Important role of energy-dependent mitochondrial pathways in cultured rat cardiac myocyte apoptosis

JUN SHIRAISHI, TETSUYA TATSUMI, NATSUYA KEIRA, KAZUKO AKASHI, AKIKO MANO, SATOSHI YAMANAKA, SATOAKI MATOBA, JUN ASAYAMA, TAKESHI YAOI, SHINJI FUSHIKI, HENRY FLISS, and MASAO NAKAGAWA. Important role of energy-dependent mitochondrial pathways in cultured rat cardiac myocyte apoptosis. Am J Physiol Heart Circ Physiol 281: H1637–H1647, 2001.—Recent studies have suggested that apoptosis and necrosis share common features in their signaling pathway and that apoptosis requires intracellular ATP for its mitochondrial/apoptotic protease-activating factor-1 suicide cascade. The present study was, therefore, designed to examine the role of intracellular energy levels in determining the form of cell death in cardiac myocytes. Neonatal rat cardiac myocytes were first incubated for 1 h in glucose-free medium containing oligomycin to achieve metabolic inhibition. The cells were then incubated for another 4 h in similar medium containing staurosporine and graded concentrations of glucose to manipulate intracellular ATP levels. Under ATP-depleting conditions, the cell death caused by staurosporine was primarily necrotic, as determined by creatine kinase release and nuclear staining with ethidium homodimer-1. However, under ATP-replenishing conditions, staurosporine increased the percentage of apoptotic cells, as determined by nuclear morphology and DNA fragmentation. Caspase-3 activation by staurosporine was also ATP dependent. However, loss of mitochondrial transmembrane potential (∆Ψm), Bax translocation, and cytochrome c release were observed in both apoptotic and necrotic cells. Moreover, cyclosporin A, an inhibitor of mitochondrial permeability transition, attenuated staurosporine-induced apoptosis and necrosis through the inhibition of ∆Ψm, reduction, cytochrome c release, and caspase-3 activation. Our data therefore suggest that staurosporine induces cell demise through a mitochondrial death signaling pathway and that the presence of intracellular ATP favors a shift from necrosis to apoptosis through caspase activation.

cytoskeleton; mitochondria; necrosis; staurosporine

APOTOPSIS IS A GENETICALLY determined form of cell death, which can be triggered by a number of physiologial and pathological conditions. Apoptosis is characterized by condensation, margination, and degrada
tion of chromatin, as well as cytoskeletal disruption, cell shrinkage, and membrane blebbing. Ultimately, the cell is fragmented into membrane-enclosed apoptotic bodies (4, 50). In contrast, necrosis is a passive process characterized morphologically by mitochondrial swelling and the loss of plasma membrane integrity without concomitant severe damage to the nuclei (3). Apoptosis and necrosis are therefore considered to be conceptually and morphologically distinct forms of cell death. However, recent reports (1, 21, 22, 40) have indicated that these two processes can occur simultaneously in tissues or cell cultures exposed to the same stimulus.

It is well documented (6, 18) that myocardial ischemia triggers cardiac cell death, which may possess the properties of both apoptosis and necrosis. Studies from our laboratory and studies by others have demonstrated that myocardial apoptosis is observed in the early phases of ischemic injury, whereas necrosis appears in the later stages. Moreover, the data also suggest that apoptotic myocytes are generally absent from profoundly ischemic regions of the myocardium but are more prevalent in moderately perfused regions such as the hypoperfused border zone between the central infarct area and the noninjured myocardial tissue and that apoptotic death of ischemic myocytes may be hastened by reperfusion (10, 11, 37, 39, 47). These data therefore suggest that intracellular energy production may be required to fuel the apoptotic machinery in ischemically injured myocytes. It was recently reported (9, 23) that intracellular ATP levels are a determinant of cell death modes in actively proliferating cells (i.e., Jurkat and HeLa). However, it is still uncertain whether the intracellular energy levels also regulate the manifestation of cell death in postmitotic cells such as cardiac myocytes and whether the mitochondrial permeability transition (PT) is an important step in the induction of myocyte death similarly to those used in mitotic cells.
The aim of the present study was, therefore, to investigate the role of intracellular energy levels in determining whether injured cardiac myocytes die by apoptosis or necrosis through the mitochondrial death signaling pathway. For the injury agent, we selected staurosporine, a protein kinase inhibitor and a well-established inducer of apoptosis that causes the release of mitochondrial cytochrome c into the cytosol (49). To examine the role of energy status in myocyte cell death, we first blocked both mitochondrial ATPase with oligomycin and glycolysis with glucose-free medium and then manipulated the level of intracellular ATP by incubating the staurosporine-injured cells in a medium containing different concentrations of glucose. We determined the type of cell death by histochemical and biochemical methods. We also assessed the time course of mitochondrial transmembrane potential (ΔΨₘ), Bax translocation from the cytosol to the mitochondria, and cytochrome c release from the mitochondria into the cytosol. In addition, we investigated whether cyclosporin A, an inhibitor of mitochondrial PT (54), suppresses staurosporine-induced loss of ΔΨₘ, Bax translocation, cytochrome c release, and cell death. Finally, we examined whether the activation of caspase-3, an effector caspase (28, 36), is also dependent on energy levels in the myocytes.

MATERIALS AND METHODS

Chemicals

Staurosporine was a generous gift from Kyowa Medex. Oligomycin was purchased from Nacalai Tesque. Acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) and cyclosporin A were from Biomol and Sigma, respectively. Stock solutions of staurosporine and DEVD-CHO were prepared in dimethyl sulfoxide (DMSO). Oligomycin and cyclosporin A were dissolved in ethanol. The final concentration of ethanol and DMSO was 0.1% (vol/vol). Stock solutions were stored at −80°C.

Neonatal Rat Cardiac Myocytes

Primary cultures of neonatal rat cardiac myocytes were prepared as previously described with some modifications (29). Briefly, 1- to 2-day-old Wistar rats were anesthetized with ether, and the hearts were removed under aseptic conditions and placed in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS). The atria and aorta were discarded, and the ventricles were minced into 1- to 3-mm³ fragments and were enzymatically digested four times for 10–15 min each with 7.5 ml of PBS containing 0.2% type I collagenase (Sigma). Cell suspensions from each digestion were pooled, centrifuged at 300 g for 5 min, and resuspended in HEPES buffer containing (in mM) 116 NaCl, 5.4 KCl, 0.8 MgSO₄, 1.0 NaH₂PO₄, 20 HEPES, and 5.5 glucose, with a pH of 7.35. The density 1.059/1.082) and were centrifuged at 1,000 at 37°C in a humidified atmosphere containing 5% CO₂/95% room air. Bromodeoxyuridine (BrdU; 100 μM) was added during the first 48 h to inhibit proliferation of nonmyocytes. The myocytes were then incubated in DMEM containing 0.5% FBS without BrdU, and all experiments were done 36–48 h after this incubation. By using this approach, we routinely obtained contractile cultures with >95% myocytes, as assessed by immunofluorescence staining with a monoclonal antibody against β-myosin heavy chain (29). Desmin staining was also used to distinguish cardiac myocytes from nonmyocytes as described previously (2). Cardiac myocytes on type I collagen-coated coverslips were fixed with 2% paraformaldehyde at 0°C, permeabilized with ice-cold methanol-acetone (1:1, vol/vol), blocked with 10% heat-inactivated goat serum, and sequentially incubated with anti-desmin polyclonal antibody [1:50 dilution in 1% bovine serum albumin (BSA)-buffered PBS, Monosan], fluorescein isothiocyanate-labeled goat anti-rabbit IgG (ab’)(1:100 dilution in 1% BSA-buffered PBS, ICN) and 0.5 μg/ml Hoechst 33258. The cells were visualized using fluorescence microscopy, and the images were generated using dual-exposure photography.

Experimental Protocols

Cell cultures were washed twice with PBS before the start of metabolic inhibition (MI), which was achieved by incubating the myocytes with 0.05 μM oligomycin in glucose-free DMEM (GIBCO-BRL), pH 7.4, at 37°C for 1 h. The myocytes were then washed twice with PBS and were incubated for another 4 h in DMEM containing 10 (MI10 group), 30 (MI30 group), 50 (MI50 group), or 100 (MI100 group) mg/dl of glucose, without oligomycin, to manipulate the intracellular ATP content. Staurosporine (1 μM) was added at the start of this 4-h incubation period to induce cell death. Myocytes that were treated with staurosporine and 100 mg/dl glucose for 4 h but without the prior MI were designated as the MI(−) group. Control myocytes were incubated in DMEM containing 100 mg/dl glucose but were not subjected to either MI or staurosporine treatment (control group). To examine whether staurosporine induces either apoptosis or necrosis through the mitochondrial PT, myocytes were treated with cyclosporin A (0.2 μM) 1 h before exposure to staurosporine and then were treated with staurosporine in the presence of cyclosporin A.

Measurement of ATP Content

The ATP content of myocytes was measured before, immediately after, or 2 and 4 h after MI without staurosporine. Cardiac myocytes (0.96 × 10⁶ cells/cm²) were treated with 0.25 ml of 0.6 N ice-cold perchloric acid and centrifuged at 1,000 g for 5 min at 4°C. The supernatant was neutralized with KOH to pH 5.0–7.0 and, after 10 min, was centrifuged at 8,000 g for 5 min at 4°C to remove the KClO₄. The supernatant was used for the assays. ATP was measured by high-performance liquid chromatography (LC-9A liquid chromatograph, Shimadzu) with a column of STR ODS-M (Shimadzu) (45). The protein content of the samples was determined in the acid precipitated by the method of Lowry, using BSA as standard.

Histochemical Determination of Cell Viability and Apoptosis

Living and dead cells were distinguished by using the Viability/Cytotoxicity Kit (Molecular Probes) (12). Myocytes were grown on type I collagen-coated glass coverslips. The culture medium was replaced with 2 μM calcein-acetoxy-
methylthionium and 4 μM ethidium homodimer-1, and the cells were incubated for 45 min at room temperature. Cells with permeabilized membranes (indicating the up ethidium homodimer-1 and their nuclei fluoresce red, whereas viable cells with intact membranes show green fluorescence. The number of viable and necrotic cells in 10 random microscopic fields was counted in each coverslip, and the percentage of viable cells was calculated. Apoptotic cells were identified by the distinctive condensed or fragmented nuclear morphology in cells stained with Hoechst 33258. An average of 800–1,000 nuclei from random fields were analyzed for each experiment, and apoptotic cell counts were expressed as a percentage of the total number of nuclei counted.

**Creatine Kinase Release**

Creatine kinase (CK) activity in culture media was measured spectrophotometrically as an index of necrotic cell death after the 4-h incubation with staurosporine, according to Rosalki's procedure. [CK activity was expressed as IU·1⁻¹·mg protein⁻¹ (29).]

**Agarose Gel Electrophoresis**

Double-stranded DNA breaks were assessed by agarose gel electrophoresis of low-molecular-weight genomic DNA from myocytes (14). Briefly, the culture medium was removed. Myocytes were then treated with a lysis buffer (10 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.5% Triton X-100), collected by scraping, and centrifuged at 18,000 g for 20 min. The supernatant was treated with RNase A (400 μg/ml) for 1 h at 37°C and was then treated with proteinase K (400 μg/ml) for 1 h at 37°C. The DNA was precipitated with 0.5 mM NaCl-isopropanol solution (1:1 vol/vol) at −20°C. The precipitated DNA was centrifuged at 18,000 g for 15 min, and the isopropanol was thoroughly removed. The nucleic acid pellet was resuspended in 10 mM Tris-HCl buffer containing 1 mM EDTA (pH 8.0). The samples were normalized for cell number and subjected to electrophoresis on 2% agarose gel. Gels were stained with SYBR green (Molecular Probes), and DNA was detected by visualization under ultraviolet light.

**Mitochondrial Transmembrane Potential**

Loss of ΔΨₘ was assessed with the use of the dye JC-1 (Molecular Probes) (1). Cells grown on coverslips were incubated in PBS containing 10 μM JC-1 at 37°C for 5 min. Fluorescence emission at 527 and 590 nm was determined after excitation at 480 nm.

**Immunoblotting**

For detection of cytochrome c and Bax, myocytes were scraped and pelleted by centrifugation at 800 g for 5 min. The cells were suspended in 150 μl of cold lysis buffer containing (in mM) 250 sucrose, 20 HEPES, 10 KCl, 1 MgCl₂, 1 EDTA, 1 EGTA, 1 dithiothreitol, and 1 phenylmethylsulfonyl fluoride, pH 7.5, and were incubated for 5 min on ice. The cells were then homogenized, and the suspension was centrifuged at 750 g for 10 min at 4°C to sediment the nuclear fraction. The supernatant was centrifuged at 12,000 g for 10 min at 4°C to sediment the mitochondrial fraction and then centrifuged for 60 min at 100,000 g for 4°C. The resultant supernatant was used as the cytosolic fraction (51). To detect the cleavage of procaspase-3, the scraped and pelleted myocytes were resuspended in 50 μl of cold lysis buffer (2× PBS, 1% NP40, 0.5% deoxycholic acid, and 0.1% sodium dodecyl sulfate; pH 7.5) and incubated for 10 min on ice. The suspension was centrifuged at 14,000 g for 10 min at 4°C, and the supernatant was collected for analysis. Immunoblotting was performed using standard protocols. Samples containing equal amounts of protein were subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel and blotted onto polyvinylidene difluoride membrane (AE-6665, ATTO; Tokyo, Japan). After being blocked with 5% skim milk in PBS containing 0.1% Tween 20 at room temperature for 1 h, the membranes were probed with antibodies specific to cytochrome c (7H8.2C12, PharMingen; San Diego, CA), Bax (sc-526, Santa Cruz Biotechnology, Santa Cruz, CA), or caspase-3 (sc-7148, Santa Cruz Biotechnology) at 4°C overnight, followed by horseradish peroxidase-conjugated antiserum IgG or horseradish peroxidase-conjugated anti-rabbit IgG (Amersham; Little Chalfont, UK) at room temperature for 1 h. Detection of chemoluminescence was performed with ECL Western blot detection kits (Amersham) according to the supplier's recommendations.

**Caspase-3 Activity**

Caspase-3 enzymatic activity was determined with the CPP32 assay kit (MBL), which detects the production of the chromophore p-nitroanilide after its cleavage from the peptide substrate DEVD-p-nitroanilide as previously described (7). In brief, 2.7 × 10⁶ cells were solubilized and aliquots of lysate containing equal amounts of protein were reacted with 200 μM DEVD-p-nitroanilide at 37°C for 2 h. The activity was read in a microtiter plate reader at 400 nm. DEVD-CHO was used as a specific caspase-3 inhibitor (48).

**Statistical Analysis**

Data are expressed as means ± SE of >6 samples derived from >6 separate experiments. Differences were analyzed by one-way analysis of variance combined with Bonferroni test. A P value of <0.05 was considered to be statistically significant.

**RESULTS**

**Effect of Glucose Concentration on ATP Content**

In contrast to the MI(−) group, in which ATP levels remained constant at ~22.4 ± 0.7 nmol/mg protein for the duration of the 5-h experiment, the ATP content in the MI-treated groups declined to 24.6 ± 1.6% of baseline (Fig. 1). Subsequent incubation of the MI-treated cells for 4 h with varying concentrations of glucose resulted in a time- and concentration-dependent restoration of ATP, such that 10, 30, 50, and 100 mg/dl of glucose increased the ATP to 25.6 ± 2.5, 50.4 ± 3.3, 66.6 ± 2.3, and 89.0 ± 4.8% of baseline, respectively (Fig. 1).

**Cell Viability in Staurosporine-Treated Myocytes**

The effect of staurosporine treatment on cell viability is illustrated in Fig. 2A. The MI10 group contained a high fraction of nonviable cells (red fluorescent nuclei), compared with the control group or MI(−) group, in which the fraction of viable cells (green fluorescent) was consistently >95%. However, with increasing glucose availability, the fraction of viable cells increased and reached levels not significantly different from control. In the absence of staurosporine, incubation of the MI-treated cells for 4 h with 10, 30, 50, or 100 mg/dl glucose (MI and recovery) alone did not significantly
decrease the viability of myocytes, such that 10, 30, 50, and 100 mg/dl glucose resulted in 97.3 ± 0.1, 96.9 ± 0.3, 97.8 ± 0.4, and 97.6 ± 0.5% of viability, respectively. The MI10 group also showed a significant release of CK into the culture media compared with control (Table 1). No significant increase in CK was observed with any of the other groups. Similarly, MI and recovery alone did not significantly increased CK release (data not shown).

Apoptosis in Staurosporine-treated Myocytes

Histochemical staining of the myocytes with Hoechst 33258 and an antibody to desmin showed typical fragmented nuclei in staurosporine-treated groups, as illustrated in Fig. 2B. However, the frequency of apoptotic cells increased with increasing glucose concentration (Fig. 2B). The highest percentage of apoptotic myocytes (23.5 ± 2.0%) was observed in the MI(−) group, when contrasted with control (1.2 ± 0.2%). In the MI10 group, the percentage of apoptotic myocytes was only 2.2 ± 0.3%. However, the percentage of apoptotic nuclei increased significantly to 6.5 ± 0.4, 9.1 ± 1.0, and 15.7 ± 1.0% in the MI30, MI50, and MI100 groups, respectively. MI and recovery, without staurosporine treatment, did not result in myocyte apoptosis.
such that 10, 30, 50, and 100 mg/dl of glucose resulted in 1.7 ± 0.4, 1.6 ± 0.1, 1.6 ± 0.3, and 1.7 ± 0.2% of apoptosis, respectively. The histological evidence for apoptosis in staurosporine-treated groups was confirmed by the DNA analysis data. As shown in Fig. 3, control myocytes showed no DNA fragmentation. In contrast, DNA isolated from cardiac myocytes treated with 1 μM staurosporine for 4 h exhibited extensive DNA fragmentation, thus producing the characteristic DNA ladders. The intensity of the ladders in the MI groups increased with increasing glucose concentration. MI and recovery alone did not cause DNA fragmentation (data not shown).

Mitochondrial Transmembrane Potential

JC-1, a potential sensitive mitochondrial probe, exists as a green fluorescent monomer at low membrane potential. However, at higher potentials, JC-1 forms red fluorescent “J-aggregates.” The fluorescent emission of this dye can therefore be used to monitor ∆Ψm in apoptotic cardiac myocytes (8). In the present study, control myocytes showed red-orange mitochondrial staining, indicative of normal high membrane potentials (Fig. 4). Staurosporine treatment caused loss of ∆Ψm in the first hour of treatment (Fig. 4). Moreover, myocytes treated with staurosporine in either the ATP-

Table 1. CK release from cardiac myocytes into culture media

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>MI(-)</th>
<th>MI10</th>
<th>MI30</th>
<th>MI50</th>
<th>MI100</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK release, IU·1^{-1}·mg protein^{-1}</td>
<td>0.23 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>0.28 ± 0.01*</td>
<td>0.24 ± 0.01</td>
<td>0.25 ± 0.01</td>
<td>0.24 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6. Cardiac myocytes were subjected to metabolic inhibition (MI), followed by staurosporine treatment (see Fig. 2A). Creatine kinase (CK) release from the myocytes was measured as described in MATERIALS AND METHODS. *P < 0.01 vs. control.

Translocation of Bax and Cytochrome c

The time-dependent effects of staurosporine on the intracellular localization of Bax and cytochrome c in the MI(-) group are illustrated in Fig. 4. Bax was constitutively expressed in the cytosolic fraction of the myocytes. However, staurosporine treatment caused a rapid loss of Bax from the cytosolic fraction in the first hour of treatment (to 12.4 ± 0.9% of 0 h) and a concomitant increase in Bax in the mitochondrial fraction during the same period (to 1264.2 ± 257.9% of 0 h). The mitochondrial Bax immunoreactivity was depressed after 4 h of staurosporine treatment. In contrast, before staurosporine treatment, cytochrome c was detected exclusively in the mitochondrial fraction. Staurosporine treatment caused a time-dependent decrease in cytochrome c immunoreactivity in the mitochondrial fraction (to 19.6 ± 1.8% of 0 h after 4 h of treatment), with a concomitant increase in the cytosolic fraction, reaching maximum levels after 4 h of staurosporine treatment (to 636.8 ± 64.5% of 0 h). To examine the effect of intracellular ATP content on the translocation of Bax and cytochrome c, a similar analysis was done with the control, MI(-), and MI10 groups at 1 h (for Bax) and 4 h (for cytochrome c) after the initiation of staurosporine treatment (Fig. 5). The data show that staurosporine induced a translocation of Bax from the cytosol to the mitochondrial fraction and a translocation of cytochrome c from the mitochondrial fraction into the cytosolic fraction under conditions of both high [MI(-)] group or low ATP content (MI10 group).

Activation of Caspase-3

Caspase-3 activity increased as a function of glucose concentration after 4 h of treatment with staurosporine. Whereas caspase-3 activity in the MI10 group increased by a factor of 1.9 over control, it increased 4.5-fold in the MI(-) group (Fig. 6A). The addition of DEVD-CHO, a specific caspase-3 inhibitor, to the medium of the MI(-) group 1 h before the initiation of staurosporine treatment suppressed the activities of caspase-3 to control level (Fig. 6A). The formation of caspase-3 from its precursor was also analyzed by Western blots. As shown in Fig. 6B, procaspase-3 (32 kDa) was constitutively expressed in the myocytes. No cleavage of procaspase-3 was apparent in the control group, and only modest cleavage of procaspase-3 was detected in the MI10 group. However, significant cleav-
age of procaspase, and the concomitant appearance of the 17-kDa caspase-3 subunit, was observed in the MI(−) group.

Effect of Cyclosporin A on Cell Death

Cyclosporin A inhibited the staurosporine-induced loss of ΔΨm in the MI(−) group (data not shown). Moreover, it suppressed cytochrome c release (Fig. 7), caspase-3 activity [3.00 ± 0.07-fold vs. 4.52 ± 0.14-fold for the MI(−) group, P < 0.0001] and the percentage of apoptosis [9.3 ± 0.5 vs. 23.5 ± 2.0% for the MI(−) group, P < 0.0001]. Furthermore, cyclosporin A significantly increased cell viability (68.0 ± 1.4 vs. 30.0 ± 2.1% for the MI10 group, P < 0.0001), estimated by calcein and ethidium homodimer-1 staining. However, cyclosporin A did not suppress Bax translocation (Fig. 7).

DISCUSSION

The present study demonstrates for the first time that staurosporine can cause both apoptotic and necrotic cell death in neonatal rat cardiac myocytes. Both types of cell death are associated with a loss of ΔΨm, a translocation of Bax from the cytosol into the mitochondria, and a release of cytochrome c from the mitochondria into the cytosol. Significantly, our data show that intracellular energy levels regulate the form of cell death, with low cellular ATP effecting necrosis, and high ATP causing the activation of caspase-3 and promoting apoptosis. Our data therefore suggest that myocyte apoptosis and necrosis share common mitochondrial death signaling pathways, primarily those mediated by the mitochondrial PT, and that ATP-dependent steps in the apoptotic signal transduction pathway exist upstream of caspase-3 activation, as well as downstream of cytochrome c release.

In the present study, the intracellular ATP level in the myocytes was adjusted by first inhibiting mitochondrial energy production and subsequently by varying the glucose content in the medium during the staurosporine injury phase. With glucose concentrations depleted to <30 mg/dl, the fraction of nonviable...
cells increased significantly after staurosporine treatment, accompanied by a significant increase in CK release, and enhanced staining by nuclear dyes. In our study, the increase in CK was quite modest compared with the loss of cell viability, estimated by calcein-acetoxymethyl ester and ethidium homodimer-1 staining. Modest CK increase was probably due to the lower sensitivity compared with staining for cell viability. These data suggest that under conditions of low ATP, the injured cells lost membrane integrity and succumbed to necrotic cell death. In contrast, with glucose concentrations greater than 30 mg/dl, there was an increase in cellular ATP, which resulted in a concomitant increase in the frequency of apoptotic cell death as illustrated by the increased nuclear and DNA fragmentation.

Apoptosis differs from necrosis in that apoptosis is an active, genetically regulated, and energy-requiring process, whereas necrosis is generally viewed as an unregulated, passive phenomenon normally caused by catastrophic and overwhelming injury. Several recent studies (9, 23) have confirmed that increased ATP levels favor an apoptotic form of cell death, whereas low energy levels promote necrosis in several forms of injury, including staurosporine. In an effort to identify the ATP-requiring steps within the apoptotic death signaling pathway in injured myocytes, we examined the activity of caspase-3, a cysteine protease, because of its established critical role in apoptosis in general and staurosporine-induced apoptosis in particular (28, 36, 53). In the present study, the activity of caspase-3 was significantly increased in myocytes treated with staurosporine, as confirmed by both enzymatic activity assays and cleavage of the 32-kDa precursor to the 17-kDa caspase-3 isoform. More importantly, the level of caspase-3 activity appeared to correlate with the percentage of apoptotic cells, as well as with the myo-
cyte ATP content. The data, therefore, strongly suggest that caspase-3 activation is involved in staurosporine-induced myocyte apoptosis and that ATP-dependent steps exist upstream of this activation.

Besides the well-established CD95 or tumor necrosis factor receptor/caspase-8-mediated apoptotic pathway, recent reports (56) have identified a novel cascade that is regulated by the apoptotic protease activating factor-1 (Apaf-1), thereby suggesting an important role for mitochondria in the induction of apoptosis (46, 51). With cardiac myocytes, an important mitochondrial contribution has already been reported (5, 8) under conditions of serum or glucose deprivation as well as oxidative stress. Recent observations (1, 15, 19) have also shown that an early critical event of both apoptotic and necrotic cell death appears to be mitochondrial PT, which is associated with several potentially lethal consequences, including the reduction of ΔΨₘ (38), the uncoupling of the respiratory chain with increased production of reactive oxygen species (ROS) (27), and the liberation of preformed apoptogenic proteins such as cytochrome c and apoptosis-inducing factor (43, 55). In addition, recent reports (41) have suggested that one of the triggers of mitochondrial PT is translocation of Bax, a proapoptotic protein, from the cytosol into mitochondria, and Bax may form selective channels for cytochrome c release. In the present study, we show that staurosporine caused the loss of ΔΨₘ as estimated by JC-1 staining, a concomitant translocation of Bax into the mitochondria, and the release of cytochrome c into the cytosol, under ATP-supplying and ATP-depleting conditions. Moreover, cyclosporin A, an inhibitor of mitochondrial PT, suppressed the staurosporine-induced apoptosis or necrosis through the inhibition of the ΔΨₘ reduction, cytochrome c release, and caspase-3 activation, whereas it did not prevent Bax translocation. This suggests that staurosporine caused Bax translocation into the mitochondria, raising the mitochondrial PT, reduced ΔΨₘ, and liberated apoptogenic proteins from the mitochondria independently of intracellular ATP levels. Our data also suggest that mitochondrial PT is an early common step in the death signaling pathways of apoptosis and necrosis in the myocytes, in agreement with the previous observations (1, 15, 19), and that the ATP-dependent steps occur downstream of the release of cytochrome c from the mitochondria in staurosporine-induced apoptosis. Therefore, under conditions in which intracellular energy levels are preserved, mitochondrial PT induced by staurosporine can trigger apoptosis, whereas under conditions in which protease activation is precluded by loss of ATP, PT causes necrosis.

The precise mechanism by which ATP regulates the apoptotic pathway remains unclear. However, recent in vitro analysis has indicated that cytochrome c can bind to Apaf-1 and this event unmasks the caspase recruitment domain (CARD) motif in Apaf-1 permit-
A conformational change in the Apaf-1/cytochrome c ATP is present, suggesting that the nucleotide induces complex will not bind procaspase-9 unless dATP or dATP (or ATP). Furthermore, although cytochrome c can bind to Apaf-1 in the absence of dATP, the complex will not bind procaspase-9 unless dATP or ATP is present, suggesting that the nucleotide induces a conformational change in the Apaf-1/cytochrome c complex that exposes the CARD domain of Apaf-1 and allows procaspase-9 to bind (24). In the present study, although we did not examine the activation of caspase-9, we have confirmed that staurosporine causes a translocation of Bax into the mitochondria, the loss of ΔΨm, and a release of cytochrome c into the cytosol and that myocyte apoptosis subsequently occurs through the activation of caspase-3 under ATP-rich conditions. The data, therefore, strongly suggest the possibility that the mitochondria/Apaf-1 system for activating caspase-3 operates in an ATP-dependent manner in staurosporine-induced myocyte apoptosis.

It is well documented that the pathological activation of apoptosis is now thought to contribute to a variety of cardiac disease, including arrhythmogenic right ventricular dysplasia (26), heart failure (34), and myocardial infarction (37, 39). The precise mechanism by which myocyte apoptosis occurs in these pathological conditions remains largely uncertain. However, recent observations have indicated that the Bcl-2-to-Bax ratio may play an important pathophysiological role in the protection or acceleration of apoptosis in human myocytes after ischemia and/or reperfusion (31) and that ROS derived from impaired electron transport in the mitochondria is detected in heart failure and may contribute its progression (17). Furthermore, Narula et al. (35) recently demonstrated that there is a significant accumulation of cytochrome c in the cytosol, activation of caspase-3, and cleavage of its substrate, protein kinase C-δ, in human end stage cardiomyopathy and suggested that cytochrome c-dependent activation of cysteine proteases underlies the phenomenon of apoptosis in the myopathic process. Thus the accumulating data suggest an important involvement of the mitochondrial/Apaf-1 pathway in the pathogenesis of many heart diseases.

Study Limitations

Neonatal versus adult cardiac myocytes. Hypoxia-ischemia, with or without reoxygenation, is a potent stimulator of apoptotic death in both neonatal and adult cardiac myocytes in vitro and in vivo. In most of the reports (25, 33, 52) on the induction of apoptosis, neonatal myocytes are more resistant to either ischemia or hypoxia than adult ventricular myocytes. For staurosporine-induced apoptosis, there are no data indicating comparison between neonatal and adult cardiac myocytes. As described in the previous reports (25, 32), both hypoxia and staurosporine stimulation cause apoptosis via a mitochondria-dependent release of cytochrome c. Taken together, it is therefore speculated that neonatal myocytes are more resistant to staurosporine-treatment than adult ventricular myocytes.

Effect of staurosporine. The bacterial alkaloid staurosporine is reported to induce apoptosis in a variety of cells. Staurosporine was initially described as an inhibitor of protein kinase C (44) and has been shown to inhibit many different protein kinases (13). Although it is well known that activation of protein kinases, such as the mitogen-activated protein kinase family, affects the induction of apoptosis, we have no data demonstrating the effect of protein kinases modulated by staurosporine on apoptosis in the present study. The mechanisms of staurosporine-induced apoptosis are largely unknown. However, previous reports (20) indicate that staurosporine can cause mitochondrial ROS production and elevation of intracellular free calcium levels very early in the apoptotic process that might be relevant to hypoxic or ischemia-reperfusion injury.

Effect of oligomycin. Apoptosis induced by overexpression of Bax has been reported to be prevented by oligomycin-induced inhibition of F0F1-ATPase (30). As described in previous reports (9, 23), inhibition of F0F1-ATPase by oligomycin in glucose-containing media does not necessarily prevent the induction of apoptosis.

In conclusion, the present study demonstrates that staurosporine induces the translocation of cytosolic Bax into the mitochondria and the loss of ΔΨm with a concomitant release of mitochondrial cytochrome c into the cytosol, and that it accelerates myocyte apoptosis through the activation of caspase-3 under ATP-rich conditions, but that it induces necrosis under ATP-depleting conditions. Thus, although mitochondrial PT is an early common critical event in both types of myocyte demise, intracellular ATP levels are an important determinant in the regulation of myocyte cell death.

REFERENCES


42. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, and Thompson CB. Bcl-xL regulates the mem-


