Potentiation of Fas-mediated apoptosis by attenuated production of mitochondria-derived reactive oxygen species

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Abstract

The role of reactive oxygen species (ROS) production in death receptor-mediated apoptosis is ill-defined. Here, we show that ROS levels play a role in moderating Fas-dependent apoptosis. Treatment of Jurkat T cells with oligomycin (ATP-synthase inhibitor) or (mitochondrial uncoupler) and Fas-activating antibody (CH11) facilitated rapid cell death that was not associated with decreased ATP production or increased DEVDase activity and cytochrome *c* release. However, a decrease in cellular ROS production was associated with CH11 treatment, and combinations of CH11 with oligomycin or FCCP further inhibited cellular ROS production. Thus, decreased ROS production is correlated with enhanced cell death. A transition from state 3 to state 4 mitochondrial respiration accounted for the attenuated ROS production and membrane potential. Similar observations were demonstrated in isolated rat liver mitochondria. These data show that ROS production is important in receptor-mediated apoptosis, playing a pivotal role in cell survival.

Abbreviations: MPT, mitochondrial permeability transition; ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; PI, propidium iodide; DCF, dichlorofluorescein; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; TBH, *t*-butylhydroperoxide; FADD, fas-associated protein with death domain; $H_2DCF-DA$, dichlorodihydrofluorescein-diacetate

Introduction

In addition to their critical function in energy metabolism, mitochondria are known to regulate cell viability as well as necrotic and apoptotic cell death.[1–5] Four interrelated mitochondrial pathways have been suggested to facilitate cell death: (I) mitochondrial permeability transition (MPT) and the release of apoptotic cell death promoting factors; (II) cytochrome *c* release by proapoptotic members of the BCL-2 family of proteins;[6] (III) disruption of ATP production, and (IV) alteration of the cell's redox status and overproduction of reactive oxygen species (ROS).[7–10]

Two major apoptotic pathways are known: the death receptor pathway that responds to external triggering, such as cytokines, through caspase 8 activation, and the mitochondrial pathway that responds primarily to internal insults such as DNA damage, oxidative stress and kinase inhibitors. The two apoptotic pathways can be linked via the BCL-2 family member Bid protein, which upon cleavage by caspase 8 can interact with other family members to activate the mitochondrial apoptotic pathway.[11–14]

The contribution of the redox status of cells undergoing apoptosis is debatable and generally ill-defined. ROS are constantly produced in the living cell, and small changes in the redox status of cells can have profound effects on signal transduction pathways.[15–17] Increased intracellular levels of ROS (oxidative stress) facilitate apoptosis, while further increases have been reported to oxidize elements of the apoptotic pathway and to promote necrotic cell death.[18] However, during Fas-mediated apoptosis, enhancing the reducing environment using redox-active α-lipoic acid can potentiate receptor-mediated apoptosis in Jurkat T cells.[17] This suggests an important role for redox modulation in the regulation of receptor-mediated apoptotic cell death.

Mitochondria are considered to be the main source of ROS.[19–21] Mitochondria from postmitotic cells use O_2 at a high rate and may release oxygen radicals that exceed cellular antioxidant defenses.[22] Indeed, mitochondria are the major source of superoxide anion production in cells.[19,20] The electron-transfer chain may produce a flux of superoxide radicals via the one-electron reduction of molecular oxygen, which is then dismuted by manganese superoxide dismutase (MnSOD)[21] 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. However, the present study indicates that ROS production can attenuate Fas-mediated apoptosis, and explains the inability of antioxidant strategies to inhibit cell death. Thus, by attenuating mitochondria-derived ROS fluxes, the rate of cell death can be intensified.

Results

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 h of treatment with CH11, approximately 10% of the cells were
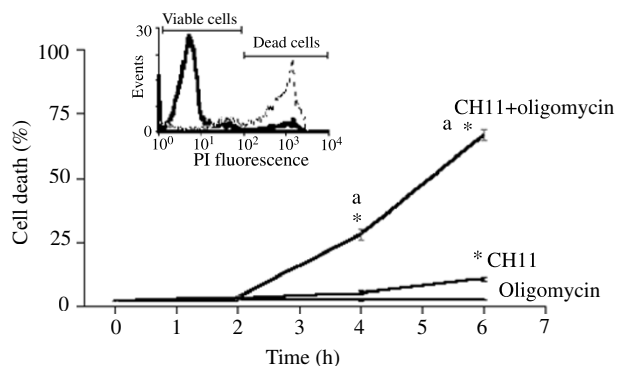
Figure 1 Potentiation of Fas-mediated cell death in Jurkat cells by oligomycin. CH11 (0.1 μg/ml million cells) was used as the Fas-activating antibody. Oligomycin (10 μg/ml) was added to the cell culture medium 5 min before CH11. $^*P < 0.05$, difference compared to beginning of treatment (0 time). $^aP < 0.05$, difference compared to CH11 alone at the time of treatment. Histograms: Representative flow cytometer histogram of cell viability following 6 h of treatments as indicated in the figure. Solid line, CH11; dashed line, CH11+oligomycin

propoidium iodide (PI) positive and considered dead cells (Figure 1). 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 almost all cells in culture within the first 6 h. Cell death began within 2 h of addition of the various apoptotic stimuli (Figure 1).

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 proapoptotic factors such as cytochrome c that activate caspase 9 to further activate downstream caspases and (III) redox-dependent alterations in mitochondria, promoting cell death. To determine which of these above pathways contribute to the synergistic increase in apoptosis, 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 (Figure 2a). CH11 treatment resulted in the release of cytochrome c and strong activation of DEVDase activity (Figure 2b,c). However, no further augmentation in cytochrome c release or DEVDase activity was recorded in cells that were treated with oligomycin+CH11 (Figure 2b,c). 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 (Figure 2b,c). However, during Fas activation, it became clear that ROS production is attenuated by CH11. Treatments with oligomycin and CH11 further
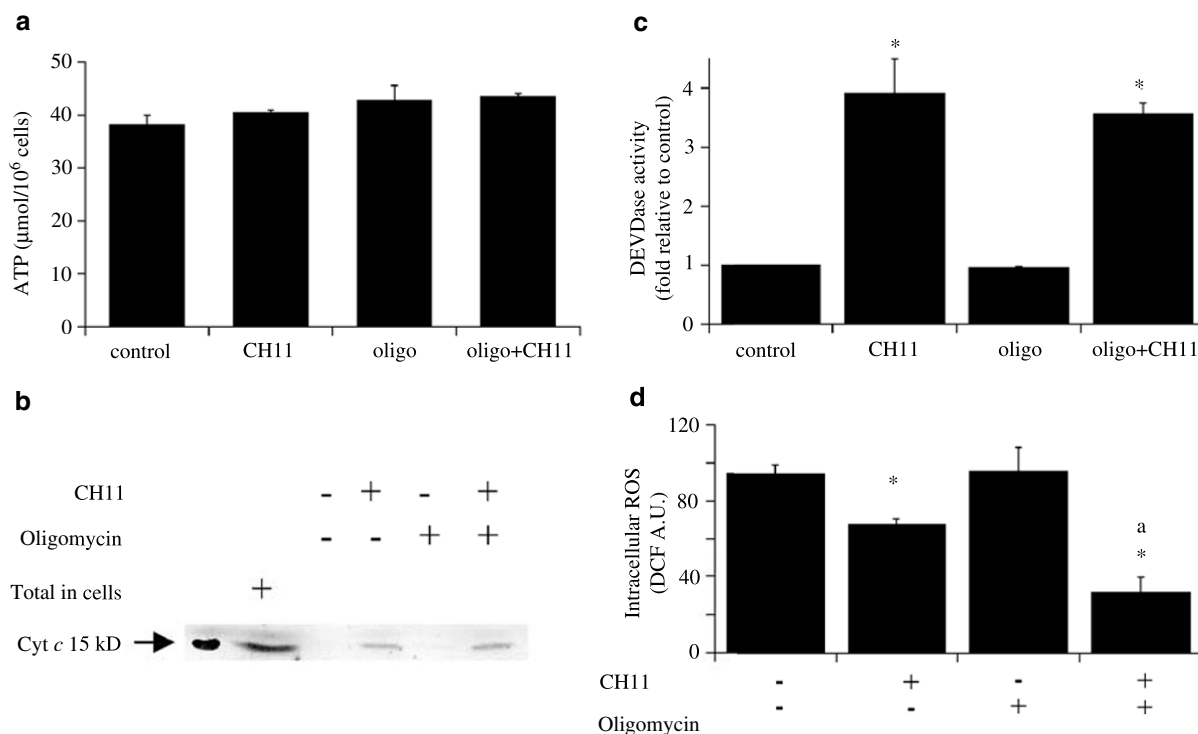
Figure 2 Early apoptotic events. Intracellular changes in apoptotic (CH11 treated) Jurkat cells in the presence or absence of oligomycin. Measurements were made following 2 h of treatment, before the cells started dying: (**a**) cellular ATP levels, (**b**) cytochrome c release from apoptotic cells, (**c**) DEVDase activity. $^*P < 0.05$, difference compared to control untreated cells, (**d**) intracellular ROS production measured by DCF fluorescence. $^*P < 0.05$, difference compared to control untreated cells. $^aP < 0.05$, difference compared to CH11-treated cells

inhibited cellular ROS production (Figure 2d) and correlated with the increased rate of cell death (Figure 1). ROS production was decreased early in the death cascade and was observed before any significant cell death occurred (Figure 2d).

Isolated mitochondria: oligomycin and state 4 respiration attenuate mitochondrial ROS production

Isolated rat liver mitochondria in state 3 (ADP replete) respiration were treated with 1 μg/ml oligomycin, and ROS production was strongly inhibited, as evaluated using kinetic analysis of the ROS-sensitive fluorophore dichlorofluorescein diacetate (DCF) fluorescence (Figure 3a). In addition to the effect of oligomycin that blocks protons from going into the
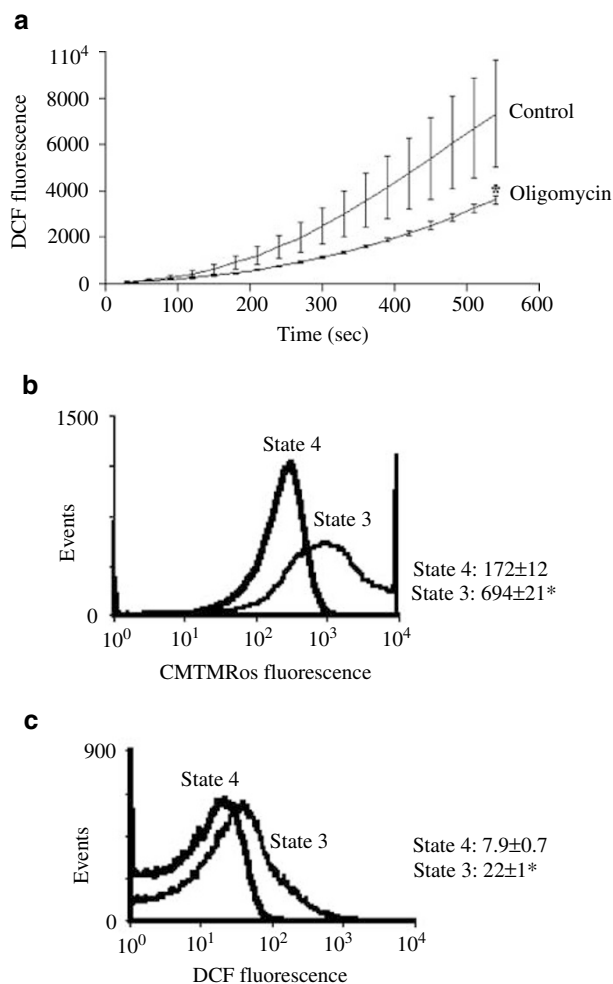
a

b

c

Figure 3 Inhibition of ROS production in isolated rat liver mitochondria by oligomycin. (**a**) Mitochondria in state 3 respiration were incubated with 1 μg/ml oligomycin, and DCF-sensitive ROS production was evaluated. Fluorescence data were collected by microfluorometer plate reader. $^*P < 0.05$, difference compared to control untreated mitochondria. (**b**) Mitochondria in two different respiration states (3 and 4) were evaluated for membrane potential using CMTMRos and ROS production using DCF. Data from 50 000 mitochondria were collected by flow cytometer. Histograms and total fluorescence (AU) data are presented. $^*P < 0.05$, difference compared to state 4 mitochondria

matrix, we suspected that there are basic differences in the capacity of isolated mitochondria to convert their superoxide to hydrogen peroxide during state 3 *versus* state 4 respiration. Mitochondria in state 3 showed an increase in ROS-dependent DCF fluorescence and higher membrane potential than state 4 respiring mitochondria. The difference in membrane potential was nearly one order of magnitude (Figure 3b), suggesting that a higher membrane potential is reached in order to maintain a balanced proton motive force to compensate for the decreased delta pH in the presence of ADP.

Do Jurkat T cells shift to state 4 mitochondrial respiration during apoptosis?

Analysis of ADP levels demonstrated that they were approximately one-tenth of the ATP level (Figure 4). 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 h (Figure 4a) 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 (Figure 4b). In digitonin-permeabilized cells, oligomycin and CH11 decreased ADP levels with an opposite effect on ATP (Figure 4c). 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[5] or increased mitochondrial oxidative damage during apoptosis.[23]

Role of mitochondrial membrane potential in apoptosis

Treatment of nonapoptotic Jurkat T cells with the mitochondrial uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) resulted in a rapid loss of the ability of mitochondria to produce ROS (Figure 5a). However, loss of cellular viability was not observed (not shown). Cellular ROS production was prolonged following 4 h of incubation with FCCP (Figure 5b). Cells treated with CH11+FCCP accelerated DNA fragmentation compared to treatment with either of them alone (Figure 5c). Decreased mitochondrial membrane potential and ROS production were observed after 4 h of incubation (Figure 5d,e). Cell death was also dramatically enhanced following prolonged CH11+FCCP treatment (Figure 5f). DEVDase activity was dependent on Fas activation by CH11. Such activity was also observed in the presence of FCCP (Figure 5g). These data indicate that the maintenance of a partial level of membrane potential is crucial for the generation of mitochondria-derived ROS during apoptosis.

Type of cell death

Using fluorescence microscopy, chromatin aggregation and nuclear fragmentation were documented following treatments with CH11, CH11+oligomycin or CH11+FCCP, indicating
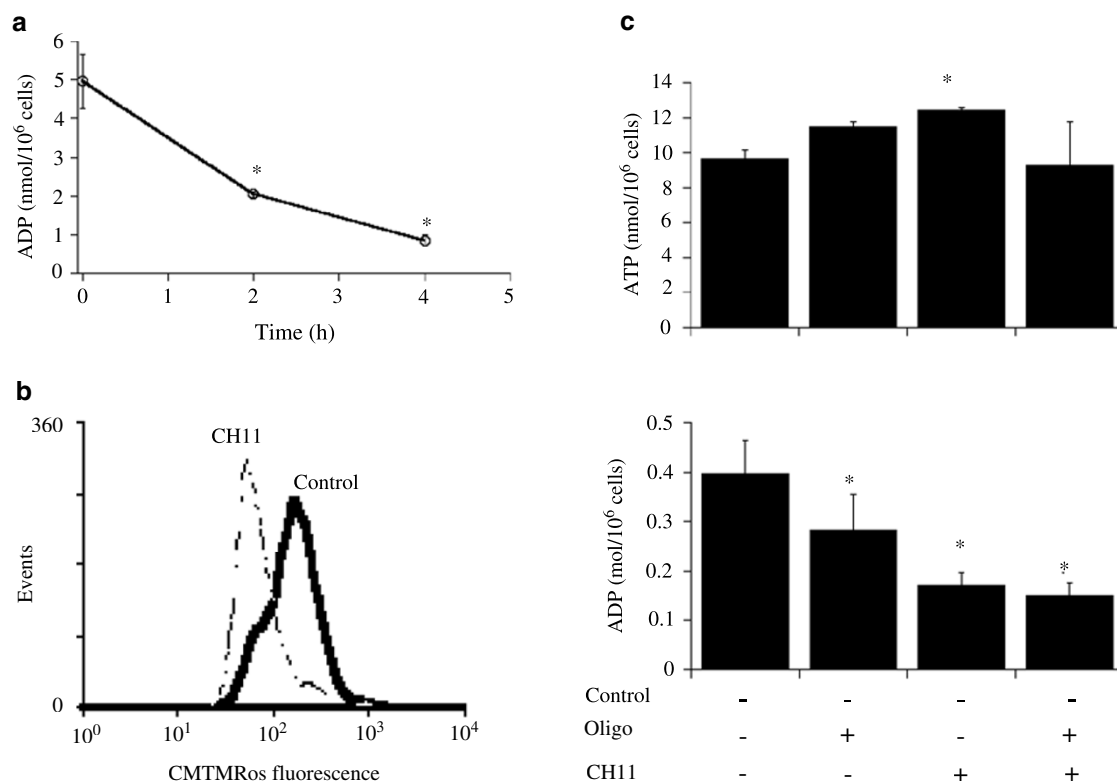
Figure 4 CH11 induces loss of ADP and mitochondrial membrane potential in Jurkat cells. (**a**) Treatment with CH11 for up to 4 h induced over 90% loss in cellular ADP content. $^*P < 0.05$, difference compared to untreated control. (**b**) Flow cytometer histogram demonstrating loss of mitochondrial membrane potential in apoptotic Jurkat cells. CMTMRos fluorescence reported loss in membrane potential that was observed in all cell populations following 4 h of exposure to CH11 (an early apoptotic event). Data were collected from 10 000 cells. (**c**) Treatment with digitonin permeabilized the Jurkat cells, thereby allowing measurements which represent mitochondrial ATP/ADP levels. Treatment with CH11 for up to 4 h induced over 60% loss in mitochondrial ADP content. CH11 significantly increased mitochondrial ATP. $^*P < 0.05$, difference compared to untreated control

apoptotic characteristics of cell death (Figure 6). No apoptosis was observed in CH11 untreated cells.

Cellular transfection with MSP-CAT plasmid

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.[24] Mitochondrial expression of catalase resulted in loss of the cells' ability to maintain normal levels of ROS (Figure 7b) and resulted in enhanced cell death (Figure 7a). 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 (not shown).

Can exogenous ROS provide protection?

We used *t*-butylhydroperoxide (TBH) as an exogenous source of ROS. At 50 μM, TBH did not induce cell death in the time frame of the experiment (up to 6 h). Addition of TBH resulted in elevated ROS in the control non-apoptotic cell population. The elevation of ROS affected all the cells in the culture (Figure

8a,b). Addition of TBH restored mean ROS levels in the apoptotic cells and elevated ROS production to that of nonapoptotic control cells. However, exogenous ROS did not fully compensate for the loss of endogenous mitochondria-derived ROS production observed in apoptotic cells (Figure 8b).

The ability of sublethal concentrations of TBH to protect against the lethal combination of CH11 and oligomycin was also tested. Protection by TBH was partial, and caspase activation (Figure 9a) was blocked by TBH, as measured by DEVDase activity assay (performed under nonreducing conditions without DTT). In addition, nuclear protection was also observed (Figure 9b). TBH prevented DNA fragmentation but was unable to prevent the accumulation of PI in the apoptotic cells (Figure 9c).

Discussion

Type of cell death

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
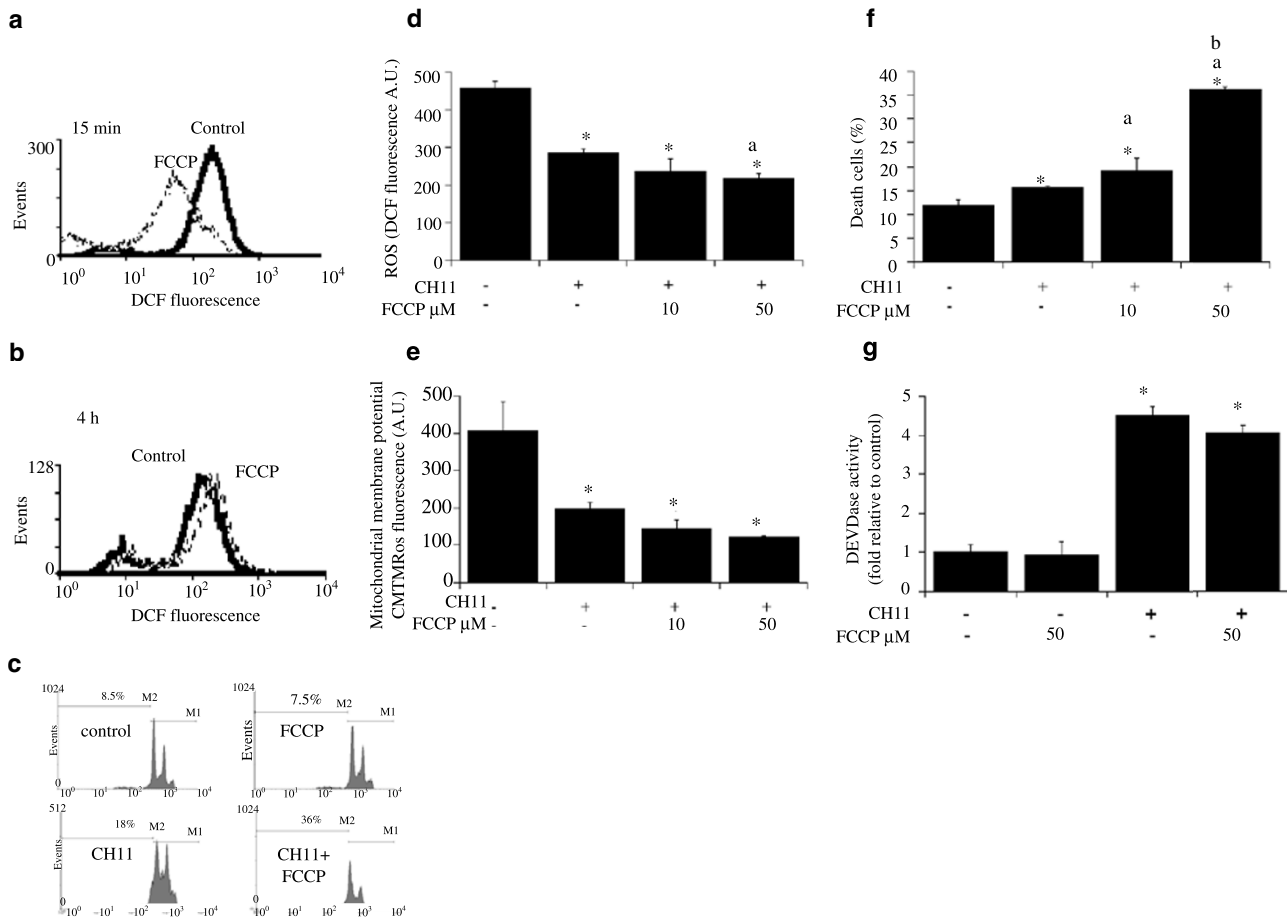
Figure 5 FCCP potentiates cell death and attenuates ROS production and mitochondrial membrane potential in apoptotic Jurkat cells. Cells were treated with FCCP. (a) Treatment with FCCP alone for 15 min induced a reduction in mitochondria-derived ROS compared to untreated control, indicating that most of cellular ROS are derived from the mitochondria. (b) Treatment with FCCP for 4 h caused an increase in mitochondrial ROS production owing to adaptation (as compared to 15 min treatment) as a result of compensation mechanism of the cell. (c) Combination of 50 μM FCCP+CH11 exacerbated apoptosis as evaluated by the level of DNA fragmentation in Jurkat cells. Treatment was for 4 h. (d) ROS production was measured by flow cytometry using DCFH-DA. Four hour of treatment with CH11 and CH11 together with FCCP reduced ROS production compared to control. $^*P < 0.05$, difference compared to untreated control. $^aP < 0.05$, difference compared to CH11-treated cells. (e) Membrane potential was measured by flow cytometry using CMTMRos fluorescence as membrane potential sensitive probe. Reduction in membrane potential was observed in the cells treated with CH11 or CH11 together with FCCP for 4 h. $^*P < 0.05$, difference compared to untreated control. $^aP < 0.05$, difference compared to CH11-treated cells. (f) Measurement of cell viability by flow cytometry using PI demonstrated increased death of the cells treated with CH11 or CH11 and FCCP. The increase in FCCP concentration caused an increase in cell death rate. $^*P < 0.05$, difference compared to untreated control. $^aP < 0.05$, difference compared to CH11-treated cells. $^bP < 0.05$, difference compared to CH11+FCCP (10 μM) treated cells. (g) DEVDase activity in cells treated with FCCP and CH11 for 4 h. $^*P < 0.05$, difference compared to untreated control

killing in cells that do not express caspase 8 and do not activate caspase in response to oligomerization of Fas-associated protein with death domain (FADD)[25] or in the absence of active caspase.[26] In the present study, DEVDase activity and chromatin aggregation were not compromised by oligomycin/CH11 or FCCP/CH11 treatment indicating that apoptosis is occurring.

Regulation of apoptosis by ATP and ADP levels

Oligomycin has been reported to protect against BAX, staurosporine and UV-induced apoptosis but not against Fas.[27–29] 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 owing to decreased consumption and/or a decrease in proton motive force-dependent adenine nucleotide translocase matrix-ATP exchange with cytosolic-ADP.

Redox regulation of apoptosis

Direct measurement of ROS production indicated that CH11 significantly attenuates ROS production and that treatment
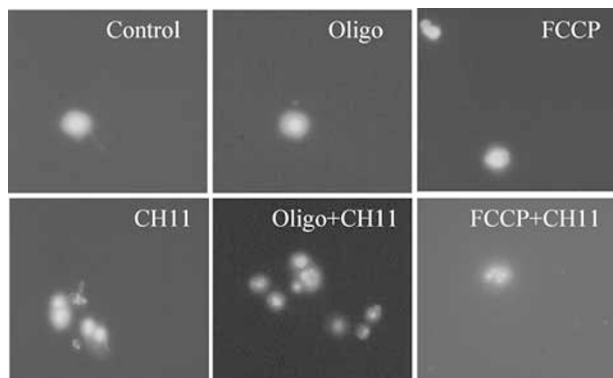
Figure 6 Visualization of chromatin changes in apoptotic cells. All treatments were for 4 h. Cells were then fixed and permeabilized with 0.1% Triton-X-100 and stained with PI (10 μM). Pictures were taken at × 40 magnification. Chromatin aggregation and fragmentation are characteristics of apoptotic cell death
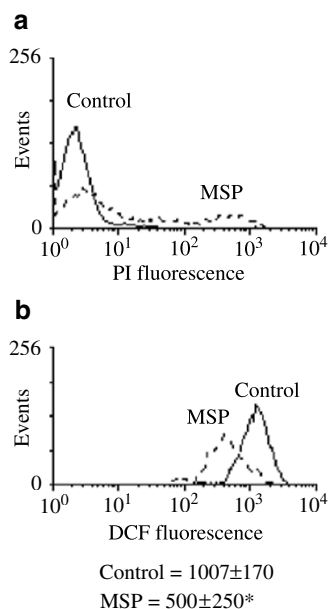

Figure 7 ROS production and cell viability in Jurkat T cells transfected with a catalase encoding plasmid. Jurkat T cells were transfected with pZeoSV/MSP-CAT (MSP), a plasmid that encodes mitochondria-targeted catalase. (**a**) Flow cytometry histogram demonstrating cell viability measured using PI 48 h after transfection. Loss of cell viability as a result of nontransfection is observed compared to untransfected cells. Data were collected from 10 000 cells. (**b**) Flow cytometry measurement of ROS production with the use of DCFH. Cells were gated or live cell population. Treatment resulted in the reduction of ROS production compared with untransfected cells. Data were collected from 10 000 cells. Total fluorescence (AU) data are presented. $^*P < 0.05$, difference compared to control

Figure 8 Distribution profile of exogenous ROS in apoptotic and nonapoptotic cells. (**a**) Fluorescence values collected by flow cytometer show that TBH was able to restore mean ROS levels in apoptotic cells compared to control untreated cells, but that level did not reach that of control TBH-treated cells. $^*P < 0.05$, difference compared to untreated control. (**b**) Histograms showing that exogenous ROS cannot compensate for the loss of endogenous ROS. Different profile of ROS distribution in apoptotic cells represented by low homogeneity compared to control cells

with oligomycin+CH11 further decreased mitochondria-derived ROS. The Fas (CD95) apoptotic pathway has not been shown to be associated with the production of ROS or the induction of oxidative stress. Furthermore, it has been reported that ROS inhibit Fas-induced cell death. The few studies indicating involvement of ROS in Fas-mediated apoptosis propose the use of SOD-mimicking compounds as protective agents.[30] However, such compounds are not only antioxidants that remove superoxide anions, but that can
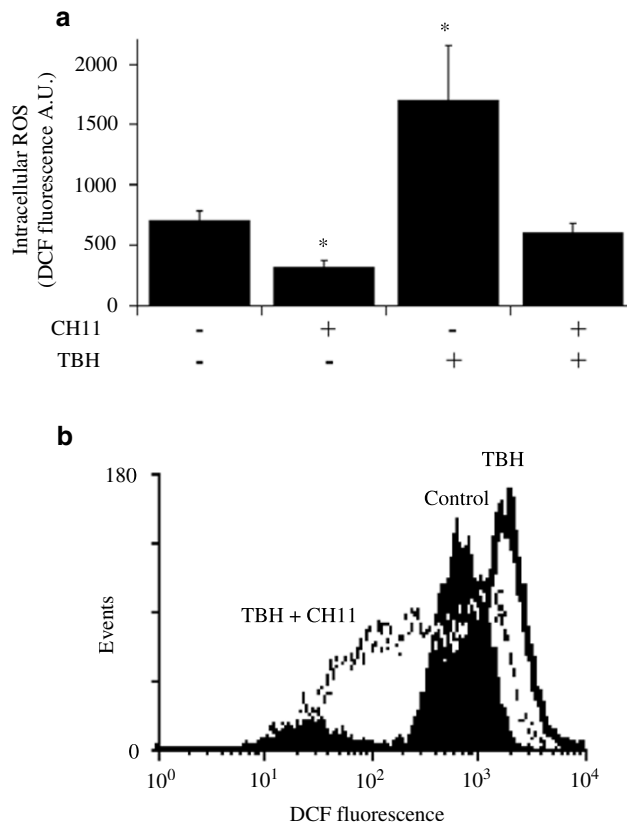
also serve as a source of H_2O_2. In fact, conventional antioxidants are virtually ineffective in controlling the death process.[31] Moreover, long preincubation with lipoic acid, a thiol lipophilic antioxidant, can stimulate the Fas death pathway.[17]

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.[18] Measurable levels of intracellular ROS are always produced in cells and also serve as signaling molecules for numerous signal transduction pathways.[16] Tumor necrosis factor receptor-associated factor (TRAF)-mediated signal transduction facilitated ROS production from the mitochondrial electron-transfer chain resulting in enhanced NF-kB activation.[32] Therefore, mitochondria-derived ROS may orchestrate the redox tone of the cell 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.
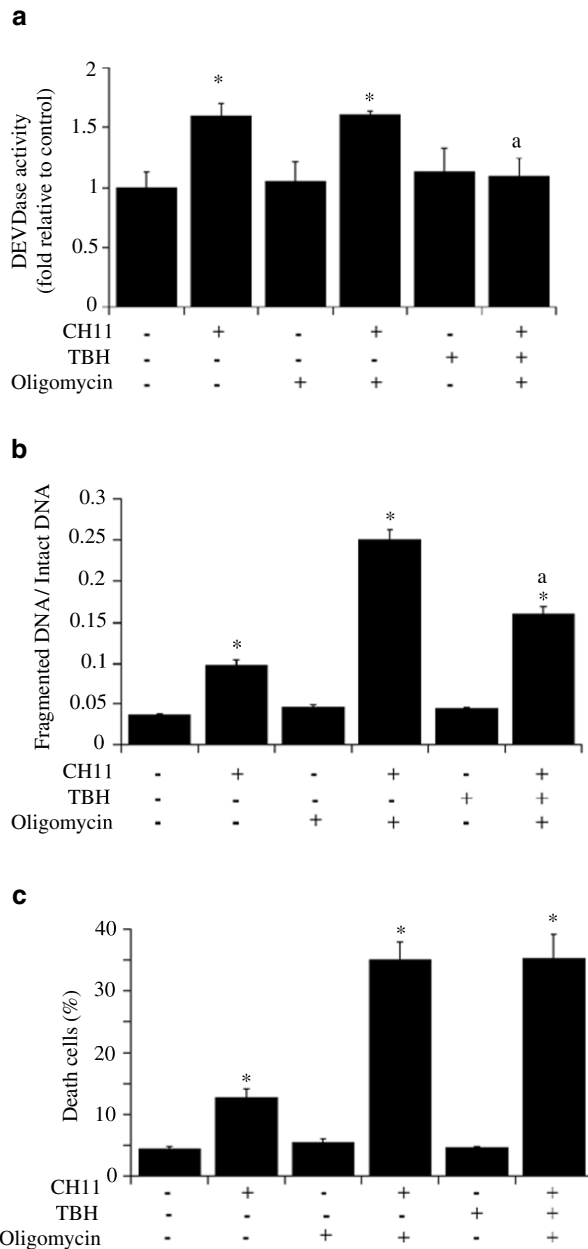
a

that ROS production is enhanced during state 4 respiration[19] was not observed in the present study using isolated mitochondria. ROS production was directly correlated with, ψ (state 4: no ATP, ΔpH 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 in apoptosis via a mechanism that does not involve direct mitochondrial damage.

Superoxide has been suggested to serve as a natural inhibitor of Fas-mediated cell death.[33] In contrast, SOD has also been shown to protect from apoptotic cell death, suggesting that superoxide facilitates and exacerbates this process.[30] Elevated SOD levels have been shown to increase the intracellular production of H_2O_2 via its dismuting activity, and may explain the apoptosis inhibitory effects of both superoxide and SOD.[15] In order to exclude the possibility that oligomycin shifts ROS production from hydrogen peroxide to superoxide, thereby enhancing cell death, apoptotic cells were treated with FCCP, a mitochondrial uncoupler. In agreement with our findings linking mitochondrial membrane potential and ROS production, FCCP attenuated ROS levels and enhanced cell death. Control cells were insensitive to the effect, most likely because of their ability to adapt and to rescue ROS production.

Transfection of Jurkat T cells with a plasmid that encodes a mitochondria-compartmentalized catalase also potentiated cell death. Mitochondrial catalase overexpression alone initiated cell death as a result of the inability to compensate for the loss of mitochondrial ROS (as measured in viable cells 48 h after transfection). CH11 did not further enhance apoptotic cell death in mitochondrial catalase overexpressing cell lines (data not shown). These data indicate that endogenous ROS levels are rate limiting in the process of receptor-mediated apoptotic cell death.

Endogenous *versus* 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 establish that dissipation of intracellular ROS is an important step in facilitating Fas receptor-mediated cell death.

b

c

Figure 9 Effect of exogenous ROS: TBH (50 μM) affects Jurkat T cells treated with CH11 and oligomycin. (**a**) Caspase-3-like activity: TBH prevented DEVDase activity in cells treated with CH11+oligomycin. DEVDase activity was measured under nonreducing conditions with no DTT. $^*P<0.05$, difference compared to untreated control. (**b**) Fragmented DNA: Level of DNA fragmentation measured by flow cytometry using PI to stain DNA was reduced by addition of TBH to cells treated with CH11 and oligomycin. $^*P<0.05$, difference compared to untreated control. $^aP<0.05$, difference compared to CH11+oligomycin-treated cells. (**c**) Membrane permeability: TBH was unable to protect cells treated with CH11 and oligomycin. Cell viability was measured by flow cytometry using PI. Data were collected from 10 000 cells. $^*P<0.05$, difference compared to untreated control

Treatment of isolated rat liver mitochondria during state 3 respiration attenuated DCF-sensitive ROS production (peroxides). The use of mitochondria in state 3 *versus* state 4 respiration revealed an attenuation of the mitochondrial membrane potential and ROS production. In apoptotic cells, rapid loss of membrane potential was also observed, accompanied by decreased ROS production. The proposal

Materials and Methods

Cell culture

Human Jurkat T cells (ATCC) were grown in RPMI medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/

ml) and 1 × glutamine at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2. When cell density reached 1 million cells/ml, the cells were exposed to different apoptotic stimuli. CH11, a Fas-activating antibody, at a concentration of 0.1 μg/ml was used to initiate the receptor death domain dependent apoptotic process.

Determination of cell viability

Cell membrane integrity was detected by flow cytometry (FACSort, BD) as a measurement of cell viability. For this assay, the nonpermeant DNA interchelating dye PI that is excluded by viable cells was used. Fluorescence setting was excitation at 488 nm and emission at 575 nm.[17,34]

DNA integrity

Cells exposed to apoptotic stimuli were centrifuged (600 × *g*, 5 min) and collected. The pellet was resuspended in a solution containing 50 μg/ml PI, 0.1% (w/v) sodium citrate and 0.1% (v/v) Triton-X-100. The permeabilized cells were kept in the dark for 2 h at 4°C and their DNA integrity was analyzed using a flow cytometer. An argon-ion laser was used for excitation at 488 nm and the emission was recorded at 575 nm. Data were collected from at least 10,000 cells.[7]

Caspase 3-like (DEVDase) activity

Cell pellets were lysed in PBS containing 0.2% Triton X-100 on ice for 10 min. The cell lysates were centrifuged at 10000 × *g* for 5 min. The clear supernatant was collected and placed on ice. Cell extracts (50 μg/ml protein) were incubated with 60 μM fluorogenic caspase-3 substrate (Ac-DEVD-AMC, Calbiochem, La Jolla, CA, USA) in incubation buffer (PBS) with or without 5 mM dithiothreitol (DTT). Fluorescence was recorded following 30 min of incubation at ambient temperature in the dark using a microfluorometer plate reader (GENios, TECAN, AU), at 360 nm excitation and 460 nm emission.[17]

Cytochrome *c* released assay

Mitochondrial intermembrane space release of cytochrome *c* into the cytosol has been evaluated following induction of an apoptotic stimulus as described previously.[35]

Digitonin-permeabilized cells

Jurkat T cells (10^6/ml) were permeabilized with digitonin (0.1% v/v) in a medium that contained 100 mM KCl, 5 mM KPi, 5 mM EDTA, 20 mM HEPES, 1 μM Rotenone and 1 μg/ml oligomycin. Permeabilized cells were centrifuged at 3000 rpm, washed and treated with acid for ATP/ADP extraction.

Intracellular ATP and ADP

Jurkat cells were washed with ice-cold PBS and treated with 4% (w/v) phosphoric acid. All samples were immediately frozen in liquid nitrogen and stored at −80°C, until HPLC analysis. Immediately before the assay, samples were thawed, vortexed and then centrifuged at 15 000 × *g* for 5 min. The clear supernatant was removed and injected into the HPLC system. UV detection of intracellular ATP and ADP was performed using an ESA (Chelmsford, MA, USA) UV detector at 260 nm and a stationary phase of C-18 cyano (GL Science). The mobile phase consisted of

phosphate buffer pH 2.7, 50 mM+4 mM tetrabutylammonium hydrogen sulfate 95%, and 5% methanol. The flow rate was 1 ml/min.

Intracellular ROS

Intracellular ROS were detected using H_2DCF-DA.[7] After the different treatments, the cells were washed three times with PBS. Cells were centrifuged (600 × *g*, 5 min) and resuspended in PBS and incubated with dichorodihydrofluoresceindia cetate (H_2DCF-DA) (25 μM) for 30 min at 37°C. To detect intracellular fluorescence the fluorochrome-loaded cells were excited using a 488 nm argon-ion laser in a flow cytometer (FACSort, BD). The dichlorofluorescein (DCF) emission was recorded at 530 nm. Data were collected from at least 10 000 cells.

Isolation of rat liver mitochondria

After overnight fasting, the animals were anesthetized and killed by decapitation. Livers were removed with scissors and immediately immersed in ice-cold 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.35 (MSH buffer) containing 1 mM EDTA. Livers were freed of fat and connective tissue, cut to pieces with scissors and homogenized in 60 ml MSH/EDTA buffer per liver using a glass homogenizer with a Teflon pestle. Mitochondria were isolated by conventional differential centrifugation:[36] The homogenate was centrifuged for 10 min at 1 000 × *g* at 4°C to sediment unbroken cells, cell debris and nuclei. The supernatant was centrifuged for 10 min at 10 000 × *g* at 4°C to give the first mitochondrial pellet. All manipulations of the mitochondria outside of the centrifuge were performed at 4°C. The fat layer floating on top of the supernatant was removed with a tissue and the supernatant was carefully decanted. The pellet was suspended in 80 ml of MSH buffer per liver using a cold pestle and given another low spin 1000 × *g* at 4°C for 5 min.The supernatant was centrifuged for 10 min at 10 000 rpm at 4°C, the pellet resuspended in MSH buffer and the last centrifugation step was repeated. The final mitochondrial pellet was suspended in about 1 ml of MSH buffer to give a final concentration of 80–100 mg mitochondrial protein/ml. Mitochondria were stored on ice and used within 3–6 h after isolation. The protein content of the final mitochondrial suspension was determined by the Bradford method using bovine serum albumin as the standard.

Standard incubation procedure

Isolated mitochondria (0.5–1 mg protein/ml) were incubated at ambient temperature in a buffer consisting of 210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4. Mitochondria were energized with 5 mM succinate as the respiration substrate (state 4 respiration). For state 3 respiration, a different incubation medium was prepared as follows, 200 mM sucrose, 20 mM potassium chloride , 3 mM potassium monophosphate, 5 mM Tris base and 6 mM magnesium chloride. ADP 80 μM was used to initiate state 3 respiration in energized mitochondria.

Mitochondrial ROS (hydrogen peroxide) measurements

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[21] to produce a constant flux of hydrogen peroxide. Intramitochondrial peroxides were detected using H_2DCF-DA.[37] The mitochondria were resuspended and incubated with 25 μM H_2DCF-DA for 10 min at room temperature for the detection of intramitochondrial fluorescence. The

fluorochrome-loaded mitochondria were excited using a 488-nm argon-ion laser in a flow cytometer. DCF emission was recorded at 530 nm. Data were collected from at least 50 000 mitochondria.

Determination of mitochondrial membrane potential

Mitochondrial membrane potential was detected by flow cytometry. For this assay, the membrane potential sensitive fluorescent probe MitoTracker Orange CMTMRos was used at the following fluorescence settings: excitation at 488 nm and emission at 575 nm (FL2 channel). Isolated mitochondria (1 mg) were stained with $0.5\,\mu$M CMTMRos, and samples were exposed to various treatments as described in the figure legends and analyzed for membrane potential. Under the experimental conditions used in this article, MitoTracker Orange CMTMRos did not induce any significant mitochondrial high-amplitude swelling (not shown). The probe was found to be highly sensitive to changes in the energy status of the mitochondria.[37]

Evaluation of mitochondrial membrane potential in cells

Jurkat T cells (1 million cells/ml) were stained with $0.1\,\mu$M CMTMRos for 30 min at 37°C. Accumulation of the dye in the mitochondria was evaluated by flow cytometer analysis as already described. Data were collected from 10 000 cells.

Micrographs pictures of the cells

To determine the type of cell death (necrotic or apoptotic), control cells and cells treated with oligomycin or FCCP and CH11 alone or in combination were rinsed twice in PBS fixed with 1% PFA for 30 min on ice and exposed to the DNA-binding fluorochrome PI (10 μM) dissolved in PBS containing 0.1% Triton-X-100. Emitted red fluorescence for PI was registered in a model U-VPT-P (Olympus Optical Co. Ltd., Japan) fluorescence microscope using a blue/green/red triple-pass filter. The results were documented using an optical camera.

Cell transfection

Before transfection, 10^6 Jurkat T cells were seeded. The expression plasmid vector pZeoSV2(+) containing human catalase cDNA with an MnSOD mitochondrial leader sequence (pZeoSV/MSP-CAT) as previously described by Bai et al.[38] was transfected into Jurkat cells using the X-tremeGENE Q2 transfection reagent (Roche Diagnostics) according to the instructions provided by the manufacturer. Control cells and those exposed to CH11 treatment were analyzed for their capacity to generate ROS and for cell viability 48 h after transfection.

Statistics

Data are expressed as mean $\pm$ S.D. of at least three experiments. Two-way ANOVA was used in multivariable analyses. Differences were considered significant at probability levels of $P < 0.05$ using the Fisher's protected least significant difference method.

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