Activation of c-Jun N-terminal kinase promotes survival of cardiac myocytes after oxidative stress

Christopher J. DOUGHERTY*, Lori A. KUBASIAK*, Howard PRENTICE†, Peter ANDREKA*, Nanette H. BISHOPRIC* and Keith A. WEBSTER*[1]

*Department of Molecular and Cellular Pharmacology, University of Miami Medical Center, 1600 NW 10th Avenue, RMSB 6038, Miami, FL 33136, U.S.A., and †Biomedical Sciences, Charles E. Schmidt College of Science, Florida Atlantic University, Boca Raton, FL 33431, U.S.A.

Reperfusion injury occurs when ischaemic tissue is reperfused. It involves the generation and release of reactive oxygen that activates numerous signalling pathways and initiates cell death. Exposure of isolated cardiac myocytes to chronic hypoxia followed by reoxygenation results in the early activation of c-Jun N-terminal kinase (JNK) and death by apoptosis of approx. 30% of the myocytes. Although JNK activation has been described in a number of models of ischaemia/reperfusion, the contribution of JNK activation to cell fate has not been established. Here we report that the activation of JNK by reoxygenation correlates with myocyte survival. Transfection of myocytes with JNK pathway interfering plasmid vectors or infection with adenoviral vectors support the hypothesis that JNK is protective. Transfection or infection with JNK inhibitory mutants increased the rates of apoptosis by almost 2-fold compared with control cultures grown aerobically or subjected to hypoxia and reoxygenation. Caspase 9 activity, measured by LEHD cleavage, increased > 3-fold during reoxygenation and this activity was enhanced significantly at all times in cultures infected with dominant negative JNK adenovirus. Hypoxia–reoxygenation mediated a biphasic (2.6- and 2.9-fold) activation of p38 mitogen-activated protein kinase, as well as a small increase of tumour necrosis factor α (TNFα) secretion, but treatments with the p38 MAPK-specific inhibitor SB203580 or saturating levels of a TNFα-1 blocking antibody provided only partial protection against apoptosis. The results suggest that JNK activation is protective and that the pathway is largely independent of p38 MAPK or secreted TNFα.

Key words: apoptosis, caspase, hypoxia, ischaemia, reoxygenation.

INTRODUCTION

Reperfusion injury to the myocardium results, at least in part, from oxygen free radicals that are released from the ischaemic tissue upon reoxygenation. This damage may be reversible or irreversible depending on the severity and duration of the ischaemic period (reviewed in [1]). Reversible damage includes arrhythmia and myocardial 'stunning', both of which may be caused by combinations of free-radical attack and transient calcium overload [2]. Irreversible damage occurs when the ischaemic period is extended and severe; it is also mediated by oxidative stress and involves myocardial cell death through necrosis, apoptosis and/or oncosis [3–5]. Apoptotic cell death in response to ischaemia–reperfusion or hypoxia–reoxygenation has been described in multiple cell types including neuronal, kidney and tumour cells, as well as cardiac myocytes [6–10]. Mitochondrial damage, cytochrome *c* release and activation of caspases 9 and 3 have been implicated in these pathways of cell death (reviewed in [11]). The factors that modulate these signalling pathways have not been described fully in any system.

Numerous reports have documented changes of kinase signalling pathways in redox-stressed cells and tissues, including cardiac myocytes and intact myocardium (reviewed in [12,13]). Many such studies focus on the relative activities of mitogen-activated protein kinase/extracellular-signal-regulated protein kinase (MAPK/ERK) and the stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 MAPK, because the balance of these may determine cell fate [14]. Reports on the contributions of MAPK, JNK and p38 MAPK to cardiac myocyte apoptosis after oxidative stress are conflicting. These include evidence that JNK and/or p38 MAPK activation promote apoptosis [15–22] or that upstream activators of JNK may prevent apoptosis, possibly by modulating the production of tumour necrosis factor α (TNFα) [23]. JNK is induced strongly by oxidative stress in primary cardiac myocyte cultures as well as in the intact heart [24]. Hypoxia–reoxygenation *in vitro* and ischaemia–reperfusion *in vivo* result in loss of cardiac myocytes, a process in which apoptosis probably plays a significant role [3,25–27]. We report here that JNK activation in neonatal cardiac myocytes subjected to hypoxia–reoxygenation does not promote apoptosis and may be protective.

A preliminary report of this work has been published in abstract form [27a].

MATERIALS AND METHODS

Materials

Antibodies to JNK1, JNK2, MAPK/ERK kinase kinase 1 (MEKK1), TNFα and haemagglutinin antigen (HA) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies

Abbreviations used: JNK, c-Jun N-terminal kinase; TNFα, tumour necrosis factor α; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal-regulated protein kinase; MEKK1, MAPK/ERK kinase kinase 1; MKK4, MAPK kinase 4; MAPKAP kinase 2, MAPK-activated protein kinase 2; HA, haemagglutinin antigen; Hsp27, heat-shock protein 27; TUNEL, terminal transferase deoxytidyl uridine end labelling; dn, dominant negative; ca, constitutively active; GFP, green fluorescent protein; PI, propidium iodide.

[1] To whom correspondence should be addressed (e-mail kwebster@chroma.med.miami.edu).

against phospho-ERK, phospho-JNK, p38 MAPK and phospho-p38 MAPK were from New England Biolabs (Beverly, MA, U.S.A.). Anti-MAPK-activated protein kinase 2 (anti-MAPKAP kinase 2) antibody and heat-shock protein 27 (Hsp27) were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-vertebrate sarcomeric myosin antibody (MF-20) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A. Secondary antibodies (FITC- and rhodamine-tagged) were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.). Recombinant human interleukin-1β and SB203580, as well as Hoechst 33342 and propidium iodide (PI) dyes were from CalBiochem (La Jolla, CA, U.S.A.). TUNEL (terminal transferase deoxytidyl uridine end labelling) assays were implemented using an ApopTag® Fluorescein *In Situ* Apoptosis Detection Kit (Intergen, Purchase, NY, U.S.A.) and used as described by the manufacturer. Polycationic liposomes (GeneFector™) were a gift from Dr Alberto Haces (VennNova, LLC, Pompano Beach, FL, U.S.A.) and LipofectAMINE™ was from Life Technologies (Rockville, MD, U.S.A.). TNFα and interferon-γ were from Genzyme (Cambridge, MA, U.S.A.). Reagents for constructing recombinant adenoviruses [28] were provided by Dr Bert Vogelstein (Howard Hughes Medical Institute, Baltimore, MD, U.S.A.).

Plasmids expressing MAPK-interfering genes, including dominant negative (dn) JNK1, dn MAPK kinase 4 (MKK4; stress-activated protein kinase/ERK kinase 1), wild-type MEKK1 and constitutively active (ca) MEKK1 [29], were gifts from Dr Gary Johnson (Division of Basic Sciences, National Jewish Medical and Research Center, Denver, CO, U.S.A.). The Thr-183 and Tyr-185 sites in dnJNK1 were mutated to Ala and Phe, respectively; in dnMKK4, the active-site Lys-116 of MKK4 was mutated to Arg; caMEKK1 was a truncated form of wild-type MEKK1 consisting of amino acids 1171–1493. The plasmid encoding HA-tagged JNK1 (HA-JNK1) [30] was a gift from Dr Silvio Gutkind (Oral and Pharyngeal Cancer Branch, National Institutes of Health, Bethesda, MD, U.S.A.). Caspase 9 activity was measured using an LEHD cleavage colorimetric assay kit, and TNFα was quantitated using a TNFα ELISA Kit™, both from R&D Systems (Minneapolis, MN, U.S.A.). All other reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Construction of Ad-dnJNK and Ad-caMEKK1

Procedures for construction of recombinant adenoviral vectors were as described previously [28]. Briefly, the cDNA inserts from pdnJNK or pcaMEKK1 were subcloned into the multiple cloning site of the shuttle plasmid pAdTrack-CMV. The purified recombinant plasmids were linearized and co-electroporated with pAdEasy-1 adenoviral backbone vector into *Escherichia coli* BJ5183. Purified recombinant adenoviral vector DNAs were linearized and lipofected into HEK-293 cells. Lipofected cells were monitored for green fluorescent protein (GFP) expression, harvested after 7–10 days, lysed and used to re-infect HEK-293 cells. Viral particles were purified and titres were determined using GFP-positive cells. Cardiac myocyte cultures were infected in serum-free medium at 20 plaque-forming units (p.f.u.)/cell for 24 h before the experiments began.

Cell culture

Methods for primary culture of neonatal rat cardiac myocytes have been described previously [31,32]. Briefly, enriched cultures of myocyte and non-myocyte cells were obtained from 1–2-day-old neonatal rats by stepwise trypsin dissociation and plated at a density of 4×10^6 cells/60 mm dish or 3×10^5 cells/well on four-well glass dishes (Nunc) in minimal essential medium supplemented with 5% fetal calf serum, penicillin and streptomycin. After 3–5 days, cells were transferred to a defined serum-free Dulbecco's modified Eagle's medium/M-199 (4:1) medium supplemented with transferrin, vitamin B_{12} and insulin. The final cultures contained $> 97\%$ cardiac myocytes contracting at > 200 beats/min. To inhibit fibroblast growth, 0.1 mM bromodeoxyuridine was included in the medium for the first 3–5 days after plating.

Hypoxia and reoxygenation

Cultures in defined serum-free Dulbecco's modified Eagle's medium/M-199 (4:1) containing 3.8 g/l glucose were exposed to hypoxia as described previously [32–34]. Oxygen was monitored continuously with an oxygen electrode (Controls Katharobic, Philadelphia, PA, U.S.A.) inside of the hypoxia chamber and contractility was monitored by edge detection as described previously [31,32]. The chamber oxygen tension was maintained at < 10 mm Hg. For replacement of the culture medium under hypoxia, fresh medium was made hypoxic before it was added to the dishes. Cultures exposed only to hypoxia (i.e. without reoxygenation) were lysed under hypoxia using ice-cold deoxygenated buffers. For reoxygenation, plates were removed from the chamber and reoxygenated by replacing the medium with oxygenated medium and incubating under 21% O_2 (95% air/5% CO_2).

Quantitative analysis of apoptotic nuclei

Cells were examined for morphological evidence of apoptosis or necrosis after staining with the fluorescent DNA-binding dyes Hoechst 33342 and PI, as described previously [33,35]. Treated and control cell monolayers grown on uncoated Nunc two-well coverslip dishes were rinsed with PBS and stained with 5 μg/ml Hoechst 33342 and 5 μg/ml PI for 15 min and visualized at a magnification of $\times 400$. Cells were scored as apoptotic if they exhibited unequivocal nuclear chromatin condensation and/or fragmentation, whereas PI-stained cells with normal nuclear morphology were scored as necrotic. In some experiments, apoptotic nuclei were localized within cardiac myocytes by staining with a monoclonal antibody against sarcomeric myosin (MF-20). Cells were fixed in ice-cold methanol, rinsed and stained with the MF-20 antibody and Hoechst 33342, followed by a rhodamine- or FITC-tagged anti-mouse IgG secondary antibody. GFP was visualized at 520 nm. TUNEL analyses were conducted using the ApopTag® Fluorescein *In Situ* Apoptosis Detection Kit, exactly as described by the manufacturer. Cells were imaged and photographed on a Zeiss IM inverted-phase fluorescence microscope using a mounted Contax 35 mm camera and 400 ASA Kodak colour transparency film. To quantify apoptosis, an average of 400 nuclei from random fields were analysed and apoptotic cell counts were expressed as a percentage of the total number of nuclei counted.

Analysis of DNA fragmentation

Cells were lysed for 5 h at 37 °C in a buffer containing 100 mM NaCl, 10 mM Tris/HCl, pH 8, 5 mM EDTA, 0.5% SDS and 1 μg/ml proteinase K. Proteins were precipitated with 0.8 M NaCl and DNA was extracted with phenol/chloroform and precipitated with isopropanol. The resulting pellet was resuspended in Tris/EDTA buffer and treated with DNase-free RNase for 30 min at 37 °C. The DNA content was quantitated by spectrophotometry at 260/280 nm. Samples (5 μg) were subjected to electrophoresis in 2% agarose gels and imaged by ethidium bromide staining and digital photography.

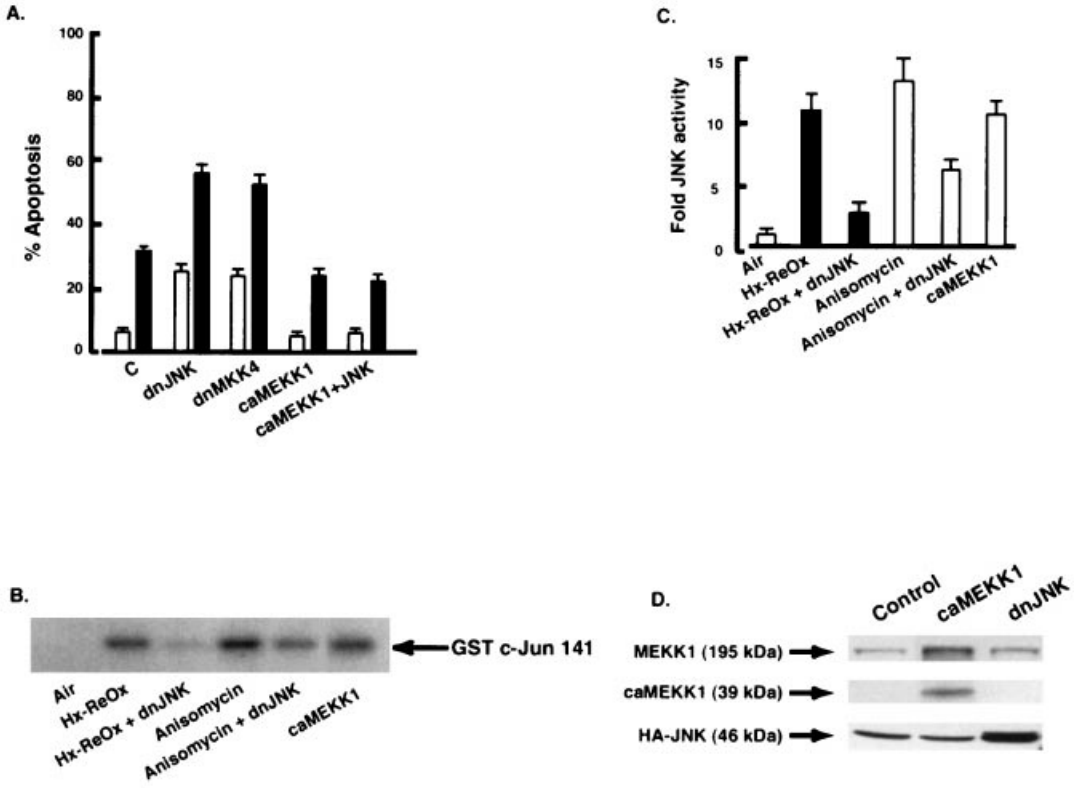

Figure 1 Modulation of apoptosis by JNK-interfering plasmids

(**A**) Cardiac myocytes were co-transfected with a reporter (GFP) and dnJNK1, dnMKK4, caMEKK1 or caMEKK1 + HA-JNK1 and subjected to aerobic incubation (open bars) or 20 h hypoxia and 20 h reoxygenation (solid bars) as indicated. GFP-positive cells were counted under the microscope after treatments and rates of apoptosis are expressed as the percentage of apoptotic cells (means ± S.E.M.), determined by Hoechst 33342 and PI staining as described in the Materials and methods section. PI-positive (necrotic) cells were $< 1\%$ in all cases (results not shown). (**B**) Cells were co-transfected with HA-JNK1 and an empty pcDNA3 plasmid (lanes 1, 2 and 4), dnJNK1 (lanes 3 and 5) and caMEKK1 (lane 6) and subjected to hypoxia and reoxygenation (Hx-ReOx) or anisomycin treatment (10 μg/ml for 1 h) as indicated. HA-JNK1 was assayed as described in the Materials and methods section. (**C**) Quantitation of JNK activity from three separate experiments; open bars represent aerobic cultures, solid bars represent hypoxia–reoxygenation treatment. (**D**) Western blots of cardiac myocytes transfected with HA-JNK1 and empty vector (control), caMEKK1 or dnJNK1 as indicated were probed with anti-MEKK1 or anti-HA antibodies as described in the Materials and methods section. The caMEKK1 band migrated to 72 kDa because it is a small fragment of the wild-type MEKK1 (amino acids 1171–1493). Two bands can be seen in the dnJNK1 lane that probably represent HA-JNK1 and HA-dnJNK1.

Western-blot analysis

Our methods for Western-blot analyses have been described previously [33]. Cells were harvested in ice-cold lysis buffer containing 1 mM sodium vanadate, 0.5 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin and 2 μg/ml aprotinin. Protein content was determined using a Pierce BCA kit. Equal amounts of protein (50–100 μg) were fractionated on SDS/polyacrylamide (12%) gels and electroblotted on to nitrocellulose (BioRad). Blots were stained with Ponceau Red to monitor the transfer of proteins. Membranes were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (25 mM Tris/-137 mM NaCl/2.7 mM KCl) containing 0.05% Tween-20 and incubated with specific antibodies for 2–4 h in the same buffer. After washing, the blots were incubated for 1 h with a 1:7500 dilution of horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG and visualized using an enhanced chemiluminescence (ECL) detection system (Pierce).

JNK and p38 MAPK kinase assays

These assays have been described in detail elsewhere [25]. Cultures were lysed in 150 μl of ice-cold lysis buffer (20 mM Tris/HCl, pH 7.5, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA and 25 mM β-glycerophosphate). Equal amounts of pre-cleared extract were incubated on ice with 6 μl of antibody (anti-JNK1/JNK2, anti-HA, anti-p38 MAPK or anti-MAPKAP kinase 2) and Protein A–agarose beads. The beads were pelleted and washed twice with lysis buffer and resuspended in kinase buffer (25 mM Hepes, pH 7.4, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM sodium vanadate and 25 mM β-glycerophosphate) with 5 μg of purified c-Jun_{1-141}-glutathione S-transferase fusion protein, 5 μg of myelin basic protein or 2 μg of Hsp27 and 20 μM ATP with 10 μCi of [^{32}P]ATP. Samples were incubated at 30 °C for 30 min. Reactions were stopped by addition of 20 μl of 4 × SDS sample buffer, boiled and electrophoresed on SDS/polyacrylamide (12%) gels. The gels were fixed, dried and subjected to autoradiography. JNK activity was quantitated by analysing densitometry of fragments on digitized images using NIH Image 1.60 with Adobe Photoshop 4.0.

Transfections

Cardiac myocytes were transfected on day 1 or 2 after isolation using polycationic liposomes. Cells were treated with 10 μl of the liposome reagent (GeneFector™) and 5–10 μg of DNA for 5 h in

serum-free medium, followed by 18–24 h in medium supplemented with 10 % fetal bovine serum. Transfection efficiency was 11.5 ± 1.6 % ($n = 20$), as estimated by GFP expression from a transfected GFP plasmid (three separate experiments). The transfection procedure alone did not affect the level of apoptosis during treatments (results not shown).

RESULTS

Modulation of apoptosis by transfection of dnJNK1 and caMEKK1

We demonstrated previously that hypoxia–reoxygenation of cardiac myocytes resulted in both the rapid activation of JNK and the apoptosis of approx. 30 % of the myocytes 16–24 h after reoxygenation [25,33]. To determine whether activated JNK was promoting survival or death in this model, myocytes were transfected with the dnJNK1 or caMEKK1 plasmids and subjected to hypoxia–reoxygenation. Transfected cells were identified by co-transfecting a GFP reporter as described in the Materials and methods section. These results are shown in Figure 1. Hypoxia–reoxygenation caused apoptosis in 33.3 ± 0.8 % ($n = 6$) of the transfected myocytes, in close agreement with our previous report of untransfected myocytes [33]. When myocytes were transfected with the JNK-inhibitory plasmids, dnJNK1 and dnMKK4, the incidence of apoptosis in the transfected cells increased in both aerobic and hypoxia–reoxygenation-treated myocytes. Apoptosis rates in aerobic myocytes rose to 25.2 ± 1.4 % ($n = 4$) and 23.0 ± 2.4 % ($n = 4$) in cultures transfected with dnJNK1 and dnMKK4, respectively, and to 58.0 ± 1.4 % ($n = 4$) and 53.3 ± 1.4 % ($n = 6$) respectively in reoxygenated myocytes. All values were significantly different from control uninfected cultures ($P < 0.01$). Conversely, myocytes transfected with caMEKK1 with or without co-transfected HA-JNK1 experienced significantly less apoptosis than control reoxygenated myocytes (Figure 1A; $P < 0.02$, $n = 3$). The transfection procedure did not affect the level of apoptosis, as determined by measuring the apoptotic index in cultures co-transfected with pGFP and empty DNA vector (results not shown).

The effect of transfected plasmids on JNK activity is shown in Figure 1(B). Co-transfected HA-JNK1 was activated by reoxygenation or anisomycin (positive control) treatments, and this was inhibited by co-transfecting dnJNK1. Transfection of caMEKK1 activated HA-JNK1. Figure 1(C) shows quantification of JNK activity; transfection of the dnJNK1 plasmid repressed reoxygenation-mediated JNK activation by 78 ± 12 % ($n = 3$). JNK activation by anisomycin was repressed 52 ± 11 % by dnJNK1 plasmid co-transfection. Expression of the co-transfected vectors containing wild-type MEKK1, caMEKK1 and HA-tagged dnJNK1 are shown in Figure 1(D). The HA-tag was present on both dnJNK1 and HA-JNK1; therefore, the intense double band shown in the dnJNK1-transfected cells represents the contribution from both proteins. These results show that over-expression of dnJNK1 and caMEKK1 modulates JNK pathway kinase activity.

Modulation of apoptosis by infection with dnJNK1 and caMEKK1 adenovirus

To confirm the co-transfection results, recombinant adenoviral vectors expressing dnJNK1 and caMEKK1 were created and the apoptosis and kinase assays were repeated in cultures infected with the virus (Figure 2). The rates of apoptosis measured by the number of condensed nuclei in cardiac myocytes pre-infected with Ad-dnJNK1 were 20 ± 1.9 % ($n = 4$) and 58 ± 2.6 % ($n = 4$) in aerobic and hypoxia–reoxygenation-treated cultures, respectively. The corresponding rates in myocytes pre-infected with Ad-caMEKK1 were 6.5 ± 0.7 % ($n = 4$) and 19.3 ± 1.5 % ($n = 4$). These results support the transfection data illustrated in Figure 1. dnJNK over-expression significantly enhanced, whereas caMEKK1 significantly reduced reoxygenation-mediated apoptosis compared with uninfected or empty-vector-infected cultures ($P < 0.01$ in both cases).

Figure 3 shows representative results obtained from Hoechst- (Figure 3A) and TUNEL- (Figure 3B) stained cultures. Immunostaining with sarcomeric myosin antibody confirmed that the majority of GFP-positive and Hoechst-stained nuclei under all conditions were myocytic. This confirms our previous observations that these cultures contain > 97 % cardiac myocytes. The white arrows in Figure 3(Ai) identify GFP-, sarcomeric myosin- and Hoechst-positive cardiac myocyte nuclei. The yellow arrows identify two non-myocytic (sarcomeric myosin-negative) normal nuclei. Figure 3(Aii) shows a higher magnification field, where intense Hoechst staining of condensed nuclei clearly co-localized with cells staining positive for sarcomeric myosin. Figure 3(B) shows TUNEL-stained (red) nuclei in the left-hand panels and anti-sarcomeric myosin (FITC/green) stain in the right-hand panels. The white arrows identify examples of TUNEL-positive nuclei co-localized with a sarcomeric myosin-positive background; yellow arrows show several TUNEL-negative nuclei that are also positive for sarcomeric myosin. Note that cardiac myocytes undergoing extensive cell death in response to Ad-dnJNK1 infection and hypoxia–reoxygenation treatment tend to become loosely attached to the culture plate and clumped so that the photomicrographs of anti-sarcomeric myosin stains tend to be blurred because of the increased field depth. In Figure 3(A) the sarcomeric myosin secondary antibody was rhodamine-tagged to distinguish GFP, whereas in Figure 3(B) it was FITC-tagged to distinguish it from the TUNEL stain.

Quantitation of TUNEL assays revealed: 4.3 ± 1.4 % ($n = 3$) and 26.0 ± 2.7 % ($n = 3$) positive cells for control aerobic and hypoxia–reoxygenated myocytes, respectively; 5.3 ± 0.8 % ($n =$

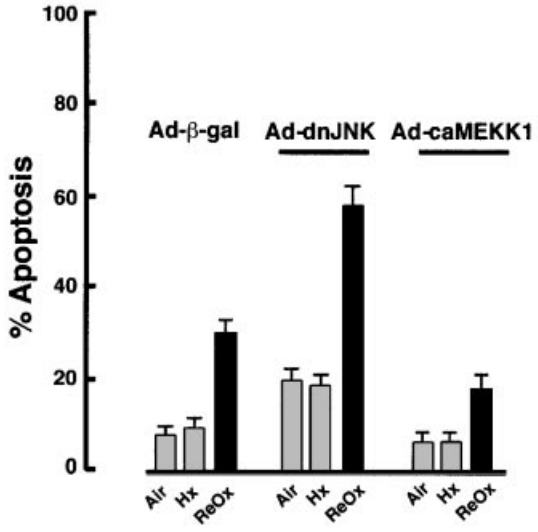

Figure 2 Modulation of apoptosis by Ad-dnJNK1 and Ad-caMEKK1 infection

Cardiac myocytes were infected with 20 p.f.u./cell Ad-GFP, Ad-dnJNK1 or Ad-caMEKK1 and subjected to aerobic (Air), hypoxic (Hx) or reoxygenation (ReOx) treatments as described in Figure 1; apoptotic rates (means ± S.E.M.) were determined by quantifying condensed nuclei as described in the Materials and methods section.

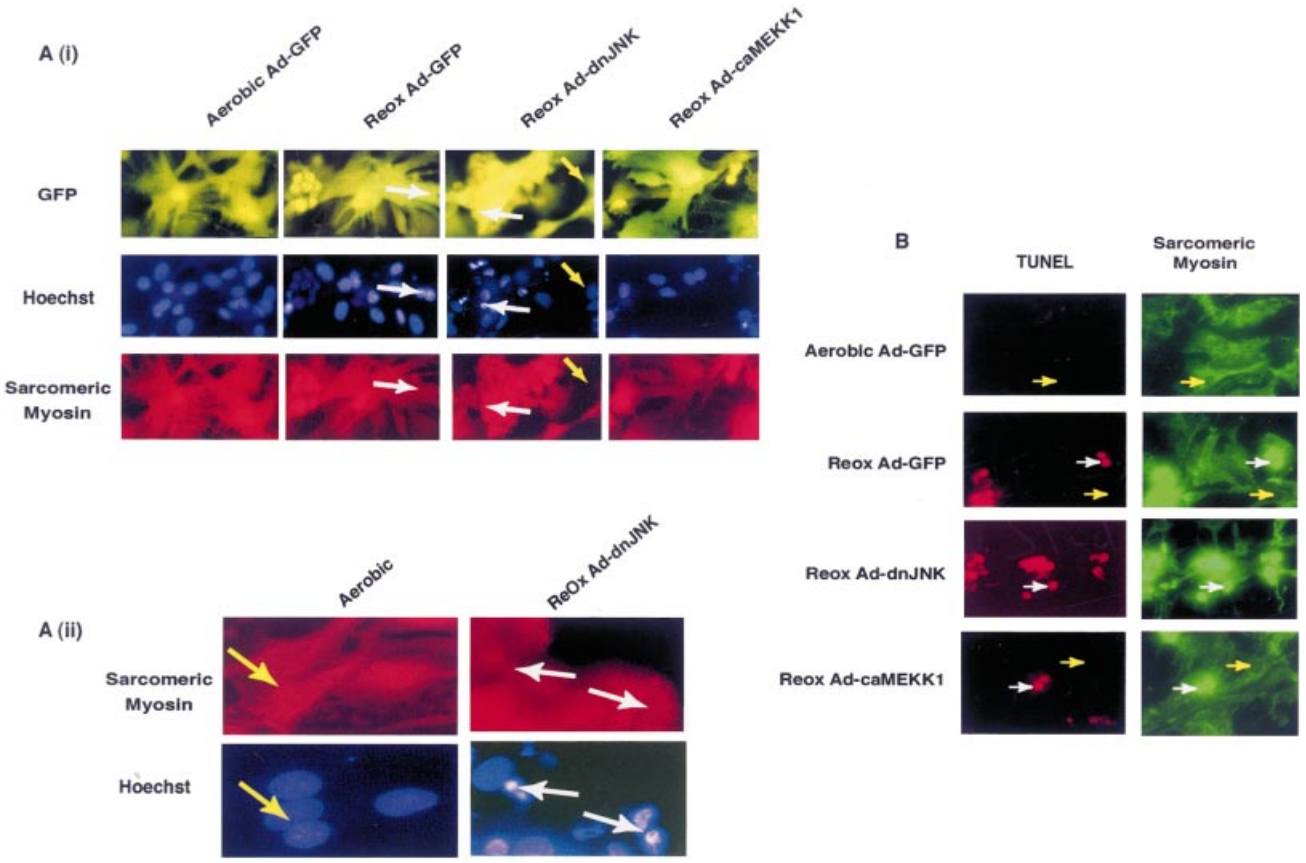

Figure 3 Analysis of apoptotic nuclei by Hoechst and TUNEL staining

Representative plates of Hoechst 33342- (**A**) and TUNEL- (**B**) stained cultures infected with 20 p.f.u./cell of the indicated adenovirus, cultured aerobically or subjected to hypoxia and reoxygenation (Reox) as indicated. GFP expression in cultures infected with adenoviruses, indicating the level of infected cells is shown in (**A**, top panel); infection was $> 95\%$ in all cases. GFP, Hoechst and anti-sarcomeric myosin stains show the same fields of cardiac myocytes. The white arrows indicate examples of apoptotic nuclei that are positive for GFP, Hoechst and anti-sarcomeric myosin. Yellow arrows indicate examples of nuclei that are positive for GFP but not sarcomeric myosin, and are scored as normal non-myocytes. Non-myocyte nuclei represented $< 3\%$ of total nuclei in these cultures (results not shown). (**A**, ii) shows a higher magnification field of anti-sarcomeric myosin- and Hoechst-stained cells; yellow arrows indicate normal non-myocyte nuclei squeezed between two myocyte (sarcomeric myosin-positive) nuclei in aerobic cultures; white arrows show two out of at least four intensely stained, condensed nuclei co-localized with sarcomeric myosin in Ad-dnJNK1-infected/reoxygenated cultures. Panels in (**B**) show the fields of cells stained sequentially for TUNEL (red) and sarcomeric myosin (FITC/green). The yellow arrows here indicate examples of normal (non-apoptotic) sarcomeric myosin-positive nuclei. The white arrows indicate TUNEL- and sarcomeric myosin-positive nuclei. All fields were imaged and photographed using a Zeiss IM inverted-phase fluorescence microscope at final magnification of $\times 400$ ($\times 1200$ for the field in (**A**, ii). Images were scanned and reproduced using Adobe Photoshop and Adobe Illustrator software.

3) and $16.3 \pm 1.5\%$ ($n = 3$) for the corresponding Ad-caMEKK1-infected cells; and $18.0 \pm 2.0\%$ ($n = 3$) and $55.0 \pm 3.8\%$ ($n = 3$) for the corresponding Ad-dnJNK1-infected cultures. The results obtained from the TUNEL and Hoechst-stain assays were similar; in both cases caMEKK1 significantly inhibited and dnJNK1 significantly increased the rates of apoptosis of hypoxia–reoxygenated cardiac myocytes ($P < 0.01$ in all instances, $n = 3$). The effect of caMEKK1 infection on the apoptosis level of aerobic control cultures was not significant.

Figure 4(A) shows the respective DNA ladders resulting from treatments in the presence and absence of Ad-dnJNK1 or Ad-MEKK1 infection. These results also support the Hoechst and TUNEL assays showing activation of apoptosis by dnJNK1 and inhibition by caMEKK1. The kinase assays shown in Figure 4(B) confirm that dnJNK1 repressed JNK activation in infected reoxygenated myocytes. The corresponding Western blot (Figure 4B, lower panel) shows strong expression of JNK1 protein, with both the p54 and p46 alternative splice products in the Ad-dnJNK1-infected cultures as expected. JNK activation by anisomycin treatment was also repressed. Quantitation of the JNK activity data is shown in Figure 4(C); reoxygenation-mediated JNK activity was inhibited $87 \pm 7\%$ ($n = 4$) by Ad-dnJNK1 and anisomycin-stimulated JNK activity was inhibited by $51 \pm 9\%$ ($n = 4$). Figure 4(D) shows Western blots probed for the potential targets of Ad-caMEKK1; infection with Ad-caMEKK1 selectively activated JNK with no significant effect on ERK or p38 MAPK. This result is in agreement with other reports showing the preferential activation of JNK by MEKK1 [36]. Ad-caMEKK1 infection increased the expression levels of caMEKK1 and phospho-JNK by (6.2 ± 6)-fold ($n = 2$) and (11.6 ± 7)-fold ($n = 2$), respectively, relative to aerobic controls. Anisomycin treatment is shown as a positive control for JNK and p38 MAPK activation.

Activation of caspase activity by reoxygenation and dnJNK1 infection

The results described in Figures 1–4 indicate that JNK activity correlates more closely with survival than apoptosis after hypoxia–reoxygenation exposure of cardiac myocytes. This is

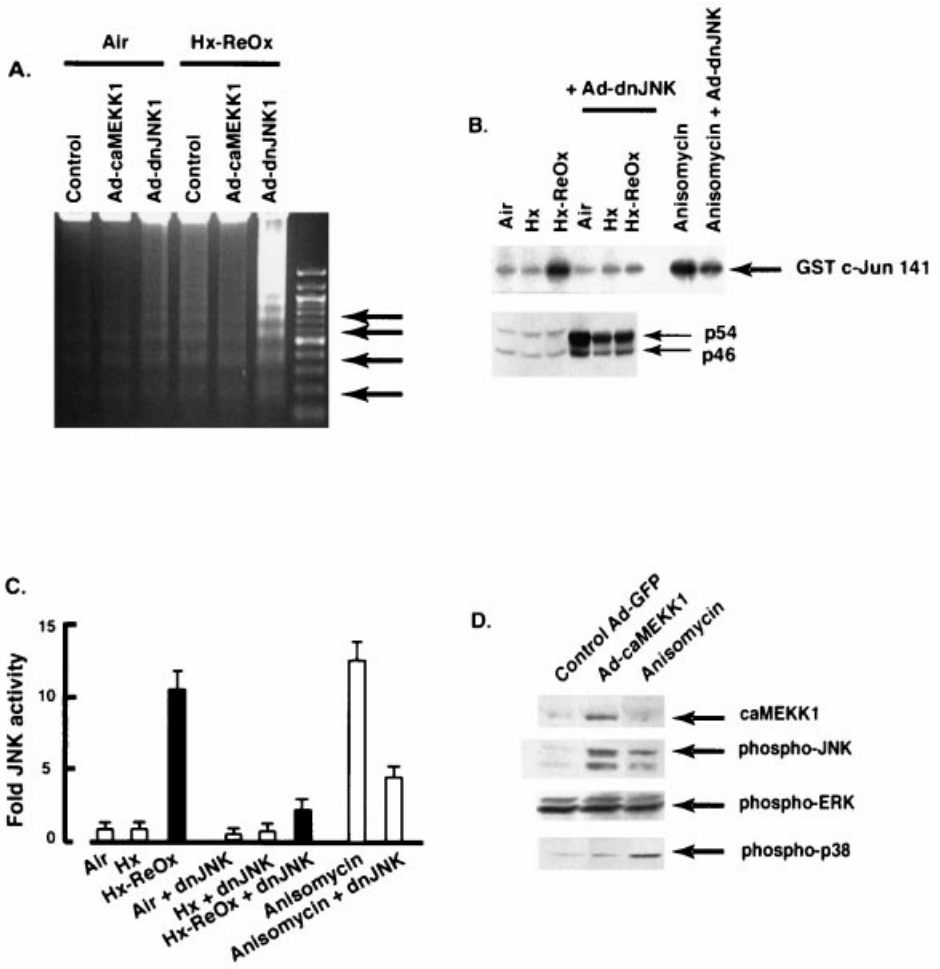

Figure 4 DNA fragmentation and JNK activity in myocytes infected with adenovirus

(**A**) Analysis of genomic DNA from control, Ad-caMEKK1- and Ad-dnJNK1-infected cardiac myocytes subjected to normoxic incubation (Air) or hypoxia and reoxygenation (Hx-ReOx) as indicated. Arrows indicate the positions of approx. 240 bp DNA fragments. (**B**) Upper panel; control and infected myocytes were subjected to the treatments indicated and JNK activity was measured as described in the Materials and methods section; anisomycin treatments (two lanes on the right; controls) were as described in Figure 1. Lower panel; representative Western blot of extracts from cardiac myocytes subjected to the same treatments as in the upper panel and probed with anti-JNK1/JNK2 antibody. (**C**) Quantitation of JNK activity from three separate experiments; open bars represent aerobic or hypoxic cultures, solid bars represent hypoxia–reoxygenation treatment. (**D**) Representative Western blots of control infected myocytes, Ad-caMEKK1-infected myocytes (20 p.f.u./cell) and anisomycin-treated myocytes probed sequentially with antibodies directed against MEKK1, phospho-JNK1/2, phospho-ERK-1 and phospho-p38 MAPK as indicated. The anti-MEKK1 antibody recognizes both the intact and cleaved (ca) MEKK1 protein.

supported by three separate assays of apoptosis, Hoechst staining, TUNEL assays and genomic DNA ladders. Previous studies have implicated the activation of caspases 9 and 3 in the death pathway of neuronal cells, kidney cells and cardiac myocytes subjected to hypoxia–reoxygenation [7,8,37–39]. Therefore, we used an LEHD cleavage assay, as described in the Materials and methods section, to estimate the activity of caspase 9 in control, Ad-GFP-infected and Ad-dnJNK1-infected cultures subjected to hypoxia and reoxygenation. These results are shown in Figure 5. LEHD cleavage activity increased rapidly in both control and Ad-dnJNK1-infected cultures after reoxygenation. In control myocytes the activity increased by (3.2 ± 0.2)-fold ($n = 3$) compared with untreated cells after 8 h of reoxygenation. Infection with Ad-dnJNK1 increased basal LEHD cleavage activity under both air and hypoxia and promoted a (4.3 ± 0.34)-fold ($n = 3$) activation after 8 h of reoxygenation. Caspase activity declined between 8 and 20 h of reoxygenation. In staurosporine-treated cultures used as a positive control for caspase 9 [40,41], the caspase activity increased (4.8 ± 0.5)-fold ($n = 3$) over untreated controls after 8 h (Figure 5). These results support previous work that has demonstrated the activation of caspase 9 in cells subjected to hypoxia and reoxygenation [7,37], and supports the morphological and DNA-ladder results described above that indicate a possible protective role for JNK in this model.

The roles of p38 MAPK and TNFα in JNK and reoxygenation-mediated apoptosis

Activated JNK has been assigned negative, positive or neutral roles in the survival of different cell types, including cardiac myocytes exposed to stress [42,43]. A recent report demonstrated that H_2O_2-mediated apoptosis was enhanced in embryonic stem cell-derived cardiac myocytes with a disrupted MEKK1 gene [23]. In contrast, other recent reports suggest that JNK activation promotes apoptosis of myocytes exposed to hypoxia–reoxygena-

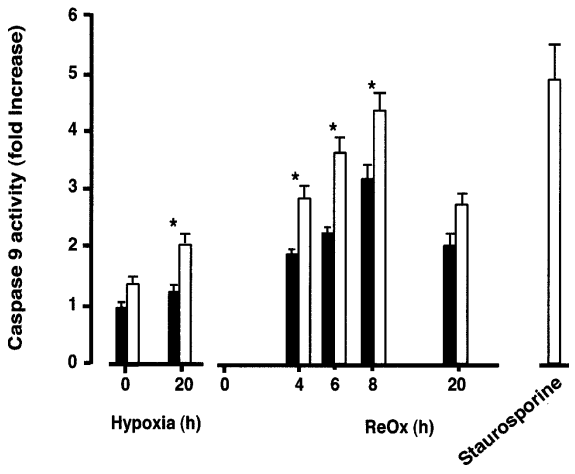

Figure 5 Modulation of LEHD cleavage activity by hypoxia–reoxygenation and Ad-dnJNK1

Caspase-9 activity was estimated using a colorimetric assay as described in the Materials and methods section. Solid and open columns represent GFP- (control) and Ad-dnJNK1-infected cultures respectively. Staurosporine treatment (100 nM for 8 h) was included as a positive control. All values were normalized to aerobic control-infected cultures. * indicates a significant difference between control- and Ad-dnJNK1-infected samples at each time point ($P = 0.01$, $n = 3$). ReOx, hypoxia–reoxygenation treatment.

tion, reperfusion or metabolic inhibitors [16,19,21,22,44]. Some of these reports implied roles for p38 MAPK and secreted TNFα in initiating apoptosis. Therefore, we analysed p38 MAPK and TNFα activities under the conditions of hypoxia and reoxygenation described above. Figure 6(A) shows a representative blot of p38 MAPK; the activity increased during the early stages of hypoxia and then declined. During reoxygenation, p38 MAPK activity increased again after 2–4 h and remained elevated for at least 8 h. Reoxygenation-mediated p38 MAPK activation peaked at (2.9 ± 0.5)-fold above the basal aerobic activity between 4–8 h ($n = 3$). Quantification of p38 MAPK is shown in Figure 6(B) ($n = 3$). The kinetics of p38 MAPK changes were quite different from the response of JNK, which did not change under hypoxia, was activated abruptly by reoxygenation and declined to basal levels after 2–4 h of reoxygenation [25,34]. Therefore, reoxygenation is characterized by an early peak in the activity of JNK and a delayed but sustained activation of p38 MAPK.

Previous studies have shown that p38 MAPK can positively regulate TNFα production, and TNFα is known to have a role in ischaemic cardiomyopathy (reviewed in [45]). To determine the roles of p38 MAPK and TNFα in this model we measured TNFα production and tested the effects on apoptosis of a TNFα-blocking antibody, as well as the p38 MAPK-specific inhibitor SB203580. Cardiac myocytes were subjected to hypoxia–reoxygenation and secreted TNFα was measured in the extracellular medium under the same conditions that mediate apoptosis. TNFα was not detected in cell extracts by Western blots, suggesting that intracellular levels are low (results not shown), but extracellular TNFα accumulated during hypoxia and reoxygenation in both Ad-β-galactosidase- and Ad-dnJNK1-infected cultures. Ad-dnJNK1 infection caused an approx. 1.5-fold increase in the rates of secreted TNFα at all time points compared with controls during exposure to both hypoxia and reoxygenation (Figure 7A). The maximum level of extracellular TNFα attained under these conditions was never greater than 20 pg/ml.

To directly determine the contribution of secreted TNFα in this model, cardiac myocytes were subjected to hypoxia–reoxygenation or treatment with a cocktail of TNFα, interferon-γ and interleukin-1β; the latter conditions have been shown previously by this laboratory to cause apoptosis of neonatal cardiac myocytes [46]. The combination of three cytokines is required because neonatal cardiac myocytes are resistant to TNFα treatment alone. Parallel cultures were pre-incubated with a TNFα-blocking antibody and apoptosis was assessed by DNA fragmentation. These results are shown in Figure 7(B). The blocking antibody only marginally reduced reoxygenation-mediated apoptosis in uninfected cells (Figure 7B, lanes 5 and 6 from the left). Similarly, the enhanced apoptosis in Ad-dnJNK1-infected myocytes was only partially reduced (Figure 7B, lane 12). In contrast, anti-TNFα antibody pretreatment effectively blocked cytokine-mediated apoptosis, even though these treatments involved $>$ 1000-fold more extracellular TNFα than the reoxygenation conditions, with or without Ad-dnJNK1 infection. These results suggest a relatively minor role for secreted TNFα in the activation of apoptosis by hypoxia–reoxygenation or by JNK inhibition. This conclusion is supported further by the observation that spent medium from reoxygenated myocytes does not stimulate apoptosis of fresh cultures (results not shown). The effects of

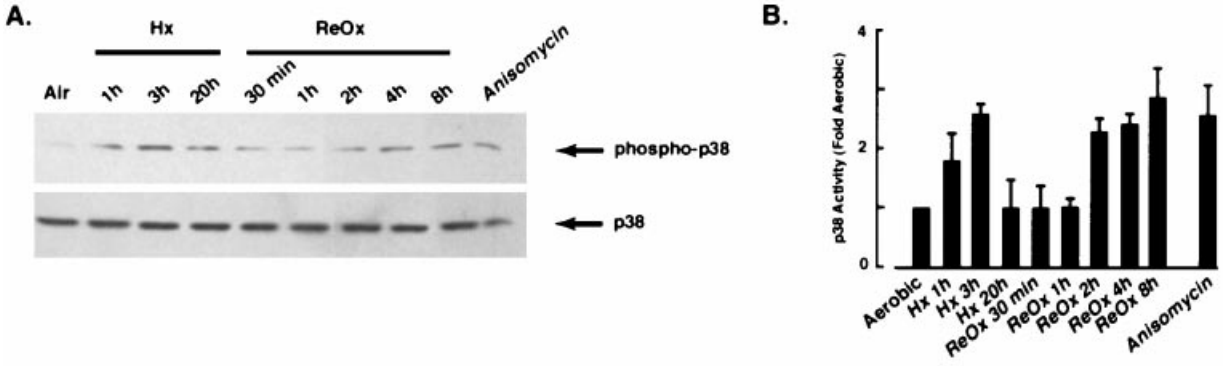

Figure 6 Activation of p38 MAPK by hypoxia and reoxygenation

(**A**) Representative Western blot of cardiac myocyte extracts probed with a phospho-specific p38 MAPK antibody and a p38 MAPK antibody on the lower panel. (**B**) Quantitation of p38 MAPK phosphorylation from three separate experiments. Hx, hypoxia treatment only; ReOx, hypoxia–reoxygenation treatment.

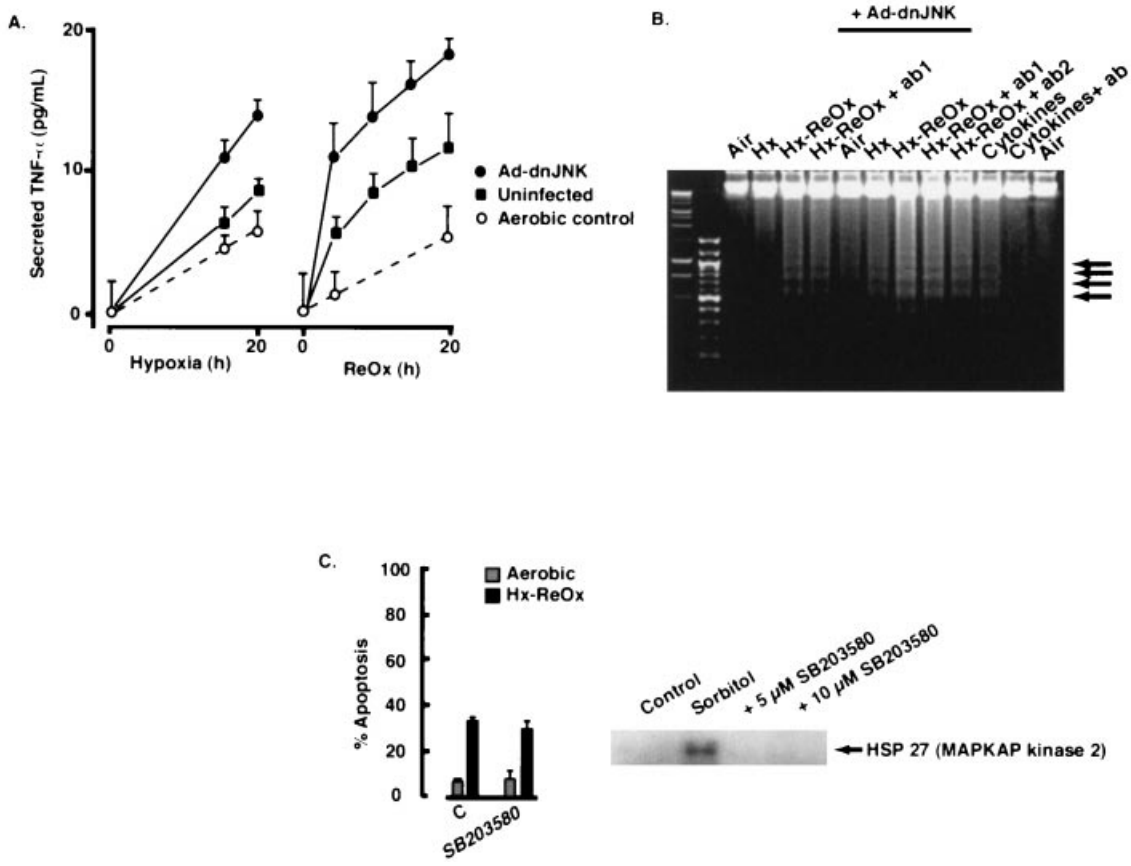

Figure 7 Contributions of TNFα and p38 MAPK to reoxygenation-mediated apoptosis

(**A**) Secretion of TNFα into the culture medium was measured by ELISA at the indicated times during exposure of myocytes to hypoxia and hypoxia–reoxygenation (ReOx) as described in the Materials and methods section. The culture medium was changed immediately before exposure to hypoxia and again at the time of reoxygenation. Therefore, TNFα levels start close to zero at the beginning of each phase. The dashed line represents TNFα levels in control myocytes subjected to continuous aerobic incubation in parallel with the treatments. Ad-dnJNK1 infections were as described in the Materials and methods section. Approx. 1.5-fold more TNFα was secreted at all time points by Ad-dnJNK1-infected myocytes, but the levels never exceeded 20 pg/ml under any conditions. (**B**) Cardiac myocytes were subjected to hypoxia and reoxygenation (lanes 5, 6 and 9–11) in the presence or absence of a TNFα-neutralizing antibody (ab1, 5 μg/ml; ab2, 10 μg/ml) as indicated. In lanes 12 and 13 myocytes were incubated for 72 h with a cytokine cocktail (25 ng/ml TNFα/5 ng/ml interleukin-1β/100 units/ml interferon-γ), and also in the presence or absence of 5 μg/ml TNFα-neutralizing antibody as indicated (ab). Arrows indicate positions of approx. 240 bp DNA fragments. (**C**) Rates of apoptosis of hypoxia–reoxygenated cardiac myocytes preincubated with the p38 MAPK-selective inhibitor SB203580 (10 μM). Apoptosis was measured by Hoechst 33342 staining as described in Figure 1. In the right-hand panel myocytes were exposed to sorbitol (400 mM for 20 min) in the presence or absence of SB203580, and p38 MAPK-dependent activation of MAPKAP kinase 2 was measured by phosphorylation of Hsp27 as described in the Materials and methods section. MAPKAP kinase 2 activity was undetectable in the presence of 5 or 10 μM SB203580.

pretreating myocytes with the p38 MAPK inhibitor SB203580 are shown in Figure 7(C). Only a small decrease in the level of apoptosis was observed by SB203580 treatment at a concentration that fully blocked the activation of p38 MAPK by sorbitol.

DISCUSSION

The results described here support the following conclusions. (i) Activation of JNK by oxidative stress under the conditions used does not promote apoptosis of primary neonatal cardiac myocytes and may be protective. (ii) p38 MAPK activity increases after reoxygenation, and there is a small increase in secreted TNFα, but these factors are not the principal initiators of apoptosis in this model. Three conditions were shown to block JNK activation and induce apoptosis simultaneously, including (i) transfection with dnJNK1, (ii) transfection with dnMKK4 and (iii) infection with Ad-dnJNK1. A fourth condition, transfection or infection with caMEKK1, reduced apoptosis while increasing JNK activity, but not that of either ERK or p38 MAPK. In experiments involving adenoviral delivery of dnJNK1 and caMEKK1, apoptosis measurements were confirmed by nuclear condensation, TUNEL and DNA-fragmentation assays; dnJNK1 infection was also shown to enhance LEHD cleavage activity in cell extracts from untreated and hypoxia–reoxygenated cultures, indicating increased activity of caspase 9 (and possibly caspases 4 and 5, which can also cleave LEHD). Caspase 9 is activated by cytochrome *c* and apoptosis protease-activating factor-1, implicating mitochondria as direct or indirect targets of this pathway [7,8,37–39]. These results eliminate the possibility that JNK activation is required for apoptosis by oxidative stress in this model, and suggest that JNK activation is protective. It is noteworthy that the over-expression of caMEKK1 had a relatively small effect on apoptosis following reoxygenation (Figures 1, 2 and 4). One possible reason for this is that the JNK pathway is already close to fully activated by reoxygenation and increasing the MEKK1 component by over-expressing caMEKK1 adds only marginally to this activity. Indeed, the level of JNK activity

after reoxygenation is almost equal to that caused by anisomycin treatment (Figure 1). Alternatively, the ability of JNK to override reoxygenation-mediated cell death may be limited even when the activity is optimal and sustained. The observation that apoptosis also increased in aerobic myocytes expressing dnJNK1-interfering genes is not surprising and reflects the importance of balanced MAPK activities for cellular integrity under most conditions. The relatively high basal rate of apoptosis ($> 5\%$) suggests that these cells are subjected to continuous stress even under normal aerobic conditions.

The possibility that JNK activation promotes survival is supported by a recent report showing that embryonic stem cell-derived cardiac myocytes lacking MEKK1, and with significantly suppressed JNK activity, were sensitized to H_2O_2 treatment [23]. In this study, apoptosis of the MEKK1$^{-/-}$ myocytes could be blocked by treatment with the p38 MAPK inhibitor SB203580 or by pretreatment with a TNFα-blocking antibody before exposure to H_2O_2. The results supported a pathway in which p38 MAPK stimulated, and JNK blocked, the generation of TNFα. The authors concluded that TNFα was the direct mediator of H_2O_2-initiated apoptosis. In contrast, apoptosis of primary cardiac myocytes induced by hypoxia–reoxygenation in our study was not blocked by either SB203580 or TNFα antibody treatments. Furthermore, the TNFα levels in the extracellular medium following reoxygenation or infection with Ad-dnJNK1 were only a fraction of the levels required to cause apoptosis by exogenous cytokines. Additionally, the spent medium from these former cultures did not cause apoptosis of fresh myocytes (results not shown), indicating that lethal levels of cytokines were not accumulating under these conditions. We reported previously that the exposure of isolated cardiac myocytes to multiple combined cytokines can induce apoptosis, but TNFα alone at a concentration of 25 ng/ml did not [46]. We conclude that the protective actions of JNK activation appear to be shared in both embryonic stem cell-derived and primary cultures of cardiac myocytes, but the signalling pathways differ. The difference may be related to the respective stage of development; it may be a consequence of the MEKK1$^{-/-}$ knockout phenotype, or it may be related to the use of reoxygenation instead of H_2O_2 as the stimulus for apoptosis. H_2O_2 treatment strongly activates the MAPK/ERK pathway as well as p38 MAPK and JNK [12,24].

The results of the co-transfection experiments described in Figure 1 are also at variance with a dominant role for secreted cytokines, including TNFα, because they demonstrate responses of individual cells to the transfected gene products. In our experiments, the transfection efficiency of cardiac myocytes was routinely 10% or less; therefore transfection would not be expected to significantly change the extracellular accumulation of cytokines secreted from the other $> 90\%$ of cells during treatments (hypoxia–reoxygenation). Cells transfected with dnJNK1 plasmids experienced a > 2-fold-increased rate of apoptosis compared with non-transfected cells, even though the extracellular milieu (secreted TNFα) was the same. Therefore, if secreted TNFα were responsible for this effect, the mechanism must involve a highly restricted, local action of the cytokine in which the secreting cell itself is the target. The low (pg/ml) levels of secreted TNFα observed and the relative insensitivity of neonatal cardiac myocytes to exogenously added TNFα [46] make this possibility unlikely.

Numerous previous studies have implicated pro-apoptotic roles for activated JNK and/or p38 MAPK using a range of cells and stimuli [14,47]. Wang et al. [48] reported that adenovirus-mediated over-expression of p38 MAPK induced hypertrophy and apoptosis of primary cardiac myocytes. Mackay and Mochly-Rosen [19] demonstrated that p38 MAPK was activated in primary cardiac myocytes by hypoxia and glucose starvation, and they attributed the subsequent apoptosis to p38 MAPK activation. Several other groups correlated increased p38 MAPK and JNK activity with apoptosis in stressed H9c2 cells, neonatal cardiac myocytes, adult myocytes and reperfused hearts [16,22,44]. Some studies report significant effects of the inhibitors PD98059 and SB203580 [16,19], whereas others, including ourselves, have seen only minor effects of these inhibitors on apoptosis [17,20]. Other studies using different cell models have reported neutral or protective roles for JNK and/or p38 MAPK [23,42,43,49]. JNK activation has been reported to promote the survival of cells treated with epidermal growth factor or phorbol esters [50], of lymphocytes or hepatocytes undergoing apoptosis by the Fas activation pathway [51,52], and of serum-starved cells responding to integrin stimulation [53]. Embryos with mutated JNK1/JNK2 genes exhibit increased apoptosis in the developing forebrain, a consequence that was attributed to JNK deficiency [54,55]. Our group recently reported that JNK activation correlates with protection against NO-mediated cardiac myocyte cell death [56]. The different results may reflect the cell- and stimulus-specific nature of these signalling pathways, and are likely to include such factors as the developmental stage and metabolic state of the cells at the time of stimulation.

The targets that determine the roles of JNK or p38 MAPK in cell survival are not clear. Several reports have documented that activated JNK and p38 MAPK can translocate to the mitochondria where they modulate functions of the Bcl-2 family of proteins. JNK has been reported to phosphorylate the anti-apoptosis factor Bcl-X_L on Thr-47 and Thr-115, causing inactivation of Bcl-X_L, increased release of cytochrome *c*, activation of caspases 9 and 3 and enhanced apoptosis [57]. Similarly, p38 MAPK has been shown to translocate to mitochondria and phosphorylate Bcl-2 on Ser-87 and Thr-56 [58], or it can stimulate the caspase-8-dependent cleavage of Bid [59]; in both cases cytochrome *c* is released and downstream caspases are activated. Conversely, interleukin-3 withdrawal or treatment of cells with okadaic acid can also mediate the translocation of activated JNK to the mitochondria with targeting of Bcl-2, but in this case Bcl-2 is phosphorylated on Ser-70, and the cells are protected from apoptosis [60]. In yet other studies, the microtubule-damaging agent paclitaxel was shown to stimulate phosphorylation of Bcl-2 on Thr-69, Ser-87 and Ser-70, resulting in its inactivation and increased apoptosis [61]. Therefore, phosphorylation may activate or inactivate the anti-apoptotic function of Bcl-2 family proteins in a manner that is determined by the protein targets and the specific residues phosphorylated. Regulation of Bcl-2 functions through phosphorylation is thought to involve protein stability and the modulation of interactions with other family members [62]. It is not yet known whether JNK targets mitochondria in cardiac myocytes subjected to oxidative stress. Clearly this is a possibility and the targets may include Bcl-2 family proteins. The conflicting reports on the role of JNK, including demonstrations of both pro- and anti-apoptotic actions in similar cells and models may reflect subtle differences in the experimental treatments that result in different intracellular targets for JNK.

Alternatively the protective actions of JNK may be independent of Bcl-2 and involve cross-talk with other anti-apoptotic signalling pathways, such as phosphoinositide 3-kinase and Akt [63]. Transfer of the insulin-like growth factor-1 or Akt gene by adenovirus has been shown recently to protect cardiac myocytes from hypoxia and ischaemia-activated apoptosis [64], and reoxygenation has been shown to stimulate Akt expression [65]. Another possible pathway that has not been explored fully in cardiac myocytes involves the Fos-Jun transcription factor,

activator protein 1. Activator protein 1 can be increased by JNK and can promote apoptosis or survival depending on the cell type and stimulus [66]. JNK and p38 MAPK are both activated by ischaemia–reperfusion in the intact heart but neither has been shown definitively to be pro- or anti-apoptotic. Our results indicate that activated JNK can be protective under some circumstances.

This work was supported by National Institutes of Health grant no. HL44578, and grant no. BM034 from the Chiles Endowment Biomedical Research Program of the Florida Department of Health (to K.A.W.), and by an Established Investigator award from the American Heart Association (to N.H.B.). The opinions, findings and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the Biomedical Research Program or the Florida Department of Health.

REFERENCES

1 Buja, L. M. (1998) Modulation of the myocardial response to ischemia. Lab. Invest. **78**, 1345–1373
2 Kusuoka, H. and Marban, E. (1992) Cellular mechanisms of myocardial stunning. Annu. Rev. Physiol. **54**, 243–256
3 Kajstura, J., Cheng, W., Reiss, K. and Clark, W. A. (1996) Apoptotic and necrotic myocyte cell death are independent contributing variables of infarct size. Lab. Invest. **74**, 86–107
4 Kajstura, J., Cheng, W., Reiss, K., Clark, W. A., Sonnenblick, E. H., Krajewski, S., Reed, J. C., Olivetti, G. and Anversa, P. (1996) Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. Lab. Invest. **74**, 86–107
5 Ohno, M., Takemura, G., Ohno, A., Misao, J., Hayakawa, Y., Minatoguchi, S., Fujiwara, T. and Fujiwara, H. (1998) ''Apoptotic'' myocytes in infarct area in rabbit hearts may be oncotic myocytes with DNA fragmentation: analysis by immunogold electron microscopy combined with in situ nick end-labeling. Circulation **98**, 1422–1430
6 Kuida, K. (2000) Caspase-9. Int. J. Biochem. Cell Biol. **32**, 1–4
7 Wang, G. W., Zhou, Z., Klein, J. B. and Kang, Y. J. (2001) Inhibition of hypoxia/reoxygenation-induced apoptosis in metallothionein-overexpressing cardiomyocytes. Am. J. Physiol. Heart Circ. Physiol. **280**, H2292–H2299
8 Tamatani, M., Mitsuda, N., Matsuzaki, H., Okado, H., Miyake, S., Vitek, M. P., Yamaguchi, A. and Tohyama, M. (2000) A pathway of neuronal apoptosis induced by hypoxia/reoxygenation: roles of nuclear factor-kappaB and Bcl-2. J. Neurochem. **75**, 683–693
9 Saikumar, P., Dong, Z., Weinberg, J. M. and Venkatachalam, M. A. (1998) Mechanisms of cell death in hypoxia/reoxygenation injury. Oncogene **17**, 3341–3349
10 Bossenmeyer-Pourie, C., Koziel, V. and Daval, J. L. (1999) CPP32/CASPASE-3-like proteases in hypoxia-induced apoptosis in developing brain neurons. Brain Res. Mol. Brain Res. **71**, 225–273
11 Bishopric, N. H., Andreka, P., Slepak, T. and Webster, K. A. (2001) Molecular mechanisms of apoptosis in the cardiac myocyte. Curr. Opin. Pharmacol. **1**, 141–150
12 Sugden, P. H. and Clerk, A. (1998) ''Stress-responsive'' mitogen-activated protein kinases in the myocardium. Circ. Res. **83**, 345–352
13 Franklin, R. A. and McCubrey, J. A. (2000) Kinases: positive and negative regulators of apoptosis. Leukemia **14**, 2019–2034
14 Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. and Greenberg, M. E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270**, 1326–1331
15 Tue, T. L., Ma, X. L., Wang, X., Romanic, A. M., Liu, G. L., Louden, C., Gu, J. L., Kumar, S., Poste, G., Ruffolo, R. R. and Feuerstein, G. Z. (1998) Possible involvement of stress-activated protein kinase signaling pathway and Fas receptor expression in prevention of ischemia/reperfusion-induced cardiomyocyte apoptosis by carvedilol. Circ. Res. **82**, 166–174
16 He, H., Li, H.-L., Lin, A. and Gottlieb, R. A. (1999) Activation of the JNK pathway is important for cardiomyocyte death in response to simulated ischemia. Cell Death Differ. **6**, 987–991
17 Zechner, D., Craig, R., Hanford, D. S., McDonough, P. M., Sabbadini, R. A. and Glembotski, C. C. (1998) MKK6 activates myocardial cell NF-kB and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. J. Biol. Chem. **273**, 8232–8239
18 Yin, T., Sandhu, G., Wolfgang, C. D., Burrier, A., Webb, R. L., Rigel, D. F., Hai, T. and Whelan, J. (1997) Tissue-specific pattern of stress kinase activation in ischemia/reperfused heart and kidney. J. Biol. Chem. **272**, 19943–19950
19 Mackay, K. and Mochly-Rosen, D. (1999) An inhibitor of p38 mitogen-activated protein kinase protects neonatal cardiac myocytes from ischemia. J. Biol. Chem. **274**, 6272–6279
20 Turner, N. A., Xia, F., Azhar, G., Zhang, X., Liu, L. and Wei, J. Y. (1998) Oxidative stress induces DNA fragmentation and caspase activation via the c-Jun NH2-terminal kinase pathway in H9c2 cardiac muscle cells. J. Mol. Cell. Cardiol. **30**, 1789–1801
21 Crenesse, D., Gugenheim, J., Hornoy, J., Tornieri, K., Laurens, M., Cambien, B., Lenegrate, G., Cursio, R., De Souza, G., Auberger, P. et al. (2000) Protein kinase activation by warm and cold hypoxia-reoxygenation in primary-cultured rat hepatocytes-JNK(1)/SAPK(1) involvement in apoptosis. Hepatology **32**, 1029–1036
22 Hreniuk, D., Garay, M., Gaarde, W., Monia, B. P., McKay, R. A. and Cioffi, C. L. (2001) 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. Mol. Pharmacol. **59**, 867–874
23 Minamino, T., Yujiri, T., Papst, P. J., Chan, E. D., Johnson, G. L. and Terada, N. (1999) MEKK1 suppresses oxidative stress-induced apoptosis of embryonic stem cell-derived cardiac myocytes. Proc. Natl. Acad. Sci. U.S.A. **26**, 15127–15132
24 Clerk, A., Fuller, S. J., Michael, A. and Sugden, P. H. (1998) Stimulation of stress-regulated mitogen-activated protein kinases (stress-activated protein kinases/c-Jun-N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. J. Biol. Chem. **273**, 7228–7234
25 Laderoute, K. R. and Webster, K. A. (1997) Hypoxia/reoxygenation stimulates Jun kinase activity through redox signaling in cardiac myocytes. Circ. Res. **80**, 336–344
26 Gottlieb, R. A., Burleson, K. O., Kloner, R. A., Babior, B. M. and Engler, R. L. (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J. Clin. Invest. **94**, 1612–1628
27 Fliss, H. and Gattinger, D. (1996) Apoptosis in ischemic and reperfused rat myocardium. Circ. Res. **79**, 949–956
27a Dougherty, C., Hernandez, O., Prentice, H., Andreka, P., Bishopric, N. H. and Webster, K. A. (2000) Activation of c-Jun N-terminal kinase through the Rac1/cdc42-TAK-1 pathway promotes survival of reoxygenated cardiac myocytes. Circulation **102**, no. 18
28 He, T. C., Zhou, S., Da Costa, L. T., Yu, J., Kinzler, K. W. and Vogelstein, B. (1998) A simplified system for generating recombinant adenovirus. Proc. Natl. Acad. Sci. U.S.A. **95**, 2509–2514
29 Johnson, L. N., Gardner, A. M., Diener, K. M., Lange-Carter, C. A., Gleavy, J., Jarpe, M. B., Minden, A., Karin, M., Zon, L. I. and Johnson, G. L. (1996) Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death. J. Biol. Chem. **271**, 3229–3237
30 Coso, O. A., Chiariello, M., Yu, J., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J. S. (1995) The small GTP-binding proteins rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell **81**, 1137–1146
31 Webster, K. A. and Bishopric, N. H. (1992) Molecular regulation of cardiac myocyte adaptations to chronic hypoxia. J. Mol. Cell. Cardiol. **24**, 741–751
32 Webster, K. A., Discher, D. and Bishopric, N. H. (1993) Induction and nuclear accumulation of fos and jun proto-oncogenes in hypoxia cardiac myocytes. J. Biol. Chem. **268**, 16852–16859
33 Webster, K. A., Discher, D. J., Kaiser, S., Hernandez, O. M., Sato, B. and Bishopric, N. H. (1999) Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. J. Clin. Invest. **104**, 239–252
34 Hernandez, O. M., Discher, D. J., Bishopric, N. H. and Webster, K. A. (2000) Rapid activation of neutral sphingomyelinase by hypoxia-reoxygenation of cardiac myocytes. Circ. Res. **86**, 198–204
35 Bishopric, N. H., Zeng, G., Sato, B. and Webster, K. A. (1997) Adenovirus E1A inhibits cardiac myocyte-specific gene expression through its amino terminus. J. Biol. Chem. **272**, 20584–20594
36 Masuda, Y., Nakaya, M., Aiuchi, T., Hashimoto, S., Nakajo, S. and Nakaya, K. (2000) The mechanism of geranylgeraniol-induced apoptosis involves activation, by a caspase-3-like protease, of a c-Jun N-terminal kinase signaling cascade and differs from mechanisms of apoptosis induced by conventional chemotherapeutic drugs. Leukemia Res. **24**, 937–950
37 Saikumar, P., Dong, Z., Patel, Y., Hall, K., Hopfer, U., Weinberg, J. M. and Venkatachalam, M. A. (1998) Role of hypoxia-induced Bax translocation and cytochrome c release in reoxygenation injury. Oncogene **17**, 3401–3415
38 Dong, Z., Saikumar, P., Patel, Y., Weinberg, J. M. and Venkatachalam, M. A. (2000) Serine protease inhibitors suppress cytochrome c-mediated caspase-9 activation and apoptosis during hypoxia-reoxygenation. Biochem. J. **347**, 669–677
39 Liu, W., Kato, M., Akhand, A. A., Hayakawa, A., Suzuki, H., Miyata, T., Kurokawa, K., Hotta, Y., Ishikawa, N. and Nakashima, I. (2000) 4-Hydroxynonenal induces a cellular redox status-related activation of the caspase cascade for apoptotic cell death. J. Cell Sci. **113**, 635–641
40 Rocha, S., Soengas, M. S., Lowe, S. W., Glanzmann, C., Fabbro, D., Winterhalter, K., Bodis, S. and Pruschy, M. (2000) Protein kinase C inhibitor and irradiation-induced apoptosis: relevance of the cytochrome c-mediated caspase-9 death pathway. Cell Growth Differ. **11**, 491–499

41 Stepczynska, A., Lauber, K., Engels, I. H., Janssen, O., Kabelitz, D., Wesselborg, S. and Schulze-Osthoff, K. (2001) Staurosporine and conventional anticancer drugs induce overlapping, yet distinct pathways of apoptosis and caspase activation. Oncogene **20**, 1193–1202

42 Assefa, Z., Vantieghem, A., Declercq, W., Vandenabeele, P., Vandenabeele, J. R., Merlevede, W., de Witte, P. and Agostinis, P. (1999) The activation of the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. J. Biol. Chem. **274**, 8788–8796

43 Khwaja, A. and Downward, J. (1997) Lack of correlation between activation of Jun-NH_2-terminal kinase and induction of apoptosis after detachment of epithelial cells. J. Cell Biol. **139**, 1017–1023

44 Yue, T.-L., Wang, C., Gu, J.-L., Ma, X.-L., Kumar, S., Lee, J. C., Feuerstein, G. Z., Thomas, H., Maleef, B. and Ohlstein, E. H. (2000) Inhibition of extracellular signal-regulated kinase enhances ischemia/reperfusion-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. Circ. Res. **86**, 692–699

45 Meldrum, D. R. (1998) Tumor necrosis factor in the heart. Am. J. Physiol. **274**, R577–R595

46 Ing, D. J., Zang, J., Dzau, V. J., Webster, K. A. and Bishopric, N. H. (1999) Modulation of cytokine-induced cardiac myocyte apoptosis by nitric oxide, Bak, and Bcl-x. Circ. Res. **84**, 21–33

47 Harding, T. C., Xue, L., Bienemann, A., Haywood, D., Dickens, M., Tolkovsky, A. M. and Uney, J. B. (2001) Inhibition of JNK by overexpression of the JNL binding domain of JIP-1 prevents apoptosis in sympathetic neurons. J. Biol. Chem. **276**, 4531–4534

48 Wang, Y., Huang, S., Sah, V. P., Ross, J., Heller Brown, J., Han, J. and Chien, K. R. (1998) Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. J. Biol. Chem. **273**, 2161–2168

49 Yujiri, T., Sather, S., Fanger, G. R. and Johnson, G. L. (1998) Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science **282**, 1911–1914

50 Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. and Davis, R. J. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76**, 1025–1037

51 Yeh, W. C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N. et al. (1997) Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. Immunity **7**, 715–725

52 Diao, J., Khine, A. A., Sarangi, F., Hsu, E., Iorio, C., Tibbles, L. A., Woodgett, J. R., Penninger, J. and Richardson, C. D. (2001) X protein of hepatitis B virus inhibits Fas-mediated apoptosis and is associated with up-regulation of the SAPK/JNK pathway. J. Biol. Chem. **276**, 8328–8340

53 Almeida, E. A., Ilic, D., Han, Q., Hauck, C. R., Jin, F., Kawakatsu, H., Schlaepfer, D. D. and Damsky, C. H. (2000) Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase. J. Cell Biol. **149**, 741–754

54 Kuan, C. Y., Yang, D. D., Samanta Roy, D. R., Davis, R. J., Rakic, P. and Flavell, R. A. (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. Neuron **22**, 667–676

55 Sabapathy, K., Jochum, W., Hochedlinger, K., Chang, L., Karin, M. and Wagner, E. F. (1999) Defective neural tube morphogenesis and altered apoptosis in the absence of both JNK1 and JNK2. Mech. Dev. **89**, 115–124

56 Andreka, P., Zang, J., Dougherty, C. J., Slepak, T. I., Webster, K. A. and Bishopric, N. H. (2001) Cytoprotection by Jun kinase during nitric oxide-induced cardiac myocyte apoptosis. Circ. Res. **88**, 305–312

57 Kharbanda, S., Saxena, S., Yoshida, K., Pandey, P., Kaneki, M., Wang, Q., Cheng, K., Chen, Y. N., Campbell, A., Sudha, T. et al. (2000) Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage. J. Biol. Chem. **275**, 322–327

58 Torcia, M., De Chiara, G., Nencioni, L., Ammendola, S., Labardi, D., Lucibello, M., Rosini, P., Marlier, L. N., Bonini, P., Sbarba, P. D. et al. (2001) Nerve growth factor inhibits apoptosis in memory B lymphocytes via inactivation of p38 MAPK, prevention of Bcl-2 phosphorylation, and cytochrome c release. J. Biol. Chem. **276**, 39027–39036

59 Zhuang, S., Demirs, J. and Kochevar, I. (2000) p38 mitogen-activated protein kinase mediates Bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. J. Biol. Chem. **275**, 25939–25948

60 Deng, X., Xiao, L., Lang, W., Gao, F., Ruvolo, P. and Way, Jr, W. S. (2001) Novel role for JNK as a stress-activated Bcl2 kinase. J. Biol. Chem. **276**, 23681–23688

61 Yamamoto, K., Ichijo, H. and Korsmeyer, S. J. (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol. Cell. Biol. **19**, 8469–8478

62 Breitschopf, K., Haendeler, J., Malchow, P., Zeiher, A. M. and Dimmeler, S. (2000) Posttranslational modification of Bcl-2 facilitates its proteasome-dependent degradation: molecular characterization of the involved signaling pathway. Mol. Cell. Biol. **20**, 1886–1896

63 Pham, F. H., Sugden, P. H. and Clerk, A. (2000) Regulation of protein kinase B and 4E-BP1 by oxidative stress in cardiac myocytes. Circ. Res. **86**, 1252–1258

64 Matsui, T., Li, L., del Monte, F., Fukui, Y., Hajjar, R. J. and Rosenzweig, A. (1999) Adenoviral gene transfer of activated phosphatidyl kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes *in vitro*. Circulation **100**, 2373–2379

65 Yamashita, Y., Kajstura, J., Discher, D. J., Wasserlauf, B. J., Bishopric, N. H., Anversa, P. and Webster, K. A. (2001) Reperfusion-activated Akt kinase prevents apoptosis in transgenic mouse hearts over-expressing IGF-1. Circ. Res. **88**, 609–614

66 Shaulian, E. and Karin, M. (2001) AP-1 in cell proliferation and survival. Oncogene **20**, 2390–2400

Received 12 March 2001/13 December 2001; accepted 11 January 2002