inadequate peripheral vasoconstriction and insufficient heart rate increase. Precipitating factors are vasodilators, high temperature of food or of environment and diets high in carbohydrates (Luciano et al., 2010).

CAROTID SINUS HYPERSENSITIVITY

It is an extrinsic sinus node disease that is characterized by pre-syncope or syncope exacerbated by the carotid sinus reflex response. Its incidence is 35–40 patients/year/million individuals, with a predominance in males (male:female ratio of 4:1) and more frequent in the elderly, especially diabetics with coronary or carotid atherosclerosis. Precipitating factors are sudden movements of the head and neck, cervical compressions and use of tight neck tie (Healey et al., 2004). The approach of the

carotid sinus syndrome includes the implantation of a pacemaker if cardioinhibitory response, or use of volumetric expansion, if vasodepressor response.

NEUROLOGICAL CAUSES

Neurological causes are cerebrovascular disease, autonomic dysfunction and subclavian steal syndrome. Focal neurological deficits in stroke, vertebrobasilar transient ischemic stroke, migraine (for vasospasm or vasovagal reflex) may be presented as syncope.

The primary autonomic dysfunction occurs in pure primary dysfunction syndrome (Bradbury–Eglleston), in central nervous system diseases (Parkinson's disease, multiple system atrophy or Shy–Drager syndrome, Huntington's disease and Guillain–Barré syndrome). Secondary dysfunctions occur by changes of aging, due to the involvement of the peripheral nervous system in diabetes mellitus, renal failure, alcoholism, amyloidosis; infections of the nervous system by Chagas disease, human immunodeficiency virus; metabolic diseases such as vitamin B12 deficiency, porphyria; autoimmune diseases such as rheumatoid arthritis and others. And as antihypertensive drugs (diuretics, vasodilators), anti-depressants may also cause autonomic dysfunction (Azhar and Lipsitz, 1998).

The first report of subclavian steal syndrome was described in 1960 by Contorni. It shows a prevalence of up to 6.4%. There is malformation or obstruction by atherosclerosis of the proximal subclavian artery to the origin of vertebral artery, resulting in retrograde flow in this artery. It occurs mainly in the left subclavian artery. Neurological symptoms, such as dizziness, paresthesia and syncope, occur during exercise performed by the arm, but patients may present framework of transient ischemic attacks (Osiro et al., 2012; Potter and Pinto, 2014).

OTHERS CAUSES

There are endocrinological causes that can be presented as orthostatic hypotension to cause autonomic dysfunction or hypovolemia. As examples, chronic adrenal insufficiency and hypopituitarism can be cited. Diabetes insipidus and salt-losing nephropathies since they result in volume depletion, and pheochromocytoma and carcinoid syndrome, due to vasoactive substances, can also be causes of syncope. Hyperventilation and psychiatric disorders, due to cerebral hypoperfusion, are other causes of syncope (Kapoor, 2002; Moya et al., 2009).

PROGNOSIS

The pathophysiology, approach, prognosis and treatment depend on the cause of syncope, and mandatory their identification, since their annual mortality can reach between 18 and 33% if cardiac cause, and between 0 and 12% if the non-cardiac cause (Kapoor, 2002).

There are studies that compared mortality among patients with syncope of cardiac origin and non-cardiac origin. Among participants in the Framingham heart Study, 7814 patients were included from 1971 to 1998 and 822 presented syncope of which 9.5% were cardiac. Adjusted multivariate analysis showed that the risk of death increased by 31% among all patients with syncope and was doubled among those with syncope of cardiac origin, compared with those without syncope. Neurological cause of syncope was also associated with a threefold risk of stroke. Moreover, there was no association with death or major adverse events among those with vasovagal syncope (Soteriades et al., 2002).

In the study by Ungar et al. with 380 patients included, death from any cause occurred in 35 (9.2%) patients during the mean follow-up of 614 days. Death was considered cardiovascular in 9 patients (26%). And among the patients who died, 82% were older and had cardiac risk factors such as abnormal ECG and/or heart disease. In contrast, among those with no abnormal ECG and/or heart disease, only six (3%) deaths occurred, resulting in a negative predictive value of 97% (Ungar et al., 2010).

Another study of 200 patients with syncope, cardiac origin was associated with adverse events defined as death, recurrence of syncope, cardiovascular events, and major procedures during the following short-term (1 month) and long (1 year) (Numerosos et al., 2013).

A study of 37,017 patients with syncope in the period 2001–2009, without previous hospitalization for comorbidities, and with a control group of 185,085 individuals, demonstrated increased risk of all-cause mortality, stroke, cardiovascular hospitalization, device implantation, and recurrent syncope in healthy individuals after first admission for syncope (Ruwald et al., 2013). Another study more recent from the same team, with 70,819 patients hospitalized between 2001 and 2009, aged between 19 and 90 years, with a mean follow up of 3.9 years, showed that recurrence of syncope, which occurred in 16.4%, was associated to 3.2 times the risk of death within a year (Ruwald et al., 2014).

Regarding the recurrence of syncope, risk stratification cannot predict it. The incidence of recurrence of syncope was similar regardless of cause of syncope. Its rate was 0.3% in the first month, of 0.8% per month during the first year and 0.5% per month during the second year. On univariate analysis, predictors of recurrence were male sex, presence of prodrome and absence of palpitations (Ungar et al., 2010). On the other hand, the number of vasovagal syncope events in the preceding year may be a predictor of recurrence. Those patients with less than two previous episodes, the recurrence was 22% vs. 69% those with more than 6 episodes, with a probability of 46% (Sumner et al., 2010).

Thus, prognosis is determined by the underlying etiology specifically the presence and severity of cardiac disease. And it is imperative to identify its cause and risk stratification for positive impact in reducing morbidity and mortality.

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