

Inhibition of C-Jun N-Terminal Kinase 1, but Not c-Jun N-Terminal Kinase 2, Suppresses Apoptosis Induced by Ischemia/Reoxygenation in Rat Cardiac Myocytes

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ABSTRACT

In the present study, rat cardiac myocytes were used as an *in vitro* ischemia/reperfusion injury model to delineate the role of c-Jun N-terminal kinase (JNK) 1 and JNK2 isoforms in ischemia/reoxygenation-induced apoptosis using an antisense approach. Exposure of rat cardiac myocytes to ischemia did not induce apoptosis as detected by staining with either acridine orange/ethidium bromide or annexin-V-fluorescein/propidium iodide. In contrast, a time-dependent increase in the number of apoptotic cells was noted after reoxygenation of ischemic myocytes, whereas the level of necrotic cells remained unaltered. Reoxygenation, but not ischemia alone, also caused a time-dependent increase in JNK activation that preceded apoptosis induction. Treatment of cardiac myocytes with antisense (AS) oligonucleotides that specifically targeted either JNK1 or JNK2 significantly reduced both mRNA and protein expression of the

target isoform but had no effect on the expression of the alternate isoform. Pretreatment of cardiac myocytes with JNK1 AS, but not JNK2 AS, resulted in almost complete attenuation of reoxygenation-induced apoptosis. Furthermore, control oligonucleotides for JNK1 AS or JNK2 AS had no effect on JNK mRNA or protein expression or reoxygenation-induced apoptosis, indicating a sequence-specific mode of action. Additional studies revealed that apoptosis induced by other JNK-activating stimuli, including ceramide, heat shock, and UV irradiation, was partly suppressed after treatment with JNK1 AS but not JNK2 AS. These findings demonstrate that the JNK1 isoform plays a preferential role in apoptosis induced by ischemia/reoxygenation as well as diverse JNK-activating cellular stresses.

Ischemia/reperfusion injury has been identified as a major stimulus for organ dysfunction and cellular death in several disease states including angina and myocardial infarction (Ambrosio and Tritto, 1999). Although timely restoration of blood flow serves to improve myocardial viability, the reperfusion process itself may exacerbate myocardial injury. Several mechanisms may contribute to reperfusion injury, including postischemic inflammation, generation of oxygen free radicals, and alterations in intracellular calcium hemostasis (Ambrosio and Tritto, 1999). Cardiac myocyte cell death after ischemia/reperfusion can occur by both apoptosis and necrosis, two distinct modes of cellular death. Although cell death after prolonged periods of ischemia is attributable predominantly to necrosis (Umansky et al., 1995), apoptosis occurs in cells and tissues exposed to reoxygenation after ischemia. Reperfusion after transient myocardial ischemia activates apoptosis in cardiac myocytes grown in culture (Laderoute and Webster, 1997; Webster et al., 1999) or in animal models of myocardial ischemia/reperfusion (Gottlieb et al., 1994; Buerke et al., 1995).

The intracellular signaling pathways that mediate stress responses of the myocardium have not been fully delineated. However, substantial evidence has demonstrated that p38 mitogen-activated protein (MAP) kinases and c-Jun N-terminal kinases (JNK) are activated in cardiac myocytes in response to diverse cellular stresses resulting in apoptosis. In the isolated perfused rat heart, p38 MAP kinase is activated by ischemia and maintained during reperfusion (Bogoyevitch et al., 1996; Yin et al., 1997). In contrast, activation of JNK occurs during the reperfusion phase and not during ischemia (Bogoyevitch et al., 1996; Knight and Buxton, 1996; Laderoute and Webster, 1997; Yin et al., 1997; Clerk et al., 1998). Furthermore, it has been demonstrated in rat heart that JNK translocates to the nucleus during ischemia to be phosphorylated during the reperfusion period (Mizukami and Yoshida, 1997). A strong correlation between JNK activation and apoptosis induction has been described previously in heart and kidney exposed to ischemia/reperfusion (Yin et al., 1997; Yue et al., 1998). Cook et al. (1999) have noted an enhanced activation of JNK and p38 MAP kinase in hearts

ABBREVIATIONS: MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; BME, basal medium Eagle; DMEM, Dulbecco's modified Eagle's medium; AS, antisense.

obtained from patients with heart failure caused by ischemic heart disease, suggesting that activation of these kinases may contribute to the pathophysiology of the disease.

The MAP kinase family of mitogen-activated, serine/threonine kinases comprises extracellular signal-regulated kinases, p38 MAP kinases, and JNKs, which play important roles in diverse cellular processes. These kinase families are differentiated based on activating stimuli, substrate specificity, and distinct physiological responses (Bogoyevitch, 2000). Whereas extracellular signal-regulated kinases participate in cell growth and differentiation, p38 MAP kinases and JNKs play a crucial role in the cellular response to environmental stress including inflammatory cytokines, UV irradiation, heat shock, and ischemia/reperfusion (Sugden and Clerk, 1998). However, based on cell type and activating stimuli, the JNK signaling pathway can also participate in cellular processes unrelated to stress responses, such as proliferation and differentiation (Bost et al., 1999; Potapova et al., 2000). After activation by dual phosphorylation on tyrosine and threonine residues, JNKs phosphorylate the transcription factors c-Jun, ATF-2, and Elk-1 (Kyriakis et al., 1994; Gupta et al., 1996). JNKs are alternatively known as stress-activated protein kinases (SAPKs), although the JNK and SAPK terminology originally referred to human and rat enzymes, respectively (see Sugden and Clerk, 1998). Molecular cloning has revealed three human JNK genes (*JNK1*, *JNK2*, and *JNK3*) that correspond to *SAPK γ* , *SAPK α* , and *SAPK β* in rat. Each of these genes produces alternatively spliced transcripts that encode proteins of 46 and 54 kDa (Kyriakis et al., 1994; Gupta et al., 1995). Although JNK1 and JNK2 are widely expressed, JNK3 is predominately found in brain and, to a lesser extent, in heart and testes (Mohit et al., 1995; Gupta et al., 1996). Although the JNK signaling pathway is known to play a role in ischemia/reperfusion injury, the distinct role of JNK isoforms in ischemia/reoxygenation-induced apoptosis has not been reported previously. The fact that the various isoforms differ in their specificity for downstream transcription factors (Gupta et al., 1996) as well as in stress-induced activation (Yin et al., 1997; Butterfield et al., 1999) suggests that JNK isoforms may exhibit different physiological roles.

The aim of the present study was to examine the roles of the JNK1 and JNK2 isoforms in reoxygenation-induced apoptosis using a cardiac myocyte model of ischemia/reperfusion injury. Treatment of cells with highly specific antisense oligonucleotides targeting either JNK1 or JNK2 reduced the expression of the respective target mRNA and protein without effecting expression of the alternative isoform. Oligonucleotide-mediated reduction of JNK1 protein expression resulted in suppression of apoptosis induced by ischemia/reoxygenation whereas inhibition of JNK2 protein expression had no effect on reoxygenation-induced apoptosis. We provide additional evidence that apoptosis triggered by diverse stimuli including heat shock, UV irradiation, or ceramide is also inhibited after treatment with JNK1 AS, but not JNK2 AS.

Experimental Procedures

Preparation of Cardiac Myocytes. Cardiac myocytes were prepared from 3-day old neonatal Sprague-Dawley rats using the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical

Corporation, Lakewood, NJ). Cells were resuspended in basal medium Eagle (BME; Life Technologies, Grand Island, NY) supplemented with 5% newborn calf serum, 5% horse serum, 1% BME vitamin solution, 1% nonessential amino acids, 100 U/ml penicillin, and 1,000 μ g/ml streptomycin, and plated onto 15 cm² tissue culture dishes (Nalge; Nunc Corporation, Rochester, NY) for 1 h in a humidified incubator (95% air/5%CO₂ at 37°C) to selectively remove contaminating nonmyocytes. Nonadherent cells were collected and replated onto Primaria (Becton-Dickinson, Franklin Lakes, NJ) culture dishes (100,000–125,000 cells/cm²) in supplemented BME. The nonmyocyte population amounted to <10% of the total cell population as determined by immunofluorescence staining with an anti-myosin antiserum (Sigma, St. Louis, MO). Spontaneously contracting cells were used for experiments at days 3 to 4 after isolation.

Exposure of Cardiac Myocytes to Ischemia/Reoxygenation and Other Apoptosis-Inducing Stresses. Ischemia was induced by replacing culture medium with 95% N₂/5% CO₂ pre-equilibrated, serum- and glucose-free Dulbecco's modified Eagle's medium (DMEM, Life Technologies) and placing the cells into a 37°C incubator in a humidified atmosphere perfused with 95% N₂/5% CO₂. Oxygen level was <1% and was monitored with a Fyrite Gas Analyzer (Bacharach, Pittsburgh, PA). After the indicated time periods of ischemia, cells were reoxygenated with warm, complete growth medium (containing glucose and serum) and placed at 37°C in a 95% air/5% CO₂ humidified atmosphere.

In some experiments, cells were plated into 35-mm culture dishes and exposed to heat shock, UV irradiation, or ceramide. Cardiac myocytes were heat shocked by immersion into a temperature-controlled water bath (Precision Scientific, Chicago, IL) at 42°C for 30 min. UV irradiation (80 J/m²) was performed using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). After exposure to heat shock or UV irradiation, the cells were incubated at 37°C in a CO₂ incubator for 6 h and then analyzed for apoptosis induction. Cells were treated with 10 μ M C6-ceramide for 8 h (*N*-hexanoylsphingosine) (Biomol Research Laboratories, Plymouth Meeting, PA) or 100 nM staurosporine for 6 h (Biomol Research Laboratories) in serum-free medium and then analyzed for apoptosis induction.

Treatment with Oligonucleotides. 2'-Methoxyethyl mixed backbone oligonucleotides were prepared as described by Monia et al. (1996). These oligonucleotides contain a central phosphorothioate deoxyoligonucleotide region that supports RNase H activity, flanked by 2'-methoxyethyl modified phosphodiester wings (Altmann et al., 1996). Antisense oligonucleotides complementary to JNK1 or JNK2, termed JNK1 AS or JNK2 AS, had sequences of 5'-CTCATGATG-GCAAGCAATTA-3' and 5-GCTCAGTGGACATGGATGAG-3', respectively. Control oligonucleotides to JNK1 and JNK2 had sequences of 5'-GCTCGGTGGAAATGGATCAG-3' and 5'-GCTAAGCGGTCAAG-GTTGAG-3', respectively. Areas in the sequence containing 2'-methoxyethyl modifications are indicated by underlines. The transfection of cells was performed as described previously (Garay et al., 2000). Briefly, cells were incubated with oligonucleotides at concentrations up to 1250 nM in BME containing *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride/dioleoylphosphatidylethanolamine (Lipofectin; Life Technologies) at a concentration of 0.25 μ g/10 nmol oligonucleotide. After 4 h, the medium was removed and replaced with supplemented BME.

Northern Blot Analysis. Total RNA was prepared from cells by the QIAGEN RNeasy method (QIAGEN, Inc., Valencia, CA) according to the manufacturer's directions. RNA samples were quantified spectrophotometrically and electrophoresed through 1.2% agarose-formaldehyde gels and transferred to Hybond-N+ nucleic acid transfer membranes (Amersham, Piscataway, NJ) by capillary diffusion for 12 to 14 h. Immobilized RNA was cross-linked to the membrane by exposure to UV light using a Stratalinker (Stratagene, La Jolla, CA) and hybridized using ³²P-labeled JNK1, JNK2, or glyceraldehyde-3-phosphate dehydrogenase specific cDNA probes which were prepared by asymmetric polymerase chain reaction using specific cDNA templates. Probes hybridized to mRNA transcripts were visu-

alized and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Blots were routinely stripped of radioactivity by boiling and reprobed with a ^{32}P -radiolabeled glyceraldehyde-3-phosphate dehydrogenase probe to confirm equal loading.

Western Blot Analysis. For Western blot analysis, cells were grown in 60 mm Primaria culture dishes at a density of $125,000/\text{cm}^2$. Cells were subjected to the indicated stresses, lysed and centrifuged and total protein concentration was determined. Extracts were boiled in SDS-polyacrylamide gel electrophoresis sample buffer and proteins ($30 \mu\text{g}/\text{lane}$) were separated on a 10% SDS-polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane and treated with blocking buffer [PBS containing 10% (w/v) dry milk (Carnation; Nestlé USA, Glendale, CA) and 0.2% Tween-20]. Membranes were probed with either anti-phospho-specific JNK antibody (1:1000 dilution, New England Biolabs, Beverly, MA) which detects only dually-phosphorylated JNK (Thr183/Tyr185), anti-JNK1 (C-17)-G antibody (1:1000 dilution, Santa Cruz Corp., Santa Cruz, CA), or anti-JNK2 (D-2) antibody (1:1000 dilution, New England Biolabs) in blocking buffer. Anti-rabbit IgG conjugated with horseradish peroxidase was used as the second antibody (1:1000 dilution, 1 h, room temperature) and immune complexes were visualized using enhanced chemiluminescence according to the manufacturer's instructions (LumiGLO reagent, New England Biolabs). Blots were quantified with the use of laser scanning densitometry.

Measurement of Apoptosis and Necrosis. Cells were examined for morphological features of apoptosis (chromatin condensation and fragmentation) and necrosis by fluorescence microscopy using acridine orange and ethidium bromide uptake as described previously (Garay et al., 2000). Cardiomyocytes were plated into 1-well Permanox chamber slides (Nalge; 100,000 cells/chamber) or 35-mm culture dishes in 2 ml of growth media. After exposure to ischemia or ischemia/reoxygenation, medium was aspirated from the cells and 50 μl of a 1:1 stock solution of ethidium bromide and acridine orange was added to 1 ml of media and a coverslip was attached to the cells. Treated cells were quantified by fluorescence microscopy according to the following descriptions: normal nuclei (bright green chromatin with organized structure), early apoptotic (bright green chromatin that is highly condensed or fragmented), late apoptotic (bright orange chromatin that is highly condensed or fragmented), or necrotic (bright orange chromatin with organized structure). At least 200 cells from randomly selected fields were counted and quantified for each data point. The apoptotic index [percentage of apoptotic (or necrotic) cells] was calculated as number of apoptotic (or necrotic) cells/total cells counted $\times 100$. Sample identities were concealed during scoring.

Apoptosis was also monitored using an Annexin-V-FLUOS (Roche Molecular Biochemicals, Mannheim, Germany) staining kit according to the manufacturer's instructions. Cellular viability was determined using a propidium iodide exclusion assay. Briefly, cells were exposed to treatments as described above and detached from the chamber slide with a 30-s rinse with cell dissociation buffer (Life Technologies) prewarmed to 37°C followed by addition of 1 ml of PBS. Slides were placed into a 37°C incubator until cells rounded up and detached (~ 5 min). Cells were pelleted by centrifugation (800 g, 5 min) and resuspended in 25 μl of Annexin Staining Solution consisting of 1 ml of HEPES buffer containing 20 μl of Annexin-V-fluorescein labeling reagent and 20 μl propidium iodide. Five microliters of this suspension was removed to the etched ring of a Gold Seal fluorescent antibody slide, a coverslip was attached, and the slide was viewed on a microscope equipped with a fluorescein isothiocyanate filter at $600\times$. The apoptotic index was calculated based on a minimum count of 200 cells per observation.

Data Analysis. Student's *t* test was used to determine statistical significance. Data analysis and graph generation were performed with Prism (GraphPAD Software, San Diego, CA).

Results

Specific Inhibition of JNK1 and JNK2 mRNA and Protein Expression after Treatment with Oligonucleotides Targeted to Either JNK1 or JNK2. The effect of antisense oligonucleotides targeting either rat JNK1 (JNK1 AS) or JNK2 (JNK2 AS) on JNK isoform mRNA expression in cultured neonatal rat cardiac myocytes was monitored by Northern blot analysis. The expression of JNK3/SAPK β mRNA in these cells was not detected by Northern analysis (W. Gaarde, unpublished observations). Treatment of cardiac myocytes with increasing concentrations of JNK1 AS or JNK2 AS resulted in a dose-dependent reduction in JNK1 or JNK2 mRNA levels (IC_{50} values ~ 500 nM for both oligonucleotides) (data not shown). Exposure of cardiac myocytes to 750–1250 nM JNK1 AS resulted in a significant inhibition of JNK1 mRNA while having no effect on JNK2 mRNA (Fig. 1). Conversely, 750–1250 nM JNK2 AS significantly inhibited JNK2 mRNA expression, whereas the expression of JNK1 remained unchanged (Fig. 1). Moreover, JNK mRNA expression was not decreased after exposure to a JNK1 mismatch control oligonucleotide indicating that the antisense oligonucleotides reduce JNK mRNA levels in a sequence-specific manner (Fig. 1).

Using lysates obtained from oligonucleotide-treated cardiac myocytes, JNK1 and JNK2 protein levels were assessed by Western blot analysis with antibodies that specifically recognize either JNK1 (Fig. 2A) or JNK2 (Fig. 2B). Differential processing of the 3' coding region of the corresponding mRNA of both JNK1 and JNK2 results in the expression of 46-kDa (p46-JNK1/p46-JNK2) and 54-kDa proteins (p54-JNK1/p54-JNK2) (Gupta et al., 1996; Ip and Davis, 1998). However, as in previous observations (Clerk et al., 1998; Potapova et al., 2000), p46-JNK1 is predominantly labeled by

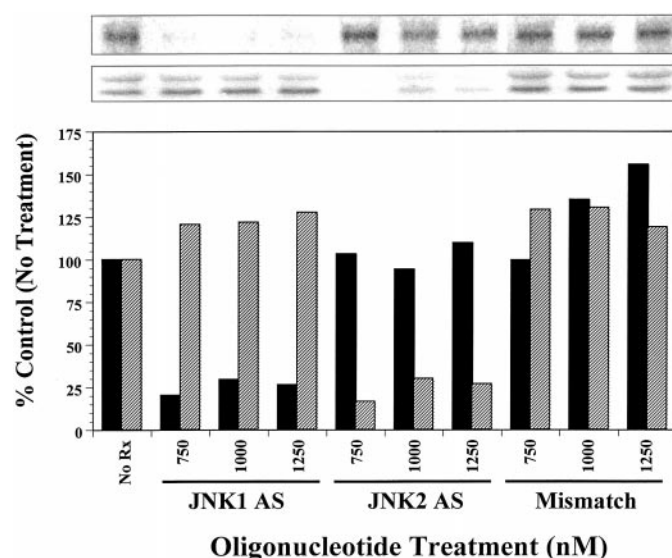


Fig. 1. Inhibition of JNK mRNA expression by antisense oligonucleotides targeted to JNK1 or JNK2 in rat cardiac myocytes. Top, cells were exposed to increasing concentrations (750–1250 nM) of antisense oligonucleotides directed against rat JNK1 (JNK1 AS), rat JNK2 (JNK2 AS), or a JNK1 mismatch control oligonucleotide and total RNA was prepared 48 h later. Normalized RNA was analyzed for JNK1 or JNK2 levels in duplicate by Northern blot analysis. Bottom, JNK mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and quantified by PhosphorImager analysis as described. The results are representative of two independent experiments.

the JNK1-selective antibody (Fig. 2A), whereas the p54-JNK2 band is primarily labeled using the JNK2-selective antibody (Fig. 2B). Using a JNK1-selective antibody, a marked reduction in p46-JNK1 and p54-JNK1 protein levels was observed after 48 h of treatment with 750 to 1250 nM JNK1 AS but not with a mismatch control oligonucleotide (Fig. 2A). Quantitative analysis of Western blots revealed that treatment with 1250 nM JNK1 AS inhibited p46-JNK1 expression by $77 \pm 3\%$ and p54-JNK1 expression by $69 \pm 7\%$. Similarly, a corresponding reduction in p46-JNK2 and p54-JNK2 protein expression was observed in JNK2 AS-treated cells with no effect on protein expression after treatment with a mismatch control oligonucleotide (Fig. 2B). Treatment of cells with 1250 nM JNK2 AS reduced p46-JNK2 and p54-JNK2 expression by $73 \pm 10\%$ and $71 \pm 2\%$, respectively. There was no reduction in protein expression when lysates from JNK2 AS-treated cells were probed with an anti-JNK1 antibody or when lysates from JNK1-treated cells were probed with an anti-JNK2 antibody, which confirms the target-selectivity of JNK1 AS and JNK2 AS as shown in the Northern analysis.

Induction of Apoptosis by Ischemia/Reoxygenation in Rat Cardiac Myocytes. Time course experiments for reoxygenation-induced apoptosis were performed to determine an appropriate time point at which to evaluate the effect of the oligonucleotides targeted to JNK isoforms. Cardiac myocytes were subjected to increasing periods of ischemia followed by 15 h of reoxygenation and apoptosis was assessed using fluorescent DNA binding dyes. Few apoptotic cells ($5.5 \pm 0.6\%$) were observed in cardiac myocytes grown under normoxic conditions and this number was not significantly altered after exposure to increasing periods of ischemia (Fig. 3A). In contrast, when cells were exposed to ischemia, ranging from 30 min to 8 h and then reoxygenated for 15 h, the number of apoptotic cells increased significantly (Fig. 3A). Subsequent experiments examined the time course for both apoptosis and necrosis after exposure of cardiac myocytes to either 4 h of ischemia or 4 h of ischemia followed

by reoxygenation (up to 24 h). Increasing periods of reoxygenation resulted in a time-dependent increase in the number of apoptotic cells that reached 19% at 24 h postreoxygenation (Fig. 3B). In contrast, there was no difference in the level of necrotic cells between cultures subjected to normoxia, ischemia only, or ischemia/reoxygenation, suggesting that the predominant form of cell death is apoptosis in this model (Fig. 3B). The augmentation of apoptotic, but not necrotic cells, after reoxygenation of ischemic cardiac myocytes was confirmed using annexin-V-fluorescein and propidium iodide, which detect early apoptotic and necrotic cells, respectively. (Fig. 3C). Exposure of cardiac myocytes to 4 h ischemia followed by 4 h reoxygenation resulted in $31 \pm 5\%$ apoptotic cells as detected by annexin-V-fluorescein staining.

Induction of JNK Activation by Ischemia/Reoxygenation in Rat Cardiac Myocytes. JNK phosphorylation was investigated under the same conditions of 4 h ischemia followed by increasing periods of reoxygenation using an anti-active JNK antibody that detects the dual phosphorylated active forms of both p46-JNK and p54-JNK. Phosphorylation of JNK was not detected in cells exposed to up to 24 h of ischemia, which is in agreement with previous reports (Mackay and Mochly-Rosen, 1999; Garay et al., 2000). Phosphorylation of JNK was observed within 15 min of reoxygenation; this phosphorylation peaked at 2 h postreoxygenation and then decreased significantly by 4 h (Fig. 4A). Quantitative analysis of Western blots by densitometry revealed a ~ 7 -fold and 8-fold increase in phosphorylated p46-JNK and p54-JNK, respectively, observed at 2 h postreoxygenation compared with cells exposed to 4 h of ischemia only (Fig. 4B). The total level of JNK, as detected by an antibody that measures all JNK isoforms, regardless of the phosphorylation state, remained constant throughout the entire period of reoxygenation (data not shown). In some cardiac myocyte preparations, the anti-active JNK antibody, as well as a non-isoform-specific JNK antibody, detected an additional band migrating at ~ 48 kDa (Fig. 4A and W. Gaarde, unpublished observations) whereas this additional band was not detected using specific JNK1 or JNK2 antibodies (Fig. 2, A and B). Although the identity of this band is unknown, it may correspond to JNK3/SAPK β , which is known to migrate at 49 kDa (Mohit et al., 1995; Butterfield et al., 1997). Treatment of cardiac myocytes with either JNK1 AS or JNK2 AS followed by ischemia/reoxygenation resulted in a marked attenuation of JNK phosphorylation, whereas the JNK1 mismatch control oligonucleotide had no effect (Fig. 4C).

Effect of Oligonucleotides Targeting JNK1 or JNK2 on Apoptosis Induced by Ischemia/Reoxygenation and Other JNK-Activating Stresses. To delineate the contribution of JNK isoforms to reoxygenation-induced apoptosis, rat cardiac myocytes were treated with JNK1 AS, JNK2 AS, or a mismatch oligonucleotide for 48 h before exposure to ischemia/reoxygenation. As noted previously, a similar percentage of apoptotic cells was observed after exposure of cardiac myocytes to either normoxia or 4 h of ischemia (Fig. 5A). However, when these ischemic cells were reoxygenated for 4 h, a significant increase in the number of apoptotic cells was observed (Fig. 5A). Pretreatment of cardiac myocytes with JNK1 AS resulted in a significant attenuation of reoxygenation-induced apoptosis. An $86 \pm 6\%$ ($n = 3$) and $76 \pm 9\%$ ($n = 3$) decrease in the number of apoptotic cells was noted after treatment with 1000 nM or 1250 nM JNK1 AS, respec-

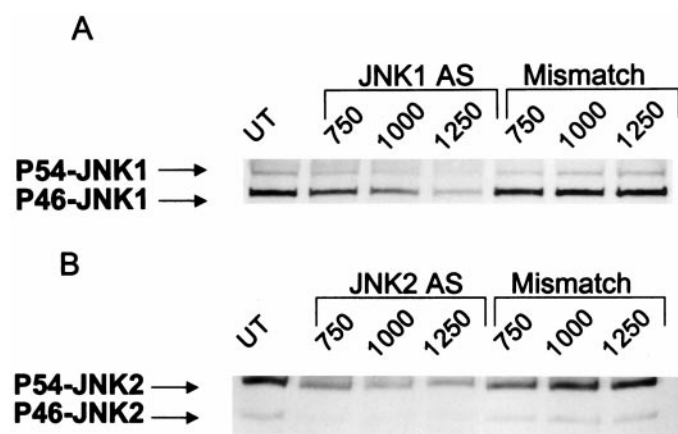


Fig. 2. Inhibition of JNK protein expression and JNK phosphorylation by antisense oligonucleotides targeted to JNK1 and JNK2 in rat cardiac myocytes. Western blot analysis of JNK1 and JNK2 protein levels in cardiac myocytes treated with an antisense oligonucleotide directed against rat JNK1 (JNK1 AS), rat JNK2 (JNK2 AS), or the corresponding control oligonucleotides for 48 h. Protein extracts (30 μ g) were prepared from cardiac myocytes and analyzed by Western blot analysis using either a JNK1-selective antibody (shown in A) or a JNK2-selective antibody (shown in B). Shown are representative results from one of three independent experiments. UT, untreated.

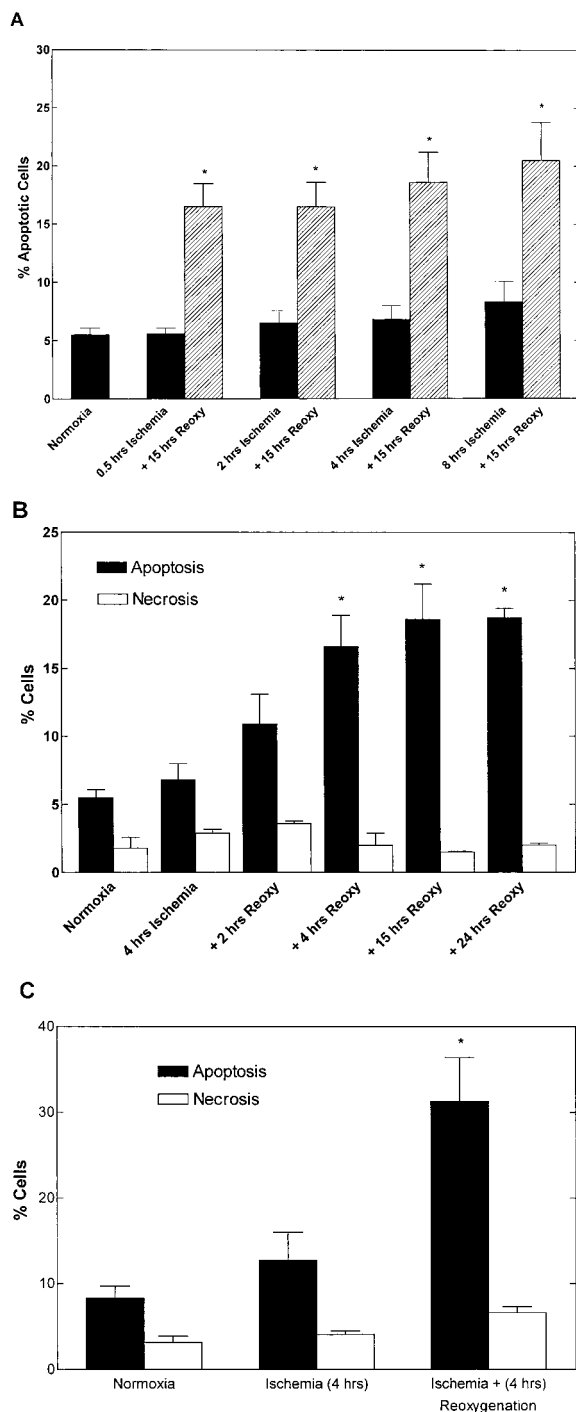


Fig. 3. Effect of ischemia and ischemia/reoxygenation on apoptosis and necrosis induction in rat cardiac myocytes. A, cells were subjected to the indicated periods of ischemia followed by 15 h of reoxygenation. Results shown are means \pm SEM from three to five independent experiments. B, cells were exposed to 4 h of ischemia followed by the indicated periods of reoxygenation. Results shown are means \pm SEM from three to four independent experiments. For A and B, apoptotic and necrotic cells were determined by fluorescence microscopy using ethidium bromide and acridine orange and are shown as a percentage of the total cells counted. At least 200 cells from randomly selected fields were counted and quantified for each data point. *, Significantly different from untreated cells, $p < 0.05$. C, cells were subjected to normoxia, 4 h of ischemia, or 4 h of ischemia/15 h of reoxygenation. Apoptosis and necrosis were quantified by fluorescence microscopy using annexin-V-fluorescein and propidium iodide and are shown as a percentage of the total cells counted. Results shown are means \pm SEM from three independent experiments. *, Significantly different from untreated cells, $p < 0.05$.

tively (Fig. 5A). In contrast, treatment of cardiac myocytes with JNK2 AS or a mismatch control oligonucleotide had no effect on the number of apoptotic cells after reoxygenation (Fig. 5A). Using annexin-V-fluorescein staining, a similar reduction in reoxygenation-induced apoptosis was observed in JNK1 AS-treated cells, but not JNK2 AS-treated cells (data not shown). To delineate whether inhibition of JNK1 actually prevents apoptotic cell death or merely delays its onset, apoptosis was measured after longer periods of reoxy-

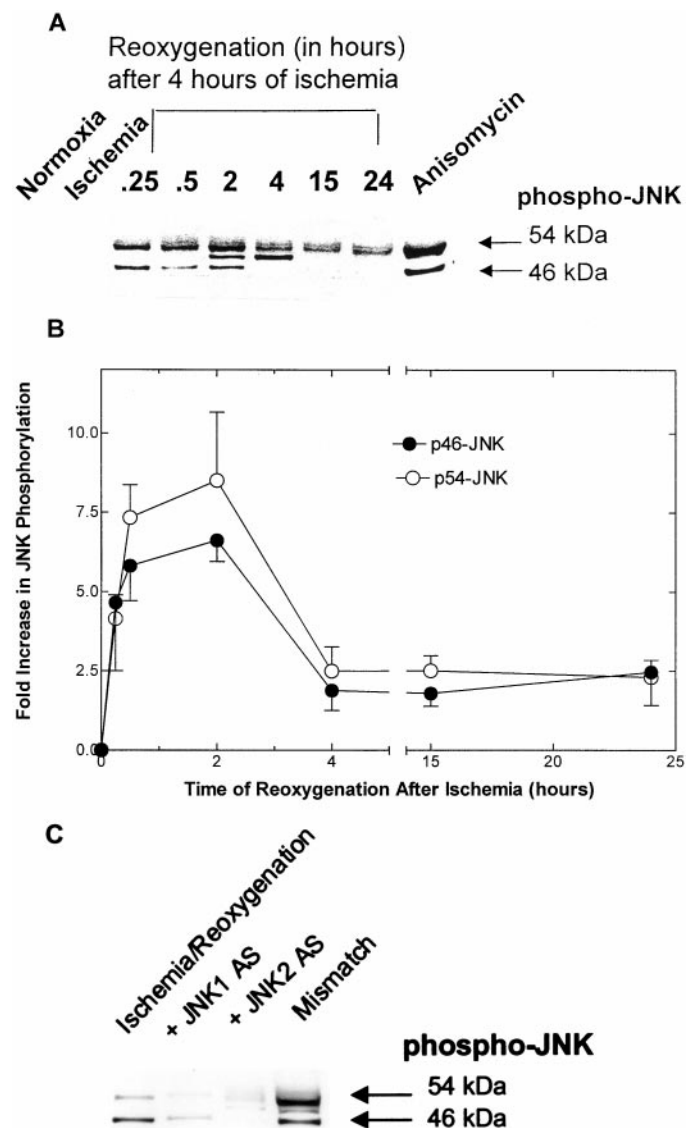


Fig. 4. Reoxygenation of ischemic rat cardiomyocytes results in phosphorylation of JNK. A, Western blot analysis of cell lysates from rat cardiomyocytes exposed to 4 h of ischemia followed by the indicated periods of reoxygenation. As a positive control, cells were exposed to 10 μ g/ml anisomycin for 1 h. Cell lysates were resolved by Western blot analysis on 10% SDS-polyacrylamide gels and probed using a phospho-specific JNK (Thr183/Tyr185) antibody. B, densitometric quantitative analysis of JNK phosphorylation after 4 h of ischemia followed by the indicated periods of reoxygenation. Results are the mean \pm SEM from four separate experiments. Data is expressed relative to cells exposed to only ischemia. C, cells were treated with 1250 nM JNK1 AS, JNK2 AS, or a JNK1 mismatch control oligonucleotide for 48 h and then exposed to 4 h ischemia/4 h reoxygenation. Lysates were resolved by Western blot analysis on 10% SDS-polyacrylamide gels and probed using a phospho-specific JNK (Thr183/Tyr185) antibody. The lumigram is representative of three separate experiments.

generation after ischemia. For these experiments, cardiac myocytes were treated with 1000 nM JNK1 AS and then exposed to 4 h of ischemia followed by either 4 or 72 h of reoxygenation. As shown in Fig. 5B, the number of apoptotic cells was significantly reduced in the JNK1 AS-treated cells after either 4 or 72 h of reoxygenation, suggesting that cell death is prevented by pretreatment with the JNK1 oligonucleotide. Under these experimental conditions, the level of necrotic cells was not significantly increased in JNK1 AS-treated cells reoxygenated for 72 h (data not shown).

To further investigate the role of JNK isoforms in apopto-

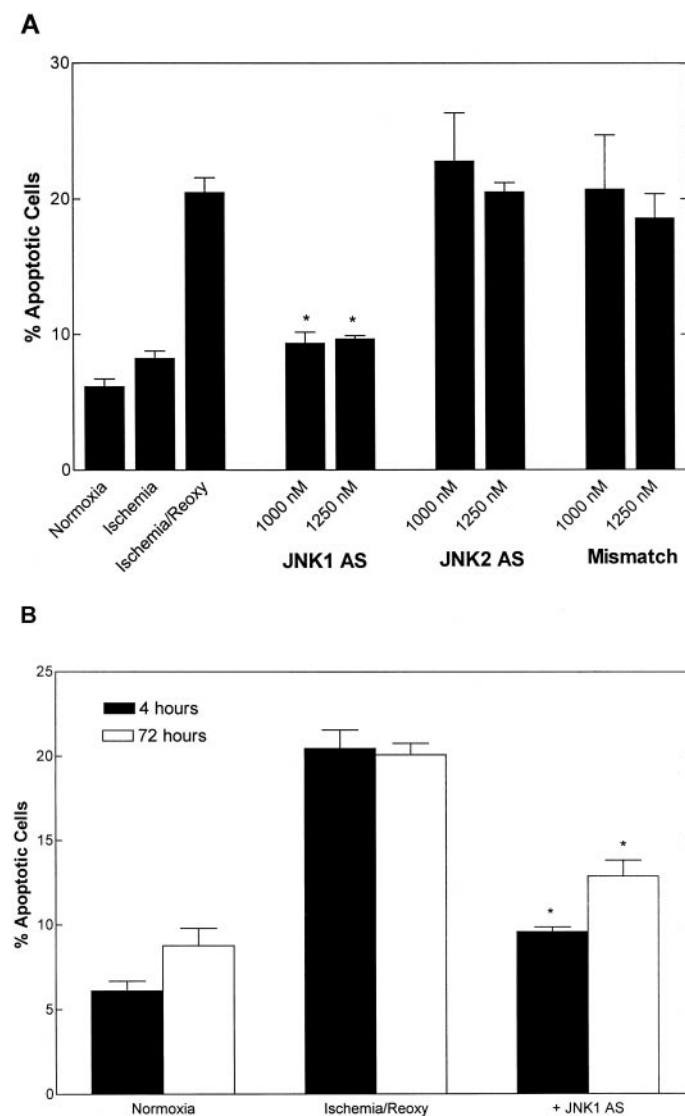


Fig. 5. Inhibition of reoxygenation-induced apoptosis by an antisense oligonucleotide directed against JNK1, but not JNK2, in rat cardiac myocytes. A, cells were incubated with 1000 or 1250 nM JNK1 AS, JNK2 AS, or a control oligonucleotide for 48 h before exposure to either ischemia (4 h) or ischemia (4 h)/reoxygenation (15 h). B, after exposure to 1000 nM JNK1 AS, cardiac myocytes were subjected to 4 h of ischemia followed by either 4 or 72 h of reoxygenation. For A and B, the percentage of apoptotic cells were determined by fluorescence microscopy using ethidium bromide and acridine orange and are shown as a percentage of the total cells counted. At least 200 cells from randomly selected fields were counted and quantified for each data point. The apoptotic index (percentage of apoptotic cells) was calculated as number of apoptotic cells/total cells counted \times 100. Values shown are the means \pm SEM obtained from three to six experiments. *, Significantly different from untreated cells, $p < 0.05$.

sis, we examined the effect of JNK1 AS and JNK2 AS pretreatment on cell death induced by other apoptosis-inducing stresses. Exposure of cardiac myocytes to either heat shock or UV irradiation strongly stimulated JNK phosphorylation, whereas ceramide-stimulated phosphorylation was induced to a lesser extent (Fig. 6A). Treatment of rat cardiac myocytes with 1000 nM JNK1 AS or JNK2 AS before stimulation with ceramide, UV irradiation or heat shock, resulted in a significant attenuation in JNK phosphorylation induced by these stresses (Fig. 6A). Furthermore, a significant number

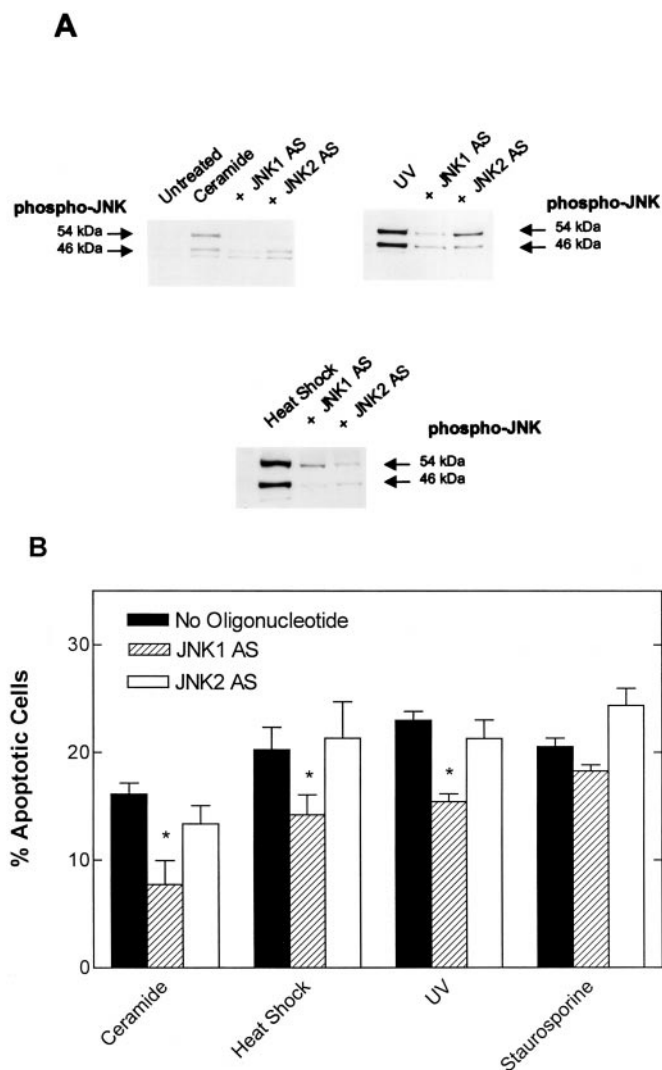


Fig. 6. Inhibition of stress-induced apoptosis by an antisense oligonucleotide directed against JNK1, but not JNK2, in rat cardiac myocytes. A, Western blot analysis of cell lysates from rat cardiac myocytes treated with 1000 nM JNK1 AS or JNK2 AS for 48 h and then exposed to no stress (untreated), ceramide (10 μ M, 30 min), UV irradiation (80 J/m²), or heat shock (30 min, 40°C). The lumigram is representative of two separate experiments. Cell lysates were resolved by Western blot analysis on 10% SDS-polyacrylamide gels and probed using a phospho-specific JNK (Thr183/Tyr185) antibody. B, cells were pretreated with either 1000 nM JNK1 AS or JNK2 AS for 48 h before exposure to the indicated treatments: normoxia, ceramide (10 μ M), heat shock (30 min at 40°C), UV irradiation (80 J/m²) or staurosporine (100 nM) as described under *Experimental Procedures*. Apoptotic cells were determined by fluorescence microscopy using ethidium bromide and acridine orange and are shown as a percentage of the total cells counted. In these experiments, apoptosis in normoxic cultures was $6.5 \pm 0.7\%$. Results shown are means \pm SEM from four to five independent experiments. *, Significantly different from untreated cells, $p < 0.05$.

of apoptotic cells was noted after exposure of cardiac myocytes to ceramide, heat shock, or UV irradiation compared with the level of $6.4 \pm 0.7\%$ found in normoxic cultures (Fig. 6B). Apoptosis was also induced by the protein kinase C inhibitor, staurosporine, which does not result in JNK phosphorylation (Fig. 6B). Pretreatment of cardiac myocytes with 1000 nM JNK1 AS resulted in a significant attenuation of apoptosis induced by ceramide ($75 \pm 5\%$), heat shock ($47 \pm 6\%$), or UV irradiation ($48 \pm 3\%$) whereas exposure to JNK2 AS did not alter apoptosis induced by any of these cellular stresses. Staurosporine-induced apoptosis was not significantly effected by treatment with either oligonucleotide. There was no alternation in stress-induced JNK phosphorylation or apoptosis after treatment with the JNK1 mismatch control oligonucleotide (data not shown).

Discussion

In the present study, an antisense approach was used to delineate the contribution of JNK1 and JNK2 isoforms in ischemia/reoxygenation-induced apoptosis. Treatment of cardiac myocytes from neonatal rats with antisense oligonucleotides targeted to either rat JNK1 or JNK2 specifically reduced the mRNA and protein expression of the respective JNK isoform in a target-selective manner. Antisense oligonucleotide-mediated reduction of JNK1 protein expression inhibited reoxygenation-induced apoptosis whereas suppression of JNK2 protein expression had no effect on reoxygenation-induced cell death, demonstrating that JNK1, and not JNK2, plays a critical role in reoxygenation-induced apoptosis. Furthermore, apoptosis induced by other JNK-activating stresses, including ceramide, heat shock, or UV irradiation, was also suppressed by the antisense oligonucleotide targeting JNK1.

Exposure of ischemic tissues to reoxygenation greatly augments tissue damage and the JNK signaling cascade has been implicated as playing a pathological role in this process. Reoxygenation after ischemia induces both JNK activation and apoptosis using perfused rat heart (Bogoyevitch et al., 1996; Knight and Buxton 1996; Clerk et al., 1998) and primary cardiac myocytes (Laderoute and Webster, 1997; Webster et al., 1999). Two previous studies have measured both apoptosis and JNK activation concurrently in ischemic/reperfused heart and reported a good correlation between these two processes (Yin et al., 1997; Yue et al., 1998). In the present study, apoptosis was observed after ischemia/reoxygenation but, not with ischemia alone, as assayed using methods which detect nuclear condensation and cell-surface phosphatidylserine exposure. A larger proportion of apoptotic cells ($31 \pm 5\%$) was detected by annexin-V-fluorescein staining, when compared with ethidium bromide/acridine orange staining ($20 \pm 1.5\%$), consistent with the fact that the binding of annexin-V-fluorescein to translocated phosphatidylserine on the cell surface measures an early apoptotic event (Van Engeland et al., 1998). Although a slight increase in apoptotic cells was noted after 2 h of reoxygenation, a significant increase in apoptosis was not detected until 4 h after reoxygenation. The time course for JNK activation, which occurred rapidly and peaked at 2 h after reoxygenation, preceded apoptosis induction. The small percentage of necrotic cells noted in normoxic or ischemic myocyte cultures was not significantly altered after reoxygenation, indicating

that the predominant mode of cell death in this cardiac myocyte model is apoptosis. It should be noted that nonmyocyte cells in the primary culture may also contribute to apoptosis induced by ischemia/reoxygenation.

This is the first study to investigate the contribution of JNK1 and JNK2 to reoxygenation-induced cell injury. Treatment of cardiac myocytes with specific antisense oligonucleotides targeted to either JNK1 (JNK1 AS) or JNK2 (JNK2 AS) significantly reduced both mRNA and protein expression of the target JNK isoform but had no effect on the expression of the alternate isoform. Moreover, control oligonucleotides for JNK1 AS or JNK2 AS had no effect on JNK protein expression or reoxygenation-induced apoptosis, indicating a sequence-specific mode of action. Ablation of JNK1 protein expression by JNK1 AS treatment resulted in almost complete suppression of reoxygenation-induced apoptosis. In contrast, there was no effect on reoxygenation-induced apoptosis in JNK2 AS-treated cells. These data indicate that JNK1, but not JNK2, is the predominant isoform involved in reoxygenation-induced apoptosis in cardiac myocytes. Previously, we demonstrated that inhibition of JNK protein expression by a JNK1 antisense oligonucleotide inhibits hypoxia-mediated apoptosis in human kidney cells although the oligonucleotide directed against human JNK1, in contrast to those used in the present study, did not display JNK isoform-specific inhibition (Garay et al., 2000). Treatment of cardiac myocytes with JNK1 AS suppressed apoptotic cell death both at 4 and 72 h after reoxygenation, suggesting that JNK1 AS prevented, not just delayed, the apoptotic response to reoxygenation. The percentage of necrotic cells was not augmented at either reoxygenation time, indicating that the cells were not dying through an alternate mode of cell death. In the present study, the majority of JNK1 AS-treated cardiac myocytes exposed to ischemia/reoxygenation were viable, as detected by Trypan Blue exclusion, and were contracting synchronously. Nonetheless, further experimentation is needed to determine whether suppression of apoptosis by signal transduction inhibitors, such as JNK1 AS, results in a surviving myocyte population that exhibits normal cardiac function.

The JNK signaling cascade is also activated by diverse cellular stresses including proinflammatory cytokines (Guo et al., 1998), ultraviolet irradiation (Chen et al., 1996; Tourner et al., 2000; Zanke et al., 1996), heat shock (Zanke et al., 1996) and ceramide (Hernandez et al., 2000). Stress-induced JNK activation and apoptosis has also been noted in cardiac myocytes (Clerk et al., 1998; Webster et al., 1999). In the present study, we show that exposure of rat cardiac myocytes to heat shock, UV irradiation or ceramide results in both JNK activation and apoptosis. More importantly, pretreatment of cells with JNK1 AS, but not JNK2 AS, resulted in almost complete protection of ceramide-induced apoptosis while apoptosis induced by heat shock or UV irradiation was only partly suppressed. A dominant negative JNK1 mutant resulted in partial protection against UV irradiation-induced apoptosis in small cell tumor cells (Butterfield et al., 1997) while complete suppression against apoptosis was observed in embryonic kidney cells (Chen et al., 1996). The partial suppression of apoptosis induced by heat shock or UV irradiation in the present study may occur because the cardiac myocytes were exposed to a maximal exposure of stress and because these cellular stresses also activate p38 MAP kinases (Force and Bonventre, 1998; Sugden and Clerk, 1998).

Not surprisingly, JNK1 AS pretreatment did not protect against apoptosis induced by the protein kinase C inhibitor staurosporine, which does not activate JNK. Taken together with the inhibition of ischemia/reoxygenation-induced apoptosis, these data suggest that the JNK1 isoform plays an important and preferential role in apoptosis induced by a variety of JNK-activating cellular stresses. A specific function for JNK isoforms in stress-induced apoptosis has been investigated in only a few recent studies, most notably in neurons and tumor cells. Using inhibitory JNK1 mutants or antisense oligonucleotides, JNK1, but not JNK2, was found to be the predominant isoform involved in apoptosis of tumor cells induced by either an antitumor drug (Seimiya et al., 1997) or UV irradiation (Butterfield et al., 1997). Tournier et al. (2000) demonstrated that fibroblasts derived from mice lacking the JNK1 and JNK2 genes were protected against UV radiation-induced cell death. Suppression of stress-induced apoptosis in JNK1 AS-treated myocytes may be a consequence of JNK1 pathway blockade and unchecked activation of survival-promoting proteins such as the Bcl-2 family or survival signaling pathways (e.g., the PI 3-kinase/Akt axis). Indeed, Mockridge et al. (2000) have recently demonstrated that Akt is phosphorylated during reoxygenation after ischemia/reoxygenation but not during ischemia alone in rat cardiomyocytes. Furthermore, JNK regulates Bcl-2 via phosphorylation of the loop region, which renders Bcl-2 inactive (Maundrell et al., 1997).

In summary, we have used highly specific antisense oligonucleotides targeted to either JNK1 or JNK2 to delineate isoform-specific functions in rat cardiac myocyte apoptosis induced by a variety of stress stimuli that activate JNK. Inhibition of JNK1 protein expression, but not that of JNK2, resulted in a significant suppression of reoxygenation-induced apoptosis, suggesting a preferential role for the JNK1 isoform in reoxygenation-induced apoptosis. These results suggest that inhibition of the JNK signaling pathway, and in particular the JNK1 isoform, may represent a useful strategy for the prevention of reperfusion injury.

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