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Inhibition of Hypoxia/Reoxygenation-Induced Apoptosis by an Antisense Oligonucleotide Targeted to JNK1 in Human Kidney Cells

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ABSTRACT. The present study explored the relationship between activation of c-Jun N-terminal kinase (JNK) and apoptosis following exposure of primary human kidney cells to hypoxia/reoxygenation. Apoptosis induction was apparent only after prolonged exposure of cells to hypoxia (>48 hr), when a 2-fold increase in DNA fragmentation was observed. In contrast, 15 hr of reoxygenation following either 4 or 8 hr of hypoxia was associated with a pronounced (>17-fold) increase in DNA fragmentation. Fluorescence microscopy, using DNA binding dyes, demonstrated that cell death following hypoxia/reoxygenation was due predominantly to apoptosis and not necrosis. Furthermore, reoxygenation, but not hypoxia alone, caused a time-dependent increase in the activation of JNK as monitored by western blot analysis using a phospho-specific JNK antibody. In contrast, p38 mitogen-activated protein kinase was activated following hypoxia, but this activation was not augmented during reoxygenation. Exposure of human kidney cells to a 2′-methoxyethyl mixed backbone antisense oligonucleotide directed against human JNK1 (JNK1 AS) resulted in a potent suppression of JNK mRNA and protein expression, whereas a 6-base mismatch control oligonucleotide was without effect. Moreover, a significant diminution of reoxygenation-induced apoptosis was observed in cells exposed to JNK1 AS but not to the mismatch control oligonucleotide. Taken together, these results strongly indicate that activation of the JNK signaling cascade is a major mechanism whereby hypoxia/reoxygenation induces apoptosis. BIOCHEM PHARMACOL **59**;9:1033–1043, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. apoptosis; c-Jun N-terminal kinase (JNK); hypoxia; reoxygenation; p38 MAP kinase

Exposure of heart or kidney tissue to ischemia followed by subsequent restoration of blood flow (reperfusion) results in cellular death and organ dysfunction. Cell death due to ischemia/reperfusion *in vivo* or hypoxia/reoxygenation *in vitro* typically has been assumed to be a result of necrosis, in which there is a rapid loss of cell integrity accompanied by membrane lysis and inflammation (for a review, see Ref. 1). However, more recent studies have demonstrated that whereas cell death following prolonged periods of ischemia can be attributed to necrosis [2, 3], apoptosis is the primary form of cell death that occurs following ischemia/reperfusion [3–5]. Apoptosis is a regulated process of cell death characterized by distinct morphological and biochemical events including membrane blebbing, nuclear and cytoplasmic shrinkage, DNA fragmentation, and chromatin condensation (see Ref. 1 and references cited therein). Although apoptosis plays a fundamental role in certain physiological events such as tissue homeostasis and neuronal development, deregulated apoptosis contributes to the pathogenesis of several human diseases including atherosclerosis, myocardial ischemia, and acute renal injury [6, 7].

Hypoxia induces diverse metabolic and ionic alterations including an increase in the activity of several kinases [8] and the expression of immediate-early stress genes [9]. Additional injury occurs during the reperfusion period as a result of an inflammatory response as well as oxidative damage to cellular components from reactive oxygen intermediates (for reviews, see Refs. 10 and 11). At present, the signaling pathways resulting in ischemia/reperfusion-induced apoptosis remain unclear. However, JNK,§ a stress-activated protein kinase, initially characterized for its ability to activate the transcriptional factor c-Jun [12, 13], has been postulated to play a role in reperfusion-induced cellular death. Recent evidence indicates that JNK is activated during reperfusion following ischemia, but not

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§ *Abbreviations*: AS, antisense; ERK, extracellular regulated kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; REGM, renal epithelial cell growth medium; and RPTE, renal proximal tubule epithelial.

during ischemia alone, in perfused rat heart [5, 14, 15], kidney [5, 16], and liver [17]. Similarly, an augmentation in JNK activation has also been observed in cardiomyocytes exposed to hypoxia/reoxygenation [18, 19].

JNK, ERKs, and p38 MAP kinases comprise the family of mitogen-activated serine/threonine kinases, which play important roles in diverse cellular processes. Following activation by dual phosphorylation on tyrosine and threonine residues, JNKs relay extracellular signals to the transcription factors c-Jun, ATF2 and Elk-1, which serve as downstream targets [13, 20, 21]. ERKs are activated primarily by phorbol esters and growth factors [22, 23], whereas p38 MAP kinases and JNKs typically mediate responses to a wide variety of physical stresses including ultraviolet light, heat shock, protein synthesis inhibitors, and ischemia/reperfusion [14, 24, 25]. Despite activation by similar stresses, p38 MAP kinases and JNKs exhibit differences in substrate specificity and upstream kinase cascades leading to their activation [26, 27]. For the JNK family, at least three genes (*JNK1*, *JNK2*, and *JNK3*) encoding rat and human JNKs have been identified by molecular cloning. Each of these genes produces alternatively spliced transcripts that encode proteins of ~46 and 54 kDa [13, 20]. At present, specific JNK isoforms have not been associated definitively with specific biological functions. However, JNK isoforms display differing substrate interaction *in vitro* [28] as well as differential stress-induced activation [5, 29], suggesting that the JNK isoforms may exhibit distinct physiological roles.

A correlation between the time courses for JNK activation and apoptosis induction following ischemia/reperfusion was noted in the only previous study to examine both apoptosis and JNK activation concurrently [5]. The lack of specific JNK inhibitors has hindered studies investigating the precise role of JNK in reperfusion injury *in vivo*. In the present study, we utilized an antisense approach to specifically inhibit *JNK* gene expression in order to definitively address whether the JNK signaling pathway plays a role in reoxygenation-induced apoptosis, using human kidney cells as an *in vitro* model of reperfusion injury. The present findings demonstrated that hypoxia/reoxygenation, but not hypoxia alone, activated JNK and that this activation strongly correlated with the profile of apoptosis induction that occurs predominantly following reoxygenation. More importantly, reduction in both JNK mRNA and protein expression using a 2′-methoxyethyl mixed backbone antisense oligonucleotide targeted to human JNK1 resulted in a significant inhibition of reoxygenation-induced apoptosis. These results suggest a crucial role for JNK in mediating apoptotic cell death induced by the cellular stress of hypoxia/reoxygenation.

MATERIALS AND METHODS

Cell Culture

Human RPTE cells (passages 4–7, Clonetics Corp.) were cultured in Falcon Primaria tissue culture flasks (75 cm^2/250 mL, Becton Dickinson) in Clonetics REGM supplemented with 0.5% fetal bovine serum, 10 ng/mL of human recombinant epidermal growth factor, 5 μg/mL of insulin, 0.5 μg/mL of hydrocortisone, 0.5 μg/mL of epinephrine, 6.5 μg/mL of triiodothyronine, 10 μg/mL of transferrin, 50 μg/mL of gentamicin, and 50 ng/mL of amphotericin B at 37° in a 95% air/5% CO_2 humidified atmosphere. RPTE cells were subcultured by aspiration of the growth medium followed by a 30-sec rinse with a solution of 0.01% EDTA/0.025% trypsin.

Hypoxia and Reoxygenation

Cells were 70–80% confluent at the time of hypoxic treatment. Hypoxia was induced with the use of a cell culture incubator perfused with 95% N_2/5% CO_2. The oxygen level was $< 1\%$ and was monitored with a Fyrite Gas Analyzer (Bacharach). After the indicated time periods, cells were removed from the hypoxic incubator and reoxygenated by immediate replacement of hypoxic medium with normoxic, serum-containing REGM.

Treatment with Oligonucleotides and Inhibitors

2′-Methoxyethyl mixed backbone oligonucleotides were prepared as described by Monia *et al.* [30]. These oligonucleotides contain a central phosphorothioate oligodeoxynucleotide region that supports RNase H activity, flanked by 2′-methoxyethyl modified phosphodiester wings [31]. An antisense oligonucleotide complementary to *JNK1*, termed JNK1 AS, had a sequence of 5′-CTCTCTGTAGGCCCGCTTGG-3′. A mismatch control oligonucleotide to *JNK1* had a sequence of 5′-CTTTCCGTTGGACCCCTGGG-3′. Areas in the sequence containing 2′-methoxyethyl modifications are indicated by underlines. Cells were incubated with oligonucleotides at concentrations of 50–350 nM in REGM containing DOTMA/DOPE (Lipofectin, GIBCO BRL) at a concentration of 0.25 μg/10 nmol of oligonucleotide. After 4 hr, the medium was removed and replaced with warm REGM. Cells were treated with the caspase inhibitors DEVD-CHO (Biomol Research Laboratories) or YVAD-CHO (Biomol Research Laboratories), at 50 μM, or with 10 μM of the p38 inhibitor SB203580 (Calbiochem) for the last hour of hypoxia and then were reoxygenated as described above.

Northern Blot Analysis

Total RNA was prepared from cells by the guanidinium isothiocyanate procedure or by the Qiagen RNeasy method according to the manufacturer's directions. RNA samples were quantitated spectrophotometrically, electrophoresed through 1.2% agarose–formaldehyde gels, and transferred to Hybond-N^+ nucleic acid transfer membranes (Amersham) by capillary diffusion for 12–14 hr. Immobilized RNA was cross-linked to the membrane by exposure to UV light using a Stratalinker (Stratagene) and hybridized using

^{32}P-labeled JNK1 or G3PDH specific cDNA probes, which were prepared by asymmetric polymerase chain reaction (PCR) using specific cDNA templates. Probes hybridized to mRNA transcripts were visualized and quantitated using a Molecular Dynamics PhosphorImager. Blots were routinely stripped of radioactivity by boiling and reprobed with a ^{32}P-radiolabeled G3PDH probe to confirm equal loading.

Western Blot Analysis

For western blot analysis, cells subjected to hypoxia, hypoxia/reoxygenation, or treatment with anisomycin (10 μg/mL for 1 hr) were lysed and centrifuged, and total protein concentration was determined. Extracts were boiled in SDS–PAGE sample buffer, and proteins (40 μg/lane) were separated on a 10% SDS–polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane, treated with blocking buffer [PBS containing 10% (w/v) dry milk (Carnation) and 0.2% Tween-20]. Membranes were probed with either anti-phospho-specific JNK antibody (1:1000 dilution, New England Biolabs), which detects only dually-phosphorylated JNK (Thr183/Tyr185), anti-JNK1 (F-3) antibody, which is specific for JNK1 (1:1000 dilution, Santa Cruz), or anti-phospho-specific p38 MAP kinase antibody (1:1000 dilution, New England Biolabs), which detects only dually-phosphorylated p38 MAP kinase (Thr180/Tyr182) in blocking buffer (overnight, 4°). Anti-rabbit IgG conjugated with horseradish peroxidase was used as the secondary antibody (1:1000 dilution, 1 hr, room temperature), and immune complexes were visualized using enhanced chemiluminescence according to the manufacturer's instructions (LumiGLO reagent, New England Biolabs). Blots were quantitated by laser scanning densitometry.

Measurement of DNA Fragmentation

Cells, grown in 96-well microtiter plates (4500 cells/well), were labeled overnight with 5-bromo-2′-deoxyuridine (10 μM) at 37°. After labeling, the cells were exposed to hypoxia or hypoxia/reoxygenation as indicated. Next, cells were lysed, and fragmented DNA was separated from intact chromatin by centrifugation (10 min, 250 *g*). Internucleosomal DNA fragmentation was determined quantitatively using a DNA fragmentation photometric ELISA kit (Boehringer Mannheim) according to the manufacturer's directions. The absorbance at 370 nm was recorded using a microplate reader (Ceres UV900C, Biotek Instruments).

Quantitation of Apoptosis and Necrosis

To determine that cell death was due to apoptosis, quantitation of cell viability and apoptotic index was determined by acridine orange/ethidium bromide uptake according to the procedure described by McGahon *et al.* [32] with some modifications. RPTE cells were plated into Labtek 1-well Permanox chamber slides (Nalge, 80,000 cells/chamber) in 2 mL of growth medium. Following exposure to hypoxia or hypoxia/reoxygenation, medium was aspirated from the cells, and 50 μL of a 1:1 stock solution of ethidium bromide and acridine orange was mixed with 1 mL of PBS and added immediately to the cells. Stock solutions of ethidium bromide (Sigma) and acridine orange (Sigma) were made up in PBS at a concentration of 100 μg/mL of solution. Since dye uptake is instantaneous, the morphological features of apoptosis were monitored by fluorescence microscopy using a microscope equipped with a fluorescein isothiocyanate filter at 600X. For photomicroscopy, cells were plated into Falcon Primaria 60-mm dishes (125,000 cells/dish) in 5 mL of the appropriate growth medium. Following exposure to hypoxia or hypoxia/reoxygenation, cells were detached with trypsin as described above and pelleted by centrifugation (800 *g*, 5 min). Cells were resuspended in 1 mL of ice-cold REGM, transferred to a clean 1.5-mL microcentrifuge tube, and re-centrifuged (800 *g*, 5 min). The cell pellet was resuspended into 50 μL of ice-cold growth medium and kept on ice. Each 50 μL of cell suspension was stained by the addition of 2.5 μL of a 1:1 stock solution of ethidium bromide/acridine orange. A 4-μL aliquot of stained cells was removed immediately to an etched ring of a Gold Seal fluorescent antibody slide, and a coverslip was attached.

Treated cells were quantitated 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 quantitated for each data point. The apoptotic index [percentage of apoptotic (or necrotic) cells] was calculated as the number of apoptotic (or necrotic) cells/total cells counted × 100. Sample identities were concealed during scoring.

Apoptosis was also monitored using an Annexin-V-FLUOS (Boehringer Mannheim) staining kit according to the manufacturer's instructions. Briefly, cells were exposed to treatments as described above and detached from the chamber slide with a 30-sec rinse with Cell Dissociation Buffer pre-warmed to 37°, followed by the addition of 1 mL of PBS. Slides were placed in a 37° 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 of 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 600X. The apoptotic index was calculated based on a minimum count of 200 cells per observation.

Assessment of Cell Proliferation

The trypan blue dye-staining procedure was used to quantitate the number of cells (both viable and non-viable) following the experimental treatments used in the present study. Cells were rinsed in PBS followed by a 30-sec rinse with a solution of 0.01% EDTA/0.025% trypsin. After low-speed centrifugation, the cells were suspended in PBS with an equal volume of 0.4% trypan blue stain solution for 5 min. The viable and non-viable cells were counted using a hemacytometer.

Data Analysis

Student's *t*-test was used to determine statistical significance. Data analysis and graph generation were performed with GraphPad Prism.

RESULTS

Induction of Apoptosis by Hypoxia and Hypoxia/Reoxygenation in Human Kidney Cells

Exposure of kidney cells to increasing periods of hypoxia resulted in a small, albeit significant, increase in DNA fragmentation (2.0 ± 0.3-fold over normoxic levels) after 48 hr of hypoxic treatment, whereas normoxic, time-matched control cultures were viable and showed no DNA fragmentation (Fig. 1A). In contrast, a pronounced increase in DNA fragmentation occurred in RPTE cells exposed to 4 hr of hypoxia followed by reoxygenation (for 15 hr) or 8 hr of hypoxia followed by reoxygenation (for either 15 or 24 hr) (Fig. 1B). A similar profile of DNA fragmentation was observed in cells reoxygenated for 24 hr following exposure to 15 hr of hypoxia (data not shown). Cells exposed to either normoxic conditions or hypoxia alone exhibited no increase in DNA fragmentation (Fig. 1B). These data indicated that reoxygenation of hypoxic RPTE cells markedly accelerated cellular death.

Measurement of Apoptosis and Necrosis Following Hypoxia/Reoxygenation

To determine the type of cell death induced by hypoxia and reoxygenation, the assessment of nuclear shapes and membrane integrity was determined using fluorescence microscopy. For these experiments, RPTE cells were exposed to either hypoxia or hypoxia/reoxygenation, labeled with the DNA binding dyes ethidium bromide and acridine orange, and quantitated by fluorescent microscopy. Compared with normoxic cultures, which exhibited dispersed chromatin (Fig. 2A), RPTE cells exposed to hypoxia followed by reoxygenation exhibited morphological features characteristic of apoptosis, including nuclear condensation, membrane blebbing, and the formation of apoptotic bodies (Fig. 2, B and C). Following hypoxia/reoxygenation, the majority of apoptotic cells (~95%) were classified as early apoptotic. Quantitation of these cells demonstrated that

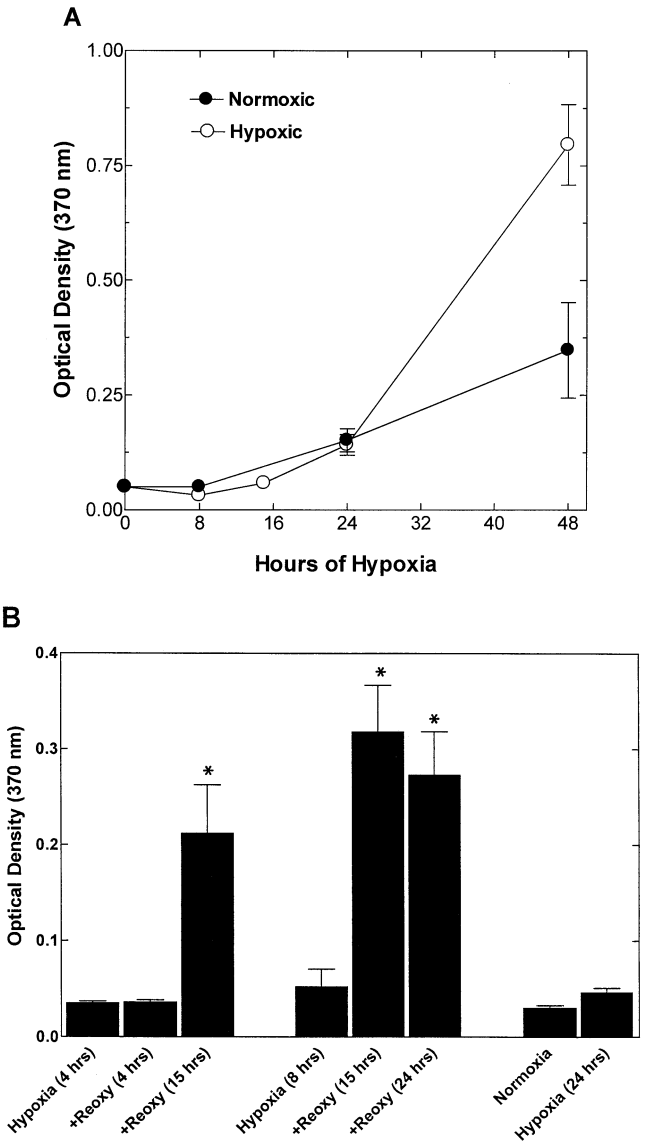

FIG. 1. Effect of hypoxia and hypoxia/reoxygenation on DNA fragmentation in human kidney cells. (A) Time course of the effects on DNA fragmentation. Following exposure of RPTE cells to hypoxia for the indicated times, cells were lysed, and cytoplasmic DNA fragments were measured by photometric ELISA. Values shown are the means ± SEM of triplicate determinations from one of three similar experiments. (B) Following exposure to either 4 or 8 hr of hypoxia, cells were reoxygenated for the indicated times. Cells were lysed and DNA fragmentation was measured by photometric ELISA. Values for normoxic (control) and 24-hr hypoxic cultures are shown for comparison. Results shown are means ± SEM of triplicate determinations from one of three similar experiments. Key: (*) significantly different from untreated cells, $P < 0.05$.

the percentages of apoptotic and necrotic cells present under normoxic conditions were 5 and 2%, respectively (Table 1). An increase in the number of apoptotic cells was observed with increased exposure to hypoxia, reaching a maximum of 15% at 48 hr of hypoxia, whereas only 4% of the cells were necrotic at this time point (Table 1). Reoxygenation of cells exposed to either 4, 8, or 15 hr of hypoxia markedly increased the fraction of cells undergoing

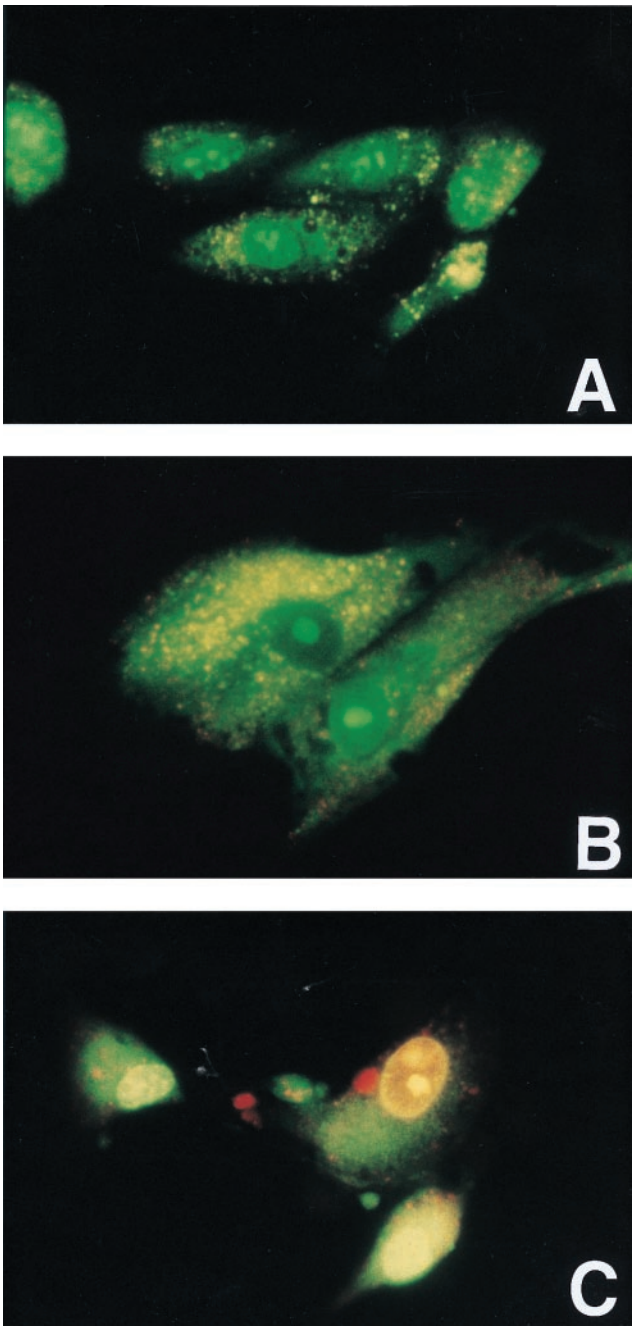

FIG. 2. Representative morphology of normoxic and hypoxic/reoxygenated RPTE cells. Cells were subjected to either normoxia or hypoxia/reoxygenation, labeled with the DNA binding dyes ethidium bromide and acridine orange, and visualized using fluorescence microscopy using a fluorescein isothiocyanate filter. (A) Normoxic RPTE cells, demonstrating dispersed nuclear chromatin and a uniform green fluorescence indicative of a viable cell; 200X. (B) RPTE cells exposed to 8 hr of hypoxia followed by 15 hr of reoxygenation, showing condensed nuclear chromatin that appears bright green, indicative of early apoptosis; 400X. Under the conditions employed in this study, the majority (~95%) of apoptotic cells were classified as early apoptotic. However, the remainder exhibited morphology of late apoptosis. (C) Nucleus fluorescing orange typical of a nonviable cell, shrunken size, and formation of red apoptotic bodies; 400X

TABLE 1. Apoptosis: the predominant form of cell death following hypoxia/reoxygenation of human kidney cells

Hours of hypoxia	Hours of reoxygenation	Apoptotic cells (% of total)	Necrotic cells (% of total
0	0	4.6 ± 0.6	1.9 ± 0.5
4	0	6.7 ± 0.9	2.0 ± 0.6
4	15	16.2 ± 0.8*	3.0 ± 0.7
8	0	6.7 ± 1.5	1.7 ± 0.5
8	15	14.0 ± 3.5*	2.4 ± 0.7
8	24	17.3 ± 1.3*	2.4 ± 0.6
15	0	7.1 ± 0.8	3.1 ± 0.9
15	8	16.3 ± 1.7*	3.1 ± 0.9
15	24	16.7 ± 1.3*	3.3 ± 0.9
24	0	9.4 ± 2.7	2.7 ± 0.4
48	0	14.8 ± 3.1	4.0 ± 0.3

Human kidney cells were exposed to hypoxia or hypoxia/reoxygenation for the indicated times. 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 quantitated for each data point. The apoptotic index [percentage of apoptotic (or necrotic) cells] was calculated as the number of apoptotic (or necrotic) cells/total cells counted × 100. Values shown are the means ± SEM from 3–4 independent experiments.

*Significantly different from cells exposed to the corresponding hypoxic treatment only ($P < 0.05$).

apoptosis but had no significant effect on the number of necrotic cells present (Table 1). Apoptosis was also quantitated using Annexin-V–fluorescein and propidium iodide to quantitate apoptotic and necrotic cells, respectively. Following normoxia or 4-hr hypoxia, 8.5 ± 2 and 9.7 ± 3% (N = 3) of cells were apoptotic, and this value increased to 20 ± 2% (N = 3) following 4 hr hypoxia/15 hr reoxygenation. Taken together, these results demonstrated that hypoxia/reoxygenation predominately induced apoptotic, but not necrotic cell death and confirmed the augmentation in apoptosis following reoxygenation.

A key process associated with apoptosis is the activation of aspartate-specific proteases, called caspases, that cleave cellular target proteins. Pretreatment of human kidney cells with the caspase inhibitors YVAD-CHO or DEVD-CHO at 50 μM for 1 hr prior to reoxygenation significantly attenuated reoxygenation-induced DNA fragmentation by 74 ± 7% (N = 3) and 76 ± 1% (N = 3), respectively. Since both cell death and proliferation contribute to the apoptotic index, direct cell counting was performed to assess the contribution of cell proliferation under the conditions employed in the present study. The extent of proliferation was increased significantly only for the longer periods of hypoxia/reoxygenation, where an increase in total cell number of 212 ± 23 and 219 ± 45% (N = 3) was observed following 8 hr hypoxia/24 hr reoxygenation and 15 hr hypoxia/24 hr reoxygenation.

Induction of JNK Activation and p38 MAP Kinase Following Hypoxia and Hypoxia/Reoxygenation in Human Kidney Cells

To determine whether apoptotic death was associated with the activation of stress kinases, cells were exposed to

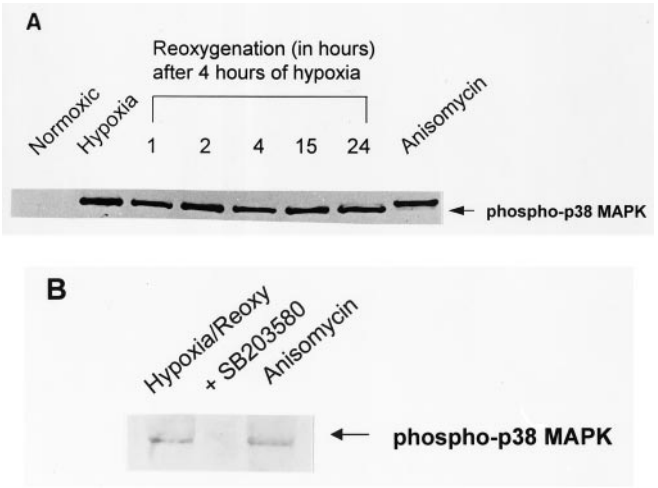

FIG. 3. Phosphorylation of p38 MAP kinase by hypoxia and hypoxia/reoxygenation. (A) Human kidney cells were exposed to either 4 hr of hypoxia or hypoxia/reoxygenation for the indicated time periods. (B) Western blot analysis of cells exposed to 10 μM SB203580 for 1 hr followed by 4 hr of hypoxia and 4 hr of reoxygenation. Cell lysates were prepared, and equal amounts of protein (40 μg) were subjected to western blot analysis using a phospho-specific p38 MAP kinase (Tyr182) antibody. Lysates from anisomycin-treated cultures are shown as a positive control.

hypoxia or hypoxia/reoxygenation, and immunoblot analysis was performed using antibodies that recognized the activated, phosphorylated forms of either p38 MAP kinase or JNK. Exposure of human kidney cells to hypoxia significantly phosphorylated Tyr182 of p38 MAP kinase, indicating the activation of this enzyme (Fig. 3A). The level of phosphorylation of p38 MAP kinase was maintained, not augmented, following increasing periods of reoxygenation. Pretreatment of cells with 10 μM SB203580, a specific inhibitor of p38 MAP kinase [33], inhibited the phosphorylation of p38 MAP kinase induced by hypoxia/reoxygenation (Fig. 3B). Furthermore, reoxygenation-induced DNA fragmentation was not suppressed (104 ± 4% of untreated values, N = 3) by 10 μM SB203580, suggesting that apoptosis induced by reoxygenation is not dependent upon p38 MAP kinase activation.

In RPTE cells exposed to increasing periods of hypoxia, there was no phosphorylation of JNK, indicating that the enzyme was not activated under hypoxic conditions (Fig. 4A). However, in cells exposed to anisomycin, a strong activator of JNK [24], a potent phosphorylation of both p46-JNK and p54-JNK was noted (Fig. 4A). In contrast to the results obtained under hypoxic conditions, a sustained and potent increase in the phosphorylation of both p46-JNK and p54-JNK was observed following exposure of RPTE cells to hypoxia/reoxygenation, indicating the activation of JNK following reoxygenation (Fig. 4B). JNK phosphorylation was detected at 1 hr after reoxygenation and increased steadily from 1 to 24 hr post-reoxygenation. Quantitation of western blots by densitometry revealed an ~20-fold increase observed at 24 hr of reoxygenation when compared with cells exposed to hypoxia only (Fig. 4C). To determine whether the increase of JNK phosphorylation was due to increased levels of JNK protein in these cells, western blotting of cellular extracts was performed. These experiments demonstrated that an equal amount of total JNK protein was detected in lysates from cells exposed to increasing periods of reoxygenation, indicating that the elevation in JNK phosphorylation was not due to an increase in the total amount of JNK protein (Fig. 4D).

Treatment of Human Kidney Cells with a JNK1 Antisense Oligonucleotide

Oligonucleotides directed against JNK have been used previously to investigate the role of the JNK pathway in caspase activation and apoptosis induction by antitumor compounds [34] and in EGF-induced cellular proliferation [35]. To address whether activation of the JNK pathway played a role in reoxygenation-induced apoptosis, human kidney cells were exposed for 24 hr to JNK1 AS (350 nM), an antisense oligonucleotide targeted against human JNK1. Northern blot analysis, performed using lysates from cells exposed to increasing concentrations of JNK1 AS, resulted in a potent and concentration-dependent reduction of JNK1 mRNA levels ($IC_{50} < 50$ nM). An almost complete inhibition of JNK1 mRNA expression occurred following treatment of cells with concentrations of JNK1 AS of 150 nM or greater. A small, dose-independent reduction of JNK2 mRNA expression was also observed following treatment with higher concentrations of JNK1 AS (Fig. 5, A and B). JNK1 or JNK2 mRNA expression was not altered following exposure to the 6-base mismatch antisense control for the JNK1 oligonucleotide, indicating that JNK1 AS reduced JNK mRNA levels in a sequence-specific manner (Fig. 5, A and B).

Quantitative analysis of western blots performed using an antibody that recognizes JNK1 revealed that treatment with increasing concentrations of JNK1 AS for 48 hr resulted in a marked, concentration-dependent inhibition of both p46-JNK1 and p54-JNK1 protein levels (Fig. 5C). In contrast, there was no significant reduction in either p46-JNK1 or p54-JNK1 protein levels in cells exposed to a 350 nM concentration of the 6-base mismatch control oligonucleotide (Fig. 5C).

Exposure of RPTE cells to 4 hr of hypoxia resulted in a similar percentage (~5%) of apoptotic cells as was found in normoxic cultures. However, when these hypoxic cells were reoxygenated for 15 hr, a significant increase in the number of apoptotic cells was observed (Fig. 6). Pretreatment of RPTE cells with JNK1 AS resulted in a significant blockade of reoxygenation-induced apoptosis. A 61 ± 8% (N = 5) and 72 ± 7% (N = 6) decrease in the number of apoptotic cells was noted following treatment with 250 or 350 nM JNK1 AS, respectively (Fig. 6). Furthermore, treatment of RPTE cells with the mismatch control oligonucleotide had no effect on the number of apoptotic cells following reoxygenation (Fig. 6). Treatment of cells with JNK1 AS

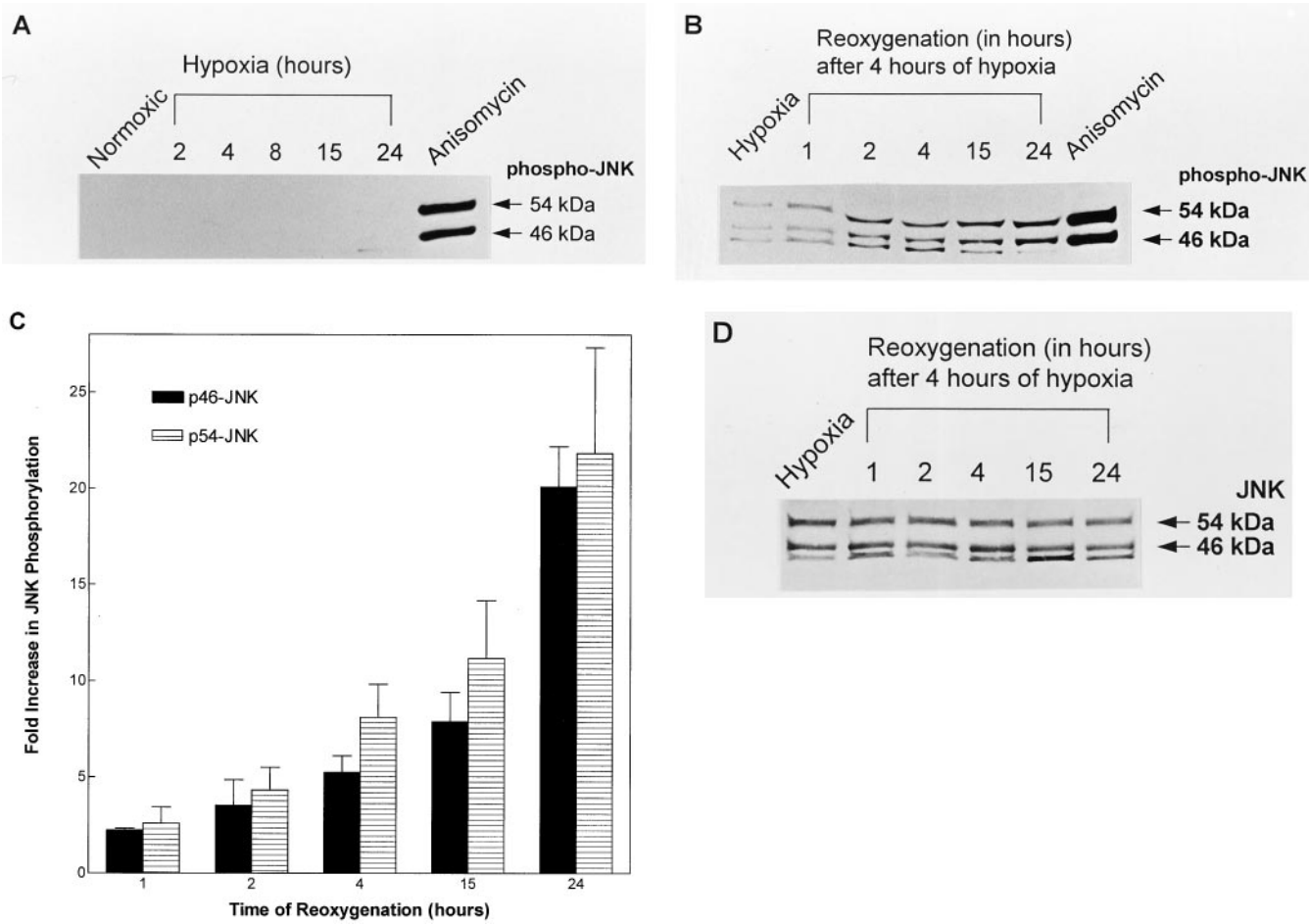

FIG. 4. Time-dependent effects of hypoxia and hypoxia/reoxygenation on JNK phosphorylation in human kidney RPTE cells. (A) Western blot analysis of cell lysates obtained from human kidney cells exposed to either increasing amounts of hypoxia or anisomycin (10 μg/mL for 1 hr). (B) Western blot analysis of cell lysates from human kidney cells exposed to 4 hr of hypoxia followed by the indicated periods of reoxygenation. Lysates from normoxic and anisomycin-treated cultures are shown for comparison. The lumigram is representative of four separate experiments. In A and B, cell lysates were resolved by western blot analysis on 10% SDS–polyacrylamide gels and probed using a phospho-specific JNK (Thr183/Tyr185) antibody. (C) Densitometric quantitation analysis of JNK phosphorylation following 4 hr of hypoxia followed by the indicated periods of reoxygenation. Results are the means ± SEM from four separate experiments. Data are expressed relative to cells that received only hypoxia. (D) Cell lysates from RPTE cells exposed to hypoxia/reoxygenation and subjected to western analysis using an anti-JNK antibody.

(350 nM), but not the mismatch oligonucleotide, significantly inhibited reoxygenation-induced apoptosis by 57 ± 5%, as monitored by Annexin-V staining. Under these experimental conditions, treatment of cells with the oligonucleotides had no significant effect on cell proliferation.

DISCUSSION

The present results demonstrated that both JNK activation and apoptosis induction occur following hypoxia/reoxygenation of human kidney cells when compared with cells exposed to only hypoxia. More importantly, using a 2′-methoxyethyl mixed backbone antisense oligonucleotide that inhibits JNK1 mRNA and protein expression, we demonstrated a potent suppression of reoxygenation-induced apoptosis. Our current data provide definitive evidence that JNK was involved in the induction of apoptotic cell death following hypoxia/reoxygenation.

Exposure of human kidney cells to hypoxia followed by reoxygenation produced a marked increase in apoptotic, but not necrotic, cell death. Kidney cells exposed to hypoxia/reoxygenation exhibited distinct morphological changes associated with apoptosis, including chromatin condensation, membrane blebbing, DNA fragmentation, and the formation of apoptotic bodies. An essential component of apoptotic cell death is activation of cysteine proteases, called caspases, which mediate cellular proteolysis. In the present study, the caspase inhibitors YVAD-CHO and DEVD-CHO significantly attenuated reoxygenation-induced DNA fragmentation in human kidney cells, providing further evidence for an apoptotic mode of cell death following reoxygenation. In contrast, the cells appeared resistant to the effects of hypoxia, as only hypoxic periods greater than 48 hr resulted in apoptosis. These findings are consistent with studies performed using cardiomyocytes and perfused heart or kidney, which show that

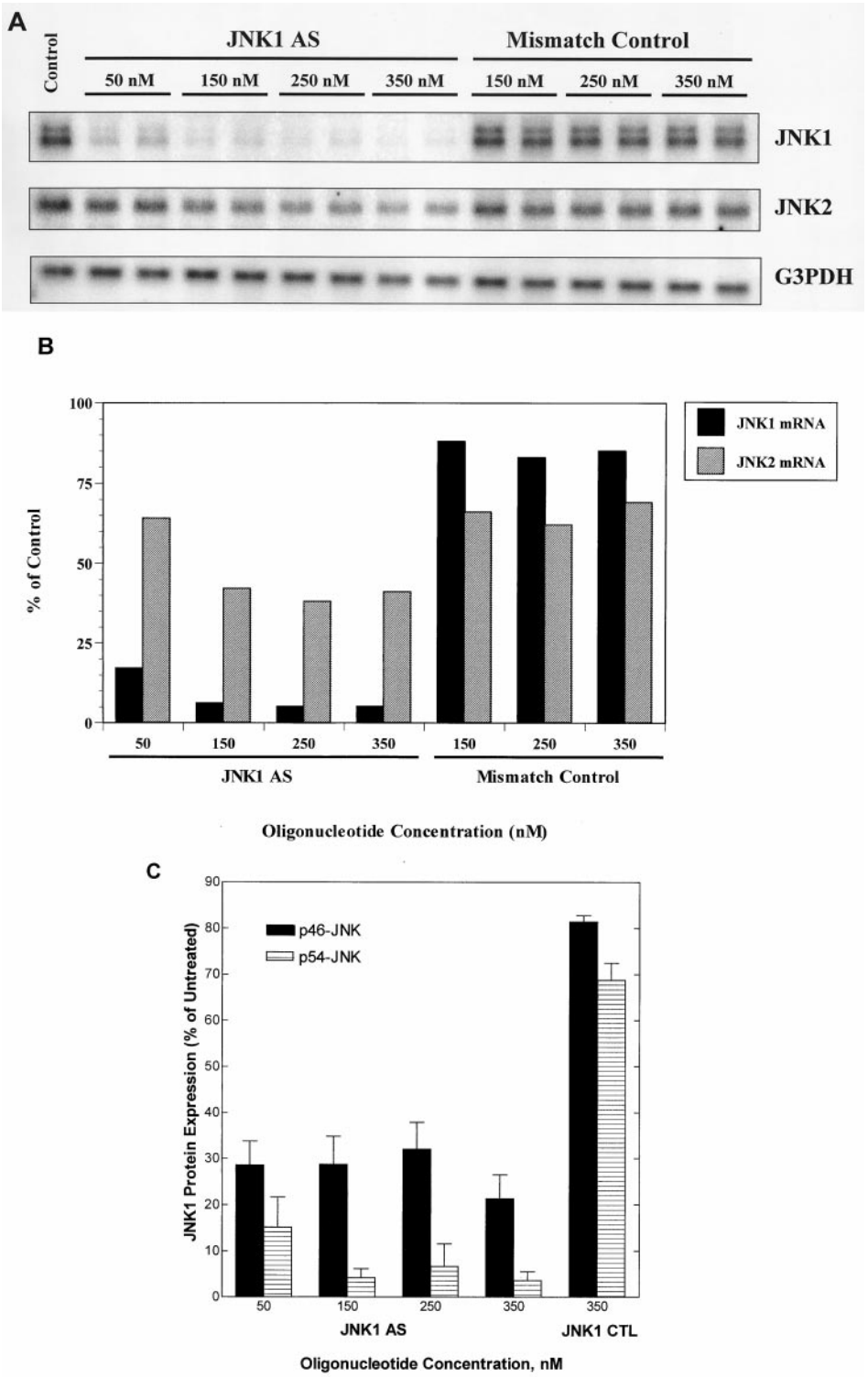

FIG. 5. Inhibition of JNK mRNA and protein expression by JNK1 antisense oligonucleotide and mismatched control in human kidney cells. (A) Cells were treated with increasing concentrations (50–350 nM) of an antisense oligonucleotide directed against human JNK1 (JNK1 AS) or a 6-base mismatch control oligonucleotide, and total RNA was prepared 24 hr later. Normalized RNA was analyzed for JNK1, JNK2, or G3PDH levels in duplicate by northern blotting. "Control" indicates cells treated with cationic lipid only. (B) Quantitation of JNK mRNA levels from panel A. JNK mRNA levels were normalized to G3PDH mRNA levels and quantitated by PhosphorImager analysis as described. The results are representative of two independent experiments. (C) Western blot analysis of JNK1 protein levels in RPTE cells treated with an antisense oligonucleotide directed against human JNK1 (JNK1 AS) or mismatched control oligonucleotide (JNK1 CTL). Protein extracts were prepared from RPTE cells exposed to increasing concentrations of the indicated oligonucleotide for 48 hr, analyzed by western blotting, and probed using a specific JNK1 antibody. Shown is a quantitative representation of JNK1 protein expression, which was obtained by densitometric scanning of lumigrams and expressed as a percentage of the level in cells not treated with oligonucleotide. The results are the means ± SEM from three independent experiments.

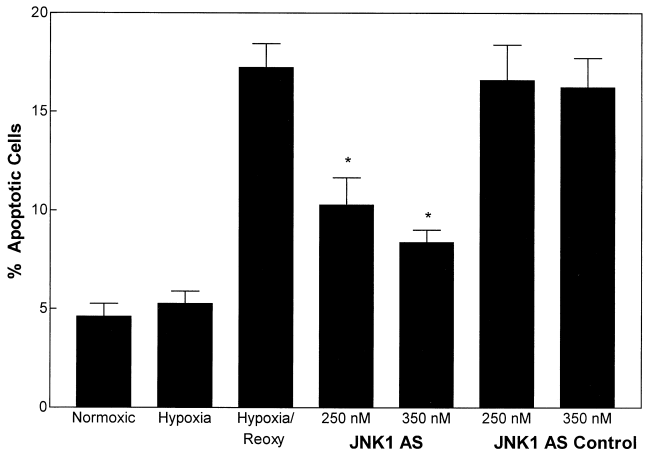

FIG. 6. Inhibition of reoxygenation-induced apoptosis by an antisense oligonucleotide directed against JNK1 in human kidney cells. Cells were incubated with 350 nM JNK1 oligonucleotide (JNK1 AS) or the 6-base mismatch control oligonucleotide for 48 hr prior to exposure to either hypoxia (4 hr) or hypoxia (4 hr)/reoxygenation (15 hr). The percentages of apoptotic cells were determined by fluorescence microscopy and are shown as a percentage of the total cells counted. At least 200 cells from randomly selected fields were counted and quantitated for each data point. The apoptotic index (percentage of apoptotic cells) was calculated as the number of apoptotic cells/total cells counted x 100. Values shown are the means ± SEM obtained from three to six experiments. Key: (*) significantly different from untreated cells, $P < 0.05$.

apoptotic cell death is enhanced following ischemia/reperfusion [2, 4, 5].

JNK activation following hypoxia and hypoxia/reoxygenation exhibited an identical profile to that of apoptosis induction. Whereas JNK was not activated following hypoxia alone, a potent, albeit prolonged, pattern of JNK activation was noted following reoxygenation of hypoxic kidney cells. The pattern of JNK phosphorylation occurred steadily over the course of several hours, whereas a more short-lived (usually 1–2 hr) period of JNK activation has been noted after ischemia/reperfusion using perfused heart or cardiomyocytes [5, 14, 18]. The delay in the onset of JNK phosphorylation observed in the human kidney cells may be a consequence of the hypoxic, instead of ischemic, conditions employed in the current study. Although hypoxia serves as a major component of ischemia, the onset and type of cellular stress resulting from these two conditions may differ [36]. More importantly, sustained JNK activity may serve as a critical initiator of apoptotic death. A persistent and prolonged JNK activation has been implicated previously in the initiation of apoptotic cell death in diverse cell types such as 293 kidney, U937, and Jurkat T cells as well as primary striatal cultures [37–39].

Only two previous studies have examined JNK activation and apoptosis in the same tissue following ischemia/reperfusion. Yin *et al.* [5] demonstrated a good correlation between the time courses for JNK activation and apoptosis induction following reperfusion injury in kidney and heart. Carvedilol, a β-adrenoceptor antagonist and antioxidant, inhibited apoptosis, Fas receptor expression, and JNK activation in rabbit hearts exposed to ischemia/reperfusion [15]. Whereas the data from these correlative studies suggest that JNK activation plays a role in apoptotic cell death following reperfusion injury, the precise role of JNK in reperfusion-induced apoptosis has not been examined directly. In the present study, we have utilized an antisense approach to provide definitive evidence for the role of JNK in apoptotic cell death following hypoxia/reoxygenation. Treatment of human kidney cells with a 2′-methoxyethyl mixed backbone antisense oligonucleotide targeted against human JNK1 resulted in a potent suppression of JNK mRNA and protein expression. The principal finding to emerge from these experiments was that a dramatic 61 and 72% reduction in reoxygenation-induced apoptosis was observed in cells treated with either 250 or 350 nM JNK1 AS, respectively. In contrast, there was no alteration in the number of apoptotic cells induced by reoxygenation following exposure of cells to a 6-base mismatch control oligonucleotide, indicating a sequence-specific mode of action. The present data conclusively demonstrate that activation of the JNK signaling pathway plays a predominant role in reoxygenation-induced apoptotic cell death. Whereas the data suggest that inhibition of JNK1 mRNA expression is sufficient to suppress reoxygenation-induced apoptosis, the partial involvement of JNK2 cannot be ruled out at present, since a small, albeit concentration-independent, suppression of JNK2 mRNA levels was also noted following treatment of kidney cells with JNK1 AS. Specific roles for JNK isoforms in the induction of stress-induced apoptosis have been addressed in only a few recent studies. Antitumor drug-induced apoptosis in myeloid leukemia U937 cells was inhibited by an antisense oligonucleotide targeted to JNK1 [34]. Furthermore, dominant negative mutants for JNK1, but not JNK2, suppressed apoptosis initiated by UV radiation in small cell lung cancer cells [40]. Finally, Yang *et al.* [41] demonstrated that mice lacking the *JNK3* gene, which is expressed predominantly in the brain, were protected against excitotoxicity-induced seizures and apoptotic cell death.

It has been suggested that p38 MAP kinase activation plays a role in the induction of apoptosis [42, 43]. Although JNK and p38 MAP kinase are activated by similar apoptosis-inducing stresses, these stress kinases exhibit diverse substrate specificity and are differentially activated via upstream kinases [26, 27]. The present findings demonstrate that p38 MAP kinase was phosphorylated markedly during hypoxia and that the phosphorylation was maintained during reoxygenation of human kidney cells, indicating a distinct pattern of activation for this stress kinase when compared with JNK. These data are in agreement with previous published reports in rat heart and kidney, which showed that p38 MAP kinase was activated following ischemia only [5, 14]. Furthermore, reoxygenation-induced DNA fragmentation in these human kidney cells was not attenuated by pre-exposure to SB203580, a p38 MAP kinase inhibitor [33], providing further evidence that p38

MAP kinase does not play a role in reoxygenation-induced apoptosis.

In conclusion, hypoxia/reoxygenation, but not hypoxia alone, activated both the JNK signaling pathway and apoptosis in human kidney cells. Ablation of JNK mRNA and protein expression with an antisense oligonucleotide against JNK1 resulted in a significant inhibition of apoptosis in human kidney cells exposed to hypoxia/reoxygenation. These data indicated that JNK is a crucial component in apoptosis induction following reoxygenation.

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