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# Mitochondrial state 3 to 4 respiration transition during Fas-mediated apoptosis controls cellular redox balance and rate of cell death

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**Abstract**

The role of reactive oxygen species (ROS) production in death receptor-mediated apoptosis is ill defined. We show that ROS levels play a novel role in moderating the rate of cell death in Fas-dependent apoptosis. Treatment of Jurkat T cells with oligomycin (ATP-synthase inhibitor) or FCCP (mitochondrial uncoupler) and Fas activating antibody (CH11), facilitated rapid cell death. ATP levels, DEVDase activity and cytochrome *c* release were not account for the synergistic killing effect. However, a decrease in cellular ROS production was associated with CH11 treatment and combinations of CH11 with oligomycin or FCCP further inhibited cellular ROS levels. Thus, decreased ROS production is correlated with accelerated cell death. A transition from state 3 to state 4 mitochondrial respiration following apoptotic stimuli accounted for an attenuated membrane potential and as a results mitochondria-derived ROS production capacity diminished. Similar observations were demonstrated in isolated rat liver mitochondria. Transfection with mitochondrial targeted catalase inhibited mitochondrial ROS production and potentiated cell death. These data show that ROS production is important in receptor-mediated apoptosis and may play a pivotal role in cell survival.


*Keywords:* Fas; Apoptosis; Miochondrial membrane potential; Respiration; Reactive oxygen species; Redox

## 1. Introduction

### 1.1. Cellular ROS and Fas-mediated apoptosis

Mitochondria are considered to be the main source of ROS [1–3]. Mitochondria from post-mitotic cells use $O_2$ at a high rate and may release oxygen radicals that exceed cellular antioxidant defenses [4]. Indeed, mitochondria are the major source of superoxide anion production in cells [1,2]. The electron-transfer chain may produce a flux of superoxide radicals via the one-electron reduction of molecular oxygen, which is then dismuted by MnSOD [3] to produce a constant flux of hydrogen peroxide.

It is generally accepted that activation of Fas-mediated cell death is not responsive to inhibition by antioxidants. We show that ROS production can attenuate Fas-mediated apoptosis and therefore explains the inability of antioxidant strategies to inhibit Fas-mediated cell death. Thus, by attenuating mitochondrial-derived ROS fluxes, the rate of cell death can be intensified.

## 2. Results and discussion

### 2.1. Potentiation of Fas-mediated apoptosis in Jurkat T cells by the mitochondrial ATP synthase inhibitor oligomycin

Activation of the Fas-death domain pathway using the Fas-activating antibody CH11 resulted in cell death. Following 6 hr of treatment with CH11 approximately 10% of the cells were propoidium iodide (PI) positive and considered dead cells. Addition of 10 μg/mL oligomycin did not facilitate cell killing in the Jurkat T cell populations. However, a combination of oligomycin and CH11 facilitated rapid cell death of more than 75% of cells in culture within the first 6 hr (Fig. 1 show a typical synergistic cell death experiment of the combination CH11 + oligomycin).

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*Abbreviations:* ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

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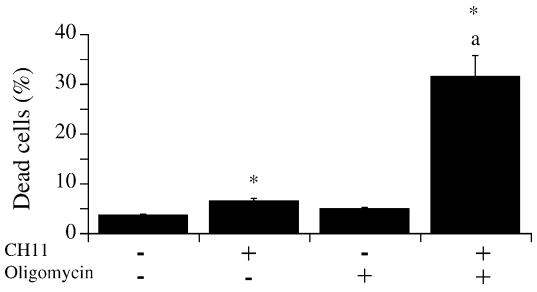


Fig. 1. Potentiation of Fas-mediated cell death in Jurkat cells by oligomycin. CH11 (0.1 μg/mL × million cells) was used for 4 hr as the Fas-activating antibody. Oligomycin (10 μg/mL) was added to the cell culture medium 5 min before CH11. $^{*}P < 0.05$, difference compared to untreated control. $^{a}P < 0.05$, difference compared to CH11 treatment.

Cell death began within 2 hr of addition of the various apoptotic stimuli. Exposure of Jurkat T cells to the ATP synthase (complex 5) inhibitor oligomycin or the mitochondrial uncoupler FCCP potentiated the effect of CH11 Fas-activating antibody in facilitating cell death.

In addition to apoptosis Fas activation was reported previously to facilitate necrotic pathway of cell killing in cells that do not express caspase 8 and do not activate caspase in response to oligomerization of FADD (Fas-associated protein with death domaine) [5] or in the absence of active caspase [6]. In the present study, DEVDase activity and chromatin aggregation were not compromised by oligomycin/CH11 or FCCP/CH11 treatment indicating that apoptosis is occurring.

It has been reported that mitochondria can affect cell death by three interrelated mechanisms: (I) loss of the ability to generate ATP, leading to cell death; (II) mitochondrial release of pro-apoptotic factors such as cytochrome *c* that activate caspase 9 to further activate downstream caspases; (III) and redox-dependent alterations in mitochondria, promoting cell death. To determine which of these above pathways contribute to the synergistic increase in apoptosis of the combination treatments. ATP levels, cytochrome *c* release, caspase activation and ROS levels were evaluated in response to treatments with oligomycin, CH11 or their combination. HPLC analysis demonstrated no loss of ATP under any of the conditions that induced apoptosis. CH11 treatment resulted in the release of cytochrome *c* and strong activation of DEVDase activity. However, no further augmentation in cytochrome *c* release or DEVDase activity was recorded in cells that were treated with CH11 + oligomycin. Thus ATP depletion, increased mitochondrial cytochrome *c* release or enhanced caspase activation could not account for the synergistic increase in apoptotic cell death following treatment with CH11 + oligomycin. During Fas activation it became clear that ROS production is attenuated by CH11. Treatments with oligomycin and CH11 further inhibited cellular ROS production and correlated with the increased rate of cell death. ROS production was decreased early in the death cascade and was observed before any significant cell death occurred.

To elucidate the reason for the diminished ROS production capacity in the apoptotic cells we analyzed the ATP and ADP cellular levels. Analysis of ADP levels in control untreated cells demonstrated that they were approximately one-tenth of the ATP level. It is therefore, likely that ADP is the rate-limiting compound controlling mitochondrial respiration. Activation of the apoptotic process resulted in rapid consumption of cellular ADP within the first 4 hr and thus, mitochondria in apoptotic cells were unable to sustain full state 3 respiration. The respiration shift was reflected by the observed loss of mitochondrial membrane potential in the apoptotic cells.

Using digitonin-permeabilized cells mitochondrial ATP and ADP were evaluated, oligomycin and CH11 decreased ADP levels with an opposite effect on ATP. The cell pellet-associated ADP levels were low, supporting the state 3 to state 4 respiration shift. This finding may explain why decreased membrane potential is observed without a requirement for mitochondrial permeability transition pore opening [7] or increased mitochondrial oxidative damage during apoptosis [8].

Oligomycin has been reported to protect against BAX, staurosporine and UV-induced apoptosis but not against Fas [9–11]. The increased rate of cell death observed in the present study following oligomycin treatment is unrelated to the cellular energetic status. No ATP depletion was observed at early time points when apoptosis was apparent. In fact, ATP levels increased in digitonin permeabilized cells following treatment with CH11 or oligomycin. The reason why ATP was not consumed in the mitochondria of cells treated with oligomycin, CH11 or a combination of both is not fully understood. It is possible that mitochondrial ATP is preserved due to decreased consumption and/or a decrease in proton motive force-dependent adenine nucleotide translocase matrix-ATP exchange with cytosolic-ADP.

### 2.2. Role of mitochondrial membrane potential in apoptosis

Treatment of non-apoptotic Jurkat T cells with the mitochondrial uncoupler FCCP resulted in a rapid loss of the ability of mitochondria to produce ROS. However, loss of cellular viability was not observed. Cellular ROS production was prolonged following 4 hr of incubation with FCCP. Cells treated with CH11 + FCCP accelerated DNA fragmentation compared to either treatment of them alone. Decreased mitochondrial membrane potential and ROS production were observed after 4 hr of incubation. Cell death was also dramatically enhanced following prolonged CH11 + FCCP treatment. DEVDase activity was dependent on Fas activation by CH11. Such activity was also observed in the presence of FCCP alone and was not augmented in the combination teatment compared to CH11. These data indicate that the maintenance of a partial level of membrane potential is crucial for the generation of

mitochondria-derived ROS during apoptosis and that such low ROS level are natural regulators of Fas-induced progression of apoptosis.

To further elucidate the role of ROS as survival factors a Mitochondria targeted catalase plasmid was transfected into Jurkat T cells and used to determine whether ROS are indeed important in the death process. Mitochondria-targeted catalase has previously been shown to potentiate apoptotic cell death in response to TNF and cycloheximide in HepG2 cells [12]. In the Fas model mitochondrial expression of catalase resulted in loss of the cells' ability to maintain normal levels of ROS and resulted in enhanced cell death. Addition of extracellular catalase to the incubation medium (1000 U/mL) did not result in enhanced cell death or a loss in the capacity to produce ROS.

The Fas (CD95) apoptotic pathway has not been shown to be associated with production of ROS or the induction of oxidative stress. The few studies indicating involvement of ROS in Fas-mediated apoptosis propose the use of SOD-mimicking compounds as protective agents [13]. However, such compounds are not only antioxidants that remove superoxide anions, they can also serve as a source of $H_2O_2$. In fact, conventional antioxidants are virtually ineffective in controlling the death process [14]. Moreover, long pre-incubation with lipoic acid, a thiol lipophilic antioxidant can stimulate the Fas death pathway [15].

It is widely held that cells possess strong redox buffering capacity to maintain viability. Increased oxidative stress will eventually lead to apoptosis and later on to necrotic cell death [16]. Measurable levels of intracellular ROS are always produced in cells and also serve as signaling molecules for numerous signal transduction pathways [17]. TRAF (tumor necrosis factor receptor)-mediated signal transduction facilitated ROS production from the mitochondrial electron-transfer chain resulting in enhanced NF-κB activation [18]. Therefore, mitochondria-derived ROS may orchestrate the redox tone and activate number of survival pathways of the cell including NF-κB and the AKT/PKB and thereby serve a function in cell survival. During Fas-mediated apoptosis, the capacity to produce ROS is impaired. The conversion of superoxide to hydrogen peroxide appears to be more dependent on the presence of protons in the matrix and therefore is oligomycin-sensitive. Such an effect is unique to apoptotic cells since oligomycin-dependent inhibition of ROS production was not observed in control cells.

### 2.3. Can similar effect be observed in isolated mitochondria?

Treatment of isolated rat liver mitochondria during state 3 respiration attenuated DCF-sensitive ROS production (peroxides). The use of mitochondria in state 3 vs. state 4 respiration revealed an attenuation of the mitochondrial membrane potential ($\Delta\psi$) and ROS production. In apoptotic cells, rapid loss of membrane potential was also observed, accompanied by decreased ROS production. The proposal that ROS production is enhanced during state 4 respiration [1] was not observed in the present study using isolated mitochondria. ROS production was directly correlated with $\Delta\psi$ (state 4: no ATP, $\Delta$pH is maximal $\Delta\psi$ is minimal; state 3 is the opposite). Cellular ADP makes up approximately 10% of the ATP concentration. Therefore, intracellular degradation of ADP may shift cells from state 3 to state 4 respiration. This study indicates that an early event associated with cell death is ADP degradation. The respiration state could explain the observed loss of mitochondrial membrane potential and ROS production in apoptosis via a mechanism that does not involve direct mitochondrial damage.

### 2.4. Endogenous vs. exogenous ROS

Treatment of Jurkat T cells with TBH elevated total intracellular ROS in the cell population. The distribution of exogenous ROS affected the entire cell population. TBH treatment increased ROS levels in apoptotic cells. However, lack of homogeneity in the ROS levels in apoptotic cells following TBH treatment suggests that exogenous ROS cannot compensate fully for the loss of endogenous ROS production and only partially protects from apoptosis cell death.

In conclusion, we link the mitochondrial respiratory state to ROS production in apoptotic cells and established that dissipation of intracellular ROS is an important step in facilitating Fas receptor-mediated cell death.

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