DIABETES AND HEART FAILURE: PATHOGENESIS AND NOVEL THERAPEUTIC APPROACHES

EDITED BY: Celestino Sardu, Claudio de Lucia, Laurent Metzinger and Coert J. Zuurbier

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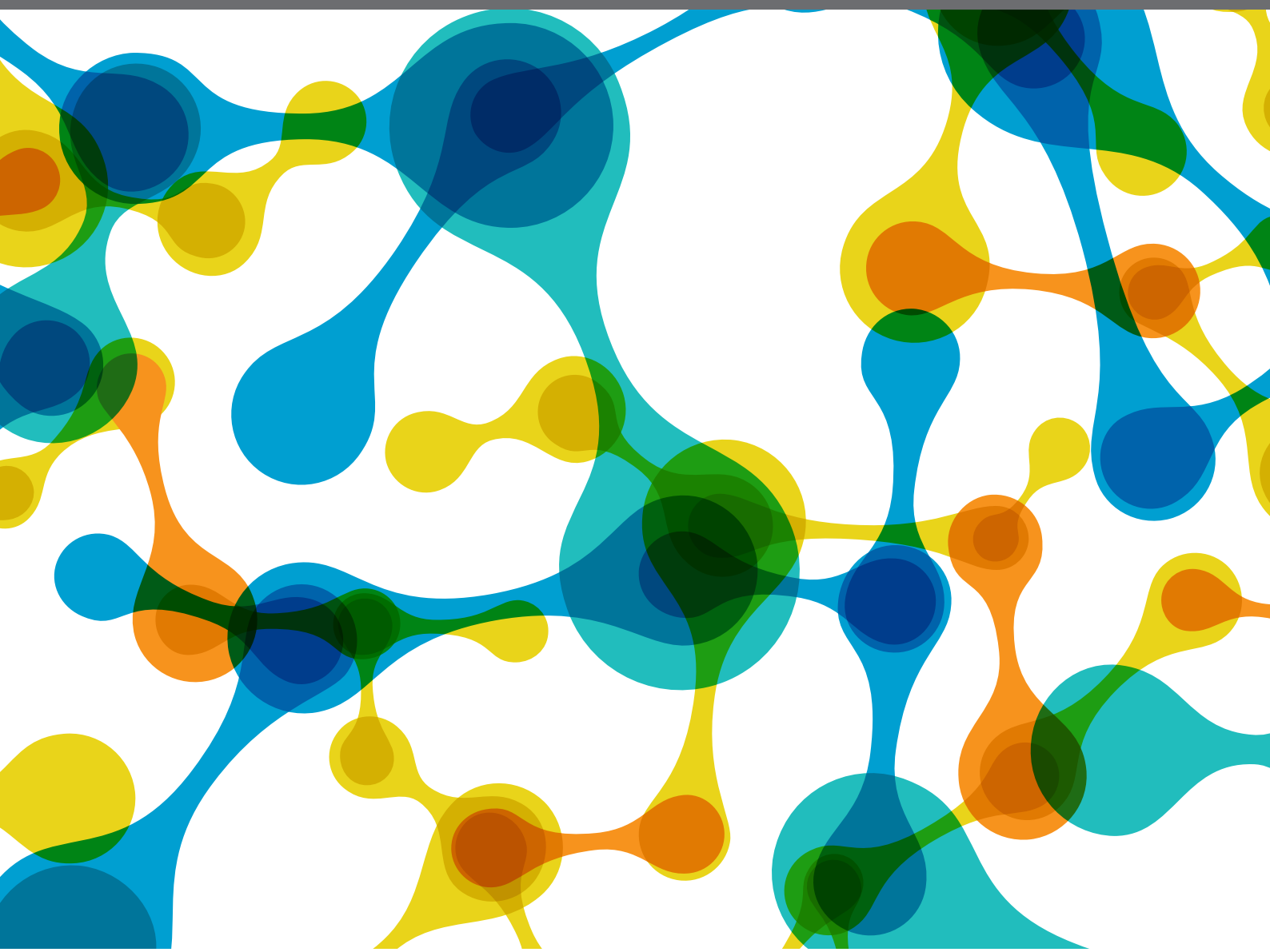

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DIABETES AND HEART FAILURE: PATHOGENESIS AND NOVEL THERAPEUTIC APPROACHES

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EDITORIAL
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Editorial: Diabetes and Heart Failure: Pathogenesis and Novel Therapeutic Approaches

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Keywords: diabetes, heart failure, diastolic dysfunction, arrhythmia, SGLT2 (sodium-glucose cotransporter 2) inhibitor, GLP-1-glucagon-like peptide-1

Editorial on the Research Topic

Diabetes and Heart Failure: Pathogenesis and Novel Therapeutic Approaches

Type 2 diabetes mellitus (T2DM) and Heart Failure (HF) are two complex multifactorial diseases that can coexist and strongly amplify each other, suggesting overlapping mechanisms contributing to disease state. T2DM patients with HF have a higher risk of mortality and hospitalization for HF than HF patients without T2DM. Therefore, there is an increasing necessity to find new diagnostic instruments and treatments to improve clinical outcomes in T2DM subjects with HF (Maack et al., 2018). Several complex pathological mechanisms such as altered cardiac ionic homeostasis, oxidative stress, hyperglycemia-induced cellular damage, and mitochondrial dysfunction are implicated. Importantly, altered cardiac ionic homeostasis is an established signature and driver of HF pathology (Pogwizd et al., 2003), but has been largely neglected in T2DM pathology. Insulin resistance and disturbances in glucose and fatty acid metabolism are currently viewed as major instigators of T2DM (Jia et al., 2018). However, older and recent researches indicate that cardiac ionic disturbances may actually provide an important common ground for both diseases and explain, at least in part, why they mutually amplify each other. Intriguingly, these changes may lead to electrical and mechanical alterations in systolic and diastolic electrical phases. Therefore, in this Research Topic we have tried to bring the two major diseases together by creating a collection of articles written by authors that have focused on common molecular pathways and mechanisms, electrical/mechanical alterations, and subsequently on clinical outcomes in both T2DM and HF.

In the first review by Borghetti et al. emphasis is placed on focusing of diabetic therapies beyond glucose control. Although anti-hyperglycemic drugs are crucial in the management of diabetes by effectively reducing microvascular complications, preventing renal failure, retinopathy, and nerve damage, they have little effect on diabetic cardiomyopathy. Interestingly, several novel drugs have now shown cardiovascular beneficial effects beyond their ability to control glycemia, such as GLP-1 receptor agonists and sodium-glucose co-transporter 2 inhibitors. In addition, the recent development of modulating the expression of specific cardiac genes or non-coding RNAs *in vivo* for therapeutic purpose, has opened up the possibility to regulate the expression of key players in the development/progression of diabetic cardiomyopathy.

The review by Bajpai and Tilley discusses the roles of leukocytes and particularly neutrophils, macrophages, and lymphocytes in the appearance of myocardial infarction and heart failure during diabetes. Cardiac injury in diabetes, a chronic inflammatory disease, is linked to increased leukocyte mobilization and the expression of pro-inflammatory cytokines and appearance of oxidative stress. The lessons learned from experimental diabetes models in rodents, including the popular

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streptozotocin-induced Type I diabetes rodent model, are implemented to human patients, and the authors conclude that further studies are necessary to fully apprehend the potential alterations in leukocyte phenotypes and the molecular mechanisms responsible for diabetes.

The review by Grisanti highlights the impact of diabetes on the electrical conduction system in the heart, resulting in atrial fibrillation and ventricular arrhythmias, with a focus on molecular mechanisms, cardiac alterations and therapeutic ameliorations, with a particular emphasis on the contribution of oxidative stress to the pathogenesis of cardiac arrhythmias. The author states that modifications induced by diabetes within the heart change the electrical signaling and conduction in turn altering ion channels and gap junctions' expression and function. Still, antiarrhythmic drugs are effective in the course of diabetes but their mode of action remains to be better characterized.

Uthman et al. dive into direct cardiovascular effects of the novel drug class of SGLT2 inhibitors, the first antidiabetic drugs that were able to reduce hospitalization of heart failure. Although SGLT2 inhibitors were develop to specifically target the kidney (SGLT2 mainly present in kidney), current research demonstrate many additional important and possibly beneficial direct effects of these drugs on endothelial cells and cardiomyocytes. SGLT2 inhibitors can normalize elevated sodium and calcium levels in cardiomyocytes through inhibition of the cardiac sodium-hydrogen exchanger, improve vascular function and activate anti-oxidant systems and intracellular stress signals such as AMPK and reduce inflammatory pathways. Further research will have to demonstrate to what extent these cellular cardiac mechanisms contribute to the large beneficial effects of SGLT2i's in clinical trials. Despa then goes on to describe that sodium is often increased in the failing and diabetic cardiac cell. Elevated intracellular sodium can then cause oxidative stress and augments the sarcoplasmic reticulum Ca^{2+} leak, thus amplifying the risk for arrhythmias and promoting heart dysfunction. Alterations in Na^+ extrusion and/or Na^+ uptake that underlie the $[Na^+]_i$ increase in heart failure and diabetes are discussed, and emphasis is placed on the emerging role of Na^+ -glucose cotransporters in the diabetic heart. Doliba et al. subsequently demonstrate the functional and energetics consequences of elevated sodium in the diabetic cardiomyocyte in their pioneering studies in this field. Their work clearly indicates that raising sodium in the cardiac cell will directly result in impaired mitochondrial function, possibly explaining the often observed energetic problems in the diabetic heart. These reviews all point to the importance of ionic homeostasis and disturbances in the setting of diabetic cardiomyopathy, cardiac arrhythmias, and the elevated incidence of heart failure in T2DM patients.

The article by Liu et al. evaluated the clinical effects of Black garlic in patients with ischemic heart failure. They found that black garlic treatment improved cardiac function in terms of left ventricular ejection fraction and the scores of quality of life (QOL) and decreased circulating BNP precursor N-terminal (Nt-proBNP) by increasing antioxidant levels.

Koyani et al. studied the effects of saxagliptin and sitagliptin (anti-hyperglycemic drugs that have been shown to inhibit dipeptidyl peptidase 4 -DPP4) in mouse ventricular cardiomyocytes. The authors showed that saxagliptin (but not sitagliptin) impaired Ca^{2+} transient relaxation and prolonged action potential duration in cardiomyocytes. They suggested that these results are linked to saxagliptin-DPP9 interaction and following impairment in Ca^{2+}/calmodulin- dependent protein kinase II (CaMKII)- phospholamban (PLB) and protein kinase C (PKC) signaling.

Trotta et al. investigated the anti-hypertrophic effect of the melanocortin receptor 5 (MC5R) stimulation in cardiomyocytes exposed to high glucose. The authors showed that MC5R agonists increased viability and reduced total protein in cell stimulated with high glucose via reduced GLUT1/GLUT4 ratio at the plasma membrane and increased intracellular phosphoinositide 3-kinase (PI3K) activity. In addition, MC5R stimulation showed beneficial effects on cardiac function and hypertrophy paralleled by significantly reduced blood glucose levels in a rat model of diabetes (streptozotocin-induced).

We have arrived at exciting times in the world of diabetes and heart failure. At last, several novel antidiabetic drugs have shown large cardiovascular benefits in T2DM patients. Understanding the causal mechanism of these effects are now crucial in order to further our understanding of the interaction between diabetes and heart failure, possibly also offering new therapeutic strategies to combat heart failure in the absence of diabetes.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Diabetes and Arrhythmias: Pathophysiology, Mechanisms and Therapeutic Outcomes

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The prevalence of diabetes is rapidly increasing and closely associated with cardiovascular morbidity and mortality. While the major cardiovascular complication associated with diabetes is coronary artery disease, it is becoming increasingly apparent that diabetes impacts the electrical conduction system in the heart, resulting in atrial fibrillation, and ventricular arrhythmias. The relationship between diabetes and arrhythmias is complex and multifactorial including autonomic dysfunction, atrial and ventricular remodeling and molecular alterations. This review will provide a comprehensive overview of the link between diabetes and arrhythmias with insight into the common molecular mechanisms, structural alterations and therapeutic outcomes.

Keywords: diabetes mellitus, arrhythmia, atrial fibrillation, cardiac fibrosis, autonomic dysregulation

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INTRODUCTION

Diabetes mellitus is a group of metabolic disorders where there are high blood sugar levels over time. Prolonged elevations in sugar levels lead to a number of health complications including cardiovascular disease and kidney disease (Forbes and Cooper, 2013). There are two main forms of diabetes mellitus including type 1, which has an unknown etiology and is characterized by a loss of insulin-producing β-cells in the pancreas resulting in the inability of the pancreas to produce enough insulin (van Belle et al., 2011). Type 1 diabetes is often juvenile in onset, insulin dependent, and comprises roughly 10% of the diabetic patient population. Type 2 diabetes results from insulin resistance and the body's inability to respond to insulin (Kahn et al., 2014). It is generally adult-onset and is a result of genetics and lifestyle choices including excessive body weight, lack of exercise and poor diet. Type 2 diabetes is rapidly increasing in incidence (Centers for Disease C and Prevention, 2008). As of 2015 there were an estimated 415 million people with diabetes worldwide (Federation, 2014).

Cardiac arrhythmia is a group of conditions where the heart beats too fast (tachycardia), too slow (bradycardia) or irregularly (Roberts-Thomson et al., 2011). While most arrhythmias are not serious acutely, prolonged arrhythmic episodes increase an individual's likelihood of stroke, heart failure and cardiac arrest (Nattel et al., 2014). Arrhythmias arise due to a problem in the electrical conduction of the heart however, the cause of these complications is not fully defined. Atrial fibrillation is the most common type of arrhythmia and is associated with significant morbidity and mortality (Kannel et al., 1983). It is becoming increasingly apparent that diabetes mellitus is a significant promoter of cardiac arrhythmias (Kannel et al., 1998).

While diabetes likely contributes to multiple types of cardiac arrhythmias, the connection between diabetes and atrial fibrillation has been the most extensively studied to date. Observational studies looking at the association between diabetes mellitus and atrial fibrillation have been

inconclusive and inconsistent (Benjamin et al., 1994; Psaty et al., 1997; Wilhelmsen et al., 2001; Nichols et al., 2009; Pallisgaard et al., 2016; Dahlqvist et al., 2017). The incidence of diabetes is most commonly associated with coronary artery disease however, electrical conduction complications are also an important cardiovascular problem associated with both type 1 and type 2 diabetes (Huxley et al., 2011; Dahlqvist et al., 2017). In a 38 year follow up of Framingham heart study patients, diabetes mellitus was identified as an independent risk factor of atrial fibrillation (Benjamin et al., 1994). However, other studies failed to see a connection between atrial fibrillation and diabetes (Wilhelmsen et al., 2001). Discrepancies in these studies may be a result of the populations examined since differences in the association of diabetes and atrial fibrillation appear to be variable depending on age (Pallisgaard et al., 2016), gender (Nichols et al., 2009; Dahlqvist et al., 2017) and ethnicity (Lipworth et al., 2012; Dewland et al., 2013; Rodriguez et al., 2016; O'Neal et al., 2017). In a comprehensive meta-analysis, diabetic patients were found to have an ∼40% greater risk for developing atrial fibrillation compared to non-diabetic patients (Huxley et al., 2011) and a more recent meta-analysis, identified a 20% increase in the risk of developing atrial fibrillation for prediabetic patients whereas in patients with diabetes, this number was elevated to 28% greater change of atrial fibrillation development (Aune et al., 2018). Furthermore, this meta-analysis identified a dose dependent relationship between increased blood glucose levels and atrial fibrillation suggesting that rises in glucose may be an important contributor to atrial fibrillation. In a large study, over 845,000 patients, diabetes was found to be a strong, independent risk factor for atrial fibrillation and other cardiovascular diseases mellitus (Movahed et al., 2005). However, obesity, which is common in patients with diabetes mellitus is independently associated with atrial fibrillation and might also be a contributing factor (Grundvold et al., 2015; Baek et al., 2017). Levels of pericardial fat have been linked to atrial fibrillation in humans (Al Chekakie et al., 2010). Though not as extensively characterized, diabetes likely also contributes to ventricular arrhythmias since there is electrocardiographic evidence of this in humans and the underlying mechanisms linking diabetes with atrial fibrillation would apply to other types of arrhythmias (Cardoso et al., 2003). **Table 1** summarizes the clinical studies examining the correlation between diabetes and arrhythmias. While there is a clear link between diabetes and cardiac arrhythmias, the mechanisms underlying these changes are not fully elucidated. Potential causes include changes in glucose levels, the autonomic nervous system, structural and electrical remodeling, mitochondrial alterations and inflammation will be reviewed herein (**Figure 1**).

BLOOD GLUCOSE LEVELS

Meta-analysis of clinical populations suggests a dose-dependent relationship between blood glucose levels and atrial fibrillation, implying that glucose levels may be an important contributor to atrial fibrillation onset (Aune et al., 2018). However, this may not be the case since intensive glucose control has not been shown to be beneficial in reducing death from cardiovascular causes or all-cause death in multiple large trials (Group et al., 2008; Duckworth et al., 2009) and has been associated with increased mortality in another (Action to Control Cardiovascular Risk in Diabetes Study et al., 2008). Duration of pharmacological treatment (Dublin et al., 2010) and poorly controlled diabetes have also been linked to increased incidence of atrial fibrillation (Huxley et al., 2012). However, in a prospective study of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial cohort of patients, intensive glycemic control did not impact the rate of new-onset atrial fibrillation (Fatemi et al., 2014). Animal studies investigating the involvement diabetes in atrial fibrillation suggest it may be due to glucose fluctuations rather than hyperglycemia (Saito et al., 2014). In a streptozotocin-induced rat model, glucose fluctuations increased incidence of atrial fibrillation, atrial fibrosis, and reactive oxygen species (Saito et al., 2014).

There is increasing evidence that hypoglycemic states may contribute to atrial fibrillation. Several reports of hypoglycemic triggered atrial fibrillations have been reported clinically (Odeh et al., 1990; Celebi et al., 2011; Ko et al., 2018) and information gathered from the Framingham Heart Study suggests insulin resistance does not play a role (Fontes et al., 2012). Severe hypoglycemia in type 2 diabetics was associated with a range of adverse clinical outcomes including death from cardiovascular cause (Chow et al., 2014) and in patients from the Outcomes Reduction with an Initial Glargine Intervention (ORIGIN) trial, severe hypoglycemia was associated with greater risk for all-cause mortality and arrhythmic death (Investigators et al., 2013). In a study using 30 patients with type 2 diabetes and known cardiovascular disease, glucose monitoring in conjunction with electrocardiograms showed patients taking insulin and/or sulfonylurea had a high incidence of severe (<3.1 mmol/L) but asymptomatic hypoglycemia whereas patients taking metformin and/or dipeptidyl peptidase-4 inhibitors did not and patients with severe hypoglycemia had more ventricular arrhythmias (Stahn et al., 2014). In a separate but similar study, type 2 diabetic patients with a history or risk of cardiovascular disease were monitored for interstitial glucose and ambulatory electrocardiogram simultaneously (Chow et al., 2014). Bradycardia and atrial and ventricular ectopic counts were higher during episodes of nocturnal hypoglycemia further suggesting a role for hypoglycemia in arrhythmic events.

AUTONOMIC DYSFUNCTION

The autonomic nervous system is an important regulator of heart rhythm through innervation by sympathetic and parasympathetic nerves. Dysfunction of the autonomic nervous system is recognized as a risk for development of atrial fibrillation and a contributing factor to disease progression (Agarwal et al., 2017). A link between type 2 diabetes and autonomic dysfunction has also been well established and is recognized as a complication that damages multiple organs including the heart (Mäkimattila et al., 1997; Oberhauser et al., 2001). While the etiology of diabetic autonomic neuropathy is not fully understood, it is thought that metabolic insult, neurovascular insufficiency, autoimmune

TABLE 1 | Characterization of studies evaluating the correlation between diabetes and arrhythmias. Statistics are reported as [risk ratio (95% confidence interval)].

Study	Location	Duration	Population characteristics	Findings
Benjamin et al., 1994	United States	38 years	2090 males 2641 females 55-94 years old	Follow-up from the Framingham Heart Study, diabetes was significantly associated with the development of atrial fibrillation (1.4 for men, 1.6 for women)
Dahlqvist et al., 2017	Sweden	10.2 years (non-diabetics) 9.7 years (diabetics)	179,980 non-diabetics 35.4±14.5 years old 36,253 type 1 diabetics 35.6±14.6 years old	Slight increased risk in males [1.13 (1.01–1.25)] and greater increased risk [1.50 (1.30–1.72)] in females
Dublin et al., 2010	United States	N/A	2203 control 68 years median age 1410 atrial fibrillation 74 years median age	Increased risk of developing atrial fibrillation in pharmacologically treated diabetic patients [1.40 (1.15–1.71)] compared with control (1.00) whereas non-treated diabetics had no difference [1.04 (0.75–1.45)]
Fatemi et al., 2014	United States and Canada	4.68 years	5042 diabetic-standard glycemic control 5040 diabetic-intensive glycemic control	Intensive glycemic control had no impact on atrial fibrillation incidence compared with standard therapy in diabetic patients
Fontes et al., 2012	United States	∼10 years	3023 59.2±6.9 years old	Insulin resistance was no associated with risk of atrial fibrillation
Huxley et al., 2011	Multiple Countries	N/A	1,686,097	Meta-analysis associated diabetes with atrial fibrillation [1.39 (1.10–1.75)]
Huxley et al., 2012	United States	N/A	13,025	Pre-diabetic and untreated diabetes were not associated with increased risk for atrial fibrillation. Type 2 diabetics had an increased risk of atrial fibrillation [1.35 (1.14–1.60)]. No association was observed between fasting glucose or insulin and atrial fibrillation but there was a positive association between HbA1c levels and atrial fibrillation in both diabetic and non-diabetic subjects.
Ko et al., 2018	Korea	8.5 years	1,509,280 30-75 years old	Severe hypoglycemia was associated with increased risk of atrial fibrillation [1.10 (1.01–1.19)]
Lipworth et al., 2012	United States	9 years	3026 white 5810 black >65 years old	Diabetes was associated with an increased risk for atrial fibrillation in both white [1.38 (1.15–1.66)] and black [1.25 (0.98–1.59)] subjects with an elevated incidence in white subjects.
Movahed et al., 2005	United States	10 years	552,624 non-diabetic 293,124 diabetic Primarily male 65 year old average	There was a significant association between type 2 diabetes and development of atrial fibrillation [2.13 (2.10–2.16)] and atrial flutter [2.20 (2.15–2.26)]
Nichols et al., 2009	United States	7.2 years	7159 non-diabetics 10,213 diabetics 58.4±11.5 years old	Positive association of diabetes with atrial fibrillation among women [1.26 (1.08–1.46)] but not men [1.09 (0.96–1.24)]
O'Neal et al., 2017	United States	10 years	8611 white 5077 black 63 year old average	Diabetes was associated with a slightly elevated risk for atrial fibrillation in white subjects [1.21 (1.01–1.45)] but not black subjects [1.06 (0.79–1.43)]
Psaty et al., 1997	United States	3.28 years	4844 combined gender >65 years old	Elevated blood glucose was associated with atrial fibrillation [1.10 (1.04–1.17)]
Pallisgaard et al., 2016	Denmark	16 years	4,827,713 non-diabetics 253,374 diabetics	Diabetes is associated with incidence of atrial fibrillation, particularly in young patients 2.34 relative risk with a 1.52–3.60 (95% confidence level) in 18–39 year olds, 1.52 (1.47–1.56) in 40–64 year olds, 1.20 (1.19–1.23) in 65–74 year olds and 0.99 (0.97–1.01) in 75–100 year olds
Investigators et al., 2013	Multiple Countries	6.2 year median	12,537 50+ years old	Severe hypoglycemia was associated with risk of arrhythmic death [1.77 (1.17–2.67)]
Rodriguez et al., 2016	United States	13.7 years	114,083 non-Hispanic white 11,876 African American 5174 Hispanic 3803 Asian 63 year old average age Females	Diabetes was associated with a slightly elevated risk for atrial fibrillation in women (1.33 for non-Hispanic whites, 1.42 for African American, 1.25 for Hispanic, 1.42 for Asian) with no notable difference dependent on ethnicity
Wilhelmsen et al., 2001	Sweden	25.2 years	7495 males 47-55 years old	No association

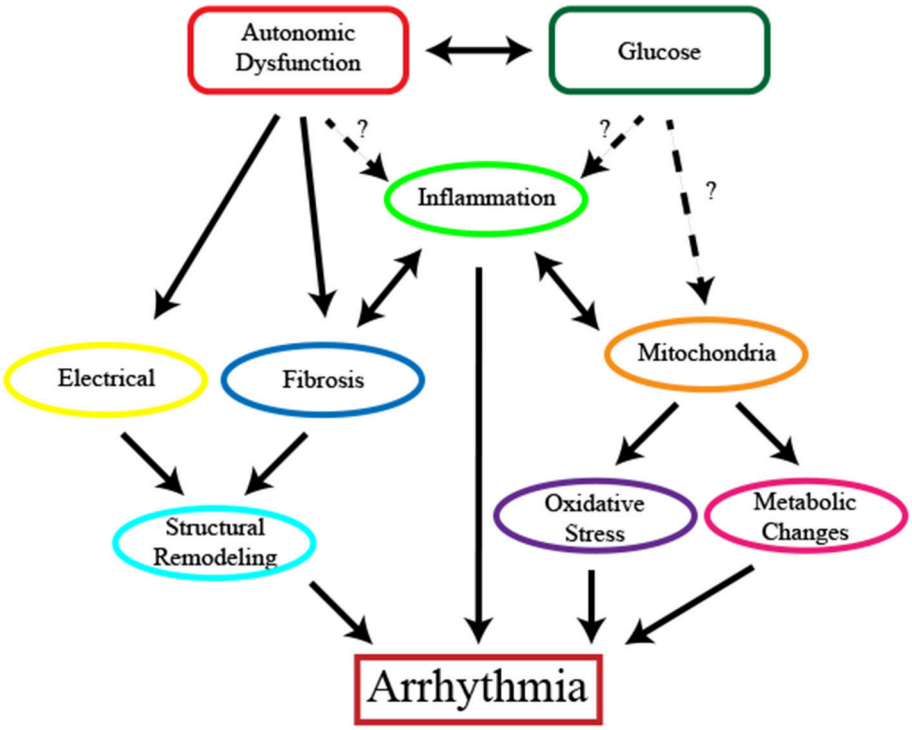

FIGURE 1 | The complex relationship between diabetes and cardiac arrhythmias. Potential contributors to the induction of cardiac arrhythmias including hypoglycemia, hyperglycemia or glucose fluctuations and autonomic dysfunction activate multiple mechanisms to contribute to the development of cardiac arrhythmias. Structural remodeling including changes in the electrical conduction of the heart and fibrosis promote and potentiate the progression of the disease. Mitochondrial dysfunction leads to changes in cardiomyocyte function and metabolism and contributes to disease progression through oxidative stress. Inflammation is present and may arise as a result of oxidative stress and structural changes.

damage and deficiency in neurohormonal growth factors may be contributing factors to the damage or loss of nerves (Vinik et al., 2003). Despite the fact that cardiovascular autonomic neuropathy has been extensively studied, it remains largely overlooked and serious complication of diabetes (Vinik et al., 2003).

In a study of nearly 2,000 men and women from the Framingham Offspring Study, heart rate variability, an indicator of autonomic nervous system function, was associated with plasma glucose levels and reduced in diabetic and patients with impaired fasting glucose levels (Singh et al., 2000). In healthy, non-diabetic adults, impaired heart rate recovery, another measure of autonomic dysfunction, and a predictor of all cause death in diabetic patients (Wheeler et al., 2002; Cheng et al., 2003), was more common in participants with abnormal fasting plasma glucose levels, which was also true in diabetic patients (Panzer et al., 2002). A closer examination of the link between diabetes and autonomic neuropathy using heart rate recovery as measure of autonomic dysfunction also associated diabetes and autonomic dysfunction with new-onset atrial fibrillation and heart failure independent of other cardiovascular risk factors (Negishi et al., 2013). Interestingly, this study demonstrated an incremental and predictive association between diabetes, heart rate recovery and new-onset atrial fibrillation. There is some evidence that the presence of cardiac autonomic neuropathy in asymptomatic type 1 and type 2 diabetes patients could predict major cardiovascular events including arrhythmias (Valensi et al., 2001). The recurrence of atrial fibrillation is increased in diabetic patients with autonomic neuropathy, which was determined by Ewing's test. Electrocardiographic measurements from diabetic patients with autonomic neuropathy had a longer P-wave duration and dispersion compared to control patients or diabetic patients, suggesting that autonomic neuropathy is causing inhomogeneous atrial depolarization to trigger atrial fibrillation (Bissinger et al., 2011). In a study investigating the changes in autonomic function and repolarization that occurring during prolonged hypoglycemia in type 2 diabetic patients, twelve type 2 diabetic patients and eleven age and body mass index-matched control patients had their glucose levels maintained by hyperinsulinemic clamps at euglycemia (6 mmol/L) or hypoglycemia (2.5 mmol/L; Chow et al., 2017). Differences in autonomic regulation during periods of hypoglycemia, as indicated by heart rate, heart rate variability and blood pressure, occurred between patients with type 2 diabetes and controls during hypoglycemia, suggesting that changes in cardiac autonomic regulation in diabetic patients may occur during hypoglycemic episodes and may contribute to arrhythmias (Chow et al., 2017).

Animal models of diabetes show alterations in cardiac innervation (Gando et al., 1993; Otake et al., 2009; Švíglerová et al., 2011; Thaung et al., 2015). In a streptozotocin-induced

diabetic rat model, diabetic rats had increased heterogeneity of sympathetic nerves as measure by immunohistochemistry for tyrosine hydroxylase (sympathetic nerves) and acetylcholinesterase (parasympathetic nerves; Otake et al., 2009). This study also found that sympathetic nerve stimulation increased incidence of atrial fibrillation in diabetic rats. Zucker diabetic fatty rats have elevated resting cardiac sympathetic nerve activity (Thaung et al., 2015). Signs of chronic β-adrenergic stimulation were observed in hearts from these rats including impaired responses to dobutamine stimulation, downregulation of β1-adrenergic receptors and increases in Gαi proteins. These studies demonstrate dysfunction of the autonomic nervous system in diabetes, confirming the findings from human studies.

STRUCTURAL REMODELING

Structural remodeling likely plays a large role in which diabetes mellitus and obesity promote cardiac arrhythmias. Atrial hypertrophy, fibrosis and fat deposits are observed in the hearts of obese and type II diabetes patients (Tadic and Cuspidi, 2015). Extensive atrial fibrosis is a hallmark of atrial fibrillation and is thought to play a role in both initiating and perpetuation the arrhythmia. Fibrotic tissue in the myocardium disrupts the geometry of the heart and alters the mechanical, electrical and chemical composition (**Figure 2**). There is extensive evidence of cardiac fibrosis in both type 1 Sutherland et al., 1989 and type 2 diabetes (Regan et al., 1977; Fischer et al., 1984; Nunoda et al., 1985; van Hoeven and Factor, 1990; Shimizu et al., 1993; Kawaguchi et al., 1997) however, the contribution of fibrosis to atrial fibrillation is not fully understood in the context of diabetes. Structural remodeling is particularly relevant in diabetic cardiomyopathy where architectural changes including fibrosis and cardiomyocyte length changes as a result of cardiac dilation increased axial resistance in cardiomyocytes which exasperates conduction dysfunction (Aromolaran and Boutjdir, 2017).

Animal models of diabetes also exhibit signs of increased cardiac fibrosis (Kato et al., 2006; Liu et al., 2012). In Goto-Kakizaki rats, a genetic non-overweight type 2 diabetes model with slight impairments of glucose tolerance, Goto-Kakizaki rats had significantly greater atrial arrhythmogenicity and increased atrial fibrosis compared with control rats (Kato et al., 2006). However, this study did not examine the progression of the disease making it difficult to determine if arrhythmias arose due to atrial fibrosis or if fibrosis is a contributing factor to atrial fibrillation. In a rabbit alloxan-induced diabetic model, diabetic rabbits had increased atrial interstitial fibrosis and electrophysiological changes including a prolonged inter-atrial conduction time and increased atrial effective refractory period which increased the inducibility of atrial fibrillation (Liu et al., 2012).

Advanced glycation end products (AGEs) are proteins or lipids that become glycated as a result of sugar exposure and have become recognized as a major contributor to complications from diabetes (Ramasamy et al., 2011). AGEs and AGE receptors (RAGEs) may contribute to the structural remodeling seen in the diabetic heart. AGEs and RAGEs are increased in streptozotocin-induced diabetic rat atrial and inhibition of AGE formation significantly reduced elevated levels of connective tissue growth factor and atrial fibrosis observed in the diabetic rats (Kato et al., 2008). Elevated RAGE has been associated with atrial fibrillation in humans, however the link between RAGE and diabetes was not observed in this study (Lancefield et al., 2016). However, other mechanisms likely also contribute to the fibrosis seen in the diabetic heart. Studies using fasudil, a Rho-kinase (ROCK) inhibitor, and a high-fat/low dose streptozotocin rat model of diabetes, implemented the RhoA/ROCK pathway in cardiac fibrosis through decreasing RhoA, ROCK and collagen expression (Chen et al., 2014).

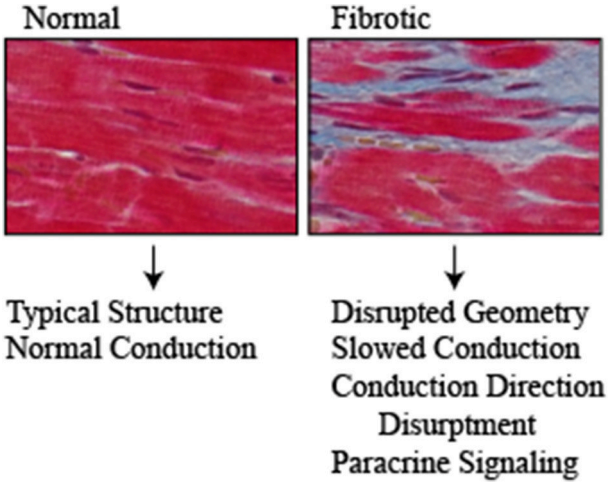

FIGURE 2 | Normal and fibrotic cardiac tissue highlights the structural changes that occur with fibrosis (Red=cardiomyocytes, Blue=fibrosis). Structural changes that occur with diabetes contribute to the pathogenesis of arrhythmias through disrupting the normal architecture of the heart. Fibrosis and fat deposits slow the electrical conduction and disrupt the direction. Furthermore, they serve as a source of paracrine signaling molecules including cytokines/chemokines, adipokines and pro-fibrotic that exasperate the disease.

While not as extensively investigated as fibrosis, increases in epicardial and pericardial fat is associated with type 2 diabetes (Rosito et al., 2008; Noyes et al., 2014; Levelt et al., 2016) and are correlated with left atrial enlargement, cardiac structural changes, and has been associated with increased atrial fibrillation risk (Al Chekakie et al., 2010; Batal et al., 2010; Wong et al., 2011). Increased epicardial fat is associated with adipocyte infiltration into the myocardium, which contributes to changes in the electrical conduction between cardiomyocytes due to physical disruption and slowing of the conduction time (Friedman et al., 2014; Mahajan et al., 2015; Haemers et al., 2017). Epicardial and pericardial fat is also an abundant source of adipokines and cytokines which have pro-fibrotic and pro-inflammatory effects on the heart. Studies have shown that the secretome from human epicardial fat, including TGF-β family members and matrix metalloproteinases, produces a pro-fibrotic response in rat atrial myocardium (Venteclef et al., 2015). Additionally, pericardial

and epicardial fat have increased markers of inflammation including C-reactive protein, IL-6, IL-1β, and TNF-α, which are associated with increased incidence, severity and reoccurrence of atrial fibrillation (Abe et al., 2018). Furthermore, inflammatory mediator production is not reversed by standard therapies including angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (Mazurek et al., 2003).

ROLE OF ELECTRICAL CONDUCTION

Studies consistently demonstrate prolonged action potentials in diabetic patients and animal models. Type 1 (Sivieri et al., 1993) and type 2 (Jermendy et al., 1990; Veglio et al., 2002; Ramirez et al., 2011) diabetic patients have been identified as having slowed conduction velocity and an increased prevalence of prolonged QT interval. In some cases, this has been linked with autonomic neuropathy (Ewing and Neilson, 1990). This is also observed in numerous animal models of type 1 and type 2 diabetes (Xu et al., 2002; Lengyel et al., 2007; Huang et al., 2013). Studies in Goto-Kakizaki (Howarth et al., 2008) and Zucker Diabetic Fatty rats (VanHoose et al., 2010) also identified prolonged QT intervals with additional changes in the R wave amplitudes and signs of autonomic neuropathy. While prolongation of action potentials consistently occurs throughout studies, the mechanisms responsible are less clear. Potassium channels have been the most widely identified change leading to slowed action potentials in diabetic hearts. Prolongation of the QT interval is observed in diet-induced obese mice, which was attributed to a protein kinase D-dependent reduction in voltage gated potassium channel expression (Huang et al., 2013). Oxidative stress-induced alterations in GsH redox state, which will be discussed in more detail in subsequent sections, may regulate K+ channels, that have been shown to be decreased in myocytes isolated from diabetic rats and be reversed by insulin application (Xu et al., 2002). This has also been further supported in follow-up studies, corroborating the involvement of glucose metabolism in these changes (Xu et al., 1996). Changes in outward K+ currents are observed early after streptozotocin injection and corresponded with increases in glucose levels, which were prevented by blocking hyperglycemia (Shimoni et al., 1994). Periodic changes in K+ ion current have been linked to oscillations in energy metabolism in cardiomyocytes, which could be modulated by changing glucose metabolism (O'Rourke et al., 1994). Studies have shown that in cardiomyocytes, glycolysis is more effective than oxidative phosphorylation at regulating K+ channel opening (Weiss and Lamp, 1987). These changes in K+ channels observed in small animals appear to also hold true in larger, more human relevant animal species. In a type 1 diabetes model in dogs, while only slight lengthening in ventricular repolarization was observed, there were decreases in transient outward K+ and slow delayed rectifier potassium currents (Lengyel et al., 2007). In a streptozotocin-induce type 1 diabetes mouse model, prolongation of the QT interval were observed along with increased susceptibility to arrhythmia and decreased K+ currents (Meo et al., 2016). Many of these studies make it difficult to determine the mechanisms responsible for changes in electrical conduction since animal models often have a number of alterations in metabolism including hyperglycemia and hyperlipidemia. However, studies using a cardiac-specific insulin receptor knockout mouse model, several K+ channel components that are important for ventricle repolarization were identified as being decreased, in particular components of the transient outward K+ current fast component, which were also associated with a reduction in the current (Lopez-Izquierdo et al., 2014). Similar with what is seen in other diabetic models and human patients, cardiac-specific insulin receptor knockout mice also had a longer ventricular action potential duration due to a prolonged QT interval, substantiating the role of insulin signaling in diabetes-induced arrhythmias.

Contrarily, not all research implicates K+ currents in action potential changes with diabetes. In a fructose-fat fed rat model of pre-diabetes, QRS prolongation was present, slower conduction velocity, and increased propensity for ventricular fibrillation (Axelsen et al., 2015). There were no changes in Na+ or K+ currents, fibrosis or gap junctions, suggesting another mechanism for dysfunction. In an alloxan-induced diabetic rabbit model, no changes in action potential duration were observed, but there was a reduction in conduction velocity (Stables et al., 2014). A reduction in cell capacitance and Na+ channel density were present in diabetic hearts however, no changes in gap junctions or fibrosis were observed. In obesity-induced QT interval prolongation, there is extensive literature connecting abnormal calcium conduction with arrhythmias (Aromolaran and Boutjdir, 2017). However, this mechanism of arrhythmogenesis is not clear in diabetes and if Na+ or Ca+ channels play an important role in diabetes-induced arrthymias remains to be determined. Energy in the form of ATP is necessary for maintaining membrane potential and generating action potentials, which is mainly supplied by oxidative phosphorylation in the mitochondria, and oxidative phosphorylation to a lesser extent (Barth and Tomaselli, 2009). It is likely that metabolic activity and arrhythmias are interdependent since changes in the cellular energy promotes arrhythmias however arrhythmias also influence metabolic activity. Whole transcriptome analysis of human atrial tissue revealed an upregulation of metabolic process related genes with atrial fibrillation (Barth et al., 2005). This was confirmed in a study using human atrial appendages where metabolomics and proteomics were performed comparing patients with sinus rhythm compared to patients that developed persistent atrial fibrillation following cardiac surgery (Mayr et al., 2008). Patients with atrial fibrillation had an elevation in substrates and enzymes for ketogenic metabolism and other metabolic processes. Additionally, mutantion or knockout of important ion channel genes cause both prolonged ventricular repolarization as well as diabetes (Hu et al., 2014). Hypoglycaemia is also associated with hypokalemia, which could contribute to delayed repolarization (Petersen et al., 1982; Heller and Robinson, 2000; Christensen et al., 2009). As mentioned above, there is extensive clinical and experimental evidence to suggest that structural alterations contribute to the occurrence and persistence of atrial fibrillation (Nattel and Harada, 2014). Changes in gap junctions, which are important for the electrical

impulse propagation and synchronization in the heart, are observed with fibrosis and hypertrophy and affect the electrical conduction of the heart (Spach et al., 1988; Saffitz and Kléber, 2004; Ten Tusscher and Panfilov, 2007; dos Santos et al., 2016). In the adult heart, connexin-43 is the main cardiac gap junction component and changes in expression, distribution or post-translational modifications contribute to heart rhythm disturbances (Boengler et al., 2006). Therapies to restore connexin levels are capable of improving conduction disturbances in atrial fibrillation models, further supporting the importance of connexins in the pathogenesis of atrial fibrillation (Igarashi et al., 2012).

There may be alterations in gap junctions in the heart with diabetes. Decreases in phosphorylated and overall connexin-43 levels, a major gap junction protein which has been linked with atrial fibrillation, have been shown in a streptozotocin-induced diabetes model (Mitasíková et al., 2009) which may occur through protein kinase Cε-dependent mechanisms (Lin et al., 2006). These changes were associated with a decreased in connexin-43 phosphorylation and ventricular conduction abnormalities. However, in a different study that also used a streptozotocin-induced diabetes model, connexin-43 levels were elevated and distribution changes were evident (Hage et al., 2017). Other studies using diabetic (db/db) mice show decreased connexin-43 expression that can be reversed with exercise (Veeranki et al., 2016). In this study, exercise resulted in improvements in mitochondrial oxygen consumption rate, tissue ATP levels and reduced cardiac fibrosis with diabetes. Further convoluting the involvement of cardiac connexin-43 in diabetes-induced arrhythmias, an obese diabetic (db/db) mouse model of diabetes showed was atrial hypertrophy and fibrosis without alterations in connexin-43 staining (Hanif et al., 2017). No alterations in connexin-43 levels were also observed in a Zucker Diabetic Fatty rat model of type 2 diabetes, where the conduction velocity was significantly slower in diabetic rats, but levels of connexin-43 were unchanged (Olsen et al., 2013). However, this study did observe distribution changes in connexin-43, which may contribute to functional changes.

Changes in other connexins may also contribute to the pathogenesis of cardiac arrhythmia with diabetes. In a streptozotocin-induced diabetic rat model where connexin-40, 43, and 45 mRNA expression was measured in the sinoatrial node, right ventricle and right atrium, connexin-45 expression was significantly elevated in the sinoatrial node with no changes seen in the atrial or ventricles (Howarth et al., 2007). Using a streptozotocin-induced diabetic rat model, the duration of atrial tachyarrhythmia induced by atrial stimulation was extended in diabetic rats while the conduction velocity was decreased (Watanabe et al., 2012). Increased atrial fibrosis was also observed in diabetic rats compared with controls and had decreased connexin 40 expression with no significant differences in connexin 43. A separate study examining mRNA changes in sinoatrial node of streptozotocin-induced diabetic rats failed to see differences in connexin 40 expression but identified increased transcript expression for connexin 45 among changes in numerous other ion channels including transient receptor potential channel (TRPC) 1, TRPC6, voltage gated calcium channel (Ca_v) 3.1, $Ca_v\beta3$, ryanodine receptor 3 and $Ca_v\gamma4$ (Ferdous et al., 2016). This same group identified a unique profile of ion channel alterations in the atrioventricular node with no changes in connexins (Howarth et al., 2017).

ROLE OF MITOCHONDRIA AND OXIDATIVE STRESS

The contribution of oxidative stress to the pathogenesis of cardiac arrhythmias is becoming increasingly recognized (Yang and Dudley, 2013; Samman Tahhan et al., 2017). There are a number of signs of oxidative stress with atrial fibrillation including increased levels of superoxide and hydrogen peroxide (Dudley et al., 2005; Kim et al., 2005; Reilly et al., 2011; Zhang et al., 2012), decreased nitric oxide bioavailability (Cai et al., 2002; Bonilla et al., 2012), changes in the ratio of oxidized glutathione disulfide to reduced glutathione and differences in the ratio of oxidized cysteine to reduced cysteine (Neuman et al., 2007). There is also known to be increased oxidative stress in diabetes, which contributes to the damage of multiple tissue types throughout the body including the heart (Giacco and Brownlee, 2010; Rochette et al., 2014). In diabetes, there is a known increase in superoxide production, which contributes to a reduction in vascular nitric oxide bioactivity through increased NADPH oxidases and dysfunction endothelial nitric oxide synthase (Guzik et al., 2002) which is similar decreases in endothelial nitric oxide synthase and nitric oxide bioavailability are associated with atrial fibrillation (Cai et al., 2002).

Changes in oxidative stress that are present in the heart during diabetes are likely mitochondrial in origin since in diabetic human atrial tissue, where mitochrondrial changes in metabolism of multiple substrates are observed (Anderson et al., 2009). Hydrogen peroxide emissions are increased regardless of the substrate, suggesting alterations in the electron transport system or antioxidant capacity (Anderson et al., 2009). In permeabilized myofibers from right atrial appendages obtained from non-diabetic and type 2 diabetic patients, mitochondria from diabetic patients had a decreased capacity for glutamate and fatty acid-supported respiration, increased content of myocardial triglycerides and increased mitochondrial hydrogen peroxide emission during oxidation of carbohydrate and lipid based substrates (Anderson et al., 2009). There is some evidence that oxidative stress contributes to the atrial remodeling and inflammation seen with atrial fibrillation. In a rabbit alloxan-induced diabetes model, Langendorff perfused diabetic hearts had greater induction of atrial fibrillation following burst pacing, which was decreased with use of the antioxidant probucol (Fu et al., 2015). Antioxidant administration also attenuated atrial interstitial fibrosis and signs of decreased oxidative stress including reductions in serum and tissue malonaldehyde, NF-κB, TGF-β, and TNF-α. However, several studies in humans have shown little or no cardiovascular benefits from antioxidant supplementation demonstrating the need for better understanding of the mechanisms that oxidative stress contributes to atrial fibrillation in the context of diabetes (Sesso et al., 2008; Violi et al., 2014).

There has been limited investigation into the mechanisms linking oxidative stress, diabetes and arrhythmias. One of the most extensively studied mechanisms is Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). CaMKII is a serine-threonine kinase that has emerged as an important nodal point to allow cardiomyocytes to respond to perturbances in calcium and reactive oxygen species through the activation of a diverse group of downstream targets to regulate membrane excitability and calcium cycling (Voigt et al., 2012; Mesubi and Anderson, 2016). CaMKII is increased in atria of atrial fibrillation patients and in mouse models of susceptible to atrial fibrillation (Purohit et al., 2013). CaMKII has been shown to influence calcium dynamics through several different mechanisms in the diabetic heart. In obese Zucker rats and high-fat-fed rodents, there is increased muscle mitochondrial content and CaMKII activation (Jain et al., 2014). Increases in mitochondrial reactive oxygen species and S-nitrosylation of the ryanodine receptor lead to increased SR calcium leak and activation of CAMKII. CaMKII may also contribute to connexin alterations and electrical conduction changes observed in diabetes (Zhong et al., 2017). In ApoE knockout mice fed a high fat diet, downregulation of the ion channels, connexin-43 upregulation and ventricular remodeling could be prevented by administration of a CaMKII antagonist.

Post-translational modifications of CamKII may contribute to its role in diabetes-induced arrhythmias. Animal studies show that mitochondria isolated from streptazocin treated rat hearts have increased total *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) and *O*-GlcNAc transferase levels, with *O*-GlcNAc transferase being localized in the mitochondrial matrix as opposed to an inner membrane localization in control rats (Banerjee et al., 2015). Mislocalization of *O*-GlcNAc transferase results in decreased interactions with complex IV of the electron transport chain, resulting in impairments of its activity. Acute hyperglycemia in cardiomyocytes has been shown to result in covalent and persisting modifications of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) by *O*-GlcNAc, which can also be observed in the heart and brain of diabetic humans and rats (Erickson et al., 2013). *O*-GlcNAc modification of CaMKII leads to increased activation of spontaneous sarcoplasmic reticulum Ca^{2+} release resulting in arrhythmias (Erickson et al., 2013). In diabetic humans and mouse models of diabetes, there are increased levels of oxidized CaMKII (Luo et al., 2013). Oxidized CaMKII has been linked with ventricular arrhythmia (Wang et al., 2018) and atrial fibrillation (Purohit et al., 2013) however, how oxidized CaMKII contributes to atrial fibrillation with diabetes is not currently defined. In addition to changes in CaMKII, abnormal calcium handling has been observed in diabetic hearts however, how this relates to arrhythmogenesis has no yet been investigated (Belke and Dillmann, 2004; Lacombe et al., 2007).

ROLE OF INFLAMMATION

Inflammation has been identified as a risk factor for cardiac arrhythmias due to the increased frequency of incidence following cardiac surgery (Bruins et al., 1997), genetic studies (Gaudino et al., 2003) and increased occurrence during myocarditis (Spodick, 1976). In human patients, C-reactive protein (Aviles et al., 2003) has been associated with incidence of atrial fibrillation and was able to predict future development and polymorphisms in the interleukin-1 family affect risk for atrial fibrillation (Cauci et al., 2010; Gungor et al., 2013). Inflammation has also been suggested as an underlying pathogenic mediator for diabetes. Since it was identified that TNF-α secretion by adipocytes played a role in the body's update of glucose and response to insulin which contributes to the development of diabetes (Hotamisligil et al., 1993), extensive research has been done looking at the role of inflammation in diabetes (Wellen and Hotamisligil, 2005; Calle and Fernandez, 2012).

The connection between inflammation, arrhythmias and diabetes is not currently well characterized and an ongoing area of research however, hypoglycemia, which has been suggested to trigger atrial fibrillation (Odeh et al., 1990; Celebi et al., 2011; Ko et al., 2018), increases markers of inflammation (Investigators et al., 2013). In a recent study, toll-like receptor (TLR) 2 knockout mice have decreased incidence of arrhythmias compared to wild-type mice in a streptozotcin model of diabetes mellitus (Monnerat et al., 2016). This is thought to occur through IL-1β production by macrophages since macrophages from TLR2 knockout animals had lower levels of $MCHII^{high}$ macrophages and NLRP3 inflammasome. IL-1β was decreased in the hearts of TLR2 knockout animals and IL-1β could decreased potassium current and increase calcium sparks in isolated cardiomyocytes. Furthermore, inhibition of the NLRP3 inflammasome or IL-1β reversed diabetes-induced arrhythmias.

THE IMPACT OF DIABETES THERAPIES ON ARRHYTHMIAS

Current type 2 diabetes therapies aim to treat hyperglycemia to reduce and maintain glucose concentration to normal levels in an effort to prevent the development of complications (Kahn et al., 2014). Since the process through which diabetes causes arrhythmias is not currently know, the impact of current therapies is just beginning to be understood. Studies suggest that merely controlling glucose levels is not beneficial (Group et al., 2008; Duckworth et al., 2009) and potentially detrimental (Action to Control Cardiovascular Risk in Diabetes Study et al., 2008) in controlling cardiovascular complications. This lack of benefit from intense glycemic control includes the rate impact the rate of new-onset atrial fibrillation (Fatemi et al., 2014). However, poorly controlled diabetes has also been linked to increased incidence of atrial fibrillation showing that the role of glycemic control is not fully understood at this time (Huxley et al., 2012).

Metformin is currently the most widely used medication to treat type 2 diabetes and acts to suppress gluconeogenesis thus lowering glucose levels. Metformin has been associated with decreased atrial fibrillation risk compared with diabetic patients not taking medication (Chang et al., 2014). *In vitro* studies using an atrial cell line demonstrated that metformin decreased reactive oxygen species in response to pacing and prevented cardiomyocyte remodeling (Chang et al., 2014). In

diabetic Goto-Kakizaki rats, metformin treatment decreased cardiac fibrosis and arrhythmias (Fu et al., 2018). Alterations in small conductance calcium-activated potassium channels were observed in the atria of these animals, which was corrected with metformin treatment, suggesting that metformin may restore the atrial electrophysiology. Metformin has also been shown to prevent high glucose induction of apoptosis, autophagy and connexin-43 downregulation in H9C2 cells, a ventricular myoblast cell line (Wang et al., 2017). However, there have been reported incidences of onset of atrial fibrillation with metformin use in diabetic patients (Boolani et al., 2011), which may be attributed to lactic acidosis, which occurs rarely with metformin treatment (Salpeter et al., 2010).

Thiazolinediones are peroxisome proliferator-activated receptor-γ agonists, which decrease glucose levels by increasing storage of fatty acids in adipocytes, also decrease incidences of atrial fibrillation onset, which might also be influenced by their anti-inflammatory actions (Chao et al., 2012; Pallisgaard et al., 2017; Zhang Z. et al., 2017). However, other studies have shown in patients with coronary disease, thiazolinediones have no improvements in atrial fibrillation compared with other diabetes treatments including metformin, insulin, sulfonylurea or meglitinides, suggesting that the anti-inflammatory effects of thiazolinediones does not further improve anti-arrhythmia effects of controlling glucose levels (Pallisgaard et al., 2018).

Dipeptidyl peptidase-4 (DPP-4) inhibitors, such as alogliptin, which increases incretin levels to inhibit glucagon release leading to increased insulin secretion are also a common treatment for type 2 diabetes. In an alloxan-induced rabbit model of diabetes mellitus, diabetic rabbits had increased left ventricular hypertrophy and left atrial dilation (Zhang X. et al., 2017). Diabetic hearts had a higher level of atrial fibrillation inducibility and treatment with alogliptin prevented morphological changes and increased propensity for atrial fibrillation. Additionally reactive oxygen species, mitochondrial membrane depolariazation and mitochondrial biogenesis were improved with alogliptin. In a Taiwanese population, DPP-4 inhibitors, in conjunction with metformin, decreased the onset of atrial fibrillation compared to diabetics taking metformin and other second-line therapies (Chang et al., 2017). Other standard diabetes therapies may also have beneficial effects on cardiac arrhythmias. Retrospective studies suggest that there may be differences in sudden cardiac arrest and ventricular arrhythmias between types of sulonylurea medications, where glyburide was found to have lower risk for sudden cardiac arrest and ventricular arrhythmias compared with glipizide (Leonard et al., 2018).

ARRHYTHMIA THERAPIES IN DIABETIC PATIENTS

Pharmacological therapies for arrhythmia include agents that control rate and rhythm. Currently, there has been no research examining the efficacy of anti-arrhythmic medications in patients with diabetes (Dobbin et al., 2018). Surgically, catheter ablation is an established therapeutic option for heart rhythm control in drug resistant patients. Catheter ablation has been shown in a large study composed of 1,464 patients to have the same efficacy and safety in diabetes patients as the general population (Anselmino et al., 2015). However, due to the presence of other atrial fibrillation recurrence predictors such as alterations in the electrical and anatomical composition of the atrial myocytes, metabolic alterations and other comorbidities (D'Ascenzo et al., 2013), the need to redo ablation is more common (Chao et al., 2010; Anselmino et al., 2015). However, smaller studies have shown that while ablation is equally effective in diabetic patients, there are increased numbers of thrombotic or hemorrhagic complications (Tang et al., 2006).

Thromboprophylaxis therapies are also commonly used in patients with atrial fibrillation to decrease risk of stroke and other complications. A number of clinical trials investigating the effectiveness of various theromboprophylaxis therapies have included diabetic subpopulations. In the Rivaroxaban Once Daily Oral Direct Factor Xa Inhibition Compared with Vitamin K Antagonism for Prevention of Stroke and Embolism Trial in Atrial Fibrillation (ROCKET-AF) trial, 40% of the patients had diabetes (Patel et al., 2011). The Randomized Evaluation of Long-Term Anticoagulation Therapy (RE-LY) trial comparing warfarin and high dose dibagatran was composed of about 23% diabetic patients (Connolly et al., 2009). The Effective Anticoagulation with Factor Xa Next Generation in Atrial Fibrillation-Thrombolysis in Myocardial Infarction (ENGAGE AF-TIMI) trial had ∼36% diabetic participants (Giugliano et al., 2013). No differences were observed between diabetic and non-diabetic patients in any of these studies. The Apixaban for Reduction in Stroke and Other Thromoembolic Events in Atrial Fibrillation (ARISTOTLE) trail, composed of ∼25% diabetic patients, found no difference in the primary outcome of decreased stroke and thromboembolic events between diabetic and non-diabetic patients (Granger et al., 2011). However, diabetic patients had an increased risk of bleeding compared with non-diabetic participants. Taken together, these studies suggest that anti-arrhythmic and anti-thromboprophylaxis therapies are effective in diabetic patients with few adverse effects.

The renin-angiotensin system is involved in the genesis of arrhythmias through its impact on structural and electrical remodeling (Iravanian and Dudley, 2008). Therapies targeting this pathway including angiotensin-converting enzyme (ACE) inhibitors and angiotensin-II receptor blockers (ARB) have been hypothesized to be beneficial in preventing atrial fibrillation occurrence and are currently the focus of numerous studies (Iravanian and Dudley, 2008). Activation of the renin-angiotensin system is often associated with diabetes where it is thought to impact the initiation and progression of the disease (Giacchetti et al., 2005). While there have not been direct studies linking the renin-angiotensin system and diabetes-induced arrhythmias, it is likely that they are intertwined since the renin-angiotensin system impacts nearly all contributing factors including cardiac remodeling, electrical remodeling and inflammation (Iravanian and Dudley, 2008). Inhibitors of the renin-angiotensin system have been shown to reduce cardiovascular events, decrease diabetic complications and can reduce incidence of new onset diabetes (Hansson et al.,

1999; Heart Outcomes Prevention Evaluation Study, 2000; Brenner et al., 2001; Dahlof, 2002; Bangalore et al., 2011). This has been confirmed in a large clinical trial investigating the effects of the ARB valsartan on diabetes development in patients with impaired glucose tolerance where valsartan was found to reduce incidence of diabetes but did not reduce the rate of cardiovascular events (Group et al., 2010). However, in patients with impaired fasting glucose or impaired glucose tolerance, the ACE inhibitor Ramipril was not able to reduce new incidence of diabetes in patients with impaired fasting glucose but did promote normoglycemia (Investigators et al., 2006). While renin-angiotensin system targeted therapies show promise in reducing incidence of cardiac arrhythmias and diabetes, additional research is necessary to further understand the mechanisms involved and confirm studies performed in small patient populations.

CONCLUSIONS

The impact of diabetes on the electrical conduction of the heart and development of cardiac arrhythmias is becoming increasingly apparent. Due to the complex, multifactorial nature of diabetes, the relationship between diabetes and cardiac arrhythmias is not yet fully understood however, correlations between increased blood glucose levels, glucose fluctuation and hypoglycemia, and arrhythmias have been observed and are a likely initiator of the disease. Autonomic dysfunction, which is known to contribute to diabetic complications in other tissues potentiates disease progression through changes in the heart's energy needs, the production of paracrine signaling factors and alterations in receptors that influence ion channel activity in the heart. Alterations in the architecture of the heart including fibrosis, fat deposition and hypertrophy change the electrical conduction of the heart and disrupt the pattern of the electrical signal. They are also an important source of paracrine factors that enhance disease progression. Taken together, these alterations within the heart change the electrical conduction by regulating ion channels and gap junctions between cardiomyocytes changing the electrical signaling. While anti-arrhythmic therapies appear to be effective in diabetic patients, the effectiveness of diabetes therapeutics on prevention of cardiac arrhythmias is unclear. Due to the complex nature of diabetes and cardiac arrhythmias, further experimental and clinical research is necessary to fully elucidate the relationship between diabetes and arrhythmias in the hope of developing improved therapeutic strategies in the future.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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Direct Cardiac Actions of Sodium Glucose Cotransporter 2 Inhibitors Target Pathogenic Mechanisms Underlying Heart Failure in Diabetic Patients

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Sodium glucose cotransporter 2 inhibitors (SGLT2i) are the first antidiabetic compounds that effectively reduce heart failure hospitalization and cardiovascular death in type 2 diabetics. Being explicitly designed to inhibit SGLT2 in the kidney, SGLT2i have lately been investigated for their off-target cardiac actions. Here, we review the direct effects of SGLT2i Empagliflozin (Empa), Dapagliflozin (Dapa), and Canagliflozin (Cana) on various cardiac cell types and cardiac function, and how these may contribute to the cardiovascular benefits observed in large clinical trials. SGLT2i impaired the Na^+/H^+ exchanger 1 (NHE-1), reduced cytosolic [Ca^{2+}] and [Na^+] and increased mitochondrial [Ca^{2+}] in healthy cardiomyocytes. Empa, one of the best studied SGLT2i, maintained cell viability and ATP content following hypoxia/reoxygenation in cardiomyocytes and endothelial cells. SGLT2i recovered vasoreactivity of hyperglycemic and TNF-α-stimulated aortic rings and of hyperglycemic endothelial cells. Anti-inflammatory actions of Cana in IL-1β-treated HUVEC and of Dapa in LPS-treated cardiofibroblast were mediated by AMPK activation. In isolated mouse hearts, Empa and Cana, but not Dapa, induced vasodilation. In ischemia-reperfusion studies of the isolated heart, Empa delayed contracture development during ischemia and increased mitochondrial respiration post-ischemia. Direct cardiac effects of SGLT2i target well-known drivers of diabetes and heart failure (elevated cardiac cytosolic [Ca^{2+}] and [Na^+], activated NHE-1, elevated inflammation, impaired vasorelaxation, and reduced AMPK activity). These cardiac effects may contribute to the large beneficial clinical effects of these antidiabetic drugs.

Keywords: SGLT2 inhibitors, cardiomyocyte, endothelial cell, smooth muscle cell, cardiac fibroblast, isolated heart, 2 type diabetes, heart failure

SGLT2 INHIBITORS IN DIABETES AND HEART FAILURE: EXPLORING DIRECT CARDIAC EFFECTS

Sodium glucose cotransporter 2 (SGLT2) inhibitors (SGLT2i) are kidney-targeted anti-diabetic agents that have exhibited marked reductions in cardiovascular events and mortality of type 2 diabetes (T2D) patients. In two large clinical trials, Empa, (Zinman et al., 2015) and Cana, (Neal et al., 2017) reduced the risk of heart failure related hospitalization by 35 and 33%, respectively. These trials demonstrate that SGLT2i may serve as an effective treatment strategy against heart failure in a T2D setting. SGLT2i were designed to inhibit the kidney-specific SGLT2 and to induce glycosuria. SGLT2 is part of a family of proteins facilitating glucose translocation in a variety of tissues. SGLT2 is mainly expressed in the kidney, with some expression in the pancreatic alpha cells. In the kidney, SGLT2 is located in the first part of the proximal tubule, where it enables ∼90% of glucose reabsorption from the urine; the other 10% reabsorption is through SGLT1, which is located in the distal part of the proximal tubule. SGLT2i use is associated with ∼50 g/day of glucose loss, which corresponds with ∼200 kcal/day (Ferrannini et al., 2015). Indeed, administration of a SGLT2i on top of standard glucose lowering therapy (metformin, insulin and sulfonylureas) modestly reduced (∼0.4%) glycated hemoglobin plasma levels and was associated with small decreases in body weight, plasma insulin and blood pressure (Zinman et al., 2015 Neal et al., 2017). However, it is unlikely that the small changes in these parameters can explain the large beneficial actions of SGLT2i, and thus preclinical studies were sought to explore possible underlying mechanisms.

In vivo preclinical studies have shown decreased ROS levels, inflammatory cytokines and vascular dysfunction after SGLT2i administration in animals with diabetes (Oelze et al., 2014; Andreadou et al., 2017; Tanajak et al., 2018). Moreover, preserved cardiac function has been observed with administration of SGLT2i Empa in a non-diabetic model of chronic heart failure (Byrne et al., 2017). In microvascular coronary endothelial cells of mice with STZ-induced diabetes, Empa administration resulted in activation of AMPK, improved mitochondrial function through inhibition of Drp1-mediated fission, restoration of vascular barrier function, eNOS phosphorylation, adhesion molecules expression and reduced mitochondrial ROS levels (Zhou et al., 2018). However, the *in vivo* animal studies cannot discern whether the cardiovascular beneficial effects of Empa and other SGLT2i are due to kidney-related systemic alterations or due to direct cardiovascular effects, or both. To examine whether SGLT2i operate directly on cardiac specific pathophysiological mechanisms without interference of other mediating factors, including plasma circulating glucose and insulin, isolated cardiac cell and organ studies are required. In this review we summarize the results of these studies and discuss how these direct cardiac effects of the inhibitors may contribute to the effects seen on heart failure. We restrict ourselves to cardiac cellular studies using clinically relevant SGLT2i concentrations ($\leq$10 μM).

PATHOGENESIS LINKING DIABETES AND HEART FAILURE

T2D is associated with a two to five fold higher risk of developing heart failure (Kannel et al., 1974; Cavender et al., 2015). Vice versa, chronic heart failure patients have an increased risk of developing T2D and metabolic abnormalities, including increased insulin resistance (Riehle and Abel, 2016). Thus, the presence of one disease impacts the development or progression of the other. Patients with heart failure and T2D have a worse prognosis than those with either one of the diseases (Cubbon et al., 2013).

Heart failure is a syndrome characterized by fatigue, dyspnea and the inability to exercise. It often develops after MI, valvular disease, chronic tachycardia, hypertension, diabetic cardiomyopathy, or in the setting of various genetic or acquired cardiomyopathies. The initial remodeling process associated with heart failure primarily includes left ventricular hypertrophy, fibrosis and diastolic dysfunction. Although the exact causes of T2D are still debated, hyperinsulinemia, insulin resistance, hyperglycemia and dyslipidemia are considered early contributors to the development of T2D (Jia et al., 2018). In T2D patients, insulin resistance and increased salt sensitivity lead to hypertension by increased sodium retention, activation of sympathetic nervous system and atherosclerosis (Feldstein, 2002; Lastra et al., 2010). Atherosclerosis in turn may cause MI and heart failure. Furthermore, the T2D heart is considered to be in a state of metabolic overload, with elevated glycogen and lipid stores within the heart (Varma et al., 2018). T2D is only one of several diseases that can develop into heart failure (see above). Early on in the development of heart failure, there are several key mechanisms that instigate functional and structural cardiac impairments, which are shared among heart failure and

Abbreviations: ΔG_{ATP}, Gibbs free energy of ATP hydrolysis; $[Ca^{2+}]_c$, cytoplasmatic calcium; $[Na^+]_c$, cytoplasmatic sodium; 2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose; AGE, advanced glycation end-product(s); Akt, protein kinase B; AMP/ADP, adenosine monophosphate/adenosine diphosphate; AMPK, 5′ adenosine monophosphate-activated protein kinase; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; CABG, coronary artery bypass graft; Cana, Canagliflozin; C_{max}, maximal serum concentration; Dapa, Dapagliflozin; Drp1, dynamin-related protein; Empa, Empagliflozin; eNOS, endothelial nitric oxide synthase; ETC, electron transport chain; HAEC, human aortic endothelial cells; HbA1c, hemoglobin A1c; HFpEF, heart failure with preserved ejection fraction; HUVEC, human umbilical cord vein endothelial cells; ICAM-1, intercellular Adhesion Molecule 1; IL-1β, interleukin 1β; IL-10, interleukin 10; IL-6, interleukin 6; IR, ischemia-reperfusion; IRS-1, insulin receptor substrate 1; JNK, c-Jun N-terminal kinases; LPS, lipopolysaccharides; LTCC, L-type calcium channel; MCP-1, monocyte chemoattractant protein 1; MI, myocardial infarction; Na^+/K^+, ATPase sodium-potassium ATPase; NCX, Na^+/Ca^{2+}-exchanger; NFκB, nuclear factor-kappa B; NHE, sodium hydrogen exchanger; NLRP3, NACHT, LRR and PYD domains-containing protein 3; NO-cGMP-PKG, nitric oxide - cyclic guanosine monophosphate - protein kinase G; NOX2, NADPH oxidase 2; PA, palmitic acid; PCr/ATP, phosphocreatine/adenosine triphosphate; PTCA, percutaneous transluminal coronary angioplasty; ROS, reactive oxygen species; SGLT1, sodium glucose cotransporter 1; SGLT2, sodium glucose cotransporter 2; SGLT2i, sodium glucose cotransporter 2 inhibitor(s); SMC, smooth muscle cell(s); SMIT-1, sodium-myo-inositol co-transporter 1; SR, sarcoplasmic reticulum; STAT3, signal transducer and activator of transcription 3; STZ, streptozotocin; T2D, type 2 diabetes; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion protein 1.

all these diseases, including T2D (Bugger and Abel, 2014; Packer, 2017). Therefore, targeting these early overlapping mechanisms is a promising strategy to treat heart failure. Several of these mechanisms with potential relevance to direct myocardial effects of SGLT2i are summarized in **Figure 1**.

First, elevated cytosolic $[Na^+]$ and $[Ca^{2+}]$ ($[Na^+]_c$ and $[Ca^{2+}]_c$) are observed in cardiomyocytes from failing and T2D hearts (Despa et al., 2002; Lambert et al., 2015; Despa, 2018). Elevations in $[Na^+]_c$ and $[Ca^{2+}]_c$ are coupled to reduced mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) in cardiomyocytes, which will decrease the energetic and redox function of mitochondria (Murphy and Eisner, 2009; Bay et al., 2013). Elevated $[Ca^{2+}]_c$ is perceived in hyperglycemia- and LPS-induced endothelial dysfunction (Wang et al., 2008; Cui et al., 2013), atrial fibrillation (Neef et al., 2010) and in myocytes during IR injury (Garcia-Dorado et al., 2012). Increases in $[Na^+]_c$ and $[Ca^{2+}]_c$ are caused by perturbed ion fluxes due to altered activity of ion channels and/or transporters, including the sodium-calcium exchanger (NCX), the sodium-hydrogen exchanger 1 (NHE-1), the ryanodine receptor regulating SR-calcium release and the sodium-potassium ATPase (Na^+/K^+ ATPase), and targeting these ion regulating transporters has been proposed to improve cardiovascular function (Baartscheer et al., 2003a; Despa et al., 2012; Karmazyn, 2013; Luo et al., 2013; Sasahara et al., 2013).

Second, the development of an energy crisis, best reflected by decreases in cardiac PCr/ATP, is present in both heart failure and diabetic cardiomyopathy. As a consequence, the cellular energy sensor AMPK is altered. AMPK activity is increased in failing myocytes (Tian et al., 2001), whereas in T2D divergent AMPK alterations have been found (Varma et al., 2018). AMPK acts as an energy sensor, and activates catabolic actions such as increasing glucose uptake and glycolysis, and impairing anabolic reactions (Beauloye et al., 2011). Targeting the AMPK pathway by increasing its activity have been shown to combat cardiac hypertrophy and heart failure (Gélinas et al., 2018). Besides, AMPK activity is relevant in non-metabolic cellular processes, including the regulation of vascular tone and the suppression of inflammation (Beauloye et al., 2011; He et al., 2015; Cordero et al., 2018). Metformin, the first-line drug in the treatment of T2D for the last two decades, is known to increase AMPK (Foretz et al., 2014), an effect that can also be observed in the human non-diabetic heart tissue (El Messaoudi et al., 2015).

Third, early increased oxidative stress figures prominently in both heart failure (Dey et al., 2018) and T2D pathogenesis (Shah and Brownlee, 2017). The highly reactive ROS can oxidize many proteins in its vicinity, with consequently altered function of that protein. ROS is also an upstream driver of endothelial dysfunction associated with heart failure, by the increase of nitric oxide synthase (NOS) uncoupling, switching the production of NO into that of peroxynitrite (Paulus and Tschöpe, 2013; Joshi et al., 2014; Münzel et al., 2015). Reducing ROS therefore will normalize the function of many proteins of cardiac cells and may so undermine the development of heart failure.

Fourth a chronic low-grade state of inflammation is present in both heart failure and diabetes. Cellular ion dysregulation, AMPK inactivity and ROS production are relevant factors in the development of inflammation (Frati et al., 2017). Other mechanisms that underlie the progression of inflammation in diabetes may include increased *O*-GlcNAcylation, formation of AGEs, activation of the renin-angiotensin-aldosterone system, damage-associated molecular patterns (DAMPs) and the production of cytokines and adipokines (Frati et al., 2017). Inflammation may lead to fibrosis, cell death (pyroptosis) and cardiac remodeling, and its suppression may therefore be a relevant mechanism in the prevention and treatment of diabetes-associated heart failure.

These four mechanisms are not separate entities but are all intrinsically interrelated, and together may induce contractile, vascular and mitochondrial dysfunction, and cell death in the heart, which may evolve into left ventricular concentric or

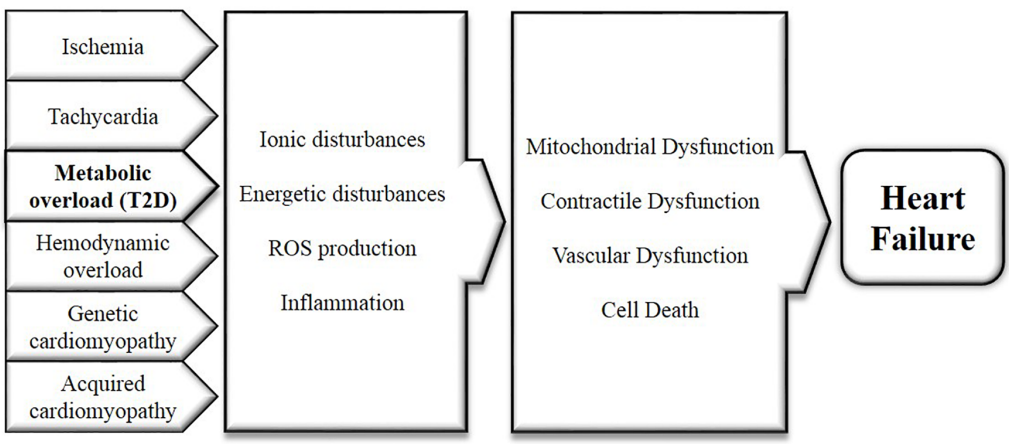

FIGURE 1 | Mechanisms underlying and linking diabetes and heart failure, caused by cardiac metabolic overload and all other diseases with elevated risk for developing heart failure. These mechanisms include the disturbances of cellular ion levels, energetic disturbances, excessive ROS production and inflammation. Consistent dysregulation of these factors will sequentially instigate contractile, endothelial and mitochondrial dysfunction as well as cell death, as observed in the pathogenesis of heart failure.

eccentric hypertrophy and heart failure (Boudina et al., 2007; Van Heerebeek et al., 2008; Miki et al., 2013; Sena et al., 2013; Cassidy et al., 2015; Munasinghe et al., 2016). In the subsequent paragraphs, SGLT2i effects in the major cardiac cell types will be discussed in relation to these four early signatures (ion homeostasis, energy balance, oxidative stress and inflammation) of both heart failure development and diabetic cardiomyopathy.

SGLT2 INHIBITORS IN CARDIOMYOCYTES

Intracellular Ion Regulation

Several reports have documented that SGLT2i modify ionic homeostasis in cardiomyocytes. Hamouda et al. (2014) showed that 1–10 μM Dapa reduced the amplitudes of cell shortening and L-type Ca^{2+} current in ventricular cardiomyocytes from STZ-treated and control rats. Dapa only lowered systolic $[Ca^{2+}]_c$, but not diastolic $[Ca^{2+}]_c$, in STZ-treated rat cardiomyocytes, however, this effect was absent in control rat cardiomyocytes. These changes were observed at t = 5 min and were absent after 1–3 h of incubation with 1 μM Dapa (Hamouda et al., 2014). These data suggest that Dapa may confer acute negative inotropic effects in the diabetic cardiomyocyte. In support of this, Empa (0.25–1 μM) reduced $[Na^+]_c$, diastolic and systolic $[Ca^{2+}]_c$, and impaired the NHE-1 activity in healthy rabbit cardiomyocytes (Baartscheer et al., 2017). In that study, Empa also increased $[Ca^{2+}]_m$ in rat cardiomyocytes. This finding may reflect improved mitochondrial capacity to synthesize ATP and target oxidants, which would be beneficial to restore the energetic state of myocytes that is known to be decreased in heart failure. The lowering of $[Na^+]_c$ by Empa was observed at 5 mM and 11 mM glucose incubations and inhibition of NHE-1 occurred in the presence and absence of extracellular glucose, supporting the notion that glucose and SGLT's were not involved in these direct Empa effects. In addition, we recently showed that Dapa (1 μM) and Cana (3 μM) also reduced $[Na^+]_c$ and impaired NHE-1 activity in mouse cardiomyocytes (Uthman et al., 2018a). Further prove for SGLT2i interaction with NHE-1 was obtained by molecular binding studies, showing SGLT2i to exhibit high binding affinities with the extracellular Na^+-binding site of the NHE (Uthman et al., 2018a). These data indicate that the SGLT2i exert an off-target effect on the NHE-1.

Mitochondrial Function

Empa increased cell viability and preserved ATP levels following hypoxia/reoxygenation in cultured H9C2 embryonic heart-derived cells (Andreadou et al., 2017). These effects were equally present in myocytes stimulated by AGE, and Empa did not change the expression level of RAGE (receptor for AGE), suggesting that these pro-survival mechanisms of Empa were not mediated through AGE/RAGE signaling. In mitochondria derived from cardiac muscle fibers obtained from isolated IR rat hearts, Empa increased complex II respiration, and permeabilized and uncoupled the inner mitochondrial membrane (Jespersen et al., 2017). The authors discuss that these mitochondrial changes may improve remodeling following IR conditions.

Expression of SGLT's

Although the SGLT2 has not been detected in cardiomyocytes and the heart, SGLT1 appears to be highly expressed in human, rat and mouse hearts and cardiomyocytes, and may even be upregulated in ischemic, hypertrophic, failing and diabetic hearts (Banerjee et al., 2009; Kashiwagi et al., 2015; Lambert et al., 2015; Vrhovac et al., 2015; Di Franco et al., 2017; Van Steenbergen et al., 2017). Currently, the relationship between SGLT2i and SGLT1 in failing and diabetic hearts has not been investigated.

Overall, SGLT2i directly affect cardiomyocytes by reducing $[Na^+]_c$ and $[Ca^{2+}]_c$, by inhibition of NHE-1, and by increasing mitochondrial function and cell viability (**Figure 2**). The exact membrane receptors/transporters mediating these effects in cardiomyocytes have not yet been identified, with the exemption of NHE-1 and LTCC.

SGLT2 INHIBITORS IN VASCULAR CELLS

Vascular function may be directly affected by SGLT2i through the changing of endothelial or vascular smooth muscle cell (SMC) function. Various reports have investigated SGLT2i effects on AMPK activity, mitochondrial function, vasodilation and on the expression of adhesion molecules in hyperglycemic and inflamed vascular cells (**Figure 3**).

AMPK Activity

Cell studies with SGLT2i in various disease models have generally shown that SGLT2i improve vascular function and/or reduce markers of atherogenesis, by directly acting on vascular cells. Increased AMPK activity was observed in intact HUVEC and HAEC, after 15–30 min treatment with Cana (≥10 μM) but not with Empa or Dapa (Mancini et al., 2018). Similarly, phosphorylation of ACC as an indicator of AMPK activation was increased acutely with 10 μM Cana in cultured human aortic vascular SMC (Mancini et al., 2018).

Cell Viability and Mitochondrial Function

In HUVEC subjected to hypoxia/reoxygenation, Empa increased cell viability and preserved ATP levels in the presence and absence of AGE (Andreadou et al., 2017). In healthy cells, viability remained unaltered with 24 h Empa or Dapa treatment, but was modestly reduced with Cana treatment, which is suggested to be caused by reduced glucose uptake causing compromised ATP synthesis (Mancini et al., 2018). Empa (1 μM) did prevent mitochondrial dysfunction in hyperglycemic mouse aortic rings, i.e., increased oxygen consumption rates and reduced proton leakage (El-daly et al., 2018). It is uncertain, however, whether Empa would affect the vasculature at physiologically more relevant hyperglycemic glucose concentrations (<20 mM). Together, these data suggest that SGLT2i directly improve cell viability under conditions of high glucose, and that this may be due to mitochondrial alterations.

Vasodilation

The effects of Dapa on endothelial cell- and SMC- dependent vascular tone in various models have been studied. Acute Dapa

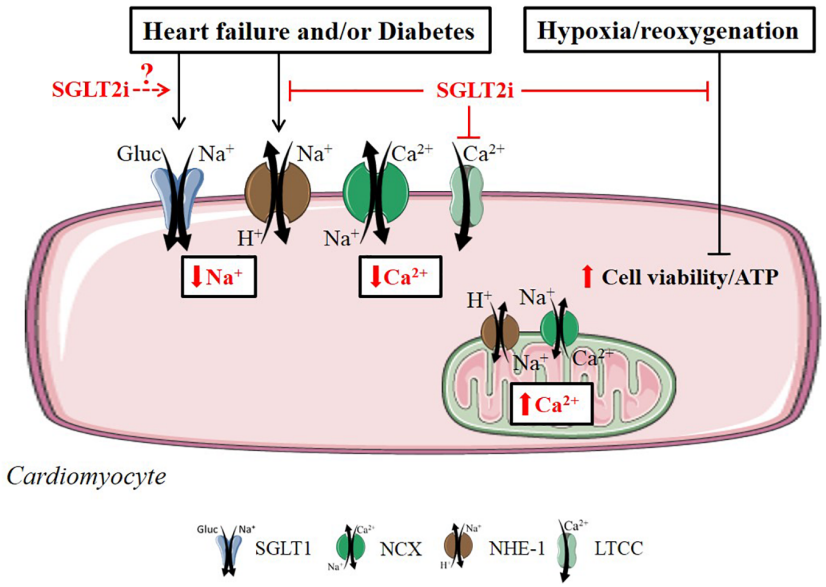

FIGURE 2 | Direct SGLT2i effects related to diabetes, heart failure and hypoxia/reoxygenation in the cardiomyocyte. SGLT2i exert beneficial cardiac cell effects in by directly regulating ionic homeostasis, mitochondrial respiration and cell viability in the cardiomyocyte. Reported intracellular SGLT2i effects are indicated in red arrow symbols. The relationship between SGLT2i and cardiac SGLT1 in the diabetic or failing cardiomyocyte is unknown (illustrated by dashed arrow line in the figure).

administration (1 nM–100 μM) increased vasorelaxation in an endothelial cell-independent manner in healthy mouse aortic rings, while in a hyperglycemic setting (44 mM glucose) 1 h pre-incubation with 1 μM Dapa restored relaxation without influencing SMC vasoreactivity (Gaspari et al., 2017). Acute treatment with high dose Cana (10 μM) caused vascular relaxation in pulmonary, but not coronary artery rings from single STZ-injected mice (Han et al., 2015), indicating vascular bed specific effects of SGLT2i. Pre-treatment with 0.5–1 μM Empa, 50 nM Dapa or 50 nM Cana for 24 h resulted in a stronger vasorelaxation response in hyperglycemic (25 mM glucose) mice aortic rings following application of acetylcholine or a proteinase-activated receptors 2 agonist (El-daly et al., 2018). Likewise, exposure of 1 μM Empa for 3 days promoted eNOS activity (nitrite formation) in hyperglycemic HUVEC, yet cell density was not restored at this physiologically relevant Empa dosage (Steven et al., 2017), suggesting that the functional improvement by Empa did not directly correlate to increased cell viability.

Cell Adhesion

In IL-1β-stimulated HUVEC and HAEC, 10 μM Cana attenuated secretion of MCP-1 and IL-6, which was at least partly associated with increased AMPK activity (Mancini et al., 2018). Dapa treatment (1–1000 nM) resulted in lower ICAM-1 and VCAM-1 protein levels and NFκB mRNA expression in HUVEC subjected to TNF-α or to a hyperglycemic insult. However, low dose Dapa (≤10 nM), but not 100 nM and 1 μM Dapa, seemed to reduce the inflammatory responses from hyperglycemia or TNF-α (Gaspari et al., 2017). Knowing that the maximal plasma concentrations of Dapa healthy volunteers taking 10 mg Dapa fluctuates between ∼6–300 nM (Kasichayanula et al., 2011), it raises the possibility of Dapa having anti-inflammatory actions in the T2D and/or heart failure patient. Contrary to Dapa application, Mancini et al. (2018) reported that adhesion molecule expression, JNK and NFκB signaling of LPS-stimulated HAEC were not altered after Cana administration (Mancini et al., 2018), although adhesion to pro-monocytic cells was reduced. Our own results indicated that SGLT2i pre-incubation does not prevent ICAM and VCAM expression and the increase in permeability in TNF-α stimulated HUVEC and human coronary artery endothelial cells [HCAEC, (Uthman et al., 2018b)].

Expression of SGLT's

As several studies reported absence of SGLT2 expression in the heart (Han et al., 2015; Vrhovac et al., 2015; Di Franco et al., 2017; Van Steenbergen et al., 2017), SGLT2 may be excluded as a possible explanation for the direct vascular effects of SGLT2i. However, others have proposed that the SGLT2 mRNA is ubiquitously expressed in most human tissue (Zhou et al., 2003) and that the SGLT2 protein is also present in endothelial cells (El-daly et al., 2018; Li et al., 2018). In HUVEC, both SGLT1 and SGLT2 expressions were demonstrated at the protein and mRNA level and their quantities were elevated in the presence of PA, which also resulted in reduced Akt, IRS-1 and eNOS phosphorylation and nitric oxide concentration (Li et al., 2018). PA-induced endothelial dysfunction was partially restored with administration of the classic SGLT inhibitor phlorizin, and knockdown of SGLT1 or SGLT2. Additionally, Empa reduced glucose uptake in cultured endothelial cells similar to SGLT1/2 inhibitor sotagliflozin, measured by reduced glucose analog 2-NBDG uptake (El-daly et al., 2018). So far, no direct target of SGLT2i on endothelial cells has been identified, although the

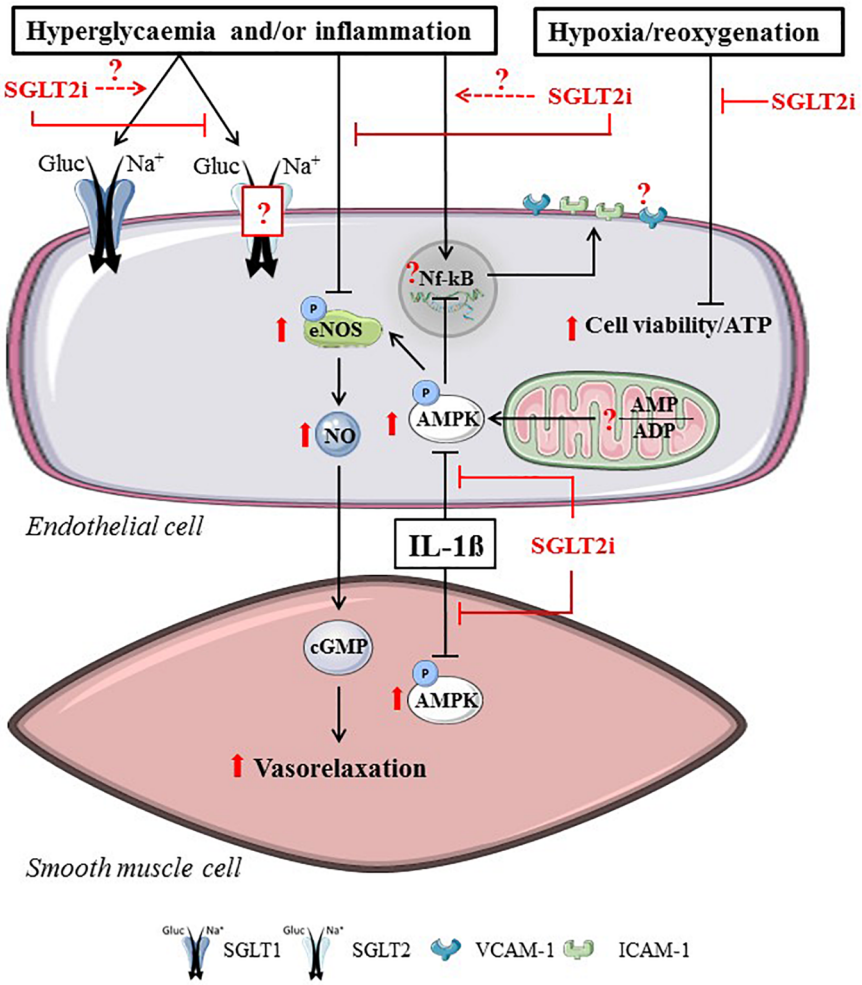

FIGURE 3 | Direct SGLT2i effects related to hyperglycemia, inflammation or hypoxia/reoxygenation in vascular cells. SGLT2i directly alter endothelial cells and smooth muscle cells by reducing SGLT2-mediated glucose uptake, ameliorating vasorelaxation, increasing AMPK activity and maintaining cell viability in vascular cells. Reported intracellular SGLT2i effects are indicated in red arrow symbols. The presence of SGLT2 in endothelial cells, the interaction between SGLT1 and SGLT2i, the effects of SGLT2i on AMP/ADP and the attenuation of NF-kB-mediated adhesion molecule expression remains uncertain (illustrated by dashed arrow lines and question marks in the figure).

presence of SGLT1 and SGLT2 in endothelial cells might be connected to the direct vascular effects summarized above.

SGLT2 INHIBITORS IN CARDIAC FIBROBLASTS

Cardiac fibroblasts are valuable targets for therapeutic applications due to their role in cardiac remodeling after MI. Ye et al. (2017) studied SGLT2i effects in mouse cardiac fibroblasts stimulated with LPS. Pre-incubation with Dapa (0.3–0.5 μM) showed attenuation of LPS-induced upregulation of NLRP3, ASC, IL-1β and caspase-1 mRNA levels (Ye et al., 2017). Administration of phlorizin (100 μM) did not exhibit similar reponses as Dapa and this indicates that SGLT-independent mechanisms were affected by Dapa. Effects of Dapa on NLRP3, TNF-α and caspase-1 were similar to A769662, an AMPK activator, while co-administration of Dapa with Compound C, an AMPK inhibitor, abrograted the anti-inflammatory effects, including phosphorylation of AMPKα at Thr172. Thus, Dapa induces anti-inflammatory responses in myofibroblasts, mediated through increased AMPK activation without the involvement of SGLT (**Figure 4**).

SGLT2 INHIBITORS IN ISOLATED HEARTS

Data retrieved from isolated heart studies indicate that SGLT2i have direct cardiac effects at organ level, i.e., acute vasodilation, delayed ischemic contracture onset and increased post-ischemic P-STAT3 expression. The degree of IR injury and vasodilation, however, relies upon the concentration administered as well as the type of SGLT2i.

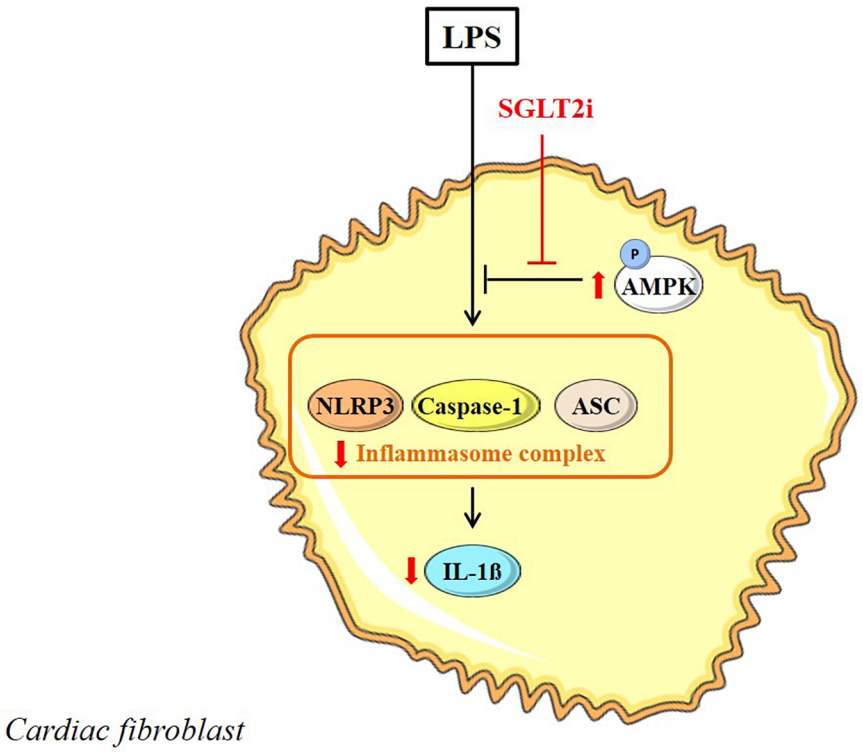

FIGURE 4 | Direct SGLT2i in the LPS-stimulated myofibroblast. SGLT2i increase AMPK activity and inhibit inflammasome activation in the myofibroblast. Reported intracellular SGLT2i effects are indicated in red arrow symbols.

Vasodilation

Several studies have addressed the effects of SGLT2i in the isolated perfused heart. Acute coronary vasodilation occurs in intact mouse hearts following Empa (1 μM) or Cana (3 μM), but not after Dapa (1 μM) (Uthman et al., 2018a). These findings may reflect the differences in drug efficacy since Dapa did show a trend toward vasodilation, which may suggest that a higher concentration of Dapa could induce vasodilation in the healthy heart.

Ischemia-Reperfusion

Empa (4.75 μM) administration prior to ischemia did not change infarct size after 40 min ischemia and 30 min reperfusion in the isolated rat heart (Jespersen et al., 2017). However, during a 25 min ischemia and 2 h reperfusion protocol in isolated mouse hearts, Empa (1–10 μM) delayed ischemic contracture development similar to the effects of a classic NHE-1 inhibitor Cariporide. Reperfusion injury remained unaltered or even worsened for the Empa-treated hearts under these conditions (Uthman et al., 2018c). These effects may indicate an ATP-sparing effect of SGLT2i that is caused by inhibition of NHE-1 during ischemia, although NHE-1 inhibition during reperfusion may differ between Empa and Cariporide. Furthermore, the detrimental outcomes of high-dosage Empa (higher than the maximal concentration observed in clinical trials), may possibly refer to potential cell toxic effects of this drug used at a higher concentration during IR injury. Furthermore, Empa did not change mitochondrial calcium retention capacity in mitochondria isolated from mouse hearts (Andreadou et al., 2017). This suggests that Empa does not directly affect the mitochondrial permeability transition pore, which is in line with findings reflecting that Empa does not protect the isolated heart against IR injury (Jespersen et al., 2017; Uthman et al., 2018c).

Fibrosis

Perfusion with a high dose of Dapa (10 μM) for 60 min increased STAT3 phosphorylation in the remote area of post-ischemic rat hearts (Lee et al., 2017). Increased p-STAT3 levels were associated with higher IL-10 levels in Dapa treated hearts, which is suggested to facilitate the conversion of macrophages from an inflammatory M1 to anti-inflammatory M2 phenotype and therefore inhibit myofibroblast differentiation and extracellular matrix formation.

DISCUSSION

This review provides an overview of the direct effects of SGLT2i in the heart and in the most prominent cell types present in the heart, including cardiomyocytes, endothelial cells, vascular SMC and cardiofibroblasts. **Figures 2–4** graphically summarize the described effects of SGLT2i in these cardiac cell types. They show that SGLT2i positively affect the Na^+ and Ca^{2+} homeostasis, inhibit NHE-1 activity, improve mitochondrial

respiration, and increase post-hypoxic ATP levels and cell viability in cardiomyocytes. In vascular cells, SGLT2i can directly improve vasorelaxation, increase AMPK activity, eNOS phosphorylation and, although inconsistently, adhesion molecule expression in hyperglycemic and/or inflamed conditions. In LPS-treated cardiofibroblasts, SGLT2i exert anti-inflammatory actions similar to AMPK activation pathways. Finally, in isolated hearts SGLT2i improved vasodilation, delayed ischemic contracture and increased post-ischemic STAT3 phosphorylation. These preclinical cell/organ studies clearly show that there are direct cardiac effects of SGLT2i, offering several off-target, cardioprotective mechanisms that may contribute to the beneficial effects observed in the clinical trials.

Ionic Homeostasis in Diabetes and Heart Failure

A growing number of pre-clinical studies have demonstrated beneficial cardiovascular effects of SGLT2i independent of their systemic glucose-lowering actions (Andreadou et al., 2017; Baartscheer et al., 2017; Byrne et al., 2017; Ye et al., 2017; Uthman et al., 2018a). In light of these results, the direct lowering of $[Na^+]_c$ by SGLT2i may constitute part of the beneficial effects of SGLT2i through attenuation of inflammation, endothelial dysfunction, oxidative stress and cardiac remodeling.

To reduce the global cardiovascular risk, the World Health Organization advises to lower daily salt intake within the population to <5 g (WHO, 2012). High salt (i.e., Na^+) intake evidently predicts the development of heart failure and diabetes (He and MacGregor, 2018) as well as an increased incidence of T2D in individuals with high caloric intake (Lanaspa et al., 2018). Na^+ loading contributes to elevated oxidative stress and activation of the NLRP3 inflammasome (inflammation), consequently leading to the development of diabetes in humans (Wan et al., 2018). In endothelial cells, a mild increase of extracellular Na^+ led to elevated levels of adhesion molecules and the transmigration of peripheral blood mononuclear cells (Dmitrieva and Burg, 2015). An exact molecular mechanism of Na^+ loading that causes inflammation has not been experimentally determined, although it has been reported that the efflux of K^+ via the Na^+/K^+-ATPase is a necessary step that triggers NLRP3 activation (Muñoz-Planillo et al., 2013). Increased Na^+/K^+-ATPase through elevated extracellular Na^+ may therefore cause K^+ efflux and activate NLRP3. Notably, increased extracellular Na^+ also led to activation of NLRP3 independent of K^+ efflux (Muñoz-Planillo et al., 2013). Taken together, extracellular Na^+ is an important determinant of inflammation and may therefore be relevant in targeting cardiovascular disorders.

A modest increase in extracellular glucose (from 5.5 to 11 mM) acutely elevated $[Na^+]_c$ in isolated cardiomyocytes (Baartscheer et al., 2017). Chronic hyperglycemia, as occurring in T2D and heart failure, may thus at least partly be accountable for the increase in cardiac $[Na^+]_c$. Increased $[Na^+]_c$ at a steady extracellular glucose concentration was recently observed in T2D rat cardiomyocytes, which could be ascribed to elevated Na^+-glucose cotransport through increased expression of SGLT1 (Lambert et al., 2015). Cardiac SGLT1 expression is increased in conditions of T2D and heart failure, in both animal and man (Banerjee et al., 2009; Lambert et al., 2015; Di Franco et al., 2017). Thus, $[Na^+]_c$ in cardiomyocytes can be increased due to increased extracellular glucose levels as well as increased SGLT1 expression.

Elevation of $[Na^+]_c$ results in a secondary rise of $[Ca^{2+}]_c$ via the Na^+/Ca^{2+}-exchanger (NCX) (Baartscheer et al., 2003a). Studies support the contention that increases in $[Na^+]_c$ and $[Ca^{2+}]_c$ induce signaling cascades that lead to dysregulation of mitochondrial homeostasis, including impaired energetics and elevated ROS production, and as such reduced cardiac hypertrophy and remodeling [reviewed in detail elsewhere: (Bay et al., 2013)].

The reduction in $[Ca^{2+}]_c$ by SGLT2i appears to be directly correlated to the lowering of $[Na^+]_c$. The $[Na^+]_c$ lowering effects of SGLT2i can be explained by inhibition of NHE-1. Cardiomyocytes express high amounts of NHE-1 (Yokoyama et al., 2000), which is also of paramount importance for pH regulation during pathological conditions, including diabetes, IR injury and heart failure (ten Hove et al., 2003; Packer, 2017). Preclinical studies have shown that pharmacological interventions targeting sarcolemmal sodium ion transporters proved effective in ameliorating heart failure (Kusumoto et al., 2001; Engelhardt, 2002; Baartscheer et al., 2003b, 2005, 2008; Baartscheer and Van Borren, 2008). However, these promising preclinical studies in the setting of HF on the use of NHE-1 inhibition never translated into clinical testing for HF, because of negative results obtained with the use of these inhibitors in the setting of acute cardiac ischemia. Clinical trials on the short-term use (<7 days) of NHE-1 inhibitors in the setting of acute MI in CABG and PTCA patients showed a reduction in the incident of non-fatal MI without an overall benefit on cardiovascular mortality. These clinical studies were halted due to the occurrence of an increased incidence in cerebrovascular events in these acute cardiovascular conditions following treatment with the NHE-1 inhibitors [Control vs. NHE inhibitor; EXPEDITION 3.0% vs. 5.2% ($p < 0.001$); GUARDIAN-trial 1.0% vs. 1.5%; ESCAMI-trial 0.2 vs. 2.2% (not significant)] (Théroux et al., 2000; Zeymer et al., 2001; Mentzer et al., 2008). In addition, the optimal dosage, timing and duration of the intervention and the patient populations that could most likely benefit from NHE-1 inhibitors were insufficiently investigated by phase 2 clinical trials, which may explain the divergence between the highly promising preclinical results of NHE-1 inhibitors and the ambiguity hereof in the known clinical trials. Furthermore, it is known that mild acidosis, but not severe acidosis, may be protective against ischemia in neuronal tissue (Vornov et al., 1996). Most importantly, to date no clinical trial has investigated the effects of using NHE-1 inhibitors as chronic therapy for the treatment of heart failure. Possibly, the risk of stroke may be less pronounced or even absent if NHE-1 inhibitors are used chronically, not immediately halted, used at milder dosages and in combination with platelet inhibitors. Therefore, the clinical efficacy of inhibiting NHE-1 and lowering $[Na^+]_c$ in different chronic cardiac disorders remains to be tested.

Previous experiments with Empa have shown that NHE-1 inhibition also occurred in glucose-free conditions, thus eliminating SGLT's as mediators for the lowering of $[Na^+]_c$ (Baartscheer et al., 2017). Whether SGLT2i also inhibit NHE-1 activity in diabetic cardiomyocytes has not yet been investigated, although increased activity of NHE-1, accompanied by higher $[Ca^{2+}]_c$ and hypertrophy has been observed in the diabetic myocardium (Darmellah et al., 2007). Others have reported elevated NHE-1 expression in glucose-induced hypertrophy in isolated rat cardiomyocytes, which may be prevented by treatment with Cariporide (Chen et al., 2007).

In diabetic and failing hearts, the cellular energetics (PCr/ATP) are impaired (Beer et al., 2002; Levelt et al., 2016). ATP generation is directly linked to $[Ca^{2+}]_m$ as it determines the regulation of dehydrogenases, generation of reducing equivalents for oxidative phosphorylation, ΔG_{ATP} and the inner mitochondrial membrane potential (Glancy and Balaban, 2012). It has been demonstrated that $[Ca^{2+}]_m$ is reduced when $[Na^+]_c$ is elevated from 5 to 15 mM as the exchange of $[Ca^{2+}]_m$ and $[Na^+]_c$ through the mitochondrial NCX is raised (Liu and Rourke, 2008). Conversely, Empa led to elevated $[Ca^{2+}]_m$ in myocytes (Baartscheer et al., 2017), probably because of reduced exchange of $[Na^+]_c$ for $[Ca^{2+}]_m$ due to lower $[Na^+]_c$. The increased $[Ca^{2+}]_m$ is likely to increase cardiac energetics resulting in an increased PCr/ATP ratio (Bertero and Maack, 2018). Although in healthy hearts SGLT2i were without effect on PCr/ATP levels (Uthman et al., 2018a), it cannot be excluded that SGLT2i will increase PCr/ATP levels in diabetic and failing myocytes, especially since it is already observed that ATP levels are preserved in cultured cardiomyocytes subjected to hypoxia/reoxygenation (Andreadou et al., 2017).

AMPK Activation to Improve Energetics and Prevent Inflammation

5′ AMPK is a regulator of cardiac energy metabolism. Given that myocardial energetic status is severely compromised in failing (Neubauer, 2007; Ingwall, 2009) and diabetic (Levelt et al., 2016) hearts, AMPK activation could enhance cardiac energy metabolism and hence restore myocardial energy levels (Beauloye et al., 2011). Furthermore, activation of AMPK reduced inflammation and oxidative stress levels in PA-stimulated endothelial cells (Li et al., 2015). Cana in endothelial cells and Dapa in myofibroblasts activated AMPK, resulting in reduced inflammatory responses in these cells (Ye et al., 2017; Mancini et al., 2018). A possible mechanism by which SGLT2i change AMPK activity is through complex I inhibition and increased AMP/ADP ratio, which was examined in mouse hepatocytes (Hawley et al., 2016). However, this effect was only observed with high doses of Cana, and not with Empa or Dapa. One may also postulate that the inhibition of glucose uptake by SGLT2 inhibition may activate AMPK as a compensatory feedback mechanism to restore cell metabolism. Limited data are available as of yet that explains how SGLT2i could activate AMPK. Further research with, e.g., knock-down models of SGLT2 could elaborate on the cellular mechanism of SGLT2i to directly activate AMPK.

Whether the SGLT2i convey through similar AMPK activation pathways in cardiac cells needs further investigation. Based on the existing research, lower dosages of Dapa ($\leq$0.5 μM) seemed to be most effective for AMPK activation and the suppression of inflammation, while higher Cana concentrations activated AMPK and reduced inflammatory responses in AMPK-dependent and -independent manners. These results imply that the different SGLT2i activate AMPK at different concentrations.

Inflammation and Oxidative Stress

Inflammation is considered an essential driving factor of cardiovascular disease in diabetes, whereas elevated levels of extracellular glucose alone is insufficient to induce a pro-atherogenic state (Sharma et al., 2018). Instead, the combination of glucotoxic and inflammatory stimuli are needed to establish an environment prone to developing atherosclerosis (Azcutia et al., 2010). Current understandings of the pathophysiology of heart failure, in particular with preserved ejection fraction (HFpEF) postulate that the presence of comorbidities cause microvascular inflammation that ultimately leads to the development of heart failure (Paulus and Tschöpe, 2013). Importantly, vascular inflammation can be induced by reduced AMPK activity (He et al., 2015) as well as high Na^+ loading, the latter resulting in elevation of fasting blood glucose levels, oxidative stress and insulin resistance (Wan et al., 2018). Endothelial inflammation then leads to perturbed NO-cGMP-PKG signaling and increased leukocyte trafficking, which subsequently induces cardiomyocyte hypertrophy and myofibroblast differentiation, and as such cardiac remodeling. Since SGLT2i have demonstrated direct anti-inflammatory actions in vascular cells, targeting this new paradigm may potentially and at least partly explain the positive results observed in the clinical trials and may identify novel therapeutic implications for SGLT2i.

A Window for SGLT2i as Anti-arrhythmic Therapy

In heart failure, cardiac arrhythmias primarily depend on triggered activity (Coronel et al., 2013). Elevated levels of diastolic $[Ca^{2+}]_c$ increase the open probability of the Ryanodine receptor resulting in spontaneous release of calcium from the SR. This in turn activates the NCX, leading to the transient inward current responsible for delayed after depolarizations (Baartscheer et al., 2003c). In addition, it has been shown that the number of after-transients measured in isolated myocytes is related to the occurrence of ventricular tachycardia *in vivo* (Janse et al., 2001). No data are available on the effects of SGLT2i on arrhythmogenesis. However, under pathologic conditions where myocardial $[Na^+]_c$ is increased, such as in heart failure, a reduction of $[Na^+]_c$ is associated with a reduction of $[Ca^{2+}]_c$, the number and amplitude of Ca^{2+} after-transients and the associated arrhythmias (Despa et al., 2002; Pogwizd et al., 2003; Bers, 2014). In the same vein of thought, we speculate that the inhibitory effect of SGLT2i on Na^+ influx during heart failure is antiarrhythmic.

Erickson et al. (2013) have documented that Ca^{2+}/calmodulin-dependent kinase II (CaMKII) is elevated in diabetes and that it is associated with increased Ca^{2+}-release events from the SR. Thus, diabetes directly impacts arrhythmogenesis. Recent work by Mustroph et al. (2018) has demonstrated that Empa reduced CAMKll activity and CaMKII-dependent SR Ca^{2+} leak. Thus, Empa is likely to directly reduce Ca^{2+}-release and arrhythmias. In addition, indirect effects of SGLT2i on arrhythmogenesis can be mediated through attenuation of the cardiac remodeling and hypertrophic phase known to occur in diabetes (Byrne et al., 2017).

The Presence of SGLT2 in Endothelial Cells?

Given that several studies have reported that SGLT2i directly reduced glucose uptake in endothelial cells, it may be possible that this occurred as a result of SGLT2 inhibition. Conflicting results regarding the expression of SGLT2 in the coronary vasculature have been reported. While SGLT2 has not been detected at all in the heart (Van Steenbergen et al., 2017), increasing evidence demonstrate the existence of SGLT2 in non-cardiac endothelial cells (El-daly et al., 2018; Li et al., 2018). Existing data that suggested the absence of SGLT2 in the endothelium had only attempted to detect SGLT2 at mRNA levels. However, more recent studies have postulated the presence of SGLT2 in endothelial cells at protein level and that its expression levels are amendable by exposure to SGLT2-specific siRNA or PA (El-daly et al., 2018; Li et al., 2018), providing further support for a novel mechanism of SGLT2 and its inhibitors in vascular cells. A major limitation for the identification of SGLT2 at protein level is the lack of knowledge on the quality and specificity of the antibodies used. Therefore, further development of these techniques are highly warranted. Whether diabetic or failing conditions may evoke SGLT2 expression in cardiac endothelial cells has so far not been investigated.

SMIT-1 as Potential Target for SGLT2i

Another possible target for SGLT2i is the sodium-myoinositol cotransporter 1 (SMIT-1), a member of the SGLT receptor family. Overexpression of SMIT-1 leads to activation of NOX2, ROS production and increased glucose sensitivity in cardiomyocytes, and its deletion triggered opposite effects (Van Steenbergen et al., 2017). The effects of increased SMIT-1 expression were not associated with increased glucose uptake with higher extracellular glucose concentration, and the authors suggest that SMIT-1 effects relate to extended glucose sensitization that could alter downward signaling events related to $[Na^+]_c$ and $[Ca^{2+}]_c$. Considering the $[Na^+]_c$ lowering effect of SGLT2i, SGLT2i targeting SMIT-1 during hyperglycemic condition to attenuate hyperglycemia-induced damage might be assumed. However, Baartscheer et al. (2017) showed Na^+-lowering with Empa, even in the absence of glucose. Furthermore, the IC50 of Empa and Cana for SMIT-1 were estimated at 8.3 μM and 5.6 μM, respectively, which is far off the C_{max} for Empa (0.6 μM) (Suzuki et al., 2012; Scheen, 2014).

Future Directions

Our current understanding is that cardiac $[Na^+]_c$ is raised in conditions of heart failure and diabetes, while SGLT2i cause the reduction of $[Na^+]_c$, through inhibition of NHE-1. Studies considering the impact of SGLT2i on NHE-1 activity, $[Na^+]_c$, $[Ca^{2+}]_c$, $[Ca^{2+}]_m$ and mitochondrial energetics in diabetic and failing cardiomyocytes are warranted. Furthermore, whether the reduction of inflammation observed with SGLT2i is a direct consequence of Na^+ lowering is as of yet unknown. It remains uncertain whether the SGLT2 is expressed in cardiac endothelial cells and, if so, is involved in the cardiac effects of SGLT2i. Likewise, since SGLT1 expression in cardiomyocytes is increased in diabetic, heart failure and hypertrophic conditions, the effect of SGLT2i on SGLT1 under these circumstances should be investigated. The identification of SGLT2 in the endothelium with existing techniques requires accurate validation. Moreover, SMIT-1 might be a valuable target to study in the field of heart failure and SGLT2i.

CONCLUSION

In conclusion, increased intracellular sodium concentration is an early hallmark and driver in the pathogenesis of heart failure and T2D. Besides other mechanisms, SGLT2i lower $[Na^+]_c$ in cardiomyocytes, activate AMPK in endothelial cells and cardiofibroblasts, and inhibit cellular glucose uptake in endothelial cells, to favorably interfere with $[Ca^{2+}]_c$ homeostasis, improve mitochondrial function, reduce inflammation and ROS production and restore nitric oxide formation. These effects may explain the beneficial effects of SGLT2i to prevent heart failure and other related cardiac complications in the diabetic as well as the non-diabetic heart.

AUTHOR CONTRIBUTIONS

LU conducted the literature review, drafted the article, provided critical revision of the article, and final approval of the version to be published. AB, CS, JF, MK, MH, RC, and NW provided critical revision of the article and final approval of the version to be published. CZ drafted the article, provided critical revision of the article, and final approval of the version to be published.

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frontiers in Physiology

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Saxagliptin but Not Sitagliptin Inhibits CaMKII and PKC via DPP9 Inhibition in Cardiomyocytes

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Some oral anti-hyperglycemic drugs, including gliptins that inhibit dipeptidyl peptidase 4 (DPP4), have been linked to the increased risk of heart failure (HF) in type-2 diabetic patients. While the cardiovascular safety trial, TECOS, revealed no link between sitagliptin and the risk of HF, a substantial 27% increase in the hospitalization for HF was observed in type-2 diabetic patients treated with saxagliptin within the SAVOR-TIMI 53 trial. A previous *in vitro* study revealed that saxagliptin impairs the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-phospholamban (PLB)-sarcoplasmic reticulum Ca^{2+}-ATPase 2a axis and protein kinase C (PKC) activity in cardiomyocytes leading to impaired cardiac contractility and electrophysiological function. However, the link between saxagliptin and its target proteins (CaMKII and PKC) remains to be explored. Since DPP8 and DPP9 (but not DPP4) are expressed by cardiomyocytes and saxagliptin is internalized by cardiomyocytes, we investigated whether DPP8/9 contribute to saxagliptin-mediated inhibition of CaMKII and PKC activity. Structural analysis revealed that the DPP4-saxagliptin interaction motif (S630, Y547) for the cyanopyrrolidine group is conserved in DPP8 (S755, Y669) and DPP9 (S730, Y644). Conversely, F357 that facilitates binding of the anchor lock domain of sitagliptin in the S2 extensive subsite of DPP4 is not conserved in DPP8/9. In parallel, unlike saxagliptin, sitagliptin did not affect phosphorylation of CaMKII/PLB or activity of PKC in HL-1 cardiomyocytes. These findings were recapitulated by pharmacological inhibition (TC-E-5007, a DPP8/9 antagonist) and knock-down of DPP9 (but not DPP8). In primary mouse ventricular cardiomyocytes, saxagliptin (but not sitagliptin) impaired Ca^{2+} transient relaxation and prolonged action potential duration (APD). These results suggest that saxagliptin-DPP9 interaction impairs the CaMKII-PLB and PKC signaling in cardiomyocytes. We reveal a novel and potential role of DPP9 in cardiac signaling. The interaction of saxagliptin with DPP9 may represent an underlying mechanism for the link between saxagliptin and HF. Elucidation of saxagliptin-DPP9 interaction and downstream events may foster a better understanding of the role of gliptins as modulators of cardiac signaling.

Keywords: diabetes, heart failure, gliptins, Ca^{2+} transient, cardiac electrophysiology

INTRODUCTION

Prevalence of diabetes mellitus (DM) is increasing worldwide and about 90% of all DM patients suffer from type 2 DM (T2DM). Hyperglycemia induces micro- and macro-vascular complications resulting in cardiac, kidney, eye and vessel dysfunction (Peter et al., 2008). One of the major causes of T2DM-associated mortality is heart failure (HF) (McMurray et al., 2014), though the exact underlying pathophysiological events responsible for DM-induced HF are still not clearly understood.

Both, HF and DM are multifactorial diseases that share some common etiological and risk factors including imbalanced ionic homeostasis, ion channel dysfunction, oxidative stress and aberrant metabolic homeostasis (Miki et al., 2013; Kasznicki and Drzewoski, 2014). Diabetic cardiomyopathy leads to impaired cardiac relaxation and systolic and/or diastolic dysfunction that are observed also in failing hearts (Bleske, 2000; Mandinov et al., 2000). Moreover, prolongation of cardiac action potential duration (APD) is often observed during chronic DM and HF (Wang and Hill, 2010).

Apart from DM being a predisposing factor for the development of HF, therapeutic remedies of DM are linked to the risk of HF as well (Udell et al., 2015). Anti-diabetic drugs, including thiazolidinediones and gliptins, are reported to increase the rate of hospitalization for HF during clinical trials (Udell et al., 2015). Therefore, along with DM-HF pathophysiology, mechanistic insight into anti-diabetic drug-induced HF has become essential for cardiovascular safety management of DM patients.

Gliptins are inhibitors of dipeptidyl peptidase 4 (DPP4), a serine peptidase, that degrades glucagon like peptide-1 and in turn augments incretin effect by increasing prandial insulin and reducing glucagon secretion from pancreatic beta and alpha cells, respectively. During the cardiovascular safety trial, TECOS, sitagliptin did not show any effect on the hospitalization of HF in patients suffering from T2DM (Green et al., 2015). However, the SAVOR-TIMI 53 trial reported a 27% increase in the risk of HF in diabetes patients undergoing saxagliptin therapy (Scirica et al., 2013). This clinical observation is supported by our previous data that link saxagliptin to cardiac dysfunction and HF under *in vitro* and *ex vivo* conditions (Koyani et al., 2017). At molecular level, saxagliptin inhibited Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-mediated phospholamban (PLB) phosphorylation as well as intracellular protein kinase C (PKC) activity in cardiomyocytes.

Apart from DPP4, the DPP4 gene family also includes DPP8 and DPP9 (Yu et al., 2010). DPP8 and DPP9, expressed in various tissues and cells are cytosolic enzymes that have the ability to cleave DPP4 substrates including glucagon-like peptide-1 (Yu et al., 2010). Given the fact that the gliptin-target, DPP4, is not expressed by cardiomyocytes, we set out to explore a potential role of the two cardiomyocyte-resident DPP isoforms, DPP8 and DPP9, in gliptin-mediated signaling events. Despite structural homology to DPP4 (Yu et al., 2010), the role of DPP8/9 in cardiac function is yet unclear. Therefore, we aimed to examine and compare the effects of saxagliptin and sitagliptin on intracellular signaling that may lead to contractile/electrophysiological dysfunction and HF.

MATERIALS AND METHODS

Cell Culture

HL-1 cells (a murine cardiomyocyte cell line, Sigma-Aldrich, Vienna, Austria) were cultured in fibronectin (0.5% [w/v])/gelatin (0.02% [w/v]) coated flasks and maintained in Claycomb medium (Sigma-Aldrich) (Claycomb et al., 1998) containing 10% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific, IL, United States), 0.1 mM norepinephrine, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), and kept at 37°C under 5% CO_2, as previously described (Scheruebel et al., 2014).

Incubation Protocol

HL-1 cells were treated with indicated (in respective Figures and Figure legends) concentrations of saxagliptin or sitagliptin (Adooq Bioscience, CA, United States, dissolved in DMSO, final concentration of DMSO – 0.01% [v/v]) for indicated time periods.

Cardiomyocyte Isolation and Patch Clamp

Cardiomyocytes were isolated from adult mice (14–18 weeks old, either sex). The experimental procedure and number of used animals were approved by the ethics committee of the Federal Ministry of Science, Research and Economy of the Republic of Austria (BMWF-66.010/0038-V/3b/2018). Mice were euthanized (40 mg/kg ketamine and 10 mg/kg xylazine), hearts were quickly removed and cardiomyocytes were isolated as previously described (Ackers-Johnson et al., 2016) using collagenase 2 and 4 (Worthington Biochemical Corporation, NJ, United States). After isolation cardiomyocytes were kept in standard external solution (containing in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 2.2, NaH_2PO_4 0.4, HEPES/Na^+ 10, D(+)-glucose 5.6, pH 7.4 adjusted with NaOH). All experiments were performed on the day of isolation.

Action potentials (APs) were recorded in the whole cell configuration of the patch clamp technique using Axopatch 200B amplifier (Molecular Devices, CA, United States) and the A/D – D/A converters Digidata 1322A (Molecular Devices). To record APs, cardiomyocytes were superfused with the standard external solution at 37°C and pipettes were filled with an internal solution (containing in mM: KCl 110, ATP/K^+ 4.3, $MgCl_2$ 2, $CaCl_2$ 1, EGTA 11, HEPES/K^+ 10, pH 7.4 adjusted with KOH, estimated free $[Ca^{2+}] < 10^{-8}$ M). For AP recordings cells were stimulated with minimal suprathreshold current pulses (5 ms) at a frequency of 1 Hz, as previously described (Koyani et al., 2017). In order to exclude any initial transient behavior, first 10 APs were excluded from analysis. Ten consecutive APs were analyzed using LabChart 7.0 (Cardiac action potential analysis module, ADInstruments Ltd., Oxford, United Kingdom).

Ca^{2+} Transient (CaT) Measurements

After incubation with saxagliptin, sitagliptin or TE-C 5007 at indicated concentration for 4 h, cells were washed twice with the standard external solution and incubated with standard external solution containing 1 μM Fura-2-AM and 1 μM Pluronic F-127 (Thermo Fisher Scientific) for 30 min at 25°C. CaT was assessed by field stimulation (platinum electrode distance: 1 cm; pulse duration: 5 ms; suprathreshold pulse amplitude: 4 V/cm) at a frequency of 1 Hz using a video-based sarcomere length detection system (IonOptix Corporation, MA, United States) at 37°C. Fluorescence intensities were measured at 340 and 380 nm of excitation and at 510 nm of emission wavelengths using a dual excitation light source. The F340/F380 ratio was used as an index of cytosolic Ca^{2+} concentration and to calculate CaT relaxation tau (τ_{CaT}). Data were analyzed using Clampfit 10.2 (Molecular Devices) and LabChart 7.0 (peak analysis module, ADInstruments Ltd., Oxford, United Kingdom).

Fluorescence Microscopy

Fluorescamine (50 mg/ml in acetone, Sigma-Aldrich) and saxagliptin/sitagliptin were mixed in borate buffer (pH 8.5) at indicated concentrations (at 25°C). The reaction mixture was evaporated to dryness and dissolved in the standard external solution (Koyani et al., 2017). HL-1 cells were incubated with saxagliptin/sitagliptin-fluorescamine adduct for 4 min and washed with the standard external solution (see above) at 37°C. Fluorescence images were captured at excitation/emission wavelengths of 390/470 nm.

Western Blot Analysis

HL-1 cells or mouse left ventricle (LV) were lysed in ice-cold lysis buffer (containing in mM: HEPES 50, NaCl 150, EDTA 1, $Na_4P_2O_7$ 10, Na_3VO_4 2, NaF 10, 1% [v/v] Triton X-100, 10% [v/v] glycerol, pH 7.4, Sigma-Aldrich) containing a Protease Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Pellets were separated by centrifugation at 13,000 rpm (4°C, 10 min). Protein estimation of whole cell was performed using BCA protein assay kit (Thermo Fisher Scientific). Whole cell protein lysates (50 μg) were added to 10 μl of NuPAGE LDS sample buffer (Invitrogen) containing 2 μl of NuPAGE sample reducing agent (Invitrogen). After heating for 10 min at 70°C, proteins were separated by electrophoresis on NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen, 0.2 μm) (Jain et al., 2015). Membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing Tween 20 (TBST, 25°C, 2 h) and incubated with primary antibodies overnight at 4°C. The following primary antibodies (diluted in 5% [w/v] BSA-TBST) were used: phospho-CaMKII (pCaMKII, T286, 1:1000, Abcam, ab32678), phospho-PLB (pPLB, T17, 1:5000, Badrilla, A010-13), DPP8 (1:500, Santa Cruz, sc-376399), DPP9 (1:500, Santa Cruz, sc-271634), CaMKII (1:500, Santa Cruz, sc-9035), and PLB (1:2000, Thermo Fisher Scientific, MA3-922). After washing, the membranes were incubated with HRP-conjugated goat anti-mouse IgG (1:10,000, Cell Signaling, 7076) or goat anti-rabbit IgG (1:10,000, Cell Signaling, 7074) (25°C, 2 h). Immunoreactive bands were visualized using Immobilon Western Chemiluminescent HRP substrate (Merck Millipore, Vienna, Austria) and developed by Bio-Rad ChemiDoc MP Imaging System. For normalization, membranes were stripped with stripping buffer (58.4 g/l NaCl, 7.5 g/l glycine, pH 2.15), blocked and incubated with a primary antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000, Santa Cruz, sc-25778). Densitometric evaluation of immunoreactive bands was performed using Image Lab 4.1 software.

RNA Isolation and Quantitative Real-Time PCR (qPCR)

QIAshredder and RNeasy Mini Kit (Qiagen, Hilden, Germany) were used to isolate RNA from HL-1 cardiomyocytes according to the manufacturer's protocol. After determining RNA concentration, one μg RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States) according to the supplier's manual. For gene quantification, six ng cDNA per template was used with GoTaq qPCR Master Mix (Promega, Vienna, Austria) and gene specific primers. The qPCR protocol was performed by LightCycler 480 system (Roche Diagnostics, Vienna, Austria). The following gene specific primers from Qiagen were used: GAPDH (QT01658692), DPP8 (QT00143388), and DPP9 (QT00144165). Relative gene expression levels were normalized to GAPDH and calculated using $\Delta\Delta$CT method (Koyani et al., 2016).

Gene Silencing

HL-1 cells were transfected with siRNAs (four different constructs) specific for DPP8 or DPP9 (40 nM each, Qiagen), or scrambled negative control siRNA (40 nM si-scr; Allstars negative control siRNA, Qiagen) (Koyani et al., 2014). The siRNA transfection was performed using Lipofectamine 3000 (Invitrogen) according to the supplier's manual. Briefly, HL-1 (50% confluent) were transfected with 1 ml medium (composition of medium is given above, without FBS and antibiotics) containing 2 μl of Lipofectamine 3000 and the respective siRNA for 6 h at 37°C. Transfection medium was replaced with medium (containing FBS and antibiotics) and cells were grown for another 24 h. The mRNA expression levels of silenced genes were measured using qPCR (see above). In parallel, transfected cells were treated with saxagliptin or sitagliptin to follow protein expression using Western blot (see above) or PKC activity assay (see below).

PKC Activity Assay

After transfection and/or treatment with saxagliptin, sitagliptin or TC-E 5007, HL-1 cells were washed and lysed in lysis buffer containing Protease Phosphatase Inhibitor Cocktail. Estimation of active PKC was performed using a PKC kinase activity kit (ADI-EKS-420A, Enzo Life Sciences, NY, United States) according to the manufacturer's protocol.

Structural Analysis

The 3D structures of DPP4 bound to saxagliptin (PDB code: 3bjm) and sitagliptin (PDB code: 1×70) were aligned based on the 3D structure of DPP4 using pymol 1.8.2. As a result, theoretical models of DPP4 bound to both saxagliptin and sitagliptin were created allowing the direct comparison of the DPP4 amino acids involved in binding of gliptins. This 3D model was subsequently aligned with either the 3D structure of DPP8 (PDB code: 6eoo) or DDP9 (PDB code: 6eoq) based on structural similarity between DPP4 and DPP8/9 using pymol 1.8.2. As a result, theoretical models of DPP8 and DPP9 bound to both saxagliptin and sitagliptin were created. The amino acids of DPP8/9 present in the gliptin binding interface were then compared to those of DDP4 in order to access the potential for sitagliptin- and/or saxagliptin-binding to DPP8/9.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 23 software. Approximate normal distribution of data was assessed by visual (histograms and normal Q-Q plots) and numerical investigation (*z*-value of skewness and kurtosis; *p*-value of Shapiro–Wilk test). After checking homogeneity of variance by Levene's test, between groups comparisons were evaluated by unpaired Student's *t*-test, one-way ANOVA (followed by Tukey's *post hoc* test) or ANCOVA to compare the effect of saxagliptin/sitagliptin vs. rundown by adjusting the matched control values (covariates). ANCOVA was only applied when covariates and regression slopes were not different between the compared groups. *P*-values ≤ 0.05 were considered statistically significant. All tests were 2-sided.

RESULTS

Fluorescamine-Derivatives of Saxagliptin and Sitagliptin Are Internalized by Cardiomyocytes

As saxagliptin is internalized by cardiomyocytes (Koyani et al., 2017), we aimed to investigate whether sitagliptin is also internalized by cardiomyocytes. For these experiments, both compounds were coupled with fluorescamine (a non-fluorescent dye that yields fluorescence upon reaction with primary amines in a 1:1 stoichiometry) in order to visualize cellular uptake and localization.

Untreated (**Figure 1A**) or fluorescamine-treated (**Figure 1B**) HL-1 cells showed no fluorescence (unbound fluorescamine has a very short half-life (5–10 s) in an aqueous solution; Udenfriend et al., 1972), while incubation with the saxagliptin-fluorescamine

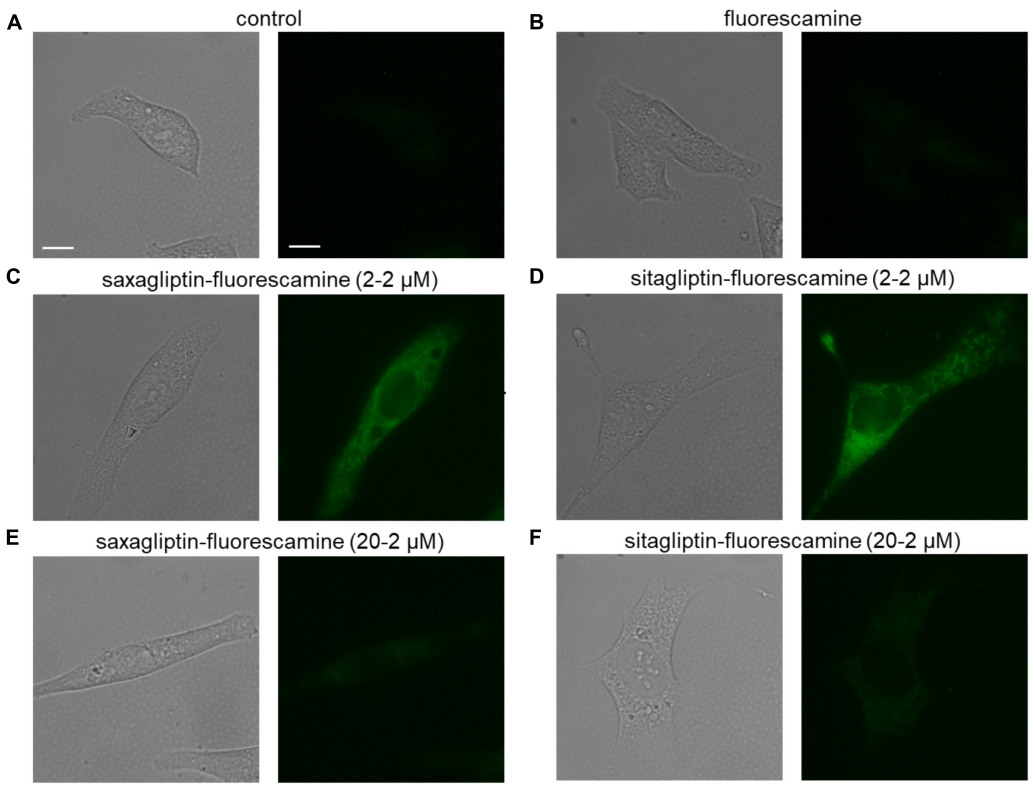

FIGURE 1 | Saxagliptin and sitagliptin internalizes into cardiomyocytes. Representative bright-field (left panels, **A–F**) and corresponding fluorescent images (right panels, **A–F**) of HL-1 cardiomyocytes treated with standard external solution **(A)** alone (control) or containing **(B)** fluorescamine (2 μM), saxagliptin-fluorescamine adduct [**(C)** 2–2 μM or **(E)** 20–2 μM] or sitagliptin-fluorescamine adduct [**(D)** 2–2 μM or **(F)** 20–2 μM] for 5 min (*n* = 6, scale bar: 10 μm). "n" represents the number of experiments.

(2–2 μM) complex showed pronounced fluorescence that is located exclusively in the cytosol (**Figure 1C**). A similar intracellular fluorescence pattern was observed in cells incubated with the sitagliptin-fluorescamine complex (**Figure 1D**). To determine specificity of drug uptake, we added a 10-fold molar excess (20–2 μM) of unlabeled saxagliptin/sitagliptin to investigate competition between free and fluorescamine-bound drugs. Under these conditions, fluorescence intensity was reduced almost to basal level (**Figures 1E,F**). These data indicate that sitagliptin is internalized by cardiomyocytes and localizes in the cytosol, similarly, as reported for saxagliptin (Koyani et al., 2017).

Saxagliptin but Not Sitagliptin Inhibits the CaMKII-PLB Axis

Since both gliptins are internalized by cardiomyocytes (**Figures 1C,D**) and saxagliptin was reported to attenuate phosphorylation of CaMKII and PLB, we aimed to evaluate whether sitagliptin displays similar pharmacological effects on the CaMKII-PLB axis. In line with our previous data (Koyani et al., 2017), incubation of HL-1 cells with 2 μM saxagliptin resulted in reduced CaMKII phosphorylation (T286) starting from 2.5 min (**Figure 2A**). In contrast, 2 μM sitagliptin failed to inhibit pCaMKII expression (**Figure 2B**). Concentration-dependent experiments demonstrate that saxagliptin (but not sitagliptin) inhibits CaMKII phosphorylation starting from 200 nM (**Figures 2C,D**, respectively). Additionally, sitagliptin treatment (up to 10 μM) had no effect on the CaMKII phosphorylation (**Figure 2D**).

As phosphorylation of CaMKII promotes PLB phosphorylation (T17), we examined pPLB expression level. Treatment of HL-1 cardiomyocytes with saxagliptin resulted in reduced immunoreactive pPLB band starting from 15 min (**Figure 2E**). In parallel, concentration-dependent experiments show saxagliptin-reduced pPLB expression starting from 200 nM dose (**Figure 2G**). Conversely, neither time- (**Figure 2F**) nor concentration-dependent experiments (**Figure 2H**) showed an effect of sitagliptin on PLB phosphorylation levels. Moreover, total CaMKII or PLB levels did not change in response to gliptin treatment (**Figure 2I**). Densitometric evaluation of Western blots and statistical analysis is shown in **Supplementary Figures 1–3**.

Structural Analysis of Gliptins-DPP4/8/9 Interaction and Effect of DPP8/9 Inhibition

The primary target of gliptins, DPP4, is not expressed by cardiomyocytes (Shigeta et al., 2012; Koyani et al., 2017). However, cytosolic localization of gliptins (**Figures 1C,D**) may lead to their interaction with the cardiomyocyte resident DPP isoforms (DPP8 and DPP9) due to their structural homology with DPP4. To test this hypothesis, we first investigated the expression of DPP8/9 in HL-1 cells and mouse LV by Western blotting.

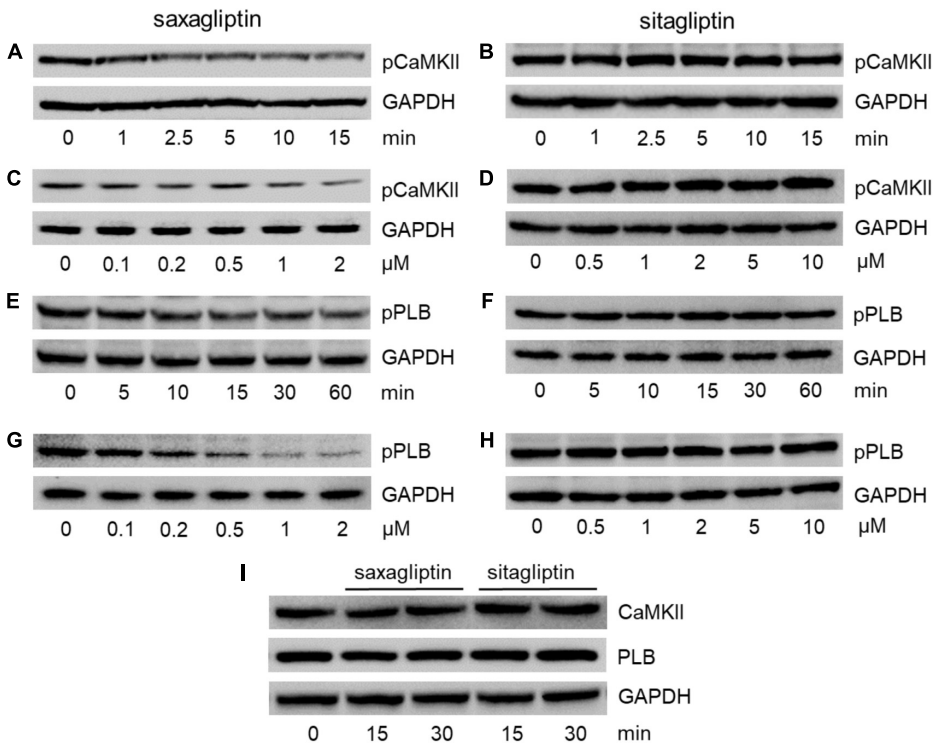

FIGURE 2 | Saxagliptin but not sitagliptin inhibits the CaMKII-PLB axis. Representative Western blots ($n = 6$) for **(A–D)** pCaMKII (T286), **(E–H)** pPLB (T17), and **(I)** CaMKII and PLB expression in HL-1 cardiomyocytes treated with saxagliptin **(A,C,E,G)** or sitagliptin **(B,D,F,H)** for the indicated time points (**A**,**B**,**E**,**F**,**I**, 2 μM) and concentrations (**C,D**, 5 min; **G,H**, 30 min). GAPDH was used as a loading control. "n" represents the number of experiments.

Figure 3A confirms the presence of DPP8/9 in HL-1 cells and mouse LV.

Based on the three-dimensional structure of DPP4 bound to saxagliptin (PDB code: 3bjm; Metzler et al., 2008) and sitagliptin (PDB code: 1×70; Kim et al., 2005) we identified key amino acids of DPP4 that are involved in gliptin binding (**Figures 3B,C**). Close inspection of the interaction modes shows that DPP4-saxagliptin interaction motif (S630, Y547) is conserved in DPP8 (S755, Y669) and DPP9 (S730, Y644). However, the region around DPP4-sitagliptin binding comprises of amino acids (S209, F357) that are neither conserved in DPP8 nor in DPP9. Additionally, DPP8 and DPP9 harbor two charged amino-acids in the surrounding of F357 (D278/R524 for DPP8 and D251/R499 for DPP9) that might interfere with binding of sitagliptin to DPP8/9 either via steric clashes and/or charge repulsion. These structural analyses suggest that binding of saxagliptin is conserved in DPP8/9 whereas binding of sitagliptin is weakened or even abolished.

To investigate the role of DPP8/9 in saxagliptin-induced intracellular signaling, we used TC-E 5007, a DPP8/9 inhibitor with IC_{50} of 145 and 242 nM, respectively (Lankas et al., 2005). Treatment of HL-1 cardiomyocytes with 2 μM TC-E 5007 resulted in a time-dependent inhibition of pCaMKII (T286) and pPLB (T17) expression starting from 30 and 60 min, respectively (**Figures 3D,E**). In contrast, TC-E 5007 did not affect total CaMKII and PLB protein levels (**Figure 3F**). Densitometric evaluation of blots and statistical analysis is shown in **Supplementary Figure 4**.

Saxagliptin Inhibits the CaMKII-PLB Axis Dependent on DPP9

To further clarify the individual role(s) of DPP8 and/or DPP9 in saxagliptin-reduced pCaMKII and pPLB expression in cardiomyocytes, a RNA interference approach was used. HL-1 cells were transfected with specific siRNA against DPP8 or DPP9. qPCR analyses revealed that siRNA transfection significantly reduced mRNA levels of the target DPP isoform (∼52% of DPP8 and ∼58% of DPP9) without affecting expression of the non-target DPP isoform (**Figure 4A**). In contrast, at protein level we observed ∼64 and 79% reduction of DPP8 and DPP9 expression, respectively (**Figure 4B**). There was no difference in the expression of DPP8/9 between non-transfected and si-scr transfected cells (**Figures 4A,B**).

Next, we evaluated the effect of DPP knock-down on phosphorylation of CaMKII and PLB. Treatment of control cardiomyocytes with saxagliptin (but not sitagliptin) reduced

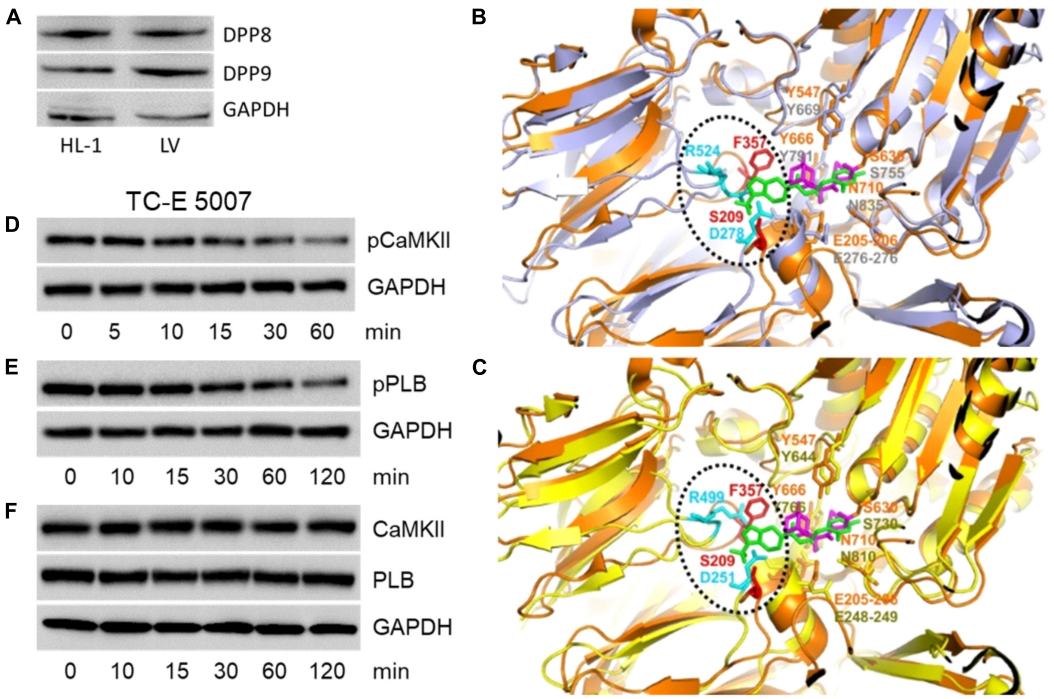

FIGURE 3 | Structural analysis of gliptins-DPP4/8/9 binding. **(A)** A representative Western blot (n = 3) showing expression of DPP8 and DPP9 in total protein lysate (50 μg) of HL-1 cardiomyocytes and mouse LV. **(B)** 3D structural alignment of saxagliptin bound to DPP4 (PDB code: 3bjm) and sitagliptin (PDB code: 1×70) aligned with the 3D structure of DPP8 (PDB code: 6eoo) using pymol. The 3D structure of DPP4 and DPP8 are shown as cartoon representation and colored in orange and gray, respectively. The DPP4-bound forms of saxagliptin and sitagliptin are shown in sticks and colored in magenta and green, respectively. The DPP4 and DPP8 amino-acids present in the gliptin-binding surface of DPP4 are shown in sticks and colored in orange and gray, respectively, if conserved in the structural model or, in red and cyan, if not. **(C)** 3D structural alignment as described in **(B)** but using DPP9 (PDB code: 6eoq, colored in yellow) in place of DPP8. Representative Western blots (n = 6) for **(D)** pCaMKII (T286), **(E)** pPLB (T17), and **(F)** CaMKII and PLB expression in HL-1 cardiomyocytes treated with TC-E 5007 (2 μM) for the indicated time points. GAPDH was used as a loading control. "n" represents the number of experiments.

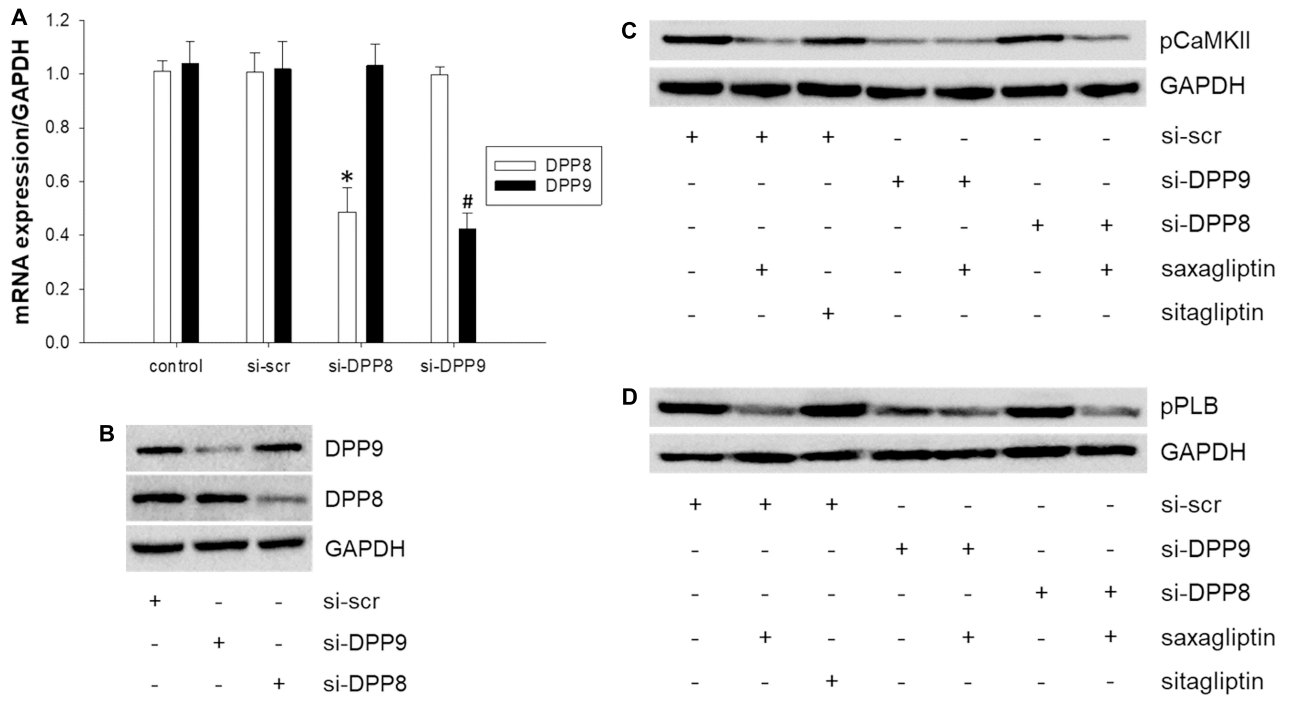

FIGURE 4 | Saxagliptin inhibits the CaMKII-PLB axis via DPP9 inhibition. **(A)** mRNA and **(B)** protein expression of DPP8 and DPP9 in HL-1 cardiomyocytes transfected with si-scr, si-DPP8 or si-DPP9. GAPDH was used as a house keeping gene/protein. A representative Western blot showing **(C)** pCaMKII (T286), and **(D)** pPLB (T17) expression in HL-1 cardiomyocytes transfected with si-DPP8 or si-DPP9, and/or treated with saxagliptin or sitagliptin (2 μM each) as indicated for **(C)** 5 min and **(D)** 30 min. All values are expressed as mean ± SEM ($n = 6$). $^{*}p < 0.05$ vs. si-scr DPP8 and $^{\#}p < 0.05$ vs. si-scr DPP9 by one-way ANOVA followed by Tukey's *post hoc* test. "n" represents the number of experiments.

pCaMKII expression (**Figure 4C**). Furthermore, silencing of DPP9 (but not DPP8) resulted in reduction of CaMKII phosphorylation in a similar manner as observed with saxagliptin alone. The efficacy of saxagliptin to inhibit CaMKII phosphorylation is blunted in cardiomyocytes transfected with si-DPP9. Conversely, saxagliptin effectively inhibited pCaMKII expression in DPP8 knocked down cardiomyocytes.

Further, we tested these conditions for PLB phosphorylation. Similar to CaMKII phosphorylation, pPLB expression was dampened by saxagliptin (but not sitagliptin) and DPP9 (but not DPP8) knock-down (**Figure 4D**). In line, the efficacy of saxagliptin was abolished in DPP9 (but not DPP8) silenced cells (**Figure 4D**). Densitometric evaluation of blots and statistical analysis is shown in **Supplementary Figure 5**. These data demonstrate that saxagliptin targets DPP9 and thereby impairs the CaMKII-PLB axis in cardiomyocytes.

Saxagliptin Inhibits PKC Activity, Dependent on DPP9

As reported previously (Koyani et al., 2017) and here (**Figures 5A,B**), saxagliptin significantly reduced PKC activity in cardiomyocytes in a time- (from 2.5 min) and concentration-dependent manner (from 1 μM). In contrast, our present data show that the treatment of cardiomyocytes with sitagliptin had no effect on intracellular PKC activity (**Figures 5A,B**). In parallel, the DPP8/9 inhibitor TC-E 5007 reduced active PKC levels in a time-dependent manner starting from 10 min in HL-1 cardiomyocytes (**Figure 5C**).

Next, we silenced DPP8/9 expression to get an indication whether one or both DPP isoforms might play a role in saxagliptin-reduced PKC activity. Ablation of DPP9 resulted in decreased PKC activity, whereas si-DPP8 had no effect (**Figure 5D**). The efficacy of saxagliptin was significantly abolished in DPP9 (but not DPP8) silenced cardiomyocytes. These data reveal that DPP9 links saxagliptin with reduced PKC activity in cardiomyocytes.

Saxagliptin and TC-E 5007 Impairs CaT Relaxation and Prolongs APD

To evaluate the effects of saxagliptin- and TC-E 5007-evoked intracellular signaling on cardiomyocyte function, we performed CaT and patch clamp analysis. Saxagliptin and TC-E 5007 impaired the CaMKII-PLB axis that regulates sarcoendoplasmic reticulum (SR) Ca^{2+}-ATPase 2a (SERCA2a) function in Ca^{2+} removal during cardiac relaxation. Therefore, reciprocal of τ_{CaT} was analyzed to assess cardiac relaxation. Treatment of mouse ventricular cardiomyocytes with saxagliptin and TC-E 5007 resulted in impaired cardiac relaxation, whereby sitagliptin was ineffective (**Figures 6A,B**). In parallel, electrophysiological analysis showed prolongation

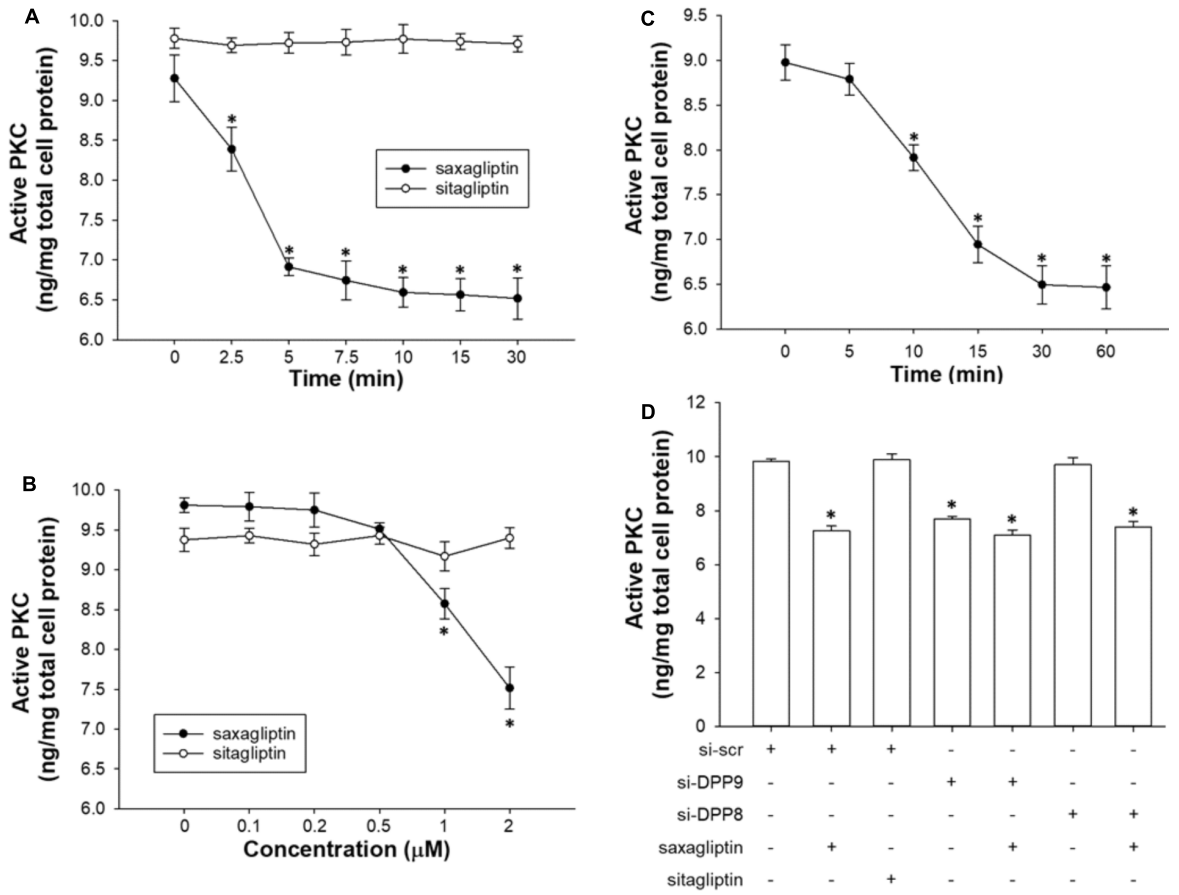

FIGURE 5 | Saxagliptin inhibits PKC via DPP9 inhibition. Quantification of active PKC levels in HL-1 cardiomyocytes treated with saxagliptin or sitagliptin in a **(A)** time-dependent (2 μM) or **(B)** concentration-dependent manner (10 min), and **(C)** TC-E 5007 (2 μM) for indicated time periods. **(D)** HL-1 cardiomyocytes were transfected with si-DPP8 or si-DPP9, and/or treated with saxagliptin or sitagliptin (2 μM) for 10 min as indicated to follow measurements of active PKC. All values are expressed as mean ± SEM ($n = 6$). *$p < 0.05$ vs. **(A,C)** 0 min, **(B)** 0 μM, and **(D)** si-scr by one-way ANOVA followed by Tukey's *post hoc* test. "n" represents the number of experiments.

of APD upon treatment with saxagliptin but not sitagliptin (**Figures 6C,D**).

DISCUSSION

In the present study we identified DPP9 as a primary target of saxagliptin that in turn initiates intracellular signaling contributing to cardiac contractile and electrophysiological dysfunction (**Figure 7**). Both, saxagliptin and sitagliptin internalize into cardiomyocytes and localize exclusively in the cytosol. Basically, the primary target of gliptins, DPP4, is not expressed by cardiomyocytes. However, cardiomyocytes do express other DPP isoforms, namely DPP8 and DPP9. Structural analysis reveals favorable interaction of saxagliptin (but not sitagliptin) with both the DPP isoforms. Moreover, saxagliptin (but not sitagliptin) treatment resulted in reduced phosphorylation of CaMKII and PLB, and PKC activity. These results were recapitulated while using a DPP8/9 inhibitor, TC-E 5007. In contrast, these effects were observed only in DPP9 (but not DPP8) silenced cardiomyocytes. Furthermore, the efficacy of saxagliptin to inhibit the CaMKII-PLB axis and PKC was abolished in cardiomyocytes transfected with si-DPP9 (but not si-DPP8). At functional level, saxagliptin and TC-E 5007 impaired CaT relaxation and saxagliptin prolonged APD of mouse ventricular cardiomyocytes.

Although gliptins have high aqueous solubility, *in vitro* studies show a significant membrane permeability of saxagliptin (Koyani et al., 2017), sitagliptin (Lee et al., 2016) as well as vildagliptin (He et al., 2009). Lee et al. (2016) have reported that sitagliptin permeated endothelial membrane in a dose-dependent manner and activates the Src/VE-cadherin pathway. This intracellular cascade was directly linked to vascular leakage in the retina (Lee et al., 2016). Internalization of saxagliptin into cardiomyocytes was further reported to inhibit cardiac kinases including CaMKII and PKC that lead to cardiac dysfunction (Koyani et al., 2017). Like saxagliptin, we observed that fluorescamine-bound sitagliptin is internalized by cardiomyocytes and localized in the cytosol.

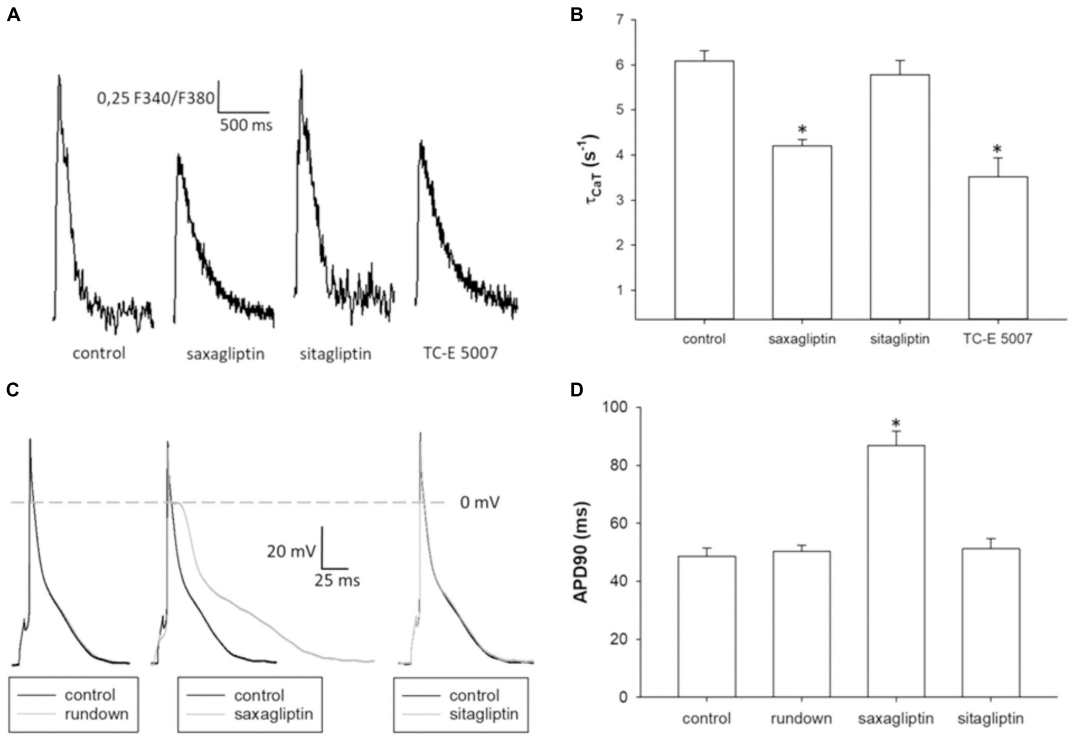

FIGURE 6 | Saxagliptin and TC-E 5007 impairs CaT relaxation and prolongs APD. **(A)** Representative CaT traces and **(B)** CaT decay tau (τ_{CaT}) of control and a saxagliptin-, sitagliptin- or TC-E 5007 (2 μM and 2 h for all)-treated mouse ventricular cardiomyocytes stimulated at 1 Hz frequency (n = 14). **(C)** Representative action potentials (AP) and **(D)** AP duration at 90% repolarization of mouse ventricular cardiomyocytes before (control) and after 5 min superfusion with either external solution alone (rundown), saxagliptin or sitagliptin (2 μM for both) and stimulated at 1 Hz frequency (n = 10/5). Controls of rundown, saxagliptin and sitagliptin groups are combined to improve readability of the figure **(D)**. All values are expressed as mean ± SEM. "n" represents the number of **(B)** experiments and **(C)** cardiomyocytes/animals. *$p < 0.05$ vs. control by **(B)** one-way repeated-measure ANOVA followed by Tukey's *post hoc* test or **(D)** ANCOVA.

DPP8 and DPP9 are primarily localized in the cytosol and nucleus in T-cells (Ross et al., 2018). Despite structural similarities and cellular localization, increasing evidence suggests distinct roles of both DPP isoforms. We report that both DPPs are expressed in HL-1 cardiomyocytes and mouse LV. A previous study showed high expression of DPP9 in human heart using Northern blot analysis (Olsen and Wagtmann, 2002). Although functions of these DPPs in cardiac signaling are largely unknown, both DPPs play a role in monocytes/macrophage pyroptosis (Okondo et al., 2017) as well as in leukemia (Spagnuolo et al., 2013). Overexpression of DPP9 correlates with poor 5-year overall survival in patients with non-small cell lung cancer (Tang et al., 2017).

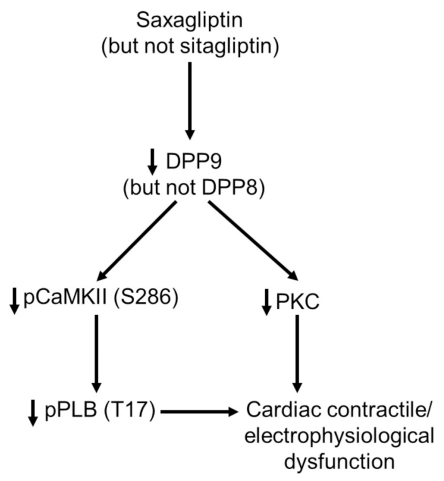

FIGURE 7 | A schematic presentation of the observed DPP9-mediated effects of saxagliptin on cardiac signaling, contractility and electrophysiology.

Here, we show a specific role of DPP9 in cardiac signaling where knock-down of DPP9 resulted in inhibition of the CaMKII-PLB axis and PKC activity. Although the exact mechanism of DPP9-induced cardiac signaling remains to be elucidated, a previous report addresses a potential role of protein phosphatase 2A (PP2A) in the observed effects (Zhang et al., 2015). Zhang and coworkers have reported phosphatase 2A inhibitor (I2PP2A) as a substrate of DPP9 (Zhang et al., 2015). Moreover, PP2A is known to regulate both CaMKII (Erickson, 2014; Lubbers and Mohler, 2016) and PKC (Sontag et al., 1997; Boudreau et al., 2002). Therefore, one may predict a plausible role of PP2A in DPP9-mediated regulation of CaMKII and PKC in cardiac signaling. However, further studies are required to confirm these signaling events.

Although gliptins are developed in order to inhibit activity of DPP4 specifically, partial inhibition of DPP8/9 by gliptins occurs due to the structural homology of these DPP isoforms. Structural analysis reveals that amino acids involved in saxagliptin-DPP4 binding are also conserved in DPP8/9 (**Figure 3B**). Apparently, the triazolopyrazine moiety with the trifluoromethyl substituent of sitagliptin represents the "anchor lock domain" of this inhibitor. The anchor lock domain was shown to tightly bind to the S2 extensive subsite in DPP4 (Berger et al., 2018). Most importantly, the triazolopyrazine group binds to F357 in the S2 extensive subsite of DPP4 and this residue may apparently facilitate high affinity binding of sitagliptin. Additionally, two charged amino acids present in DPP8/9 (**Figure 3C**) may repel the binding of sitagliptin to DPP8/9. These observations obtained from molecular modeling in the present study are in line with *in vitro* inhibition of DPP8/9 by gliptins. The K_i values for inhibition of human DPP8/9 by saxagliptin are 508/98 nM and those for sitagliptin are 33,780/55,142 nM, respectively (Wang et al., 2012). Based on these data (Wang et al., 2012) and the present structural analysis, it is likely to assume an inhibition of DPP8/9 by saxagliptin (but not sitagliptin) under *in vivo* conditions. Moreover, the observed effects of saxagliptin on the CaMKII-PLB axis and PKC activity are dependent on DPP9. Saxagliptin inhibits DPP9 *in vitro* at K_i of 98 nM (Wang et al., 2012), a concentration that is relevant to the plasma C_{max} of saxagliptin following a 5 mg single dose (24 ng/ml, equivalent to ∼76 nM) (Onglyza, 2016).

CaMKII is one of the major multifunctional protein kinases that contribute to the development and progression of HF. Increasing evidence suggests that aberrant CaMKII signaling is a core mechanism in HF and related arrhythmias (Hasegawa et al., 2016). CaMKII regulates function of ion channels involved in cardiac electrophysiology and Ca^{2+} homeostasis. The latter phenomenon includes PLB that is regulated by CaMKII-mediated phosphorylation. Upon dephosphorylation, PLB inhibits SERCA2a, which pumps ∼70% of cytosolic Ca^{2+} into SR during cardiac relaxation (Bers, 2000). Saxagliptin-reduced PLB phosphorylation resulted in reduced Ca^{2+} removal by SERCA2a as well as Na^{+}-Ca^{2+} exchanger (Koyani et al., 2017). These effects were supported by elevated end-diastolic LV pressure and reduced end-systolic LV pressure. One or both of these parameters are observed in failing hearts. Our present data show that saxagliptin interferes with the CaMKII-PLB axis in a DPP9-dependent manner, while sitagliptin had no effect on these signaling events. Furthermore, saxagliptin (but not sitagliptin) and TC-E 5007 impaired τ_{CaT} in mouse ventricular cardiomyocytes.

Apart from contractile dysfunction, prolonged APD and QT interval are considered hallmarks of HF (Wang and Hill, 2010). In the present study, we observed prolongation of APD in mouse ventricular cardiomyocytes after superfusion with saxagliptin (but not sitagliptin). Reduced PKC activity, as observed with saxagliptin, was correlated to impaired delayed-rectifier K^{+} current, prolonged APD and QT interval (Koyani et al., 2017). In the present study, we further discovered that DPP9 is involved in saxagliptin-reduced PKC activity. On the contrary, sitagliptin treatment had no effect on PKC activity in cardiomyocytes. This observation is in line with a previous study where sitagliptin was ineffective to PKC activity in human endothelial cells (Hasegawa et al., 2016). However, in THP-1 macrophages, saxagliptin inhibited oxidized low-density lipoprotein-induced pPKC levels (Dai et al., 2014), indicating a link between saxagliptin and PKC pathway.

Our *in vitro* data demonstrating ineffectiveness of sitagliptin on cardiac signaling, contractility and electrophysiology may provide a molecular mechanism for *in vivo* data showing no link between sitaglitpin treatment and risk of HF (Green et al., 2015). Moreover, this is the first study to reveal the role of DPP9 in cardiac signaling, contractility and electrophysiology. Our data reveal DPP9 as a novel saxagliptin-target that initiates intracellular signaling cascades leading cardiac contractile and electrophysiological dysfunction.

Limitations

The DPP8/9 inhibitor, TC-E 5007, has a slow onset of action on PKC inhibition (10 min, **Figure 5C**) that lead to prolongation of APD, and we have performed patch clamp experiments in a "before and after superfusion" setting. Therefore, we could not perform patch clamp experiments using TC-E 5007 according to our experimental setting. Moreover, adult primary mouse cardiomyocytes are notoriously hard to keep in culture for prolonged periods of time. This precluded silencing experiments with sufficient knockdown efficiency and cell viability.

AUTHOR CONTRIBUTIONS

CK, CT, SS, SK, and IP performed experiments and analyzed the data. BB and TM performed structural analysis. All authors interpreted and discussed data, and contributed to manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01622/full#supplementary-material

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frontiers in Physiology

REVIEW
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The Role of Leukocytes in Diabetic Cardiomyopathy

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Diabetes is predominant risk factor for cardiovascular diseases such as myocardial infarction and heart failure. Recently, leukocytes, particularly neutrophils, macrophages, and lymphocytes, have become targets of investigation for their potential role in a number of chronic inflammatory diseases such as diabetes and heart failure. While leukocytes contribute significantly to the progression of diabetes and heart failure individually, understanding their participation in the pathogenesis of diabetic heart failure is much less understood. The present review summarizes the role of leukocytes in the complex interplay between diabetes and heart failure, which is critical to the discovery of new targeted therapies for diabetic cardiomyopathy.

Keywords: diabetes, heart failure, leukocyte, lymphocyte, inflammation

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INTRODUCTION

Diabetes is a metabolic syndrome that manifests a low grade of systemic inflammation, leads to an increase in all-cause mortality and contributes to the development of number of cardiovascular complications (Duncan et al., 2003). Cardiovascular diseases remain the leading cause of deaths in the United States and in many countries globally, including coronary heart disease, stroke, high blood pressure, and arterial diseases (Benjamin et al., 2018). Notably, death rates among adults with both heart disease and diabetes mellitus are 2–4 times higher than those with heart disease alone, and the mortality rate of patients with heart disease >65 years of age is ∼68% in conjunction with diabetes (Benjamin et al., 2018). Clearly diabetes very negatively impacts the progression and outcome of heart disease, thus understanding the interplay between the two is an important endeavor for advancing treatment strategies of patients with diabetic cardiomyopathy (DCM).

The mechanisms contributing to diabetic cardiac dysfunction are complex and involve a number of molecular phenotypes including insulin resistance, oxidative/nitrative stress (Vita and Keaney, 2002; Creager et al., 2003; Widlansky et al., 2003), activation of mitogen-activated protein kinase (MAPK) (Malek et al., 1999; Vita, 2002), pro-inflammatory, poly (adenosine diphosphate [ADP]-ribose) polymerase (PARP) (Calles-Escandon and Cipolla, 2001), transcription factors (Kim et al., 2006; Bakker et al., 2009), as well as changes in the composition of extracellular matrix (Heil and Schaper, 2004) and inactivation of pro-survival pathways (Silver and Vita, 2006), eventually leading to cell death (Korshunov et al., 2007), which have been reviewed elsewhere (Jia et al., 2018). At a cellular level, high glucose levels negatively impact the function of several cell populations such as cardiac progenitor cells (Salabei et al., 2016), cardiomyocytes, adipocytes (Wang et al., 2006), fibroblasts (Russo and Frangogiannis, 2016) and leukocytes (Burke et al., 2004). For instance, higher levels of glucose and free fatty acids stress pancreatic islets and insulin-sensitive tissue such as adipose tissue, which leads to local production of the cytokines interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) and chemokines CC-chemokine ligand 2 (CCL2), CCL3

and CXC-chemokine ligand 8 (CXCL8). Exposure to glucose also results in increased levels of advanced glycation (glycosylation or glycoxidation) end products (AGEs) that can directly regulate endothelial cell permeability, monocyte migration, and ultimately promotes inflammatory gene expression, contributing to microvascular and macrovascular complications (Goldin et al., 2006). Glucose levels also correlate with mitochondrial transmembrane potential in peripheral blood leukocytes attained from human Type I diabetics (Matteucci et al., 2011), an increase of which results in elevated superoxide production that may directly contribute to cell damage (Brownlee, 2001).

Numerous studies have shown that leukocytes and their subsets (neutrophils, monocytes, and lymphocytes) are involved in both the initiation and progression of cardiovascular diseases (Madjid et al., 2004; Hansson, 2005; Sarndahl et al., 2007). Diabetic cardiac injury is characterized by increased leukocyte mobilization and secreted pro-inflammatory cytokines, adhesion molecules, oxidative stress (Yu et al., 2011; Hernandez-Mijares et al., 2013) and stimulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Lorenzo et al., 2011). Higher leukocyte counts are associated with predicting the risk of cardiovascular disease in diabetic patients (Hong et al., 2014), suggesting a key role of these cells in worsening diabetes-associated cardiovascular disease.

A number of review articles have summarized the role of leukocytes in either diabetes or cardiovascular disease (for instance please refer to: Donath and Shoelson, 2011; Frangogiannis, 2014); however, increasing rates of heart failure in diabetic patients warrants an examination of the literature regarding the role of leukocytes in diabetic cardiovascular disease. Therefore, this review focuses on the role and behavior of leukocytes in the pathogenesis of diabetic heart failure.

LEUKOCYTES, INFLAMMATION, AND DIABETES

Leukocytes are essential mediators of the immune system that fight against foreign elements and maintain tissue homeostasis (Fearon and Locksley, 1996). Leukocytes work in an organized fashion with an impressive range of action (Odegaard and Chawla, 2008). They are derived from hematopoietic stem cells (progenitor cells) in the bone marrow. These pluripotent stem cells produce two distinct lineages: lymphoid progenitor cells and myeloid progenitor cells. Lymphoid progenitors are the precursors of T- and B- lymphocytes (T- and B-cells) and myeloid progenitors are the precursors of neutrophils, basophils, eosinophils, monocytes, macrophages, erythrocytes, dendritic cells, and platelets (Kondo, 2010). Monocytes/macrophages, neutrophils, and lymphocytes in particular have been demonstrated to both regulate and be impacted by the pathogenesis of diabetes (Hong et al., 2014).

Chronic inflammatory diseases, including diabetes, are characterized by dysfunctional and uncontrolled leukocyte behavior (Graves and Kayal, 2008; Swirski and Nahrendorf, 2013). Leukocyte recruitment is triggered by inflammation and they can produce a plethora of cytokines, chemokines, and reactive oxygen/nitrogen species to act systemically during diabetes (Naguib et al., 2004), and at local sites during myocardial infarction- or atherosclerosis-induced cardiac injury (Hansson and Libby, 2006; Eming et al., 2007), thereby contributing to sustained inflammation. Early inflammatory events in diabetes triggers the release of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 (Medzhitov and Janeway, 2000), which gradually increase as the disease progresses (Pickup et al., 1997). Several studies have demonstrated that initial elevated levels circulating IL-6, plasminogen activator inhibitor-1 (PAI-1), C-reactive protein (CRP) and fibrinogen, are associated with the manifestation of diabetes (Pradhan et al., 2001; Festa et al., 2002; Meigs et al., 2004). Pro-inflammatory cytokines downregulate the major anabolic cascades involved in insulin signaling and impair glucose homeostasis (Hotamisligil et al., 1995; Lumeng et al., 2007b). In response to pro-inflammatory mediators, the endothelial lining of the microvasculature will increase expression of intracellular adhesion molecule 1 (ICAM-1) and/or vascular cell adhesion molecule (VCAM-1) that interact with leukocyte-expressed integrins to capture them and allow their migration to the injured area (Chan et al., 2001; Henderson et al., 2001). These inflammatory cascades are tightly regulated by nuclear transcription factors including NF-κB, a master molecule of inflammation and tissue hemostasis (Lawrence, 2009). NF-κB activation leads to or boosts the expression of cytokines, chemokines and adhesion molecules and more prominent leukocyte recruitment. Thus, the inflammatory cascades - from leukocyte activation to NF-κB stimulation – work in a positive feedback loop fashion (Monaco et al., 2004; Lawrence, 2009).

Many leukocyte subsets are involved in diabetes-associated chronic inflammation, in particular neutrophils, macrophages, and T-cells. Neutrophils react to and secrete higher levels of cytokines and growth factors in diabetic patients relative to healthy controls, including IL-8, IL-1β, TNF-α, and IL-1ra, which contribute to further migration of neutrophils to inflammatory sites, phagocytic activity, release of lytic proteases, production of reactive oxygen species and apoptosis (Werner and Grose, 2003; Komesu et al., 2004; Baum and Arpey, 2005; Hatanaka et al., 2006). The excessive production of cytokines and exacerbation of neutrophil and macrophage activation may contribute to further tissue damage and increased susceptibility to invasive microorganisms (Tennenberg et al., 1999).

Macrophages are well-established phagocytic cells, which renders them effective at the clearance of apoptotic and necrotic cells (Gordon, 2003; Gordon and Martinez, 2010), but exist along a continuum of phenotypes that makes them difficult to definitively classify. As such, various classifications exist including classically activated macrophages (CAMφs) vs. alternatively activated macrophages (AAMφs) (Gordon and Martinez, 2010), and the more broad pro-inflammatory (M1) vs. pro-reparative (M2) macrophages (Nahrendorf et al., 2007; Mosser and Edwards, 2008; Bajpai et al., 2018). Under diabetic conditions, macrophages are recruited into adipose tissue (AT) and activated via local cytokine secretion (TNF-α, IL-12, and IL-6) (Vachharajani and Granger, 2009), contributing to the establishment of an inflammatory profile and insulin resistance within the tissue. A deficiency of MCP-1 (CCL2) or CCR2 (CCL2

receptor) in mice results in the impairment of pro-inflammatory macrophage recruitment to adipose tissue, thus impeding the induction of insulin resistance (Kanda et al., 2006; Yu et al., 2006) and suggesting an important role for pro-inflammatory macrophages in the initiation and development of diabetes. Further, free fatty acids can be recognized by Toll-like receptors (TLRs), leading to the activation of macrophages, which release more TNF-α (Shi et al., 2006; Davis et al., 2008). TNF-α, one of the cytokines most abundantly secreted by CAMφs, has the ability to reduce the expression of important genes in the glucose regulation process, such as the glucose transporter GLUT-4 (Lumeng et al., 2007a); in fact, TNF-α receptor KO mice are resistant to diabetes stimulation (Uysal et al., 1997), suggesting the endocrine function of adipose tissue (AT) directly impacts the development of insulin resistance via recruitment and activation of CAMφs. Secretion of cytokines by CAMφs further activates the JNK and NF-κB signaling pathways in various leukocytes, thereby promoting the further production of IL-1β, TNF-α, and MCP-1 and increasing the expression of iNOS, all of which contribute to insulin resistance in different tissues (Kaneto et al., 2005a,b; Andreasen et al., 2011). Myeloid-specific Iκκ-β (an activator of NF-κB)-deficient mice have shown decreased NF-κB activation and pro-inflammatory cytokine production (IL-1β, IL-6, TNF-α, and MCP-1), leading to inhibition of the development of insulin resistance (Arkan et al., 2005). Of note, it has been shown that IL-10 produced by AAMφs blocks the pathological effects of TNF-α in AT (Lumeng et al., 2007b; Prieur et al., 2011), suggesting that while CAMφs have insulin resistance-inducing effects, AAMφs have a protector role within AT. Indeed, A-ZIP transgenic mice (that are insulin-resistant and hyperlipidemic), which have a deficiency in MCP-1, displayed decreased hyperglycemia, hyperinsulinemia, and hepatomegaly; moreover, these mice had increased levels of AAMφs markers, such as Arg1 and Chi313 (Nio et al., 2012). Notably, AAMφ development is dependent on IL-4/IL-13 stimulation, which activates the transcription factor STAT-6, and STAT-6-deficient mice are more prone to obesity, oxidative stress in their AT and susceptibility to T2D development, which, in turn, is associated with the absence of AAMφs (Ricardo-Gonzalez et al., 2010).

Recent studies suggest adaptive immune cells, especially T lymphocytes, also play a pivotal role in diabetes. As with macrophages, $CD4^+$ effector T cells can be divided into proinflammatory Th1, Th17, and anti-inflammatory Th2 and $Foxp3^+$ regulatory T cell (Treg) subtypes based on their functionality and cytokine production (Raphael et al., 2015). Once activated, Th1 and Th2 cells show many significant signs of inflammation, such as cytokine release. For instance, Th1 cells produce interferon gamma- (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor beta (TNF-β), triggering cell-mediated immunity and phagocyte-dependent inflammation (Raphael et al., 2015). Th2 cells, in contrast, produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 to regulate antibody responses (Kahn et al., 2006). Studies have shown that Th1 and Th2 cells have key functional roles in regulating inflammatory processes, although they are activated later than macrophages during inflammation (Cintra et al., 2008; Martinez et al., 2008). Th17 cells, important pro-inflammatory $CD4^+$ T cell subtypes that secrete IL-17 and IL-22, have also been associated with diabetes (Zuniga et al., 2010; Zhang et al., 2014). It was shown that macrophages from AT express the IL-22 receptor (IL-22R) and respond to Th17-released IL-22 to secret more IL-1β, thereby further promoting AT inflammation (Dalmas et al., 2014; Zhao R. et al., 2014). In all, leukocytes clearly contribute to the pathogenesis of diabetes, and herein we will discuss the impact of leukocyte regulation in diabetic cardiomyopathy.

LEUKOCYTES IN DIABETIC CARDIOMYOPATHY

Heart failure associated with diabetes, or DCM, is a common hallmark of diabetes progression. As discussed above, diabetes is associated with chronic systemic inflammation, which leads to leukocyte activation and recruitment to various organs and further inflammatory tissue remodeling over time. In general, this results in organ fibrosis as resident fibroblasts become activated in response to pathophysiologic conditions, which for the heart leads to wall stiffening and decreased contractility (Russo and Frangogiannis, 2016). Reduced cardiac output ultimately stimulates further cardiac inflammation and fibrosis, leading to dilation and established heart failure. Leukocytes are known to modulate cardiac fibroblasts by virtue of secreted mediators of fibrosis, including transforming growth factor-β (TGF-β) (Bugger and Abel, 2014; Russo and Frangogiannis, 2016), however, whether DCM-induced fibrosis is preceded by leukocyte infiltration and activation has not been reported.

Several factors contribute to DCM and the potential leukocyte responsiveness during its progression, including chronic hyperglycemia, which leads to obesity, high cholesterol levels, as well as high blood pressure and coronary artery diseases. Recent evidence suggests cross-talk between inflammation and insulin signaling, highlighting a strong relationship between insulin-resistant states, inflammation, and heart failure (Kim et al., 2005). For example, altered microvascular endothelial ICAM-1 expression in diabetic rats has been shown to be restored with insulin treatment (Anjos-Valotta et al., 2006). There are also multiple molecular pathways involved in the induction of diabetic heart failure including oxidative/nitrative stress (Vita and Keaney, 2002; Creager et al., 2003; Widlansky et al., 2003), activation of mitogen-activated protein kinase (MAPK) (Malek et al., 1999; Vita, 2002), pro-inflammatory, poly (adenosine diphosphate [ADP]-ribose) polymerase (PARP) (Calles-Escandon and Cipolla, 2001) and transcription factors signaling pathways (Kim et al., 2006; Bakker et al., 2009), as well as changes in the composition of extracellular matrix (Heil and Schaper, 2004) and inactivation of pro-survival pathways (Silver and Vita, 2006).

In the early phase of inflammation, proinflammatory cytokines including TNFα, IL-6 (Dinh et al., 2009) IL-1β (Masters et al., 2011), Interferon (IFN)-γ, TGF-β (Biernacka et al., 2015 are secreted by macrophages and/or lymphocytes and may cause or exacerbate cardiac injury. In addition, these locally produced cytokines have been found to possess autocrine

and paracrine properties that can influence neighboring tissues to enhance vascular permeability (Salt et al., 2003), recruitment of invasive leukocytes (Hokama et al., 2000; Pettersson et al., 2011) and reactive oxygen species (ROS) production (Giacco and Brownlee, 2010; Mann, 2015; Low Wang et al., 2016). Altogether, disturbances in metabolic and inflammatory signaling pathways during diabetes progression are associated with alterations in leukocyte activation and enhanced cardiac inflammation (**Figure 1**). Therefore, in this section of review, we will discuss the role of leukocytes subsets in DCM.

Neutrophils

Neutrophils often provide the first line of defense at sites of inflammation. These are considered short-lived effector cells, possessing limited capacity for biosynthetic activity and ROS generation, but have been shown to be crucially involved in cardiac repair by polarizing macrophages toward a reparative phenotype (Horckmans et al., 2017). In addition, they secrete a number of factors that regulate inflammation, including peroxidases, cytokines, microparticles (MPs), and neutrophil extracellular traps (NETs). The activity of myeloperoxidase (MPO), stored in azurophilic granules of neutrophils and released during inflammation (Anatoliotakis et al., 2013), has been shown to be increased in the plasma of patients with diabetes concomitant with coronary heart disease (Gorudko et al., 2012). Neutrophil gelatinase-associated lipocalin (NGAL) is one of the cytokines solely produced by neutrophils and its expression is increased following acute myocardial infarction and during chronic heart failure (Yndestad et al., 2009; Villacorta et al., 2015). NGAL modulates the enzymatic activity of matrix metalloproteinase-9 (MMP-9) and is an important mediator of plaque instability in atherosclerosis, suggesting that it might play a role in thrombo-inflammation (Sivalingam et al., 2017). MPs are small vesicles (0.1–1.0 μm) released from stimulated and/or apoptotic endothelial cells, platelets, and leukocytes (monocytes and neutrophils) (Boulanger et al., 2017). Neutrophil-derived MPs, which can be regulated by endothelium-derived MPs and depend on locally released nitric oxide (Muller, 2014), contain the functionally active anti-inflammatory protein annexin 1, which inhibits the interaction between leukocytes and endothelial cells *in vitro* and *in vivo* (Hayhoe et al., 2006; Sugimoto et al., 2016). The changes and roles of MPs in either diabetes, heart failure or DCM remains largely unknown.

A recently identified process involving NET formation, which involves the release of DNA and granule proteins of neutrophils that prime other immune cells to augment inflammation, may contribute to the development of DCM since studies have indicated that NET formation is enhanced in diabetic patients and ultimately contributes to impaired wound healing (Papayannopoulos, 2015; Wong et al., 2015). The release of NETs,

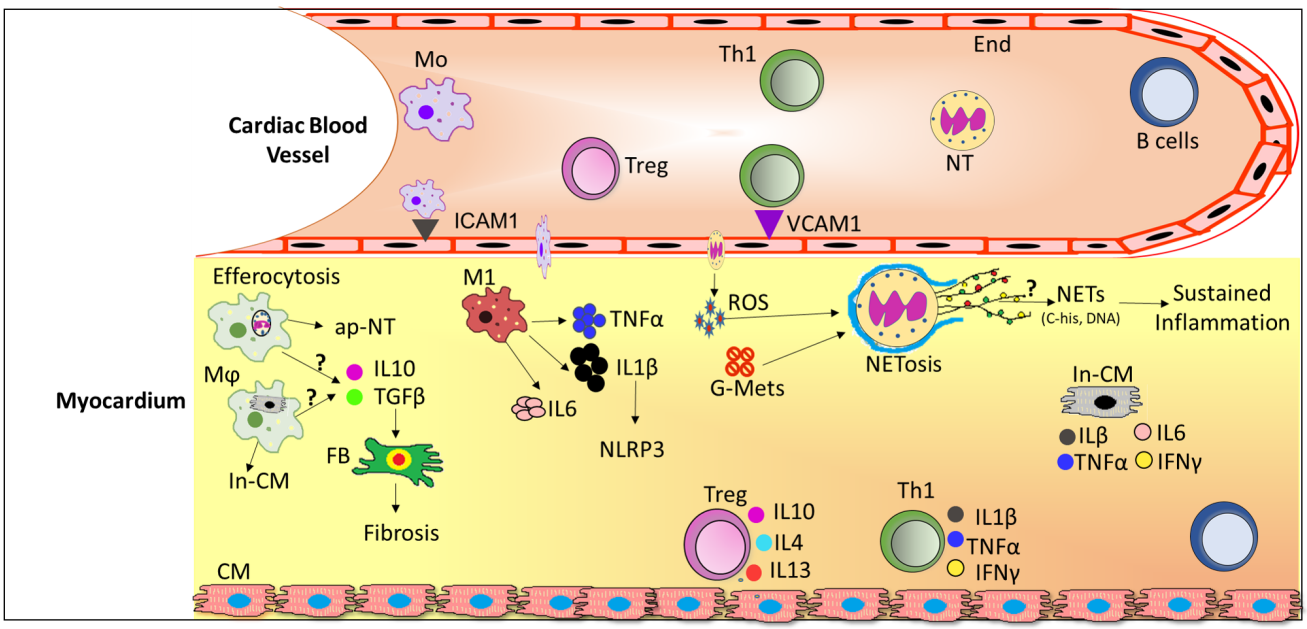

FIGURE 1 | Schematic diagram depicting infiltration of leukocytes from the circulation and their role in the diabetic cardiomyopathy (DCM). In DCM, a number of local processes are activated by glucose metabolites, reactive oxygen species (ROS) and pro-inflammatory cytokines together with accumulation of neutrophils and macrophages into the lesion site. Upon infiltration, neutrophils release extracellular traps (NETs) which induce sustained inflammation. Activated macrophages phagocytose cellular debris and also release pro-inflammatory cytokines and growth factors which activates fibroblasts to induce fibrosis. Th1 cells secrete pro-inflammatory cytokines which further exacerbate the inflammation in DCM whereas Treg cells secrete anti-inflammatory cytokines, where the ratio of pro-/anti-inflammatory cytokines may predict the progression of DCM. Abbreviations: ap-NT, apoptotic neutrophils; B cells, B lymphocytes; CM, cardiomyocytes; End, endothelial cells; FB, Fibroblast; G-Mets, Glucose metabolites; In-CM, Injured cardiomyocytes; ICAM1, Intracellular adhesion molecule 1; IL6, Interleukin 6; IL1β, Interleukin 1 beta; IFNγ, Interferon gamma; M1, pro-inflammatory macrophages; Mo, monocytes; Mϕ, activated macrophages; NETs, Neutrophils extracellular traps; NT, neutrophils; ROS, reactive oxygen species; Th1, T helper cells 1; Treg, T regulatory cells; VCAM1, vascular cell adhesion molecule 1.

termed NETosis, is a proposed cell death mechanism, which, if dysregulated, can contribute to pathogenesis (Fadini et al., 2016; Papayannopoulos, 2018). During NETosis, mitochondrial ROS, inflammatory cytokines and glucose metabolites may each participate in the activation of NF-κB to transcriptionally up-regulate peptidyl arginine deiminase 4 (PAD-4), which acts to promote histone processing, an important event in NET formation (Azroyan et al., 2015; Wong et al., 2015). Subsequently the digestion products and granule proteins contents are released into the extracellular space, providing an extremely strong pro-inflammatory stimulus (Wong et al., 2015; Silk et al., 2017). Future studies will be required to determine the specific impact of NETosis in diabetes progression, and more specifically in DCM.

Macrophages

Macrophages have been implicated in the pathogenesis of diabetes, wherein they display impaired phagocytic activity (Tan et al., 1975; Khanna et al., 2010), reduced release of lysosomal enzymes (McManus et al., 2001), and reduced chemotactic activity (Khanna et al., 2010; Raj et al., 2018) in diabetic patients. These traits are significantly correlated with increased blood glucose levels (Jakelic et al., 1995) and reversed by decreasing blood glucose levels in both humans (Jakelic et al., 1995) and rats (Alba-Loureiro et al., 2006). Normally in injured tissue, macrophages engulf apoptotic cells and cellular debris to reduce inflammation, a phenomenon called efferocytosis (DeBerge et al., 2017). Several molecular processes contribute to this mechanism and in particular the metalloproteinase disintegrin and metalloproteinase domain-containing protein 9 (ADAM-9) was shown to be upregulated in macrophages under conditions of high glucose, secondary to decreased expression of miR-126, which increased MER proto-oncogene, tyrosine kinase (MerTK) cleavage to ultimately reduce efferocytosis (Suresh Babu et al., 2016). Importantly, human diabetic hearts displayed the same molecular signatures in terms of miR-126, ADAM9, and cleaved MerTK expression, suggesting this process may be involved in regulating human DCM progression. Thus, impaired efferocytosis would be expected to prolong cardiac inflammation as dead cardiomyocytes and debris would not be efficiently removed.

As discussed above, macrophages have been demonstrated to exist along a spectrum of phenotypes book-ended by either pro-inflammatory (M1) or pro-reparative (M2) descriptors, and certainly a regulated balance between the two subtypes is necessary for homeostasis of inflammation (Nahrendorf et al., 2007; Mosser and Edwards, 2008; Bajpai et al., 2018). During diabetes the balance favors the M1 phenotype, which acts to promote a low level of chronic tissue inflammation and insulin resistance (Rao et al., 2014). M1 macrophages have been shown to be upregulated in the myocardium prior to the onset of cardiac dysfunction (Nahrendorf et al., 2007) and early non-selective macrophage depletion with clodronate liposomes has been demonstrated to reduce cardiac inflammation (Schilling et al., 2012). Conversely, macrophages of the M2 phenotype are associated with reduced cardiac inflammation under conditions of experimental diabetes (Jadhav et al., 2013), however, further investigation is required to elucidate the impact of phenotype-specific depletion or activation of macrophages in the context of DCM. Notably, the M1 and M2 classification system is now thought to be oversimplified, with recognition of a spectrum of multiple macrophage phenotypes (Xue et al., 2014) that have been recently identified and which have unknown impact on DCM.

T-Lymphocytes

Distinct T-lymphocytes subtypes, including T-helper subsets (Th) and T regulatory cells (Treg), regulate inflammation and insulin resistance. Increased frequency of Th1, Th17, and Th22 subsets were shown to contribute to coronary artery disease onset in diabetic patients after adjusting for age, sex, and duration of diabetes (Zhao R.X. et al., 2014). In another study, increased serum levels of Th1-associated cytokines (IL-12 and IFN-γ) with strong suppression of Th2-associated cytokines (IL-4, -5) were found to be correlated with diabetic coronary artery disease (Madhumitha et al., 2014). Several clinical studies have confirmed that Th1-associated cytokines are upregulated in the peripheral blood from pre-diabetic or T2DM (type 2 diabetes) patients (Zeng et al., 2012; McLaughlin et al., 2014), whereas the activation of Th2 cell-mediated immunity is delayed and impaired in diabetes (Wu et al., 2011). IL17- secreting Th17 cells are also increased in T2DM patients and may be associated with dysregulated lipid metabolism (Zuniga et al., 2010; Zhang et al., 2014; Garidou et al., 2015).

As their name suggests, Treg cells regulate inflammatory responses and tissue impairment (Sakaguchi et al., 2008; Nosbaum et al., 2016). In T2DM, Treg cells can suppress Th1, Th2, and Th17 responses by various pathways, such as the suppression of cytokine secretion, modulation of the microenvironment, and altering the expression of surface receptors to improve insulin resistance (Guzman-Flores et al., 2013; Bluestone et al., 2015). $Foxp3^+$ Treg cells have been demonstrated effective in the control of autoimmune disease (Buckner, 2010), and in DCM patients, a significant reduction in peripheral TGF-β and IL-10 with decreased Foxp3 expression contributed to an imbalance in the Treg/Th17 ratio (Li et al., 2010, 2017; Tang et al., 2010). Given the decreased number of Treg cells (Jagannathan-Bogdan et al., 2011), as well as altered Treg/Th17 and Treg/Th1 ratios in patients with T2DM (Zeng et al., 2012), an appropriate balance between pro-inflammatory (Th17 or Th1) and regulatory (Treg) subsets of T cells may be required to maintain overall T cell homeostasis and prevent chronic inflammation. While it is evident that T cells play an important role in mediating cardiac injury (Bansal et al., 2017), and genetic depletion of T cells protects against cardiac fibrosis and decreased LV function (Laroumanie et al., 2014; Weirather et al., 2014; Nevers et al., 2015), further delineation of the role of each T-lymphocyte subset would be worthwhile exploring specifically in the context of diabetic heart failure.

B-Lymphocytes

B-lymphocytes are antigen-presenting cells and autoantibody secretors. B-lymphocyte-deficient mice demonstrated less inflammation and exhibited improved glucose tolerance (Winer

et al., 2011). Additionally, Nishimura et al. demonstrated that mice deficient of programmed cell death protein-1 (PD-$1^{-/-}$, a key factor for B-cell differentiation) expressed elevated levels of circulating autoantibodies that bound specifically to cardiomyocytes and were associated with progression of dilated cardiomyopathy (Nishimura et al., 2001). In another study, B cells from diabetes mellitus patients had elevated pro-inflammatory IL-8 levels but failed to secrete the anti-inflammatory IL-10 under a variety of pro-inflammatory conditions (Jagannathan et al., 2011). In contrast, a recent study demonstrated that naturally occurring B-regulatory cells mediate protection against autoimmune destruction of pancreatic islets by selectively suppressing autoreactive T-cell responses (Kleffel et al., 2015). Given that B cells are the earliest cell type that infiltrate pancreatic islets in mice and directly regulate islet T cell infiltration, B cell-directed therapy could be effective to protect against diabetes, however, much more insight into their action under these conditions is required.

THERAPEUTIC STRATEGIES

Since numerous signaling pathways activated during DCM ultimately contribute to fibrosis, preclinical studies have focused on mitigating this effect via targeting of various fibrogenic aspects. Several studies by the Tschöpe group showed that pre-clinical streptozotocin-induced DCM rodent models are associated with increased pro-inflammatory cytokine and adhesion molecule expression in the heart, as well as leukocyte accumulation and fibrosis, effects that were sensitive to treatment with a variety of treatments, including statin, interleukin converting enzyme inhibitor and monoclonal antibody-mediated inhibition of TNFα (Van Linthout et al., 2007; Westermann et al., 2007a,b). In addition, another group previously demonstrated that the antifibrotic agent tranilast, and its derivatives FT011 and FT23, act to oppose TGFβ-mediated fibrosis in a streptozotocin-induced transgenic (mRen-2)27 hypertensive rat model of DCM (Martin et al., 2005; Kelly et al., 2007; Tan et al., 2012; Zhang et al., 2012). These compounds acted to attenuate diastolic cardiac dysfunction, which was associated with decreased fibrosis and, notably, macrophage accumulation within the myocardium. Since therapeutic strategies for the treatment of cardiac fibrosis have been reviewed elsewhere (Russo and Frangogiannis, 2016), here we focus more specifically on clinical and preclinical evidence for potential therapies that could mitigate DCM via regulation of leukocytes themselves. As discussed above, both neutrophils and B-lymphocytes may offer potential therapeutic targets for the treatment of DCM, however, more preclinical studies will be required to assess this concept. As such, the remainder of the discussion will focus on reported responses to therapeutic strategies involving modulation of macrophage and T cell activities.

Macrophages

Although inhibition of pro-fibrotic processes appears capable of decreasing the progression of DCM and cardiac leukocyte accumulation, reduced leukocyte accumulation within the diabetic heart has conversely been demonstrated to decrease cardiac fibrosis during experimental diabetes in rodents. For instance, treatment of either streptozotocin-induced mice, as a model for Type I diabetes, or Israeli sand rats, as a model for Type II diabetic cardiomyopathy, with the CXCR4 antagonist AMD3100 was able to decrease fibrosis, suggesting that inhibition of leukocyte recruitment to the heart during development of DCM is sufficient to decrease pro-fibrotic signaling (Chu et al., 2015). Additionally, a recent study reported that β2-adrenergic receptor (β2AR) stimulation of macrophages under conditions of high glucose inhibited pro-inflammatory NF-κB-dependent production of TNFα and that long-term treatment of Zucker diabetic fatty (ZDF) rats with the β2AR agonist salbutamol decreased monocyte activation, cardiac macrophage, collagen and fibronectin accumulation, as well as preserved cardiac function compared to non-salbutamol-treated ZDF rats (Noh et al., 2017). Notably, β2AR stimulation-mediated inhibition of macrophage activation *in vitro* and cardiomyopathy progression *in vivo* was context-dependent, occurring only under hyperglycemic but not normal glucose conditions, while our own studies have shown that β2AR agonism increases, while antagonism or deletion decreases, leukocyte responsiveness (Grisanti et al., 2016a,b). Thus, disease-specific environmental factors may play a key role in determining the effectiveness of potential therapeutics.

Additional studies support the involvement of macrophages in DCM, wherein clodronate-liposome-mediated depletion of macrophages was demonstrated to reduce the expression of macrophage and inflammatory markers in the heart and partially preserve cardiac function in a transgenic mouse model of cardiac lipotoxity (Schilling et al., 2012). Further, in streptozotocin-treated mice, pro-inflammatory cytokine expression, oxidative stress, fibrosis and cardiac dysfunction were associated with enhanced monocyte accumulation within the heart, all of which were reduced by treatment with bone morphogenetic protein 7 (BMP7), the supposition being that this promoted monocyte conversion into anti-inflammatory macrophages favoring survival signaling (Urbina and Singla, 2014). Similarly, fibroblast growth factor-9 administration to infarcted db/db diabetic mice was shown to enhance M2 macrophage polarization, which was associated with decreased inflammatory cytokine expression, reduced cardiac remodeling and improved cardiac function (Singla et al., 2015). Further, activation of peroxisome proliferator–activated receptor gamma (PPARγ), a ligand-activated transcription factor that controls the expression of key genes involved in lipid and glucose metabolism and inflammation (Blaschke et al., 2006), has been shown to reduce human monocyte chemotaxis (Kintscher et al., 2000) and suppress macrophage pro-atherosclerotic osteopontin expression (Oyama et al., 2002), suggesting that clinically used glitazones may be able to reduce the infiltration or phenotypic conversion of pro-inflammatory macrophages.

A more recent study similarly reported alterations in streptozotocin-treated mouse hearts, including enhanced pro-inflammatory cytokine expression, fibrosis and decreased

function that was associated with macrophage accumulation, but notably highlighted the negative impact of estrogen deficiency on these processes through the use of ovariectomized female mice (Jia et al., 2017). These changes were also associated with increased expression of pro-M1/anti-M2 macrophage miR155. However, exacerbation of DCM in the absence of estrogen was prevented via either clodronat liposome-mediated macrophage depletion or treatment with gold nanoparticle-conjugated antago-Mir155, which promoted M2 macrophage marker expression and improved cardiac structure and function. Finally, induction of heme oxygenase-1 (HO-1) was shown to enhance M2 macrophage polarization *in vitro* and in rodent models, including high fat diet-fed C57BL/6 mice and ZDF rats, which led to the amelioration of pro-inflammatory cytokine generation and cardiac dysfunction in the face of diabetic cardiomyopathy (Sierra-Filardi et al., 2010; Jadhav et al., 2013; Tu et al., 2014). Altogether, these studies suggest that a balance between M1 and M2 macrophage phenotypes within the heart may be an essential component of controlling DCM progression.

T-Lymphocytes

Similar to targeting macrophages, studies have highlighted the potential therapeutic effectiveness of targeting T lymphocytes for preventing the development of DCM. For instance, streptozotocin-treated mice displayed enhanced cardiac T cell infiltration associated with increased fibrosis and decreased cardiac function, each of which were augmented by T cell-specific deletion of hypoxia inducible factor 1α (HIF-1α) (Lin et al., 2016). Further, genetic depletion of T cell trafficking protected cardiac fibrosis and LV function by reducing S1P1 and TGF-β1 expression (Laroumanie et al., 2014; Weirather et al., 2014; Nevers et al., 2015). Additionally, Rag1KO mice, which lack mature T lymphocytes, are protected against streptozotocin-induced cardiac fibrosis (Abdullah et al., 2016). The same group has also reported that T-cell-specific sphingosine 1-phosphate receptor 1 (S1P1)-mediated signaling is essential for the streptozotocin-induced fibrosis as the S1PR1 antagonist FTY720 was able to attenuate this response, as was T cell-specific deletion of S1PR1 (Abdullah et al., 2016; Abdullah and Jin, 2018). Notably, while depletion of T cell-specific expression of S1PR1 exerted protection against cardiac fibrosis in the diabetic model, non-streptozotocin-treated T cell-specific S1PR1 knockout mice exhibited enhanced cardiac fibrosis, suggesting that S1P1R-dependent T lymphocyte signaling differentially alters cardiac remodeling outcomes in a pathologically contextual manner.

FUTURE PERSPECTIVES AND UNANSWERED QUESTIONS

Although scientists have explored new phenotypes and functions of leukocytes in the context of heart failure, their role in diabetic cardiomyopathy is still developing and there remain several important avenues of research for the future. First, although the role(s) of leukocytes in regulating DCM in different experimental rodent models may overlay, the predominant use of the streptozotocin-induced Type I diabetes rodent model to investigate the leukocytes in the development and progression of DCM potentially leads to limited applicability to the clinically relevant and highly prevalent type II diabetes-associated DCM (Holscher et al., 2016). Thus, further studies are required to understand the potential differences in leukocyte phenotypes and their underlying mechanisms for promoting DCM using rodent models that better mimic conditions observed during the development of type II diabetes mellitus. Second, B-lymphocytes clearly contribute to cardiac remodeling during the development of heart failure since systemic B-lymphocyte depletion has been shown to reduce T cell–, macrophage- and neutrophil-induced tissue damage by reducing the systemic amplification of the inflammatory response after myocardial infarction (Zouggari et al., 2013). However, the role of B-lymphocytes specifically in the progression of DCM is unknown, therefore additional studies within this context are needed. Third, there are known differences between males and females in the progression of DCM (Natarajan et al., 2003; Laverty et al., 2017). It is evident that females are protected from cardiovascular diseases due to multiples factors including estrogen receptor signaling (Pare et al., 2002), reduced ROS production, and higher antioxidants (Barp et al., 2002; Ide et al., 2002). As such, future work would be immensely beneficial in understanding potential sex-specific leukocyte behaviors during the development and progression of DCM.

AUTHOR CONTRIBUTIONS

AB and DT wrote the manuscript.

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Black Garlic Improves Heart Function in Patients With Coronary Heart Disease by Improving Circulating Antioxidant Levels

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Background: Black garlic (BG) has many health-promoting properties.

Objectives: We aimed to explore the clinical effects of BG on chronic heart failure (CHF) in patients with coronary heart disease (CHD).

Design: The main components of BG were measured by gas chromatography–mass spectrometry (GC–MS) and its antioxidant properties were determined by the clearance rate of free radicals. One hundred twenty CHF patients caused by CHD were randomly and evenly assigned into BG group and placebo group (CG). The duration of treatment was 6 months. Cardiac function was measured according to the New York Heart Association (NYHA) functional classification system. The following parameters were measured, including walking distance, BNP precursor N-terminal (Nt-proBNP), left-ventricular ejection fraction (LVEF) value, and the scores of quality of life (QOL). The circulating antioxidant levels were compared between two groups.

Results: There are 27 main compounds in BG with strong antioxidant properties. BG treatment improved cardiac function when compared with controls ($P < 0.05$). The QOL scores and LVEF values were higher in the BG group than in the CG group while the concentration of Nt-proBNP was lower in the BG group than in the CG group ($P < 0.05$). Circulating antioxidant levels were higher in the BG group than in the CG group. Antioxidant levels had positive relation with QOL and LVEF values, and negative relation with Nt-proBNP values.

Conclusion: BG improves the QOL, Nt-proBNP, and LVEF in CHF patient with CHD by increasing antioxidant levels.

Keywords: coronary heart disease, congestive heart failure, quality of life, left-ventricular ejection fraction, black garlic

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INTRODUCTION

Chronic heart failure (CHF) may be caused by myocardial abnormalities, which result in systolic and/or diastolic ventricular dysfunction, abnormalities of the valves, pericardium, endocardium, heart rhythm, a reduced cardiac output, or brain abnormalities (12). Vasomotor function cannot meet the needs of systemic metabolism, resulting in hemodynamic abnormalities and neurohormonal activation (Hammadah et al., 2017). There are 26 million CHF patients

worldwide and the prevalence of CHF is still increasing with population aging (Schmid et al., 2017). CHF is a common cause of death in the elderly (Andres et al., 2018; Clark, 2018). Five-year mortality rate of CHF is more than 20%, and seriously threatens human life (Nakajima et al., 2014). Considering its poor prognosis, it is critical to prevent the occurrence and development of CHF and to promote early rehabilitation of CHF patients.

Garlic is a kind of valuable atherosclerosis-preventing functional food (Alali et al., 2017). Many reports showed that garlic had lipid-lowering, plasma anticoagulant and antioxidant activities, and improves endothelial injuries (Gorinstein et al., 2007). The extract of garlic was effective to reduce blood pressure, arterial stiffness, inflammation, and other cardiovascular diseases (Ried et al., 2016). Garlic is a kind of feasible and promising functional food for individuals with cardiovascular disease (Aslani et al., 2016; Siddiqui et al., 2017).

Black garlic (BG) is a kind of deep-processed food made of fresh garlic under high temperatures and humidity. It can improve immune activity with fewer side effects (Nakasone et al., 2016). BG has many health-promoting properties: BG prevented the growth and induced apoptosis of HT29 colon cancer cells via phosphatidylinositol 3-kinase (PI3K)/Akt pathway, suggesting that BG may be effective in the therapy of colon cancer (9); BG had potential beneficial effects in the treatment of diabetes by increasing in the numbers of monocytes and granulocytes, and decreasing lymphocyte proliferation (10); BG has anti-allergic actions and may be beneficial as functional food in the prevention of allergic disorders (11). BG has various biological functions, including antioxidant (Lu et al., 2017; Sun and Wang, 2018), anti-inflammatory (Jeong et al., 2016), anticancer (Dong et al., 2014), antidiabetic (Abel-Salam, 2012), anti-allergic action (Yoo et al., 2014), and the improvement of lipid metabolism (Ha et al., 2015), cardiac (Czompa et al., 2018), and hepatic protection (Kim et al., 2011; **Figure 1**). However, the effects of BG on the CHF patients and the related molecular mechanisms remain unknown.

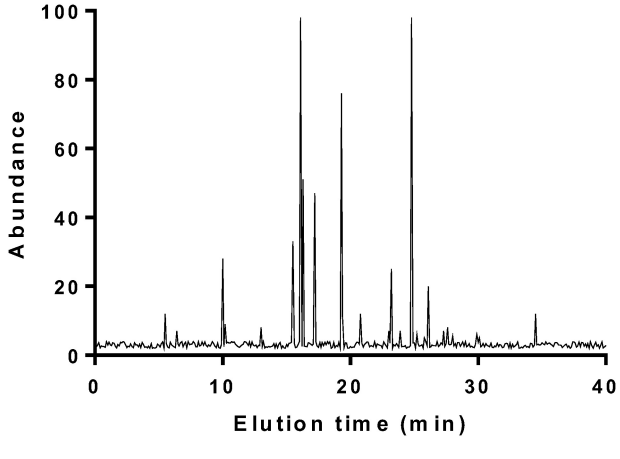

FIGURE 1 | GC analysis of main components of BG.

Chronic heart failure is a complex, and dynamic development process. Neuro-hormones, inflammation, and cytokines play an important role in this process. A large number of studies have shown that brain natriuretic peptide (BNP) is involved in the pathophysiological process of the development of CHF (Du et al., 2012). BNP is a neuro-hormone secreted by ventricular myocytes. Under normal circumstances, there is little BNP in the atria and ventricle. In many pathological conditions, such as the ventricular volumetric load and pressure overload, BNP concentration will be increased in the blood (Kou et al., 2016). The higher severity of heart failure will result in higher concentration of BNP (Hahn et al., 2016; Feng et al., 2017). BNP shows good specificity in differential diagnosis of CHF (Jin et al., 2018), cardiogenic dyspnea (Golshani et al., 2016), and lung-derived respiratory distress (Sun et al., 2015).

After being stimulated by cardiomyocytes, BNP is cleaved by proteases into Nt-proBNP and active BNP. The clinical application of Nt-proBNP and bioactive hormone BNP to CHF is similar. However, BNP has a 20 min half-life and is poorly stable *in vitro*. Comparatively, the half-life of Nt-proBNP is 60–120 min and stable *in vitro* (Mukherji et al., 2017). Therefore, the quantitative detection of plasma Nt-proBNP is more feasible. In this study, we explored the effects of BG on CHF patients with coronary heart disease (CHD). The improvement of quality of life (QOL) of CHF patients was compared with controls and the levels of Nt-proBNP were measured.

MATERIALS AND METHODS

Measurement of the Component of Black Garlic

Raw garlic and BG were purchased from Shandong Sanjin Black Garlic Industry Co., Ltd. (Jinxiang, China). The difference for the main components between raw garlic and BG was listed in **Table 1**. BG was prepared from fresh garlic via the fermentation (60–80°C, 70–95% relative humidity) for 50 d. Thirty grams of raw garlic and BG was weighed, respectively, ground by a mortar, and placed in a 1000-mL round-bottomed flask. Thirty milliliters of sodium chloride and distilled water was added, and heated by temperature-controlled electric heating apparatus. After distillation, it was concentrated to 1.0 mL and transferred to a chromatography flask for gas chromatography–mass spectrometry (GC–MS) analysis.

Agilent 7890 Gas Chromatograph and Agilent 7890 GC/5975CMS GC/MS were purchased from Agilent (Foster City, CA, United States). The following chromatographic conditions were used: Column: HP-5MS (60 m × 0.25 mm × 0.25 μm); inlet temperature, 250°C; injection quantity, 10 μL; split ratio, 4:1; carrier gas, He, 1.0 mL/min; temperature program, 50 (2 min) and 220°C (30 min) and heating rate, 4°C/min. The following mass spectrometry conditions were used: transmission line temperature, 240°C; EI source electron energy, 70 eV; electron multiplier voltage, 1635 V; mass scanning range, 0–450 amu; ion source temperature, 230°C; and quadrupole temperature, 150°C. GC–MS results were analyzed manually and compared

TABLE 1 | Comparison for the ingredients between raw garlic and BG (100 g).

Ingredients	Raw garlic	BG
Moisture (g)	65.31 ± 1.75	37.12 ± 3.70
Total sugar (g)	27.23 ± 0.52	48.47 ± 1.75
Reducing sugar (g)	0.47 ± 0.03	39.49 ± 0.74
Protein (g)	5.32 ± 0.08	10.26 ± 0.76
Crude fat (g)	0.35 ± 0.06	0.16 ± 0.05
Acid value (g)	0.47 ± 0.06	2.13 ± 0.14
5-HMF (mg)	0	8.732 ± 0.17
Total phenols (mg)	184.35 ± 14.12	482.46 ± 20.04
Amino acid (mg)		
Aspartic acid	14.66 ± 0.81	30.81 ± 1.43
Glutamate	60.5 ± 0.27	38.52 ± 1.14
Serine	75.06 ± 0.5	41.28 ± 0.69
Glycine	102.58 ± 10.36	13.17 ± 0.75
Histidine	73.42 ± 1.07	14.8 ± 0.21
Threonine	131.47 ± 0.68	36.71 ± 0.82
Arginine	1079.88 ± 1.05	845.16 ± 20.29
Alanine	27.3 ± 1.02	108.59 ± 11.05
Valine	41.71 ± 0.39	13.84 ± 0.72
Tyrosine	96.68 ± 8.45	90.19 ± 10.67
Valine	40.89 ± 2.25	56.72 ± 0.33
Methionine	491.38 ± 78.84	3.48 ± 1.01
Cysteine	3.75 ± 0.27	18.08 ± 0.79
Isoleucine	14.08 ± 1.03	21.93 ± 0.29
Leucine	9.2 ± 0.8	28.72 ± 0.85
Phenylalanine	30.55 ± 0.16	62.86 ± 1.73
Tryptophan	23.51 ± 0.67	7.65 ± 0.54
Lysine	120.39 ± 13.45	61.83 ± 0.27
Total	1943.77 ± 161.22	1486.65 ± 112.62

TABLE 2 | The main component of polyphenols of BG.

Components	Molecular formula	Molecular weight	Retention time (min)	Percent components
Allyl alcohol	C3H6O	58.08	5.5	1.65
Allyl sulfide	C6H10S	114.21	6.4	0.83
Allyl methyl disulfide	C4H8S2	120.24	10.0	4.58
1,3-Dithiane	C4H8S2	120.24	10.2	1.12
Dimethyl trisulfide	C2H6S4	126.26	13.0	0.82
Tetrahydro-2H-1,4,6-oxodiazocine ring	C5H10N2OS	146.00	15.5	5.52
Diallyl disulfide	C8 H12O4	172.18	16.1	17.32
2-Vinyl-1,3-dithiane	C6H10S2	146.00	16.3	8.85
N,*N*-Dimethylthiourea	C3H8N2S	104.17	17.2	8.00
Ethylthiourea	C3H8N2OS	120.17	17.7	0.12
2-Ethyltetrahydrothiophene	C6H12S	116.22	19.2	13.24
(Methylthio)acetonitrile	C3H5NS	87.14	19.3	1.40
H1-Propyl-1-(fringyl)-butane	C7H1S	118.00	20.8	1.35
5-Methyl-1,2,4-triazole-3-decanol	C3H5N3S	115.16	23.0	0.50
3-Vinyl-3,4-dihydro-1,2-dithiazolone	C6H8S2	144.26	23.2	3.88
2-Ethylene-1,3-dithiane	C6H10S2	146.00	23.9	0.67
3-Vinyl-3,4-dihydro-1,2-dithiane	C6H8S2	144.26	24.8	17.42
3,5-Diethyl-1,2,4-tritetrahydrothiophene	C6H12S3	180.35	25.2	0.54
1,3,5-Trithiane	C3H6S3	138.27	25.8	0.29
(2-Arylthio)-acetonitrile	C5H7NS	113.00	25.9	0.19
Diallyl trisulfide	C6H10S3	178.34	26.1	2.98
1,3,5-Trithiane	C3H6S3	138.27	27.3	0.76
2-*n*-Propylthiophene	C7H10S	126.22	27.6	0.85
(2-Arylthio)-acetonitrile	C5H7NS	113.00	28.0	0.45
1,3,5-Trithiane	C3H6S3	138.27	29.9	0.36
1,2-Dithiocyclopentane	C3H6S2	106.21	30.7	0.39
2-Thiophene	$C4H3NO_2S$	129.14	34.5	1.34

with standard mass spectra to determine the chemical structure of each separated components.

The Measurement of Antioxidant Properties of Garlic

Preparation of Extracts of Black Garlic

The fresh raw garlic and BG were dried to a constant mass and pulverized. One gram of raw garlic and BG sample mechanical powder, and 20 mL of a 50% ethanol solution were added at a ratio of 1:20 to the stock solution. The main components were extracted via ultrasonic at 30°C for 30 min, centrifuged, filtered, and diluted with 50% ethanol to 20 mL as a sample solution.

Determination of Antioxidant Capacity of Black Garlic

One hundred microliters of raw garlic and BG extracts was added to the 96-well transparent plates with different concentrations (0.25, 0.50, 1.00, 2.00, 4.00, and 8.00 mg/mL) and 100 μL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution was added. With the same method, the different concentrations of DPPH and methanol were mixed as a blank group, and solution and methanol were mixed as a control group. After being kept at room temperature for 30 min in the dark, the absorbance was measured at 517 nm and repeated three times. DPPH clearance = $(1-(D1-D2)/D3) \times 100\%$, where $D1$ was the sum of the absorbance of the DPPH solution and the sample solution; $D2$ was the sum of the absorbance of the sample solution and the extraction solvent; $D3$ was the sum of the absorbance of the DPPH solution and the extraction solvent.

Determination of the Reducibility of Fe^{3+}

Two hundred microliters of raw garlic and BG extract (0.625, 1.250, 2.500, 5.000, and 10.000 mg/mL) was placed in 5-mL centrifuge tubes, respectively, and 0. 5-mL 0.2 mol/L phosphate-buffered saline (PBS), pH 6.6, and 0.5-mL 1% potassium ferricyanide solution were added. After being mixed, bath at 50°C for 20 min, and then 0.5 mL 10% ferric chloride solution (TCA) was added and centrifuged at 5000 r/min for 5 min. 0.5-mL supernatant was taken, and 0.5-mL distilled water and 0.1-mL 0.1% ferric chloride solution were added. The absorbance values were measured at 700 nm and repeated three times.

Clearance Rate of 2,2′-Azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS)-Free Radicals

Seven millimolar ABTS solution was prepared with PBS (pH = 7.4), and 7 mM ABTS and 2.45 mM potassium phosphate were mixed in equal volume. The solution was diluted with PBS until the absorbance reached 0.66 ± 0.03 at 734 nm. Twenty microliters of different concentrations of raw garlic and BG extracts (0.25, 0.50, 1.00, 2.00, 4.00, and 8.00 mg/mL) was added to the 96-well plate, and 150 μL of 0.2 mmol/ABTS stock solution was used as a control. After the reaction was performed at room temperature for 10 min, and the absorbance was measured at 517 nm. ABTS clearance rate = $(D0-D)/D0 \times 100\%$, where $D0$ was the sum of absorbance of ABTS working solution and PBS; D was the sum of absorbance of ABTS working solution and sample solution.

Determination of Oxygen Radical Absorption Capacity (ORAC)

With different concentrations of raw garlic, BG extract (0.25, 0.50, 1.00, 2.00, 4.00, and 8.00 mg/mL) and 20 μL Trolox standard (diluted with 75 mmol/L PBS, pH = 7.4) was added to a 96-well plate, incubated at 37°C for 20 min, and then 20 μL of 119 mM 2,2-azo-bis(2-amidino-propa) hydrochloride (ABAP) solution was added. The fluorescence intensity was measured with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurement time interval was 5 min and 35 measurements were continuously performed. The ORAC values were expressed in Trolox equivalents in mmol TE/gDW (Trolox equivalent per gram of dry weight).

Participants

All procedures were approved by the human research ethical committee of Jilin University (Changchun, China) (Approval No. 20160713F). This trial was registered at http://www.chictr.org.cn/searchprojen.aspx, Clinical number: ChiCTR1800017999[1]. All patients agreed with consent form and signed their names. CHF patients were determined according to Framingham or modified Boston criteria for heart failure (Remes et al., 1992). The total scores of CHF were 8. Cardiac function was measured according to the New York Heart Association (NYHA) (Bredy et al., 2018). Grade I – the patients suffered from heart disease and physical activity was not limited. General physical activity would not cause fatigue, palpitations, dyspnea, or angina; Grade II – the patients suffered from heart disease, and physical activity was slightly limited. General physical activity could cause fatigue, heart palpitations, difficulty in breathing, or angina; Grade III – the patients had heart disease, and physical activity was significantly limited. Light physical labor could cause fatigue, heart palpitations, difficulty in breathing, or angina; Grade IV – the patients suffered from heart disease, and physical activity was completely lost. There were the symptoms of heart failure or angina during rest. Any physical activity could make the symptoms worse.

[1]http://www.chictr.org.cn/showprojen.aspx?proj=30330

Inclusion Criteria

At the same time Framingham's heart failure qualitative diagnostic criteria and Boston heart failure quantitative diagnostic criteria; age 35–75 years; CHD was measured by using Doppler echocardiography (GE, Fairfield, CT, United States); left ventricular ejection fraction (LVEF) ≤ 50; heart function grades II–III.

Exclusion Criteria

The participants would be excluded if they had a history of knee surgery within past 3 months, a systemic arthritic condition, and any other muscular, joint, or neurological condition affecting lower limb function. The patients had acute coronary syndrome, severe valvular heart disease, combined shock, methicillin-resistant *Staphylococcus aureus* (MRSA) infections, planned extra-cardiac surgery, combined hepatorenal, and other systemic diseases.

Patients Grouping

From May 1, 2016 to June 30, 2017, a total of 489 CHF patients were screened. CHF patients caused by CHD were selected and evenly assigned into BG (received 20 g garlic daily) and placebo (CG) groups. Primary endpoints were based on the 1-month observation after randomization. The first primary endpoint consisted of mortality, stroke, and myocardial infarction to define the sample size 120. The duration of treatment was 6 months. The following parameters were measured: 6-min walking distance, BNP, EF value, scores of QOL, and blood lipid profiles. QOL was assessed by using the Minnesota Living with Heart Failure Questionnaire (MLHFQ) (Mogle et al., 2017). Routine treatment included oxygen inhalation, angiotensin-converting enzyme inhibitors (ACE-in), angiotensin receptor blockers (ARBS), beta-blockers, digitalis preparations (except digitalis contraindications), intermittent application of diuretics, cardiac resynchronization therapy (CRT), and implantable cardioverter defibrillator (ICD). All the CHF patients received a 30-min walking exercise for 5 days a week on a flat surface at their comfortable speed.

Lipid Profile Analysis

Triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) is associated with CHF risk or progression (Jin et al., 2018). Serum TG was measured by using an immunometric assay (Immulite 2000 Thyroglobulin, Los Angeles, CA, United States). Serum TC, LDL-C, and HDL-C were analyzed by using an automated chemistry analyzer (Olympus, Japan).

Measurement of Circulating Antioxidant Levels

Five milliliters of blood was obtained from each patient. Circulating antioxidant levels were investigated by measuring the levels of malondialdehyde (MDA), and nitric oxide (NO), glutathione peroxidase (GSH-Px), and superoxide dismutase

(SOD) via ELISA kits (Beyotime Institute of Biotechnology, Beijing, China).

Clinical Examination of Therapeutic Results

Cardiac function was measured according to the functional classification system of the NYHA. Significantly effective: heart function was improved by two levels or more; Valid: heart function was improved by one level but less than two levels; Invalid: heart function was improved less than one level; Deterioration: heart function was reduced at one level or above. Total efficiency = (significant + effective)/total number of cases × 100%. Six minutes walking test was used to compare the patient's walk distance before and after garlic consumption between the two groups. LVEF was measured by radionuclide ventriculography with patients in the supine position (Naar et al., 2014).

Blood urea nitrogen (BUN) was measured on an automatic biochemistry analyzer (Beckman Coulter LX20, Beckman, CA, United States) by using a BUN kit (Beckman Coulter, Inc., Brea, CA, United States). Serum was isolated from blood sample via centrifugation. Serum creatinine was measured by a creatinine kit (Biovision, Milpitas, CA, United States). BUN and creatinine were assessed at before and after BG therapy.

Detection of BNP Precursor N-Terminal (Nt-proBNP)

Two milliliters of venous blood was collected in a standard tube containing an anticoagulant from all patients with an empty stomach. Roche Elecsys Nt-proBNP kit (Roche, Indianapolis, IN, United States) was used to measure the level of Nt-proBNP by using electrochemiluminescence immunoassay on Elecsys 1010/2010/modular analytics E170 immunoassay system (Roche Diagnostics GmbH, Mannheim, Germany).

Statistical Analysis

All data were statistically analyzed by using SPSS20.0 statistical software. The measurement data were first tested for normal distribution and homogeneity of variance. If there was a normal distribution and homogeneity of variance, paired *t*-tests were used before and after treatment between the two groups. *T*-test was used for the comparison between the two groups, and those that did not meet the homogeneity of variance. Count data were examined using the χ^2-test. Continuous data were ranked using the rank sum test. $P < 0.05$ was considered statistically significant.

RESULTS

The Main Components of Black Garlic

There are 27 kinds of volatile components in fermented BG (**Figure 1** and **Table 2**). Among them, higher volatile components are 3-vinyl 3,4-dihydro-1,2-dithiane and diallyl compounds, such as disulfide, 2-ethyltetrahydrothiophene, 2-vinyl-1,3-dithiane, *N*,*N*″-dimethyl thiourea, etc. BG has a high content of 2-ethyl tetrahydrothiophene, which gives fragrance.

The Antioxidant Properties of Black Garlic

Clearance Rate of DPPH-Free Radicals of Black Garlic

2,2-Diphenyl-1-picrylhydrazyl is a free radical with a single electron, stable nitrogen center, and is widely used for evaluating the antioxidant properties of plant extracts. When a free radical scavenger is present, the DPPH radical accepts an electron or hydrogen atom to form a stable compound that changes its solution from deep purple to pale yellow, and the degree of discoloration is quantitatively related to the number of electrons. In the present study, the absorbance value was measured with a microplate reader. As shown in **Figure 2A**, BG had DPPH-free radical scavenging ability, and as the concentration increasing, the DPPH scavenging ability increased. This may be due to the increase of polyphenols content in BG processing, and enhanced antioxidant capacity and free radical scavenging capacity.

Reduction Ability of Black Garlic

Reducing ability is a commonly used method for evaluating antioxidant activity. According to the reduction effect of the sample, electrons are scavenged free radicals. As shown in **Figure 2B**, the reducing ability of BG at the same concentration was significantly higher than that of raw garlic. In this experiment, the total phenol content of raw garlic was 0.49 mg GAeq/g (equivalents of gallic acid per gram of the sample), and the total phenol content of BG reached 2.60 mg GAeq/g, which was five times as much as raw garlic. The results suggested that BG had stronger reducing ability than raw garlic.

Clearance Rate of ABTS Radicals of Black Garlic

As shown in **Figure 2C**, ABTS clearance rate of BG was significantly higher than raw garlic. There was a significant difference in increase clearance rate. At 0.05 mg/mL, raw garlic and BG extracts had no scavenging effect on ABTS. With the increase of concentration, the clearance rate of ABTS was increased. Clearance rate of BG was stronger than that of raw garlic. Generally, the ABTS-free radical scavenging capacity of the BG extract was better than that of raw garlic. On the one hand, it might be related to the content of polyphenols.

Clearance Rate of Oxygen-Free Radicals of Black Garlic

The antioxidant principle of ORAC refers to the fact that free radicals can destroy the fluorescent probe and change the fluorescence intensity. The magnitude of its change reflects the degree of free radical damage. Antioxidants can inhibit the change of fluorescence caused by free radicals, and the degree of inhibition can reflect the magnitude of their antioxidant capacity against free radicals. As shown in **Figure 2D**, both raw garlic and BG could scavenge oxygen-free radicals, but BG had stronger scavenging ability than raw garlic. Raw garlic had an oxygen radical absorption capacity of 324.43 μmol TE/gDM. The absorption capacity of BG reached 984.56 μmol TE/gDM, and the difference was significant ($P < 0.05$). This was consistent with the results of DPPH and Fe^{3+} reducing ability, which was due to the

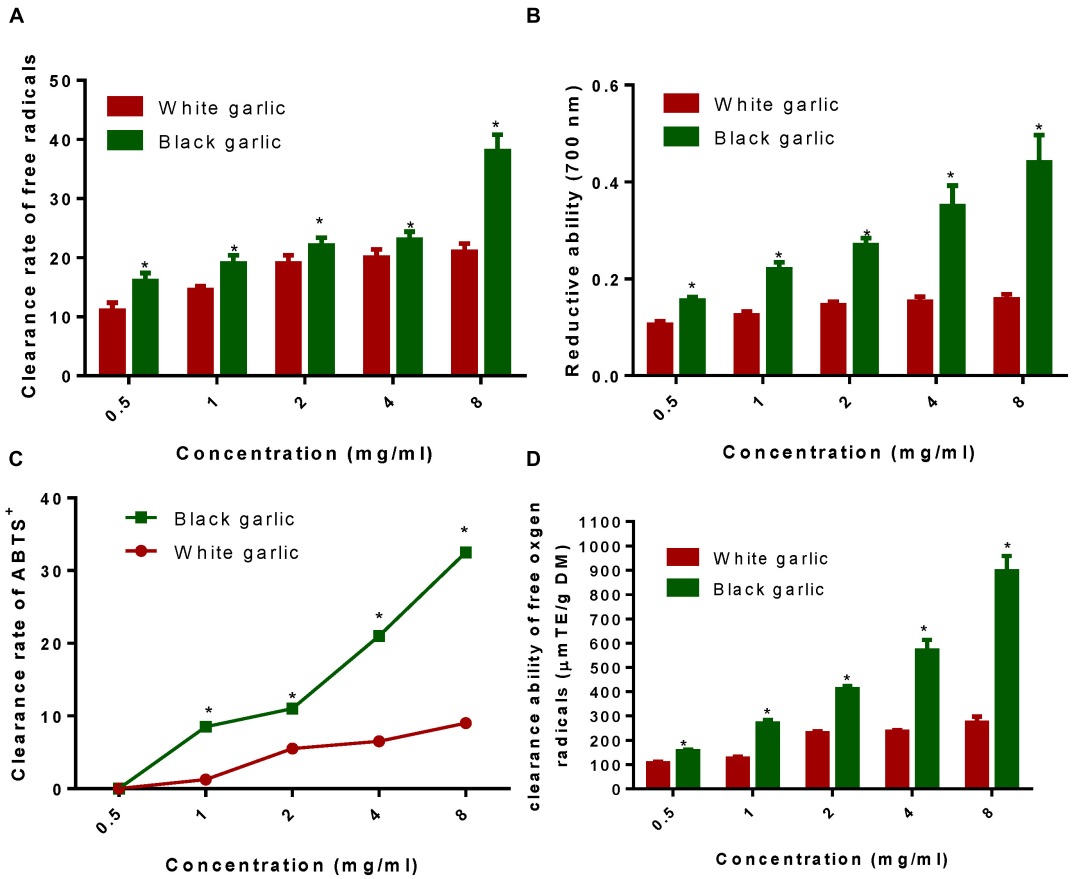

FIGURE 2 | The antioxidant properties of BG. **(A)** Clearance rate of DPPH-free radicals of BG. **(B)** Reduction ability of BG. **(C)** Clearance rate of ABTS radicals of BG. **(D)** Clearance rate of oxygen-free radicals of BG. *Stands for $P < 0.05$ vs. white garlic.

fact that the polyphenol content in BG was significantly higher than that of raw garlic.

Clinical Characteristics

There was no significant statistical differences for clinical characteristics of CHF patients between BG and CG groups, including gender distribution, body mass index (BMI), age, diastolic blood pressure (DBP), and systolic blood pressure (SBP) (**Table 3**, $P > 0.05$). The cases for taking ACE-In, ARBS, beta-blockers, diuretics, and performing CRT and ICD therapies were comparable between two groups ($P > 0.05$, **Table 3**).

Therapeutic Results of Black Garlic

There was no significant difference ($P > 0.05$) in the mean values of BUN and before BG therapy ($P < 0.05$, **Table 4**). After therapy, the values of mean BUN and serum creatinine were significantly reduced in both groups ($P < 0.05$, **Table 4**). Meanwhile, the values of mean BUN and serum creatinine were significantly reduced when compared with placebo groups ($P < 0.05$, **Table 4**).

Comparison of Two Sets of Lee Scores

Before BG treatment, the statistical difference for Lee scores was insignificant between BG and CG groups (**Table 5**, $P > 0.05$). After BG consumption, the statistical differences for Lee scores were significant in both BG and CG groups when compared with before treatments (**Table 5**, $P < 0.05$). BG reduced more Lee scores than CG (**Table 5**, $P < 0.05$).

The QOL Scores

Before BG consumption, the statistical difference for the QOL scores was insignificant between BG and CG groups (**Table 6**, $P > 0.05$). After 6-month BG consumption, the statistical differences for the QOL scores were significant in both BG and CG groups (**Table 6**, $P < 0.05$). BG increased more QOL scores than CG (**Table 6**, $P < 0.05$).

Comparison of 6-Min Walk Distance Between Two Groups

Before BG consumption, the statistical difference for 6-min walk distance was insignificant between BG and CG groups (**Table 7**, $P > 0.05$). After 6-month BG consumption, the statistical differences for 6-min walk distance were significant in both BG and CG groups (**Table 7**, $P < 0.05$). Meanwhile, BG increased more 6-min walk distance than CG (**Table 7**, $P < 0.05$).

TABLE 3 | Clinical characteristics between BG and placebo groups.

Parameters	BG	Placebo	χ^2-and *t*-value	*P*-value
Gender (male/female)	32/28	34/26	0.135	0.714
Age (year)	39.89 ± 13.26	40.23 ± 12.78	−1.303	0.179
SBP (mm Hg)	126.21 ± 11.52	130.54 ± 12.76	−1.684	0.086
DBP (mm Hg)	87.23 ± 7.16	86.53 ± 7.48	−1.296	0.158
BMI	24.91 ± 1.74	24.52 ± 1.48	−1.563	0.098
TC (mmol/L)	5.52 ± 0.64	5.72 ± 0.87	−0.618	0.274
TG (mmol/L)	2.24 ± 0.81	2.32 ± 0.94	−2.158	0.109
LDL-C (mmol/L)	2.01 ± 0.64	2.35 ± 0.83	−1.864	0.187
HDL-C (mmol/L)	1.84 ± 0.46	1.65 ± 0.32	−2.619	0.074
Cr (μmol/L)	85.24 ± 13.58	87.04 ± 14.51	−1.244	0.136
HbA1C (%)	8.42 ± 0.73	8.72 ± 0.86	−0.654	0.246
ACE-In	6	5	3.07	0.38
ARBS	3	7		
Beta-blockers	4	3		
Diuretics	8	4		
CRT	6	9	0.68	0.41
ICD	2	4	0.18	0.68

Chi-square test and t-test were used to compare the significant difference between COG and CG groups. BMI, body mass index; ACE-In, angiotensin-converting enzyme; ARBS, angiotensin receptor blockers; CRT, cardiac resynchronization therapy; ICD, implantable cardioverter defibrillator. All data were presented as mean value ± SD. There were significantly statistical differences between two groups if P < 0.05.

TABLE 4 | The therapeutic results of BG.

		Before treatment	After treatment	*t*-values	*P*-values
Blood urea nitrogen (mg/dL)	BG	19.03 ± 6.85	15.23 ± 5.24	6.41	0.01[a]
	Placebo	18.71 ± 6.34	17.05 ± 5.98	2.16	0.03[a]
	t-values	0.37	3.28		
	P-values	0.51	0.02		
Serum creatinine (mg/dL)	BG	1.41 ± 0.32	1.04 ± 0.27	8.65	0.01[a]
	Placebo	1.36 ± 0.29	1.20 ± 0.19	2.39	0.04[a]
	t-values	0.24	4.31		
	P-values	0.63	0.02		

[a]*Stands for P < 0.05 vs. before treatment.*

TABLE 5 | The comparison of Lee scores between two groups.

	Before treatment	After treatment	*t*-values	*P*-values
BG	4.02 ± 1.81	1.18 ± 0.40	11.38	0.01[a]
Placebo	4.23 ± 1.76	2.19 ± 0.73	9.25	0.01[a]
t-values	0.25	4.12		
P-values	0.68	0.02[b]		

n = 60 for each group. [a]P < 0.05 vs. before treatment and [b]P < 0.05 vs. a placebo group.

Comparison of Nt-proBNP Concentration Between Two Groups

Before BG consumption, the statistical difference for Nt-proBNP concentration was insignificant between BG and CG groups (**Table 8**, $P > 0.05$). After 6-month BG consumption, the statistical differences for Nt-proBNP concentration were significant in both BG and CG groups when compared with before treatments (**Table 8**, $P < 0.05$). Meanwhile, the concentration of Nt-proBNP was reduced by 18.47 ± 2.69% in the BG group when compared with the CG group (**Table 8**, $P < 0.05$).

TABLE 6 | The comparison of QOL between two groups.

	Before treatment	After treatment	*t*-values	*P*-values
BG	43.68 ± 3.38	21.76 ± 4.18	23.40	0.01[a]
Placebo	42.55 ± 3.05	29.39 ± 4.16	14.85	0.01[a]
t-values	0.34	2.19		
P-values	0.78	0.02[b]		

n = 60 for each group. [a]P < 0.05 vs. before treatment and [b]P < 0.05 vs. a placebo group.

TABLE 7 | The comparison of 6-min walk distance between two groups (m).

	Before treatment	After treatment	*t*-values	*P*-values
BG	356.22 ± 41.93	426.16 ± 29.96	8.47	0.01[a]
Placebo	348.32 ± 36.79	372.83 ± 28.16	2.12	0.08
t-values	0.298	2.41		
P-values	0.649	0.02[b]		

n = 60 for each group. [a]P < 0.05 vs. before treatment and [b]P < 0.05 vs. a placebo group.

TABLE 8 | The comparison of Nt-proBNP between two groups (pg/mL).

	Before treatment	After treatment	*t*-values	*P*-values
BG	1895.12 ± 249.34	1291.64 ± 207.04	5.94	0.01[a]
Placebo	1861.63 ± 257.13	1536.54 ± 216.32	2.65	0.04[a]
t-values	0.10	1.95		
P-values	0.87	0.03[b]		

n = 60 for each group. [a]P < 0.05 vs. before treatment and [b]P < 0.05 vs. a placebo group.

Comparison of LVEF Volume Between Two Groups

Before BG consumption, the statistical difference for LVEF volume was insignificant between BG and CG groups (**Table 9**, $P > 0.05$). After 6-month BG consumption, the statistical differences for LVEF volume were significant in both BG and CG groups when compared with before treatments (**Table 9**, $P < 0.05$). Meanwhile, the values of LVEF volume were improved by 14.29 ± 4.38% in the BG group when compared with the CG group (**Table 9**, $P < 0.05$).

Adverse Reaction

No obvious adverse reactions occurred during the whole experiment, suggesting that the drug is safe in treatment.

Circulating Antioxidant Levels

Circulating antioxidant levels were investigated between two groups. The statistical difference for the biomarkers was

TABLE 9 | The comparison of LVEF between two groups.

	Before treatment	After treatment	*t*-values	*P*-values
BG	29.36 ± 9.34	36.82 ± 10.43	5.03	0.01[a]
Placebo	28.24 ± 8.15	32.73 ± 10.21	3.10	0.01[a]
t-values	0.98	2.14		
P-values	0.36	0.03[b]		

n = 60 for each group. [a]P < 0.05 vs. before treatment and [b]P < 0.05 vs. a placebo group. LVEF, left-ventricular ejection fraction.

insignificant between two groups before therapy (**Figure 3**, $P > 0.05$). After therapy, circulating levels of NO (**Figure 3A**) and MDA (**Figure 3B**) were lower in BG than in CG group while the circulating levels of SOD (**Figure 3C**) and GSH-PX (**Figure 3D**) were higher in BG than in CG group ($P < 0.05$).

DISCUSSION

Recent studies have shown that BNP has a high reliability in the diagnosis of CHF and provides an important method for heart failure diagnosis (Booth et al., 2014). BNP has been listed as one of the diagnostic criteria for heart failure by the American College of Cardiology (ACC) and the European Society of Cardiology (ESC) (Fonseca et al., 2004; Emdin et al., 2007). According to an earlier report, the measuring limits of BNP, proBNP, and NT-proBNP were 0.4, 3, and 10 pg/mL, respectively (Seferian et al., 2007). The study showed that BNP concentrations were elevated, and cardiac function indicators were highly expressed. There was a significant correlation between cardiac dysfunction and NT-proBNP concentration, suggesting that BNP concentration measurement can be used as an effective method to screen patients with CHF, and it will be effective in the early diagnosis of CHF.

The concentration of plasma BNP was positively correlated with NYHA classification. The worse of heart function, the higher the severity of heart failure and the higher concentration of plasma BNP. In the present experiment, the most patients with cardiac function III and IV and Nt-proBNP were significantly decreased after receiving BG (**Table 8**). This study showed that the plasma BNP concentration value could be used as a reliable indicator to judge the severity of heart failure, and it was easy to operate.

The determination of plasma BNP concentration has been considered as a powerful indicator in prognostic evaluation of CHF risk. High-level plasma BNP, especially before treatment, showed a poor prognosis. Comparatively, the BNP concentration decreased (average 215 pg/mL) in patients who did not have a cardiac endpoint (Cheng et al., 2001). In addition, BNP concentration was reported to be an independent risk

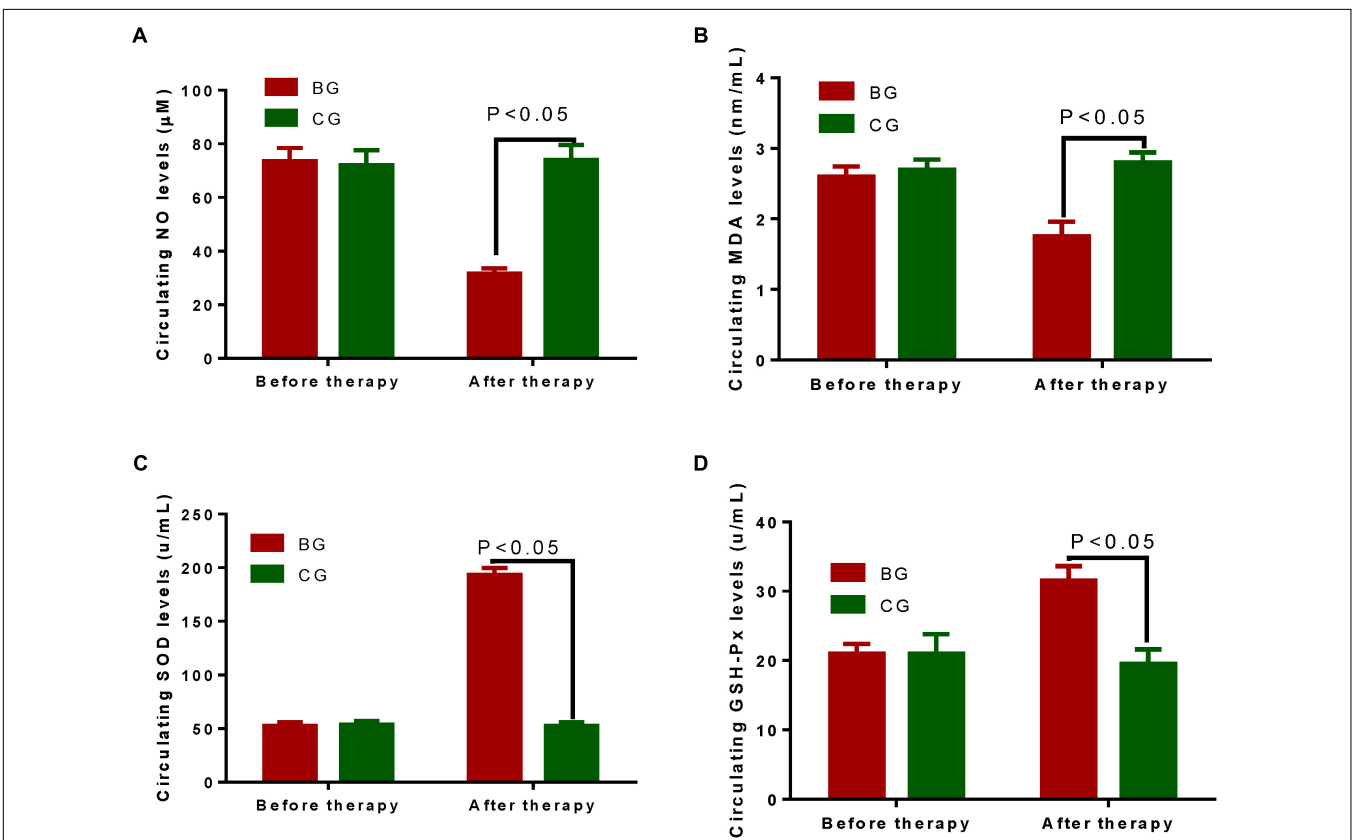

FIGURE 3 | Circulating antioxidant levels between two groups. **(A)** Circulating levels of NO between two groups. **(B)** Circulating levels of MDA between two groups. **(C)** Circulating levels of SOD between two groups. **(D)** Circulating levels of GSH-PX between two groups. The statistical difference was significant if $P < 0.05$.

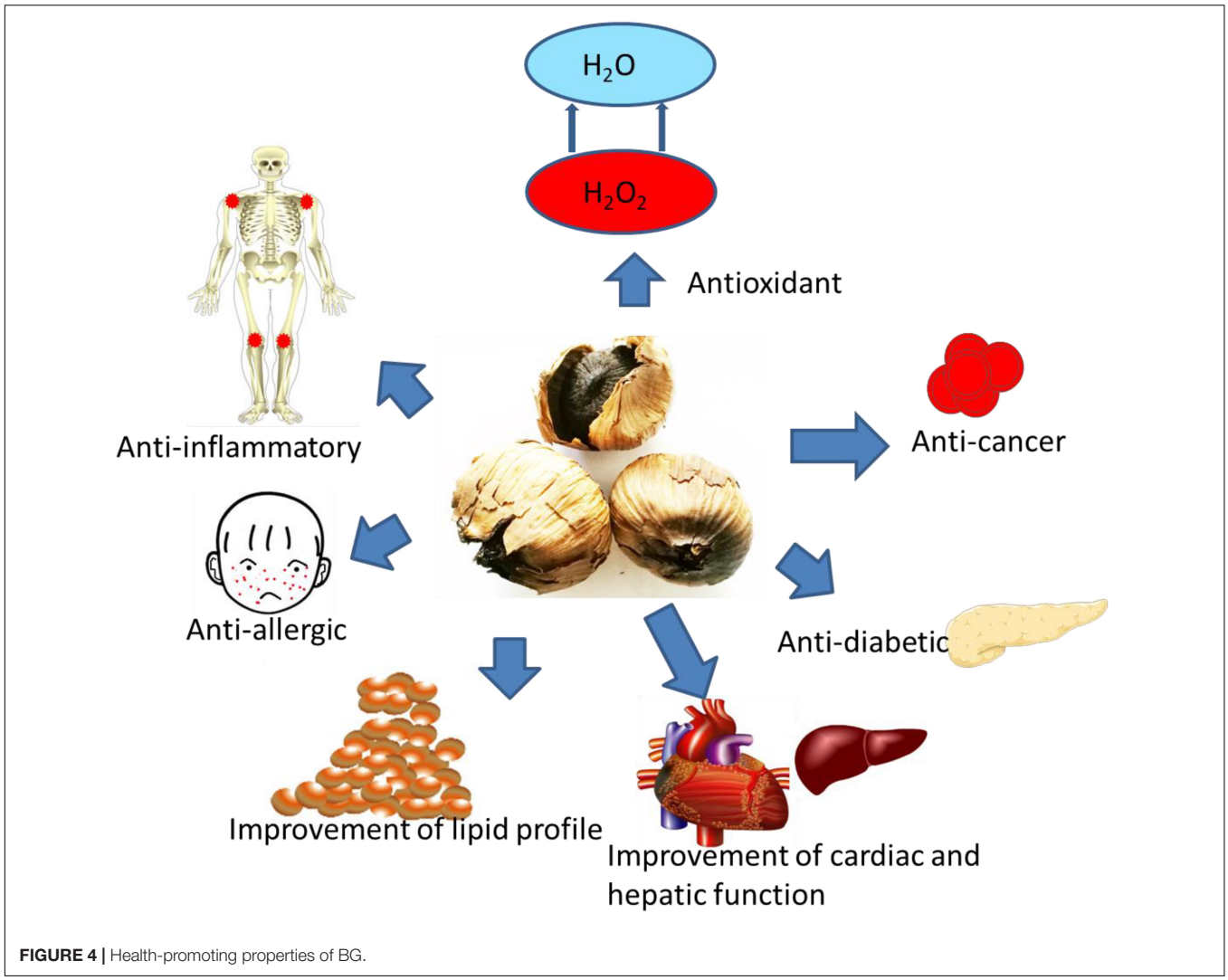

FIGURE 4 | Health-promoting properties of BG.

predictor of death in CHF patients (Richards et al., 2001). The heart failure patients with BNP >700 pg/mL and the 120-day mortality rate and readmission rate were 80%. In contrast, the patients with BNP values < 350 pg/mL had mortality and the readmission rate was <10% (Logeart et al., 2004). The level of BNP not only reflects the severity of heart failure, but also is an effective prognostic indicator of heart failure. The results of this study showed that the Nt-proBNP levels were significantly higher in the patients with heart failure. BNP levels were powerful indicators for the diagnosis of heart failure. The results also showed that BG consumption resulted in significant reduction in the level of Nt-proBNP compared with that before the treatment and CG group ($P < 0.05$), suggesting that BG consumption can reduce plasma N-terminal BNP levels in heart failure patients, antagonize neuroendocrine activation, and improve cardiac function. Meanwhile, therapeutic results of BG were approved by reducing the values of mean BUN and serum creatinine when compared with placebo groups ($P < 0.05$, **Table 4**).

Hambrecht study found that rehabilitation exercise on CHF lowered the resting heart rate during submaximal exercise, and increased maximal oxygen uptake, exercise tolerance and anaerobic threshold, physical activity, and QOL (Hambrecht et al., 2000). CHF can cause skeletal muscle mechanoreceptor activation leading to increased ventilation and chest tightness sensation, as well as fatigue and sympathetic activation, and at least one-fourth of CHF patients are caused by skeletal muscle abnormalities (Rogers, 2001). Thus, all patients received 30-min walking exercise daily.

The results of this study also showed that the levels of LVEF in both BG and CG groups were improved when compared with before treatment ($P < 0.05$). The scores of the diagnosis of heart failure were decreased ($P < 0.05$). Medicine combined with BG effectively improved heart failure patients with lower LVEF ($P < 0.05$).

In this study, the 6-min walk test was used as a performance evaluation indicator for CHF patients. The 6-min walk test was used as an objective indicator to evaluate the activity, physical fitness, and drug intervention effects of patients with heart failure.

The 6-min walking experiment has been studied in more depth and widely used (Brehm et al., 2017; Fakhro et al., 2017; Omar and Guglin, 2017).

The results of this study showed that the QOL scores were improved after walking exercise in both groups compared with before the excise ($P < 0.05$); and after BG treatment, the improvement was better in the BG group than that in CG group ($P < 0.05$); suggesting that BG will be better than CG for improving the living ability of patients with heart failure.

After therapy, circulating levels of NO (**Figure 3A**) and MDA (**Figure 3B**) were lower in BG than in CG group while the circulating levels of SOD (**Figure 3C**) and GSH-PX (**Figure 3D**) were higher in BG than in CG group ($P < 0.05$). The results suggest that BG improves the antioxidant properties of CHD patients. Antioxidant levels had positive relation with QOL and LVEF values, and negative relation with Nt-proBNP values. The improvement of QOL has been demonstrated in the patients who have received antioxidant therapy (Shah et al., 2010). Nt-proBNP is an important biomarker for reflecting total oxidized stress in the patients with acute myocardial infarction (Kasap et al., 2007). On the other hand, antioxidant therapy can improve LVEF values (de Lorgeril et al., 2001). Thus, BG may improve the symptoms of CHD patients by increasing antioxidant activities. Black garlic has many health-promoting properties (**Figure 4**). Considering the short time of the present study, the small sample size and other influencing factors, further work is highly demanded to confirm the present result.

CONCLUSION

Black garlic improved blood circulation in the treatment of CHD patients. BG combined with conventional treatment improved the LVEF and heart function, and reduced the heart failure diagnosis. BG treatment improved the QOL, the patient's actual living ability, and QOL. BG consumption increased the distance of 6-min walking test in CHD patients and showed obvious advantages in the recovery of physical function. BG combined with medicine treatment reduced plasma N-terminal pro-body BNP levels and antagonized neuroendocrine activation in CHD patients.

AUTHOR CONTRIBUTIONS

JL and CW designed the study. JL, GZ, XC, and CW performed the experiments. GZ analyzed the data. CW wrote the manuscript. JL and XC revised and corrected the manuscript. All authors read and approved the final manuscript.

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frontiers in Physiology

REVIEW
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Diabetic Cardiomyopathy: Current and Future Therapies. Beyond Glycemic Control

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Diabetes mellitus and the associated complications represent a global burden on human health and economics. Cardiovascular diseases are the leading cause of death in diabetic patients, who have a 2–5 times higher risk of developing heart failure than age-matched non-diabetic patients, independent of other comorbidities. Diabetic cardiomyopathy is defined as the presence of abnormal cardiac structure and performance in the absence of other cardiac risk factors, such coronary artery disease, hypertension, and significant valvular disease. Hyperglycemia, hyperinsulinemia, and insulin resistance mediate the pathological remodeling of the heart, characterized by left ventricle concentric hypertrophy and perivascular and interstitial fibrosis leading to diastolic dysfunction. A change in the metabolic status, impaired calcium homeostasis and energy production, increased inflammation and oxidative stress, as well as an accumulation of advanced glycation end products are among the mechanisms implicated in the pathogenesis of diabetic cardiomyopathy. Despite a growing interest in the pathophysiology of diabetic cardiomyopathy, there are no specific guidelines for diagnosing patients or structuring a treatment strategy in clinical practice. Anti-hyperglycemic drugs are crucial in the management of diabetes by effectively reducing microvascular complications, preventing renal failure, retinopathy, and nerve damage. Interestingly, several drugs currently in use can improve cardiac health beyond their ability to control glycemia. GLP-1 receptor agonists and sodium-glucose co-transporter 2 inhibitors have been shown to have a beneficial effect on the cardiovascular system through a direct effect on myocardium, beyond their ability to lower blood glucose levels. In recent years, great improvements have been made toward the possibility of modulating the expression of specific cardiac genes or non-coding RNAs *in vivo* for therapeutic purpose, opening up the possibility to regulate the expression of key players in the development/progression of diabetic cardiomyopathy. This review summarizes the pathogenesis of diabetic cardiomyopathy, with particular focus on structural and molecular abnormalities occurring during its progression, as well as both current and potential future therapies.

Keywords: diabetic cardiomyopathy, anti-hyperglycemic drug, SGLT-2 inhibitors, incretin-based therapy, heart failure, pathogenesis, treatment

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INTRODUCTION

Diabetes mellitus is a major public health problem and represents a huge health concern for the global population. In 2010, 285 million people were affected, and this number is estimated to increase to almost 700 million people by 2040 (Shaw et al., 2010). Type 2 diabetes (T2DM) is a chronic metabolic disorder characterized by hyperglycemia and insulin resistance, also representing one of the major risks for developing heart failure (HF) (Schocken et al., 2008). In 1974, the Framingham study showed that diabetic patients have a 2–5 times higher risk of developing HF than age-matched, non-diabetic patients, and independent of other comorbidities. This suggests a specific intrinsic mechanism that drives the pathological cardiac remodeling in this population (Kannel et al., 1974). The United Kingdom Prospective Diabetes Study (Group) indicated an association between the risk of cardiovascular complications and glycemia, observing that for every 1% decrease in HbA1c there was an 18% reduction in myocardial infarction (MI) events (Group, U. P. D. S. U., 1998).

Heart failure is a multifactorial disease in diabetic patients. Both type 1 diabetes mellitus (T1DM) and T2DM are associated with an increase in macrovascular and microvascular dysfunction, resulting in ischemic events and altered vascular permeability (Krentz et al., 2007; Calcutt et al., 2009). Atherosclerosis and hypertension are often present in diabetic patients and contribute to coronary artery disease (CAD) and peripheral vascular disease, both of which affect the heart. However, besides these well-known pathological triggers, diabetes contributes to the development of HF through a more disease-specific variety of mechanisms, which are mostly driven by hyperglycemia, hyperinsulinemia, metabolic changes, and oxidative stress (Davidoff et al., 2004).

The aim of this review is to summarize molecular, structural, and functional changes occurring during the pathogenesis of diabetic cardiomyopathy. We will discuss management strategies, with particular focus on the therapeutic effect of glucose lowering drugs on HF development/progression, merging basic research and clinical observations. Emerging potential new targets and future prospects to improve the cardiovascular health of diabetic patients will be discussed as well.

THE PATHOGENESIS OF DIABETIC CARDIOMYOPATHY

Diabetic cardiomyopathy is defined as the existence of abnormal cardiac structure and performance in the absence of other cardiac risk factors, such CAD, hypertension, and significant valvular disease (Jia et al., 2018). It was first described more than four decades ago (Rubler et al., 1972), with hyperglycemia and impaired cardiac insulin signaling pathway having pivotal roles in its progression/onset (Jia et al., 2018). Clinically, the diabetic heart is characterized by diastolic dysfunction with preserved ejection fraction. These alterations are caused by the pathological remodeling of the heart. Increases in interstitial and perivascular fibrosis, as well as left ventricle (LV) hypertrophy are structural hallmarks associated with the diabetic heart (Tate et al., 2017). However, the underlying pathogenic mechanisms remain unclear; it includes but is not limited to abnormal extracellular matrix (Perge et al., 2017) deposition, an increase in oxidative stress and inflammation, in conjunction with mitochondrial dysfunction, and changes in the metabolic profile and energy production (Isfort et al., 2014; De Rosa et al., 2018; **Figure 1**).

An increase in fibrosis is the result of an increase in collagen deposition coupled with abnormalities in ECM protein structure and turnover (Tate et al., 2017). In the diabetic heart, upregulation in the expression of profibrotic factors, such as transforming growth factor beta 1 and connective tissue growth factor, can cause abnormal ECM protein deposition (Mizushige et al., 2000; Way et al., 2002; D'Souza et al., 2011). At the same time, a decrease in the activity of the ECM-degrading enzyme metalloproteinase (Westermann et al., 2007) can lead to ECM accumulation. Hyperglycemia induces advanced glycation end product (AGEs) formation, as a result of a non-enzymatic binding between amine residues of proteins or lipids and sugars (Kilhovd et al., 1999; Goh and Cooper, 2008; Yamagishi et al., 2012). AGEs damaging potential is correlated with their ability to cross-link collagen molecules, which increases their resistance to proteolysis and slows down their turnover (Aronson, 2003). AGEs may also bind to receptor for advanced glycation end products on the cardiac cell membranes, further promoting both pro-fibrotic and pro-inflammatory signaling, and increasing the expression of oxidative stress mediators (Candido et al., 2003; Haidara et al., 2006; Yamagishi et al., 2012).

Left ventricle hypertrophy is the main morphological change observed in the diabetic heart. Echocardiograms of diabetic patient's hearts have shown an increase in LV posterior and septal wall thickness (Eguchi et al., 2008). LV hypertrophy can occur as an adaptive response to elevated hemodynamic stress (Ritchie et al., 2009). However, this morphological change can also occur independent of pressure-overload in diabetic patient (Galderisi et al., 1991; Eguchi et al., 2008). LV hypertrophy develops as a result of myocyte hypertrophy, an increase in interstitial and perivascular fibrosis, and thickening of the myocardial capillary basement membrane (Voulgari et al., 2010; Velic et al., 2013).

Metabolic dysfunction, hyperinsulinemia, oxidative stress, and inflammation are among the prevalent causes of this increase in LV mass seen in diabetic patients (Huynh et al., 2014). High glucose levels have been found to induce an increase in cardiomyocyte size *in vitro* (Feng et al., 2008). The diabetic heart is characterized by an upregulation in hypertrophic gene expression, such as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and B-myosin heavy chain (Candido et al., 2003; Chang et al., 2006; Connelly et al., 2007; Ritchie et al., 2007; Huynh et al., 2010). Hyperglycemia activates the systemic and intracardiac renin–angiotensin–aldosterone system pathway resulting in an increase of angiotensin II (Ang II) levels (Frustaci et al., 2000). Ang II stimulates proliferation of cardiac fibroblasts and cardiomyocyte hypertrophy (Kumar et al., 2012). High levels of plasma aldosterone and overexpression of the mineralocorticoid receptor, along with increased Ang II activity, can exacerbate insulin resistance, hyperlipidemia, and hypertension (Baudrand et al., 2016).

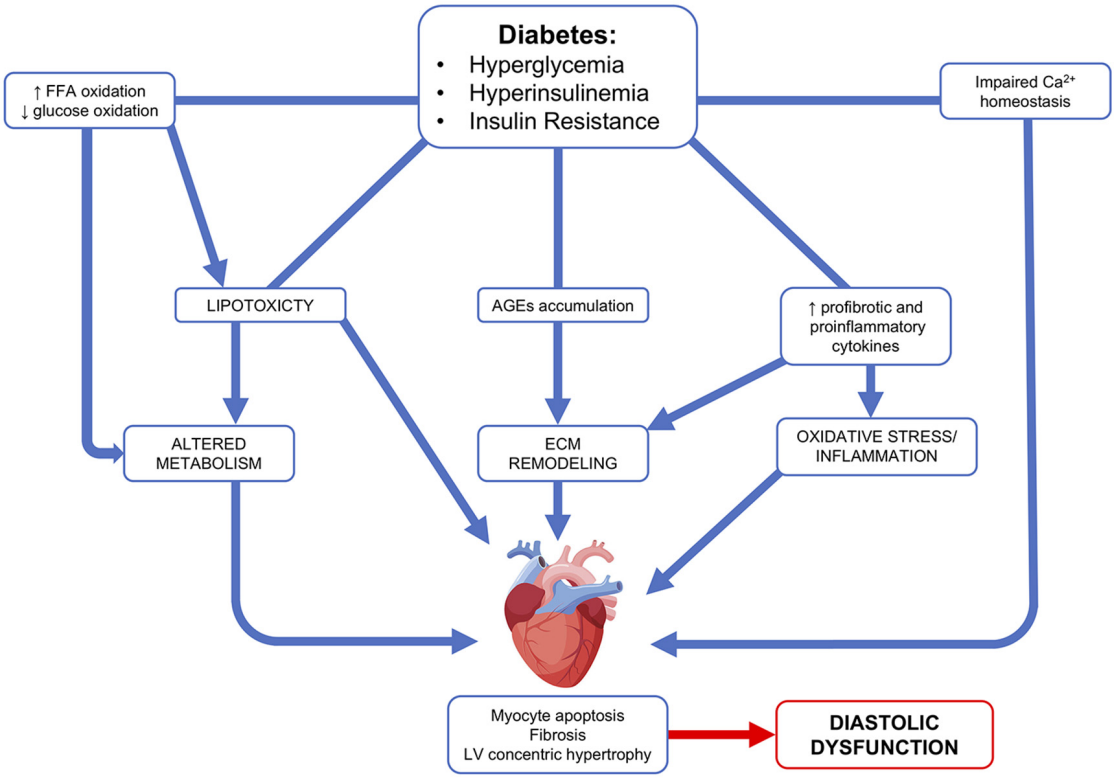

FIGURE 1 | Diabetic cardiomyopathy pathophysiological mechanisms. Hyperglycemia, hyperinsulinemia, and insulin resistance lead to an increase in free fatty acid (FFA) oxidation, profibrotic and proinflammatory cytokines, as well as an accumulation of advanced glycation end products (AGEs). These abnormalities lead to altered metabolism, extracellular remodeling, oxidative stress, and inflammation. Ultimately, this leads to cardiac effects, such as myocyte apoptosis, fibrosis, and LV concentric hypertrophy.

Under normal physiological conditions, the adult heart can use a variety of substrates to produce ATP, a phenomenon called "metabolic substrate flexibility". Free fatty acids (FFAs) are the preferred energy substrate of the adult heart, although other substrates, such as glucose, lactate, ketone bodies, and select amino acids can be used (Jia et al., 2016). Hyperglycemia and insulin resistance lead to a complete loss of this flexibility. A decrease in glucose transporter type 4 recruitment to the sarcolemma reduces the ability to use glucose as an energy source. At the same time, an increase in FFAs released from adipose tissue and FFAs transporter translocation to the sarcolemma leads to an internalization of this substrate in the cardiomyocytes (Harmancey et al., 2012). The loss of metabolic flexibility and the increase in fatty acid oxidation results in a loss of efficiency between substrate use and ATP production in the diabetic heart (Levelt et al., 2018). The energy source switch is accompanied by impaired oxidative phosphorylation and boosted mitochondrial ROS generation. This increase in mitochondrial uncoupling leads to increased mitochondrial O_2 consumption, but this is not accompanied by a proportional increase in ATP synthesis, leading to a decrease in cardiac energy efficiency (Bugger and Abel, 2010; Rider et al., 2013). Moreover, the inability to switch to glucose oxidation makes the heart susceptible to damage and dysfunction under hypoxic conditions, such as in myocardial ischemia (Stanley et al., 1997).

An excessive accumulation of FFAs is detrimental to cardiomyocytes, as they are not equipped to store lipids. This highlights the concept of lipotoxicity as a mechanism for the development of diabetic cardiomyopathy through a decrease in myocyte physiological autophagy and an increase in apoptosis (Mandavia et al., 2013; Levelt et al., 2018).

Besides lipotoxicity, oxidative stress and inflammation are also mechanisms that trigger programmed cell death. An increase in the number of apoptotic cardiomyocytes was found in biopsies from diabetic patients in comparison to non-diabetic patients (Kuethe et al., 2007). In myocardial tissue of diabetic patients, metabolic and oxidative stress cause an increase in sensitivity to Ca^{2+} of mitochondrial permeability transition pore, that result in cardiomyocytes autophagy and cardiac necrosis (Anderson et al., 2011).

Maladaptive proinflammatory response furthers the progression of diabetic cardiomyopathy. Diabetes causes immune cell migration in the myocardium and an increase in macrophage pro-inflammatory M1 polarization, whereas the M2 anti-inflammatory phenotype is decreased (Jia et al., 2015). Upregulation of several proinflammatory cytokines, such as tumor necrosis factor (TNFα), interleukins 6 and 8, and

monocyte chemotactic protein 1 is characteristic of the diabetic heart. These cytokines affect several cardiac cell populations, including cardiomyocytes, endothelial cells, fibroblasts, and smooth muscle cells, which all contribute to pathological remodeling and oxidative stress (Jia et al., 2018).

Proteotoxic stress, caused by accumulation of misfolded proteins and/or proteasome inhibition, has recently been discovered as an additional pathologic trigger for the diabetic heart. The cardiac ubiquitin proteasome system (Kruk-Bachonko et al., 2017) is responsible for the maintenance of protein homeostasis by degrading the misfolded or oxidized proteins (Gilca et al., 2017). UPS dysfunction occurs early on in the onset of diabetic cardiomyopathy and promotes cardiac maladaptive remodeling, whereas increasing UPS activity through PA28α overexpression has been shown to reduce cardiac dysfunction in a STZ-induced diabetes model (Li et al., 2017).

In addition, calcium handling machinery is directly compromised, as has been found in several animal models of both T1DM and T2DM. Hyperglycemia correlates with enhanced spontaneous calcium release from the sarcoplasmic reticulum, reduced cytoplasmic Ca^{2+} clearance, decreased SR Ca^{2+} load, and prolongation of action potential duration (Belke et al., 2004; Sorrentino et al., 2017). Some of the molecular changes responsible for the dysfunctional calcium handling have been studied in animal models and include: lower activity levels of the sarco/endoplasmatic reticulum Ca^{2+} ATPase 2 (SERCA2a) (Belke et al., 2004) and sodium (Na^{+})–Ca^{2+} exchanger (NCX) (Chattou et al., 1999), impaired ryanodine receptor (RyR2) function (Yaras et al., 2005), and reduced phospholamban phosphorylation (Gando et al., 1997). All of these abnormalities contribute to the defective excitation–contraction coupling associates with diabetes (Lebeche et al., 2008).

DIABETIC CARDIOMYOPATHY: CLINICAL MANIFESTATION AND DIAGNOSIS

Cardiac remodeling occurs in several different phases during the progression of diabetic cardiomyopathy, which is often asymptomatic during the early stages. The pathogenesis starts at a subcellular level, as described above, and the clinical manifestation of this dysfunctional remodeling is hypertrophy. Concentric LV hypertrophy is a strong predictor of adverse cardiovascular outcomes (Bluemke et al., 2008). The correlation between diabetes and hypertrophy was once reported as a result of other secondary comorbidities, such as aging, obesity, and hypertension (Kuperstein et al., 2001). However, several studies have shown a direct correlation between T2DM and an increase in LV mass, independent of hypertension and body mass (Eguchi et al., 2008). The next pathophysiological change is the development of interstitial and perivascular fibrosis, which have been identified as a more advanced stage in the disease progression. Hypertrophy and fibrosis cause impaired relaxation and passive filling of the LV and LV diastolic stiffness. As previously mentioned, diastolic dysfunction represents a major functional abnormality in diabetic patients, which can be asymptomatic during the earlier stages. Systolic dysfunction is less frequent and develops only in a small percentage of patients in the later stages of diabetic cardiomyopathy (Palomer et al., 2018). Furthermore, diabetes-associated fibrosis, found in both T1DM and T2DM, may contribute to the development of atrial fibrillation and arrhythmic events (Russo and Frangogiannis, 2016).

Little is known about the difference in the pathogenesis of diabetic cardiomyopathy in T1DM vs. T2DM. Both types of diabetes affect cardiovascular health. A common denominator seems to be the development of diastolic dysfunction. However, clinical presentation of HF is relatively rare in T1DM in comparison to T2DM, which may be due to patients being younger and being treated with insulin (Miki et al., 2013). There are fewer studies correlating T1DM with hypertrophy and an increase in LV mass compared to T2DM. No studies have found myocardial steatosis in T1DM (Levelt et al., 2018), opposed to what has been found in the hearts of T2DM patients, where steatosis precedes the diastolic dysfunction (McGavock et al., 2007). It should be noted that the underlying mechanism of diabetic cardiomyopathy may be different between the two distinct types of diabetes. Both T1DM and T2DM are characterized by hyperglycemia and dyslipidemia, but only T2DM also have hyperinsulinemia. This could explain the difference in cardiac morphology and clinical features found in patients affected by T1DM vs. T2DM, as well as differences between animal models (Holscher et al., 2016).

Currently, there are no specific morphological changes, biochemical markers, or clinical manifestations needed to secure a diabetic cardiomyopathy diagnosis. This pathology is often asymptomatic throughout the early stages and usually overlaps with other complications in diabetic patients, making a definitive diagnosis challenging.

In the past 20 years, incredible improvements have been made in non-invasive imaging technologies, such as echocardiography and magnetic resonance imaging (MRI), which provide detailed information about cardiac morphology and functions (Lee and Kim, 2017). Both transmitral Doppler and Tissue Doppler imaging are used to quantify the functional myocardial abnormalities. The ratio between early passive transmitral inflow velocity (E) and velocity of the medial mitral annulus (e') is a substitute for invasively measured left ventricular filling pressure and a reliable prognostic marker for diabetic patients (Levelt et al., 2018). Abnormalities in E/e' correlate with the development of HF and increased mortality, independent of other risk factors, such as hypertension and CAD (From et al., 2010).

Magnetic resonance imaging is capable of detecting abnormalities in cardiac morphology more accurately than echocardiography. This technique allows us to acquire precious information on myocardial fibrosis, steatosis, LV mass, and diastolic function. Positron emission tomography has been used to assess myocardial metabolic abnormalities. These new imaging techniques could be incredibly helpful for diagnosing diabetic cardiomyopathy at very early stages, but they are still only used for research purposes due to their cost, time demand, and level of expertise required to interpret results (Palomer et al., 2018).

CURRENT THERAPIES: NOVEL GLUCOSE-LOWERING DRUGS

Hyperglycemia and chronic sustained hyperinsulinemia cause microvascular complications leading to renal failure, retinopathy, and nerve damage. Thus, lowering blood glucose levels is fundamental in the treatment regimen for diabetes. However, several observational studies fail to demonstrate a reduction in HF hospitalizations in diabetic patients treated with anti-hyperglycemic therapy. Moreover, in 2007 a meta-analysis showed a potentially increased risk of MI with the glucose lowering drug rosiglitazone, highlighting the necessity of thoroughly assessing the safety of this drug on the cardiovascular system (Nissen and Wolski, 2007). For this reason, the Federal Drug Administration (Hussain et al., 2018) and European Medicine Agency now require cardiovascular outcome trials for newly developed anti-hyperglycemic drugs in order to gain approval. This new regulation has resulted in a high number of cardiovascular outcome trials and increased the availability of important information on the effect of these drugs on cardiovascular health. Interestingly, several drugs currently in use can improve cardiac health beyond their ability to control glycemia (von Lewinski et al., 2017). However, there is not much data available regarding the mechanisms by which these drugs exert their pleiotropic effects other than these clinical/epidemiological studies.

GLP-1 Receptor Agonists

Glucagon-like peptide 1 (GLP-1) is a gut-derived peptide hormone primarily secreted after food intake. This so-called incretin has the ability to decrease glycemia by increasing the release of insulin and repressing glucagon expression in a glucose-dependent manner (Mojsov et al., 1986).

Glucagon-like peptide 1 receptor is a G-protein coupled receptor that catalyzes the conversion of ATP in cAMP upon activation. Increased cytosolic cAMP in β-pancreatic cells leads to insulin secretion (Holst, 2007). Besides this, activation of the GLP-1 receptor in different tissues leads to a broad spectrum of effects, including deceleration of gastric emptying, suppression of appetite with consequent weight loss, reduction of circulating lipoprotein, and a decrease in blood pressure. However, endogenous secreted GLP-1 [GLP-1 (7–36)] has a very short half-life, which is approximately 2–3 min in the circulation. This active isoform is rapidly degraded primarily by dipeptidyl peptidase-4 (DPP-4) to GLP-1 (9–36), a receptor antagonist. Thus, several synthetic GLP-1 receptor agonists (GLP-1RA$_s$) have been developed to provide prolonged *in vivo* action and subsequently have beneficial effects for T2DM patient (Meier, 2012). These drugs are able to increase insulin release only in the context of hyperglycemia. GLP-1RA$_s$ have, therefore, a very low risk of inducing severe hypoglycemia, which is detrimental to the health of diabetic patients subjected to glucose-lowering treatment because it has been associated with an increase in cardiovascular events and mortality in this population (Zoungas et al., 2010).

Thus, GLP-1RA$_s$ potential cardioprotective effects are derived from their ability to attenuate established cardiovascular risk factors, such as hyperglycemia, obesity, high blood pressure, and dysfunctional lipid profile. However, a more direct effect of this drug on the myocardium cannot be excluded and represents an area of growing interest. Several studies have shown the presence of GLP-1 receptor in atrial tissue of rodents and non-human primates (Wohlfart et al., 2013; Pyke et al., 2014; Richards et al., 2014). Wallner et al. (2015) reported GLP-1 receptor expression in both human atrial and ventricular tissue, although exenatide, a GLP-1RA$_s$, exerts its inotropic effect only on atrial myocardium. Several studies have shown a GLP-1R-dependent activation of Epac-2, which is then able to translocate to the membrane and increase ANP secretion (Kim et al., 2013) and troponin I phosphorylation, with a consequent increase in myocyte contractility (Cazorla et al., 2009). Treatment with GLP-1RA$_s$ or infusion of exogenous GLP-1 has been shown to decrease infarct size and improve cardiac function in several animal models of ischemic heart disease (Bose et al., 2005; Timmers et al., 2009; Liu et al., 2010; DeNicola et al., 2014). Interestingly, a small pilot study involving diabetic and non-diabetic patients found that 72 h of intravenous GLP-1 infusion in patients undergoing percutaneous revascularization after MI improved cardiac function (Nikolaidis et al., 2004). GLP-1RA$_s$ have been found to be beneficial for the endothelium as well: treatment with exenatide resulted in reduction of glucose-induced ROS generation and apoptosis in endothelial cells of diabetic rats, and stimulates proliferation and NO synthase activity in human endothelia cells (Ding and Zhang, 2012). Thus, the modulation of GLP-1 signaling has exiting potential as a treatment option for diabetic patients, which goes far beyond its ability to reduce hyperglycemia.

The cardiovascular safety of GLP-1RA$_s$ has been evaluated in several randomized clinical trials. All recent outcome trials have proven non-inferiority as requested by the authorities. However, cardiovascular outcome was heterogeneous between the trials with some neutral ones on the one side and others even proving superiority (**Table 1**).

ELIXA trail was the first study to evaluate the CV safety of lixisenatide in 6,068 diabetic patients who had recently been hospitalized for acute coronary syndrome. There were no significant differences in cardiovascular events or hospitalizations after 25 months of follow-up between the lixisenatide and placebo group. Thus, this study demonstrated that the use of this drug is safe in patients with T2DM and recent acute coronary syndrome (Pfeffer et al., 2015). The cardiovascular safety of liraglutide was evaluated in the LEADER trial, in which 9,340 diabetic patients at high risk for cardiovascular events (having established cardiovascular disease, either CAD or chronic HF, and/or cerebrovascular disease, peripheral vascular disease, and chronic kidney disease) were enrolled. This study showed not only the safety but also the beneficial cardiovascular effect of this drug. Liraglutide was associated with a significant reduction in the primary composite endpoint, which includes CV mortality, non-fatal MI, non-fatal stroke as well as in all-cause mortality. Hospitalizations for HF were not different between the liraglutide and the placebo group (Marso et al., 2016b). Interestingly,

TABLE 1 | Principle characteristics of clinical trials evaluating effect of diabetes treatments on heart failure/cardiovascular outcomes (2010–2019).

Drug class	Name of drug	NCT identifier	Study name	Results	Clinical Trial Phase (total # of patients)	Trial duration
GLP-1 receptor agonists	Lixisenatide vs. placebo	NCT01147250	ELIXA	CV safety	Phase III (6,068)	25 months
	Exenatide vs. placebo	NCT01144338	EXSCEL	CV safety ↓All-cause mortality	Phase III (14,782)	38 months
	Liraglutide vs. placebo	NCT01179048	LEADER	↓3-MACE ↓All-cause mortality	Phase III (9,340)	45 months
	Semaglutide vs. placebo	NCT01720446	SUSTAIN 6	↓3-MACE	Phase III (3,297)	25 months
DPP-4 inhibitors	Sitagliptin vs. placebo	NCT00790205	TECOS	CV safety	Phase III (14,761)	36 months
	Alogliptin vs. placebo	NCT00968708	EXAMINE	CV safety	Phase III (5,380)	18 months
	Saxagliptin vs. placebo	NCT01107886	SAVOR-TIMI 53	CV safety ↑In HHF	Phase IV (16,492)	25 months
	Linagliptin vs. Glimepiride	NCT01243424	CAROLINA	Results expected 2019	Phase III (6,115)	Ongoing
	Linagliptin vs. placebo	NCT01897532	CARMELINA	Results expected 2018	Phase IV (8,300)	54 months
SGLT2-inhibitors	Canagliflozin vs. placebo	NCT01032629	CANVAS	↓3-MACE ↓HFF ↑Lower extremity amputations	Phase III (10,142)	43 months
	Empagliflozin vs. placebo	NCT01131676	EMPA-REG OUTCOME	↓3-MACE ↓All-cause mortality ↓HHF	Phase III (7,020)	37 months

CV safety = cardiovascular safety, 3-mace = 3-point major adverse cardiovascular events, HHF = hospitalized heart failure. Blue: CV safety, Red: negative results, Green: positive results, Gray: trial still in progress/results expected in future.

subgroup analysis revealed that patients with more severe kidney disease, older or with established cardiovascular disease may have greater benefit from liraglutide treatment in comparison with other patient groups (Roder, 2018).

In the much smaller SUSTAIN-6 trial, patients with T2DM and established cardiovascular and renal disease were enrolled and randomized to receive either the longer acting semaglutide or placebo. The inclusion criteria were very similar to the LEADER study and the follow-up was 25 months. This trial, designed as a non-inferiority safety study, revealed that treatment with semaglutide decreased the combined primary outcome (cardiovascular death, non-fatal MI, and non-fatal stroke), however, there was no difference in the secondary endpoint of cardiovascular death alone (Marso et al., 2016a). The results of the primary outcome were driven by a reduction in non-fatal MI and stroke. However, in light of its smaller non-inferiority design, this trial may underestimate the difference in CV mortality compared with the much larger LEADER trial (Asleh et al., 2018).

The results of the large-scale EXSCEL trial were recently presented. Treatment with exenatide failed to demonstrate a significant reduction in the primary composite including death from cardiovascular causes, non-fatal MI, and non-fatal stroke (Holman et al., 2017). However, exenatide significantly reduced the secondary outcome of all-cause mortality. The reason for this divergence in results are still not fully understood, but the diabetic population studied in EXSCEL was more heterogeneous in terms of age and CV risks with the respect of the ones analyzed in the other trials. Moreover, a shorter follow-up period, lower baseline HbA1c level, a high discontinuation rate, together with a more frequent use of SGLT-2 inhibitor in the placebo group (Roder, 2018), could at least partly explain why there was no difference in CV events among treated and placebo group. Further studies are needed to determine which population of diabetic patients may benefit most from this therapy and investigate mechanisms leading to improved CV outcomes (**Table 1**).

DPP-4 Inhibitors

Dipeptidyl peptidase-4 is expressed in most parts of cells/tissue and exhibits exopeptidase activity against GLP-1 and several other peptide hormones and chemokines. Thus its activity is not only limited to glucose metabolism but also regulates several processes, including inflammation, vascular function, cell homing, and survival (Mulvihill and Drucker, 2014).

Dipeptidyl peptidase-4 plasma activity correlates with cardiac dysfunction in humans and experimental models of HF (dos Santos et al., 2013), indicating a direct link between the DPP-system and cardiovascular health. In various models of HF, DPP-4 inhibition has improved ventricular remodeling, severity of HF, and even survival (Shigeta et al., 2012; Takahashi et al., 2013). Interestingly, a small study conducted with non-diabetic patients affected by non-ischemic myopathy has shown that the use of a DPP-inhibitor increases myocardial glucose uptake, opening up the possibility that this compound could be potentially beneficial in the progression of diabetic cardiomyopathy as well

(Witteles et al., 2012). DPP-4 inhibitors prevent cardiac diastolic dysfunction by attenuating fibrosis and oxidative stress in mouse models of insulin resistance and obesity (Bostick et al., 2014).

Therefore, it was surprising that clinical outcomes with DPP-4 inhibitors showed heterogeneous and partially negative effects (or no effect) in cardiovascular health in large outcome trials, despite several smaller studies and translational data from animal models have suggested potential beneficial effects. Three DPP-4 inhibitors have been tested in large clinical trials: sitagliptin, alogliptin, and saxagliptin (**Table 1**). The use of these drugs was not associated with any increase (but neither a decrease) in the composite primary outcome, including CV mortality, non-fatal MI, and non-fatal stroke with hazard ratios very close to 1.00. However, HF hospitalization was modestly yet still significantly increased by 27% in the SAVOR-TIMI trial with saxagliptin (3.5% for saxagliptin vs. 2.8% for placebo). A similar, although not statistically significant trend could be detected in EXAMINE trial with 3.1% HF hospitalizations in the alogliptin group vs. 2.9% in the placebo group, whereas no difference was observed in the TECOS trial with an incidence of 3.1% for hospitalizations for HF in the sitagliptin and placebo group, respectively (Scirica et al., 2013; White et al., 2013; Green et al., 2015). The observation of increased HF hospitalization did not result in elevated mortality, but it was even more pronounced in subgroups with impaired renal function. More extensive data will soon be available from the outcome data for linagliptin from the CARMELINA and CAROLINA trials. No large outcome trial was performed using vildagliptin, which is not marketed for sale in the United States. These controversial data have been the starting point for contradicting interpretations of recent meta-analyses, ranging from no increase in the risk for the hospitalization of HF after DPP-4 inhibitor use (Savarese et al., 2016) to an increased risk (Kongwatcharapong et al., 2016), indicating that there are considerable differences between substances within the class of DPP-4 inhibitors. Moreover, DPP-4 inhibitors could have a different effect in different populations (relatively "healthy" diabetic population vs. higher risk subjects). However, sub-group analyses failed to identify a specific population in which beneficial cardiovascular effect were evident (Luconi et al., 2017).

Dipeptidyl peptidase-4 inhibitors have been shown to have an impact on various organ systems, including the heart, kidneys, vascular system, and the neuroendocrine system. It impacts hormones or second messengers like BNP, stromal cell-derived factor 1, neuropeptide Y (NPY), and substance P, causing partially activation of the sympathetic nervous system and stimulation of β-adrenergic receptors (Packer, 2018). However, long-term DPP-4 inhibitor treatment in a diabetic mouse model undergoing transverse aortic constriction resulted in an impairment of cardiac function due to an increase in proinflammatory and profibrotic gene expression (Mulvihill et al., 2016). Thus, upregulation of inflammatory cytokines could play a role in the potential interplay of DPP-4 inhibitors and increased hospitalization for HF (Luconi et al., 2017).

A recent study has shown that DPP-4 is absent in cardiomyocytes, but saxagliptin is internalized in these cells where it inhibits SERCA2a/Ca^{2+}/Calmodulin-dependent protein kinase II/phospholamban axis and reduces Ca^{2+}-extrusion *via* NCX. This results in depleted SR Ca^{2+}-content and cytosolic Ca^{2+}-overload, as seen in HF, which consequently leads to impaired myocardial function. Moreover, saxagliptin induced prolonged action potential duration and consequently QTc interval *via* reduced protein-kinase C-mediated delayed rectifier K^+ current (Koyani et al., 2017).

Some of the drugs used in the clinic also inhibit DPP-8 and to a lesser extent DPP-9. These two DPPs are also located in the cytosol of cardiomyocytes and might therefore directly affect myocardial function, energetics, or metabolism. DPP-8 and DPP-9 were reported to have an impact on cellular homeostasis and energy metabolism *via* cytosolic calreticulin and adenylate kinase 2 (Wilson et al., 2013). In animal models, inhibition of DPP-8 or DPP-9 has even been described to cause a variety of symptoms ranging from alopecia over gastrointestinal disorders and blunted hematopoiesis to increased mortality in rats (Lankas et al., 2005). However, these side effects were not observed in the large clinical trials with saxagliptin (SAVOR-TIMI 53) or the previously completed smaller studies using vildagliptin, although these drugs also strongly inhibit DPP-8 and DPP9.

In conclusion, underlying signal transduction mechanisms derived from animal data must be discussed with caution. The possible therapeutic role of various DPP-4 inhibitors in patients with HF, and more specifically diabetic cardiomyopathy, is not yet fully understood.

SGLT-2 Inhibitors

Since regulatory agencies began requiring cardiovascular outcome trials for newly developed anti-hyperglycemic drugs, the EMPA-REG-OUTCOME trial (Zinman et al., 2015) was the first to report remarkably improved CV outcomes including all-cause mortality in patients treated with one particular anti-diabetic drug (**Table 1**). This double-blind, placebo-controlled trial randomized 7,020 patients with T2DM at high CV risk to either once-daily empagliflozin treatment (10 or 25 mg) or placebo treatment. The investigators reported a significant 14% reduction of the combined primary endpoint encompassing CV death, non-fatal MI, and non-fatal stroke in patients treated with empagliflozin (pooled analysis) during a mean follow-up of 3.1 years, which was mainly driven by a reduction in CV deaths (hazard ratios, 0.62; 95% CI, 0.49–0.77; $P < 0.001$). Furthermore, a 35% relative reduction in the rate of HF hospitalization was observed in the empagliflozin group ($p < 0.002$). The CANVAS program (Neal et al., 2017a), which analyzed data from two sister trials, CANVAS and CANVAS-R (Neal et al., 2017b), included 10,142 patients with T2DM and a high CV risk and was designed to study the CV safety and efficacy of canagliflozin (SGLT2i). Consistent with the findings from the EMPA-REG-OUTCOME trial, canagliflozin significantly reduced the rate of primary outcome events (composite of CV death, non-fatal MI, and stroke) by 14% and HF hospitalization by 33%. However, all-cause and CV mortality were not significantly reduced by canagliflozin. The cardiovascular outcome trial investigating the effects of dapagliflozin has not been reported yet, however, the CVD-REAL (comparative effectiveness of cardiovascular outcomes in new users of SGLT-2 inhibitors) although not

being a randomized controlled trial also suggests a reduction in mortality and HF hospitalization the real world setting, confirming the findings from the EMPA-REG-OUTCOME trial and the CANVAS program, suggesting a potential drug class effect at least on some of the outcome parameters (Kosiborod et al., 2017). However, the CANVAS program reported an unexpected increase in the risk of lower-extremity amputation in the canagliflozin-treated group. The mechanism of action is not understood, and furthermore it is not known if the higher amputation risk is specific to canagliflozin (Tanaka and Node, 2017). The striking and unexpected findings of SGLT2i regarding cardiovascular outcomes has initiated many discussions on how the cardiovascular benefits could be explained mechanistically, generating many hypotheses that are difficult to address with the limited data available. The described glucose lowering effect (Zinman et al., 2015), weight loss (Riggs et al., 2015), and blood pressure reduction (Baker et al., 2014), hemodynamic effects, direct vascular effects, osmotic diuresis, and natriuresis (Inzucchi et al., 2015; Butler et al., 2017) may contribute to the CV effects (Lytvyn et al., 2017). However, even a combination of these factors is unlikely to fully explain the results of EMPA-REG OUTCOME and the CANVAS program.

Recently, despite the lack of myocardial SGLT-2-expression, direct effects of the drug on heart muscle cells have even been suggested. There is evidence that the sodium hydrogen exchanger (NHE) may play an important role in the interplay of HF and diabetes since renal and cardiac isoforms of the NHE are upregulated in both conditions (Baartscheer et al., 2017; Packer, 2017). Inhibition of NHE by SGLT-2 inhibitors and modulation of intramyocardial Ca^{2+} and Na^{+} fluxes seems to have a beneficial impact on diastolic myocardial function (Baartscheer et al., 2017). Two recently published studies suggested that empagliflozin improves diastolic dysfunction in diabetic mouse models, which was linked to enhanced SERCA activity and anti-fibrotic effects (Habibi et al., 2017; Hammoudi et al., 2017).

Another potential explanation for the beneficial CV effects of SGLT-2 inhibition is a modulation of myocardial energy metabolism (Ferrannini et al., 2016). The myocardium of diabetic HF-patients loses the ability to properly oxidize fatty acids and metabolize glucose. It has been suggested that SGLT-2 inhibition slightly increases levels of ketone bodies independent of the presence of diabetes, which can then be oxidized in preference to fatty acids. This metabolic substrate shift might improve myocardial work efficiency and oxygen consumption (Bonner et al., 2015; Ferrannini et al., 2016; Al Jobori et al., 2017). However, the impact of SGLT-2 inhibitors on cardiac metabolism has not yet been carefully studied and it remains unclear how SGLT-2 inhibitors exert their beneficial CV effects through myocardial metabolism modulation (Ussher et al., 2016). Direct assessment of myocardial metabolism after SGLT-2 inhibition *in vivo* is challenging but would reveal new insights. Although there is a general consensus that SGLT-2 inhibition improves CV outcomes, several questions remain unanswered regarding the exact mechanisms of action, optimal timing, and whether or not these drugs would exert similar effects in non-diabetic patients with a high risk of CVD (Kaplan et al., 2018). Since the CV benefits of SGLT-2 inhibition seem to be independent of glucose control, SGLT-2 inhibitors might be both safe and effective in non-diabetic HF patients (Butler et al., 2017). Currently, several Phase III outcome trials with SGLT2 inhibitors in non-diabetic HF patients with preserved (HFpEF) and reduced ejection fraction (HFrEF) are planned and the field is eagerly awaiting the results.

NEW POTENTIAL TARGETS FOR FUTURE THERAPIES

Gene Therapy

In recent years, great improvements have been made toward the possibility of modulating the expression of specific cardiac genes *in vivo* for therapeutic purpose. Thus, the idea to up or down-regulate the expression of key players in the development of diabetic cardiomyopathy may not be that far from an actual therapeutic approach.

The E3 ubiquitin ligase mitsugumin 3 expression is increased in the cardiac tissue of T2DM animal models. Cardiac-specific overexpression of this molecule leads to proteasomal degradation of both insulin receptor and IRS-1 resulting in insulin resistance (Liu et al., 2015). Furthermore, an increase in fibrosis was observed in transgenic mice, suggesting that the inhibition of E3 ubiquitin ligase mitsugumin 3 could represent an overall potential therapeutic strategy for the prevention of diabetic cardiomyopathy.

Forkhead box-containing protein 1, O subfamily (FoxO1) is another molecule involved in the regulation of IRS-1/Akt signaling pathway. Metabolic stress induces constant activation of FoxO1 that results in blunted Akt signaling and insulin resistance. Cardiomyocyte-specific deletion of FoxO1 rescued cardiac dysfunction and preserved insulin responsiveness in mice fed with a high-fat diet (Battiprolu et al., 2012). Thus, FoxO1 may be a potential target for future therapeutic approach.

Reduced chamber compliance is a hallmark change in HFpEF associated with T2DM and is partly due to altered phosphorylation of the structural sarcomeric protein titin with a consequent increase of cardiomyocytes stiffness. A recent study showed how treatment with Neuregulin1 (NRG-1) was able to rescue titin-based cardiomyocyte stiffening in diabetic mouse hearts (Hopf et al., 2018), *via* increased PKG and ERK1/2 activity and reduced PKCα activity, which reversed the changes in titin-phosphorylation associated with diabetes.

As mentioned above, diabetes is strongly associated with mitochondrial dysfunction. A recent study showed that diabetes disrupts mitochondrial proteomic signature. 99% of mitochondrial protein are encoded in the nucleus and then imported in this organelle by a complex, in which mitochondrial heat shock protein 70 (mtHsp70) is a key component. Diabetes correlates with a downregulation of this protein. Interestingly, mtHsp70 overexpression is able to restore cardiac function in diabetic mice through attenuation of mitochondrial dysfunction (Shepherd et al., 2018).

Modulation of Oxidative Stress

Oxidative stress is one of the major contributors in the pathogenesis of the diabetic heart. A number of studies have evaluated different strategies to decrease ROS accumulation.

Sulforaphane, a dietary isothiocyanate compound is an activator of Nrf2, a transcription factor that regulates the expression of several antioxidant proteins. Sulforaphane treatment resulted in a decrease in ROS production in arterioles of diabetic mice (Velmurugan et al., 2013) and attenuated cardiac remodeling and dysfunction induced by high fat diet (Zhang et al., 2014).

Several studies conducted in the past 40 years have explored the efficacy of coenzyme Q_{10} in reducing oxidative stress and pathological remodeling of the heart (Huynh et al., 2014). Coenzyme Q_{10} treatment results in reduction of systolic and diastolic blood pressure in diabetic patients (Hodgson et al., 2002; Chew et al., 2008) acting as vasodilator. Moreover, supplementation with coenzyme Q_{10} decreases cardiac inflammation, fibrosis, and hypertrophy in mouse models of T1DM and T2DM (Huynh et al., 2010, 2013).

miRNA and lncRNA-Based Treatment

miRNA-based treatment is a multi-target therapy causing simultaneous regulation of crucial pathways, making it an excellent candidate to modulate complex networks, such as those involved in the pathogenesis of diabetic cardiomyopathy.

miRNAs expression undergoes changes in the diabetic heart. miRNAs modulation can be a response to several pathological insults, including hyperglycemia, hyperinsulinemia, oxidative stress, and inflammation. Interestingly, glycemic control is unable to rescue hyperglycemia-induced alterations of miRNAs in the heart of streptozotocin-induced diabetic mice, suggesting that diabetic cardiomyopathy and the miRNA alterations associated with it, can progress despite normalization of blood glucose level (Costantino et al., 2016).

miR-1, the most expressed miRNA in the heart, constantly increases from the early to later phases of diabetic cardiomyopathy. It negatively regulates the expression of Pim1 and Bcl-2, which are anti-apoptotic and cardioprotective proteins. Remarkably, transfection with anti-miR1 activates pro-survival signals in cardiomyocytes and cardiac progenitor cell exposed to high glucose (Katare et al., 2011). On the other hand, miR-133a expression was drastically decreased in hearts of streptozotocin-induced diabetic mice. This downregulation correlates with the increase of fibrotic markers, such as TGFβ, fibronectin, and collagen. Interestingly, overexpression of miR-133a attenuates the development of fibrosis, suggesting that this miRNA could be a potential therapeutic target for diabetes-induced cardiac fibrosis and related cardiac dysfunction. Diabetes correlates with a decreased expression of miR-30c and miR-181a in human samples and animal models, and overexpression of these miRNAs in cardiomyocytes exposed to high glucose attenuated p53-induced apoptosis and hypertrophy (Raut et al., 2016).

Hyperglycemia reduces miR-146a expression in cardiac endothelial cells. Endothelial-specific overexpression of miR-146a attenuates the pathological remodeling in the diabetic heart and decreases the inflammatory response (Feng et al., 2017).

Finally, long non-coding RNA (lncRNAs) is a novel class of RNA that does not code for proteins and are important regulators of gene expression. Recent studies have found that diabetes correlates with an aberrant expression of these molecules (Raut and Khullar, 2018). Briefly, lncRNA-myocardial infarction-associated transcript (MIAT) expression is upregulated in models of diabetic cardiomyopathy and when knocked down, there are improvements in cardiac function and a decrease in cardiomyocyte apoptosis (Zhang et al., 2016; Zhou et al., 2017).

Non-coding RNAs as Biomarkers

The possibility to easily detect different miRNAs in plasma and how stable they are opens up the opportunity to use these molecules as biomarkers for several pathologies, including diabetic cardiomyopathy (Marfella et al., 2013; Ono, 2015; Sardu et al., 2016a; de Lucia et al., 2017). The lack of specific biomarkers coupled with the fact that the earlier stages of this pathology are mostly asymptomatic makes detecting diabetic cardiomyopathy a challenge in clinical practice. miRNAs have been proposed as a new and very specific biomarker for several cardiovascular disease. A recent study determined that both circulating and cardiac miR-19b-3p and miR-181b-5p levels were associated with cardiac dysfunction during the development of diabetic cardiomyopathy in mice fed a high fat, suggesting that these miRNAs could be suitable biomarkers for this disease in asymptomatic diabetic patients (Copier et al., 2017). Furthermore, circulating miR-1 and miR-133a levels are associated with myocardial steatosis in T2DM patients, independent of confounding factors (de Gonzalo-Calvo et al., 2017).

A recent study conducted in patient suggested that also lncRNAs can be used as circulating biomarkers in diabetic cardiomyopathy. lncRNAs, such as long intergenic non-coding RNA predicting cardiac remodeling (LIPCAR), MIAT, and smooth muscle and endothelial cell-enriched migration/differentiation-associated (SENCR) were found to be independent predictors of diastolic dysfunction in diabetic patients (de Gonzalo-Calvo et al., 2016).

Further studies conducted in patients are needed to investigate the real potential of these molecules as biomarkers.

CONCLUSION

Diabetes mellitus represents one of the greatest burdens on the global health care system and is one of the major risks for cardiovascular disease. Interestingly, cardiovascular risk is higher in diabetic women than in diabetic men, suggesting that diabetes affects cardiovascular health to greater degree in women. This difference is probably due to sex hormones and neurohormonal diversity, coupled with gender-specific activation of molecular pathway involved in the cardiac metabolism/remodeling (Toedebusch et al., 2018).

Diabetic cardiomyopathy is an important complication of diabetes and represents a distinct form of HF that is independent from other comorbidities. Its pathogenesis is not completely understood, although hyperglycemia and cardiac insulin resistance are key players. Further studies are vital for understanding the precise pathophysiological mechanism involved and to unravel the complex molecular and structural abnormalities leading to fibrosis, hypertrophy, mitochondrial dysfunction, steatosis, oxidative stress, impaired Ca^{2+} handling, inflammation, and metabolic switch observed in diabetic cardiomyopathy.

Despite a growing interest in the pathophysiology of the diabetic cardiomyopathy, there are no specific guidelines for diagnosing patients or structuring a treatment strategy in clinical practice. Currently, treatment plans are based on controlling the underlying diabetes and improving the risk factors associated with the progression of the cardiovascular disease. Several studies have found that glycemic control improved LV diastolic function in T2DM (Gaede et al., 2003; von Bibra et al., 2004). However, cardiovascular disease also occurs in diabetic patients who are well managed under treatment, highlighting the necessity for targeted therapeutic strategies for this population (Tate et al., 2017).

Changes in lifestyle favoring exercise, balanced caloric intake, anti-diabetic medications, lipid-lowering therapies, and the management of HF are the current treatment strategies available for these patients. Administration of beta-blockers, angiotensin converting enzyme inhibitors (ACEi)/angiotensin receptor blockers (ARB) are currently the standard treatment for chronic HF with or without diabetes. If patients remain symptomatic with LV EF <35%, it is recommended to add a mineralocorticoid receptor antagonist. If patients are still symptomatic, the ACEi/ARB should be replaced by an angiotensin receptor neprilysin inhibitor. Furthermore, cardiac resynchronization therapy should be considered in patients with sinus rhythm and wide QRS complex (≥130 ms) (Ponikowski et al., 2016; Sardu et al., 2016b, 2017). An in-depth description of therapeutic approaches for HF was beyond the scope of this review.

In this review, we focused mainly on anti-hyperglycemic drugs and their benefit on cardiovascular outcomes. Besides modulating diabetes as a cardiovascular risk, several new glucose lowering drugs have been found to have a direct effect on myocardial tissue. Although particularly promising, a more comprehensive analysis of cardiovascular outcome data together with translational studies is necessary to elucidate the benefit/risk and the mechanism of action of these glucose-optimizing agents. Specifically, understanding the real effect of glucose lowering drugs on diabetic cardiomyopathy based on cardiovascular outcome trial data is challenging. These trials were designed for safety purpose and include a very heterogeneous population of diabetic patients from the cardiovascular prospective. However, despite the lack of information on the direct effect on diabetic cardiomyopathy, these trials provide the suggestion of possible cardiovascular protection. Novel therapeutic approaches, including gene and non-coding RNA-based therapy, are currently being investigated in models and represent an exciting and promising new direction for treating diabetic patients. These therapies can eventually regulate common factors of diabetic cardiomyopathy and HF retaining a great potential in the cardiovascular field. However, further studies are needed to investigate the real translational potential in clinical practice.

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frontiers in Physiology

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The Melanocortin MC5R as a New Target for Treatment of High Glucose-Induced Hypertrophy of the Cardiac H9c2 Cells

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The study explored the anti-hypertrophic effect of the melanocortin MC5R stimulation in H9c2 cardiac myocytes exposed to high glucose. This has been done by using α-MSH and selective MC5R agonists and assessing the expression of GLUT4 and GLUT1 transporters, miR-133 and urotensin receptor levels as a marker of cardiac hypertrophy. The study shows for the first time an up-regulation of MC5R expression levels in H9c2 cardiomyocytes exposed to high glucose medium (33 mM D-glucose) for 48 h, compared to cells grown in normal glucose medium (5.5 mM D-glucose). Moreover, H9c2 cells exposed to high glucose showed a significant reduction in cell viability (−40%), a significant increase in total protein per cell number (+109%), and an increase of the urotensin receptor expression levels as an evidence of cells hypertrophy. The pharmacological stimulation of MC5R with α-MSH (90 pM)of the high glucose exposed H9c2 cells increased the cell survival (+50,8%) and reduced the total protein per cell number (−28,2%) with respect to high glucose alone, confirming a reduction of the hypertrophic state as per cell area measurement. Similarly, PG-901 (selective agonist, 10^{-10} M) significantly increased cell viability (+61,0 %) and reduced total protein per cell number (−40,2%), compared to cells exposed to high glucose alone. Interestingly, the MC5R agonist reduced the GLUT1/GLUT4 glucose transporters ratio on the cell membranes exhibited by the hypertrophic H9c2 cells and increased the intracellular PI3K activity, mediated by a decrease of the levels of the miRNA miR-133a. The beneficial effects of MC5R agonism on the cardiac hypertrophy caused by high glucose was also observed also by echocardiographic evaluations of rats made diabetics with streptozotocin (65 mg/kg i.p.). Therefore, the melanocortin MC5R could be a new target for the treatment of high glucose-induced hypertrophy of the cardiac H9c2 cells.

Keywords: cardiac hypertrophy, melanocortin 5 receptor agonism, glucose content, PI3K, GLUT1, GLUT4

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INTRODUCTION

Cardiac hypertrophy is caused by an increased glucose uptake into the cardiac myocytes that determines a high glucose-mediated oxidative stress into the cardiomyocytes (Kagaya et al., 1990; Zhang et al., 1995; Leong et al., 2002; Nascimben et al., 2004; Wang et al., 2009; Han et al., 2015; Wei et al., 2018). This increased glucose uptake is mostly due to an imbalance of the translocation

of GLUT1 and GLUT4 glucose transporters from intracellular membranes to the cell surface of the myocytes with a GLUT1/GLUT4 ratio favoring GLUT1 (Slot et al., 1991; Abel et al., 1999; Paternostro et al., 1999; Tian et al., 2001; Kolwicz and Tian, 2011; Shao and Tian, 2015). In a normal adult heart GLUT4 is the primary glucose transporter translocating on plasma membrane after insulin stimulation, while the mediator of basal cardiac glucose uptake GLUT1 is downregulated after birth. Conversely, pathological hypertrophic condition links a GLUT4 depletion, resulting in a direct increase in GLUT1 levels (Slot et al., 1991; Abel et al., 1999; Paternostro et al., 1999; Tian et al., 2001; Kolwicz and Tian, 2011; Shao and Tian, 2015). Among these, GLUT4 expression and translocation is regulated by miR-133 both in skeletal muscle and in cardiac myocytes (Horie et al., 2009).

It is well known that regulation of the glucose homeostasis and insulin sensitivity involves the central melanocortin system, mostly through the hypothalamic proopiomelanocortin (POMC) which is well-established regulator of insulin secretion, glucose utilization, and glucose production. However, scant data exist about the role of peripheral melanocortin peptides or peptidomimetics in this regulation (Fan et al., 2000; Costa et al., 2006; Hill and Faulkner, 2017). Recently, it has been shown that peripheral α-melanocyte stimulating hormone (α-MSH) promotes glucose uptake in the skeletal muscle via melanocortin receptor 5 (MC5R) pathway (Enriori et al., 2016), suggesting a key role of this peptide and this receptor in the glucose transport and pathologies related to an altered glucose uptake. Interestingly, a recent human study showed that single-nucleotide polymorphism in the MC5R was associated with type 2 diabetes in obese subjects (Valli-Jaakola et al., 2008).

Therefore, in light of these evidences the aim of this study was to assess the anti-hypertrophic potential of the melanocortin MC5 receptor in H9c2 cardiomyocytes exposed to high glucose. Particularly, it has been investigated the phosphoinositide 3-kinase (PIK3) activity as a possible intracellular target of the MC5R stimulation. PI3K is a major player for mediating cardiac glucose since it is a regulator of glucose transporters (Egert et al., 1997; Vlavcheski et al., 2018), it is involved in reduction of cardiac hypertrophy (Weeks et al., 2017), and it is stimulated by the MC5R after α-MSH activation in HEK293 cells (Rodrigues et al., 2009).

MATERIALS AND METHODS

Cell Culture and Treatments

Embryonic rat cardiac H9c2 (2-1) cells (ECACC, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (DMEM; AU-L0101Aurogene, Italy), containing 5.5 mM D-glucose and supplemented with 10% Heat Inactivated Fetal Bovine Serum (AU-S181H Aurogene, Italy), 5% L-Glutamine (AU-X0550 Aurogene, Italy) and 5% Penicillin-Streptomycin Solution (AU-L0022 Aurogene, Italy), at 37°C under an atmosphere of 5% CO_2 (Trotta et al., 2017). Then, H9c2 (2-1) cells were exposed to 5.5 mM D-glucose (Normal control group, NG); 5.5 mM D-glucose + Angiotensin II (1 μM; A9525 Sigma, Italy; Ang II group, positive control for cardiomyocytes hypertrophy) (Stuck et al., 2008); 33 mM D-glucose (A24940-01 Life Technologies, Italy; High glucose group, HG) (Wei et al., 2018); 33 mM D-glucose + α-MSH (90 pM; M4135 Sigma, Italy; HG + α-MSH group) (Enriori et al., 2016); 33 mM D-glucose + MC5R agonist PG-901 (10^{-10} M), dissolved in PBS (HG + PG-901 group) (Maisto et al., 2017); 33 mM D-glucose + MC5R antagonist PG-20N (130 nM), dissolved in PBS (HG + PG-20N group) (Rossi et al., 2016). H9c2 cardiomyocytes were stimulated with high glucose medium, with or without α-MSH,PG-901, and PG-20N treatment, for 48 h, being the 33 mM high glucose concentration reported to induce cardiac hypertrophy in H9c2 cardiomyocvtes after 2 days of exposure (Han et al., 2015; Zhong et al., 2015; Wei et al., 2018). Different cell numbers were used in the different experiments: for the viability assay, cells were seeded at 5×10^3 cells/cm^2 (Wei et al., 2018); the cells used for total RNA and protein detection were seeded at 1×10^6 cells/cm^2 (Han et al., 2015); for immunofluorescence assay, cells were seeded at 1×10^4 cells/cm^2 (Suhaeri et al., 2015); for isolation of membrane fractions cells were seeded at 4.1×10^3 cells/cm^2 (Yu et al., 2000). Three independent experiments were performed, each done in triplicate. H9c2 cell morphology was daily observed with optic microscope (Leica DMi1, Germany) and cell area was quantified using Image J software, by determining the average area per cell following a treatment and counting over 250 cells per well examined across triplicate wells (Wang et al., 2009).

Anti-miR-133a Transfection

H9c2 cells were transfected with Anti-rno-miR-133a (MIN0000839 Qiagen, Italy) or negative control (1027271 Qiagen, Italy) using Lipofectamine 2000 reagent (11668-027 Life Technologies, Italy), according to the manufacturer's protocol.

Cell Viability Assay

H9c2 viability was measured by 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT)assay. 5×10^3 cells/well were seeded in 96-well plates and treated as previously described. Briefly, MTT solution (1:10 in culture medium) was added to each well, incubated for 4 h and then removed. Each well was then washed for 20 min with isopropanol-HCl 0.2 N. Optical density (OD) values were measured at 570 nm using a 96-well plate reader (iMark, Bio-Rad Laboratories, Italy).

MC5R mRNAs and miR-133a Levels Determination

For MC5R mRNAs determination levels, total RNA isolation was performed according the RNeasy Mini kit (74134 Qiagen, Italy), following the Purification of Total RNA from Animal Cells RNA concentration and purity were determined using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Italy). According to Siniscalco et al. (2013), 1 μg of RNA was reverse transcripted following the manufacturer's protocol by using Superscript III reverse transcriptase system (4367659 Invitrogen, Italy) and oligo(dT)15. Real-time PCR was performed with Reddy Mix PCR Master Mix (AB-0575/DC/LD/B ThermoScientific,

Italy), each reaction consisting in 1 μl of diluted cDNA, 22.5 μl of 1.19 ReddyMix PCR MasterMix, 1 μl of ddH_2O and 1 μl of rat MC5R primer assay (QT01701920 Qiagen, Italy). The amplification profile used was the following: 95°C for 2 min; 35 cycles 94°C for 30 s, 55°C for 35 s, and 72°C for 65 s, followed by final elongation step at 72°C for 5 min. MC5R data were normalized relative to GAPDH and then used to calculate expression levels, according the $2^{-\Delta\Delta Ct}$ method.

For miR-133a determination levels, miRNAs isolation was performed according to the miRNeasy Mini kit (217004 Qiagen, Italy), following the supplementary protocol Purification of Total RNA, including Small RNAs, from Animal Cells. 5 μl of Syn-cel-miRNA-39 miScript miRNA Mimic 5 nM (MSY0000010 Qiagen, Italy) was spiked into each sample, before nucleic acid preparation, in order to monitor the miRNA recovery efficiency and to normalize miRNA expression in the Real-time PCR analysis. NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Italy) was used to determine RNA concentration and purity. Mature miRNA reverse transcription was performed according to the miScript II RT kit (218161 Qiagen, Italy). Then miR-133a expression levels were detected using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Italy). Each reaction, carried out in triplicate, was set according the SYBR Green PCR Kit (218073 Qiagen, Italy) and by using specific miScript Primer Assays for miR-133a (MS00033208 Qiagen, Italy) and Syn-cel-miR-39 (MS00080247 Qiagen, Italy). Δ*C*t value for each miRNAas *C*t miRNA–*C*t Syn-cel-miR-39 was calculate to perform the relative quantization of miRNA expression; fold change was then obtained as $2^{-\Delta\Delta Ct}$. *P*-values are calculated with a Student's *t*-test of the replicate $2^{-\Delta\Delta Ct}$ values for each miRNA in the different groups. A *P*-value less than 0.05 was considered significant.

Immunocytochemistry

H9c2 cells were fixated with 4% paraformaldehyde, washed with PBS (AU-L0615 Aurogene, Italy) and then incubated for 30 min in blocking solution (1% BSA in PBS), in order to inhibit non-specific antibody binding. The primary antibodies, diluted in PBS blocking buffer and incubated overnight at 4°C, were: anti-GPR14 for Urotensin II receptor detection (sc-28998 Santa Cruz, United States) (Johns et al., 2004; Wang et al., 2009; Wei et al., 2018) and anti-actin (a-5441 Sigma, Italy). Specific antigens in each side were located using a Fluorescein Isothiocyanate (FITC) – conjugated anti-rabbit (GTXRB-003-D488N Immunoreagents, United States) and Tetramethylrhodamine (TRITC) – conjugated anti-mouse (GTXMU-003-D594N Immunoreagents, United States) secondary antibodies. H9c2 cells were counterstained with pentahydrate bisbenzimide (Hoechst 33258 Sigma, Italy) and then mounted with mounting medium (90% glycerol in PBS). Immunofluorescence images, obtained from the observation at a fluorescence microscope (Leica, Germany) and at a fluorescence confocal microscope (LSM 710 Zeiss, Germany), were analyzed with Leica FW4000 (Leica, Germany) and with Zen Zeiss (Zeiss, Germany) softwares. An observer blind to the treatment performed the labeling quantization for each microscope field, the percentage of positive cells was calculated by the number of labeled positive cells of 300 cells in four different microscope fields. In order to avoid overcounting cells, only bisbenzimide counterstained cells were considered as positive profiles, performing on each digitized image the cell positive profile quantization. Data are reported as the intensity means ± SEM of the percentages of positive cells / total cells counted in each analyzed field for each treatment. Three independent experiments were performed, each done in triplicate.

Cell Lysate Preparation for Protein Quantization

Cells were washed with cold phosphate-buffered saline (PBS; AU-L0615 Aurogene, Italy); then 150 μl of cold RIPA lysis buffer (R0278 Sigma-Aldrich, United States) supplemented with a complete protease inhibitor cocktail (11873580001 Roche, United States) was added to each well. Lysates were collected and then centrifugated at 12,000 rpm for 10 min at 4°C. Total protein concentration in supernatants was measured using the Bio-Rad Protein Assay (500-0006 Bio-Rad Laboratories, Italy) and used for the determination of hypertrophy marker as total protein/viable cell number at direct cell counting (Wang et al., 2009), as well as for Western Blotting and ELISA assays.

MC5R, Urotensin II Receptor and $K_{IR}6.1$ Protein Levels Assessment

Western Blotting assay was performed in a 12% PAGE separation gel, electro-transferring 30 μg of protein sample onto a PVDF membrane (IPFL10100 Merck Millipore, Italy), blocked for 1 h at room temperature with 5% non-fat dry milk (EMR180500 Euroclone, Italy). Then blots were incubated over-night with the following specific primary antibodies: anti-MC5R (sc-28994 Santa Cruz, United States), anti-GPR14 for Urotensin II receptor detection (sc-288998 Santa Cruz, United States), anti K_{IR} 6.1 (P0874 Sigma, Italy), and anti-actin (sc-8432 Santa Cruz, United States). This step was followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated secondary anti-rabbit (sc-2004 Santa Cruz, United States) or anti-mouse (sc-2005, Santa Cruz, United States) antibodies. The signal was expressed as Densitometric Units (D.U.).

PI3K Activity Determination

PI3K activity measurement was performed by using the PI3K Activity ELISA (K-1000s Echelon, Italy). The activity assay was performed following the immunoprecipitation of PI3K from cells, as suggested by the manufacturer's instructions.

Plasma Membrane GLUT1 and GLUT4 Levels Determination

H9c2 plasma membrane-enriched fractions were obtained by subcellular fractionation according to Yu et al. (2000). GLUT1 and GLUT4 levels from these fractions were measured using ELISA assays, according to the manufacturer's instructions (MBS720405 and MBS451402 My BioSource, United Kingdom).

Adenosine Triphosphate (ATP) Levels Determination

Rat ATP levels as marker of intracellular glucose content (Kuznetsov et al., 2015) were assayed in cell lysates by using Rat ATP Elisa kit (E02A0038BlueGene Biotech, China) according to the manufacturer's protocol.

Statistical Analysis

The results are presented as mean ± S.E.M. of three independent experiments, performing the triplicate of all the treatments in a single experiment. Statistical significance was determined using ANOVA followed by Bonferroni's test. A *P*-value less than 0,05 was considered significant to reject the null hypothesis.

In vivo Proof of Concept

To confirm the role of MC5R agonism in modulating cardiac hypertrophy induced by high-glucose exposure, we translated the *in vitro* experiments in a setting of *in vivo* ones, by investigating the effects of α-MSH and PG-901 in diabetic Sprague-Dawley rats. Male Sprague-Dawley rats (8 weeks of age), housed in a 12-h light/dark cycle animal room and fed with a standard chow diet and tap water *ad libitum*, were randomly divided into the following four groups (n = 5 for each group): (i) non-diabetic rats (CTRL); (ii) STZ-diabetic rats (STZ); (iii) STZ treated with α-MSH (STZ + α-MSH); and (iv) STZ treated with PG-901 (STZ + PG-901). Diabetes was induced in animals by a single intraperitoneal injection of 70 mg/kg STZ in 10 mM citrate buffer (pH 4.5; Sigma Chemical Co., United States) and 15 h later, human regular insulin (1.5 ± 0.5 units/day) was administered intraperitoneally yielding blood glucose levels of ∼22 mmol/l for 8 days (Di Filippo et al., 2005). Blood glucose greater than 300 mg/dL were verified 1 week after the STZ injection (Glucometer Elite XL; Bayer Co., Elkhart, IN, United States), in order to confirm diabetes development (Di Filippo et al., 2016). Then, diabetic rats received weekly intraperitoneal injections of 500 μg/kg α-MSH (Forslin Aronsson et al., 2007) (M4135 Sigma, Italy) or 50 – 500 – 5000 μg/kg PG-901. Animals were treated for 3 weeks after diabetes confirmation, and blood glucose levels were checked intermittently throughout the study to confirm diabetes maintenance. After the 3-week

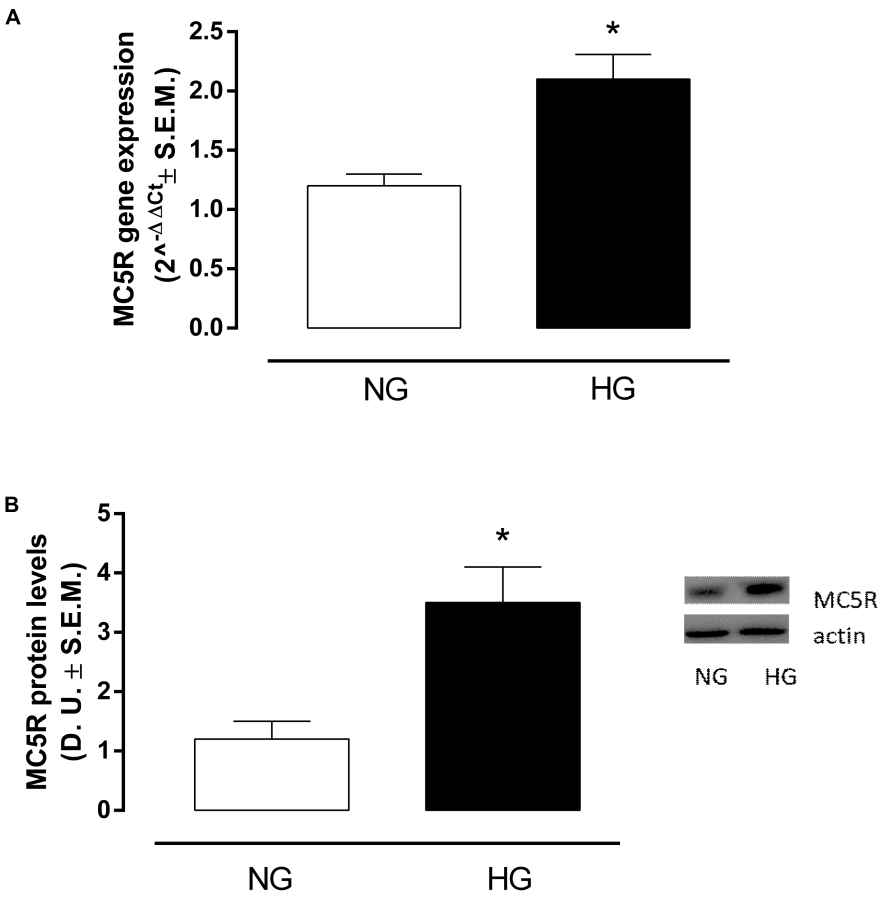

FIGURE 1 | MC5R mRNA and protein levels. **(A)** RT-PCR analysis showed a significant up-regulation of MC5R in H9c2 cells exposed to high glucose (33 mM D-glucose) compared to cardiomyocytes exposed to normal glucose (5.5 mM D-glucose). **(B)** The significantMC5Rup-regulation in HG group was confirmed also by detection of MC5R protein levels by Western Blotting assay. Values are expressed as mean of $2^{-\Delta\Delta Ct}$ or D.U. ± S.E.M. of n = 9 values, obtained from the triplicates of three independent experiments. NG, normal glucose; HG, high glucose; D.U., Densitometric Units; *P < 0,01 vs. NG.

treatments, transthoracic echocardiography (Visualsonics Vevo 2100, Canada) was performed according to Di Filippo et al. (2014), using a 10–14 MHz linear transducer to obtain the images for the measurement of morphometric parameters, based on the average of three consecutive cardiac cycles for each rat. This study was carried out in accordance with to the guidelines of the Ethic Committee for animal experiments at the University of the Studies of Campania "Luigi Vanvitelli."

RESULTS

High Glucose Exposure Increases MC5R Levels in H9c2 Cells

RT-PCR analysis showed that in H9c2 cells exposed to high glucose stimulus MC5R gene expression was significantly increased ($P < 0,01$ vs. NG) compared to control cells (**Figure 1A**). This was confirmed also by Western Blot Assay, showing a significant elevation of MC5R protein expression in H9c2 exposed to high glucose ($P < 0,01$ vs. NG), compared to control cells (**Figure 1B**).

MC5R Agonism Reduces H9c2 Hypertrophy Induced by High Glucose, Increasing Cell Survival

H9c2 cell area quantization showed an evident increase in cell area in cardiomyocytes exposed to high glucose (HG) compared to cells exposed to normal glucose (NG; +58,2%, $P < 0,01$ vs. NG), indicating a hypertrophic condition (**Figure 2**). Agonism at MC5R with α-MSH (90 pM) and PG-901 (10^{-10} M) significantly reduced cell area in cells exposed to high glucose. This reduction was absent in H9c2 cells grown in high glucose and treated with MC5R antagonist (−28,8 and −29,6%, respectively, $P < 0,01$ vs. HG) PG-20N (130 nM) (**Figure 2**). GPR-14 immunofluorescence

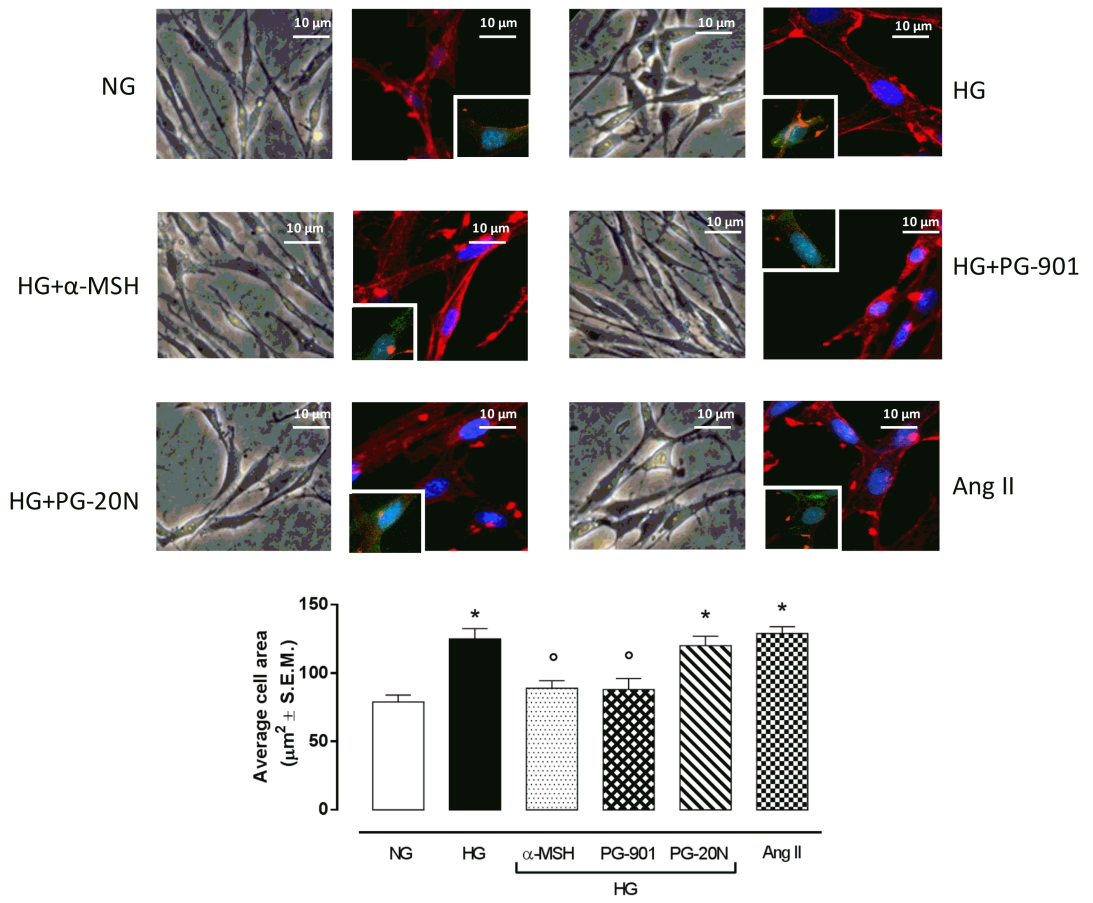

FIGURE 2 | Cell size visualization and quantization. Representative optic microscope (40X) and immunofluorescence images (40X; in blue Hoechst for nucleus and in red actin for cytoskeleton labeling) show that H9c2 cells exposed to high glucose (33 mM D-glucose) exhibited an evident increase in cell area compared to cells exposed to normal glucose (5.5 mM D-glucose). MC5R agonism with α-MSH (90 pM) and PG-901 (10^{-10} M) treatment significantly decreased cell area in H9c2 cells exposed to high glucose. Conversely, H9c2 cells treated with MC5R antagonist PG-20N and exposed to high glucose showed a cell area similar to HG cells. Also representative images of cell nucleus (blue), actin (red), and urotensin II receptor (anti-GPR14, green) co-localization are shown, evidencing that the increased cell area in H9c2 cells exposed to high glucose is paralleled by a high Urotensin II receptor labeling. This is decreased by α-MSH (90 pM) and PG-901 (10^{-10} M) treatments. Cell area was calculating by analyzing 300 cells in four different microscope fields. Values are expressed as mean ± S.E.M. of $n = 9$ values, obtained from the triplicates of three independent experiments. NG, normal glucose; Ang II, angiotensin II; HG, high glucose; $^{*}P < 0,01$ vs. NG; $^{\circ}P < 0,01$ vs. HG.

labeling confirmed the hypertrophy shown by H9c2 cells grown in HG compared to cells exposed to NG, showing a significant increase in GPR14levels (+111,1%, $P < 0,01$ vs. NG) (**Figure 3**). The high Urotensin II receptor levels in H9c2 cells exposed to high glucose were decreased by α-MSH (90 pM; −37,9%, $P < 0,01$ vs. HG) and PG-901 (10^{-10} M; −40,0%, $P < 0,01$ vs. HG), while they were not modified by PG-20N treatment (**Figure 3A**). Also GPR-14 Western Blotting Assay evidenced the same Urotensin II protein expression pattern in the different experimental settings (**Figure 3B**). These results were confirmed also by total protein/ cell number ratio, a sensible marker of hypertrophy (**Figure 4**): H9c2 cells exposed to high glucose showed a significant increase in total protein per cell number (+109%, $P < 0,05$ vs. NG), a significant reduction in cell viability (−40%, $P < 0,01$ vs. NG). α-MSH treatment reduced the total protein per cell number (−28,2%, $P < 0,01$ vs. HG) and increased cell survival (+50,8%, $P < 0,01$ vs. HG), confirming the reduction of hypertrophic state. Also PG-901 treatment reduced total protein per cell number (−40,2%, $P < 0,01$ vs. HG) and significantly increased cell viability (+61,0 %,$P < 0,01$ vs. HG) compared to cells exposed to high glucose. In contrast, an antagonism at MC5R with PG-20N did not produce effect on the parameters recorded in HG (**Figure 4**).

Activation of MC5R Reduces Glucose Uptake and Increases $K_{IR}6.1$ Expression in H9c2 Cells Exposed to High Glucose

In order to confirm the increase in glucose uptake, a feature of pathological cardiac hypertrophy, ATP levels were determined as marker of intracellular glucose content. As expected, H9c2 cells exposed to high glucose showed a significant increase in ATP level compared to cells exposed to normal glucose

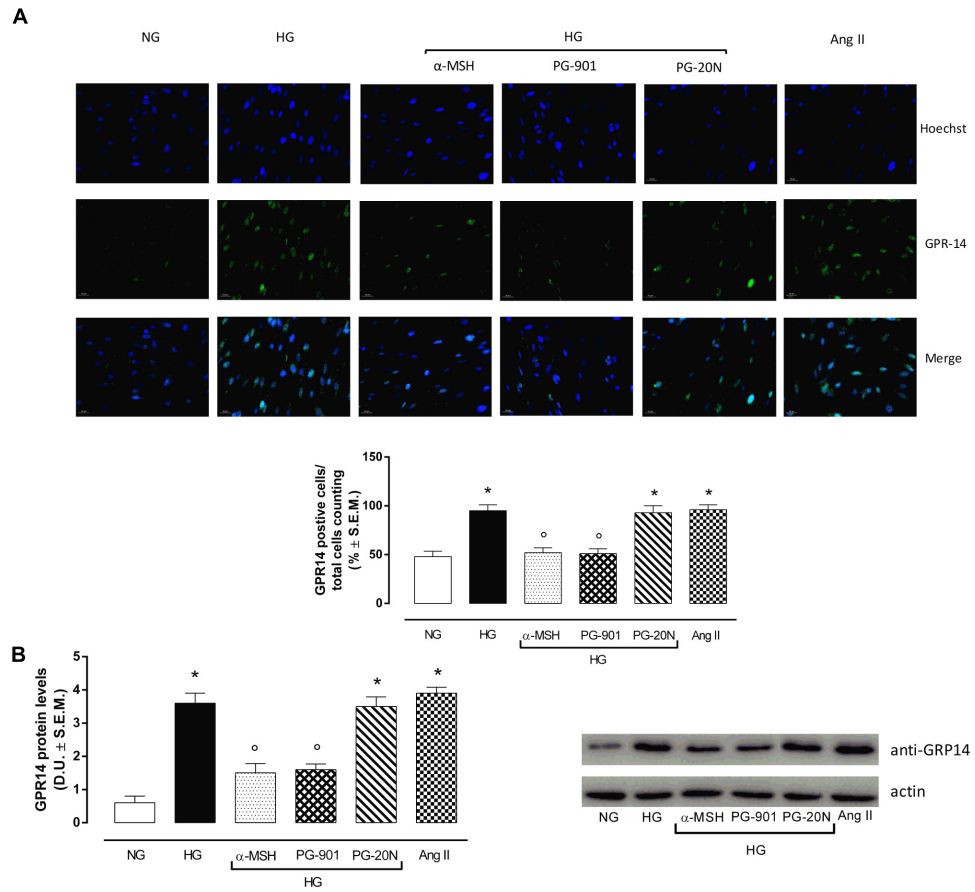

FIGURE 3 | Urotensin II receptor immunocytochemistry and Western Blotting Assay. **(A)** Representative immunofluorescence images (20X; in blue Hoechst for nucleus and in green GRP14 for Urotensin II receptor labeling) show that H9c2 cells exposed to high glucose (33 mM D-glucose) exhibited an evident increase Urotensin II levels, highly expressed in hypertrophic cardiomyocytes compared to cells exposed to normal glucose (5.5 mM D-glucose). MC5R agonism with α-MSH (90 pM) and PG-901 (10^{-10} M) treatment significantly decreased Urotensin II labeling in H9c2 cells exposed to high glucose. MC5R antagonist PG-20N treatment in H9c2 cells exposed to high glucose showed high Urotensin II levels. The percentage of positive cells was calculated by the number of labeled positive cells of 300 cells in four different microscope fields. **(B)** Western Blots analysis detecting GPR-14 protein levels confirmed the elevated protein expression of Urotensin II receptor in hypertrophic H9c2 cells; this was significantly reduced in H9c2 cells exposed to high glucose and treated with MCR5 agonists. Values are expressed as mean ± S.E.M. of $n = 9$ values, obtained from the triplicates of three independent experiments. NG, normal glucose; Ang II, angiotensin II; HG, high glucose; D.U., Densitometric Units; $^{*}P < 0,01$ vs. NG; $^{\circ}P < 0,01$ vs. HG.

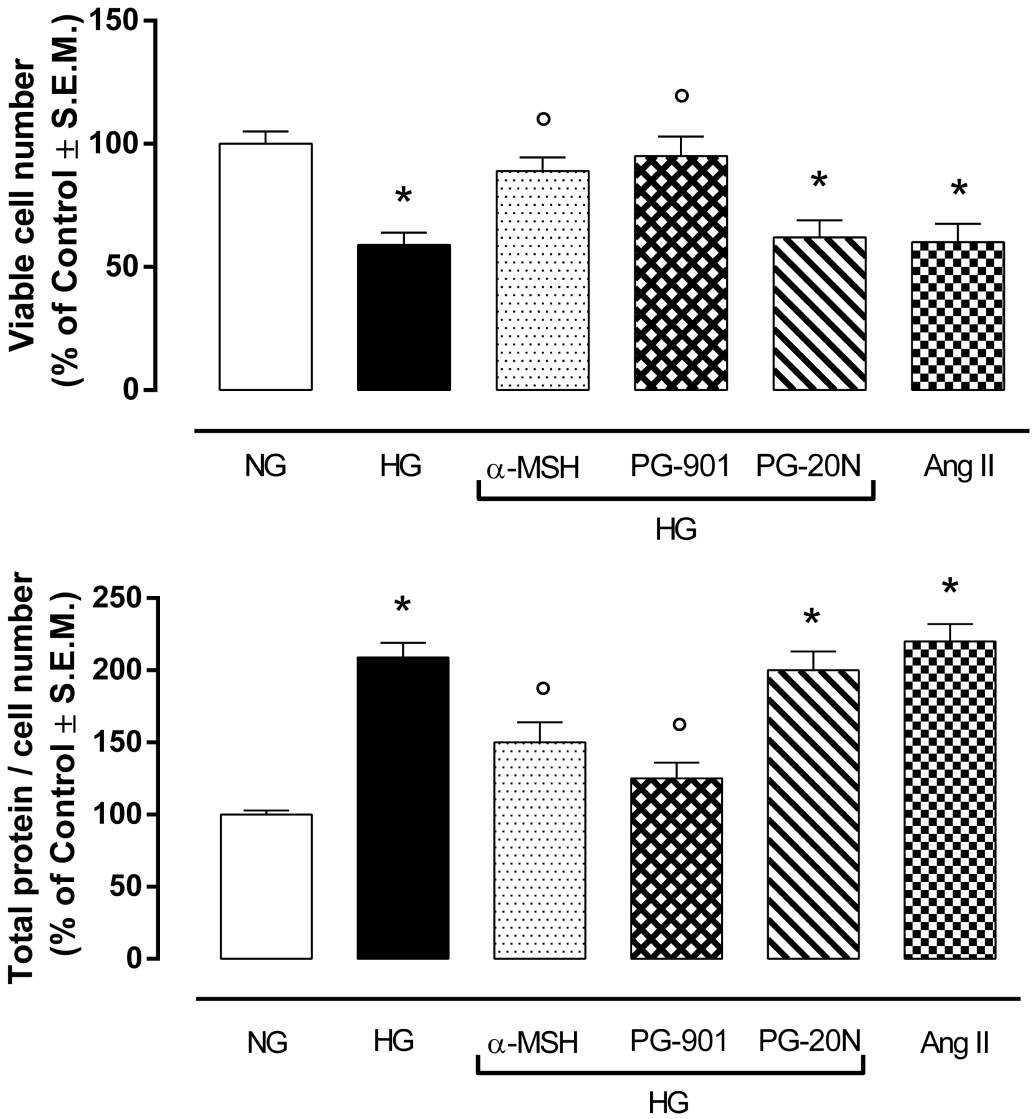

FIGURE 4 | MTT assay and determination of total protein / viable cell number at direct cell counting. H9c2 cells exposed to high glucose (33 mM D-glucose) exhibited a significant decrease in cell viability and higher total protein / viable cell number ratio, as a marker of cardiac hypertrophy, compared to cell exposed to normal glucose (5.5 mM D-glucose). Agonism at MC5R with a-MSH (90 pM) and selectively with PG-901 (10^{-10} M) increased cell viability and decreased total protein / viable cell number ratio in cells exposed to high glucose. Cell viability values and total protein / cell number ratio exhibited by H9c2 cells exposed to high glucose were not affected by PG-20N MC5R antagonist (130 nM). Values are expressed as mean ± S.E.M. of $n = 9$ values, obtained from the triplicates of three independent experiments. NG, normal glucose; Ang II, angiotensin II; HG, high glucose; *$P < 0,01$ vs. NG; °$P < 0,01$ vs. HG.

(+123,4%, $P < 0,01$ vs. NG). The agonism at MC5R with α-MSH and PG-901 significantly reduced cellular glucose uptake, as measured from ATP levels compared to HG cells (−40,5 and 45,2%, respectively, $P < 0,01$ vs. HG). The PG-20N antagonist did not lead to any change of cellular glucose content (**Figure 5A**). H9c2 cells exposed to high glucose were also characterized by a significant reduction in $K_{IR}6.1$ protein levels (−56,0%, $P < 0,01$ vs. NG) compared to control cells, probably due to the high ATP intracellular levels. In contrast, the reduction in intracellular ATP content due to α-MSH and PG-901 MC5R agonism was paralleled by a significant increase in $K_{IR}6.1$ protein expression (+90,9 and +100,0%, respectively, $P < 0,01$ vs. HG), compared to HG cells (**Figure 5B**).

Reduction of miR-133a Levels by MC5R Agonists in H9c2 Cells Exposed to High Glucose Stimulus and Consequent PI3K Activation

qRT-PCR analysis showed an overexpression of miR-133a in H9c2 exposed to high glucose stimuli and transfected with negative control mina inhibitor, compared to control cells (+95,4%, $P < 0,01$ vs. NG) (**Figure 6A**). As expected, this was

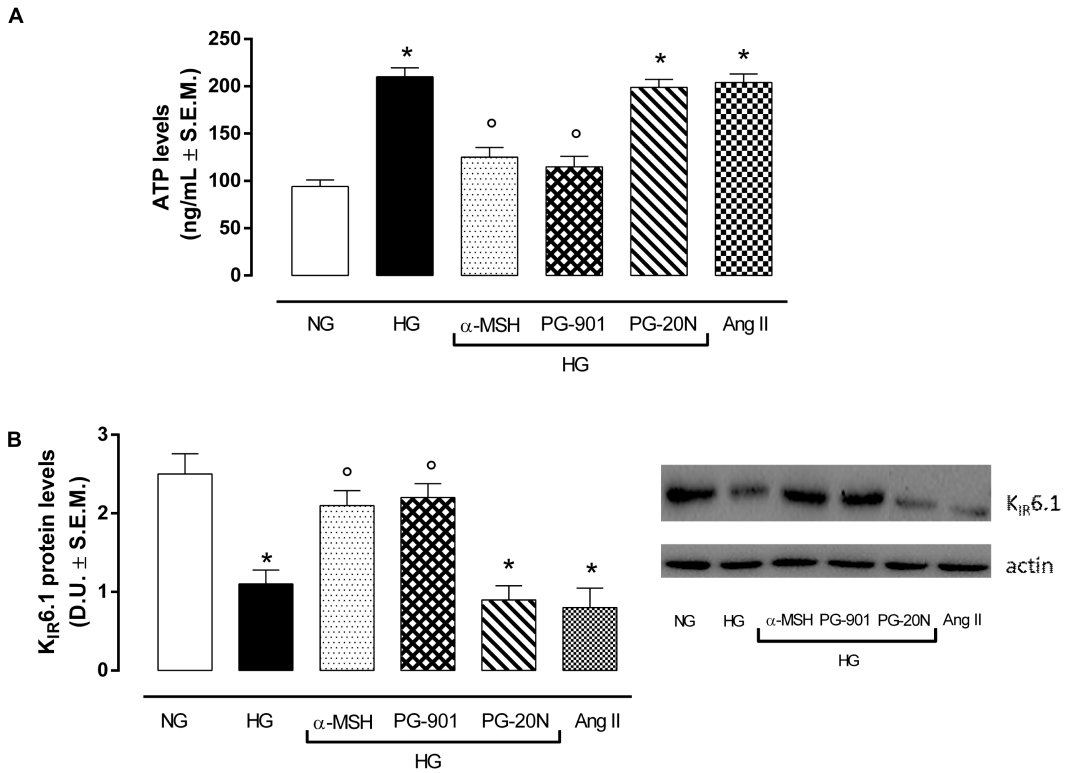

FIGURE 5 | ATP levels as a marker of glucose content andKIR6.1 protein expression. **(A)** As expected, H9c2 cells exposed to high glucose (33 mM D-glucose) showed increased ATP levels compared to cell exposed to normal glucose (5.5 mM D-glucose). a-MSH (90 pM) and PG-901 (10^{-10} M) significantly decreased ATP levels, reducing the high cellular glucose uptake exhibited by HG cells. PG-20N antagonist did not modify ATP levels. **(B)** Western Blotting analysis of KIR6.1 protein levels showed a significant KIR6.1 down-regulation in H9c2 cells exposed to high glucose, probably due to high ATP intracellular content. The ATP levels reduction caused by MC5R agonists α-MSH and PG-901in H9c2 cells exposed to high glucose was paralleled by a significant increase in $K_{IR}6.1$ protein expression. Values are expressed as mean ± S.E.M. of $n = 9$ values, obtained from the triplicates of three independent experiments. NG, normal glucose; Ang II, angiotensin II; HG, high glucose; D.U., Densitometric Units;*$P < 0,01$ vs. NG; °$P < 0,01$ vs. HG.

paralleled by a decreased PI3K activity in H9c2 cells exposed to HG (−57,0%, $P < 0,01$ vs. NG), being PI3K targeted by miR-133a (**Figure 6B**). Interestingly, α-MSH significantly reduced the miR-133a expression over by −47.0%, ($P < 0,01$ vs. HG) and consequently recovering PI3K activity (+82,1%, $P < 0,01$ vs. HG). These evidences on miR-133a and PI3K by α-MSH were copied by MC5R agonist PG-901 (−45,8 and +67,4%, respectively, $P < 0,01$ vs. HG). No sign of significative change was seen with PG-20N on the parameters recorded in HG (**Figures 6A,B**). H9c2 cells exposed to all experimental conditions and transfected with anti-miR-133a evidenced reduced miR-133a levels, as expected. These were paralleled by increased PI3Kactivity showed by H9c2 cells exposed to high glucose with or without MC5R agonists and antagonist (**Figures 6A,B**).

MC5R Agonism Reduces the Elevated GLUT1/GLUT4 Ratio Induced by High Glucose in H9c2 Hypertrophy

An elevated GLUT1/GLUT4 ratio, a characteristic feature of cardiac hypertrophy, was found to be significant in cells exposed to high glucose (+137,2%, $P < 0,01$ vs. NG). Interestingly, GLUT1/GLUT4 ratio was significantly reduced in H9c2 exposed to high glucose treated with α-MSH (−55,6%, $P < 0,01$ vs. HG) and with MC5R agonist PG-901 (−56,7, $P < 0,01$ vs. HG). PG-20N, a selective MC5R antagonist, did not change the GLUT1/GLT4 ratio observed in HG cells (**Figure 7**).

MC5R Agonism Modulates *in vivo* High Glucose-Induced Cardiac Alterations

As shown in **Table 1** by M-mode measurements, diabetic rats (STZ) had a significantly greater left ventricular mass per body weight, compared to non-diabetic rats (+82,6%, $P < 0,05$ vs. CTRL). This was significantly reduced by α-MSH (−30,9%, $P < 0,05$ vs. STZ) and by the doses of 500 and 5000 μg/kg PG-901 (−33,3 and −35,7%, respectively, $P < 0,05$ vs. STZ). While systolic, diastolic and relative wall thicknesses were similar between non-diabetic and diabetic rats, systolic and diastolic left ventricular cavity dimensions were significantly increased in STZ animals, compared to CTRL group (+23,6%, $P < 0,05$ vs. CTRL and +21,6%, $P < 0,01$ vs. CTRL, respectively). Left ventricular internal dimensions in diastole were significantly reduced by MC5R agonists (−13,3% by α-MSH, −13,8% by PG-901 500 μg/kg and −15,1% by PG-901 5000 μg/kg, $P < 0,05$

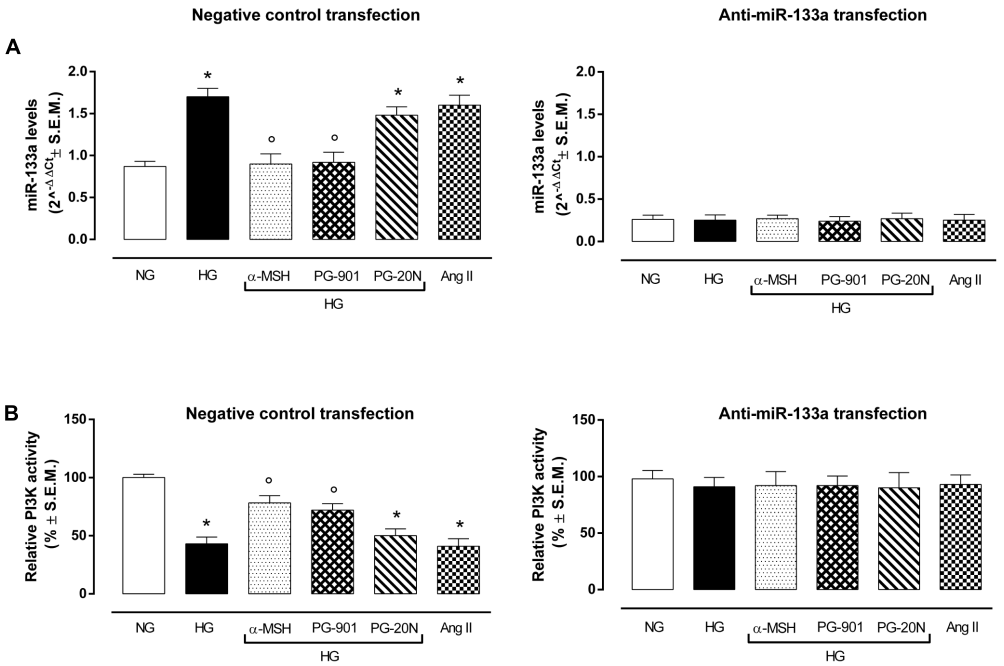

FIGURE 6 | miR-133a levels and PI3K activity. **(A)** H9c2 cells exposed to high glucose (33 mM D-glucose) and transfected with negative control miRNA inhibitor showed increased miR-133a levels compared to cell exposed to normal glucose (5.5 mM D-glucose). a-MSH (90 pM) and PG-901 (10^{-10} M) significantly decreased miR-133a levels, while PG-20N antagonist didn't modify miR-133a expression. As expected, miR-133a knockdown abolished this differential expression pattern in our experimental setting. Values are expressed as mean of $2^{-\Delta\Delta Ct} \pm$ S.E.M. of $n = 3$ independent experiments, performing each treatment in triplicate in a single experiment. **(B)** Fitting with these evidences, PI3K activity was decreased in H9c2 cells exposed to high glucose, but it was significantly reverted by a-MSH (90 pM) and PG-901 (10^{-10} M).miR-133a knockdown reverted PI3K activity to values expressed by control cells in all the experimental conditions. Values are expressed as mean ± S.E.M. of $n = 9$ values, obtained from the triplicates of three independent experiments. NG, normal glucose; Ang II, angiotensin II; HG, high glucose; $^*P < 0{,}01$ vs. NG; $^\circ P < 0{,}01$ vs. HG.

vs. STZ), as well as the left ventricular internal dimensions in systole (−8,1% by α-MSH, −9,7% by PG-901 500 μg/kg and −12,9% by PG-901 5000 μg/kg, $P < 0{,}01$ vs. STZ). Diabetic rats showed a significant reduction in left ventricular fractional shortening (LVFS; −26,3%, $P < 0{,}01$ vs. CTRL), ejection fraction (LVEF; −11,1%, $P < 0{,}01$ vs. CTRL) and circumferential fiber shortening values (VCF; −28,9%, $P < 0{,}01$ vs. CTRL), compared to CTRL animals. These values were increased by α-MSH (+22,2% in LVFS,+6,9% in LVEF and +21,9% in VCF, $P < 0{,}05$ vs. STZ), PG-901 500 μg/kg (+23,8% in LVFS,+7,8% in LVEF and +25,0% in VCF, $P < 0{,}05$ vs. STZ) and PG-901 5000 μg/kg (+29,2% in LVFS and +28,1% in VCF, $P < 0{,}05$ vs. STZ;+8,8 in LVEF, $P < 0{,}01$ vs. STZ). Isovolumetric relaxation time (IVRT) and the ratio of maximal early diastolic peak velocity / late peak velocity of mitral flow (E/A ratio) were significantly decreased in STZ group compared to non-diabetic rats (−26,7 and −20%, respectively, $P < 0{,}01$ vs. CTRL) and significantly increased by treatment with MC5R agonists (−24,5% in IVRT and −17,4% in E/A with α-MSH, $P < 0{,}05$ vs. STZ; −26,5% in IVRT and −18,9% in E/A with PG-901 500 μg/kg, $P < 0{,}05$ vs. STZ; −31,7% in IVRT, $P < 0{,}01$ vs. STZ and −20,4% in E/A, $P < 0{,}05$ vs. STZ with PG-901 5000 μg/kg). Myocardial performance index was greater in the diabetic group compared to the non-diabetic animals (+53,6%, $P < 0{,}01$ vs. CTRL), and it was reduced both by α-MSH administration (−18,6%, $P < 0{,}05$ vs. STZ) that by PG-901 at the doses of 500 μg/kg (−20,9%, $P < 0{,}05$ vs. STZ) and of 5000 μg/kg (−23,2%, $P < 0{,}01$ vs. STZ). These improved echocardiographic parameters exhibited by diabetic rats treated with MC5R agonists were paralleled also by significantly reduced blood glucose levels (**Table 1**).

DISCUSSION

Melanocortin receptor 5, (MC5R) predominantly activated by α-MSH and then equally by ACTH, β-MSH, and γ-MSH, is highly expressed in skeletal muscles, exocrine and sebaceous glands, while at a lower level MC5R mRNA is been detected in rodent adipocytes (Chen et al., 1997; Abdel-Malek, 2001; Getting, 2006). Although MC5R functions and signaling are still poorly understood, they are speculated to regulate aldosterone secretion and to mediate neuro-myotrophic, gastric, and anti-inflammatory effects (Gantz et al., 1994; Griffon et al., 1994; Fathi et al., 1995). Interestingly, these G-protein receptors functionally coupled to adenylate cyclase have been recently showed to mediate the skeletal muscle glucose uptake through protein kinase A regulation: the authors demonstrated for the first time that peripheral α-MSH significantly increases glucose uptake via the activation of MC5R and PKA on soleus and

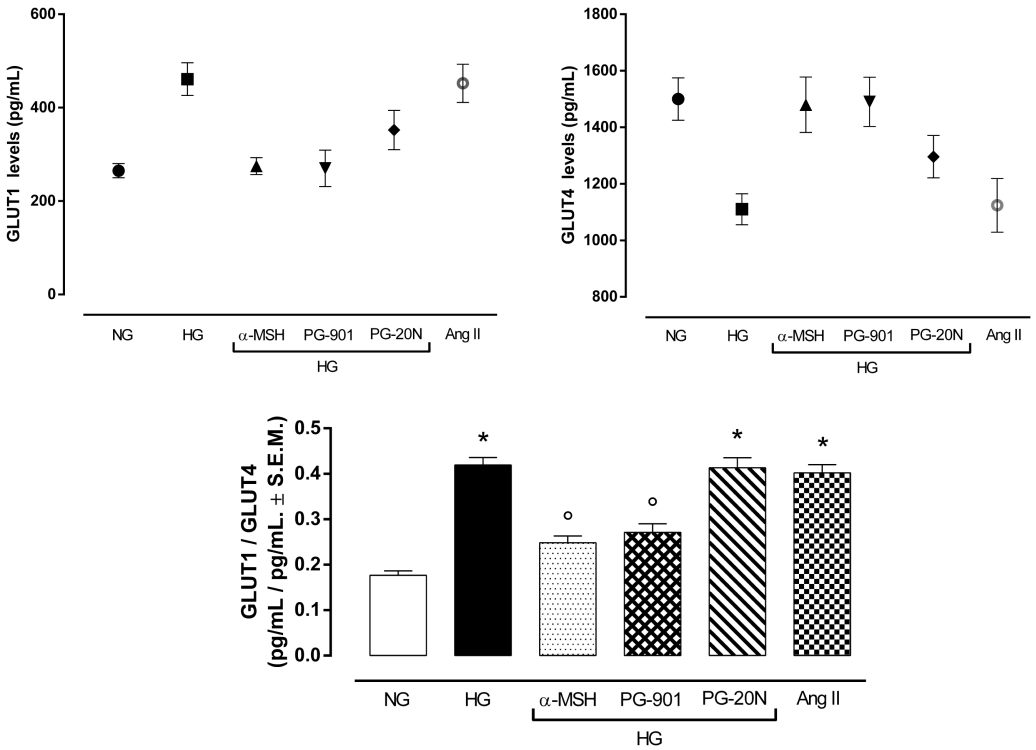

FIGURE 7 | Plasma membrane GLUT1 and GLUT4 levels. In line with previous evidences, H9c2 cells exposed to high glucose (33 mM D-glucose) exhibited lower GLUT4 levels and increased GLUT1 levels in plasma membrane, resulting in a higher GLUT1/GLUT4 levels compared to cells exposed to normal glucose (5.5 mM D-glucose). a-MSH (90 pM) and PG-901 (10^{-10} M) significantly restored GLUT1/GLUT4 ratio, by increasing GLUT4 and consequently decreasing GLUT1 plasma membrane levels. PG-20N MC5R antagonist (130 nM) did not lead to any modification of the high GLUT1/GLUT4 ratio induced by high glucose. Values are expressed as mean ± S.E.M. of $n = 9$ values, obtained from the triplicates of three independent experiments. NG, normal glucose; Ang II, angiotensin II; HG, high glucose; $^{*}P < 0{,}01$ vs. NG; $^{\circ}P < 0{,}01$ vs. HG.

TABLE 1 | *In vivo* cardiac parameters evaluated by echocardiography.

	CTRL	STZ	STZ + α-MSH	STZ + PG-901		
		65 mg/kg	500 μg/Kg	50 μg/Kg	500 μg/Kg	5000 μg/Kg
Blood glucose (mg/dl)	90 ± 8	351 ± 12*	284 ± 9°	331 ± 11	290 ± 13°	274 ± 9°
LV mass/BW (mg/g)	2, 3 ± 0, 4	4, 2 ± 0, 5**	2, 9 ± 0, 5°°	3, 1 ± 0, 4	2, 8 ± 0, 6°°	2, 7 ± 0, 5°°
AWTd/TB (cm/cm)	0, 42 ± 0, 12	0, 46 ± 0, 11	0, 41 ± 0, 15	0, 43 ± 0, 14	0, 40 ± 0, 10	0, 44 ± 0, 12
AWTs/TB (cm/cm)	0, 76 ± 0, 15	0, 74 ± 0, 12	0, 72 ± 0, 14	0, 75 ± 0, 11	0, 78 ± 0, 14	0, 71 ± 0, 21
PWTd/TB (cm/cm)	0, 41 ± 0, 15	0, 45 ± 0, 18	0, 44 ± 0, 13	0, 42 ± 0, 16	0, 44 ± 12	0, 46 ± 0, 11
PWTs/TB (cm/cm)	0, 74 ± 0, 12	0, 71 ± 0, 16	0, 75 ± 0, 22	0, 73 ± 0, 14	0, 75 ± 0, 19	0, 74 ± 0, 18
LVd/TB (cm/cm)	1, 82 ± 0, 1	2, 25 ± 0, 1**	1, 95 ± 0, 1°°	1, 99 ± 0, 2	1, 94 ± 0, 1°°	1, 91 ± 0, 1°°
LVs/TB (cm/cm)	0, 51 ± 0, 008	0, 62 ± 0, 007*	0, 57 ± 0, 009°	0, 61 ± 0, 01	0, 56 ± 0, 01°	0, 54 ± 0, 008°
RWT	0, 41 ± 0, 11	0, 39 ± 0, 09	0, 40 ± 0, 10	0, 42 ± 0, 08	0, 38 ± 0, 11	0, 41 ± 0, 12
LVFS (%)	44, 5 ± 2, 5	32, 8 ± 2, 1*	40, 1 ± 2, 4°°	37, 2 ± 2, 8	40, 6 ± 2, 2°°	42, 4 ± 2, 5°°
LVEF (%)	76, 3 ± 1, 6	67, 8 ± 1, 1*	72, 5 ± 1, 8°°	70, 7 ± 1, 6	73, 1 ± 1, 4°°	73, 8 ± 1, 2°
VCF (circ/sec)	0, 0045 ± 0.0002	0, 0032 ± 0, 0003*	0, 0039 ± 0, 0002°°	0, 0037 ± 0, 0004	0, 0040 ± 0, 0002°°	0, 0041 ± 0, 0002°°
IVRT (msec)	41, 2 ± 1, 52	30, 2 ± 1, 68*	37, 6 ± 1, 56°°	33, 8 ± 1, 55	38, 2 ± 1, 62°	39, 8 ± 1, 50°
E/A ratio (msec)	1, 65 ± 0, 05	1, 32 ± 0, 08*	1, 55 ± 0, 09°°	1, 50 ± 0, 09	1, 57 ± 0, 07°°	1, 59 ± 0, 08°°
MPI	0, 28 ± 0, 028	0, 43 ± 0, 016*	0, 35 ± 0, 026°°	0, 039 ± 0, 028	0, 34 ± 0, 024°°	0, 33 ± 0, 019°

LV mass, left ventricular mass; BW, body weight; AWTd, anterior wall thickness in diastole; TB, tibial length; AWTs, anterior wall thickness in systole; PWTd, posterior wall thickness in diastole; PWTs, posterior wall thickness in systole; LVd, left ventricular internal dimensions in diastole; LVs, left ventricular internal dimensions in systole; RWT, relative wall thickness; LVFS, left ventricular fractional shortening; LVEF, left ventricular ejection fraction; VCF, left ventricular circumferential fiber shortening; IRVT, isovolumetric relaxation time; E/A ratio, ratio of maximal early diastolic peak velocity / late peak velocity of mitral flow; MPI, myocardial performance index. Values are expressed as mean ± S.E.M. of n = 5 animals; for each rat the average was obtained from three consecutive cardiac cycles. CTRL, non-diabetic rats; STZ, diabetic rats; $^{**}P < 0{,}05$ and $^{*}P < 0{,}01$ vs. CTRL; $^{\circ\circ}P < 0{,}01$ and $^{\circ}P < 0{,}05$ vs. STZ.

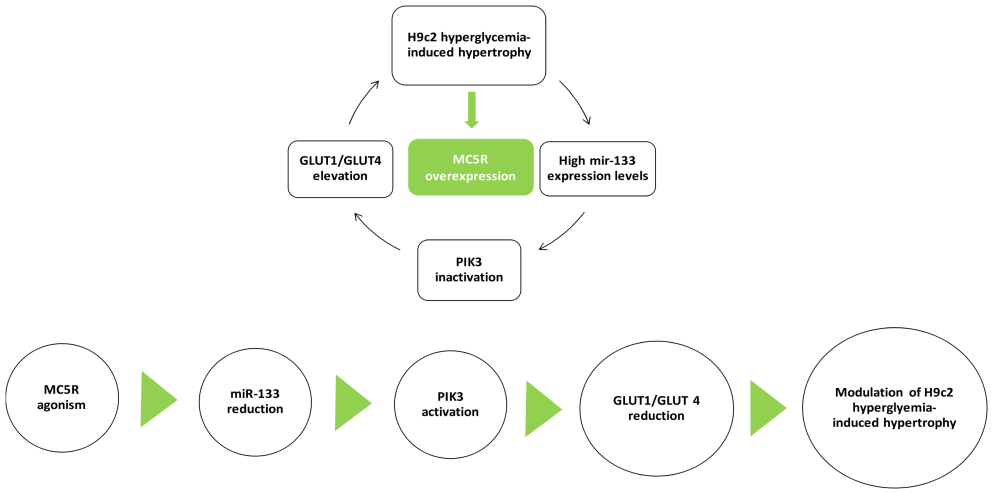

FIGURE 8 | MC5R signaling in cardiac hypertrophy induced by high glucose. H9c2 hypertrophy induced by hyperglycemia is characterized by increased miR-133a levels, leading to PI3K inactivation and GLUT1/GLUT4 elevation. Here, we show for the first time an up-regulation of MC5R, involved in glucose-uptake, as a defensive and anti-hypertrophic response to the high-glucose induced damage. MC5R agonism reduced H9c2 hypertrophic markers by decreasing miR-133a levels, consequently restoring PI3K activity and GLUT1/GLUT4 ratio.

gastrocnemius muscles (Abdel-Malek, 2001; Enriori et al., 2016; Hill and Faulkner, 2017).

Although glucose is a primary substrate for heart metabolism and alterations of glucose uptake are tightly associated with cardiovascular diseases, particularly cardiac hypertrophy induced by hyperglycemia, the role of melanocortin receptors in mediating cardiac glucose uptake has been never explored before (Fathi et al., 1995; Wang et al., 2013; Han et al., 2015; Szablewski, 2017). Considering these evidences, we investigated the role of MC5R in mediating cardiac glucose-uptake in H9c2 exposed to high glucose and the role played by MC5R in the glucose-induced hypertrophy. Recent evidences showed that the exposure of rat H9c2 cardiac myocytes to high glucose can be considered an useful *in vitro* model of myocardial hypertrophy, since high glucose levels rapidly induce connective tissue growth factor (CTGF) mRNA that mediates hypertrophy on H9c2 cells (Wang et al., 2009; Han et al., 2015; Li et al., 2017; Wei et al., 2018). Interestingly, H9c2 cells showed a significant up-regulation of MC5R mRNA and protein content following high glucose exposure compared to cells exposed to normal glucose. This increased MC5R expression probably being a defensive and anti-hypertrophic response to the high-glucose induced damage, since by activating MC5Rwith α-MSH or with PG-901 the cell viability was increased and the cardiac hypertrophy markers, including the high intracellular glucose content (detected by ATP levels determination), were decreased compared to cells exposed to high glucose only. Interestingly, the modulation of intracellular glucose content was paralleled by changes in K_{ATP} Inward Rectifier K^+ Channel 4 ($K_{IR}6.1$ or KCJN8) expression. The *KCNJ8*-encoded Kir6.1 (K_{ATP}) channel is an important regulator of vascular tone and cardiac adaptive response to metabolic stress (Tester et al., 2011), activated by low intracellular ATP levels (Takano and Noma, 1993). In our setting, $K_{IR}6.1$ protein levels were significantly decreased by H9c2 exposure to high glucose, in line with previous works (Liang et al., 2016, 2017; Trotta et al., 2017), and were restored by a-MSH and PG-901 treatments. This may be due to the modulation of ATP intracellular levels exerted by MC5R agonists: while the high ATP intracellular content in H9c2 cells exposed to high glucose was paralleled by a decreased $K_{IR}6.1$ protein expression, a reduction in ATP levels was paralleled by an increase in $K_{IR}6.1$ protein levels. Therefore, MC5R stands as a receptor to target in order to modulate the high glucose-induced hypertrophy.

From the molecular point of view and in order to ascertain the intracellular MC5R signaling, our study focused on phosphoinositide 3-kinase (PI3K). This lipid kinase, activated by G protein-coupled receptors (GPCRs) through the direct binding of Gβγ subunits and the small GTPase Ras to PI3K, is a major player for mediating insulin-induced glucose uptake in skeletal and cardiac muscle (Le Marchand-Brustel et al., 1995; Schwindinger and Robishaw, 2001; Riley et al., 2006). Here, we show that both the α-MSH and PG-901 increase the PI3K activity within the H9c2 cells exposed to high glucose, on HEK293 cells (Rodrigues et al., 2009). Moreover, being PI3K also involved in the regulation of H9c2 survival through Akt phosphorylation, leading to a reduction of cell death (Wang et al., 2015; Liu et al., 2017), the protective action of MC5R agonism on cell viability could be just linked to PI3K activation.

PI3K activity is usually modulated by miR-133a, a miRNA involved in regulation of cardiac hypertrophy: increased levels of miR-133aare paralleled by PI3K inactivation (Horie et al., 2009; Abdellatif, 2010; Josse et al., 2014) or viceversa low levels of miR-133a link to PI3K activation. Fitting with this, the high glucose exposure markedly increased miR-133a levels in our setting and reduced PI3K activity. In contrast, the MC5R activation by α-MSH and more selectively with the PG-901 agonist, significantly reduced miR-133a levels, consequently

increasing PI3K activity. Moreover, miR-133a knockdown in H9c2 cells exposed to high glucose alone or with MC5R agonists reverted PI3K activity to the levels exposed by normal cells. This confirmed also MC5R modulation of miR-133a levels and consequently, of PI3K activity, evidenced in our experimental setting. However, the molecular mechanisms by which MC5R activation regulates miR-133a levels have to be further investigated.

PI3K inactivation leads to increase of plasma membrane GLUT1/GLUT4 ratio, a feature of pathological cardiac hypertrophy. In a normal adult heart GLUT4 is the primary glucose transporter translocating on plasma membrane after insulin stimulation, while the mediator of basal cardiac glucose uptake GLUT1 is downregulated after birth. Conversely, a pathological hypertrophic condition links a GLUT4 depletion, resulting in a direct increase in GLUT1 levels (Slot et al., 1991; Abel et al., 1999; Paternostro et al., 1999; Tian et al., 2001; Kolwicz and Tian, 2011; Shao and Tian, 2015). PI3K activation appears to be necessary for GLUT4 translocation in the heart, being insulin stimulated GLUT4 translocation blocked by PI3K inhibitor (Egert et al., 1997; Vlavcheski et al., 2018). In line with these evidences, our results furtherly showed that PI3K inactivation induced by high glucose exposure in H9c2 cells lead to a significant increased plasma membrane GLUT1/GLUT4 ratio compared to cells exposed to high glucose. This was reverted by the PI3K activation induced by the stimulation of MC5R with α-MSH and PG-901, thus significantly decreasing GLUT1/GLUT4 ratio.

Therefore, the MC5R is pivotal for cardiac hypertrophy. However, the role exerted by MC5R could have limitations due to the nature of the cells used: H9c2 cells are still physiologically far from being primary cardiomyocytes. On another note, however, these cells presents advantages linked to the fact that they can be easily manipulated and exhibit longer survival and growth with respect to adult cardiomyocytes, that can only be maintained for a short time in culture after a technically challenging isolation (Peter et al., 2016). Moreover, cultures of rat neonatal cardiomyocytes have recently become the standard experimental *in vitro* system used to investigate the aberrant molecular processes occurring during cardiac hypertrophy (Watkins et al., 2011). However, the role of MC5R agonism in modulating cardiac alterations induced by high-glucose was here confirmed also by echocardiographic evaluations in STZ-diabetic Sprague Dawley rats treated with α-MSH and PG-901. The increase shown by STZ-diabetic rats in left ventricular mass per body weight and myocardial performance index, in line with previous evidences (Wichi et al., 2007; Di Filippo et al., 2014), was significantly reduced by α-MSH and PG-901 treatment. Although the increase in LV mass can be interpreted as a consequence of increased myocyte volume and thus hypertrophy it cannot be ruled out that this parameter has changed as a consequence of edema or alterations in the homeostasis of different cell types. Noteworthy, cardiac hypertrophy is the abnormal enlargement, or thickening, of the heart muscle, resulting from increases in cardiomyocyte size and changes in other heart muscle components, such as extracellular matrix. Moreover, the reduced values of left ventricular internal dimensions, fractional shortening, ejection fraction, and circumferential fiber shortening evidenced by STZ-diabetic rats and confirmed by previous works (Joffe et al., 1999; Wichi et al., 2007) clearly evidence a sort of dilated cardiomyopathy, together with a probable fibrosis not measured here, that occurred in these animals, in line with many recent studies in STZ models (Chengji and Xianjin, 2018). These parameters were significantly improved by α-MSH and PG-901, as well as the isovolumetric relaxation time and E/A ratio values.

CONCLUSION

The MC5R seems to be a new target in high glucose-induced cardiac myocytes derangements, and an agonism at this receptor can be a strategic tool to reduce these conditions. *In vitro*, the selective MC5R agonism seems to reduce GLUT1/GLUT4 ratio through PI3K activation, mediated by a decrease in miR-133a levels (**Figure 8**). These evidences open new possibilities for therapeutic interventions through peripheral melanocortin pathways.

AUTHOR CONTRIBUTIONS

MT, RM, and AH performed the research. NA contributed to immunofluorescence analysis and results interpretation. MD analyzed the data. CT and CD designed and wrote the research study.

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REVIEW
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The Role of Sodium in Diabetic Cardiomyopathy

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Cardiovascular complications are the major cause of mortality and morbidity in diabetic patients. The changes in myocardial structure and function associated with diabetes are collectively called diabetic cardiomyopathy. Numerous molecular mechanisms have been proposed that could contribute to the development of diabetic cardiomyopathy and have been studied in various animal models of type 1 or type 2 diabetes. The current review focuses on the role of sodium (Na^+) in diabetic cardiomyopathy and provides unique data on the linkage between Na^+ flux and energy metabolism, studied with non-invasive ^{23}Na, and ^{31}P-NMR spectroscopy, polarography, and mass spectroscopy. ^{23}Na NMR studies allow determination of the intracellular and extracellular Na^+ pools by splitting the total Na^+ peak into two resonances after the addition of a shift reagent to the perfusate. Using this technology, we found that intracellular Na^+ is approximately two times higher in diabetic cardiomyocytes than in control possibly due to combined changes in the activity of Na^+–K^+ pump, Na^+/H^+ exchanger 1 (NHE1) and Na^+-glucose cotransporter. We hypothesized that the increase in Na^+ activates the mitochondrial membrane Na^+/Ca^{2+} exchanger, which leads to a loss of intramitochondrial Ca^{2+}, with a subsequent alteration in mitochondrial bioenergetics and function. Using isolated mitochondria, we showed that the addition of Na^+ (1–10 mM) led to a dose-dependent decrease in oxidative phosphorylation and that this effect was reversed by providing extramitochondrial Ca^{2+} or by inhibiting the mitochondrial Na^+/Ca^{2+} exchanger with diltiazem. Similar experiments with ^{31}P-NMR in isolated superfused mitochondria embedded in agarose beads showed that Na^+ (3–30 mM) led to significantly decreased ATP levels and that this effect was stronger in diabetic rats. These data suggest that in diabetic cardiomyocytes, increased Na^+ leads to abnormalities in oxidative phosphorylation and a subsequent decrease in ATP levels. In support of these data, using ^{31}P-NMR, we showed that the baseline β-ATP and phosphocreatine (PCr) were lower in diabetic cardiomyocytes than in control, suggesting that diabetic cardiomyocytes have depressed bioenergetic function. Thus, both altered intracellular Na^+ levels and bioenergetics and their interactions may significantly contribute to the pathology of diabetic cardiomyopathy.

Keywords: sodium, calcium–sodium exchanger, NMRS, oxygen consumption, mitochondrial bioenergetics

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DIABETIC CARDIOMYOPATHY

Diabetic cardiomyopathy is a multi-faceted disease. Diabetes is associated with an increased incidence of atherosclerotic heart disease, which results in ischemic cardiomyopathy. In addition to ischemic – heart – disease associated cardiomyopathy, there are other metabolic changes in the heart that are not necessarily related to myocardial ischemia. There is altered substrate utilization and mitochondrial dysfunction; insulin resistance; decreased flexibility in substrate use; changes in oxidative phosphorylation and the citric acid cycle; abnormalities in ketogenesis and glucose free fatty acid (FFA) cycling; and altered Ca^{2+} handling (Boudina and Abel, 2010; Veeranki et al., 2016; Jia et al., 2018).

Changes in mitochondrial morphology are associated with remodeling of the mitochondrial proteome and decreased respiratory capacity. There are changes in mitochondrial bioenergetics with decreased phosphocreatine (PCr)/ATP (shown with ^{31}P NMR); decreased oxygen consumption and increased H_2O_2 production; defects in the ATP sensitive K^+ channel (K_{ATP}); mitochondrial uncoupling resulting in increased state 4 respiration and decreased ATP synthesis and increased oxygen consumption without increased ATP production; and mitochondrial generation of reactive oxygen species (ROS) and lipid peroxides that may activate uncoupling proteins (Boudina and Abel, 2010; Veeranki et al., 2016; Jia et al., 2018).

The combination of these various insults results in left ventricular hypertrophy, interstitial fibrosis, left ventricular diastolic and systolic dysfunction, right ventricular dysfunction, and impaired contractile reserve. The purpose of this paper is to show the importance of maintaining intracellular sodium ($[Na^+]$) homeostasis in the heart and to review some of our early work of the effects of diabetes on metabolism (and vice versa) that are related to ion fluxes (Na^+, H^+, Ca^{2+}).

SODIUM TRANSPORT SYSTEMS IN CARDIOMYOCYTES

Sodium transport processes and $[Na^+]$ concentration play important roles in cellular function. $[Na^+]_i$ concentration regulates Ca^{2+} cycling, contractility, metabolism, and electrical stability of the heart (Lambert et al., 2015). In the normal cell, there is a large steady-state electrochemical gradient favoring Na^+ influx. This potential energy is used by numerous transport mechanisms, including Na^+ channels and transporters which couple Na^+ influx to either co- or counter-transport of other ions and solutes (Bers et al., 2003). Myocardial $[Na^+]_i$ is determined by the balance between Na^+ influx down a trans-sarcolemmal electrochemical gradient, via Na^+/Ca^{2+} exchanger, Na^+/H^+ exchanger 1 (NHE1), Na^+/Mg^{2+} exchange, Na^+/HCO_3^- cotransport, $Na^+/K^+/2Cl^-$ cotransport and Na^+ channels, and Na^+ efflux against an electrochemical gradient, mediated by Na^+/K^+ pump (Ottolia et al., 2013; Shattock et al., 2015). Under normal conditions, Na^+/Ca^{2+} exchange and Na^+ channels are the dominant Na^+ influx pathway; however, other transporters may become important during pathological conditions. The Na^+/Ca^{2+} exchanger transports three Na^+ ions into the cytoplasm in exchange for one Ca^{2+} ion using the energy generated from the Na^+ gradient as a driving force, and it is one of the main mechanisms for Na^+ influx in cardiomyocytes (Shattock et al., 2015). The eukaryotic Na^+/Ca^{2+} exchanger protein, as exemplified by the mammalian cardiac isoform NCX1.1, is organized into 10 transmembrane segments (TMSs; Liao et al., 2012; Ren and Philipson, 2013) and contains a large cytoplasmic loop between TMS 5 and 6 which play a regulatory role (Philipson et al., 2002). Regulation of the mammalian Na^+/Ca^{2+} exchanger has been clearly shown both at the functional and structural levels. Allosteric regulation of the Na^+/Ca^{2+} exchanger, by cytoplasmic Na^+ and Ca^{2+} ions, occurs from within the large cytoplasmic loop that separates TMS 5 from TMS 6 (Philipson et al., 2002). The structures of the two regulatory domains within this region of the eukaryotic exchanger have been described (Hilge et al., 2006; Nicoll et al., 2006; Besserer et al., 2007; Wu et al., 2010). These two contiguous stretches of residues bind cytoplasmic Ca^{2+}, leading to an increase in exchanger activity (Hilgemann et al., 1992; Matsuoka et al., 1995; Chaptal et al., 2009; Ottolia et al., 2009), and are identified as Ca^{2+} binding domains 1 and 2. Na^+ ion regulation of the Na^+/Ca^{2+} exchanger is less well studied; however, it is known that high cytoplasmic Na^+ inactivates the exchanger (Hilgemann et al., 1992). Whether Na^+/Ca^{2+} exchanger modulation by cytoplasmic Na^+ is relevant to cardiac physiology remains to be established since relatively high intracellular Na^+ concentrations ($\geq$20 mM) are required to significantly inactivate the exchanger experimentally (Hilgemann et al., 1992; Matsuoka et al., 1995). Recently, Liu and O'Rourke (2013) revealed a novel mechanism of Na^+/Ca^{2+} exchanger regulation by cytosolic NADH/NAD^+ redox potential through a ROS-generating NADH-driven flavoprotein oxidase. The authors proposed that this mechanism may play key roles in Ca^{2+} homeostasis and the response to the alteration of protein kinase C (PKC) in the cytosolic pyrine nucleotide redox state during cardiovascular diseases, including ischemia–reperfusion (Liu and O'Rourke, 2013). Acting in the opposite direction, the Na^+/K^+ pump moves Na^+ ions from the cytoplasm to the extracellular space against their gradient by utilizing the energy released from ATP hydrolysis. One of the strongest drivers for the activation of the Na^+/K^+ pump is the elevation of $[Na^+]_i$ (Shattock et al., 2015). A fine balance between the Na^+/Ca^{2+} exchanger and the Na^+/K^+ pump controls the net amount of $[Na^+]_i$, and aberrations in either of these two systems can have a large impact on cardiac function (Shattock et al., 2015). While the relevance of Ca^{2+} homeostasis in cardiac function has been extensively investigated (Ottolia et al., 2013), the role of Na^+ regulation in heart function and metabolism is often overlooked. Small changes in the cytoplasmic Na^+ content have multiple effects on the heart by influencing intracellular Ca^{2+} and pH levels thereby modulating heart contractility and function. Therefore, it is essential for heart cells to maintain Na^+ homeostasis. Despite the large amount of work done in the evaluation of Na^+ transport, there is little data that defines the metabolic support (oxidative phosphorylation, glycolysis, and ATPase

activity) of Na^+ transport under normal and pathophysiological conditions.

$[Na^+]_i$ and Na^+ transport are altered in several diseases, including diabetes mellitus (DM) (Kjeldsen et al., 1987; Makino et al., 1987; Warley, 1991; Regan et al., 1992; Schaffer et al., 1997; Devereux et al., 2000; Hattori et al., 2000; Taegtmeyer et al., 2002; Villa-Abrille et al., 2008; Young et al., 2009; Boudina and Abel, 2010). It has been shown in heart failure myocytes, that resting $[Na^+]_i$ was increased from 5.2 ± 1.4 to 16.8 ± 3.1 mmol/L (Liu and O'Rourke, 2008). Decreased activity of the Na^+/K^+ pump (Greene, 1986; Kjeldsen et al., 1987; Hansen et al., 2007) and Na^+/Ca^{2+} exchanger (Chattou et al., 1999; Hattori et al., 2000) were reported in hearts from animals with type 1 diabetes (T1DM). Many studies have also shown that the function and/ or expression of the Na^+/K^+ pump is reduced in cardiac hypertrophy (Pogwizd et al., 2003; Boguslavskyi et al., 2014). Previously shown, the Na^+/Ca^{2+} exchanger protein and mRNA expression levels were significantly depressed in diabetic animal models (Makino et al., 1987; Hattori et al., 2000) and Na^+/Ca^{2+} exchanger activity, but not mRNA, was decreased in streptozotocin-treated neonatal rats (Schaffer et al., 1997). Because the Na^+/Ca^{2+} exchanger is the main mechanism for systolic Ca^{2+} removal, the significant reduction in exchanger activity could increase intracellular Ca^{2+} and may contribute to diabetic cardiomyopathy as a result of altered diastolic Ca^{2+} removal (Dhalla et al., 1985; Villa-Abrille et al., 2008). It has been shown that the Na^+/Ca^{2+} exchanger activity can be restored by insulin (Villa-Abrille et al., 2008). The myocardial NHE1 was found to be enhanced in the hypertrophied Goto-Kakizaki diabetic rat heart (Darmellah et al., 2007) and led to higher $[Na^+]_i$ gain during ischemia–reperfusion (Kuo et al., 1983; Pieper et al., 1984; Pierce and Dhalla, 1985; Tanaka et al., 1992; Williams and Howard, 1994; Doliba et al., 1997; Avkiran, 1999; Xiao and Allen, 1999; Babsky et al., 2002; Anzawa et al., 2006, 2012; Williams et al., 2007). It has been suggested that elevated glucose concentrations in DM significantly influence vascular NHE1 activity via glucose induced PKC-dependent mechanisms, thereby providing a biochemical basis for increased NHE1 activity in the vascular tissues of patients with hypertension and DM (Williams and Howard, 1994). In work done by David Allen's group, it was demonstrated that the major pathway for Na^+ entry during ischemia appears to be the so-called persistent Na^+ channel and the major pathway for Na^+ entry on reperfusion is NHE1 (Xiao and Allen, 1999; Williams et al., 2007). These changes in $[Na^+]_i$ affect the Na^+/Ca^{2+} exchanger and contribute to Ca^{2+} influx and to ROS generation, which are the major causes of ischemia/reperfusion damage (Avkiran, 1999). It has been also shown that Na^+–glucose cotransport is enhanced in type 2 diabetes (T2DM), which increases Na^+ influx and causes $[Na^+]_i$ overload (Lambert et al., 2015).

One of the causes of altered Na^+ transport and increased $[Na^+]_i$ concentration can be related to the downregulation of bioenergetics. For example, in diabetes, alterations in oxidative phosphorylation may compromise ion transport (Kuo et al., 1983; Pieper et al., 1984; Pierce and Dhalla, 1985; Tanaka et al., 1992; Doliba et al., 1997). Sarcolemmal Na^+, K^+-ATPase function may also be depressed or down-regulated due to increased serum and intracellular fatty acids (Pieper et al., 1984). Resultant changes in intracellular cation concentrations, specifically Na^+ and Ca^{2+}, may in turn cause changes in cellular metabolism (Makino et al., 1987; Allo et al., 1991). In addition, changes in local (autocrine and paracrine) and circulating neurohormones, such as ouabain (OUA)-like (Blaustein, 1993) and natriuretic factors (Kramer et al., 1991), can exacerbate the initial changes in ion transport and result in functional abnormalities found in diabetes.

This review discusses the interdependence of Na^+ transport and bioenergetics in the cardiac myocyte. While an energy deficit effects Na^+ transport, on other hand, $[Na^+]_i$ has a strong effect on bioenergetics as evidenced by decreased free concentration of ATP and PCr and reduced mitochondrial respiration and oxidative phosphorylation related to changes in $[Na^+]_i$.

CARDIOMYOCYTE STUDIES IN DIABETIC HEARTS

Dr. Osbakken's laboratory employed unique non-invasive nuclear magnetic resonance spectroscopy (NMRS) methods for the simultaneous assessment of $[Na^+]_i$ by ^{23}Na NMRS and adenine nucleotides by ^{31}phosphorus (^{31}P) NMRS in cardiomyocytes embedded in agarose beads (Ivanics et al., 1994; Doliba et al., 1998, 2000). ^{23}Na NMRS allows for the determination of total Na^+ signal, and $[Na^+]_i$ and extracellular Na^+ ($[Na^+]_e$) pools by splitting into two resonances after the addition of a shift reagent to the perfusate (Doliba et al., 1998; Doliba et al., 2000; Holloway et al., 2011). This method allowed evaluation of changes in $[Na^+]_i$ in a rat model of streptozotocin-induced DM. Streptozotocin was injected intraperitoneally (60 mg/kg body wt, dissolved in citrate buffer). Myocytes were harvested four weeks after streptozotocin injection. It was found that the baseline $[Na^+]_i$ in DM cardiomyocytes increased to 0.076 ± 0.01 mmoles/mg protein (or 16.37 mmol/L) from control (Con) levels of 0.04 ± 0.01 mmoles/mg protein (or 9.3 mmol/L); $P < 0.05$ (Doliba et al., 2000). This observation is similarly reported for heart failure myocytes (Liu and O'Rourke, 2008). Of note, in DM, baseline ATP and PCr were lower compared to Con (peak area/methylene diphosphonate standard area; Doliba et al., 2000): ATP-Con: 0.67 ± 0.08, ATP-DM: 0.31 ± 0.06, $P < 0.003$; PCr-Con: 0.92 ± 0.08; PCr-DM: 0.46 ± 0.12, $P < 0.009$. This suggests that DM cardiomyocytes have depressed bioenergetics function, which may contribute to abnormal Na^+, K^+-ATPase function and thus result in increased $[Na^+]_i$.

To further explore these findings, we measured ^{23}Na and ^{31}P spectra from superfused cardiomyocytes subjected to three metabolic inhibitors: 2-deoxyglucose (2DG), 2, 4-dinitrophenol (DNP), and OUA (**Figures 1A,B**; Doliba et al., 2000).

Inhibition of glycolysis with 2-DG was associated with minimal or no change in $[Na^+]_i$ in DM cardiomyocytes compared to an increase in $[Na^+]_i$ in Con cardiomyocytes (DM 2DG: −4.6 ± 6%, Con 2-DG: 32.9 ± 8.1% $p < 0.05$). The Na^+, K^+-ATPase inhibitor, OUA, produced a smaller change from baseline in $[Na^+]_i$ in DM cardiomyocytes compared to Con (DM OUA 21.2 ± 9.2%; vs Con OUA: 50.5 ± 8.8% $p < 0.05$;

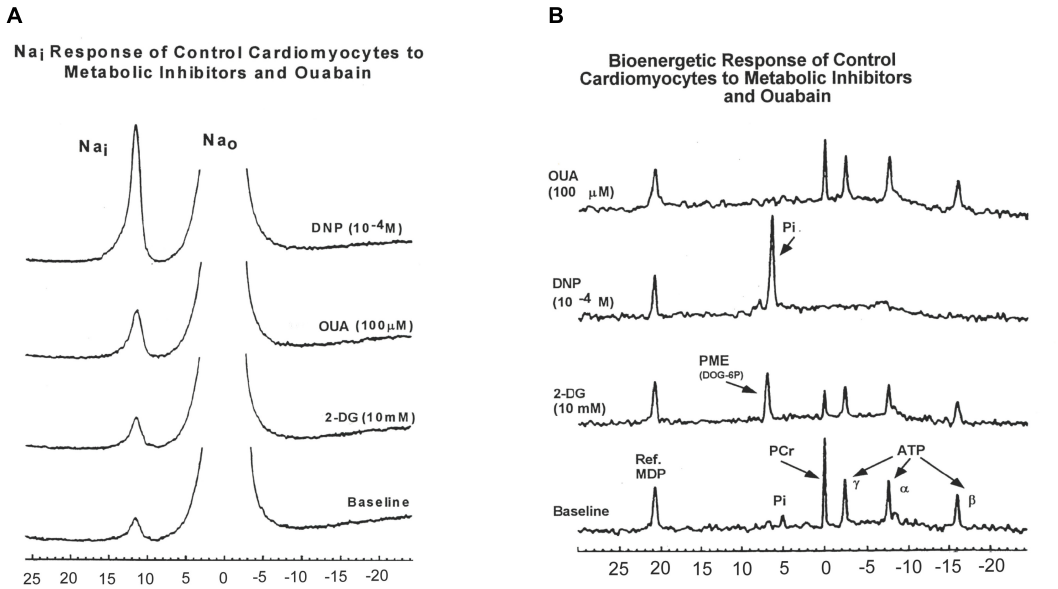

FIGURE 1 | (A) A typical 23Na spectra obtained from control rat cardiomyocytes showing intra- and extra-cellular sodium during baseline conditions and during administration of 2-deoxyglucose (2-DG, 10 mM); 2,4-dinitrophenol (DNP, 10-4 M); and ouabain (OUA, 100 μM). **(B)** Effects of 2-DG, DNP, and OUA on ^{31}P spectra obtained from control rat cardiomyocytes (typical spectra presented). MDP, methylene diphosphonate standard; PME, phosphomonoester; Pi, inorganic phosphate; PCr, phosphocreatine; ATP, adenosine triphosphate (α, γ, β); Na_i, intracellular sodium; Na_0, extracellular sodium. Data reprinted with permission from Doliba et al. (2000) Translated from Biokhimiya. 2000:65(4) 590-97. Copyright 2000 by MAIK "Nauka/Interperiodica"; DOI 0006-2979/00/6504-0502$25.00; Copyright permission granted by Pleiades Publishing, LLC.

Doliba et al., 2000). However, despite this apparent lower effect of OUA on DM cardiomyocytes, the absolute $[Na^+]_i$ after treatment with OUA was still 41% higher in DM cardiomyocytes compared to control due to the higher baseline $[Na^+]_i$.

In both animal models, uncoupling of oxidative phosphorylation with DNP was associated with similar large increases in $[Na^+]_i$; Con, 119.0 ± 26.9%; DM, 138.2 ± 12.6 (**Figure 1A**).

Figure 1B presents examples of ^{31}P-NMR spectra for control cardiomyocytes obtained during baseline and 2-DG, OUA, and DNP interventions. In control cardiomyocytes, 2-DG caused a 26.4 ± 4.8% decrease of β-ATP and 35.4 ± 4.9% decrease of PCr compared to baseline. In diabetic cardiomyocytes, 2-DG caused slightly smaller decreases in β-ATP (16.2 ± 5.9%) and PCr (27.96 ± 1.7%) when compared to control. Uncoupling of oxidative phosphorylation with DNP caused apparent complete depletion (i.e., to total NMR invisibility) of both β-ATP and PCr (–100%) in both control and diabetic cardiomyocytes. The large $[Na^+]_i$ increase due to DNP intervention suggests that both groups of cardiomyocytes require oxidative ATP synthesis to support the cell membrane ion gradient.

Unexpectedly, inhibition of Na, K-ATPase with OUA produced minimal change in bioenergetic parameters in cardiomyocytes from both animal models.

In diabetic cardiomyocytes, the decreased response of $[Na^+]_i$ to OUA and 2-DG can be related to prior inhibition of Na^+/K^+ pump (Greene, 1986; Kjeldsen et al., 1987; Hansen et al., 2007) and glycolysis (Boden et al., 1996; Boden, 1997).

ISOLATED MITOCHONDRIAL STUDIES IN DIABETIC HEARTS

The $[Na^+]_i$ is tightly coupled to Ca^{2+} homeostasis and is increasingly recognized as a modulating force of cellular excitability, frequency adaptation, and cardiac contractility (Faber and Rudy, 2000; Grandi et al., 2010; Despa and Bers, 2013; Clancy et al., 2015). Mitochondrial ATP production is continually adjusted to energy demand through increases in oxidative phosphorylation and NADH production mediated by mitochondrial Ca^{2+} (Liu and O'Rourke, 2008). Mitochondria in cardiac myocytes have been recognized as a Ca^{2+} storage site, as well as functioning as energy providers that synthesize a large proportion of ATP required for maintaining heart function. In cardiac mitochondria, Ca^{2+} uptake and removal are mainly mediated via the mitochondrial Ca^{2+} uniporter and the mitochondrial Na^+/Ca^{2+} exchanger (mNa^+/Ca^{2+}) (Gunter and Pfeiffer, 1990; Bernardi, 1999; Brookes et al., 2004; Liu and O'Rourke, 2008; Palty et al., 2010), respectively. The Ca^{2+} concentration for half-Vmax of the Ca^{2+} uniporter was estimated as ∼10–20 mM in studies of isolated mitochondria, which far exceeds cytosolic Ca^{2+} (1–3 mM; Liu and O'Rourke, 2009). By catalyzing Na^+-dependent Ca^{2+} efflux, the putative electrogenic mNa^+/Ca^{2+} exchanger plays a fundamental role in regulating mitochondrial Ca^{2+} homeostasis (Gunter and Gunter, 2001; Liu and O'Rourke, 2008), oxidative phosphorylation (Cox and Matlib, 1993a,b; Cox et al., 1993; Liu and O'Rourke, 2008), and Ca^{2+} crosstalk among mitochondria, cytoplasm, and

the endoplasmic reticulum (ER; Szabadkai et al., 2006). The dependence of the mNa^+/Ca^{2+} exchanger on $[Na^+]_i$ is sigmoidal with half-maximal velocity ($K_{0.5}$) at ∼5–10 mM, which covers the range of physiological $[Na^+]_i$ in the heart (Bers et al., 2003; Saotome et al., 2005). Mitochondrial Ca^{2+} activates matrix dehydrogenases (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and the NAD^+-linked isocitrate dehydrogenase) (Hansford and Castro, 1985; McCormack et al., 1990; Balaban, 2002; Gunter et al., 2004) and may also activate F_0/F_1-ATPase (Yamada and Huzel, 1988; Territo et al., 2000, 2001), and the adenine nucleotide translocase (ANT; Moreno-Sanchez, 1985). The $K_{0.5}$ for Ca^{2+} activation of these three dehydrogenases is in the range of 0.7–1 mM (McCormack et al., 1990; Hansford, 1991). The overall effect of elevated mitochondrial Ca^{2+} may be the upregulation of oxidative phosphorylation and the acceleration of ATP synthesis (McCormack et al., 1990; Balaban, 2002; Matsuoka et al., 2004; Jo et al., 2006). Activation of Ca^{2+}-dependent dehydrogenases by Ca^{2+} increases NADH production, which is the primary electron donor of the electron transport chain. $NADH/NAD^+$ potential is the driving force of oxidative phosphorylation and an increase in $NADH/NAD^+$ potential leads to a linear increase of maximal respiration rate in isolated heart mitochondria (Moreno-Sanchez, 1985; Mootha et al., 1997). On the other hand, the excessive rise in mitochondrial Ca^{2+} triggers the mitochondrial permeability transition pore (PTP), resulting in pathological cell injury and death (Hajnoczky et al., 2006). Insufficient mitochondrial Ca^{2+} accumulation, secondary to cytoplasmic Na^+ overload, decreases $NAD(P)H/NAD(P)^+$ redox potential, resulting in compromised NADH supply for oxidative phosphorylation (Liu and O'Rourke, 2008). Since NADPH is required to maintain matrix antioxidant pathway flux, its oxidation causes a cellular overload of ROS (Kohlhaas and Maack, 2010; Kohlhaas et al., 2010; Liu et al., 2010; Clancy et al., 2015). ROS accumulation then contributes to oxidative modification of Ca^{2+} handling and ion channel targets to promote arrhythmias. This cascade of failures, stemming from $[Na^+]_i$ overload, is thus hypothesized to provoke triggered arrhythmias (Liu et al., 2010), which, in the context of the altered electrophysiological substrate in HF, may induce sudden cardiac death (SCD). Interestingly, chronic inhibition of the mNa^+/Ca^{2+} exchanger during the induction of HF prevents these mitochondrial defects and abrogates cardiac decompensation and sudden death in a guinea pig model of HF/SCD (Liu et al., 2014). Therefore, the mitochondrial Ca^{2+} concentration must be kept within the proper range to maintain physiological mitochondrial function.

To further evaluate the pathophysiology of DM, our group studied mitochondrial respiratory function [state 3 and state 4 respiration, respiratory control index (RCI), ADP/O ratio, and rate of oxidative phosphorylation (ROP), using different substrates, and ion transport (calcium uptake)] in DM hearts compared to Con hearts. State 3 and RCI and ROP of DM rat heart were decreased when using pyruvate plus malate as substrates (**Table 1**; Doliba et al., 1997; Babsky et al., 2001). State 3 and ROP were also decreased when α-ketoglutarate was used as substrate (**Table 1**). The phosphorylation capacity, expressed as ADP/O ratio, appeared to be normal with both sets of substrates. The greatest decrease in substrate oxidation was observed with pyruvate, suggesting that pyruvate dehydrogenase activity is depressed in DM. It should be pointed out that in DM mitochondria, the decrease in state 3 was dependent on the concentration of pyruvate; and that the Km for pyruvate was higher in DM (0.058 ± 0.01 mM) compared to Con (0.0185 ± 0.0014), with no significant difference in V_{max} (Doliba et al., 1997). RCI was decreased approximately 35% at all pyruvate concentrations.

To determine whether changes in Ca^{2+} transport might be the cause of change in oxidative function presented above, state 3 respiration was initially stimulated by ADP, and then by $CaCl_2$ in Con and DM mitochondria during pyruvate plus malate oxidation; Ca^{2+} uptake was recorded using the change in H^+ flux (i.e., $Ca^{2+}/2H^+$ exchange; **Figure 2**; Doliba et al., 1997). Stimulation of oxygen consumption by ADP or Ca^{2+} was approximately 50% lower in DM mitochondria compared to Con. In order to measure Ca^{2+} capacity, 100 mM $CaCl_2$ was added to the incubation medium and Ca^{2+} uptake was followed by changes in pH. In contrast to Con mitochondria, mitochondria from DM animals did not completely consume even the first

TABLE 1 | Substrate oxidation by heart mitochondria of normal and diabetic rats.

	Rate of respiration (ng-atoms of O/min/mg protein)		Respiratory control		Rate of oxidative phosphorylation,
	State 3	State 4	(State 3/State 4)	ADP/O ratio	(nmoles ADP/s/mg protein)
Pyruvate					
Con	192.50+16.09	29.36+4.68	6.70+1.20	2.79+0.18	9.10+1.57
DM	115.29+18.15*	26.44+5.01	4.40+0.54*	2.74+0.24	5.07+1.42*
α-Ketoglutarate					
Con	174.26+4.59	16.11+2.68	11.76+1.63	2.86+0.26	8.09+0.65
DM	156.81+3.45*	14.28+2.83	11.58+1.86	2.71+0.18	6.58+0.20*

*Mitochondria (1.2 mg of protein in mL) were prepared as described earlier (Doliba et al., 1998); respiration in state 3 was measured in the presence of 0.3 mM-ADP, and respiration in state 4 was measured after ADP was completely phosphorylated. Data reprinted by permission from Nature/Springer/Palgrave: (Doliba et al., 1997). *Significantly different from control $P \leq 0.01$. Copyright 1997 by Springer Nature; License Number 4385511236859. Originally published by Plenum Press, New York 1997 (DOI 10987654321).*

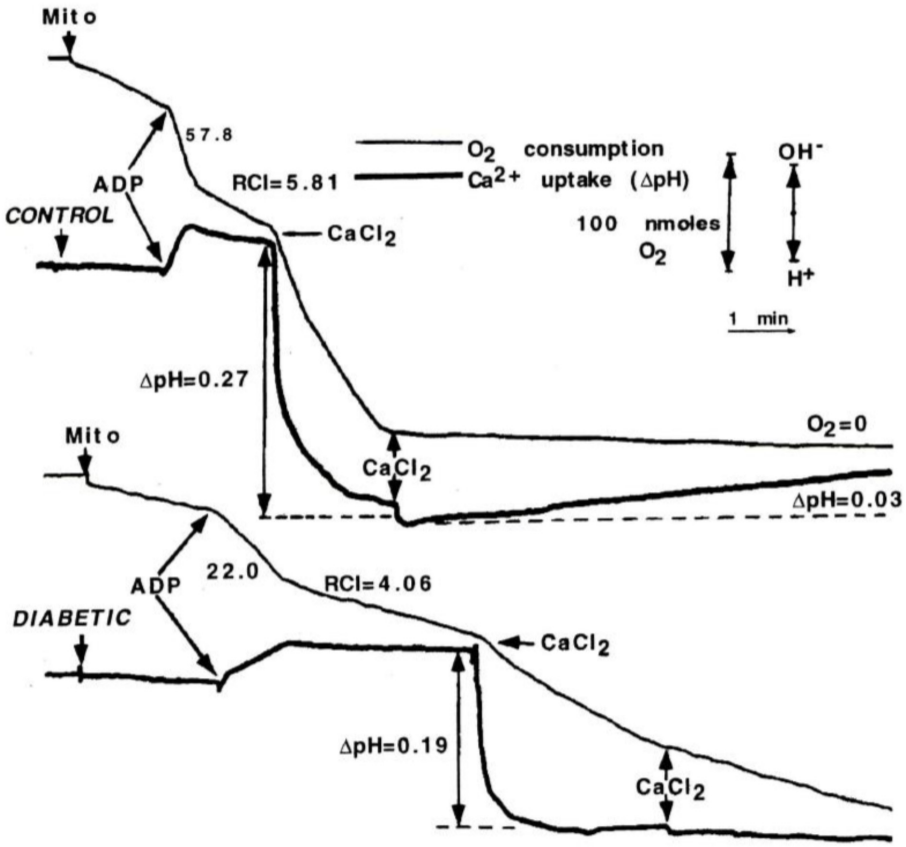

FIGURE 2 | ADP and Ca^{2+}-stimulated respiration of mitochondria from control and diabetic rats. Mitochondria (2 mg) were added to assay medium supplemented with 3 mM pyruvate plus 2.5 mM malate. ADP (0.3 mM) or $CaCl_2$ (50 μM) was used to initiate state 3 respiration and Ca-uptake. Ca^{2+} uptake by mitochondria was monitored by using the change in H^+ flux. Stimulation of oxygen consumption by ADP or Ca^{2+} was approximately 50% lower in DM mitochondria compared to Con. Data reprinted by permission from Nature/Springer/Palgrave: Doliba et al. (1997). Copyright 1997 by Springer Nature; License Number 4385511236859. Originally published by Plenum Press, New York 1997 (DOI 10987654321).

addition of $CaCl_2$. These data suggest that the Ca^{2+} capacity in heart in DM rats is greatly decreased compared to Con.

RESPIRATORY FUNCTION AND SUBSTRATE USE STUDIED BY MASS SPECTROSCOPY

Previous studies in our laboratory and laboratories of other investigators have shown abnormalities in pyruvate oxidation in animal models of DM, possibly related to effects of abnormal Ca^{2+} content on enzymes such as pyruvate dehydrogenase. To evaluate the possible role of abnormal pyruvate dehydrogenase function on respiratory function of heart mitochondria from diabetic rats, mass spectroscopy determination of O_2 consumption and $^{13}C^{16}O_2$ production from [1-^{13}]pyruvate were measured in heart mitochondria from Con (n = 8) and DM (4 weeks after streptozotocin injection; n = 8) rats (Doliba et al., 1997). **Figure 3** presents the time course of $^{13}C^{16}O_2$ production (curve 1) and oxygen consumption (MVO_2) (curve 2) during oxidation of [1-^{13}C]pyruvate by heart mitochondria from Con and DM rats (Doliba et al., 1997). Both the $^{13}C^{16}O_2$ production and MVO_2 stimulated by ADP (**Figure 3A**) or carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP), an uncoupler of respiration and oxidative phosphorylation (**Figure 3B**), were much less in DM mitochondria compared to Con (with ADP, 35–50% less; FCCP, 20–30% less). Addition of Ca^{2+} caused minimal changes in $^{13}C^{16}O_2$ production in DM; whereas Ca^{2+} increased $^{13}C^{16}O_2$ production by 33–40% in Con (**Figure 4**; Doliba et al., 1997). This lack of stimulation of a key enzyme by Ca^{2+} may be a factor in the development and progression of pathophysiological sequelae in DM and may be related to abnormal Ca^{2+} transport function. The data presented in the next two paragraphs suggest that abnormal mitochondrial Ca^{2+} transport and bioenergetics in DM cardiac mitochondria can be related to abnormalities in Na^+ flux.

Na^+ Regulation of Mitochondrial Energetics: DM Modeling Effort

Previous data reported above, and by others suggest that the etiology of DM end organ damage may be related to abnormalities in Na^+ transport. We and others (Cox and

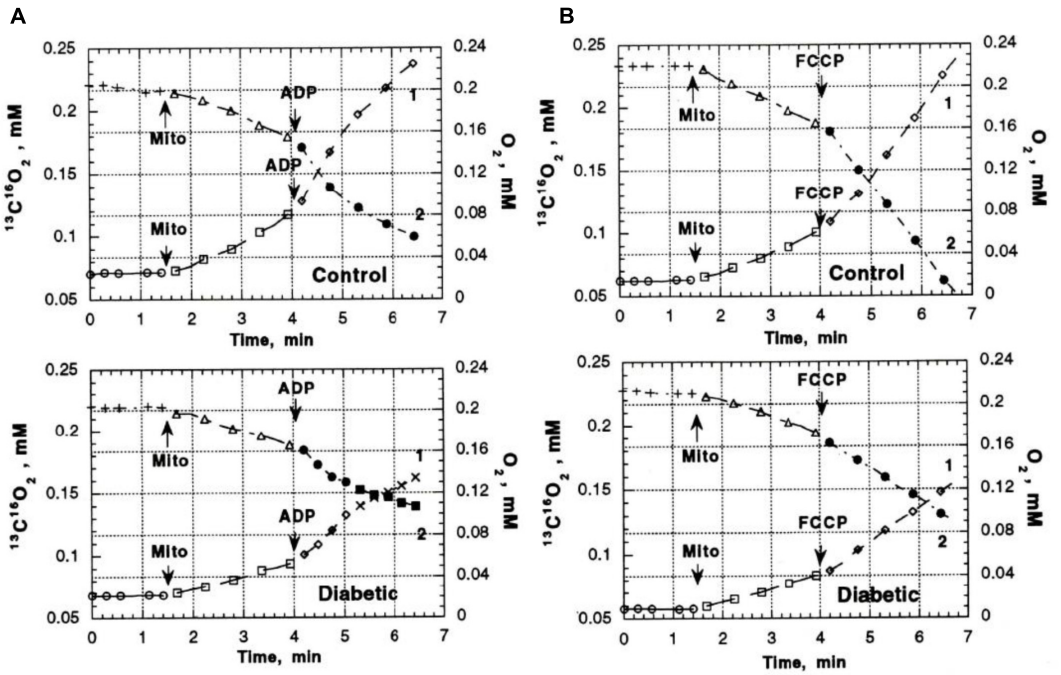

FIGURE 3 | $^{13}C^{16}O_2$ production (Boudina and Abel, 2010) and O_2 consumption (MVO_2) (Veeranki et al., 2016) during oxidation of [1-^{13}C] pyruvate by heart mitochondria from control and diabetic rats. **(A)** $^{13}C^{16}O_2$ production and MVO_2 after addition of ADP. **(B)** $^{13}C^{16}O_2$ production and MVO2 after addition of FCCP to uncouple oxidative phosphorylation. Both the $^{13}C^{16}O_2$ production and MVO_2 stimulated by ADP FCCP were much less in DM mitochondria compared to Con. Data reprinted by permission from Nature/Springer/Palgrave: Doliba et al. (1997). Copyright 1997 by Springer Nature; License Number 4385511236859. Originally published by Plenum Press, New York 1997 (DOI 10987654321).

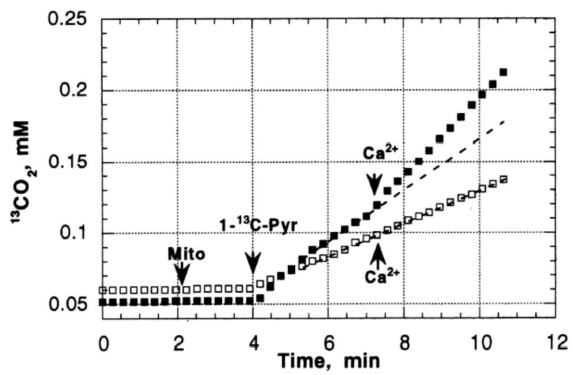

FIGURE 4 | The effect of Ca^{2+} on CO_2 production in Con (black squares) and DM mitochondria (open squares). Addition of Ca^{2+} caused minimal changes in $^{13}C^{16}O_2$ production in DM; whereas Ca^{2+} increased $^{13}C^{16}O_2$ production by 33–40% in Con. Data reprinted by permission from Nature/Springer/Palgrave: Doliba et al. (1997). Copyright 1997 by Springer Nature; License Number 4385511236859. Originally published by Plenum Press, New York 1997 (DOI 10987654321).

Matlib, 1993b; Cox et al., 1993; Maack et al., 2006; Liu and O'Rourke, 2008) proposed that increased $[Na^+]_i$ is involved in the regulation of mitochondrial oxidative phosphorylation through the Ca^{2+} metabolism. Mitochondrial Ca^{2+} ($[Ca^{2+}]_m$) plays a key role in linking ATP production to ATP demand (i.e., mechanical activity) and as Ca^{2+} rises in the cell, so does $[Ca^{2+}]_m$; this activates mitochondrial enzymes to step-up ATP production (Liu and O'Rourke, 2008; Kohlhaas and Maack, 2010). This relationship, which crucially matches ATP supply to demand, is blocked when $[Na^+]_i$ is elevated (Liu et al., 2010). The rise in $[Na^+]_i$ activates Na^+/Ca^{2+} exchange in the inner mitochondrial membrane and keeps $[Ca^{2+}]_m$ low preventing ATP supply from meeting demand, leaving the heart metabolically compromised. Not only might this contribute to the known metabolic insufficiency in failing hearts but Kohlhaas et al. (2010) have shown that this mechanism increases mitochondrial free radical formation in failing hearts, further exacerbating injury.

To test this hypothesis, different concentrations of NaCl (in mM: 0.05; 0.1; 0.5; 1; 3; 10) were added to Con and DM mitochondria while respiratory function was monitored (Babsky et al., 2001); 1 mM a-ketoglutarate was used as substrate and mitochondrial respiration was stimulated by 200 mM ADP. Ruthenium red (1 mM), a blocker of Ca^{2+} uptake, was added to the polarographic cell before Na^+ was added. Na^+ in concentrations higher than 0.5–1 mM significantly decreased ADP-stimulated mitochondria oxygen consumption (**Figure 5**; Babsky et al., 2001). Mitochondria from DM rats were more sensitive to increasing extramitochondrial Na^+ as demonstrated by more rapid and larger decrease in state 3 respiration (Babsky et al., 2001). The decrease in state 3 in both Con and DM mitochondria was abolished by addition of 10 mM $CaCl_2$ to the

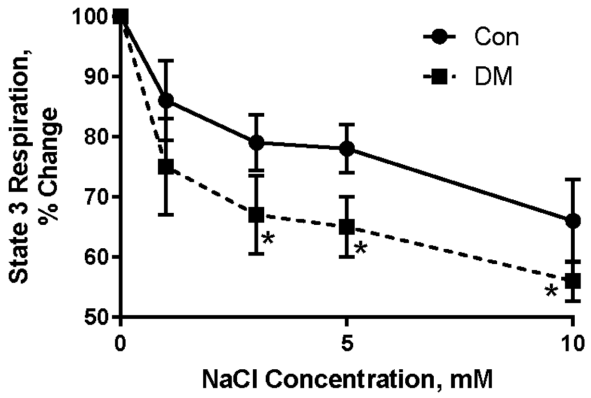

FIGURE 5 | Effect of different concentrations of Na^+ on ADP stimulated mitochondrial oxygen consumption (state 3) in control (CON) and diabetic (DM) heart mitochondria (means ± SE, $n = 5$). * < 0.05 CON vs DM. Baseline of state 3 (without Na^+) is assumed to be 100%. Data reprinted with permission from Babsky et al. (2001). Copyright 2001 by the Society for Experimental Biology and Medicine; DOI: 0037-9727/01/2266-0543$15.00.

polarographic cell before adding NaCl (Babsky et al., 2001). Our data agree with the studies of O'Rourke and colleagues who have shown that the elevation of $[Na^+]_i$ can impair mitochondrial energetics (Liu and O'Rourke, 2008, 2013; Kohlhaas et al., 2010; Liu et al., 2010).

Effect of Na^+ on Adenine Nucleotides and Pi in Con and DM Mitochondria

In support of polarographic data, we used ^{31}P NMRS to study the influence of different concentrations of NaCl on ATP synthesis in mitochondria isolated from Con and DM (Babsky et al., 2001). Exposure of DM mitochondria superfused at a rate of 2.7 cc/min with buffer containing Na^+ (5–30 mM) led to greater decreases of β-ATP/Pi ratio than that found in Con (**Figure 6A**; Babsky et al., 2001). Diltiazem (DLTZ), an inhibitor of mitochondrial Na^+/Ca^{2+} exchange, abolished the Na^+ (5–30 mM) initiated decrease of β-ATP in DM mitochondria and reduced the increase of Pi with resultant values of β-ATP/Pi similar in both Con and DM mitochondria (**Figure 6B**; Babsky et al., 2001).

ISCHEMIA, PRECONDITIONING (IPC), AND THE DIABETIC HEART

One of most important factors of diabetic cardiomyopathy is post-ischemic myocardial injury that is associated with oxygen free radical generation, intracellular acidosis, bioenergetic depletion, as well as with abnormalities in Na^+, H^+, and Ca^{2+}-transport in cardiomyocytes. Ca^{2+} overload and ischemic acidosis are also important intracellular alterations that could cause damage to ischemic cardiomyocytes (Bouchard et al., 2000). Sodium ions are involved in regulating both H^+ and Ca^{2+} levels in cardiomyocytes through NHE1, Na^+/Ca^{2+}, Na^+-K^+-$2Cl^-$, and Cl^-/HCO_3^- ion transporters. Furthermore, Na^+ is an important regulator of bioenergetic processes in healthy and diseased cardiomyocytes (Babsky et al., 2001).

Ischemic preconditioning (IPC) is a powerful protective mechanism by which exposure to prior episodes of ischemia protects the myocardium against longer and more severe ischemic insults (Murry et al., 1986). The relationship between DM and myocardial IPC is not yet clear (Miki et al., 2012). Some studies have demonstrated that diabetes may impair IPC by producing changes in both sarcolemmal and mitochondrial K-ATP channels, which then alters mitochondrial function (Hassouna et al., 2006). These changes may lead to an elevated superoxide production which produces cellular injuries.

Ishihara et al. (2001) show in 611 patients (including 121 patients with non-insulin treated diabetes) that DM prevents the IPC effect in patients with an acute myocardial infarction. However, a study of Rezende et al. (2015) showed that T2DM was not associated with impairment in IPC in coronary artery disease patients. In fact, there is some evidence that prior short episodes of ischemia that can often occur in the diabetic heart

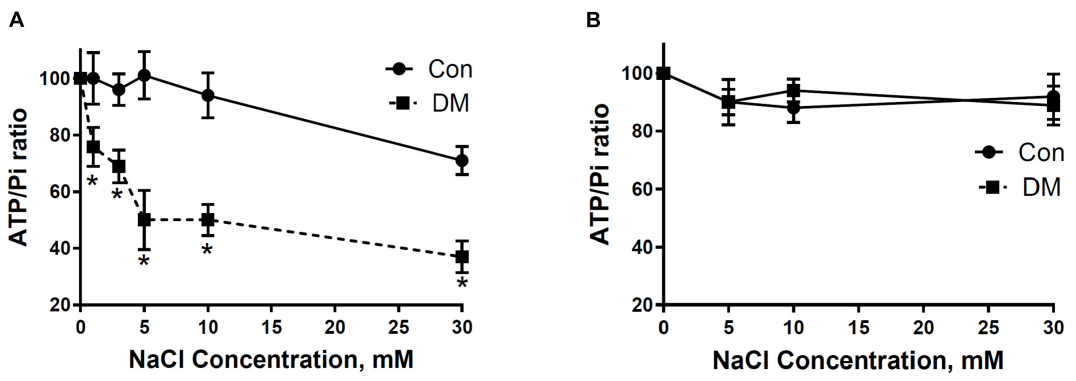

FIGURE 6 | (A) The effect of extramitochondrial Na^+ on ATP and Pi ratios in CON and DM heart mitochondria. **(B)** 250 μM DLTZ, an inhibitor of mitochondrial Na^+–Ca^{2+} exchange, was added to perfusate. Baseline (without Na^+) is assumed to be 100% (means ± SE. $n = 4$). Significance: DM vs CON: *$P < 0.05$. Data reprinted with permission from Babsky et al. (2001). Copyright 2001 by the Society for Experimental Biology and Medicine; DOI: 0037-9727/01/2266-0543$15.00.

are the substrate for IPC, whereby the heart is protected during longer episode of ischemia.

Tsang et al. (2005) hypothesized that in diabetic hearts, IPC depends on intact signaling through the phosphatidylinositol 3-kinase (PI3K)-Akt pro-survival pathway. The authors concluded that diabetic hearts are less sensitive to the IPC protective effects related to defective components in the PI3k-Akt pathway. For example, in animal models of diabetes, exposure to more prior episodes of IPC were needed to activate PI3K-Akt to a critical level and thus provide cardioprotection during exposure to longer episodes of ischemia–reperfusion than in Con.

Our group studied the effect of IPC on $[Na^+]_i$ levels in isolated perfused rat hearts (**Figure 7**; Babsky et al., 2002). We have shown that 20 min ischemia increased the $[Na^+]_i$ in Con hearts by ∼50% compared to baseline. During 10–20 min of post-ischemic reperfusion the $[Na^+]_i$ significantly decreased, but was still ∼20% higher compared to baseline levels. Even though IPC significantly improved the post-ischemic recovery of cardiac function (LVDP and heart rate), unexpectedly the $[Na^+]_i$ levels were higher than Con at end IPC, and during ischemia, and were similar to Con during reperfusion. These results are in agreement with the data reported by Ramasamy et al. (1995). While our studies did not include a DM model, Ramasamy's studies did; and showed that the % change in $[Na^+]_i$ from baseline was lower during ischemia in DM than in Con, and that the effect of the NHE1 inhibitor EIPA (similar to preconditioning ischemia) was less in DM than in Con. This suggests that the NHE1 activity was impaired in DM. The topic of NHE1 and ischemia is discussed further below.

Although diabetes mostly poses higher cardiovascular risk, the pathophysiology underlying this condition is uncertain. Moreover, though diabetes is believed to alter intracellular pathways related to myocardial protective mechanisms, it is still controversial whether diabetes may interfere with IPC, and whether this might influence clinical outcomes. We believe that ischemia developed in diabetic heart does not produce the same conditions that are developed in animal models when two–three 5-min ischemic episodes are each followed by 5–10 min of reperfusion. This difference may be a reason for the many controversies concerning relationship of IPC and the diabetic heart.

To conclude this discussion, it is likely that the changes in $[Na^+]_i$ may contribute to ischemic and reperfusion damage, possibly through their effects on Ca^{2+} overload (Allen and Xiao, 2003; Xiao and Allen, 2003; Williams et al., 2007).

ISCHEMIA AND NHE1

Ischemic conditions may activate the NHE1. There are data that show that hyperactivity of NHE1 results of the increase in $[Na^+]_i$ that leads to Ca^{2+} overload through the Na^+/Ca^{2+} exchanger, myocardial dysfunction, hypertrophy, apoptosis, and heart failure (Cingolani and Ennis, 2007). David Allen's group showed that two inhibitors of NHE1, amiloride and zoniporide, cause cardioprotection which was judged by the recovery of LVDP and by the magnitude of the reperfusion contracture (Williams et al., 2007). The authors also showed that there were two different mechanisms for Na^+ entry during ischemia and

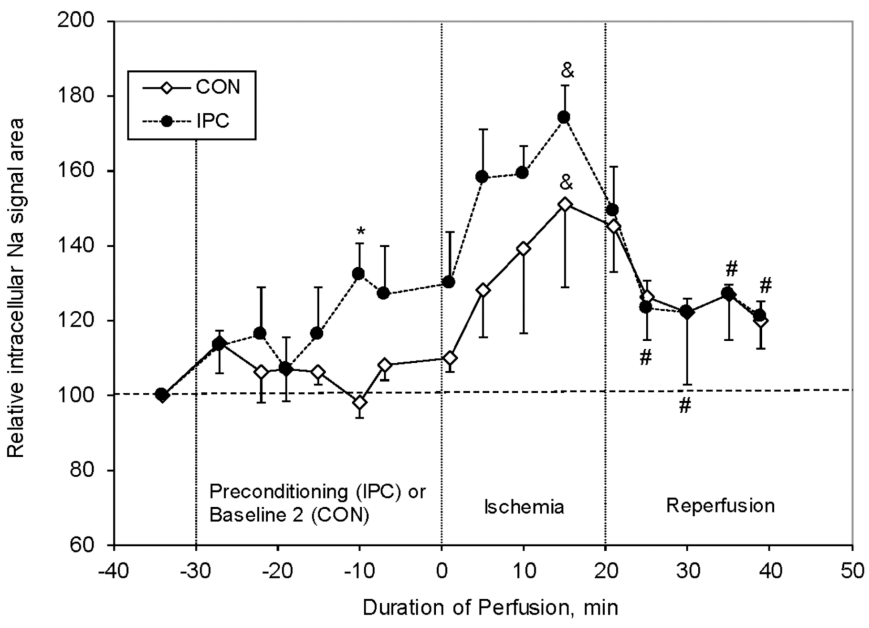

FIGURE 7 | Relative changes in intracellular sodium (Na_i) resonance areas as a function of time in control (CON, $n = 6$) and preconditioned (IPC, $n = 4$) rat hearts. Na_i baseline is normalized to 100. Significance: $^*P < 0.01$ (IPC vs CON), $^{\#}P < 0.05$ (IPC group vs end of ischemia), and $^{\&}P < 0.01$ (vs pre-ischemic level for each group). Data reprinted with permission from Babsky et al. (2002). Copyright 2002 by the Society for Experimental Biology and Medicine; DOI: 1535-3702/02/2277-0520$15.00.

reperfusion: a major pathway for Na^+ entry during ischemia is the persistent Na^+ channels ($I_{Na,P}$) and the major pathway for Na^+ entry on reperfusion is NHE1 (Williams et al., 2007). The optimal therapy may require blocking both pathways. Pisarenko et al. (2005) show that inhibition of NHE1, similar to IPC, protects rat heart. In rabbit hearts, inhibition of NHE1 has been shown to be associated with significant protection during ischemia/reperfusion injury in immature myocardium, mostly by reducing myocardial calcium overload (Cun et al., 2007; Zhou et al., 2008). Furthermore, NHE1 inhibition leads to a decrease of infarct size after coronary artery thrombosis and thrombolysis and provides a comparable to preconditioning degree of cardioprotection against 60 min of regional ischemia (Hennan et al., 2006). NHE1 inhibition attenuates the cardiac hypertrophic response and heart failure in various experimental models. For example, early and transient administration of a NHE1 inhibitor inhibits cardiomyocyte hypertrophy in cultured cells, as well as *in vivo* cardiac hypertrophy and heart failure, suggesting a critical early NHE1-dependent initiation of hypertrophy (Kilic et al., 2014). However, in a dog model, one NHE1 inhibitor such as EMD 87580 did not protect against ischemia–reperfusion injury, and no additive protection beyond preconditioning was obtained (Kingma, 2018). It appears that NHE1 activity has a biphasic effect on myocardial function. Total blockage of activity provides a beneficial effect, but overexpression also provides cardioprotection. It is important to point out that the mitochondrial K_{ATP} channel also plays an important role during ischemia and reperfusion damage (Garlid et al., 1997; Sato and Marban, 2000). The mitochondrial damage, which is in part a consequence of closure of K_{ATP} channels, can be partially reversed by mitochondrial K_{ATP} channel openers (Xiao and Allen, 2003). Combined treatment of NHE1 by Cariporide and K_{ATP} channels by diazoxide provide the most beneficial effect (Xiao and Allen, 2003).

It is interesting to note that the cardioprotective effects of the NHE1 inhibitor, Cariporide, were tested in several clinical trials to protect the heart from ischemia during coronary artery bypass surgery (CABG; Boyce et al., 2003; Mentzer et al., 2008). While Cariporide (at its highest dose of 120 mg) provided protection against all-cause mortality and myocardial infarction at day 36 and 6 months after CABG compared to placebo, there was an increased mortality in the form of cerebrovascular events. Thus, Cariporide was not further developed for clinical use as a cardioprotection agent.

SODIUM TRANSPORT INHIBITORS IN TREATMENT OF DIABETIC CARDIOMYOPATHY

The NHE1 are integral membrane proteins that may have multiple activities in the heart. Nine different NHEs have been identified. NHE1 is the major isoform found in the heart, and plays an integral role in regulation if intracellular pH, Na^+ and Ca^{2+}. Aberrant regulation and over-activation of NHE1 can contribute to heart disease and appears to be involved in acute ischemia–reperfusion damage and cardiac hypertrophy. Changes in intracellular pH related to changes in NHE1 function can stimulate the Na^+/Ca^{2+} exchanger to eliminate intracellular Na^+ and increase intracellular Ca^{2+} (Levitsky et al., 1998; Odunewu-Aderibigbe and Fliegel, 2014).

Pharmacological overload caused by angiotensin II, endothelin-1, and a1-adrenergic agonists can enhance the activity of the NHE1, which leads to an extrusion of H^+ and an increase in intracellular Na^+. Inhibition of NHE1 can reverse these effects and lead to regression of myocardial hypertrophy that can produce a beneficial effect in heart failure, and can protect against ischemic injury in genetic diabetic rat and non-diabetic rat hearts. However, at present, there are no NHE1 inhibitors that have been found to be therapeutically useful in the treatment of heart disease (Ramasamy and Schaefer, 1999; Cingolani and Ennis, 2007).

More recently, during studies of newer anti-diabetic drugs on cardiac function, it was found that Na^+-glucose exchangers used in the treatment of diabetes provided significant cardiac protection. Further investigation into the potential etiology of this protection suggests that at least one of these drugs, Empagliflozin (EMPA) may produce this affect via inhibition of NHE1. This protective effect is apparently unrelated to EMPA effect on HbA1C. In two animal models (rabbit and rat), the effect appears to be related to decreases in cytoplasmic Na^+ and Ca^{2+} and an increase in mitochondrial Ca^{2+}. It is unclear what the effects are due to in humans, but some evidence suggests that they may be similar (Baartscheer et al., 2017; Lytvyn et al., 2017; Packer, 2017; Packer et al., 2017; Bertero et al., 2018; Inzucchi et al., 2018).

SUMMARY

The data presented in this review paper suggest that while changes in bioenergetic function may be a cause of ion transport abnormalities, it is as likely that abnormalities of ion content and transport may contribute to metabolic (bioenergetics and respiratory function) abnormalities. The results also suggest that increased $[Na^+]_i$ concentration in DM cardiomyocytes may be a factor, leading to chronically decreased myocardial bioenergetics. Further studies in this area may provide insight into some possible cellular and mitochondrial mechanisms which contribute to progressive pathophysiological processes as disease progresses and may set the stage for better therapies in future.

AUTHOR CONTRIBUTIONS

ND contributed to five sections related to sodium transport and cellular and mitochondrial bioenergetics, AB to "Effect of Sodium on Adenine Nucleotides and Pi", AB and MO to "Ischemia and NHE1" and "Ischemia, Preconditioning and the Diabetic Heart", MO to "Diabetes Cardiomyopathy" and "Sodium Transport Inhibitors in Treatment of Diabetic Cardiomyopathy."

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Myocyte $[Na^+]_i$ Dysregulation in Heart Failure and Diabetic Cardiomyopathy

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By controlling the function of various sarcolemmal and mitochondrial ion transporters, intracellular Na^+ concentration ($[Na^+]_i$) regulates Ca^{2+} cycling, electrical activity, the matching of energy supply and demand, and oxidative stress in cardiac myocytes. Thus, maintenance of myocyte Na^+ homeostasis is vital for preserving the electrical and contractile activity of the heart. $[Na^+]_i$ is set by the balance between the passive Na^+ entry through numerous pathways and the pumping of Na^+ out of the cell by the Na^+/K^+-ATPase. This equilibrium is perturbed in heart failure, resulting in higher $[Na^+]_i$. More recent studies have revealed that $[Na^+]_i$ is also increased in myocytes from diabetic hearts. Elevated $[Na^+]_i$ causes oxidative stress and augments the sarcoplasmic reticulum Ca^{2+} leak, thus amplifying the risk for arrhythmias and promoting heart dysfunction. This mini-review compares and contrasts the alterations in Na^+ extrusion and/or Na^+ uptake that underlie the $[Na^+]_i$ increase in heart failure and diabetes, with a particular emphasis on the emerging role of Na^+ - glucose cotransporters in the diabetic heart.

Keywords: heart failure, type-2 diabetes, Na^+-glucose cotransporter, Na^+/H^+ exchanger, Na^+/K^+-ATPase, Na^+/Ca^{2+} exchanger

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MAINTENANCE OF MYOCYTE Na^+ HOMEOSTASIS IS VITAL FOR PRESERVING HEART FUNCTION

All mammalian cells maintain a low intracellular Na^+ concentration ($[Na^+]_i$) by actively extruding Na^+ through the Na^+/K^+-ATPase (NKA) at the expense of metabolic energy. The energy stored in the electrochemical Na^+ gradient is then used for the transmembrane transport of other ions (e.g., Ca^{2+} through the Na^+/Ca^{2+} exchanger, NCX, H^+ via the Na^+/H^+ exchanger, NHE, etc.), uptake of energy substrates (glucose through the family of Na^+-glucose cotransporters, SGLTs, and aminoacids through Na^+-aminoacid cotransporter) and, in the case of excitable cells, generation of action potentials (via voltage-gated Na^+ channels). Changes in $[Na^+]_i$ critically affect the function of these transporters, therefore $[Na^+]_i$ homeostasis is essential for numerous cellular processes.

In cardiac myocytes, NCX is the main route for Ca^{2+} extrusion from the cells (Bers, 2001), which intimately links Ca^{2+} cycling to $[Na^+]_i$. Even a small (few mM) increase in $[Na^+]_i$ alters Ca^{2+} fluxes through NCX, resulting in higher Ca^{2+} levels in the cytosol and sarcoplasmic reticulum (SR) and consequently larger contractions (**Figure 1**). This mechanism is responsible for the inotropic effect of cardiac glycosides such as digoxin. However, as demonstrated clinically with digoxin, the beneficial effect of enhanced contractility is counteracted by a higher risk for

ectopic arrhythmias, as larger SR Ca^{2+} load increases the incidence of spontaneous Ca^{2+} waves and delayed afterdepolarizations (Bers, 2014).

$[Na^+]_i$ also controls the level of Ca^{2+} in the mitochondria ($[Ca^{2+}]_m$) through the mitochondrial Na^+/Ca^{2+} exchanger (mitoNCX), which is the main route for mitochondrial Ca^{2+} extrusion in the heart (Griffiths, 2009). MitoNCX is half-maximally activated at $[Na^+]_i$ in the physiological range (5–10 mM) (Boyman et al., 2013). Therefore, mitoNCX is very sensitive to changes in $[Na^+]_i$. An increase in $[Na^+]_i$ accelerates mitochondrial Ca^{2+} efflux and thus reduces $[Ca^{2+}]_m$ (Cox and Matlib, 1993; Maack et al., 2006). Because $[Ca^{2+}]_m$ stimulates several dehydrogenases involved in the tricarboxylic acid cycle (McCormack et al., 1990), regeneration of NADH and NADPH from their oxidized forms slows down at lower $[Ca^{2+}]_m$. Slower restoration of the NADH pool limits the rate of electron transport and thus diminishes mitochondrial ATP production. Notably however, glycolytic ATP also drives cellular processes in the heart, particularly NKA (Glitsch and Tappe, 1993). NADPH is utilized to neutralize the H_2O_2 produced by the electron transport chain and lower NADPH levels may result in oxidative stress (Bertero and Maack, 2018). Abnormally low $[Na^+]_i$ is likely to have the opposite effect and cause mitochondrial Ca^{2+} overload, which also has detrimental effects on myocyte function. This notion is supported by the recent finding that elimination of mitochondrial Ca^{2+} efflux through deletion of the gene encoding mitoNCX results in increased generation of superoxide and necrotic cell death leading to heart failure (Luongo et al., 2017).

In summary, $[Na^+]_i$ regulates Ca^{2+} cycling, electrical activity, and oxidative stress in cardiac myocytes. Thus, maintenance of myocyte Na^+ homeostasis is vital for preserving heart function.

MYOCYTE $[Na^+]_i$ IS ELEVATED IN HEART FAILURE AND TYPE-2 DIABETES

$[Na^+]_i$ is in the 4–8 mM range in resting ventricular myocytes from healthy rabbit, guinea-pig, dog and human hearts (Harrison et al., 1992; Yao et al., 1998b; Gray et al., 2001; Despa et al., 2002a; Pieske et al., 2002; Gao et al., 2005) and somewhat higher (10–15 mM) in rat and mouse myocytes (Donoso et al., 1992; Yao et al., 1998a; Despa et al., 2002a, 2005). $[Na^+]_i$ increases in a frequency dependent manner when myocytes are excited electrically. This $[Na^+]_i$ rise is caused by enhanced Na^+ entry due to the regular opening of the voltage-gated Na^+ channels and activation of NCX during Ca^{2+} transients. As $[Na^+]_i$ rises, NKA is activated to extrude more Na^+ and a new steady-state is reached when the higher Na^+ influx is balanced by an elevated Na^+ efflux.

The balance between the passive Na^+ entry and Na^+ pumping out of the cell is perturbed in both humans and animal models with heart failure (HF), resulting in elevated $[Na^+]_i$. Pieske et al. (2002) found that $[Na^+]_i$ is ∼4 mM higher in myocytes from failing human myocardium compared to non-failing hearts. A comparable $[Na^+]_i$ increase was reported in myocytes from rabbits with heart failure induced by volume and pressure overload (Despa et al., 2002b; Baartscheer et al., 2003) or by rapid pacing (Schillinger et al., 2006). $[Na^+]_i$ is also elevated in myocytes from mice with heart failure caused by conditional, cardiomyocyte-specific deletion of SERCA gene (Louch et al., 2010). These studies found that $[Na^+]_i$ was elevated at all stimulation rates in the 0–3 Hz range. While a few mM rise in $[Na^+]_i$ may seem modest, Despa et al. (2002b) showed that, compared to other HF-induced alterations such as smaller Ca^{2+} transients and longer action potentials, it has the greatest impact on NCX function, and thus on cellular and SR Ca^{2+} load.

Diabetes is a systemic disease that leads to structural, contractile and electrical abnormalities of the heart, even in the absence of coronary artery disease or hypertension (Taegtmeyer et al., 2002; Young et al., 2002, 2009; Guha et al., 2008; Boudina and Abel, 2010). Diabetic cardiomyopathy is characterized by diastolic dysfunction (>50% prevalence) that progresses to systolic dysfunction and heart failure at more advanced diabetic stages (Ingelsson et al., 2005; Kostis and Sanders, 2005; Masoudi and Inzucchi, 2007; Pataky et al., 2011; Chaudhary et al., 2015). Some studies reported alterations in myocyte Na^+ transport consistent with elevated $[Na^+]_i$ in animal models of both type-1 and type-2 diabetes (see below). However, whether or not $[Na^+]_i$ is altered in diabetic hearts was largely unknown until we recently found higher $[Na^+]_i$ in resting and contracting myocytes from rats with late-onset type-2 diabetes that display a cardiac phenotype that closely resembles the diabetic cardiomyopathy in humans (HIP rats) (Lambert et al., 2015). Interestingly, the $[Na^+]_i$ rise that we measured in diabetic HIP rat hearts is comparable to the increase in $[Na^+]_i$ that occurs in HF.

As discussed above, high $[Na^+]_i$ may amplify the risk for arrhythmias and cause oxidative stress. Indeed, elevated $[Na^+]_i$ was shown to cause oxidation of NAD(P)H and to increase the H_2O_2 level in myocytes from guinea-pigs with heart failure induced by aortic constriction (Liu and O'Rourke, 2008). These effects were prevented by pharmacological inhibition of mitoNCX (Liu and O'Rourke, 2008). In a guinea pig model of heart failure and sudden cardiac death (aortic constriction + daily β-adrenergic receptor stimulation), mitoNCX inhibition attenuated cardiac hypertrophic remodeling and prevented cardiac dysfunction and sudden cardiac death, likely through normalizing $[Ca^{2+}]_m$ (Liu et al., 2014). Therefore, the increase in $[Na^+]_i$ is an active contributor to heart dysfunction in HF. Similar mechanisms are likely also in play in diabetic cardiomyopathy. In support of this assertion, Babsky et al. (2001) found that higher $[Na^+]$ had a larger negative effect on state 3 respiration, rate of oxidative phosphorylation and ATP production in mitochondria isolated from rats with streptozotocin-induced diabetes compared to control.

MECHANISMS UNDERLYING THE $[Na^+]_i$ RISE IN HEART FAILURE AND DIABETES

Since $[Na^+]_i$ is at steady-state when the active Na^+ efflux through the Na^+/K^+-ATPase and the total Na^+ influx through various pathways (**Figure 1**) are at equilibrium, the $[Na^+]_i$ rise in HF and

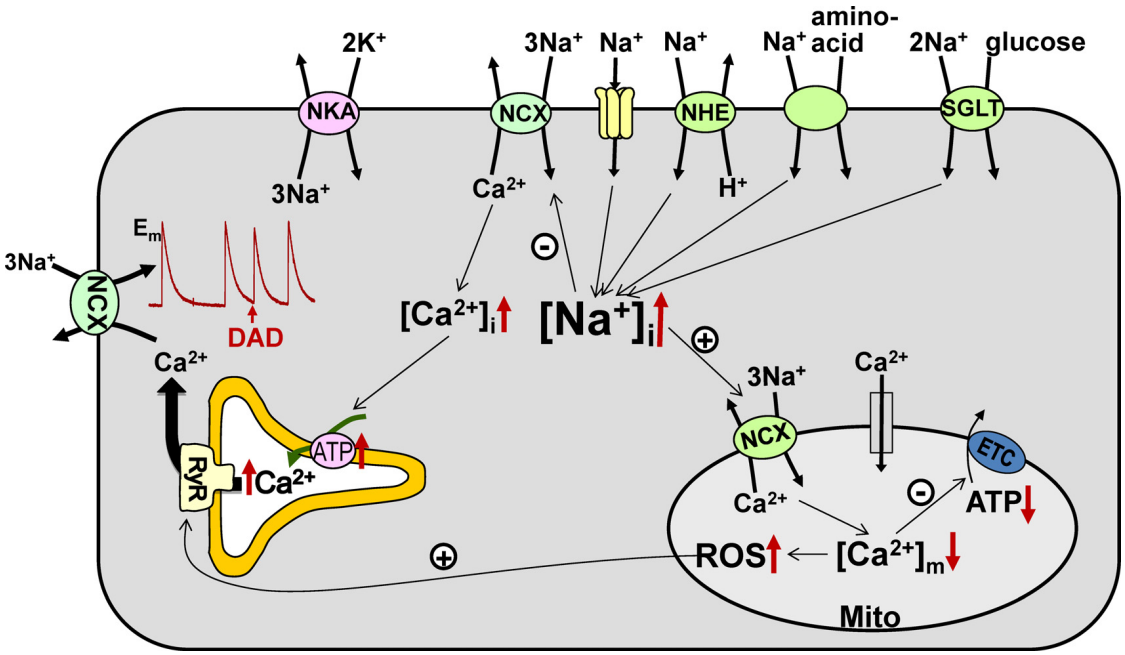

FIGURE 1 | Na^+ transport pathways and consequences of elevated $[Na^+]_i$ in cardiac myocytes. Several pathways, including the Na^+/Ca^{2+} exchanger (NCX), Na^+ channels, Na^+/H^+ exchanger (NHE), Na^+-glucose cotransporter (SGLT), and Na^+-aminoacid cotransporter, contribute to the entry of Na^+ into cardiac myocytes, while the Na^+/K^+-ATPase (NKA) is the essential route for Na^+ efflux. Either enhanced Na^+ influx or impaired Na^+ extrusion result in higher $[Na^+]_i$. Elevated $[Na^+]_i$ leads to (i) increased cellular and SR Ca^{2+} load, which augments the risk for the occurrence of delayed afterdepolarizations (DADs), and (ii) reduced mitochondrial Ca^{2+} levels, which leads to lower ATP production and causes oxidative stress. ETC – electron transport chain.

diabetic cardiomyopathy could be caused by both reduced Na^+ extrusion and enhanced Na^+ entry.

Na^+/K^+-ATPase Expression and Function in Heart Failure and Diabetes

Numerous studies in human myocardium and animal models reported lower protein expression of various NKA subunits in HF. Schwinger et al. (1999) found reduced expression of NKA α_1-, α_3-, and β_1-subunits in failing human hearts. Protein expression of all three α-subunit isoforms is decreased in hearts from rabbits with pressure and volume overload-induced HF (Bossuyt et al., 2005). In contrast, expression of α_1 isoform is unchanged while α_2 is reduced and α_3 is increased in most rat HF models (Verdonck et al., 2003a). These data suggest that NKA activity might be reduced in HF. However, NKA function depends strongly on regulation by various modulators, including the endogenous inhibitor phospholemman. Indeed, Boguslavskyi et al. (2014) reported hypophosphorylation of phospholemman with no change in NKA expression following aortic constriction in mice, which resulted in a progressive decline in NKA current and elevation of $[Na^+]_i$. Thus, functional measurements in live cells and intact beating hearts are needed in order to compare NKA activity in failing and control hearts. By measuring the rate of $[Na^+]_i$ decline as a function of $[Na^+]_i$ in live myocytes, we found no changes in either the maximal Na^+ transport rate or the apparent affinity for internal Na^+ in myocytes from failing rabbit hearts compared to controls (Despa et al., 2002b). Decreased maximal Na^+ extrusion rate (mainly through NKA-α2 isoform) but unchanged $[Na^+]_i$-affinity were reported in myocytes from rats with HF following myocardial infarction (Semb et al., 1998; Swift et al., 2008) as well as in mice with end-stage HF following genetic deletion of SERCA2 (Louch et al., 2010). In contrast, myocytes from dogs with chronic atrioventricular block and hypertrophy have unaltered maximal NKA current but reduced NKA $[Na^+]_i$-affinity (Verdonck et al., 2003b). Overall, NKA activity is decreased in some but not all HF models investigated, which may contribute to the rise in $[Na^+]_i$.

There are significantly fewer studies of NKA expression and function in diabetic cardiomyopathy. NKA activity is reduced by 21% in the myocardium of rats with streptozocin-induced type-1 diabetes (Kjeldsen et al., 1987). Hansen et al. (2007) found decreased NKA current measured with 10 mM Na^+ but not with 80 mM Na^+ in the pipette solutions in myocytes from rabbits with alloxan-induced type-1 diabetes, which indicates a reduction in the affinity of NKA for internal Na^+ but no change in the maximal NKA activity. Myocytes from type-2 diabetic HIP rats showed no change in NKA-mediated Na^+ extrusion for $[Na^+]_i$ in the physiological range (0–20 mM) compared to their control littermates (Lambert et al., 2015). In agreement with this result, we also found that NKA-α1 expression is unaltered, while there is ∼50% decrease in NKA-α2 (Lambert et al., 2015). Since NKA-α2 represents less than 25% of the total NKA in rat myocytes (Despa and Bers, 2007; Swift et al., 2007), a ∼50% reduction in its expression has only a minor effect on total NKA function. However, NKA-α2 has a preferential localization in the t-tubules

(Despa and Bers, 2007; Swift et al., 2007, 2010; Despa et al., 2012a) and therefore reduced NKA-α2 expression might affect Ca^{2+} cycling and contractility through local, subcellular, effects.

Na^+ Influx Pathways in Heart Failure and Diabetes

We (Despa et al., 2002b) and others (Baartscheer et al., 2003) found that higher [Na^+]$_i$ is caused by enhanced Na^+ influx rather than reduced NKA activity in hearts from rabbits with pressure and volume-overload induced HF. This is also the case in myocytes from type-2 diabetic HIP rats, where Na^+ influx is increased by ∼40% compared to myocytes from control rats (Lambert et al., 2015). While not directly measuring the rate of Na^+ entry, several other studies reported upregulation of membrane transporters that facilitate Na^+ import in HF and diabetic cardiomyopathy (see below), supporting an essential role for Na^+ influx in the [Na^+]$_i$ rise that occurs in these pathological conditions.

Na^+ Current in Heart Failure and Diabetes

We found that the excess Na^+ influx in myocytes from rabbits with HF is TTX-sensitive, which suggests that it is carried by Na^+ channels (Despa et al., 2002b). There are numerous reports of increased late Na^+ current in HF (Maltsev et al., 1998; Valdivia et al., 2005; Mishra et al., 2015; Hegyi et al., 2018), a slowly inactivating current that may be carried by both cardiac and neuronal Na^+ channels present in myocytes. While the amplitude of late Na^+ current is small (∼0.1–0.5% of the amplitude of the peak Na^+ current), the current is long lasting (hundreds of milliseconds) and thus may contribute to myocyte Na^+ homeostasis. However, the role of late Na^+ current in elevating Na^+ influx and [Na^+]$_i$ in HF is controversial. On one hand, ranolazine, a late Na^+ current inhibitor, reduced [Na^+]$_i$, and diastolic Ca^{2+} overload in failing human hearts (Sossalla et al., 2008). Moreover, the CaMKII-dependent increase in late Na^+ current produced by exogenous reactive oxygen species was associated with a TTX and ranolazine-dependent rise in [Na^+]$_i$ (Wagner et al., 2011). On the other hand, computational modeling predicts that higher late Na^+ current measured in myocytes from failing hearts generates only a modest increase in [Na^+]$_i$, smaller than measured experimentally (Wagner et al., 2011; Cardona et al., 2016). Alternatively, HF may also enhance a background Na^+ channel conductance that is responsible for the higher rate of Na^+ entry.

Independent of a potential effect on [Na^+]$_i$, increased late Na^+ current contributes to the prolongation of the action potential in HF, which may result in early afterdepolarizations. Moreover, via NCX, longer action potentials favor Ca^{2+} loading of the myocyte, which increases the propensity for delayed afterdepolarizations. Indeed, ranolazine significantly abbreviated the action potential and prevented the occurrence of delayed afterdepolarizations in myocytes from mice with HF induced by aortic constriction (Toischer et al., 2013). Ranolazine prevented ventricular fibrillation in rabbits with pacing-induced HF (Frommeyer et al., 2012) Thus, while the contribution of increased late Na^+ current to the rise in [Na^+]$_i$ is not fully elucidated, late Na^+ current inhibition has proven beneficial effects in HF.

Na^+/Ca^{2+} Exchanger in Heart Failure and Diabetes

NCX, which exchanges three Na^+ ions for one Ca^{2+}, is the main route for Ca^{2+} extrusion (Bers, 2001) and the most prominent contributor to Na^+ influx (Despa et al., 2002a) in cardiac myocytes. Cardiac NCX expression is generally increased in both animal models of HF (O'Rourke et al., 1999; Pogwizd et al., 1999; Louch et al., 2010) and failing human hearts (Hasenfuss et al., 1999). However, higher NCX expression does not necessarily translate into higher rate of NCX-mediated Na^+ entry. This is because the higher [Na^+]$_i$ and smaller Ca^{2+} transients typically seen in HF shift the balance of fluxes through NCX to disfavor the Ca^{2+} out/Na^+ in mode of function and may even cause the reversal of the exchanger during an action potential. In agreement with this reasoning, we found no change in the NCX-mediated Na^+ influx in failing rabbit myocytes (Despa et al., 2002b) despite a 100% increase in NCX expression (Pogwizd et al., 1999).

In contrast to HF, NCX expression and function is decreased in rats with streptozotocin-induced type-1 diabetes (Chattou et al., 1999; Hattori et al., 2000) and in type-2 diabetic HIP rats (Despa et al., 2012b). It is thus unlikely that NCX contributes to the enhanced myocyte Na^+ entry in diabetes.

Na^+/H^+ Exchanger in Heart Failure and Diabetes

NHE is markedly upregulated in HF (Yokoyama et al., 2000; Leineweber et al., 2007; Fliegel, 2009; Packer, 2017) and its inhibition improved heart function in various animal models of HF (Kusumoto et al., 2001; Engelhardt et al., 2002; Aker et al., 2004; Kilić et al., 2014). Baartscheer et al. (Baartscheer et al., 2003) reported that increased Na^+/H^+-exchange activity is responsible for elevated [Na^+]$_i$ in myocytes from failing rabbit hearts. Moreover, chronic treatment with cariporide, an NHE inhibitor, prevented the onset of HF in rabbits with pressure and volume overload (Baartscheer et al., 2005). The activity of myocardial Na^+/H^+ exchanger (NHE) is enhanced and contributes to left ventricular hypertrophy in the Goto-Kakizaki rat model of T2D (Darmellah et al., 2007). Increased NHE activity leads to higher [Na^+]$_i$ gain during ischemia-reperfusion in hearts from T2D db/db mice (Anzawa et al., 2006). Moreover, reducing [Na^+]$_i$ gain during ischemia-reperfusion by NHE inhibition was associated with a lower incidence of ventricular tachycardia and fibrillation in db/db hearts (Anzawa et al., 2006). These data point to an important contribution of NHE to the excess cardiac Na^+ influx in HF and diabetic cardiomyopathy.

Na^+-GLUCOSE COTRANSPORTER AND [Na^+]$_i$ DYSREGULATION IN DIABETIC HEARTS

One transporter known to be present in the heart but rarely discussed in the context of myocyte Na^+ homeostasis is the Na^+-glucose cotransporter (SGLT), which couples Na^+ transport to glucose uptake and thus to energy substrate metabolism. The major SGLT isoforms, SGLT1, and SGLT2, have distinct tissue

distribution and systemic role. SGLT1 is found predominantly in epithelial cells from the intestine, where it participates in dietary glucose absorption, whereas SGLT2 is the major isoform expressed in renal epithelial cells and is essential for glucose reabsorption from the forming urine. Highly specific SGLT2 inhibitors are the latest class of blood glucose lowering drugs. Recently, SGLT2 inhibitors were demonstrated to have beneficial cardiac effects in patients with type-2 diabetes and HF (Zinman et al., 2015). However, reports from several labs (Nishimura and Naito, 2005; Wright and Loo, 2011; Van Steenbergen et al., 2017) indicate that SGLT2 is not expressed in the heart. This suggests that the cardioprotection conferred by SGLT2 inhibitors is mediated by interaction with a different cardiac target and/or by effects in extracardiac tissues. Intriguingly, two recent studies (Baartscheer et al., 2017; Uthman et al., 2018) found that SGLT2 inhibitors (empagliflozin, dapagliglozin, and canagliflozin) block the Na^+/H^+ exchanger, possibly by binding to the Na^+-binding site of NHE, and thus lower myocyte $[Na^+]_i$ in multiple species. Furthermore, empagliflozin and canagliflozin also induced vasodilation (Uthman et al., 2018). While these measurements were performed in healthy hearts/myocytes, the effects uncovered are likely to play a part in the improvement of heart function by SGLT2 inhibitors that was observed clinically in patients with HF and type-2 diabetes.

In contrast to SGLT2, SGLT1 is highly expressed in the heart (Zhou et al., 2003; Banerjee et al., 2009, 2010; Wright and Loo, 2011). Recently, SGLT1 upregulation was causally linked to PRKAG2 cardiomyopathy that is caused by mutations in the gene encoding the γ2 subunit of AMP-activated protein kinase (Banerjee et al., 2010) and cardiac-specific SGLT1 deletion attenuated the cardiomyopathy (Ramratnam et al., 2014). Moreover, cardiac-specific overexpression of SGLT1 in mice causes hypertrophy and left-ventricular dysfunction (Ramratnam et al., 2014). Thus, there is increasing evidence that enhanced SGLT1 activity harms the heart.

Banerjee et al. (2009) reported that the mRNA level of SGLT1 is increased in hearts from humans and mice with type-2 diabetes and ischemic cardiomyopathy. In agreement with this result, we found higher SGLT1 protein expression in failing hearts from T2D patients compared to failing hearts from lean, non-diabetic individuals and in hearts from type-2 diabetic HIP rats vs. control rats (Lambert et al., 2015). Obesity, in the absence of type-2 diabetes, was also associated with elevated levels of cardiac SGLT1 in human hearts (Lambert et al., 2015). Moreover, the presence of HF alone resulted in higher cardiac SGLT1 protein expression in both lean and obese individuals (Lambert et al., 2015). Consistent with augmented SGLT1 expression, we found that the SGLT-mediated glucose uptake is significantly increased in hearts from diabetic vs. control rats (Lambert et al., 2015). Moreover, SGLT inhibition (both pharmacological and through omission of glucose from external solution) greatly reduced the rate of Na^+ entry in myocytes from diabetic rats while it had no significant effect in myocytes from control rats. SGLT-mediated Na^+ influx was ≈7 times higher in diabetic vs. control hearts (Lambert et al., 2015). Together, these results indicate that SGLT1 is upregulated in diabetic rat hearts and its activation is largely responsible for the excess Na^+ influx that accounts for the increase in $[Na^+]_i$.

Other members of the Na^+-glucose cotransporter family may also play a role in the development of diabetic cardiomyopathy. Van Steenbergen et al. (2017) found that hyperglycemia stimulates the production of reactive oxygen species in the heart through activation of NADPH oxidase and this mechanism is prevented in myocytes from mice with genetic deletion of the sodium-myoinositol cotransporter-1 (SMIT1). This finding suggests that SMIT1 is activated by high glucose, which may also contribute to the $[Na^+]_i$ rise in diabetic hearts.

In summary, $[Na^+]_i$ is augmented in both HF and diabetic cardiomyopathy and higher $[Na^+]_i$ further exacerbates heart dysfunction. An enhancement of Na^+ influx is at least partially responsible for the $[Na^+]_i$ rise in both conditions. However, the Na^+ entry pathways that mediate the excess Na^+ entry may be distinct in the two pathologies, with Na^+ channels and NHE being activated in HF and SGLT isoforms and NHE having the main contribution in diabetic cardiomyopathy.

AUTHOR CONTRIBUTIONS

SD reviewed the literature in the field, and wrote and edited the manuscript.

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