Acute Metformin Therapy Confers Cardioprotection Against Myocardial Infarction Via AMPK-eNOS–Mediated Signaling

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OBJECTIVE—Clinical studies have reported that metformin reduces cardiovascular end points of type 2 diabetic subjects by actions that cannot solely be attributed to glucose-lowering effects. The therapeutic effects of metformin have been reported to be mediated by its activation of AMP-activated protein kinase (AMPK), a metabolite sensing protein kinase whose activation following myocardial ischemia has been suggested to be an endogenous protective signaling mechanism. We investigated the potential cardioprotective effects of a single, low-dose metformin treatment (i.e., 286-fold less than the maximum antihyperglycemic dose) in a murine model of myocardial ischemia-reperfusion (I/R) injury.

RESEARCH DESIGN AND METHODS—Nondiabetic and diabetic (*db/db*) mice were subjected to transient myocardial ischemia for a period of 30 min followed by reperfusion. Metformin (125 μg/kg) or vehicle (saline) was administered either before ischemia or at the time of reperfusion.

RESULTS—Administration of metformin before ischemia or at reperfusion decreased myocardial injury in both nondiabetic and diabetic mice. Importantly, metformin did not alter blood glucose levels. During early reperfusion, treatment with metformin augmented I/R-induced AMPK activation and significantly increased endothelial nitric oxide (eNOS) phosphorylation at residue serine 1177.

CONCLUSIONS—These findings provide important information that myocardial AMPK activation by metformin following I/R sets into motion events, including eNOS activation, which ultimately lead to cardioprotection. ***Diabetes*** **57:696–705, 2008**

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AAT, area at risk; AMPK, AMP-activated protein kinase; eNOS, endothelial nitric oxide; FAO, fatty acid oxidation; Inf, infarct size; I/R, ischemia-reperfusion; LCA, left coronary artery; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter.

Metformin is one of the most commonly prescribed antihyperglycemic agents for the treatment of type 2 diabetes (1). Its major effects in terms of blood glucose are mediated through a reduction in hepatic glucose output and an increase in insulin-dependent peripheral glucose utilization (2). The therapeutic effects of metformin, however, are not limited to its ability to lower blood glucose, as evidence supports direct vascular effects (3,4). Additionally, two large-scale clinical trials have reported that metformin improves vascular function and reduces mortality and cardiovascular end points of type 2 diabetic subjects (5,6) by actions that cannot be attributed entirely to its antihyperglycemic effects (7).

Recent studies suggest that the pleotropic effects of metformin may be mediated by its activation of AMP-activated protein kinase (AMPK) (1,8,9), a protein kinase that is activated in response to alterations in cellular energy levels (10). Its activation is mediated by increases in AMP-to-ATP ratios through mechanisms involving allosteric regulation of AMPK subunits, making it a better substrate for upstream AMPK kinases (AMPKK) and a worse substrate for competing protein phosphatases (11). Metabolic activators of AMPK include ischemia, oxidative stress, exercise, and glucose deprivation (12). When activated, AMPK stimulates fatty acid oxidation (13), promotes glucose transport (14), accelerates glycolysis (15), and inhibits triglyceride (16) and protein synthesis (17). Additionally, the activation of AMPK has been shown to increase the phosphorylation and activity of endothelial nitric oxide synthase (eNOS) (18,19).

Studies using the isolated perfused working heart model have reported that metformin at concentrations known to lower blood glucose provides cardioprotection in diabetic hearts against increasing preload and in nondiabetic hearts against global ischemia (20,21). However, questions regarding the protective mechanisms of metformin in in vivo models of myocardial ischemia-reperfusion (I/R) remain to be answered. The purpose of the present study was to investigate the potential cardioprotective effects of a single, low-dose administration of metformin in a murine model of myocardial I/R. We report significant reductions of myocardial infarct size in nondiabetic and diabetic mice with acute metformin therapy. Furthermore, our results demonstrate that the cardioprotective actions of metformin are mediated by an AMPK-eNOS signaling pathway.

RESEARCH DESIGN AND METHODS

Male C57BL6/J nondiabetic and B6.Cg-m$^{+/+}$ Lepr^{db}/J (*db/db*) diabetic mice, 8–10 weeks of age, were used (Jackson Labs, Bar Harbor, ME). Additionally,

we used mice (8–10 weeks old) completely deficient in eNOS (eNOS$^{-/-}$). The generation of cardiac-specific transgenic mice overexpressing a dominant-negative AMPKα2 subunit (AMPKα2 dn Tg) has been described previously (22). AMPKα2 dn Tg and nontransgenic (NTg) littermates were bred in our colony and used at 8–10 weeks of age. All experimental mouse procedures were approved by the animal care and use committee at Albert Einstein College of Medicine and conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH pbl. no. 86–23, rev. 1996) and with federal and state regulations.

Metformin (1,1-dimethylbiguanide hydrochloride), purchased from Sigma (St. Loius, MO), was dissolved in saline and administered via an intraperitoneal injection at a dose of 125 μg/kg in a final volume of 100 μl to nondiabetic and diabetic mice 18 h before myocardial ischemia (preconditioning group). In separate groups of nondiabetic and diabetic mice, metformin was injected directly into the lumen of the left ventricle at the time of reperfusion at a final concentration of 125 μg/kg in final volumes of 100 μl (reperfusion). Blood obtained via a tail snip was screened using a Sure Step glucose-monitoring system (Lifescan).

Myocardial I/R protocol and myocardial area-at-risk and infarct size determination. Surgical ligation of the left coronary artery (LCA) was performed similar to methods described previously (23). Mice were anesthetized with ketamine (50 mg/kg) and pentobarbital sodium (50 mg/kg), intubated, and connected to a rodent ventilator. A median sternotomy was performed, and the LCA was ligated. Mice were subjected to 30 min (45 min for the AMPKα2 dn Tg and NTg littermates) of LCA ischemia followed by different periods of reperfusion. At 24 h of reperfusion, left ventricular area at risk (AAR) and infarct size (Inf) were determined by methods previously described (24). In an additional group of mice, a blood pressure catheter (Millar Mikro-Tip) was placed in the aorta after mice were anesthetized, intubated, and connected to a rodent ventilator. Mean arterial blood pressure and heart rate were then evaluated following an injection of saline or metformin into the lumen of the left ventricle.

Serum Troponin T. At 4 h of reperfusion, a group of mice was anesthetized with ketamine (100 mg/kg) and xylazine (8 mg/kg), and a blood sample was collected. Serum was obtained and sent to the clinical lab at Jacobi Medical Center (Bronx, NY) to measure Troponin-T using an enzyme-linked immunosorbent assay–based assay.

Echocardiograhic assessment of left ventricular structure and function. Baseline two-dimensional echocardiography images were obtained 1 week before LCA ischemia. The mice were lightly anesthetized with isoflourane in 100% O_2, and in vivo transthoracic echocardiography of the left ventricle using a 30-MHz RMV scanhead interfaced with a Vevo 770 (Visualsonics) was used to obtain high-resolution, two-dimensional electrocardiogram-based kilohertz visualization; B mode images were acquired at a rate of 1,000 frames/s over 7 min. These images were used to measure left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD). The B-mode images were also used to calculate left ventricular ejection fraction. One week after the baseline images were acquired, the mice were subjected to 30 min of LCA occlusion followed by reperfusion as described above. At 1 week of reperfusion, post I/R echocardiographic images were obtained and analyzed.

AMPK activity assay and Western blot analysis. Samples of the left ventricle (75 mg) were homogenized, and lysates were used for AMPK activity assays and Western blot analysis (25,26). Protein concentrations were measured with the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Heart AMPK activity was assessed with a kinase assay measuring the incorporation of [γ^{32}P]-ATP into the synthetic AMPK substrate with the sequence HMR-SAMSGLHLVKRR (SAMS peptide). Activity was expressed as incorporated ATP (in picomoles) per milligram of protein per minute. For Western blot analysis, equal amounts of protein were loaded into lanes of polyacrylamide-SDS gels. The gels were electrophoresed, followed by transfer of the protein to a polyvinylidine fluoride membrane. The membrane was then blocked and probed with primary antibodies overnight at 4°C. Immunoblots were next processed with secondary antibodies (Amersham) for 1 h 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.

Statistical analysis. All data in this study are expressed as the mean ± SEM. Differences in data between the groups were compared using Prism 4 (GraphPad Software) with Student's paired two-tailed t test or one-way ANOVA where appropriate. For the ANOVA, if a significant variance was found, the Tukey test or Dunnett's multiple comparison test was used as the post hoc analysis. A P value <0.05 was considered significant.

RESULTS

Metformin limited the extent of myocardial injury following ischemia-reperfusion in the nondiabetic heart.

Nondiabetic mice were subjected to 30 min of LCA ischemia followed by reperfusion. Metformin or vehicle was administered either 18 h before ischemia or at the time of reperfusion. The extent of myocardial infarction was then evaluated at 24 h of reperfusion. The AAR per left ventricle was similar (P = NS) in all of the groups (Fig. 1*A*). Metformin administered before ischemia (preconditioning group) decreased the Inf relative to the AAR (Inf/AAR) by 62% (50.64 ± 0.86 vs. 19.35 ± 1.92%, $P <$ 0.001). Administration of metformin at the time of reperfusion decreased Inf/AAR by 49% (50.64 ± 0.86 vs. 25.9 ± 2.73%, $P <$ 0.001). The extent of infarct size reduction between the metformin groups was similar (P = NS). Clinically, patients generally receive medical treatment for acute myocardial ischemia after the onset of symptoms. Therefore, to further investigate the cardioprotective effects of metformin, mice were treated at the time of reperfusion in all subsequent experiments. Representative photomicrographs of myocardial tissues from vehicle- and metformin-treated mice are shown in Fig. 1*B*.

Serum levels of Troponin-T were measured in a group of mice at 4 h of reperfusion (Fig. 1*C*). Following myocardial I/R, serum levels of Troponin-T rose from 1.13 ± 0.31 ng/ml (sham) to 13.63 ± 3.12 ng/ml in the vehicle-treated group ($P <$ 0.01 vs. sham). Metformin significantly ($P =$ 0.026) attenuated the rise in serum Troponin-T by 56% (13.63 ± 3.12 vs. 5.99 ± 0.49 ng/ml).

The effects of metformin on left ventricular structure and function following myocardial I/R were evaluated using in vivo transthoracic echocardiography. For these experiments, mice were subjected to 30 min of myocardial ischemia and 7 days of reperfusion. Myocardial I/R increased both LVEDD (3.25 ± 0.11 to 3.96 ± 0.13 mm for I/R + vehicle, $P <$ 0.01; 3.27 ± 0.13 to 3.67 ± 0.08 for I/R + metformin, $P <$ 0.05) and LVESD (1.87 ± 0.13 to 2.96 ± 0.19 mm for I/R + vehicle, $P <$ 0.001; 1.98 ± 0.12 to 2.50 ± 0.07 mm for I/R + metformin, $P <$ 0.01). Metformin improved LVEDD by 44% and improved LVESD by 52% ($P <$ 0.05 vs. I/R + vehicle). Following myocardial I/R, the ejection fraction (Fig. 1*D*) decreased in both groups. Metformin treatment did, however, significantly improve ejection fraction by 52% ($P =$ 0.007 vs. I/R + vehicle). In addition, mice in the vehicle-treated group had a significant increase in heart rate from baseline ($P <$ 0.01 vs. baseline).

An injection of either saline or metformin into the lumen of the left ventricle transiently (<30 s) reduced mean arterial blood pressure (~20%; 85 ± 6 to 72 ± 4 and 89 ± 7 to 73 ± 8 mmHg for vehicle and metformin, respectively; n = 4) and heart rate (~10%; 399 ± 13 to 370 ± 18 and 412 ± 14 to 388 ± 19 bpm for vehicle and metformin, respectively; n = 4). This reduction was not statistically different from the baseline readings in either group, and no differences were observed between the mice that received saline or metformin. Additionally, no differences in blood glucose were observed following treatment with metformin (not shown).

The cardioprotective actions of metformin are mediated through the activation of AMPK.

We next investigated whether the dose of metformin used in this study could activate AMPK in the myocardium. Mice were anesthetized and intubated. A median sternotomy was performed, and metformin was injected into the lumen of the left ventricle of naïve mice. Mice were then killed, and their heart tissue was used to assess the phosphorylation status and activity of AMPK (Fig. 2). A significant ($P <$ 0.05

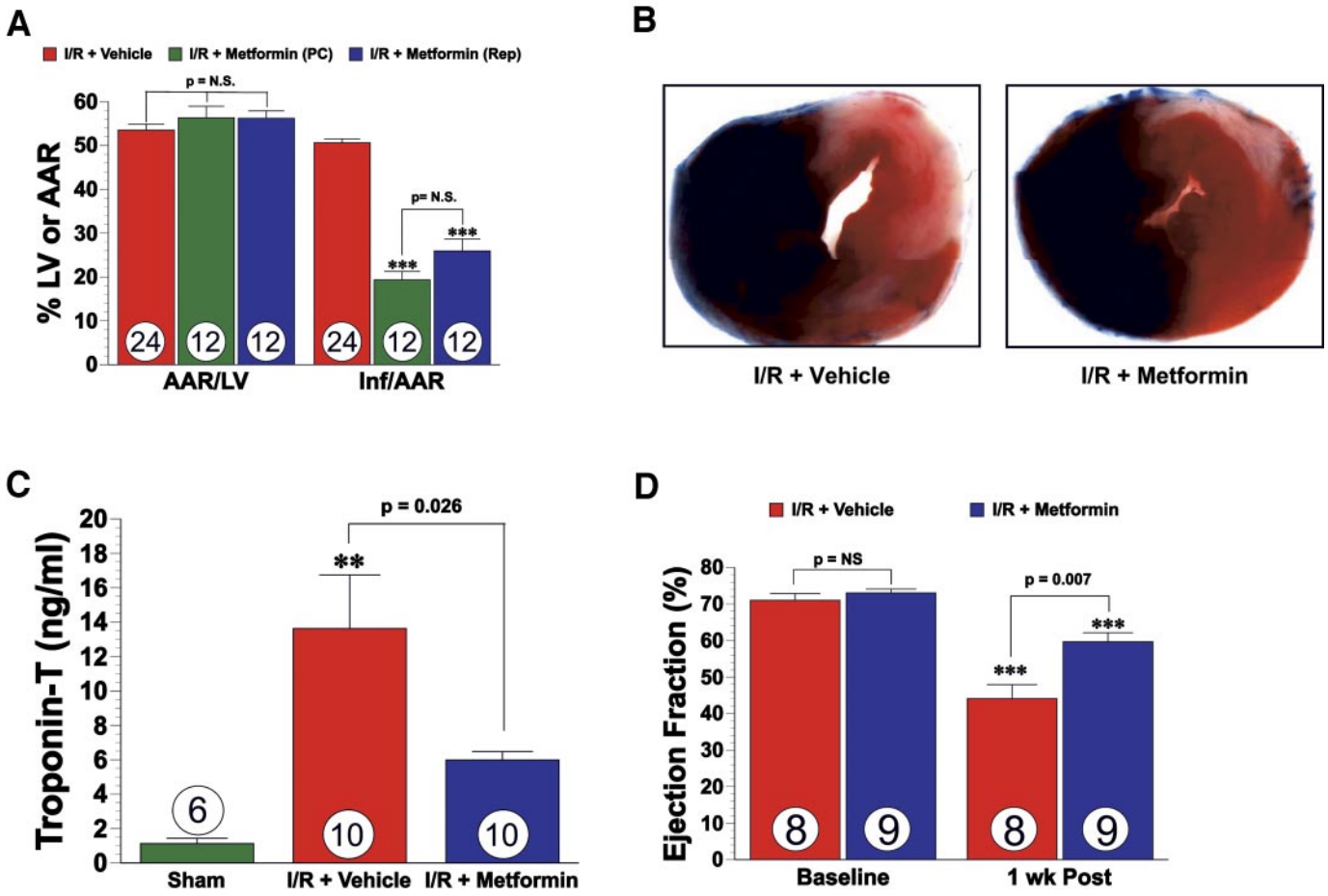

FIG. 1. Metformin reduced the extent of injury and improved left ventricular function in nondiabetic mice following myocardial ischemia and reperfusion. *A*: AAR with respect to the left ventricle (LV) was similar between all groups. Metformin (125 μg/kg) administered before ischemia (preconditioning group [PC]) or at the time of reperfusion (Rep) significantly attenuated myocardial infarct size with respect to the AAR (Inf/AAR). *B*: Representative mid-ventricular photomicrographs of hearts treated with vehicle and metformin at the time of reperfusion. *C*: Circulating levels of Troponin-T were measured 4 h after reperfusion. *D*: Ejection fraction was calculated using high-resolution, two-dimensional B-mode echocardiography images at baseline and following myocardial ischemia. Values are means ± SEM. Numbers inside bars indicate the number of animals that were investigated in each group. ***$P < 0.001$ vs. I/R + vehicle or baseline.

vs. sham) increase in the phosphorylation of AMPK at threonine residue 172 and the activation of AMPK was observed as early as 30 min following the injection of metformin and remained at this elevated state for up to 24 h. To examine the role of AMPK in the cardioprotective action of metformin, we assessed the phosphorylation and activation of AMPK following myocardial I/R (Fig. 3). Myocardial I/R significantly ($P < 0.05$ vs. sham) increased the phosphorylation as well as the activation of AMPK. Treatment with metformin significantly ($P < 0.05$ vs. I/R + vehicle) augmented the I/R-induced increase in both the phosphorylation and activation of AMPK.

We next investigated whether AMPK was critical for the cardioprotective actions of metformin. Cardiac-specific AMPKα2 dominate-negative transgenic (AMPKα2 dn Tg) and nontransgenic (NTg) littermates were subjected to 45 min of LCA ischemia followed by 24 h of reperfusion. An ischemic time of 45 min was used because of the background strain of the AMPKα2 dn Tg mice. Preliminary studies (data not shown) revealed that mice on an FVB background are more resistant to ischemia compared with mice on a C57BL/6J background. Initial experiments were performed to confirm the cardioprotective effects of metformin for this background strain (Fig. 3*D*). The administration of metformin at the time of reperfusion decreased Inf/AAR by 33% (37.91 ± 2.87 vs. 25.41 ± 2.97%, $P = 0.01$) in NTg mice. The Inf/AAR in the AMPKα2 dn Tg mice was found to be exacerbated by 34% compared with the NTg littermates (50.67 ± 1.42 vs. 37.91 ± 2.87%, $P = 0.005$). Metformin failed to provide protection in the AMPKα2 dnTg (50.67 ± 1.42 vs. 44.67 ± 2.88%), suggesting that AMPKα2 plays a critical role in the cardioprotection actions mediated by metformin.

The cardioprotective actions of metformin are also mediated through eNOS. The phosphorylation of eNOS at serine residue 1177 ($eNOS^{Ser1177}$) close to the carboxy-terminal is a critical requirement for eNOS activation and has been reported to be mediated by AMPK during myocardial ischemia (19). The activity of eNOS is also influenced by phosphorylation at threonine residue 495 ($eNOS^{Thr495}$). Phosphorylation at this site inhibits NO synthesis, whereas dephosphorylation can promote NO synthesis (27). We, therefore, examined whether the cardioprotective actions of metformin were mediated through eNOS. The ability of metformin to alter the phosphorylation status of eNOS ($eNOS^{Ser1177}$ and $eNOS^{Thr495}$) was first evaluated in naïve mice (Fig. 4*A*–*C*). Metformin significantly increased $eNOS^{Ser1177}$ phosphorylation over basal levels (sham) for an observed period of 24 h. Metformin did not alter $eNOS^{Thr495}$ phosphorylation at any time point

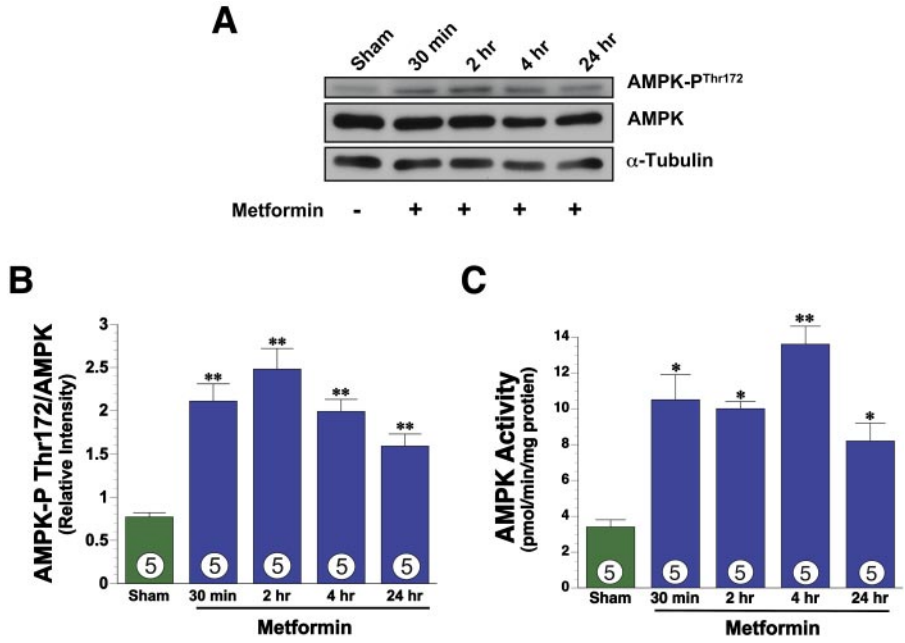

FIG. 2. Single administration of metformin activates AMPK in the left ventricle. *A*: Representative immunoblots of phosphorylated AMPK at residue threonine 172 (AMPK-P^{Thr172}) and total AMPK at 30 min to 24 h following a single injection of metformin into the lumen of the left ventricle. *B*: Densitometric analysis of the phosphorylated state of AMPK^{Thr172}. Bars represent the ratio of phosphorylated AMPK to total AMPK. *C*: Time course of total AMPK activity following a single injection of metformin into the lumen of the left ventricle. Values are means ± SEM. Numbers inside bars indicate the number of animals that were investigated in each group. *$P < 0.05$ compared with sham, **$P < 0.01$ compared with sham.

investigated. Metformin also failed to increase eNOS^{Ser117} phosphorylation when administered to AMPKα2 dn Tg mice (Fig. 4*D–E*). Similarly, eNOS^{Thr495} phosphorylation remained unchanged in AMPKα2 dn Tg mice administered metformin (Fig. 4*D* and *F*). We next investigated whether metformin altered the phosphorylation status of eNOS (eNOS^{Ser1177} and eNOS^{Thr495}) following myocardial I/R (Fig. 5*A–C*). No change in the eNOS^{Ser1177} phosphorylation over basal levels was detected at 30 min and 2 h of reperfusion in the vehicle-treated mice. However, an increase ($P < 0.01$ vs. sham) in eNOS^{Ser1177} phosphorylation was observed in this group at 4 h of reperfusion. In contrast, metformin significantly increased eNOS^{Ser1177} phosphorylation over basal levels at both 2 and 4 h of reperfusion ($P < 0.01$ vs. sham). This corresponded to a 93 and 41% increase over the vehicle-treated group at 2 and 4 h of reperfusion, respectively. No change in the phosphorylation status of eNOS^{Thr495} over basal levels was detected at 30 min and 2 h of reperfusion in the vehicle-treated or metformin-treated mice. However, a similar decrease ($P < 0.05$ vs. sham) in eNOS^{Thr495} phosphorylation was observed in both groups at 4 h of reperfusion. Metformin failed to increase eNOS^{Ser1177} phosphorylation at 2 and 4 h of reperfusion (Fig. 5*D* and *E*) in AMPKα2 dn Tg mice. Interestingly, I/R alone did not increase eNOS^{Ser1177} at 4 h of reperfusion in either group. eNOS^{Thr495} phosphorylation remained unchanged in AMPKα2 dn Tg mice treated with vehicle or metformin at 2 h of reperfusion following I/R, but in both groups a decrease in phosphorylation was observed at 4 h of reperfusion (Fig. 5*D* and *F*).

We next investigated the potential role of eNOS-derived NO in the cardioprotective effects of metformin. Mice deficient in eNOS (eNOS$^{-/-}$) were subjected to 30 min of myocardial ischemia followed by reperfusion (Fig. 6). Myocardial infarct size analysis at 24 h of reperfusion revealed that metformin did not affect infarct size in eNOS$^{-/-}$ mice (P = NS vs. I/R + vehicle), suggesting that eNOS and NO also plays an important a role in the cardioprotective actions of metformin.

Metformin does not increase Akt. Akt/protein kinase B has been reported to phosphorylate eNOS at serine residue 1177 (1). Therefore, we investigated the possibility that metformin increased eNOS activation by increasing Akt phosphorylation. The ability of metformin to increase the phosphorylation of Akt at serine residue 473 (Akt^{Ser473}) was first evaluated in naïve mice and then evaluated following myocardial I/R. Metformin did not alter the phosphorylation of Akt^{Ser473} for an observed period of 24 h (Fig. 7*A* and *B*). Myocardial I/R significantly ($P < 0.05$ vs. sham) increased the phosphorylation of Akt^{Ser473} at all time points examined (Fig. 7*C* and *D*). However, no differences in Akt^{Ser473} phosphorylation were observed between the vehicle- and metformin-treated groups, suggesting that in our model metformin does not signal through Akt.

Metformin limited the extent of myocardial injury following in vivo ischemia-reperfusion in the diabetic heart. We next investigated the potential cardioprotective actions of a single dose of metformin in the diabetic heart. Diabetic (*db/db*) mice were subjected to 30 min of LCA ischemia and reperfusion. Metformin (125 μg/kg) or vehicle was administered either 18 h before ischemia or at the time of reperfusion. The extent of myocardial infarction was then evaluated at 24 h of reperfusion. The AAR/LV was similar (P = NS) in all of the groups (Fig. 8). Metformin administered before ischemia (preconditioning group) decreased the Inf/AAR by 34% (67.32 ± 2.5 vs. 44.1 ± 5.9%, $P < 0.001$), whereas the administration of metformin at the time of reperfusion decreased Inf/AAR by 17% (67.32 ± 2.5 vs. 56 ± 2.14%, $P < 0.05$). The extent of infarct size reduction between the groups administered metformin

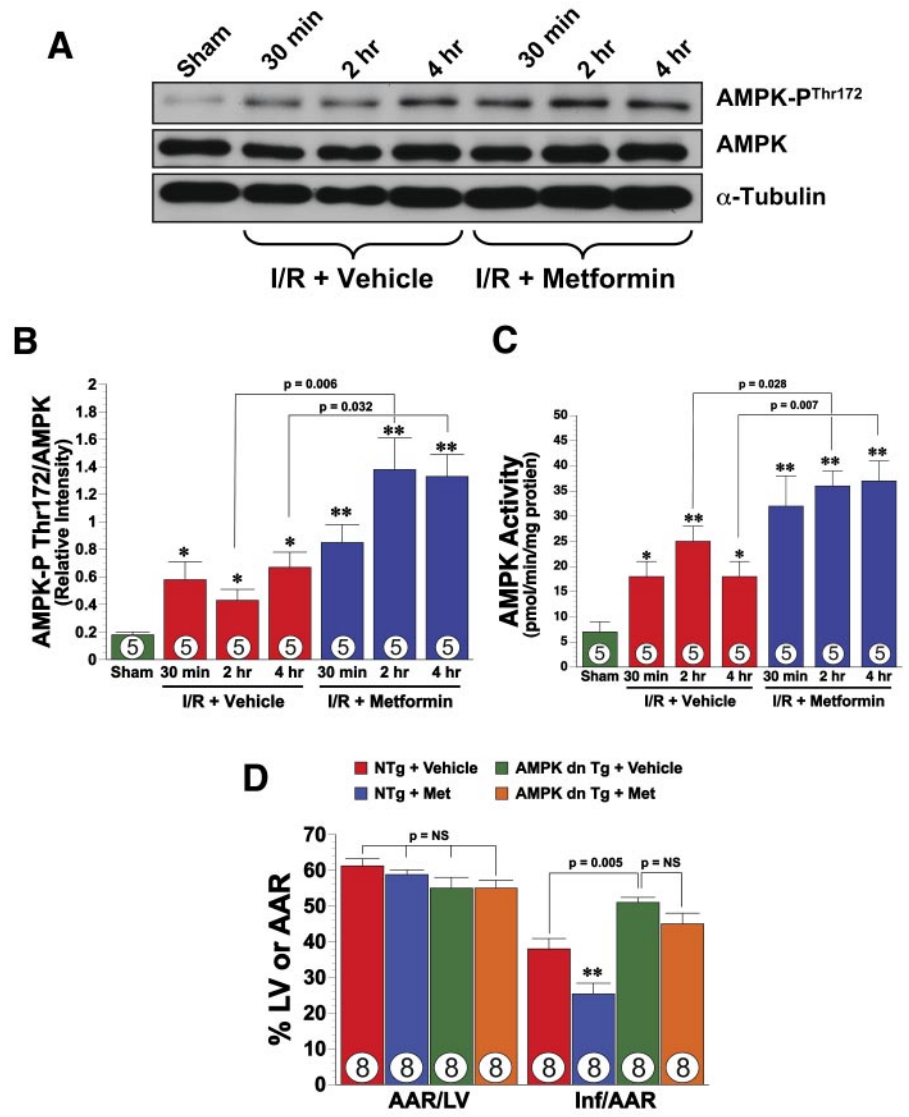

FIG. 3. Metformin augments the phosphorylation and activation of AMPK in the left ventricle following in vivo I/R. *A*: Representative immunoblots of phosphorylated AMPK at residue threonine 172 (AMPK-P^{Thr172}) and total AMPK at 30 min to 4 h of reperfusion following myocardial ischemia and metformin treatment (at reperfusion). *B*: Densitometric analysis of the phosphorylated state AMPK^{Thr172}. Bars represent the ratio of phosphorylated AMPK to total AMPK. *C*: Time course of total AMPK activity following myocardial ischemia-reperfusion and metformin treatment (at reperfusion). *D*: Myocardial infarct size was determined 24 h after 45 min of LCA ischemia in cardiac specific AMPKα2 dominant-negative transgenic mice (AMPK dn Tg) and nontransgenic (NTg) littermates receiving either vehicle or metformin at the time of reperfusion. AAR with respect to the left ventricle (LV) was similar between all groups. In the NTg mice, metformin treatment significantly attenuated myocardial infarct size. Conversely, Tg mice were found to have exacerbated injury when compared with the NTg littermates, and the cardioprotective effects of metformin were found to be ablated in these mice. Values are means ± SEM. Numbers inside bars indicate the number of animals that were investigated in each group. *$P < 0.05$ compared with sham, **$P < 0.01$ compared with sham or NTg + vehicle.

was found to be significant ($P < 0.05$). However, there was no significant difference in the serum blood glucose values between the groups (365 ± 27 vs. 350 ± 42 vs. 373 ± 26, mg/dl, respectively).

DISCUSSION

Experimental and clinical studies have reported that metformin possesses cardioprotective actions in the setting of diabetes (6,20). Specifically, the UK Prospective Diabetes Study (UKPDS) has revealed a reduction in the incidence of myocardial infarction by up to 39% in diabetic patients treated with metformin (5,28). At first glance it may seem that metformin reduces cardiovascular risk factors through an effective control of glycemic levels. However, a closer look at the UKPDS studies reveals that metformin reduced A1C values in treated patients to the same extent as in the other cohort of patients treated with conventional therapies, suggesting that metformin might have additional cardioprotective actions beyond its antihyperglycemic effects (7). This notion is further supported by the observation that metformin does not affect glucose values in nondiabetic rodents (20) yet improves cardiac function following in vitro global ischemia (21). This is further supported by the findings of the current study, which is the first to provide strong evidence that a single administration of metformin to nondiabetic and diabetic mice profoundly reduces infarct size without lowering blood glucose levels. In addition, our study elucidates the mechanisms involved in metformin-induced cardioprotection and answers important questions regarding its dosage, time of administration, and use as a cardioprotective agent. Regarding dosage, 125 μg/kg is 286-fold less than

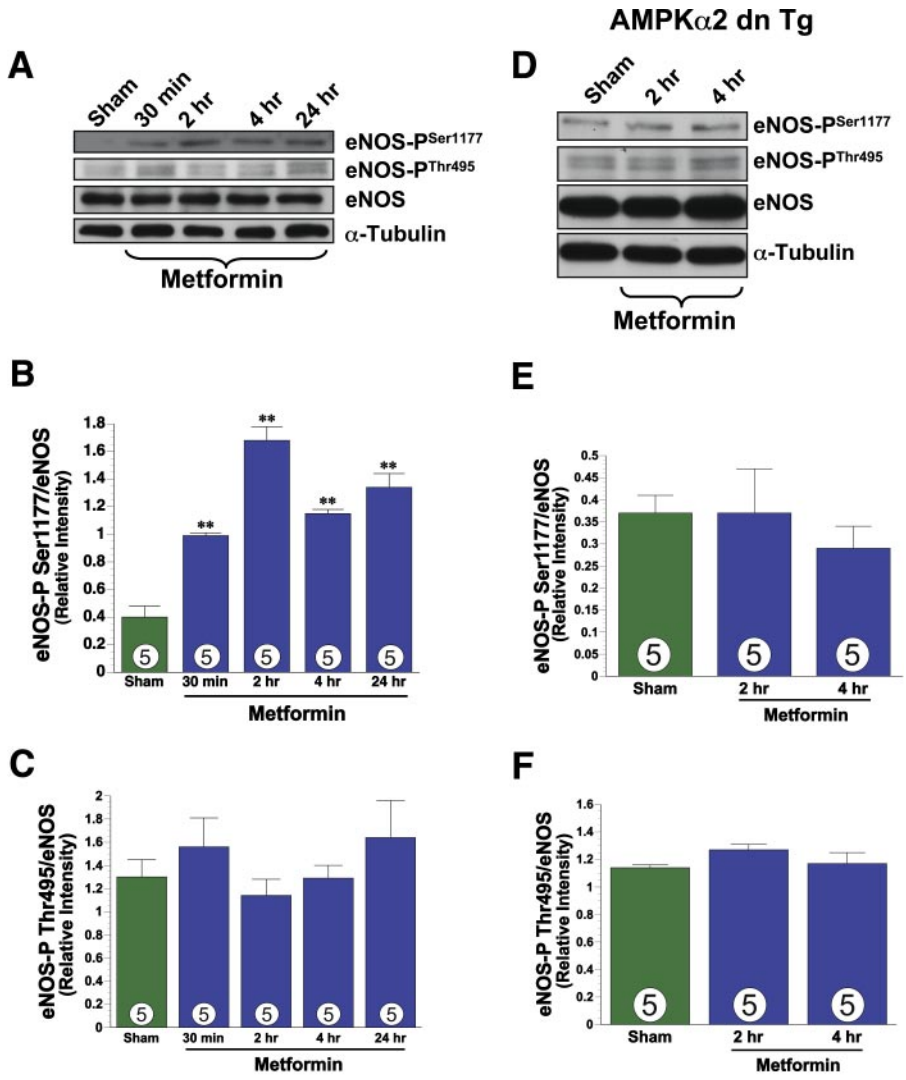

FIG. 4. Single administration of metformin increases the phosphorylation of eNOS at serine residue 1177. *A*: Representative immunoblots of phosphorylated eNOS at serine residue 1177 (eNOS-P^{Ser1177}), theronine residue 495 (eNOS-P^{Thr495}), and total eNOS at 30 min to 24 h following a single injection of metformin into the lumen of the left ventricle. *B*: Densitometric analysis of the phosphorylated state of eNOS^{Ser1177}. *C*: Densitometric analysis of the phosphorylated state of eNOS^{Thr495}. *D*: Representative immunoblots of eNOS-P^{Ser1177}, eNOS-P^{Thr495}, and total eNOS at 2 and 4 h following a single injection of metformin into the lumen of the left ventricle of AMPKα2 dn Tg mice. *E*: Densitometric analysis of the phosphorylated state of eNOS^{Ser1177} in AMPKα2 dn Tg hearts. *F*: Densitometric analysis of the phosphorylated state of eNOS^{Thr495} in AMPKα2 dn Tg hearts. Bars represent the ratio of either phosphorylated eNOS^{Ser1177} or eNOS^{Thr495} to total eNOS. Values are means ± SEM. Numbers inside bars indicate the number of animals that were investigated in each group. **$P < 0.01$ compared with sham.

the maximum antihyperglycemic dose (2,500 mg/day) of metformin, making the current study the first to clearly demonstrate that a subtherapeutic dose of metformin can provide cardioprotection. Regarding the time of administration, we demonstrate that in contrast to previous studies (20,21), which reported the cardioprotective effects of a chronic administration or constant perfusion of metformin, an acute administration of metformin given once either before ischemia or at the time of reperfusion can provide protection. These findings are of clinical relevance since they demonstrate that acute metformin treatment could potentially be initiated at the time of coronary artery reperfusion to patients experiencing myocardial ischemia.

One mechanism by which metformin protects the myocardium is via activation of AMPK (9). AMPK is an important regulator of diverse cellular pathways (29) and is considered to be a "fuel gauge" or "master switch" for cellular energy levels (12). This is of particular importance in the setting of myocardial ischemia due to the very-high-energy demands and low-energy reserves of the heart (30). The phosphorylation and activity of AMPK are increased within minutes of the onset of myocardial ischemia and remain elevated for at least 48 h following reperfusion (25,31,32). It has been suggested, however, that activation of AMPK during early reperfusion may be detrimental to the myocardium, as a result of high fatty acid oxidation (FAO) rates and subsequent inhibition of glucose oxidation (30). Conversely, lending support for a protective role of AMPK in the setting of I/R are studies showing that the transduction of dominant-negative AMPK impairs ischemia-stimulated glucose transport and fatty acid metabolism leading to increased susceptibility to cellular damage and increased left ventricular dysfunction (22,33). The results of the current study support a protective role for the activation of AMPK following myocardial I/R. First, we provide evidence that AMPK activity in the left ventricle increases within 30 min of a single injection of metformin and remains at this elevated level for at least 24 h. Second,

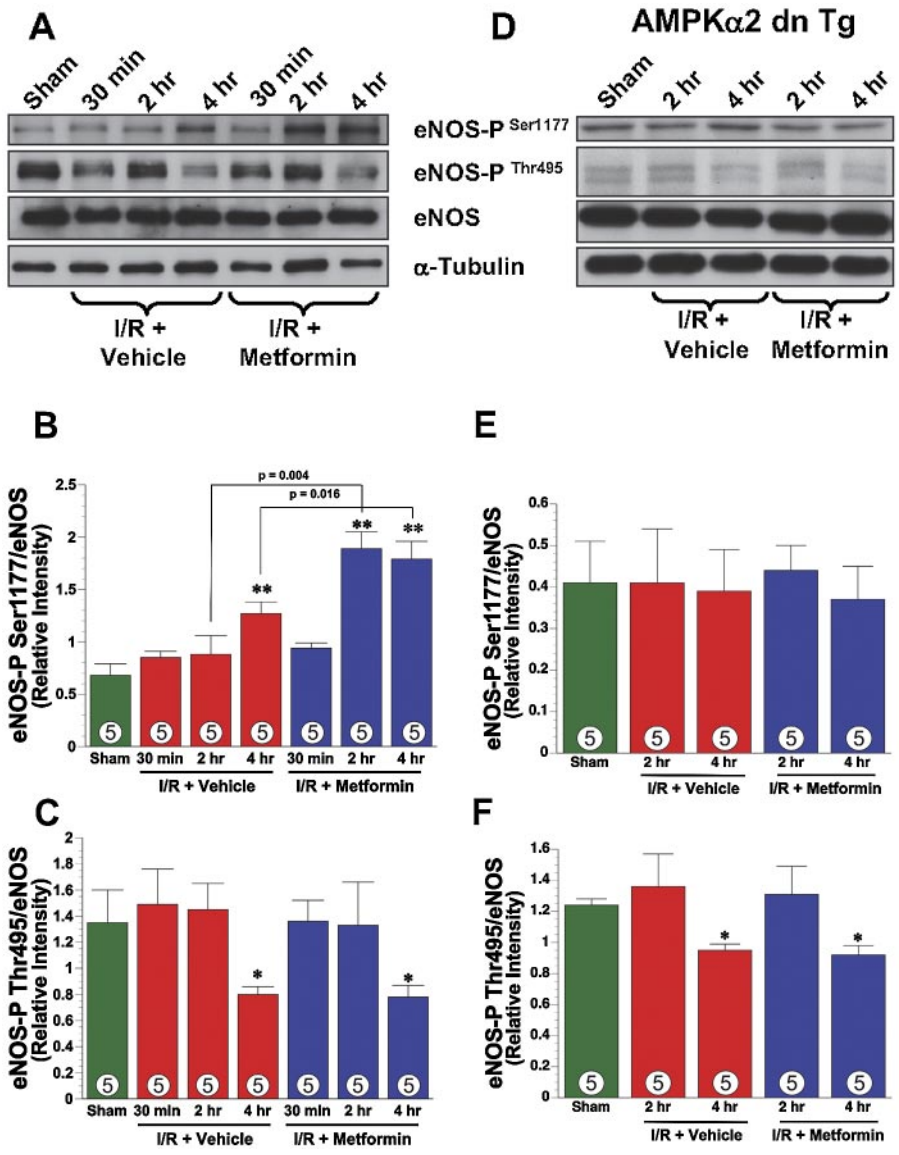

FIG. 5. Metformin increases the phosphorylation of eNOS^{Ser1177} in the left ventricle following in vivo ischemia-reperfusion. *A*: Representative immunoblots of phosphorylated eNOS^{Ser1177}, eNOS^{Thr495}, and total eNOS at 30 min to 4 h of reperfusion following myocardial ischemia and metformin treatment (at reperfusion). *B*: Densitometric analysis of the phosphorylated state of eNOS^{Ser1177}. *C*: Densitometric analysis of the phosphorylated state of eNOS^{Thr495}. *D*: Representative immunoblots of eNOS-P^{Ser1177}, eNOS-P^{Thr495}, and total eNOS at 2 and 4 h of reperfusion from the hearts of AMPKα2 dn Tg mice. *E*: Densitometric analysis of the phosphorylated state of eNOS^{Ser1177} in AMPKα2 dn Tg hearts. *F*: Densitometric analysis of the phosphorylated state of eNOS^{Thr495} in AMPKα2 dn Tg hearts. Bars represent the ratio of either phosphorylated eNOS^{Ser1177} or eNOS^{Thr495} to total eNOS. *P < 0.05 compared with sham, **P < 0.01 compared with sham.

our data are the first to demonstrate that when metformin is administered at the time of reperfusion it has the ability to augment the I/R-induced increase in AMPK activity. Amplifying signaling through AMPK by metformin during early reperfusion is beneficial to the injured myocardium due to the ability of AMPK to promote ATP generation (34,35) and to attenuate cardiomyocyte apoptosis (32,36). Third, our results provide direct evidence that AMPKα2 deficiency in the myocardium exacerbates injury following myocardial I/R and that the cardioprotective actions of metformin are ablated in these mice. Although, our study is limited by the fact that we did not measure FAO; we did demonstrate that metformin reduced infarct size in *db/db* diabetic mice, which have high circulating fatty acid levels (37). Therefore, if metformin and AMPK stimulate excessive FAO at the expense of glucose oxidation during early reperfusion, the beneficial effects of AMPK signaling seem to out weigh the potential detrimental effects associated with FAO. Taken together, these findings suggest that the activation of AMPK during I/R is an endogenous protective mechanism that can be augmented to mediate the cardioprotective actions of metformin.

Davis et al. (1) recently reported that metformin increased the phosphorylation of eNOS in an AMPK-dependent manner, which in turn increased eNOS activity and NO bioavailability. Additional studies (18) also suggest an AMPK-eNOS pathway in response to shear stress (38) and myocardial ischemia (19). Similarly, our data suggests an increased activation of myocardial eNOS secondary to the phosphorylation and activation of AMPK in response to myocardial I/R and metformin treatment. Specifically, metformin altered the phosphorylation status of eNOS^{Ser1177}. In agreement with a previous report (1), we also found that metformin did not alter Akt phosphorylation or eNOS^{Thr495}

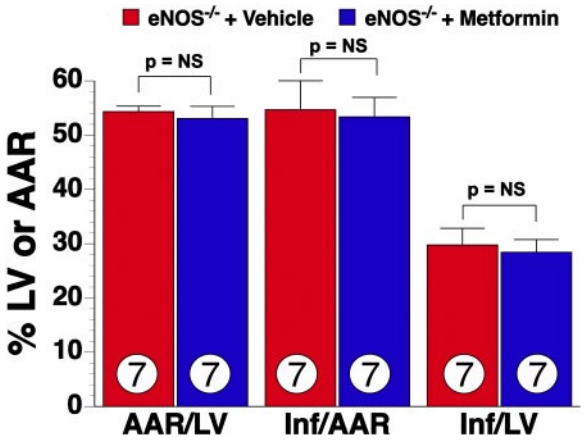

FIG. 6. eNOS mediates the cardioprotective effects of metformin. Myocardial infarct size was determined 24 h after 30 min of LCA ischemia in eNOS-deficient mice receiving either vehicle or metformin (125 μg/kg) at the time of reperfusion. AAR with respect to the left ventricle (LV) was similar between both groups. No significant differences in myocardial infarct size were observed between the study groups. Values are means ± SEM. Numbers inside bars indicate the number of animals that were investigated in each group.

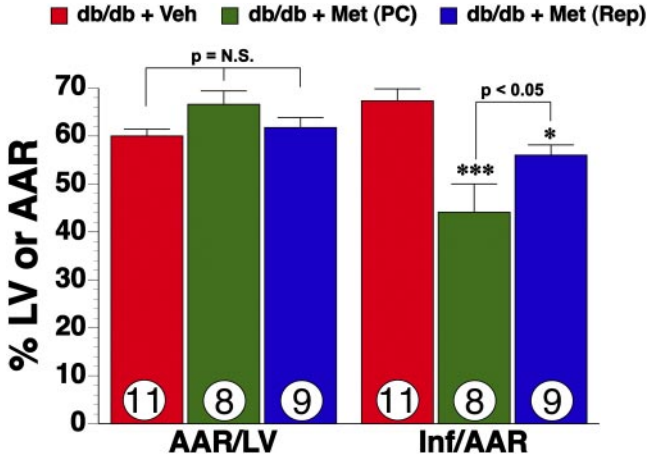

FIG. 8. Metformin reduced infarct size in the diabetic heart following myocardial ischemia and reperfusion. Myocardial infarct size was determined 24 h after 30 min of LCA ischemia in diabetic (*db/db*) mice receiving either vehicle or metformin (125 μg/kg) before ischemia or at the time of reperfusion. AAR with respect to the left ventricle (LV) was similar between all groups. Metformin administered before ischemia (preconditioning group [PC]) or at the time of reperfusion (Rep) significantly attenuated myocardial infarct size with respect to the AAR (Inf/AAR). Values are means ± SEM. Numbers inside bars indicate the number of animals that were investigated in each group. *$P < 0.05$ compared with I/R + vehicle, ***$P < 0.01$ compared with I/R + vehicle.

and failed to increase eNOS^{Ser1177} phosphorylation in AMPKα2 dn Tg hearts, suggesting that the metformin-induced eNOS activation is AMPK-dependent. In the heart, eNOS is expressed not only by vascular endothelial cells, but also by cardiomyocytes (39,40). Importantly, in recent years, it has been suggested that the regulation of NO synthesis by eNOS in the cardiomyocyte represents a critical final common pathway to explain the benefit of several effective treatments for both acute myocardial ischemia and chronic congestive heart failure (27). Our results suggest a primary role for cardiomyocyte eNOS activation, not endothelial cell eNOS activation, in the observed metformin-induced cardioprotection. This is evidenced by the finding that metformin failed to induce eNOS^{Ser1177} phosphorylation in the hearts of the cardiomyocyte specific AMPKα2 dn Tg mice. However, we cannot rule out the activation of coronary vascular eNOS with subsequent NO generation as a potential cardioprotective mechanism following metformin treatment. Additionally, in agreement with previous reports (41,42), the cardioprotection induced by metformin was abolished in the absence of eNOS. Therefore, the ability of metformin to increase the phosphorylation of eNOS through AMPK and increase NO bioavailability provides cardioprotective mechanisms that are complementary to the AMPK-medi-

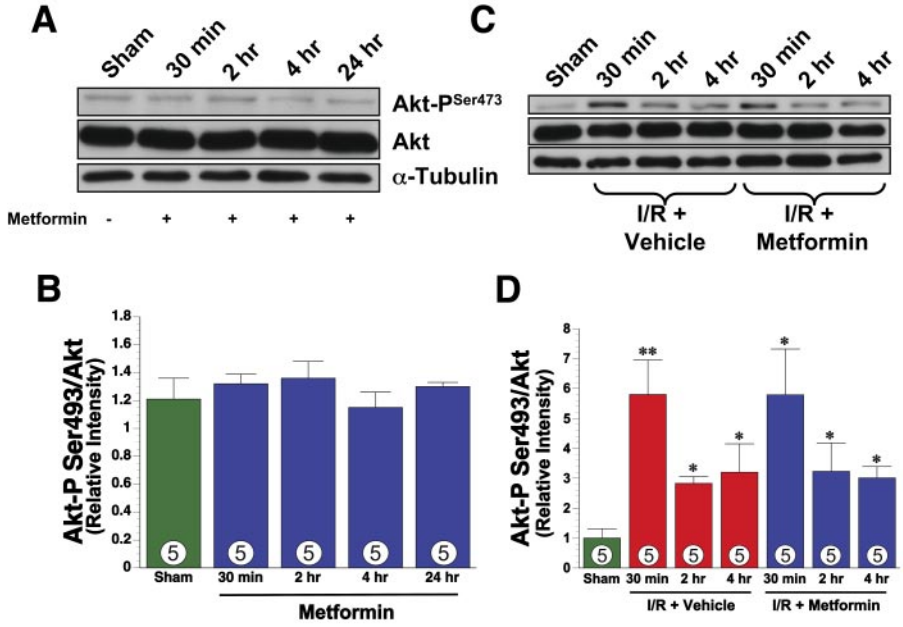

FIG. 7. Metformin does not increase Akt phosphorylation. *A*: Representative immunoblots of phosphorylated Akt at serine residue 473 (Akt-P^{Ser473}) and total Akt at 30 min to 24 h following a single injection of metformin into the lumen of the left ventricle. *B*: Densitometric analysis of the phosphorylated state of Akt^{Ser473}. *C*: Representative immunoblots of phosphorylated Akt-P^{Ser473} and total Akt at 30 min to 4 h of reperfusion following myocardial ischemia and metformin treatment (at reperfusion). *D*: Densitometric analysis of the phosphorylated state of Akt^{Ser473}. Bars represent the ratio of phosphorylated Akt^{Ser473} to total eNOS. *$P < 0.05$ compared with sham, **$P < 0.01$ compared with sham.

ated effects on apoptosis and ATP generation. Although we did not demonstrate the direct actions of NO in this current study, we (23,43,44) and others (45) have shown that NO possesses a number of physiological properties, such as vasodilation, inhibition of oxidative stress, platelet aggregation, leukocyte chemotaxis, and apoptosis, which make it a potent cardioprotective-signaling molecule (46).

The cardioprotective actions of metformin are not solely limited to its ability to reduce myocardial infarct size, as demonstrated by the acute administration in the current study. Rather, as mentioned, metformin reduces the risk factors of cardiovascular disease in diabetic patients when administered chronically (5,28). In these patients, metformin has been reported to improve lipoprotein profiles, reduce oxidative stress, and improve vascular stability (7). Combined these effects could lead to a reduced incidence of atherosclerosis, which in turn prevents the risk of myocardial infarction. Therefore, metformin has the ability to reduce the incidence and size of myocardial infarction depending on when it is administered.

In summary, our findings demonstrate that a single, low dose of metformin confers significant cardioprotection against myocardial I/R injury in nondiabetic and diabetic animals when administered either before ischemia or at the time of reperfusion. This suggests that metformin therapy does not have to be solely limited to the treatment of diabetic subjects, but rather indicates that an acute administration of metformin may have a practical clinical use following myocardial ischemia in all patient populations. These findings also reemphasize the notion that many current therapeutic agents possess pleiotropic actions that can be initiated at doses much lower than those currently recommended.

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