A peptide inhibitor of c-Jun NH2-terminal kinase reduces myocardial ischemia-reperfusion injury and infarct size in vivo

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Milano, G. and Bonny, C. and Samaja, M. and von Segesser, L. and Nicod, P. and Vassalli, G. A peptide inhibitor of c-Jun NH2-terminal kinase reduces myocardial ischemia-reperfusion injury and infarct size in vivo. Am J Physiol Heart Circ Physiol 292: H1828–H1835, 2007. First published December 8, 2006; doi:10.1152/ajpheart.01117.2006.—The c-Jun NH2-terminal kinase (JNK) pathway of the mitogen-activated protein kinase (MAPK) signaling cascade regulates cell function and survival after stress stimulation. Equally robust studies reported dichotomous results suggesting both protective and detrimental effects of JNK during myocardial ischemia-reperfusion (I/R). The lack of a highly specific JNK inhibitor contributed to this controversy. We recently developed a cell-penetrating, protease-resistant peptide inhibitor of JNK, D-JNKI-1. Here we report on the effects of D-JNKI-1 in myocardial I/R. D-JNKI-1 was tested in isolated-perfused adult rat hearts. Increased activation of JNK, p38-MAPK, and extracellular signal-regulated kinase-1/2 (ERK1/2), as assessed by kinase assays and Western blotting, occurred during I/R. D-JNKI-1 delivered before onset of ischemia prevented the increase in JNK activity while not affecting ERK1/2 and p38-MAPK activation. JNK inhibition reduced ischemic injury, as manifested by increased time to contracture (P < 0.05) and decreased left ventricular end-diastolic pressure during ischemia (P < 0.01), and enhanced posthypoxic recovery of systolic and diastolic function (P < 0.01). D-JNKI-1 reduced mitochondrial cytochrome-c release, caspase-3 activation, and the number of apoptotic cells determined by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (P < 0.05), indicating suppression of the mitochondrial machinery of apoptosis. D-JNKI-1 delivered at the time of reperfusion did not improve functional recovery but still prevented apoptosis. In vivo, D-JNKI-1 reduced infarct size after coronary artery occlusion and reperfusion by ~50% (P < 0.01). In conclusion, D-JNKI-1 is an important compound that can be used in preclinical models to investigate the role of JNK signaling in vivo. Inhibition of JNK during I/R is cardioprotective in anesthetized rats in vivo.

myocardium; mitogen-activated protein kinases; apoptosis

THE MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) play fundamental roles in cell function, growth, differentiation, and stress responses by transmitting extracellular signals from the cell membrane to nuclear and cytoplasmic targets. The MAPK signaling cascade consists of at least three distinct kinase pathways that lead to the activation of extracellular signal-regulated kinase (ERK), p38-MAPK, and c-Jun NH2-terminal kinase (JNK). The JNK pathway is primarily activated by environmental stress and comprises a series of successively acting kinases. An upstream MAPK kinase kinase, MEKK-1, phosphorylates two MAPK kinase, MKK-4 and MKK-7, which directly phosphorylate JNK, which, in turn, phosphorylates a number of cellular targets, including c-Jun.

Previous studies of JNK activation in myocardial ischemia-reperfusion (I/R) have provided conflicting results. Several studies in isolated cardiomyocytes (20, 26), isolated-perfused rat hearts (10, 18, 22, 30), and rat hearts in vivo (14) suggested that activation of JNK occurs specifically on reoxygenation-reperfusion. However, other studies in isolated rat hearts (31) as well as in rat and rabbit hearts in vivo (24, 28) suggested that ischemia by itself can activate the JNK pathway. Likewise, the role of JNK in myocardial I/R remains controversial, since equally robust studies have reported dichotomous results suggesting both cardioprotective (1, 12, 13, 27) and detrimental (2, 15, 16, 19, 21, 25) effects.

Curcumin, the inhibitor of JNK employed in most previous studies, has antioxidant activities that extend beyond JNK inhibition (7). Thus lack of a highly specific pharmacological inhibitor has hampered the elucidation of the role of JNK in I/R. In addition, the p38-MAPK inhibitor SB-203580 can also inhibit JNK at high concentrations (9).

Recently, a cell-penetrating and protease-resistant peptide inhibitor of JNK, D-JNKI-1, has been developed by one of us (C. Bonny). JNKI-1 is a two-domain peptide containing a 20-amino acid sequence of the minimal JNK-binding domain of Iset-Brain-1/JNK-interacting protein-1 (a scaffold protein), linked to a 10-amino acid TAT sequence of the human immunodeficiency virus TAT protein that mediates intracellular translocation (5). JNKI-1 blocks selectively the access of JNK to c-Jun and other substrates by a direct competitive mechanism (4, 5). The all-D-retroinverso form (D-JNKI-1) made of D-amino acids in reversed sequence is resistant to degradation and acts at the JNK binding site (4). JNKI-1 shows prosurvival effects in vitro or acoustic trauma in vivo (29). Recent studies have suggested prosurvival effects of D-JNKI-1 in several models, including insulin-secreting β-cells exposed to IL-1β (5), neurons exposed to excitotoxic stimuli in vitro or ischemia in vivo (6), and auditory hair cells exposed to aminoglycoside in vitro or acoustic trauma in vivo (29).

Given the specific inhibition of JNK by D-JNKI-1 (6), this peptide is of great interest for investigating the role of JNK in I/R.

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and other stress conditions. We have characterized the effects of D-JNKI-1 in ex vivo and in vivo rat myocardial I/R models.

MATERIALS AND METHODS

Inhibitory peptide. The D-JNKI-1 peptide has been described previously (5, 6). D-JNKI-1 linked to a FITC fluorochrome (D-JNKI-1-FITC) was used to visualize cell entry and accumulation. A d-TAT peptide lacking the c-Jun-binding domain sequence was used as a control.

I/R injury in isolated-perfused hearts. Animal experimentation conformed to the Guide for the Care and Use of Laboratory Animals, Fig. 1. Schematic of the study protocol. Isolated hearts were stabilized for 20 min (baseline), followed by 20-min global ischemia (Isch) and 90-min reperfusion (Reperf). A: D-JNKI-1 (white arrow), d-TAT, or vehicle was delivered at baseline. JNK activation was determined immediately after heart isolation before baseline stabilization (time 0), and at 10-min ischemia (black arrows). B: MAPK activation was determined at 15-min reperfusion. C: cytochrome-c, caspase-3 activity, and terminal deoxynucleotidyl transferase-mediated dUTP-rhodamine nick-end labeling (TUNEL)-positive cells were determined at 90-min reperfusion. D: D-JNKI-1 was delivered at the time of reperfusion. LV, left ventricular.

Fig. 2. D-JNKI-1 accumulation in rat myocardium visualized by epifluorescence microscopy. Top: D-JNKI-1-FITC; myocardium exhibits homogeneous green fluorescence. Bottom: d-TAT (weak autofluorescence of normal heart tissue).

Fig. 3. JNK-specific in vitro kinase activity during ischemia (10 min; the Western blot for phospho-c-Jun is shown at top). Vehicle (shaded bar), d-TAT (50 μM; solid bar), or D-JNKI-1 (50 μM; hatched bar) was delivered at baseline. Data are mean fold-increases (± SE) above baseline (open bar; n = 4/group). *P < 0.05 vs. baseline; #P < 0.05 vs. vehicle and d-TAT.
formulated by the National Research Council, 1996, and the Swiss law on animal protection; it was authorized by the Veterinarian Office of Canton Vaud, Lausanne (Switzerland). Hearts were isolated from adult male Sprague-Dawley rats and perfused in a modified Langendorff system under constant pressure (70 mmHg) with gassed (94% O₂-6% CO₂) Krebs-Henseleit buffer solution at 37°C, as described previously (23). Left ventricular (LV) and coronary perfusion pressures were monitored with pressure transducers (mpc-500, Millar Instruments). Hearts were stabilized for 20 min (baseline), during which the balloon volume was set to an LV end-diastolic pressure (LVEDP) of 10 mmHg and subsequently exposed to 20 min of global ischemia, followed by 90 min of reperfusion (Fig. 1). JNK inhibition was assessed at 10-min ischemia and at 20-min ischemia plus 15-min reperfusion. Hearts were perfused with varying concentrations (10, 50, and 250 μM) of D-JNKI-1 or D-TAT in a 2-ml volume over 2 min (n = 6/group), delivered either at baseline or immediately before reperfusion. Heart rate (HR), peak LV systolic pressure (LVSP), and LVEDP were measured throughout the study protocol. LV developed pressure (LVDP) was calculated as LVSP – LVEDP, and the rate-pressure product was calculated as LVDP x HR.

c-Jun Western blots. Nuclear proteins were isolated as described previously (15). Briefly, frozen heart samples (n = 6/group) were homogenized in lysis buffer solution, centrifuged to separate cytosolic and nuclear proteins, frozen in liquid nitrogen, and stored at −80°C. Equal amounts of total protein, measured by a modified Lowry assay, were loaded for all samples. Samples were separated on a 12% denaturing gel and electroblotted onto nitrocellulose membranes, which were incubated with a primary antibody to phospho-specific c-Jun (Santa Cruz Biotechnology) followed by an appropriate horse-radish peroxidase-conjugated secondary antibody. Membranes were also incubated with a primary polyclonal antibody to histone deacetylase-1 (HDAC-1; Santa Cruz Biotechnology) as a control nuclear protein, according to previous studies (15). The same rat heart sample exposed to I/R in the absence of treatment was loaded on all gels to compare different gels. Immunoblots were developed with the Lumiglo reagent/peroxide chemiluminescent system (Cell Signaling Biotechnology). Densitometry was performed using the National Institutes of Health (NIH) AutoExtractor-1.51 software. Data are shown as fold-increases above baseline values.

In vitro kinase assays. Kinase assays were performed on heart tissues (n = 6/group) using Cell Signaling Technologies kits (nos. 9810, 9820, and 9800), as described previously (23). Briefly, JNK-specific kinase activity was determined by incubating tissue extracts with immobilized c-Jun fusion protein beads at 4°C, followed by

Fig. 4. MAPK activation by ischemia (20 min) and reperfusion (15 min). A: JNK-specific in vitro kinase activity (the Western blot for phospho-c-Jun in the context of the in vitro kinase assay is shown at top). B: amounts of phosphorylated c-Jun protein [Western blots for phospho-c-Jun and histone deacetylase-1 (HDAC-1) in nuclear extracts are shown at top]. C: p38-specific in vitro kinase activity (the Western blot for phospho-ATF-2 in vitro is shown at top). D: ERK1/2-specific in vitro kinase activity (the Western blot for phospho-Elk-1 in vitro is shown at top). Vehicle (shaded bars), D-TAT (50 μM; solid bars), or D-JNKI-1 (10, 50, and 250 μM; hatched bars) was delivered at baseline. Data are mean fold-increases (± SE) above baseline (open bars). *P < 0.05 vs. baseline; #P < 0.05 vs. vehicle and D-TAT.
microcentrifugation and incubation with kinase buffer solution supplemented with 200 μM ATP, p38-MAPK and ERK1/2-specific kinase activities were determined by incubating tissue extracts with immobilized phospho-p38-MAPK (Thr180/Thr182) and phospho-ERK1/2 (Thr202/Thr204) antibodies, respectively. Following electrophoretic, nitrocellulose membranes were incubated with antibodies against phospho-c-Jun (Ser63), phospho-ATF-2 (Thr71), or phospho-Erk-1 (Ser383).

Cytochrome-c Western blots. Cytosolic and mitochondrial protein separation was performed on fresh tissues using a kit specially adapted to heart muscle (Mitochondria Isolation kit, Mito-Isolate 1; Sigma). Mitochondrial and cytosolic fractions were immunoblotted using anti-cytochrome-c antibody (Santa Cruz) and analyzed as described above.

Caspase-3 activity assay. Caspase-3 activity was determined by using the caspase-3/CPP32 colorimetric assay kit (MBL). Protein (150 μg) from cardiac extracts was added to 50 μl reaction buffer and 5 μl acetyl-Asp-Glu-Val-Asp-p-nitroanilide substrate. Samples were incubated at 37°C for 2 h, and p-nitroanilide production was measured at 405 nm.

In situ detection of nuclear DNA fragmentation. DNA fragmentation was assessed on formaldehyde-fixed, paraffin-embedded heart sections by terminal deoxynucleotidyl transferase-mediated dUTP-rhodamine nick-end labeling (TUNEL) using the In Situ Cell Death detection kit, Fluorescein (Intergen, Oxford, UK). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1/1,000 in methanol). TUNEL-positive nuclei were counted by fluorescence microscopy (6 random fields/section; 3 sections/heart cut from the apical, mid-ventricular and basal portions of the heart) and normalized to total nuclei counts.

In vivo I/R injury. Rats were anesthetized with halothane, intubated, and ventilated (tidal volume, 2.5 ml at 50 strokes/min) using a Harvard ventilator. After left thoracotomy, a silk suture (5-0) was passed around the proximal segment of left coronary artery, and a polyethylene catheter was used to form a snare. Rats were allowed to reach steady state during 10 min before D-JNKI-1 (11 mg/kg ip; n = 7), D-TAT (same dose as D-JNKI-1; n = 4), or vehicle (n = 5) administration. The left coronary artery was occluded by pulling the snare, which was released 30 min later (reperfusion).

Analysis of infarct size. Rats were killed 24 h postreperfusion. The heart was removed, the left coronary artery was occluded as was done in vivo, and Evans blue solution was infused to demarcate ischemic areas. Frozen hearts were cut into six to seven slices and incubated in in vivo, and Evans blue solution was infused to demarcate ischemic heart was removed, the left coronary artery was occluded as was done

### Statistical analysis

- Statistical analysis of differences in MAPK activation, cytochrome-c release, apoptosis, and infarct size between individual groups was performed by using the Mann-Whitney U-test. Analysis of LV function was performed by use of one-way ANOVA with Bonferroni’s post hoc test. Significance was set at P < 0.05.

### RESULTS

#### Myocardial D-JNKI-1 uptake.

Five minutes after instillation of D-JNKI-1-FITC or D-TAT into isolated hearts, followed by a 10-min perfusion with PBS to eliminate unbound peptide, myocardial peptide accumulation was detectable by epifluorescence microscopy (Fig. 2).

D-JNKI-1 inhibits JNK activity during ischemia and reperfusion. Both ischemia and reperfusion were associated with increases in JNK-specific in vitro kinase activity by 3.5- and 6.6-fold, respectively. D-JNKI-1 (50 μM) delivered before onset of ischemia reduced the increases in JNK-specific in vitro kinase activity during ischemia and reperfusion by 81% (Fig. 3) and 57% (Fig. 4A), respectively. Loading of equal amounts of protein on Western blots was confirmed by densitometric determination of HDAC-1 protein levels in nuclear extracts [<9% variation between samples; not significant (NS); data not shown]. Amounts of phosphorylated c-Jun protein increased by 60% on reperfusion (P < 0.05). This increase was fully prevented by D-JNKI-1 (P < 0.05; Fig. 4B). Increases in phospho-c-Jun normalized to HDAC-1 protein levels (to correct for minor changes in protein loading) were 54%, 60%, and 6% in the D-TAT, vehicle, and D-JNKI-1 (50 μM) group, respectively (P < 0.05 for D-JNKI-1 vs. controls). p38-MAPK- and ERK1/2-specific in vitro kinase activities were increased after I/R but were unaffected by D-JNKI-1 up to 250 μM (Fig. 4, C and D).

D-JNKI-1 attenuates ischemic myocardial dysfunction. D-JNKI-1, but not D-TAT, delivered before onset of ischemia...
markedly increased the time to onset of ischemic myocardial contracture, defined as the time by which LVEDP has increased by 50% (Fig. 5A), and reduced LVEDP after 20-min ischemia ($P < 0.05$; Fig. 5B).

$\alpha$-JNKI-1 improves myocardial recovery on reperfusion. Changes in LVEDP and LVDP × HR during I/R are shown in Fig. 6. $\alpha$-JNKI-1 administered before onset of ischemia reduced LVEDP ($P < 0.001$; Fig. 6A) and enhanced LVDP × HR ($P < 0.001$; Fig. 6B) on reperfusion.

$\alpha$-JNKI-1 inhibits apoptosis after I/R. The number of TUNEL-positive cells was markedly increased after I/R but was decreased 4.3-fold by $\alpha$-JNKI-1 delivered before onset of ischemia ($P < 0.05$; Fig. 7A). Cytochrome-c release and, to a lesser extent, caspase-3 activity were increased after I/R but were markedly reduced by $\alpha$-JNKI-1 (50 $\mu$M; $P < 0.05$; Fig. 7, B and C). A higher concentration of $\alpha$-JNKI-1 (250 $\mu$M) achieved only a marginal incremental reduction in caspase-3 activity compared with 50 $\mu$M (data not shown).

Fig. 6. Effects of $\alpha$-JNKI-1 delivered at baseline (B) on postischemic myocardial function. LVEDP (A) and LV developed pressure (LVDP) × heart rate (HR) (B) throughout the study protocol (left) and at 90-min reperfusion (right; bar graphs) are shown. Bars: shaded, vehicle; solid, $\alpha$-TAT (50 $\mu$M); hatched, $\alpha$-JNKI-1 (10, 50, and 250 $\mu$M). Data are means ± SE; $n = 6$ / group. **$P < 0.001$ vs. vehicle and $\alpha$-TAT.

$\alpha$-JNKI-1 delivered at the time of reperfusion inhibits apoptosis. $\alpha$-JNKI-1 (50 $\mu$M) delivered after 20 min of ischemia before reperfusion did not affect LV functional recovery (Fig. 8, A and B) but reduced numbers of TUNEL-positive nuclei 7.8-fold and fully suppressed caspase-3 activation on reperfusion (Fig. 8, C and D).

$\alpha$-JNKI-1 reduces infarct size in vivo. The normalized LV surface area at risk was comparable in the three groups (Fig. 9A). Infarct size was similar in the vehicle and $\alpha$-TAT groups (49.90 ± 5.91% vs. 56.19 ± 2.23%; NS) but decreased in the $\alpha$-JNKI-1 group (26.65 ± 4.45%; $P < 0.01$; Fig. 9B).

DISCUSSION

Conflicting results have been reported about the role of JNK in myocardial I/R injury. Nonspecific antioxidant effects of curcumin (7), the inhibitor of JNK generally used in previous studies, have been a methodologically limiting factor. Recently, a cell-penetrating and protease-resistant peptide inhibitor of JNK, $\alpha$-JNKI-1, has been developed by one of us (C. Bonny)
D-JNKI-1 delivered before the onset of ischemia prevented the increase in JNK-specific in vitro kinase activity during ischemia and markedly attenuated the increase in JNK activity during reperfusion. D-JNKI-1 also prevented the increase in phosphorylated c-Jun protein on reperfusion. The apparently more complete prevention of c-Jun phosphorylation compared with the increase in JNK kinase activity by D-JNKI-1 may be due, again, to the higher sensitivity of the kinase assay versus Western blotting. An alternate explanation is the higher binding affinity of D-JNKI-1 for c-Jun compared with that for JNK-binding domains contained in MKK-4 and MKK-7 (6). D-JNKI-1 did not affect ERK1/2 and p38-MAPK activation after I/R, indicating specific inhibition of the JNK subfamily of MAPKs.

D-JNKI-1 attenuated ischemic injury, as manifested by increased time to onset of ischemic contracture and decreased LV end-diastolic pressures at the end of ischemia. Moreover, D-JNKI-1 dramatically enhanced posts ischemic recovery of cardiac function. These effects were accompanied by suppression of cytochrome-c release, caspase-3 activation, and DNA fragmentation induced by I/R. These results are consistent with inhibition of the mitochondrial machinery of apoptosis by the peptide. D-JNKI-1 delivered at the time of reperfusion failed to improve posts ischemic recovery, however, while still preventing apoptosis. These findings are consistent with recent reports showing that mitochondrial signals initiate the activation of JNK by hypoxia-reoxygenation in isolated cardiomyocytes exposed to oxidative stress (11) and that active JNK localized on mitochondria can directly induce the release of cytochrome c (2). Moreover, our results are in agreement with the observation that targeted mice lacking either the jnk1 or the jnk2 gene exhibit decreased cytochrome-c release and apoptosis after myocardial I/R (17). Ironically, however, the same study reported that transgenic mice overexpressing MKK-7, a direct upstream activator of JNK, similarly showed decreased cytochrome-c release and apoptosis after I/R. These results suggested that JNK signaling has both pro- and antiapoptotic ramifications and that the overall dominance of either effect depends on the pathophysiological conditions. In our model, caspase-3 activation was moderate relative to cytochrome-c release and DNA fragmentation, raising the possibility that caspase-independent apoptosis might be involved. A developmental switch from caspase-dependent to caspase-independent apoptosis in cardiomyocytes around birth has been described recently (3).

A previous in vitro study used JNKI-1 to block JNK in the HL-5 cardiac atrial myocyte cell line exposed to serum/glucose depletion and H2O2 to mimic reactive oxygen species (ROS) generated during ischemia, then replaced in their standard culture medium to simulate reperfusion (8). However, JNK inhibition by JNKI-1 was not characterized in that study. 1-JNKI-1 (1 μmol/l) slightly enhanced ROS-induced apoptosis in HL-5 cells. Obviously, our results in isolated-perfused hearts and previous data in HL-5 cells in vitro are not directly comparable, also because different concentrations and forms of JNKI-1 (the protease-resistant all-D-retroinverso form versus L-JNKI-1) were used in the two studies.

Following the functional characterization of D-JNKI-1 in isolated-perfused rat hearts, we tested whether the protective effects of D-JNKI-1 against myocardial I/R injury were also...
present in vivo. In anesthetized adult rats, systemic administration of d-JNKI-1, but not d-TAT, at the time of left coronary artery occlusion decreased infarct size after reperfusion by approximately one-half.

In conclusion, d-JNKI-1 is an important tool compound that can be used in preclinical models to investigate the role of JNK signaling in vivo. Moreover, a reduction in JNK activity and phosphorylation is important in the preservation of cardiac function in rats in the face of ischemia, an effect mediated by reductions in cytochrome-c distribution, caspase 3 activity, and apoptosis.

GRANTS

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REFERENCES


