Regulation of Necrosis of H9c2 Myogenic Cells upon Transient Energy Deprivation

RAPID DEENERGIZATION OF MITOCHONDRIA PRECEDES NECROSIS AND IS CONTROLLED BY REACTIVE OXYGEN SPECIES, STRESS KINASE JNK, HSP72, AND ARC*

Received for publication, June 27, 2003, and in revised form, September 30, 2003
Published, JBC Papers in Press, September 30, 2003, DOI 10.1074/jbc.M306903200

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Subjecting myogenic H9c2 cells to transient energy deprivation leads to a caspase-independent death with typical features of necrosis. Here we show that the rupture of cytoplasmic membrane, the terminal event in necrosis, is shortly preceded by rapid depolarization of mitochondrial membranes. The rapid deenergization of mitochondria critically depended upon prior generation of reactive oxygen species (ROS) during ATP depletion stage. Accordingly, expression of catalase prevented mitochondrial depolarization and averted subsequent necrosis. Interestingly, trifluoperazine, a compound that protects cells from ischemic insults, prevented necrosis of H9c2 cells through inhibition of ROS production. Other factors that regulated the mitochondrial membrane depolarization and subsequent loss of plasma membrane integrity include a stress kinase JNK activated at early steps of recovery from ATP depletion, as well as an apoptotic inhibitory protein ARC. Accordingly, inhibition of JNK or overexpression of ARC prevented mitochondrial depolarization and rescued H9c2 cells from necrosis. ROS and JNK affected mitochondrial deenergization and necrosis independently of each other since inhibition of ROS production did not prevent activation of JNK, whereas inhibition of JNK did not suppress ROS accumulation. Therefore, JNK activation and ROS production represent two independent pathways that control mitochondrial depolarization and subsequent necrosis of cells subjected to transient energy deprivation. Overexpression of ARC, although preventing mitochondrial depolarization, did not affect either JNK activation or production of ROS. The major heat shock protein Hsp72 inhibited JNK-related steps of necrotic pathway but did not affect ROS accumulation. Interestingly, mitochondrial depolarization and subsequent necrosis can be suppressed by an Hsp72 mutant Hsp72AEEVD, which lacks chaperone function but can efficiently suppress JNK activation. Thus, Hsp72 is directly implicated in a signaling pathway, which leads to necrotic death.

Heart ischemia followed by reperfusion causes tissue damage due to both apoptotic and necrotic cell death. Apoptosis is characterized by nuclear condensation, chromatin fragmentation, and breakdown of cells into multiple apoptotic bodies that retain membrane integrity. Research from many laboratories has established that apoptosis is a highly coordinated process subject to complex regulation (see e.g. Ref. 1). Necrosis, a morphologically distinct mode of cell death, is characterized by cell swelling, loss of plasma membrane integrity, and leakage of cellular constituents to the extracellular medium (2). In contrast to apoptotic cell death, necrosis was considered until recently to be an unregulated event triggered by severe stress that damages cells beyond their capability to repair. However, recent evidence started to emerge that under conditions of moderate stress that does not cause irreparable damage to cellular structures, cells could still die via necrosis (2, 3). This death appeared to be controlled by a specific program(s) that involves many signaling elements also implicated in early stages of apoptosis (for review see Ref. 3). It appears that early events in these two modes of cell death could be the same, and signals bifurcate later leading to either apoptosis or necrosis.

An interesting example of a regulated necrosis is death of cardiomyocytes after myocardial ischemia/reoxygenation. This type of cell death is associated with activation of stress kinases p38 and JNK.† Using isolated, Langendorff-perfused rabbit hearts, it was demonstrated that inhibition of p38 kinase with a pharmacological inhibitor SB 203580 greatly reduced not only myocardial apoptosis but also necrosis triggered by ischemia/reperfusion (4). These results clearly implicated p38 kinase in execution of both apoptotic and necrotic modes of cell death. Similarly, ischemia/reperfusion of kidney resulted in activation of stress kinases p38 and JNK (5). Preconditioning of kidney by exposure to transient ischemia led to both suppression of JNK and p38 and reduction of necrosis triggered by a second more severe ischemic insult imposed 8–15 days later (5). Therefore, it appears that mitogen-activated protein kinases regulate not only apoptotic mode of cell death but necrosis as well.

Activation of three classes of mitogen-activated protein kinases, ERK, JNK, and p38, was observed in simulated myocardial ischemia in vitro, where primary cardiomyocytes or H9c2 myogenic cells were subjected to transient energy deprivation (6, 7). Whether cells survive the treatment or die depends upon

Received, June 27, 2003, and in revised form, September 30, 2003. Published, JBC Papers in Press, September 30, 2003.
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† This work was supported by a National Institutes of Health training grant (to J. A. Y.) and a grant from American Heart Association (to V. L. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; PI, propidium iodine; TMRE, tetramethylrhodamine; methyl ester, perchlorate; PBS, phosphate-buffered saline; DHE, dihydroethidium; GFP, green fluorescent protein; TFP, trifluoperazine; CPZ, chlorpromazine; AIF, apoptosis-inducing factor.
the intricate balance of the magnitude and duration of activation of individual kinases. ERK is generally implicated in cell survival, since specific inhibition of ERK dramatically decreased cell viability (6, 8, 9). In contrast, JNK and p38 appeared to promote cell death, since inhibition of these kinases led to cell protection (10–12). Prior data from this laboratory using H9c2 cells established that inhibition of ERK enhances while inhibition of JNK strongly reduces necrosis after transient energy deprivation, indicating that JNK activation facilitates this type of cell death (7).

In apoptosis, JNK promotes cell death most likely through phosphorylation and activation of a number of pro-apoptotic Bcl-2 family members that serve as key regulators of programmed cell death (12). For example, in tumor necrosis factor-induced apoptosis, JNK was shown to activate cleavage of BH3-only protein Bid, which stimulated the mitochondrial pathway of apoptosis (13). In UV-stimulated apoptosis, JNK phosphorylated a distinct set of BH3-only proteins, Bim and Bmf, which resulted in the Bax/Bak-dependent activation of the mitochondrial apoptotic pathway (14). These data indicated that in the apoptotic pathway JNK acts upstream of mitochondria. However, the mechanism of involvement of JNK in regulation of cell necrosis is yet unclear.

Mitochondria, being the source of both pro-apoptotic and pro-necrotic factors (e.g. cytochrome c, Smac, or AIF), plays a key role in determining the fate of cells exposed to stress (15). Release of these factors may be associated with the opening of a nonselective pore, known as mitochondrial permeability transition (MPT) pore. Opening of the MPT pore, which could be regulated by Bcl2-related proteins, results in rapid uncoupling of mitochondria. In certain models MPT was implicated in both apoptotic and necrotic cell death (16). For example, inhibition of MPT by cyclosporin A protected L929 cells from tumor necrosis factor-induced necrosis (17) and primary rat hepatocytes from tumor necrosis factor-induced apoptosis (18). Ischemia/reperfusion-mediated cell death appeared to be associated with MPT opening since isolated hearts as well as cardiomyocytes can be protected from ischemia/reperfusion insult by the MPT inhibitor, cyclosporin A (19–21). It should be noted, however, that the type of cell death, i.e. apoptosis or necrosis, that is suppressed by cyclosporin A was not specifically addressed in these works.

More evidence for the role of mitochondria in regulation of cell death pathways comes from studies of the apoptotic regulatory protein ARC. ARC is specifically expressed in cells of myogenic origin, and its ectopic expression has a strong pro-apoptotic effect on cardiac cells under various types of deleterious conditions (22–24). Very importantly, overexpression of ARC prevented deenergization of mitochondrial membrane of H9c2 cells exposed to oxidative stress and potently rescued cells from necrotic-like death under these conditions (25).

Oxidative stress has been implicated in both cell apoptosis and necrosis (2, 3). Indeed, in many systems it was shown that in the process of cell death, reactive oxygen species (ROS) are generated, and suppression of ROS production by anti-oxidants and the detoxifying enzymes catalase or superoxide dismutase has a protective effect (2, 3). However, how ROS enhance cell death is yet unknown.

The outcome of ischemic injury of cardiac cells also depends upon accumulation of heat shock proteins (Hsps), which are induced in response to ischemia or transient energy deprivation. Induction of Hsp72 and other Hsps was suggested to play an important role in preconditioning, a phenomenon of protection of a heart from strong ischemic insult by prior exposure to mild ischemia or other mild stresses. Importantly, it was demonstrated that both cardiomyocytes and isolated hearts could be protected from ischemic damage by adenovirus-mediated expression of Hsp72 (26, 27). Moreover, constitutive expression of Hsp72 in transgenic animals strongly enhanced functional recovery and reduced infarct size in hearts after transient ischemia (28–30).

The mechanism of Hsp72-mediated cardio-protection is not well understood. Energy-deprived ischemic cells accumulate misfolded proteins, and Hsps, being molecular chaperones, can assist in their refolding or rapid degradation (31). In addition to their function as molecular chaperones Hsps also can directly interfere with signaling events that trigger apoptotic program, for example, through suppression of activation of JNK or p38. This effect of Hsp72 on suppression of stress kinases was shown to play an important role in prevention of necrosis of H9c2 cells after transient ATP depletion (7).

Therefore, it seems that execution of both apoptotic and necrotic modes of cell death depends upon many signaling events, such as activation of stress kinases, depolarization of mitochondria, generation of ROS, and induction of heat shock proteins. However, in contrast to apoptosis, very little is known about specific pathways that lead to necrosis since there have been no systematic studies that define relations between these players and establish the consequences of events. Here we undertake an investigation of pathways implicated in necrotic death of H9c2 myogenic cells triggered by transient energy deprivation. Specifically we studied the involvement of mitochondrial depolarization in this process as well as the roles of ROS, Hsp72, and JNK in regulation of mitochondrial depolarization during necrosis.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Materials**—Rat heart myocardium H9c2 cells were obtained from ATCC. H9c2 cells expressing dominant negative JNK1 and parental H9c2 clone were a kind gift from Dr. J. Wei (32). H9c2 cells expressing ARC (ARC24) and parental H9c2Neo line were described before (22). All cells were grown in Dulbecco’s modified Eagle’s medium (ATCC) supplemented with 15% fetal bovine serum. Cells were grown at 37 °C in an atmosphere of 5% CO2 to 85% confluence. Rotenone, propidium iodine (PI), and anisomycin were from Sigma. Tetramethylrhodamine, methyl ester, perchlorate (TMRM) were from Molecular Probes. JNK inhibitor SP600125 (used at 15 μM) was from Biomol. Trifluoperazine and chlorpromazine were from Alexis.

**Antibodies and Western Blotting**—In this study we used antibodies against anti-phaspho-JNK (Promega; rabbit polyclonal) and anti-γ-tubulin (Santa Cruz; mouse monoclonal). After immunoblotting analysis secondary antibodies conjugated with peroxidase were visualized with ECL substrates (Amersham Biosciences), and films were quantified by densitometry. Anti-Bax antibody specific for N terminal of the protein used for immunocytochemistry was from Zymed Laboratories Inc. (6A7 mouse monoclonal).

**Anti-Bax Immunocytochemistry**—Immunocytochemistry was performed according to standard technique. Briefly, cells were fixed in 3.7% paraformaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min, blocked with 5% bovine serum albumin in PBS, and incubated with primary anti-Bax antibody diluted 1:50 in 3% bovine serum albumin, PBS at 4 °C overnight. Primary antibody was detected with Alexa Fluor 647 signal amplification kit (Molecular Probes).

**TMRM Double Staining**—Live cells were incubated with TMRM at a final concentration of 200 nM for 15 min. TMRM was washed out, and 4 μg/ml PI was added for an additional 5 min.

**Exposure to Metabolic Inhibitor**—60–85% confluent H9c2 cells were washed twice in PBS and incubated in PBS supplemented with 5 μM rotenone for the indicated period of time. Cells were then washed twice with complete medium and recovered in complete medium for the required period of time.

**Detection of ROS Generation**—To detect ROS generation cells were labeled with 2 μM dihydroethidium (DHE) for 15 min. DHE was prepared in PBS stock in MeSO, separated into aliquots, and stored at 20 °C for not longer than 1 week. The intensity of DHE fluorescence was analyzed with Image-Pro Plus software from Media Cybernetics.
(AdTR5-DC/HSP70-GFP) were previously described (7, 13). Inoculation of cells with $3 \times 10^7$ plaque-forming units of each virus per 35-mm dish was sufficient to infect almost 100% of cells. Thirty-six hours after infection, cells accumulated Hsp72 and Hsp72EEVD, as judged by immunoblotting of cytosol extracts (not shown). As a control, adenovirus-expressing GFP under the regulation of tTA was used.

Cytosol Extract Preparation and JNK Kinase Assay—Cells were washed twice with PBS, aspirated, and lysed in 200 μl of lysis buffer per 35-mm dish (40 mM HEPES, pH 7.5, 50 mM KCl, 1% Triton X-100, 2 mM dithiothreitol, 1 mM Na3VO4, 50 mM β-glycerophosphate, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml each leupeptine, pepstatin A, aprotinin). The lysates were clarified by centrifugation in a microcentrifuge at 15,000 rpm for 7 min. Total protein concentration was measured in the supernatants, after which they were diluted with lysis buffer to achieve equal protein concentrations in all samples. All procedures were performed at 4 °C. JNK activity was assayed by immunoblotting of cell lysates with anti-phospho-JNK antibody. The same membranes were stripped and re-probed with anti-tubulin antibody to control for equal loading.

RESULTS

Depolarization of Mitochondria Precedes Necrosis upon Transient Energy Deprivation of H9c2 Cells—To address the mechanism of cell necrosis after transient energy deprivation, we utilized the myogenic cell line H9c2. Previously, we showed that incubation of these cells in glucose-free PBS in the presence of the mitochondrial respiratory chain inhibitor rotenone led to more than 80% reduction of ATP levels within 15 min (7). H9c2 cells were incubated in PBS/rotenone for 2.5 h, and then rotenone was washed out, and cells were allowed to recovered in complete media supplemented with glucose, where the ATP levels were restored within 15–30 min (7). As we have reported previously, these treatments led to cell death with features typical of necrosis, where no activation of caspases was observed, and the pan-caspase inhibitor benzoyloxy carbonyl-Val-Ala-Asp-Val (VAD) could not protect cells (7). Furthermore, cells died within 2–3 h, indicating that death was not due to the secondary necrosis, which follows apoptosis. The major hallmark of cell death was the breakdown of the plasma membrane, as manifested by the appearance of membrane permeability to the fluorescent dye PI, which binds to chromosomal DNA and, thus, mainly accumulates in the nucleus (Fig. 1A).

The number of necrotic cells critically depended upon the duration of ATP depletion. Indeed, ATP depletion for 2 h triggered almost no necrotic death, 2.5 h of depletion led to necrosis in 40% of cells, and incubation in the PBS/rotenone medium for 3 and 4 h resulted in 80 and 100% of necrosis, respectively (Fig. 1B). The number of PI-positive cells gradually increased upon incubation in complete medium. For example, the extent of necrosis right at the beginning of recovery after 2.5 h of metabolic inhibition was about 10% (data not shown). Upon further recovery in complete media, the fraction of necrotic cells gradually increased and by 3 h reached about 40% (Fig. 1B). No further increase was observed, so that even after an overnight incubation the fraction of necrotic cells remained close to 40% (data not shown).

Mitochondrial collapse is a common and essential step in cell death pathways activated by certain death-promoting signals. Therefore, we sought to investigate whether depolarization of mitochondrial membrane takes place during necrotic death of H9c2 cells triggered by transient energy deprivation. Live H9c2 cells were incubated with the fluorescent dye TMRM, which accumulates in energized mitochondria and rapidly leaks out upon depolarization of mitochondrial membrane (see e.g. Ref. 33). Accordingly, when control cells were loaded with TMRM for 15 min, we observed characteristic mitochondrial punctuate staining (Fig. 2A, left panel). Upon 15 min of incubation of cells with rotenone in PBS, punctuate staining disappeared, and instead, TMRM showed diffuse cytoplasmic distribution (Fig. 2A, right panel). Importantly, within 15 min after the start of recovery in complete medium with glucose almost 100% of cells again acquired typical punctuate mitochondrial TMRM staining (Fig. 2A, right panel) (in the last panel, cells looked somewhat shrunken due to prior incubation with rotenone; however, the intensity of mitochondrial TMRM staining was similar to that in control cells). These data indicate that recovery of cells in complete media led to restoration of mitochondrial potential to normal levels. Unexpectedly, prolonged incubation of H9c2 cells in complete medium led to the secondary loss of TMRM fluorescence in a fraction of cells, and by 3 h ~40% of cells became TMRM-negative. When H9c2 cells were stained simultaneously with TMRM and PI, there were no double-stained cells observed; cells with energized mitochondria (i.e. those with mitochondrial TMRM staining) had intact cytoplasmic membrane and were PI-negative, whereas cells with de-energized mitochondria (TMRM-negative) had their cytoplasmic...
membrane permeable for PI (Fig. 2B). Of note, TMRM and PI accumulate in different cellular compartments (PI is nuclear, whereas TMRM is cytoplasmic/mitochondrial), allowing easy differentiation between these two dyes (see Fig. 2B). These data suggest that either deenergization of mitochondrial membrane precedes the loss of plasma membrane integrity, or mitochondrial deenergization is an immediate consequence of plasma membrane rupture and cell death. To distinguish between these two possibilities we took advantage of time-lapse microscopy. H9c2 cells were incubated in PBS/rotenone for 2.5 h, recovered in complete medium for an additional 3 h. Alternatively, cells were treated with anisomycin (15 μg/ml) in the presence of benzoxycarbonyl-VAD (50 μM) for 12 h. Cells were fixed and stained with an antibody that recognizes the mitochondria-associated form of Bax, as described under “Experimental Procedures.”

**Fig. 2.** Depolarization of mitochondria precedes rupture of cytoplasmic membrane and is independent of Bax translocation to mitochondria. A, H9c2 cells were incubated in either PBS for 2 h (Control) or PBS/rotenone (5 μM) for 2 h (rotenone) or were incubated in PBS/rotenone for 2.5 h and then recovered in complete media (Recovery). Cells were loaded with TMRM for 15 min, and then TMRM was washed out, and cells were observed under the fluorescence microscope in the red channel. B, H9c2 cells were incubated in PBS/rotenone (5 μM) for 2.5 h and then transferred to complete media for 2 h 45 min. Cells were then loaded with TMRM for 15 min and, after washing out TMRM, were stained with PI for the additional 5 min. Arrows indicate PI-stained cells. Arrowheads show TMRM-stained cells. C, same as in B, but H9c2 cells were incubated in complete media for 2 h before TMRM/PI staining. A series of pictures were taken with 2-min intervals. Shown are selected time points from three independent experiments. D, necrosis of H9c2 cells does not depend upon Bax translocation to mitochondria. H9c2 cells were placed either in PBS (control) or PBS/rotenone (5 μM) for 2.5 h and then recovered in complete medium for an additional 3 h. Alternatively, cells were treated with anisomycin (15 μg/ml) in the presence of benzoxycarbonyl-VAD (50 μM) for 12 h. Cells were fixed and stained with an antibody that recognizes the mitochondria-associated form of Bax, as described under “Experimental Procedures.”
fluorescent microscope on the temperature-controlled table, and a series of pictures with 2-min intervals were taken (Fig. 2C). Cells that undergo necrosis (marked with arrows) originally had punctuate mitochondrial TMRM staining, and their nuclei were devoid of PI (see the first column), indicating that these cells were alive and had energized mitochondria. At a certain time point these cells lost TMRM staining, which was followed by rapid accumulation of PI within 4–6 min. Therefore, in the last column marked cells have nuclei brightly stained with PI and no mitochondrial TMRM staining (Fig. 2C). In no instances did we observe PI accumulation in cells with energized mitochondria, indicating that the depolarization of mitochondria precedes the rupture of the plasma membrane.

Necrosis of H9c2 Cells Does Not Depend upon Alterations of Bax, Cytochrome c, and Mitochondrial Permeability Transition—In the course of apoptosis, depolarization of mitochondria is often triggered by translocation of Bcl-2 family member Bax from the cytoplasm to mitochondrial membrane (34). To investigate whether Bax plays a role in the observed depolarization of mitochondrial membrane after transient energy deprivation, we performed immunocytochemistry with anti-Bax antibody specific for Bax N-terminal domain, which becomes exposed only upon translocation of Bax from the cytoplasm to mitochondria. As seen in Fig. 2D, no Bax-specific immunofluorescence was detected in H9c2 cells incubated either in PBS (control) or PBS/rotenone for 2.5 h and recovered in complete media for either 1 or 3 h (compare the left and middle panels). We observed some nonspecific nuclear staining that was not changed with the omission of primary antibody (data not shown). In contrast, when H9c2 cells were exposed to an apoptotic stimulus anisomycin in the presence of a broad-spectrum caspase inhibitor benzylxoycarbonyl-VAD to prevent cell shrinkage, typical mitochondrial punctuate staining of cytoplasm, indicative of Bax translocation to mitochondria, was readily detected (right panel). These data indicated that the loss of mitochondrial membrane potential caused by transient energy deprivation of H9c2 cells was not associated with Bax translocation. Interestingly, depolarization of the mitochondrial membrane also did not lead to cytochrome c release. In fact, as observed by immunocytochemistry with anti-cytochrome c antibodies, no cytochrome c release from mitochondria to cytoplasm was detected in H9c2 cells transferred from PBS/rotenone to complete media for either 1 or 3 h (data not shown). This result is consistent with our previous data that necrotic death of H9c2 cells under these conditions is not accompanied by caspase activation (7), which is the usual consequence of the cytochrome c release.

To address the possibility that rapid depolarization of mitochondrial membrane may result from activation of the MPT pore, we administered cyclosporin A, a compound that efficiently blocks the MPT pore in other systems and protects cells from certain forms of apoptosis and necrosis (17, 33). Cyclosporin A had no detectable effect on membrane depolarization and necrotic death of H9c2 cells (data not shown). These data indicate that the mechanism of rapid depolarization of the mitochondrial membrane, which precedes permeabilization of plasma membrane, is distinct from the MPT pore opening.

ATP Depletion of H9c2 Cells Triggers ROS Production—Because production of ROS and oxidative stress were reported to contribute to apoptotic and necrotic death of cardiomyocytes exposed to ischemia/reperfusion (35–37), we investigated whether ROS are produced upon transient energy deprivation of H9c2 cells and are implicated in necrosis. To assay ROS production in H9c2 cells we utilized DHE, which becomes fluorescent upon oxidation by ROS. H9c2 cells incubated in PBS/rotenone for different time periods were loaded with DHE for 15 min and observed under a fluorescent microscope. As seen in Fig. 3A, no ROS production was detected at early time points of ATP depletion; however, at later time points, ROS started to accumulate. In fact, dim DHE fluorescence appears in all cells by 1 h 45 min of ATP depletion. DHE fluorescence reached its maximum 15–30 min later and was maintained at this level for the rest of the ATP depletion period. Importantly, when cells were transferred to complete medium, ROS production ceased rapidly (Fig. 3A, last panel). It should be noted that incubation of cells in PBS/rotenone for less than 2 h not only failed to trigger significant ROS production but also did not lead to necrosis (data not shown). Therefore, there was a good correlation between the duration of ATP depletion, the time span of increased ROS production, and the number of necrotic cells. In fact, when H9c2 cells were incubated in PBS/rotenone for 2 h 15 min, increased ROS production was seen for only a short time period, and necrosis during subsequent transfer of cells to complete medium did not exceed 15–20% (Fig. 3B). However, upon incubation of H9c2 cells in ATP depletion media for 2 h 30 min, increased ROS production was detected for a longer time period, and the fraction of necrotic cells was ~40% (Fig. 3B). Upon ATP depletion for 3 h, ROS were produced for even a longer period, and necrosis of about 80% of cells was seen (Fig. 3B).

To study if ROS production is involved in cell death observed upon transient energy deprivation, we tested whether prevention of ROS accumulation can rescue H9c2 cells from necrosis. We utilized an adenoviral vector expressing catalase, which converts toxic H₂O₂ into water and molecular oxygen. As a control we used an adenovirus expressing GFP. H9c2 cells were infected with adenoviruses expressing either catalase or GFP, and 36 h after infection cells placed in PBS/rotenone for 2.5 h followed by incubation in complete medium. After 2.5 h in complete media cells were stained with PI and observed under the microscope to monitor necrotic death. As seen in Fig. 3C, 40% of GFP expressing cells became necrotic and accumulated PI in their nucleus. However, only 7% of catalase-expressing cells became necrotic under these conditions. Therefore, catalase, which eliminates ROS upon transient energy deprivation, efficiently rescues cells from necrotic death.

To investigate whether ROS production regulates mitochondrial membrane depolarization, which precedes the breakdown of the plasma membrane in the process of necrosis, we tested for the effects of expression of the catalase on the loss of mitochondrial membrane potential. H9c2 cells expressing either catalase or GFP were incubated in the PBS/rotenone medium and then were transferred to a complete medium in the presence of PI and TMRM. The double PI/TMRM staining allowed us to monitor both cell necrosis and the status of their mitochondria. If ROS were critical for depolarization of mitochondria, H9c2 cells rescued by catalase from necrosis would be TMRM-positive and PI-negative. Alternatively, if ROS acts downstream of mitochondria, expression of catalase should lead to accumulation of TMRM and PI double negative cells. As mentioned above, expression of catalase reduced the number of necrotic cells (see Fig. 3C), and almost all surviving cells were TMRM-positive, indicating that they retained energized mitochondria. Almost no PI-negative viable cells with de-energized mitochondria (TMRM-negative) were seen in catalase-expressing cells (Fig. 3D). These data indicate that catalase prevents depolarization of mitochondrial membrane, and therefore, ROS appear to play a critical role in regulation of mitochondrial depolarization.

JNK and Hsp72 Regulate Mitochondrial Membrane Depolarization in Cell Necrosis—We have shown previously that death
of H9c2 cells triggered by transient energy deprivation is positively regulated by a stress kinase JNK (7). JNK was inactive, while H9c2 cells were incubated in the ATP depletion medium but became rapidly activated upon recovery in complete media (7). Moreover, when H9c2 cells were incubated in the presence of a specific inhibitor of JNK, SP600125, during the course of the experiment, the number of necrotic H9c2 cells was reduced 2-fold (Fig 4A). Even stronger protection (up to 70%) was observed in an H9c2 cell line, which constitutively expresses a dominant negative form of JNK1, as compared with the control parental cell line (Fig. 4B and Ref. 7). These experiments confirmed an important role of JNK activation in necrosis of H9c2 cells and allowed us to investigate the involvement of JNK activation in depolarization of mitochondria triggered by

**Fig. 3.** Cell necrosis triggered by transient ATP depletion correlates with the magnitude of ROS production. **A**, H9c2 cells were incubated either in PBS for 1 h (Cont.) or in PBS/rotenone (Rot.) for 1, 1.5, or 2 h. Cells were then stained with DHE for an additional 15 min and photographed. In the last panel cells were incubated in PBS/rotenone for 2.5 h and then allowed to recover in complete media supplemented with DHE for 15 min. **B**, H9c2 cells were incubated in PBS (Cont.) or PBS/rotenone (5 μM) for 2.25, 2.5, or 3 h. Cells were then recovered in complete medium for 3 h and stained with PI for 5 min. Three hundred cells were counted, and the fraction of necrosis was determined as the percent of PI positive cells (left y axis). In parallel, (right y axis) H9c2 cells were incubated in PBS/rotenone, stained with DHE at indicated time points, and observed under fluorescent microscope. Enhanced ROS production became visible at 1-h 45-min time point and had not ceased during further incubation in PBS/rotenone medium. The histogram represents an average of three independent experiments. **C**, H9c2 cells were infected with adenovirus expressing either catalase or GFP. 36 h after infection cells were placed either in PBS (control) or PBS/rotenone for 2.5 h and then recovered in complete medium for additional 3 h. Cells were then stained with PI, and percent of necrosis was determined as in Fig. 1B. **D**, H9c2 cells treated as in panel C were stained with TMRM/PI as described under “Experimental Procedures.” Arrows point to PI-positive cells. Three separate fields are shown.
transient ATP depletion. If inhibition of JNK prevents depolarization of mitochondria, H9c2 cells rescued by SP600125 from necrosis would be TMRM-positive and PI-negative. Alternatively, if JNK acts downstream of mitochondria, inhibition of JNK should lead to accumulation of TMRM and PI double negative cells. In the experiment no TMRM and PI double negative cells.
negative cells were detected upon incubation with SP600125 (not shown). Similarly, cells expressing dominant negative JNK1 did not accumulate TMRM and PI double negative cells upon transient energy deprivation and retained a significantly higher proportion of TMRM-positive cells than H9c2 parental control (data not shown). These data indicate that inhibition of JNK activation protects mitochondria from depolarization, suggesting that JNK is an upstream player in the necrotic pathway.

In various cell types, JNK activity under stressful conditions could be down-regulated by the heat shock protein Hsp72, leading to inhibition of both apoptotic and necrotic modes of cell death. Indeed, ectopic expression of Hsp72 protected primary cardiomyocytes and H9c2 cells from transient ischemia or transient energy deprivation (7, 38), and at least with H9c2 cells, this protection was associated with inhibition of JNK (7). To further elucidate the mechanisms of Hsp72-mediated protection from necrosis, H9c2 cells were infected with adenovirus expressing either Hsp72 along with GFP or GFP alone, as a control. Conditions were found that achieve infection of almost 100% of cells. As seen in Fig. 4C, expression of GFP did not significantly change the fraction of necrotic PI-positive H9c2 cells after transient energy deprivation, whereas populations of H9c2 cells expressing Hsp72 showed 50% reduction of necrosis (Fig. 4C). Importantly, when TMRM was added to these cells together with PI, we found that, as with inhibition of JNK, all cells without TMRM staining were PI-positive, whereas all cells with TMRM staining were PI-negative (data not shown). These results indicate that Hsp72 acts upstream of mitochondria and suggest that this inhibition could be associated with inhibition of JNK.

Energy deprivation was shown to cause massive misfolding and aggregation of cytosolic proteins, probably due to malfunction of the chaperone machinery, and protein damage was suggested to be a direct cause of cell necrosis (see Ref. 31). Because protein damage can also lead to activation of JNK (39), Hsp72 may protect cells from transient ATP depletion by protecting cytoplasmic proteins from misfolding and aggregation, which would lead to reduced JNK activation and, thus, protection from necrosis. An alternative possibility is that Hsp72 is directly implicated in the signaling process, and Hsp72-mediated repair of damaged proteins is not critical for protection from necrosis. To address this question, we employed an Hsp72 mutant Hsp72\textsubscript{EEVD}, which does not have a chaperone activity but still can regulate JNK (40). H9c2 cells were infected with adenovirus expressing the Hsp72\textsubscript{EEVD} mutant and exposed to transient energy deprivation. Hsp72\textsubscript{EEVD} provided similar protection from necrosis as normal Hsp72 (Fig. 4C). Of note, normal Hsp72 and Hsp72\textsubscript{EEVD} mutant were expressed at similar levels (data not shown) and efficiently suppressed activation of JNK upon recovery as well as background JNK activity (Fig. 4D). Furthermore, as with normal Hsp72, upon expression of Hsp72\textsubscript{EEVD}, all cells with energized mitochondria were PI-negative, whereas all cells with de-energized mitochondria were PI-positive (data not shown). Thus, expression of the Hsp72\textsubscript{EEVD} mutant also prevents mitochondrial depolarization. These data indicate that Hsp72-mediated protection of H9c2 cells from necrotic death does not depend on Hsp72 chaperone activity. Therefore, the protective function of Hsp72 appears to be related to its role in cell signaling, e.g. in suppression of JNK, rather than to repair of proteins damaged by energy deprivation.

Accumulation of ROS Triggers Necrosis of H9c2 Cells Independent of JNK Activation—Because ROS production at the ATP depletion stage was critical for mitochondrial depolarization and necrosis, the simplest hypothesis was that ROS triggers activation of JNK, which in turn causes mitochondrial collapse, culminating in the breakdown of the plasma membrane and cell necrosis. Therefore, we tested whether ROS production is required for activation of JNK and subsequent cell death. Catalase-expressing cells as well as GFP-expressing control cells were incubated in PBS/rotenone for 2.5 h and transferred to complete medium, and JNK activity was assayed 60 min later. As seen in Fig. 5A JNK was activated to a similar extent in catalase-expressing cells and GFP control. Therefore, we concluded that ROS production was not necessary for JNK activation upon recovery from transient energy deprivation.

We next addressed the possibility that JNK activation during early stages of ATP depletion is a prerequisite for ROS production. H9c2 cells were incubated in PBS/rotenone with or without the JNK inhibitor SP600125, and ROS production was monitored by the appearance of DHE fluorescence. SP600125 did not affect ROS production as DHE fluorescence appeared at the same time and had similar intensity in both control and SP600125-treated cells (not shown). Similarly, no difference in ROS production was observed in H9c2 cells constitutively expressing dominant negative JNK1 as compared with parental control cells (Fig. 5B). Therefore, JNK activation and ROS production represent two independent pathways that trigger mitochondrial membrane depolarization and cell necrosis upon transient energy deprivation.

To test whether the protective effect of Hsp72 is mediated at least in part by suppression of ROS accumulation, we infected H9c2 cells with adenovirus expressing Hsp72 along with GFP or adenovirus expressing GFP alone. Thirty-six hours after infection cells were placed in PBS/rotenone, and ROS production was monitored as above. Fig. 5C shows that ROS accumulation was similar in Hsp72-expressing and control cells. Similarly, overexpression of Hsp72 mutant, Hsp72\textsubscript{EEVD}, which does not have chaperone activity, did not affect ROS production, although it strongly protected cells from necrosis. Therefore, the protective effect of Hsp72 was related to inhibition of JNK activation and not to suppression of ROS production.

Anti-ischemic Compound Trifluoperazine (TFP) Inhibits ROS Production—It was previously reported that various cell types could be protected from injury caused by ischemia/reoxygenation by TFP (41, 42). Necrotic cell death of cardiomyocytes under the conditions of transient energy deprivation has many common features with death stimulated by ischemia/reperfusion. Therefore, we tested whether TFP can protect H9c2 cells from death under these conditions. H9c2 cells were incubated in PBS/rotenone supplemented with TFP and then transferred to complete medium also supplemented with TFP. Two hours later H9c2 cells were stained with PI and observed under the microscope. As seen in Fig. 6A, incubation with TFP led to an ~2-fold reduction in the number of PI-permeable cells as compared with untreated control cells. Importantly, to protect H9c2 cells TFP should be present during the ATP depletion stage of the experiment because when TFP was present only at the recovery stage it failed to protect cells (data not shown). When H9c2 cells were transferred to a complete medium in the presence of both PI and TMRM, we observed that rescued, PI-negative cells had fully energized mitochondria with extensive TMRM staining (data not shown). Therefore, TFP prevented depolarization of mitochondria triggered by transient energy deprivation and protected H9c2 cells from necrotic death.

Because necrotic death of H9c2 under conditions of transient energy deprivation was regulated by two independent pathways, ROS production and JNK activation, we investigated the effect of TFP on these two events. H9c2 cells were placed in PBS/rotenone with TFP for 2.5 h, and JNK activation was assayed 30 and 60 min after cells were transferred to complete medium.
media also supplemented with TFP. As a control, we incubated cells in medium without TFP. JNK was activated to a similar extent when the experiment was performed in the presence or in the absence of TFP (Fig. 6B). To investigate the effects of TFP on ROS production, H9c2 cells were incubated in PBS/rotenone with or without TFP, and ROS accumulation was monitored by DHE fluorescence as above. Fig. 6C shows that in the presence of TFP ROS accumulation was suppressed about 2-fold. Therefore, TFP protected H9c2 cells most likely through suppression of ROS production and not through a JNK-dependent pathway.

The exact mechanism of TFP action is poorly understood. It was shown to cause inhibition of phospholipase A2 and calmodulin (43, 44). Both phospholipase A2 and calmodulin could potentially be implicated in ROS production. To get insights into the pathway of ROS production regulated by TFP we performed transient ATP depletion in the presence of various inhibitors of either calmodulin or phospholipase A2. No protection from necrotic death was observed when transient energy deprivation was performed in the presence of phospholipase A2 inhibitors mepacrine or aristolochic acid (data not shown). Similarly, calmodulin inhibitors, chemically different from TFP,
calmidazolium, or \( N \)-aminobutyl (W13), did not protect cells from necrosis (data not shown). These data strongly suggest that neither inhibition of phospholipase A2 nor calmodulin contributes to TFP-mediated inhibition of ROS production. Interestingly, when transient ATP depletion was performed in the presence of another calmodulin inhibitor, chlorpromazine (CPZ), we observed strong protection of cells from necrosis similar to that conferred by TFP (Fig. 6A). CPZ has high structural similarity to TFP. Therefore, it appears that TFP and CPZ protect H9c2 cells not due to inhibition of either phospholipase A2 or calmodulin but due to a distinct uncharacterized activity, which leads to suppression of ROS accumulation.

**ARC Inhibits Mitochondrial Deenergization and Protects Cells from Necrosis**—Our data imply that mitochondrial depolarization is critical for the necrotic death pathway stimulated by transient energy deprivation. Overexpression of a muscle- and heart-specific protein ARC was previously shown to prevent depolarization of mitochondria and protect cardiac and H9c2 cells from necrosis induced by oxidative stress (25).

Therefore, we investigated whether ARC can prevent depolarization of mitochondria and, thus, protect H9c2 cells from death triggered by transient ATP depletion. For these experiments we utilized the H9c2 cell line stably overexpressing the ARC protein (ARC24). As a control we used H9c2 cells transformed with vector alone, H9c2/Neo (of note, both ARC24 and control cells were constructed in the Ekhterae laboratory, and H9c2/Neo clone was somewhat more sensitive to transient energy deprivation compared with clones used in experiments described above.) As seen in Fig. 7, A and B, 40% of control H9c2/Neo cells undergo necrosis upon a 1-h 45-min incubation in PBS/rotenone followed by a 2-h incubation in complete medium. However, in ARC24 line only 10–15% of cells became necrotic under these conditions. All PI-negative cells had intensive mitochondrial TMRM staining, suggesting that ARC acts upstream of mitochondria in preventing necrotic cell death (data not shown).

To clarify relations between players in the necrotic pathway, we investigated whether ARC could suppress ROS accumulation. ARC24 as well as H9c2/Neo cells were placed in PBS/rotenone, and ROS production was monitored as above. As seen in Fig. 7C, ROS production was even stronger in ARC24 compared with H9c2/Neo control cells. Therefore, ARC acts either downstream of ROS production or is implicated in a parallel pathway that contributes to cell necrosis. It should be noted that ROS production starts earlier in ARC24 and H9c2/Neo parental cell lines from the Ekhterae laboratory than H9c2 cell line from the Sherman laboratory used in all other experiments, so that ROS production peaks at 1 h 30 min after the beginning of ATP depletion. This correlates well with these cells being more sensitive to transient energy deprivation.

ARC also might protect mitochondria from depolarization by interfering with JNK activation. Therefore, we investigated whether ARC-mediated protection of H9c2 cells involves inhibition of JNK. ARC24 cells as well as control cells were incubated in PBS/rotenone medium as described above, and JNK activity was assayed 0.5, 1, and 2 h after transfer into complete media. As seen in Fig. 7D, after transfer of H9c2/Neo control and then transferred to complete medium in the presence or absence of TFP for additional 0.5 or 1 h. JNK activity was assayed as in Fig. 4D. phJNK1 and phJNK2. C, H9c2 cells were placed either in PBS (control) or PBS/rotenone with or without TFP for 2.5 h and then transferred to complete medium in the presence or absence of TFP for additional 0.5 or 1h. JNK activity was assayed as in Fig. 4D. phJNK1 and phJNK2. C, H9c2 cells were placed either in PBS (control) or PBS/rotenone with or without TFP for 2.5 h and then transferred to complete medium in the presence or absence of TFP for additional 0.5 or 1h. JNK activity was assayed as in Fig. 4D. phJNK1 and phJNK2.
cells to complete media, JNK activation reached the peak at the 1-h time point, and by 2 h JNK activity returned to control level. In the ARC24 cell line, background JNK activity was higher than in H9c2/Neo control. When these cells were transferred to complete media, JNK became strongly and temporally activated, and by 2 h JNK activity returned to the initial level. These data show that ARC can prevent depolarization of mitochondria triggered by transient energy deprivation even in the

**Fig. 7.** ARC protects H9c2 cells from necrosis triggered by ATP depletion/repletion independent of JNK activation. **A,** H9c2 cells expressing ARC (ARC24) or parental cell line H9c2/Neo were incubated either in PBS (Cont.) or PBS/rotenone (Rot.; 5 μM) for 1 h 45 min and then were transferred to complete medium for additional 2 h. Cells were then stained with PI and photographed. **B,** experiment was performed as in panel A. Percent of necrosis was determined as in Fig. 1B. The histogram represents an average of three independent experiments. **C,** H9c2 cells expressing ARC (ARC24) or parental cells H9c2/Neo were incubated either in PBS (control) or PBS/rotenone (5 μM) for 1.5 h and ROS production was measured. **D,** H9c2 cells expressing ARC (ARC24) or parental cells H9c2/Neo were incubated either in PBS (control (C)) or PBS/rotenone (5 μM) for 1 h 45 min and then were transferred to complete medium for 0.5, 1, or 2 h. JNK activity was assayed as in Fig. 4D.
presence of active JNK. Therefore, ARC either acts downstream of both JNK and generation of ROS or is involved in a parallel pathway of a control of mitochondrial integrity and necrosis.

**DISCUSSION**

Simulation of ischemia upon incubation of H9c2 cells with the mitochondrial respiratory chain inhibitor, rotenone, leads to rapid depolarization of mitochondrial membranes (Fig. 2A). However, after the removal of rotenone, the treatment that simulates reperfusion, membrane potential is restored within 5–7 min, and mitochondria became TMRM-fluorescent. Interestingly, at later points of ATP repletion, the membrane potential suddenly collapsed in a fraction of cells, and mitochondria rapidly depolarized minutes before the terminal event in necrosis, rupture of the plasma membrane. Therefore, mitochondrial depolarization does not occur because of permanent damage of mitochondria triggered by transient energy deprivation.

Mitochondrial depolarization immediately precedes the loss of plasma membrane integrity, and all manipulations with cellular factors that lead to protection from the loss of plasma membrane integrity prevented depolarization of mitochondrial membranes (see below), suggesting that it is a critical late step in the necrotic pathway. Depolarization of mitochondria may be caused by the opening of the MPT pore. However, under our conditions inhibition of the MPT pore opening with cyclosporin A neither abrogated mitochondrial deenergization nor suppressed the appearance of PI-positive cells. Furthermore, MPT opening often leads to the release of cyt c, which was not detected in our experiments. Therefore, mitochondrial depolarization in our experiments was not mediated by the opening of MPT.

Ca$^{2+}$ influx to cytosol reportedly plays a role in necrotic death of ischemic neurons and some other cell types (45). In fact, we observed a strong increase of cytosolic Ca$^{2+}$ 4–6 min before PI penetrated into cells, which almost coincided with the mitochondrial membrane depolarization (not shown). However, the addition of EGTA to the medium, which abrogated the increase in the cytosolic Ca$^{2+}$, did not affect the rate of appearance of PI-positive cells (not shown). Also, no effect on necrosis was observed when H9c2 cells were incubated with cell-permeable Ca$^{2+}$-chelator, BAPTA-AM (not shown). Therefore, the increase of cytosolic Ca$^{2+}$ was not implicated in necrotic death.

How depolarization of mitochondrial membrane affects the subsequent steps in the necrotic process, leading to rupture of the plasma membrane, remains to be clarified. It is known that upon receiving a death signal mitochondria release a number of pro-apoptotic and pro-necrotic factors, including cyt c, Smac, and AIF (15). Furthermore, AIF was shown to localize to the nucleus and induce cell necrosis by an unknown mechanism (46). We suggest that AIF or some other mitochondrial factor is released from mitochondria upon its depolarization and rapidly induces execution stages of necrosis. Interestingly, inhibition of stress kinase JNK and expression of Hsp72 or ARC as well as detoxification of ROS or the addition of TFP seemed to rescue cells from necrotic death by inhibiting mitochondrial deenergization. Accordingly, these treatments caused dramatic reduction of PI-positive cells. Moreover, we never detected an increase in the number of viable PI-negative cells with depolarized TMRM-negative mitochondria, indicating that in the necrotic pathway, JNK, Hsp72, ARC, and ROS act upstream of mitochondria. Therefore, mitochondrial deenergization appears to be a regulated event controlled by a number of cellular factors.

We have also demonstrated that transient energy deprivation of H9c2 cells triggers production of ROS, which plays a critical role in cell necrosis. The mitochondrial respiratory chain is considered to be one of the major sources of ROS in a cell. ROS are produced at multiple points of the respiratory chain, including NADH dehydrogenase and Q-cycle (47). Because of an excessive reduction of pools of certain components of the chain, e.g. coenzyme Q, electrons could directly be transferred to oxygen in a single electron reaction, leading to production of the superoxide radical (47). Although under certain conditions inhibition of the respiratory chain directly causes production of ROS, under our conditions ROS production was not an immediate consequence of cessation of respiration. In fact, the respiratory chain was inhibited almost instantly after the rotenone addition, as judged by the drop in ATP levels (7), whereas the first signs of ROS production were seen only after 90 min of cell incubation in PBS/rotenone medium (see Fig. 3A). Similar increase in ROS production in response to rotenone was observed in HL-60 cells (48). Most likely this was also a delayed effect because ROS measurements were performed at the 40-min time point after the rotenone addition. It is unclear

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**FIG. 8. Model of necrotic death pathways activated in H9c2 cells upon transient energy deprivation.**
Deenergization of Mitochondria Precedes Necrosis

how prolonged inhibition of the respiratory chain could lead to ROS accumulation. ROS accumulation could result from the gradual decline and eventual failure of ATP-dependent ROS-scavenging systems like glutathione. ROS could be generated in several cellular compartments including mitochondria (47), plasma membrane NADH oxidase (49), or xanthine oxidase (49). Interestingly, although the primary ROS generated in these compartments are superoxide radicals, the ROS that lead to cell necrosis are probably the more stable derivatives of superoxide, \( \text{H}_2\text{O}_2 \). In fact, overexpression of catalase, which converts \( \text{H}_2\text{O}_2 \) to water and molecular oxygen but does not affect superoxide radicals, strongly protected cells from necrosis. Therefore, it appears that \( \text{H}_2\text{O}_2 \) is the critical factor in the necrotic process. Accumulation of ROS in the course of energy deprivation can explain the puzzling observation that the extent of cell necrosis strongly correlates with the length of the ATP depletion period. Findings in this work indicate that ROS production is the critical factor in induction of necrosis and that duration of cell exposure to ROS directly correlates with duration of ATP depletion (Fig. 3B).

Inhibition of ROS production appears to be related to the mechanism of action of TFP compound. In our experiments, TFP caused 2-fold protection of cells from necrosis induced by transient energy deprivation. TFP was reported to inhibit calmodulin (43), but the calmodulin-inhibiting activity of TFP appears to be unrelated to cell protection for two reasons. First, necrosis of H9c2 was not inhibited by depletion of Ca\(^{2+}\) (data not shown). Second, other inhibitors of calmodulin except for a compound that was structurally closely related to TFP, CPZ, did not protect cells (see below). TFP also was reported to inhibit PLA2, which could potentially trigger the rupture of plasma membrane (44). However, other inhibitors of PLA2 were ineffective in cell protection from necrosis. Furthermore, here we showed that TFP acts at the ATP depletion step by reducing the generation of ROS. Therefore, it appears that TFP (and its structural analog CPZ) has a specific activity against ROS production, which is distinct from its action on calmodulin and PLA2. TFP was previously reported to inhibit a cyclosporin A-insensitive mechanism of mitochondrial swelling and depolarization upon treatment with pre-oxidants (44, 50). Our data suggest that this protection could be due to inhibition of ROS production. It would be interesting to test whether in animal models of ischemia TFP also acts by preventing production of ROS.

How JNK regulates the mitochondrial membrane depolarization under these conditions is not clear. JNK activation takes place soon after the removal of rotenone, lasts for 60–90 min, and then subsides. Therefore, JNK activation precedes depolarization of mitochondrial membranes. Accordingly, JNK does not have a direct effect on the depolarization, and there should be some molecular events between JNK activation and mitochondrial depolarization. JNK could affect mitochondria by controlling the Bcl-2 family members, e.g. Bax. However, no Bax re-localization to mitochondria was seen in H9c2 cells undergoing necrosis. Potentially, other member of the Bcl-2 family could be regulated by JNK and control mitochondrial integrity.

Previously we showed that Hsp72 suppresses JNK activation and protects H9c2 cells from death triggered by transient energy deprivation (7). Furthermore, in this work we have demonstrated that Hsp72 prevents mitochondrial depolarization caused by transient energy deprivation. Importantly, Hsp72 does not require its chaperone activity for either prevention of mitochondrial deenergization or for protection from this type of necrosis. It was found in several cell types that energy deprivation leads to accumulation of abnormal proteins and protein aggregation, probably because major chaperones cannot function properly upon these conditions (31). Therefore, one possibility is that Hsp72 protects cells by facilitating refolding of the damaged proteins. However, Hsp72AAEEVD mutant that does not have a chaperone activity is very potent in protection of cells from transient energy deprivation. This is in contrast to heat-shocked cells where this mutant Hsp72 is not able to provide protection from death (13, 40). Importantly, the Hsp72AAEEVD mutant retains its capability to suppress the JNK-signaling pathway. Therefore, it appears that in protection of H9c2 cells from necrosis Hsp72 plays a signaling role.

ROS-mediated necrosis in a manner independent of JNK activity, as inhibition of ROS accumulation with catalase did not affect activation of JNK, and inhibition of JNK by either a pharmacological inhibitor SP600125 or by expression of the dominant-inhibitory form of JNK1 did not affect ROS production (Fig. 5, A and B). These data indicate that the process of cell necrosis is controlled by at least two independent pathways, one involving activation of JNK and another one involving ROS production (see the scheme in Fig. 8). On the other hand each of these treatments protects H9c2 cells from necrosis. The lack of effects of ROS on JNK activity was unexpected because in several models JNK was shown to be activated by the oxidative stress (see e.g. Ref. 51). Furthermore, activation of JNK upon transient energy deprivation is in part due to the inhibition of JNK phosphatases, which are extremely sensitive to the oxidative stress (7). Nevertheless, the lack of effect of catalase on JNK activation indicates that a ROS-independent pathway is involved. Interestingly, there is a clear temporal separation of these two pathways, since ROS is produced at the ATP depletion stage of the experiment and declines within minutes after transfer to complete medium. In contrast JNK is inactive during ATP depletion and becomes activated upon transfer to the complete medium (see the scheme in Fig. 8).

The place of ARC in this model is currently unclear. It protected cells from necrosis induced by transient ATP depletion, which is in line with the previous report that ARC protects H9c2 cells from necrosis induced by \( \text{H}_2\text{O}_2 \) (25). However, ARC did not influence either activation of JNK or production of ROS in our experiments. On the other hand, ARC certainly acts upstream of the mitochondrial membrane depolarization. Previously, it was reported that \( \text{H}_2\text{O}_2 \) causes ARC down-regulation (25). Therefore, it is likely that ARC acts as an intermediate component between ROS production and the mitochondrial collapse. However, since the levels of ARC in our cells are below the detection limit, it was impossible to directly test the possibility that ROS production regulates ARC, which in turn affects mitochondrial deenergization. Importantly, both ROS and JNK acted in the pathway upstream of rapid depolarization of mitochondrial membranes that precedes the plasma membrane rupture. The protective protein Hsp72 appeared to act in the JNK branch of the pathway, since it inhibited JNK but did not affect ROS production. In contrast, TFP prevented production of ROS but did not affect activation of JNK (Fig. 8).

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