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Cellular Characterization of Leukotoxin Diol-Induced Mitochondrial Dysfunction

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Leukotoxin, a cytochrome P450-derived epoxide of linoleic acid, has been implicated as a causative factor in acute respiratory distress syndrome. Conversion of this fatty acid epoxide to leukotoxin diol by epoxide hydrolase has been hypothesized as the critical activation step in leukotoxin-induced cellular toxicity. In both human and insect cells, we observed that leukotoxin diol causes acute cellular toxicity and that cyclosporin A, an inhibitor of the mitochondrial permeability transition, ameliorates leukotoxin diol-associated toxicity. To evaluate mitochondria as a target of leukotoxin diol, multiple aspects of mitochondrial integrity were evaluated in both cell- and organelle-based assays. Leukotoxin diol specifically activated the mitochondrial permeability transition, resulting in release of cytochrome c and subsequent cell death. Pretreatment with cyclosporin A inhibited these effects and, furthermore, limited *in vivo* toxicity. While the mechanisms underlying leukotoxin-mediated toxicity remain to be fully elucidated, the observation that leukotoxin diol disrupts mitochondrial function specifically through activation of the mitochondrial permeability transition suggests at least one mechanism through which leukotoxin diol may exert its activity in physiological contexts.

Key Words: **leukotoxin; mitochondria; cyclosporin; ards; epoxide; hydrolase; epoxyoctadecamonoenoic; epome.**

Epoxide hydrolase converts epoxides of linoleic acid (leukotoxins) into diol metabolites (leukotoxin diols), which can induce death in a variety of mammalian cell types and cause signs of acute respiratory distress in mice (1). In contrast, hydrolysis of biologically active arachidonate epoxides yields diol metabolites which are generally considered nontoxic, although they may play a role in modulating vascular function (2, 3).

Leukotoxin is considered a marker for, and a potential causative factor in, human acute respiratory distress syndrome (4–9) and is one of the few epoxide protoxins known. Despite its unique biological activity and potential importance to human health, little has been reported regarding the mechanism of leukotoxin-induced cell death.

Leukotoxin diol is reported to cause mitochondrial uncoupling, but the mechanism by which mitochondria become uncoupled has not been determined (10, 11). Recent evidence suggests that loss of mitochondrial membrane integrity is often (but not always) (12) a critical death mechanism (13, 14). Therefore we tested the hypothesis that leukotoxin diol specifically alters permeability of the mitochondrial inner membrane. Inner membrane permeabilization is characterized by rapid loss of the ion gradient across the inner membrane. Loss of this gradient, resulting in mitochondrial uncoupling, leads to loss of oxidative phosphorylation and subsequent ATP depletion (15, 16). The protein bcl-2 may inhibit this transition by direct regulation of a specific mitochondrial pore or megachannel, preventing release of cytochrome c, which activates destructive proteolytic enzymes and is required for cell death (17, 18). In this report, we provide evidence that mitochondria are a cellular target of leukotoxin diol. Furthermore, we demonstrate that pretreatment with cyclosporin A, an inhibitor of the mitochondrial permeabil-

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ity transition believed to interact with the mitochondrial pore through binding to cyclophilin D, inhibits leukotoxin diol-induced disruption of mitochondrial inner membrane permeability in both human and insect cells and, furthermore, blocks leukotoxin diol-induced mortality and morbidity in mice.

MATERIALS AND METHODS

Experimental design. To evaluate leukotoxin diol-associated cell toxicity, insect SF21 cells (cultured in 96% EX-CELL 401 supplemented with L-glutamine, 3% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin) and human HeLa cells (cultured in DMEM, 2% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) were used. In these experiments, the concentrations of the methyl ester of leukotoxin or leukotoxin diol used were chosen to correspond to those observed in clinical manifestations of acute respiratory distress syndrome (7). To evaluate whether the protective effect of cyclosporin A with respect to leukotoxin diol-associated toxicity (observed at the cellular level) paralleled an *in vivo* context, leukotoxin diol-associated toxicity, and its modulation by cyclosporin A, were evaluated in male Swiss–Webster mice in a mortality assay.

Materials. Methyl leukotoxin and methyl leukotoxin diol (*threo*) were synthesized, purified and characterized as previously described (1). The *erythro* form of methyl leukotoxin diol was synthesized by osmium tetroxide oxidation of methyl linoleate as follows: *tert*-butyl hydroperoxide (820 ml) was added dropwise to a solution of methyl linoleate (1 g) and tetramethylammonium hydroxide (40%, 130 ml) in *tert*-butanol (10 ml). OsO_4 (0.5%, 340 ml) in *tert*-butanol was then added and the solution was stirred, at room temperature, for 30 min. The reaction was quenched with 2% Na_2SO_3 (10 ml) and the aqueous layer extracted with methylene chloride. After drying over anhydrous Na_2SO_4, the extracts were purified by normal phase-silica gel chromatography and the purity and identity of the compound was confirmed using a combination of TLC, GC/MS, and ^{1}H NMR.

Cell viability assays. Cells were diluted with growth medium (for SF21 cells, 96% EX-CELL 401 with L-glutamine, 3% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin; for HeLa cells, DMEM, 2% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin) to a density of 1×10^6 cells/ml prior to performing toxicity assays in 24-well tissue culture plates (1 ml cells/well). Cell viability was determined by Trypan blue exclusion 1, 2, 4, and 8 h after addition of cyclosporin A (1 mM from an ethanolic stock solution) and/or the indicated methyl esters of fatty acid metabolites (180 μM/SF21; 210 μM/HeLa from an ethanolic stock solution).

Mitochondrial permeability assays. Isolated mouse liver mitochondria (0.5 mg) were prepared by standard differential centrifugation as described (19). Protein concentration was determined by Bradford assay (Bio-Rad). A Clark-type electrode (Yellow Springs Instruments) was utilized to measure oxygen consumption (only mitochondria with respiratory control ratios (RCR) of 3.0 or greater were accepted). Mitochondria (500 mg) were suspended in STM buffer (0.25 M sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM succinate, 2 mM rotenone, 3 mM KH_2PO_4, 10 mM EGTA) and mitochondrial swelling, as measured by absorbance at 540 nm at 27°C (20), was plotted as a function of time after addition of cyclosporin A (1 mM from an ethanolic stock solution) and/or the fatty acid methyl esters (180 μM from an ethanolic stock solution). Mitochondria were then centrifuged at 14,000 rpm in a microcentrifuge (4°C); the supernatants were collected and concentrated by ultrafiltration through Centricon 10 membranes (Amicon) at 4°C. The concentrated supernatants were loaded onto a 15% SDS–PAGE gel after calculation of protein concentration by Bradford assay. After electrophoresis, protein was transferred to a PVDF membrane (Immobilon-P, Millipore) for Western blot analysis.

Western blot analysis of cytochrome c release. Western blot analysis was conducted essentially as previously described (20). Cytochrome c monoclonal antibody (7H8.2C1, generously donated by Dr. R. Jemmerson) was used as a primary probe for cytochrome c. Horseradish peroxidase-conjugated anti-mouse IgG (Sigma) and ECL detection reagents (Amersham) were used for immunoblot analysis. A Shimadzu CS-9310PC Dual-Wavelength Flying Spot Scanning Densitometer was used to compare blot signals. A linear response was obtained between 0.05 and 0.3 mg cytochrome c.

Laser scanning confocal microscopy. Laser scanning confocal microscopy was performed with an Olympus BH2 with 60X objective, Bio-Rad (MRC 1024 ES), argon-krypton laser, 488 line, emission filter 522 DF35, dichroic T1-T2. Cells were loaded for 10 min, at room temperature, with Mitotracker Green (1 mM, Molecular Probes) after exposure for 4 h to either leukotoxin or leukotoxin diol at 180 μM. The loaded cells (5 ml) were transferred to glass slides and imaged for Mitotracker Green fluorescence.

In vivo toxicity. Male Swiss–Webster mice (25–30 g) from Charles River Laboratories received tail vein injections of lipid methyl esters as previously described (1) except that vehicle or cyclosporin A (10 mg/kg) was injected intraperitoneally 20 min prior to administration of methyl leukotoxin diol (100 mg/kg). After 4 h, surviving mice were injected with sodium pentobarbital (200 ml). The trachea was exposed and chest opened by midline thoracotomy. The lungs were inflated with air to fill the chest and to minimize displacement of edema fluid, and the trachea was ligated. The inflated lungs, trachea and other medeastinal contents were removed *en bloc* and fixed by immersion in 10% formalin for 24 h. The entire lung was then sliced into 2- to 5-mm-thick blocks, embedded in paraffin, and sections were stained with hematoxylin and eosin.

Statistical analyses. Experiments evaluating individual cell counts (cell toxicity) or mitochondrial swelling were performed at least in triplicate. The mean and population standard deviation (represented as error bars) of each data point are reported. Whole animal studies used 12 animals per treatment group; a chi-squared goodness of fit test with the appropriate degree of freedom was applied using standard statistical tables and the associated *P* value reported in the figure legend.

RESULTS AND DISCUSSION

To determine whether inhibitors of the mitochondrial membrane transition would rescue cells against leukotoxin diol-induced death, we exposed insect (SF21) and mammalian (HeLa) cells to cyclosporin A prior to leukotoxin diol treatment (the methyl esters of leukotoxin and leukotoxin diol were used throughout these studies since synthesis is straightforward and since the esters are of similar toxicity to the fatty acids). Cyclosporin A is effective in preventing mitochondrial release of cytochrome c by a variety of inducers (14), most likely through interaction with cyclophilin D. Insect ovoid cells were initially chosen because of their low endogenous levels of epoxide hydrolase. Cyclosporin A pretreatment partially rescued SF21 (Fig. 1a) and HeLa (Fig. 1b) cells from leukotoxin diol-induced death, suggesting that permeabilization of the mitochondrial inner membrane is an important component of the death pathway in diverse cell types.

To identify whether leukotoxin diol selectively induces loss of mitochondrial membrane integrity in isolated mitochondria, mouse liver mitochondria were iso-

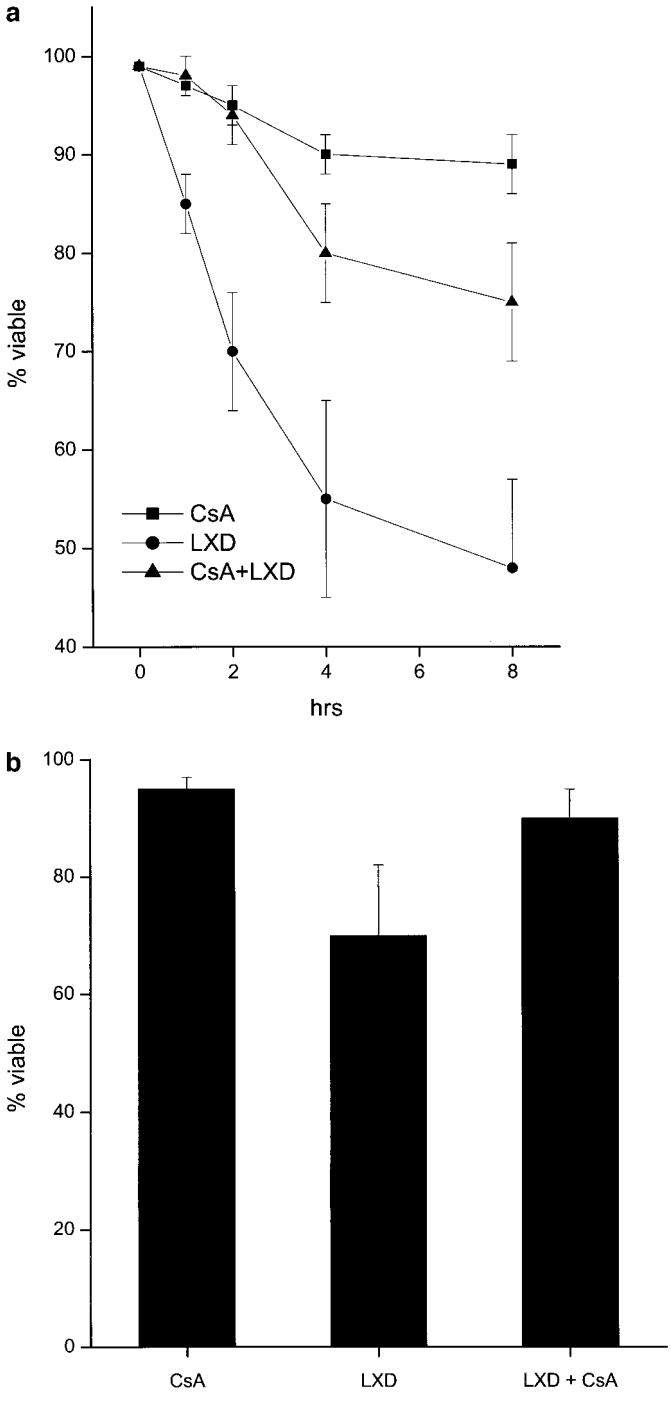


FIG. 1. Cyclosporin A protects both insect ovoid and mammalian cells from leukotoxin diol-induced death. (a) *Spodoptera frugiperda* ovum cell line 21 (SF21) cells were incubated with each of the following compounds and cell viability was determined by trypan blue exclusion at 1, 2, 4, and 8 h: CsA (cyclosporin A, 1 mM), LXD (methyl ester of leukotoxin diol, 180 μM), LXD + CsA. No mortality was observed with either cell line with the methyl esters of leukotoxin, epoxy stearic acid, palmitic acid, stearic acid, oleic acid and linoleic acid. All experiments were done at least in triplicate and the values reported are expressed as the mean ± the standard deviation. (b) Similar rescue effects (4-h time point only is shown) were observed using HeLa cells: CsA (1 mM), LXD (210 μM), LXD + CsA.

lated and mitochondrial swelling, one correlate of the mitochondrial permeability transition (20), was measured by monitoring absorbance at 540 nm (19). Leukotoxin diol, but not leukotoxin, specifically induced

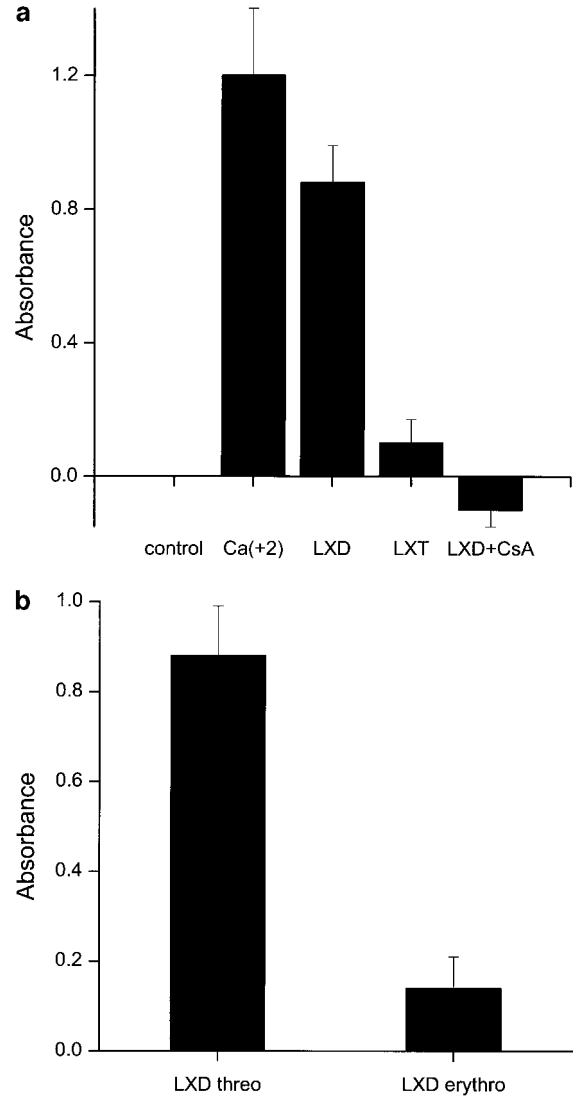


FIG. 2. Leukotoxin diol is a structurally specific inducer of mitochondrial inner membrane permeability; its action is blocked by cyclosporin. (a) Mitochondrial swelling, as measured by Abs 540 nm, was determined after 10 min following addition of the following compounds: (a) control is vehicle (ethanol) alone, 0 by definition; (b) calcium chloride (100 mM); (c) leukotoxin diol methyl ester (180 μM); (d) leukotoxin (180 μM); (e) leukotoxin diol methyl ester (180 μM) + cyclosporin A (1 mM). Cyclosporin A alone, as well as the control compounds discussed in the legend of Fig. 1, failed to induce a decrease in OD 540 nm. (b) Mitochondria were prepared as described above and were treated with: (I) *threo*-(9,10-dihydroxy-12(*Z*)-octadecenoate methyl ester) (180 μM) and (II) *erythro*-(9,10-dihydroxy-12(*Z*)-octadecenoate methyl ester) (180 μM). All experiments were done at least in triplicate and the values reported are expressed as the mean ± the standard deviation. Control absorbance changes were subtracted from the total absorbance change of each sample. Mitochondria with control absorbance changes greater than 0.3 were discarded.

swelling within 10 min (Fig. 2a). Structurally related compounds tested under these conditions and concentrations which did not induce swelling include the methyl esters of palmitic acid (C16, 0 double bonds), stearic acid (C18, 0 double bonds), epoxystearic acid, oleic acid (C18, 1 double bond), and linoleic acid (C18, 2 double bonds) (data not shown). We also examined the stereoselectivity of swelling by comparing mitochondria which had been exposed to the *threo* or the *erythro* configurations of leukotoxin diol. The *threo* form of leukotoxin diol consistently induced swelling to a greater extent than did the *erythro* form (Fig. 2b). Together, these observations suggest leukotoxin diol-induced mitochondrial swelling is not attributable to nonspecific detergent effects, but is moiety- and stereospecific.

The release of cytochrome c from mitochondria into the cytosol during apoptosis is thought to be dependent on MPT induction (19, 21). To establish whether leukotoxin diol induces cytochrome c release from mitochondria, and whether this effect, if present, could be inhibited by cyclosporin A, the mitochondria used in the swelling assays described above were pelleted and the supernatant fractions were purified by SDS–PAGE, subjected to Western transfer, and the membrane probed with anti-cytochrome c antibody (20). Leukotoxin diol induced significant release of cytochrome c. Furthermore, leukotoxin diol-induced cytochrome c release was effectively inhibited by cyclosporin A (Fig. 3). These results imply that induction of mitochondrial pore opening by leukotoxin diol is effectively blocked by pore-selective inhibitors of the mitochondrial permeability transition.

FIG. 3. Leukotoxin diol-induced release of cytochrome c is inhibited by cyclosporin A. The mitochondrial samples used in the swelling studies were centrifuged and the supernatants collected for measurement of mitochondrial cytochrome c release by Western blot analysis using a monoclonal antibody. Leukotoxin diol methyl ester (I) induced released of cytochrome c (indicated by an arrow) which was effectively inhibited by pretreatment of mitochondria with cyclosporin A (II). The upper band is due to nonspecific binding of antibody showing that equivalent levels of protein were loaded onto the gel.

To determine whether leukotoxin diol alters mitochondrial membrane integrity in the context of the cell, SF21 cells were labeled with a fluorescent mitochondria-specific dye (Mitotracker Green) and analyzed by laser scanning confocal microscopy (22). Cells exposed to leukotoxin appeared similar to controls—that is, the dye remained discretely localized within individual mitochondria (Figs. 4a and 4b). Cells exposed to leukotoxin diol, however, exhibited substantial dye leakage into the cytosol (Fig. 4c), indicating selective permeabilization of the mitochondrial membrane. Thus, using three independent measurements of mitochondrial integrity, i.e., swelling, cytochrome c release and Mitotracker Green leakage, we observed that leukotoxin diol specifically disrupts mitochondrial function. Aspects of leukotoxin-induced mitochondrial dysfunction have been noted in previous reports. Sakai *et al.* noted changes in pulmonary function in isolated lungs perfused with 200 and 600 μM, but not lower concentrations, of leukotoxin (10). In contrast with our observations, their studies revealed evidence for mitochondrial dysfunction at 600 μM leukotoxin, but not at lower leukotoxin concentrations. Possibly, the discrepancy between their observations and our observation of leukotoxin diol-induced mitochondrial dysfunction at a substantially lower dose (180 μM) may be attributable to inefficient epoxide hydrolase-mediated conversion of leukotoxin to leukotoxin diol in the perfused rat lung system.

Because leukotoxin diol, but not leukotoxin, specifically induces ARDS-like symptoms in mice (1), we next examined whether cyclosporin A is an effective inhibitor of leukotoxin diol-induced death and pulmonary edema in this animal model. Mice treated with cyclosporin A prior to leukotoxin diol exhibited dramatically lower mortality than mice that received leukotoxin diol alone (Fig. 5a). Histopathological analysis of murine pulmonary tissue revealed that cyclosporin A pretreatment blocked leukotoxin diol-induced pulmonary hemorrhage and edema, symptoms which are commonly present in ARDS (23) (Fig. 5b). To our knowledge, this is the first time that cyclosporin A has been shown to provide protection against toxic injury to the lung.

Conversion of epoxides to diols is not a universally successful strategy for decreasing toxicity (1). In the unique case of linoleate-derived epoxides, the corresponding diols may be a signal for cell destruction. Our data suggest that leukotoxin diol specifically triggers the mitochondrial permeability transition and subsequent cell death. We propose the existence of a leukotoxin diol-triggered death pathway (Fig. 6) which includes mitochondrial inner membrane permeabilization and subsequent release of cytochrome c, both of

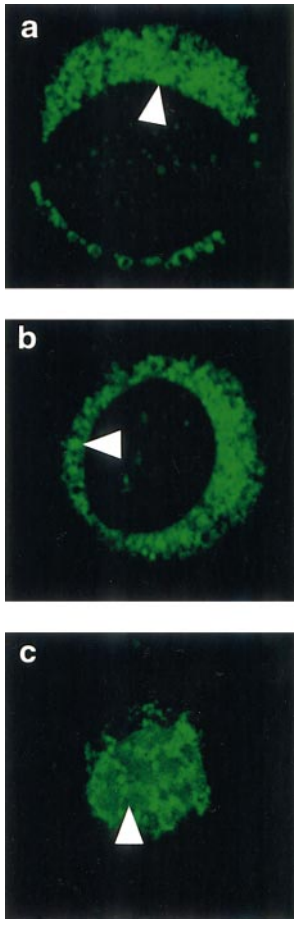
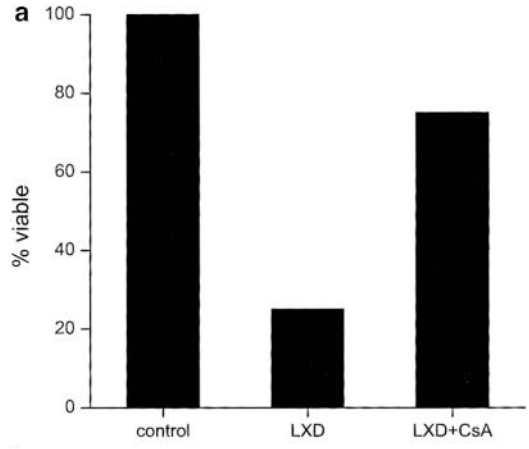


FIG. 4. Leukotoxin diol specifically induces mitochondrial membrane leakage in living cells. SF21 cells were incubated with the following compounds for 4 h: (a) vehicle (EtOH) alone; (b) leukotoxin methyl ester (180 μM); (c) leukotoxin diol methyl ester (180 μM). Cell aliquots (1 ml) were then treated for 10 min with Mitotracker Green (1 mM) prior to analysis by confocal microscopy. Discrete localization of dye within mitochondria is observed in the control (a) and leukotoxin-treated (b) cells as shown by arrows. Only the leukotoxin diol-treated cells (c) exhibited leakage of dye from mitochondria into the cytosol.



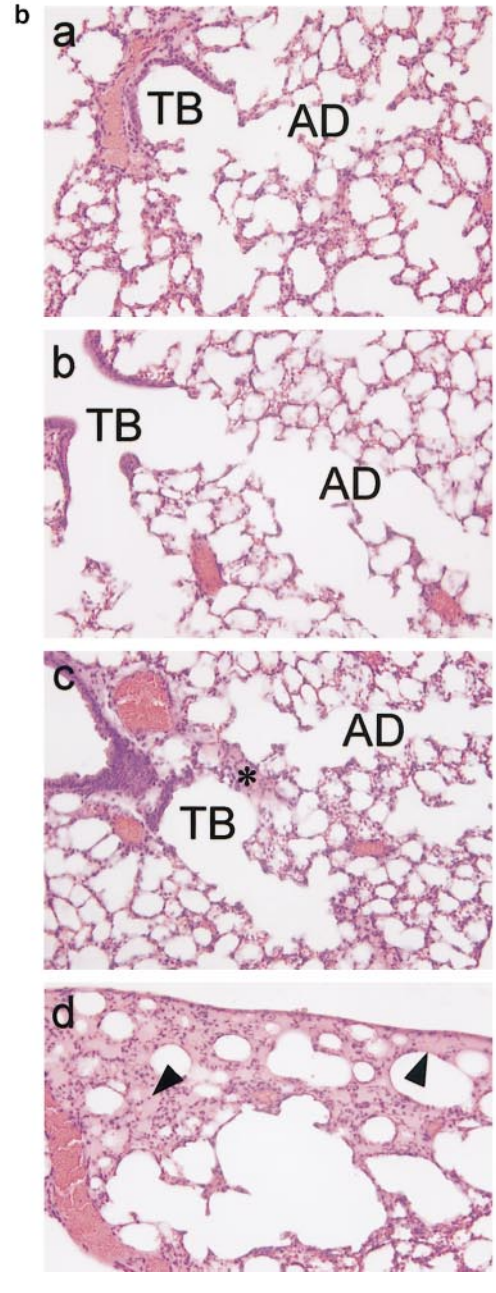


FIG. 5. Cyclosporin A protects mice from leukotoxin diol-induced death, pulmonary edema and focal hemorrhage. (a) Pretreatment of mice with cyclosporin A (10 mg/kg) prior to treatment with leukotoxin diol methyl ester (100 mg/kg) increased survival from 25 to 75% (N = 12 for each group; Chi-Square = 0.05). (b) Compared to controls (a), the lung tissue of cyclosporin pretreated mice (b) showed little difference in alveolar structure or perivascular and peribronchiolar integrity. Mice treated with leukotoxin diol methyl ester alone (c, section from central airway; d, same section from periphery) showed focal areas of hemorrhage, thickening of perivascular and peribronchiolar spaces in the terminal bronchiol region, and significant levels of focal alveolar edema. AD, alveolar duct; TB, terminal bronchiol; *, points of focal hemorrhage; arrows, focal alveolar edema.

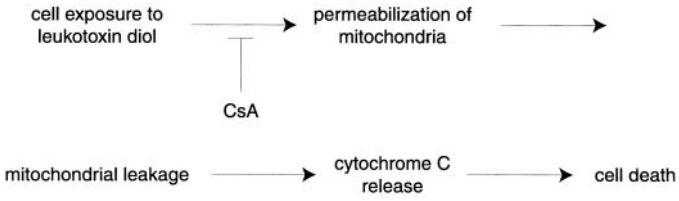


FIG. 6. Proposed mechanism of leukotoxin diol-induced cell death. Exposure of cells to leukotoxin diol results in selective permeabilization of the mitochondrial inner membrane leading to mitochondrial membrane leakage, release of cytochrome c, and subsequent cell death. Cyclosporin A inhibits this pathway by protecting mitochondria from induction of the permeabilization transition.

which are blocked by the mitochondrial pore inhibitor cyclosporin A. Since cyclosporin A also inhibits leukotoxin diol-induced death, pulmonary edema and hemorrhage *in vivo* (Figs. 1 and 6), related nonimmunosuppressive analogs may be useful in treating inflammatory disorders such as ARDS.

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