CPU0213, a novel endothelin type A and type B receptor antagonist, protects against myocardial ischemia/reperfusion injury in rats

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Abstract

The efficacy of endothelin receptor antagonists in protecting against myocardial ischemia/reperfusion (I/R) injury is controversial, and the mechanisms remain unclear. The aim of this study was to investigate the effects of CPU0123, a novel endothelin type A and type B receptor antagonist, on myocardial I/R injury and to explore the mechanisms involved. Male Sprague-Dawley rats weighing 200-250 g were randomized to three groups (6-7 per group): group 1, Sham; group 2, I/R + vehicle. Rats were subjected to in vivo myocardial I/R injury by ligation of the left anterior descending coronary artery and 0.5% sodium carboxymethyl cellulose (1 mL/kg) was injected intraperitoneally immediately prior to coronary occlusion. Group 3, I/R + CPU0213. Rats were subjected to identical surgical procedures and CPU0213 (30 mg/kg) was injected intraperitoneally immediately prior to coronary occlusion. Infarct size, cardiac function and biochemical changes were measured. CPU0213 pretreatment reduced infarct size as a percentage of the ischemic area by 44.5% (I/R + vehicle: 61.3 ± 3.2 vs I/R + CPU0213: 34.0 ± 5.5%, P < 0.05) and improved ejection fraction by 17.2% (I/R + vehicle: 58.4 ± 2.8 vs I/R + CPU0213: 68.5 ± 2.2%, P < 0.05) compared to vehicle-treated animals. This protection was associated with inhibition of myocardial inflammation and oxidative stress. Moreover, reduction in Akt (protein kinase B) and endothelial nitric oxide synthase (eNOS) phosphorylation induced by myocardial I/R injury was limited by CPU0213 (P < 0.05). These data suggest that CPU0123, a non-selective antagonist, has protective effects against myocardial I/R injury in rats, which may be related to the Akt/eNOS pathway.

Key words: Endothelin receptor antagonist; Myocardial ischemia/reperfusion injury; Akt; eNOS

Introduction

Endothelin (ET) is a 21-amino acid peptide isolated from endothelial cells with powerful vasoconstrictive properties (1). There are three isoforms of ET (ET-1, ET-2, and ET-3). ET-1 is the most prominent isoform in the cardiovascular system, involved in endothelial dysfunction, vasomotor contraction, leukocyte activation, and cellular proliferation (2). ET-1 exerts its biological effect via endothelin type A (ETA) receptors and endothelin type B (ETB) receptors. ETA receptors are predominantly located on smooth muscle cells, where they mediate vasoconstriction. ETB receptors are mainly found on both endothelial and smooth muscle cells, where they mediate vasodilation or vasoconstriction (3,4).

There are numerous reports showing that plasma ET-1 levels are elevated during myocardial ischemia/reperfusion (I/R) injury (5,6). Both exogenous and endogenous ET-1 can potentiate myocardial damage produced by I/R injury (7), and ET-1 was associated with increased long-term mortality in a high-risk ST-elevation myocardial infarction population

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reperfused by primary percutaneous coronary intervention (PCI) (8). Thus, ET-1 is considered to play an important role in the pathophysiology of myocardial I/R injury, and therapy targeted at suppression of the ET system may prevent the development of myocardial I/R injury.

It is generally accepted that ETA receptor antagonists exhibit protective effects against myocardial I/R injury (7,9,10). However, the cardioprotective actions of non-selective ET_{A}/ET_{B} receptor antagonists remain controversial, since they may block the beneficial effects of the ET_{B} receptor (11,12). Nevertheless, there is some evidence that non-selective ET_{A}/ET_{B} receptor blockade may be superior to selective ET_{A} receptor blockade in certain conditions (13).

CPU0213 is a novel non-selective ETA/ETB receptor antagonist (Figure 1). It has been reported to improve diabetic cardiac insufficiency (14), and suppress ventricular fibrillation in cardiomyopathy (15). However, the effects of CPU0213 on myocardial I/R injury are unknown. In the present study, we evaluated whether CPU0123, a non-selective ETA/ETB receptor antagonist, could ameliorate myocardial damage induced by I/R, and tried to identify the potential signaling pathway involved.

Material and Methods

Animals

Male Sprague-Dawley rats weighing 200-250 g were used. All experiments were performed in accordance with the guidelines for the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals published by the NIH (NIH Publication No. 85-23, revised 1996). The experimental protocols were approved by the Nanjing Medical University Committee on Animal Care.

Myocardial I/R model

Rats were anesthetized with 10% chloral hydrate. The heart was exposed through thoracotomy at the left fourth intercostal space, and myocardial ischemia was induced by ligation of the left anterior descending coronary artery (LAD) with 6.0 silk sutures. After 30 min of ischemia, the slipknot was released and the myocardium was reperfused for 6 h for measurement of some biochemical parameters, protein assay and cardiac function assessment, or for 24 h for infarct size evaluation. Rats were randomized to three groups: 1) Sham, animals were subjected to identical surgical procedures, without coronary occlusion; 2) I/R + vehicle, 0.5% sodium carboxymethyl cellulose (Hercules, USA) was injected intraperitoneally immediately prior to coronary occlusion, and 3) I/R + CPU0213, 30 mg/kg CPU0213 was injected intraperitoneally immediately prior to coronary occlusion.

Determination of infarct size

Infarct size, infarct area (IA) and risk area (RA) were determined by triphenyltetrazolium chloride (TTC)/Evans blue double-staining method following 24 h of reperfusion. Briefly, the heart was quickly removed and perfused with 0.9% saline on a Langendorff apparatus. The LAD was reoccluded and 1 mL 1.5% Evans blue (Amresco, USA) was injected into the aorta and the coronary artery system to determine the non-ischemic area. The heart was then isolated and placed in a freezer at -20°C for 1 h. The frozen heart was cut into 1–2-mm thick parallel transverse slices and incubated in 1.5% TTC (Amresco) at 37°C for 15 min. After fixation in 4% paraformaldehyde, slices were photographed with a digital camera. The infarct size and the risk area were assessed with the AlphaEaseFC software (Alpha Innotech, USA). Ratios of risk area to left ventricle area (RA/LV) and infarct area to risk area (IA/RA) were calculated and reported as percentages.

Evaluation of cardiac function

Rats were anesthetized by diethyl ether inhalation. In vivo cardiac function was measured by transthoracic echocardiography using a vivid 7 echocardiograph (GE Vingmed Ultrasound, Norway) equipped with a 14-MHz phase array linear transducer S12, allowing a 150 maximal sweep rate (General Electric Company, USA), at Jiangsu Province Hospital. The following M-mode measurements were performed: LV internal dimension at diastole, LV internal dimension at systole, LV posterior wall dimension at diastole, LV posterior wall dimension at systole, interventricular septal dimension at diastole, and interventricular septal dimension at systole. From these parameters, end-diastolic volume and end-systolic volume, fractional shortening (FS), ejection fraction (EF) of the LV, stroke volume, and cardiac output were calculated. All measurements were made by one observer who was blind to the treatment groups. All measurements were averaged over five consecutive cardiac cycles.

Measurement of serum CK, LDH, and MDA

Creatine kinase (CK) and lactate dehydrogenase (LDH) are enzymes expressed predominantly by myocardial tissues. Thus, we measured CK and LDH activity to evaluate myocardial damage. Arterial blood samples (1 mL) were collected...
after 6 h of reperfusion. Serum was obtained after centrifugation at 1500 g and 4°C for 10 min, and was then stored at -80°C until analysis. CK and LDH activity was detected in the serum with a Hitachi 7170A full-automatic biochemical analyzer (Hitachi, Japan). To assess oxidative stress, malondialdehyde (MDA) levels were determined with a commercial kit (Jiangcheng Biological Institute, Nanjing, China) by the thiobarbituric acid (TBA) method. The amounts of lipid peroxides were measured as the production of MDA, which in combination with TBA forms a pink chromogen compound, whose absorbance was measured at 532 nm.

Measurement of ET-1 in plasma
Systemic ET-1 levels have been recently reported to be a predictor of prognosis in patients admitted for acute myocardial infarction treated by PCI (16). In order to explore the influence of CPU0213 on ET-1 levels, we measured ET-1 levels in plasma. After 6 h of reperfusion, blood samples (2 mL) were collected with the addition of 30 µL 10% EDTA-2Na and 20 µL aprotinin. Plasma was obtained after centrifugation at 900 g at 4°C for 10 min and was then stored at -80°C until analysis. Plasma levels of ET-1 were determined by a radioimmunoassay method with a commercial kit (Beijing Northern Bioengineering Institute, Beijing, China).

Determination of MPO in myocardial tissue
Myeloperoxidase (MPO) activity in ischemic cardiac tissues was measured as a marker of neutrophil accumulation. After 6 h of reperfusion, the myocardial tissue (approximately 100 mg) was harvested from the ischemic area, homogenized in 5 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethyl ammonium bromide, and then centrifuged at 20,000 g for 30 min at 4°C before extraction. The supernatant was collected and reacted with 0.167 g/L 3,3-dimethoxybenzidine dihydrochloride and 0.0005% H2O2 in 50 mM phosphate buffer. Absorbance was measured spectrophotometrically at 460 nm at 37°C with a Spectronic GENESYS 2 UV-Vis spectrophotometer (Spectronic, USA).

Western blotting
After 6 h of reperfusion, heart samples were homogenized in cold lysis buffer (50 mM potassium phosphate buffer containing 1% Triton X-100, protease inhibitor cocktail (#04 693 132 001; Roche, Germany), phosphatase inhibitor cocktail (#04 906 845 001; Roche), and 4 mM EDTA, pH 7.2). After incubation on ice for 40 min, the homogenates were centrifuged at 13,800 g at 4°C for 10 min. Supernatants were collected and protein concentrations were determined with the Pierce reagent (#23227; Pierce, USA). The proteins were separated by 7.5% SDS–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, USA) using a Bio-Rad semidry transfer system (Bio-Rad, USA). After being blocked with 5% skimmed milk, the immunoblots were probed with anti-phospho-Akt (protein kinase B; Ser473), anti-phospho-eNOS (endothelial nitric oxide synthase; Ser1177; Cell Signaling Technology, USA), total Akt (Cell Signaling Technology), or eNOS (Santa Cruz Biotechnology, USA) antibodies overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:4000). The blots were detected with an ECL Western Blot Detection Kit (Pierce), and band density was analyzed using the Image J software (NIH, USA).

Statistical analysis
Data are reported as means ± SEM. Statistical analyses were performed by ANOVA followed by the post hoc Bonferroni test. For comparison of infarct size, differences between groups were determined by the unpaired Student t-test. A value of P < 0.05 was considered to be statistically significant.

Results
Myocardial infarct sizes
Infarct size was established by TTC/Evans blue double-staining method. Evans blue stains the myocardium blue, representing non-ischemic tissue, while the remaining area represents the risk area. TTC stains the myocardium red due to complete dehydrogenase activity, while the infarct area, lacking dehydrogenase activity, remains unstained (white; Figure 2A). CPU0213 pretreatment resulted in a significant reduction of IA/RA compared to the I/R + vehicle group (I/R + vehicle: 61.3 ± 3.2 vs I/R + CPU0213: 34.0 ± 5.5%; P < 0.05), representing a 44.5% reduction in infarct size (Figure 2B). There was no significant difference in RA/LV, indicating a similar position of the coronary artery ligation.

Cardiac function
The left ventricular function and structure were assessed by echocardiography after 30-min ischemia and 6-h reperfusion. Representative M-mode echocardiographs of the three groups are shown in Figure 3A, B, and C. The echocardiograph revealed decreased EF and FS in the I/R + vehicle group compared to the Sham group. CPU0213 pretreatment
increased EF and FS by 17.2% (I/R + vehicle: 58.4 ± 2.8 vs I/R + CPU0213: 68.5 ± 2.2%; P < 0.05) and 27.4% (I/R + vehicle: 26.6 ± 1.6 vs I/R + CPU0213: 33.9 ± 1.7%; P < 0.01), respectively, compared to the I/R + vehicle group (Figure 3D), suggesting that CPU0213 improved myocardial contractile function. There were no significant differences in other parameters between the I/R + vehicle group and the I/R + CPU0213 group (data not shown).

**ET-1 levels in plasma, and CK and LDH activity in serum**

Basal ET-1 levels in plasma of no I/R injury animals were low. After 30 min of ischemia and 6 h of reperfusion, ET-1 levels were significantly elevated above basal levels (Sham: 20.8 ± 2.1 vs I/R + vehicle: 34.1 ± 4.4 pg/mL; P < 0.05). ET-1 levels were inhibited by CPU0213 (I/R + vehicle: 34.1 ± 4.4 vs I/R + CPU0213: 20.4 ± 2.8 pg/mL; P < 0.05; Figure 4A).

Serum CK and LDH activities are additional markers of myocardial injury. Serum samples were obtained from the rats subjected to 30 min of ischemia and 6 h of reperfusion. CPU0213 pretreatment reduced CK and LDH activity compared to the I/R + vehicle group, indicating a protective role of CPU0213 (Figure 4B and C).

**MDA levels in serum and MPO activity in the myocardium**

MDA is a marker of lipid peroxidation, which is considered to be a major mechanism of tissue damage. To investigate whether CPU0213 had an effect on lipid peroxidation, we measured serum MDA levels. MDA levels were increased in the I/R + vehicle group compared to the Sham group, but were reduced by 41.9% in the I/R + CPU0213 group (I/R + vehicle: 9.2 ± 1.3 vs I/R + CPU0213: 5.4 ± 0.5 nM; P < 0.05; Figure 5A), suggesting that CPU0213 can reduce lipid peroxidation.

We also determined MPO activity in ischemic myocardial tissues after 30 min of ischemia and 6 h of reperfusion. Rats pretreated with CPU0213 had a 32.8% reduction in MPO activity compared to vehicle-treated rats (I/R + vehicle: 752.7 ± 71.0 vs I/R + CPU0213: 505.9 ± 53.0 U/mg protein; P < 0.05; Figure 5B), suggesting that CPU0213 can inhibit neutrophil infiltration.

**Akt and eNOS phosphorylation**

To explore potential signal transduction pathways activated by CPU0213, we examined the Akt/eNOS pathway since it has been reported that a non-selective ETα/ETβ antagonist increased Akt and eNOS phosphorylation in the hearts of streptozotocin-induced diabetic rats (17). We found a significant increase in the phosphorylation status of Akt in the ischemic myocardium in the I/R + CPU0213 group compared to the I/R + vehicle group (1.24-fold; Figure 6A). The phosphorylation status of Akt was suppressed by I/R, but was partly restored by CPU0123. A similar effect was observed with eNOS phosphorylation (Figure 6B). CPU0213 pretreatment had no effect on total Akt or eNOS in the myocardium of all three groups. Thus, the data suggest a role for the Akt/eNOS pathway in mediating the effects of CPU0213.

**Discussion**

The major finding of the present study was that CPU0213 pretreatment immediately prior to coronary occlusion significantly reduced myocardial infarct size, which is consistent with previous studies using other ET antagonists (7,10,11). To further investigate the effect of CPU0213 on myocardial function, we assessed left ventricular function and structure by echocardiography. Interestingly, administration of CPU0213 improved left ventricular contractile function as indicated by a significant increase in EF and FS.

ET-1 is important for the modulation of cardiac contractility. Both exogenous and endogenous ET-1 have been shown to exert acute positive inotropic effects in vivo, which may be mediated via ETα receptor activation (18,19). Furthermore, the inotropic response to ET-1 was suggested to be biphasic because of an overall positive inotropic effect of ETα receptor stimulation and an ETβ receptor-mediated decrease in contractility at low ET-1 concentrations (20). In contrast, we found that the ETα/ETβ receptor antagonist CPU0213 increased contractile function compared to the I/R + vehicle group. There are several possible reasons for this discrepancy. First, in our model, a large number of myocardial cells showed necrosis and apoptosis because of ischemia, and it is unclear whether ET-1 has positive inotropic effects under pathological conditions. Furthermore, even if ET-1 induces positive inotropy in surviving cardiomyocytes, this may be insufficient to maintain left ventricular function. In the present study, we found that CPU0213 pretreatment decreased infarct size and attenuated cardiomyocyte injury following I/R, resulting in preservation of left ventricular contraction function. These data are consistent with a previous study using a selective ETα antagonist (7). Administration of bosentan, a non-selective ETα/ETβ receptor antagonist, after occlusion of the LAD, caused a significant decline in systolic and diastolic arterial pressure and heart rate during reperfusion (11). Thus, CPU0213 may improve cardiac function by reducing both the afterload and heart rate, which may also lead to decreased myocardial oxygen demand and attenuate myocardial injury during reperfusion.
Although we demonstrated that CPU0213 resulted in an improvement of EF and FS, these improvements were not as marked as the reduction in infarct size and the inflammatory parameters. There are two explanations: myocardial stunning is characterized by viable cardiomyocytes with depressed function after brief episodes of I/R (21) and early reperfusion during acute myocardial infarction results in a mixture of infarcted and stunned myocardium (22). This is confirmed by Figure 2 of our study, where there is evidence of live myocardium in the twilight zone between the infarct area and the non-ischemic area. The tissue salvaged by reperfusion may require days or weeks to recover its contractile function (23). As such, it may take more than 6 h to show clear improvement of EF and FS in the CPU0213-treated group. Furthermore, ET-1 is only one factor in the complex system of cardiac dysfunction after I/R, and there are also numerous alternative influences that can affect the recovery of cardiac function.

Numerous studies have demonstrated that plasma ET-1 levels increase after I/R (5, 6). In the present study, plasma ET-1 levels also significantly increased after 30 min of ischemia and 6 h of reperfusion, while CPU0213 largely blocked this effect. This result is in contrast to previous studies using other ET<sub>v</sub>/ET<sub>B</sub> receptor antagonists (24, 25). The ET<sub>B</sub> Receptor is suggested to be responsible for clearing circulating ET-1, and therefore non-selective ET<sub>v</sub>/ET<sub>B</sub> receptor antagonists block the ET<sub>B</sub> receptors and increase plasma ET levels. By contrast, Feng et al. (15) showed that, in a model of L-thyroxin-induced cardiomyopathy, CPU0213 significantly inhibited elevated expression of ET-1 and dramatically decreased prepro-endothelin-1 (preproET-1) mRNA levels. Furthermore, in a model of septic shock, CPU0213 limited the up-regulation of endothelin converting enzyme (ECE) and preproET-1 mRNA compared to the untreated septic shock group (26). The ET gene encodes preproET-1, which is cleaved into a precursor termed big ET. Big ET is secreted from endothelial cells and is converted to ET-1 by ECE (27, 28). This may be explained by the improved hemodynamics and reduced ET-1 production following CPU0213. Verma et al. (29) also demonstrated that attenuating the release of ET-1 following I/R resulted in enhanced cardiomyocyte tolerance to I/R in vitro. Therefore, we measured total CK and LDH activity in serum and found that both CK and LDH activity increased after I/R, but decreased after pretreatment with CPU0213.

Oxidative stress is generally accepted to play an important role in myocardial I/R (30). In addition, oxidative stress can elevate ET-1 levels, and ET-1 has been demonstrated to increase the production of reactive oxygen species (ROS) (31). Although oxidative stress is an established consequence of ET<sub>B</sub> receptor activation, a similar action of the ET<sub>B</sub> receptor remains controversial (32, 33). To determine the actions of the non-selective endothelin antagonist CPU0213 on oxidative stress, we measured serum levels of MDA, the metabolite of lipid peroxidation (34), which reflects ROS production. Serum MDA levels increased in the I/R + vehicle group, but were markedly decreased in the I/R + CPU0213 group, indicating that CPU0213 can also reduce lipid peroxidation and oxidative stress, consistent with previous data (11).

Neutrophil accumulation and activation in cardiac tissues can damage the myocardium. During the period of reperfusion, neutrophils can rapidly plug microvessels and capillaries, thus contributing to the “no-reflow” phenomenon (35). Plasma ET-1 levels were found to predict angiographic no-reflow after successful primary or rescue PCI in patients with acute myocardial infarction (36). Therefore, we measured the peroxidase enzyme MPO found in neutrophils, reflecting neutrophil infiltration. MPO activity in the ischemic myocardium was decreased by CPU0213, indicating that CPU0213 suppresses myocardial infarction. Gonon et al. (37) reported a similar response with a selective ET<sub>B</sub> receptor antagonist, LU 135252. Thus, the anti-inflammation actions of CPU0213 may contribute to its cardioprotective action.

The relationship between ET-1 and the Akt/eNOS pathway remains unclear. In an endothelial cell system, ET<sub>B</sub> receptor activation led directly to Akt and eNOS activation (38). By contrast, administration of an endothelin receptor antagonist improved cardiac function by normalizing vascular endothelial growth factor (VEGF) levels and increased the phosphorylation status of Akt and eNOS in hearts of streptozotocin-induced diabetic rats (11). We found that myocardial I/R blunted the phosphorylation status of Akt, which was partially restored by CPU0213. These data are inconsistent with a previous study (39), but may be explained by the different durations of I/R. In that study, after 45 min of ischemia followed by 60 min of reperfusion, there were no differences in the phosphorylation status of Akt or eNOS between Sham, vehicle- and BQ123- (a selective ET<sub>A</sub> receptor antagonist) treated groups. By contrast, we showed that following 30-min ischemia and 6-h reperfusion, phospho-Akt and phospho-eNOS were impaired by I/R, but partly restored by CPU0213. Thus, it is possible that the short duration of reperfusion used by Hoshino et al. (39) was insufficient to impair phosphorylation status. In agreement with our data, non-selective ET<sub>v</sub>/ET<sub>B</sub> but not selective ET<sub>A</sub> receptor antagonists improved endothelium-dependent vasodilation in individuals with insulin resistance (40). These data suggest that a non-selective ET<sub>v</sub>/ET<sub>B</sub> receptor antagonist may have additional benefits compared to a selective ET<sub>A</sub> receptor antagonist. However, the exact mechanisms triggering the alternations in the Akt/eNOS pathway remain unclear. Multiple factors can activate the Akt/eNOS pathway, including VEGF, interleukin-6, and protein kinase C (PKC), and it is difficult to determine whether CPU0213 directly or indirectly improved deterioration of the Akt/eNOS pathway by influencing the expression of other upstream proteins. On this basis, future studies are required to determine the relationship between the Akt/eNOS pathway and non-selective endothelin antagonists in I/R.

We found that the non-selective ET<sub>v</sub>/ET<sub>B</sub> receptor antagonist CPU0213 significantly attenuated myocardial I/R injury
when administered immediately prior to coronary occlusion. CPU0213 treatment was associated with a decrease in ET-1 levels, oxidative stress, and myocardial inflammation. Moreover, CPU0213 improved the deterioration observed in the Akt/eNOS pathway following myocardial I/R injury. Thus, CPU0213 may represent a promising strategy for the treatment of acute myocardial reperfusion.

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References


Figure 1. Chemical structure of CPU0213 (1-butyl-3-(2,4-dis-(para-chlorobenzyloxy)-phenyl)-pyrazole-5-carboxylic acid).

Figure 2. Gross pathology of ischemic hearts. A, Representative mid-myocardial cross-sections of triphenyltetrazolium chloride (TTC)/Evans blue-stained hearts (blue-stained area: non-ischemic tissue; the remaining area: risk area; red-stained area: ischemic area; white area: infarct area. B, RA/LV and IA/RA ratios. RA = risk area; IA = infarct area; LV = left ventricle. Data are reported as means ± SEM for 5 rats/group. *P < 0.05 vs I/R + vehicle (unpaired Student t-test).
Figure 3. Cardiac function examined by echocardiography after ischemia-reperfusion. Representative M-mode echocardiographs of Sham (A), I/R + vehicle (B), and I/R + CPU0213 groups (C). D, CPU0213 pretreatment increased ejection fraction (EF) and fractional shortening (FS) compared to the I/R + vehicle group. I/R = ischemia/reperfusion. Data are reported as means ± SEM for 7 rats/group.

Figure 4. Endothelin-1 (ET-1) levels in plasma, and creatine kinase (CK) and lactate dehydrogenase (LDH) in serum after ischemia-reperfusion. A, ET-1 levels in plasma. B, Total CK activity in serum. C, Total LDH activity in serum. Data are reported as means ± SEM for 6 rats per group.

**Figure 5.** Malondialdehyde (MDA) levels in serum and myeloperoxidase (MPO) activity in myocardial tissues after ischemia-reperfusion. **A,** MDA levels in serum. **B,** MPO activity in myocardial tissues. Data are reported as means ± SEM for 6 rats/group. #P < 0.01 vs Sham, *P < 0.05 vs I/R + vehicle (one-way ANOVA followed by the Bonferroni post hoc test).

**Figure 6.** Representative Western blots showing (A) phospho-Akt (protein kinase B; Ser473) and total Akt and (B) phospho-eNOS (endothelial nitric oxide synthase; Ser1177) and total eNOS in ischemic myocardial tissues after ischemia-reperfusion. Quantitative densitometry data are reported as means ± SEM. N = 4. #P < 0.05 vs Sham; *P < 0.05 vs I/R + vehicle (one-way ANOVA with the Bonferroni post hoc test).