Insulin attenuates myocardial ischemia/reperfusion injury via reducing oxidative/nitrative stress

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Ji L, Fu F, Zhang L, Liu W, Cai X, Zhang L, Zheng Q, Zhang H, Gao F. Insulin attenuates myocardial ischemia/reperfusion injury via reducing oxidative/nitrative stress. Am J Physiol Endocrinol Metab 298: E871–E880, 2010. First published February 2, 2010; doi:10.1152/ajpendo.00623.2009.—It is well known that insulin possesses a cardioprotective effect and that insulin resistance is closely related to cardiovascular diseases. Peroxynitrite (ONOO–) formation may trigger oxidative/nitrative stress and represent a major cytotoxic effect in heart diseases. This study was designed to investigate whether insulin attenuates ONOO– generation and oxidative/nitrative stress in acute myocardial ischemia/reperfusion (MI/R). Adult male rats were subjected to 30 min of myocardial ischemia and 3 h of reperfusion. Rats randomly received vehicle, insulin, or insulin plus wortmannin. Arterial blood pressure and left ventricular pressure were monitored throughout the experiment. Insulin significantly improved cardiac functions and reduced myocardial infarction, apoptotic cell death, and blood creatine kinase/lactate dehydrogenase levels following MI/R. Myocardial ONOO– formation was significantly attenuated after insulin treatment. Moreover, insulin resulted in a significant increase in Akt and endothelial nitric oxide (NO) synthase (eNOS) phosphorylation, NO production, and antioxidant capacity in ischemic myocardium. Inhibition of insulin signaling with wortmannin not only blocked the cardioprotection of insulin but also markedly attenuated insulin-induced antioxidative/antinitrative effect. Furthermore, the suppression on ONOO– formation by either insulin or an ONOO– scavenger uric acid reduced myocardial infarct size in rats subjected to MI/R. We concluded that insulin exerts a cardioprotective effect against MI/R injury by blocking ONOO– formation. Increased physiological NO production (via eNOS phosphorylation) and superoxide anion reduction contribute to the antioxidative/antinitrative effect of insulin, which can be reversed by inhibiting phosphatidylinositol 3′-kinase. These results provide important novel information on the mechanisms of cardiovascular actions of insulin.

Experimental evidence has revealed that insulin resistance plays a key role in the pathogenesis of cardiovascular diseases, including endothelial dysfunction and myocardial infarction (16, 30). In recent years, there have been several landmark studies showing that glucose, insulin, and potassium significantly reduced the mortality of patients with acute myocardial infarction (5, 8, 27). Our previous studies (10, 25, 43) demonstrated that insulin may play an important role to attenuate both myocardial ischemia and reperfusion injury through the survival signaling, that is, phosphatidylinositol 3′-kinase (PI3-kinase)-Akt-dependent pathway. However, to date, the mechanisms underlying the cardiovascular actions of insulin remain largely unclear.

Reperfusion of the ischemic myocardium must be performed to prevent cellular apoptosis and necrosis. Unfortunately, abundant evidence suggests that reperfusion itself may lead to accelerated and additional myocardial injury beyond that generated by ischemia alone. The pathogenesis of reperfusion-induced myocardial injury is apparently multifactorial. Of the many theories regarding the development of reperfusion injury, the enhanced generation of highly reactive oxygen species (ROS) by the heart during the acute reperfusion phase, including superoxide anion (O2–), hydrogen peroxide (H2O2), and hydroxyl radical (OH), is an appealing one that is supported by a large foundation of experimental evidence (28, 33, 46). In the ischemic myocardium, the burst of ROS generation can overwhelm the intrinsic antioxidants and cause oxidative injury to various cellular structures, which facilitates the unabated effect of ROS. Administration of a free radical scavenger, such as superoxide dismutase (SOD), prevents O2– burst (4, 39). Recent study indicates that enzymatic antioxidant catalase overexpression may protect cardiac myocytes partly through Akt signaling (45). More importantly, clinical evidence has demonstrated that insulin potently suppress ROS generation in obese subjects (3). However, it is not known whether insulin may decrease myocardial ischemia/reperfusion (MI/R)-induced ROS generation.

Nitric oxide (NO), a simple molecule produced primarily by endothelial cells in the cardiovascular system, plays an important regulatory role in apoptotic cell death (17). High pharmacological concentrations of NO produced from inducible NO synthase (iNOS) induce apoptosis, whereas low concentrations of NO produced from endothelial NO synthase (eNOS) or pharmacological concentrations of exogenous NO released by NO donors reduce apoptosis (6, 32). In 1990, Beckman et al. (1) demonstrated that NO combines with O2– at a very fast rate (κ = 6.7 × 109 mol–1·s–1) to form peroxynitrite (ONOO–). Over the last two decades, accumulating studies suggest that the harmful effects of NO in the heart and in the vasculature are attributable to the formation of ONOO–, a strong oxidative/nitrative molecule, rather than the possible toxic effect of NO itself (19, 24, 26). ONOO– formation represents a major mechanism of myocardial injury, including substantial oxidation and potential destruction of host cellular constituents, leading to the dysfunction of critical cellular processes, disruption of cell signaling pathways, and the induction of cell death through both apoptosis and necrosis (35). The generation of ONOO– may be decreased by removing the substrates for its synthesis, NO and O2–. In addition, it has been reported that exogenously supplied NO, or that provided by NO donors, can...
antagonize the cytotoxic actions of ONOO⁻ (7). On the other hand, insulin has been previously demonstrated to enhance eNOS activity (20) and to downregulate iNOS-NO-ONOO⁻ pathway (18). However, to date, whether insulin protects hearts against MI/R injury via regulating eNOS/iNOS-NO-ONOO⁻ pathway remains unclear.

Therefore, the aims of the present study were 1) to determine whether insulin might decrease oxidative/nitrative stress in MI/R rats and, if so, 2) to investigate the mechanisms involved.

MATERIALS AND METHODS

The experiments were performed in adherence with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care.

Experimental protocol. Adult male Sprague-Dawley rats were fasted overnight and anesthetized through intraperitoneal administration of 60 mg/kg pentobarbital sodium. The right femoral vein was cannulated for the drug infusion. Myocardial ischemia was produced by exteriorizing the heart through a left thoracic incision by placing a 6–0 silk suture and making a slipknot around the left anterior descending coronary artery about 2–3 mm from its origin. Ischemia and reperfusion were monitored and confirmed by electrocardiogram (ECG) observation. After 30 min of ischemia, the slipknot was released, and the myocardium was reperfused for 3 h. Rats were randomized to receive one of the following treatments: 1) vehicle (saline); 2) insulin (60 U/l, intravenous infusion at 4 ml/kg per h for 3 h, beginning 5 min before reperfusion); or 3) insulin plus wortmannin (15 μg/kg intravenous injection 15 min before reperfusion).

Sham-operated control rats (sham MI) underwent the same surgical procedures with the exception of left anterior descending coronary artery occlusion. The dose of insulin or wortmannin has been demonstrated in our previous study (10). Our preliminary experiments have shown that the presence of wortmannin at this concentration (15 μg/kg) did not significantly alter myocardial functional parameter or cause detectable secondary effects during the time course of MI/R. At least 2 rats in each group were used for all the determinations at the end of the reperfusion.

Cardiac functional assessment. MI/R-induced cardiac dysfunction was continuously monitored before and during the entire MI/R period. A microcatheter was inserted into the left ventricle through the right carotid artery to measure the left ventricular pressure (LVP). The arterial pressure was measured by right femoral artery intubation. ECG and LVP were simultaneously recorded on a polygraph (RM-6200C; Chengdu, Instrument, Chengdu, China). Heart rate (HR), mean arterial blood pressure (MABP), left ventricular systolic pressure, left ventricular end diastolic pressure (LVEDP), and the instantaneous first derivation of LVP (+LVdP/dt_max and −LVdP/dt_max) were derived by computer algorithms.

Determination of myocardial infarct size. The myocardial infarct size (INF) was determined by a double-staining technique and was analyzed by a digital imaging system described previously (11). At the end of the reperfusion, the ligature around the coronary artery was retied and 4 ml of 2% Evans blue dye (Shanghai Chemical Reagents, Shanghai, China) was injected into the left ventricular cavity. The dye was circulated and uniformly distributed except in the region of the heart previously perfused by the occluded coronary artery (ischemic region or area at risk, AAR). The heart was quickly excised, and the atria, right ventricle, and fatty tissues were removed from the heart, frozen at −20°C, and sliced into 1-mm-thick sections perpendicular to the long axis of the heart using a heart slice chamber. Slices were incubated individually using a 12-well culture plate in 1% triphenyltetrazolium chloride (TTC, Shanghai Chemical Reagents) in phosphate buffer at pH 7.4 at 37°C for 10 min, and photographed with a digital camera. Evans blue-stained areas (area not at risk), TTC stained areas (red staining, ischemic but viable tissue), and TTC stained negative areas (infarcted myocardium) in each slice were determined by planimetry on a computer with Sigma Scan software. The myocardial infarct size was expressed as a percentage of infarcted area (INF) over total AAR (INF/AAR × 100%).

Determination of plasma creatine kinase and lactate dehydrogenase levels. Blood samples (1 ml) were drawn at the end of 3 h of reperfusion. Plasma creatine kinase (CK) and lactate dehydrogenase (LDH) levels were measured spectrophotometrically (DU 640; Beckman Coulter, Brea, CA) in a blinded manner. All measurements were assayed in duplicates.

Terminal deoxynucleotidyl nick-end labeling assay. Myocardial apoptotic index was analyzed by terminal deoxynucleotidyl nick-end labeling (TUNEL) assay as described previously (34). A double-staining technique was used, i.e., TUNEL staining for apoptotic cell nuclei and DAPI staining for all myocardial cell nuclei. TUNEL staining was performed by using an In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the protocol provided by the manufacturer. In brief, cardiomyocytes from at least four slides per block that were randomly selected were evaluated immunohistochemically to determine the number and percentage of cells exhibiting positive staining for apoptosis. For each slide, 10 fields were randomly chosen, and a total of 100 cells per field were counted by using a defined rectangular field area (×20 objective). The index of apoptosis was determined [(no. of apoptotic myocytes/total no. of myocytes counted) × 100%] from a total of 40 fields per heart, and the assays were performed in a blinded manner.

Determination of myocardial caspase-3 activity. Cardiac caspase-3 activity was performed by using a caspase-3 colorimetric assay kit (Chemicon, Temecula, CA) following the manufacturer’s instructions. In brief, myocardial tissue was homogenized in ice-cold lysis buffer for 30 s. The homogenates were centrifuged, supernatants were collected, and protein concentrations were measured by bicinchoninic acid method. To each well of a 96-well plate, supernatant containing 200 μg of protein was loaded and incubated with 25 μg caspase-3 substrate N-acetyl-Asp-Glu-Val-Asp(DEV)-d-p-nitroanilide at 37°C for 1.5 h. The optical density was measured at 405 nm with a SpectraMax-Plus microplate spectrophotometer. The activity of caspase-3 in tissue samples was calculated using a standard curve and expressed as fold increase over the mean value of sham MI.

Determination of Akt, eNOS, iNOS, and gp91phox expression by Western blot. Ischemic myocardium tissue samples were lysed with lysis buffer. After sonication, the lysates were centrifuged; proteins were separated by electrophoresis on SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking with 5% skim milk in Tris-buffered saline at room temperature for 1 h, we incubated the membrane with an antibody against Akt, phosphorylated Akt, eNOS, phosphorylated eNOS (Cell Signaling Technology, Danvers, MA), iNOS, or gp91phox (BD Bioscience Laboratories, San Jose, CA) overnight at 4°C. The membrane was then washed with PBST and incubated with horseradish peroxidase-conjugated IgG antibody (Cell Signaling Technology, Rockford, IL). The immunoblotting was visualized with ChemiDoc XRS (Bio-Rad Laboratory, Hercules, CA), and the blot densities were analyzed with LabImage software.

Measurement of NOx content in cardiac tissue. Tissue samples from AAR were rinsed, homogenized in 0.9% NaCl solution (1:10, wt/vol), and centrifuged at 3,000 g for 5 min. The pellet was discarded. NOx concentrations in the supernatant were quantified by a NO detection kit (nirate reductase).

Determination of tissue antioxidant capacity. Tissue samples from AAR were rinsed, homogenized in 0.9% NaCl solution (1:10, wt/vol), and centrifuged at 3,000 g for 5 min. The pellet was discarded. Total
antioxidant capacity was determined with a spectrophotometric assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer’s instruction. In brief, 30 μl of supernatant were added to the reaction buffer containing xanthine, xanthine oxidase, and hydroxylamine. After 40 min of incubation at 37°C, accumulation of nitrite was quantified by the Griess reaction. Tissue antioxidant capacity is inversely related to the concentration of nitrate. Results were normalized against the mean value of control and expressed as fold changes.

Quantification of tissue nitrotyrosine content. Nitrotyrosine content in the I/R cardiac tissue, a footprint of in vivo ONOO− formation and an index of nitrative stress, was determined using a nitrotyrosine ELISA kit (Cell Sciences, Canton, MA) as described in our previous study (21).

Statistical analysis. All values are presented as means ± SE. Differences were compared by ANOVA followed by Bonferroni correction for post hoc t-test, where appropriate. Probabilities of <0.05 were considered to be statistically significant. All of the statistical tests were performed with the GraphPad Prism software, version 5.0 (GraphPad Software, San Diego, CA).

RESULTS

Insulin improved cardiac functional recovery after reperfusion. No significant differences in systemic hemodynamics were observed among all groups under baseline conditions. There were no significant differences in HR and MABP among all groups during ischemia or reperfusion period. As shown in Fig. 1, treatment with insulin markedly increased +LVdP/dtmax and −LVdP/dt_max after 3 h of reperfusion (P < 0.01). In addition, LVEDP was significantly elevated in rats subjected to MI/R receiving vehicle only, whereas rats treated with insulin markedly improved functional recovery following MI/R. Pretreatment with wortmannin significantly blunted these beneficial effects of insulin (P < 0.01), whereas wortmannin itself had no significant effect on cardiac function in rats subjected to MI/R (34). These results indicated that treatment with insulin improved cardiac systolic and diastolic function in rats subjected to MI/R.

Insulin decreased myocardial injury in MI/R rats. To examine whether insulin might reduce myocardial injury, myocardial INF and plasma, CK and LDH levels were measured. As shown in Fig. 2A, no myocardial infarction was observed in the sham-operated hearts. Thirty minutes of ischemia followed by 3 h of reperfusion resulted in significant myocardial infarction in the vehicle-treated rats, as evidenced by a large area of TTC-negatively-stained myocardium. Treatment with insulin significantly reduced the myocardial INF (26.5% ± 1.6% vs. 40.8% ± 1.9% in vehicle-treated group, P < 0.01). In addition, administration of wortmannin together with insulin completely abolished the protective effect of insulin (38.0% ± 1.1% vs. insulin group, P < 0.01).

Cardiomyocyte necrosis is characterized by cell membrane disruption and cell content release. To investigate whether insulin could decrease cardiomyocyte necrosis, plasma CK and LDH levels were measured at 3 h after reperfusion. As seen in Fig. 2, B and C, at 3 h after reperfusion, CK and LDH levels

Fig. 1. Effects of insulin treatment on myocardial functions in rats subjected to 30 min of myocardial ischemia and 3 h of reperfusion (n = 6/group). Values are presented means ± SE. LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; +LVdP/dt_max, the instantaneous first derivation of left ventricle pressure; MI, myocardial ischemia/reperfusion (30 min/3 h); Sham MI, sham-operated; I, insulin; W, wortmannin. **P < 0.01 vs. Sham MI, ##P < 0.01 vs. MI, δP < 0.05, δδP < 0.01 vs. MI + I.
were increased to 5,136 ± 758 and 3,924 ± 437 U/l in MI/R rats. Insulin markedly decreased the reperfusion-associated CK and LDH elevations compared with those in the MI/R rats (2,953 ± 467 U/l and 1,947 ± 362 U/l, respectively, both P < 0.01). More importantly, pretreatment with wortmannin before reperfusion partly blunted insulin-induced decrease of plasma CK and LDH levels (both P < 0.05 vs. insulin group). All these results provided direct evidence that insulin significantly attenuates myocardial injury, and wortmannin, a selective PI3-kinase inhibitor, blocks the protective effect of insulin in rats subjected to MI/R.

**Insulin inhibited myocardial apoptosis.** Apoptosis is the major form of cell death after a short period of ischemia followed by reperfusion. To investigate whether insulin could decrease myocardial apoptosis, TUNEL staining and caspase-3 activity in I/R cardiac tissue were determined. In myocardial tissue from the sham-operated rats, a very low level of TUNEL-positive staining (AI: 1.6% ± 1.2%) was detected (Fig. 3A). In contrast, a significant number of TUNEL-positive cells (AI: 21.6% ± 1.5%) were observed in myocardial tissue from hearts subjected to MI/R and receiving vehicle. Treatment of insulin shortly before reperfusion exerted a significant antiapoptotic effect, as evidenced by reduced TUNEL-positive staining (AI: 11.0% ± 1.5%, P < 0.01 vs. vehicle). Moreover, administration of wortmannin significantly blocked the insulin-induced decrease of apoptosis (AI: 19.0% ± 1.5%, P < 0.05 vs. insulin), whereas wortmannin itself at this dosage had no effects on myocardial apoptosis in MI/R rats (10, 44).

Caspase-3 is a pivotal mediator of apoptosis, and myocardial caspase-3 activity is regarded as a marker of MI/R myocyte apoptosis. As shown in Fig. 3B, analysis of the caspase-3 activity revealed that MI/R also induced a significant rise in caspase-3 activity. Treatment with insulin substantially reduced this MI/R-induced increase in myocardial caspase-3 activity (1.8 ± 0.3 vs. 2.9 ± 0.2, P < 0.01). Wortmannin completely abolished caspase-3 inhibitory effect of insulin (2.6 ± 0.3, P < 0.01). These data, together with the TUNEL results, indicated that insulin inhibited myocardial apoptosis following MI/R in vivo.

**Insulin treatment attenuated ONOO- overproduction in I/R cardiac tissue.** Considerable evidences demonstrated that overproduction of ONOO- and resultant oxidative/nitrative stress play a causative role in posts ischemic myocardial apoptosis (14, 19, 24, 26, 36). Therefore, we examined cardiac ONOO- production. ONOO-, the biradical reaction product of O2- and NO, was determined by measurement of nitrotyrosine, a “footprint” of in vivo ONOO- production. As shown in Fig. 4, nitrotyrosine content was significantly increased in MI/R rats. Treatment with insulin significantly reduced nitrotyrosine content (P < 0.05 vs. vehicle group). Importantly, the suppressive effect of insulin on MI/R-induced ONOO- production was significantly blunted by PI3-kinase inhibitor wortmannin (P < 0.05 vs. insulin group). These data suggested that insulin exerted an antioxidative/antinitrative stress in MI/R.

**Insulin treatment resulted in eNOS phosphorylation, increased NOx content, and inhibited iNOS expression in I/R cardiac tissue.** Experimental evidences (7) demonstrating that exogenously supplied NO antagonizes the cytotoxic actions of ONOO- suggest that increased NO production may attenuate the deleterious effect of ONOO- and reduce oxidative/nitrative stress. However, as a primary substrate for ONOO- synthesis, increased NO concentration is responsible for ONOO- overproduction. Therefore, we measured NO production, eNOS/iNOS protein expression, and eNOS phosphorylation at the end of 3-h reperfusion. Consistent with previously published results (10), there was no significant difference in eNOS expression among different groups; treatment with insulin resulted in significant eNOS phosphorylation (Fig. 5B) and marked in-
crease in NOx content (Fig. 5C); pretreatment with wortmannin completely blocked eNOS phosphorylation and NOx increase induced by insulin. Interestingly, in contrast to eNOS phosphorylation, the iNOS expression was increased in MI/R rats. Treatment with insulin blocked iNOS expression in these animals, which was completely blunted by pretreatment with wortmannin (Fig. 5D).

Insulin treatment significantly reduced superoxide overproduction in I/R cardiac tissue. Earlier work showed that inhibition of either NO or ROS attenuated reperfusion injury and decreased tissue nitrotyrosine formation, a marker of ONOO⁻ (36). The major source of O₂⁻ production in the heart is NADPH oxidase (29). Because genes that encode NADPH oxidase and iNOS belong to the same inflammatory gene family, the aforementioned results (insulin inhibits iNOS expression) suggest that insulin may inhibit NADPH oxidase expression and subsequent O₂⁻ production. To test this hypothesis, gp91phox protein expression in the I/R cardiac tissue was determined. As summarized in Fig. 6A, I/R-induced overexpression of gp91phox, a major component of NADPH oxidase, was completely blocked by insulin treatment. Pretreatment with wortmannin significantly blunted this beneficial effect. In addition, antioxidant capacity was determined by the activity of SOD (O₂⁻ scavenger) in cardiac tissue. MI/R-induced reduction in total antioxidant capacity was significantly preserved after insulin treatment, which was also completely blunted by pretreatment with wortmannin (Fig. 6B). These data suggested that insulin treatment reduced O₂⁻ overproduction in MI/R rats.

Insulin treatment resulted in Akt phosphorylation. To further investigate the mechanisms underlying the insulin-induced antioxidant/antinitrative effects, we measured the Akt expression and phosphorylation by Western blotting in myocardium in rats at the end of 3-h reperfusion (Fig. 7). There was no significant difference in Akt expression among different groups, whereas treatment with insulin significantly increased the phosphorylation of Akt (P < 0.01). Pretreatment with wortmannin completely blocked Akt phosphorylation induced by insulin. The results suggested that PI 3-kinas-Akt signaling pathway contributed to the beneficial effects of insulin in MI/R.

Inhibition of ONOO⁻ formation preferentially attenuated cardiac injury in MI/R rats. Our results suggest that insulin may exert its cardioprotective effects by inhibition of the overproduction of ONOO⁻. To obtain more evidence to sup-

![Fig. 3. Myocardial apoptosis in rats subjected to MI/R with different treatments (n = 6/group). Values presented are means ± SE. A, top: representative photomicrographs of in situ detection of apoptotic myocytes by terminal deoxynucleotidyl nick-end labeling (TUNEL) staining in ischemic heart tissue from rats subjected to 30 min of ischemia and 3 h of reperfusion. Green fluorescence shows TUNEL-positive nuclei; blue fluorescence shows nuclei of total cardiomyocytes. Bottom: percentage of TUNEL-positive nuclei in heart tissue sections. B: myocardial caspase-3 activity. **P < 0.01 vs. Sham MI, ###P < 0.01 vs. MI, δP < 0.05, δδP < 0.01 vs. MI + I.](http://ajpendo.physiology.org/)

![Fig. 4. Nitrotyrosine production in rat hearts subjected to MI/R with different treatments (n = 6/group). Values presented are means ± SE. **P < 0.01 vs. Sham MI, #P < 0.05 vs. MI, δP < 0.05 vs. MI + I.](http://ajpendo.physiology.org/)
port this conclusion, an additional series of experiments was performed. Male adult rats were subjected to MI/R as described above and treated with either uric acid (5 mg/kg intravenous injection 10 min before ischemia), an ONOO\(^-\)/H\(_2\)O\(_2\) scavenger, or uric acid plus insulin. As summarized in Fig. 8A, treatment with uric acid significantly reduced MI/R-induced ONOO\(^-\)/H\(_2\)O\(_2\) production (\(P < 0.01\)). Pretreatment with insulin had no additional effect (\(P > 0.05\) vs. uric acid group). Uric acid treatment also markedly attenuated myocardial injury as determined by myocardial INF (Fig. 8B). Pretreatment with insulin afforded no additional cardioprotection. These results demonstrated that scavenging ONOO\(^-\) preferentially protected hearts against MI/R injury and that decreased production of ONOO\(^-\) plays a critical role in the cardioprotection of insulin.

**DISCUSSION**

Several important observations were made in our present experiments. First, we have observed for the first time that insulin attenuates oxidative/nitrative stress and inhibits MI/R-induced ONOO\(^-\) production (\(P < 0.01\)). Pretreatment with insulin had no additional effect (\(P > 0.05\) vs. uric acid group). Uric acid treatment also markedly attenuated myocardial injury as determined by myocardial INF (Fig. 8B). Pretreatment with insulin afforded no additional cardioprotection. These results demonstrated that scavenging ONOO\(^-\) preferentially protected hearts against MI/R injury and that decreased production of ONOO\(^-\) plays a critical role in the cardioprotection of insulin.

Under physiological conditions, there is a critical balance between cellular concentrations of NO, O\(_2\)\(^-\), and SOD, whereas pathological conditions such as ischemia and reperfusion result in ONOO\(^-\) formation. ONOO\(^-\) is a strong biological oxidant and nitrating species formed from the near-diffusion-limited reaction of the free radicals NO and O\(_2\)\(^-\). The myocardial cytotoxicity of ONOO\(^-\) involves direct oxidative damage to lipids, proteins, and DNA (31), the activation of metalloproteinases (37), and the nitration of tyrosine residues within proteins (2, 31). Rapid generation of ONOO\(^-\) during reperfusion of the ischemic heart has been detected using luminal chemiluminescence (36). It is well demonstrated that generation of ONOO\(^-\) plays a critical role in MI/R injury, and myocardial ONOO\(^-\) formation and oxidative/nitrative stress also have been shown to occur at 3 h after reperfusion (15, 41). The drugs that inhibit ONOO\(^-\) formation or antagonize its toxicity protect the heart from reperfusion injury (15). These results provide direct evidence that ONOO\(^-\) can be formed in I/R myocardium and contributes significantly to the postischemic myo-
cardiac injury. In the present study, insulin treatment significantly decreased cardiac NOOO⁻ overproduction and thus attenuated MI/R injury as evidenced by improved cardiac functions and reduced INF, cell necrosis (decreased plasma CK and LDH), and apoptosis (decreased TUNEL-positive staining and attenuated caspase-3 activity), which is the major form of early myocardial damage produced by MI/R although this death form had not peaked at the end of 3-h reperfusion. These data have provided the first evidence that the cardioprotection of insulin after MI/R involves a novel mechanism of antioxidative/antinitrative stress by decreasing NOOO⁻ overproduction. Moreover, we have previously demonstrated that the survival signaling, i.e., PI 3-kinase-Akt-dependent pathway, plays a critical role in antiapoptotic action of insulin, which is related to the cardioprotective effect of insulin in alleviating chronic adverse changes in posts ischemic cardiac structure and function (10, 40). Thus, in our present study, wortmannin, a PI 3-kinase inhibitor that did not cause detectable secondary effects during the time course of MI/R in vivo (10, 13, 34, 44), was administered to block the cardioprotective effects of insulin. Interestingly, pretreatment with wortmannin significantly reversed insulin-induced NOOO⁻ reduction. This result convincingly favors the notion that insulin may exert antioxidative/antinitrative stress in MI/R.

ROS have long been recognized to cause oxidative stress and act as the major mediators of I/R injury. Experimental studies have demonstrated that ROS released during the early phase of myocardial reperfusion strongly oxidize cardiomyocytes that have already been damaged by the ischemia (28, 33, 46). TNF-α is well known as an inflammatory cytokine in the pathogenesis of acute myocardial infarction, and it affects intracellular insulin signaling in fat, skeletal muscle, and other insulin-sensitive tissues by inhibiting kinase activity in the proximal part of the insulin signaling pathway (42). Recent study has indicated that anti-TNF-α potently blocked TNF-α-induced activation of NADPH oxidase (the most important source for O₂⁻ production) and resultant formation of nitrotyrosine in isolated coronary arterioles following I/R (12). All these suggest that inhibiting O₂⁻ generation may reduce NOOO⁻ overproduction and resultant oxidative/nitrative stress. In the present study, we have demonstrated that treatment with insulin significantly reduced gp91phox (a critical component of NADPH oxidase) expression in MI/R rats, which was blocked by wortmannin. On the other hand, SOD, O₂⁻ scavenger, has been well...
demonstrated to reduce superoxide anion-dependent damages by transforming $\text{O}_2^-/\text{H}_2\text{O}_2$ into $\text{H}_2\text{O}_2$. We have demonstrated that treatment with insulin significantly improved total antioxidant capacity (SOD activity) in I/R cardiac tissue, which was also reversed by wortmannin. On the basis of these observations, it is suggested that the reduction of $\text{O}_2^-/\text{H}_2\text{O}_2$ generation should be responsible for suppression of ONOO$^-$/H$^2\text{O}_2^-$ afforded by insulin in MI/R rats.

The most interesting finding of the present study is that insulin differentially regulates eNOS and iNOS activity in MI/R rats. It has been well demonstrated that eNOS phosphorylation and subsequent NO production are of great importance in antiapoptotic signaling pathway by insulin (10). Studies indicate that eNOS provides a constant, dynamic source of NO, whereas iNOS produces larger amounts of NO. Immunohistochemical evidence has been reported that myocardial iNOS expression and protein nitration increased in regional myocardial I/R rats in vivo (23). Nitrative stress originated from the reaction between ROS, and NO released from iNOS in a diffusion-limited rate has been shown to play a significant role in myocardial injury. Inhibiting iNOS expression or scavenging ONOO$^-$ may reduce nitrative stress and attenuate MI/R injury (38). Evidence indicated that, after 30-min ischemia, iNOS expression was undetectable at 1 h of reperfusion; however, it was markedly upregulated at 3 h of reperfusion; moreover, 30 min of ischemia followed by 1 or 3 h of reperfusion had no significant effect on eNOS expression (22). In our present study, at the end of 3-h reperfusion (when iNOS is expressed in I/R myocardial tissue) I/R-stimulated iNOS expression was inhibited after insulin treatment; in contrast, insulin treatment resulted in increased eNOS phosphorylation with a subsequent increase in NO production. Interestingly, inhibition of insulin-stimulated NO production and iNOS reduction by wortmannin abolished the insulin-induced ONOO$^-$ reduction. These data suggest that insulin increases NO production from eNOS to exert the physiological effect of NO and thus contributes to the cardioprotection of insulin. On the other hand, under pathological conditions where iNOS expression is stimulated, insulin inhibits iNOS expression and thus protects tissues from nitrative stress.

NO itself is not toxic and does not produce significant tissue injury even at a very high concentration (17). However, the presence of $\text{O}_2^-$/H$^2\text{O}_2^-$ may divert NO to a toxic pathway by leading to the formation of ONOO$^-$, which causes oxidative/nitrative stress and tissue injury (9). In our present study, we have demonstrated that insulin treatment markedly reduced I/R-induced ONOO$^-$ formation by inhibition of NADPH oxidase-derived $\text{O}_2^-$ and iNOS-derived NO. In addition, scavenging ONOO$^-$ preferentially also protected hearts against MI/R injury, whereas pretreatment with insulin had no additional effect, which further supported that insulin afforded cardioprotection via reducing ONOO$^-$-caused oxidative/nitrative stress. Physiological or pharmacological concentrations of NO produced from eNOS or NO donors exert significant cardioprotective effects and antagonize the cytotoxic actions of ONOO$^-$. Theoretically, a signaling system that possesses dual actions (i.e., stimulation of NO production and inhibition of $\text{O}_2^-$ reproduction) would provide the most protection against I/R injury. To our knowledge, insulin is the one that provides this dual-protective effect and attenuates MI/R injury.

In summary, the data in the present study in MI/R rats have demonstrated that insulin attenuates oxidative/nitrative stress by blocking ONOO$^-$ formation. Increased physiological NO production (via eNOS phosphorylation) and $\text{O}_2^-$ reduction contribute to the antioxidative/antinitrative effect of insulin.

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DISCLOSURES
No conflicts of interest are declared by the authors.

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