Vasculoprotective effect of insulin in the ischemic/reperfused canine heart: Role of Akt-stimulated NO production

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Abstract

Objectives: The objectives of this study were to investigate the vasculoprotective effects of glucose–insulin–potassium (GIK) on ischemia/reperfusion-induced coronary endothelial functional injury and to elucidate the mechanism involved.

Methods: Dogs were subjected to 50 min of coronary occlusion and 4 h of reperfusion. Vehicle, GIK, or GK were intravenously infused 5 min before reperfusion, and the coronary vascular dysfunction and endothelial apoptosis were determined. In a separate study, cultured endothelial cells were subjected to simulated ischemia/reperfusion, and the signaling pathway involved in insulin’s anti-apoptotic effect was investigated.

Results: In vivo ischemia/reperfusion caused significant coronary vascular endothelial dysfunction as evidenced by reduced endothelium-dependent vasorelaxation, decreased nitric oxide (NO) production, and endothelial cell apoptosis as determined by caspase 3 activation and TUNEL staining. Treatment with GIK, but not GK, markedly improved the endothelium-dependent coronary vasorelaxation (P<0.01 versus vehicle), increased total NO production (P<0.01), and attenuated endothelial apoptosis. In cultured endothelial cells, treatment with insulin also markedly increased NO production and reduced simulated ischemia/reperfusion-induced apoptosis. Moreover, pre-treatment with either Akt inhibitor or NO synthase inhibitor almost abolished the anti-apoptotic effect exerted by insulin but not by SNAP, an NO donor.

Conclusion: These results demonstrate that in vivo treatment with GIK at reperfusion attenuates ischemia/reperfusion-induced coronary endothelial dysfunction and endothelial apoptosis in an Akt-dependent and NO-mediated fashion. The coronary vasculoprotective effect elicited by insulin may contribute to the previously observed cardiac protective effect of GIK.

Keywords: Insulin; Endothelial dysfunction; Reperfusion injury; Apoptosis; Nitric oxide

1. Introduction

Endothelial dysfunction manifested as decreased bioactive nitric oxide (NO) levels is one of the most common pathological changes occurring in cardiovascular diseases such as ischemia/reperfusion, heart failure, and atherosclerosis [1,2]. Endothelial dysfunction contributes significantly to subsequent functional and cellular injury through a variety of pathological pathways. Disturbed balance between vasorelaxation and vasoconstriction may contribute to the “no reflow phenomenon” seen after ischemia and reperfusion. Endothelial dysfunction may also exacerbate tissue injury indirectly by increasing platelet–leukocyte–endothelium interactions [3]. Therapeutic strategies aimed at improving endothelial function have been shown to markedly retard the development of atherosclerosis and attenuate vascular and tissue injury associated with ische-
mia/reperfusion [4]. Therefore, coronary endothelial protection is an early and effective therapeutic approach that may reduce subsequent cardiomyocyte injury.

Considerable evidence exists that apoptosis plays a critical role in myocardial reperfusion injury [5]. In a recent study, Scarabelli et al. [6] reported that in isolated perfused heart subjected to ischemia and reperfusion, endothelial apoptosis occurs at an earlier time point than that of cardiomyocytes. More interestingly, the number of apoptotic cardiomyocytes progressively decreases with distance from the coronary vessels subjected to 30 min of ischemia and 60 min of reperfusion. These results suggest that soluble pro-apoptotic mediators released from apoptotic endothelial cells early after reperfusion may be a trigger for subsequent cardiomyocyte apoptosis. Therefore, treatment that reduces coronary endothelial apoptosis may be a novel approach that can reduce ultimate cardiomyocyte cell death after ischemia and reperfusion [7].

Metabolic modulation with glucose–insulin–potassium (GIK) in acute myocardial infarction (AMI) has a long and controversial history [8]. Recently, it was demonstrated that, compared with patients receiving reperfusion therapy and placebo, those receiving GIK treatment at reperfusion had a remarkable 66% reduction in their relative in-hospital mortality risk in patients with AMI. In contrast, in those receiving no reperfusion therapy, no significant difference was observed between patients receiving placebo or GIK. This landmark study suggested that GIK may attenuate myocardial reperfusion injury and thus may exert significant cardioprotection in patients with AMI who receive reperfusion [9]. However, the underlying mechanism responsible for GIK’s cardioprotective effect remains incompletely understood. In a recent study, we have demonstrated that the anti-apoptotic property of insulin is largely responsible for GIK’s cardioprotective effect when administered shortly before reperfusion, and that insulin exerts its anti-apoptotic effect primarily via an Akt-dependent eNOS activation pathway in cardiomyocytes [10].

Several recent in vitro studies performed in cultured endothelial cells have demonstrated that insulin activates eNOS and increases NO production [11]. However, whether treatment with GIK or insulin may reduce endothelial injury associated with myocardial ischemia/reperfusion (MI/R), a critical early event that contributes to the ultimate cardiac injury, has never been previously studied. Moreover, although it is well known that endothelial cells have the highest level of eNOS expression, whether GIK or insulin may exert an endothelial protective effect via Akt-mediated eNOS activation pathway remains unknown.

Therefore, the aims of the present study were: (1) to determine whether treatment with GIK may preserve coronary vascular function and attenuate endothelial injury associated with ischemia/reperfusion; and if so, (2) to investigate the signaling pathway through which GIK (or its components) exerts its anti-apoptotic effect in endothelial cells following ischemia and reperfusion.

2. Methods

2.1. Experiment protocol

The experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care. Adult mongrel dogs of either sex (10–15 kg) were anesthetized with pentobarbital sodium (30 mg/kg, i.v.) and artificially ventilated with a positive-pressure animal respirator. Blood gas was monitored, and oxygen and 5% NaHCO₃ were supplied when required to maintain the blood gas and pH within the physiological range. The right femoral artery and vein were cannulated for measurement of arterial blood pressure and infusion of drugs, respectively. A catheter was inserted into the left ventricle via the right carotid artery to measure left ventricular pressure with a hemodynamic analyzing system during the whole MI/R process. Myocardial ischemia was induced by exposing the heart through a left thoracic incision and placing a screw occluder on the left anterior descending coronary artery to produce coronary artery stenoses. After 50 min of ischemia, the occluder was released and the myocardium was reperfused for 4 h [12]. Dogs were randomly assigned to one of the following treatments: (1) vehicle (0.9% NaCl); (2) GIK (glucose 250 g/L, insulin 60 U/L, potassium 80 mmol/L, intravenous infusion at 2 ml/kg/h for 4 h, beginning 5 min before reperfusion); (3) GK. Sham myocardial ischemia and reperfusion dogs were subjected to the same surgical procedures performed on MI/R dogs, except that the coronary artery was left unoccluded.

2.2. Isolated coronary arterial rings

At the end of 4 h reperfusion period, dogs were killed by overdose injection of pentobarbital. Hearts were immediately removed and placed into ice-cold and oxygenated (5% CO₂ and 95% O₂) Krebs-Henseleit (K–H) buffer consisting of (mmol/L): NaCl 118, KCl 4.75, CaCl₂2H₂O 2.54, KH₂PO₄ 1.19, MgSO₄?7H₂O 1.19, NaHCO₃ 25, and glucose 10.0, pH=7.4. Coronary arteries distal of occlusion were carefully isolated, cleaned of fat and connective tissue and cut into rings about 2 mm in length. In some segments, endothelium was mechanically removed by pulling silk suture through the vessel. The rings were then mounted onto stainless steel hooks, suspended in 5-ml tissue baths, and connected to FORT-10 force transducers (WPI, Sarasota, FL) to record changes via a MacLab data acquisition system. The baths were filled with 5 ml of K–H buffer and aerated with 95% O₂ and 5% CO₂ at 37 °C. The rings were then stretched to an optimal preload of 9.81 mN, and allowed to equilibrate for 60 min. During this period, the K–H buffer in the tissue bath was replaced every 20 min. The optimal preload of isolated coronary rings (9.81 mN)
was determined by the length-developed tension relationship as described previously [13].

After an equilibration period of 30 min with the tension of the vascular rings maintained at 9.81 mN, the rings were exposed to endothelin (ET-1, 10 nmol/L) to generate approximately 19.6–24.5 mN of developed force. Once a stable contraction was obtained, acetylcholine (ACh), an endothelin-dependent vasodilator, was added to the bath in cumulative concentrations of $10^{-11}$, $10^{-10}$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ mol/L to determine the endothelial function. After the cumulative response stabilized, the rings were washed and allowed to equilibrate to baseline. The procedure was then repeated with an endothelium-independent vasodilator, acidified NaNO$_2$ ($10^{-8}$–$10^{-4}$ mol/L) to determine vascular smooth muscle function and the sensitivity to NO.

2.3. Immunohistological detection of caspase 3 activation and TUNEL staining

In a separate study, coronary arteries from dogs of each group were removed and fixed in 10% formalin for 24 h. Fixed coronary segments were dehydrated and embedded in paraffin, and sections were cut at 6 µm and mounted onto glass slides. Immunohistochemical procedures for detecting caspase-3 activation were performed according to the published procedure. Rabbit polyclonal antibody against cleaved caspase-3 (activated form) and Anti-rabbit IgG HRP-linked antibody were purchased from Cell Signaling Technology, Inc. (USA). The DAKO avidin-peroxidase kit (DAKO Corporation, USA) was used for caspase-3 immunostaining. TUNEL staining was performed by using an In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, USA) according to the protocol provided by the manufacturer and modified as described previously [10,14]. Apoptotic index was determined as previously described and expressed as number of positively stained cells/total number of endothelial cells counted × 100% [14].

2.4. Culture of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) were harvested enzymatically from umbilical cords with Type II collagenase (0.1 mg/ml), and propagated as described [15]. Endothelial purity was evaluated by von Willebrand factor immunostaining (>90%). At confluence, cells were replated in 1.5% gelatin-coated flasks and used at 20,000 cells/cm$^2$. Cells were used within passage 4 after primary culture. This study was approved by our institutional review board. Informed consent was given by persons donating umbilical cords.

2.5. Simulated ischemia/reperfusion (SI/R) in cultured cells

Twenty hours after incubation with normal culture medium, the HUVEC were transferred to an ischemic buffer adapted from Esumi, et al [16] containing (in mmol/L): NaCl 137, KCl 3.8, MgCl$_2$ 0.49, CaCl$_2$ 2H$_2$O 0.9, HEPES 4 supplemented with deoxyglucose 10, sodium dithionate 0.75, KCl 12, and lactate 20, pH 6.5, and incubated for 2 h at 37°C in a humidified cell culture incubator. This buffer is designed to simulate the extracellular milieu of myocardial ischemia, with the approximate concentrations of potassium, hydrogen, and lactate ions occurring in vivo. At the onset of reperfusion after 2 h of simulated ischemia (SI), the endothelial cells were randomly exposed to one of the following treatments: vehicle; insulin ($10^{-7}$, $10^{-8}$ or $10^{-9}$ mol/L); insulin plus L-NAME ($10^{-4}$ mol/L); insulin plus Akt inhibitor (1L-6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-Octadecylcarbonate, Calbiochem, 5×$10^{-5}$ mol/L). After 4 h of reperfusion, apoptosis was determined using the In Situ Cell Death Detection Kit as described above.

2.6. Determination of NO production

Total nitric oxide production (NO$_3^-$) in tissue-bath fluid or culture medium was determined by measuring the concentration of nitrite, a stable metabolite of nitric oxide, with a modified Griess reaction method [17]. Briefly, tissue-bath fluid or culture medium (100 µl) was taken and mixed with an equal volume of modified Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). The accumulation of NO production in response to ACh in tissue bath was measured 15 min after the cumulative concentrations of ACh reached $10^{-5}$ mol/L. After 10 min of incubation at room temperature, the concentration of the resultant chromophore was spectrophotometrically determined at 540 nm using a spectrophotometer (SpectraMax Plus384, Molecular Device, USA). The nitrate concentrations in the samples were calculated from freshly prepared nitrate standard curves made from sodium nitrate with the same K–H solution or culture medium.

2.7. Statistical analysis

All values (in the text and figures) were presented as mean ± S.E. (of $n$ independent experiments). All data were analyzed using ANOVA followed by the Bonferroni correction for post hoc $t$ tests. $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Endothelium-dependent vasorelaxation of coronary artery rings

In ET-1 pre-contracted coronary arterial segments from sham operated dogs, ACh produced a concentration-
dependent vasorelaxation with the maximal response occurring at $10^{-5}$ mol/L ACh (93.8±1.7%). Pre-incubation of coronary artery rings with an NOS inhibitor (L-NAME: $10^{-4}$ mol/L) for 20 min, or mechanical denudation of endothelium both attenuated ACh-induced vasorelaxation (the maximal vasorelaxation: 18.7±3.8% and 27.9±2.4%, respectively) (Fig. 1) without affecting their response to acidified NaNO₂, an endothelium-independent vasodilator. These results indicate that the ACh-induced vasorelaxation in the canine coronary artery preparation is endothelium-dependent and NO mediated.

3.2. The effects of GIK and its components on endothelial dysfunction in ischemic-reperfused coronary arteries

Endothelial dysfunction is one of the earliest pathological consequences of ischemia and reperfusion. To clarify whether treatment of GIK may protect the endothelium from ischemia-reperfusion injury, we studied the effects of GIK and its components on endothelium-dependent vasorelaxation in isolated coronary artery segments (Fig. 2). In vivo ischemia and reperfusion (vehicle) resulted in significant endothelial dysfunction as evidenced by decreased vasorelaxation in response to ACh (maximal relaxation: 28.1±2.3%, $n=7$, $P<0.01$ vs. 93.8±1.7% in sham operated dogs) with a normal response to an endothelium-independent vasodilator NaNO₂. Treatment with GIK during myocardial reperfusion in vivo significantly preserved the coronary vasodilating response to ACh (maximal relaxation: 80.3±3.8%, $n=6$, $P<0.01$ vs. vehicle). Whereas treatment with GK failed to prevent MI/R-induced endothelial dysfunction (maximal relaxation: 35.5±4.9%, $n=6$, $P>0.05$ vs. vehicle) (Fig. 2). These results provided direct evidence showing that insulin, but not glucose or potassium, is the predominant protective component of the GIK cocktail that reduces MI/R-induced coronary endothelial dysfunction.

3.3. ACh-stimulated nitric oxide release in coronary artery segments

To determine whether the decreased NO production or increased NO destruction was responsible for the endothelial dysfunction observed in ischemic/reperfused coronary artery, we measured the total NOX production in the perfusate in the tissue bath which included NO released from coronary rings in the tissue bath without (basal) and with (stimulated) ACh. As illustrated in Fig. 3, MI/R resulted in significant decrease in coronary arterial NO production in response to ACh ($10^{-5}$ mol/L) compared with
that in sham operated group (3.4 ± 1.9 μmol/L/mg dw vs. sham 16.8 ± 2.6 μmol/L/mg dw, \( P < 0.01 \)), indicating MI/R caused coronary endothelial injury. In vivo treatment with GIK (13.2 ± 2.1 μmol/L/mg dw, \( P < 0.01 \) vs. vehicle), but not GK (4.4 ± 2.3 μmol/L/mg dw, \( P > 0.05 \) vs. vehicle), significantly restored NO production in isolated coronary arteries.

3.4. Treatment with GIK/insulin significantly reduced endothelial apoptosis

Had demonstrated that treatment with GIK significantly attenuated ischemia/reperfusion induced endothelial dysfunction and improved NO production, we further determined whether GIK may reduce post-ischemic endothelial apoptosis.

Myocardial ischemic/reperfused coronary arteries from dogs with different treatments in vivo were immunostained with an antibody that selectively recognizes activated caspase 3. As illustrated in Fig. 4, activated caspase 3 was strongly expressed in MI/R coronary arteries treated with vehicle. Treatment with GIK, but not GK, significantly reduced coronary vascular caspase 3 activation.

In situ detection of coronary endothelial apoptosis with TUNEL staining also showed that MI/R resulted in a large number of endothelial TUNEL-positive cells (45 ± 7% vs. sham 8 ± 2%, \( P < 0.01 \)). As illustrated in Fig. 5, GIK treatment at the onset of reperfusion markedly reduced TUNEL-positive coronary endothelial cells (12 ± 4% vs. 45 ± 7% of vehicle, \( P < 0.01 \)). Interestingly, treatment with GK failed to show any protection against MI/R-induced coronary endothelial cell apoptosis (41 ± 8%, \( P > 0.05 \) vs. vehicle).
3.5. Insulin exerts its anti-apoptotic effect via Akt-stimulated NO production

Our in vivo results described above clearly demonstrated that GIK and insulin exerted significant vasculoprotective effects as evidenced by preserved endothelial-dependent vasorelaxation and reduced endothelial apoptosis. However, the interrelationship between these two protective phenotypes remains unknown. Specifically, whether insulin attenuates post-ischemic endothelial apoptosis via stimulating NO production, or it may preserve endothelium-dependent vasorelaxation by reducing post-ischemic endothelial apoptosis through NO-independent mechanisms, could not be answered by our in vivo experimental data. To address this critical question, cultured HUVEC were exposed to simulated ischemia and reperfusion in vitro and the role of NO in insulin’s anti-apoptotic effect was investigated. As shown in Figs. 6 and 7, simulated ischemia (2 h) and reperfusion (4 h) resulted in significant decrease in NO production by endothelial cells (8.23 ± 1.65 μmol/L vs. 45.94 ± 2.56 μmol/L in normal culture, n=10, P<0.01) and significant endothelial apoptosis (24.7 ± 2.6% vs. 0.9 ± 0.5% in normal culture, n=10, P<0.01). Treatment with insulin at the final concentration ranging from 10⁻⁹ mol/L to 10⁻⁷ mol/L produced a concentration-dependent increase in NO production. Insulin (10⁻⁷ mol/L) treated at the onset of simulated reperfusion significantly increased NO production (39.26 ± 1.22 μmol/L, P<0.01 vs. vehicle) and reduced endothelial apoptosis (9.2 ± 2.8%, P<0.01 vs. vehicle). Most interestingly, pre-treatment of endothelial cells with L-NAME (a non-selective NOS inhibitor, 100 μmol/L) or a selective Akt inhibitor (50 μmol/L) not only blocked insulin-stimulated NO production (8.11 ± 2.43 μmol/L and 6.88 ± 2.55 μmol/L, respectively, n=10, P>0.05 vs. vehicle), but also abolished insulin’s anti-apoptotic effect in cultured endothelial cells exposed to SI/R (22.3 ± 1.8% and 23.8 ± 1.4%, respectively, n=10, P>0.05 vs. vehicle). Treatment with L-NAME or Akt inhibitor alone had no
Although insulin-induced vasodilation in skeletal tissue is beneficial effects on TUNEL-positive staining. These results demonstrated that insulin exerts its endothelial protective effect via an Akt-dependent, NO mediated mechanism.

To further confirm that insulin exerts its anti-apoptotic effect through Akt-NO pathway, we performed an additional experiment by treating the endothelial cells with S-nitroso-N-acetylpencillamine (SNAP, 1 μmol/L), an NO donor. This treatment significantly reduced SI/R-induced endothelial cell apoptosis (9.8 ± 1.8%, n = 10, P < 0.01 vs. vehicle). Whereas pretreatment with Akt inhibitor at the concentration that blocked insulin’s anti-apoptotic effect had no effect on the anti-apoptotic effect exerted by SNAP.

4. Discussion

Two novel findings have been made in the present study. First, we have demonstrated for the first time in a large animal model that treatment with GIK or insulin exerted significant vasculoprotective effect as evidenced by preserved endothelium-dependent vasorelaxation and reduced endothelial apoptosis after in vivo ischemia and reperfusion. Second, using cultured endothelial cells subjected to simulated ischemia and reperfusion, we have provided direct evidence that increased NO production by insulin in ischemia/reperfused endothelial cells is the cause, rather than a result of reduced endothelial apoptosis.

Recently, the renaissance of GIK as a treatment for AMI has raised new interests in clarifying the effects that insulin acts on the heart and the mechanisms involved [18]. Although insulin-induced vasodilation in skeletal tissue is observed in healthy subjects [19], the vasculoprotective effects and the mechanisms of insulin in ischemic/reperfused coronary arteries remain unknown. Moreover, whether insulin may attenuate coronary vascular endothelial dysfunction and thus enhance myocardial blood flow in AMI patients remain poorly understood. Our previous study demonstrated that treatment with GIK at the onset of reperfusion increases the coronary blood flow and improves the recovery of cardiac function during reperfusion while there was no significant change in the mean arterial blood pressure in the MI/R dogs [12]. In the present study, it is confirmed that the endothelium-dependent vasorelaxation in ischemic/reperfused coronary artery is significantly blunted and demonstrated for the first time that in vivo treatment with GIK, but not GK, preserves coronary endothelial function in MI/R dogs. In addition, we have demonstrated that the endothelial NO production is markedly decreased in coronary arterial segments isolated from ischemic/reperfused dogs, indicating that the decreased NO production, rather than increased NO destruction, is responsible for the decreased endothelium-dependent vasorelaxation in ischemic/reperfused dog coronary artery. Since a very small increase in myocardial blood flow can significantly reduce myocardial ischemia [18], these coronary vasodilatory actions elicited by insulin may partly contribute to the beneficial effects of GIK therapy for AMI patients [8].

Our present study showed that in vivo treatment with insulin significantly preserved endothelium-dependent vasorelaxation assessed in isolated coronary artery and reduced endothelial apoptosis determined by caspase-3 activation and TUNEL labeling. Because ACh-induced vasorelaxation in isolated coronary artery is determined by three factors, i.e., NO production by each endothelial cell, total number of endothelial cells in a coronary segment, and the responsiveness of smooth muscle cells to NO, it is possible that insulin may reduce post-ischemic endothelial apoptosis by an NO-independent pathway and increase the viable endothelial cells that can respond to ACh stimulation. To dissect the interrelationship between NO production and anti-apoptotic effect of insulin, an in vitro culture cell approach was employed. Our results demonstrated that in vascular endothelial cells, administration of insulin at the onset of reperfusion following a simulated ischemia significantly reduced endothelial apoptosis. Interestingly, this anti-apoptotic effect of insulin was abolished by pretreatment with either Akt inhibitor or NOS inhibitor. This result provides strong evidence that insulin exerts its anti-apoptotic effect through the Akt-NOS signaling pathway, and is consistent with our previous findings that PI3K-Akt-eNOS pathway is required for insulin’s cardiomyocyte protective effects [10].

Compared with NO-donors, such as nitroprusside which has long been used in the treatment of acute myocardial ischemia, insulin has a longer half-life and is more tolerance-resistant for long term use. Recent studies have shown that the basis of the beneficial effects of insulin treatment for AMI is multifactorial. On the one hand, GIK/insulin attenuates MI/R-induced myocardial apoptosis, reduces myocardial infarction and improves the recovery.
of cardiac function as demonstrated in different species of animals as well as in human [9,10,20]. On the other hand, insulin preserves coronary vascular function and attenuates coronary endothelial cell apoptosis via Akt-NO pathway, the resultant increase in coronary NO production, as demonstrated in the present study, may further increase coronary blood flow and improve ischemic myocardial perfusion. Moreover, it was recently reported that insulin has anti-inflammatory properties by inhibiting the production of harmful cytokines including tumor necrosis factor-α, interleukin-1 and intercellular adhesion molecules [21]. These actions, together with GIK/insulin-elicited metabolic modulations on myocardium and in preventing stress hyperglycemia occurs in AMI, make insulin a more versatile drug compared with exogenous NO donors in the treatment of AMI. However, in the condition of insulin resistance which may occur in AMI, whether GIK treatment may also be beneficial remains to be elucidated.

In summary, we have demonstrated that insulin, a crucial component of the GIK cocktail, attenuates ischemia/reperfusion-induced endothelial apoptosis in an Akt dependent and NO mediated fashion. This anti-apoptotic effect in turn enhances the number of viable endothelial cells in ischemic/reperfused coronary vessels and thus preserves coronary vasodilatory response to endothelium-dependent vasodilators, such as ACh. This NO-stimulating and coronary vasculoprotective effect of insulin may play an important role in GIK’s overall cardioprotective effects by preventing vasospasm, inhibiting neutrophil–endothelial interaction and subsequent neutrophil-mediated heart injury as reported previously [10,22], and thus improving myocardial perfusion in AMI.

![Insulin treatment reduced simulated ischemia/reperfusion-induced endothelial cell apoptosis through Akt-NO signaling pathway.](image-url)
Acknowledgments

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