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Glucocorticoid-Induced Apoptosis of Thymocytes: Requirement of Proteasome-Dependent Mitochondrial Activity

Noriko Tonomura,*† Kelly McLaughlin,‡ Lisa Grimm,¶ Richard A. Goldsby,*§ and Barbara A. Osborne*†

Thymocytes undergo negative and positive selection during development in the thymus. During this selection process, the majority of thymocytes are eliminated by apoptosis through signaling via TCR or die by neglect, possibly mediated through glucocorticoids. In this study, we report that thymocytes require molecular oxygen to undergo apoptosis induced by dexamethasone (DEX), a synthetic glucocorticoid, and treatment with N-acetyl-L-cysteine (NAC), a thiol antioxidant, inhibits thymocyte apoptosis in vivo as well as ex vivo. We detected elevated intracellular levels of hydrogen peroxide (H$_2$O$_2$) during DEX-induced apoptosis, which is reduced by NAC treatment, indicating that the elevated levels of intracellular H$_2$O$_2$ are proapoptotic. We also show that loss of mitochondrial membrane potential, cytochrome c release, as well as caspase-3 activation induced by DEX are attenuated by NAC treatment. We identified the production site for H$_2$O$_2$ as the ubiquinone cycle at complex III of mitochondria by using various inhibitors of the mitochondrial electron transport chain, and we show that the cell death events mediated by mitochondria are also significantly reduced when the inhibitors were used. Through inhibition of the proteasome, we also show that the production of H$_2$O$_2$ and the cell death events mediated by mitochondria are regulated by proteosomal activities in DEX-induced thymocyte apoptosis. We conclude that in DEX-treated thymocytes, the increased production of H$_2$O$_2$ originates from mitochondria and is proapoptotic for cell death mediated by mitochondria. We also conclude that all the apoptotic events mediated by mitochondria are regulated by proteasomes.

immediate downstream substrate, initiating the cascade of protease activation in the execution phase of apoptosis.

In addition to the mitochondria, one of the key regulators of lymphocyte apoptosis is the proteasome (16, 17), a large proteolytic multienzyme complex residing in the cytoplasm and nucleus. We have previously observed that the inhibition of proteasome activities results in a reduction of caspase-3 activity. This finding suggests that the proteasome may exert its regulatory role upstream of cell death events mediated by mitochondria during thymocyte apoptosis. Therefore, it is possible that the proteasome may also play a role in regulating oxidative stress originating from mitochondria during apoptosis, ROS production at sites other than the mitochondria has been shown to play a role in some forms of apoptosis (18). However, it is likely that the majority of ROS are produced by mitochondria, and accumulating evidence suggests that the regulation of inner and outer membrane permeability, electron transport, and transmembrane transportation of ions and molecules such as Cyt c seems crucial for the maintenance of the mitochondrial homeostasis, as well as for the regulation of apoptosis (19–22).

In this study, we define the contribution of molecular oxygen and H$_2$O$_2$ to the apoptotic processes, as well as the site of H$_2$O$_2$ production during DEX-induced thymocyte apoptosis. Additionally, we demonstrate the regulatory role of the proteasome in modulating cell death events mediated by mitochondria and H$_2$O$_2$ production.

### Materials and Methods

#### Reagents

Dexamethasone (DEX), N-acetyl-l-cysteine (NAC), antimycin A, rotenone, and H$_2$O$_2$ were purchased from Sigma-Aldrich (St. Louis, MO). Lactacystin was purchased from Kamiya Biomedical (Seattle, WA). Anti-Cyt c, anti-CD3, and anti-CD28 mAbs were obtained from BD PharMin. Lactacystin was purchased from Kamiya Biomedical (Seattle, WA). Anti-

#### Ex vivo thymocyte culture

Thymocytes were obtained from 3- to 5-wk-old female BALB/c mice. Thymocytes were cultured at 37°C in 5% CO$_2$ in RPMI 1640 medium containing 10% FBS. All mice were maintained in the animal facility at the University of Massachusetts (Amherst, MA).

#### In vivo treatment of BALB/c mice with NAC and DEX

NAC solution was prepared at the concentration of 32 mg/ml in PBS (pH 7.2). DEX solution was prepared at the concentration of 1 mg/ml in 16% ethanol/PBS. Female BALB/c mice at 4 wk of age were weighed and given NAC injections (8 mg NAC/20-g mouse) at 6 h and 1 h before a DEX injection (250 μg DEX/20-g mouse). Mice in the control group were given an equivalent volume of PBS or 16% ethanol/PBS. Thymocytes were isolated from all mice at 10–16 h after the DEX injection and assayed for apoptosis.

#### Treatment of thymocytes with NAC

Thymocytes were incubated with 60 mM NAC starting 1 h before the induction of apoptosis and throughout the experiment. PBS was added to the control cultures.

#### Induction of apoptosis by DEX, TCR-mediated signaling, or H$_2$O$_2$

Apoptosis in thymocytes was induced by 5 μM DEX or 40 μM H$_2$O$_2$. The final dilutions were prepared from the following stock solutions: 2.5 mM DEX in 70% ethanol and 8.8 M H$_2$O$_2$ (30% solution w/w) in PBS. To the control cultures, 70% ethanol or PBS was added. To induce apoptosis via TCR, thymocytes were cultured in a tissue culture plate that was coated with anti-CD3 and anti-CD28 mAbs at 10 mg/ml each.

#### Treatments of thymocytes with mitochondrial electron transport chain inhibitors

Mitochondrial electron transport chain inhibitors were added to thymocyte cultures 30 min before the induction of apoptosis. The final concentrations of each inhibitor used for the experiments were: 10 μM rotenone and 1 μM antimycin A. In the control cultures, the appropriate solvents were added at volumes equal to the experimental.

#### Treatment of thymocyte with proteasome inhibitors

Lactacystin was added to thymocyte cultures at 8 μM 1 h before the induction of apoptosis. The stock solution was prepared as a 20-mM solution in H$_2$O.

#### Detection of H$_2$O$_2$ in thymocytes

For the detection of H$_2$O$_2$, thymocytes were preincubated with 5 μM CM-H$_2$DCFDA or 1 μM H$_2$DCFDA-di(acetoxymethyl ester) in medium for 30 min at 37°C in a 5% CO$_2$ incubator before any other treatment. These dyes get oxidized by intracellular H$_2$O$_2$ and become fluorescent. After appropriate treatments were performed, cells were harvested and assayed for the H$_2$O$_2$-associated fluorescence by flow cytometry.

#### Detection of thymocyte apoptosis by Annexin VFITC staining

Apoptosis was detected by the binding of Annexin VFITC to phosphatidylserine exposed on the outside of the plasma membrane of apoptotic cells. At designated time points, thymocytes were harvested and stained with Annexin VFITC for 15 min at room temperature in dark. The fluorescent signals were detected by flow cytometry.

#### Detection of Δψ$_m$

At each time point, thymocytes were incubated with 50 nM 3,3′-dihexyloxacarbocyanine iodide (DiOC$_6$) in medium for 15 min at 37°C in a 5% CO$_2$ incubator. After incubation, cells were washed once and resuspended in cold PBS/BSA. Stained cells were immediately analyzed by flow cytometry.

#### Detection of caspase-3 activity: PhiPhiLux assay

One hour before the desired time point, 5 × 10$^7$ thymocytes per sample were pelleted and resuspended in 50 μl of RPMI medium containing 9 nM PhiPhiLuxG$_D_2$ and G$_D_2$ (Oncoluminain, Gaithersburg, MD) and 10% PBS. After a 1-h incubation at 37°C in a 5% CO$_2$ incubator, the samples were analyzed immediately by flow cytometry. Uncleaved PhiPhiLuxG$_D_2$ and PhiPhiLuxG$_D_2$ emit background fluorescence at 552 and 505 nm, respectively, and cleaved PhiPhiLuxG$_D_2$ and PhiPhiLuxG$_D_2$ emit fluorescence at 580 and 530 nm, respectively.

#### Preparation of cytoplasmic, nuclear, and mitochondrial fractions

Preparation of all fractions was performed as described by Andrews et al. (23) with some modifications. Briefly, thymocytes were resuspended in ice cold buffer A (10 mM HEPES-potassium hydroxide (pH 7.9) at 4°C, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 1 mM leupeptin, 0.2 mM pepstatin block), and incubated for 35 min on ice for hypotonic cell lysis. After incubation, nuclei were pelleted at 1000 × g for 15 min at 4°C, and the supernatant was separated and centrifuged at 16,000 × g for 30 min at 4°C. This high-speed supernatant was stored as the cytoplasmic fraction. The high-speed pellet was then lysed in mitochondrial lysis buffer (50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 50 mM NaCl, 1% Nonidet P-40), and stored as the mitochondrial extract. Protein concentrations of all the fractions were determined by using the BCA protein assay kit (Pierce, Rockford, IL) or the Bio-Rad protein assay kit (Hercules, CA). Each fraction was aliquoted at 70 μg of total protein per tube and stored at −70°C.

#### Western blot analysis of Cyt c

Western blot analysis of Cyt c was performed using appropriate cellular fractions prepared as previously described. Each cytoplasmic extract (70 μg of total protein per lane) was separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Blots were then blocked in Blotto (0.2% Tween 20, 10% nonfat milk, and 3% BSA in PBS) and probed with anti-Cyt c mAb at 1:250 in Blotto. Sheep anti-mouse Ab conjugated to streptavidin HRP was used as secondary Ab.
at 1:10,000 in Blotto. Bands were visualized using the ECL Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to x-ray film. The bands were quantified using Kodak 1D Image Analysis software (Rochester, NY).

**Statistical analysis**

Data shown in the graphs are expressed as the mean ± SD of three samples, and represent at least two independently performed experiments. Repeated measures analysis of variance and unpaired Student’s t test were performed using JMP IN Edition, version 3.1.5 statistical analysis software (SAS Institute, Cary, NC).

**Results**

**Involvement of molecular oxygen and ROS in thymocyte apoptosis ex vivo and in vivo**

We first examined the involvement of molecular oxygen in DEX-induced thymocyte apoptosis. Thymocytes were isolated from BALB/c mice and incubated ex vivo in an anaerobic environment (95% N2; 5% CO2) for an hour before the induction of apoptosis by DEX. This anaerobic treatment of cells significantly reduced DEX-induced apoptosis (Fig. 1A). As shown in Fig. 1B, when thymocytes were treated with NAC, a thiol antioxidant, DEX-induced apoptosis was significantly reduced. These results confirmed our previous report (3) and strongly suggest that molecular oxygen and ROS play an important role in DEX-induced apoptosis ex vivo.

The possibility that ROS play a role in thymocyte apoptosis in vivo was addressed by giving i.p. injections of NAC to 4-wk-old BALB/c mice followed by a DEX injection to induce thymocytes to undergo apoptosis. Fig. 2 shows the protective effect of NAC against DEX-induced apoptosis in thymocytes in vivo. Because the amount of DEX-induced thymocyte apoptosis in vivo varied from experiment to experiment, the percentage of protection by NAC was calculated as indicated in Fig. 2 for standardized comparison. The reduction in the amount of apoptosis by NAC treatment was expressed as the percent of the amount of apoptosis by DEX. As more death was observed in thymocytes from the untreated animals over time, the protective effect of NAC became more apparent, arguing that ROS play a critical role in DEX-induced thymocyte apoptosis in vivo. Because NAC primarily scavenges H2O2 (24, 25), these initial observations provided the basis to hypothesize that H2O2 might play an important role in DEX-induced thymocyte apoptosis.

**Production of H2O2 and cell death events mediated by mitochondria**

To elucidate the relationship between intracellular levels of H2O2 and apoptotic events in thymocytes, it was desirable to monitor intracellular levels of H2O2 during the course of thymocyte apoptosis. To measure intracellular levels of H2O2 during apoptosis, thymocytes were incubated with two H2DCFDA derivative dyes, CM-H2DCFDA or H2DCFDA-di(acetoxymethyl ester), whose fluorescence is proportional to the level of intracellular H2O2. The resulting fluorescence was then analyzed by flow cytometry. When thymocytes were induced to die by DEX, elevated intracellular levels of H2O2 were observed, and this increase was reduced by NAC treatment (Fig. 3A). NAC treatment also reduced the amount of apoptosis induced by DEX (Fig. 3B). In each case, thymocytes were also induced to die by anti-CD3 and anti-CD28 mAbs, and by an appropriate amount of exogenous H2O2 sufficient to cause apoptosis as a positive control for the H2DCFDA derivative dyes.

**FIGURE 1.** Anaerobic and NAC treatments protect thymocytes from DEX-induced apoptosis. Thymocytes were isolated from 4-wk-old BALB/c mice and (A) were cultured in an anaerobic chamber (95% N2; 5% CO2), or (B) were treated with or without NAC (60 μM). Cells were induced to die by the addition of DEX (5 μM), harvested after a 6-h incubation, and stained with Annexin V-fluorescein. The amount of apoptosis was measured by FITC fluorescence by flow cytometry. Each value is presented as the mean ± SD. Statistical significance was calculated based on the differences from the control of each condition. The graph is showing a representative of two experiments. ***, p < 0.01.

**FIGURE 2.** NAC protects DEX-induced thymocyte apoptosis in vivo. One-month-old female BALB/c mice were given two doses of NAC or PBS (NAC control) via i.p. injection 6 h and 1 h before the DEX or 16% ethanol in PBS (EtOH/PBS:DEX control) injection. The four experimental groups: “PBS/(EtOH/PBS)”, “NAC/(EtOH/PBS)”, “PBS/DEX”, and “NAC/DEX” contained a minimum of two mice per group. At 13, 15, and 16 h after the DEX injection, mice were sacrificed and thymocytes were isolated to measure apoptosis. The percentage of protection was calculated as below for standardized comparison: % protection = (apoptosis by DEX – apoptosis by DEX in presence of NAC)/apoptosis by DEX) × 100; when apoptosis by DEX = apoptosis in “PBS/DEX” – apoptosis in “PBS/(EtOH/PBS)”; apoptosis by DEX in presence of NAC = apoptosis in “NAC/DEX” – apoptosis in “NAC/(EtOH/PBS)”. 2471 The Journal of Immunology
both cases, the exogenous H$_2$O$_2$- and the TCR-mediated signaling increased the intracellular levels of H$_2$O$_2$ and the amount of apoptosis, which were also attenuated by the NAC treatment (Fig. 3, A and B). Six hours after cells were induced to die by DEX (5 $\mu$M) or H$_2$O$_2$ (40 $\mu$M), or 8 h after the induction via TCR, cells were harvested to measure intracellular H$_2$O$_2$, or apoptosis by flow cytometry. C, Six hours after the induction of apoptosis by DEX or exogenous H$_2$O$_2$, or 16 h after signaling via TCR, cells were harvested and stained with DiOC$_6$ to measure $\Delta$\textphi$_{m}$ by flow cytometry. D, One hour before the desired time point outlined in C, thymocytes were harvested and resuspended in medium containing 9 nM PhiPhiLuxG$_2$D$_2$, and incubated for an hour to detect caspase-3 activity by flow cytometry. Each value is presented as mean ± SD. Each graph shows a representative of at least three experiments. Statistical significance was calculated based on the differences from the control of each condition. **, $p < 0.01$; *, $p < 0.05$. E, Cytoplasmic extracts of DEX-, H$_2$O$_2$-, or anti-TCR-Ab-treated thymocytes were prepared 5, 4, or 10 h postinduction, respectively. The amount of cytoplasmic Cyt c was visualized by Western blot analysis. Purified rat Cyt c and mitochondrial fraction (Mito) were included as positive controls. The bands representing Cyt c were quantified and expressed as arbitrary units for comparison. Each treatment was compared with its control for purposes of quantification.

DEX resulted in $\Delta$\textphi$_{m}$ depolarization, caspase-3 activation, and Cyt c release. However, when NAC was added to the cultures, these effects were significantly reduced (Fig. 3, C–E). Taken together, these results show that NAC treatment protects thymocytes from apoptosis by reducing the level of intracellular oxidative stress that promotes $\Delta$\textphi$_{m}$ depolarization, caspase-3 activation, and Cyt c release.

H$_2$O$_2$ production and mitochondria

To locate the site of H$_2$O$_2$ production during DEX-induced thymocyte apoptosis, we used various mitochondrial electron transport chain inhibitors, and observed their effect on the production of H$_2$O$_2$. These inhibitors block electron transfer from one component to the next in the transport chain, and the site of blockage is...
specific for each inhibitor. The production of ROS in the mitochondria starts with the formation of a superoxide anion through the leak of an electron from the transport chain to a molecule of oxygen. Therefore, when an inhibitor of electron transportation blocks the electron flow, the production of ROS at sites downstream of the blockage is also decreased. When rotenone (complex I inhibitor) or antimycin A (complex III at Q site) (26) were used, the production of \( \text{H}_2 \text{O}_2 \), and the amount of apoptosis induced by DEX were significantly reduced, suggesting the formation of \( \text{H}_2 \text{O}_2 \) is downstream of their blocking sites. In contrast, in the presence of exogenously introduced \( \text{H}_2 \text{O}_2 \), both the amount of apoptosis and the production of \( \text{H}_2 \text{O}_2 \) were enhanced (Figs. 4, A and B, and 5, A and B). To address the involvement of complex IV in the formation of \( \text{H}_2 \text{O}_2 \), azide, a Cyt c oxidase (complex IV) inhibitor was used, which showed no effect in \( \text{H}_2 \text{O}_2 \) production, or apoptosis (data not shown).

Taken together, these results suggest that the site for DEX-induced \( \text{H}_2 \text{O}_2 \) production is most likely at the ubiquinone cycle at complex III, because the reduction of \( \text{H}_2 \text{O}_2 \) production was observed when the electron flow was reduced by rotenone before electrons entered the ubiquinone cycle, or by antimycin A at the ubiquinone cycle.

Because antimycin A alters the level of \( \text{H}_2 \text{O}_2 \) production originating from the mitochondria, and correspondingly decreases the amount of apoptosis, we investigated the effect of antimycin A on the loss of \( \Delta \psi_m \), Cyt c release, and caspase-3 activation by DEX and exogenous \( \text{H}_2 \text{O}_2 \). Increases in the levels of \( \Delta \psi_m \) depolarization, and activity of caspase-3 induced by DEX were attenuated by rotenone and antimycin A, while those induced by exogenous \( \text{H}_2 \text{O}_2 \) were enhanced (Figs. 4, C and D, and 5, C and D). Antimycin A also attenuated DEX-induced Cyt c release by 97% (Fig. 5E). These observations correlated with the trend in changes in the levels of the intracellular \( \text{H}_2 \text{O}_2 \) and the amount of apoptosis. When cells were treated with \( \text{H}_2 \text{O}_2 \) and antimycin A, they died very quickly, and the cytoplasmic extracts from these cells did not contain enough protein for Cyt c Western blot analysis.

Depriving cultured thymocytes of molecular oxygen should reduce the intracellular molecular oxygen, which may become superoxide anion after accepting the electron from the electron transport chain in mitochondria. We hypothesized that deprivation of oxygen from thymocyte cultures should enhance the protective effect of antimycin A in DEX-treated cells. Therefore, we treated thymocytes with antimycin A in an anaerobic environment (95% \( \text{N}_2 \), 5% \( \text{CO}_2 \)). Treatment with antimycin A in an anaerobic environment resulted in an additive protective effect against DEX-induced production of \( \text{H}_2 \text{O}_2 \) and apoptosis (Fig. 6, A and B). In this study, increased levels of intracellular \( \text{H}_2 \text{O}_2 \) were observed when thymocytes were induced to die by the addition of exogenous \( \text{H}_2 \text{O}_2 \); however, we could not tell whether this increase was by the diffusion of the added \( \text{H}_2 \text{O}_2 \) or by the increased production at the mitochondria, especially because antimycin A did not show the inhibitory effect. As shown in Fig. 6A, the anaerobic conditions attenuated the enhanced \( \text{H}_2 \text{O}_2 \) production seen in the cultures treated with exogenous \( \text{H}_2 \text{O}_2 \), therefore confirming that the increase in intracellular \( \text{H}_2 \text{O}_2 \) observed in cultures treated with exogenous \( \text{H}_2 \text{O}_2 \) is not solely by result of diffusion into the cells of the added \( \text{H}_2 \text{O}_2 \), but is also mitochondria- and molecular oxygen-dependent.

**Figure 4.** Rotenone inhibits DEX-induced apoptosis, enhanced production of intracellular \( \text{H}_2 \text{O}_2 \), and cell death events. Isolated thymocytes were cultured (A) with 5 \( \mu \text{M} \) CM-H \( 2 \text{DCFDA} \) or (B–E) without the dye, then treated with or without rotenone (10 \( \mu \text{M} \)). A and B. Eight hours after they were induced to die by DEX (5 \( \mu \text{M} \)) or \( \text{H}_2 \text{O}_2 \) (40 \( \mu \text{M} \)), cells were harvested to measure intracellular \( \text{H}_2 \text{O}_2 \) or apoptosis by flow cytometry. C. Eight hours after the induction of apoptosis by DEX or exogenous \( \text{H}_2 \text{O}_2 \), cells were harvested and stained with DiOC6 to measure \( \Delta \psi_m \) by flow cytometry. D. Seven hours after the induction of apoptosis by DEX or exogenous \( \text{H}_2 \text{O}_2 \), thymocytes were harvested and resuspended in medium containing 9 \( \text{nM} \) PhiPhiLumG \( \text{D}_{2} \), and incubated for an hour to detect caspase-3 activity by flow cytometry. Each value is presented as mean ± SD. Each graph shows a representative of at least two experiments. Statistical significance was calculated based on the differences from the control of each condition. ***, \( p < 0.01 \); *, \( p < 0.05 \).

**Discussion**

Our previous report was among the first to show that molecular oxygen and ROS are involved in thymocyte apoptosis (3). Since then, accumulating evidence has clearly shown that the level of oxidative stress influences thymocyte vulnerability to apoptosis. This study was focused on elucidating the origin and the regulatory mechanism of the oxidative stress as defined by intracellular levels of \( \text{H}_2 \text{O}_2 \) during apoptosis in DEX-treated thymocytes. Our observations which demonstrated that both withdrawal of molecular oxygen and treatment with NAC inhibit DEX-induced apoptosis show that oxygen and ROS are involved in the DEX-induced apoptosis pathway, both in vivo and ex vivo. Because of its selective patterns of reactivity and its water- and intermembrane-diffusible nature, \( \text{H}_2 \text{O}_2 \) has been postulated to be an intracellular signaling...
molecule (18). NAC is a thiol antioxidant that serves as a ROS scavenger by itself or as a precursor of glutathione, an intracellular thiol protein that primarily diminishes H$_2$O$_2$ (24, 25). Therefore, we chose to measure intracellular levels of H$_2$O$_2$ as the indicator of intracellular oxidative stress. We show that DEX treatment increases the intracellular level of H$_2$O$_2$, while treatment with NAC lowers intracellular H$_2$O$_2$ levels. These changes in the levels of intracellular H$_2$O$_2$ induced by DEX and NAC treatment correlate with the amount of apoptosis, Cyt c loss, Cyt c release, and caspase-3 activation, providing direct evidence that increased oxidative stress is a proapoptotic factor in thymocytes.

In addition, the production of H$_2$O$_2$ was significantly reduced when the electron flow was reduced by rotenone before electrons entered the ubiquinone cycle, or by antimycin A at the ubiquinone cycle. These results determined that the site of H$_2$O$_2$ production during DEX-induced thymocyte apoptosis is at the ubiquinone cycle at complex III in mitochondria. This finding was not entirely unexpected, because the majority of ROS in living cells are produced by semiquinone anion species that occur as an intermediate in the ubiquinone cycle (2). At the ubiquinone cycle, ubiquinone carries two electrons to the Q$_0$ site, where one electron is transferred to cytochrome c$_1$ of the complex III and eventually to Cyt c. Ubiquinone with one electron (semiubiquinone anion) is generated as an intermediate at the Q$_0$ site, and it passes the second electron to cytochrome b of complex III. Then, cytochrome b passes the electron to a fully oxidized ubiquinone at the Q$_i$ site, generating an ubisemiquinone anion, which antimycin A inhibits. Antimycin A can be used to induce apoptosis when used at a relatively high concentration that blocks electron flow completely, and this concentration of antimycin a also induces enhanced production of ROS, due to the increased reduction of electron carriers/complexes upstream of the blockage. In our study, antimycin A was titrated before each experiment to assure that the concentration used was inhibitory to H$_2$O$_2$ production as well as apoptosis. Enhanced H$_2$O$_2$ production and apoptosis were observed with 50

**FIGURE 5.** Antimycin A inhibits DEX-induced apoptosis, enhanced production of intracellular H$_2$O$_2$, and cell death events. Isolated thymocytes were cultured (A) with 5 μM CM-H$_2$DCFDA or (B–E) without the dye, then treated with or without antimycin A (1 μM). A and B, Eight hours after they were induced to die by DEX (5 μM) or H$_2$O$_2$ (40 μM), cells were harvested to measure intracellular H$_2$O$_2$ or apoptosis by flow cytometry. C, Eight hours after the induction of apoptosis by DEX or exogenous H$_2$O$_2$, cells were harvested and stained with DiOC$_6$ to measure Δψ$_m$ by flow cytometry. D, Seven hours after the induction of apoptosis by DEX or exogenous H$_2$O$_2$, thymocytes were harvested and resuspended in medium containing 9 nM PhiPhiLuxG1, and incubated for an hour to detect caspase-3 activity by flow cytometry. Each value is presented as mean ± SD. Each graph shows a representative of at least three experiments. Statistical significance was calculated based on the differences from the control of each condition. ***, p < 0.01; **, p < 0.05. E, Cytoplasmic extracts of DEX-treated thymocytes were prepared 4 h postinduction of apoptosis by DEX. The amount of cytoplasmic Cyt c was visualized by Western blot analysis. The bands representing Cyt c were quantified and expressed as arbitrary units as described in Fig. 3.
Each condition. Significance was calculated based on the differences from the control of samples was cultured in a conventional incubator (air/5% CO₂) and the concentration used in our experiments reduced the electron flow, and did not block it completely. The generation of the free radicals generated in this study, addition of exogenous H₂O₂ by itself can cause PT, Cyt c release, caspase-3 activation, and apoptosis, suggesting that the increased production of H₂O₂ in mitochondria during apoptosis is not only a result of the mitochondrial homeostasis disruption, but is also a cause of the disruption. This observation agrees with the report that the mitochondrial PT pore that mediates PT is sensitive to ROS (11). Therefore, the production of H₂O₂ and the mitochondrial homeostasis disruption may create a self-amplifying process in the mitochondria leading to apoptosis.

We used lactacystin to address the role of the proteasome in regulating cell death events mediated by mitochondria. Inhibiting proteasome activities attenuated cell death events mediated by mitochondria observed in this study, including the production of H₂O₂, providing evidence that the proteasome plays a regulatory role upstream of the mitochondria. Because our data showed that the proteasome was capable of regulating apoptotic events in response to changes in levels of intracellular oxidative stress, it is possible that the H₂O₂ generated in mitochondria creates a negative and/or positive feedback loop to the proteasome, which in turn affects the selective degradation of Bcl-2 family members in the mitochondria. The molecular mechanism(s) by which the proteasome senses oxidative stress remains to be elucidated. One possible mechanism in which proteasome regulates apoptosis is to degrade pro- and/or antiapoptotic proteins. Recent observations suggest that the proteasome regulates the expression levels of pro- and antiapoptotic Bcl-2 family members in mitochondria (29, 30). Although the precise molecular mechanisms are yet to be elucidated, data from numerous studies suggested that proapoptotic Bcl-2 family members such as Bid, Bax, and Bak mediate Cyt c release either by forming a channel/pore themselves and thus permeabilizing the mitochondrial outer membrane, or by interacting and regulating the voltage-dependent anion channel, a resident mitochondrial membrane pore (20–22, 31). The antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL may prevent apoptosis either by physically interacting with the proapoptotic Bcl-2 family members and inhibiting their action, or by regulating voltage-dependent anion channel (20–22, 31). Therefore, changes in the ratio of pro- and antiapoptotic Bcl-2 proteins could be the mechanism by which the proteasome acts to regulate apoptosis in thymocytes at or before the mitochondrial level. Most of the proapoptotic Bcl-2 members are localized to the cytoplasm or cytoskeleton before an apoptotic signal (32–34). Upon receiving such a signal, they undergo conformational changes that allow them to migrate.
possibly by the dynein motor complex and integrate into the mitochondrial outer membrane (34). Therefore, it is possible that proteasomal activities are modifying the migration process of the pro-apoptotic Bcl-2 family members by degrading the cytoskeletal/motor proteins. Another example of proteasome-mediated degradation of antiapoptotic protein is the degradation of inhibitors of apoptosis proteins (IAPs) upon apoptotic stimuli, including DEX (35). IAPs inhibit activation and/or activities of caspases, and this degradation occurs very early in the apoptotic process through autoubiquitination (35). Slee et al. (36) have shown that in Jurkat cells, caspase-3 establishes a proapoptotic feedback loop to mitochondria through direct cleavage of Bid, causing further permeabilization of the outer membrane. Thus, it is possible that the proteasome may play a critical role in regulating apoptosis in T cells/thymocytes at several points in the cell death cascade.

Fig. 8 shows a summary of this study and a hypothesized regulatory role of H$_2$O$_2$ and proteasomes. We showed that the increased production of H$_2$O$_2$ is observed during DEX-, TCR- and exogenous H$_2$O$_2$-induced thymocytes apoptosis, and we identified the site of the production of H$_2$O$_2$ at ubiquinone cycle at complex III in the mitochondria. The deprivation of molecular oxygen and the treatment with NAC diminished the increases in the production of H$_2$O$_2$, loss of $\Delta \Psi_m$, caspase-3 activation, Cyt c release, and amount of apoptosis, therefore suggesting that higher levels of intracellular H$_2$O$_2$ promotes these apoptotic events and increases cell susceptibility to apoptosis. We showed that inhibition of the proteasome resulted in a reduction of mitochondria-mediated cell death events induced by DEX and exogenous H$_2$O$_2$. This result indicates that the proteasome plays the following roles: 1) regulation of mitochondrial membrane permeabilization, possibly by degradation of Bcl-2 family members, and/or through degradation of IAPs, and 2) regulation of apoptosis in response to the intracellular oxidative stress, therefore establishing a feedback loop with the oxidative stress originating from mitochondria during apoptosis.
Accumulating evidence has shown that in response to oxidative stress, numerous signaling pathways are activated. Such pathways include the mitogen-activated protein kinase pathway, the phosphoinositide 3-kinase/Akt pathway, p53-mediated pathway, heat shock protein-mediated pathway, extracellular signal-regulated kinase pathway, and c-Jun N-terminal kinase pathway. A common outcome of the activation of these pathways is a change in expression patterns of many genes by modulating the activities of various transcription factors. In addition, there are numerous transcription factors that are known to change their activity according to oxidative status, such as NF-κB and AP-1.

Multiple pathways and transcription factors can be activated or deactivated by the rise in intracellular H₂O₂ during DEX-induced thymocyte apoptosis, and while these direct mechanisms remain to be elucidated, our study has shed light on two important areas of oxidative stress signaling in thymocyte apoptosis: the role of molecular oxygen and H₂O₂, and the role of the proteasome in mediating and modulating the signaling pathway before a mitochondrial involvement.

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References


