The Mitochondrial Apoptotic Pathway Is Activated by Serum and Glucose Deprivation in Cardiac Myocytes

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Abstract—Many cell types undergo apoptosis under conditions of ischemia. Little is known, however, about the molecular pathways that mediate this response. A cellular and biochemical approach to elucidate such signaling pathways was undertaken in primary cultures of cardiac myocytes, a cell type that is especially sensitive to ischemia-induced apoptosis. Deprivation of serum and glucose, components of ischemia in vivo, resulted in myocyte apoptosis, as determined by nuclear fragmentation, internucleosomal cleavage of DNA, and processing of caspase substrates. These manifestations of apoptosis were blocked by zVAD-fmk, a peptide caspase inhibitor, indicating that caspase activity is necessary for the progression of apoptosis in this model. In contrast to control cells, apoptotic myocytes exhibited cytoplasmic accumulation of cytochrome c, indicating release from the mitochondria. Furthermore, both caspase-9 and caspase-3 were processed to their active forms in serum-/glucose-deprived myocytes. Caspase processing, but not cytochrome c release, was inhibited by zVAD-fmk, placing the latter event upstream of caspase activation. This evidence demonstrates that components of ischemia activate the mitochondrial death pathway in cardiac myocytes. (Circ Res. 1999;85:403-414.)

Key Words: apoptosis ■ cysteine proteinase ■ cytochrome c ■ mitochondria ■ ischemia

Myocardial ischemia is a pathological process that results in extensive cell death, a significant portion of which can be attributed to apoptosis. Myocyte apoptosis has been demonstrated in necropsy samples of humans suffering myocardial infarction as well as in rabbit, rat, and mouse models of continuous ischemia or transient ischemia followed by reperfusion. Cultured cardiac myocytes also undergo apoptosis in response to component stimuli of ischemia, such as hypoxia, serum and nutrient deprivation, and metabolic inhibition, with and without restoration of control conditions. The identities of the molecular signaling pathways that mediate ischemia-induced apoptosis are largely unknown, however. Previous experiments have implicated signaling pathways involving p53 and its transcriptional targets, growth factor withdrawal, and TNF and ceramide signaling. These pathways are likely to act in a redundant, overlapping fashion, as evidenced by the observation that abrogation of individual pathways does not prevent myocyte apoptosis during myocardial infarction in vivo.

The caspase family of cysteine proteases serves as the central executors of apoptosis. Caspases are synthesized as proenzymes that are activated by proteolytic processing to form the active large (p20) and small (p10) subunits. Once activated, caspases initiate a cascade of proteolysis that involves further processing/activation of additional caspases, and ultimately, cleavage of specific cellular proteins, such as poly(ADP-ribose) polymerase, lamin, DNA fragmentation factor (DFF)/inhibitor of caspase-activated DNase (ICAD), protein kinase C-δ (PKCδ), and focal adhesion kinase (FAK). Breakdown of these and other substrates leads to the orderly dismantling of the apoptotic cell. It has recently been demonstrated that caspases are processed in cardiac myocytes during continuous ischemia and ischemia-reperfusion in vivo. Significantly, inhibition of caspase activity with a caspase pseudosubstrate blocks myocyte apoptosis in these models. Thus, it appears that caspases are central mediators of myocyte apoptosis during ischemia.

Apoptotic stimuli activate the caspase proteolytic cascade by inducing oligomerization and subsequent autocatalytic processing of a subset of caspases known as the signaling caspases. Oligomerization occurs at distinct cell locations in the context of multiprotein structures, such as the Fas/FADD complex at the plasma membrane or the Apaf-1 complex at the mitochondria. Apaf-1 is an adaptor molecule that recruits and activates caspase-9, but only when simultaneously bound to dATP/ATP and cytochrome c. Therefore, translocation of cytochrome c from the mitochondrial...
intermembrane space into the cytoplasm, where it binds Apaf-1, serves as a trigger for apoptosis.\textsuperscript{27–30} The mechanism by which this process is initiated and executed is not understood, although it is known that antiapoptotic members of the Bcl-2 family block cytochrome c release, whereas the proapoptotic member Bax promotes it.\textsuperscript{28,29,31,32} Furthermore, cytochrome c release can result from changes in mitochondrial membrane permeability after loss of membrane potential (\(\Delta \Psi_{\text{m}}\)).\textsuperscript{33,34} Loss of \(\Delta \Psi_{\text{m}}\), a common, early characteristic of apoptosis, is thought to result from dissipation of the \(\text{H}^+\) gradient after opening of the permeability transition (PT) pore in the inner mitochondrial membrane. Although cytochrome c release can precede and occur independently of decreased \(\Delta \Psi_{\text{m}}\),\textsuperscript{28,29,35} dissipation of \(\Delta \Psi_{\text{m}}\) is sufficient to activate the apoptosis program.\textsuperscript{33,34,36} During ischemia in vivo, myocyte mitochondria exhibit decreased \(\Delta \Psi_{\text{m}}\) and reduced function.\textsuperscript{37} Thus, the mitochondria may serve as a death trigger during ischemia by promoting the release of cytochrome c. Although the mitochondrial/cytochrome c death pathway mediates apoptosis in response to many stimuli,\textsuperscript{38–41} its involvement in ischemia-induced apoptosis has not been demonstrated in any cell system. To address this issue, components of ischemia, namely serum and glucose deprivation, were examined in a cell culture model of cardiac myocyte apoptosis. We find that cytochrome c is released from the mitochondria of all apoptotic myocytes, coinciding with activation of both caspase-9 and caspase-3. Furthermore, cytochrome c release occurs independently of caspase activity. These data provide evidence implicating the caspase-9/Apaf-1 mitochondrial death pathway in myocyte apoptosis and suggest a mechanism by which ischemia leads to cardiac myocyte apoptosis.

### Materials and Methods

For all experiments, chemicals were purchased from Sigma, unless otherwise noted. Tissue culture reagents were obtained from Gibco-BRL unless stated otherwise. Pregnant female rats or 1-day-old neonatal rats were supplied by either Taconic Farms (Germantown, NY) or Charles River Laboratories (Wilmington, MA). All animal experimental protocols were approved by the review board of the Animal Institute of the Albert Einstein College of Medicine.

#### Primary Myocyte Cultures

Primary cultures of neonatal rat cardiac myocytes were prepared as described.\textsuperscript{42} Cells were plated on coated dishes (Falcon Primaria, Becton Dickinson Labware) at a density of 350 cells/mm\textsuperscript{2} in plating medium, which consisted of a 1:1 mixture of DMEM and Ham’s F12 supplemented with 10% defined FBS (HyClone Laboratories, Inc), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.25 \(\mu\)g/mL amphotericin B, and 1 mg/mL BSA. Approximately 24 hours after plating, cardiac myocytes were washed twice with PBS, and treatment medium was added. For controls, treatment medium consisted of RPMI 1640 (containing 11 mmol/L D-glucose), supplemented with 10% FBS, whereas deprivation medium consisted of glucose-free, serum-free RPMI 1640, with or without addition of 1 mmol/L 2-deoxy-D-glucose. In certain experiments, 100 \(\mu\)mol/L \(z\)-VAD-fmk (benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone) or vehicle, DMSO, was added to the medium.

#### Ladder Assays

Adherent and floating cells were collected and lysed in the following (in mmol/L): Tris (pH 8.0) 10, NaCl 100, and EDTA 25, as well as 0.5% SDS and 1.0 mg/mL protease K at either 37°C for 4 hours or overnight at room temperature. DNA was extracted from the digested cells as previously described\textsuperscript{43} and subjected to electrophoresis on 1.4% agarose gels.

#### Immunocytochemistry

Cells on coverslips were fixed in 3.7% formaldehyde, blocked with 10% normal goat serum and 0.4% Triton X-100 in PBS, and incubated with primary antibody diluted in 2% normal goat serum and 0.4% Triton X-100. Mouse monoclonal anti–cytochrome c (Pharmingen) was used at a dilution of 1:500; rabbit polyclonal anti-ventricular myosin light chain 2 antibody (MLC2v; a kind gift of Dr Kenneth Chien, University of California, San Diego), 1:50; and rabbit polyclonal CM1, which recognizes only the processed p20 subunit of activated caspase-3,\textsuperscript{44} 1:500. After washing coverslips with PBS/0.1% Tween-20, cells were incubated with secondary antibody consisting of FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc, diluted 1:75) or Texas Red–conjugated anti-mouse IgG (Molecular Probes, diluted 1:250). Finally, cells were counterstained with the DNA binding dye DAPI. To stain for mitochondria, unfixed cells were incubated with 100 \(\mu\)mol/L MitoTracker Red (Molecular Probes) for 30 minutes at 37°C. After washing and fixation in 3.7% formaldehyde, cells were stained further with anti–cytochrome c antibody as described above, using FITC-conjugated anti-mouse IgG (Molecular Probes) as secondary antibody.

#### Western Blot Analysis

A polyclonal anti–caspase-9 antibody was generated by immunizing rabbits with bacterially expressed, recombinant human caspase-9 (amino acids 139 to 416). The affinity-purified antibody recognized both the unprocessed pro–caspase-9 protein and the processed 35-kDa intermediate (lacking the p10 subunit) on Western blots. It did not cross-react with recombinant caspase-1, -2, -3, -6, -7, -8, or -10 (data not shown).

Western blotting was performed on lysates prepared from serum-/glucose-deprived or control myocytes. Adherent and floating cells were collected and resuspended in 60 mmol/L Tris-Cl (pH 6.8), 1% SDS, 10% glycerol, and 0.7 mol/L \(\beta\)-mercaptoethanol. Samples were boiled and were subjected to electrophoresis on 8% or 13% polyacrylamide gels and then transferred to Immobilon-P nylon membranes (Millipore Corp). After blocking in 5% nonfat milk and 0.05% Tween-20 in PBS, blots were incubated with antibodies to FAK (1:1000, mouse monoclonal antibody, Transduction Laboratories), PKC\(\delta\) (1:2000, rabbit polyclonal antibody, Santa Cruz Bio-technology), or caspase-9 (1:12,000). For analysis of mitochondrial proteins, membranes were blocked in 10% nonfat milk for 2 hours and incubated overnight at 4°C with mouse monoclonal antibodies to denatured cytochrome c (1:500, Pharmingen), cytochrome oxidase IV (COX IV; 0.1 \(\mu\)g/mL, Molecular Probes) or actin (1:500, Sigma). Secondary antibody consisted of a 1:75 000 dilution of horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG (for mitochondrial analysis, Sigma) and was detected with SuperSignal Ultra Chemiluminescent Substrate (Pierce) on Kodak BioMax Light film (Eastman Kodak). For other antibodies, secondary antibody consisted of 1:2000 dilutions of HRP-conjugated anti-rabbit IgG or anti-mouse IgG (Southern Biotechnology Associates, Inc), which was detected by enhanced chemiluminescence (Amersham Life Sciences, Inc).

#### Cellular Fractionation

Cardiac myocytes were washed twice with ice-cold PBS and collected by centrifugation at 200g for 10 minutes at 4°C. The cell pellets were then resuspended in 400 \(\mu\)L of extraction buffer containing (in mmol/L) mannitol 220, sucrose 68, HEPES, (pH 7.4) 20, KCl 50, EGTA 5, MgCl\(_2\) 2, EDTA 1, and DTT 1, as well as protease inhibitors. Cells were homogenized for 40 strokes using a glass Dounce homogenizer and a B pestle. Cell homogenates were then subjected to centrifugation at 14 000g for 30 seconds to pellet nuclei, and supernatants were centrifuged once more at 200 000g for 30 minutes to pellet mitochondria. Pellets were then rehomogenized and reprocessed.

#### Mitochondrial Enzyme Assays

Apolipoprotein B (APOB), a 486-amino acid protein, is synthesized in the endoplasmic reticulum and subsequently transported to the Golgi apparatus and the endoplasmic reticulum. After transport, APOB is secreted into the blood stream, where it binds to the lipid core of very low density lipoprotein (VLDL). This complex is then taken up by the liver, where it is degraded into smaller fragments, which are eventually excreted into the urine. To determine the amount of APOB in the blood, an ELISA assay was performed using anti-APOB antibodies specific for the human protein.

#### Statistical Analysis

For statistical analysis, Student’s t-test was used to determine the significance of differences between experimental and control groups. The results were expressed as mean ± SEM. A value of \(P < 0.05\) was considered statistically significant.
Measurement of Intracellular ATP Content

The intracellular ATP content of control and serum-/glucose-deprived myocytes was measured with the ATP bioluminescent assay kit (Sigma). Cells were lysed directly in somatic-cell ATP-releasing agent, and the lysate was assayed according to the manufacturer’s instructions using a 1:25 dilution of the ATP assay mix (a solution containing the firefly luciferase protein and luciferin). Light emitted was measured using the Monolight luminometer (model 2010, Analytical Luminescence Laboratory). ATP content was calculated by comparison with a standard curve derived from known concentrations of ATP, ranging from 0.01 to 10 pmol.

Mitochondrial Reductase Activity

Cellular reductase activity of live cultured myocytes was determined by measuring the reduction of MTT. At each end point, treatment medium was replaced with fresh, serum-free medium (with glucose for controls, with 1 mmol/L 2-deoxyglucose for serum-/glucose-deprived samples) containing 2.4 mmol/L MTT at pH 7.4. Cells were incubated with MTT medium for 1 hour at 37°C with occasional mixing. After solubilization in N-propanol (FisherBiotech), absorbance was measured at 560 nm using the UltraSpec III UV/visible spectrophotometer (Pharmacia Biotech, Inc).

Measurement of ΔΨm

Mitochondrial membrane potential was assessed using both JC-1 and rhodamine 123 staining. After treatment in control or deprivation medium, cardiac myocytes on coverslips were incubated in serum-free medium containing 10 μmol/L JC-1 (Molecular Probes) or 25 μmol/L rhodamine 123 at 37°C for 5 minutes (JC-1) or 10 minutes (rhodamine 123). During this step, the glucose and deoxyglucose contents of the medium were maintained as during the treatment period. After staining, cultures were washed twice with the original treatment medium. Coverslips were then removed from culture dishes and inverted onto a slide. The live (unfixed) cardiac myocytes were viewed as follows: for JC-1, excitation at 450 to 490 nm with sampling at 520 nm, and for rhodamine 123, excitation at 546 nm with sampling at 590 nm. As a positive control for loss of ΔΨm, cells were treated with 50 mmol/L sodium cyanide and 62.5 μg/mL oligomycin (mixture of oligomycin A, B, and C) at 37°C for 30 minutes.

Figure 1. Serum/glucose deprivation-induced changes in cardiac myocyte morphology. Shown are phase-contrast views of cardiac myocytes cultured for 24 hours in control (A) or serum-free, glucose-free medium containing 1 mmol/L 2-deoxyglucose (B). Note the contracted morphology of the remaining deprived cells. Rounded, floating cells can be seen above the plane of focus.

Figure 2. A and B, Time course of serum/glucose deprivation–induced internucleosomal DNA fragmentation. Genomic DNA was isolated from cardiac myocytes cultured under control or deprivation conditions for the indicated times and subjected to gel electrophoresis. A, Medium lacked serum and glucose and contained 1 mmol/L 2-deoxyglucose. B, No 2-deoxyglucose was added. C, Effect of zVAD-fmk on internucleosomal DNA fragmentation. Total cardiac DNA was isolated from cardiac myocytes that had been cultured for 30 hours in control medium or serum-free, glucose-free medium containing 1 mmol/L 2-deoxyglucose in the presence of zVAD-fmk or vehicle alone. 1 Kb indicates DNA molecular weight marker. Similar results were obtained in 2 additional independent myocyte preparations.
Statistical Analysis
For ATP and MTT studies, triplicate samples were examined at each of 6 time points. Results were subjected to ANOVA and Tukey post hoc statistical analysis with differences considered significant at $P < 0.05$. Data are reported as the mean ± SD.

Results
Deprivation of Serum and Glucose Induces Apoptosis in Cultured Cardiac Myocytes
Ischemia is a complex physiological process, in which multiple changes contribute to cellular death. Among these are deprivation of nutrients, growth, and survival factors. To study the effects of these component stimuli on myocyte apoptosis, primary neonatal rat cardiac myocytes were cultured in the absence of serum and glucose. To further limit glucose metabolism, the nonmetabolizable glucose analogue 2-deoxy-D-glucose (1 mmol/L) was added to the medium in some experiments. Myocyte contraction, an ATP-dependent process, was not observed after 4 hours of treatment. By 6 to 15 hours, myocytes exhibited a shriveled, constricted shape. Many round, floating cells were observed, and by 24 hours, there were few adherent cells remaining (Figure 1B). In contrast, myocytes incubated in control medium containing glucose and serum were confluent and well spread, with flattened morphology (Figure 1A), and beat rapidly in synchrony. The morphological changes observed were suggestive of apoptosis. In fact, internucleosomal DNA fragmentation was observed in the deprived, but not control, plates, at 24 and 30 hours (Figure 2A). Serum and glucose deprivation without addition of 2-deoxyglucose also induced apoptosis, but the process was much slower; apoptotic DNA ladders were first evident at 48 to 60 hours (Figure 2B). Serum deprivation alone did not induce apoptosis in the time course examined (data not shown), although it has been reported to cause myocyte death at later time points.12,13 Likewise, addition of 1 mmol/L 2-deoxyglucose to control medium or to serum-containing glucose-free medium had no effect on myocyte morphology (data not shown). This may be due to the presence of glucose or other energy sources in serum. Alternatively, survival factors present in serum may actively suppress the apoptotic signal.

Apoptosis was confirmed on a cell-by-cell level by examining cellular and nuclear morphology with an antibody to the sarcomeric protein MLC2v and the DNA binding dye DAPI, respectively. Control myocytes were flat and striated, with large, round nuclei (Figure 3A and 3B). This contrasted with the serum-/glucose-deprived cells, which were shrunken and lacked striations, suggestive of sarcomeric breakdown (Figure 3D). Three to six percent of myocytes that were still adherent to the plate exhibited fragmented nuclei with condensed chromatin, a clear indication of apoptosis (Figure 3C). This quantity underestimates the total extent of death, how-
ever, because the vast majority of cells had detached from the culture plate (see below).

Myocytes with nuclei in earlier stages of fragmentation, including margination of DNA at the nuclear periphery, were observed in medium containing #0.1 mmol/L 2-deoxyglucose (Figure 3C, inset). Such nuclei were seen only rarely in the presence of 1 mmol/L 2-deoxyglucose. This is consistent with the kinetics of cell death being more rapid in the presence of high concentrations of the metabolic inhibitor, such that intermediate stages of death were less likely to be observed.

Caspases Mediate Cardiac Myocyte Apoptosis During Serum/Glucose Deprivation

Proteolysis of caspase substrates provides a marker for apoptosis in general and caspase activity in particular. To determine whether caspases were activated in serum-/glucose-deprived myocytes, Western blot analysis of caspase substrates PKCδ and FAK was performed. In fact, both substrates were proteolyzed in a time-dependent manner after deprivation (Figure 4A). By 13 hours, both FAK (120 to 125 kDa) and PKCδ (80 kDa) were processed to their predicted caspase cleavage products of 77 to 85 kDa and 40 kDa, respectively.47,48 By 24 hours, the majority of the precursor proteins was proteolyzed. In control cells, a low level of processing was observed; this may reflect basal caspase activity or nonspecific proteolysis at hypersensitive sites during preparation of the cell lysates.

To determine whether caspase activity was necessary for the progression of myocyte apoptosis during ischemia, cardiac myocytes were subjected to serum/glucose deprivation in the presence of either the caspase pseudosubstrate inhibitor zVAD-fmk or vehicle alone. The caspase inhibitor completely blocked internucleosomal DNA fragmentation in serum-/glucose-deprived cells (Figure 2C). Consistent with the absence of DNA ladders, zVAD-fmk prevented nuclear fragmentation as well (see Figure 9D) and preserved sarcomeric staining (data not shown). Inhibition of caspase activity also blocked proteolysis of caspase substrates FAK and PKCδ (Figure 4B). Thus, caspases are necessary for the execution of myocyte apoptosis during serum/glucose deprivation. Despite the prevention of the terminal features of apoptosis, however, cellular morphology and contractile capability of the remaining adherent cells did not improve to normal (data not shown), unless glucose and serum were restored. This suggests that energy depletion or other caspase-independent events may also contribute to cellular damage in this model.

The Mitochondrial Death Pathway Is Activated in Apoptotic Myocytes

Treatment of myocytes with serum-free, glucose-free medium containing 2-deoxyglucose resulted in a decrease in cellular ATP levels within the first 2 hours, which ultimately declined to levels that were ≈30% of control cells (Figure 5A). This reflects the absence of the glycolytic production of ATP, as well as the limited redox potential of the mitochondria that results from the reduced supply of substrates (eg, pyruvate) for the oxidative respiratory pathway. In fact, an impairment in mitochondrial redox activity was apparent...
soon after initiation of the stimulus, as assessed by the conversion of the tetrazolium dye MTT to its reduced form, a reaction mediated by mitochondrial reductases.\textsuperscript{44} MTT reductase activity fell precipitously to 45% of control levels within 4 hours of treatment (Figure 5B). It remained steady at \approx 40% for the next 5 hours. These changes occurred before any signs of cell death were observed, indicating that the decrease in metabolism and the consequent fall in energy production was not a result of the loss of live cells, but was rather due to a metabolic impairment within a population that was still viable.

In light of these changes in mitochondrial function, we assessed whether there was any decrease in mitochondrial membrane potential using the potential-sensitive dye JC-1. Myocytes exhibited loss of $\Delta \Psi_m$ after a 6-hour incubation in medium lacking glucose and serum and containing 2-deoxyglucose (Figure 5C). Loss of membrane potential persisted at 18 hours (data not shown). In contrast, no change in $\Delta \Psi_m$ was detectable after 2 hours of deprivation conditions (data not shown). Similar results were obtained with rhodamine 123 staining (data not shown). Thus, loss of $\Delta \Psi_m$ lagged slightly behind changes in cellular ATP content and MTT reductase activity.

Because dissipation of the mitochondrial potential can be sufficient to activate the apoptotic pathway,\textsuperscript{33,34,36} we hypothesized that the mitochondrial impairments observed here might lead to the induction of apoptosis through the release of cytochrome $c$. To test this hypothesis, the subcellular localization of cytochrome $c$ was determined by Western blot analysis of fractionated cell lysates (Figure 6). Cytochrome $c$ was present exclusively in the insoluble, membrane fraction of control myocytes (compare lanes 1 and 3). In contrast, after 18 hours of serum and glucose deprivation, cytochrome $c$ was observed in both the membrane (lane 2) and cytosolic (lane 4)
The appearance of cytochrome c in the cytosol was not due to contamination of the cytosolic fraction with mitochondria, as the mitochondrial membrane protein COX IV was found exclusively in the membrane fraction (compare lanes 1 and 2 with 3 and 4). To confirm translocation of cytochrome c to the cytosol in individual apoptotic cells, immunostaining was performed (Figure 7). Control cells with normal nuclear morphology (Figure 7A) exhibited a wispy, punctate, subcytoplasmic pattern of cytochrome c immunostaining (Figure 7C), which coincided with a marker for mitochondria, MitoTracker Red (Figure 7G through 7I). In contrast, serum-/glucose-deprived myocytes stained positively with the CM1 antibody, which recognizes in apoptotic cells. The higher proportion of actin in both membrane fractions (lanes 1 and 2) compared with cytosolic fractions (lanes 3 and 4) reflects incomplete disruption of the cytoskeleton and/or the presence of insoluble polymerized actin.

Caspase-9 was processed to its active 18-kDa form (Figure 8A), and Western blot analysis to determine whether the caspase was proteolytically processed (Figure 8A). In control lanes, the full-length caspase-9 precursor was observed at ~46 kDa. Only minimal processing was observed, which may reflect basal activation of the caspase, or more likely, autocatalysis of aggregated protein after cell lysis. No differences were observed after 13 hours of serum/glucose deprivation. We cannot exclude the possibility, however, that undetectable levels of processed caspase-9 enzyme are generated that are sufficient to activate the proteolytic cascade. In fact, caspase substrates are already proteolyzed at this time (Figure 4A), which suggests that some upstream signaling caspase is active at 13 hours. By 18 hours, however, the 46-kDa caspase-9 precursor was converted to a ~35-kDa fragment, which represents the first processing intermediate (removal of p10) during caspase-9 activation. An additional band of 30 kDa was also observed; this most likely reflects removal of the prodomain. The abundance of the caspase-9 precursor and the intermediates substantially decreased by 24 hours, presumably because of further processing to the final p10 and p20 subunits, which were not detectable with this antibody.

Thus, caspase-9 is activated by serum/glucose deprivation in cardiac myocytes.

**Cytochrome C Release Occurs Independently of Caspase Activity**

Cytochrome c release from the mitochondria can occur through caspase-dependent or independent pathways. We therefore investigated the effects of inhibiting caspase activity on the mitochondrial pathway. As stated previously, treatment of serum-/glucose-deprived cells with the caspase inhibitor zVAD-fmk completely blocked nuclear fragmentation (Figure 9D). Furthermore, no CM1 staining was observed (Figure 9E), denoting inhibition of caspase-3 processing. In addition, proteolysis of caspase-9 was inhibited (Figure 8B), although this was not complete. Perhaps zVAD-fmk is not capable of completely blocking caspase-9 autocatalysis on cytochrome c release, even though it is an effective inhibitor of further caspase activity. Similar results have been reported previously with the viral caspase
inhibitor CrmA, which blocked Fas-induced apoptosis and caspase-8 activity, but not caspase-8 processing. Most notably, however, cytochrome \( c \) release from the mitochondria was not blocked (Figure 9F, cell on right). In fact, \( \sim 40\% \) of myocytes cultured in medium lacking serum and glucose but containing 2-deoxyglucose and zVAD-fmk exhibited diffuse, cytoplasmic cytochrome \( c \) immunostaining in the absence of caspase-3 activation or nuclear fragmentation. This suggests that at least 40% of the population had initiated the apoptotic program, which would have advanced to death.

Figure 7. Correlation of cytochrome \( c \) translocation, caspase-3 activation, and apoptosis in serum-/glucose-deprived cardiac myocytes. Cells that had been cultured in control medium (A through C) or serum-free, glucose-free medium containing 1 mmol/L 2-deoxyglucose (D through F) for 18 hours were costained with DAPI (A and D); CM1, an antibody against activated caspase-3 (B and E); and anti–cytochrome \( c \) antibody (C and F). Note the presence of processed caspase-3 (E) in the apoptotic cell (D). In addition, contrast the diffuse staining of cytochrome \( c \) in this same cell (F) with the punctate appearance in the control cell (C). This punctate staining is consistent with mitochondrial localization, as evidenced by panels G through I, which show, respectively, staining of control cells with the mitochondrial marker MitoTracker Red, anti–cytochrome \( c \) antibody (FITC), and the superimposition of the 2 (yellow). J and K, Cells cultured in serum-free, glucose-free medium with no 2-deoxyglucose for 36 hours and stained with DAPI (J) and CM1 (K), respectively. Note differences in the pattern of activated caspase-3 staining here under milder conditions, compared with panel E. L, Nutrient-deprived myocytes stained with the secondary antibody FITC-conjugated anti-rabbit IgG alone to control for background fluorescence.
had caspases been activated. Thus, cytochrome c translocation in this model occurs through a caspase-independent mechanism.

**Discussion**

Serum and glucose deprivation, in combination with the addition of 2-deoxyglucose, induces time-dependent apoptosis in cultured neonatal rat cardiac myocytes, as evidenced by changes in nuclear morphology, internucleosomal DNA fragmentation, caspase activation, and cleavage of caspase substrates. Caspase activation in this model occurs, at least in part, via the mitochondrial death pathway, as evidenced by the translocation of cytochrome c from the mitochondria to the cytosol and the activation of both caspase-9 and caspase-3. Moreover, the release of cytochrome c occurs independently of caspase activity. The apoptotic stimulus used in this model involves several components of ischemia in vivo, including deficiency of survival and growth factors and some nutrients. Other important aspects of ischemia, such as hypoxia, were not examined. However, the sufficiency of the component stimuli to activate the caspase-9/Apaf-1 mitochondrial death pathway in vitro suggests that this pathway mediates ischemia-induced apoptosis in vivo as well.

When cells were cultured in serum-glucose-free medium containing 2-deoxyglucose, activated caspase-3 was localized throughout the cytosol. In contrast, when 2-deoxyglucose was omitted, processed caspase-3 was observed at specific subcellular locations, namely the nucleus and periphery of the cell. One interpretation of these observations is that the less severe inhibition of glycolysis resulting from omission of 2-deoxyglucose might involve only a subset of those caspases activated by the more severe stimulus. Alternatively, the absence of 2-deoxyglucose may slow the kinetics of apoptosis, allowing the identification of intermediate stages, such as the initial activation of specific subcellular pools of caspases.

A previous study using confocal microscopy and immunoelectron microscopy concluded that pro-caspase-3 is located in both the cytoplasm and the mitochondrial intermembrane space, and apoptotic stimuli led to the activation of the mitochondrial pool. The disparity between the pattern of activation observed in the aforementioned study and the current report may reflect differences in cell type or death stimulus. More generally, however, the observations in both studies suggest that there are distinct sites at which pools of inactive procaspases reside and become activated when the cell receives an apoptotic stimulus. These sites might represent locations where effector caspases are targeted to ensure contact with either upstream signaling caspases or downstream substrates. Studies that identify these compartments as well as proteins that colocalize with activated caspase-3 should be informative in this regard.

Inhibition of caspase activity by administration of zVAD-fmk blocked processing of caspases and their substrates, nuclear fragmentation, and DNA cleavage. In contrast, translocation of cytochrome c from the mitochondria occurred independently of caspase activation during serum/glucose deprivation, consistent with a direct link between the apoptotic signals and the mitochondria. In this respect, serum/glucose deprivation resembles apoptotic stimuli such as UV irradiation, staurosporine, and chemotherapeutic drugs, in which cytochrome c release occurs independently and upstream of caspase activation. It contrasts, however, with the recently described caspase-dependent pathway leading to cytochrome c release that occurs during Fas- and TNF-mediated apoptosis, which involves caspase-8-mediated cleavage of Bid and subsequent translocation of truncated Bid to the mitochondria.

The mechanisms by which serum/glucose deprivation leads to cytochrome c release are not known. Possibilities include withdrawal of survival factors, which have been shown in other cell types to provide antiapoptotic signals through Akt-mediated phosphorylation of the proapoptotic Bcl-2 family member Bad, a Bcl-2/Xdimerization partner. When phosphorylated, Bad is sequestered away from Bcl-2/Xdimerization partner. Furthermore, caspase-9 is also phosphorylated by Akt, rendering it catalytically inactive. Thus, serum withdrawal in the cardiac myocyte model may activate a death signal through elimination of such survival signals.

Whatever upstream pathways transduce the serum/glucose deprivation signal to the mitochondria, changes intrinsic to the mitochondria are likely to ultimately mediate cytochrome c release. We observed a loss of $\Delta \Psi_m$ beginning 6 hours after the onset of serum/glucose deprivation and continuing to at

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**Figure 8.** A, Time course of caspase-9 processing during serum/glucose deprivation. Western blotting for caspase-9 was performed on lysates prepared from myocytes incubated in control medium or serum-free, glucose-free medium containing 1 mmol/L 2-deoxyglucose for 13 to 24 hours. B, Effect of zVAD-fmk on caspase-9 processing. Cells were incubated for 24 hours in control or deprivation medium in the presence (+) or absence (−) of zVAD-fmk. Proteins were resolved on 13% polyacrylamide gels that were reacted with an anti–caspase-9 antibody that detects both the 46-kDa precursor (arrow) and a 35-kDa processing intermediate (arrowhead). Asterisk denotes proteolytic band at 30 kDa that may represent another processing intermediate.
least 18 hours. Although loss of $\Delta\Psi_m$ is not necessary for cytochrome c release in some systems, it is often sufficient for cytochrome c release to occur. Therefore, it is possible that loss of $\Delta\Psi_m$ contributes to cytochrome c release in cardiac myocytes. Loss of $\Delta\Psi_m$ is indicative of the opening of large, nonselective channels in the inner membrane known as the PT pore. It has been previously proposed that opening of the PT pore would allow the entry of solutes and water into the mitochondrial matrix, thereby causing swelling of the matrix. Because of the greater surface area of the inner membrane, this swelling would, in turn, cause rupture of the outer mitochondrial membrane, liberating proteins that reside in the intermembrane space, including cytochrome c. Additional experiments are needed to determine whether loss of $\Delta\Psi_m$ causes the initial release of cytochrome c from cardiac myocytes or merely amplifies the release once initiated by other mechanisms.

The data presented here strongly indicate that apoptosis occurs after serum/glucose deprivation. This does not exclude the possibility, however, that necrosis also occurs. In fact, in our model, it is difficult to distinguish between necrosis and apoptosis, because most of the apoptotic deaths are late events in which cellular membrane integrity is eventually lost in the absence of phagocytosis. Nevertheless, the presence of apoptotic myocytes in our model is in distinct contrast to a previous in vitro model of ischemia in which the majority of myocyte death that ensued was attributed to necrosis. The ischemic stimulus used in that study involved deprivation of glucose, serum, and oxygen, conditions that inhibit both glycolysis and oxidative respiration. As a result, ATP would be more severely exhausted compared with our model in which oxygen was maintained. Under the more stringent conditions, apoptosis, an ATP-dependent process, would not be favored. In fact, apoptosis is blocked in many cell types by severe depletion of ATP and is even converted to necrosis in some circumstances. Paradoxically, although ATP is required for apoptosis, mild ATP depletion, such as that occurring after blockade of only one component of the ATP production scheme, actually induces or enhances apoptosis. This may contribute to the activation of apoptosis in our model, as well as in a second cultured myocyte model of ischemia, in which oxidative respiration, but not glycolysis, was inhibited. It remains to be determined whether mild ATP depletion itself is a primary direct activator of the cytochrome c pathway.

Because apoptotic and necrotic stimuli both lead to mitochondrial damage, this organelle appears to be a point of convergence of the pathways that mediate these morphologically distinct forms of cell death. It is possible, therefore, that necrotic stimuli also lead to the release of cytochrome c from damaged mitochondria. Whether such release can successfully activate the remainder of the pathway leading to caspase activation under conditions of severe ATP depletion is unclear, however. Even if the Apaf-1/caspase-9 pathway were activated, it is unknown whether it mediates downstream necrotic events or merely exists as an “accidental” consequence that plays no role in the pathogenesis of necrotic death. The sorting out of these issues will shed light on the mechanism of necrosis and, in so doing, may call into question the notion that necrosis and apoptosis are completely distinct processes.

In conclusion, the experiments presented here provide evidence for the involvement of the mitochondrial signaling pathway in mediating apoptosis in response to components of ischemia in cardiac myocytes. Elucidation of the details by which this pathway is activated and regulated in cardiac myocytes may suggest novel strategies for the treatment of ischemic heart disease.

Note Added in Proof
Malhotra and Brosius recently showed that hypoxia and glucose deprivation induce the release of cytochrome c from mitochondria of neonatal cardiac myocytes.
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References


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