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Activation of AMPK by Metformin Improves Left Ventricular Function and Survival in Heart Failure

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Abstract

Clinical studies have reported that the widely used anti-hyperglycemic drug metformin significantly reduces cardiac risk factors and improves clinical outcomes in patients with heart failure. The mechanisms by which metformin exerts these cardioprotective effects remain unclear and may be independent of anti-hyperglycemic effects. We tested the hypothesis that chronic activation of AMPK with low-dose metformin exerts beneficial effects on cardiac function and survival in *in vivo* murine models of heart failure. Mice were subjected to permanent left coronary artery (LCA) occlusion or to 60 min LCA occlusion followed by reperfusion for 4 wks. High-resolution, two-dimensional echocardiography was performed at baseline and 4 wk post myocardial infarction to assess left ventricular (LV) dimensions and function. Metformin (125 μg/kg) administered to mice at ischemia and then daily, improved survival by 47% ($p < 0.05$ vs. vehicle) at 4 wk following permanent LCA occlusion. Additionally, metformin given at reperfusion and then daily, preserved LV dimensions and LV ejection fraction ($p < 0.01$ vs. vehicle) at 4 wk. The improvement in cardiac structure and function was associated with increases in AMPK and eNOS phosphorylation as well as increased PGC-1α expression in cardiac myocytes. Furthermore, metformin significantly improved myocardial cell mitochondrial respiration and ATP synthesis compared to vehicle. The cardioprotective effects of metformin were ablated in mice lacking functional AMPK or eNOS. This study demonstrates that metformin significantly improves left ventricular function and survival via activation of AMPK and its downstream mediators, eNOS and PGC-1α in a murine model of heart failure.

Keywords

myocardial ischemia; heart failure; metformin; nitric oxide

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Introduction

Heart failure is the inability of the heart to meet hemodynamic demands and represents the end stage of various forms of cardiac disease. In the industrialized nations, heart failure represents a major health problem that has been increasing in prevalence and incidence. In the United States, heart failure affects more than 5 million people, with 500,000 new cases reported every year. It is responsible for almost 1 million hospital admissions and 40,000 deaths annually[1]. The most important cause of HF is coronary artery disease (CAD) and acute myocardial infarction leading to loss of functioning myocytes, development of myocardial fibrosis, and subsequent left ventricular (LV) remodeling, all of which contribute towards the development of LV dysfunction.

Metformin is an orally administered biguanide drug that is widely used to lower blood glucose concentrations in patients with diabetes mellitus. Metformin decreases blood glucose by mechanisms different from those of sulphonylureas or insulin and exerts its actions by enhancing insulin sensitivity, inducing greater peripheral uptake of glucose, and decreasing hepatic glucose output while lowering plasma insulin concentrations2. Additionally, blood glucose control is achieved without any weight gain especially in patients with obesity and metabolic syndrome[3]. Analysis of the United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that improved glycemic control in overweight patients treated with metformin was associated with a decreased risk of diabetes related cardiovascular end points and all cause deaths when compared to conventional therapies that lower blood glucose to similar levels[4]. Therefore, the cardioprotective effects of metformin cannot be attributed to its anti-hyperglycemic effects alone and may be related to the actions of metformin on lipid metabolism, vascular smooth muscle and cardiomyocyte calcium handling, endothelial function, hypercoagulation, and platelet reactivity5.

Experimental studies suggest that the pleiotropic effects of metformin are mediated in part by activation of AMP-activated protein kinase (AMPK)6, a protein kinase that is activated in response to alterations in cellular energy levels. When activated, AMPK stimulates fatty acid oxidation, promotes glucose transport, accelerates glycolysis, and inhibits both triglyceride and protein synthesis7. Activation of AMPK has also been shown to increase the phosphorylation and activity of endothelial nitric oxide synthase (eNOS)8 as well as the expression of transcriptional coactivator and metabolic regulator, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α)9, both of which are important regulators of mitochondrial biogenesis and function. Activation of AMPK by metformin may account for the reduction of cardiovascular disease risk10 and improvement of vascular function in patients with type 2 diabetes11. The purpose of the present study was to investigate the potential cardioprotective effects of a chronic low dose administration of metformin on survival and cardiac function in a murine model of heart failure.

Materials and Methods

Animals

C57BL/6J mice obtained from Jackson Laboratories (Bar Harbor, ME) were utilized for the present study. Additionally, we used mice completely deficient in eNOS ($eNOS^{-/-}$, maintained on a C57BL/6J background), as well as, cardiac-specific transgenic mice overexpressing a dominant-negative AMPKα2 subunit (AMPKα2). The generation of AMPKα2 dn mice has been described previously[12]. AMPKα2 dn Tg and non-transgenic (NTg) littermates were bred in our colony and maintained on an FVB background. All animals were used at 8-10 weeks of age and received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals published by the

National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

Materials

Metformin (1,1-dimethylbiguanide hydrochloride) was purchased from Sigma (St. Loius, MO). It was dissolved in saline and administered at a dose of 125 μg/kg in a final volume of 100 μL as an intracardiac (i.c.) injection at the time of reperfusion once (Metformin) or once at reperfusion followed by daily intraperitoneal (i.p.) injections (Metformin QD) for 4wk. Saline was used as a vehicle at reperfusion followed by daily i.p. injections for 4 wk (vehicle). In the permanent left coronary artery occlusion (LCA) occlusion model (Figure 1A), metformin was administered once at the onset of ischemia i.c. followed by daily i.p. injections for 4 wk.

Heart failure Protocols

Heart failure was induced by either permanent ligation of the LCA (Figure 1A) or by subjecting the mice to 60 min of LCA occlusion followed by reperfusion for up to 4 wk (Figure 2A). Surgical ligation of the LCA was performed according to methods described previously[13].

Myocardial Area-at-Risk and Infarct Size Determination

Left ventricular area-at-risk (AAR) and infarct size (Inf) determination was performed using Evans blue dye and 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) staining method. All of the procedures for the AAR and Inf determination have been previously described[14].

Echocardiograhic Assessment of Left Ventricular Structure and Function

Baseline echocardiography images were obtained one week prior to LCA ischemia to avoid any anesthetic effects as previously described[15]. After 4 wk following the myocardial infarction (4 wk Post), Echo images were obtained and analyzed once again.

Histological Analysis of Infarct Size

After the post myocardial infarction echocardiographic assessment, the hearts of mice were processed for histological assessment of infarct size as previously described[14].

Western Blot Analysis

Western blot analysis was performed as previously described[15].

Cardiac Mitochondria Isolation

Cardiac mitochondria were isolated from the following groups of mice as previously described[16]: sham operated, vehicle and Metformin QD treated mice.

Mitochondrial Respiratory Rate and ATP synthesis

Oxygen consumption and ATP synthesis of cardiac mitochondria was measured as previously described[1617].

Statistical Analysis

All the data in this study are expressed as mean ± standard error (SEM). Differences in data between the groups were compared using Prism 4 (GraphPad Software, Inc) with Student's paired 2-tailed t-test or one-way analysis of variance (ANOVA) where appropriate. For the

ANOVA, if a significant variance was found, the Tukey or Bonferoni test was used as the post hoc analysis. p values less than 0.05 was considered significant.

Results

Metformin improves survival following permanent LCA occlusion

Mice were subjected to permanent occlusion of LCA and metformin or vehicle was administered prior to LCA ligation and then daily for 4 wk (Figure 1A). 2-D Echo was obtained at baseline and 4 wk to assess LV function. At 4 wk, both vehicle and metformin treated mice subjected to myocardial infarction exhibited significant mortality compared with sham-operated mice. Mice receiving metformin exhibited an overall survival rate of 44% (12/27) during the 4 wk protocol compared with mice treated with vehicle, which exhibited a 30% (11/37) survival. Metformin treatment, therefore led to a 47% improvement in survival compared with the vehicle ($p < 0.05$ between groups) (Figure 1B). The extent of myocardial infarction was evaluated in mice receiving either metformin (n=6) or vehicle (n=6) at 24 hr in separate groups of mice. The AAR per LV, Inf per AAR and Inf per LV were similar (p = not significant, NS) in both metformin and vehicle treated mice (Figure 1C). Following permanent LCA occlusion, at 4 wk, EF was severely reduced ($p < 0.001$ vs. baseline) and similar in the vehicle (n=11) and metformin (n=12) treated mice (Figure 1D). These data demonstrate that metformin treatment significantly improves survival in mice subjected to permanent occlusion of LCA and the survival benefit is independent of any effect on infarct size or left ventricular function.

Metformin improves LV structure and function and attenuates cardiac hypertrophy in heart failure

In order to study the effects of metformin in a more clinically relevant model of heart failure that mimics the effects of coronary revascularization therapy, mice were subjected to 60 min of LCA occlusion followed by reperfusion (Figure 2A). In the initial set of experiments, metformin (125 μg/kg) or vehicle were administered at the time of reperfusion and the extent of myocardial infarction was evaluated at 24 hr (Online Figure I). We found that metformin treatment decreased Inf/AAR by 29% (68.63±2.34% for vehicle vs. 48.92±2.51% for Metformin, $p < 0.001$) following MI compared to vehicle. To investigate if the reduction in infarct size alone was sufficient to improve LV structure and function, mice were randomized into three groups and subjected to 60 min ischemia. The first group received saline as a vehicle at reperfusion (i.c) and then daily (i.p) injections (vehicle), the second group received metformin as a single (i.c.) bolus only once at the time of reperfusion (Metformin) and the third group received metformin (i.c.) once at reperfusion and then as daily (i.p.) injections (Metformin QD). Echo was performed at baseline and 4 wk to assess LV dimensions and EF in all groups. Following the severe ischemic insult, all groups of mice developed profound ischemia-induced cardiomyopathy as evidenced by a significant increase in both LVEDD and LVESD (Figure 2B-C) and a significant decrease in EF (Figure 2D). At 4 wk following reperfusion, a single administration of metformin at reperfusion alone did not attenuate LV dilatation or improve LV function. In contrast, daily administration of metformin starting at the time of reperfusion significantly improved LVEDD and LVESD by 28% each ($p < 0.05$ vs. vehicle) and EF by 31% ($p < 0.01$ vs. vehicle) at 4 wk. In addition, cardiac hypertrophy, as measured by heart to body weight (H/BW) ratio was increased in vehicle and Metformin groups ($p < 0.01$) but not in the Metformin QD group compared to sham (p= NS) (Figure 2E).

We also measured the infarct area relative to the entire left ventricle at 4 wk following reperfusion (Online Figure I). For each heart, we analyzed multiple sections taken from the mid-ventricle and then averaged these numbers to obtain a single Inf/LV measurement for

each animal. Vehicle-treated mice displayed a 13.62 ± 1.16% Inf/LV. Conversely, both groups treated with metformin displayed a smaller area of scar formation. Analysis from the multiple mid-ventricle sections per animal revealed that the mice in the Metformin group displayed a 9.01 ± 0.69% Inf/LV ($p<0.01$ vs. vehicle) and the mice in the Metformin QD group displayed a 8.16 ± 063% Inf/LV ($p<0.001$ vs. vehicle). The difference between the metformin treatment groups was not statistically significant (p=NS). Furthermore, no differences in blood glucose were observed following treatment with Metformin QD for 4 wks (Online Table I). Since Metformin QD caused significant improvements in LV function, LV dimension and cardiac hypertrophy, this therapeutic strategy was used in all subsequent experiments.

Metformin promotes the phosphorylation of AMPK and eNOS and increases the expression of PGC1α during heart failure

We have previously reported that a single injection of metformin phopshorylates and increases the activity of both AMPK and eNOS for up to 24hr[15]. In the present study, we investigated if Metformin QD resulted in sustained elevations in AMPK and eNOS phosphorylation as well as expression of PGC1α at 4 weeks following reperfusion of the ischemic myocardium (Figure 3A). Phosphorylation of $AMPK^{Thr172}$ was elevated in both vehicle ($p < 0.05$) and Metformin QD ($p < 0.001$) treated groups (Figure 3B). Metformin QD was found to significantly augment the ischemia-induced increase in AMPK-P^{Thr172} ($p < 0.01$ vs. vehicle). Similarly, Metformin QD treatment significantly increased phosphorylation of $eNOS^{Ser1177}$ ($p < 0.05$ vs. vehicle) (Figure 3C) and expression of PGC1α ($p < 0.05$ vs. vehicle) (Figure 3D) compared to sham and vehicle treatment. These results suggest that the activation of AMPK and its downstream mediators eNOS and PGC1α may underlay the beneficial effects of metformin in the myocardium and protect against ischemia-induced heart failure.

Metformin improves mitochondrial respiration and ATP synthesis during heart failure

Since both, eNOS and PGC1α are important regulators of mitochondrial biogenesis and mitochondrial function, we investigated if metformin treatment had any beneficial effects on mitochondrial function and ATP synthesis. Mitochondria isolated from the hearts of vehicle-treated mice were found to have a 36% reduction in maximal ADP-stimulated (state 3) oxygen consumption as compared to sham-operated animals (Figure 4A). Mitochondria from vehicle-treated mice also had an increased oligomycin-inhibited respiration (Figure 4B) and reduced respiratory control ratio (Figure 4C), suggestive of uncoupling. Additionally, ATP synthesis rates and the ATP/oxygen consumption ratio in the mitochondria from vehicle-treated mice were significantly reduced as compared to sham-operated mice (Figure 4D-E). Conversely, mitochondria from Metformin QD-treated mice were found to have significantly greater rates of oxygen consumption, lower rates of oligomycin-inhibited respiration, higher respiratory control ratios, greater ATP synthesis rates, and higher ATP/oxygen consumption ratios as compared to vehicle-treated mice. This data indicates that the respiration of cardiac mitochondria during heart failure was inefficient, likely a result of uncoupled respiration, and that metformin treatment attenuates this dysfunction.

Metformin mediated improvements in LV structure and function are abrogated in AMPK deficient mice

To investigate the role of AMPK in metformin-mediated cardioprotection, both AMPKdn and NTg mice were subjected to the ischemia-induced heart failure protocol. Metformin administration at reperfusion decreased Inf/AAR by 30% (43.2±3.6% for vehicle vs. 30.2±5.8% for Met QD, $p < 0.05$) in NTg mice but not in AMPKdn mice (48.1±5.7% for vehicle vs. 44±8.4% for Met QD, p = NS). Metformin QD did not result in any significant

improvements in LVEDD (Figure 5A), LVESD (Figure 5B) and EF (Figure 5C) in AMPKdn mice and no differences were observed in cardiac hypertrophy as measured by H/BW ratios (Figure 5E). Metformin QD however did improve all parameters of LV structure and function in NTg mice compared to vehicle and the results were consistent with the improvement seen in C57BL/6 mice (Figure 5). These data suggest that AMPKα2 plays an important role in metformin-mediated cardioprotection against HF.

Metformin mediated improvements in LV structure and function are abrogated in eNOS$^{-/-}$ mice

To investigate the role of eNOS in metformin-mediated cardioprotection, eNOS$^{-/-}$ mice were subjected to the HF protocol. Metformin administered at reperfusion did not attenuate Inf per AAR and Inf per LV (p = NS) at 24hr. At 4 wk, Echo analysis revealed LV dilation and systolic dysfunction in the hearts of both groups as evidenced by a significant increase in LVEDD (Figure 6A) and LVESD (Figure 6B) and EF (Figure 6C). Metformin QD did not result in any significant improvements in LVEDD, LVESD and EF in eNOS$^{-/-}$ mice and no differences were observed in cardiac hypertrophy as measured by H/BW ratios. (Figure 6E). These data suggest that eNOS and NO may also be important mediators of metformin-mediated cardioprotection.

Discussion

This study demonstrates that metformin therapy significantly retards the progression of ischemic cardiomyopathy and heart failure in mice after myocardial infarction. The key findings in this study are: (1) metformin therapy improved survival by 47% after permanent occlusion of the left coronary artery compared to treatment with saline, (2) metformin therapy led to significant improvements in cardiac remodeling and function during heart failure, (3) metformin-mediated cardioprotection was associated with an increase in phosphorylation of AMPK and eNOS and an increase in expression of PGC-1α and (4) metformin therapy attenuated mitochondrial dysfunction during heart failure. These data provide additional insight into the pleiotropic effects of metformin in cardiovascular disease and its therapeutic role in ischemic induced heart failure.

The pleiotrophic actions of metformin are thought to be mediated by the activation of AMPK[18,19], an important regulator of diverse cellular pathways[20]. Chronic activation of AMPK phosphorylates transcription factors altering gene expression[21] and modulates mitochondrial biogenesis[22]. *In vitro* studies have shown that AMPK activation is a key mediator of the changes in substrate utilization during cardiac ischemia and functions to maintain energy homeostasis, cardiac function and myocardial viability[23]. Our data demonstrate that metformin increases AMPK phosphorylation and that the cardioprotective actions of metformin were ablated in AMPKα2 dominant negative mice. These results suggest that the chronic activation of AMPK during the development of ischemia-induced heart failure is a critical mechanism mediating the beneficial actions of metformin.

Heart failure is associated with abnormalities of mitochondrial biogenesis[24] and mitochondrial injury correlates strongly with its severity[25]. In heart failure, there is a decrease in activity of complexes of the respiratory chain and Krebs cycle enzymes. The reduced expression of mitochondrial proteins results in decreased mitochondrial respiration efficiency and limited ATP synthesis capacity and myocardial energy production[26]. The decreased oxidative capacity of the failing myocardium therefore limits the ability of the heart to meet hemodynamic demands and leads to symptoms of heart failure. Both, eNOS and PGC-1α are important regulators of mitochondrial biogenesis and function and play important roles in the pathophysiology of HF[27,28]. For instance, targeted overexpression of the eNOS gene within the vascular endothelium has been shown to attenuate cardiac

dysfunction and improve survival in ischemic cardiomyopathy[29]. AMPK has been shown to increase the phosphorylation of eNOS leading to an increase in eNOS activity and NO bioavailability[30]. Additionally, we[15] and others[8,30] have demonstrated that metformin increases the phosphorylation of eNOS in an AMPK-dependent manner, as evidenced by the finding that metformin fails to increase eNOS phosphorylation in the hearts of AMPKα2 dominant negative mice. The results of the current study support these previous findings, as we found that metformin therapy promotes the phosphorylation of eNOS during heart failure and that the metformin-mediated improvements in LV function were abolished in the absence of eNOS. AMPK is also an upstream activator of PGC-1α and may exert its actions by increasing the expression of PGC-1α[9]. PGC-1α is a member of a family of transcription coactivators that plays a central role in the regulation of cellular energy metabolism[31]. PGC-1α is induced in response to conditions that demand increased myocardial ATP synthesis[32,33] and has been shown to drive mitochondrial biogenesis and improve mitochondrial function in cardiac myocytes and hearts of transgenic mice[28]. PGC-1α deficient mice have decreased expression of genes involved in mitochondrial oxidative phosphorylation and have decreased state 3 mitochondrial respiration rates[27,33]. In our study metformin treatment increases the expression of PGC-1α during heart failure. Furthermore, we have demonstrated that metformin improves mitochondrial oxygen consumption and ATP synthesis. These beneficial actions may be mediated by an increase in PGC1-α expression and/or eNOS phosphorylation.

The findings of the current study highlight a metformin-mediated cardioprotective signaling pathway involving AMPK, eNOS, and PGC-1α Previously, we evaluated the cardioprotective effects of a single administration of metformin in the setting of acute myocardial ischemia-reperfusion injury and found that metformin reduced infarct size when it was administered at the time of reperfusion in an AMPK-eNOS dependent fashion15. While the current study also demonstrates that metformin provides protection in a similar manner there are some important differences. First of all, the findings of the current study are significant because they demonstrate that chronic metformin therapy initiated after myocardial ischemia is beneficial for the treatment of heart failure. Importantly, we found that although a single administration of metformin at the time of reperfusion is beneficial in attenuating infarct size, this alone is not sufficient to cause a significant improvement in cardiac function after 4 wk. On the other hand, daily metformin therapy initiated at the time of reperfusion provided significant improvements in cardiac function and LV dimensions. This suggests that metformin treatment could potentially be initiated at the time of coronary artery reperfusion and then continued daily in patients undergoing myocardial ischemia to achieve a long-term improvement in cardiac function and to decrease the morbidity and mortality resulting from heart failure. These findings support several experimental and clinical studies reporting that metformin possesses significant cardioprotective actions and is safe in the setting of diabetes and HF34,[35]. Although previously contraindicated in heart failure due to the potential risk for development of lactic acidosis, the Food and Drug Administration (FDA), in response to the findings of several recent studies, has now updated the prescribing information for metformin to eliminate this contraindication[36]. A meta-analysis of controlled studies evaluating anti-diabetic agents and outcomes in patients with heart failure and diabetes found that metformin when compared to other anti-hyperglycemic therapies significantly reduced mortality and hospital admissions in treated patients despite a similar decrease in hemoglobin A_{1C} values, suggesting that metformin may have additional cytoprotective actions beyond blood glucose lowering actions[34]. This observation is further supported by experimental studies demonstrating that metformin does not affect glucose values in non-diabetic rodents[37], yet improves cardiac function following *in vitro* global ischemia[38]. Finally, the findings of the current study expand on our initial findings and provide data demonstrating that metformin can attenuate mitochondrial dysfunction through the activation of AMPK and the downstream signaling pathway involving eNOS and/or

PGC-1α. As such, this current study is timely and provides important insights into the use of metformin treatment for cardiovascular disease in all patient populations.

The current study mainly focused on the ability of metformin to improve mitochondrial function during heart failure, however, there are certainly a number of other effects mediated by AMPK, eNOS, and PGC-1α that could account for the observed cardioprotection. In particular, the ability of metformin therapy to promote the phosphorylation of eNOS and increase NO bioavailability provides numerous potential cardioprotective actions in the setting of HF, such as vasodilation and the inhibition of oxidative stress and apoptosis[39]. All of these actions in addition to the effects of NO on the mitochondria could account for the improvements in LV function following metformin treatment. In particular, the effects of NO on hemodynamics could play an important role in providing prolonged changes in afterload and coronary blood flow regulation, which could then promote LV function and improve LV ejection fraction. However, in a previous study, we found that a single administration of metformin (125 μg/kg) did not alter hemodynamics in the period immediately following its administration. Nonetheless, since we have not evaluated if chronic metformin therapy could alter hemodynamics, we cannot rule out the possibility that the activation of eNOS over the period of 4 weeks can improve outcome through changes in afterload and/or coronary blood flow. Therefore, additional studies are warranted to fully understand the cardioprotective signaling mechanisms of metformin in the treatment of heart failure.

In summary, our findings demonstrate that low dose metformin administered at the time of reperfusion and daily improves survival and affords significant cardioprotection against ischemia-induced heart failure by improving mitochondrial function via activation of AMPK and the downstream signaling pathway involving eNOS and PGC-1α. These data suggest that metformin therapy should not be limited to the treatment of hyperglycemia but may rather have practical clinical use following myocardial infarction in all patient populations to reduce the morbidity and mortality from ischemia induced heart failure.

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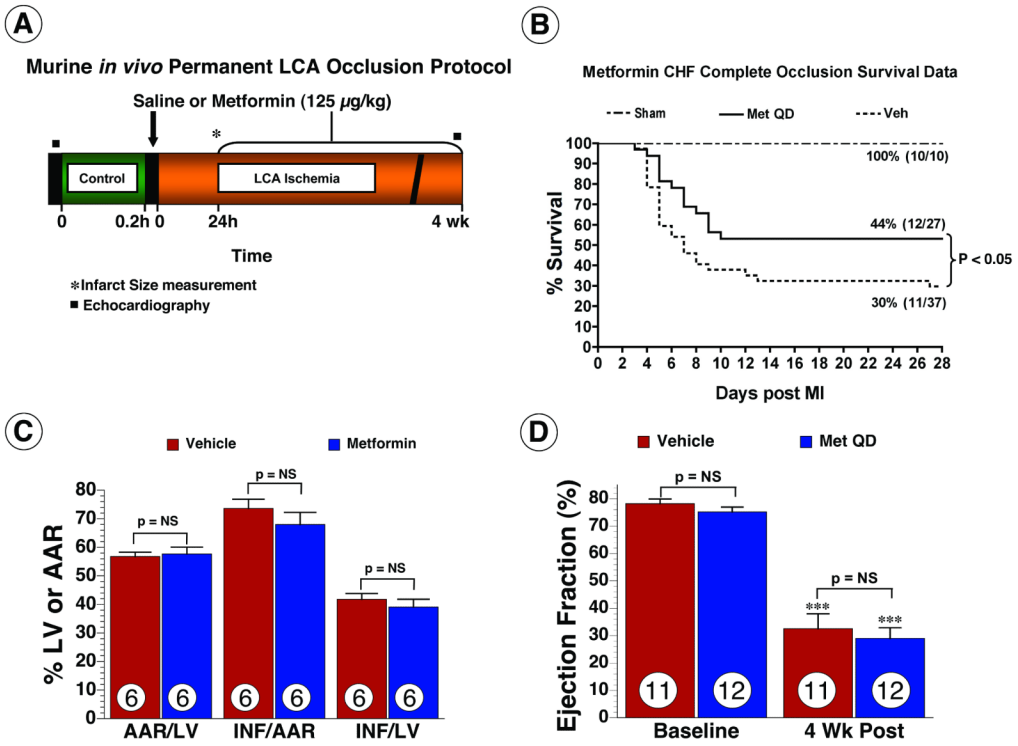

Figure 1.

Metformin improved survival following permanent occlusion of left coronary artery. **(A)** Murine *in vivo* permanent left coronary artery (LCA) occlusion protocol. **(B)** Kaplan-Meier survival curve following metformin therapy in mice during the 4 wk post myocardial infarction. Metformin significantly improved survival by 47% ($p < 0.05$) compared to vehicle treated mice. **(C)** Myocardial infarct size analysis in a subset of animals subjected to 24 hr of myocardial ischemia. Area-at-risk (AAR) as a percentage of the left ventricle (LV) was similar between both groups. Metformin (125 μg/kg) administration did not attenuate myocardial infarct size following permanent LCA occlusion. **(D)** LV function was evaluated in a subset of animals at 4 wks of myocardial ischemia. Ejection fraction at baseline and following myocardial ischemia were similar in both groups. Values are means ± S.E.M. Numbers inside bars indicate the number of animals that were investigated in each group. *** $p < 0.001$ vs. Baseline. Veh = vehicle, Met QD = Metformin QD.

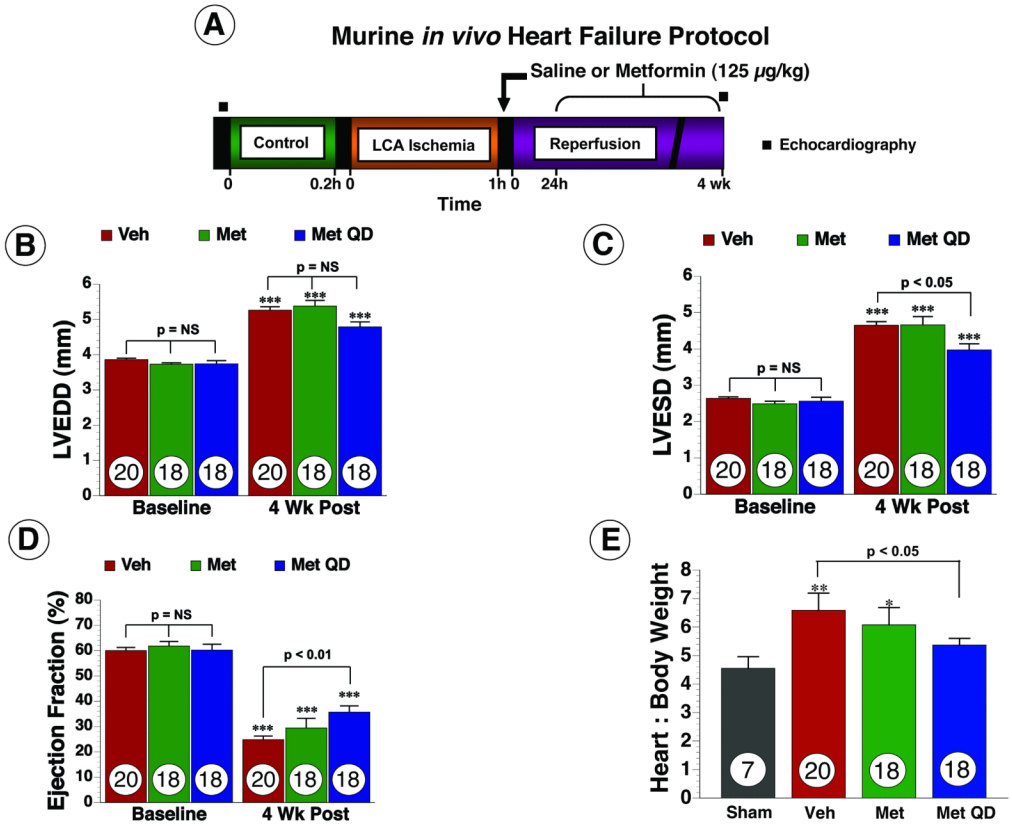

Figure 2.

Metformin improved left ventricular structure and function, as well as, cardiac hypertrophy in mice following myocardial ischemia and reperfusion. **(A)** Murine *in vivo* heart failure protocol. **(B)** Left ventricular end diastolic diameter (LVEDD) **(C)** left ventricular end systolic diameter (LVESD) and **(D)** Ejection fraction were calculated using two-dimensional B-mode echocardiography images at baseline and following myocardial ischemia in all groups **(E)** Heart to body weight (H/BW) ratio used as a measure of cardiac hypertrophy. Values are means ± S.E.M. Numbers inside bars indicate the number of animals that were investigated in each group. *** $p < 0.001$ vs. Baseline, ** $p < 0.01$ vs. Sham, * $p < 0.05$ vs. Sham. Veh = vehicle, Met = Metformin, Met QD = Metformin QD.

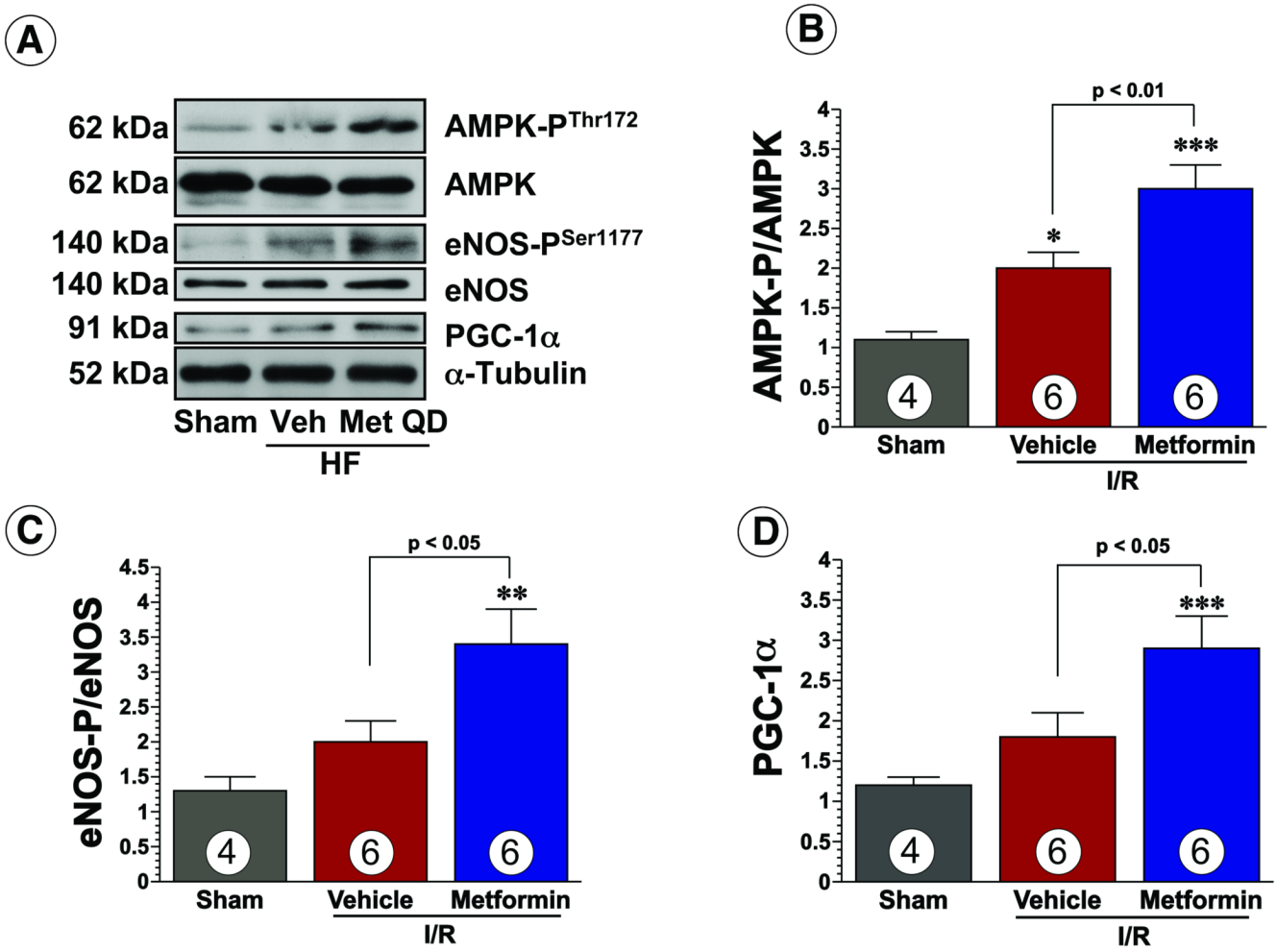

Figure 3.

Chronic administration of metformin phosphorylates AMPK and eNOS and increases the expression of PGC1α at 4 weeks during heart failure (HF). **(A)** Representative immunoblots of AMPK phosphorylated at residue threonine 172 (AMPK-P^{Thr172}) and total AMPK eNOS phosphorylated at residue Serine 1177 (eNOS-$P^{Ser1177}$), total eNOS, and PGC-1α and α-tubulin following HF protocol. Densitometric analysis of **(B)** phosphorylated state $AMPK^{Thr172}$. Bars represent the ratio of phosphorylated AMPK to total AMPK. **(C)** phosphorylated state eNOS-$P^{Ser1177}$. Bars represent the ratio of phosphorylated eNOS to total eNOS **(D)** total PGC1α Values are means ± S.E.M. Numbers inside bars indicate the number of animals that were investigated in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.01$ compared to Sham. Veh = vehicle, Met QD = Metformin QD.

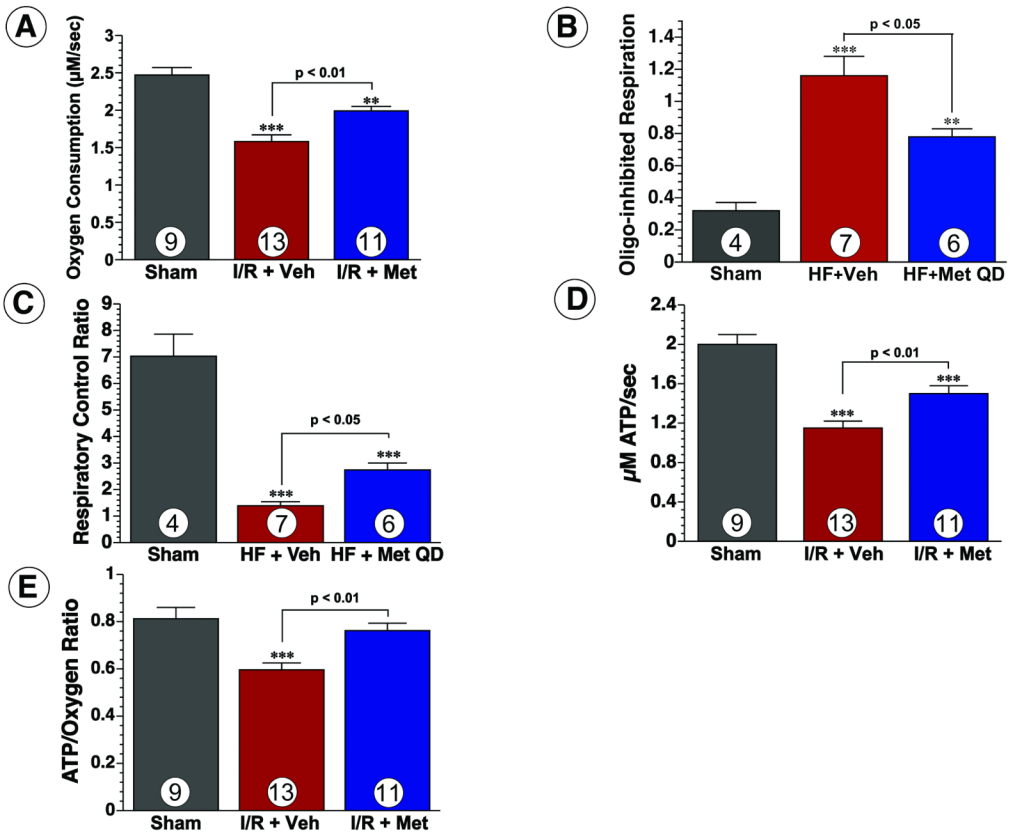

Figure 4.

Metformin therapy improves mitochondrial respiration and ATP synthesis. **(A)** Mitochondrial oxygen consumption rates, **(B)** oligomycin (oligo)-inhibited respiration, **(C)** respiratory control ratios of mitochondria isolated from sham-operated, vehicle-treated, and Metformin QD-treated mice 4 wk after myocardial infarction. Mean values (±SEM) are shown for state 3 (ADP-stimulated) respiration in the presence of succinate and glycerol-3-phospate. **(D)** ATP synthesis rates and **(E)** the ratio of ATP synthesis to maximal oxygen consumption obtained from the same isolated mitochondria shown in panel A. Values are means ± S.E.M. Numbers inside bars indicate the number of animals that were investigated in each group. *** $p < 0.001$ vs. Sham, ** $p < 0.01$ vs. Sham. Veh = vehicle, Met QD = Metformin QD.

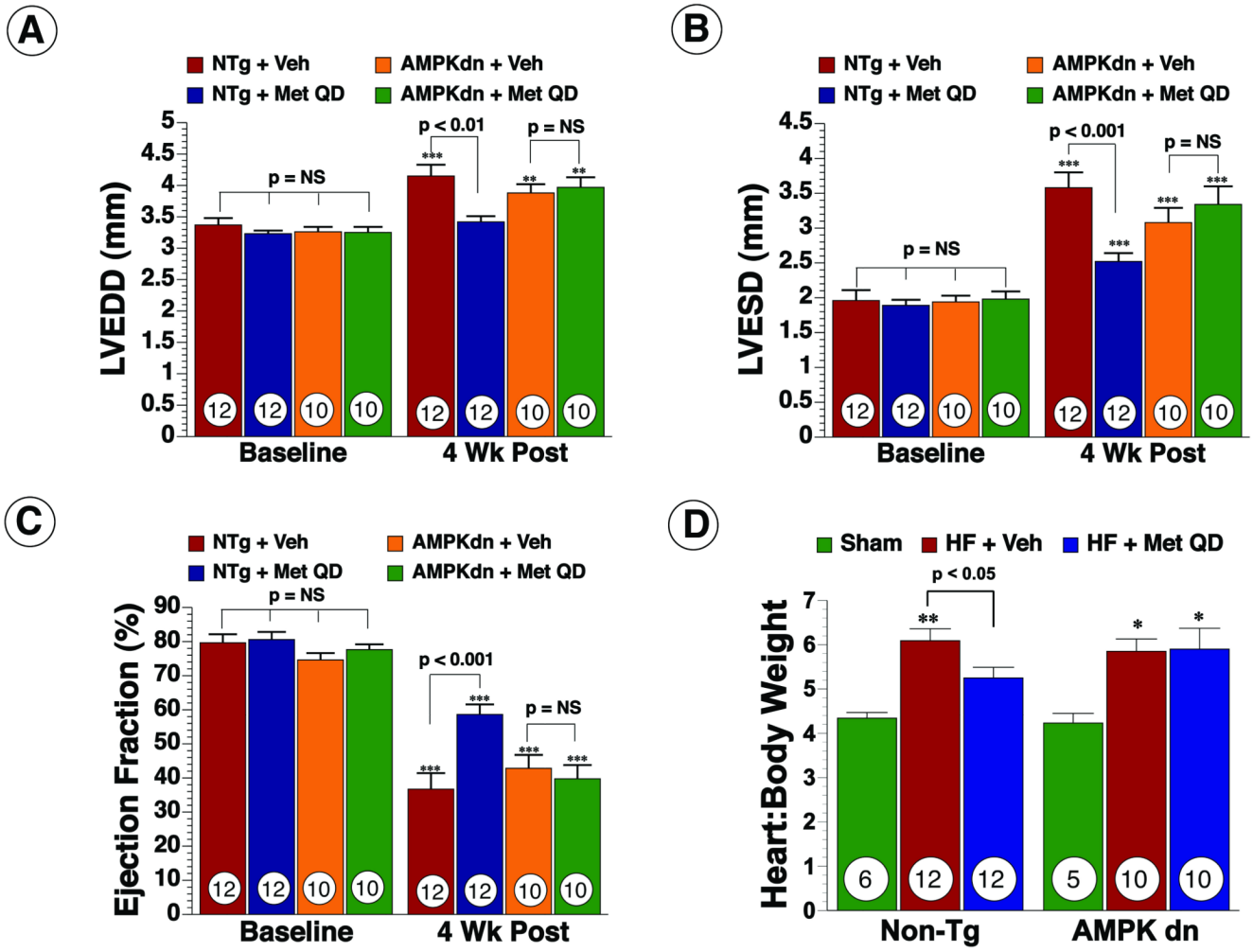

Figure 5.

AMPK mediates metformin-induced cardioprotection. **(A)** LVEDD, **(B)** LVESD and **(C)** Ejection Fraction (EF) and **(D)** H/BW ratios were measured at baseline and following myocardial ischemia in NTg and AMPKdn mice ± daily metformin therapy. Values are means ± S.E.M. Numbers inside bars indicate the number of animals that were investigated in each group. ** $p < 0.01$ vs. Baseline, *** $p < 0.001$ vs. Baseline, * $p < 0.05$ vs. Sham. Veh = vehicle, Met QD = Metformin QD.

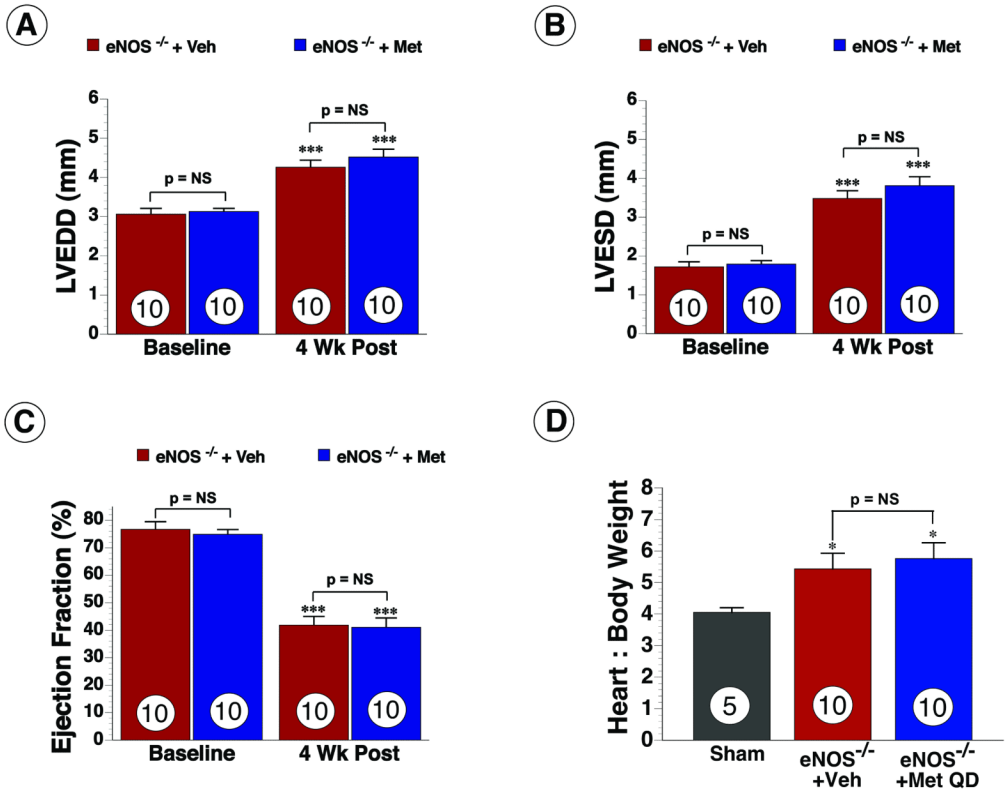

Figure 6.

eNOS mediates metformin-induced cardioprotection. **(A)** LVEDD, **(B)** LVESD and **(C)** Ejection Fraction (EF) and **(D)** H/BW ratios was measured at baseline and following myocardial ischemia in eNOS deficient mice ± daily metformin therapy. Values are means ± S.E.M. Numbers inside bars indicate the number of animals that were investigated in each group. *** $p < 0.001$ vs. Baseline, * $p < 0.05$ vs. Sham. Veh = vehicle, Met QD = Metformin QD.