Mitochondrial Impairment is a Critical Event in Anthrax Lethal Toxin-Induced Cytolysis of Murine Macrophages

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INTRODUCTION

Spores derived from the gram-positive *Bacillus anthracis*, the causative agent of the anthrax disease, can induce death in infected hosts (reviewed in refs. 1–3). *B. anthracis* releases the bipartite lethal toxin (LT), which is sufficient to kill animals even in the absence of bacterial infection.4 LT is composed of two proteins, protective antigen (PA) and lethal factor (LF). The proteolytic activity of LF, a metalloprotease, is essential for LT-induced cytopathic and lethal effects in mice.5 LF cleaves mitogen-activated protein kinase kinases (MAPKKs), thereby disrupting MAPK signaling pathways.6-10 LT uptake and subsequent MAPKK cleavage are ubiquitous, and also occur in cells that are resistant to LT killing.11,12 This suggests that either LF targets cell type-specific factors in addition to MAPKKs, or that cells differ in their response to MAPKK cleavage.13-15

LT triggers a cascade of physiological events in murine macrophages, including permeability changes in monovalent and divalent cations, arrest of protein synthesis, release of cytoplasmic proteins, including lactate dehydrogenase (LDH), and drastic morphological changes.16,17 However, events directly responsible for LT-mediated cytolyis remain to be shown.

Proteasome inhibitors abrogate LT killing of murine macrophages and dendritic cells, indicating that proteosomal degradation of protective factors is required for induction of cytopathic effects.18-20 However, it is unclear how proteasome inhibitors block LT toxicity. Proteasomal degradation of protective factors, such as XIAP, controls cell death mediated by TNF-α or Trail,21 and it is conceivable that protective factors are also targeted by proteasomes during LT killing of murine macrophages. The proteasome system, along with the lysosomal system, makes up the major cellular protein degradation machinery. Proteasomal degradation of target proteins is generally regulated by polyubiquitination, a multi-step process.22,23 Proteasomal activity is required for multiple cellular functions, including homeostasis and degradation of misfolded and short-lived proteins.24,25

Here we report that proteasome inhibitors and potassium chloride (KCl) efficiently block LT-induced cytolyis in murine J774A.1 macrophages, even when administered at a late time point post-LT exposure. The proteasome inhibitor MG132 prevented the rapid decrease in mitochondrial succinate dehydrogenase (SDH) activity. Strikingly, the mitochondrial SDH activity recovered when MG132 was added late to LT-treated cells, prior...
to membrane perturbation. In contrast to MG132, KCl treatment did not interfere with mitochondrial dysfunction, but specifically blocked membrane perturbation in LT-treated macrophages. We present evidence that mitochondrial impairment is immediately followed by cytolysis and multiple downstream epiphenomena, including a drop in ATP levels, a loss in mitochondrial integrity and deubiquitination of cellular proteins.

**MATERIALS AND METHODS**

**Cell culture and reagents.** Murine J774.A1 macrophages (TIB 67, American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal bovine serum, glucose (200 mM), penicillin G (200 µg/ml) and streptomycin (100 µg/ml) (GIBCO BRL). Recombinant anthrax PA and LF, obtained from List Biological Laboratories, were reconstituted in sterile water at a concentration of 5 µg/µl. PA was used at 500 ng/ml, and LF at 250 ng/ml. The proteasome inhibitor MG132 (Oncogene), and the fluorescent proteasome substrate (z-GGL-AMC) (Calbiochem) were reconstituted in DMSO and used at 10 µM. The final DMSO concentration in the media was 0.1%. Cycloheximide (CHX) and puromycin were obtained from Sigma. The proteasome inhibitor Velcade (bortezomib) was generously provided by Dr. Roman Perez-Soler (Albert Einstein College of Medicine).

**Cell viability assays.** Cells were treated with LT, 500 ng/ml PA and 250 ng/ml LF, unless indicated otherwise. Cell viability was measured by analysis of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cleavage to formazan by succinate dehydrogenases in living cells. For the colorimetric MTT assay, 10^5 cells/well were plated in 96 well plates. Two to three h post-plating, cells were exposed to lethal toxin. The MTT solution (5 mg/ml MTT in PBS) was added directly to wells at different time points post LT exposure, and was incubated at 37˚C for 4 h. The dye was solubilized with acidic isopropanol (25 mM HCl, 0.5% SDS in isopropanol), and the absorbance of the converted dye was measured at 570 nm.

**Analysis of mitochondrial membrane potential (DeltaPsi m) and membrane permeability.** Murine macrophages were cultured in 24 well non-tissue culturetreated plates (3.75 x 10^5 cells/well). For analysis of DeltaPsi m, cells were loaded with 7.5 µg/ml of JC-1 (T3168; Molecular Probes) for 10 min at 37˚C and washed twice with PBS. At different time points post LT-exposure, cells were detatched in a solution of 0.08 g/ml EDTA and 1 mM PMSF (Sigma). Cellular lysates were centrifuged, and supernatants were mixed with SDS sample buffer. Fifty µg of protein from each sample was loaded on precast Biorad SDS-Tris HCl polyacrylamide gels, and transferred to PVDF membranes (Amersham). Blots were developed using ECL Plus solution (Amersham).

**Transmission electron microscopy.** Samples were fixed with glutaraldehyde and postfixed with osmium tetroxide, followed by addition of uranyl acetate as described previously. After dehydration through a graded series of ethanol washes, samples were embedded in LX112 resin (LADD Research Industries). Ultrathin sections were cut on a Reichert Ultracut UCT, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80 kV.

**RESULTS**

**Proteasome inhibitors block a late step in LT killing of murine macrophages.** Proteasome inhibitors block LT killing of murine macrophages and dendritic cells, suggesting that this process is dependent on proteasomal degradation of protective factors. To identify the step in LT killing that is blocked by proteasome inhibitors, we exposed murine J774.A1 macrophages to a high dose of LT (500 ng/ml PA and 250 ng/ml LF) and added proteasome inhibitors at different time points following exposure to LT. We selected the most potent reversible proteasome inhibitor, MG132, and the water-soluble proteasome inhibitor Velcade, for these experiments. Cell viability was measured by the colorimetric MTT assay, which monitors the mitochondrial activity of living cells. The MTT assay measures the conversion of succinate to formurate by succinate dehydrogenases (SDH), which are predominantly expressed in mitochondria.

The MTT signal of murine macrophages dropped within 30 min of LT treatment, reaching background levels after 3 h (Fig. 1A). This drop in SDH activity was prevented when the proteasome inhibitor MG132 was added simultaneously with LT (Fig. 1A). Strikingly, the MTT signal reverted rapidly to levels of untreated cells when MG132 was added up to 60 min post-LT exposure (Fig. 1A). Even 90-120 min after LT exposure, MG132 caused partial recovery of the MTT signal (Fig. 1A). Like MG132, the proteasome inhibitor Velcade efficiently blocked LT killing of macrophages, and triggered a rapid recovery of the MTT signal when added post-LT exposure (data not shown). These results suggested that proteasome inhibitors are able to save LT-treated macrophages with strongly diminished mitochondrial activity.

**Recovery of SDH activity does not require protein biosynthesis.** The MTT signal returned to levels observed in untreated J774.A1 macrophages when proteasome inhibitors where added to macrophages 30-60 min post-LT exposure (Fig. 1A). To test whether the recovery of the SDH activity required de novo protein biosynthesis, we preincubated macrophages with the protein synthesis inhibitor cycloheximide (CHX) for two hours and subsequently added LT. These cells were then treated with MG132 at different time points post-LT exposure. The recovery of the MTT signal in LT-treated macrophages following late addition of MG132 occurred even in the presence of CHX (Fig. 1B). Similar results were obtained when macrophages were preincubated with CHX for up to 6 h prior to LT/MG132 challenge (data not shown). Therefore, the rapid recovery of mitochondrial SDH activity in LT-treated macrophages following late addition of proteasome inhibitors did not require protein biosynthesis.

Protein synthesis inhibitors have been reported to block LT killing of macrophages. However, we were unable to block LT killing by the protein...
Mitochondrial Impairment and LT Killing.

The rapid drop in mitochondrial SDH activity in LT-treated cells (Fig. 1A), and the recovery of this activity after late addition of proteasome inhibitors (Fig. 1A) suggested mitochondrial targeting in LT killing. To determine mitochondrial activity in this process, we exposed murine J774A.1 macrophages to a high dose of LT and analyzed the mitochondrial membrane potential ($\Delta\psi_{m}$) using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolo-carbocyanine iodide (JC-1) by flow cytometry.37 A partial reduction in $\Delta\psi_{m}$ occurred approximately 30 min post-LT exposure, followed by a complete loss in $\Delta\psi_{m}$ 90-120 min post-LT treatment (Fig. 2A). The early drop in $\Delta\psi_{m}$ (Fig. 2A) was concurrent with the rapid drop in SDH activity (Fig. 1A), indicating early targeting of mitochondria by LT.

Mitochondrial targeting was further supported by ultra-structural analysis, which revealed two subpopulations of macrophages after 1.5 h of LT treatment (Fig. 2B–E). Approximately half of the LT-treated macrophages contained mitochondria with signs of swelling (Fig. 2C), while the remaining cells showed a complete loss of organelle integrity and cellular matrix. After 4 h of LT treatment, all macrophages exhibited the latter phenotype, with highly compromised organelles (Fig. 2D-E).

To determine whether the drop in mitochondrial SDH activity was caused by protein degradation, we subjected cellular lysates of LT-treated macrophages to Western blot analysis, using antibodies against SDH and multiple house-keeping proteins. We found that levels of SDH and house-keeping proteins, including actin and cytochrome c, did not change in macrophages following LT treatment (Fig. 3A). These results also indicated that the reduction in SDH activity was not caused by degradation of this protein.

As proteasomal degradation is largely controlled by ubiquitination, we analyzed levels of ubiquitinated proteins by Western blotting, using anti-ubiquitin antibodies. Consistent with reports of changes in ubiquitinated proteins in LT-treated macrophages,38,39 we found that levels of ubiquitinated proteins dropped synthesis inhibitors CHX or puromycin (Fig. 1C), even at concentrations that are sufficient to completely block protein synthesis in multiple cell types, including murine macrophages.33-35 This is in contrast to cell death mediated by apoptosis inducers, which is drastically enhanced following transient downregulation of short-lived proteins.36 Our findings suggested that LT killing, as well as the recovery of SDH activity after late addition of proteasome inhibitors, did not require de novo protein synthesis, and is likely not regulated by short-lived factors.
2 h post-LT challenge of murine macrophages, and these proteins were undetectable 4 h post-LT treatment (Fig. 3A). As proteasomes remained active over a four-hour period in LT-treated macrophages (Fig. 3C), we hypothesized that proteasomal activity might be responsible for the loss in ubiquitinated proteins. Surprisingly, the drop in levels of ubiquitinated proteins also occurred when the proteasome inhibitor MG132 was added to J774A.1 macrophages 2 h post-LT exposure (Fig. 3B). This result indicated that the drop in ubiquitinated proteins was independent of proteasomal activity, and was rather caused by deubiquitination. Moreover, total protein staining of cellular lysates with Comasse Blue revealed no apparent differences between LT-treated and untreated macrophages (data not shown), further supporting the theory that the reduction in ubiquitinated proteins was not caused by broad protein degradation.

Loss of ATP and deubiquitination of cellular proteins in LT-treated macrophages. The early drop in 

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\Delta \psi_m \text{ and SDH activity in LT-treated macrophages indicated mitochondrial dysfunction. We found that this rapid impairment was not accompanied by a reduction in ATP levels, as analyzed by an ATP-dependent luciferase assay (Fig. 4B). However, ATP levels dropped drastically 60–90 min post-LT exposure (Fig. 4B), concurrent with a rise in cytolysis, as measured by flow cytometry using the membrane-impermeable DNA-binding dye TO-PRO-3 (Fig. 4A and 6C). Cytolysis began 60 min post-LT exposure, and by 120 min, the overwhelming majority of cells were TO-PRO-3-positive (Fig. 4A and 6C). These results suggested that the drop in ATP levels was directly linked to the increase in membrane perturbation.

To test whether the drop in ATP levels was caused by cytolysis, we attempted to prevent or delay membrane perturbation in LT-treated macrophages by using combinations of sucrose, NaCl and KCl, as reported by Hanna et al.\textsuperscript{17} We found that high KCl concentrations (150 mM KCl) efficiently blocked LT-induced membrane perturbation as determined by flow cytometry using TO-PRO-3 (Fig. 4A and 6C), while sucrose and NaCl had no impact on LT intoxication (data not shown). KCl treatment also largely prevented the drop in ATP levels in LT-treated macrophages (Fig. 4B), presumably by blocking membrane perturbation. To further analyze the link between ATP levels and cytolysis, we performed KCl wash-out experiments. When macrophages were treated with LT in the presence of KCl, and the LT/KCl-containing media was replaced with media alone, cells underwent immediate cytolysis, concurrent with a precipitous drop in ATP levels (data not shown). Finally, we found that KCl exposure also prevented the precipitous drop in ubiquitinated proteins in LT-treated macrophages (Fig. 5). These results showed that KCl is a potent inhibitor of LT-induced cytolysis. Our results suggested that the loss in ATP levels and the drop in levels of ubiquitinated proteins were directly linked to membrane perturbation, and therefore most likely represent downstream epiphenomena in LT killing.

KCl blocks membrane perturbation, but does not interfere with mitochondrial impairment of LT-treated macrophages. To identify steps in LT killing that were blocked by MG132 or KCl, we added these agents at different time points post-LT exposure, and measured cytolysis using the membrane-impermeable dye TO-PRO-3. In the absence of MG132 or KCl, we observed an increase in TO-PRO-3-positive cells, beginning 60 min post-LT treatment (Fig. 4A and 6C). When we added KCl 30 to 90 min post-LT exposure, and measured the numbers of TO-PRO-3-positive cells 120 min post-LT treatment, we found that KCl treatment prevented a further increase in cytolysis (indicated by asterisks in Fig. 6C). The signal obtained after 120 min was similar to that recorded at the time point of KCl addition (Fig. 6C). These results indicated that KCl arrested cytolysis immediately, as no further increases in TO-PRO-3-positive cells were observed following KCl addition (Fig. 6C). Similar results were obtained with MG132 (data not shown).

As described earlier, the recovery of the MTT signal was dependent on the time point of MG132 addition (Fig. 1A). LT-treated macrophages became progressively non-responsive to MG132 (Fig. 1A). This non-responsiveness to MG132/KCl directly correlated with the increase in TO-PRO-3-positive cells (Fig. 4A and 6C). These results suggested that MG132 and KCl were able to prevent cytolysis at very late time points in LT intoxication—up to the point at which cells undergo membrane perturbation. Our data suggested that cells with compromised membranes were beyond rescue, and therefore non-responsive to these inhibitors.
To test whether KCl, like MG132, was able to block mitochondrial impairment, we treated murine J774A.1 macrophages with LT in the presence of 150 mM KCl, and determined the SDH activity and DeltaPsim. Strikingly, KCl exposure did not prevent the rapid initial drops in SDH activity (Fig. 6A) and DeltaPsim (Fig. 6B) in LT-treated macrophages. These findings contrast results obtained with MG132, which efficiently blocked the reduction in both the MTT signal (Fig. 1A) and DeltaPsim (Fig. 6B).

As KCl did not inhibit mitochondrial impairment, we tested whether KCl prevents LT killing of macrophages. Intriguingly, a significant fraction of LT-treated macrophages were dead after 5 h following KCl treatment, while LT-treated cells recovered, and no cell death was detected following 5 h of MG132 treatment (Fig. 6D). Although KCl exposure specifically blocked cytolysis when measured 120 min post-LT exposure, it did not prevent LT killing, which was presumably caused by mitochondrial impairment. These results pointed to fundamental differences in the way MG132 and KCl interfere with LT toxicity. While MG132 prevented LT-induced mitochondrial impairment and was able to rescue cells, KCl was unable to interfere with mitochondrial dysfunction or to rescue these cells. As mitochondrial impairment was gradual, it is conceivable that reduced mitochondrial activity reaches a threshold at which it triggers cytolysis. Our studies distinguished, for the first time to our knowledge, essential steps in LT killing (mitochondrial impairment and cytolysis) from epiphenomena (drop in ATP levels and ubiquitinated proteins).

DISCUSSION

LT mediates a cascade of physiological events in macrophages, which ultimately results in cytolysis.17 Here we present evidence implicating mitochondrial impairment as an early and critical event in LT killing. This is indicated by an early reduction in SDH activity and DeltaPsim, recovery of SDH activity after late addition of proteasome inhibitors, swelling of mitochondria, and a loss of mitochondrial integrity in LT-treated macrophages. The mitochondrial activity recovered after late addition of proteasome inhibitors to LT-treated macrophages, pointing to a close link between proteasomal degradation and mitochondrial activity. Our findings suggest that proteasomes control continuous mitochondrial impairment, and, through this, cytolysis of LT-treated macrophages. Late addition of proteasome inhibitors also prevents killing of thymocytes by gamma radiation and dexamethasone.40

Like MG132, KCl also blocked LT-mediated cytolysis, even when added late to LT-treated cells, and immediately prior to membrane perturbation. LT-treated macrophages with compromised membranes were nonresponsive to proteasome inhibitors and KCl, and apparently reached a "point of no return". Though MG132 and KCl prevented cytolysis, both appeared to target different events in LT killing. While MG132 blocked mitochondrial impairment, KCl exposure did not interfere with this early event, and targeted only membrane perturbation. Accordingly, proteasome inhibitors were able to save LT-treated cells, while KCl exposure did not prevent LT killing, presumably caused by mitochondrial impairment.

Our findings indicate that LT triggered mitochondrial impairment and an osmotic crisis in J774A.1 macrophages, prompting multiple questions: (1) Whether and how does MAPKK-cleavage lead to mitochondrial impairment? (2) What is the connection between proteasomal activity and mitochondrial dysfunction? (3) Why does SDH activity recover in the presence of proteasome inhibitors? (4) How does mitochondrial impairment lead to cytolysis?
A release of mitochondrial factors controls apoptosis as well as necrotic cell death.\textsuperscript{41,42} and inhibitors of mitochondrial membrane fluxes, including Bcl-2 and cyclosporine, have been shown to block apoptotic as well as necrotic cell death.\textsuperscript{43} It is conceivable that LT killing of macrophages is also regulated by mitochondrial factors.

The ability of high potassium chloride concentrations to block LT-induced membrane perturbation suggests that cytosis is controlled by changes in intracellular potassium levels. Notably, treatment with high KCl concentrations also prevents cytosis mediated by several membrane-active bacterial toxins and potassium ionophores.\textsuperscript{44} As high intracellular potassium or calcium levels can cause swelling of mitochondria,\textsuperscript{45,46} changes in intracellular cation levels might lead to the mitochondrial swelling and cytosis observed in LT-treated macrophages (Fig. 2C).

Mitochondrial impairment is a critical event in anthrax lethal toxin-induced cytolysis of murine macrophages. In contrast to rapid LT killing of murine macrophages, human cell lines, such as endothelial and melanoma cells, are killed within two to three days of LT exposure.\textsuperscript{15,51} It is therefore possible that proteasome inhibitors block LT killing of human macrophages, several hours, or even days, after LT exposure. Water-soluble proteasome inhibitors, like Velcade, are already used in clinical medicine as anti-cancer agents, and might be applicable to the treatment of anthrax disease in humans.\textsuperscript{52}

Proteasome inhibitors blocked LT killing of murine macrophages in vitro, and may also prevent anthrax disease progression in vivo. The window of opportunity to prevent LT killing by proteasome inhibitors may be much wider in human cell lines. In contrast to rapid LT killing of murine macrophages,\textsuperscript{14,17,50} human cell lines, such as endothelial and melanoma cells, are killed within two to three days of LT exposure.\textsuperscript{15,51} It is therefore possible that proteasome inhibitors block LT killing of human macrophages, several hours, or even days, after LT exposure. Water-soluble proteasome inhibitors, like Velcade, are already used in clinical medicine as anti-cancer agents, and might be applicable to the treatment of anthrax disease in humans.\textsuperscript{52}

Proteasomal degradation is usually regulated by ubiquitination,\textsuperscript{38,39} and levels of ubiquitinated proteins dropped precipitously in murine macrophages 2 h post-LT exposure. The loss of ubiquitinated proteins could not be prevented by late addition of proteasome inhibitors, indicating that this process was independent of proteasomal activity, and was likely caused by deubiquitination. Broad deubiquitination has been described in synaptosomes, where it is triggered by calcium influx.\textsuperscript{53} As calcium flux peaks 2 h post-LT exposure in macrophages,\textsuperscript{17} and calcium has been linked to LT toxicity,\textsuperscript{54,55} it is conceivable that a rise in intracellular calcium triggers deubiquitination in these cells.

Apoptotic cell death is enhanced/promoted by proteasomal degradation of protective factor(s) thereby causing the release of death-promoting factors. Accordingly, proteasomal inhibitors prevent apoptosis induction by several cell death inducers. The ability of proteasomal inhibitors to efficiently block LT killing of murine macrophages suggests that this process is also controlled by proteasomal degradation of protective factors. However, findings presented here suggest that (1) proteasome might be doing something other than destroying cytoplasmic protective factors, or (2) proteasome inhibitors may be affecting other organelles besides proteasomes. The mitochondrial activity recovered completely in LT-treated macrophages following delayed addition of proteasome inhibitors. This recovery occurred even in the presence of protein biosynthesis inhibitors, arguing against the degradation of protective factors controlling mitochondrial activity and LT killing. We hypothesize that inhibition of proteasomes induces cellular conditions that prevent LT-mediated cytopathic effects. The mechanism of proteasome inhibitor-mediated blockage of LT killing awaits further investigation.
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