

Regulation of Intracellular pH Mediates Bax Activation in HeLa Cells Treated with Staurosporine or Tumor Necrosis Factor- α *

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Induction of apoptosis in HeLa cells with staurosporine produced a rise in the intracellular pH (pH_i). Intracellular alkalization was accompanied by translocation of Bax to the mitochondria, cytochrome *c* release, and cell death. The chloride channel inhibitor furosemide prevented intracellular alkalization, Bax translocation, cytochrome *c* release, and cell death. Translocation of full-length Bid to the mitochondria was also prevented by furosemide. The cleavage product of Bid degradation (truncated Bid, tBid) was not detectable in the mitochondria. Its accumulation in the cytosol was prevented by furosemide. Apoptosis induced by tumor necrosis factor- α (TNF) lowered pH_i, an effect also accompanied by Bax translocation, cytochrome *c* release, and cell killing. Furosemide prevented all of these events. TNF induced a depletion of full-length Bid from the mitochondria and the cytosol but induced an accumulation of mitochondrial tBid. Furosemide only delayed full-length Bid depletion and tBid accumulation. The caspase 8 inhibitor IETD did not prevent the translocation of Bax. Although IETD did inhibit the cleavage of Bid and the accumulation of tBid, cell killing was reduced only slightly. It is concluded that with either staurosporine or TNF a furosemide-sensitive change in pH_i is linked to Bax translocation, cytochrome *c* release, and cell killing. With TNF Bax translocation occurs as Bid is depleted and can be dissociated from the accumulation of tBid. With staurosporine a role for full-length Bid in Bax translocation cannot be excluded but is not necessary as evidenced by the data with TNF.

Bax is a proapoptotic member of the Bcl-2 family of proteins that is implicated in the pathogenesis of cell death in an increasing number of models of apoptosis both *in vivo* and *in vitro*. In particular, Bax has emerged as a mediator of the mitochondrial phase of apoptosis, a process that culminates in the release of cytochrome *c* from the intermitochondrial space and the activation of effector caspases.

Bax is constitutively present in many cell types that undergo apoptosis in response to a variety of stimuli. By contrast, in other cells Bax expression is induced by activation of p53 upon

damage to the genome or interference with the normal progression of the cell cycle (reviewed in Ref. 1). Whether constitutively expressed or induced, however, the primary action of Bax is a consequence of its translocation from the cytosol to the mitochondria. Clearly, where Bax is constitutively present in the cytosol, a mechanism that is referred to as Bax activation follows the introduction of an apoptotic stimulus to cause translocation of the protein to the mitochondria. Translocation of preformed Bax from the cytosol to the mitochondria has been reported with a variety of apoptotic stimuli (2–11). In the situation where Bax is synthesized upon introduction of an apoptotic stimulus, evidence exists that a similar mechanism of Bax activation is operative to control its translocation to the mitochondria (12).

In the absence of an activating signal, Bax translocation is likely prevented by the interaction of the C-terminal segment of the protein with its N-terminal domain, an effect that prevents insertion of the hydrophobic C terminus into the mitochondria (2, 13–16). In fact, deletion of the N terminus of Bax resulted in mitochondrial localization of the molecule in the absence of an apoptotic stimulus (13, 14).

At least two models have been proposed to account for the mechanism of Bax activation in apoptosis. An interaction of Bax with another proapoptotic protein may trigger Bax activation. In particular, the proapoptotic protein Bid induced a change in Bax conformation that resulted in exposure of its N-terminal domain and its integration into mitochondrial membranes (6, 7). The evidence that such a Bid-dependent mechanism of Bax activation operates in an intact cell is based largely on staurosporine-induced apoptosis (6). Mice deficient in Bid, however, were not resistant to the cell killing by staurosporine (17). Another concern with the Bid-dependent model is that the problem of Bax activation simply becomes the problem of Bid activation. Like Bax cytosolic Bid exists in an inactive conformation (18, 19). Caspase-8 cleaved Bid to generate an active truncated Bid (tBid)¹ that induced the release of cytochrome *c* from mitochondria (20–22). Engagement of either the TNF or Fas receptor recruited first FADD and then the proenzyme form of caspase-8. Upon treatment with an anti-Fas antibody or with TNF, hepatocytes, fibroblasts, or thymocytes derived from Bid-deficient mice activate caspase-8 but survive longer than their comparable wild-type cells (17). By contrast, other inducers of apoptosis, including staurosporine, dexamethasone, and γ -irradiation, killed Bid-deficient and wild-type cells to the same extent (17).

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¹ The abbreviations used are: tBid, truncated Bid; TNF, tumor necrosis factor- α ; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; BCECF-AM, 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; CHX, cycloheximide; STR, staurosporine; pH_i, intracellular pH; PIPES, 1,4-piperazinediethanesulfonic acid.

A second model of Bax activation proposes that a change in the pH of the cytosol alters the conformation of the protein, an effect that results in exposure of the membrane-targeting C-terminal domain and translocation to the mitochondria. Withdrawal of IL-7 from T lymphocytes (D1 cells) that are dependent on this cytokine for continued viability produced a rise in intracellular pH, Bax translocation, and apoptotic cell death (15). Similarly, alkalization and Bax translocation were observed after IL-3 withdrawal from a different cell line (15). More recently, U937 cells treated with C₂-ceramide evidenced an early rise in intracellular pH associated with a conformational change in Bax (23).

Alkalization itself induced Bax translocation, as demonstrated by two experiments with D1 cells maintained in IL-7 (15). First, Bax translocation occurred upon incubation of homogenates in buffers of pH 7.8 or higher (15). In the second experiment, nigericin was used to equilibrate intact cells with the extracellular pH. Again, Bax translocation was observed only upon incubation in alkaline buffers (15).

The present study considered the role of changes in intracellular pH in the mechanism of Bax activation in HeLa cells upon induction of apoptosis by either staurosporine or TNF. In both cases a change in intracellular pH was accompanied by translocation of Bax to the mitochondria with consequent release of cytochrome *c* and the death of the cells. The chloride channel inhibitor furosemide prevented the pH change induced by either staurosporine or TNF. At the same time, furosemide prevented Bax translocation, cytochrome *c* release, and cell death. In TNF-intoxicated cells, furosemide did not prevent the cleavage of Bid. In staurosporine-intoxicated cells, furosemide did prevent the translocation of full-length Bid to the mitochondria.

EXPERIMENTAL PROCEDURES

Cell Line—HeLa cells (ATCC-CC-1) were maintained in 25-cm² polystyrene flasks with 5 ml of Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Herndon, VA) containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (complete DMEM). The cells were 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. Following overnight incubation, the cells were washed twice with PBS and placed in DMEM without serum. The day after, the cells were washed with PBS, and the medium was replaced with Hank's balanced salt solution (HBSS) without serum, which was supplemented with 2 mM L-glutamine and 1 mM glycine and adjusted to pH 7.4. Cells were incubated for 90 min at 37 °C prior to treatment.

Treatment Protocols—All treatments were performed in HBSS medium without serum at pH 7.4. In all experiments staurosporine (Biomol) was added to a final concentration of 150 nM. STR was dissolved in Me₂SO and added to the cells in 0.2% volume. Human TNF (Calbiochem) was dissolved in sterile PBS containing 0.5% bovine serum albumin and added to the cultures at a final concentration of 10 ng/ml. Cycloheximide (Sigma) was dissolved in PBS and added at a final concentration of 10 μM. Furosemide (Sigma) was dissolved in Me₂SO and added in 0.5% volume at a final concentration of 2 mM. Where indicated, the cells were pretreated for 30 min with furosemide before addition of either STR or TNF and CHX. The cell permeable caspase-8 inhibitor IETD-fluoromethyl ketone (Kamiya Biomedical Co., Seattle, WA) was dissolved in Me₂SO 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 concentration used.

Measurement of Cell Viability—Cell viability was determined at the times indicated in the text by the release of lactate dehydrogenase into the culture medium as described previously (24).

Intracellular pH Determination—Cells (10⁵) were plated on 12-mm glass coverslips placed in a 24-well plate. HeLa cells were treated in HBSS without serum and loaded subsequently with 1 μM (2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein), acetoxymethyl ester (BCECF-AM) (Molecular Probes, Eugene, OR) for 30 min at 37 °C.

BCECF-AM was dissolved in Me₂SO according to the manufacturer's instructions. Following a 30-min incubation, each coverslip was washed with PBS and transferred to a cuvette with 2 ml of HBSS. The pH-sensitive fluorescence was assessed on a PerkinElmer Life Sciences LS4 fluorescence spectrophotometer. The excitation was set alternatively at 490 or 440 nm, and the emission was set at 535 nm. The pH_i values were determined from a standard curve based upon the ratio of fluorescence intensities measured at the two excitation wavelengths.

pH Calibration Curve—HeLa cells grown on coverslips were transferred to a high K⁺ buffer (30 mM NaCl, 120 mM KCl, 1 mM CaCl₂, 0.5 mM MgSO₄, 1 mM NaH₂PO₄, 5 mM glucose, 10 mM HEPES, 10 mM PIPES), adjusted to various pH values between 6.0 to 8.0 in the presence of 1 μM BCECF-AM. To adjust the intracellular pH to that of the extracellular buffer, the cells were treated for 30 min at 37 °C with 10 μM nigericin (Sigma). By plotting the fluorescence ratio versus the pH values, a pH calibration curve was generated.

Isolation of Cytosolic and Mitochondrial Fractions—Cells (4 × 10⁶) were plated in 75-cm² polystyrene flasks. Following treatment the cells were harvested by centrifugation at 750 × *g* for 10 min at 4 °C. The cell pellets were resuspended in 1 ml of 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 250 mM sucrose. The cells were broken open with six passages through a 26-gauge needle applied to a 1-ml syringe. The homogenate was centrifuged at 750 × *g* for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatant was transferred to a high speed centrifuge tube. Centrifugation was conducted at 10,000 × *g* for 15 min at 4 °C. The resulting mitochondrial pellet was lysed in 50 μl of 20 mM Tris, pH 7.4, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100. The supernatant from the 10,000 × *g* spin was centrifuged at 100,000 × *g* (60 min at 4 °C), and the resulting supernatant was used for preparation of cytosol. The cytosolic fraction was concentrated through a Microcon YM-10 centrifugal filter device (Millipore, Bedford, MA). Protein content of each fraction was determined by the bicinchoninic acid assay (Sigma).

Western Blot Assays—Equivalent amounts of protein were electrophoresed on SDS-polyacrylamide gels. Kaleidoscope prestained standards (Bio-Rad) were used to determine molecular weight. The gels were then electroblotted onto nitrocellulose membranes. Bid was detected with a rabbit polyclonal antibody (BIOSOURCE, Camarillo, CA). Bax was detected with a rabbit polyclonal antibody (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA). Cytochrome *c* was detected by a monoclonal antibody (Pharmingen). To monitor the purity of the subcellular preparations, the same nitrocellulose blots used for the determination of Bax, Bid, and cytochrome *c* were also stained for either COX4 (cytochrome *c* oxidase subunit IV) or β-actin (antihuman mouse monoclonal antibodies against either COX4 (Clontech, Palo Alto, CA) or β-actin (Santa Cruz Biotechnology), respectively). In each case, the relevant protein was visualized by staining with the appropriate secondary horseradish peroxidase-labeled antibody followed by enhanced chemiluminescence.

RESULTS

Staurosporine Raised Intracellular pH—The fluorescence excitation profile of the indicator dye BCECF is pH-dependent over the range of possible cytoplasmic pH and was, accordingly, used here for determining pH_i. Treatment of HeLa cells with 150 nM staurosporine produced a rise in pH_i. Fig 1 shows that an increased pH_i was detected within 3 h and reached a peak of pH 8.0 after 4 h, a rise of at least 0.6 units over the initial pH. The intracellular pH remained elevated through the fifth hour after treatment with staurosporine. The chloride channel inhibitor furosemide (25) prevented this intracellular alkalization produced by staurosporine. Fig. 1 shows that throughout the entire 5-h duration of the experiment, pH_i remained at the initial level in the presence of both staurosporine and furosemide.

Furosemide Prevented the Translocation of Bax and Bid to the Mitochondria upon Treatment with Staurosporine—The proapoptotic proteins Bax and Bid are expressed constitutively in HeLa cells. Staurosporine produced a redistribution of both Bax and Bid from the cytosol to the mitochondria (6) (Fig. 2). Fig. 2 compares the content of Bax in the mitochondria and in the cytosol within 6 h of the treatment of HeLa cells with either

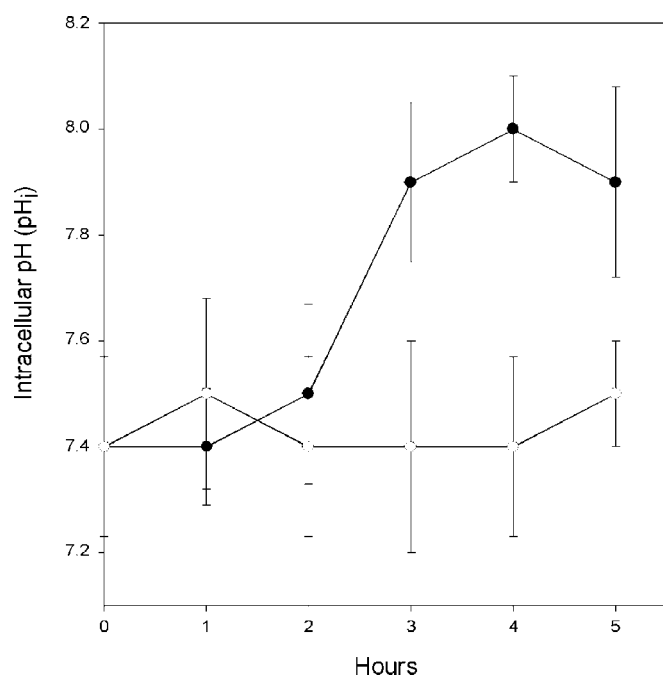


FIG. 1. **Intracellular pH (pH_i) upon treatment with staurosporine alone or staurosporine plus furosemide.** HeLa cells were treated with staurosporine alone (closed circles) or staurosporine plus furosemide (open circles). At the times indicated, pH_i was determined as described under "Experimental Procedures." Results are the mean ± S.D. of three separate experiments.

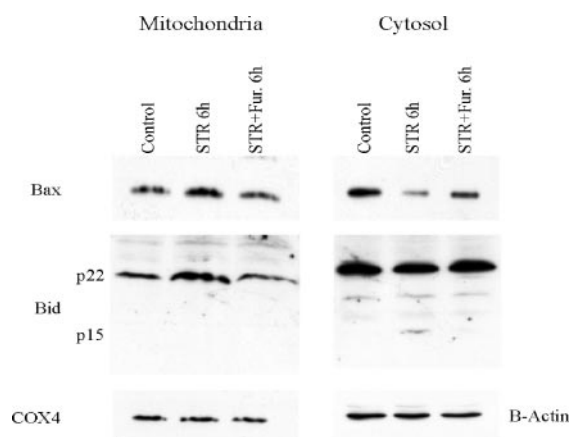


FIG. 2. **Altered intracellular distribution of Bax and Bid upon treatment with staurosporine is prevented by furosemide.** HeLa cells were treated with staurosporine alone or staurosporine plus furosemide (Fur.). Control cells were untreated. After 6 h the cells were processed to obtain a mitochondrial and cytosolic fraction as described under "Experimental Procedures." The contents of Bax, Bid, COX4, and β -actin were determined by SDS-PAGE and Western blotting.

staurosporine alone or staurosporine and furosemide. With staurosporine alone, the content of Bax increased in the mitochondria and decreased in the cytosol. Furosemide prevented this translocation of Bax. When the HeLa cells were treated with both furosemide and staurosporine, the content of Bax in the mitochondria did not increase, and that in the cytosol decreased only slightly (Fig. 2).

A similar effect of furosemide occurred with the translocation of Bid. Full-length Bid (p22 in Fig. 2) accumulated in the mitochondria in response to staurosporine. At the same time, the content of full-length Bid in the cytosol decreased (Fig. 2). The cleavage product of Bid (p15 in Fig. 2) was present in the cytosol but not detectable in the mitochondria 6 h after treatment with staurosporine alone. Furosemide prevented the

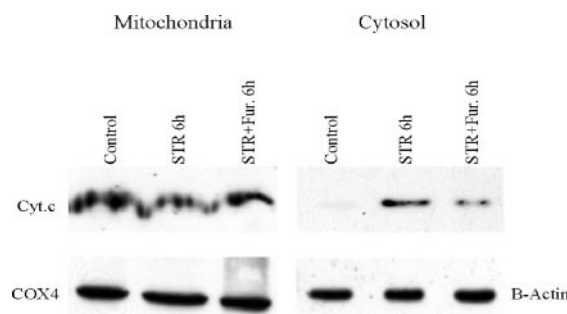


FIG. 3. **Cytochrome c release upon treatment with staurosporine and its reduction by furosemide.** HeLa cells were treated with staurosporine alone or staurosporine plus furosemide (Fur.). Control cells were untreated. After 6 h the cells were processed to obtain a mitochondrial and cytosolic fraction as described under "Experimental Procedures." The contents of cytochrome c (Cyt. c), COX4, and β -actin were determined by SDS-PAGE and Western blotting.

staurosporine-induced increase in full-length Bid in the mitochondria and the decrease in the cytosol (Fig. 2). The accumulation of tBid in the cytosol within 6 h of treatment with staurosporine alone did not occur in the presence of furosemide (Fig. 2). Importantly, the changes in the intracellular distribution of Bax and Bid cannot be interpreted as reflecting differences in either the number of mitochondria or the relative content of cytosolic proteins compared under the various conditions illustrated in Fig. 2. The content of the mitochondrial marker protein COX4 did not vary under the conditions studied (Fig. 2). Similarly, the content of the cytosolic marker protein β -actin did not vary under the conditions studied (Fig. 2).

Furosemide Reduced Cytochrome c Release from the Mitochondria—In HeLa cells treated with staurosporine, the translocation of Bax and Bid to the mitochondria is accompanied by induction of the mitochondrial permeability transition and the release of cytochrome c into the cytosol (11). In parallel with the inhibition of Bax and Bid translocation, furosemide reduced the loss of cytochrome c from the mitochondria and, in turn, its accumulation in the cytosol substantially (Fig. 3). The content of cytochrome c in the mitochondria decreased, and the content in the cytosol increased within 6 h of treatment with staurosporine (Fig. 3). Furosemide reduced this change in the distribution of cytochrome c (Fig. 3). Again, the purity of the mitochondrial and the cytosolic fractions was assessed by the presence of the COX4 and the β -actin proteins, respectively (Fig. 3).

Furosemide Prevented the Killing of HeLa Cells by Staurosporine—The biochemical effects of furosemide on staurosporine-intoxicated HeLa cells were reflected in a prevention of the cell killing. Treatment of HeLa cells with 150 nM staurosporine killed almost 50% of the cells within 20 h (Fig. 4). Addition of furosemide to the culture medium 30 min prior to treatment with staurosporine prevented this cell killing. After 20 h the number of dead cells in the presence of both furosemide and staurosporine was not significantly different from that in the control, untreated cultures (Fig. 4).

Furosemide Reduced the Killing of HeLa Cells by TNF—The next experiments considered the effect of furosemide on the another model of apoptosis in HeLa cells. Like staurosporine, TNF has been used widely to induce apoptosis in a variety of cell types. Most cells are not killed by exposure to TNF alone. Upon inhibition of RNA or protein synthesis, however, these same cells become sensitive to this cytokine. In the presence of the protein synthesis inhibitor cycloheximide, TNF killed almost 60% of the HeLa cells within 16 h (Fig. 5). Furosemide reduced the extent of this cell killing substantially. When furosemide was added to the culture medium 30 min prior to that

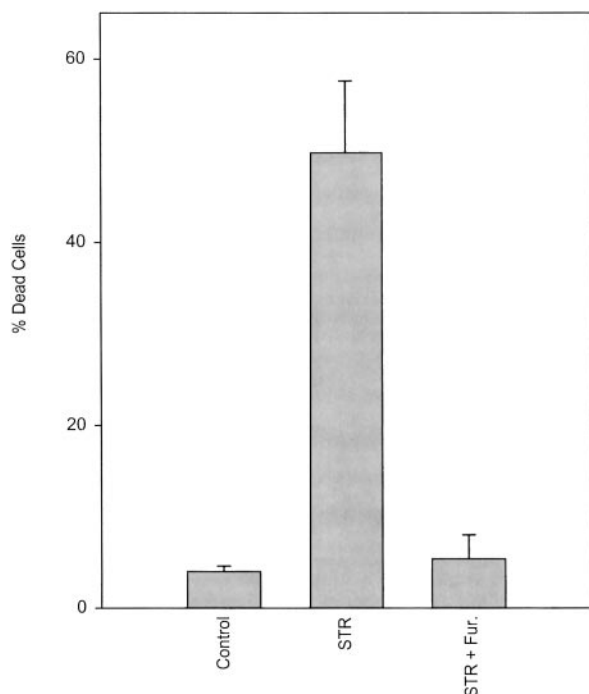


FIG. 4. Cell killing upon treatment with staurosporine and its prevention by furosemide. HeLa cells were treated for 20 h with staurosporine alone or staurosporine plus furosemide (*Fur.*). Control cells were untreated. The viability of the cells was determined as described under "Experimental Procedures." Results are the mean \pm S.D. of three separate experiments.

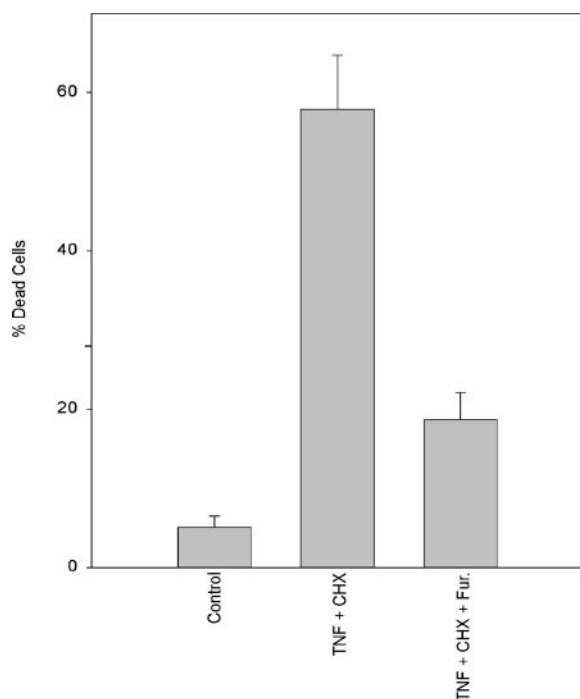


FIG. 5. Cell killing upon treatment with TNF and its reduction by furosemide. HeLa cells were treated for 16 h with TNF plus cycloheximide or TNF plus cycloheximide and furosemide (*Fur.*). Control cells were untreated. The viability of the cells was determined as described under "Experimental Procedures." Results are the mean \pm S.D. of three separate experiments.

of TNF and cycloheximide, less than 20% of the cells were dead 16 h later (Fig. 5).

TNF Lowered Intracellular pH—The fluorescent indicator dye BCECF was again used to determine the effect of TNF on the intracellular pH of HeLa cells. Table I shows that TNF in

TABLE I
Intracellular acidification upon treatment with TNF and its prevention by furosemide

HeLa cells were treated for 3 h with TNF plus cycloheximide or TNF plus cycloheximide and furosemide. Control cells were untreated. The intracellular pH (pH_i) was then determined with the use of BCECF-AM as described under "Experimental Procedures." Results are the mean \pm S.D. of three separate experiments.

Treatment	pH_i
Control	7.1 ± 0.2
TNF + CHX	6.6 ± 0.2
TNF + CHX + furosemide	7.2 ± 0.2

the presence of cycloheximide produced an intracellular acidification. Within 3 h of exposure to TNF, the pH_i decreased by one-half pH unit to 6.6 from the initial pH observed in the control cells. This intracellular acidification was prevented by furosemide. In the presence of TNF and furosemide, pH_i was essentially the same as in the controls (Table I).

Furosemide Reduced Translocation of Bax Induced by TNF—As occurred with staurosporine (Fig. 2), TNF produced a translocation of Bax from the cytosol to the mitochondria. Fig. 6 shows that the content of Bax in the mitochondria was increased within 4 h of the exposure of HeLa cells to TNF and cycloheximide. At the same time, the content of Bax in the cytosol decreased (Fig. 6). Furosemide reduced this translocation of Bax. When HeLa cells were pretreated with furosemide, the increase of Bax in the mitochondria was less than with TNF and cycloheximide alone (Fig. 6). Similarly, the decrease of Bax in the cytosol was less in the presence of furosemide than with TNF and cycloheximide alone (Fig. 6). Fig. 6 also shows that the content of the mitochondrial marker COX4 and the cytosolic marker β -actin did not vary under the conditions studied.

A trivial explanation for the effect of furosemide on Bax translocation might be an interaction of the drug directly with Bax to change its conformation independently of a change in pH_i . To rule out this possibility, control and TNF-treated cells were homogenized and fractionated in the presence or absence of furosemide. Western blots were obtained with an antibody that detected an altered Bax conformation. The presence of furosemide did not affect the content of Bax in any fraction (data not shown).

Furosemide Did Not Prevent Changes in Bid Metabolism Produced by TNF—In HeLa cells, the metabolism of Bax is similar in the two models of apoptosis studied here (see Figs. 2 and 6). By contrast, the metabolism of Bid is different in the two models, as is the effect of furosemide on these changes. Within 2 h of the treatment of HeLa cells with TNF and cycloheximide, full-length Bid is decreased in both the mitochondrial and cytosolic fractions (Fig. 7A). Within 4 h full-length Bid is almost undetectable in the mitochondria and depleted substantially in the cytosol (Fig. 7B). After 2 h, this loss of full-length Bid is accompanied by the presence of its cleavage product, tBid (p15), in the mitochondria and less so in the cytosol (Fig. 7A). After 4 h, the content of tBid in the mitochondria is less than at 2 h, and it is undetectable in the cytosol (Fig. 7B). After 4 h the presence of furosemide had no effect on the changes in Bid metabolism produced by TNF and cycloheximide. As with TNF and cycloheximide alone, full-length Bid was essentially undetectable in the mitochondria in the presence of furosemide (Fig. 7B). tBid could be observed in the mitochondria with furosemide, a result similar to that with TNF alone (Fig. 7B). In the cytosol furosemide did not prevent the substantial depletion of full-length Bid, and there was similarly no detectable presence of tBid (Fig. 7B).

The depletion of Bid that occurs with TNF is slowed by furosemide. Although by 4 h, furosemide had no effect on Bid

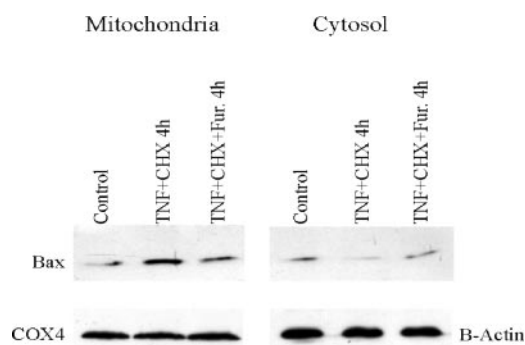
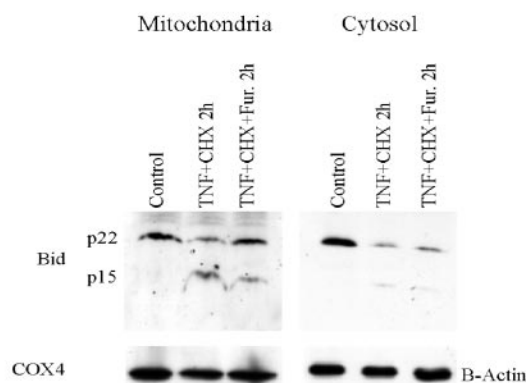


FIG. 6. Translocation of Bax to the mitochondria upon treatment with TNF is reduced by furosemide. HeLa cells were treated for 4 h with TNF plus cycloheximide or TNF plus cycloheximide and furosemide (*Fur.*). Control cells were untreated. The cells were then processed to obtain mitochondrial and cytosolic fractions as described under "Experimental Procedures." The contents of Bax, COX4, and β -actin were determined by SDS-PAGE and Western blotting.

A



B

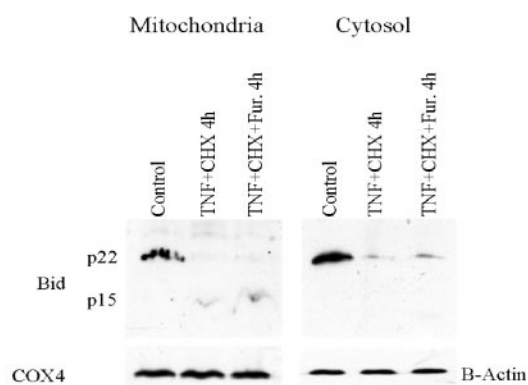


FIG. 7. The metabolism of Bid upon treatment with TNF or TNF plus furosemide. HeLa cells were treated for either 2 h (A) or 4 h (B) with TNF plus cycloheximide or TNF plus cycloheximide and furosemide (*Fur.*). Control cells were untreated. The cells were then processed to obtain mitochondrial and cytosolic fractions as described under "Experimental Procedures." The contents of Bid, COX4, and β -actin were determined by SDS-PAGE and Western blotting.

metabolism, there were differences after 2 h. In the presence of furosemide, the depletion of full-length Bid from both the mitochondria and cytosol was somewhat less than with TNF alone (Fig. 7A). At the same time, the accumulation of tBid in the mitochondria was less than with TNF alone at 2 h (Fig. 7A). All

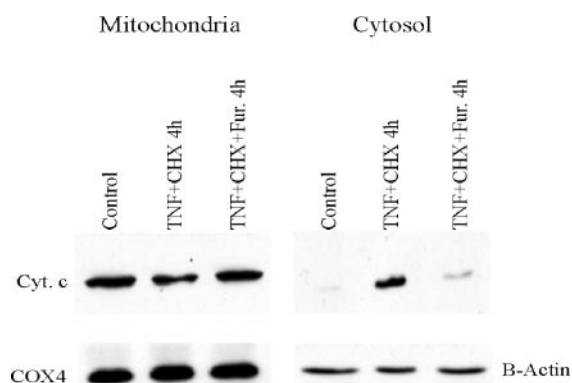


FIG. 8. Cytochrome c release upon treatment with TNF and its reduction by furosemide. HeLa cells were treated for 4 h with TNF plus cycloheximide or TNF plus cycloheximide and furosemide (*Fur.*). Control cells were untreated. The cells were then processed to obtain mitochondrial and cytosolic fractions as described under "Experimental Procedures." The contents of cytochrome c (*Cyt. c*), COX4, and β -actin were determined by SDS-PAGE and Western blotting.

of the changes in Bid metabolism illustrated in Fig. 7, A and B occurred in mitochondrial and cytosolic fractions that did not differ in their content of the mitochondrial marker COX4 or the cytosolic marker β -actin, respectively.

Furosemide Reduced Cytochrome c Release from the Mitochondria Induced by TNF—In parallel with the inhibition of Bax translocation occurring with TNF, furosemide reduced the loss of cytochrome c from the mitochondria and, in turn, its accumulation in the cytosol (Fig. 8). The content of cytochrome c in the mitochondria decreased, and the content in the cytosol increased within 4 h of treatment with TNF and cycloheximide (Fig. 8). Furosemide reduced this change in the distribution of cytochrome c substantially (Fig. 8). Again, the purity of the mitochondrial and the cytosolic fractions was assessed by the presence of the COX4 and the β -actin proteins, respectively (Fig. 8).

Dissociation of the Mitochondrial Accumulation of tBid from Bax Translocation and the Cell Killing with TNF—The depletion of mitochondrial full-length Bid in Fig. 7 would argue that it is not required for either the translocation of Bax to the mitochondria or the subsequent killing of the HeLa cells by TNF. The same data, however, do not necessarily exclude a role for tBid in both events. The role of tBid in the translocation of Bax and the killing of HeLa cells by TNF was explored with the caspase-8 inhibitor IETD (26).

IETD did not prevent the translocation of Bax to the mitochondria induced by TNF (Fig. 9). In the presence or absence of IETD, within 4 h of the treatment of HeLa cells with TNF and cycloheximide, the Bax content of the mitochondria increased (Fig. 9). At the same time, the content of Bax decreased in the cytosol, but to a lesser extent in the presence of IETD than its absence (Fig. 9). By contrast, both of the changes in Bid metabolism that occurred with TNF were prevented by IETD. In the mitochondria, there was no loss of full-length Bid and, importantly, no accumulation of tBid (Fig. 9). In the cytosol, full-length Bid was not lost in the presence of IETD in cells treated with TNF, and there was no detectable accumulation of tBid (Fig. 9). Despite this prevention of the formation of tBid, IETD did not prevent the release of cytochrome c from the mitochondria. Cytochrome c was lost from the mitochondria and accumulated in the cytosol in the presence or absence of IETD (Fig. 9). A substantial loss of viability occurred in the presence of the caspase-8 inhibitor IETD (Table II). This cell killing correlated with the Bax translocation and cytochrome c release that persisted despite the presence of IETD (Fig. 9) and contrasts with the inhibition of Bid metabolism.

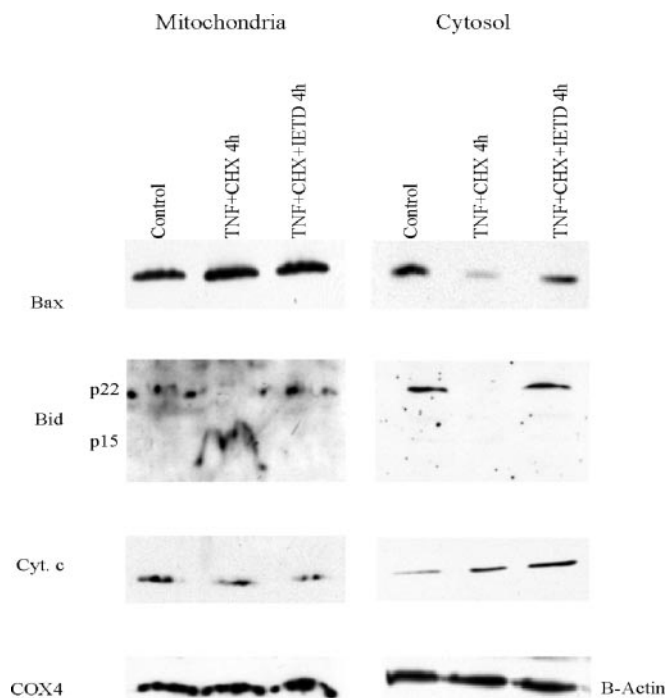


FIG. 9. The effect of the caspase-8 inhibitor IETD on Bax, Bid, and cytochrome *c* upon treatment with TNF. HeLa cells were treated for 4 h with TNF plus cycloheximide or TNF plus cycloheximide and IETD. Control cells were untreated. The cells were then processed to obtain mitochondrial and cytosolic fractions as described under "Experimental Procedures." The contents of Bax, Bid, cytochrome *c* (Cyt. *c*), COX4, and β -actin were determined by SDS-PAGE and Western blotting.

TABLE II

IETD reduces only slightly the cell killing by TNF

HeLa cells were treated for 20 h with TNF plus cycloheximide or TNF plus cycloheximide and IETD. Cell viability was determined as described under "Experimental Procedures." Results are the mean \pm S.D. of three separate experiments.

Treatment	% Dead cells
Control	4.3 \pm 0.8
TNF + CHX	68.6 \pm 4.5
TNF + CHX + IETD	49.8 \pm 5.3

DISCUSSION

The data presented above document that a change in pH_i occurs in response to the induction of apoptosis in HeLa cells by either staurosporine or TNF. In addition to the change in pH_i , three other events were monitored, namely the translocation of cytosolic Bax to the mitochondria, the release of mitochondrial cytochrome *c* from these organelles, and the loss of cell viability. The chloride channel inhibitor furosemide prevented the change in pH_i with either staurosporine or TNF. At the same time, furosemide prevented Bax translocation, cytochrome *c* release, and cell killing. It is generally accepted that Bax translocation is related causally to cytochrome *c* release, an event that activates caspases to effect the loss of cell viability. Thus, the change in pH_i with staurosporine or TNF must occur upstream of the translocation of Bax. For example, it is not possible that the change in pH_i results in caspase inhibition and, thus, preservation of cell viability. With such a scenario, furosemide would have inhibited cell killing without having an effect on Bax translocation and cytochrome *c* release. Alternatively and independently of chloride channel inhibition, furosemide could conceivably interact directly with Bax to change its conformation or alter the mitochondria in such a manner as to prevent Bax translocation. The present study, however,

found no evidence of such an interaction between furosemide and Bax. Previously, furosemide did not alter mitochondrial glutathione content nor affect respiration supported by complex I or II (27). By contrast, a case can be made for a causal association between the pH_i change and the translocation of Bax to the mitochondria. The conformation of Bax is known to change with pH, and conformational changes in Bax promote its insertion into mitochondrial membranes (15, 23). Thus, it is reasonable to conclude that the data presented here support the hypothesis that, in the apoptosis produced by either staurosporine or TNF, the inducing agent changes intracellular pH to produce a conformational change in Bax. Such Bax activation is followed by its translocation to the mitochondria, the release of cytochrome *c*, and eventually the death of the cell.

The data presented do not link directly a change in pH_i to Bax translocation. Rather they link a furosemide-sensitive event to this result. It is possible that furosemide-sensitive alterations in the internal ionic milieu other than a change in pH are responsible directly for a change in Bax conformation and are reflected in an altered pH_i . In this regard, pinacidil, an activator of ATP-sensitive K^+ channels, induced apoptosis in HepG2 cells (28). Pinacidil increased intracellular sodium and potassium concentrations. The effect of pinacidil on pH_i was not considered (28). Furosemide prevented these ionic changes and the apoptosis (28). It deserves emphasis, however, that a change in pH altered the conformation of Bax *in vitro*, as assessed by increased protease sensitivity, exposure of the N-terminal epitopes, and exposure of the C-terminal hydrophobic domain (15). In addition to the apoptotic stimuli used here, intracellular alkalinization has been described upon withdrawal of the cytokines IL-7 and IL-3 from lymphocytic cell lines (15) or the treatment of a monocytic cell line with ceramide (23). By contrast, a decrease in intracellular pH accompanied the apoptosis induced by IL-2 withdrawal from CTLL-2 cells (29) or by activation in Jurkat cells of the Fas receptor (30), a member, interestingly, of the TNF receptor superfamily. We have shown in CEM cells that Fas receptor activation is accompanied by Bax translocation to the mitochondria.² Thus, it remains likely that the change in pH_i affects Bax conformation directly, thereby promoting its translocation to the mitochondria.

Mutagenesis of Bax revealed that both positively and negatively charged amino acids contribute to the pH dependence of Bax conformation (15). Thus, a sufficiently acidic pH also should alter Bax conformation in a manner similar to the affect of alkalinization. It should be noted that the pH obtained upon TNF stimulation in the present study was more acidic than any pH assessed previously for an affect on Bax conformation (15).

The mechanism whereby both staurosporine and TNF change the intracellular pH likely relates to effects on chloride ion channels in the plasma membrane of the cell. As one of the principal intracellular inorganic anions, chloride participates as an obligatory counterion in various ion transport processes, including those involved in the regulation of intracellular pH and cell volume (31). Various chloride-transport proteins and channels have been identified. Their activity is regulated by phosphorylation, either directly or indirectly by phosphorylation of regulatory proteins, as demonstrated for the chloride transporter $Na^+-K^+-Cl^-$ -cotransporter (32). Staurosporine is a protein kinase inhibitor that has been shown to activate chloride transport (33), whereas phosphatase inhibitors inactivate transport (34). By contrast, other chloride channels are activated by kinases signaled through ceramide (35). Ceramide is known to signal TNF by activation of kinase cascades, as well

² M. Tafani and J. L. Farber, unpublished data.

as by a ceramide-activated protein phosphatase. In this regard, TNF is reported to activate K^+ and Cl^- channels in a variety of cells, including neutrophils, neurons, and hepatoma cells (36).

Currently, it is not known which of the various chloride transporters and channels are involved in the apoptotic phenomena observed in the present study. The fact that staurosporine and TNF induced opposite effects upon pH_i may reflect merely that these two agents act upon different chloride transport proteins. Typically, activation of chloride transport may result in intracellular acidification. Thus, it is worth noting that in epithelial cells an additional mechanism of chloride transport results in alkalinization (37).

The translocation of Bax to mitochondria with subsequent release of cytochrome *c* is consistent with the previous documentation that the cytotoxicity of TNF depended upon induction of the mitochondrial permeability transition (38, 39). Purified, recombinant Bax induced the mitochondrial permeability transition in isolated mitochondria *in vitro* (40), and induction of the overexpression of Bax in stably transfected cells produced apoptosis that depended upon induction of mitochondrial permeability transition (41). Accordingly, an increasingly defined understanding of the mechanisms underlying the apoptosis induced by TNF is at hand. In cells that have lost or are prevented from expressing an inductive resistance to cell killing, binding of TNF to its cell surface receptor leads to the activation of Bax by a change in intracellular pH. The signal transduction pathway responsible for the change in pH_i needs to be explored. Activated Bax translocates to the mitochondria. In turn, this translocation induces the mitochondrial permeability transition, causing the release of cytochrome *c* and the activation of effector caspases. The precise mechanism whereby caspase activity results in the loss of cell viability remains to be defined precisely.

In addition to translocation of Bax, the apoptosis induced by either staurosporine or TNF produced changes in another proapoptotic protein, namely Bid. Prevention of apoptosis with furosemide modified these changes in Bid. The effect of a caspase-8 inhibitor on the TNF-induced alterations in Bid metabolism was also considered. Taken together, the data pertaining to Bid metabolism allow a conclusion with respect to the role of this protein in the models studied.

Full-length Bid translocated from the cytosol to the mitochondria in HeLa cells treated with staurosporine, an observation in agreement with data from our and other laboratories published previously (6, 7, 11). The Bid content of the cytosol decreased, a consequence of both mitochondrial translocation and cleavage of Bid. tBid accumulated in the cytosol but was not detectable in the mitochondria. Furosemide prevented all of these changes. Thus, in the case of staurosporine, the data could be interpreted as supporting a role in the HeLa cell for the mitochondrial translocation of full-length Bid, either alone or in association with that of Bax, in the genesis of cytochrome *c* release. Two facts argue against such a function for Bid translocation. First, embryonic fibroblasts derived from Bid knock-out mice ($Bid^{-/-}$) were not resistant to the apoptosis induced by staurosporine (17). Second, in the present study the Bax translocation that occurred with TNF was not accompanied by that of full-length Bid. Rather, TNF produced a substantial depletion of full-length Bid. The fact that furosemide delayed the depletion of mitochondrial Bid by TNF does not argue in favor of a role for full-length Bid. Furosemide simply maintained the mitochondrial content of Bid at the level prior to stimulation of the cells with TNF. At the same time, furosemide prevented Bax translocation and cytochrome *c* release. Thus, the translocation of full-length Bid would not seem to be required for Bax to translocate to the mitochondria in the

pathogenesis of the apoptosis induced by either staurosporine or TNF.

Is there a role for tBid with either staurosporine or TNF? Mitochondrial accumulation of tBid was not detected with staurosporine. The presence of tBid in the cytosol with staurosporine most likely reflects the caspase activation that follows upon the release of cytochrome *c* from the mitochondria. By contrast, tBid was detected readily in the mitochondria following treatment of HeLa cells with TNF, an effect that was accompanied by Bax translocation and inhibited by furosemide. Such data might be used to support the hypothesis that tBid assists the translocation of Bax or acts alone to cause the release of cytochrome *c*. The effect of the caspase-8 inhibitor IETD argues against such an interpretation. In the presence of IETD, TNF caused the translocation of Bax and the death of the cells. There was no detectable mitochondrial accumulation of tBid, however, in the presence of IETD (Fig. 9). Thus, Bax translocation with TNF can occur either with a concurrent depletion of full-length Bid or without a concurrent accumulation of tBid. Accordingly, our data do not support a role for Bid or its metabolite tBid in the release of cytochrome *c* from mitochondria following the exposure of HeLa cells to TNF.

Other than, of course, the data relating to Bid metabolism, the findings here are similar to those reported with $Bid^{-/-}$ mice (17). Mouse embryo fibroblasts prepared from $Bid^{-/-}$ mice evidenced Bax translocation to the mitochondria upon treatment with TNF, an effect that was associated with only a slight delay in cell killing as compared with the effect of TNF on $Bid^{+/+}$ cells (42). The major difference between $Bid^{-/-}$ and $Bid^{+/+}$ mice was the relative resistance of hepatocytes from the former to apoptotic injury upon activation of the Fas receptor *in vivo* and *in vitro* (17). Cell death mediated by activation of the Fas receptor has been implicated in several important biological functions, including the removal of autoreactive lymphocytes and the cell killing by cytotoxic ($CD8^+$) lymphocytes. It is possible that Bid evolved as a specific mediator of cell death upon Fas receptor activation. Mice (43, 44) and humans (45) that are genetically deficient in the function of the Fas death receptor have been reported. Both the Fas-deficient mice and humans develop a marked lymphoproliferative disorder with generalized lymphadenopathy and splenomegaly accompanied by autoimmune disease (43–45). Although there has been no mention of such a lymphoproliferative disorder in the $Bid^{-/-}$ mice, the spleen and thymus were said to be normal with respect to weight and histology (17). It would seem that $Bid^{-/-}$ mice do not show evidence of deficient Fas function other than in the liver. Thus, the hepatocyte remains, at present, the only cell that may depend upon Bid to undergo apoptosis with activation of the Fas receptor. Liver cells express Bax, as well as Bid, and it is not at all clear why they should be restricted to using Bid to mediate Fas-induced apoptosis. Hepatocytes are clearly not restricted to using Bid in the receptor-mediated cell death produced by TNF. TNF-mediated hepatotoxicity induced by endotoxin/galactosamine treatment was delayed only slightly in $Bid^{-/-}$ mice (46). Within 6 h the level of serum enzymes that indicate liver injury was not significantly different between wild-type and Bid-deficient mice. Within 7–8 h the liver histology of $Bid^{-/-}$ mice became comparable with that of the wild-type mice (46).

In summary, a change in intracellular pH occurs upon induction of apoptosis in HeLa cells by staurosporine or TNF. The change in pH_i is followed by Bax translocation to the mitochondria, cytochrome *c* release, and cell death. The chloride channel inhibitor furosemide prevented all these changes. No necessary role for Bid translocation could be established. In fact, the translocation of Bax, the release of cytochrome *c*, and the death

of the cells were observed in the absence of mitochondrial translocation of either full-length Bid or tBid.

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Regulation of Intracellular pH Mediates Bax Activation in HeLa Cells Treated with Staurosporine or Tumor Necrosis Factor- α

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