Mitochondria are the primary source of the H$_2$O$_2$ 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.

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 lipoxygenase 5/12, 20 µM eicosatetraenoic acid (14); lipoxygenase 12, 10 µM bicaline (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$_2$O$_2$ 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 (BioTek 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 Amxax 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.

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

**Results**

**Superoxide is a major source of the H$_2$O$_2$ signal.** H$_2$O$_2$ can be produced directly or as a byproduct of superoxide dismutation. Therefore, one possible source of the H$_2$O$_2$ signal in response to glucocorticoids is increased superoxide that is metabolized into H$_2$O$_2$. To determine whether the H$_2$O$_2$ signal originates from superoxide, we measured the H$_2$O$_2$ 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$_2$O$_2$ efflux in response to a 12 h dexamethasone treatment.
Mitochondria are a source of increased oxidants following dexamethasone treatment. Measurement of H₂O₂ efflux in response to dexamethasone treatment indicated that this process 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. 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.

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 H₂O₂ is a required signal for glucocorticoid-induced apoptosis in lymphoma cells (8). The data from the current study indicate that the majority of the H₂O₂ generated by dexamethasone treatment originates...
from superoxide. To participate in apoptosis signaling the superoxide must be metabolized to H$_2$O$_2$. SODs metabolize superoxide to H$_2$O$_2$. 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$_2$O$_2$ signal during glucocorticoid-induced apoptosis in lymphoma cells. We did not find an additional source of H$_2$O$_2$ 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$_2$O$_2$ by SOD and/or other enzymes. The resulting H$_2$O$_2$ is a necessary signal for glucocorticoid-induced apoptosis (8). The production of H$_2$O$_2$ 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$_2$O$_2$ observed following glucocorticoid treatment. We previously showed that dexamethasone treatment inhibits glucose uptake (26). In
superoxide. Another possibility is that some H$_2$O$_2$ is produced directly by other enzymes in response to dexamethasone treatment. For example, p66shc is an enzyme in the intermembrane space that produces H$_2$O$_2$ 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$_2$O$_2$ signal prevents glucocorticoid-induced apoptosis (8). This indicates that there are downstream targets of H$_2$O$_2$ that are required for the apoptotic process. One potential downstream target of the H$_2$O$_2$ 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$_2$O$_2$ 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). Unleathering 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$_2$O$_2$ signal required for this process. Our previous data indicate that the removal of the H$_2$O$_2$ signal serves as a protective mechanism (8). The current study suggests that an inability of the mitochondria to generate a H$_2$O$_2$ signal in response to glucocorticoids could contribute to the glucocorticoid resistance observed in the clinic.

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