c-Jun N-terminal Kinase (JNK-1) Confers Protection against Brief but Not Extended Ischemia during Acute Myocardial Infarction*

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Brief periods of ischemia do not damage the heart and can actually protect against reperfusion injury caused by extended ischemia. It is not known what causes the transition from protection to irreversible damage as ischemia progresses. c-Jun N-terminal kinase-1 (JNK-1) is a stress-regulated kinase that is activated by reactive oxygen and thought to promote injury during severe acute myocardial infarction. Because some reports suggest that JNK-1 can also be protective, we hypothesized that the function of JNK-1 depends on the metabolic state of the heart at the time of reperfusion, a condition that changes progressively with duration of ischemia. Mice treated with JNK-1 inhibitors or transgenic mice wherein the JNK-1 gene was ablated were subjected to 5 or 20 min of ischemia followed by reperfusion. When JNK-1 was inactive, ischemia of only 5 min duration caused massive apoptosis, infarction, and negative remodeling that was equivalent to or greater than extended ischemia. Conversely, when ischemia was extended JNK-1 inactivation was protective. Mechanisms of the JNK-1 switch in function were investigated in vivo and in cultured cardiac myocytes. In vitro there was a comparable switch in the function of JNK-1 from protective when ATP levels were maintained during hypoxia to injurious when reoxygenation followed glucose and ATP depletion. Both apoptotic and necrotic death pathways were affected and responded reciprocally to JNK-1 inhibitors. JNK-1 differentially regulated Akt phosphorylation of the regulatory sites Ser-473 and Thr-450 and the catalytic Thr-308 site in vivo. The studies define a novel role for JNK-1 as a conditional survival kinase that protects the heart against brief but not protracted ischemia.

There are more than 1,000,000 cases of acute myocardial infarction (AMI) in the United States each year and many pro-

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3 The abbreviations used are: AMI, acute myocardial infarction; ROS, reactive oxygen species; IPC, ischemic preconditioning; LV, left ventricle; mPTP, mitochondrial permeability transition pore; MRI, magnetic resonance imaging; i.p., intraperitoneal.
ischemic tissue that counters the effects of other metabolite changes and can prevent mPTP opening during reperfusion (17). ROS exert the primary regulation of the mPTP during reperfusion, and it has been proposed that the secondary regulators, particularly calcium and adenine nucleotides, determine the ROS threshold required to open the pore (reviewed in Refs. 3, 17). Akt, ERK, and PKCε have been assigned protective roles, whereas JNK-1 is widely reported to increase reperfusion injury (reviewed in Refs. 9–14, 18). JNK-1 is activated by ROS and has also been assigned pivotal roles in promoting ischemia reperfusion injury of brain, kidney, liver, gastric mucosa, and lung (reviewed in Refs. 18, 19). However, it is also recognized that JNK-1 can be protective in some settings (20, 23–27). Here, we show that JNK-1 provides an essential function in protecting the heart against reperfusion injury if the period of ischemia is brief, but it increases cell death and injury when the period of ischemia is extended. We show that reperfusion after brief ischemia is equally as damaging as reperfusion after prolonged ischemia when JNK-1 is inactive. The effects are mediated at least in part by Akt that is differentially regulated by JNK-1 in a manner determined by the duration of ischemia.

EXPERIMENTAL PROCEDURES

Acute Myocardial Infarction—Male C57B6 and JNK-1 (−/−) mice were from The Jackson Laboratory and were used at age 10–12 weeks. AMI was induced by ligation of the left anterior descending coronary artery with a 4/8 silk suture after thoracotomy. The coronary artery was occluded for 5 or 20 min. Successful coronary artery occlusion was verified by elevation of the ST segment in the ECG. Reperfusion was accomplished by releasing the suture; the chest wall was closed, and the mouse was given 100% oxygen. To inhibit JNK, 30 mg/kg of the mouse was given 100% oxygen. To inhibit JNK, 30 mg/kg of

Determination of Area at Risk and Infarct Size—After reperfusion for 24 h, the coronary artery was religated and Evans blue retrogradely injected by a carotid artery catheter to delineate the area at risk. At the end of the protocol, the heart was excised, frozen, and sectioned perpendicular to the long axis into 1–2-mm portions. The sections were counterstained with 3.0 % nitro blue tetrazolium chloride solution. Each slice was weighed and visualized using a scanner, and the LV area, area at risk, and area of infarction for each slice were determined by computer planimetry using ImageJ software (version 1.57, from the National Institutes of Health). Variability in the measured area at risk was less than 15%.

MRI Imaging Protocol—Mice were anesthetized with pentobarbital. ECG signals were obtained from surface electrodes secured to the two forelimbs for amplification (CP-302; Sable Systems, Henderson, NV) so that the R-wave can be used to generate a trigger pulse for image acquisition. The fidelity of the trigger pulse to the R-wave was continuously monitored during image acquisition with a multichannel digitizing oscilloscope (Hewlett Packard). All imaging was performed on a 4.7-tesla (200 MHz) 40-cm bore magnet interfaced with a Bruker AvanceTM console employing a gradient set with an inner diameter of 70 mm and a maximum gradient strength of 1000 milliteslas/m. MRI acquisitions employed an electrocardiogram-triggered gradient echo flow and compensated cine sequence to obtain orthogonal long axis images. The second orthogonal long axis image was used to plan the subsequent short axis images. A two-dimensional cine-FLASH sequence was used to acquire sets of contiguous, 1-mm-thick, short axis images spanning the heart from apex to base. An echo time of 3.9 ms and repetition times of 10.8–16.7 ms provide for the acquisition of 12 equal phases per cardiac cycle. A 2.56 × 2.56-cm field of view was acquired using a custom Helmholz receiver coil. The inner bore of the RF coil (6 cm long × 2.5 cm in diameter) sufficed to both support the mouse and to center it in the gradient set. To improve the signal to noise ratio, the signal was averaged three times, resulting in an acquisition time for each slice of 4 min. Six to eight 1-mm-thick short axis slices were used to cover the entire heart, resulting in a total scan time of 30–45 min for each mouse at each imaging session.

Echocardiography—High definition echo imaging was implemented using a Visual Sonics, Vevo-770 system (Toronto, Ontario, Canada). Contractile parameters were calculated automatically from short axis images.

Isolation and Culture of Cardiac Myocytes—Neonatal cardiac myocytes were isolated, cultured, and exposed to hypoxia reoxygenation in serum-free medium containing 5 or 0.5 mM glucose as described previously (59–60). Infection with adenovirus (20 pfu/cell) was accomplished as described previously (25).

Assays—Total ROS were measured after treatments using an Oxiselect ROS assay kit from Cell Biolabs, San Diego, as described by the manufacturer. For ATP measurement, left ventricles were rapidly dissected and snap-frozen in liquid nitrogen. Frozen tissues were suspended in 0.4 M perchloric acid, liquidized at 4 °C, and centrifuged. Perchlorate was precipitated with 1.0 M K2HPO4 and re-centrifuged for 5 min, and the supernatant was used for measurement of [ATP] using a luciferase enzyme assay kit (Calbiochem). Glucose was measured in the media using a glucose assay kit from Sigma. Total Akt kinase was assayed in lysates of isolated cardiac myocytes or left ventricle tissue using a PKB kinase assay kit (ImmuneChem Pharmaceuticals Inc., Burnaby, British Columbia, Canada). Briefly, cultured myocytes or snap-frozen left ventricles were solubilized in lysis buffer as described previously (59–61). Akt was immunoprecipitated (i.p.) from lysates and incubated with substrate peptide (RPRAATF-NH2) and 5 μL of ATP at 32 °C for 1 h. The reaction was stopped by adding SDS sample loading buffer and boiling for 5 min. The samples were subjected to Western blot analysis and probed with a specific antibody against the phospho-substrate. Blots were quantified using ImageJ software. Samples were taken before Akt i.p. for protein determination and Western blot analysis; equal amounts of total Akt was used for each immunoprecipitation. The efficiency of i.p. was >90% (data not shown). Antibodies were from Cell Signaling Technologies, Santa Cruz Biotechnology, or R&D Systems, Inc. Kinase assays were performed using a modified kinase assay (26, 59–61). Briefly, myocytes were cultured aerobically or subjected to hypoxia reoxygenation. JNK was immunoprecipitated from cell lysates, and equal amounts of extract were incubated with purified c-Jun, IRS-1, or Akt pro-
tein (0.5 μg each) in kinase buffer with the indicated amounts of ATP for 10 min at 37 °C. Samples were subjected to Western blot analysis and probed with specific antibodies against the phospho-substrates as indicated. Blots were quantified using ImageJ software. Antibodies were from Cell Signaling Technologies, Santa Cruz Biotechnology, or R&D Systems, Inc. Glucose and ATP were measured using kits from Sigma and PerkinElmer Life Sciences, respectively. Death assays, including genomic DNA fragmentation, TUNEL, and Hoechst or DAPI staining have been described previously (59–61). DNA ELISA assays kit and caspase 3/7 assays were from Biomol International. Western blot procedures are described in detail elsewhere (59–61). In some instances, the brightness function of Photoshop was used to improve visualization without changing the relative image density or resolution.

RESULTS

JNK Protects against Acute Injury during Brief Ischemia-reperfusion—Multiple studies have shown that pharmacological inhibition of JNK-1 reduces myocardial infarction and apoptosis in animal models of AMI (13, 21, 22, 24). However, it is not possible to attribute an exclusive death-promoting role for JNK-1 because of other reports that JNK can be protective (20, 24–27). To address the possibility that the role of JNK is determined by the conditions of ischemia prior to reperfusion, we subjected mice to reperfusion following brief or extended ischemia in the presence and absence of JNK-1 inhibitor SP600125 as described under “Experimental Procedures.” As expected, we found that hearts that were reperfused after 5 min of ischemia, a period that has been used to precondition, caused only minor infarction and minimal apoptosis (Fig. 1, a–c). In contrast, when JNK-1 was inhibited during 5 min of ischemia reperfusion, both apoptosis and infarction increased markedly. The opposite results were found when ischemia was for 20 min (Fig. 1, a, top 5 panels, and b–d, left panels). Consistent with most previous reports, we found that JNK-1 inhibition during extended ischemia reduced infarction and apoptosis by about 40% (16, 17). Notably, the mean infarct size and apoptotic indices of hearts subjected to 5 min of ischemia reperfusion in the presence of SP600125 were no different from hearts subjected to 20 min of ischemia reperfusion alone. These results suggest that JNK-1 protects against brief but not extended ischemia.

SP600125 was developed as a selective inhibitor of JNK-1 and is widely used as such in vitro and in vivo (28, 29). In their original article, Bennett et al. (30) reported that SP600125 did not inhibit PKA, ERK, p38, ATF2, or IKK-1 at concentrations that optimally blocked JNK-1. However, a subsequent study using in vitro kinase assays with purified enzymes and substrates reported that SP600125 inhibits multiple other kinases in vitro.

FIGURE 1. JNK protects the heart against brief but not extended ischemia in vivo. C57B6 wild type or JNK-1−/− mice were subjected to 5 or 20 min of ischemia by coronary artery ligation followed by 2 h (for apoptosis) or 24 h (infarction) of reperfusion. JNK was inhibited where indicated by intraperitoneal delivery of SP600125 as described under “Experimental Procedures.” Mice were sacrificed and the hearts excised to quantify area at risk (AAR) and programmed death as indicated. a, representative heart sections treated as indicated and stained with Evans Blue and 2,3,5-triphenyltetrazolium chloride as described under “Experimental Procedures.” Arrows indicate blanched areas of infarction. b, infarct size was quantified from at least six hearts per group *, p < 0.05; JNK-1 inhibitor SP600125 was delivered 30 and 5 min before ischemia and 30 min after reperfusion; PI3K inhibitor LY 24002 was delivered 30 min before ischemia. c, infarct size was quantified from at least five JNK−/− hearts per group; *, p < 0.05. d, representative sections of myocardium after the indicated treatments stained for TUNEL. Pos contrl, positive control; Neg, negative. e, at least 1000 cells were counted from three separate experiments; **, p < 0.01.
including ERK, adenosine monophosphate-regulated kinase, and PKA with similar potency to JNK-1 (31). To confirm that the effects of SP600125 in our model were attributable primarily to JNK-1, mice with ablation of the JNK-1 gene (JNK-1\(^{-/-}\)) were subjected to the same periods of ischemia reperfusion. JNK-1\(^{-/-}\) mice at the age used here (10 weeks) have normal cardiac physiology and function, and previous work has shown that they sustain smaller infarcts with less apoptosis than wild type mice when subjected to severe ischemia reperfusion (24). Lower infarction correlated with significant reductions of phosphorylated c-Jun but no change in JNK-2 expression (24, 32). As shown in Fig. 1 (a, bottom panels, and c), there was minimal infarction or apoptosis in sham-operated JNK-1\(^{-/-}\) mice. In some cases sham-operated JNK-1\(^{-/-}\) hearts displayed small areas of blanching at the apex that may indicate damage without experimental AMI; however, these areas were small and constituted minimal infarction. In contrast, both infarct size and apoptotic indices increased dramatically when mice were subjected to brief or extended ischemia reperfusion. Paradoxically in JNK-1\(^{-/-}\) hearts, 20 min of ischemia caused less infarction and apoptosis than 5 min of ischemia, an unexpected effect that illustrates the extensive influence of JNK-1 on reperfusion injury (discussed further below). This effect is also supported by the inhibitor results; 5 min of ischemia caused more damage than 20 min of ischemia when SP600125 was present in both conditions (Fig. 1, a and b). These results confirm that JNK-1 is primarily responsible for the differential effects of SP600125 on infarct size shown in Fig. 1, a and b, and if indeed SP600125 inhibits other kinases in vivo, these kinases probably do not contribute significantly to the observed phenotype. As a first step to identify possible targets of JNK-1, we also tested the effects of LY 294002, a PI3K inhibitor that blocks Akt, one of the central survival kinases activated during AMI (11, 33). LY 294002 treatment increased infarction after brief ischemia thereby mimicking the effect of JNK-1 inhibition. It also abrogated protection that was conferred by JNK-1 inhibition during extended ischemia (Fig. 1b). These results are consistent with previous studies showing that Akt confers significant protection against extended ischemia reperfusion (1, 9, 11, 33). To our knowledge, the effect of PI3K inhibition on infarction subsequent to brief ischemia has not been previously reported, and our observation that LY 294002 mimics the effect of SP600125 as well as the phenotype of the JNK-1\(^{-/-}\) mice suggests common and perhaps interactive roles for JNK-1 and Akt pathways in protecting the heart against brief ischemia reperfusion injury.

Long Term Consequences of JNK-mediated Regulation of Infarction—The results described in Fig. 1 constitute the first evidence that brief ischemia generates extensive acute injury when JNK-1 is inhibited. To determine whether this injury progresses to adverse long term remodeling, hearts were subjected to ischemia reperfusion in the presence and absence of SP600125 as described in Fig. 1, and cardiac functions were analyzed by echocardiography and MRI 2 weeks after ischemia. Echo results are shown in Fig. 2, and MRI videos are shown supplement 1. Echo and MRI results were consistent, and both indicated dilated cardiomyopathy with severe contractile dysfunction of hearts that were subjected to 20 min of ischemia alone or 5 min of ischemia with JNK-1 inhibitor. The pathology was abrogated in the 20-min ischemia group by JNK-1 inhibition and was absent or muted in the 5-min ischemia vehicle group consistent with the results of acute injury described in Fig. 1. MRI quantification revealed a dramatic &gt;50% loss of ejection fraction in hearts subjected to 20 min of ischemia alone or 5 min of ischemia with JNK-1 inhibitor, with no significant difference between the groups (Table 1). Echocardiography revealed significantly decreased intraventricular septal thickness and increased LV internal diameters of hearts subjected to 20 min of ischemia alone or 5 min of ischemia with the JNK-1 inhibitor, again with no significant difference between the groups (Table 2). We observed small changes in contractile parameters and wall dimensions in the hearts of mice caused by 5 min of ischemia alone relative to unoperated controls. These changes were small compared with the same conditions when JNK-1 was inactive but may reflect minor myocardial injury sustained by brief ischemia in this model. The results demonstrate for the first time a profound role for JNK-1 in preventing

### TABLE 1

| Treatment                        | EDV (mm\(^3\)) | ESV (mm\(^3\)) | EF (%)  |
|----------------------------------|----------------|----------------|--------|
| No ischemia                      | 44.07          | 8.67           | 80.12  |
| Ischemia for 5 min               | 51.53          | 17.11          | 66.80  |
| Ischemia for 20 min              | 97.97          | 66.12          | 32.51* |
| Ischemia for 5 min + JNK inhibitor| 73.50          | 45.66          | 37.88* |
| Ischemia for 20 min + JNK inhibitor| 54.85          | 14.60          | 73.38  |

* Data are significantly different from respective values without JNK inhibitor (p &lt; 0.05).

### TABLE 2

| Treatment                        | IVSd (mm) | IVSs (mm) | LVIDd (mm) | LVIDs (mm) |
|----------------------------------|-----------|-----------|------------|------------|
| Ischemia for 5 min               | 0.62      | 1.12      | 3.38       | 2.01       |
| Ischemia for 20 min              | 0.37      | 0.66      | 4.13       | 3.23       |
| Ischemia reperfusion for 5 min + JNK inhibitor| 0.47      | 0.86      | 4.08       | 2.73*      |
| Ischemia reperfusion for 20 min + JNK inhibitor| 0.59      | 1.09      | 3.55       | 2.04       |

* Data are significantly different from respective values without JNK inhibitor (p &lt; 0.05).
infarction and negative remodeling following brief ischemia but in promoting injury when ischemia is prolonged. Many previous reports have shown that the brief period of ischemia used in these studies does not produce irreversible injury or infarction, although in many species it initiates myocardial stunning (34–36). Our observation that inhibition of JNK-1 during episodes of brief ischemia/reperfusion results in myocardial injury, which is as severe as that caused by extended ischemia when JNK-1 is active, constitutes a new and potentially important role for JNK-1 both in protecting the heart against brief ischemia and regulating the degree of infarction. Paradoxically, we found that when JNK-1 was inhibited under both conditions, brief ischemia caused even greater injury than extended ischemia.

**JNK Protects Cardiac Myocytes against Hypoxia Reoxygenation When Glucose Is Sustained**—Glucose is rapidly consumed during ischemia, and high energy phosphates, including ATP, decline rapidly during the first 20 min (37–41). To determine whether energy level may be a causal factor in reversing the function of JNK-1, we used an in vitro model of ischemia reperfusion wherein isolated cardiac myocytes are subjected to hypoxia reoxygenation. In a previous study, we reported that activated JNK-1 was protective when glucose levels were sustained before reoxygenation in this model (25). In contrast to our results, Hreniuk et al. (42) exposed cardiac myocytes to hypoxia reoxygenation in the absence of glucose and reported that activated JNK-1 increased death. To determine whether the difference between these results was caused by glucose availability, we quantified cardiac myocyte survival after hypoxia and reoxygenation in the presence of physiological (5 mM) or low (0.5 mM) glucose concentration. These conditions simulate energy levels after brief and extended ischemia when glucose and ATP are sustained or depleted, respectively. Simultaneously, JNK-1 was inhibited with a dominant negative JNK-1 (MKK4 KD) adenovirus (Ad-dnJNK) or SP600125. Results are shown in Fig. 3. Consistent with the results obtained in vivo, we found that reoxygenation caused only minimal cell injury when glucose was sustained during hypoxia, but there was extensive injury when glucose was depleted. Also consistent with the in vivo results, inhibition of JNK-1 was protective when glucose was depleted but exacerbated injury when glucose was sustained. The effect is dramatically illustrated by DNA fragmentation (Fig. 3, a and b) and TUNEL assays (Fig. 3, c and d). These results were confirmed in multiple death assays, including the activities of caspases 3/7 and 9 (Fig. 3e and supplement 2), and implicate glucose availability and/or ATP as regulating the death function of JNK-1 in this model. The results also suggest that glucose availability accounts for the differences between our earlier results and those of Hreniuk et al. (42).

Changes in glucose and ATP during hypoxia reoxygenation are shown in Fig. 4, a–c. In cultures with starting physiological glucose, the fractional change in ATP was small, and the concentration of both ATP and glucose remained high at the time of reoxygenation (Fig. 4, a and b). In contrast, when the starting glucose was low, glucose in the media was depleted within 4 h, and intracellular ATP declined in a biphasic manner to ~10% of the starting level after 8 h of hypoxia (Fig. 4b). Therefore, in the in vitro model, the switch in the function of JNK-1 from protective to injurious occurs after significant loss of glucose and ATP. For comparison, Fig. 4c presents ATP levels measured in the left ventricles of mouse hearts during progressive ischemia in vivo. Five minutes of ischemia caused only a minor decrease of ATP, but after 20 min ATP concentrations were <20% of the starting level. These results are consistent with previous estimates of ATP levels during ischemia (37–41). Therefore, the switch of JNK-1 function from protective to injurious occurs in vitro and in vivo when ATP concentrations decreased to <25% of the nonischemic level. To address in more detail the relationship between JNK-1 function and glucose/ATP, cultures with low starting glucose were supplemented with glucose at 4 or 7 h of hypoxia (arrows in Fig. 4b), and death assays were performed on cells infected with adenoviral vectors expressing GFP or dnJNK. Inhibition of JNK-1 during reoxygenation was protective (i.e. JNK-1 was injurious) when glucose was supplemented at 7 h but not at 4 h of hypoxia. Therefore, the switch of JNK-1 function occurs after glucose depletion (4 h of hypoxia in the low glucose media) and coincides with >70% loss of ATP in vitro and in vivo.

**Reperfusion ROS and JNK Activity in Vitro**—To investigate the mechanism(s) for the differential regulation of cell death by JNK-1, we measured ROS production and JNK-1 activity in cardiac myocytes exposed to hypoxia reoxygenation. In vivo, ROS are generated during the first few minutes of reperfusion, and the level of ROS production increases with prolonged ischemia (34, 35, 44). As shown in Fig. 5a, ROS production peaked between 10 and 30 min of reoxygenation and declined close to base line at 2 h. ROS were quenched by treatment with N-acetylcysteine, and significantly more ROS were generated in the glucose depletion conditions, a result that is consistent with extended ischemia reperfusion in vitro (44). JNK-1 activation as determined by phospho-JNK-1 was significantly greater in glucose-depleted cultures at 10 min and 1 h of reoxygenation, and SP600125 had only a small effect on phospho-JNK-1 consistent with its mode of inhibition by competing for ATP binding to JNK-1 downstream of MKK4/7. Also consistent with this, c-Jun phosphorylation was greater in the glucose-depleted condition and was inhibited >80% by SP600125 (Fig. 5c).

**Differential Regulation of PI3K-Akt-GSK3β by JNK**—In our in vivo studies, we found that the effects of JNK inhibition during brief ischemia were mimicked by inhibition of PI3K with LY294002 (Fig. 1). We also found that inhibition of GSK3β with the selective inhibitor SB415285 protected against infarction following extended ischemia when JNK was inhibited during brief ischemia (see supplement 3). These results suggest a mechanism wherein activated JNK-1 facilitates Akt activation and phosphorylation of GSK3β in the glucose/ATP-sustained condition but inhibits the same pathway when glucose/ATP are depleted. To further investigate whether JNK-1 differentially regulates Akt, we measured phospho-Akt and total Akt activity in cardiac myocytes at 15 min and 1 h reoxygenation ± glucose and ± JNK-1 inhibitor. Fig. 6a shows that glucose availability indeed influences the effect of JNK-1 on the phosphorylation of Akt-Thr-308. When cultures with sustained glucose were reoxygenated, Akt-Thr-308 phosphorylation was reduced by dnJNK. In contrast Akt-Thr-308 phosphorylation was enhanced by dnJNK under conditions of glucose depletion. The
same effect was seen for total Akt activity when JNK-1 was inhibited by SP600125 (Fig. 6c). When glucose was sustained, JNK-1 inhibition prevented Akt activation, but when glucose was depleted, SP600125 enhanced Akt activation. The differential effect of JNK-1 on Akt was very significant; JNK-1 inhibition in the glucose-depleted condition increased Akt-Thr-308 phosphorylation by 2.8-fold at 15 min of reoxygenation, whereas JNK-1 inhibition in the glucose-sustained condition reduced Akt-Thr-308 phosphorylation by 1.47-fold during the same time period. The same trends were seen for total Akt activity. Therefore, JNK-1 differentially regulates Akt activity in a glucose/ATP-dependent manner during reoxygenation. The effect was independent of the JNK-1 inhibitor used and paralleled the results on cell injury.

**JNK-1 Targets IRS-Ser-307**—It has been reported that JNK-1 can regulate Akt activity negatively or positively by respectively targeting IRS-1-Ser-307 or Akt-Thr-450 (23, 45, 46). IRS-1-Ser-307 negatively regulates PI3K and is phosphorylated by JNK-1 and S6 kinase as part of the negative feedback regulation of insulin signaling. To investigate a possible role...
for JNK-1-mediated suppression of Akt through IRS-1, cardiac myocytes were infected with adenoviral vectors expressing GFP or constitutively active JNK-1 as described under “Experimental Procedures.” As indicated in Fig. 7a, insulin-mediated Akt phosphorylation was reduced by >50% in Ad-caJNK-1-infected cells in parallel with about a 2-fold increase of IRS-1-Ser-307 phosphorylation. Therefore, activated JNK-1 exerts significant regulation of insulin-mediated Akt activity in cardiac myocytes. To determine whether IRS-1 phosphorylation by JNK-1 is regulated by glucose/ATP, cardiac myocytes were subjected to hypoxia reoxygenation as described above, and JNK-1 was inhibited with Ad-dnJNK or SP600125. These results are shown in Fig. 7 (b and c). IRS-1-Ser-307 was phosphorylated during reoxygenation of both glucose-sustained and glucose-depleted cultures, and in both cases the phosphorylation was blocked by inhibition of JNK-1. There were no significant differences in the levels of IRS-1-Ser-307 phosphorylation during reoxygenation of cultures in either condition (p > 0.05; n = 4). Consistent with the results described in Fig. 6, we found that JNK inhibition in the glucose-depleted but not glucose-sustained condition was associated with enhanced phosphorylation of Akt-Thr-308; however, differential regulation of IRS-1-Ser-307 cannot account for this difference. No other Ser/Thr sites on IRS-1 have been reported to be targets for JNK. As discussed above, Shao et al. (23) reported that JNK-1 phosphorylates Akt on Thr-450 conferring positive regulation of Akt. Therefore, we quantified Akt-Thr-450 phosphorylation during hypoxia reoxygenation in the presence and absence of JNK inhibitors as described above for Akt-Thr-308. In data not shown, we were unable to observe the same glucose-dependent regulation of Akt-Thr-450 as was observed for Akt-Thr-308. This may be because of high background signals seen for the Thr(P)-450 site that suggested phosphorylation by kinases other than JNK-1 (data not shown). We have not yet determined the mechanism for the JNK-1-induced increase of activity of Akt in the glucose-sustained condition.

JNK Differentially Regulates Akt in Vivo—To investigate whether Akt and IRS-1 are components of the switch in vivo, we again measured the phosphorylation of IRS-1-Ser-307, Akt-Thr-450, and their downstream effectors Akt-Thr-308 and GSK3β-Ser-9 during ischemia reperfusion in the presence and absence of SP600125. These results are shown in Fig. 8. JNK-1 inhibition decreased the phosphorylation of IRS-1-Ser-307 after both brief and extended ischemia/reperfusion confirming that this site is a JNK-1 target in vivo under both conditions. In this case, JNK-1 inhibition caused a marked decrease in the phosphorylation of Akt-Thr-450 but only when reperfusion followed brief ischemia. This suggests that JNK-1 differentially regulates Akt-Thr-450 phosphorylation in a time-of-ischemia-dependent manner and may contribute to the functional JNK switch in vivo. Differential regulation of Akt signaling was also reflected in the phosphorylation of Akt-Thr-308 and GSK3β-Ser-9. In agreement with the results in vitro, phosphorylation of Akt-Thr-308 and GSK3β-Ser-9 were both decreased by JNK-1 inhibition when ischemia was for 5 min but markedly increased when ischemia was for 20 min. The latter effect may be attributed at least in part to relief from inhibition by IRS-1-Ser(P)-307. Therefore, the positive regulatory Akt-Thr-450 site is phosphorylated by JNK-1 only when ischemia is of brief duration, but the negative IRS-1-Ser-307 site is phosphorylated under both conditions. To confirm that Akt is differentially regulated by JNK-1 and glucose, we measured total Akt activity in left ventricles following brief or extended ischemia in the presence and absence of SP600125 as described under “Experimental Procedures.” These results are shown in Fig. 8c. Consistent with the results shown in Fig. 8a, Akt activity was decreased when JNK-1 was inhibited during brief ischemia but markedly increased when JNK-1 was inhibited during extended ischemia. In both cases, inhibition of JNK with SP600125 resulted in >80% decrease in the phosphorylation
of c-Jun. These results confirm differential glucose/ATP-dependent targeting of the Akt pathway by JNK-1.

Mechanism for the Switch in JNK Function—Fig. 9 illustrates the mechanism that we propose for the time-of-ischemia-mediated switch in the function of JNK-1 in vivo. ROS production is at its highest during the first few seconds of reperfusion but lasts for several hours (34, 35, 44). Intracellular calcium levels also increase. MAPKs (JNK/ERK/p38) and Akt and PKC pathways are activated by signals associated with reperfusion (1–4). JNK-1 is stimulated by ROS, and activated JNK-1 also remains elevated in cardiac cells for several hours after reperfusion. ATP loss from the mouse heart is extensive when the period of ischemia is 20 min or longer (Fig. 4c) (3, 4). Our studies reveal that in this condition JNK-1 blocks the activation of Akt at least in part by phosphorylating IRS-1-Ser-307. Akt regulates multiple survival pathways that collectively repress mitochondrial death channels and its inactivation by JNK after extended ischemia results in the loss of this protection. Inhibition of Akt by JNK-1 in this setting may account for as much as 50% of reperfusion-mediated infarction (21, 22). When the period of ischemia is brief, ROS and kinase pathways are still activated by reperfusion, but ATP levels are maintained, and the role of JNK-1 is reversed. In this setting, JNK-1 appears to be required for the activation of Akt, and its inhibition promotes infarction. The results suggest that at least part of the mechanism of the switch in function of JNK-1 involves selective phosphorylation by JNK-1 of Akt-Thr-450 that occurs.
after brief but not extended ischemia. Although we have shown that Akt-Thr-450 phosphorylation correlates with enhanced Akt activity and cardioprotection in vivo, we have not yet directly demonstrated cause and effect. Results in vitro suggest that other pathways in addition to Akt-Thr-450 could be involved in the glucose/ATP-dependent switch in the function of JNK-1.

**DISCUSSION**

Evidence is presented that c-Jun N-terminal kinase-1 is required to protect the heart against lethal reperfusion injury following brief but not extended ischemia. We found that reperfusion following brief ischemia was equally as damaging as that caused by extended ischemia when JNK-1 was inhibited pharmacologically or by ablation of the JNK-1 gene. The increased injury caused by JNK inactivation was acutely evident with extensive apoptosis and tissue infarction and chronically with negative remodeling, contractile dysfunction, and rapid progression to dilated cardiomyopathy and heart failure. The latter results are dramatically illustrated by contractile parameters (Tables 1 and 2) and MRI images of contracting hearts (supplement 1). In each case, similar results were obtained whether JNK-1 was inactivated genetically or pharmacologically confirming a key role for JNK-1. Paradoxically, we found that brief ischemia reperfusion of JNK-1−/− mice caused sig-
**JNK Protects against Brief Ischemia**

**FIGURE 9. Proposed pathway for the functional switch of JNK-1 during ischemia.** IRS-1 Ser-307 and Akt Thr-450 are both targets for JNK. When ischemia is brief, both are phosphorylated during reperfusion, and the positive Akt-Thr-450 site preserves Akt activity and confers protection. Phosphorylation of Akt-Thr-450 is dependent on the time of ischemia and glucose/ATP availability. Akt activity decreases when reperfusion follows extended ischemia because IRS-1 Ser-307 but not Akt Thr-450 is phosphorylated by JNK-1. Akt regulates the mPTP and possibly the outer mitochondrial membrane channel (OMC) through multiple substrates and determines cell fate and infarction.

Significantly greater infarction and levels of apoptosis than those caused by extended ischemia (Fig. 1c). The same result was seen when JNK-1 was inhibited pharmacologically. The mean infarct size of hearts subjected to 5 min of ischemia in the presence of SP600125 was 37.5 ± 8% compared with 22 ± 7% for hearts subjected to 20 min of ischemia also in the presence of SP600125 (p < 0.05). This again illustrates the powerful regulation exerted by JNK-1 over myocardial injury and the consequences of the loss of protection. Five minutes of ischemia when JNK-1 was inactive caused the same amount of injury as that caused by 20 min of ischemia with active JNK-1. The 40% infarct reduction conferred by inhibition of JNK-1 during extended ischemia is consistent with previous studies wherein JNK-1 was inhibited by an ATP competitive inhibitor (AS601245), a peptide inhibitor (DJNK1-1), or by ablation of the JNK-1 gene (21, 22, 24). The results appear to be at variance with the results of Shao et al. (23). The latter group reported that JNK-1 enhanced Akt activation by direct phosphorylation of Akt-Thr-450. Shao et al. (23) subjected rats to 30 min of ischemia reperfusion in the presence and absence of a dually selective JNK-1/p38 inhibitor (V-150) and reported significantly larger infarcts when the inhibitor was present. They concluded that the protection was mediated by JNK-1 because a p38 mono-selective inhibitor was not protective. However, in light of our data as well as that of others using JNK-1 mono-selective inhibitors or JNK-1 gene deletion (21, 22, 24, 25), it seems possible that the increased injury associated with V-150 results from the combined effect of JNK-1 and p38 inhibition, and JNK-1 only protects against reperfusion injury when the ischemic period is brief. It is noteworthy that p38 was not inhibited by AS601245, DINK1-1, or SP600125 in vivo (21, 22), and these inhibitors were each shown to be protective in models of AMI similar to that used by Shao et al. (23).

During brief ischemia, we found that the increased levels of infarction and apoptosis caused by JNK inhibition were mimicked by inhibition of PI3K with the selective inhibitor LY294002 and significantly reversed by inhibition of GSK3β with SB415285. These results again highlight the importance of the Akt survival pathway in protecting the heart against brief as well as extended ischemia and implicate this activity as part of the mechanism of the switch in function of JNK-1. Akt is activated during reperfusion and has multiple targets associated with cell survival and growth (47). The survival-related targets include BCl-2 and FOXO family proteins, caspase 9 and GSK3β (reviewed in Refs. 1, 2, 47). Previous reports have shown that inhibition of GSK3β blocks mPTP opening during ischemia reperfusion and can significantly reduce infarction (43). Our observation that LY294002 mimicked and SB415285 countered the injurious effects of JNK inhibitor on brief ischemia reperfusion suggests that JNK-1 targets PI3K-Akt signaling and confers positive regulation when ischemia is brief. This possibility is supported by the results shown in Fig. 8. We found that the phosphorylation of Akt-Thr-308, Akt-Thr-450, and GSK3β-S9 were all reduced when JNK-1 was inhibited during brief ischemia reperfusion, but these same targets were more highly phosphorylated when JNK-1 was inhibited during extended ischemia reperfusion. IRS-1 Ser-307 was phosphorylated in a JNK-1-dependent manner during reperfusion under both conditions. These results provide a possible mechanism for the ischemia-dependent switch in the function of JNK-1 in vivo (see Fig. 9). When the ischemic period is short, Akt-Thr-450 and IRS-1 Ser-307 are both phosphorylated by JNK-1, and the net effect on Akt-Thr-308 and GSK3β is positive. In this case, inhibition of JNK-1 reduces Akt activation during reperfusion because Akt-P T450 dominates over IRS-1 Ser(P) T307. In contrast, when ischemia is extended, IRS-1 Ser-307 but not Akt-Thr-450 is phosphorylated by JNK-1, and the net effect on Akt activity is negative. In this case, inhibition of JNK-1 increases Akt activation during reperfusion by blocking the phosphorylation of IRS-1 Ser-307 and relieving suppression on PI3K. We do not know why Akt-Thr-450 is only phosphorylated under conditions of brief ischemia reperfusion; however, changes in cellular metabolites, low ATP and pH, that accompany long duration ischemia may influence the kinetics of kinase reactions as well as the activities of phosphatases (48).

Our *in vitro* results using hypoxia reoxygenation of cardiac myocytes under glucose-sustained and glucose-depleted conditions closely parallel the results *in vivo*. The conditions were chosen to mimic the bioenergetic state of brief and extended ischemia where glucose and ATP are sustained or depleted, respectively. Consistent with the results *in vivo*, we found that JNK-1 was protective when glucose and ATP were sustained during hypoxia but injurious when glucose and ATP were depleted. The switch in function of JNK-1 was demonstrated in multiple death assays and was independent of the mechanism of JNK inhibition by SP600125 or Ad-dnJNK-1 (Fig. 3 and supplement 2). These results are consistent with regulation of mitochondrial death channels by differential targeting of the PI3K-Akt-GSK3β pathway. This possibility was further supported by direct quantification of the phosphorylation levels of Akt and IRS-1. We found that IRS-1 was phosphorylated equally in a JNK-1-dependent manner irrespective of glucose availability (Fig. 7). However the degree of Akt activation and the phosphorylation levels of Akt Thr-308 and Akt Ser-473 were regulated by JNK-1 in a glucose-dependent manner (Figs. 6 and 7). When glucose was sustained during hypoxia, inhibition of JNK-1 decreased Akt phosphorylation and total Akt

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*4 K. Webster, unpublished observations.*
activity, whereas JNK-1 inhibition had the opposite effect when glucose was depleted. These results are again consistent with the results obtained in vivo and implicate competition between negative and positive regulation of Akt by JNK-1. We have not yet been able to confirm a role for differential JNK-1-dependent phosphorylation of Akt-Thr(P)-450 in vitro.

Our observation that the injury cause by brief ischemia of JNK-1−/− mice was even greater than that caused by extended ischemia is novel and deserves additional comment. It indicates that reperfusion injury is the principal component of infarction in this model and illustrates again the critical roles played by survival kinases. This unique phenomenon occurs because JNK-1 inactivation confers a loss of survival function when ischemia is brief but a gain of survival function when ischemia is extended. As such, the JNK−/− myocardium subjected to reperfusion after brief ischemia is highly compromised, whereas the same heart reperfused after extended ischemia is protected. In the former case, JNK-1 blocks the death pathways whereas in the latter case JNK-1 is an essential part of the death program. We propose that JNK-1 inactivation decreases the thresholds of ROS/calcium that are required to open the mPTP after brief ischemia such that signals that would normally initiate reversible stunning are sufficient to open the mPTP and cause infarction. Conversely, when ischemia is extended, JNK-1 inactivation increases the ROS/calcium thresholds required for mPTP opening, thus conferring protection. During extended ischemia, JNK-1 may be required for efficient mPTP opening, perhaps to counter the inhibitory effects of low pH or other biochemical changes associated with extended ischemia. We further propose that the switch of JNK-1 function is caused by altered substrate affinities determined by the cellular microenvironments associated with brief versus extended ischemia. One such candidate for this differential regulation is Akt-Thr-450.

Ischemia reperfusion injury involves necrosis and apoptosis, and our results suggest that both death pathways are differentially regulated by JNK-1. Multiple pathways that determine death/survival are activated during ischemia and reperfusion and are likely to contribute in parallel with or alternatively to the Akt pathway. Early studies described antagonism between JNK-1 and ERK/p38 survival pathways (49). JNK-1 has multiple substrates; it can promote apoptosis by inactivating BCI-2 (50) or the BH3-only proteins BID, Bim, and Bmf (51, 52). JNK-1 and Akt can cooperate by phosphorylating BAD on Thr-201 or the BH3-only proteins BID, Bim, and Bmf (51, 52). JNK-1 and Akt both phosphorylate caspase 9 and 14-3-3 proteins with opposing consequences (53–56). Differential regulation of GSK3β may affect apoptosis as well as necrosis through the GSK3β-mediated phosphorylation and stabilization of Mcl-1 (57). Therefore, JNK-1 can deliver both survival and death signals that may complement or antagonize Akt and other survival pathways. Our results suggest that metabolite levels, in particular the bioenergetic state of the post-ischemic myocardium, determine the cross-talk between JNK-1 and Akt causing a switch in the function of JNK-1, and this is a major contributing factor to the outcome of AMI. The switch occurs when ATP levels decline below a threshold. These effects may be clinically relevant; energy turnover in the human heart is more gradual than in the mouse, and longer periods of ischemia are tolerated before lethal injury. The mean time to reperfusion of patients with AMI is 3–4 h, and depending on the degree of ATP conservation, JNK-1 may provide critical protection from reperfusion injury during this period (58). Our results indicate that caution is warranted in the use of JNK-1 inhibitors for treatments of AMI.

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