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Mitochondria are the primary source of the H_2O_2 signal for glucocorticoid-induced apoptosis of lymphoma cells

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Abstract. Glucocorticoids are a class of steroid hormones commonly used for the treatment of hematological malignancies due to their ability to induce apoptosis in lymphoid cells. An understanding of the critical steps in glucocorticoid-induced apoptosis is required to identify sources of drug resistance. Previously, we found that an increase in hydrogen peroxide is a necessary signal for glucocorticoid-induced apoptosis. In the current study, we found that mitochondria are the source of the signal. Glucocorticoid treatment inhibited Complex I and Complex III of the electron transport chain (ETC). Mitochondrial matrix reactive oxygen species (ROS) increased concomitantly with the oxidation of the mitochondrial glutathione pool. Treatment with Tiron, a superoxide scavenger, inhibited the signal. This suggests that the hydrogen peroxide signal originates as superoxide from the mitochondria and is metabolized to hydrogen peroxide. An inability to generate mitochondrial oxidants in response to glucocorticoids could cause drug resistance.

Introduction

Glucocorticoid-induced apoptosis in lymphocytes consists of the signaling phase, the commitment step marked by the release of cytochrome *c* from the mitochondria, and the execution phase in which caspases degrade cellular proteins (1). The execution phase has been well-characterized and is common to many agents that induce apoptosis (1). The signaling phase of glucocorticoid-induced apoptosis is not completely understood. Identifying the critical signals is key to determining sources of resistance in the clinic since glucocorticoids are a primary drug in the treatment of lymphoid malignancies.

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Abbreviations: ETC, electron transport chain; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; SOD, superoxide dismutase; Tiron, 4,5-dihydroxy-m-benzenedisulfonic acid

Key words: apoptosis, lymphoma mitochondria, reactive oxygen species

The commitment to undergo apoptosis following glucocorticoid treatment is determined by a complex interaction of pro-apoptotic and survival signals (reviewed in refs. 2-4). Transcriptional events, including the upregulation of Bim (5), and metabolic events, such as an increase in Ca^{2+} flux (6) and increased neutral sphingomyelinase activity (7), are required. Recently, we showed that an increase in hydrogen peroxide (H_2O_2) is also a required signaling event (8). Glucocorticoid treatment of WEHI7.2 murine thymic lymphoma cells causes an increase in H_2O_2 during apoptosis signaling. Cells that overexpress catalase also produce increased H_2O_2 following glucocorticoid treatment; however, if the quantity of catalase is sufficient to remove the H_2O_2, the cells do not undergo apoptosis.

In our previous studies, we established the necessity of an H_2O_2 signal, but we did not determine the source of the increased H_2O_2. Previous work by Tonomura *et al* shows that glucocorticoid treatment of immature thymocytes causes an increase in reactive oxygen species (ROS) from Complex III of the electron transport chain (ETC) (9). The study by Tonomura *et al* did not determine whether there are additional ROS sources or whether tumor cells have the same glucocorticoid response. These details are significant as tumor cells often metabolize ROS differently than non-transformed cells (10). The goal of the current study was to use the WEHI7.2 model to identify the source of the H_2O_2 produced during the signaling phase of glucocorticoid-induced apoptosis in lymphoma cells.

Materials and methods

Reagents. 15-Lipoxygenase inhibitor, baicalein, piriprost and 8,11,14-eicosatriynoic acid were purchased from Cayman Chemical Co. (Madison, WI, USA). JC-1 and DAPI were purchased from Invitrogen/Molecular Probes (Carlsbad, CA, USA). All other drugs and chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise noted.

Cell culture. The WEHI7.2 mouse thymic lymphoma cell line (11) was maintained in suspension cultures in DMEM (Invitrogen) with 10% calf serum (Hyclone Laboratories, Ogden, UT, USA) in a humidified 37˚C environment with 5% CO_2 (12).

Drug treatments. Response to dexamethasone, a synthetic glucocorticoid, was determined by incubating cells in a final

concentration of 1 μM dexamethasone in an ethanol vehicle (final concentration of ethanol = 0.01%) or an equivalent amount of vehicle alone.

Enzymes were inhibited as follows: xanthine oxidase, 100 μM allopurinol (13); cyclooxygenase 1/2, 200 μM ibuprofen (14); cyclooxygenase 1/2 plus lypoxygenase 5/12, 20 μM eicosatriynoic acid (14); lypoxygenase 12, 10 μM bicalein (14); lipoxygenase 15, 10 μM 15-lipoxygenase inhibitor; lipoxygenase 5, 50 μM piriprost (15); NAD(P)H oxidase, 10 μg/ml apocynin (13); cytochrome P450, 15 μM cimetidine (13) or 2.5 μM ketokonazole (16). All of the inhibitors and vehicles were first titrated for toxicity in the presence and absence of dexamethasone. The inhibitors were used at concentrations that showed no toxicity. All drug treatments were continuous.

Protein and enzyme/ETC complex activity measurements. Cellular protein was measured in clarified lysates using the BCA Protein Assay kit (ThermoScientific, Rockford, IL, USA) according to the manufacturer's instructions. Superoxide dismutase (SOD) activity was measured as previously described (17). Clarified whole cell lysates were used for measurements of Complex I (18) and Complex III (19) activities. Complex IV activity was measured by first isolating mitochondria using the ThermoScientific Mitochondrial Isolation kit for Cultured Cells (ThermoScientific) and then measuring the activity using the method of Zhang *et al* (20). Values were normalized to cellular or mitochondrial protein as appropriate and expressed as the percentage of the vehicle-treated control.

Amplex® Red measurements. The rate of H_2O_2 efflux was determined by measuring the rate of oxidation of the fluorogenic indicator Amplex® Red (Invitrogen) in the presence of horseradish peroxidase. Briefly, cells were resuspended in phenol red-free DMEM (Invitrogen) with 10% calf serum (Hyclone Laboratories) containing 50 μM Amplex® Red and 0.1 unit/ml horseradish peroxidase. The rate of increase in fluorescence (Ex 510/Em 590) was measured using a Synergy HT plate reader (Bio Tek Instruments, Winooski, VT, USA). Rates were normalized to cellular protein measured as described above.

MitoSOX measurements. Cells were incubated in a final concentration of 5 μM MitoSOX (Molecular Probes/Invitrogen) in DMEM with 10% calf serum at 37˚C for 3 h. The rate of increase in MitoSOX fluorescence (Ex 530/Em 590) was measured using a Synergy HT plate reader (BioTek Instruments, Inc.). Rates were normalized to the sample protein measured as described above. Localization of the MitoSOX signal was determined by treating cells with MitoSOX, 0.1 μM JC-1 and 0.1 μM DAPI for 30 min. Fluorescent images were captured using the DeltaVision Restoration Microscopy System (Applied Precision, Inc., Issaquah, WA, USA) at the following wavelengths; MitoSOX (Ex 530/Em 590), JC-1 (Ex 360/Em 568), DAPI (Ex 358/Em 461). Data were collected and processed using Scion Image (Scion, Frederick, MD, USA).

roGFP2 measurements. The redox sensitive GFP plasmid, p-EGFP-N1/roGFP2 with a mitochondrial targeting sequence (21) (a donation from Dr S. James Remmington), was electroporated into WEHI7.2 and variant cells using the Amaxa Nucleofactor™ II (Amaxa GmbH, Germany). Following transfection, cells were incubated in phenol red-free DMEM (Invitrogen) supplemented with 10% calf serum for 24 h, then treated with 1 μM dexamethasone or vehicle control for 12 h. Cells were imaged using the DeltaVision Restoration Microscopy System using excitation lines at 407 nm and 488 nm and a 510/21 nm emission filter. Data were collected and processed using Scion Image. Images were corrected for background fluorescence by subtracting the intensity of a nearby cell-free region. Fluorescence excitation ratios were then calculated by dividing the integrated intensities of the cells at the different excitation wavelengths using the formulas described in Hanson *et al* (21). Between 20 and 35 cells were analyzed per treatment. Mitochondrial localization of the roGFP2 was measured as described for the MitoSOX.

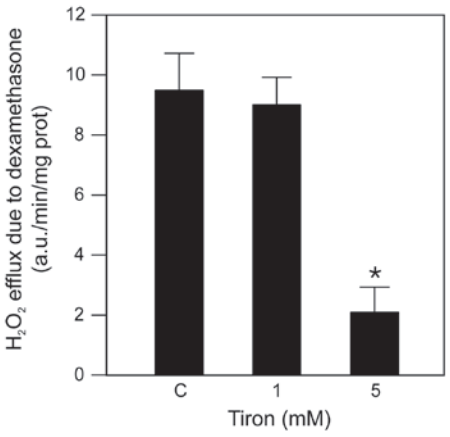


Figure 1. Scavenging superoxide decreases H_2O_2 efflux following a 12-h dexamethasone treatment. Measurement of H_2O_2 efflux in the presence of indicated concentrations of Tiron. Values were corrected for the H_2O_2 efflux in the absence of dexamethasone for each concentration of Tiron. Values are the mean + SEM. (n=3). *Significantly different from C, cells treated with dexamethasone alone ($P \leq 0.05$). Tiron, 4,5-dihydroxy-m-benzenedisulfonic acid.

Statistics. Means were compared using t-tests or ANOVA, where appropriate, using the algorithms in Excel (Microsoft, Redmond, WA, USA). $P \leq 0.05$ was considered to indicate a statistically significant result.

Results

Superoxide is a major source of the H_2O_2 signal. H_2O_2 can be produced directly or as a byproduct of superoxide dismutation. Therefore, one possible source of the H_2O_2 signal in response to glucocorticoids is increased superoxide that is metabolized into H_2O_2. To determine whether the H_2O_2 signal originates from superoxide, we measured the H_2O_2 signal due to dexamethasone, a synthetic glucocorticoid, in the presence of Tiron (4,5-dihydroxy-m-benzenedisulfonic acid), which scavenges superoxide (22). For these measurements, we chose a 12 h dexamethasone treatment as we have previously determined that this time point is in the signaling phase of dexamethasone-induced apoptosis in WEHI7.2 cells (8). As shown in Fig. 1, Tiron caused a dose-dependent decrease in H_2O_2 efflux in response to a 12 h dexamethasone treatment.

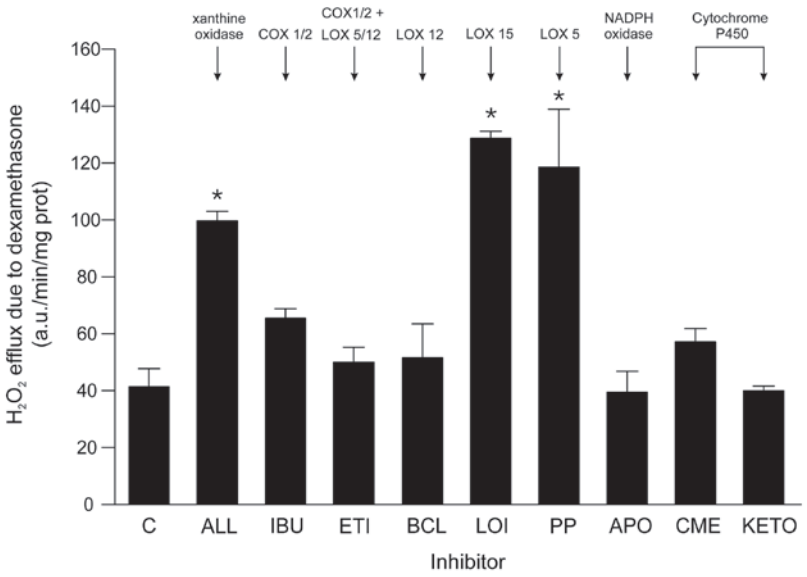


Figure 2. Enzyme inhibition did not decrease H_2O_2 efflux due to a 12-h dexamethasone treatment. Measurement of H_2O_2 efflux in the presence or absence of enzyme inhibitors. Target enzymes are listed above each column. Values have been corrected for the H_2O_2 efflux in the presence of inhibitor alone. Inhibitor concentrations were as follows: 100 μM allopurinol (ALL); 200 μM ibuprofen (IBU); 20 μM eicosatriynoic acid (ETI); 10 μM bicalein (BCL); 10 μM 15-lipoxygenase inhibitor (LOI); 50 μM piriprost (PP); 10 μg/ml apocynin (APO); 15 μM cimetidine (CME); 2.5 μM ketokonazole (KETO) and dexamethasone in the absence of inhibitor is labeled (C). Values are the mean + SEM (n≥3). *Significantly different from C, cells treated with dexamethasone alone. COX, cyclooxygenase; LOX, lipoxygenase.

All the values were corrected for H_2O_2 efflux in vehicle-treated cells, thus the values represent only the increase in H_2O_2 efflux in response to dexamethasone treatment. Although there was still a residual signal following Tiron treatment, addition of 5 mM Tiron eliminated approximately 80% of the signal. This suggests that superoxide is a major source of the H_2O_2 signal in response to dexamethasone.

Cytosolic enzymatic sources do not increase H_2O_2 production in response to dexamethasone. A number of cytosolic or membrane bound enzymes produce superoxide or H_2O_2 either as a product of their enzymatic activities or from leakage due to the one electron reduction of oxygen. These include xanthine oxidase, NAD(P)H oxidase, cyclooxygenase, lipoxygenase and cytochrome P450. To determine whether these enzymes contribute to the increased H_2O_2 produced in response to dexamethasone treatment we measured the H_2O_2 efflux due to dexamethasone treatment in the presence of inhibitors of these enzymes. The use of the inhibitors was expected to decrease the H_2O_2 efflux if the target enzyme was producing increased superoxide/H_2O_2 in response to dexamethasone. As shown in Fig. 2, inhibition of xanthine oxidase, cyclooxygenases 1 and 2, lipoxygenases 5, 12 and 15, cytochrome P450 or NAD(P)H oxidase did not reduce the H_2O_2 signal. The plotted values were corrected for H_2O_2 efflux in control cells treated with inhibitor only. This indicates that these enzymes are unlikely to be sources of increased superoxide/H_2O_2 in response to dexamethasone. Treatment with inhibitors of the lipoxygenases and xanthine oxidase significantly increased the H_2O_2 efflux indicating that these enzymatic activities may reduce oxidative stress caused by dexamethasone treatment.

Mitochondria are a source of increased oxidants following dexamethasone treatment. Mitochondria are widely considered a source of ROS in the cell. Components of the ETC leak superoxide, particularly when oxidative phosphorylation is interrupted (23,24). We tested whether dexamethasone treatment increased mitochondrial matrix oxidants by measuring the fluorescence of MitoSOX, a mitochondrial matrix-targeted probe that fluoresces after reacting with ROS. Twelve hours after the addition of dexamethasone, the mitochondrial matrix ROS was higher than that in the control cells (Fig. 3A). As shown in Fig. 3B, 95.97±0.45% (n=14) of the fluorescent MitoSOX signal co-localized with JC-1, a mitochondrion-specific dye.

We then used a mitochondrially targeted roGFP2 probe to measure the mitochondrial redox environment in response to dexamethasone. This probe measures the relative oxidation of the mitochondrial glutathione pool (21). Quantitation of the oxidized roGFP2 signal indicated that the mitochondrial glutathione pool became oxidized in response to dexamethasone (Fig. 3C). As shown in Fig. 3D, 97.21±0.24% (n=16) of the mitochondrially targeted roGFP2 co-localized with JC-1.

Glucocorticoids inhibit ETC complex activity. When oxidative phosphorylation is inhibited, the ROS produced from the ETC increases (23,24). To determine whether glucocorticoids inhibited oxidative phosphorylation, we measured the effect of glucocorticoid treatment on the activity of Complexes I, III and IV. As shown in Fig. 4, glucocorticoid treatment inhibited Complex I and Complex III activity, but had no effect on Complex IV activity.

Mn superoxide dismutase (MnSOD) activity increases in response to glucocorticoid treatment. We previously demonstrated that an increase in H_2O_2 is a required signal for glucocorticoid-induced apoptosis in lymphoma cells (8). The data from the current study indicate that the majority of the H_2O_2 generated by dexamethasone treatment originates

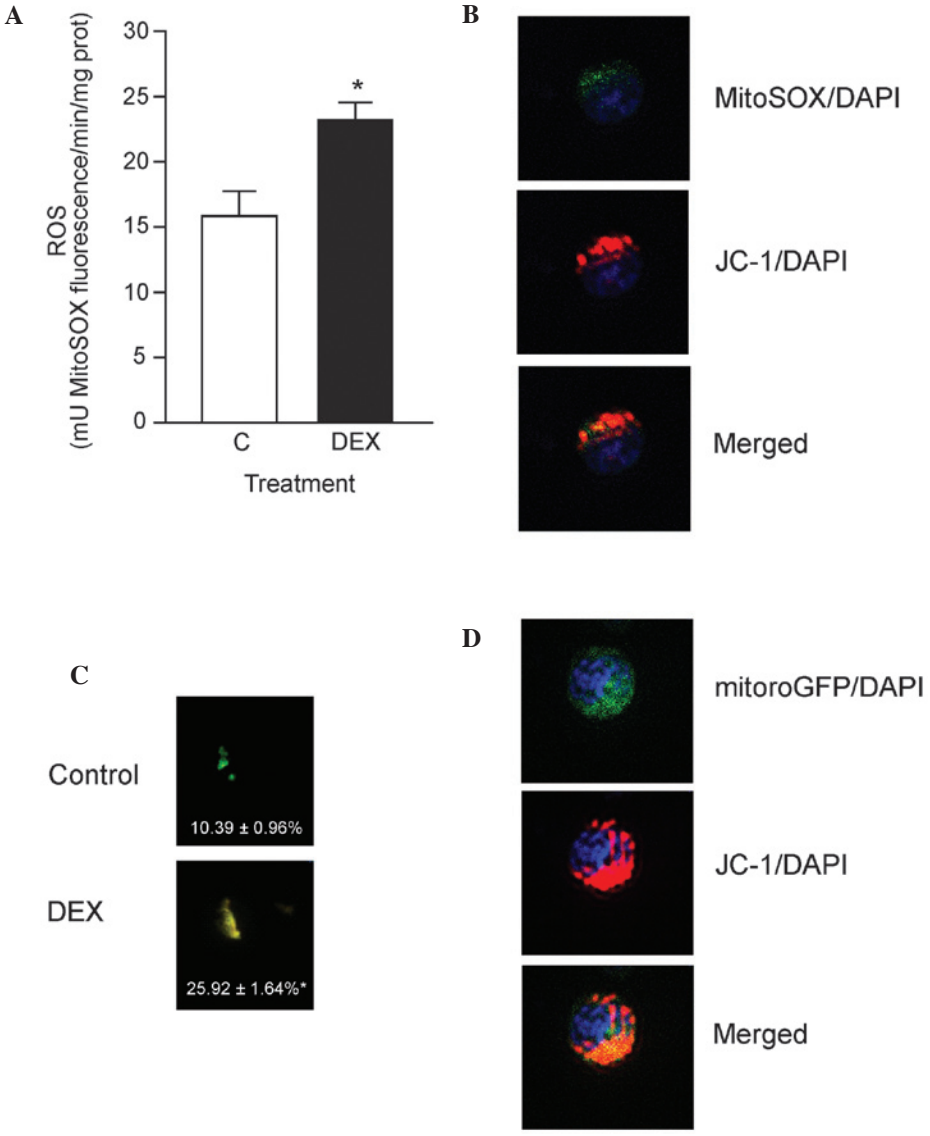


Figure 3. Dexamethasone treatment increases mitochondrial matrix ROS. (A) Rate of increase in MitoSOX fluorescence in C, vehicle-treated cells, compared to those treated for 12 h with dexamethasone (DEX). Values represent the means + SEM. (n=3). *Significantly different from control values ($P \leq 0.05$). (B) Representative images showing the localization of the MitoSOX signal (green) and mitochondrial specific JC-1 (red); cells were counterstained with DAPI (blue) to show nuclear location. A representative merged image shows the co-localization of the MitoSOX and JC-1 signals. (C) Representative overlay images showing the amount of reduced (green) and oxidized (red) mitochondrial roGFP2 in the presence or absence of dexamethasone. Values indicate the mean % roGFP2 oxidized + SEM (n=25-30 cells). *Significantly different from control values. (D) Representative images showing the locatization of the roGFP2 (green) and mitochondrial specific JC-1 (red); cells were counterstained with DAPI (blue) to show nuclear location. A representative merged image shows the co-localization of the roGFP2 and JC-1 signals.

from superoxide. To participate in apoptosis signaling the superoxide must be metabolized to H_2O_2. SODs metabolize superoxide to H_2O_2. Therefore, we measured the activities of MnSOD, which is located in the mitochondrial matrix, and Cu,Zn superoxide dismutase (Cu,ZnSOD) which is located in the intermembrane space and cytosol. As shown in Fig. 5, MnSOD activity increased in response to dexamethasone treatment but there was no change in Cu,ZnSOD activity in response to dexamethasone. These data indicate that SOD is available to metabolize superoxide.

Discussion

Our data indicate that mitochondria are the primary source of the H_2O_2 signal during glucocorticoid-induced apoptosis in lymphoma cells. We did not find an additional source of H_2O_2 among the enzymes we tested. The current study characterizes the ROS signal in lymphoma cells and fits a model whereby glucocorticoids inhibit Complexes I and III of the ETC causing an increase in mitochondrial superoxide production. The superoxide is metabolized to H_2O_2 by SOD and/or other enzymes. The resulting H_2O_2 is a necessary signal for glucocorticoid-induced apoptosis (8). The production of H_2O_2 is concomitant with the oxidation of the mitochondrial glutathione pool. Oxidation of mitochondrial glutathione can contribute to apoptotic signaling (25).

The ability of dexamethasone to disrupt mitochondrial metabolism likely contributes to the increase in H_2O_2 observed following glucocorticoid treatment. We previously showed that dexamethasone treatment inhibits glucose uptake (26). In

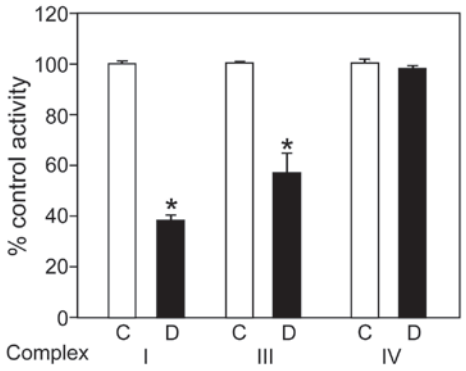


Figure 4. ETC Complexes I and III are inhibited by a 12-h dexamethasone treatment. Activity of Complexes I, III and IV in D, the presence of dexamethasone, compared to C, vehicle-treated cells. Values have been corrected for protein and normalized to control values for each complex. Values are the mean + SEM. (n=3). *Significantly different from control values (P≤0.05).

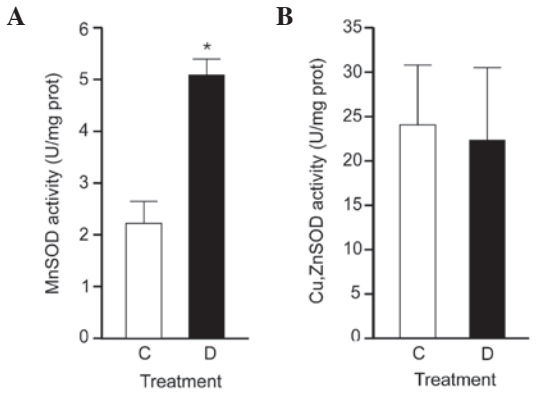


Figure 5. Manganese superoxide dismutase activity increases in response to a 12 h-dexamethasone treatment. (A) MnSOD or (B) Cu,ZnSOD activity in the presence of D, dexamethasone, compared to C, vehicle-treated cells. Values are the mean + SEM. (n=3). *Significantly different from control values (P≤0.05).

the absence of glucose, lymphocytes utilize glutamate as an energy source (27). In other cell types when glutamate is used as a substrate, mitochondrial ROS generation increases under conditions where Complex I and, in particular, Complex III are inhibited (24). The combination of a switch to glutamate metabolism and the inhibition of Complex III caused by dexamethasone treatment is likely to contribute to the increase in mitochondrial ROS generation.

Our data suggest that the main species of oxidant generated by dexamethasone treatment is superoxide. This is consistent with the ability of dexamethasone to inhibit Complexes I and III. Inhibition of Complexes I and III is expected to produce superoxide via the reverse flow of electrons and from the Q cycle in Complex III (23). Although Tiron treatment reduced the H_2O_2 signal, there was residual H_2O_2 that persisted even after the addition of 5 mM Tiron. One possible explanation for this observation is that the superoxide is metabolized to H_2O_2 quickly by enzymes in the mitochondria that compete with Tiron for superoxide. MnSOD in the mitochondrial matrix and Cu,ZnSOD in the mitochondrial intermembrane space are positioned close to the source and could compete for superoxide. Another possibility is that some H_2O_2 is produced directly by other enzymes in response to dexamethasone treatment. For example, p66shc is an enzyme in the intermembrane space that produces H_2O_2 via interaction with cytochrome *c* in response to apoptotic signals (28). Although we did not find additional sources of ROS in this study, other possibilities remain to be tested.

Removal of the H_2O_2 signal prevents glucocorticoid-induced apoptosis (8). This indicates that there are downstream targets of H_2O_2 that are required for the apoptotic process. One potential downstream target of the H_2O_2 increase is BAX. Oxidation of cys62 in BAX causes conformational activation and translocation of BAX to the mitochondria (29). Activated BAX plays a role in mitochondrial membrane pore formation for the release of cytochrome *c* (30). An increase in H_2O_2 also stimulates the peroxidase activity of cytochrome *c*. This results in oxidation of cardiolipin and release of cytochrome *c* from the outer surface of the inner mitochondrial membrane (31). Unteathering cytochrome *c* from the membrane is required to allow cytochrome *c* to move into the cytosol through the pores in the outer mitochondrial membrane (30,31). Our data suggest an additional downstream target, the oxidation of the mitochondrial glutathione pool. In other cells types, oxidation of the mitochondrial glutathione pool is an apoptotic signal (25).

Our data indicate that mitochondria are central to apoptotic signaling during glucocorticoid-induced apoptosis. In addition to the release of apoptotic proteins, mitochondria are the source of the H_2O_2 signal required for this process. Our previous data indicate that the removal of the H_2O_2 signal serves as a protective mechanism (8). The current study suggests that an inability of the mitochondria to generate a H_2O_2 signal in response to glucocorticoids could contribute to the glucocorticoid resistance observed in the clinic.

Acknowledgements

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