

**Inhibition of Ca²⁺ influx is required for mitochondrial Reactive Oxygen
Species-induced Endoplasmic Reticulum Ca²⁺ depletion and cell death in
leukemia cells**

Yicheng Zhang, Jonathan Soboloff, Ziping Zhu & Stuart A. Berger

*From the Arthritis and Immune Disorder Research Centre, University Health
Network and the Department of Immunology, University of Toronto; Toronto,
Ontario, Canada M5G 1L7 (YZ, JS, ZZ, SAB).*

*Current Address: Department of Hematology, Tongji Hospital, Tongji Medical
College, & Huazhong University of Science And Technology, Wuhan China,
430030 (YZ)*

*Current Address: Department of Biochemistry and Molecular Biology, University
of Maryland School of Medicine, Baltimore, MD 21201, USA (JS)*

Running Title: Role of mitochondrial ROS in ER Stress

Address all correspondence and reprint requests to:

Dr. Stuart A. Berger

Arthritis and Immune Disorder Research Centre

University Health Network

Toronto Medical Discovery Tower

8th Floor, room 8-356

101 College Street

Toronto, Ontario, Canada, M5G 1L7

(416) 581-7457 (Tel & Fax)

Berger@UHNRES.UTORONTO.CA (E-mail)

Number of Tables: 0

Figures: 10

References: 48

Abstract Word Count: 249

Introduction Word Count: 518

Discussion Word Count: 1,311

Abbreviations: ANT: Adenine Nucleotide Transporter, AA: Antimycin A, ATRA: Atractyloside, BHT: Butylated hydroxytoluene, Ec: Econazole, ER: Endoplasmic Reticulum, MMP: Mitochondrial Membrane Potential, Nac: N-Acetyl-L-cysteine, OS: Oxidative Stress, PMA: Phorbol 12-myristate 13-acetate, ROS: Reactive Oxygen Species, Tg: Thapsigargin, Tu: Tunicamycin, UPR: Unfolded Protein Response

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) play 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 SERCA 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 BHT and Nac 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 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.

Introduction

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 accumulation of unfolded or misfolded proteins in the ER lumen and initiate the unfolded protein response (UPR) (Ferri and Kroemer, 2001; Kaufman, 1999; 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. Pre-conditioning with sublethal levels of ER stress have been shown to protect cells, in part through upregulation of chaperones (Hung et al., 2003; Liu et al., 1998). 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) 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 and Berger, 2004; Zhang et al., 2002). 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 anti-tumour drugs or by environmental toxic substances (Orrenius, 1985; Orrenius and Nicotera, 1987; Slater et al., 1995) (Feinendegen, 2002; Ueda et al., 2002). Since 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 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 containing 10% heat-inactivated fetal bovine serum (FBS) and 2.5mM L-glutamine. HL-60 cells were treated with Ec (15 μ M) in the presence or absence of AA (125 nM), BHT (5 μ M), or Nac (1mM) at 37°C for 2 hours, in medium containing 1% FBS. Drugs were washed out and the cells were recovered in 10% FBS drug-free RPMI 1640 for 6 hours. Samples were co-stained with Annexin V-Cy5 (BioVision) and propidium iodide (P.I.), and cell death was determined by flow cytometry. Early (Annexin V positive, P.I. negative) and late (Annexin V positive, P.I. positive) events were scored as dead cells. Caspase 3/7 activation was measured using the Vybrant FAM caspase assay kit from Molecular Probes-Invitrogen 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 hrs and then loaded with the OS indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA, 8 μ M, 37°C, 30 minutes, Molecular Probes). Fluorescence was measured by flow cytometry using excitation at 488nm and emission at 530nm at indicated intervals following 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 580nm.

Measurement of cytosolic Ca²⁺ concentration

Cytosolic Ca²⁺ measurements ([Ca²⁺]_c) were performed by flow cytometry. HL-60 cells were serum-deprived for 2 hours in Tyrode's buffer [HEPES (10 mM), NaCl (100 mM), KCl (5 mM), CaCl₂ (1.4 mM), MgCl₂ (1 mM), glucose (5.6 mM), BSA (0.05%)] containing Ec, atractyloside, antioxidants and/or hydrogen peroxide where indicated. Cells were then incubated in loading buffer (37°C, 30 min; 5 μM indo-1AM, 0.03% pluronic F-127 in Tyrode's buffer, both from Molecular Probes), washed and incubated (15 min; room temperature) to allow for the complete conversion of indo-1AM to Ca²⁺-sensitive indo-1 through hydrolysis. Measurements were performed using a laser tuned to 338 nm while monitoring emissions at 405 nm and 450 nm. FloJo software (Treestar Software; Oregon) was used to analyze the flow cytometric data. The concentration of intracellular free Ca²⁺ was calculated according to the following formula (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \times (F_{min}/F_{max}) \times (R - R_{min}) / (R_{max} - R),$$

where R is the ratio of the fluorescence intensities measured at 405 nm 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 4μM ionomycin in the absence (R_{min} and F_{min}; 10 mM EGTA) and presence of (R_{max} and F_{max}) of Ca²⁺. 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)

MMP measurements were performed by flow cytometry. Cells (5 x 10⁵ cells/ml) were growth factor-deprived for ~2 hours in Tyrode's buffer and then incubated with JC-1

(Molecular Probes; Eugene, Oregon, 5 μ g/ml; 15 min, room temperature in Tyrode's buffer) and washed (3 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):

$$\text{MMP} = (\text{R}-\text{R}_{\text{FCCP}})/(\text{R}_0-\text{R}_{\text{FCCP}}) \times 100$$

where R is the ratio of the fluorescence intensities measured at 530 nm and 585 nm during the experiments, R_0 is the fluorescence ratio of untreated cells and R_{FCCP} was the fluorescence intensity measured after the addition of carbonyl cyanide p-(trifluoromethoxy) Phenylhydrazone (FCCP, Sigma; 2 μ M), a procedure which collapses the mitochondrial membrane potential and performed at the beginning of each experiment.

ANT-dependent ADP import assay

Adenosine nucleotide transfer across the mitochondrial membrane in ER stressed cells was measured as described by Vander Heiden et al. (Vander Heiden et al., 1999). Briefly, 5×10^7 HL-60 cells were disrupted by mechanical lysis through homogenization in a mitochondria isolation buffer (200 mM mannitol, 70mM sucrose, 10 mM HEPES pH 7.4, 1 mM EGTA). Following centrifugation (750 X g 10 min. to remove debris followed by 10,000 X g, 10 min of the supernatant), mitochondrial pellets were resuspended in ADP import buffer (250 mM Sucrose, 20 mM HEPES pH7.2, 10 mM KCl, 5 mM succinate, 3 mM KH_2PO_4 , 1.5 mM MgCl_2 , 1 mM EGTA, 5 μ M rotenone) with or without the adenine nucleotide transporter inhibitor atractyloside (50 μ M, Sigma). ^{14}C -ADP (NEN; 1 μ Ci) was added to the mitochondrial suspension and incubated (10 min) on ice. After washing (2x) in ADP import buffer, the samples were resuspended in scintillant (Packard) and quantified using a Beta Counter (Wallac). ANT-dependent ADP transport

activity was determined by calculating the difference in counts between samples that were or were not pre-incubated with atractyloside.

ATP Bioluminescence Assay

ATP levels were measured by using ATP bioluminescence assay kit CLS II (Roche). Briefly, Cells (5×10^5 cells/ml) treated with Ec (15 μ M), Antimycin A (125 nM), or both at 37° C for 2 hours or 6 hours in RPMI 1640 with 0.05% BSA. After washing in cold PBS, cells were resuspended in 100 μ l of dilution buffer, add 900 μ l of boiling buffer (100mM Tris, 4mM EDTA, pH7.5), boiled for 2 minutes, and centrifuged at 1000 X g for 1 minute. The supernatants were transferred to a fresh tube and kept on ice until measurements. ATP concentration was measured with a luminometer (Luminoskan, Labsystems) by adding 100 μ l of luciferase reagent to 100 μ l of sample or ATP standards (10^{-9} M to 10^{-4} M). ATP values were calculated from a log-log plot of the standard curve.

Statistical Analysis and Reproducibility

Data was analyzed by ANOVA with Bonferroni's Post tests and are expressed as means \pm SEM. All measurements were performed in triplicate and repeated at least twice with similar results. *p<0.05, **p<0.01, ***p<0.005.

Results

Oxidative Stress generation by ER stress agents

To investigate the possibility that ER stress inducers generate ROS, we exposed HL60 cells to the agents Econazole (Ec), Thapsigargin (Tg) and Tunicamycin (Tu) for 2 hours 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. 1A-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 3 agents induce ER stress within the chosen 2 hr time period, as indicated by induction of EIF2 α phosphorylation and Bip expression, along with suppression of protein synthesis (Soboloff and Berger, 2002; Zhang and Berger, 2004). However, this is achieved differently 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 Ca²⁺ stores (Thastrup et al., 1990) leading to rapid depletion of ER Ca²⁺ stores, followed by influx of Ca²⁺ from the extracellular milieu. While Ec is best known as an inhibitor of Ca²⁺ influx, it also promotes ER Ca²⁺ depletion (Jan et al., 1999), although the mechanism of this Ca²⁺ release has not previously been addressed.

To determine the kinetics of OS generation by Ec, we followed CM-H₂DCFDA fluorescence over time. As shown in Fig 1E, fluorescence changes were observed with a short delay following 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 (Eckert et al., 2003; Inoue et al., 2003; Kadenbach, 2003; Rego and Oliveira, 2003; Ueda et al., 2002). 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, while 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 the MitoSox Red dye, a mitochondrial-specific superoxide indicator (Invitrogen). To confirm that MitoSox measures ROS generated at the mitochondria, we used the phorbol ester PMA, which stimulates oxidase activity at the plasma membrane (Dooley et al., 2004; Teufelhofer et al., 2003) and atractyloside, which generates mitochondrial ROS via inhibition of adenine nucleotide activity (see below) as controls. As shown in Fig. 2B, the CM-H₂DCFDA dye detects similar levels of fluorescence by Ec, PMA and atractyloside over a 2 hr time period. In contrast, Ec and atractyloside, but not PMA, generate the superoxide anion, as detected with MitoSox (Fig. 2C).

Interestingly, 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 fluorescence 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 adenine nucleotide transporter (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 while 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

Since 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 TTFA, 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 two hours. 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 appears 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 hours and ROS content was measured as 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 hours, and measured total ATP content. As shown in Fig. 4B, exposure to Ec for 2 hours resulted in a decline in ATP content of 50%. Although a 2 hr 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 hours (Fig. 4C) resulted in even greater depletion of ATP by Ec or Antimycin A. However as with the 2 hour 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. Specifically, are increased ROS levels required for Ec-induced changes in membrane potential? To address this question, we pre-treated 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 to 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 over 2 hours. Therefore, it is likely that the generation of ROS occurs either in parallel with, or downstream of changes in mitochondrial membrane potential.

ER Ca²⁺ depletion by Ec is inhibited by Antimycin A and antioxidants

We have previously documented that Ec, in addition to blocking Ca²⁺ influx also causes Ca²⁺ depletion from the ER (Soboloff and Berger, 2002; Zhang and Berger, 2004). In order to determine the role of Ec's mitochondrial activity in ER depletion, we treated cells with Ec or Ec plus Antimycin A, and then measured the amount of Ca²⁺ present in the ER by releasing it into the cytoplasm with Tg while blocking the influx of Ca²⁺ with the non-specific Ca²⁺ channel blocker Ni²⁺. As shown in Fig. 6A, no Ca²⁺ was released into the cytoplasm by Tg after pre-treatment of cells with Ec, demonstrating that Ec depletes the ER. Antimycin A alone had little effect on Tg-releasable Ca²⁺. 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 Ca²⁺ depletion. In order to assess the role of mitochondria on Ca²⁺ influx, experiments were then performed in the absence of Ni²⁺.

The difference between the magnitude of Tg-induced Ca^{2+} influx in the presence of Ni^{2+} vs. the absence of Ni^{2+} reflects Ca^{2+} influx. Since 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 to that observed in experiments when Ni^{2+} was present in Fig 6A. Hence, Antimycin A neutralized Ec-induced ER Ca^{2+} depletion, but 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 (Figs. 6E&F). These results indicate that Ec-induced ER Ca^{2+} depletion is likely 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

Since 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 hours. The agents were then removed and the cells were

incubated in medium for an additional 6 hours. Antimycin A, BHT and Nac were non-toxic at the concentrations used under these conditions (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.

Antimycin A and antioxidants prevent caspase activation in response to Ec

Previously, we had demonstrated in other cell types that Ec-induced cell death was associated with a variety of apoptotic indicators including PARP 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 non-apoptotic 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 HL60 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 hours followed by a 6 hour incubation in medium (not shown). However as shown in Fig. 7B, extensive caspase 3/7 activation was observed if the incubation in medium was extended to 18 hours. Furthermore, the number of cells displaying caspase activation could be reduced significantly by antimycin A or the antioxidants. These observations therefore 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 HL60 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 likely from the same intracellular store. Interestingly, 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 refilling process.

Atractyloside (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 ROS levels were observed following Atra treatment. Furthermore, we observed that Atra could stimulate Ca^{2+} mobilization in HL60 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 HL60 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 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.

Since Antimycin A reverses Ec-induced hyperpolarization, ROS generation, ER Ca^{2+} depletion and cell death, complex III activity is likely 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). While 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. Previously, we 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). However we suspect that this activity is unrelated to the ability of Antimycin A to inhibit Ec function since 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 Ec's mechanism of action. 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 Ca²⁺ depletion.

We previously demonstrated that exposure to Ec for 2 hours was sufficient to initiate an irreversible cell death process. It is of interest to note that after 2 hours 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, while 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). Following exposure to Ec, HL60 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 hours following Ec exposure (Fig. 7B), but not at 6 hours. 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).

Alternatively, apoptosis may be a secondary or indirect consequence of the ER stress process and is likely not absolutely required for cell death. Previously, we observed in a variety of cell systems that 2 hour 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 likely 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 remain unknown. However one likely target is the SERCA, since 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 is 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 discernable 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. Similarly, 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, while 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 which 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 transition 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.

References

- Babich LG, Shlykov SG, Borisova LA and Kosterin SA (1994) Energy-dependent Ca²⁺-transport in intracellular smooth muscle structures. *Biokhimiia* **59**:1218-29.
- Barnes KA, Samson SE and Grover AK (2000) Sarco/endoplasmic reticulum Ca²⁺-pump isoform SERCA3a is more resistant to superoxide damage than SERCA2b. *Mol Cell Biochem* **203**:17-21.
- Dooley CT, Dore TM, Hanson GT, Jackson WC, Remington SJ and Tsien RY (2004) Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *J Biol Chem* **279**:22284-93.
- Dorner AJ, Wasley LC, Raney P, Haugejorden S, Green M and Kaufman RJ (1990) The stress response in Chinese hamster ovary cells. Regulation of ERp72 and protein disulfide isomerase expression and secretion. *J Biol Chem* **265**:22029-34.
- Downey JM (1990) Free radicals and their involvement during long-term myocardial ischemia and reperfusion. *Annu Rev Physiol* **52**:487-504.
- Eckert A, Keil U, Marques CA, Bonert A, Frey C, Schussel K and Muller WE (2003) Mitochondrial dysfunction, apoptotic cell death, and Alzheimer's disease. *Biochem Pharmacol* **66**:1627-34.
- Feinendegen LE (2002) Reactive oxygen species in cell responses to toxic agents. *Hum Exp Toxicol* **21**:85-90.
- Ferri KF and Kroemer G (2001) Organelle-specific initiation of cell death pathways. *Nat Cell Biol* **3**:E255-63.

- Franzius D, Hoth M and Penner R (1994) Non-specific effects of calcium entry antagonists in mast cells. *Pflugers Arch* **428**:433-8.
- Gommerman JL and Berger SA (1998) Protection from apoptosis by steel factor but not interleukin-3 is reversed through blockade of calcium influx. *Blood* **91**:1891-900.
- Grynkiewicz G, Poenie M and Tsien RY (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* **260**:3440-3450.
- Halliwell B and Whiteman M (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol* **142**:231-55.
- Hampton MB, Fadeel B and Orrenius S (1998) Redox regulation of the caspases during apoptosis. *Ann N Y Acad Sci* **854**:328-35.
- Hayashi T, Saito A, Okuno S, Ferrand-Drake M, Dodd RL, Nishi T, Maier CM, Kinouchi H and Chan PH (2003) Oxidative damage to the endoplasmic reticulum is implicated in ischemic neuronal cell death. *J Cereb Blood Flow Metab* **23**:1117-28.
- Hung CC, Ichimura T, Stevens JL and Bonventre JV (2003) Protection of renal epithelial cells against oxidative injury by endoplasmic reticulum stress preconditioning is mediated by ERK1/2 activation. *J Biol Chem* **278**:29317-26.
- Inoue M, Sato EF, Nishikawa M, Park AM, Kira Y, Imada I and Utsumi K (2003) Mitochondrial generation of reactive oxygen species and its role in aerobic life. *Curr Med Chem* **10**:2495-505.
- Jan CR, Ho CM, Wu SN and Tseng CJ (1999) Multiple effects of econazole on calcium signaling: depletion of thapsigargin-sensitive calcium store, activation of

- extracellular calcium influx, and inhibition of capacitative calcium entry.
Biochim Biophys Acta **1448**:533-42.
- Kadenbach B (2003) Intrinsic and extrinsic uncoupling of oxidative phosphorylation.
Biochim Biophys Acta **1604**:77-94.
- Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum:
coordination of gene transcriptional and translational controls. *Genes Dev*
13:1211-33.
- Kozutsumi Y, Segal M, Normington K, Gething MJ and Sambrook J (1988) The
presence of malfolded proteins in the endoplasmic reticulum signals the induction
of glucose-regulated proteins. *Nature* **332**:462-4.
- Kuo SC and Lampen JO (1974) Tunicamycin--an inhibitor of yeast glycoprotein
synthesis. *Biochem Biophys Res Commun* **58**:287-95.
- Lai MT, Huang KL, Chang WM and Lai YK (2003) Geldanamycin induction of grp78
requires activation of reactive oxygen species via ER stress responsive elements
in 9L rat brain tumour cells. *Cell Signal* **15**:585-95.
- Le Bras M, Clement MV, Pervaiz S and Brenner C (2005) Reactive oxygen species and
the mitochondrial signaling pathway of cell death. *Histol Histopathol* **20**:205-19.
- Liu H, Miller E, van de Water B and Stevens JL (1998) Endoplasmic reticulum stress
proteins block oxidant-induced Ca²⁺ increases and cell death. *J Biol Chem*
273:12858-62.
- Nathan C (2003) Specificity of a third kind: reactive oxygen and nitrogen intermediates
in cell signaling. *J Clin Invest* **111**:769-78.

- Nicotera P and Orrenius S (1998) The role of calcium in apoptosis. *Cell Calcium* **23**:173-80.
- Orrenius S (1985) Oxidative stress studied in intact mammalian cells. *Philos Trans R Soc Lond B Biol Sci* **311**:673-7.
- Orrenius S and Nicotera P (1987) Biochemical mechanisms of oxidative liver cell injury. *Bull Eur Physiopathol Respir* **23**:291-5.
- Orrenius S, Zhivotovsky B and Nicotera P (2003) Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol* **4**:552-65.
- Patil C and Walter P (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr Opin Cell Biol* **13**:349-55.
- Rego AC and Oliveira CR (2003) Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem Res* **28**:1563-74.
- Richter C, Schweizer M, Cossarizza A and Franceschi C (1996) Control of apoptosis by the cellular ATP level. *FEBS Lett* **378**:107-10.
- Robinson BH, Cheema-Dhadli S and Halperin ML (1975) The effect of adenosine triphosphate on the tricarboxylate transporting system of rat liver mitochondria. *J Biol Chem* **250**:3639-43.
- Rottenberg H and Wu S (1998) Quantitative assay by flow cytometry of the mitochondrial membrane potential in intact cells. *Biochim Biophys Acta* **1404**:393-404.

- Samali A, Nordgren H, Zhivotovsky B, Peterson E and Orrenius S (1999) A comparative study of apoptosis and necrosis in HepG2 cells: oxidant-induced caspase inactivation leads to necrosis. *Biochem Biophys Res Commun* **255**:6-11.
- Slater AF, Stefan C, Nobel I, van den Dobbelen DJ and Orrenius S (1995) Signalling mechanisms and oxidative stress in apoptosis. *Toxicol Lett* **82-83**:149-53.
- Soboloff J and Berger SA (2002) Sustained ER Ca²⁺ depletion suppresses protein synthesis and induces activation-enhanced cell death in mast cells. *J Biol Chem* **277**:13812-20.
- Soboloff J, Zhang Y, Minden M and Berger SA (2002) Sensitivity of myeloid leukemia cells to calcium influx blockade: application to bone marrow purging. *Exp Hematol* **30**:1219-26.
- Szegezdi E, Fitzgerald U and Samali A (2003) Caspase-12 and ER-stress-mediated apoptosis: the story so far. *Ann N Y Acad Sci* **1010**:186-94.
- Teufelhofer O, Weiss RM, Parzefall W, Schulte-Hermann R, Micksche M, Berger W and Elbling L (2003) Promyelocytic HL60 cells express NADPH oxidase and are excellent targets in a rapid spectrophotometric microplate assay for extracellular superoxide. *Toxicol Sci* **76**:376-83.
- Thastrup O, Cullen PJ, Drobak BK, Hanley MR and Dawson AP (1990) Thapsigargin, a tumor promoter, discharges intracellular Ca²⁺ stores by specific inhibition of the endoplasmic reticulum Ca²⁺(+)-ATPase. *Proc Natl Acad Sci U S A* **87**:2466-70.
- Tzung SP, Kim KM, Basanez G, Giedt CD, Simon J, Zimmerberg J, Zhang KY and Hockenbery DM (2001) Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. *Nat Cell Biol* **3**:183-91.

- Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M and Yodoi J (2002) Redox control of cell death. *Antioxid Redox Signal* **4**:405-14.
- Vander Heiden MG, Chandel NS, Schumacker PT and Thompson CB (1999) Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. *Mol Cell* **3**:159-67.
- Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM and Ron D (1996) Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). *Mol Cell Biol* **16**:4273-80.
- Watanabe Y, Suzuki O, Haruyama T and Akaike T (2003) Interferon-gamma induces reactive oxygen species and endoplasmic reticulum stress at the hepatic apoptosis. *J Cell Biochem* **89**:244-53.
- Zhang Y and Berger SA (2004) Increased calcium influx and ribosomal content correlate with resistance to endoplasmic reticulum stress-induced cell death in mutant leukemia cell lines. *J Biol Chem* **279**:6507-16.
- Zhang Y, Crump M and Berger SA (2002) Purging of contaminating breast cancer cells from hematopoietic progenitor cell preparations using activation enhanced cell death. *Breast Cancer Res Treat* **72**:265-78.

Footnotes

This work was supported by grants from the National Cancer Institute of Canada to SAB.

Figure Legends

Figure 1. Generation of OS in ER stress agent treated cells. HL-60 cells were incubated with different ER stress agents: **A:** Ec (15 μ M), **B:** Tg (1 μ M) or **C:** Tu (1 μ g/ml) for 2 hours, then loaded with H₂DCFDA (8 μ M) for 30 minutes at 37 °C and rinsed with PBS. Fluorescence was monitored by flow cytometry at FL-1. The grey tracing is the untreated control. The data depicted is a representative experiment. **D:** Comparison of DCF fluorescence in arbitrary units for Ec, Tg or Tu treated cells compared to control. Reported values are averages of 3 independent determinations and are reported +/- S.E.M. ** $p < 0.01$ compared to control. **E:** Time course of DCF fluorescence in response to Ec (15 μ M).

Figure 2. Ec hyperpolarizes mitochondrial membrane potential (MMP), generates mitochondrial ROS and inhibits ADP transport. **A:** JC-1 loaded HL-60 cells were exposed to Ec (15 μ M), Tg (1 μ M), Tu (1 μ g/ml) or FCCP (2 μ M), respectively, and the membrane potential was monitored by flow cytometry as described in the Methods section. **B:** HL60 cells were exposed to Ec (15 μ M), PMA (2 μ M) or Atractyloside (50 μ M) for 2 hours then loaded with H₂DCFDA (8 μ M) for 30 minutes at 37 °C and rinsed with PBS. The mean fluorescence intensity was measured by flow cytometry. **C:** HL60 cells were treated with ROS-generating agents as in B, but loaded with MitoSox (5 μ M) for 30 minutes 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 Ec (15 μ M). **E:** Cell-free mitochondria were prepared as described in Materials and Methods. Samples were incubated with ER stress agents for 10 minutes in the presence or absence of atractyloside followed by the addition of ¹⁴C-ADP. ANT-dependent ADP transport activity was determined by calculating the atractyloside inhabitable counts.

ANT-dependent ADP transport was inhibited by Ec (15 μ M), but not by Tg (1 μ M) or Tu (1 μ g/ml). Values are reported as mean \pm S.E.M.

Figure 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 pre-treated for 20 minutes with 10 μ M rotenone, 10 μ M TTFA or 400 μ M KCN. The arrow indicates where 15 μ M Ec or 2 μ M FCCP was added to the cells. **C:** The effect of Antimycin A on membrane hyperpolarization, Cells stimulated with Antimycin A (125 nM), Ec or FCCP. Mitochondrial membrane potential was recorded for 2 hours.

Figure 4. Antimycin A inhibits and reverses OS generation induced by Ec and partially restores ATP production. A: Cells were incubated with Ec (15 μ M), Antimycin A (125 nM), or both at 37 $^{\circ}$ C for 2 hours, and DCF fluorescence was measured as described in the Methods section. Data are shown as mean \pm S.E.M.

* p <0.05, ** p <0.01. **B&C:** Intracellular ATP was measured in cells (0.5 - 1.0×10^6 cells/ml) treated with Ec (15 μ M), Antimycin A (125 nM), or both, at 37 $^{\circ}$ C, for 2 hours (panel **B**) or 6 hours (panel **C**). Values shown are reported as mean \pm S.E.M. * p <0.05, ** p <0.01.

Figure 5. Antioxidants do not reverse MMP hyperpolarization by Ec. Cells were pre-treated with or without BHT (5 μ M) or Nac (1 μ M) at 37 $^{\circ}$ C for 20 minutes followed by JC-1 loading and flow cytometry procedures described in the Methods section. Mitochondrial potential was followed for 2 hours after Ec (15 μ M) or FCCP (2 μ M) was added.

Figure 6. Ec-generated OS targets ER Ca²⁺ stores. Cells were loaded with Indo-1AM in Tyrode's buffer as described in Materials and Methods. Ca²⁺ concentration was monitored by flow cytometry. **A:** Cells were pre-incubated in the absence (Ctl) or presence of Ec (15 μM) or Ec + AA (125 nM) for 2 hours. Ni²⁺ (5 mM) was added to the cells to non-specifically prevent influx. Ca²⁺ released by Tg (3 μM) into the cytoplasm was used as an indicator of ER Ca²⁺ content. **B:** As in A, however Ni²⁺ was not added to cells before Tg. **C:** As in A except 5 μM BHT was used. **D:** As in C however Ni²⁺ was not added before Tg. **E:** As in A except 1 mM Nac was used. **F:** As in E however Ni²⁺ was not added before Tg.

Figure 7. Ec-induced cell death and caspase activation is suppressed by Antimycin A, BHT and Nac. Cells were treated with Ec (15 μM) in the presence or absence of AA (125 nM), BHT (5 μM), or Nac (1mM) at 37 °C for 2 hours, in medium containing 1% FBS. Drugs were washed out and the cells were recovered in RPMI plus 10% FBS for **A:** 6 hours. Samples were co-stained 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 to Ec. **B:** Cells were treated as above except the recovery time was extended to 18 hours. Cells were then incubated with the FLICA reagent (Invitrogen) to detect active caspases 3 or 7 and analyzed by flow cytometry. Reported values are % of cells that are positive for activated caspase 3/7 and are averages of two repeat measurements with standard errors.

Figure 8. Ec and H₂O₂ both target the Tg-releasable Ca²⁺ store. **A:** H₂O₂ (200 μM) - induced changes in cytoplasmic Ca²⁺ concentration. Indo-1AM-loaded cells were exposed to H₂O₂ and cytoplasmic Ca²⁺ was followed over time. **B:** As in A except cells were pre-incubated in Ec (15 μM). **C:** Tg-induced release of ER Ca²⁺ content can still be

detected in cells pre-treated with H_2O_2 (200 μM ; 2 hours). Ni^{2+} (5 mM) was used to demonstrate that this was release of Ca^{2+} from intracellular stores and not Ca^{2+} entry.

Figure 9. Atractyloside-generated ROS targets ER Ca^{2+} stores. A: HL-60 cells were incubated with atractyloside (50 μM) for 2 hours, then loaded with H_2DCFDA (8 μM) for 30 minutes at 37 °C. H_2DCFDA fluorescence was measured by flow cytometry. **B:** Indo-1 loaded HL60 cells were treated with Atractyloside (50 μM) and cytoplasmic Ca^{2+} was followed by flow cytometry. **C:** Cells were pre-treated with atractyloside (50 μM) for 2 hours. ER Ca^{2+} content was measured by releasing ER Ca^{2+} into the cytoplasm with Tg (3 μM) in the presence of Ni^{2+} to prevent Ca^{2+} influx.

Figure 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; **5)** Ec also blocks Ca^{2+} influx resulting in an ER stress response.

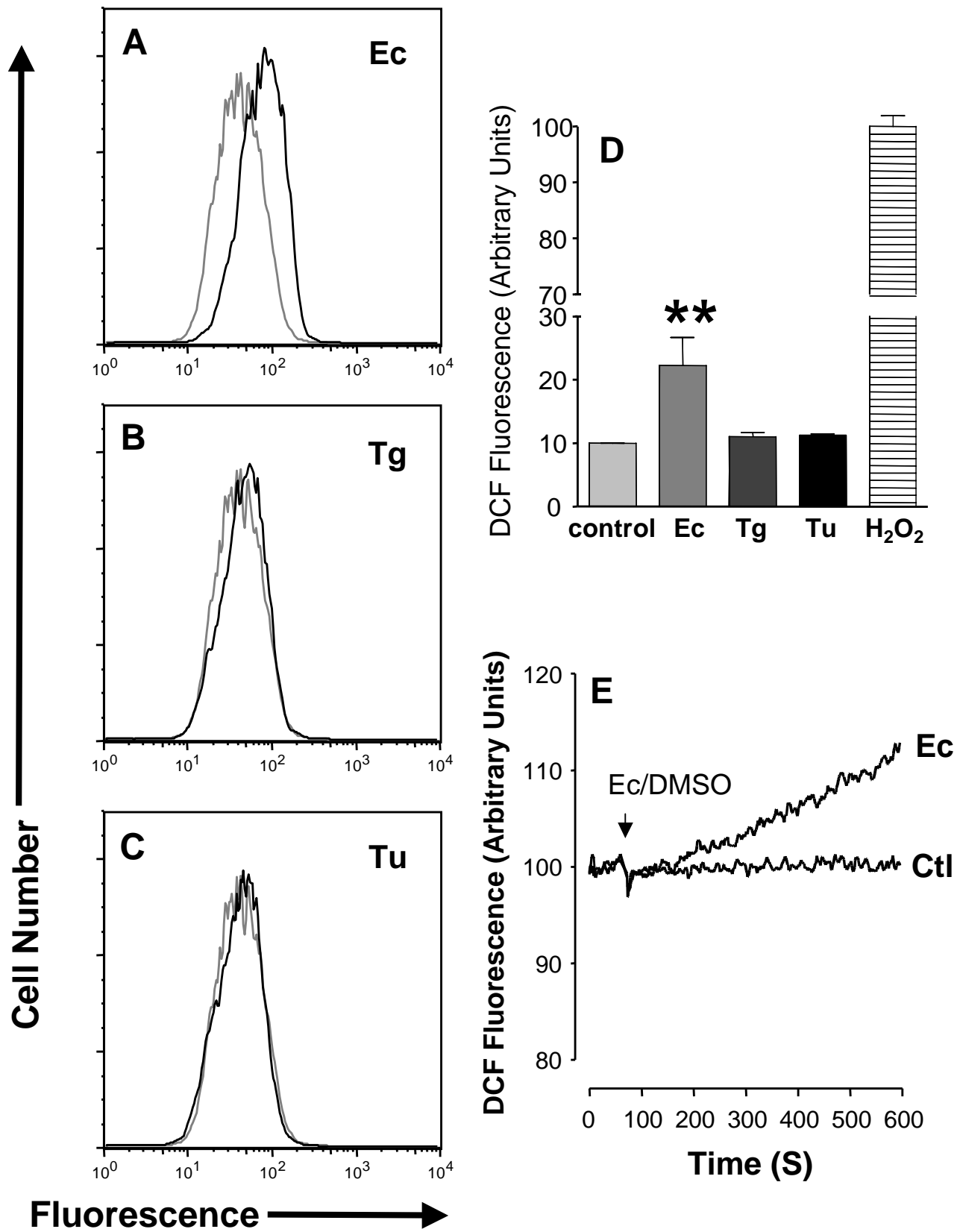


Figure 1

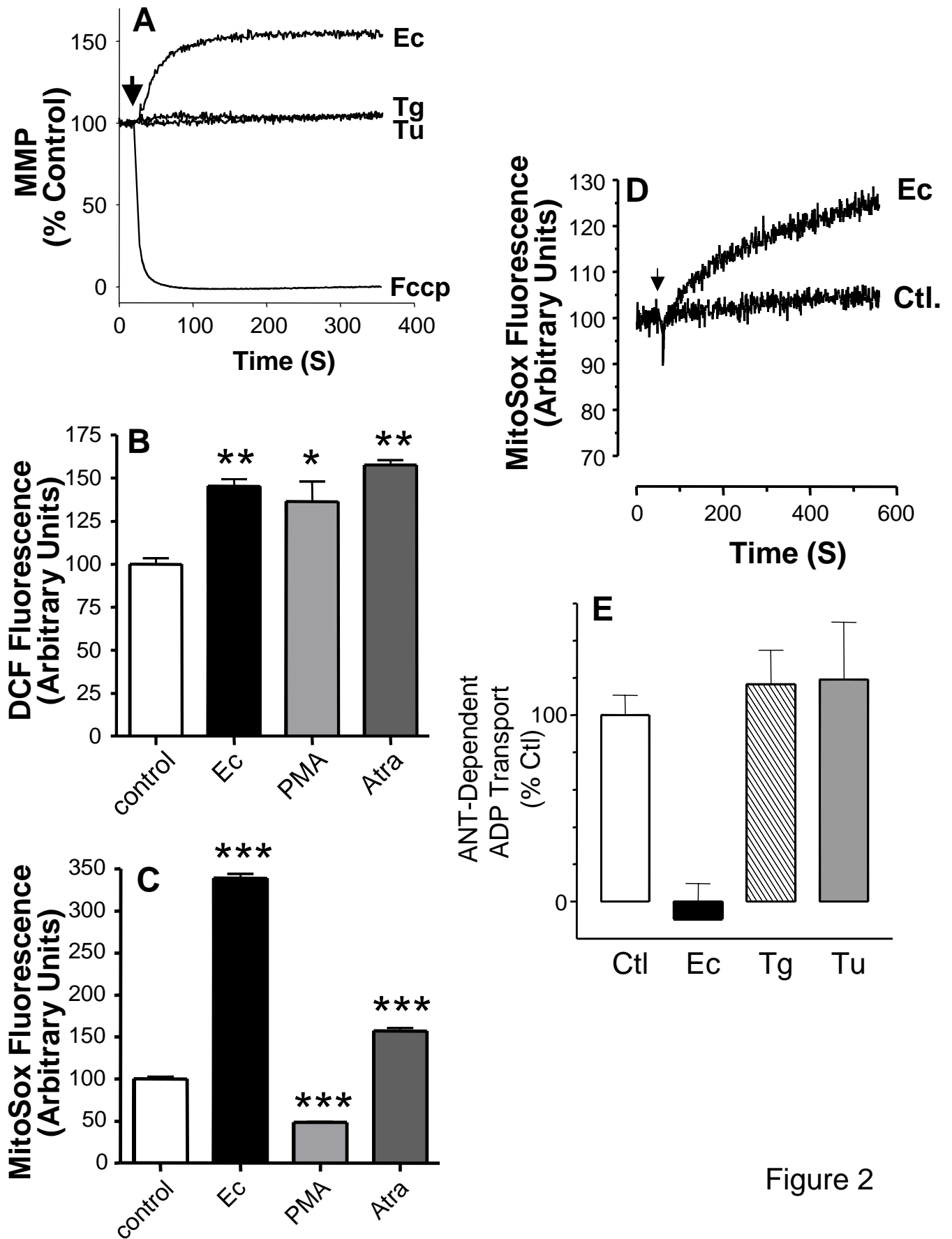


Figure 2

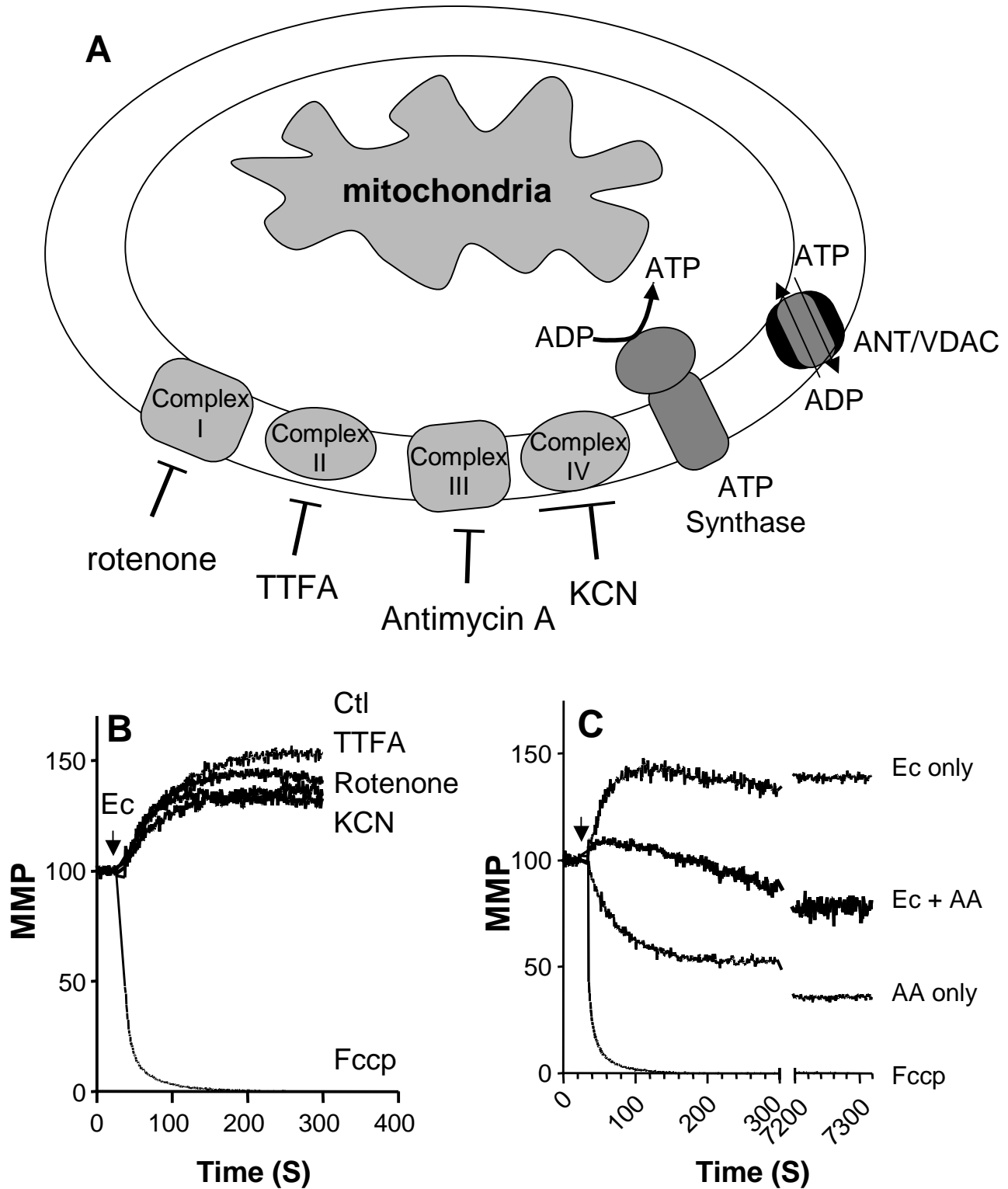


Figure 3

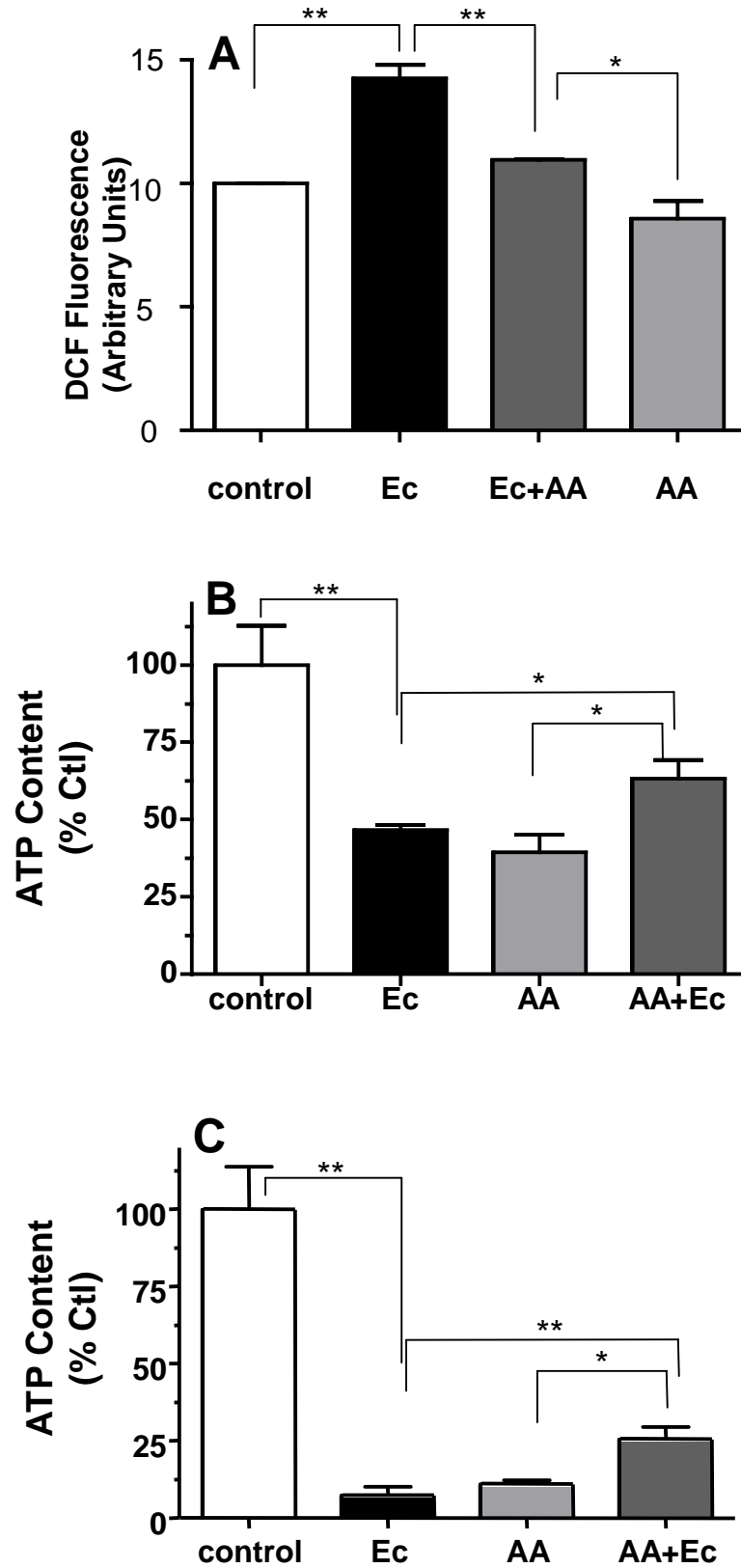


Figure 4

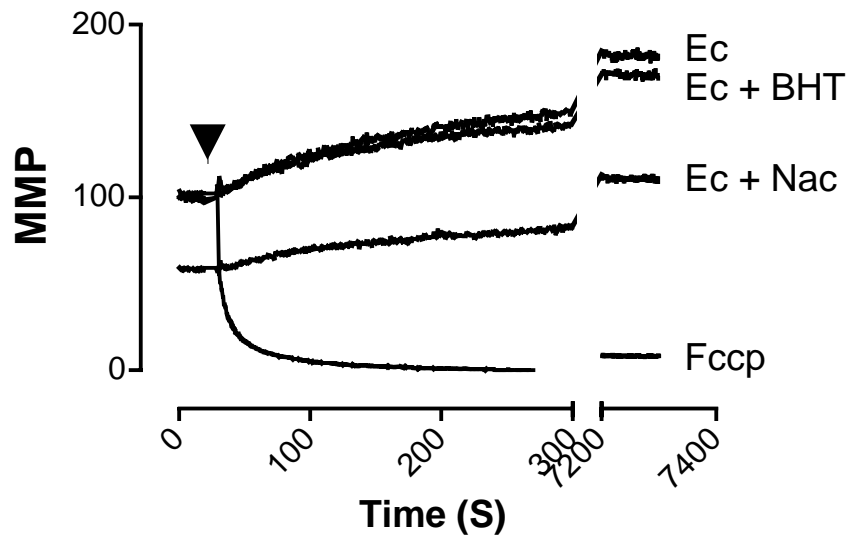


Figure 5

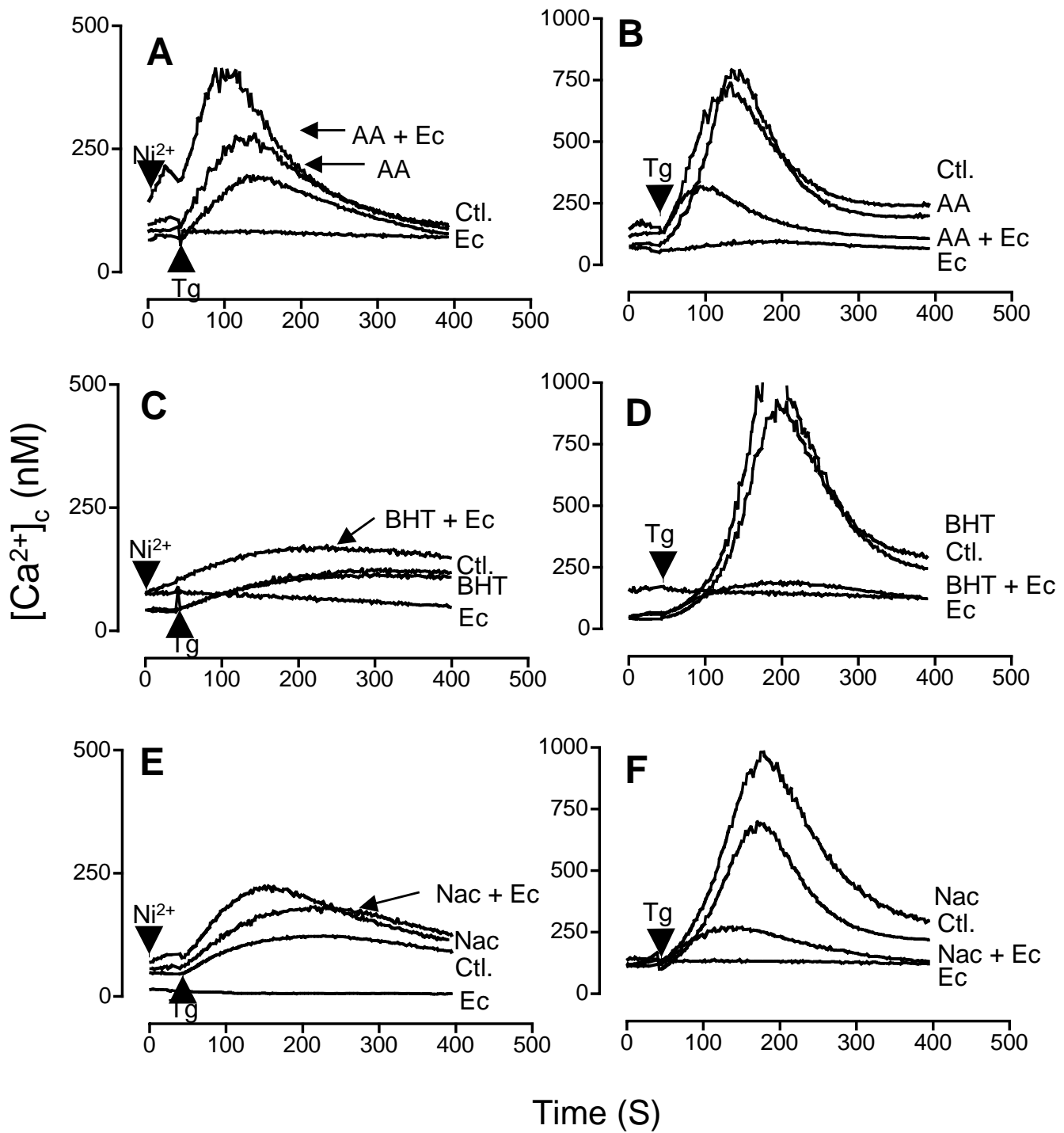


Figure 6

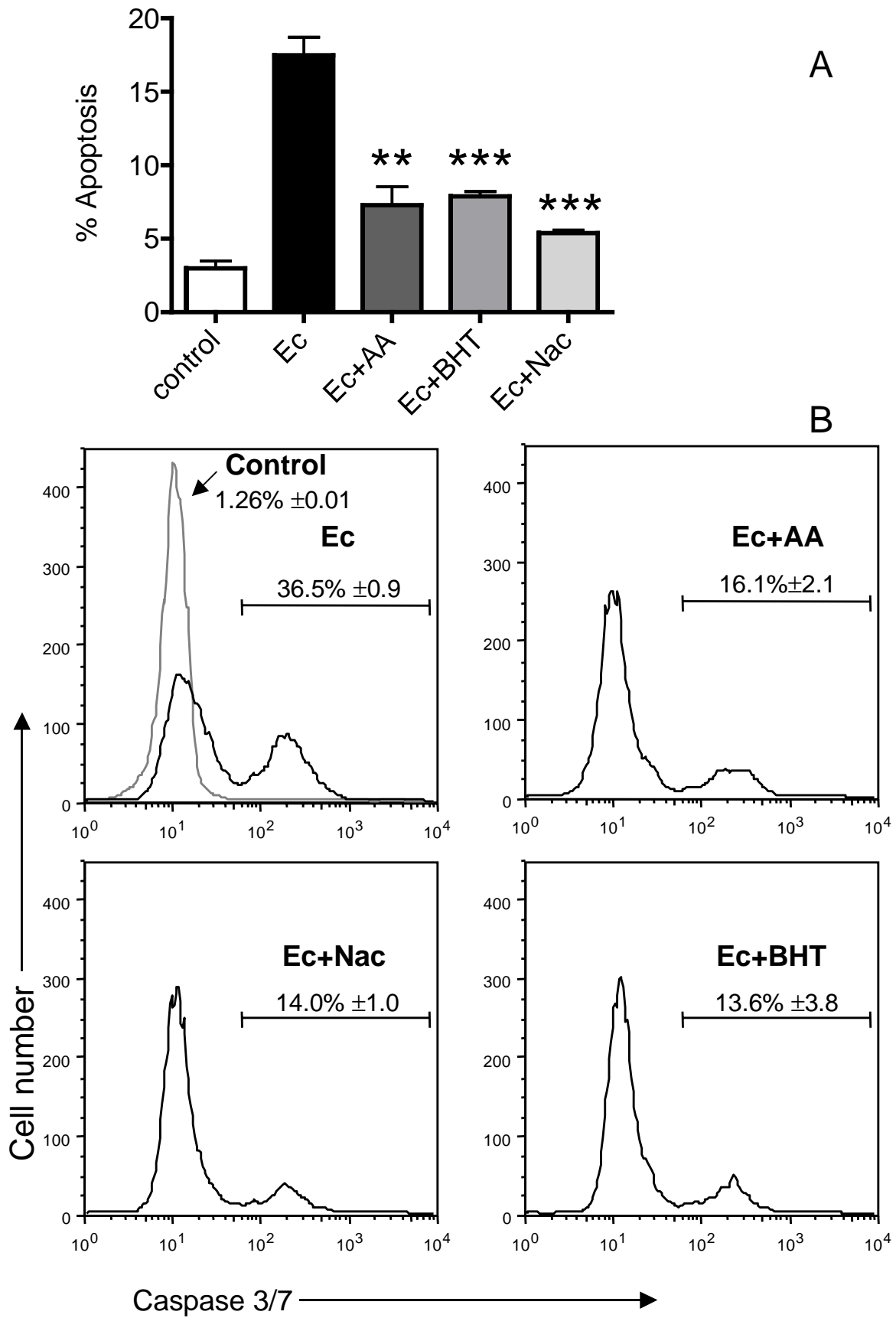


Figure 7

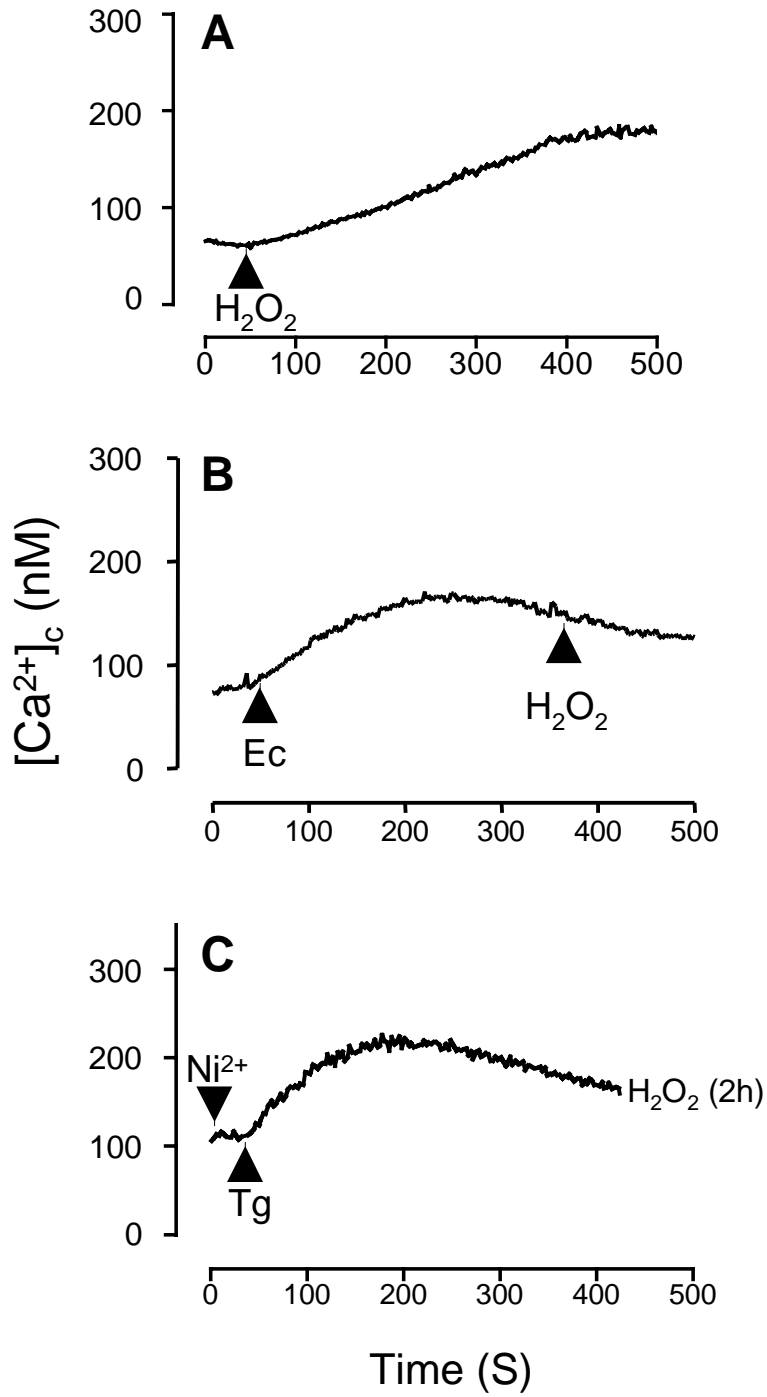


Figure 8

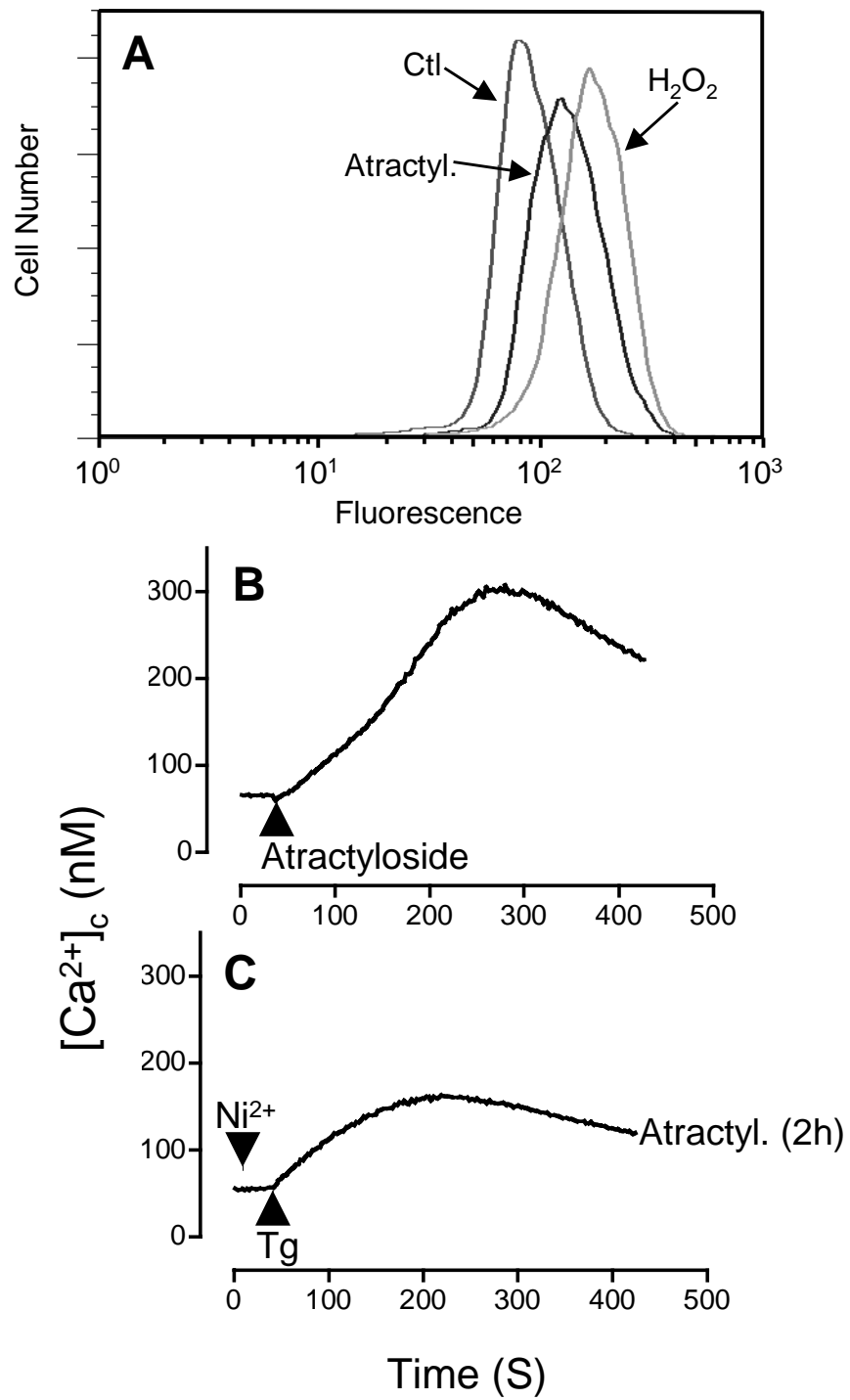


Figure 9

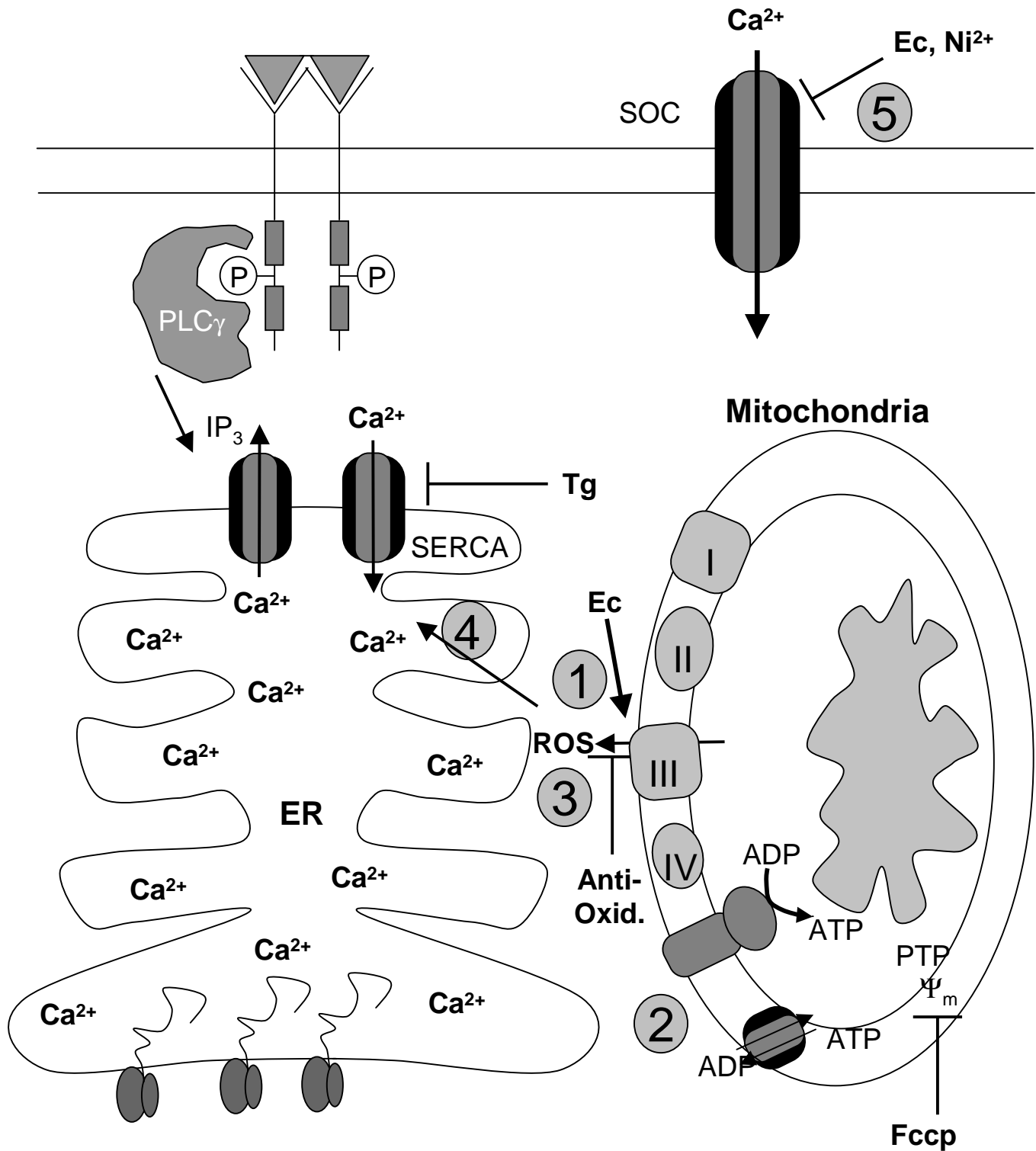


Figure 10