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Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis

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During apoptosis, the permeabilization of the mitochondrial outer membrane allows the release of cytochrome c, which induces caspase activation to orchestrate the death of the cell. Mitochondria rapidly lose their transmembrane potential ($\Delta\Psi$m) and generate reactive oxygen species (ROS), both of which are likely to contribute to the dismantling of the cell. Here we show that both the rapid loss of $\Delta\Psi$m and the generation of ROS are due to the effects of activated caspases on mitochondrial electron transport complexes I and II. Caspase-3 disrupts oxygen consumption induced by complex I and II substrates but not that induced by electron transfer to complex IV. Similarly, $\Delta\Psi$m generated in the presence of complex I or II substrates is disrupted by caspase-3, and ROS are produced. Complex III activity measured by cytochrome c reduction remains intact after caspase-3 treatment. In apoptotic cells, electron transport and oxygen consumption that depends on complex I or II was disrupted in a caspase-dependent manner. Our results indicate that after cytochrome c release the activation of caspases feeds back on the permeabilized mitochondria to damage mitochondrial function (loss of $\Delta\Psi$m) and generate ROS through effects of caspases on complex I and II in the electron transport chain.

Introduction

The activation of caspase proteases is fundamental to apoptotic cell death, although their mode of action in promoting death is not fully understood. In vertebrate cells, inhibition of caspases does not necessarily prevent cell death but profoundly delays and alters the process (Xiang et al., 1996; McCarthy et al., 1997; Amarante-Mendes et al., 1998). Activated caspases, especially caspase-3, orchestrate DNA fragmentation (Enari et al., 1998), nuclear condensation (Sahara et al., 1999), and membrane blebbing (Coleman et al., 2001; Sebbagh et al., 2001) through cleavage of specific substrates, but these are unlikely to fully account for the rapidity of caspase-dependent death once the proteases are activated. Here we will explore the impact of caspases on another critical compartment in dying cells, the mitochondria.

The central role of mitochondria in the process of apoptosis has been a focus of cell death research since the observations that the antiapoptotic Bcl-2 protein localizes to the outer membrane of this organelle (Nguyen et al., 1993), a mitochondria-rich fraction was required for the induction of apoptotic changes in a cell-free system (Newmeyer et al., 1994), and mitochondrial transmembrane potential ($\Delta\Psi$m)* is lost during an early stage of apoptosis (Zamzami et al., 1995). Over the past several years, it has become clear that a major event during apoptosis is the permeabilization of the mitochondrial outer membrane to release proteins from the intermembrane space (Waterhouse et al., 2002). Several of these, including cytochrome c, AIF, Smac/DIABLO, Omi/Htra2, and EndoG, have roles in subsequent cell death (Susin et al., 1999b; Du et al., 2000; Verhagen et al., 2000; Li et al., 2001; Suzuki et al., 2001). In particular, the release of cytochrome c induces the activation of caspase proteases through the induction of apoptosome formation (Li et al., 1997).

Mitochondrial functions including protein import, ATP generation, and lipid biogenesis depend on the maintenance of $\Delta\Psi$m (Voisine et al., 1999), and loss of $\Delta\Psi$m during apoptosis is likely to contribute to the death of the

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Key words: apoptosis; mitochondria; caspases; transmembrane potential; ROS

*Abbreviations used in this paper: $\Delta\Psi$m, mitochondrial transmembrane potential; ActD, actinomycin D; 2-HE, dihydroethidium; KCN, potassium cyanide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; PI, propidium iodide; ROS, reactive oxygen species; tBid, truncated Bid; TMRE, tetramethylrhodamine ethyl ester; TMPD, tetrametyl-*p*-phenylenediamine; zVAD-fmk, *N*-benzoylcarbanyl-Val-Ala-Asp-fluoro methylketone.

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cell through loss of these functions. In addition, mitochondrial production of reactive oxygen species (ROS) also appears to play a role in cell death (Tan et al., 1998). The relationships between these events, release of mitochondrial proteins, and caspase activation remain controversial. Although models of mitochondrial function during apoptosis often predict hypo- or hyperpolarization of the inner membrane before outer membrane permeabilization (Gottlieb et al., 2000; Martinou and Green, 2001), we have found that in the absence of caspase activation $\Delta\Psi$m does not necessarily change before and remains intact after this event (Waterhouse et al., 2001b). Single cell analysis in HeLa and other cells provided evidence that a persistent loss of $\Delta\Psi$m rapidly follows cytochrome c release only when caspases are activated, and otherwise this loss follows a variable (and slow) kinetics. The maintenance of $\Delta\Psi$m under these conditions appears to be via electron transport supported by the cytochrome c diffusely available in the cytosols of the cells that had undergone mitochondrial outer membrane permeabilization. In HeLa cells, loss of $\Delta\Psi$m corresponds to a rapid decline in ATP levels before cell death, and this is profoundly enhanced by caspase activation (Waterhouse et al., 2001b).

Here we explore the role of caspase activation in loss of $\Delta\Psi$m and generation of ROS during apoptosis. Although caspase-3 can cause permeabilization of the mitochondrial outer membrane, this is at least partially dependent on the function of the proapoptotic Bcl-2 family protein Bid and is blocked by Bcl-xL. However, the caspase then has a further effect on the mitochondria through disruption of the functions of complex I and II of the electron transport chain, resulting in loss of $\Delta\Psi$m and generation of ROS. This rapid effect of caspases on the function of the electron transport chain is therefore likely to be a major contributing factor to the process of caspase-dependent cell death.

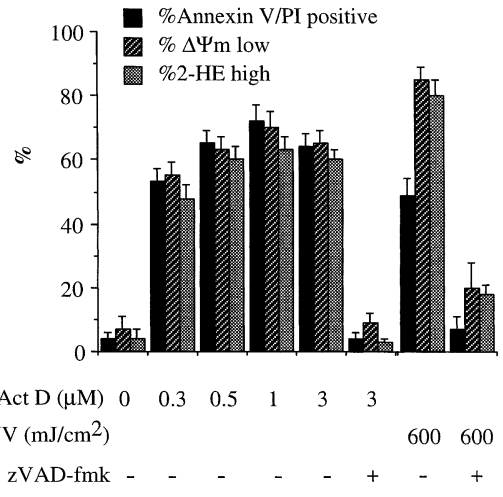


Figure 1. **Apoptosis, loss of $\Delta\Psi$m, and ROS production are caspase dependent.** HeLa cells were treated with actinomycin D or UV (as indicated) in the presence or absence of 50 μM of zVAD-fmk, harvested, and stained with annexin V–FITC and PI to assess cell death, TMRE to measure $\Delta\Psi$m, and 2-HE to measure ROS production, and then analyzed by flow cytometry.

Results

To examine the role of caspases in mitochondrial functions during apoptosis, we first examined the effects of their inhibition on two important parameters, $\Delta\Psi$m (using tetramethylrhodamine ethyl ester [TMRE] [Farkas et al., 1989]) and the generation of ROS (using dihydroethidium [2-HE] [Heibein et al., 1999]). HeLa cells were treated with actinomycin D (ActD) or UV to induce apoptosis, which was assessed by annexin V–FITC/propidium iodide (PI) staining (Fig. 1). Cell death, loss of $\Delta\Psi$m, and production of ROS showed a close correspondence in each case. Addition of the caspase inhibitor *N*-benzoylcarbanyl-Val-Ala-Asp-fluoro methylketone (zVAD-fmk) delayed both cell death and loss of $\Delta\Psi$m as observed previously (Waterhouse et al., 2001b). Interestingly, inhibition of caspase activation also blocked ROS production. Therefore, caspase functions appeared to be required for the rapid onset of these events during apoptosis.

During apoptosis, the mitochondrial outer membrane becomes permeable due to the action of pro-apoptotic Bcl-2 family proteins. Using isolated mitochondria, we asked whether such outer membrane permeabilization, with or without caspase activity, is sufficient to account for the loss of $\Delta\Psi$m in mitochondria during apoptosis. $\Delta\Psi$m was examined by uptake of TMRE (Fig. 2). The uncoupler, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP), caused a dissipation of $\Delta\Psi$m as expected. To induce outer membrane permeabilization as it occurs during apoptosis, we treated the mitochondria with the activated BH3-only protein, truncated Bid (tBid) (caspase-free; see Materials and methods), which caused a rapid release of cytochrome c (Fig.

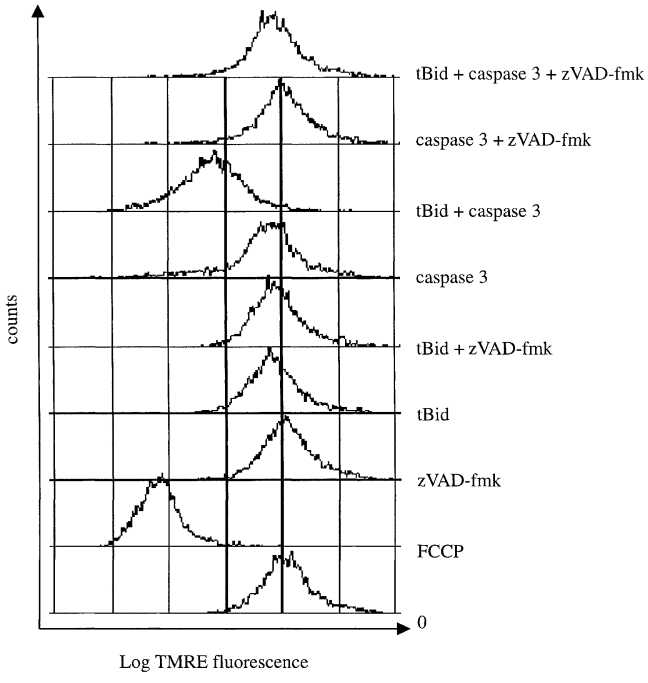


Figure 2. **Caspase-3 induces loss of $\Delta\Psi$m in isolated, tBid-treated mitochondria.** Mouse liver mitochondria (20 μg) were incubated with FCCP (10 μM), tBid (20 μg/ml), cytochrome c (100 μM), caspase-3 (0.25 μg/ml), and/or zVAD-fmk (100 μM) as indicated, then stained with TMRE, and analyzed by flow cytometry. Low fluorescence indicates a loss of $\Delta\Psi$m.

3 B, inset) but did not disrupt $\Delta\Psi$m (Fig. 2). This effect required that the mitochondria be maintained at high density (as in the experiment shown), since dilution of the treated organelles caused a loss of $\Delta\Psi$m as the cytochrome c is diluted (Waterhouse et al., 2001b). Although apoptosis in most cases does not depend on the action of tBid (Yin et al., 1999), we employed this protein as a model, since it is likely that the mitochondrial permeabilization induced by tBid is similar to that occurring during many forms of apoptosis (Korsmeyer et al., 2000). Treatment of the isolated mitochondria with caspase-3 also failed to affect $\Delta\Psi$m (Fig. 2). However, addition of both tBid and caspase-3 caused a loss of $\Delta\Psi$m, and this effect was dependent on the caspase activity as zVAD-fmk inhibited the effect. Since tBid induces the permeabilization of the mitochondrial outer membrane (Kuwana et al., 2002), it is therefore likely that it acts to permit caspase-3 access to the intermembrane space, necessary for the protease to affect $\Delta\Psi$m. Although the mechanism is unknown, tBid can cause the outer mitochondrial membrane to become permeable to dextrans of higher molecular weight than that of active caspase-3 (Kuwana et al., 2002).

Mitochondrial $\Delta\Psi$m is generated by the components of the electron transport chain, which consume O_2 and pump protons across the mitochondrial inner membrane to produce ATP (Fig. 3 A). Addition of specific substrates for each complex together with inhibitors for upstream complexes can drive respiration that can be detected using an oxygen (Clarke) electrode. As shown in Fig. 3 B, untreated and tBid-treated mitochondria consumed oxygen upon addition of substrates for complex I (malate/o-palmitoyl-L-carnitine), complex II (succinate), or cytochrome c (tetrametyl-*p*-phenylenediamine [TMPD]/ascorbate; complex IV then consumes oxygen), and this respiration was stopped by the appropriate inhibitors in each case. Nevertheless, tBid permeabilized the mitochondrial outer membrane, seen as a loss of pellet-associated cytochrome c but not matrix HSP60 (Fig. 3 B, inset). Similarly, untreated and caspase-3–treated mitochondria displayed similar oxygen uptake upon stimulation (Fig. 3 C). In each of these experiments (Figs. 3, B–E), the respiration was measured in the same mitochondria under two conditions in parallel (see Materials and methods), and therefore the pair in each figure are directly comparable (in contrast, differences in the mitochondrial preparations produced some differences in the extent of respiration between separate experiments, although the control patterns were qualitatively comparable).

In contrast, mitochondria treated with tBid plus caspase-3 consumed no oxygen in response to complex I substrates, a difference that was more pronounced in the presence of ADP (Fig. 3 D). Similarly, tBid plus caspase-3–treated mitochondria showed no increase in oxygen consumption in response to the complex II substrate succinate. Caspase-3 treatment resulted in an 88% inhibition of complex I function and a 94% inhibition of the oxygen consumption by complex II. In contrast, respiration via complex IV was similar in tBid-treated mitochondria with or without caspase-3 treatment.

The effect of caspase-3 on mitochondrial respiration was not dependent on the use of tBid. Treatment of mitochondria with calcium induces a permeability transition that causes the matrix to swell and ultimately rupture the mitochondrial outer membrane (Zamzami et al., 1996), and this was seen as a decrease in pellet-associated cytochrome c but not matrix HSP60 (Fig. 3 E, inset). It has been suggested that this effect occurs during apoptosis to result in loss of $\Delta\Psi$m and release of intermembrane proteins from mitochondria (Bernardi et al., 1998). Unlike tBid, disruption of the mitochondrial outer membrane by calcium treatment had some inhibitory effect on respiration, particularly complex I function. This effect of calcium on complex I has been described (Fontaine et al., 1998). Nevertheless, treatment of calcium-permeabilized mitochondria with caspase-3 caused a loss of complex I and II activity (Fig. 3 E). The simplest interpretation of these results is that caspase-3 enters permeabilized mitochondria and then acts to disrupt respiration by targeting proteins that are exposed to the intermembrane space.

Although we observed intact complex IV activity after caspase treatment of mitochondria, the reduction in oxygen consumption in response to substrates for complex I or II might nevertheless be due to a loss of function of complex III (Fig. 3 A). Therefore, to assess complex III function we examined the ability of accessible complex III to reduce cytochrome c (Kluck et al., 1999). As shown in Fig. 4, intact mitochondria did not reduce exogenously added cytochrome c, whereas tBid-treated mitochondria did (Kluck et al., 1999). This effect was dependent on complex III activity, since the inhibitor antimycin A blocked cytochrome c reduction in this system. Permeabilized mitochondria (by tBid or by hypotonic lysis) treated with caspase-3 displayed full complex III activity in this assay, and thus the function of complex III (at least that of cytochrome c reduction) was not damaged by caspase-3. These results support the idea that caspases damage the function of complexes I and II without affecting those of complex III or complex IV.

Caspase-3 can cleave and activate Bid and in this manner trigger mitochondrial outer membrane permeabilization (Li et al., 1998), and it was therefore likely that treatment of digitonin-treated cells with caspase-3 would induce cytochrome c release. Therefore, we compared $Bid^{+/+}$ and $Bid^{-/-}$ cells to further assess the possible role of tBid in the dissipation of $\Delta\Psi$m induced by caspase-3. We observed previously that activated Bid (tBid) can induce loss of $\Delta\Psi$m in digitonin-permeabilized cells, and this was restored by addition of exogenous cytochrome c (Waterhouse et al., 2001b) consistent with the lack of effect of tBid on electron transport function we have observed here (Fig. 3 B). In the experiment shown in Fig. 5 A, caspase-3 induced a loss of $\Delta\Psi$m but only in cells containing Bid; $Bid^{-/-}$ cells did not show a drop in $\Delta\Psi$m in response to caspase-3 treatment. This is consistent with our observations that caspase-3 did not act directly on isolated mitochondria to disrupt $\Delta\Psi$m (Fig. 2) or respiration (Fig. 3). Addition of tBid plus caspase-3 induced a loss of $\Delta\Psi$m in both wild-type and $Bid^{-/-}$ cells, which was not restored by addition of cytochrome c. Thus, caspase-3 appeared to have two effects: activation of Bid to permeabilize the outer membrane without disrupting $\Delta\Psi$m, as described (Waterhouse et al., 2001b), and an action on the permeabilized mitochondria to disrupt $\Delta\Psi$m.

To extend these findings, we examined $\Delta\Psi$m in digitonin-permeabilized cells treated with caspase-3 as in Fig. 5 A.

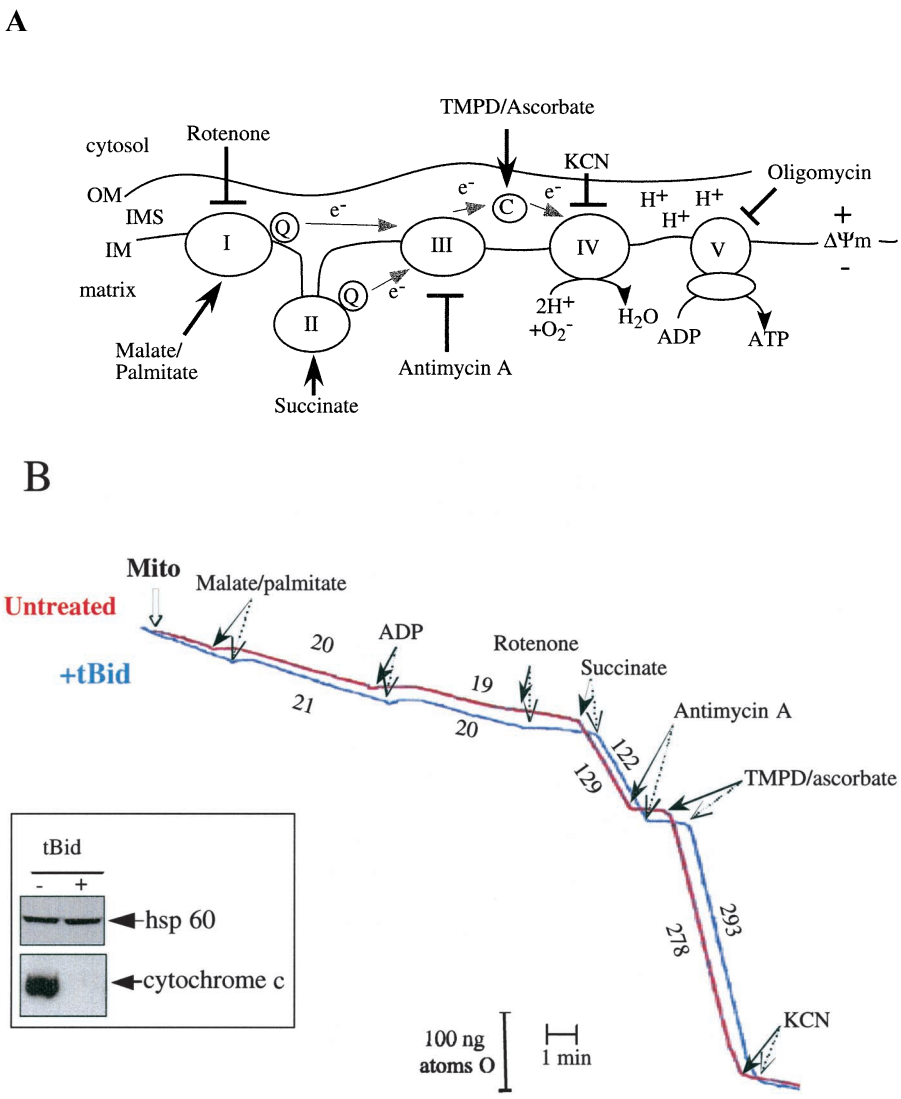
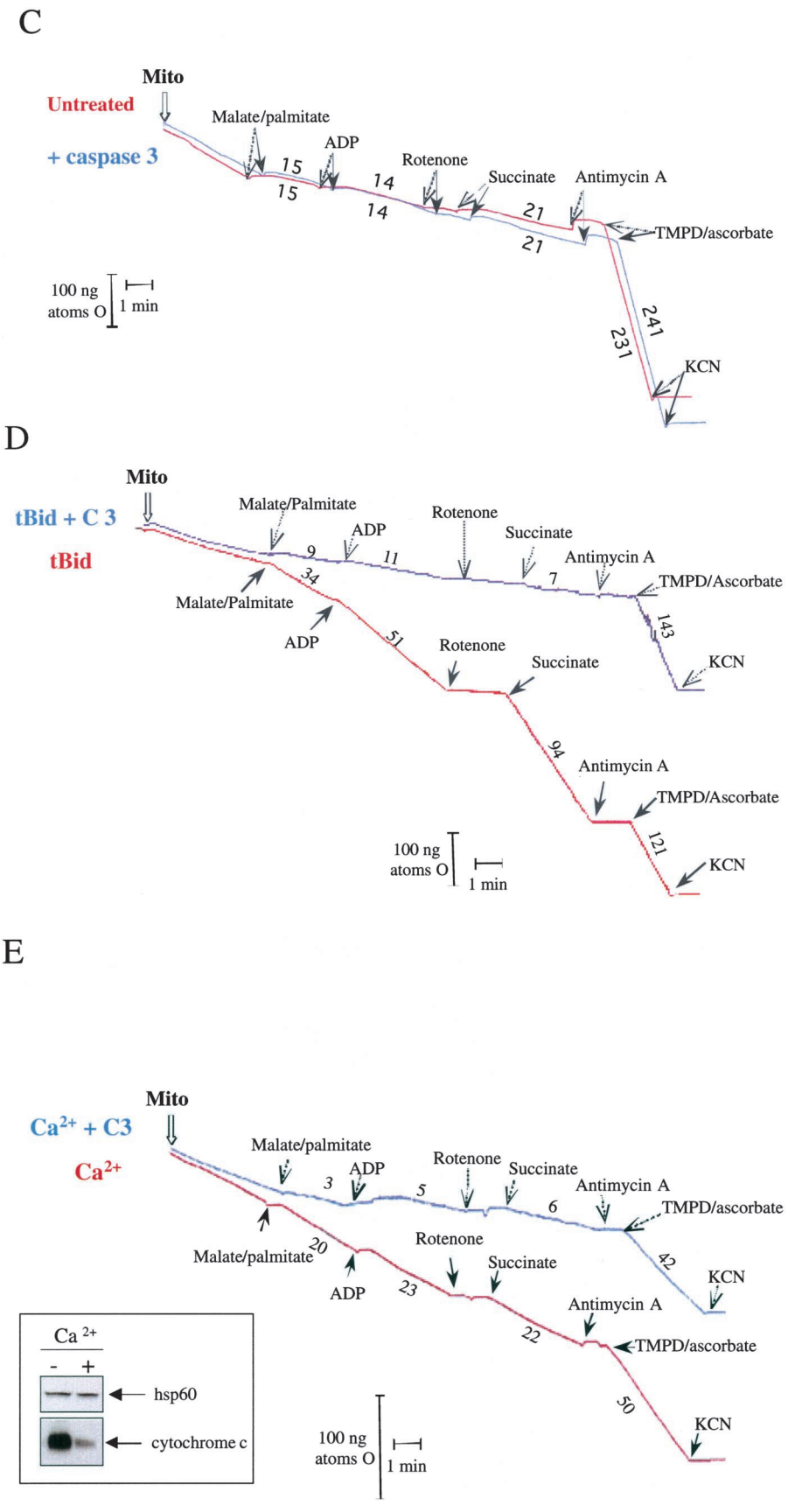


Figure 3. **Caspase-3 inhibits oxygen uptake in response to substrates for complex I or II.** (A) Simplified scheme of the respiratory chain. Briefly, the electron transport chain is composed of four multisubunit complexes referred to as complexes I–IV. The electrons enter the system through either complex I (NADH-ubiquinol oxidoreductase) or complex II (Succinate-ubiquinol dehydrogenase). The electrons are passed to complex III via ubiquinone (Q). Complex III (ubiquinol/cytochrome c oxido-reductase) transfers electrons to cytochrome c (C), which in turn donates them to complex IV (cytochrome oxidase). Complex IV will finally transfer 4 electrons to a molecular oxygen leading to the generation of H_2O. Upon this transfer of electrons, complexes I, III, and IV pump protons out of the matrix leading to the generation of $\Delta\Psi m$. Complex V (F_0F_1-ATP synthase) uses this potential to convert ADP into ATP. OM, outer membrane; IM, inner membrane; IMS, intermembrane space. Complex substrates: malate/palmitate for complex I, succinate for complex II, TMPD/ascorbate for cytochrome c. Several respiratory inhibitors used in our studies are indicated: rotenone (complex I inhibitor), antimycin A (complex III inhibitor), KCN (complex IV inhibitor), and oligomycin (complex V inhibitor). (B–E) Mouse liver mitochondria (400 μg) were incubated in dual oxygen electrode chambers in the presence of 100 μM of cytochrome c, with in control or tBid (20 μg/ml) (B), in control or caspase-3 (20 μg/ml) (C), in tBid (20 μg/ml) with or without caspase-3 (20 μg/ml) (D), and in 100 μM Ca^{2+} with or without caspase-3 (20 μg/ml) (E).

However, in this case electron transport was driven by substrate/inhibitor combinations to assess the contribution to $\Delta\Psi m$ of each complex (Fig. 5 B). Complexes I, III, and IV pump protons to generate $\Delta\Psi m$ upon electron transport, and under our conditions each complex contributes to this potential directly (complex I and IV) and/or through downstream electron transport events (complex I and II) (Fig. 3 A). As shown in Fig. 5, C–E, caspase-3 destroyed the contribution to $\Delta\Psi m$ from complex I (Fig. 5 C) or complex II (Fig. 5 D) but had no effect on the function of complex IV (Fig. 5 E). The effect of caspase-3 on the functions of complex I and II were inhibited by the addition of zVAD-fmk or/and Bcl-xL-ΔC (Fig. 5, C and D). The effect of Bcl-xL is again consistent with an inability of caspase-3 to influence mitochondrial function without access to the intermembrane space, since Bcl-xL blocks mitochondrial membrane permeabilization by Bid and other proapoptotic Bcl-2 family members (Bossy-Wetzel and Green, 1999; Kuwana et al., 2002).

One consequence of a caspase-mediated disruption in electron transport may be the zVAD-fmk–inhibitable generation of ROS discussed above (Fig. 1). Therefore, we examined if substrates for complex I (Fig. 6 B) or complex II (Fig. 6 C) drive caspase-dependent ROS generation in digitonin-permeabilized Jurkat cells. Addition of substrates for complexes I or II fueled the production of ROS in untreated mitochondria, and this was not increased by treatment with tBid. In contrast, treatment with caspase-3 (with or without addition of recombinant tBid) resulted in significant ROS production with either substrate (Fig. 6, B and C) (but not without substrates; Fig. 6 A). The increase was inhibited by Bcl-xL-ΔC, probably via inhibition of the caspase-activated, Bid-mediated permeabilization of the mitochondrial outer membrane as discussed above. Therefore, it is likely that the caspase-mediated disruption of complex I and complex II function contributes to high ROS production during apoptosis. This would account for the effect of caspase inhibition on apoptosis-associated ROS generation we observed in the experiment in Fig. 1.

Therefore, we sought to determine if complex I and II are targeted for caspase-mediated disruption during the process of apoptosis. HeLa cells were treated with actinomycin D to induce apoptosis, with and without zVAD-fmk to block caspase activation. Cells were then treated with digitonin in order to provide substrates with access to the mitochondria. Different substrates with or without exogenous cytochrome c were added, and $\Delta\Psi m$ was assessed. As shown in Fig. 7,



Then drugs are added in the following order: malate/palmitate (2.5 mM and 40 μM, respectively), 2 mM ADP, 2 μM rotenone, 5 mM succinate 1 μM antimycin A, 0.4 mM TMPD with 1 mM ascorbate and 1 mM KCN. Values for oxygen consumption are represented on the curves as ng-atoms of oxygen/min/mg of protein. Western blots in Fig. 3, B and E, represent the amount of cytochrome c or hsp60 present in the mitochondrial pellet.

ActD-treated cells lost $\Delta\Psi$m driven by complex I, II, or IV substrates. In the absence of substrates, $\Delta\Psi$m was minimal (unpublished data) as in Fig. 5 B. Addition of cytochrome c restored complex IV activity in this system (Fig. 7 C) but not the activities of complex I or II (Fig. 7, A and B). This is consistent with our results with caspase-treated mitochon-

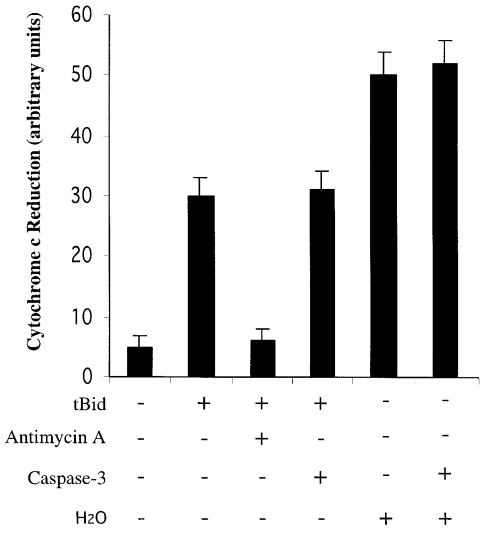


Figure 4. **Caspase-3 does not destroy the capacity of complex III to reduce cytochrome c.** Isolated mitochondria (500 μg) were incubated in the presence of tBid (25 μg/ml), caspase-3 (25 μg/ml), and/or antimycin A (1 μM) as indicated for 60 min at 37°C. As a control, 500 μg of mitochondria were incubated in water for 20 min at 4°C then incubated ± caspase-3. Reduction of exogenous cytochrome c by complex III was measured as described in Materials and methods.

dria and permeabilized cells, supporting the idea that mitochondrial outer membrane permeabilization and caspase activation during apoptosis disrupted the function of complex I and II. Consistent with this idea, inhibition of caspase activation protected $\Delta\Psi$m in each case.

One way in which $\Delta\Psi$m can be maintained in the absence of electron transport is through ATP-dependent reversal of complex V (F_0F_1-ATPase) activity (Fig. 3 A). Therefore, we examined the effects of the complex V inhibitor oligomycin under each of our conditions (Fig. 7). Although oligomycin further depressed the reduced $\Delta\Psi$m in apoptotic cells under each of our conditions, it had no effect on $\Delta\Psi$m driven by complex IV substrates plus cytochrome c. This provides further support that complex IV remains functional after caspase activation in apoptotic cells. In the absence of caspase activation, addition of oligomycin did not dissipate $\Delta\Psi$m, consistent with our previous observations (Goldstein et al., 2000; Waterhouse et al., 2001b). In addition, the observable effect of oligomycin in some cases in apoptotic cells (Fig. 7) provides circumstantial evidence that complex V activity (at least that of the F_1 component) remains intact after caspase activation; that is, ATP can drive $\Delta\Psi$m via proton pumping by the F_1-ATPase.

Oxygen consumption in apoptotic cells followed a similar pattern. Jurkat cells were treated with etoposide or stauro-

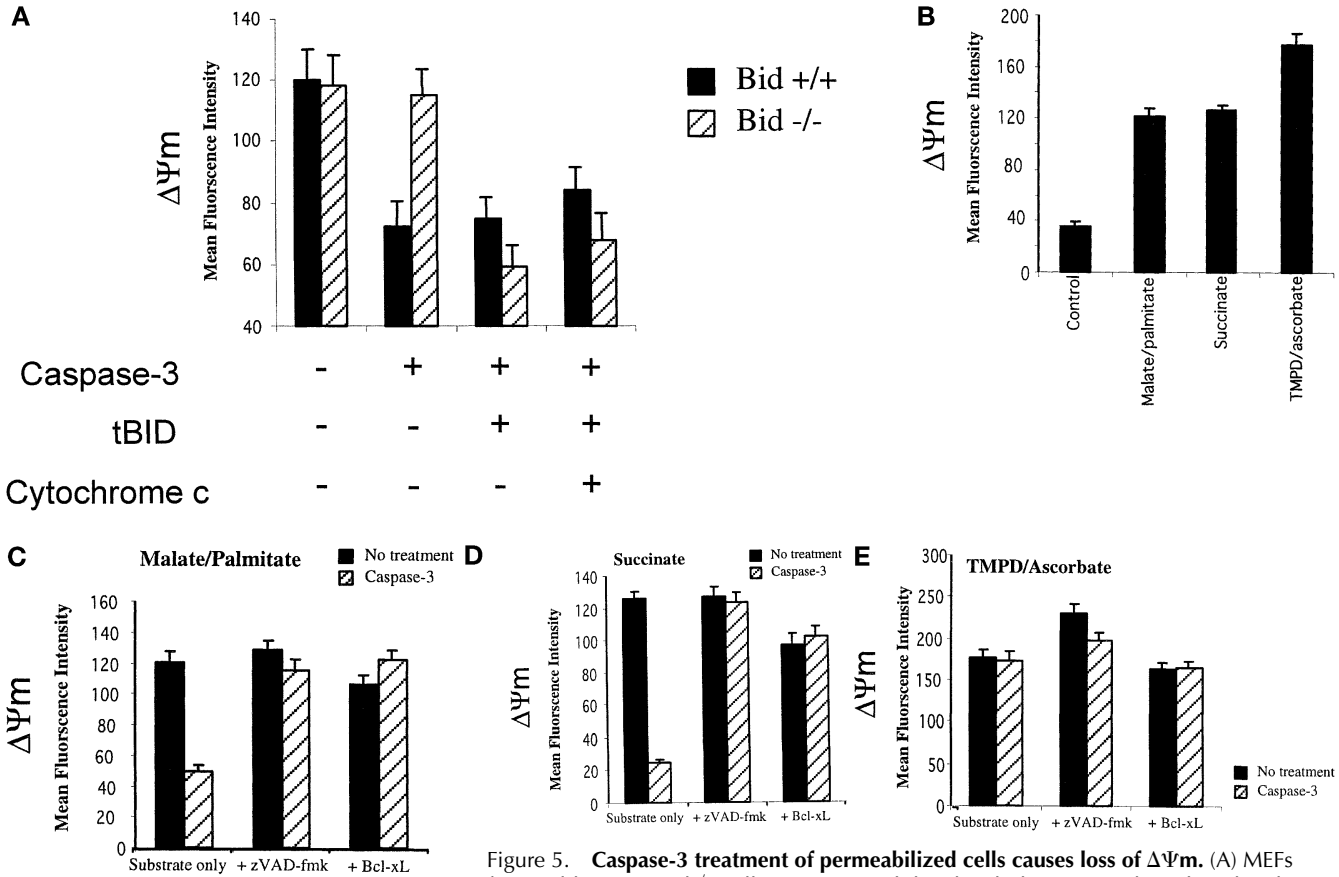


Figure 5. **Caspase-3 treatment of permeabilized cells causes loss of $\Delta\Psi$m.** (A) MEFs from wild-type or $Bid^{-/-}$ cells were permeabilized with digitonin and incubated in the presence of caspase-3 (0.5 μg/ml), tBid (20 μg/ml), and/or cytochrome c (100 μM) as indicated plus TMRE at 37°C for 30 min in the presence of succinate as substrate. (B–E) Permeabilized HeLa cells (10^6) were incubated with or without caspase-3 (0.5 μg/ml) in the presence of cytochrome c (100 μM) and TMRE with or without zVAD-fmk (100 μM) or $BclX_L$-Δc (20 μg/ml) as indicated. (B) $\Delta\Psi$m generated by the incubation of permeabilized cells with the substrates. (C) Effect of caspase-3 on $\Delta\Psi$m in the presence of malate/palmitate. (D) Effect of caspase-3 on $\Delta\Psi$m in the presence of rotenone and succinate. (E) Effect of caspase-3 on $\Delta\Psi$m in the presence of antimycin A and TMPD/ascorbate. Substrates and inhibitors were added at the concentrations described in the legend to Fig. 3 B. Cells were analyzed for $\Delta\Psi$m by flow cytometry. In each case, the MFI for cells treated with FCCP to dissipate $\Delta\Psi$m was set as 0.

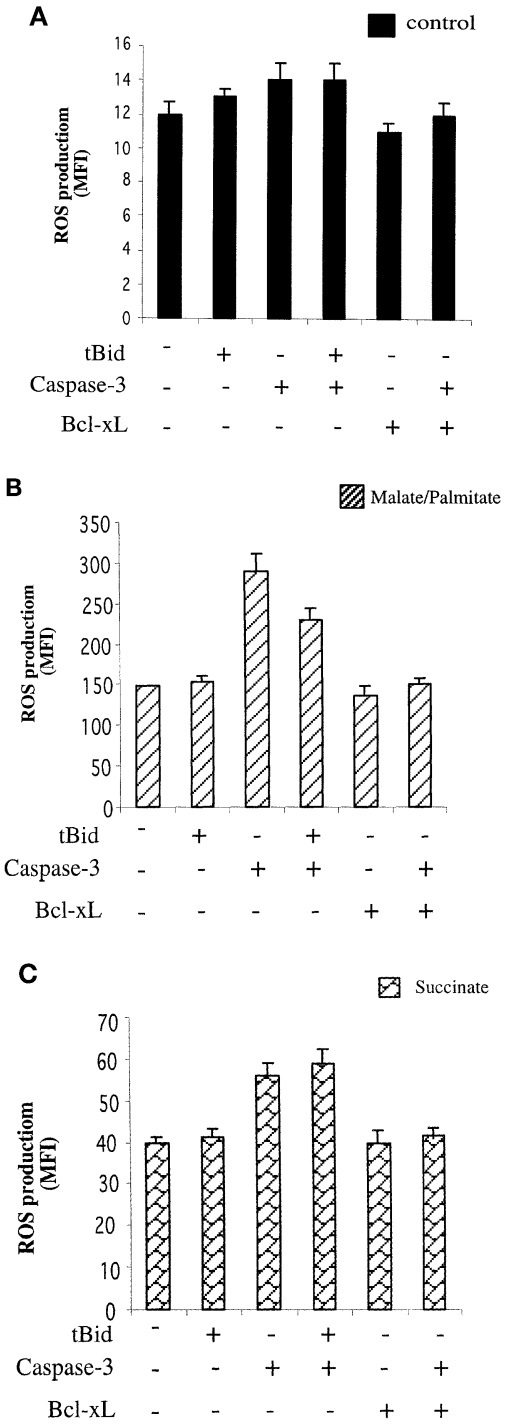


Figure 6. **ROS production in the presence of malate/palmitate or succinate is enhanced by caspase-3.** Jurkat cells (10^6) were permeabilized and incubated in the presence of dihydroethidium (2 μM) and caspase-3 (0.5 μg/ml), tBid (20 μg/ml), or Bcl-xL-ΔC (20 μg/ml) for 30 min at 37°C. (A) Control; (B) in the presence of malate/palmitate, and (C) in the presence of succinate. Then the ROS production was monitored by flow cytometry. Note that the scales differ for each condition.

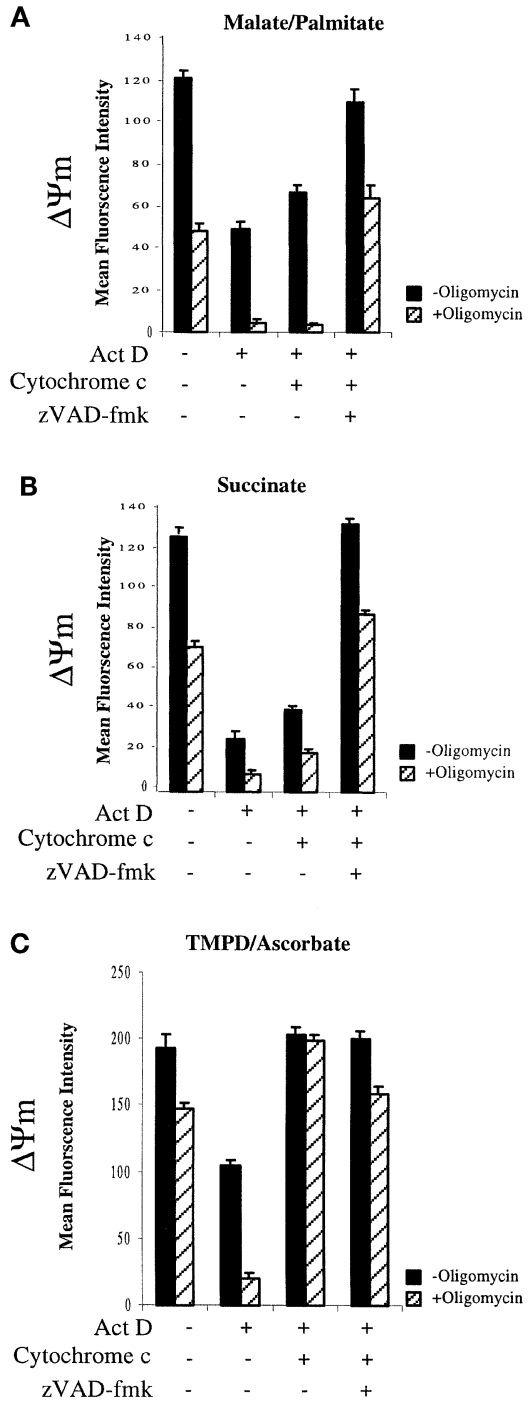


Figure 7. **Apoptotic cells maintain ΔΨm in the presence of TMPD/ascorbate but not in the presence of malate/palmitate or succinate as substrates.** HeLa cells were treated with or without 0.3 μM actinomycin D (18 h), permeabilized with digitonin, and incubated with the indicated substrates. Oligomycin (10 μg/ml) was added as indicated. Cells were stained with TMRE and analyzed by flow cytometry. (A) Malate/palmitate; (B) rotenone and succinate; (C) antimycin A and TMPD/ascorbate. Concentrations for inhibitors and substrates were the same as for the experiment in Fig. 3 B. Cells were analyzed for ΔΨm by flow cytometry. In each case, the MFI for cells treated with FCCP to dissipate ΔΨm was set as 0.

sporine to induce apoptosis. The cells were then digitonin-permeabilized to provide access of substrates to the mitochondria. We found that oxygen consumption in the presence of complex I or complex II substrates was destroyed by the apoptotic process (Fig. 8 A). This effect was caspase dependent, as it was blocked by the caspase inhibitor zVAD-fmk. In contrast, oxygen consumption by complex IV remained largely intact after caspase activation. However,

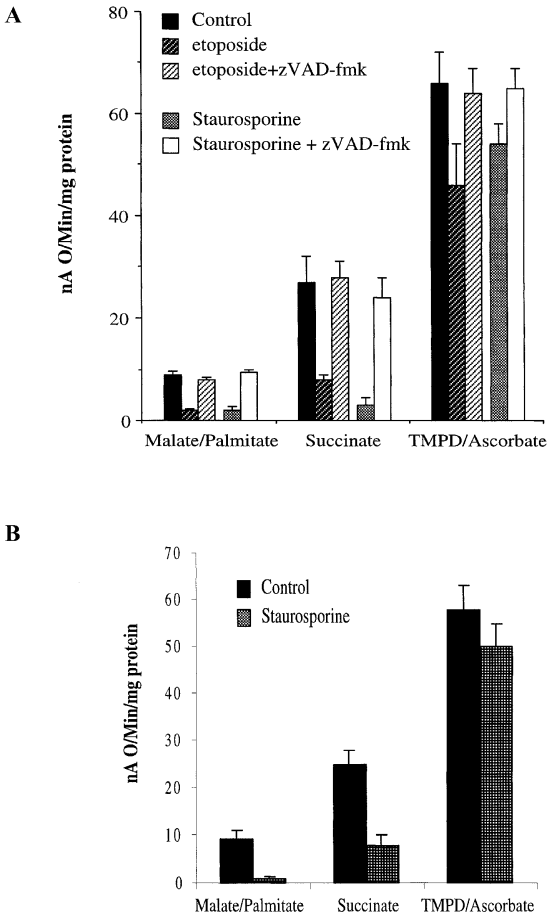


Figure 8. **Oxygen consumption in apoptotic cells.** (A) Jurkat cells were treated for 18 h with etoposide (10 μM) or staurosporine (0.05 μM) in the presence or absence of 100 μM of zVAD-fmk as indicated. The cells were then permeabilized with digitonin. The equivalent of 400 μg of protein was loaded into respiratory chambers and oxygen consumption in the presence of different substrates and inhibitors (malate/palmitate for complex I, rotenone with succinate for complex II, antimycin A with TMPD/ascorbate for cytochrome c/complex IV) was measured as in the legend to Fig. 3 B. (B) HeLa cells were treated with staurosporine (0.05 μM) for 18 h, then treated with digitonin, and analyzed for oxygen consumption as in A.

there was a small but reproducible drop in complex IV activity that was seen in this assay, and this was also blocked by zVAD-fmk. This small decrease in complex IV activity (versus large decreases in those of complex I and II) was similarly seen in apoptotic HeLa cells (Fig. 8 B). This small caspase-dependent effect is likely to be indirect, based on our results in isolated mitochondria (Fig. 3 D) or may involve caspases other than caspase-3. Further, this may represent a small decrease in oxygen consumption without a decrease in $\Delta\Psi$m, since $\Delta\Psi$m did not decrease under the same conditions (Fig. 7). Similar results were obtained in HeLa cells treated with staurosporine to induce apoptosis (Fig. 8 B). Again, oxygen consumption in the presence of complex I or complex II substrates was destroyed by the apoptotic process, although the function of complex IV remained largely intact.

Our findings show that after mitochondrial outer membrane permeabilization and the activation of caspases, the caspases target complexes I and II of the electron transport chain. This results in a sustained loss of $\Delta\Psi$m and production of ROS, both of which may then contribute to the rapid dismantling of the cell.

Discussion

During apoptosis, proapoptotic members of the Bcl-2 family induce (and some may directly cause) permeabilization of the mitochondrial outer membrane (Kuwana et al., 2002; Waterhouse et al., 2002). Although our studies often used only one of these, tBid, many other proapoptotic Bcl-2 family members have been implicated in different forms of apoptosis (Waterhouse et al., 2002), and any of these is likely to trigger outer membrane permeabilization we have studied here. The release of proteins from the intermembrane space coincides with a transient drop in $\Delta\Psi$m, which in the absence of subsequent caspase activation can recover to normal levels for hours or even days thereafter (Waterhouse et al., 2001b). This maintenance of $\Delta\Psi$m is through electron transport, since it is inhibited by azide or cyanide (complex IV inhibitors) but not by oligomycin (complex V inhibitor) (Goldstein et al., 2000; Waterhouse et al., 2001b), and this depends on the cytochrome c that is now diffuse in the cell (Waterhouse et al., 2001b). The maintenance of $\Delta\Psi$m after cytochrome c release in the absence of caspase activation has been observed in diverse cell types under different conditions of apoptosis (Bossy-Wetzel et al., 1998; Deshmukh et al., 2000; Goldstein et al., 2000; Waterhouse et al., 2001b).

Such observations suggested that caspases target mitochondrial function within minutes of cytochrome c release. In vitro, cytochrome c can induce apoptosome formation and caspase activation in ~10 min or less, consistent with this idea (Cohen, G., and K. Cain, personal communication). Since the highest concentration of cytosolic cytochrome c might be expected to be in the region of the mitochondria immediately after cytochrome c release, it is not unreasonable that caspase activation near mitochondria would be one of the first consequences of outer membrane permeabilization. Therefore, mitochondria may be among the earliest targets of caspase activation during apoptosis.

In the present study, we have analyzed the impact of caspases on the permeabilized mitochondria. Isolated intact mitochondria did not lose $\Delta\Psi$m in response to caspase-3 (Fig. 2), but mitochondria in which the outer membrane was permeable showed a disruption in complex I and II activities in response to caspase-3. In cells undergoing apoptosis via the mitochondrial pathway, mitochondrial outer membrane permeabilization occurs before caspase activation (Martinou and Green, 2001), and as noted above this event by itself does not interfere with the function of the electron transport chain unless caspases are subsequently activated. Analysis of this effect indicated that caspase-3 acts on the permeabilized mitochondria to disrupt $\Delta\Psi$m and respiration and induce ROS production via action on complexes I and II.

In digitonin-treated cells, we observed that addition of active caspase caused a disruption of $\Delta\Psi$m and production of ROS, and this was blocked by Bcl-xL (Figs. 5 and 6). This appeared to be largely dependent on the presence of Bid in the cells. Although in most cases engagement of the mitochondrial pathway is caspase independent (as noted above),

Bid is activated by caspases and therefore can link other routes of caspase activation (e.g., death receptor signaling) to mitochondrial outer membrane permeabilization (Martinou and Green, 2001). Under such circumstances, caspase activation can precede cytochrome c release. However, our results would suggest that even in those cases, the disruptive effect on mitochondrial function would require both the permeabilization of the mitochondrial outer membrane followed by the action of the protease on the intermembrane space.

Targeting of mitochondrial functions upon caspase activation has been described previously. In examining oxygen consumption during Fas-mediated apoptosis, Krippner et al. (1996) observed a loss of cytochrome c function without a substantial loss of function of complex IV. Although this is most easily explained by the release of cytochrome c, an examination of their data also showed an inhibition of complex I and II function as we observed. In an earlier study, cell death induced by TNF (a pathway with similarities to that of Fas) was shown to coincide with loss of complex I and II activity (Schulze-Osthoff et al., 1992). These observations support our conclusions that caspase-dependent loss of mitochondrial function during apoptosis involves a disruption of complexes I and II.

The simplest way in which caspases can disrupt mitochondrial function is via cleavage of molecules important for electron transport. A survey of the components of the electron transport complexes reveals several potential caspase cleave sites based on known specificities of the caspases (Stennicke et al., 2000). Whether these are actual caspase substrates or not and their accessibility to caspases during apoptosis are currently unknown. Other alternative targets may be transport molecules or other systems that impact on the function of the electron transport chain.

During apoptosis, proteins of the intermembrane space are released, but those of the matrix are not (von Ahsen et al., 2000), suggesting that the inner membrane remains intact (which is also supported by our observation that $\Delta\Psi$m is maintained). Without mitochondrial outer membrane permeabilization, caspase-3 had no effect on $\Delta\Psi$m or respiration (Figs. 2 and 3). Therefore, the relevant caspase substrates are presumably accessible on the outside of the inner membrane (i.e., exposed to the inter-membrane space). Alternatively, activation of caspases within the mitochondria, which has been described (Susin et al., 1999a; Mannick et al., 2001), may play a role here. How these would become activated upon exposure of the mitochondria to exogenous caspase-3 is, however, unclear.

Our observations that production of ROS during apoptosis can be caspase dependent (Fig. 1) suggest that they are not required for apoptosis per se as shown using other methods by others (Jacobson and Raff, 1995; Shimizu et al., 1995). However, the production of ROS during apoptosis is likely to contribute to cell death (Tan et al., 1998). Scavenging of ROS can delay or prevent cell death during apoptosis in several systems. Therefore, caspase-induced ROS production may play roles in the dismantling of the cell after caspase activation. Similarly, the loss of electron transport activity and $\Delta\Psi$m would impact on all other mitochondrial functions, further contributing to the dismantling of the cell after the activation of caspases.

Materials and methods

Chemicals and recombinant proteins

FCCP (10 μM), oligomycin (10 μg/ml), cytochrome c (100 μM), and all of the substrates and inhibitors of the electron transport chain (see below) were from Sigma-Aldrich. Recombinant Bcl-xL-ΔC and caspase-3 were produced in bacteria as previously described (Bossy-Wetzel and Green, 1999). Bcl-xL-ΔC was used at 20 μg/ml.

Caspase-free tBid was obtained as described (von Ahsen et al., 2000). Briefly, Amino acids 57–62 were replaced by the thrombin cleavage sequence LVPRGS using site-directed mutagenesis (overlap extension method). The resulting fusion protein was activated by thrombin cleavage, producing the same COOH-terminal fragment of Bid that results from caspase-8 cleavage of wild-type full-length Bid. In addition, a 6-histidine tag was attached to the COOH terminus to facilitate purification of the active fragment. The plasmid was then transformed into *Escherichia coli* BL21 (DE3) (Invitrogen), and protein expression was induced by addition of IPTG (0.5 mM). After lysis, the recombinant protein was purified using glutathione–Sepharose-4B beads (Amersham Biosciences). After three washes each with lysis buffer containing 0.1% Triton X-100 and PBS, the beads were incubated with 100 U of thrombin in 4 ml PBS for 2 h at 22°C to cleave off the COOH-terminal portion corresponding to tBid (aa 61–195) with a 6 × His tail. The supernatant of the cleavage reaction, containing tBid-His_6, was bound to 4 ml Ni-NTA resin. This resin was loaded into a column and washed sequentially with PBS, PBS containing 300 mM additional NaCl, and finally PBS, pH 6.0, containing 300 mM NaCl. The tBid was eluted with 100 mM imidazole in PBS, pH 6.0, containing 300 mM NaCl and dialyzed against PBS containing 10% glycerol for 6 h before storage at –80°C.

Cell culture and induction of apoptosis

HeLa cells were cultured in DME (GIBCO BRL) and Jurkat cells in RPMI-1640 (GIBCO BRL) supplemented with 2 mM glutamine, 200 μg/ml penicillin, 100 μg/ml streptomycin sulfate, and 10% FBS. Cells were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO2. For passage, adherent cells were incubated in 0.25% trypsin (GIBCO BRL), washed, and subcultured in growth medium. Jurkat cells were subcloned 1:10 when they reached 10^6 cells/ml.

To induce death, cells were preincubated or not with 50 μM zVAD-fmk (Kamiya Biomedical Company) and treated with staurosporine, actinomycin D, or with UV (as indicated) and then incubated for 18 h at 37°C. For UV treatment, cells were washed in PBS and irradiated with UV light in PBS at 37°C. The PBS was then aspired, and medium was replaced.

Isolation of mitochondria

Mitochondria were isolated as described in detail previously (Waterhouse et al., 2001a). The isolation procedure was performed at 4°C. Briefly, mouse liver was resuspended in 10 ml of mitochondrial isolation buffer (MIB: 220 mM mannitol, 68 mM sucrose, 10 mM Hepes-KOH, pH 7.4, 70 mM KCl, 1 mM EGTA, 1 mM PMSF, and 2 μM aprotinin) and dissociated using a 15 ml dounce with a tight fitting teflon pestle. Mitochondria were isolated by multiple steps of centrifugation in a Sorvall centrifuge with a swinging bucket rotor (HB4). The cellular lysates were centrifuged at 600 *g* for 10 min, and the supernatants were centrifuged at 3,500 *g* for 15 min. The mitochondrial pellets were resuspended in 15 ml of fresh MIB, centrifuged at 1,500 *g* for 5 min, and the supernatant centrifuged at 5,500 *g* for 10 min. The last two steps were repeated twice. The final pellets were resuspended in 400 μl of ice cold MIB.

Analysis of $\Delta\Psi$m in permeabilized cells and isolated mitochondria

For $\Delta\Psi$m analysis of isolated mitochondria (Fig. 2), 20 μg of mitochondria was resuspended in buffer A (200 mM mannitol, 50 mM sucrose, 10 mM succinate, 10 mM Hepes-KOH, pH 7.4, 5 mM potassium phosphate pH 7.4, 5 mM DTT, and 50 nM TMRE) and incubated in the presence of tBid, caspase-3, and zVAD-fmk or FCCP as indicated, for 45 min at 37°C.

For permeabilization, HeLa cells were trypsinized for 5 min at RT and washed with PBS. Jurkat cells were harvested and washed with PBS. The cells were resuspended in ice cold MIB containing 30 μg/ml digitonin until >95% of the cells were permeable to Trypan blue. Then the cells are washed twice in MIB (4°C).

For $\Delta\Psi$m measurement, permeabilized cells (10^6/ml) were incubated in buffer A (Fig. 5 A) or in buffer B (Fig. 5, B–E, and Fig. 7) (MIB + 2 mM ADP + 2 mM DTT + 50 nM TMRE) and then incubated in the presence of substrates for the electron transport chain (see concentrations below). In Fig. 5, cells were first permeabilized, then incubated for 30 min at 37°C in the presence or absence of recombinant active caspase-3 (0.5 μg/ml) in buffer

B containing cytochrome c (100 μM) or zVAD-fmk (100 μM), or Bcl-xL-ΔC (20 μg/ml), or both and finally incubated in the presence of the substrates for the electron transport chain. In Fig. 7, oligomycin (10 μg/ml) is added when indicated. Cells and isolated mitochondria were then analyzed by flow cytometry on a FACScan (Becton Dickinson) measuring TMRE fluorescence in FL-2.

Oxygen electrode measurement

Two independent Clark oxygen electrodes (Instech Laboratories) with two independent thermojacketed chambers were used. This dual system allowed us to analyze two samples in parallel. For isolated mitochondria, the respiration buffer (RB) was 140 mM KCl, 10 mM $MgCl_2$, 10 mM MOPS (pH 7.4), 5 mM KH_2PO_4, DTT 5 mM, 1 mM EGTA (or 0.2 mM EGTA in case of Fig. 3 E). For permeabilized cells, the RB was 250 mM sucrose, 2 mM EDTA, 30 mM KH_2PO_4, 5 mM $MgCl_2$, and 50 mM Tris (pH 7.4). The volume corresponding to 400 μg of protein was injected into the chambers containing 600 μl of air-saturated RB prewarmed at 37°C. To rule out an effect of dilution of cytochrome c, all measurements were performed in the presence of 100 μM cytochrome c. Substrates and inhibitors were added in the following order and final concentration: 2.5 mM malate, 40 μM O-palmitoyl-L-carnitine, 2 mM ADP, 2 μM rotenone, 5 mM succinate, 1 μM antimycin A, 1 mM ascorbate with 0.4 mM TMPD, and 1 mM potassium cyanide (KCN). Oxygen concentration was calibrated with air-saturated buffer, assuming 390 ng-atoms of oxygen/ml of buffer (Schulze-Osthoff et al., 1992). Rates of oxygen consumption are expressed as ng-atoms of oxygen/min/mg of proteins.

Measurement of ROS and apoptosis

HeLa cells (Fig. 1) were treated as indicated, harvested, and washed in PBS. The pellet was resuspended in 30 μl of annexin buffer (Hepes 10 mM, NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM) and then divided into three groups: one third each for $\Delta\Psi m$ measurement (as described above), ROS measurement using 2 μM of 2-HE in MIB buffer, and cell death measurement using annexin V–FITC (Calbiochem). Cells were incubated for 30 min at 37°C in the dark. Analysis was made by flow cytometry; $\Delta\Psi m$ was measured in FL2, 2-HE in FL2, annexin V in FL1, and propidium iodide (PI; 0.5 μg/ml added at the last minute to the sample) in FL3. The percentage of cell death in Fig. 1 represents the total of annexin V–positive and annexin V/PI double positive cells. In Fig. 6, Jurkat cells (10^6/ml) were permeabilized as described above and incubated in MIB plus 2 mM ADP, 2 mM DTT, 2 μM 2-HE, and caspase-3 (0.5 μg/ml), tBid (20 μg/ml), and/or Bcl-xL-Δc (20 μg/ml) as indicated.

Complex III assay

Measurements were performed as described previously (Kluck et al., 1999). Briefly, 500 μg of mitochondria were incubated in 100 μl of buffer C (125 mM sucrose, 60 mM KCl, 20 mM Tris-HCl, pH 7.4) in the presence of tBid (25 μg/ml) or recombinant caspase-3 (25 μg/ml) (as indicated) for 60 min at 37°C, or in 1 ml of water (20 min at 4°C), then pelleted by centrifugation (5,500 rpm, 10 min) and resuspended in 100 μl of buffer C. Then the samples were mixed with 300 μl of buffer C containing 3 mM of KCN (to block oxidation by complex IV) and decyl benzoquinol (55 μM final). Finally, ferricytochrome c (80 μM) was added and the rate of cytochrome c reduction at 550 nm was integrated over 30 s. Where indicated, 1 μM of antimycin A was added.

Western blotting

To determine mitochondrial content of hsp60 and cytochrome c, incubation aliquots were centrifuged (6,000 *g*, 10 min), and the pellet was resuspended in 1× loading buffer. Samples were heated at 95°C and loaded on a 15% SDS–polyacrylamide gel for electrophoresis and then transferred to nitrocellulose (Bio-Rad Laboratories). Membrane were blocked 1 h in TBST (25 mM Tris, 140 mM NaCl, 27 mM KCl and 0.02% Tween 20) containing 5% nonfat dried milk. Membranes were then probed with monoclonal anti–cytochrome c (clone 7H8.2C12; PharMingen) or hsp60 (clone LK-1; Stressgen). Recognized proteins were detected using HRP-labeled secondary antibodies (Amersham Biosciences) and ECL (Amersham Biosciences).

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