c-Jun N-Terminal Kinases Mediate Reactivation of Akt and Cardiomyocyte Survival After Hypoxic Injury
In Vitro and In Vivo

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Abstract—Akt is a central regulator of cardiomyocyte survival after ischemic injury in vitro and in vivo, but the mechanisms regulating Akt activity in the postischemic cardiomyocyte are not known. Furthermore, although much is known about the detrimental role that the c-Jun N-terminal kinases (JNKs) play in promoting death of cells exposed to various stresses, little is known of the molecular mechanisms by which JNK activation can be protective. We report that JNKs are necessary for the reactivation of Akt after ischemic injury. We identified Thr450 of Akt as a residue that is phosphorylated by JNKs, and the phosphorylation status of Thr450 regulates reactivation of Akt after hypoxia, apparently by priming Akt for subsequent phosphorylation by 3-phosphoinositide–dependent protein kinase. The reduction in Akt activity that is induced by JNK inhibition may have significant biological consequences, as we find that JNKs, acting via Akt, are critical determinants of survival in posthypoxic cardiomyocytes in culture. Furthermore, in contrast to selective p38–mitogen-activated protein kinase inhibition, which was cardioprotective in vivo, concurrent inhibition of both JNKs and p38–mitogen-activated protein kinases increased ischemia/reperfusion injury in the heart of the intact rat. These studies demonstrate that reactivation of Akt after resolution of hypoxia and ischemia is regulated by JNKs, and suggest that this is likely a central mechanism of the myocyte protective effect of JNKs. (Circ Res. 2006;98:111-118.)

Key Words: Akt ■ apoptosis ■ c-Jun NH2-terminal kinase ■ hypoxia ■ ischemia ■ signal transduction

The families of stress-activated protein kinases (SAPKs) consist of the c-Jun N-terminal kinase (JNK) family and the p38–mitogen-activated protein kinase (MAPK) family.1 They are potently activated by a number of cellular stresses and produce a number of biological responses that vary by the stimulus and the cell type. These kinases are activated by ischemia (especially p38-MAPKs2) and by reperfusion of ischemic tissues (JNKs and to a lesser extent p38-MAPKs3). Recently, a potent and relatively selective inhibitor of the α and β p38-MAPK isoforms has demonstrated reductions in ischemic injury in animal models of myocardial infarction and stroke.4,5 In addition, overexpression of dominant negative mutants of components of the p38-MAPK pathway in transgenic mice protected hearts from ischemia/reperfusion injury (I/R) injury.6 However, the roles played by the JNKs in ischemic injury are much less clear than those of p38-MAPKs. This is due in large part to the fact that potent and selective inhibitors of the JNKs have only very recently been developed, are not widely available, and, to our knowledge, have not been used to study I/R injury either in vivo or in cultured cardiomyocytes.

In support of a deleterious role for JNKs in ischemic injury, studies in mice in which the JNK3 gene has been deleted and studies with a peptide inhibitor of JNKs demonstrated markedly reduced ischemic injury and excitotoxicity in the brain, a process that is believed to play a major role in neuronal death after I/R injury.7,8 In cultured cells not of neuronal origin, JNKs are capable of transducing proapoptotic signals by phosphorylating 14–3–3 proteins, thereby releasing Bax to translocate to the mitochondria, where Bax mediates release...
of cytochrome c and activation of the mitochondrial death pathway. These data suggest that JNK inhibition could be cardioprotective, an hypothesis supported by a number of studies (reviewed in Manning and Davis). In contrast, other studies clearly suggest that JNKs are capable of transducing proapoptotic signals, but the mechanisms of these pro-survival effects are much less clear than the mechanisms promoting cell death. We used novel small-molecule inhibitors to demonstrate that JNKs are necessary for the full activation of Akt, a key regulator of survival of posts ischemic cardiomyocytes. Consistent with the idea of this effect of JNKs being biologically significant, inhibition of JNK activity leads to increased apoptosis in cultured cardiomyocytes and increased infarct size in vivo. Thus, we have identified a novel mechanism of regulation of Akt and, in so doing, identified a mechanism by which JNKs may transduce proapoptotic signals and mediate survival in the posts ischemic cardiomyocyte.

Materials and Methods

Characterization of Inhibitors

Enzymatic profiles for V-100, V-150, and VX702 are contained in Table I of the online-only Data Supplement, available at http://circres.ahajournals.org. Inhibitor potencies were assessed at Vertex Pharmaceuticals or by Upstate Biotechnology. A complete description of methods used to determine inhibitor potencies can be found in the Data Supplement.

Hypoxia/Reoxygenation Protocol

For studies of signaling pathways, neonatal rat cardiomyocytes or human embryonic kidney (HEK293) cells were placed in serum-free media that had been pre-equilibrated with 5% CO2/95% N2 for 4 hours. Cells were placed in an airtight chamber that was purged at 25 L/min with N2 and were then exposed to 5% CO2/95% N2 for 4 hours. Cells were removed from the chamber and placed in serum-free media that had been pre-equilibrated in air. Cells were harvested at times noted in the Figures and lysates were processed for immunoblotting. For assessment of apoptosis after hypoxia/reoxygenation by TUNEL staining, neonatal rat cardiomyocytes were subjected to 12 hours of hypoxia followed by 20 hours of reoxygenation in serum-free media.

The remainder of the Materials and Methods can be found in the Data Supplement.

Results

Concurrent JNK/p38-MAPK Inhibition Enhances Hypoxia/Reoxygenation-Induced Injury In Vitro

The dual JNK/p38 inhibitor V-150 (supplemental Table I) at a concentration of 5 μM/L significantly inhibited hypoxia/reoxygenation (H/R)-induced activation of the JNKs in neonatal rat cardiomyocytes exposed to 4 hours of hypoxia followed by 1 hour of reoxygenation as determined by immunoblotting for phosphorylation of c-Jun (Figure 1A). Of note, there was a small but consistent increase in c-Jun phosphorylation at low concentrations (~1 μM/L) of the drug, an effect that was ablated at the 5 μM/L concentration (Figure 1A). Time-course studies demonstrated that JNK activation was maximal at 1 hour after reoxygenation, and JNK activity was inhibited at all time points by 5 μM/L V-150 (Figure 1B), the concentration used in all subsequent studies except where noted. The inhibitor also prevented reoxygenation-induced upregulation of c-Jun expression, consistent with the known regulation of induction of the c-jun gene by c-Jun and the stabilization of c-Jun protein that occurs on JNK-mediated phosphorylation of c-Jun (Figure 1B). Activation of p38-MAPKs, as determined by blotting for phosphorylation of the p38-MAPK substrate MAPKAPK-2, was also blocked by V-150 (data not shown).

Given the reported roles of p38-MAPKs and JNKs in H/R-induced injury in cardiomyocytes, we found, to our surprise, that H/R-induced apoptosis was significantly enhanced by dual JNK/p38-MAPK inhibition with V-150. This was true whether apoptosis was assessed by TUNEL staining or immunoblotting for cleavage of caspase 3 (Figure 2).

Mechanisms of Enhanced H/R-Induced Cardiomyocyte Death by Concurrent JNK/p38 Inhibition: Inhibition of Akt Reactivation

To define the mechanisms by which dual JNK/p38 inhibition enhances H/R injury, we investigated the possibility of cross-talk between the JNK/p38-MAPK and Akt pathways, given the latter’s central role in regulation of cardiomyocyte survival after H/R and I/R. We found that hypoxia led to significant inactivation of Akt as determined by immunoblotting for phosphorylation of either Thr308 or Ser473 (Figure 3A and 3B). Reoxygenation induced rapid reactivation of Akt.
This reactivation was markedly reduced in the presence of the dual JNK/p38 inhibitor (Figure 3A and 3B). In addition, phosphorylation of the Akt target GSK-3β was significantly reduced, consistent with reduced activity of Akt in cells treated with the JNK/p38 inhibitor (Figure 3B). Subtle decreases in total Akt were also observed in some of the experiments (Figure 3B), though the effect was less dramatic than that observed on Akt phosphorylation. These data suggest that the stress-activated MAPKs are important regulators of Akt in the setting of H/R.

We next asked whether reduced reactivation of Akt in posthypoxic cardiomyocytes might be important in the enhanced apoptosis seen in V-150–treated cells. We found that gene transfer of a constitutively active myristoylated form of Akt decreased the enhanced apoptosis seen in V-150-treated cardiomyocytes (Figure 4). Thus, the reduced reactivation of Akt when JNKs and p38-MAPKs are inhibited concurrently likely is important for the enhanced apoptosis seen in the V-150-treated cells.

**JNKs Are Required for Maximal Reactivation of Akt After Reoxygenation**

The effects of V-150 on Akt phosphorylation could have been mediated by the JNK and/or the p38-MAPK inhibitory effects of the compound. To dissect the relative contributions of each MAPK to Akt activity after reoxygenation, we used a number of different approaches. First, we used 3 additional compounds. Two of these, SP600125 and V-100, are relatively selective for JNKs versus p38-MAPKs and one, VX702, is highly selective for p38-MAPKs (supplemental Table I).
SP600125 reduced reactivation of Akt at concentrations as low as 2 μmol/L, a concentration identical to that at which inhibition of H/R-induced JNK activation, as determined by inhibition of c-Jun phosphorylation, was seen (Figure 5A). Importantly, this concentration of compound does not significantly inhibit p38-MAPKs (24,25 and data not shown). In contrast, the selective p38-MAPK inhibitor VX702 had no effect on reactivation of Akt after hypoxia/reoxygenation, whereas the JNK inhibitor V-100, like SP600125 and V-150, markedly reduced reactivation of Akt (Figure 5B).

The above studies, using 3 distinct JNK inhibitors, suggested that inhibition of JNKs was primarily responsible for the reduced reactivation of Akt. However, concerns remain about off-target effects of small molecule inhibitors. Therefore, to confirm that the JNKs were the critical regulators of post-H/R reactivation of Akt, we used adenovirus-mediated gene transfer of dominant inhibitory (MKK4[KR])26 and constitutively active (MKK4[ED]) mutants of MKK4, a MEK immediately upstream of the JNKs. Gene transfer of MKK4(KR) reduced reactivation of Akt (Figure 5C, bottom panel). In contrast, expression of MKK4(ED) enhanced basal (Figure 5C, top panel) and H/R-induced (Figure 5C, bottom panel) phosphorylation of Akt. The effect of MKK4(ED) on Akt phosphorylation was blocked by SP600125 (Figure 5C). Thus, using multiple approaches, both directly inhibiting JNKs (inhibition with SP600125 and with V-100, and with gene transfer of MKK4[KR]), and activating JNKs (via gene transfer of MKK4[ED]), we demonstrate that JNKs, but not p38-MAPKs, positively regulate reactivation of Akt after H/R.

We then asked whether the decrease in Akt activation caused by JNK inhibition enhanced apoptosis in cardiomyocytes after H/R. We found that JNK inhibition with V-100 increased H/R-induced cardiomyocyte apoptosis (Figure 5D). In contrast, inhibition of p38-MAPKs with VX702 did not affect apoptosis, compatible with its lack of an effect on reactivation of Akt after reoxygenation (Figure 5B). Combined inhibition of JNKs and p38-MAPKs did not increase apoptosis further (Figure 5D), suggesting the proapoptotic effect of the dual JNK/p38 inhibitor V-150 was likely due to inhibition of JNKs as opposed to p38-MAPKs.

Mechanisms of JNK-Mediated Reactivation of Akt After H/R
We then explored how JNKs regulate reactivation of Akt. Although JNK1 phosphorylated Akt (Figure 6A, top panel), JNK1 did not phosphorylate T308 (Figure 6A, middle panel). This is not surprising, as the consensus JNK phosphorylation site sequence is S/T-P, and T308 is not followed by a Pro residue. We identified 6 candidate JNK phosphorylation sites in Akt. Cardiomyocytes were transduced with adenoviruses encoding GFP (G), MKK4[KR], or MKK4[ED], and then were exposed to normoxia (Control) or H/R, in the presence of SP600125 or vehicle (−). Lysates were immunoblotted for phospho-S473-Akt or total Akt. D. Effect of inhibition of p38-MAPKs, JNKs, or both on H/R-induced cell death. Cardiomyocytes were exposed to H/R either in the presence of vehicle, VX702 (7.5 μmol/L), V-100 (6 μmol/L), or both inhibitors. TUNEL-positive cells are expressed as a percent of total cells. Data are from n=4 experiments. A minimum of 250 cells per condition per experiment were scored. *P<0.05 vs both vehicle-treated and VX702-treated cells. P=NS for V-100–treated vs V-100/VX702—treated cells.
Structs were then incubated with GST-PDK1 (PDK1), GST-JNK1, or GST-PDK1 were incubated with purified kinase inactive Akt (Akt-K719M) or no Akt (-) and γ-[32P]-ATP (100 μmol/L) for 20 minutes at 30°C. Reaction mixtures were run on a gel and subjected to autoradiography. Top 2 arrows identify autophosphorylated GST-PDK1 and GST-JNK1, respectively. Bottom arrow identifies Akt (K719M) phosphorylated by PD1 and JNK1. Middle, Akt was phosphorylated as above and then was immunoblotted for phospho-T308-Akt. Note that although JNK does not phosphorylate T308 (lane 2; compare to PD1 phosphorylation of T308 in lane 3), JNK readily phosphorylates Akt (top panel, lane 5), and this is sufficient to retard mobility of Akt in the gel (middle panel, lane 2 vs lanes 1 and 3). Bottom, Activated GST-JNK1 was incubated with γ-[32P]-ATP (100 μmol/L) and hemagglutinin (HA)-tagged Akt or the HA-tagged mutants which had previously been incubated with protein phosphatase 2a (PP2a). The reaction was allowed to proceed for 20 minutes at 30°C. Anti-HA immunoblot (below autoradiogram) demonstrates equivalent expression of the Akt mutants in the lysates. Despite lower expression, wild-type (WT) Akt is phosphorylated significantly more than Akt(T450A), B, T450 phosphorylation status modulates activity of Akt. HEK293 cells transfected with WT Akt, Akt(T450A), or Akt(T450D) were subjected to hypoxia (4 hours) or hypoxia (4 hours) and reoxygenation (1 hours) followed by immune complex kinase assay using a GSK-3 peptide as substrate (see Materials and Methods). Kinase activity is normalized to the activity of WT Akt control for each experiment, and this activity was given a value of 1. n=3 experiments for each condition. *P<0.01 vs WT and Akt(T450A). **P<0.01 vs Akt(T450A). C, Reactivation of Akt(T450D) after ischemia is resistant to JNK inhibition. HEK293 cells, which were transfected with either HA-tagged WT Akt or HA-tagged Akt(T450D), were subjected to H/R in the presence of V-100 (5 μmol/L) or vehicle. The anti-HA immunoprecipitates were immunoblotted for total Akt and for phospho-Akt (T308 or S473). V-100 reduced reactivation of WT Akt after hypoxia (compare lane 3 vs 4) as determined by phosphorylation of T308 and S473, but had little effect on reactivation of Akt(T450D) (compare lane 7 vs 8). Graph depicts band density of phospho-T308-Akt in H/R conditions normalized to total Akt from n=3 experiments. *P<0.05 vs WT Akt treated with V-100. D, PD1 kinase phosphorylation of T308 of WT Akt vs Akt(T450D). HA-tagged WT Akt or Akt(T450D) were incubated with PP2a for 20 minutes. PP2a was then inactivated with okadaic acid. The Akt constructs were then incubated with GST-PDK1 (PDK1 +) or GST only (PDK1 -) together with ATP (100 μmol/L) for the times noted. Blots were probed for phospho-T308 and total Akt. E, Effect of T450 phosphorylation status on H/R-induced apoptosis. HEK293 cells were transfected with HA-tagged Akt(T450A) or Akt(T450D), and then were exposed to H/R as above. Percent apoptosis was determined for transfected cells (HA positive). The apoptosis rate in cells transfected with Akt(T450D) was <25% of the rate in cells transfected with Akt(T450A). Immunoblots for cleaved caspase 3 and HA (to confirm equivalent expression of the Akt constructs) are below the graph.

Figure 6. Mechanisms of JNK-mediated reactivation of Akt after H/R. A, Phosphorylation of Akt and Akt mutants by JNK1. Top, Purified glutathione S-transferase (GST), GST-JNK1, or GST-PDK1 were incubated with purified kinase inactive Akt (Akt-K719M) or no Akt (-) and γ-[32P]-ATP (100 μmol/L) for 20 minutes at 30°C. Reaction mixtures were run on a gel and subjected to autoradiography. Top 2 arrows identify autophosphorylated GST-PDK1 and GST-JNK1, respectively. Bottom arrow identifies Akt (K719M) phosphorylated by PD1 and JNK1. Middle, Akt was phosphorylated as above and then was immunoblotted for phospho-T308-Akt. Note that although JNK does not phosphorylate T308 (lane 2; compare to PD1 phosphorylation of T308 in lane 3), JNK readily phosphorylates Akt (top panel, lane 5), and this is sufficient to retard mobility of Akt in the gel (middle panel, lane 2 vs lanes 1 and 3). Bottom, Activated GST-JNK1 was incubated with γ-[32P]-ATP (100 μmol/L) and hemagglutinin (HA)-tagged Akt or the HA-tagged mutants which had previously been incubated with protein phosphatase 2a (PP2a). The reaction was allowed to proceed for 20 minutes at 30°C. Anti-HA immunoblot (below autoradiogram) demonstrates equivalent expression of the Akt mutants in the lysates. Despite lower expression, wild-type (WT) Akt is phosphorylated significantly more than Akt(T450A), B, T450 phosphorylation status modulates activity of Akt. HEK293 cells transfected with WT Akt, Akt(T450A), or Akt(T450D) were subjected to hypoxia (4 hours) or hypoxia (4 hours) and reoxygenation (1 hours) followed by immune complex kinase assay using a GSK-3 peptide as substrate (see Materials and Methods). Kinase activity is normalized to the activity of WT Akt control for each experiment, and this activity was given a value of 1. n=3 experiments for each condition. *P<0.01 vs WT and Akt(T450A). **P<0.01 vs Akt(T450A). C, Reactivation of Akt(T450D) after ischemia is resistant to JNK inhibition. HEK293 cells, which were transfected with either HA-tagged WT Akt or HA-tagged Akt(T450D), were subjected to H/R in the presence of V-100 (5 μmol/L) or vehicle. The anti-HA immunoprecipitates were immunoblotted for total Akt and for phospho-Akt (T308 or S473). V-100 reduced reactivation of WT Akt after hypoxia (compare lane 3 vs 4) as determined by phosphorylation of T308 and S473, but had little effect on reactivation of Akt(T450D) (compare lane 7 vs 8). Graph depicts band density of phospho-T308-Akt in H/R conditions normalized to total Akt from n=3 experiments. *P<0.05 vs WT Akt treated with V-100. D, PD1 kinase phosphorylation of T308 of WT Akt vs Akt(T450D). HA-tagged WT Akt or Akt(T450D) were incubated with PP2a for 20 minutes. PP2a was then inactivated with okadaic acid. The Akt constructs were then incubated with GST-PDK1 (PDK1 +) or GST only (PDK1 -) together with ATP (100 μmol/L) for the times noted. Blots were probed for phospho-T308 and total Akt. E, Effect of T450 phosphorylation status on H/R-induced apoptosis. HEK293 cells were transfected with HA-tagged Akt(T450A) or Akt(T450D), and then were exposed to H/R as above. Percent apoptosis was determined for transfected cells (HA positive). The apoptosis rate in cells transfected with Akt(T450D) was <25% of the rate in cells transfected with Akt(T450A). Immunoblots for cleaved caspase 3 and HA (to confirm equivalent expression of the Akt constructs) are below the graph.

Other (T92 and T311) have not been shown to be phosphorylated in situ. Of the remaining 2 residues, T450 is conserved across species, whereas S124 is conserved only in vertebrates. However, both undergo phosphorylation in the cell and both are conserved between Akt1, Akt2, and Akt3. Therefore, we focused on the potential role of T450 and S124 in JNK-mediated reactivation of Akt after H/R.

Phosphorylation of Akt with a T450 to Ala mutation (Akt[T450A]) by JNK1 was approximately 20% less than phosphorylation of wild type Akt (Figure 6A, bottom panels). In contrast, phosphorylation of Akt(S124A) by JNK1 was not significantly reduced. These data suggest that T450 is a potentially relevant JNK target.

We next explored the functional relevance of Akt phosphorylation at T450 on Akt activation after H/R. Although the T450A mutation had no effect on the basal activity of Akt or on the hypoxia-induced decline in Akt activity, reactivation of wild type Akt with reoxygenation was approximately 2-fold greater than that seen with Akt(T450A) (Figure 6B). Furthermore, the phosphomimetic Akt(T450D) mutant exhibited higher basal activity, as well as posthypoxic activity that was approximately 3-fold greater than that of the Akt(T450A) mutant (P<0.01; Figure 6B). In agreement with the kinase activity data, T308 phosphorylation was increased in Akt(T450D) (Figure 6C, compare lane 2 with 6 for control and 4 with 8 for H/R). These data suggest that phosphorylation at T450 plays a role in regulating basal activity of Akt, maintaining activity in the face of hypoxia, and regulating reactivation of Akt with reoxygenation.

If phosphorylation at T450 is mediated by JNK or a JNK-dependent kinase and is important in the reactivation of Akt after ischemia, reactivation of Akt(T450D) after ischemia should be relatively resistant to JNK inhibition, as the D mutation would mimic phosphorylation. This was indeed the
case. Compared with vehicle, JNK inhibition reduced H/R-induced phosphorylation of wild type Akt at T308 by 53% (P<0.05; Figure 6C top panel; Compare lane 3 with lane 4; see bottom panel for quantitation). In contrast, JNK inhibition reduced T308 phosphorylation by only 17% in Akt(T450D) (P=NS; Figure 6C: Compare lane 7 with lane 8 and see bottom panel). Taken together, these data demonstrate that phosphorylation of T450 is important in the reactivation of Akt after hypoxia and suggest that this is mediated by JNKs or a JNK-dependent kinase.

We then explored the mechanism by which phosphorylation of T450 might regulate T308 phosphorylation. The above data suggested that JNK-mediated phosphorylation of T450 might prime Akt for subsequent phosphorylation at T308 by PDK1. A confounding factor in evaluating this hypothesis was the increased baseline level of T308 phosphorylation in the T450D mutant compared with wild type (Figure 6C). To control for these differences in baseline phosphorylation of Akt, immunoprecipitates prepared from cells expressing wild type Akt or Akt(T450D) were dephosphorylated with protein phosphatase 2a (PP2a), reducing T308 phosphorylation in wild type and Akt(T450D) to equivalent and almost undetectable levels (Figure 6D, top panel and lanes 1,3,5,7,9, and 11 of lower panel). Dephosphorylated wild type and Akt(T450D) were then incubated in a reaction mix that included PDK1 and ATP. There were minimal differences between wild type and Akt(T450D) in phosphorylation by PDK1 at 20 minutes, but with 40 and 60 minutes incubations, phosphorylation of Akt(T450D) at T308 was significantly enhanced compared with that of wild type Akt (Figure 6D, bottom panel). These data suggest that phosphorylation of T450 primes Akt for subsequent phosphorylation by PDK1 and that inhibition of T450 phosphorylation by JNK inhibition is, at least in part, responsible for the impaired reactivation of Akt after resolution of hypoxia when JNKs are inhibited.

Finally, to determine the biological significance of T450 phosphorylation, we compared rates of H/R-induced apoptosis in HEK293 cells that had been transfected with HA-tagged Akt(T450A) or Akt(T450D). Apoptosis rates and caspase 3 cleavage were significantly lower in cells transfected with Akt(T450D) compared with cells transfected with Akt(T450A) (Figure 6E). These data indicate that phosphorylation status of T450 not only plays a role in reactivation of Akt after H/R, but also modulates H/R-induced apoptosis.

**Dual JNK/p38 Inhibition Blunts Reactivation of Akt and Enhances I/R Injury In Vivo**

We next asked whether the detrimental effects of dual JNK/p38 inhibition seen in cardiomyocytes exposed to H/R had a correlate in vivo. We confirmed that V-150 blocked I/R-induced activation of JNKs and p38-MAPKs by immunoblotting for phosphorylated targets of the JNKs (c-Jun) and the p38-MAPKs (MAPKAPK-2) (Figure 7A). In addition, the increase in total c-Jun protein typically seen with reperfusion was also blocked by V-150 (Figure 7A). However, it is important to note that although the inhibitor markedly reduced the amount of phosphorylated c-Jun, there remained a level of phosphorylated c-Jun that was elevated compared with control. These data are compatible with marked but incomplete inhibition of JNKs.

We then determined whether dual JNK/p38 inhibition reduced reactivation of Akt after reperfusion. Activity of Akt within the ischemic zone, as determined by immunoblotting for phosphorylation of T308 and S473, was reduced by JNK/p38 inhibition (Figure 7B). When T308 phosphorylation was quantified for ischemia and 45, 60, and 90 minutes of reperfusion (n=3 hearts per condition), all time points showed trends toward less phosphorylation in the V-150–treated hearts, and 90 minutes of reperfusion achieved statistical significance (Figure 7B). However, when the 3 reperfusion time points were grouped or when ischemia and reperfusion were grouped, T308 phosphorylation was significantly reduced in the V-150–treated hearts (P=0.02 and 0.003, respectively). Thus, dual JNK/p38 inhibition with V-150 reduced reactivation of Akt after I/R in vivo.

To determine whether these effects on reactivation of Akt might lead to enhanced I/R injury, rats were subjected to 30 minutes of ischemia followed by 20 hours of reperfusion to determine the effect of dual JNK/p38 inhibition on infarct size. Region at risk, as determined by injection of fluorescent microspheres at the time of the occlusion,23 was similar in the drug- versus vehicle-treated groups (Figure 7C). However, infarct size, expressed as a percent of the region at risk, was significantly greater in the JNK/p38 inhibitor-treated animals (Figure 7C). Furthermore, hemodynamics in the inhibitor-treated animals exposed to ischemia/reperfusion were significantly worse than vehicle-treated animals, consistent with the larger infarct sizes (supplemental Table II), an effect that was not observed when the compounds were dosed in the absence of ischemia (data not shown).

In distinct contrast to dual JNK/p38 inhibition, selective p38-MAPK inhibition with VX702 (Figure 7D) led to a significant reduction in infarct size (Figure 7E and supplemental Table III). These data, when combined with the results of studies in cardiomyocytes in culture, suggest that inhibition of the JNKs, but not p38-MAPKs, resulted in the enhanced injury after V-150 treatment.

**Discussion**

Herein we report that reactivation of Akt after either reperfusion of ischemic cardiomyocytes or reoxygenation of hypoxic cardiomyocytes is regulated by JNKs. In addition, we identify a specific residue of Akt, T450, that is phosphorylated by JNKs, and show that the phosphorylation status of T450 determines, in part, basal activity of Akt, how efficiently Akt is reactivated after ischemia, and cell survival after ischemia. Furthermore, we demonstrate the biological significance of this effect of the JNKs by showing that both dual JNK/p38 inhibition and selective JNK inhibition lead to increased H/R-induced apoptosis, an effect that can be rescued by gene transfer of constitutively active Akt. Finally, we demonstrate the potential pathophysiological importance of this role of the JNKs in vivo by showing that whereas selective p38-MAPK inhibition leads to myocardial protection, concurrent inhibition of JNKs and p38-MAPKs, using a dual inhibitor, leads to enhanced cardiomyocyte loss and worsened hemodynamics after reperfusion of ischemic heart.
JNKs Regulate Reactivation of Akt After Hypoxia or Ischemia

We explored several mechanisms by which JNKs might regulate reactivation of Akt after cellular stress. JNKs readily phosphorylate PDK1, but overexpression of a PDK1 construct mutated at the JNK phosphorylation sites does not significantly alter tumor necrosis factor-α- or oxidant stress-induced activation of Akt (W.H., Z.S., and T.F., unpublished observations, 2005). These data suggest that JNKs do not regulate Akt phosphorylation by targeting PDK1. Furthermore, the T450A mutation did not reduce membrane translocation (and thus colocalization with PDK1) that had been hypothesized earlier28 (Data Supplement). However, we found that JNKs readily phosphorylate Akt (Figure 6A) and that phosphorylation of T450 plays a role in JNK-dependent reactivation of Akt, possibly by priming Akt for subsequent phosphorylation by PDK1. These findings are reminiscent of those in reports demonstrating that phosphorylation of S473 somehow primes Akt for PDK-1-mediated phosphorylation of T308.29,30 Given that both S473 and T450 are localized within the hydrophobic tail of Akt, it appears that phosphorylation of residues in this region may be critical for optimal interaction of PDK1 with Akt. In the setting of ischemia, wherein S473 is significantly dephosphorylated (Figure 3), phosphorylation of T450 may assume greater importance. Although the molecular mechanism by which phosphorylation of T450 primes Akt is not known at this time, it appears that in posthypoxic cells, T450 phosphorylation status is critical in the reactivation of Akt and plays a role in cell survival.

JNKs Regulate Cardiomyocyte Survival After H/R and I/R

Our data suggest that the biological consequences of JNK inhibition and the reduced reactivation of Akt that follows are significant, leading to enhanced cell death in cultured cardiomyocytes and likely accounting for at least part of the increase in infarct size in the heart of the intact rat. Our results are somewhat surprising, given the ability of JNKs to activate the mitochondrial cell death pathway in mouse embryo fibroblasts and in cardiomyocytes.9,18 That said, although several groups using various in vitro models of ischemia have reported that JNKs mediate cell death,18–21 others have reported that JNKs are protective.12–15 In vivo, JNKs appear to protect from pressure overload-induced apoptosis.31 These disparate conclusions may be due not only to the cell type...
chosen and the model chosen to mimic ischemia, but also to the methods used to manipulate activity of the JNK pathway (eg, drugs leading to partial inhibition versus gene knockouts leading to complete inhibition).

This is the first attempt of which we are aware to address the role of JNKs in I/R injury in the heart in vivo using pharmacological inhibitors. Because concurrent inhibition of JNKs and p38-MAPKs using a dual inhibitor was detrimental, whereas selective p38-MAPK inhibition was protective, we conclude that the JNK-mediated activation of pro-survival signals predominates over JNK-mediated activation of pro-death signals in this particular model. The fact that our studies in vivo agree closely with the results of our studies in cultured cardiomyocytes strengthens our conclusion of the potential cardioprotective role played by JNKs. However, until truly selective JNK inhibitors are able to be used in vivo (V-100 cannot be used because of poor bioavailability), one cannot formally exclude the possibility of an off-target effect of V-150 (ie, inhibition of an as yet unidentified kinase) as contributing to the increase in infarct size. In contrast, on the basis of our findings and other published reports, there is support in the literature for the concept that inhibition of p38-MAPKs may be protective in the setting of acute injury and cell death following ischemia-reoxygenation in rat cardiomyocytes. Mol. Cell. Biol. 2001;15:867–874.


References


17. Wang Y, Huang S, Sah VP, Ross J, Brown JL, Han J, Chien KR. Cardiac muscle cell death signals in this particular model. The fact that our studies in vivo agree closely with the results of our studies in cultured cardiomyocytes strengthens our conclusion of the potential cardioprotective role played by JNKs. However, until truly selective JNK inhibitors are able to be used in vivo (V-100 cannot be used because of poor bioavailability), one cannot formally exclude the possibility of an off-target effect of V-150 (ie, inhibition of an as yet unidentified kinase) as contributing to the increase in infarct size. In contrast, on the basis of our findings and other published reports, there is support in the literature for the concept that inhibition of p38-MAPKs may be protective in the setting of acute ischemia and that the antiinflammatory effects of p38-MAPK inhibition may be key.4

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