

Inhibition of Ca^{2+} Influx Is Required for Mitochondrial Reactive Oxygen Species-Induced Endoplasmic Reticulum Ca^{2+} Depletion and Cell Death in Leukemia Cells

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ABSTRACT

Disturbances of endoplasmic reticulum (ER) Ca^{2+} homeostasis or protein processing can lead to ER stress-induced cell death. Increasing evidence suggests that oxidative stress (OS) plays an important role in a variety of cell death mechanisms. To investigate the role of OS in ER stress, we measured OS in response to three ER stress agents: econazole (Ec), which stimulates ER Ca^{2+} release and blocks Ca^{2+} influx; thapsigargin (Tg), a sarco(endoplasmic reticulum Ca^{2+} ATPase inhibitor that releases ER Ca^{2+} and stimulates Ca^{2+} influx; and tunicamycin (Tu), a glycosylation inhibitor that causes protein accumulation in the ER. Ec, but not Tg or Tu, caused a rapid increase in OS. Reactive oxygen species (ROS) generation was observed within mitochondria immediately after exposure to Ec. Furthermore, Ec hyperpolarized the mitochondrial membrane and inhibited adenine nucleotide transport in cell-free

mitochondria, suggesting a mitochondrial target. Antimycin A, an inhibitor of complex III in electron transport, reversed mitochondrial hyperpolarization, OS generation, ER Ca^{2+} depletion, and cell death by Ec, suggesting complex III dependence for these effects. Antioxidants butylated hydroxytoluene and *N*-Acetyl-L-cysteine prevented ER Ca^{2+} depletion and cell death by Ec. However, inhibition of Ca^{2+} influx by Ec was unaffected by either antimycin A or the antioxidants, suggesting that this target is distinct from the mitochondrial target of Ec. Atractyloside, an adenine nucleotide transport inhibitor, generated ROS and stimulated ER Ca^{2+} release, but it did not block Ca^{2+} influx, deplete the ER or induce cell death. Taken together, these results demonstrate that combined mitochondrial ROS generation and Ca^{2+} influx blockade by Ec is required for cell death.

The endoplasmic reticulum (ER) is a major intracellular calcium store and the organelle responsible for the synthesis and post-translational modification of proteins destined for secretion or surface expression. These post-translational processes include protein folding, glycosylation, disulfide bond formation, and ER-Golgi protein trafficking. Disturbances of ER calcium homeostasis and protein processing cause accu-

mulation of unfolded or misfolded proteins in the ER lumen and initiate the unfolded protein response (Kaufman, 1999; Ferri and Kroemer, 2001; Patil and Walter, 2001). Cells exhibit a variety of responses in their attempt to mitigate such ER stress. These include increased expression of ER-resident Ca^{2+} -dependent molecular chaperones such as GRP78 (BiP) and GRP94 (Kozutsumi et al., 1988) and suppression of protein synthesis to reduce the unfolded protein load. Preconditioning with sublethal levels of ER stress has been shown to protect cells, in part through up-regulation of chaperones (Liu et al., 1998; Hung et al., 2003). However, sustained ER stress will eventually result in prolonged protein synthesis inhibition that leads to cell death (Soboloff and Berger, 2002; Zhang and Berger, 2004). Important mediators of ER stress-associated death include the cleavage and activation of the ER-associated caspase-12 (Szegezdi et al., 2003)

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ABBREVIATIONS: ER, endoplasmic reticulum; SERCA, sarco(endoplasmic reticulum Ca^{2+} ATPase; Tg, thapsigargin; Ec, econazole; Atr, atractyloside; ROS, reactive oxygen species; OS, oxidative stress; Tu, tunicamycin; FBS, fetal bovine serum; AA, antimycin A; BHT, butylated hydroxytoluene; Nac, *N*-Acetyl-L-cysteine; PI, propidium iodide; CM-H₂DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; AM, acetoxymethyl ester; MMP, mitochondrial membrane potential; JC-1, 5,5',6,6'-tetraethylbenzimidazolcarbocyanine iodide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; ANT, adenine nucleotide transporter; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; DCF, 2',7'-dichlorofluorescein.

and increased expression of CHOP/GADD153 (Wang et al., 1996), a transcription factor that sensitizes cells to apoptosis.

Disruption of Ca^{2+} homeostasis in the ER, such as after treatment with the SERCA inhibitor thapsigargin (Tg) (Thastrup et al., 1990), can stimulate sustained Ca^{2+} influx from the extracellular milieu. In some cell types, this leads to both cytosolic and mitochondrial Ca^{2+} overload (Babich et al., 1994; Soboloff and Berger, 2002), triggering apoptosis (Orrenius et al., 2003). Econazole (Ec), an imidazole antifungal, also stimulates depletion of the Tg-sensitive ER calcium store. However unlike Tg, Ec additionally blocks Ca^{2+} influx, resulting in sustained ER Ca^{2+} depletion (Franzius et al., 1994; Jan et al., 1999; Soboloff and Berger, 2002). In previous studies, we have shown that the Ca^{2+} depletion caused by Ec induces activation enhanced cell death in leukemic cells, breast cancer cells, and murine bone marrow-derived mast cells through sustained inhibition of protein synthesis (Gommerman and Berger, 1998; Soboloff and Berger, 2002; Soboloff et al., 2002; Zhang et al., 2002; Zhang and Berger, 2004). However, the mechanism of how this compound affects the intracellular ER store and the subsequent fate of the cell remains unknown.

Increasing evidence suggests that reactive oxygen species (ROS) and the oxidation-reduction (redox) state play important roles in a variety of cell death mechanisms induced by widely used antitumor drugs or by environmental toxic substances (Orrenius, 1985; Orrenius and Nicotera, 1987; Slater et al., 1995) (Feinendegen, 2002; Ueda et al., 2002). Because oxidative damage to the ER has been implicated in some forms of cell death (Hayashi et al., 2003; Lai et al., 2003; Watanabe et al., 2003), we investigated the possible role of oxidative stress (OS) in the induction of ER stress by different agents. Here, we show that ER Ca^{2+} depletion and cell death induced by Ec but not Tg or tunicamycin (Tu) is dependent on ROS production at the mitochondria, thus identifying a mediating role for ROS in communicating mitochondrial disruption to the ER.

Materials and Methods

Cell Culture, Cell Death, and Caspase Assays. Human promyelocytic leukemia HL-60 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 2.5 mM L-glutamine. HL-60 cells were treated with 15 μM Ec in the presence or absence of 125 nM AA, 5 μM BHT, or 1 mM Nac at 37°C for 2 h, in medium containing 1% FBS. Drugs were washed out, and the cells were recovered in 10% FBS drug-free RPMI 1640 medium for 6 h. Samples were costained with Annexin V-Cy5 (BioVision, Mountain View, CA) and propidium iodide (PI), and cell death was determined by flow cytometry. Early (Annexin V-positive, PI-negative) and late (Annexin V-positive, PI-positive) events were scored as dead cells. Caspase-3/7 activation was measured using the Vybrant 5-carboxyfluorescein caspase assay kit from Invitrogen (Carlsbad, CA) as described by the manufacturer.

Detection of Oxidative Stress and Mitochondrial ROS. To examine the generation of OS, HL-60 cells were incubated with the indicated concentrations of Ec, Tu, or Tg in RPMI 1640 containing 2% FBS at 37°C for 2 h and then loaded with the OS indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (8 μM CM-H₂DCFDA; Invitrogen, Carlsbad, CA) at 37°C for 30 min. Fluorescence was measured by flow cytometry using excitation at 488 nm and emission at 530 nm at indicated intervals after treatment. Mitochondrial-specific ROS generation was measured using the MitoSox Red fluorescent dye (Invitrogen) as described by the

manufacturer. This dye accumulates in the mitochondria, is oxidized by superoxide, and emits at 580 nm.

Measurement of Cytosolic Ca^{2+} Concentration. Cytosolic Ca^{2+} measurements were performed by flow cytometry. HL-60 cells were serum-deprived for 2 h in Tyrode's buffer (10 mM HEPES, 100 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, and 0.05% bovine serum albumin (0.05%) containing Ec, atractyloside (Atra), antioxidants, and/or hydrogen peroxide where indicated. Cells were then incubated in loading buffer (5 μM indo-1AM and 0.03% Pluronic F-127 in Tyrode's buffer; both from Invitrogen) at 37°C for 30 min and washed and incubated (15 min at room temperature) to allow for the complete conversion of indo-1AM to Ca^{2+} -sensitive indo-1 through hydrolysis. Measurements were performed using a laser tuned to 338 nm while monitoring emissions at 405 and 450 nm. FloJo software (Treestar Software, Ashland, OR) was used to analyze the flow cytometric data. The concentration of intracellular free Ca^{2+} was calculated according to the following formula (Gryniewicz et al., 1985): $[\text{Ca}^{2+}]_i = K_d \times (F_{\min}/F_{\max}) \times (R - R_{\min})/(R_{\max} - R)$, where R is the ratio of the fluorescence intensities measured at 405 and 450 nm during the experiments, and F is the fluorescence intensity measured at 450 nm. R_{\min} , R_{\max} , F_{\min} , and F_{\max} were determined from in situ calibration of viable cells using 4 μM ionomycin in the absence (R_{\min} and F_{\min} ; 10 mM EGTA) and presence of (R_{\max} and F_{\max}) of Ca^{2+} . K_d (250 nM) is the dissociation constant for indo-1 at 37°C. R_{\min} , R_{\max} , F_{\min} , and F_{\max} varied depending upon settings and were determined at the beginning of each experimental procedure and experimental condition.

Potentiometric Measurement of Mitochondria Membrane Potential. MMP measurements were performed by flow cytometry. Cells (5×10^5 cells/ml) were growth factor-deprived for ~2 h in Tyrode's buffer and then incubated with 5 $\mu\text{g}/\text{ml}$ JC-1 (Invitrogen) for 15 min at room temperature in Tyrode's buffer. Then, cells were washed (three times) to remove extracellular JC-1. Measurements were performed using a laser tuned to 488 nm while monitoring the emissions of JC-1 monomers at 530 nm and JC-1 aggregates at 585 nm. MMP measurements were normalized using a modification of a formula from Rottenberg and Wu (1998): $\text{MMP} = (R - R_{\text{FCCP}})/(R_0 - R_{\text{FCCP}}) \times 100$, where R is the ratio of the fluorescence intensities measured at 530 and 585 nm during the experiments, R_0 is the fluorescence ratio of untreated cells, and R_{FCCP} is the fluorescence intensity measured after the addition of 2 μM carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich, St. Louis, MO), a procedure that collapses the mitochondrial membrane potential and was performed at the beginning of each experiment.

Adenosine Nucleotide Transfer-Dependent ADP Import Assay. Adenosine nucleotide transfer (ANT) across the mitochondrial membrane in ER-stressed cells was measured as described by Vander Heiden et al. (1999). In brief, 5×10^7 HL-60 cells were disrupted by mechanical lysis through homogenization in a mitochondria isolation buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, and 1 mM EGTA). After centrifugation of the supernatant (750g for 10 min to remove debris followed by 10,000g for 10 min), mitochondrial pellets were resuspended in ADP import buffer (250 mM sucrose, 20 mM HEPES, pH 7.2, 10 mM KCl, 5 mM succinate, 3 mM KH_2PO_4 , 1.5 mM MgCl_2 , 1 mM EGTA, and 5 μM rotenone) with or without the adenine nucleotide transporter inhibitor atractyloside (50 μM ; Sigma-Aldrich). [^{14}C]ADP (1 μCi ; PerkinElmer Life and Analytical Sciences, Boston, MA) was added to the mitochondrial suspension and incubated for 10 min on ice. After washing two times in ADP import buffer, the samples were resuspended in scintillant (PerkinElmer) and quantified using a beta counter (PerkinElmer Wallac, Gaithersburg, MD). ANT-dependent ADP transport activity was determined by calculating the difference in counts between samples that were or were not preincubated with atractyloside.

ATP Bioluminescence Assay. ATP levels were measured by using ATP bioluminescence assay kit CLS II (Roche Diagnostics,

Indianapolis, IN). In brief, cells (5×10^5 cells/ml) were treated with 15 μ M Ec, 125 nM antimycin A, or both at 37°C for 2 or 6 h in RPMI 1640 medium with 0.05% bovine serum albumin. After washing in chilled PBS, cells were resuspended in 100 μ l of dilution buffer, added to 900 μ l of boiling buffer (100 mM Tris and 4 mM EDTA, pH 7.5), boiled for 2 min, and centrifuged at 1000g for 1 min. The supernatants were transferred to a fresh tube and kept on ice until measurements. ATP concentration was measured with a luminometer (Luminoskan; Thermo Electron Corporation, Waltham, MA) by adding 100 μ l of luciferase reagent to 100 μ l of sample or ATP standards (10^{-9} – 10^{-4} M). ATP values were calculated from a log-log plot of the standard curve.

Statistical Analysis and Reproducibility. Data were analyzed by analysis of variance with Bonferroni's post tests and are expressed as means \pm S.E.M. All measurements were performed in triplicate and repeated at least twice with similar results (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$).

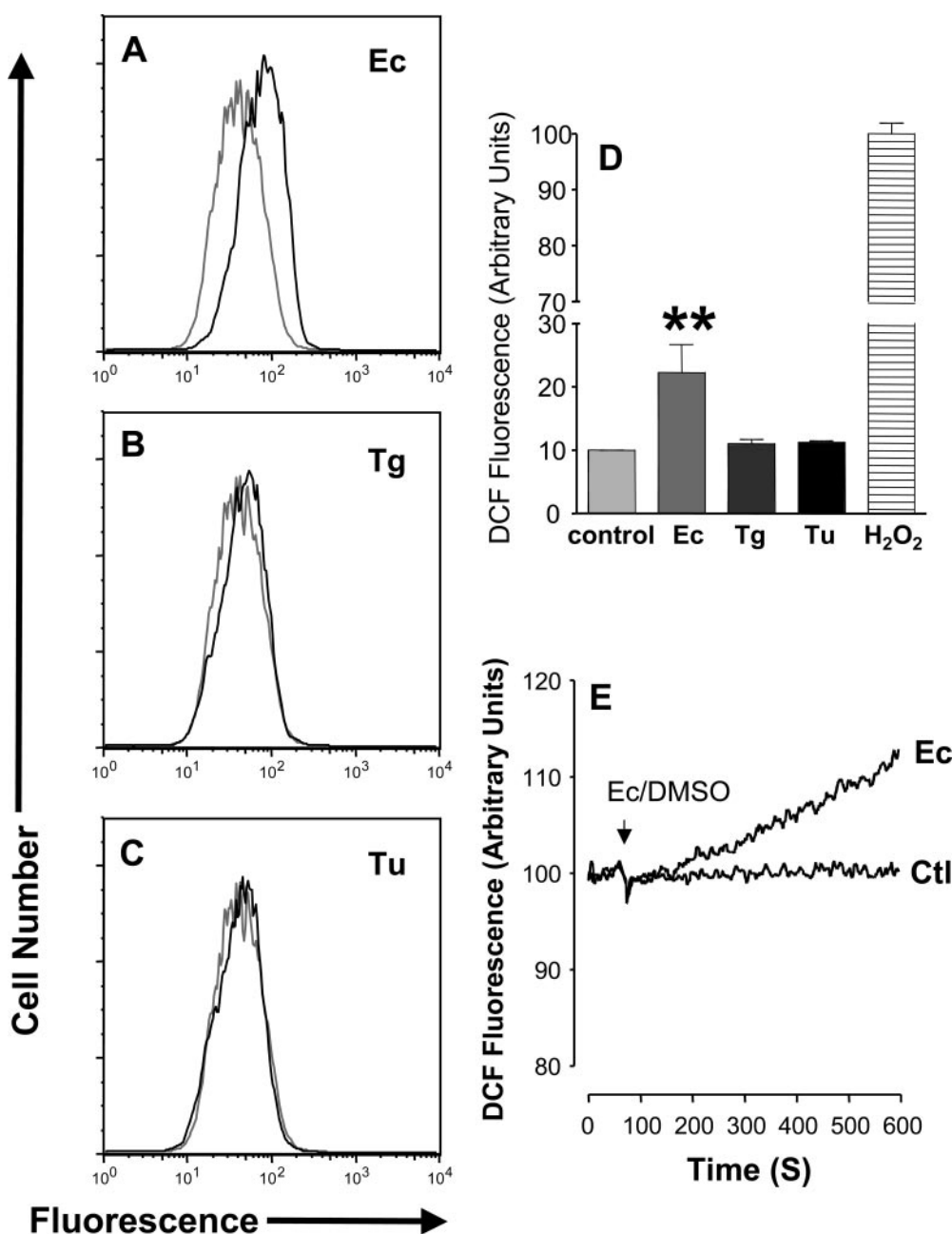


Fig. 1. Generation of OS in ER stress agent-treated cells. HL-60 cells were incubated with ER stress agents Ec (15 μ M) (A), Tg (1 μ M) (B), or Tu (1 μ g/ml) (C) for 2 h and then loaded with 8 μ M H₂DCFDA for 30 min at 37°C and rinsed with PBS. Fluorescence was monitored by flow cytometry at FL-1. The gray tracing is the untreated control. The data depicted are a representative experiment. D, comparison of DCF fluorescence in arbitrary units for Ec-, Tg-, or Tu-treated cells compared with control. Reported values are averages of three independent determinations and are reported \pm S.E.M. **, $p < 0.01$ compared with control. E, time course of DCF fluorescence in response to 15 μ M Ec.

Results

Oxidative Stress Generation by ER Stress Agents. To investigate the possibility that ER stress inducers generate ROS, we exposed HL-60 cells to the agents Ec, Tg, and Tu for 2 h and measured OS levels in the cells by flow cytometry using the generalized OS-sensitive fluorescent dye CM-H₂DCFDA (Halliwell and Whiteman, 2004). As shown in Fig. 1, A to D, increased fluorescence levels, indicating increases in OS levels, was observed for cells treated with Ec but not Tg or Tu. We have previously demonstrated that all three agents induce ER stress within the chosen 2-h time period, as indicated by induction of eukaryotic translation initiation factor 2 subunit α phosphorylation and BiP expression, along with suppression of protein synthesis (Soboloff and Berger, 2002; Zhang and Berger, 2004). However, this is achieved differ-

ently for each agent. Thus, Tu is an inhibitor of glycosylation (Kuo and Lampen, 1974) and causes ER stress through the accumulation of protein in the ER (Dorner et al., 1990). Tg inhibits the SERCA pump responsible for refilling ER Ca^{2+} stores (Thastrup et al., 1990), leading to rapid depletion of ER Ca^{2+} stores, followed by influx of Ca^{2+} from the extracellular milieu. Although Ec is best known as an inhibitor of Ca^{2+} influx, it also promotes ER Ca^{2+} depletion (Jan et al., 1999), although the mechanism of this Ca^{2+} release has not previously been addressed.

To determine the kinetics of OS generation by Ec, we followed CM- H_2DCFDA fluorescence over time. As shown in Fig. 1E, fluorescence changes were observed with a short delay after addition of Ec, suggesting a relatively rapid generation of reactivity.

Hyperpolarization of Mitochondrial Membrane Potential by Ec. The most common mechanism of OS generation by toxic agents is the disruption of electron transport in mitochondria (Ueda et al., 2002; Eckert et al., 2003; Inoue et al., 2003; Kadenbach, 2003; Rego and Oliveira, 2003). To investigate the effect of Ec on mitochondrial function, we first measured mitochondrial membrane potential, a useful indicator of mitochondrial activity and status, after exposure to Ec. As shown in Fig. 2A, the addition of Ec to cells caused an

immediate hyperpolarization of the mitochondrial membrane. In contrast, Tg and Tu had no effect, whereas the protonophore FCCP caused an immediate collapse of the potential. These observations suggested that Ec, but not Tg or Tu, had a mitochondrial target.

To further investigate the possibility of a mitochondrial target for Ec-induced ROS generation, we used MitoSox Red dye (Invitrogen), a mitochondrial-specific superoxide indicator. To confirm that MitoSox measures ROS generated at the mitochondria, we used the phorbol ester PMA, which stimulates oxidase activity at the plasma membrane (Teufelhofer et al., 2003; Dooley et al., 2004) and atractyloside, which generates mitochondrial ROS via inhibition of adenine nucleotide activity (see below) as controls. As shown in Fig. 2B, the CM- H_2DCFDA dye detects similar levels of fluorescence by Ec, PMA, and atractyloside over a 2-h time period. In contrast, Ec and atractyloside, but not PMA, generate the superoxide anion, as detected with MitoSox (Fig. 2C). It is noteworthy that a nearly 3.5-fold increase in superoxide accumulation was observed in the presence of Ec, a far greater change than observed when DCF fluorescence was assessed. Cells were then stained with MitoSox, and the effect of Ec addition was followed over time. As shown in Fig. 2D, Ec stimulated a rapid and sustained increase in MitoSox fluo-

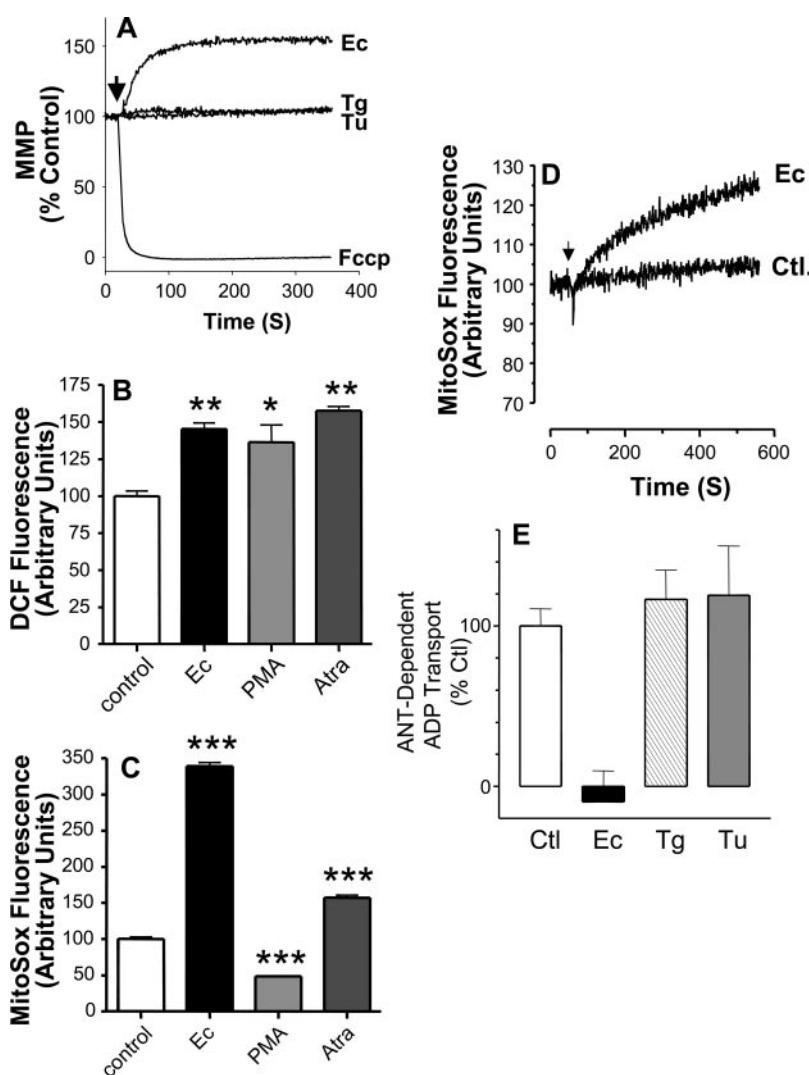


Fig. 2. Ec hyperpolarizes MMP, generates mitochondrial ROS, and inhibits ADP transport. A, JC-1 loaded HL-60 cells were exposed to 15 μM Ec, 1 μM Tg, 1 $\mu\text{g/ml}$ Tu, or 2 μM FCCP, respectively, and the membrane potential was monitored by flow cytometry as described under *Materials and Methods*. B, HL-60 cells were exposed to 15 μM Ec, 2 μM PMA, or 50 μM atractyloside for 2 h and then loaded with 8 μM H_2DCFDA for 30 min at 37°C and rinsed with PBS. The mean fluorescence intensity was measured by flow cytometry. C, HL-60 cells were treated with ROS-generating agents described as in B but loaded with 5 μM MitoSox for 30 min at 37°C and rinsed with PBS. The mean fluorescence intensity was measured by flow cytometry. D, time course of MitoSox-sensitive ROS in response to 15 μM Ec. E, cell-free mitochondria were prepared as described under *Materials and Methods*. Samples were incubated with ER stress agents for 10 min in the presence or absence of atractyloside followed by the addition of [^{14}C]ADP. ANT-dependent ADP transport activity was determined by calculating the atractyloside inhabitable counts. ANT-dependent ADP transport was inhibited by 15 μM Ec but not by 1 μM Tg or 1 $\mu\text{g/ml}$ Tu. Values are reported as mean \pm S.E.M.

rescence in contrast to the somewhat slower change in total DCF fluorescence (Fig. 1E). These observations are consistent with DCF, indicating ROS generation and mitochondria as the source of Ec-induced ROS.

Ec Inhibits Adenine Nucleotide Transport. Adenine nucleotide transport, performed by the mitochondrial ANT, is one of the final steps in the generation of ATP, and its inhibition has been shown to cause mitochondrial membrane hyperpolarization (Vander Heiden et al., 1999). We therefore investigated the effect of Ec, Tg, and Tu on adenine nucleotide transport. Cell-free mitochondria were prepared, exposed to the ER stress agents, and adenine nucleotide transport was measured. As shown in Fig. 2E, at the concentrations used to treat cells, Ec is a very potent inhibitor of ANT function, whereas Tg or Tu have no effect. This observation suggests that Ec acts directly on mitochondria to inhibit ANT, either directly or indirectly.

Antimycin A Reverses Mitochondrial Hyperpolarization by Ec. Because adenine nucleotide transport is coupled to electron transport, it is possible that a member of the electron transport chain is the direct target of Ec, leading to ROS production and suppression of ANT function. To further investigate this possibility, we determined the effect of various inhibitors of electron transport on the ability of Ec to hyperpolarize the mitochondrial membrane. The inhibitors used are illustrated in Fig. 3A. As shown in Fig. 3B, pre-

exposure of cells to 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedi-one, rotenone, or KCN, inhibitors of electron transport complexes I, II, and IV, respectively, had no effect on the ability of Ec to hyperpolarize the mitochondrial membrane. In contrast, the complex III inhibitor antimycin A both partially reduced mitochondrial membrane potential on its own and completely neutralized mitochondrial membrane hyperpolarization by Ec (Fig. 3C). Furthermore, the ability of antimycin A to neutralize Ec-induced hyperpolarization persisted for 2 h. These results therefore suggest that Ec is targeting the electron transport chain in a complex III-dependent manner, resulting in ROS generation and inhibition of ANT function.

Antimycin A Reverses ROS Generation by Ec. The fact that antimycin A neutralizes Ec-induced hyperpolarization seems somewhat paradoxical given that antimycin A on its own has been reported to generate reactive oxygen. To further investigate the interaction between Ec and antimycin A, we measured ROS production after treatment with Ec, antimycin A, or both compounds together. Cells were incubated with the two compounds for 2 h, and ROS content was measured as described above. As shown in Fig. 4A, antimycin A alone, at the concentration used in this study, did not have any effects on ROS levels. However, it clearly suppressed ROS generation by Ec. This result therefore correlates Ec-induced hyperpolarization with ROS production and provides further evidence that the ability of Ec to generate ROS is dependent on complex III activity.

ATP Depletion by Ec. Given the inhibition of adenine nucleotide transport by Ec and its other effects on electron transport, it was of interest to determine the effect of Ec treatment on ATP levels. We therefore exposed cells to Ec for 2 h, and measured total ATP content. As shown in Fig. 4B, exposure to Ec for 2 h resulted in a decline in ATP content of 50%. Although a 2-h exposure to antimycin A caused a similar depletion of ATP content, exposure to both agents simultaneously resulted in significantly less ATP depletion than either agent alone. Extending the incubation time to 6 h (Fig. 4C) resulted in even greater depletion of ATP by Ec or antimycin A. However, as with the 2-h endpoint, combined exposure to both agents resulted in less ATP depletion than with either agent alone. These observations suggest that Ec and antimycin A both target complex III, with distinct and opposing effects.

Antioxidants Do Not Reverse Mitochondrial Membrane Hyperpolarization by Ec. The results described above show that Ec hyperpolarizes the mitochondrial membrane and generates ROS. Moreover, both of these endpoints could be reversed by the complex III inhibitor antimycin A. These results, however, do not address the causal nature of ROS production. In particular, are increased ROS levels required for Ec-induced changes in membrane potential? To address this question, we pretreated cells with the antioxidants BHT or Nac and then followed mitochondrial membrane potential after exposure to Ec. Control experiments demonstrated that both antioxidants suppressed ROS generation by Ec (data not shown). As shown in Fig. 5, Nac slightly lowered the resting mitochondrial membrane potential compared with untreated cells. However, neither Nac nor BHT prevented Ec from hyperpolarizing the membrane above the resting level. Furthermore, the hyperpolarizing effect of Ec persisted for more than 2 h. Therefore, it is likely that the

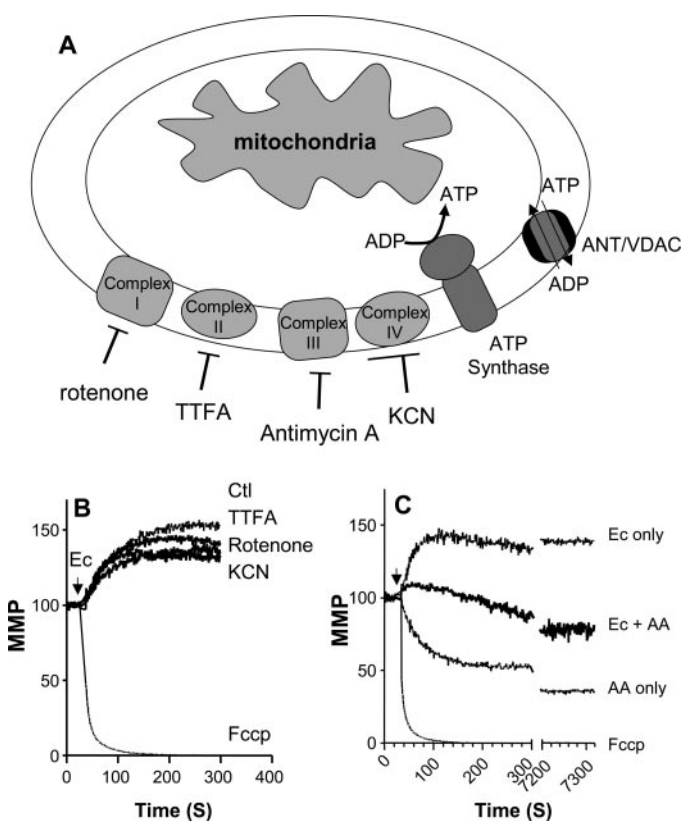


Fig. 3. Antimycin A reverses mitochondrial membrane hyperpolarization by Ec. A, illustration of the sites of action of inhibitors of mitochondrial electron transport. B, JC-1-loaded cells were pretreated for 20 min with 10 μ M rotenone, 10 μ M 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedi-one, or 400 μ M KCN. The arrow indicates where 15 μ M Ec or 2 μ M FCCP was added to the cells. C, effect of antimycin A on membrane hyperpolarization. Cells stimulated with antimycin A (125 nM), Ec, or FCCP. Mitochondrial membrane potential was recorded for 2 h.

generation of ROS occurs either in parallel with or downstream of changes in mitochondrial membrane potential.

ER Ca^{2+} Depletion by Ec Is Inhibited by Antimycin A and Antioxidants. We have previously documented that Ec, in addition to blocking Ca^{2+} influx, also causes Ca^{2+} depletion from the ER (Soboloff and Berger, 2002; Zhang and Berger, 2004). To determine the role of the mitochondrial activity of Ec in ER depletion, we treated cells with Ec or Ec plus antimycin A and then measured the amount of Ca^{2+} present in the ER by releasing it into the cytoplasm with Tg while blocking the influx of Ca^{2+} with the nonspecific Ca^{2+} channel blocker Ni^{2+} . As shown in Fig. 6A, no Ca^{2+} was released into the cytoplasm by Tg after pretreatment of cells with Ec, demonstrating that Ec depletes the ER. Antimycin A alone had little effect on Tg-releasable Ca^{2+} . However, Ec-induced depletion of the Tg-sensitive store was reversed by antimycin A, revealing a key role for the mitochondrial target of Ec in ER Ca^{2+} depletion. To assess the role of mitochondria on Ca^{2+} influx, experiments were then performed in the absence of Ni^{2+} . The difference between the magnitude of Tg-induced Ca^{2+} influx in the presence of Ni^{2+} versus the absence of Ni^{2+} reflects Ca^{2+} influx. Because Ec blocks Ca^{2+} influx in addition to depleting the ER of Ca^{2+} , no Tg-induced changes in Ca^{2+} concentration occur after Ec pretreatment, even when Ni^{2+} is not present (Fig. 6B). Antimycin A alone had no effect on Tg-induced Ca^{2+} release or influx. However, the Tg-induced Ca^{2+} concentration observed with both antimycin A and Ec present was comparable with that observed in experiments when Ni^{2+} was present (Fig. 6A). Hence, antimycin A neutralized Ec-induced ER Ca^{2+} depletion, but it had little or no effect on Ec-induced inhibition of Ca^{2+} influx.

We also assessed the ability of the antioxidants BHT and Nac to prevent Ec-mediated ER Ca^{2+} depletion and/or Ca^{2+} influx. As shown in Fig. 6C, in the presence of Ni^{2+} , BHT alone had no effect on Tg-releasable Ca^{2+} . Similar to antimycin A, however, BHT prevented Ec from fully depleting the Tg-sensitive store. Moreover, BHT did not alter Ec-induced inhibition of Ca^{2+} influx, as illustrated in Fig. 6D, where, in the presence of BHT and Ec, Tg-releasable Ca^{2+} was similar in magnitude to that released by Tg in the presence of Ni^{2+} (Fig. 6C). Similar results were observed with the antioxidant Nac (Fig. 6, E and F). These results indicate that Ec-induced ER Ca^{2+} depletion is probably due to its ROS production, presumably via its action on complex III within the mitochondria. They further demonstrate that Ec-induced ER Ca^{2+} depletion is a distinct phenomenon from Ec-induced block of Ca^{2+} influx, which can be separated via the actions of antimycin A, BHT, and Nac.

Antimycin A and Antioxidants Protect Cells from Ec-Induced Cell Death. Because antimycin A and the antioxidants BHT and Nac prevent ER Ca^{2+} depletion by Ec, we tested its effect on Ec-induced toxicity. Cells were exposed to Ec plus antimycin A or the antioxidants for 2 h. The agents were then removed, and the cells were incubated in medium for an additional 6 h. Antimycin A, BHT, and Nac were nontoxic at the concentrations used under these conditions (data not shown). The cells were then evaluated by flow cytometry using Annexin V staining. As shown in Fig. 7A, antimycin A, BHT, and Nac provided significant protection from Ec-induced cell death, revealing a key role for mitochondrially generated ROS and the release of ER Ca^{2+} in Ec toxicity.

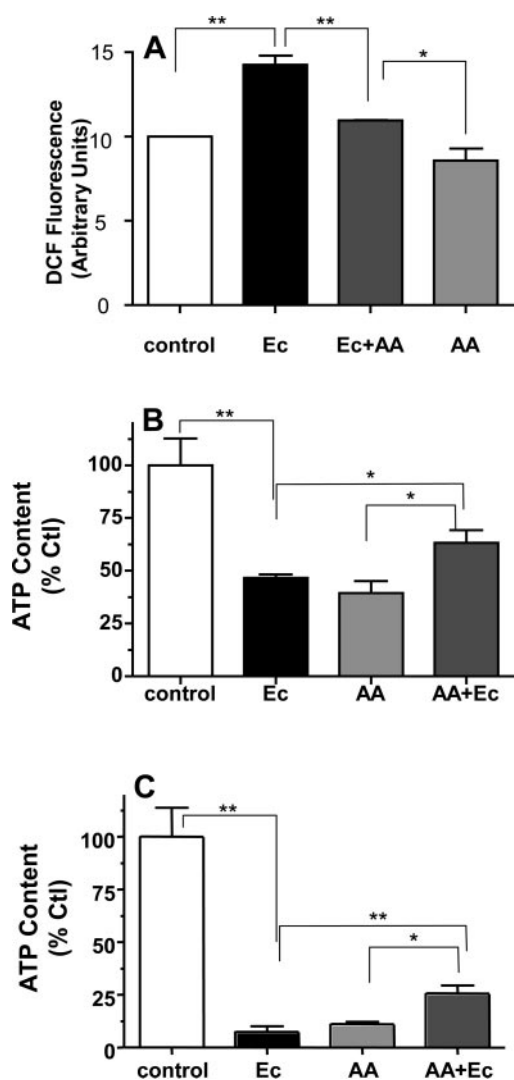


Fig. 4. Antimycin A inhibits and reverses OS generation induced by Ec and partially restores ATP production. A, cells were incubated with 15 μM Ec, 125 nM antimycin A, or both at 37°C for 2 h, and DCF fluorescence was measured as described under *Materials and Methods*. Data are shown as mean \pm S.E.M. *, $p < 0.05$; **, $p < 0.01$. B and C, intracellular ATP was measured in cells ($0.5\text{--}1.0 \times 10^6$ cells/ml) treated with 15 μM Ec, 125 nM antimycin A, or both, at 37°C for 2 h (B) or 6 h (C). Values shown are reported as mean \pm S.E.M. *, $p < 0.05$; **, $p < 0.01$.

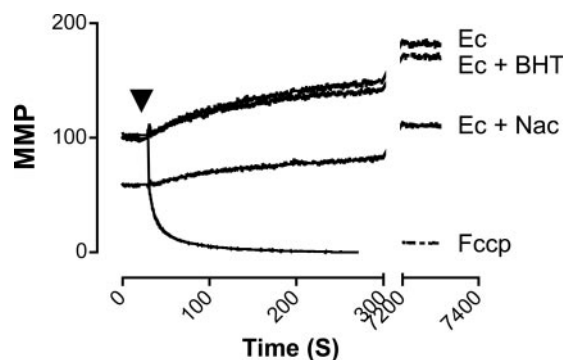


Fig. 5. Antioxidants do not reverse MMP hyperpolarization by Ec. Cells were pretreated with or without 5 μM BHT or 1 mM Nac at 37°C for 20 min followed by JC-1 loading and flow cytometry procedures described under *Materials and Methods*. Mitochondrial potential was followed for 2 h after 15 μM Ec or 2 μM FCCP was added.

Antimycin A and Antioxidants Prevent Caspase Activation in Response to Ec. We have demonstrated in other cell types that Ec-induced cell death was associated with a variety of apoptotic indicators, including poly(ADP-ribose) polymerase cleavage and protection from cell death by caspase inhibitors (Gommerman and Berger, 1998). However, we have also observed that some cells such as human MCF-7 breast cancer cells undergo a nonapoptotic form of cell death (Zhang et al., 2002) associated with a lack of DNA fragmentation and a necrotic phenotype. We previously observed that Ec-treated HL-60 cells display classic apoptotic endpoints, including membrane blebbing and nuclear condensation (Zhang and Berger, 2004). However, caspase activation in these cells was not investigated. We therefore examined the degree of caspase activation in these cells in response to Ec, focusing on caspase-3/7, the major effectors of apoptotic cell death. Initial experiments indicated that caspase-3/7 were not activated after exposure to the drug for 2 h followed by a 6-h incubation in medium (data not shown). However, as shown in Fig. 7B, extensive caspase-3/7 activation was observed if the incubation in medium was extended to 18 h. Furthermore, the number of cells displaying caspase activation could be reduced significantly by antimycin A or the

antioxidants. These observations indicate that caspase activation is a relatively late event that is at least partially dependent on the mitochondrial activity of Ec.

Hydrogen Peroxide and Atractyloside Mobilize Ca^{2+} from the ER without Depleting the Store. Other studies have shown that ROS stimulate Ca^{2+} release from the ER (Downey, 1990). Consistent with these observations, we observed that H_2O_2 caused a slow, but significant Ca^{2+} transient in HL-60 cells (Fig. 8A). When cells were exposed to Ec, followed by H_2O_2 , we observed that H_2O_2 failed to stimulate additional release of Ca^{2+} (Fig. 8B). This observation suggests that Ca^{2+} released by Ec and H_2O_2 are probably from the same intracellular store. It is noteworthy that when cells were treated for 2 h with H_2O_2 , we observed that significant levels of Ca^{2+} could still be mobilized from the ER with Tg (Fig. 8C). This observation suggests that, unlike Ec (Fig. 6), ROS alone stimulate Ca^{2+} release without fully depleting the ER of Ca^{2+} , presumably because they do not block the refill process.

Atra is an inhibitor of the adenine nucleotide transporter (Robinson et al., 1975). Our results with Ec predict that Atra should also generate ROS, which would stimulate ER Ca^{2+} mobilization. To test this possibility, we exposed cells to Atra and measured ROS content. As shown in Fig. 9A, increased

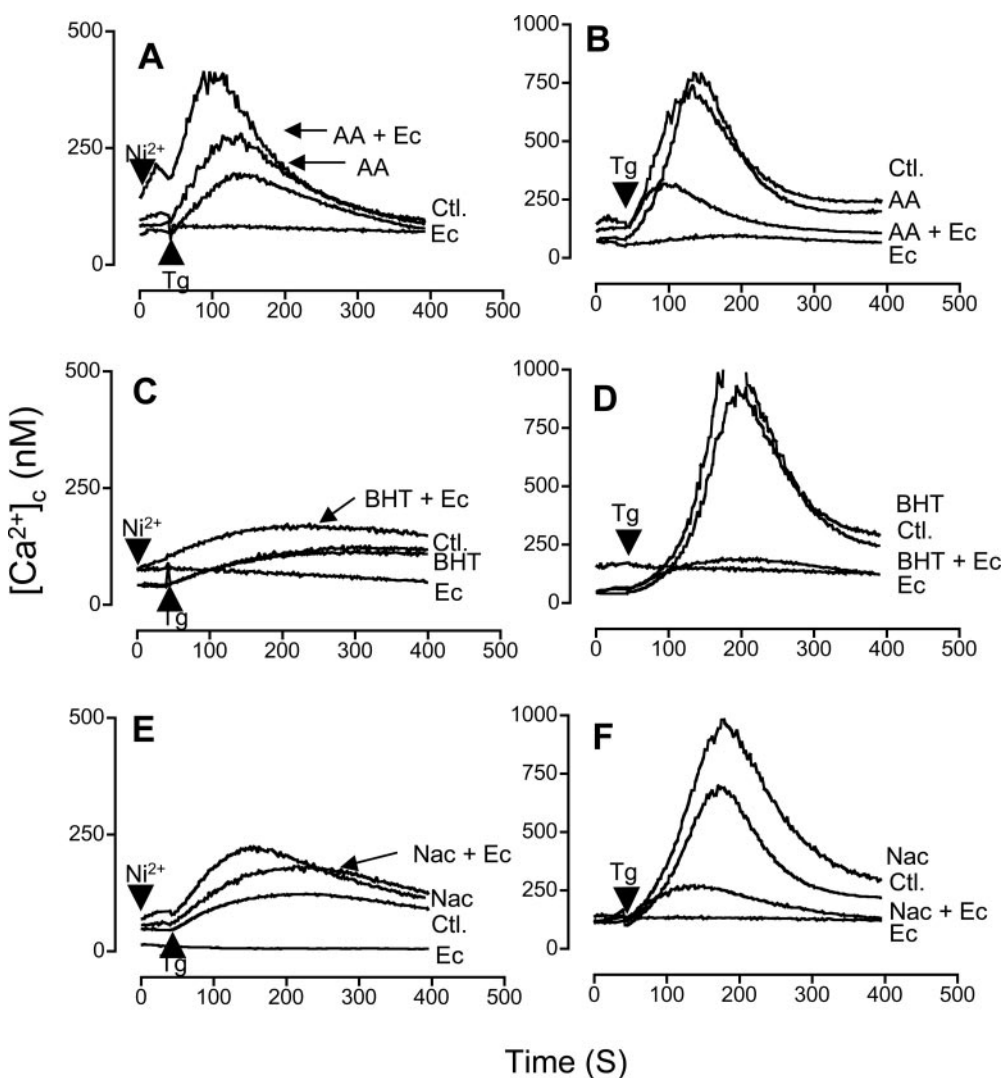


Fig. 6. Ec-generated OS targets ER Ca^{2+} stores. Cells were loaded with indo-1AM in Tyrode's buffer as described under *Materials and Methods*. Ca^{2+} concentration was monitored by flow cytometry. A, cells were preincubated in the absence (Ctl) or presence of 15 μM Ec or Ec + 125 nM AA for 2 h. Ni^{2+} (5 mM) was added to the cells to nonspecifically prevent influx. Ca^{2+} released by 3 μM Tg into the cytoplasm was used as an indicator of ER Ca^{2+} content. B, as in A, however, Ni^{2+} was not added to cells before Tg. C, as in A, except 5 μM BHT was used. D, as in C, however, Ni^{2+} was not added before Tg. E, as in A, except 1 mM Nac was used. F, as in E, however, Ni^{2+} was not added before Tg.

ROS levels were observed after ATRA treatment. Furthermore, we observed that ATRA could stimulate Ca^{2+} mobilization in HL-60 cells (Fig. 9B). However, unlike Ec, extended incubation with ATRA failed to fully deplete the Tg-sensitive store (Fig. 9C). Furthermore, despite generating ROS and stimulating Ca^{2+} release from the ER, ATRA did not induce cell death in HL-60 cells (data not shown), consistent with the concept that the cell death associated with ER Ca^{2+} release requires ER Ca^{2+} depletion.

Discussion

High levels of ROS have multiple effects on proteins, lipids, and nucleic acids in key organelles such as the mitochondria, ER, and nucleus. Emerging evidence also supports the concept that subtoxic levels of ROS act as endogenous signaling messengers, especially in calcium signaling and protein phosphorylation (Nathan, 2003). In the course of investigating the role of OS in ER stress, we observed that Ec, an

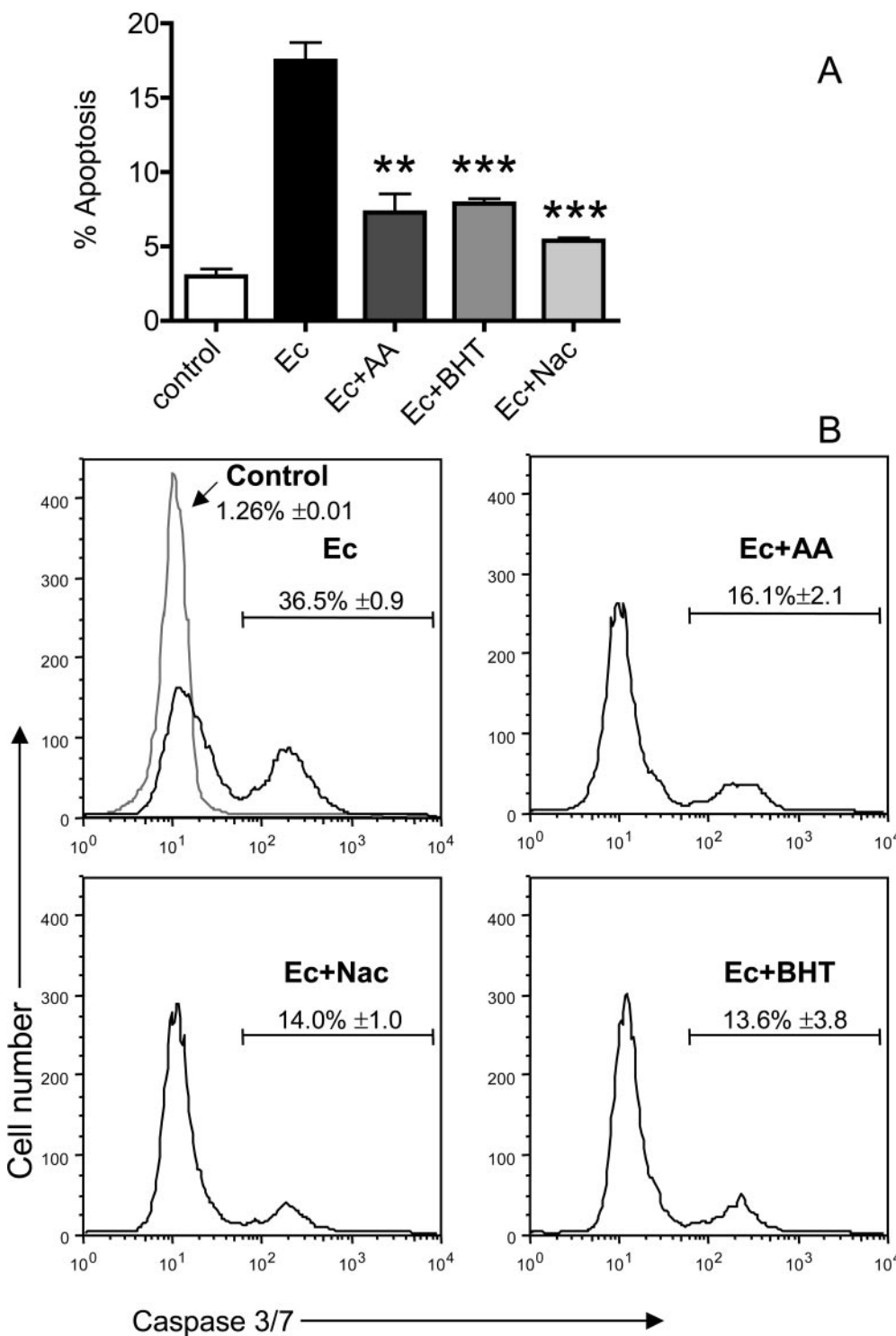


Fig. 7. Ec-induced cell death and caspase activation are suppressed by antimycin A, BHT, and Nac. Cells were treated with 15 μM Ec in the presence or absence of 125 nM AA, 5 μM BHT, or 1 mM Nac at 37°C for 2 h, in medium containing 1% FBS. Drugs were washed out, and the cells were recovered in RPMI 1640 medium plus 10% FBS for 6 h (A). Samples were costained with Annexin V-Cy5 and propidium iodide, and cell death was determined by flow cytometry. Values include both early- and late-stage apoptotic cells. **, $p < 0.01$; ***, $p < 0.001$ compared with Ec. B, cells were treated as described above, except the recovery time was extended to 18 h. Cells were then incubated with the fluorochrome inhibitor of caspases reagent (Invitrogen) to detect active caspases-3 or -7 and analyzed by flow cytometry. Reported values are percentage of cells that are positive for activated caspase-3/7 and are averages of two repeat measurements with standard errors.

imidazole that blocks Ca^{2+} influx and stimulates ER Ca^{2+} depletion, increased OS levels in cells. In contrast, Tg or Tu, two other agents that also induce ER stress-related cell death, did not generate increased OS. Ec was also observed to cause rapid hyperpolarization of the mitochondrial membrane. This hyperpolarization was associated with inhibition of adenine nucleotide transport, observed in cell-free mitochondria. The complex III inhibitor antimycin A neutralized Ec-induced hyperpolarization of the mitochondrial membrane, suppressed ROS increases by Ec, partially reversed ATP depletion by Ec, prevented ER Ca^{2+} depletion, and protected cells from Ec-induced cell death. The antioxidants BHT and Nac also blocked Ca^{2+} mobilization from the ER by Ec and protected from Ec-induced cell death, consistent with a role for ROS in ER depletion. Taken together, these results suggest that Ec decouples complex III from downstream steps in electron transport, resulting in relatively rapid ROS generation, inhibition of adenine nucleotide transport, and ATP depletion. The reactive oxygen produced is responsible for stimulating Ca^{2+} release from the ER. The combination of ROS generation stimulating ER Ca^{2+} mobilization with Ca^{2+} influx blockade results in the sustained ER Ca^{2+} depletion associated with cell death. This model is illustrated in Fig. 10.

Because antimycin A reverses Ec-induced hyperpolariza-

tion, ROS generation, ER Ca^{2+} depletion, and cell death, complex III activity is probably required for ROS generation by Ec. Furthermore, the inhibition of ANT activity by Ec suggests that electron transport does not continue down the chain to the transporter. Taken together, these observations suggest that Ec decouples complex III from complex IV, presumably by providing an alternative outlet for complex III electrons (directly or indirectly). Although further work will be required to identify how Ec targets complex III causing ROS generation, our observations show that the mitochondrial target is critical for Ec-induced cell death. We have demonstrated that cancer and leukemia cells are highly sensitive to Ec-induced cell death (Gommerman and Berger, 1998; Soboloff et al., 2002; Zhang et al., 2002). Our identification of a mitochondrial target for Ec may prove to be useful in further identifying the cellular factors associated with sensitivity to this compound.

In addition to its inhibitory effects on complex III function, antimycin A has also been reported to bind to and inhibit bcl-2 and related family members (Tzung et al., 2001). How-

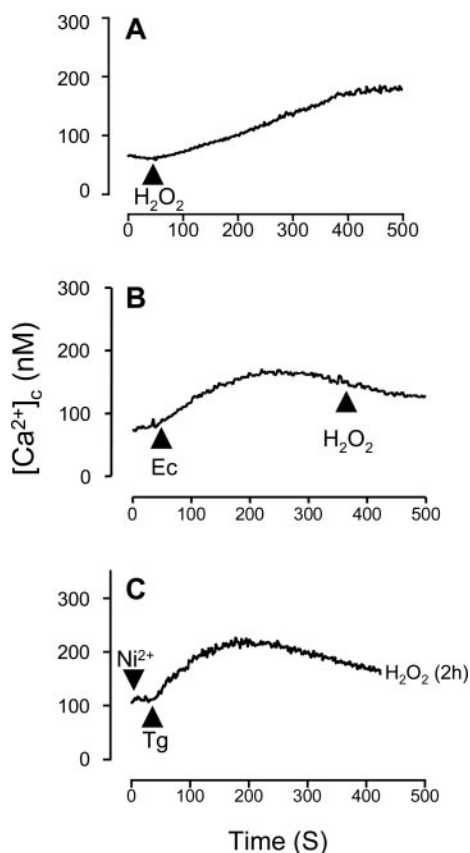


Fig. 8. Ec and H_2O_2 both target the Tg-releasable Ca^{2+} store. A, 200 μM H_2O_2 -induced changes in cytoplasmic Ca^{2+} concentration. Indo-1AM-loaded cells were exposed to H_2O_2 , and cytoplasmic Ca^{2+} was followed over time. B, as described in A, except cells were preincubated in 15 μM Ec. C, Tg-induced release of ER Ca^{2+} content can still be detected in cells pretreated with 200 μM H_2O_2 for 2 h. Ni^{2+} (5 mM) was used to demonstrate that this was release of Ca^{2+} from intracellular stores and not Ca^{2+} entry.

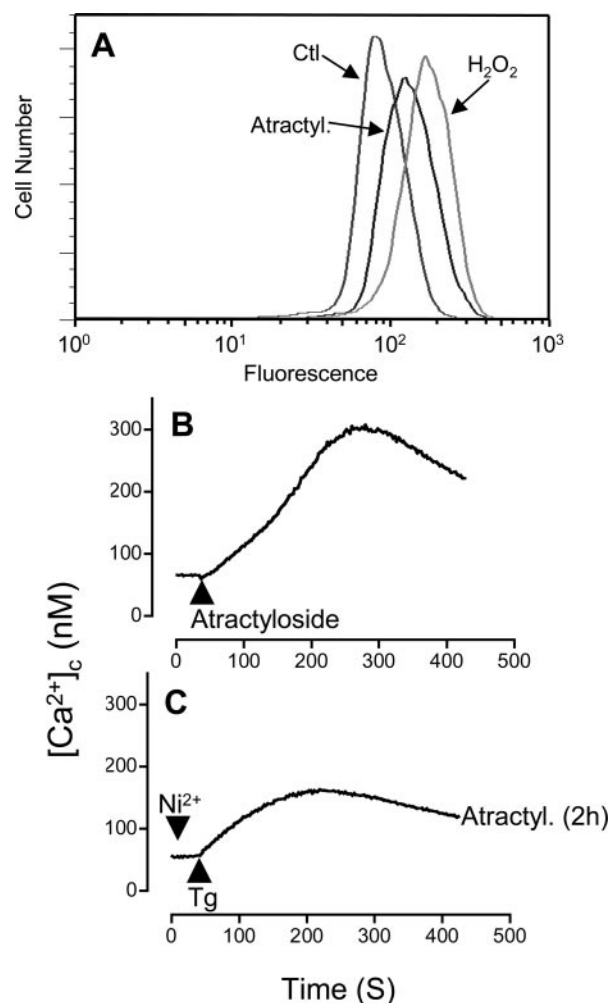


Fig. 9. Atractyl. generated ROS targets ER Ca^{2+} stores. A, HL-60 cells were incubated with 50 μM atractyl. for 2 h and then loaded with 8 μM H_2DCFDA for 30 min at 37°C. H_2DCFDA fluorescence was measured by flow cytometry. B, indo-1-loaded HL-60 cells were treated with 50 μM atractyl. and cytoplasmic Ca^{2+} followed by flow cytometry. C, cells were pretreated with 50 μM atractyl. for 2 h. ER Ca^{2+} content was measured by releasing ER Ca^{2+} into the cytoplasm with 3 μM Tg in the presence of Ni^{2+} to prevent Ca^{2+} influx.

ever, we suspect that this activity is unrelated to the ability of antimycin A to inhibit Ec function, because the concentration of the drug required for bcl-2 inhibition is much higher than that used in the current study. As well, we previously reported that cell death induced by Ec was unaffected by high levels of bcl-2 in cells (Gommerman and Berger, 1998), suggesting that bcl-2 does not affect the mechanism of action of Ec. Nevertheless, given the importance of bcl-2 family members and their effects on mitochondrial function, it would be of interest to further investigate a potential role for these proteins in ROS generation and Ca^{2+} depletion.

We previously demonstrated that exposure to Ec for 2 h was sufficient to initiate an irreversible cell death process. It is of interest to note that after 2 h of exposure, the mitochondrial membrane remains hyperpolarized and ATP content is reduced, but only by approximately 50%. Antimycin A exposure also results in a similarly reduced ATP level, whereas the combination of the two agents results in a slightly increased ATP content. This increase may be due to reduced ATP consumption associated with decreased ROS levels rather than a restoration of ATP production.

Cells exposed to lethal agents generally undergo variations of necrotic or apoptotic cell death (Orrenius et al., 2003). After exposure to Ec, HL-60 cells exhibit clear endpoints associated with apoptosis, such as membrane blebbing, Annexin positivity, and nuclear condensation (Zhang and Berger, 2004); however, these endpoints occur late in the cell death process. In the present study, caspase activation was observed at 18 h after Ec exposure (Fig. 7B), but not at 6 h.

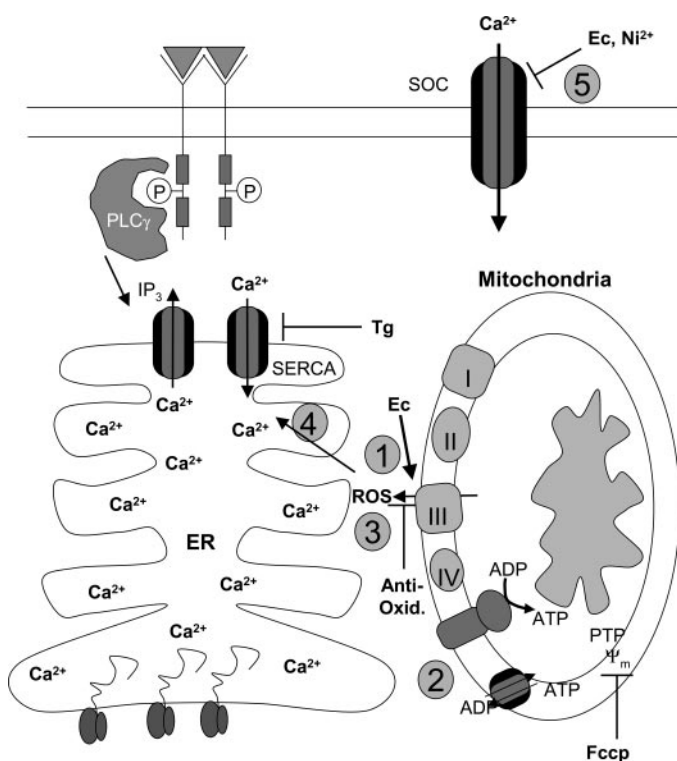


Fig. 10. Model for how Ec-stimulated mitochondrial ROS contributes to ER stress. 1) Ec targets complex III in the electron transport chain resulting in hyperpolarization of the mitochondrial membrane; 2) decoupling of electron transport from adenine nucleotide transport and ATP production; 3) inhibition of ANT and ROS generation; 4) ROS stimulates ER Ca^{2+} release; and 5) Ec also blocks Ca^{2+} influx, resulting in an ER stress response.

The inability to detect caspase activation early may be partly due to inactivation of caspases by ROS as has been documented in other cell systems (Hampton et al., 1998; Samali et al., 1999). On the other hand, apoptosis may be a secondary or indirect consequence of the ER stress process and is probably not absolutely required for cell death. We have observed, in a variety of cell systems, that 2-h exposure to Ec optimizes the difference in sensitivity between cancer or leukemia cells and normal cells (Soboloff et al., 2002; Zhang et al., 2002). We have also found that ER Ca^{2+} release is stimulated at lower Ec concentrations than those required for blocking influx (Soboloff and Berger, 2002). Taken together, these observations suggest that longer term exposure to lower doses of Ec may result in a qualitatively different form of cell death that is more dependent on mitochondrial dysfunction than ER Ca^{2+} depletion.

The ER Ca^{2+} store is a dynamic entity requiring constant energy and SERCA activity to maintain the large concentration gradient across the ER membrane. It is probable that as cells undergo various forms of cell death, depleted energy levels of the cell will be insufficient to maintain the Ca^{2+} gradient resulting in indirect ER Ca^{2+} depletion (Nicotera and Orrenius, 1998). In contrast, agents such as the ANT inhibitor atractyloside and others (Le Bras et al., 2005) generate ROS and cause Ca^{2+} release from the ER rapidly, well before energy levels are depleted. If the cell can maintain sufficient energy levels, then these agents will not be toxic. However, if the oxidative burden is too high, eventually, as energy levels deplete, the cell will undergo cell death (Richter et al., 1996).

Our observations are consistent with a general role for ROS generated at the mitochondria in stimulating ER Ca^{2+} release. The full spectrum of ROS targets in the ER remains unknown. However, one likely target is the SERCA, because ROS have been reported to inhibit SERCA function (Barnes et al., 2000). Our observations that Ec and H_2O_2 both targeted the Tg-sensitive store are consistent with the concept that SERCA is the ROS target responsible for ER Ca^{2+} release.

Both antimycin A and the antioxidants neutralized the ability of Ec to deplete the ER with no discernible effect on Ec-induced inhibition of Ca^{2+} influx. Furthermore, antimycin A and the antioxidants provided significant protection from cell death and caspase activation induced by Ec. Likewise, although atractyloside caused Ca^{2+} release from the ER, this did not lead to ER Ca^{2+} depletion or significant cell death. These observations are in agreement with our model that sustained Ca^{2+} depletion of the ER is required for lethality (Soboloff and Berger, 2002; Zhang and Berger, 2004). This would seem to suggest that although Ec releases ER Ca^{2+} content via ROS just like atractyloside, blocking store-operated Ca^{2+} is required for its toxicity and conversely, blocking Ca^{2+} entry is only fully toxic when ER Ca^{2+} content is depleted. This is consistent with our previous observation in mast cells and breast cancer cells, where Ec-induced cell death was strongly enhanced by the addition of agents that stimulate Ca^{2+} release (Gommerman and Berger, 1998; Soboloff and Berger, 2002; Zhang et al., 2002).

Large increases in cytoplasmic Ca^{2+} caused by ER depletion coupled with influx can result in rapid increases in mitochondrial Ca^{2+} . Excessive mitochondrial Ca^{2+} has been linked to activation of the mitochondrial permeability tran-

sition and induction of apoptosis (Orrenius et al., 2003). Our results suggest that ROS can perform the opposite function, namely, communicating mitochondrial dysfunction to the ER through the stimulation of Ca^{2+} release.

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