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p70s6 kinase is a functional target of insulin-activated Akt cell-survival signaling

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Abstract

Insulin administration attenuates cardiac ischemia–reperfusion apoptosis via activation of Akt-mediated cell-survival signaling. As p70s6 kinase is a cognate Akt-mediated phosphorylation target we evaluated whether p70s6 kinase activation is a functional requirement in insulin-mediated cell survival program during post-ischemic reoxygenation. Human cardiac-derived girardi cells were subjected to 6 h of simulated ischemia and 2 h of reoxygenation ± insulin treatment [0.3 mU/ml]. Concurrently, cells were pre-treated with anti-sense oligodeoxynucleotides (ODNs) corresponding to the initiation start-site of human p70s6 kinase mRNA. Sense ODN and scrambled ODN were used as controls. Cell viability was measured using lactate dehydrogenase (LDH) release and propidium iodide (PI) exclusion. Insulin at reoxygenation enhanced cell viability with attenuated LDH release ($\geqslant 50\%$, $p < 0.001$ vs. ischemic controls) and reduced PI uptake by $\geqslant 30\%$ vs. ischemic controls. The protection afforded by insulin was abolished by anti-sense ODN targeting p70s6 kinase, but not by the sense or scrambled ODNs. In parallel, insulin administration at reoxygenation significantly increased p70s6 kinase levels and activity compared with controls. P70s6 kinase activity was abolished by pre-treatment with anti-sense ODNs. Collectively, these data demonstrate that p70s6 kinase activation is a functional target of Akt following insulin-activated cytoprotection during ischemia–reoxygenation-induced injury.
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Clinical data support a mortality and morbidity reducing effect of glucose, insulin, and potassium (GIK) infusion as an adjunct to post-infarction reperfusion therapy [1,2]. Until recently, the prevailing consensus was that the beneficial effects of GIK were based on optimization of cardiac glucose metabolism with a concurrent suppression of cardiac fatty acid uptake [3]. The exclusivity of the hypothesis was challenged when we demonstrated that transient GIK administration at reperfusion resulted in reduced infarct size, despite a lack of effect on the free fatty acid and glucose levels in an in vivo rat exposed to ischemia and reperfusion [4]. Subsequently, we demonstrated that insulin alone, via induction of phosphatidylinositol (PI) 3-kinase and Akt signaling, directly promotes cytoprotective effects at reperfusion via anti-apoptotic effects [5,6]. A role for Akt in attenuating post-ischemic reperfusion apoptosis is now well established [7–11]. The phosphorylation of p70s6 kinase, eNOS, and BAD in response to Akt activation identifies these peptides as putative targets in the induction of this insulin-induced cytoprotective program [6,11]. The objective of this study was to evaluate the functional requirement of p70s6 kinase activation in the insulin-mediated cell survival program during post-ischemic reoxygenation.

p70s6 kinase is an intriguing signaling kinase that it is known to regulate a variety of cellular functions including mRNA translation, cell cycle progression, and recently has been identified as an anti-apoptotic signaling intermediate [12–14]. To explore the role of this signaling kinase, we utilized the human girardi cell line. We initially demonstrated the cytoprotective role of insulin at reoxygenation and implicated the contribution

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of PI-3-kinase and Akt signaling using pharmacologic inhibitors. The functional role of p70s6 kinase in this cytoprotective program was then evaluated using an anti-sense oligonucleotide directed against the p70s6 kinase transcript. Cell survival, peptide levels, and kinase activities were measured to establish the role of this regulatory kinase in simulated ischemia/reoxygenation injury.

Materials and methods

Simulated ischemia–reoxygenation protocol. Girardi cells (European Collection of Cell Cultures) were used in a simulated ischemia–reoxygenation protocol depicted in Fig. 1. Simulated ischemia was achieved by replacing maintenance media with a modified Esumi hypoxic buffer [5] and incubated for 6 h in a hypoxic chamber with 5% CO_2, 1% O_2, and 94% N_2 before being reoxygenated with normal girardi growth media (GGM) with or without the addition of 0.3 mU/ml of insulin.

Measurement of cell viability. Cell viability was evaluated by lactate dehydrogenase (LDH) release into the medium and via measurement of nuclear propidium iodide uptake by flow cytometry as described [15]. Furthermore, to evaluate the signaling pathway underlying the cytoprotective effect of insulin at reoxygenation in girardi cells, the following inhibitors (Calbiochem, USA) with or without insulin treatment were studied: tyrosine kinase inhibitor lavendustin A (lav; 0.1 μM); PI3-kinase inhibitor wortmannin (wort; 1 μM); the Akt inhibitor 1L-6-hydroxymethyl-chiro-inositol-2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate; abbreviated to HIMO (HIMO; 5 μM) [16]; and the mTOR/p70s6-kinase inhibitor rapamycin (rap; 0.5 nM).

Transfection protocol. For transient transfection, cells were seeded at 30% confluency 24 h prior to ODN transfection. To examine the effect of suppressed induction of p70s6 kinase, groups were treated with a 39-mer phosphorothioated derivative of anti-sense oligodeoxynucleotide (As-ODN ± insulin), 5′-TCC TCA GCT TCC CTG TCT CGG AAG TCC GGG GCT GGG TAA-3′, targeting the initiation site of human p70s6 kinase mRNA. To control for non-specific effects of treatment with sense-ODN (S-ODN), 2 similar ODNs with the same length as the As-ODN were used. Groups were treated with sense ODNs ± insulin (S-ODN ± insulin), 5′-TTA CCC AGC CCC GGA CTT CCG AGA CAG GGA AGC TGA GGA-3′, and scrambled ODNs ± insulin (Scr-ODN ± insulin), 5′-ATG CAC CGC CTT TGT CAG GTT CCA GTC CCT TCA AGA TCT-3′. The ODNs were dissolved in sterile water and transiently transfected into the cell using calcium phosphate transfection at a concentration of 12 μg/well. All subsequent experiments were performed 18 h after ODN transfection.

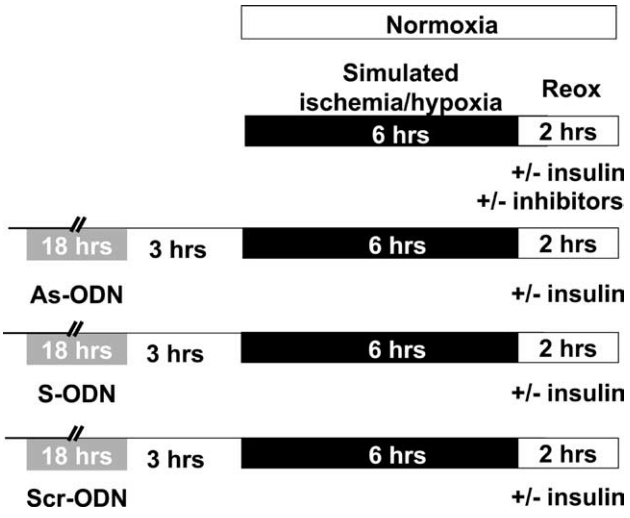

Fig. 1. Hypoxia/reoxygenation experimental protocols. Normoxic controls were subjected to media changes in parallel with other groups. The experimental groups were subjected to 6 h of lethal simulated ischemia/hypoxia and reoxygenated for 2 h with or without 0.3 mU/ml of insulin. The inhibitors were administered throughout the 2 h reoxygenation period in full growth media ± insulin at the time of reoxygenation. The inhibitors included 0.1 μM lavendustin A, 1.0 μM wortmannin, 5 μM HIMO, and 0.5 nM rapamycin. Transfection experiments were performed as described in Materials and methods. As-ODN, anti-sense-oligodeoxynucleotides; S-ODN, sense-ODN; and Scr-ODN, scrambled-ODN.

Immunoblot analysis. Phosphorylation of p70s6 kinase (phospho-p70s6 kinase, Thr 389), Akt phosphorylation (phospho-Akt, Ser 473), mTOR phosphorylation (phospho-mTOR, Ser 2448), and BAD phosphorylation (phospho-BAD, Ser 136) were determined by SDS–PAGE electrophoresis (all antibodies from Cell Signaling Technology, MA, USA). Cells treated with 0.3 mU/ml insulin (for 15 min) or vehicle served as baseline controls. Girardi cells were homogenized in a lysis buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Hepes–NaOH, pH 7.4, protease mix, and 1 mM DTT), and tissue debris was removed by centrifugation at 2000 rpm (3 × 5 min). Protein quantification, sample preparation, and electrophoresis were performed as described [6]. Ponceau Red staining (Sigma, St. Louis, USA) confirmed equal loading.

p70s6 kinase immunoprecipitation and kinase assay. p70s6 kinase antibody (5 μg) was added to 200 μg lysate (lysis buffer; 10 mM K_2HPO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DDT, 40 μg/ml PMSF, and 0.1% NP-40) and incubated for 60 min at 4 °C with constant mixing. Fifty microliters of BSA treated protein A–agarose beads (Santa Cruz) was then added to the cell lysate and incubated for 60 min at 4 °C with constant mixing. Immunoprecipitates were collected by centrifugation at 2500 rpm for 5 min at 4 °C. Pellet was washed two times with lysis buffer and once with kinase buffer (20 mM Tris–HCl, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 10 mM DTT, and 0.1 μg/ml protein kinase inhibitor cocktail). The pellet was suspended in 30 μl of the kinase buffer with ATP-mix (100 μM ATP, 75 mM $MgCl_2$, 1 μCi/μl [γ-^{32}P]ATP, and 2 mg/ml S6 substrate). The reaction was allowed to proceed for 15 min at 30 °C, terminated by adding 20 μl of 250 mM EDTA, and boiled for 5 min. Following brief centrifugation, 25 μl of the supernatant was spotted onto P81 phosphocellulose paper (Upstate Biotechnology) and the radioactivity was determined using a liquid scintillation counter.

Statistical analysis. Values are presented as means ± standard error of the mean (SEM). Comparison of multiple groups was tested for differences by one-way analysis of variance (ANOVA) combined with Fisher's post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

Improved cell viability by insulin-mediated activation of p70s6 kinase at reoxygenation in girardi cells

Reoxygenation treatment with insulin after simulated ischemia reduced LDH release by 58% and the number of propidium iodide positive cells by 32% as compared to hypoxic controls ($p < 0.001$, Figs. 2A and B). Co-administration of the tyrosine kinase inhibitor lavendustin A; the PI3-kinase inhibitor wortmannin; the Akt inhibitor HIMO; or the p70s6 kinase inhibitor rapamycin completely abolished insulin-induced cytoprotection

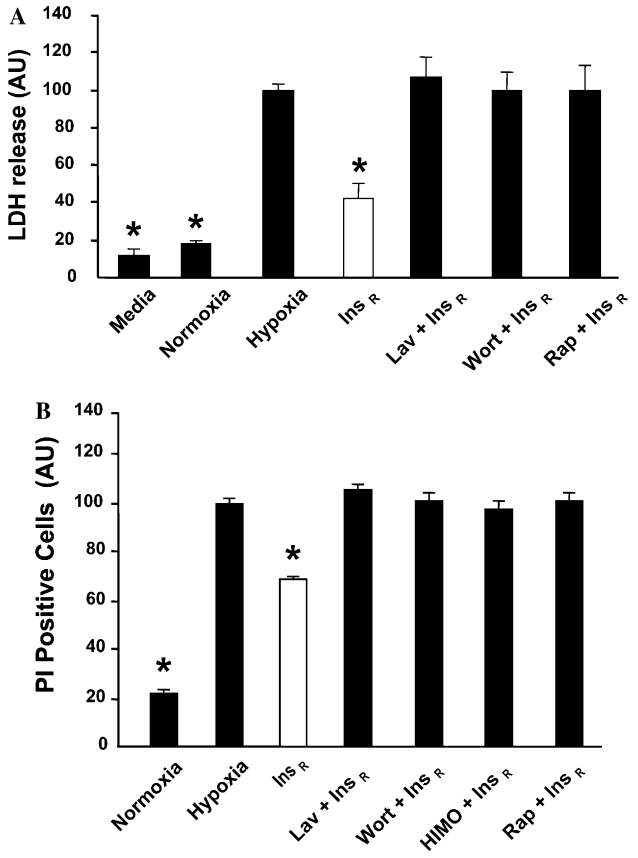

Fig. 2. Cell viability following insulin administration at reoxygenation. (A) As measured by LDH release. Insulin, Ins_R; lavendustin A, lav (tyrosine kinase inhibitor); wortmannin, wort (phosphatidylinositol 3-kinase blocker); and rapamycin, rap (mTOR blocker). (B) As measured by the number of propidium iodide (PI) positive cells at reoxygenation. Bars represent means ± SEM. $^*p < 0.001$ vs. hypoxic group in arbitrary units (AU) with hypoxia = 100 ($n > 6$ at all time points).

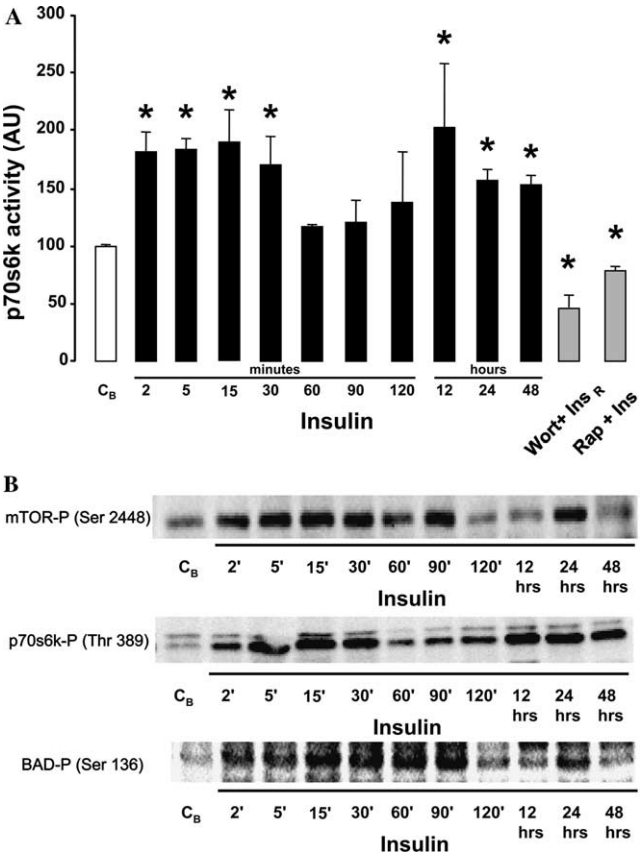

Fig. 3. Temporal effects of insulin administration on p70s6 kinase activity. (A) Activation of p70s6 kinase by insulin in arbitrary units (AU) expressed as percentage of baseline control ($C_B = 100$); the temporal effects studied from 2 min up to 48 h including the effect of co-administration of wortmannin and rapamycin on p70s6 kinase activity. Bars represent means ± SEM ($n \geqslant 4$ for all time points). $^*p < 0.001$ vs. control baseline (C_B) group. (B) Representative Western blot showing the temporal effect of insulin on mTOR, p70s6 kinase, and BAD phosphorylation.

(Figs. 2A and B). Moreover, none of the individual pharmacological antagonists modified cell viability vs. hypoxic controls (data not shown).

Coordinate activation of p70s6 kinase, upstream signaling intermediate (mTOR), and a cognate target (BAD) of p70s6 kinase in response to insulin

Insulin treatment of human girardi cells leads to an immediate and approximately 2-fold activation of p70s6 kinase (2 min of insulin exposure: 182 ± 16%, $p < 0.001$ vs. control baseline), which is sustained for the first 30 min of insulin exposure ($p < 0.001$ vs. control baseline) (Fig. 3A). The activation is blunted between 60 and 90 min of constant insulin exposure (60 min: 116 ± 2.5%; 90 min: 120 ± 19%; and 120 min: 137 ± 42%, ns vs. control baseline) and appears to be reactivated 2-fold after 12 h of insulin therapy (203 ± 55%, $p < 0.001$ vs. control baseline). This delayed reactivation is sustained up to 48 h of insulin treatment (24 h: 156 ± 10; 48 h: 152 ± 10, $p < 0.001$ vs. baseline control). The insulin-induced activation of p70s6 kinase is completely abolished by the PI3-kinase inhibitor wortmannin and the p70s6 kinase inhibitor rapamycin (45 ± 12% and 77 ± 4%, respectively, $p < 0.001$ vs. 2 min insulin exposure) (Fig. 3A). p70s6 kinase phosphorylation parallels the in vitro kinase activity with the same temporal pattern as p70s6 kinase activity (Fig. 3B).

Interestingly, both upstream regulators of p70s6 kinase, i.e., mTOR and a downstream target of p70s6 kinase (BAD) demonstrate parallel temporal phosphorylation in response to insulin administration (Fig. 3B).

Genetic attenuation of p70s6 kinase expression diminishes insulin-induced cell viability

The effect of suppression of p70s6 kinase was evaluated by transfection of girardi cells with anti-sense ODN. Treatment with As-ODN completely abolished the cytoprotective effect of insulin when measured by LDH release (As-ODN + Ins_R 85 ± 6% vs. Ins_R 42 ± 8.4%, $p < 0.001$)

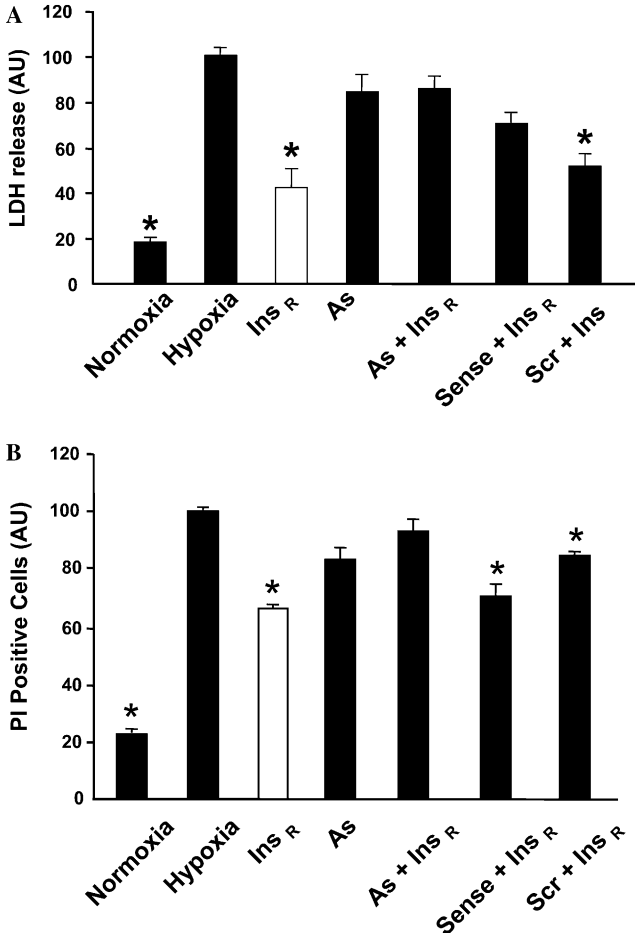

Fig. 4. Measurement of cell viability in As-ODN transfected girardi cells subjected to simulated ischemia/reoxygenation. (A) Represent LDH release and (B) uptake of PI in the cells given in arbitrary units (AU) expressed as percentage of hypoxic control (hypoxia = 100). Bars represent means ± SEM ($n > 6$ for all time points). *$p < 0.001$ vs. hypoxic group.

(Fig. 4A) and by the number of PI positive cells (As-ODN + Ins_R 93 ± 3.6% vs. Ins_R 66 ± 1.2%, $p < 0.001$) (Fig. 4B). Cell viability was not altered in the ODN treated cells in the presence of vehicle (LDH: As-ODN 83 ± 8%) (PI: As-ODN 84 ± 3.4%). Importantly, transfection of S-ODN or Scr-ODN did not affect the cytoprotection observed with insulin at reoxygenation (LDH: Scr-ODN + Ins_R 51 ± 6%, ns vs. Ins_R) (PI: S-ODN + Ins_R 71 ± 3.2% and Scr + Ins_R 85 ± 1.3%, $p < 0.001$ vs. hypoxic control). Transfection of As-ODN, S-ODN, and Scr-ODN had no significant effect on cell viability (LDH: Scr-ODN 70 ± 5% vs. hypoxic control, p = NS) (PI: S-ODN 89 ± 2.9% and Scr-ODN 93 ± 2.6%, vs. hypoxic control, p = NS).

Anti-sense ODN transfection attenuated p70s6 kinase activity

Insulin treatment augmented p70s6 kinase activity by ⩾40% compared with vehicle treated controls ($p < 0.001$)

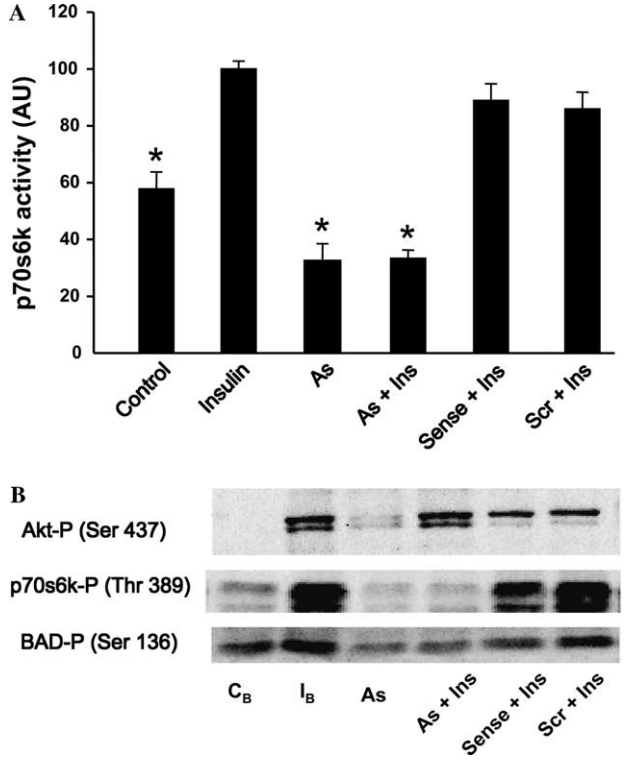

Fig. 5. Activation and phosphorylation status following genetic depletion of p70s6 kinase. (A) Activity of the p70 s6 kinase expressed in arbitrary units (AU) as percentage of insulin group (insulin = 100). Bars represent means ± SEM ($n > 6$ for all time points). *$p < 0.001$ vs. insulin group. (B) Representative Western blot showing the effect of As-ODN treatment on Akt, p70s6 kinase, and BAD phosphorylation.

(Fig. 5A) during post-ischemic reoxygenation. This kinase activation was attenuated to below vehicle control treated cells in the presence of As-ODN (As-ODN 32 ± 5.9% and As-ODN + Ins 33 ± 2.6%, vs. insulin control, $p < 0.001$) (Fig. 5A). Transfection with S-ODN or Scr-ODN did not blunt insulin-activated p70s6 kinase activity (S-ODN + Ins 89 ± 5.6% and Scr-ODN + Ins 86 ± 5.8% vs. insulin control, p = NS). S-ODN and Scr-ODN had no effect on p70s6 kinase activity (S-ODN 72 ± 6.5% and Scr-ODN 60 ± 9.1% vs. control baseline, p = NS, data not shown). As would be expected, the phosphorylation status of p70s6 kinase parallels the in vitro kinase activity and p70s6 kinase phosphorylation is significantly blunted by As-ODN transfection (Fig. 5B). Consistent with the anti-sense target, the phosphorylation status of Akt was not altered by p70s6 kinase attenuation (Fig. 5B). However, insulin's ability to phosphorylate a downstream target, i.e., BAD was significantly attenuated by As-ODN administration (Fig. 5B).

Discussion

The present study demonstrates that insulin administration at reoxygenation protects against ischemia–

reoxygenation injury in girardi cells. The pharmacological inhibition studies implicate PI-3-kinase, Akt, and mTOR in this cell-survival signaling cascade. Finally, the anti-sense studies demonstrate that p70s6 kinase is a functional target of insulin activated cell-survival signaling when administered at reoxygenation in girardi cells.

The role of p70s6 kinase has been extensively studied with respect to cell cycle regulation and in the regulation of ribosomal peptide translation [17,18]. However, this regulatory kinase has only recently been identified as a signaling intermediate in promoting cell survival [14]. This effect is via the phosphorylation and subsequent sequestration of BAD to the cytosol, thereby inhibiting its pro-apoptotic function in the outer mitochondrial membrane. In our study we demonstrate that this signaling kinase is activated in a transformed cell line. In data not shown we have also demonstrated that p70s6 kinase activity is augmented in response to insulin-reperfusion therapy in an isolated rat heart ischemia–reperfusion model. The short duration of reperfusion in the intact perfused heart and the insignificant rate of cell division in this intact heart model diminish the possibility that the cytoprotective effect of p70s6 kinase activation is due to translational or cell-cycle regulatory effects. Our data support the fact that p70s6 kinase promotes cytoprotective signaling events at least, in part, via the phosphorylation and putative sequestration of BAD in the cytosol.

The fact that insulin has profound metabolic modulatory effects that, via PI-3 kinase and Akt, promote glucose uptake and utilization has favored the metabolic modulation paradigm of insulin-mediated cardioprotection. The role of p70s6 kinase in glucose metabolism has not been extensively investigated, however, it is interesting to note that rapamycin administration does not inhibit deoxyglucose uptake in cell culture [19]. This finding is in contrast to the effect of PI-3 kinase and Akt antagonist studies with respect to glucose uptake [20,21]. Hence, this study supports the growing body of literature that supports substrate-independent and direct cell-survival activated signaling in insulin activated cardioprotection when administered at reperfusion [4–6,11].

We have previously demonstrated that insulin administration at reperfusion confers cardioprotective effect within 15 min, as evident by similar cardioprotection whether insulin was present for the first 15 min or for the 2 h duration of reperfusion in the rat heart [6]. Interestingly, in this study we see two distinct temporal periods of phosphorylation of mTOR, p70s6 kinase, and of BAD in the course of prolonged insulin exposure. Biphasic activation of p70s6 kinase is well documented following stimulation by numerous mitogens [22]. With regard to insulin, the acute first phase of p70s6 kinase activation (within minutes) has been shown to be PI 3-kinase and Akt dependent [23]. The later insulin-induced p70s6 kinase activation has been found to be independent of both PI 3-kinase and PKC signaling [22,23]. Taken together, these findings support our pharmacologic data, which support an acute PI 3-kinase dependent activation of p70s6 kinase in conferring the cytoprotective effects of insulin at reperfusion/reoxygenation. The role of the second peak of p70s6 kinase activation has not been explored in the context of hypoxia/reoxygenation. However, in other cell systems prolonged insulin exposure results in the translational induction of GLUT1 and GLUT3 as a putative adaptive process in response to hyperinsulinemia [24,25]. The relevance, if any of this second peak in response to acute hypoxia/reoxygenation, is still unknown.

In our study, it should be noted that the cytoprotective effect of p70s6 kinase attenuation is only partial, and as expected, that insulin is still able to induce Akt phosphorylation following As-ODN transfection. These observations support the fact that p70s6 kinase is not completely degraded using this technique, and thus, the protection is only partially lost. An alternate and equally feasible explanation is that the phospho-Akt activates p70s6 kinase-independent cytoprotective effects including the augmentation of glucose uptake. Moreover, it is currently unknown whether the anti-apoptotic effect of eNOS activation in response to insulin administration is dependent on or independent of p70s6 kinase signaling.

In summary, insulin appears to directly protect girardi cells from hypoxia reoxygenation injury via PI 3-kinase and Akt activated mTOR and p70s6 kinase. These data support the emerging paradigm implicating the activation of direct cell survival signaling programs in response to insulin in mediating reoxygenation/reperfusion cellular protection. Cell survival proteins targeted by p70s6 kinase activation need to be identified as potential modifiers to attenuate reperfusion injury [26].

Acknowledgments

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