Direct Activation of Mitochondrial Apoptosis Machinery by c-Jun N-terminal Kinase in Adult Cardiac Myocytes*

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Although oxidative stress causes activation of c-Jun N-terminal kinase (JNK) and apoptosis in many cell types, how the JNK pathway is connected to the apoptosis pathway is unclear. The molecular mechanism of JNK-mediated apoptosis was investigated in adult rat cardiac myocytes in culture as a model system that is sensitive to oxidative stress. Oxidative stress caused JNK activation, cytochrome c release, and apoptosis without new protein synthesis. Oxidative stress-induced apoptosis was abrogated by dominant negative stress-activated protein kinase/extracellular signal-regulated kinase-1 (SEK1)-mediated inhibition of the JNK pathway, whereas activation of the JNK pathway by constitutively active SEK1 was sufficient to cause apoptosis. Inhibition of caspase-9, an apical caspase in the mitochondrial apoptosis pathway, suppressed oxidative stress-induced apoptosis, whereas inhibition of caspase-8 had no effect, indicating that both the JNK pathway and the mitochondrial apoptosis machinery are central to oxidative stress-induced apoptosis. Both JNK and SEK1 localized on mitochondria where JNK was activated by oxidative stress. Furthermore, active JNK caused the release of apoptogenic factors such as cytochrome c from isolated mitochondria in a cell-free assay. These findings indicate that the JNK pathway is a direct activator of mitochondrial death machinery without other cellular components and provides a molecular linkage from oxidative stress to the mitochondrial apoptotic machinery.

Apoptosis is a highly regulated and ordered process that plays a central role in eliminating unnecessary cells in normal development and homeostasis. In addition, cells injured by environmental stress undergo apoptosis in pathophysiological conditions. The ordered process in apoptosis is exemplified by Fas-mediated activation of the caspase cascade (for review, see Ref. 1). Ligand-mediated aggregation of Fas receptor generates the center of “death signaling,” which activates caspase-8 that cleaves and activates downstream caspases such as caspase-3. Recent progress unraveled the fact that mitochondria are central to death signaling (for review, see Ref. 2). In certain situations, death stimuli induce an increase in the permeability of the outer mitochondrial membrane that releases apoptosis inducers such as cytochrome c (3) and apoptosis-inducing factor (AIF) (4). Cytochrome c activates caspase-9 to initiate the activation and amplification of death signaling. Despite the advance in understanding the molecular mechanisms of apoptosis, it remains unclear how environmental stress activates apoptosis machinery.

c-Jun N-terminal kinase (JNK, also known as stress-activated protein kinase (SAPK)) is one of the leading candidate signal transduction mechanisms that transmits and converts stress signaling into apoptosis signaling in various cell types (for review, see Ref 5). Thus, the JNK pathway is activated by various apoptosis-inducing environmental stresses that cause apoptosis. Inhibition of the JNK pathway abrogates apoptosis induced by these stresses, whereas activation of the JNK pathway is often sufficient to induce apoptosis. The JNK pathway was reported to activate receptor-mediated apoptosis by induction of the Fas ligand in T lymphocyte (6). More recently, it has been proposed that the JNK pathway is necessary for UV irradiation-induced activation of mitochondrial death machinery in embryonic fibroblast (7). However, the molecular linkage between the JNK pathway and apoptosis pathway is still missing.

Recent evidence indicates that apoptosis plays an important role in the loss of cardiac myocytes in pathophysiological conditions such as ischemia (8) and ischemia/reperfusion (for review, see Ref. 9). We previously reported that hypoxia and reoxygenation of adult cardiac myocytes in culture induces activation of mitochondrial death machinery by oxidative stress (10). The current study was designed to elucidate the role of JNK in environmental stress-induced apoptosis and the mechanism of JNK-mediated apoptosis in adult cardiac myocytes in culture as a model system. Apoptosis was induced by oxidative stress as an environmental stress relevant to this cell type experienced in pathophysiological conditions including ischemic heart disease. Our results indicate that JNK resides on mitochondria in cardiac myocytes where oxidative stress

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1 The abbreviations used are: AIF, apoptosis-inducing factor; JNK, c-Jun N-terminal kinase; AnV, annexin V; PI, propidium iodide; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase kinase-1; GST, glutathione S-transferase; GDI, guanine nucleotide dissociation inhibitor.
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causes sustained activation of JNK. Activation of JNK is crucial for oxidative stress-induced apoptosis. Furthermore, activated JNK phosphorylates several mitochondrial proteins and causes the release of cytochrome c and AIF by selective permeabilization of the outer mitochondrial membrane in the absence other cellular components.

EXPERIMENTAL PROCEDURES

Adult Rat Ventricular Myocyte Culture, Oxidative Stress, and Apoptosis Assay—Culture of adult rat cardiac ventricular myocytes was performed as described previously (10). Experiments were started 24 h after plating with the exception of adenovirus infection, which was carried out on the day of the plating. Adult rat ventricular myocytes were treated with 0.1 mM H2O2 and 0.1 mM FeSO4 as a superoxide-generating system for 1 h (11). Myocytes were then cultured in fresh media for the indicated period. DNA fragmentation and cell viability assays by trypan blue staining were performed as described previously (10). For quantification of apoptotic cells, myocytes were stained with annexin V (AnV) and propidium iodide (PI) at the end of the experiments as described previously (10). This method has been shown to correlate well with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (10).

For specific inhibition of caspases, myocyte cultures were treated with 20 μM z-IETD-fmk or z-LEHD-fmk (Calbiochem) for 30 min before, during and after oxidative stress or adenoviral infection. These conditions have been reported to specifically inhibit caspase-8 (z-IETD-fmk) (12) or caspase-9 (z-LEHD-fmk) (13) and apoptosis mediated by these caspases in various cell systems including cardiac myocytes.

Adenoviral Vectors—Adenoviral vectors for FLAG-tagged dominant negative (Ad/dnSEK1) and constitutively active (Ad/caSEK1) stress-activated protein kinase/extracellular signal-regulated kinase kinase-1 (SEK1; also known as MKK4) were generated by site-directed mutagenesis and adenovirus recombination system (courtesy of Dr. S. Hardy) as described previously (14). In control experiments, an adenoviral vector encoding nuclear localizing β-galactosidase (Ad/LacZ) was used at the same multiplicity of infection.

Immunostaining, Confocal Microscopy, and Electron Microscopy—Cardiac myocytes were stained with Mitotracker Red (Molecular Probes) and anti-SEK1 (Transduction Laboratories) or anti-JNK1 antibody and observed by laser-scanning confocal microscopy (Bio-Rad MRC1024). Transmission electron microscopy of the mitochondrial fraction was performed as described previously (10).

Recombinant Proteins and in Vitro Activation of JNK—Recombinant constitutively active SEK1 (rSEK1 ED) and kinase-inactive SEK1 (rSEK1 D) were produced as hexahistidine-tagged proteins and purified by nickel nitrilotriacetic acid beads (Qiagen). Wild type (rJNK WT) and kinase-inactive JNK (rJNK KR) were produced as GST fusion proteins (courtesy of Dr. J. Kyriakis). Recombinant rJNK WT and rJNK KR were phosphorylated by rSEK1 ED in the presence of 100 μM ATP and purified by glutathione beads (Amersham Biosciences, Inc.).

Subcellular Fractionation and Cell-free Assay of Cytochrome c Release—Subcellular fractionation was performed using a previously published method (10), with further purification of the mitochondria by Percoll density gradient (15). Isolated mitochondria were incubated with or without rSEK1 ED, rSEK1 KR, or in vitro phosphorylated rJNK WT or rJNK KR. After centrifugation (15,000 rpm, 10 min at 4 °C) to remove mitochondria, the supernatant and mitochondrial pellets were analyzed for various mitochondrial proteins.

Mitochondrial Inner Membrane Potential—Mitochondrial inner membrane potential was assessed by a mitochondrial voltage-sensitive dye JC-1 (Molecular Probes) and flow cytometry (FACSCalibur, Becton Dickinson).

Western Blotting and in Vitro Assay of Mitogen-activated Protein Kinases—Western blotting and in vitro immune complex kinase assay of mitogen-activated protein kinases were performed as described previously (16).

Statistics—All data are expressed as the means ± S.E. Statistical analyses were performed using analysis of variance (ANOVA). The post-test comparison was performed by the method of Bonferroni. P values of <0.05 were accepted as significant.

RESULTS

Oxidative Stress-induced Apoptosis Is Mediated by the JNK Pathway—Transient oxidative stress (0.1 mM H2O2, and 0.1 mM FeSO4) caused prolonged activation of JNK in adult ventricular myocytes in culture as demonstrated by in vitro immune complex kinase assay during and after oxidative stress (Fig. 1A). To elucidate the role of the JNK pathway in oxidative stress-induced apoptosis of adult cardiac myocytes, we utilized SEK1 (also known as MKK4), which specifically regulates JNK activity without affecting other mitogen-activated protein kinases (17). The JNK activities in adult cardiac myocytes were effectively manipulated by adenoviral vectors encoding a dominant negative mutant (Ad/dnSEK1) or a constitutively active mutant (Ad/caSEK1) of SEK1. Expression of dnSEK1 suppressed oxidative stress-induced JNK1 activation by 58.9 ± 10.9% (p < 0.05 compared with oxidative stress alone), whereas expression of caSEK1 resulted in 42.6 ± 9.3% of JNK activity (n = 4, p < 0.05 compared with control) without oxidative stress. Adenoviral expression of SEK1 mutants did not affect ERK or p38 activity (data not shown).

The involvement of the SEK1/JNK pathway in cardiac myo-
cytotoxic apoptosis was demonstrated using the DNA fragmentation assay. As shown in Fig. 1B, oxidative stress increased DNA fragmentation (Fig. 1B, lane 2), indicating an increase in apoptosis. Expression of dnSEK1 suppressed oxidative stress-induced DNA fragmentation (Fig. 1B, lane 4), whereas expression of caSEK1 resulted in an increase in fragmented DNA at base line (Fig. 1B, lane 5), which was further increased by oxidative stress (Fig. 1B, lane 6).

Quantitative analysis of apoptosis was performed 6 h after oxidative stress by staining cultured cardiac myocytes with AnV and PI, as shown in Fig. 1C. Cells with AnV staining but without nuclear PI staining were counted as apoptotic cells (Fig. 1C, inset, arrow). AnV stained plasma membranes with an irregular shape, suggesting that these cells are undergoing the process of plasma membrane blebbing (10). In the control culture, oxidative stress caused a significant increase in apoptosis (Fig. 1C, 8.7 ± 0.4 to 48.4 ± 8.6%, p < 0.001, n = 5). In myocytes expressing dnSEK1, the percentage of basal apoptotic cells did not change significantly (Fig. 1C, 13.4 ± 3.8%, not significant compared with control without oxidative stress, n = 5), but oxidative stress-induced apoptosis was significantly suppressed (Fig. 1C, 29.3 ± 3.2%, p < 0.05 compared with control with oxidative stress, n = 5). The expression of caSEK1 caused an increase in basal apoptosis (Fig. 1C, 27.0 ± 3.2%, p < 0.05 compared with control without oxidative stress, n = 5), which was further increased upon oxidative stress (Fig. 1C, 55.8 ± 3.2%, p < 0.001 compared with caSEK1 without oxidative stress).

As shown in Fig. 1D, oxidative stress caused a significant decrease in cell viability (93.0 ± 0.5 versus 36.9 ± 15.9%, p < 0.001, n = 5), which was partially blocked by dnSEK1 (63.6 ± 7.5%, p < 0.05 compared with Ad/LacZ with oxidative stress, n = 5). On the other hand, caSEK1 alone caused a decrease in cell viability at base line (65.8 ± 7.3%, p < 0.05 compared with Ad/LacZ alone, n = 5), which was further decreased by oxidative stress (38.7 ± 8.9%, not significant compared with Ad/LacZ plus oxidative stress, p < 0.001 compared with Ad/LacZ). These results indicate that inhibition of the JNK pathway by dnSEK1 suppresses oxidative stress-induced apoptosis and cell death, whereas activation of the JNK pathway results in an increase in apoptosis and cell death.

Oxidative Stress-induced Apoptosis Does Not Require New Protein Synthesis—One of the proposed mechanisms of JNK-mediated apoptosis is the phosphorylation of c-Jun and transactivation of c-Jun target genes, which may include c-Jun itself and pro-apoptotic molecules such as Fas ligand (18). To explore this possibility in oxidative stress-induced apoptosis of cardiac myocytes, the effect of protein synthesis inhibition was examined by treating adult myocytes with cycloheximide. Oxidative stress caused a 2.4-fold induction of c-Jun protein, a well characterized target of the JNK pathway, which was inhibited by 20 μM cycloheximide, indicating that oxidative stress-induced new protein synthesis was effectively blocked (Fig. 2A). Treatment of adult cardiac myocytes with cycloheximide did not affect the basal or oxidative stress-induced activity of JNK1 (9.4 ± 1.7 versus 9.1 ± 1.3-fold without or with cycloheximide, respectively, n = 3) (Fig. 2B) as reported in T lymphoma cells (19), although this kinase was initially identified by virtue of cycloheximide-induced activation (20). Inhibition of protein synthesis did not affect basal (8.7 ± 1.6 versus 9.3 ± 0.8%, control versus cycloheximide, n = 4) or oxidative stress-induced (34.9 ± 5.2 versus 43.2 ± 7.8%, control versus cycloheximide, n = 4) apoptosis in adult myocytes (Fig. 2C). Cycloheximide did not affect cell viability either in the culture without (94.0 ± 5.0 versus 92.6 ± 2.3%, control versus cycloheximide, n = 4) or with oxidative stress (63.7 ± 9.9 versus 50.4 ± 7.9%, control versus cycloheximide, n = 4) (Fig. 2D). These results indicate that oxidative stress-induced apoptosis or cell death is largely independent of new protein synthesis. In addition, suppression of protein synthesis does not cause or enhance apoptosis in this condition.

Oxidative Stress- and SEK1/JNK-induced Apoptosis Requires Caspase-9, but Not Caspase-8-like Activity—To further elucidate the mechanism of oxidative stress-induced apoptosis, we examined the role of caspase-9, a regulatory caspase in the mitochondrial pathway. Adult cardiac myocyte cultures were treated with CHX or without (Control) 20 μM cycloheximide and stimulated with (+) or without (-) oxidative stress for 60 min. A, the protein levels of c-Jun were examined 2 h after oxidative stress. The protein levels of troponin T (TnT) are also shown as an internal control to confirm equal loading of proteins. An identical result was obtained in another independent observation. B, cardiac myocyte cultures were stimulated with (closed columns) or without (open columns) oxidative stress for 60 min in the presence or absence of cycloheximide (CHX). The activity of JNK1 was measured using the in vitro immune complex kinase assay 1 h after oxidative stress. Data are the means ± S.E. of three independent experiments. The double asterisk indicates p < 0.01 compared with the control without oxidative stress. C, cardiac myocyte cultures treated as described in panel B were analyzed for apoptosis. The percentage of apoptotic (AnV-positive/PI-negative) cells was determined 6 h after oxidative stress. D, cardiac myocyte cultures treated as described in panel B were analyzed for cell viability 24 h after oxidative stress. Data are the means ± S.E. of four independent experiments. * and ** indicates p < 0.05 and p < 0.01, respectively, compared with the control without oxidative stress.

Fig. 2. Effect of protein synthesis inhibition on JNK activation, apoptosis, and cell death induced by oxidative stress. Cardiac myocyte cultures were treated with (CHX) or without (Control) 20 μM cycloheximide and stimulated with (+) or without (-) oxidative stress for 60 min.
but not caspase-8-dependent apoptotic machinery, most likely involving the mitochondrial pathway.

To investigate the molecular order of the SEK1/JNK pathway and caspase-9, the effect of caspase-9 inhibition was examined in adult cardiac myocytes infected with Ad/caSEK1. As shown in Fig. 3B, treatment of myocytes with z-LEHD-fmk attenuated apoptosis from 29.1 ± 2.7% (Ad/caSEK1) to 16.9 ± 2.2% (z-LEHD-fmk plus Ad/caSEK1, p < 0.01 compared with Ad/caSEK1, n = 4). This result indicates that SEK1/JNK induces apoptosis mostly in a caspase-9-dependent manner in adult cardiac myocytes, making a hierarchical signaling pathway. The activation of mitochondrial death machinery in oxidative stress-induced apoptosis was further supported by the observation that oxidative stress caused an increase in cytosolic cytochrome c content in cardiac myocyte cultures (Fig. 3C). These results indicate that oxidative stress activates SEK1/JNK pathway, which in turn activates mitochondrial death machinery to cause cytochrome c release.

Subcellular Localization of SEK1 and JNK1 in Adult Cardiac Myocytes—Because our results suggest the involvement of mitochondrial death machinery in oxidative stress-induced SEK1/JNK-dependent apoptosis, subcellular localization of endogenous SEK1 and JNK1 was examined using immunofluorescence staining and laser-scanning confocal microscopy. JNK1 staining showed a lattice-like pattern of extranuclear staining, which partly colocalized with mitochondria (Fig. 4A, d–f, arrowheads) in addition to nuclei (Fig. 4A, a–c, arrowheads) and Z-bands (Fig. 4A, e and f). Merged staining in the merged images (c, f, i, and l). The area of high magnification views (d–f and j–l) are indicated by white rectangles in the corresponding low magnification views (a–c and g–i). The bars indicate 20 μm. Arrows, arrowheads, and double arrowheads indicate nuclei, mitochondria, and Z-bands, respectively. B, subcellular fractionation of adult cardiac myocyte cultures was performed to obtain mitochondrial (mito) and cytosolic (cytosol) fractions. Equal amounts of proteins (5 μg/lane) was loaded on each lane of SDS-PAGE. Endogenous SEK1, JNK1, cytochrome c (Cyt c, mitochondrial protein), and Rho-GDI (cytosolic protein) were probed with corresponding antibodies. C, mitochondrial (mito) and cytosolic (cytosol) fractions were obtained from myocyte cultures treated with oxidative stress and analyzed by Western blotting using anti-active JNK (p-JNK) and JNK1 antibodies. Representative Western blotting is shown from at least three independent observations for each protein.
The purity of mitochondrial and cytosolic fractions was verified by Western blotting for cytochrome c, a mitochondrial protein, and Rho-GDI, a cytosolic protein (Fig. 4B). As shown in Fig. 4B, SEK1 and JNK1 were detected in both mitochondrial and cytosolic fractions. The subcellular distribution of JNK1 (Fig. 4C) or SEK1 (data not shown) did not change significantly upon oxidative stress, as determined by subcellular fractionation. However, when myocytes were exposed to oxidative stress, JNK on mitochondria was phosphorylated and, hence, activated, whereas cytosolic JNK remained non-phosphorylated (Fig. 4C) as determined by Western blotting for phosphorylated (active) JNK. Therefore, both SEK1 and JNK1 colocalize with mitochondria in part, and JNK on mitochondria is preferentially activated in response to oxidative stress. These findings raise the possibility for the interaction of the SEK1/JNK pathway with mitochondrial death machinery.

Activation of Mitochondrial Death Machinery by JNK in a Cell-free System—The effect of active JNK on mitochondrial death machinery was examined in the cell-free assay. Mitochondria were isolated from rat heart by differential centrifugation (10) and purified by Percoll density gradient (15). The purity of mitochondria was verified by electron microscopy (Fig. 5A) and Western blotting for mitochondrial, cytoskeletal, and cytosolic proteins (cytochrome oxidase subunit IV, troponin T, and Rho-GDI, respectively, data not shown). Both recombinant wild type JNK (rJNK WT) and catalytically inactive JNK (rJNK KR) were phosphorylated in vitro by rSEK1 ED and purified using glutathione beads. The activity of the in vitro activated rJNK was verified by in vitro kinase assay using GST-c-Jun-(1–79) as a substrate (Fig. 5B).

Purified mitochondria were incubated with in vitro phosphorylated rJNK WT or rJNK KR in the presence of ATP. After removing mitochondria by centrifugation, proteins in the supernatant were analyzed by Western blotting using a panel of antibodies for mitochondrial proteins. As shown in Fig. 6A, active rJNK WT caused the release of cytochrome c from purified mitochondria. In contrast, rJNK KR, which was phosphorylated by rSEK1 ED but lacking catalytic activity, did not cause cytochrome c release, indicating that the catalytic activity of JNK is essential for cytochrome c release. Other intermembranous proteins, namely AIF (4) and adenylate kinase 2 (21), were also found to be released from mitochondria by rJNK WT (Fig. 6A). The addition of the cytosolic fraction to the reaction did not affect JNK-induced cytochrome c release (data not shown), suggesting that cytosolic factors may not be involved in JNK-induced cytochrome c release. When the supernatant was analyzed for inner membrane proteins (cytochrome oxidase subunit IV, prohibitin, and ATP synthase subunit a) or a mitochondrial matrix protein, mitochondrial Hsp70 (mtHsp70), only a trace amount was detected (Fig. 6A). In addition, the amount of these proteins did not differ among control, rJNK WT, and rJNK KR. These data indicate that active JNK causes permeabilization of the outer membrane but not the inner membrane of mitochondria.

Because endogenous JNK was found to localize on mitochondria (Fig. 4), we also examined whether recombinant constitutively active SEK1 (rSEK1 ED), which activates JNK, can activate mitochondrial death machinery. Incubation of isolated mitochondria with rSEK1 ED also resulted in the release of intermembranous proteins into the supernatant (Fig. 6A). In contrast, the inactive form of SEK1 (rSEK1 KR) did not cause significant cytochrome c release (Fig. 6A).

We examined whether JNK treatment of mitochondria induced a change in the amount of anti-apoptotic Bcl-2 family proteins (Fig. 6B). When the mitochondrial fraction was analyzed by Western blotting, the amount of Bcl-2 or Bcl-xL was not changed by JNK. We did not detect Bid in the mitochondrial fraction (data not shown), probably because Bid resides in the cytosol under basal conditions. In addition, we did not detect the migration shift of Bcl-2 or Bcl-xL, suggesting that the phosphorylation status of these anti-apoptotic Bcl-2 family proteins may not significantly change under this condition.

Treatment of purified mitochondria with recombinant active JNK or catalytically inactive JNK did not cause detectable mitochondrial depolarization, as determined by a mitochondrial voltage sensitive dye JC-1 and flow cytometry (Fig. 6C). In contrast, treatment of mitochondria with 500 μM Ca^{2+} (data not shown) or 1 μM valinomycin in the presence of K⁺ caused a marked decrease in the intensity of JC-1 fluorescence incorporated into mitochondria (Fig. 6C), indicating the depolarization of the mitochondrial inner membrane. Therefore, JNK seems to cause a selective permeabilization of the outer mitochondrial membrane without the loss of mitochondrial potential, as reported in Bid- and Bax-induced cytochrome c release (22).

DISCUSSION

The JNK pathway has been implicated in apoptosis signaling. Although recent report showed the linkage of the JNK pathway and mitochondrial death machinery (7), whether the linkage is direct or indirect remained to be elucidated. Our current study indicates that JNK induces the release of pro-apoptotic molecules such as cytochrome c and AIF from mitochondria in the absence of other cellular factors in a cell-free assay by selective permeabilization of outer mitochondrial membrane. Although the amount of the released cytochrome c...
and AIF seems to be moderate (~5% of the total amount in the purified mitochondria, see Fig. 6A), it may have a significant impact in cardiac myocytes considering the exceptionally high amount of mitochondria and relatively small cytosolic space of cardiac myocytes compared with other cell types.

Several mechanisms have been proposed for JNK-mediated apoptosis in various cell systems. One potential mechanism is the activation of c-Jun transcriptional activity by phosphorylation reported in neuronal cells (23) and T lymphocytes (18, 24), where Fas ligand is up-regulated in a MEKK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1)- and c-Jun-dependent manner. However, Fas/ Fas ligand may not be sufficient to activate cell death signaling in cardiac myocytes (25). Our data indicate that oxidative stress-induced apoptosis in cardiac myocytes does not require new protein synthesis, similar to UV-induced apoptosis in mouse embryonic fibroblasts (7). Therefore, JNK-mediated gene expression of pro-apoptotic molecules does not seem to be a major mechanism for oxidative stress-induced apoptosis of cardiac myocytes. Furthermore, inhibition of protein synthesis did not induce or enhance apoptosis, suggesting that suppression of anti-apoptotic protein expression does not explain SEK1/JNK-mediated cell death. Our data indicate that oxidative stress-induced SEK1/JNK-mediated apoptosis requires caspase-9, the regulatory caspase in the mitochondrial pathway (Ref. 26; for review, see Ref. 2), but not caspase-8, the regulatory caspase in receptor-mediated apoptosis signaling. Interestingly, recent reports showed that JNK is anti-apoptotic in cardiac myocytes derived from neonatal rat (27) or from in vitro differentiated mouse embryonic stem cells (28). The opposite effect of JNK on apoptosis in these studies and ours may be explained by the difference in the cell culture system, i.e., neonatal versus adult cardiac myocytes, because numerous studies indicate that JNK can be either pro-apoptotic or anti-apoptotic, depending on the cell type (for review, see Ref. 5). Notably, anti-apoptotic JNK function in embryonic stem-derived cardiac myocytes, which resemble fetal or neonatal cardiac myocytes, depends on transcriptional control (28), whereas pro-apoptotic effect of JNK does not in our study and others (7), suggesting that different molecular mechanisms underlie the opposite effects of JNK in cardiac myocytes. Our results indicate that post-translational modification of preexisting molecules in the mitochondrial apoptosis pathway plays a major role in oxidative stress-induced, JNK-mediated apoptosis in adult cardiac myocytes.

Recently, a novel mechanism of JNK-mediated apoptosis has been proposed. Anti-apoptotic Bcl-2 family proteins, namely Bcl-2 (29) and Bcl-xL (30), were found to be phosphorylated by JNK, which inactivates these anti-apoptotic proteins. Because one of the major anti-apoptotic effects of Bcl-2 family proteins is to suppress the release of cytochrome c from mitochondria (31, 32), Bcl-2 family members are attractive candidates as potential JNK targets in cardiac myocyte apoptosis by oxidative stress. However, another group showed an anti-apoptotic role of Bcl-2 phosphorylation by JNK on the same Ser-70 (33). Therefore, the biological consequence of Bcl-2 phosphorylation by JNK remains to be elucidated (for review, see Ref. 5). In addition, we could not detect Bcl-2 family phosphorylation, as determined by migration shift on SDS-PAGE. Very recently, the critical role of JNK in UV-induced apoptosis was shown using Jnk1−/−/Jnk2−/− double null mouse embryonic fibroblasts (7). The JNK pathway is necessary for UV-induced Bid translocation, cytochrome c release, mitochondrial dysfunction, and apoptosis. These findings and ours are consistent in that the JNK pathway activates mitochondrial apoptotic signaling, which does not require new synthesis of pro-apoptotic proteins. Phosphorylation of Bcl-2 family proteins was not detected in the double null embryonic fibroblasts (7) and in our system, suggesting the presence of as yet unidentified JNK targets in the mitochondrial pathway (for review, see Ref. 5).
The release of cytochrome c and AIF in the cell-free assay occurs in the absence of other cellular factors, indicating that JNK targets must be on the mitochondria. The JNK target seems to be its substrate, as catalytic activity of JNK is essential. Therefore, JNK is likely to phosphorylate and activate its target on mitochondria, which is directly or indirectly linked to the release mechanism of the apoptosis inducers. Both JNK and its target are readily accessible from outside the mitochondria and are likely to be exposed on the outer surface of mitochondria because exogenously added JNK and SEK1 cause the release of apoptosis inducers. In fact, exogenously added active JNK caused phosphorylation of several mitochondrial proteins. The nature of the phosphorylated proteins and whether these proteins are direct substrates of JNK remains to be investigated.

Although it is well established that the release of apoptosis inducers such as cytochrome c and AIF is a critical step in the execution of apoptosis, the signal transduction from cellular stress to this step and the molecular mechanism of its regulation have been unclear (for review, see Ref. 2). The identification of JNK pathway as a direct activator of mitochondrial death machinery provides the direct linkage from cellular stress to the mitochondrial death machinery. Further characterization and identification of the JNK substrate(s) on mitochondria should uncover the molecular mechanism of the release of the apoptosis inducers from mitochondria.

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REFERENCES
1. Nagata, S. (1999) Annu. Rev. Genet. 33, 29–55
2. Kroemer, G., and Reed, J. C. (2000) Nat. Med. 6, 513–519
3. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Cell 86, 147–157
4. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacobot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebersold, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Nature 397, 441–446
5. Davis, R. J. (2000) Cell 103, 239–252

2 H. Aoki, K. Yoshimura, P. M. Kang, J. Hampe, T. Noma, M. Matsuzaki, and S. Izumo, unpublished observation.