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Myocardial Protection by Insulin at Reperfusion Requires Early Administration and Is Mediated via Akt and p70s6 Kinase Cell-Survival Signaling

Anne K. Jonassen, Michael N. Sack, Ole D. Mjøs, Derek M. Yellon

Abstract—The “metabolic cocktail” comprising glucose-insulin-potassium administered at reperfusion reduces infarct size in the in vivo rat heart. We propose that insulin is the major component mediating this protection and acts via Akt prosurvival signaling. This hypothesis was studied in isolated perfused rat hearts (measuring infarct size to area of risk [%]) subjected to 35 minutes regional myocardial ischemia and 2 hours reperfusion. Insulin administered at the onset of reperfusion attenuated infarct size by $\geq 45\%$ versus control hearts ($P < 0.001$). Insulin-mediated cardioprotection was found to be independent of the presence of glucose at reperfusion. Moreover, the cell survival benefit of insulin is temporally dependent, in that insulin administration from the onset of reperfusion and maintained for either 15 minutes or for the duration of reperfusion reduced infarct size. In contrast, protection was abrogated if insulin administration was delayed until 15 minutes into reperfusion. Pharmacological inhibition of both upstream and downstream signals in the Akt prosurvival pathway abolished the cardioprotective effects of insulin. Here coadministration of insulin with the tyrosine kinase inhibitor lavendustin A, the phosphatidylinositol3-kinase (PI3-kinase) inhibitor wortmannin, and mTOR/p70s6 kinase inhibitor rapamycin abolished cardioprotection. Steady-state levels of activated/phosphorylated Akt correlated with insulin administration. Finally, downstream prosurvival targets of Akt including p70s6 kinase and BAD were modulated by insulin. In conclusion, insulin administration at reperfusion reduces myocardial infarction, is dependent on early administration during reperfusion, and is mediated via Akt and p70s6 kinase dependent signaling pathway. Moreover, BAD is maintained in its inert phosphorylated state in response to insulin therapy. (*Circ Res.* 2001; 89:1191-1198.)

Key Words: cardioprotection ■ insulin ■ Akt ■ p70s6 kinase ■ BAD

The management of patients with acute myocardial infarction has improved dramatically with the restoration of arterial perfusion with thrombolytic and antiplatelet therapy. Attention has turned to adjunctive pharmacological treatments to enhance myocardial tolerance to ischemia/reperfusion injury. This strategy is being pursued in an attempt to further reduce mortality in conjunction with reperfusion therapy.¹ Ideally, as this cytoprotective therapy would usually be administered after the onset of ischemia, candidate agents would need to be effective when administered during reperfusion. In a pilot randomized, controlled clinical study, administration of the “metabolic cocktail” comprising glucose, insulin, and potassium (GIK) has been shown to reduce mortality in patients with acute myocardial infarction undergoing reperfusion.² This was supported in experimental studies where we demonstrated that GIK infusion at reperfusion reduces myocardial infarct size in the in vivo rat.³ Interestingly, in this in vivo rat study, we observed that the

early reperfusion free fatty acid and glucose levels were similar in the GIK-treated and vehicle-control-treated rats. This experimental observation questioned the exclusivity of the previously hypothesized glucose/fatty acid hypothesis concerning the cardioprotective effect of GIK.⁴ Moreover, as insulin itself is a mitogen and is known to promote cell survival,⁵ we began to investigate whether insulin, when administered at reperfusion, could enhance tolerance to ischemia. In our initial study, we utilized rat neonatal cardiocytes to study the effects of insulin in response to simulated ischemia and reoxygenation. In that study, we demonstrated that insulin administration at reoxygenation reduced cardiomyocyte injury and attenuated the incidence of apoptosis during the reoxygenation period.⁶

Collectively, these data support a role of insulin in the promotion of cell survival in the context of the postischemic reperfusion period. We propose that this enhanced cell survival may be independent of glucose and via insulin-acti-

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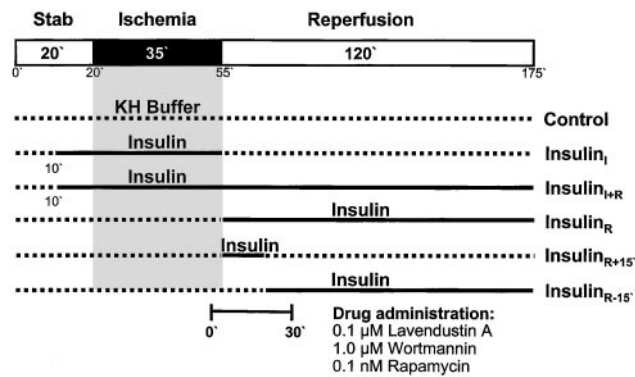


Figure 1. Experimental protocol. Stab indicates stabilization; dotted lines, buffer perfusion; solid lines, insulin infusion; $Ins_{I,I}$, insulin administration for the last 10 minutes of stabilization and for the duration of ischemia; $Ins_{I,I+R}$, insulin for 10 minutes prior to ischemia, during ischemia and reperfusion; $Ins_{R,I}$, insulin during reperfusion; $Ins_{R,I+15}$, insulin for the 15 first minutes of reperfusion; $Ins_{R,-15}$, insulin infusion started 15 minutes after the onset of reperfusion. The inhibitors (lavendustin A, wortmannin, and rapamycin) were administered for 30 minutes; from 30 minutes of ischemia until 25 minutes of reperfusion.

vated, Akt-mediated cell survival signaling. The objectives of this study were to evaluate the temporal requirements of insulin and the requirement of glucose in insulin-mediated protection against myocardial infarction. Furthermore, we characterized the role of the Akt signaling and proposed downstream prosurvival targets in mediating this cardioprotection. To enable us to dissect out the relative dose and temporal contribution of insulin to cardioprotection when administered at reperfusion and to assess its dependence on exogenous glucose at the time of reperfusion, we have performed these studies in the isolated perfused rat heart.

Materials and Methods

Langendorff Perfusion Procedure

The investigation conforms to the Home Office *Guidance on the Operation of Animals (Scientific Procedures) Act 1986* (published by HMSO 1986, London). Male Wistar rats (250 to 350 g, $n=136$; Harlan-Olac, Bicester, United Kingdom, and University of Cape Town Animal Unit, South Africa) fed a standard diet were heparinized (200 IU) and anesthetized with sodium pentobarbital (50 mg/kg IP). The hearts were studied using a Langendorff perfusion system, and regional myocardial ischemia and reperfusion was performed as described previously.⁷

Experimental Protocol

The experimental protocol is shown in Figure 1. Baseline values for functional parameters were obtained after stabilization. Initially, the temporal effects of insulin (Novo Nordisk) administration were studied at 0.3, 1.0, and 5.0 mU/mL during 3 temporally distinct administration periods. These included insulin administration from 10 minutes prior to index ischemia to include the full duration of ischemia ($Ins_{I,I}$), during reperfusion alone ($Ins_{R,I}$), and finally from 10 minutes prior to ischemia and continued for the duration of ischemia and reperfusion ($Ins_{I,I+R}$). Subsequently, we also evaluated the effect of insulin administration for only the first 15 minutes of reperfusion ($Ins_{R,I+15}$) versus delayed administration from 15 minutes of reperfusion to the end of the experiment ($Ins_{R,-15}$) (Figure 1). Furthermore to explore the Akt signaling pathway underlying the cardioprotective effect of insulin at reperfusion, the following inhibitors (Calbiochem) with or without insulin treatment were studied: tyrosine kinase inhibitor lavendustin A (lav; 0.1 μ mol/L); PI3-kinase inhibitor

wortmannin (wort; 1 μ mol/L), and the mTOR-kinase inhibitor rapamycin (rap 0.5 nmol/L). Moreover, as the low dose of insulin was the only dose not to effect basal cardiac contractile function, all subsequent experiments were performed using 0.3 mU/mL of insulin. The measurement of ischemic risk zone and infarct size were performed in a blinded fashion as described previously.⁷

Immunoblot Analysis

Myocardial Akt phosphorylation (Phospho-Akt, Ser 473), p70s6 kinase phosphorylation (Phospho-p70s6k, Thr 421/Ser 424), and BAD phosphorylation (Phospho-BAD, Ser 136) in the area at risk of infarction was determined by SDS-PAGE electrophoresis (all antibodies from New England Biolabs). Hearts perfused with 0.3 mU/mL of insulin (for 15 minutes) or KH-buffer served as baseline controls. The other hearts underwent the protocol as previously described, and the area at risk tissue was collected at the end of ischemia and at 2, 5, and 15 minutes of ischemic-reperfusion. Cardiac ventricular tissue were homogenized in lysis buffer and tissue debris were removed by centrifugation at 3000 rpm (10 minutes). This supernatant was again centrifuged at 21 000 rpm (60 minutes). The supernatant from this spin was decanted and contained the cytosolic fraction. The pellets were resuspended, sonicated, and recentrifuged at 21 000 rpm (60 minutes) and the supernatant (membrane fraction) decanted. Protein quantification, sample (22 μ g/lane) preparation, and electrophoresis were performed as previously described.⁸ Ponceau S staining (Sigma) confirmed equal loading.

Statistical Analysis

Values are presented as mean \pm standard error of the mean (SEM). Infarct size and SDS-PAGE electrophoresis results were tested for group differences by one way analysis of variance (ANOVA) combined with Fisher's post hoc test. Comparisons of coronary flow, heart rate, and left ventricular developed pressure (LVDP) between groups were performed with repeated-measures general linear model (GLM) and within-group differences were tested by the paired Student's *t* test. A value of $P<0.05$ was considered statistically significant.

Results

Insulin Reduces Infarct Size When Given at Reperfusion

The cardioprotective effect of insulin at reperfusion ($Ins_{R,I}$) was examined. Administration of 0.3, 1.0, or 5.0 mU/mL of insulin to the heart at the onset of reperfusion significantly reduced infarct size compared with control ($Ins_{0,3}$ 24.9 \pm 2.1%, $Ins_{1,0}$ 25.8 \pm 2.8%, $Ins_{5,0}$ 23.2 \pm 4.6% versus controls 47.2 \pm 1.7%, $P<0.001$) (Figure 2). The administration of 0.3 mU/mL of insulin for 10 minutes prior to ischemia and continued throughout ischemia and reperfusion ($Ins_{I,I+R}$) resulted in a similar reduction in infarct size as compared with controls ($Ins_{0,3}$ 33.5 \pm 2.6% versus control 47.2 \pm 1.7%, $P<0.001$) (Figure 2). Interestingly, the administration of the higher doses of insulin (1 mU/mL and 5 mU/mL) for 10 minutes prior to ischemia and continued throughout ischemia and reperfusion did not confer cardioprotection in our model ($Ins_{1,0}$ 48.3 \pm 5.0%, $Ins_{5,0}$ 51.9 \pm 5.6% versus control 47.2 \pm 1.7%, NS) (Figure 2). Of note, the LVDP increased from 139 \pm 13% to 161 \pm 12% ($P<0.005$) in the $Ins_{1,0}$ group and from 130 \pm 3% to 154 \pm 7% ($P<0.02$) in the $Ins_{5,0}$ group after 10 minutes of insulin perfusion during stabilization. Conversely, the LVDP was not altered by the low dose of insulin administration (0.3 mU/mL).

Finally, the insulin treatment from 10 minutes prior to ischemia until the end of ischemia ($Ins_{I,I}$) did not result in any

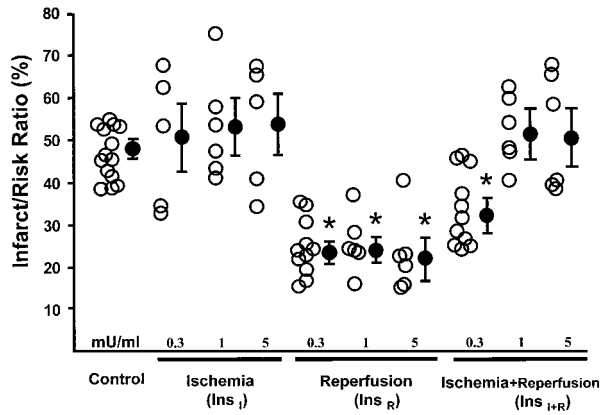


Figure 2. The temporal effects of insulin treatment on myocardial infarct size. Infarct size is expressed as percentage of the region at risk of infarction. Three different concentrations (0.3, 1.0, and 5.0 mU/mL) of insulin were administered at 3 different time points during the protocol: 10 minutes prior to and during ischemia (Insulin_I); from the onset of reperfusion (Insulin_R); and 10 minutes prior to ischemia and throughout ischemia+reperfusion (Insulin_{I+R}). Open circles represent single hearts; black circles with error bars, group mean ± SEM. **P* < 0.001 vs the control group.

significant changes in infarct size compared with controls (Ins_{0.3} 50.4 ± 7.1%, Ins_{1.0} 53.3 ± 5.1%, Ins_{5.0} 53.7 ± 6.7% versus control 47.2 ± 1.7%, NS)(Figure 2). Because the coronary flow and LVDP during reperfusion were not significantly different between groups, it is unlikely that these factors contributed toward the insulin-mediated reperfusion effects on infarct size (data not shown).

Acute Insulin Administration at the Moment of Reperfusion Reduces Infarct Size

Administration of insulin (0.3 mU/mL) for the first 15 minutes of reperfusion (Ins_{R+15'}) and for the duration of reperfusion (Ins_R) significantly reduced infarct size as compared with controls (Ins_{R+15'} 27.6 ± 4.8% and Ins_R 24.9 ± 2.1 versus control 47.2 ± 1.7%, *P* < 0.001) (Figure 3). The cardioprotective effect of insulin at reperfusion was completely

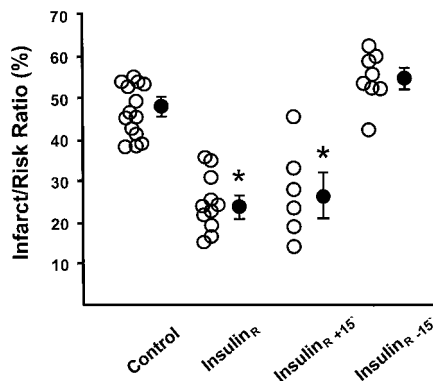


Figure 3. Timing of insulin treatment (0.3 mU/mL) at reperfusion. Treatment with insulin for the first 15 minutes (Insulin_{R+15'}) resulted in reduced infarct size, whereas postponement of the treatment for 15 minutes (Insulin_{R-15'}) resulted in abolition of the cardioprotective effects of insulin when administered at reperfusion. Open circles represent single hearts; black circles with error bars, group mean ± SEM. **P* < 0.001 vs the control group.

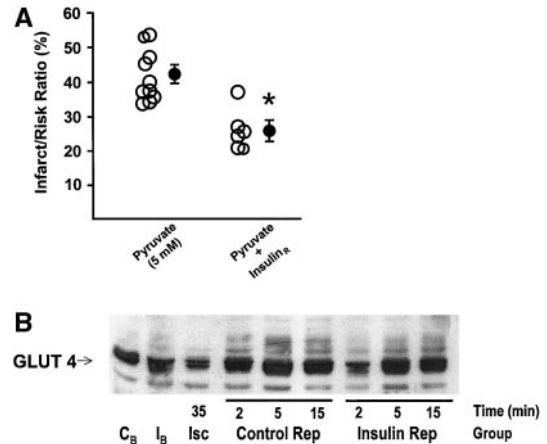


Figure 4. The effect of substrate availability at reperfusion. A, Cardioprotective effect of insulin administration at reperfusion was not affected if pyruvate was substituted for glucose as substrate at reperfusion. Open circles represent single hearts; black circles with error bars, group mean ± SEM. **P* < 0.001 vs the pyruvate group. B, Representative Western blot showing the effect of insulin on GLUT 4 translocation to the membrane when administered at reperfusion. C_B indicates control baseline; I_B, insulin baseline; Isch, end of 35 minutes of regional ischemia; Control Rep, ischemic reperfusion for 2, 5, and 15 minutes with vehicle; Insulin Rep, ischemic reperfusion for 2, 5, and 15 minutes with insulin.

abrogated if the administration was started 15 minutes after the onset of reperfusion (Ins_{R-15'} 54.8 ± 2.2% versus control 47.2 ± 1.7%, NS) (Figure 3).

Insulin-Mediated Cardioprotection Is Independent of Glucose at Reperfusion

To evaluate the requirement of glucose in this cardioprotection, we used an alternative substrate during postischemic reperfusion. Here glucose was replaced with pyruvate (5 mmol/L) in the perfusion buffer as used in previous studies as an alternate fuel substrate for cardiac perfusion.^{9,10} The cardioprotection seen in the glucose-supplemented perfusion buffer (Ins_{0.3} 24.9 ± 2.1%, Figure 2) and the buffer substituted with pyruvate showed similar degrees of infarct size reduction with coadministration of insulin versus vehicle controls (Ins_R + pyruvate 27.3 ± 2.5% versus pyruvate control 43.1 ± 2.4%, *P* < 0.001) (Figure 4). Interestingly, it has been demonstrated that ischemia reperfusion itself, as well as insulin treatment, result in translocation of GLUT 4 to the sarcolemma to facilitate glucose transport.^{11–14} Here, we demonstrate (Figure 4B) using semiquantitative immunoblot analysis that no appreciable difference in sarcolemmal GLUT 4 steady state levels could be demonstrated in the presence or absence of insulin after ischemia when measured at numerous time points of reperfusion in the isolated rat heart.

Insulin-Induced Cardioprotection Is Mediated by Tyrosine Kinase, Phosphatidylinositol 3-Kinase, and mTOR-kinase

To elucidate whether insulin exerts its cardioprotective effect through a tyrosine kinase-dependent pathway, we treated the isolated heart with lavendustin A (lav), a selective tyrosine kinase inhibitor,¹⁵ for 30 minutes (Figure 5). The protective

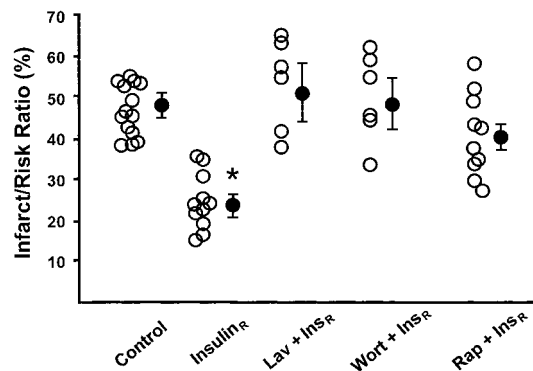


Figure 5. Insulin signaling at reperfusion. The classical insulin signaling pathway at reperfusion can be blocked by using the tyrosine kinase inhibitor lavendustin A (lav; 0.1 μ mol/L), the phosphatidylinositol 3-kinase blocker wortmannin (wort; 1 μ mol/L), and the Akt/FRAP/mTOR/p70s6k inhibitor rapamycin (rap; 0.5 nmol/L). Administration of the inhibitors alone did not alter infarct size compared with ischemic controls (data not shown). Open circles represent single hearts; black circles with error bars, group mean \pm SEM. * P <0.001 vs control group.

effect of insulin at reperfusion was completely abolished in the group that received lav (Ins_R 24.9 \pm 2.1% versus lav+Ins_R 50.3 \pm 6.5%, P <0.001) (Figure 5). Next, we examined the involvement of phosphatidylinositol 3-kinase (PI3-kinase) in the insulin-mediated protection using the PI3-kinase inhibitor wortmannin (wort).¹⁶ Wort abrogated the cardioprotective effect induced by insulin at reperfusion (Ins_R 24.9 \pm 2.1% versus wort+Ins_R 47.3 \pm 5.3%, P <0.001) (Figure 5). In order to investigate whether the downstream kinase Akt/mTOR/p70s6k was involved in the insulin-mediated protection, the FRAP/mTOR inhibitor rapamycin was coadministered and also abolished the protection offered by insulin at reperfusion (Ins_R 24.9 \pm 2.1% versus rap+Ins_R 40.2 \pm 3.2%, P <0.001) (Figure 5). Neither lavendustin A, wortmannin, or rapamycin administration alone had an effect on the degree of infarction (lav 50.3 \pm 3.2%, wort 51.1 \pm 5.5%, rap 54.1 \pm 5.8% versus control 47.2 \pm 1.7%, NS) (data not shown).

Insulin Maintains Akt Phosphorylation During Early Reperfusion

Insulin is known to activate the prosurvival kinase Akt, and the baseline insulin-perfused hearts showed the highest degree of Akt phosphorylation (Figures 6A through 6C). Akt is activated in the proximity of the cell membrane with subsequent translocation to the cytosol. Ischemia itself is shown to induce Akt phosphorylation in excess of 4-fold (cytosolic fraction) above baseline in the absence of insulin (Figure 6B). The administration of insulin at reperfusion maintains activated/phosphorylated Akt in both the cytosolic and membrane fraction of the myocardium as compared with vehicle-treated control hearts (P <0.001, Figures 6A through 6C). As would be expected, insulin-induced Akt phosphorylation at reperfusion was abolished by inhibiting the upstream signaling protein PI3-kinase (using wortmannin), whereas rapamycin, an inhibitor of the Akt target mTOR, did not alter the ability of insulin to phosphorylate Akt (Figure 6D).

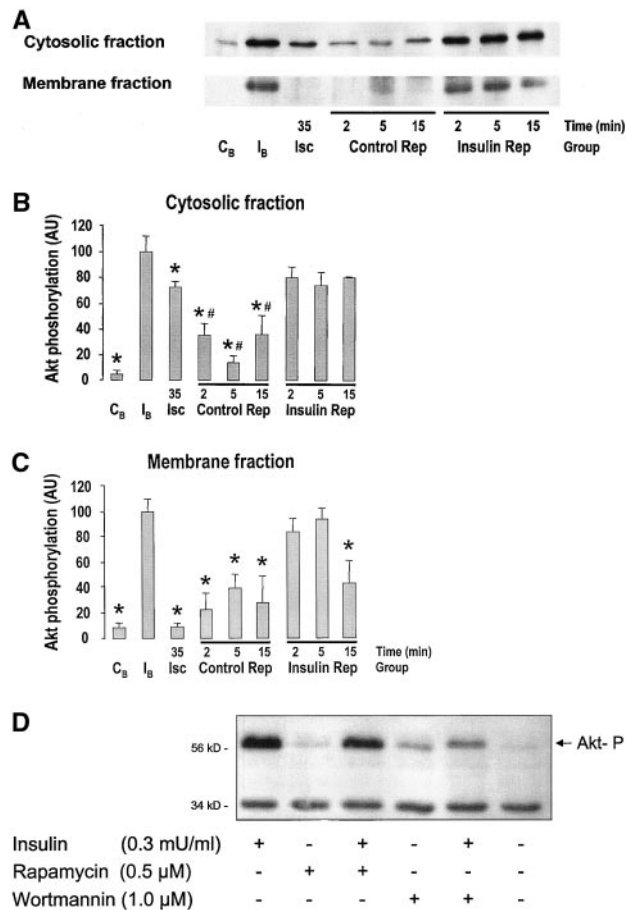


Figure 6. Insulin's effect on Akt phosphorylation when administered at reperfusion. A, Representative Western blots showing the effect of insulin on Akt phosphorylation in cytosol and membrane fraction when administered at reperfusion. C_B indicates control baseline; I_B, insulin baseline; Isch, end of 35 minutes of regional ischemia; Control Rep, ischemic reperfusion for 2, 5, and 15 minutes with vehicle; Insulin Rep, ischemic reperfusion for 2, 5, and 15 minutes with insulin. B, Densitometric analysis of mean \pm SEM of immunoblot signals of Akt phosphorylation in the cytosolic fraction and (C) in the membrane fraction. Bars represent mean \pm SEM. * P <0.001 vs insulin baseline (I_B) group; # P <0.001 vs ischemic group (Isch) in arbitrary units (AU) with I_B=100. D, A representative immunoblot of phosphorylation of Akt in response to coadministration of PI3 kinase and mTOR/p70s6k inhibitors with insulin as assessed using protein isolated from the cytosolic fraction of isolated perfused rat hearts. The nonspecific band at approximately 34 kDa demonstrates equal loading of cytosolic protein (n ≥3 at all time points).

Regulation of Akt Targets by Insulin Administration at Reperfusion

Multiple and divergent pathways that are activated by Akt are postulated to promote cell survival.¹⁷ One such pathway, the mTOR/p70s6k pathway, may be activated by insulin as suggested by the attenuation of the effect of insulin by rapamycin. mTOR/p70s6k is thought to regulate translational protein synthesis and is central in mammalian cellular growth.¹⁸ Moreover, a prosurvival effect of p70s6k activation has recently been described.¹⁹ In concordance with the steady-state Akt phosphorylation status in our study, the baseline insulin-perfused hearts showed the highest degree of

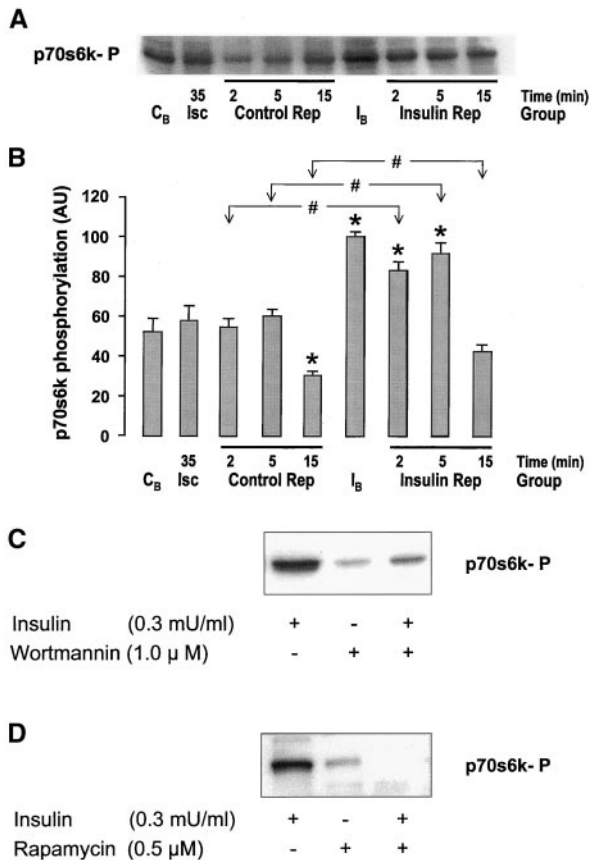


Figure 7. Effect of Insulin on p70s6k phosphorylation when administered at reperfusion. A, Representative Western blot showing the effect of insulin on p70s6k phosphorylation in cytosol when administered at reperfusion (denomination as in Figure 6A). B, Densitometric analysis of Western blot showing p70s6k phosphorylation, expressed in arbitrary units (AU) with $I_B=100$. Bars represent mean \pm SEM. * $P<0.001$ vs insulin baseline (I_B) group; # $P<0.001$ vs corresponding control group. C, A representative immunoblot of PI3-kinase inhibition with wortmannin and (D) representative immunoblot of mTOR/p70s6k inhibition with rapamycin on insulin stimulated p70s6k phosphorylation ($n\geq 3$ for all time points).

p70s6k phosphorylation as measured by SDS-PAGE electrophoresis (Figures 7A and 7B). There is always a basal activity of p70s6k in the cell, but insulin administration induced this phosphorylation by approximately 2-fold at baseline (I_B) and sustained this level of activation for the first 5 minutes of reperfusion in the presence of insulin ($P<0.001$, Figures 7A and 7B). The activity of p70s6k was significantly blunted at 15 minutes of reperfusion in the control group as compared with the control baseline group (Figures 7A and 7B). Furthermore, the p70s6k phosphorylation after 15 minutes of insulin administration at reperfusion had also diminished to levels similar to baseline control (Figure 7B). As might be expected, the insulin-induced p70s6k phosphorylation at reperfusion was abolished by PI3-kinase inhibition in the presence of wortmannin (Figure 7C) and with the coadministration of the Akt target mTOR/p70s6k inhibitor rapamycin (Figure 7D).

Insulin-Mediated Regulation of BAD Phosphorylation During Reperfusion

An additional target of Akt-directed cytoprotection is known to be mediated via the phosphorylation of the apoptotic regulator BAD.²⁰ Phosphorylated BAD is sequestered in the cytosol by 14-3-3 protein, precluding its inhibition of the prosurvival peptide Bcl-x1.²¹ To evaluate the phosphorylation status of BAD as a candidate regulatory event in response to insulin administration in the heart at reperfusion, Western blot analysis was done using a specific phospho-specific anti-BAD antibody. Comparing basal levels of BAD phosphorylation demonstrated that insulin resulted in an approximate 30% induction of BAD phosphorylation compared with vehicle-treated controls in perfused rat heart tissue (Figure 8). Interestingly, this enhanced phosphorylation status with insulin treatment at reperfusion was sustained for the first 15 minutes of reperfusion. In stark contrast, postischemic reperfusion resulted in a dephosphorylation of BAD in the vehicle-treated control heart samples to phosphorylation levels below baseline and significantly lower than the levels in insulin reperfusion hearts (Figure 8; $P<0.001$ between corresponding reperfusion time points).

Discussion

To summarize, our data demonstrate that insulin administration at reperfusion results in a significant reduction in infarct size in the isolated perfused rat heart. Moreover, these data suggest that this cardioprotection is independent of glucose and is mediated, in part, via Akt, p70s6k, and BAD cell survival effects.

Cellular protection or tolerance against ischemia has been postulated as the new challenge for patient management in cardiovascular diseases.¹ The most practical therapeutic approach to achieve this cardioprotection would be if the

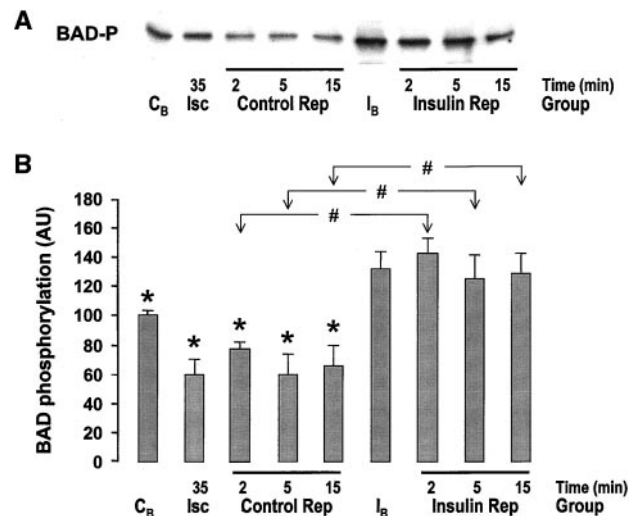


Figure 8. Effect of Insulin on BAD phosphorylation when administered at reperfusion. A, Representative Western blot showing the effect of insulin on BAD phosphorylation in cytosol when administered at reperfusion (denomination as in Figure 6A). B, Densitometric analysis of steady-state BAD phosphorylation levels expressed in arbitrary units with $C_B=100$. Bars represent mean \pm SEM. * $P<0.001$ vs insulin baseline (I_B) group. # $P<0.001$ vs corresponding control group ($n\geq 3$ for each time point).

candidate therapy could be administered during reperfusion therapy after acute myocardial ischemia. In this study, we demonstrate that insulin given at the onset of reperfusion reduces infarct size in the isolated perfused rat heart. Moreover, the administration of this mitogen was only required for a 15 minute period to confer this cardiac-protected phenotype. Conversely, the delay in administration of insulin by 15 minutes after the onset of reperfusion abrogated these cardioprotective effects.

The concept that the metabolic cocktail GIK may protect ischemic cardiomyocytes was initially introduced by Sodi-Pallares et al in 1962.²² The rationale for the use of this metabolic therapy was further delineated by Opie⁴ in 1970, where he described two chief mechanisms: ie, the promotion of cardiac glycolysis and the inhibition of free fatty acids (FFA) in the serum. The hypothesis we investigated was that insulin, in a fuel substrate-independent manner promotes cardioprotection, in part, via cell survival-activated programs. Using pyruvate as a substitute for glucose we demonstrated that the insulin-mediated cardioprotection at reperfusion was glucose-independent. These data support the concept of direct cell survival signaling effects of insulin. Moreover, a direct cardioprotective effect of insulin in the absence of glucose has been described previously.²³ Here, insulin administration attenuated LDH release in the isolated perfused working rat heart during sustained ischemia in the absence of glucose or glycolytic intermediates in the perfusate.²³

The cardioprotective effect of insulin at reperfusion was completely abolished by addition of the tyrosine kinase inhibitor lavendustin A, the PI3-kinase inhibitor wortmannin, and the mTOR-kinase inhibitor rapamycin. Lavendustin A is a potent and extremely selective inhibitor of tyrosine kinases¹⁵ and acts as a noncompetitive inhibitor at both the ATP binding site as well as at the substrate binding site.²⁴ At lower doses, lavendustin A is selective for receptor-type tyrosine kinases,¹⁵ although at higher concentrations, it will also inhibit nonreceptor tyrosine kinases (IC₅₀ 0.5 μ mol/L).²⁴ This implies that the concentration used in this study (0.1 μ mol/L) would be selective for receptor tyrosine kinases, including the insulin receptor tyrosine kinase. Next, we investigated the potential involvement of PI3-kinase in the cardioprotective effect of insulin at reperfusion. Wortmannin is widely used as a selective PI3-kinase inhibitor²⁵ and the addition of wortmannin to the perfusate together with insulin treatment at reperfusion effectively blocked the reduction in infarct size seen with insulin alone. The role of PI3-kinase in insulin-mediated myocardial protection has also been previously demonstrated by Downey and colleagues.²⁶ PI3-kinase appears to be part of a cascade and can activate Akt,^{27,28} which in turn might activate mTOR and p70s6k.²⁹

Because pharmacological inhibitors of Akt are not yet available, the role of Akt in the insulin-induced protection at reperfusion cannot easily be investigated in the intact isolated heart. However, a downstream mediator of Akt-p70s6k that is important in regulating a variety of cellular functions including mRNA translation and cell cycle progression has been shown to be activated by insulin.³⁰ Moreover, this kinase has been shown to be completely blocked by the specific immu-

nosuppressant rapamycin in adult rat cardiomyocytes.^{31,32} Rapamycin was used to evaluate the role of this signaling pathway at reperfusion. The pharmacological antagonist study with rapamycin supports a role for p70s6k activation in insulin-mediated cardioprotection against lethal reperfusion injury. The ability of insulin to phosphorylate both Akt and p70s6k supports the pharmacological data in implicating the requirement of this cell survival signaling cascade in promoting reperfusion cardioprotection.

An additional cell survival target of Akt is the cytosolic peptide BAD. This proapoptotic peptide can be sequestered in the cytosol if maintained in a phosphorylated state on either of the two serine residues (Ser 112 and 136) embedded in the 14-3-3 consensus binding sites.²¹ Dephosphorylation and, hence, activation of BAD results in translocation of BAD to the mitochondria with subsequent heterodimerization with Bcl-x1 or Bcl-2 to promote cell death.^{20,21,33} Recent data demonstrate that both Akt and p70s6k are capable of phosphorylating Ser 136 and thereby inactivating BAD.^{19,34,35} In this study, we demonstrate that insulin administration at reperfusion does indeed maintain BAD in a phosphorylated and putative inactive status during the first 15 minutes of reperfusion.

Collectively, these data strongly suggest that the classic tyrosine kinase, PI3-kinase, and Akt mediated cell survival programs are activated by insulin when administered at the moment of reperfusion following an ischemic insult in the isolated perfused rat heart. These data are supported by the recent study by Walsh and colleagues,³⁶ where the expression of a constitutively active Akt in mice protected against myocyte apoptosis in response to ischemia-reperfusion injury. Moreover, the persistent phosphorylation of p70s6k and BAD in the presence of insulin supports these additional Akt mediated effects that could promote cell survival.

A potential discrepancy in our data is the fact that when insulin was administered at the higher doses (1 mU/mL and 5 mU/mL) from 10 minutes prior to ischemia to the end of the study, we could not elicit any protection against myocardial infarction. It is unlikely that these higher doses would deplete cardiac energetic status. Despite the significant increase in LVDP with insulin treatment, previous investigators have demonstrated that energetic reserve is maintained in these circumstances.^{37,38} However, albeit unresolved, numerous studies have suggested that enhanced preischemic glycogen depletion³⁹⁻⁴¹ or inhibition of glycogenolysis⁴² will reduce infarct size. These investigators postulate that reduced glycogenolysis during ischemia will attenuate proton production with a resultant cardioprotective effect. Accordingly, we can speculate that the doses of insulin used in our study, when administered prior to the ischemic insult, may enhance glycogen stores, an effect that may counterbalance the pro-survival effects of insulin at reperfusion. Finally, recent work demonstrates that insulin signaling, including Akt and p70s6k activity, are inhibited during no-flow ischemia,⁴³ and would support the concept that cardioprotective effects of insulin are probably driven by events during reperfusion as opposed to events during the ischemic period.

The isolated perfused heart preparation was used in this study, because this would enable us to answer the long-

standing debate about the relative contribution of glucose and insulin in the cardioprotective effects of GIK therapy following acute myocardial infarction.⁴⁴ However, the perfused heart preparation model does have limitations in that postischemic reperfusion can only be maintained for a few hours: a time frame that probably does not enable the heart to reach its postinfarction steady-state of cell viability. Hence, although this limitation should be recognized, we believe that our data strongly supports and adds mechanistic perspective to the results of the pilot ECLA trial and the recent meta-analysis which demonstrate the benefits of GIK administered in patients following acute myocardial infarction.^{2,45}

An additional observation that was not fully characterized is that ischemia itself results in the phosphorylation of Akt, which is then maintained by insulin at reperfusion but significantly attenuated in the absence of insulin at reperfusion. The maintenance of Akt phosphorylation by insulin supports the subsequent data regarding the reduction in infarct size, the activation of p70s6k, and the phosphorylation status of BAD. However, a possible alternate explanation could be that the loss of viable tissue in the absence of insulin at reperfusion results in either enhanced phosphatase activation or reduced Akt itself, which could result in the same regulation described above. This latter scenario was not investigated and may be considered as a possible limitation in the conclusions regarding the signaling cascade regulation discussed in this article.

In summary, insulin appears to directly protect the myocardium by reducing infarct size if given at the onset of reperfusion. This cardioprotection appears to be independent of glucose uptake. In addition, the temporal requirement of insulin, the phosphorylation status of Akt, p70s6k, and BAD suggest that insulin attenuates early injurious events at reperfusion via orchestrating putative cell-survival signaling events. Furthermore, these data support the concept of early reperfusion injury and suggest a mechanism whereby GIK treatment at reperfusion may be beneficial in subjects undergoing reperfusion therapy following a myocardial infarction.

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