Activation of AMP-Activated Protein Kinase by Metformin Improves Left Ventricular Function and Survival in Heart Failure

Susheel Gundewar, John W. Calvert, Saurabh Jha, Iris Toedt-Pingel, Sang Yong Ji, Denise Nunez, Arun Ramachandran, Mauricio Anaya-Cisneros, Rong Tian, David J. Lefer

Abstract—Clinical studies have reported that the widely used antihyperglycemic 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 antihyperglycemic effects. We tested the hypothesis that chronic activation of AMP-activated protein kinase (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 occlusion or to 60 minutes left coronary artery occlusion followed by reperfusion for 4 weeks. High-resolution, 2D echocardiography was performed at baseline and 4 weeks after myocardial infarction to assess left ventricular dimensions and function. Metformin (125 μg/kg) administered to mice at ischemia and then daily improved survival by 47% (P<0.05 versus vehicle) at 4 weeks following permanent left coronary artery occlusion. Additionally, metformin given at reperfusion and then daily preserved left ventricular dimensions and left ventricular ejection fraction (P<0.01 versus vehicle) at 4 weeks. The improvement in cardiac structure and function was associated with increases in AMPK and endothelial nitric oxide synthase (eNOS) phosphorylation, as well as increased peroxisome proliferator-activated receptor-γ coactivator (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. (Circ Res. 2009;104:403-411.)

Key Words: myocardial ischemia • heart failure • metformin • nitric oxide

Heart failure (HF) 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, HF represents a major health problem that has been increasing in prevalence and incidence. In the United States, HF 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 and acute myocardial infarction, leading to loss of functioning myocytes, development of myocardial fibrosis, and subsequent left ventricular (LV) remodeling, all of which contribute toward 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 sulfonylureas 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 concentrations.2 Additionally, blood glucose control is achieved without any weight gain especially in patients with obesity and the metabolic syndrome.3 Analysis of the UKPDS (United Kingdom Prospective Diabetes Study) 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 antihyperglycemic 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 reactivity.5

Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/CIRCRESAHA.108.190918

403
Experimental studies suggest that the pleiotropic effects of metformin are mediated in part by activation of AMP-activated protein kinase (AMPK), 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 synthesis. Activation of AMPK has also been shown to increase the phosphorylation and activity of endothelial nitric oxide synthase (eNOS) and the expression of the transcriptional coactivator and metabolic regulator peroxisome proliferator-activated receptor-δ coactivator (PGC)1α, both of which are important regulators of mitochondrial biogenesis and function. Activation of AMPK by metformin may account for the reduction of cardiovascular disease risk and improvement of vascular function in patients with type 2 diabetes. 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 HF.

Materials and Methods

Animals
C57BL/6J mice obtained from the Jackson Laboratory (Bar Harbor, Me) were used 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 (Tg) mice over-expressing a dominant-negative AMPKα2 subunit (AMPKα2 dn). The generation of AMPKα2dn mice has been described previously. AMPKα2dn Tg and nontransgenic (NTg) littermates were bred in our colony and maintained on an FVB background. All animals were used at 8 to 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 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 Louis, 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 (IC) injection at the time of reperfusion once (metformin) or once at reperfusion followed by daily intraperitoneal (IP) injections (metformin QD) for 4 weeks. Saline was used as a vehicle at reperfusion followed by daily IP injections for 4 weeks (vehicle). In the permanent left coronary artery (LCA) occlusion model (Figure 1A), metformin was administered once at the onset of ischemia IC, followed by daily IP injections for 4 weeks.

HF Protocols
HF was induced by either permanent ligation of the LCA (Figure 1A) or by subjecting the mice to 60 minutes of LCA occlusion followed by reperfusion for up to 4 weeks (Figure 2A). Surgical ligation of the LCA was performed according to methods described previously.

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 TTC (2,3,5-triphenyl-tetrazolium chloride) (Sigma) staining method. All of the procedures for the AAR and Inf determination have been previously described.

Echocardiographic Assessment of Left Ventricular Structure and Function
Baseline echocardiography (Echo) images were obtained 1 week before LCA ischemia to avoid any anesthetic effects as previously described using a Visualsonic VEVO 770 high-resolution ultrasound system. Four weeks following the myocardial infarction (post–myocardial infarction), Echo images were obtained and analyzed once again.

Histological Analysis of Inf
After the post–myocardial infarction echocardiographic assessment, the hearts of mice were processed for histological assessment of Inf as previously described.

Western Blot Analysis
Western blot analysis was performed as previously described.

Cardiac Mitochondria Isolation
Cardiac mitochondria were isolated from the following groups of mice as previously described: sham-operated, vehicle-treated, and Metformin QD–treated mice.

Mitochondrial Respiratory Rate and ATP Synthesis
Oxygen consumption and ATP synthesis of cardiac mitochondria was measured as previously described.

Statistical Analysis
All the data in this study are expressed as means ± SEM. Differences in data between the groups were compared using Prism 4 (GraphPad Software Inc) with Student’s paired 2-tailed t test or 1-way ANOVA where appropriate. For the ANOVA, if a significant variance was found, the Tukey or Bonferroni test was used as the post hoc analysis. Probability values of P < 0.05 were considered significant.

Results

Metformin Improves Survival Following Permanent LCA Occlusion
Mice were subjected to permanent occlusion of LCA and metformin or vehicle was administered before LCA ligation and then daily for 4 weeks (Figure 1A). Two-dimensional Echo was obtained at baseline and 4 weeks to assess LV function. At 4 weeks, 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 weeks 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 hours in separate groups of mice. The AAR per left ventricle, Inf per AAR, and Inf per left ventricle were similar (P=NS) in both metformin- and vehicle-treated mice (Figure 1C). Following permanent LCA occlusion, at 4 weeks, ejection fraction (EF) was severely reduced (P < 0.001 versus baseline) and similar in the vehicle-treated (n=11) and metformin-treated (n=12) 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 Inf or LV function.
Metformin Improves LV Structure and Function and Attenuates Cardiac Hypertrophy in HF

To study the effects of metformin in a more clinically relevant model of HF that mimics the effects of coronary revascularization therapy, mice were subjected to 60 minutes of LCA occlusion followed by reperfusion (Figure 2A). In the initial set of experiments, metformin (125 μg/kg) or vehicle was administered at the time of reperfusion and the extent of myocardial infarction was evaluated at 24 hours (Figure I in the online data supplement, available at http://circres.ahajournals.org). We found that metformin treatment decreased Inf per AAR by 29% (68.63 ± 2.34% for vehicle versus 48.92 ± 2.51% for Metformin, P < 0.001) following myocardial infarction compared to vehicle. To investigate whether the reduction in Inf alone was sufficient to improve LV structure and function, mice were randomized into 3 groups and subjected to 60 minutes of ischemia. The first group received saline as a vehicle at reperfusion (IC) and then daily (IP) injections (vehicle), the second group received metformin as a single (IC) bolus only once at the time of reperfusion (Metformin), and the third group received metformin (IC) once at reperfusion and then as daily (IP) injections (Metformin QD). Echo was performed at baseline and 4 weeks 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 LV end diastolic diameter (LVEDD) and LV end systolic diameter (LVESD) (Figure 2B and 2C) and a significant decrease in EF (Figure 2D). At 4 weeks 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 versus vehicle) and EF by 31% (P < 0.01 versus vehicle) at 4 weeks. 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 weeks following reperfusion (supplemental Figure I). For each heart, we analyzed multiple sections taken from the midventricle and then averaged

**Figure 1.** Metformin improved survival following permanent occlusion of LCA. A, Murine in vivo permanent LCA occlusion protocol. B, Kaplan-Meier survival curve following metformin therapy in mice during the 4 weeks after myocardial infarction. Metformin significantly improved survival by 47% (P < 0.05) compared to vehicle-treated mice. C, Myocardial Inf analysis in a subset of animals subjected to 24 hours of myocardial ischemia. AAR as a percentage of the left ventricle was similar between both groups. Metformin (125 μg/kg) administration did not attenuate myocardial Inf following permanent LCA occlusion. D, LV function was evaluated in a subset of animals at 4 weeks of myocardial ischemia. EF at baseline and following myocardial ischemia were similar in both groups. Values are means ± SEM. Numbers inside bars indicate the number of animals that were investigated in each group. ***P < 0.001 vs baseline. Veh indicates vehicle; Met QD, Metformin QD.
these numbers to obtain a single Inf per left ventricle measurement for each animal. Vehicle-treated mice displayed a 13.62 \pm 1.16\% Inf per left ventricle. Conversely, both groups treated with metformin displayed a smaller area of scar formation. Analysis from the multiple midventricle sections per animal revealed that the mice in the Metformin group displayed a 9.01 \pm 0.69\% Inf per left ventricle (\(P<0.01\) versus vehicle), and the mice in the Metformin QD group displayed a 8.16 \pm 0.63\% Inf per left ventricle (\(P<0.001\) versus 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 weeks (supplemental Table I). Because Metformin QD caused significant improvements in LV function, LV dimension, and cardiac hypertrophy, this therapeutic strategy was used in all subsequent experiments.

**Figure 2.** Metformin improved LV structure and function, as well as cardiac hypertrophy, in mice following myocardial ischemia and reperfusion. A, Murine in vivo HF protocol. LVEDD (B), LVESD (C), and EF (D) were calculated using high-resolution 2D B-mode echocardiography images obtained at baseline and following myocardial ischemia in all groups. E, H/BW ratio was used as a measure of cardiac hypertrophy. Values are means \pm SEM. 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 indicates vehicle; Met, Metformin; Met QD, Metformin QD.

**Metformin Promotes the Phosphorylation of AMPK and eNOS and Increases the Expression of PGC-1α During HF**

We have previously reported that a single injection of metformin phosphorylates and increases the activity of both AMPK and eNOS for up to 24 hours. In the present study, we investigated whether Metformin QD resulted in sustained elevations in AMPK and eNOS phosphorylation, as well as expression of PGC-1α, at 4 weeks following reperfusion of the ischemic myocardium (Figure 3A). Phosphorylation of AMPK\(^{Thr172}\) was elevated in both vehicle-treated (\(P<0.05\)) and Metformin QD–treated (\(P<0.001\)) groups (Figure 3B). Metformin QD was found to significantly augment the ischemia-induced increase in AMPK-\(P^{Thr172}\) (\(P<0.01\) versus vehicle). Similarly, Metformin QD treatment significantly increased phosphorylation of eNOS\(^{Ser1177}\) (\(P<0.05\) versus vehicle) (Figure 3C) and expression of PGC-1α (\(P<0.05\) versus vehicle).
versus vehicle) (Figure 3D) compared to sham and vehicle treatment. These results suggest that the activation of AMPK and its downstream mediators eNOS and PGC-1α may underlie the beneficial effects of metformin in the myocardium and protect against ischemia-induced HF.

Metformin Improves Mitochondrial Respiration and ATP Synthesis During HF

Because both eNOS and PGC-1α are important regulators of mitochondrial biogenesis and mitochondrial function, we investigated whether 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 and 4E). 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 those from vehicle-treated mice. These data indicate that the respiration of cardiac mitochondria during HF 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 HF protocol. Metformin administration at reperfusion decreased Inf per AAR by 30% (43.2 ± 3.6% for vehicle versus 30.2 ± 5.8% for Met QD, \( P < 0.05 \)) in NTg mice but not in AMPKdn mice (48.1 ± 5.7% for vehicle versus 44 ± 8.4% for Met QD, \( P = \text{NS} \)). Metformin QD did not result in any significant improvements in LVEDD (Figure 5A), LVESD (Figure 5B), or 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.

Figure 3. Chronic administration of metformin phosphorylates AMPK and eNOS and increases the expression of PGC-1α at 4 weeks during HF. A, Representative immunoblots of AMPK phosphorylated at residue threonine 172 (AMPK-PThr172) and total AMPK eNOS phosphorylated at residue serine 1177 (eNOS-PSer1177), total eNOS, and PGC-1α and α-tubulin following HF protocol. B, Densitometric analysis of phosphorylated state AMPKThr172. Bars represent the ratio of phosphorylated AMPK to total AMPK. C, Phosphorylated state of eNOS-PSer1177. Bars represent the ratio of phosphorylated eNOS to total eNOS. D, Total PGC-1α. Values are means ± SEM. 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 indicates vehicle; Met QD, Metformin QD.
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 or Inf per left ventricle (P<NS) at 24 hours. At 4 weeks, Echo analysis revealed LV dilation and systolic dysfunction in the hearts of both groups, as evidenced by a significant increase in LVEDD (Figure 6A), LVESD (Figure 6B), and EF (Figure 6C). Metformin QD did not result in any significant improvements in LVEDD, LVESD, or 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 HF in mice after myocardial infarction. The key findings in this study are: (1) metformin therapy improved survival by 47% after permanent occlusion of the LCA compared to treatment with saline; (2) metformin therapy led to significant improvements in cardiac remodeling and function during HF; (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 HF. These data provide additional insight into the pleiotropic effects of metformin in cardiovascular disease and its therapeutic role in ischemic induced HF.

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 expression21 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 AMPKε2dn mice. These results suggest that the chronic activation of AMPK during the development of ischemia-induced HF is a critical mechanism mediating the beneficial actions of metformin.

HF is associated with abnormalities of mitochondrial biogenesis24 and mitochondrial injury correlates strongly with its severity.25 In HF, 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. The decreased oxidative capacity of the failing myocardium therefore limits the ability of the heart to meet hemodynamic demands and leads to symptoms of HF. Both eNOS and PGC-1α are important regulators of mitochondrial biogenesis and function and play important roles in the pathophysiology of HF. 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. AMPK has been shown to increase the phosphorylation of eNOS, leading to an increase in eNOS activity and NO bioavailability. Additionally, we and others 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 AMPKdn mice. The results of the present study support these previous findings, because we found that metformin therapy promotes the phosphorylation of eNOS during HF and that the metformin-mediated improvements in LV function were

Figure 5. AMPK mediates metformin-induced cardioprotection. LVEDD (A), LVESD (B), EF (C), and H/BW ratios (D) were measured at baseline and following myocardial ischemia in NTg and AMPKdn mice with or without daily metformin therapy. Values are means±SEM. 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 indicates vehicle; Met QD, Metformin QD.

Figure 6. eNOS mediates metformin-induced cardioprotection. LVEDD (A), LVESD (B), EF (C), and H/BW ratios (D) were measured at baseline and following myocardial ischemia in eNOS-deficient mice with or without daily metformin therapy. Values are means±SEM. 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 indicates vehicle; Met QD, Metformin QD.
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 induced in response to conditions that demand increased myocardial ATP synthesis32,33 and has been shown to drive mitochondrial biogenesis and improve mitochondrial function in cardiac myocytes and hearts of Tg mice.36 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 HF. Furthermore, we have demonstrated that metformin improves mitochondrial oxygen consumption and ATP synthesis. These beneficial actions may be mediated by an increase in PGC1-ε expression and/or eNOS phosphorylation.

The findings of the present 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 Inf when it was administered at the time of reperfusion in an AMPK-eNOS-dependent fashion.15 Although the present study also demonstrates that metformin provides protection in a similar manner, there are some important differences. First of all, the findings of the present study are significant because they demonstrate that chronic metformin therapy initiated after myocardial ischemia is beneficial for the treatment of HF. Importantly, we found that although a single administration of metformin at the time of reperfusion is beneficial in attenuating Inf, this alone is not sufficient to cause a significant improvement in cardiac function after 4 weeks. 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 HF. These findings support several experimental and clinical studies reporting that metformin possesses significant cardioprotective actions and is safe in the setting of diabetes and HF.34,35 Although previously contraindicated in HF because of the potential risk for development of lactic acidosis, the Food and Drug Administration, in response to the findings of several recent studies, has now updated the prescribing information for metformin to eliminate this contraindication.46 A metaanalysis of controlled studies evaluating antidiabetic agents and outcomes in patients with HF and diabetes found that metformin when compared to other antihyperglycemic therapies significantly reduced mortality and hospital admissions in treated patients despite a similar decrease in hemoglobin A1C 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 nondiabetic rodents,37 yet improves cardiac function following in vitro global ischemia.38 Finally, the findings of the present 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 present study is timely and provides important insights into the use of metformin treatment for cardiovascular disease in all patient populations.

The present study mainly focused on the ability of metformin to improve mitochondrial function during HF; 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 EF. 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.15 Nonetheless, because we have not evaluated whether 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 HF.

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 HF 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, rather, may have practical clinical use following myocardial infarction in all patient populations to reduce the morbidity and mortality from ischemia induced HF.

Acknowledgments

We thank Konstantinos P. Koulogiannis, MD, and Liran Blum, MD, for expert technical assistance during the course of these studies.

Sources of Funding

Supported by NIH grant 2 R01 HL-060849-08 and American Diabetes Association grant 7-04-RA-59 (to D.J.L.) and NIH grant F32 DK 077380-01 (to J.W.C.).

Disclosures

None.
References


Activation of AMP-Activated Protein Kinase by Metformin Improves Left Ventricular Function and Survival in Heart Failure

Susheel Gundewar, John W. Calvert, Saurabh Jha, Iris Toedt-Pingel, Sang Yong Ji, Denise Nunez, Arun Ramachandran, Mauricio Anaya-Cisneros, Rong Tian and David J. Lefer

Circ Res. 2009;104:403-411; originally published online December 18, 2008;
doi: 10.1161/CIRCRESAHA.108.190918

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/104/3/403

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/12/18/CIRCRESAHA.108.190918.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supplemental Material

Expanded Materials and Methods

Echocardiographic 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\(^1\). The mice were lightly anesthetized with isoflurane in 100% O\(_2\) and \textit{in vivo} transthoracic echocardiography of the left ventricle (LV) using a 30-MHz RMV scanhead interfaced with a Vevo 770 (Visualsonics) was used to obtain high-resolution two-dimensional ECG based kilohertz visualization (EKV) B mode images (Echo) acquired at the rate of 1000 frames/sec over 7 minutes. These images were used to measure LV end-diastolic dimensions (LVEDDs), LV end-systolic dimensions (LVESDs) and ejection fraction (EF). 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 mice were re-anesthetized, intubated, and connected to a rodent ventilator as previously described\(^2\). A median sternotomy was performed and the heart was rapidly excised and fixed in conventional fixing solutions (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer). After 12 hours in 4% paraformaldehyde, the heart was cut into 1 mm
thick as detailed above. The slices were dehydrated and embedded in paraffin, then cut into 4 µm slices which were heated overnight in 60°C incubator. The sections were dewaxed and stained with hematoxylin and eosin (H&E). Digital images of the slides were then captured and analyzed using computer-assisted planimetry with NIH ImageJ 1.37 software to measure the area of infarct or scar relative to the left ventricle.

**Blood Glucose Determination.** Blood obtained via a tail snip was screened using a Sure Step glucose-monitoring system (Lifescan).

**Western Blot Analysis.** Myocardial tissue samples (75 mg) taken from the area-at-risk portion of the LV were homogenized and lysates were used for Western blot analysis. Protein concentrations were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels. The gels were electrophoresed, followed by transfer of the protein to a PVDF membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. The following primary antibodies were used: AMPKα (1:300, Cell Signaling Technology); Phospho-AMPKα Thr172 (1:3000, Cell Signaling Technology); eNOS (1:5000, BD Biosciences); Phospho-eNOS Ser1177 (1:1500, Cell Signaling Technology); PGC-1α (1:3000, Cell Signaling Technology); α-Tubulin (1:20,000, Santa Cruz Biotechnology). Immunoblots were next processed with secondary antibodies.
(Amersham) for 1 hr at room temperature. Immunoblots were then probed with an ECL+Plus chemiluminescence reagent kit (Amersham) to visualize signal, followed by exposure to X-ray film.

**Cardiac Mitochondria Isolation.** Cardiac mitochondria were isolated from the following groups of mice: sham operated, vehicle and Metformin QD treated mice. Briefly, the heart was quickly excised and washed in buffer containing 250 mM sucrose, 10 mM Tris, 1 mM EGTA, pH 7.4 at 4°C. After changes of buffer, the cardiac samples were cut into small pieces and homogenized. The samples were centrifuged at 3,000 X g for 3 min to remove debris, and mitochondria were obtained by a differential centrifugation technique as previously described\(^{16}\). All isolated mitochondria were kept on ice and used within 3 h of isolation.

**Mitochondrial Respiratory Rate and ATP synthesis.** Oxygen consumption of cardiac mitochondria was measured in a sealed chamber magnetically stirred at 37°C by using calibrated Clark-type electrodes in the presence of succinate (8 mmol/L) and glycerol-3-phosphate (4 mmol/L) as previously described\(^{16}\). Maximal (ADP-stimulated) respiration was measured after the addition of ADP (1 mmol/L). Additionally, respiration in the absence of ADP phosphorylation was determined in the presence of 1 mg/ml oligomycin. Respiratory control ratios were determined as the ratio of oligomycin to state 3 respirations. To evaluate ATP synthesis, aliquots were taken from the respiration chamber over a 2-minute period after the addition of ADP. ATP was then quantified with a bioluminescence
assay using an ATP determination kit (A-22066; Molecular Probes, Eugene, OR). The ATP/O ratio was calculated with the state 3 respiratory rate for each sample\textsuperscript{17}.

### Online Table I. Blood Glucose Levels.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glucose (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/R + Vehicle</td>
<td>212.36 ± 11.51</td>
</tr>
<tr>
<td>I/R + Metformin QD</td>
<td>209.23 ± 14.55</td>
</tr>
</tbody>
</table>

Blood glucose levels were determined 4 weeks following 60 minutes of ischemia and reperfusion. Values are means ± SEM.
References


Online Figure Legend

**Online Figure I. Metformin therapy decreased infarct size at 24 hr and scar formation at 4 week following myocardial ischemia and reperfusion.** (A) Myocardial infarct size was determined following 60 min of LCA ischemia and 24 hr of reperfusion. Area-at-risk (AAR) with respect to the left ventricle (LV) was similar between all groups. Metformin (125 µg/kg) administered at reperfusion significantly attenuated myocardial infarct size with respect to the area-at-risk (Inf/AAR) and with respective to the left ventricle (Inf/LV). (B) Myocardial infarct size (Inf) with respect to the entire left ventricle (LV) was also calculated from hematoxylin and eosin (H&E) slides following 60 min of left coronary artery ischemia and 4 wk of reperfusion. Metformin therapy also significantly attenuated scar formation following myocardial ischemia and reperfusion. Values are means ± S.E.M. Numbers inside bars indicate the number of animals that were investigated in each group. Veh = vehicle, Met = Metformin, Met QD = Metformin QD. **p<0.01 compared to vehicle, ***p<0.001 compared to Vehicle.
Online Figure I

A

Vehicle
Metformin (125 μg/kg) at rep

% LV or AAR

p = NS

p < 0.001

p = 0.002

10 10 10 10 10 10

AAR/LV INF/AAR INF/LV

B

Infarct/LV

Veh Met Met QD

20 18 18

p = N.S.

** ***