Nitric Oxide Mediates the Antiapoptotic Effect of Insulin in Myocardial Ischemia-Reperfusion
The Roles of PI3-Kinase, Akt, and Endothelial Nitric Oxide Synthase Phosphorylation

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Background—Recent evidence from cultured endothelial cell studies suggests that phosphorylation of endothelial nitric oxide synthase (eNOS) through the PI3-kinase–Akt pathway increases NO production. This study was designed to elucidate the signaling pathway involved in the antiapoptotic effect of insulin in vivo and to test the hypothesis that phosphorylation of eNOS by insulin may participate in the cardioprotective effect of insulin after myocardial ischemia and reperfusion.

Methods and Results—Male Sprague-Dawley rats were subjected to 30 minutes of myocardial ischemia and 4 hours of reperfusion. Rats were randomized to receive vehicle, insulin, insulin plus wortmannin, or insulin plus L-NAME. Treatment with insulin resulted in 2.6-fold and 4.3-fold increases in Akt and eNOS phosphorylation and a significant increase in NO production in ischemic/reperfused myocardial tissue. Phosphorylation of Akt and eNOS and increase of NO production by insulin were completely blocked by wortmannin, a PI3-kinase inhibitor. Pretreatment with L-NAME, a nonselective NOS inhibitor, had no effect on Akt and eNOS phosphorylation but significantly reduced NO production. Moreover, treatment with insulin markedly reduced myocardial apoptotic death (P<0.01 versus vehicle). Pretreatment with wortmannin abolished the antiapoptotic effect of insulin. Most importantly, pretreatment with L-NAME also significantly reduced the antiapoptotic effect of insulin (P<0.01 versus insulin).

Conclusions—These results demonstrated that in vivo administration of insulin activated Akt through the PI3-kinase–dependent mechanism and reduced postischemic myocardial apoptotic death. Phosphorylation of eNOS and the concurrent increase of NO production contribute significantly to the antiapoptotic effect of insulin. (Circulation. 2002;105:1497-1502.)

Key Words: apoptosis • reperfusion • nitric oxide • signal transduction

Metabolic modulation with glucose, insulin, and potassium (GIK) in acute myocardial infarction (AMI) has a long and controversial history.1 A recent clinical trial has demonstrated that compared with patients receiving reperfusion therapy and placebo, those patients receiving GIK treatment before reperfusion had a remarkable 66% reduction in their relative in-hospital mortality risk. In contrast, in those patients 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 are receiving reperfusion.2

The mechanisms through which GIK exerts its cardioprotection remain largely speculative. It was hypothesized that metabolic modulations probably are the mechanisms of protection of GIK during the ischemic period.1,3 However, a recent study has demonstrated that GIK did not affect the pattern of myocardial substrate uptake or oxygen consumption during reperfusion, suggesting that metabolic manipulation is unlikely to be the major mechanism by which GIK exerts its protective effects during reperfusion.3

There is growing evidence that apoptosis plays a key role in myocardial reperfusion injury.4,5 In cultured neonatal rat cardiac myocytes, administration of insulin alone at reoxygenation reduced TUNEL-positive myocardial cells exposed to hypoxia/reoxygenation. On the basis of this result, the authors speculated that insulin may be the predominant protective component of the GIK metabolic cocktail.6 However, to date, the effect of each component of GIK on ischemia/reperfusion-induced myocardial apoptosis has not been directly compared in an in vivo ischemia-reperfusion...
model. More importantly, the signaling pathway by which GIK or its components may exert their antiapoptotic effect has not been investigated.

Recent experimental results suggest that nitric oxide (NO) plays an important regulatory role in apoptotic cell death. High, pathological 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. On the other hand, insulin has been previously demonstrated to exert its vasodilator effect through an endothelium-dependent, NO-mediated mechanism. Several recent studies have demonstrated that insulin and other hormones such as estrogen – Akt-dependent pathway. However, to date, the connection between the antiapoptotic and NO stimulatory effects of insulin has not been identified.

The aims of this study were to (1) directly compare the antiapoptotic effect of different components of GIK in an in vivo myocardial ischemia-reperfusion model and determine the predominant protective component of the previously used GIK metabolic cocktail in AMI with reperfusion; (2) investigate the signaling pathway through which GIK (or its components) exerts its antiapoptotic effect in myocardial cells subjected to ischemia and reperfusion in vivo; and (3) determine the role of NO in cardioprotection afforded by insulin.

Methods

Experiment Protocol
Male Sprague-Dawley rats (ACE Inc) were anesthetized with 2% isoflurane. Myocardial ischemia was produced by exteriorizing the heart through a left thoracic incision and placing a 4-0 silk and making a slipknot around the left anterior descending coronary artery. After 30 minutes of ischemia, the slipknot was released and the myocardium was reperfused for 4 (apoptosis and Western blotting) or 24 hours (infarct size). Rats were randomized to receive one of the following treatments: (1) vehicle (0.9% NaCl); (2) GIK (glucose: 200 g/L, insulin: 60 U/L, potassium: 60 mEq/L, intravenous infusion at 4 mL/kg per hour for 4 hours, beginning 5 minutes before reperfusion); (3) GK; (4) insulin; (5) insulin plus wortmannin (0.5 mmol/L); or insulin plus D-NAME (0.5 mmol/L). After 4 hours of reoxygenation, myocyte apoptosis was determined by means of a procedure published recently. Five hundred α-sarcomeric actin-positive cardiomyocytes were counted in each treatment group, and the number of TUNEL-positive cells was presented as a percentage. Control cells were left in normoxic incubator at 37°C with a normal glucose for 6 hours.

Determination of Myocardial Apoptosis and Myocardial Infarction
Myocardial apoptosis was analyzed by detection of DNA ladder formation and TUNEL assay, as described previously. To determine myocardial infarct, the heart was reperfused for 24 hours and the infarct size was determined by means of a double-staining technique and a digital imaging system (infarct area/area-at-risk × 100%).

Western Blotting
Heart tissue samples were lysed with lysis buffer. After sonication, the lysates were centrifuged; proteins were separated by electrophoresis on SDS-PAGE and transferred onto a polyvinylidene difluoride-plus membrane. After being blocked with 5% milk, the immunoblots were probed with anti-pAkt or anti-peNOS antibodies overnight at 4°C followed by incubation with the corresponding secondary antibodies at room temperature for 1 hour. The blots were visualized with ECL-plus reagent. pAkt or peNOS immunoblots were then stripped with strip buffer at 50°C for 30 minutes and reblootted for total Akt or eNOS. Akt kinase activity was determined with the use of an Akt kinase assay kit (Cell Signaling).

Measurement of NOX Content in Cardiac Tissue
Total NOX content in cardiac tissue was measured in an additional experiment (n=9 to 13 per group). Tissue samples from AAR were rinsed, homogenized in deionized water (1:10, w/v), and centrifuged at 14 000g for 10 minutes. The nitric oxide concentrations in the supernatant were quantified by a chemiluminescence detector (Stevens 2808 NO Analyzer).

Isolation of Adult Rat Cardiomyocytes and Anoxia/Reoxygenation Treatment
Calcium-tolerant ventricular myocytes were isolated from adult Sprague-Dawley rats and cultured for 18 to 24 hours, as described previously. Simulated ischemia/reperfusion (SI/R) was performed as described in a recent publication from our laboratory. At the onset of SR after 2 hours of SI, the myocytes were randomized to receive one of the following treatments: vehicle, insulin (0.01 IU/mL), insulin plus wortmannin (60 mmol/L), insulin plus L-NAME (0.5 mmol/L), or insulin plus D-NAME (0.5 mmol/L). After 4 hours of reoxygenation, myocyte apoptosis was determined by means of a procedure published recently. Five hundred α-sarcomeric actin-positive cardiomyocytes were counted in each treatment group, and the number of TUNEL-positive cells was presented as a percentage. Control cells were left in normoxic incubator at 37°C with a normal glucose for 6 hours.

Statistical Analysis
All values in the text and figures are presented as mean±SEM of n independent experiments. All data were subjected to ANOVA followed by Bonferroni correction for post hoc t test. Probabilities values of ≤0.05 were considered to be statistically significant.

Results
Insulin, but Not Glucose and Potassium, Reduced Myocardial Apoptosis and Infarct
In myocardial tissue from the normal control hearts without myocardial ischemia/reperfusion (MI/R), no DNA ladder formation and a very low level of TUNEL-positive staining (0.6±0.1%) were detected. In contrast, clear DNA ladder formation (10/12) and a significant number of TUNEL-positive cells (19.7±1.3%) were observed in myocardial tissue from hearts subjected to ischemia and reperfusion and receiving vehicle. Administration of GIK shortly before reperfusion exerted a significant antiapoptotic effect, as evidenced by lower incidence of DNA ladder formation (3/11) and reduced TUNEL-positive staining (7.4±1.1%, P<0.01 versus vehicle). Most interestingly, administration of insulin alone reduced myocardial apoptotic death to an extent that was comparable to that exerted by GIK combination (7.2±1.3%, P<0.01 versus vehicle, P>0.5 versus GIK), whereas treatment with glucose alone, potassium alone, or their combination exerted no significant protective effect on ischemia/reperfusion-induced myocardial apoptosis (GK treatment: 17.8±1.6%, P>0.5 versus vehicle). To determine whether the protective effects of insulin were sustained, an additional group of animals was subjected to 30 minutes of ischemia and 24 hours of reperfusion, and the effect of GIK and insulin on myocardial infarct size was determined. Consistent with our apoptosis results, treatment with GIK or
insulin alone (18.6±1.9% and 19.3±2.4% versus 42±3.8% in vehicle-treated hearts, P<0.01) but not with GK (39±4.2%), significantly reduced myocardial infarct size. These results provided direct evidence that insulin but not glucose or potassium is the protective component of the GIK cocktail that reduces myocardial injury after ischemia and reperfusion.

**Insulin Treatment Activates Akt Through Phosphatidylinositol 3'-Kinase**

Although in vitro experiments have demonstrated that insulin activates Akt in a phosphatidylinositol 3'-kinase (PI3-kinase)-dependent fashion, it has not been previously investigated whether the same pathway exists in the ischemic-reperfused heart in vivo. As shown in Figure 1, treatment with insulin resulted in a 2.6-fold increase in Akt phosphorylation and a 2.5-fold increase in Akt activity. Cotreatment with wortmannin, a PI3-kinase inhibitor, blocked Akt phosphorylation and activation induced by insulin. There was no difference in total Akt among the groups studied (data not shown). These results demonstrated that in vivo treatment with insulin activated Akt through the PI3-kinase pathway.

**Insulin Treatment Resulted in eNOS Phosphorylation and Increased NOx Content in Ischemic-Reperfused Myocardial Tissue**

It has recently been reported that eNOS is a novel substrate for Akt and that phosphorylation of eNOS by Akt results in calcium-independent NO production. To determine whether insulin-induced Akt activation may in turn result in eNOS phosphorylation and NO production, the effect of insulin treatment on eNOS phosphorylation and NOx content in ischemic-reperfused myocardial tissue was observed. As shown in Figure 2, treatment with insulin resulted in significant eNOS phosphorylation and marked increase in NOx content. Cotreatment with PI3-kinase inhibitor wortmannin completely blocked eNOS phosphorylation and NOx increase induced by insulin. Treatment with L-NAME or L-NMMA had no effect on eNOS phosphorylation but significantly reduced NOx in insulin-treated hearts. In contrast, treatment with L-NAME or L-NMMA completely blocked eNOS phosphorylation and NOx increase in vehicle-treated hearts (3.2±0.29 μmol per gram of protein, P>0.05 versus MI plus insulin). These results demonstrated that in vitro treatment with insulin activated eNOS and increased NO production through the PI3-kinase-Akt pathway.

**Involvement of NO in Antiapoptotic Effect of Insulin After Ischemia and Reperfusion**

Having demonstrated that insulin activated eNOS and increased NO production through the PI3-kinase-Akt pathway in myocardial tissue exposed to ischemia and reperfusion, we then investigated the contribution of the insulin-Akt-eNOS-NO pathway to the antiapoptotic effect of insulin. As described above, treatment with insulin significantly reduced myocardial apoptosis, as evidenced by reduced DNA ladder formation and decreased TUNEL-positive myocyte nuclei staining. Cotreatment with wortmannin, a PI3-kinase inhibitor, almost completely abolished the antiapoptotic effect of insulin. Most interestingly, although treatment with L-NAME had no effect on either Akt or eNOS phosphorylation induced by insulin, this treatment also significantly reduced the antiapoptotic effect of insulin (Figures 3 and 4). Treatment with D-NMMA had no effect on NOx contents in insulin-treated hearts (3.2±0.29 μmol per gram of protein, P>0.05 versus MI plus insulin). There was no difference in total eNOS among the groups studied (data not shown). These results demonstrated that in vivo treatment with insulin activated eNOS and increased NO production through the PI3-kinase-Akt pathway.

![Figure 1. Phosphorylation (A) and activation (B) of Akt by in vivo insulin treatment and its modification by wortmannin and L-NAME. Insets: Representative blots. Data obtained from quantitative densitometry were presented as mean±SEM of at least 5 independent experiments. *P<0.01 compared with vehicle; **P<0.01 vs insulin alone. V indicates vehicle; I, insulin; I+W, insulin plus wortmannin pretreatment; and I+L, insulin plus L-NAME pretreatment.](image1)

![Figure 2. Phosphorylation (p) of eNOS (A) and increase in NO production (B) in myocardial tissue subjected to ischemia and reperfusion by in vivo insulin treatment and its modification by wortmannin and L-NAME (L-NMMA for NOx measurement because L-NAME interferes with the assay). Inset: Representative blots. Data obtained from quantitative densitometry were presented as mean±SEM of at least 5 independent experiments. *P<0.05, **P<0.01 compared with vehicle. ***P<0.01 vs insulin alone. With D-NMMA had no effect on NOx contents in insulin-treated hearts (3.2±0.29 μmol per gram of protein, P>0.05 versus MI plus insulin). There was no difference in total eNOS among the groups studied (data not shown). These results demonstrated that in vitro treatment with insulin activated eNOS and increased NO production through the PI3-kinase-Akt pathway.](image2)
To further ensure that insulin exerts its antiapoptotic effect through PI3-kinase–induced NO release, another three groups of rats were studied (wortmannin alone, S-nitroso-N-acetylpenicillamine [SNAP] alone, or their combination, n=8 in each group). As summarized in the Table, administration of wortmannin alone had no effects on postischemic myocardial apoptosis. Consistent with previous reports, administration of 1 μmol/L SNAP, a concentration that resulted in a minor (∼6±1 mm Hg) and transient (<10 minutes) decrease in mean arterial blood pressure, significantly reduced postischemic myocardial apoptosis. Most importantly, although pretreatment with wortmannin abolished the protection exerted by insulin, wortmannin had no effect on the antiapoptotic effect exerted by SNAP. These results provide further evidence that NO acts as an execution molecule in the insulin antiapoptotic signaling pathway.

To ascertain whether the effect of wortmannin and L-NAME on the antiapoptotic action of insulin resulted from blockade of their targeted molecules rather than from their nonspecific systemic effects when administered in vivo, the effect of insulin on myocardial apoptosis and its involvement of PI3-kinase–Akt–NO signaling was investigated in cultured adult myocytes exposed to simulated reperfusion. As illustrated in Figure 5, simulated ischemia/reperfusion resulted in significant cardiomyocyte apoptosis. Treatment with insulin at the onset of simulated reperfusion markedly reduced TUNEL-positive cells. This protective effect was virtually abolished by cotreatment with wortmannin, a PI3-kinase inhibitor. Most important, although treatment with L-NAME alone had no effect on simulated ischemia/reperfusion-induced apoptotic death (32.9±3.1%), treatment with L-NAME significantly, though not completely, blocked the antiapoptotic effect of insulin. Another NOS inhibitor, L-NMMA, exerted the same effect as that exerted by L-NAME (26.1±2.4%). These data provided further support that the PI3-kinase–Akt–NO pathway plays a critical role in the antiapoptotic effect of insulin.

Discussion

Recently, there has been a resurgence of interest in GIK as a treatment for patients with AMI who are undergoing reperfusion therapy. However, many fundamental questions remain unanswered. What is the predominant protective component of the GIK cocktail? Does GIK treatment reduce postischemic myocardial apoptosis, a predominant pathway of cell death after reperfusion? If so, what is the signaling pathway by which GIK exerts its anti-apoptosis effect?

We have made several novel observations in our present experiment. First, we have directly compared the cardioprotective effects of the individual GIK components and have demonstrated for the first time in an in vivo model that insulin
but not glucose or potassium is the predominant protective component of the previously used GIK metabolic cocktail. Second, we have demonstrated that insulin exerts its antiapoptotic effect in a PI3-kinase–Akt–pathway–dependent manner. Although insulin has been shown to activate PI3-kinase and Akt in cultured cells, we have demonstrated for the first time that this signaling mechanism is preserved in the ischemic-reperfused myocardium in vivo. Third, and most important, we have provided evidence that phosphorylation of eNOS by Akt with subsequent increase in NO production is an important downstream effector in the antiapoptotic signaling by insulin in myocardial ischemia and reperfusion. To the best of our knowledge, this is the first demonstration of a critical role for this signaling pathway in cardiomyocyte survival after ischemia and reperfusion.

Considerable evidence demonstrates that insulin and IGF-1 exert significant antiapoptotic effects through the PI3-kinase–Akt signaling pathway in cultured neonatal myocytes. A number of pro-apoptotic proteins have been identified as direct Akt substrates, including Bad, Caspase-9, apoptosis signal-regulating kinase 1 (ASK1), and Forkhead transcription factors, FKHRL1. Phosphorylation of these molecules by Akt may reduce cell apoptotic death by inhibiting caspase-9 activity, releasing antiapoptotic molecule Bcl-2, blocking pro-apoptotic molecule Fas ligand expression, and inhibiting pro-apoptosis molecule p38 MAPK activation. However, given the growing number of Akt substrates, it is unlikely that multiple substrates are equally phosphorylated by Akt in a given cell type under a given pathological condition. Indeed, involvement of Bad in an antiapoptotic pathway by Akt has been contradicted and appears to be cell type and stimulation dependent. In cultured neonatal cardiac cells exposed to H2O2, treatment with insulin exerted a significant antiapoptotic effect through Akt-dependent Bad phosphorylation. In contrast, in endothelial cells, insulin-activated Akt exerts its antiapoptotic effect through phosphorylation of caspase-9 but not Bad. More importantly, to date, there is no direct evidence demonstrating that any specific downstream signaling molecules of Akt reported from in vitro experiments participate in the antiapoptotic effect of insulin after ischemia and reperfusion in vivo.

Strong evidence exists that eNOS is a novel Akt target. Early studies suggested that the phosphorylation of eNOS may regulate enzyme activity and may be responsible for shear stress–induced NO production. In 1999, two investigative groups reported that Akt, a serine/threonine protein kinase, phosphorylates human eNOS on a critical serine 1177 in a PI3-kinase–dependent manner. Shear stress or estrogen stimulation resulted in PI3-kinase–Akt–dependent eNOS phosphorylation, thus enhancing NO production in an endothelium-dependent but calcium-independent manner. Recent in vitro studies have demonstrated that like shear stress and estrogen, insulin also increases NO production through the PI3-kinase–Akt signaling pathway. In 1996, Zeng and Quon provided direct evidence that insulin stimulates production of NO in human umbilical vein endothelial cells, and this insulin-stimulated NO production is blocked by wortmannin. Subsequent study by the same group further demonstrated that inhibition of Akt activity by overexpression of an inhibitory mutant of Akt also completely blocked NO production induced by insulin. However, the molecular link between Akt activation and NO production was not identified in this study. In our present experiment, we have demonstrated that insulin increased NO production through eNOS phosphorylation and have provided novel direct evidence that in vivo administration of insulin resulted in eNOS phosphorylation and increased NO production through the PI3-kinase–Akt pathway.
Figure 6. Proposed antiapoptotic signaling pathway by insulin.

The precise mechanisms by which insulin-induced NO production exerts its antiapoptotic effects were not directly investigated in the present study. Several interactions of NO with apoptotic signaling machinery have been postulated to explain the apoptosis-inhibitory effects of NO. NO has been shown to nitrosate not only the apoptosis executing enzyme caspase-3 but also caspase-6, -7, and -8.7 Furthermore, NO has been implicated in the inhibition of caspase-dependent Bcl-2 cleavage and, consequently, the release of mitochondrial cytochrome c.28 In addition, in a recent study, Rossig et al.8 demonstrated that after stimulation with TNFα, low levels of NO downregulate MKP-3 mRNA levels, thereby preventing the inactivation of ERK1/2, an antiapoptotic member of the MAPK family, and reducing apoptotic cell death. Further experiments to investigate the mechanisms by which the insulin-NO pathway interferes with the apoptotic pathway are currently under investigation in our laboratory.

In summary, we have demonstrated that insulin is the primary protective component of the GIK cocktail that protects against myocardial ischemia/reperfusion injury. In vivo administration of insulin activated Akt through the PI3-kinase–dependent mechanism and reduced postischemic myocardial apoptotic death. Phosphorylation of eNOS and the concurrent increase of NO production contributes significantly to the antiapoptotic effect of insulin (Figure 6).

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