Induction of the Mitochondrial Permeability Transition Mediates the Killing of HeLa Cells by Staurosporine

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ABSTRACT

The role of the mitochondrial permeability transition (MPT) in the killing of HeLa cells by staurosporine (STR) was assessed with the use of bongkrekic acid (BK), an inhibitor of the MPT. BK prevented cell killing as well as biochemical manifestations of the MPT: (a) the loss of the mitochondrial membrane potential (ΔΨm); (b) the release of cytochrome c from the intramembranous space to the cytosol; and (c) the release of malate dehydrogenase from the mitochondrial matrix. Stable transfectants that overexpressed Akt were also resistant to cell killing and did not develop an MPT. STR inhibited the phosphorylation of Bad, whereas Bad phosphorylation was preserved in cells that overexpress Akt. In wild-type HeLa cells treated with STR, the content of Bax in the cytosol decreased as that in the mitochondria increased, a result that was again prevented by overexpression of Akt. Bid accumulation in the mitochondria with STR was not affected by overexpression of Akt. The pan-caspase inhibitor Z-Val-Ala-Val-Asp(Ome) fluoromethylketone prevented cell killing but not induction of the MPT. The data document the central role of the MPT in the killing of HeLa cells by STR. The data are consistent with the hypothesis that induction of the MPT is a consequence of the movement of Bax to the mitochondria. Phosphorylation of Bad prevents Bax translocation. Caspases participate in the events related to cell killing that occur subsequent to induction of the MPT.

INTRODUCTION

The mitochondria are increasingly implicated as playing a critical role in the pathogenesis of apoptosis (1–6). A dominant concern has been the mechanism of release of cytochrome c from the intramembranous space of the mitochondria and, in turn, on the mode of action of cytochrome c in the cytosol. Upon its release to the cytosol, cytochrome c acts to control the assembly of an “apoptosome,” a complex composed of apoptotic protease-activating factor-1 and procaspase-9 (7–10). The subsequent activation of caspase-9 and other caspases is responsible for the controlled degradation processes that are characteristic of apoptotic cell death.

The mechanism that mediates the release of cytochrome c from the mitochondria during apoptosis is the subject of considerably more debate. Central to this controversy is the mechanism of permeabilization of the outer mitochondrial membrane so as to allow release of cytochrome c. One hypothesis implicates induction of the MPT as a consequence of the opening of a megachannel called the PTP, which spans both the inner and outer mitochondrial membranes at contact sites where the two membranes are apposed (11). Alternatively, cytochrome c release is attributed to the formation of nonspecific holes, tears, or pores in the mitochondrial outer membrane by an as yet poorly defined mechanism that nevertheless does not involve induction of the MPT (12, 13).

By whatever mechanism cytochrome c is released, the proapoptotic protein Bax and other members of its family (Bak, Bok, Bik, Bad, Bid, and others) are implicated in the mediation of this phenomenon (5, 14). One argument is that Bax directly forms channels for cytochrome c release and other factors from the intramembranous space. On the other hand, Bax may regulate the activity of preexisting channels, such as the PTP, rather than form them de novo.

In the present study, we have used a well known model of apoptotic cell death, the killing of HeLa cells by STR, in an attempt to resolve some of the above controversy. We have used a specific inhibitor, BK, of the MPT to define the role of the PTP in both the release of cytochrome c and cell killing. We use three criteria to document induction of the MPT: loss of the ΔΨm, release of MDH from the mitochondrial matrix, and release of cytochrome c from the intramembranous space. In addition to preventing the loss of viability, BK prevented all evidence of induction of the MPT. We have reported previously that purified Bax induces the MPT in isolated mitochondria (15), and here it is shown that Bax translocates to the mitochondria upon treatment with STR. Stably transfect HeLa cells that overexpress Akt, a serine/threonine kinase that phosphorylates Bad (16, 17), did not show evidence of Bax movement in response to STR and were resistant to cell killing. We conclude that STR causes the translocation of Bax to the mitochondria, an event that is accompanied by induction of the MPT with the release of cytochrome c and the initiation of the execution phase of the apoptotic death of the cells.

MATERIALS AND METHODS

Cell Line. HeLa cells (human cervix adenocarcinoma, ATCC-CC-1) were maintained in 25-cm² polystyrene flasks (Corning Costar Corp., Oneonta, NY) with 5 ml of DMEM (high glucose; without pyruvate; Mediatech, Inc., Herdon, VA), containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (complete DMEM) and incubated under an atmosphere of 95% air and 5% CO2. For all experiments, HeLa cells were plated at a density of 30,000 cells/cm² in complete DMEM. After overnight incubation, the cells were washed twice with PBS and placed in DMEM without serum.

Treatment Protocols. In all experiments, STR (Biomol) was added to a final concentration of 50 nM. STR was dissolved in DMSO and added to the cells in 0.2% volume. Where indicated, the cells were pretreated for 1 h with the following reagents before addition of STR. BK (Biomol; dissolved in water) was added to the cell culture medium to give a final concentration of 150 μM. The cell permeable, broad-spectrum caspase inhibitor Z-Val-Ala-Val-Asp(Ome) fluoromethylketone (Z-VAD-FMK; Kamyia Biomedical Co., Seattle, WA) was dissolved in DMSO and added in a 0.2% volume to give a final concentration of 50 μM. In all cases, the vehicles used to prepare stock solutions of the reagents had no effect on the cells or the parameters measured at the concentrations used.

Measurement of Cell Viability. Cell viability was determined by trypan blue exclusion. After treatment, the cells were trypsinized, and 10 μl of a 0.5% solution of trypan blue were added to 100 μl of treated cells. The suspension was then applied to a hemocytometer. Both viable and nonviable cells were counted. A minimum of 200 cells was counted for each data point in a total of eight microscopic fields.

Generation of Stable Akt Transfectants. HeLa cells were plated in 24-well plates. After an overnight incubation, the cells were washed twice in PBS. Transfections were performed using Lipofectamine-Plus (Life Technologies,
Inc.) according to the manufacturer’s instructions. The cells were transfected with 0.5 μg of pCDNA-Akt (generously provided by Drs. Morris J. Birnbaum and Randall N. Pittman, Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA). After 4 h, the cells were washed twice with PBS and placed in complete DMEM. After 48 h of further incubation, the cells were washed with PBS and trypsinized. Cells from 4 wells were plated in 75-cm² polystyrene flasks in complete DMEM supplemented with 600 μg/ml of G418 (Life Technologies, Inc.). Stable transfectants were generated and cultured in 25-cm² polystyrene flasks. The overexpression of HA-tagged Akt was confirmed by Western Blot analysis. Briefly, 5 × 10⁵ cells were pelleted at 700 × g (5 min at 4°C) and resuspended in 100 μl of cell lysis buffer [20 mM Tris (pH 7.4), 100 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. Protein (20 μg) was separated on a 10% SDS-PAGE. The gel was electroblotted onto a nitrocellulose membrane. The blot was probed with an anti-hemagglutinin antigen rabbit polyclonal antibody (Y-11; Santa Cruz Biotechnology, CA) at a dilution of 1:4,000. The secondary anti-goat horseradish peroxidase-labeled antibody (1:20,000) was visualized by enhanced chemiluminescence.

Isolation of Cytosol and Mitochondrial Fractions and Determination of MDH or Cytochrome c Content. Cells were plated in 75-cm² polystyrene flasks. After treatment, the cells were harvested by trypsinization. Soybean trypsin inhibitor was used to neutralize trypsin after harvest. Cells were centrifuged at 750 × g for 10 min at 4°C. The pellets were washed with SHE-PI [10 mM HEPES-KOH (pH 7.4), 1 mM EDTA, PI, and 250 mM sucrose]. The cell pellets were resuspended in SHE-PI. The cell suspension was transferred to a Dounce homogenizer, and the cells were broken open with 25 strokes of the pestle. The homogenate was transferred to a high-speed centrifuge tube containing SHE-PI and 0.5% phenol red. The supernatant from the 10,000 × g spin was centrifuged at 700 × g for 10 min at 4°C. The pellets were washed once with SHE-PI and 0.5% phenol red. The supernatant from the 10,000 × g spin after washing was centrifuged at 10,000 × g for 30 min at 4°C. The resulting mitochondrial pellet was resuspended and centrifuged a second time into 750 mM sucrose in SHE-PI. The mitochondria were lysed in 25 mM Tris (pH 7.4), 100 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Protein (40 μg) was separated on 12% SDS-polyacrylamide electrophoresis gels and electroblotted onto nitrocellulose membranes. Phospho-Bad-136 and Phospho-Bad-112 were detected by a rabbit polyclonal antibody at a dilution of 1:500 (New England Biolabs). The secondary anti-goat horseradish peroxidase-labeled antibody (1:10,000) was visualized by enhanced chemiluminescence. The total Bad content was assessed with a rabbit anti-Bad polyclonal antibody (New England Biolabs).

All experiments using Western blotting were repeated three times (cytochrome c, MDH, Bax, Bid, Cox4, β-actin, and Bad as above). The immunoblots from each experiment were scanned with a Image Station-440CS densitometer (Eastman Kodak, Rochester, NY). The relative densities with respect to the appropriate controls were calculated. The significance of the resulting data are indicated in the individual figures.

Measurement of the Mitochondrial Membrane Potential. HeLa cells were plated in six-well plates. After overnight incubation, the cells were washed twice in PBS and placed in DMEM without serum. After another overnight incubation, the cells were treated as described in the text. During the last 30 min of treatment, the cells were exposed to 100 nM DiOC₆(3) (Molecular Probes, Inc., Eugene, OR). At the end of the experiment, the cells were trypsinized, transferred to microcentrifuge tubes, and centrifuged at 8000 × g for 10 min at 4°C. The supernatant was removed, and the pellet was washed with 500 μl of PBS. After a second centrifugation, the pellet was resuspended in 600 μl of distilled deionized water, followed by homogenization. The resulting emission fluorescence was read, and the concentration of DiOC₆(3) was calculated with the use of a Perkin-Elmer LS-5 fluorescence spectrophotometer at 488 nm (excitation) and 500 nm (emission). Values for mitochondria with depleted ΔΨₘ were determined by simultaneous treatment of cells with 10 μM carbonyl cyanide CCCP and DiOC₆(3). CCCP was dissolved in ethanol and added at a volume of 0.5%. The extent of mitochondrial de-energization as determined by the cellular content of DiOC₆(3) was corrected for the extent of cell killing at each time point analyzed.

Alternatively, the membrane potential was assessed using the green fluorescent dye JC-1 (Molecular Probes Inc., Eugene, OR). JC-1 exists as a green fluorescent monomer at low membrane potential; i.e., in the cytosol. However, at higher potentials, i.e., within the mitochondrial matrix, it forms red fluorescent aggregates (18).

Briefly, HeLa cells were plated in 35-mm dishes. After an overnight incubation, the cells were washed twice in PBS and placed in DMEM without serum. After overnight incubation again, the cells were treated as described in the text. During the last 30 min of treatment, the cells were loaded with 10 μM JC-1. At the end of the experiment, the cells were washed twice with PBS and placed in 2 ml of PBS. The cells were viewed with a long-pass filter, which allows the visualization of red and green fluorescence simultaneously, on a Nikon Optiphot fluorescence microscope. Fluorescence photomicrographs were taken at ×400 using Ektachrome-64T tungsten professional color reversal film (Eastman Kodak).

RESULTS

Induction of the MPT by STR. HeLa cells were treated with 50 nM STR, and the viability of the cells was determined by the ability to exclude trypan blue. Fig. 1 details the time course of the accumulation of dead cells. A loss of viability was evident within 3 h and progressed steadily thereafter to affect over 60% of the cells at the end of 24 h.
BK is an inhibitor of the ANT of the inner mitochondrial membrane (19–21). By stabilizing the transporter in the m-state, BK prevents induction of the MPT (19, 22, 23), an event that is associated with the c-state of the ANT. We evaluated the effect of BK on four distinct consequences of the opening of the mitochondrial permeability pore to document the role of the MPT in the killing of HeLa cells by STR: (a) the loss of viability; (b) the loss of the ΔΨm; (c) the release from the intramitochondrial space to the cytosol of cytochrome c; and (d) the release from the mitochondrial matrix of MDH (Figs. 2–5).

Fig. 2 shows that BK substantially reduced the extent of cell killing by STR. Whereas 60% of the cells were killed by STR alone, in the presence of BK and STR a little less than 80% of the cells were still alive at the end of the experiment. Importantly, atractyloside, another ANT inhibitor that does not prevent induction of the MPT, did not protect against the cell killing by STR (data not shown).

The MPT causes the loss of the ΔΨm (24–26). The fluorescent dye DiOC6(3) localizes to mitochondria as a consequence of ΔΨm, and the MPT reduces the accumulation of DiOC6(3) upon the loss of ΔΨm. Treatment of HeLa cells with STR produced a steady decline in ΔΨm (Fig. 3), and the time course of the loss of ΔΨm preceded that of the loss of viability (Fig. 1). Within 3 h, 15% of the dye was lost from the cells. This loss increased to almost 30% by 6 h and then increased to 45% by 10 h.

The time-dependent loss of DiOC6(3) fluorescence that resulted from the treatment with STR was prevented by BK. After 10 h of exposure to STR in the presence of BK, DiOC6(3) fluorescence was 93% of the control value (Fig. 3). Importantly, BK had no effect on the rate or extent of the loss of DiOC6(3) fluorescence caused by CCCP, a result demonstrating the specificity of BK in preventing the loss of ΔΨm as a consequence of the MPT. The effect of STR on ΔΨm was also assessed with the use of the fluorescence probe JC-1. Upon treatment with STR, the prominent, granular orange fluorescence became a diffuse green (data not shown).

The MPT releases cytochrome c from the intramembranous space (27, 28) and other proteins (29) from the mitochondrial matrix. STR produced a release of cytochrome c to the cytosol and a concomitant decrease in the content of cytochrome c in the mitochondria, effects that were again prevented by BK (Fig. 4). MDH is a soluble protein present in the mitochondrial matrix (28). Fig. 4 shows that cytosolic extracts from control HeLa cells exhibited some detectable MDH. This baseline level is attributable to the MDH normally present in the cytosol as part of the acetyl-CoA cycle and used to transport acetyl-CoA across the mitochondrial membrane for utilization in fatty acid synthesis. However, the MDH content of the cytosol increased substantially upon treatment of the HeLa cells with STR (Fig. 4). This accumulation of MDH in the cytosol was accompanied by a loss of the enzyme from the mitochondria (Fig. 4). Treatment of the HeLa cells with STR in the presence of BK prevented both the accumulation of MDH in the cytosol and its loss from the mitochondria (Fig. 4).
The content of COX4 was unchanged in any of the mitochondrial fractions, and there was no mitochondrial contamination of the cytosolic fractions (Fig. 4). Similarly, there was no change in the content of \( \beta \)-actin in the cytosolic fractions (Fig. 4). Thus, changes in the relative purity of the respective subcellular fractions did not affect the redistribution of cytochrome c and MDH illustrated in Fig. 4.

Overexpression of Akt Prevents Induction of the MPT by STR.

The serine/threonine kinase Akt promotes cell survival as a consequence of the phosphorylation of Bad (16, 17). Bad is capable of forming heterodimers with the antiapoptotic proteins Bcl-X\(_L\) or Bcl-2 (30). Phospho-Bad cannot bind either Bcl-X\(_L\) or Bcl-2 (31). By binding to Bcl-X\(_L\) and Bcl-2, Bad antagonizes their antiapoptotic activity. Upon its phosphorylation, Bad dissociates from Bcl-X\(_L\) and Bcl-2, thereby promoting cell survival by allowing the unhindered action of these proteins.

We produced clones of stably transfected HeLa cells in which Akt is overexpressed (Fig. 5). The sensitivity of clone 2 to STR is illustrated in Fig. 6. Whereas >60% of the wild-type cells were killed by 50 nM STR after 24 h, only a little more than 20% of HeLa cells that overexpress Akt were killed by the same dose of STR.

The phosphorylation of Bad at both Ser-112 and Ser-136 (32) was reduced upon treatment of the wild-type HeLa cells with STR (Fig. 7). HeLa cells maintained a basal level of phosphorylation of Bad at Ser-136 and Ser-112. Within 30 min of treatment with STR, however, there was a decrease in Bad phosphorylation at both serines (Ser-136 and Ser-112 in Fig. 7), an effect that was also evident after 90 min. By contrast, in clone 2 of HeLa cells that overexpress Akt, phosphorylation of Bad at Ser-136 actually increased slightly upon treatment with STR (Fig. 7). Phosphorylation of Bad at Ser-112 decreased slightly after 30 min in cells that overexpressed Akt. However, within

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**Fig. 4.** Release of cytochrome c (Cyt. c) and MDH from mitochondria in HeLa cells treated with STR. HeLa cells were treated with 50 nM STR or with STR and 150 \( \mu \)M BK. Control cells were left untreated. After 10 h, the content of cytochrome c, MDH, COX4, and \( \beta \)-actin was determined in both cytosolic and mitochondrial subcellular fractions by Western blot analysis as described in “Materials and Methods.” The experiment was repeated three times. The average intensity (mean ± SD) of the blots relative to the respective control was determined by densitometric analysis. * data points that are significantly different from the control \((P < 0.05)\).

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**Fig. 5.** Akt overexpression in HeLa cells. HeLa cells (clones 1 and 2) stably transfected with an Akt expression vector as described in “Materials and Methods” are shown. The expression of Akt was determined by Western blot analysis. WT, wild-type cells.

**Fig. 6.** Overexpression of Akt prevents the cell killing (**left panel**) and the loss of the \( \Delta \psi_m \) (**right panel**) in HeLa cells treated with 50 nM STR. Cell death was determined after 24 h by trypan blue exclusion, and \( \Delta \psi_m \) with the fluorescent dye DiOC\(_6\)(3) after the times indicated as described in “Materials and Methods.” Cells that were not treated with STR were given 10 \( \mu \)M CCCP for 30 min. The emission fluorescence of the untreated controls was 364.7 ± 14.5. The results are the means of the three separate experiments; bars, SD. **Left panel:** * \( P < 0.05 \) versus STR alone. **Right panel:** * \( P < 0.05 \) versus Akt control.
90 min there was an increase in Ser-112 phosphorylation of Bad (Fig. 7). There was no change in the total content of Bad after either 30 or 90 min treatment with STR (Fig. 7).

The biochemical evidence of the MPT induced by STR was prevented by the overexpression of Akt. Fig. 6 shows that HeLa cells that overexpress Akt did not react to STR with a loss of the $\Delta V_m$, as occurred in the wild-type cells (Fig. 3). HeLa cells that overexpress Akt did, however, react to CCCP with a loss of $\Delta V_m$ as measured again by DiOC$_6$(3) fluorescence. There was no release from the mitochondria to the cytosol of cytochrome c in HeLa cells that overexpress Akt. Fig. 8 shows that upon treatment with STR, no decrease was evident in the mitochondrial content of cytochrome c. Similarly, no increase was observed in the amount of cytochrome c in the cytosol (Fig. 8). Finally, in HeLa cells that overexpress Akt, the mitochondrial content of MDH did not change in response to STR. As a consequence, no increase in the cytosolic content of MDH could be detected. Again, the content of COX4 was unchanged in any of the mitochondrial fractions, and there was no mitochondrial contamination of the cytosolic fractions (Fig. 8). Similarly, there was no change in the content of $\beta$-actin in the cytosolic fractions (Fig. 8). Thus, the increased phosphorylation of Bad in HeLa cells that overexpress Akt is reflected in a prevention of the MPT and a preservation of cell viability upon treatment with STR.

**Overexpression of Akt Prevents the Movement of Bax to the Mitochondria in Response to STR.** The overexpression of proapoptotic Bax killed Jurkat cells as a consequence of induction of the MPT (32), and purified, recombinant Bax induced the MPT in isolated mitochondria in vitro (15). Bax and Bcl-X$_L$ are present constitutively in HeLa cells. Bax is located in the cytosol and, upon initiation of the apoptotic process, has been reported to translocate to the mitochondria (33–39).

Fig. 9 details the change in response to STR of the intracellular distribution of Bax. Over a time course in hours, the Bax content in
the cytosol decreased, a result paralleled by an increase in the Bax content of the mitochondria. The overexpression of Akt prevented this redistribution of Bax (Fig. 9). Importantly, there was no change in the total Bax content in the whole-cell lysate (Fig. 9).

The incorporation of Bax into the mitochondrial membrane is reported to require the participation of Bid, a protein that contains a BH3 domain and that binds Bax (37, 38, 40). Fig. 10 shows that Bid translocated to the mitochondria in response to STR. The baseline content of Bid in the mitochondria was less in HeLa cells that overexpressed Akt (Fig. 10). However, there was an accumulation of Bid in the mitochondria in response to treatment with STR (Fig. 10), an effect that occurred despite the inhibition of induction of the MPT with overexpression of Akt (Figs. 6 and 8). There was no change in the total Bid content in the whole-cell lysate (Fig. 10).

Inhibition of Caspases Prevents Cell Killing but not Induction of the MPT. Z-VAD-FMK is a pan-caspase inhibitor (caspases 1, 3, 5, 7, 8, and 9 but not 2), which offers protection against the apoptosis produced under a variety of experimental conditions (41). Fig. 11 shows that Z-VAD-FMK provided substantial protection against the killing of HeLa cells by STR. The number of dead cells was reduced from >60% to ~20% of the total population.

Despite this protection against the loss of viability, Z-VAD-FMK did not prevent induction of the MPT. In the presence of STR and Z-VAD-FMK, there still occurred a release of cytochrome c from the intramembranous space and a release of MDH from the mitochondrial matrix (Fig. 12). These data differ noticeably from the prevention of both of these effects of the MPT that occurred with BK (Fig. 4). The content of COX4 was unchanged in any of the mitochondrial fractions, and there was no mitochondrial contamination of the cytosolic fractions (Fig. 12). Similarly, there was no change in the content of β-actin in the cytosolic fractions (Fig. 12).

DISCUSSION

We have shown that the MPT is induced in HeLa cells upon treatment with STR, a protein kinase inhibitor that causes apoptosis. In addition to an effect on viability (Fig. 2), the sensitivity to BK of three mitochondrial alterations was used to document the MPT, the loss of the ΔΨm (Fig. 3), the release from the mitochondrial matrix to the cytosol of MDH (Fig. 4), and the release from the intramembranous space of cytochrome c (Fig. 4). In parallel with the protection from the loss of viability, BK preserved mitochondrial integrity, as assessed by the three criteria adumbrated.

BK has two known biochemical actions. It inhibits the ANT (19–21) and blocks induction of the MPT (19, 22, 23). The loss of the ΔΨm (24–26), the release from the mitochondrial matrix to the cytosol of MDH (27), and the release from the intramembranous space of cytochrome c (27–28) are documented consequences of induction of the MPT. Nevertheless, it was possible that the loss of viability was related to the inhibition of the ANT. However, another inhibitor (attractyloside) of the ANT (19–21) that does not prevent induction of the MPT did not prevent the toxicity of STR. Accordingly, it can be concluded that the killing of HeLa cells by STR is a consequence of induction of the MPT.

The previous literature on this subject has generally favored a different conclusion, i.e., that the release of cytochrome c by STR is not a consequence of the MPT (12, 13, 42, 43). In this regard, it deserves emphasis that only one previous study assessed this concern with the use of an inhibitor of the MPT. In this case, the release by
Akt prevents the interaction of this proapoptotic protein with Bcl-XL from the mitochondria in HeLa cells treated with STR. HeLa cells were treated with isolated mitochondria in vitro (15). The phosphorylation of Bad by Akt prevents the interaction of this proapoptotic protein with Bcl-XL or Bcl-2, thereby allowing these proteins to complex with Bax (16, 17, 30, 31). When Bax is bound to either Bcl-XL or Bcl-2, it would be incapable of inducing the MPT and, thus, of killing the cells. Conversely, when the phosphorylation of Bad is inhibited, the continued interaction of Bad with either Bcl-XL or Bcl-2 prevents the interaction of these proteins with Bax. Bax then moves to the mitochondria and induces the MPT, a result that causes the death of the cells. This is exactly the scenario observed in either the Akt transfectants or wild-type cells. In the latter case, STR inhibited the phosphorylation of Bad (Fig. 7), an effect that was accompanied by translocation of Bax to the mitochondria (Fig. 9), induction of the MPT (Figs. 3 and 4), and the death of the cells (Fig. 1). Overexpression of Akt maintained Bad phosphorylation (Fig. 7), prevented the movement of Bax (Fig. 9), prevented induction of the MPT (Figs. 6 and 8), and preserved the viability of the cells (Fig. 6).

By attributing induction of the MPT to Bax, we can, in turn, propose an additional mechanism to account for the previous reports of an apparent release of cytochrome c despite maintenance of ΔΨm and the absence of organelle swelling (12, 13). We observed previously that at low concentrations Bax caused the release from isolated mitochondria the intramembranous proteins cytochrome c and adenylate kinase and the release from the matrix of sequestered calcinein, effects prevented by the inhibitor of the PTP cyclosporin A and that occurred despite maintenance of ΔΨm (15). These effects were interpreted as the consequence of transient, nonsynchronous activation of the PTP, followed by a prompt recovery of mitochondrial integrity. We would argue that the effects of a low concentration of Bax in vitro may be similar to the action of this proapoptotic protein early in the course of the cell killing by STR. Together with the problem of the redistribution of fluorescent dye from mitochondria with depleted ΔΨm to mitochondria with intact ΔΨm, transient opening of PTP by Bax early in the development of STR-induced cell injury can account for the previous conclusions that argued against the participation of the MPT.

Two further findings reported here deserve some comment. A similar movement of Bid accompanied the translocation of Bax to the mitochondria (Fig. 10). Bid has been reported to release cytochrome c from isolated mitochondria in vitro and has, accordingly, been proposed to relay directly an apoptotic signal to the mitochondria (48–50). Alternatively, it has been argued that Bid interacts with Bax to trigger a change in Bax conformation that leads to its integration into the outer mitochondrial membrane (37, 38). By contrast with Bax (Fig. 9), the translocation of Bid to the mitochondria that occurred in response to STR was not prevented by overexpression of Akt (Fig. 10). Thus, the translocation of Bid to the mitochondria is not necessarily accompanied by induction of the MPT and the release of cytochrome c. Furthermore, the translocation of Bid is not sufficient for the movement of Bax to occur. Our data do not permit, however, a conclusion as to whether it is even necessary. Additional studies to explore this critical concern are in progress.

Finally, our data do not implicate the caspases in the induction of the MPT by STR with the consequent release of cytochrome c. Despite a substantial reduction in the extent of cell killing with the pan-caspase inhibitor Z-VAD-FMK (Fig. 11), there was no effect on the release of either cytochrome c or MDH (Fig. 12). A similar result has been reported previously (13, 39). Thus, the mechanism that mediates the translocation of Bax to the mitochondria, whether it involves Bid, would not seem to be a caspase-dependent phenomenon. In this regard, it deserves noting that full-length Bid is reported to be inactive. Upon cleavage by caspase-8, the COOH-terminal part of Bid then translocates to mitochondria (48–50). It will clearly be of interest to determine whether the translocation of Bid to the mitochondria observed here is caspase dependent. Inhibition of Bid translocation by
Z-VDADFMK would clearly suggest that there is no role for this protein in the apoptosis produced by STR.

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