Cytochrome *c* release and caspase activation in hydrogen peroxide- and tributyltin-induced apoptosis

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Abstract The ability of H_2O_2 and tributyltin (TBT) to trigger pro-caspase activation via export of cytochrome *c* from mitochondria to the cytoplasm was investigated. Treatment of Jurkat T lymphocytes with H_2O_2 resulted in the appearance of cytochrome *c* in the cytosol within 2 h. This was at least 1 h before caspase activation was observed. TBT caused cytochrome *c* release already after 5 min, followed by caspase activation within 1 h. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) showed that both H_2O_2 and TBT dissipated $\Delta\Psi_m$, but with different time courses. TBT caused a concomitant loss of $\Delta\Psi_m$ and release of cytochrome *c*, whereas cytochrome *c* release and caspase activation preceded any apparent $\Delta\Psi_m$ loss in H_2O_2-treated cells. Thus, our results suggest that different mechanisms are involved in triggering cytochrome *c* release with these apoptosis-inducing agents.

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Key words: Cytochrome *c*; Caspase; Hydrogen peroxide; Tributyltin; Mitochondrion; Membrane potential

1. Introduction

The caspase family of proteases plays a crucial role in apoptosis [1,2]. One of the earliest detectable changes in cells about to undergo apoptosis is the conversion of cytoplasmic pro-caspases to their active form. Active caspases then cleave a number of defined target proteins at aspartate residues, leading to the irreversible dismantling of the cell. Over-expression of caspases induces apoptosis in various cell types, and peptide inhibitors of the caspases block apoptosis induced by a wide range of stimuli, emphasising the importance of caspase activation for successful execution of apoptosis.

The ability of caspases to cleave and activate other cytoplasmic pro-caspases sets off a destructive cascade of events [1,2]. This cascade is likely to be a common pathway in apoptosis; however, the sequence of events leading to activation of the first caspase(s) will vary depending on the apoptotic stimulus. The best characterised systems are the cytotoxic T lymphocyte component granzyme B, which can directly cleave several pro-caspases, including pro-caspase-10 [3], and ligation of the Fas (APO-1/CD95) receptor, which initiates protein interactions via 'death domains' and activation of caspase 8 [4]. For many other stimuli, the targeting of mitochondria to release pro-apoptotic factors has been hypothesised to be a primary event in caspase activation [5]. The release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol has been detected in apoptotic cells and functions in activation of caspase-3 [6–8]. Recent studies have shown that microinjection of cytochrome *c* is sufficient to induce apoptosis in some but not all cells [9,10].

The present study was to determine whether hydrogen peroxide or tributyltin, agents known to disrupt mitochondrial function and induce apoptosis, stimulates mitochondrial cytochrome *c* release and activation of caspase-3. Hydrogen peroxide induces apoptosis [11], and is also known to interfere with mitochondrial functions. One prominent effect of oxidants is to activate the permeability transition pore with resultant loss of the mitochondrial membrane potential ($\Delta\Psi_m$) [12]. Tributyltin is an immunotoxicant believed to cause thymic atrophy by excessive stimulation of thymocyte apoptosis [13]. The mechanism by which it induces apoptosis is currently unclear, although earlier reports suggested that it may be linked to an alteration in cellular calcium metabolism [14]. Toxicological studies demonstrated that the organotin family of compounds are potent mitochondrial poisons [15]. The present results show that both hydrogen peroxide and tributyltin induced cytochrome *c* release and activation of caspase-3 with time courses consistent with a central role in activation of apoptosis.

2. Materials and methods

2.1. Materials

Jurkat cells were obtained from the European Type Culture Collection. Tributyltin chloride was from Merck (Darmstadt, Germany), hydrogen peroxide from Fluka (Buchs, Switzerland), anti-Fas IgM (CH-11) from Medical and Biological Laboratories (Nagoya, Japan), z-VAD-fmk from Enzyme Systems Products (Livermore, CA, USA), DEVD-AMC from Peptide Institute, Inc. (Osaka, Japan), JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbon cyanine iodide) from Molecular Probes (Leiden, Netherlands), and anti-mouse polyclonal cytochrome *c* antibody was a kind gift of Ronald Jemmerson, University of Minnesota, MN, USA. All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Jurkat cells were maintained in logarithmic phase culture in RPMI-1640 with 10% heat-inactivated foetal calf serum and 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, at 37°C in humidified air with 5% carbon dioxide. Before use, cells were harvested at $500\times g$ for 5 min and re-suspended at 1×10^6/ml in fresh medium.

2.3. Caspase activity

The measurement of DEVD-AMC cleavage was modified from Nicholson et al. [16]. Cells were pelleted and frozen on microtitre plates at 1×10^6 cells per 25 μl. Fifty μl of buffer (100 mM HEPES, 10% sucrose, 5 mM dithiothreitol, 10^{-6}% NP-40, and 0.1% CHAPS at pH 7.25) was added to each well along with 50 μM of DEVD-AMC. Substrate cleavage to release free AMC (ex. 355 nm, em. 460 nm) was

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monitored at 37°C. Fluorescent units were converted to pmoles of AMC using a standard curve generated with free AMC.

2.4. Cytochrome c release

Cells were harvested by centrifugation at 500×*g* for 5 min. Cytoplasmic extracts were prepared as described by Liu et al. [6]. The buffer included 20 mM HEPES (pH 7.5 with KOH), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μg/ml calpain inhibitor I. Protein concentrations varied from 2–5 μg/μl of extract (Bio-Rad protein assay with BSA as the standard).

2.5. Western blotting

Sample extracts (20 μg/ml) were loaded onto a 15% SDS-polyacrylamide gel and electrophoresed at 130 V for 2 h, then transferred to PVDF membranes (Bio-Rad) at 100 V over a further 2 h. Membranes were blocked in 50 mM Tris (pH 7.5) with 500 mM NaCl, 1% BSA, and 5% non-fat dried milk. The membranes were then probed with anti-cytochrome *c* antibodies (1:500) in an identical solution, followed by peroxidase labelled anti-mouse antibodies (1:10 000) and visualised by ECL (Amersham, Buckinghamshire, UK).

2.6. Mitochondrial membrane potential

Cells were incubated with JC-1 (10 μg/ml) for 10 min in the dark at room temperature, then washed twice in PBS and analysed in a Becton Dickinson FACScan flow cytometer, using the parameters described by Cossarizza et al. [18].

3. Results

We quantitated caspase activity in Jurkat cells by measuring cleavage of the fluorescent peptide Ac-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin) (DEVD-AMC) [16]. This peptide mimics the target sequence of the group II effector caspases, and as such, is an ideal substrate for determining if the caspase cascade has been triggered in the cells of interest.

The first apoptotic stimulus tested was hydrogen peroxide. We have previously documented its ability to activate the caspases and induce apoptosis [11], and Kluck et al. [8] have reported cytochrome *c* release in hydrogen peroxide-treated CEM cells. However, there has been no report of whether cytochrome *c* release occurs before caspase activation in any of these systems. Treatment of Jurkat cells (10^6/ml) with 50 μM hydrogen peroxide resulted in caspase activity above control levels within 3–4 h (Fig. 1A). Cytosolic extracts were obtained from the same cells, and Western blot analysis of cytochrome *c* levels was performed. Cytochrome *c* appearance in the cytoplasm was clearly detectable by 2 h, i.e. 1–2 h before caspase activation. Export was not affected by addition of the general caspase inhibitor z-VAD-fmk (Fig. 1B). Therefore, release of mitochondrial cytochrome *c* occurred early enough to be responsible for caspase activation by hydrogen peroxide.

The mechanism for the export of cytochrome *c* from mitochondria is unknown. Hydrogen peroxide can induce the mitochondrial permeability transition (MPT) and cause a loss in the mitochondrial membrane potential ($\Delta\Psi_m$) [17] in a process thought to be important in activation of apoptosis [5]. To

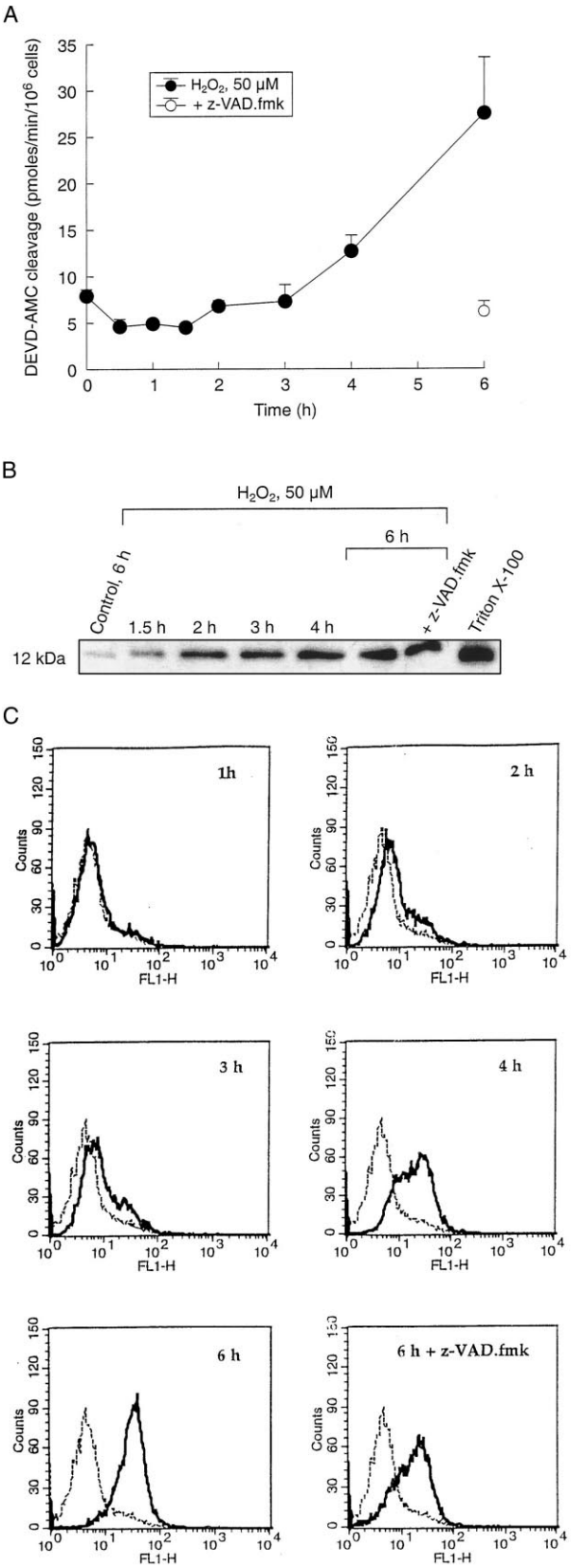


Fig. 1. Time course of caspase activation, cytochrome *c* release and mitochondrial membrane potential in Jurkat cells treated with hydrogen peroxide. Jurkat cells (10^6/ml) were treated with 50 μM hydrogen peroxide in the presence or the absence of the protease inhibitor z-VAD-fmk. At various times after treatment the cells were harvested. A: Caspase activity was measured by the ability of cellular extracts to cleave DEVD-AMC. B: The presence of cytochrome *c* in cytoplasmic extracts was measured by Western blot with an anti-cytochrome *c* antibody. C: The JC-1 monomers were measured by flow cytometry in 10 000 cells. The dotted lines represent untreated cells at each time. Results are representative of at least 3 experiments.

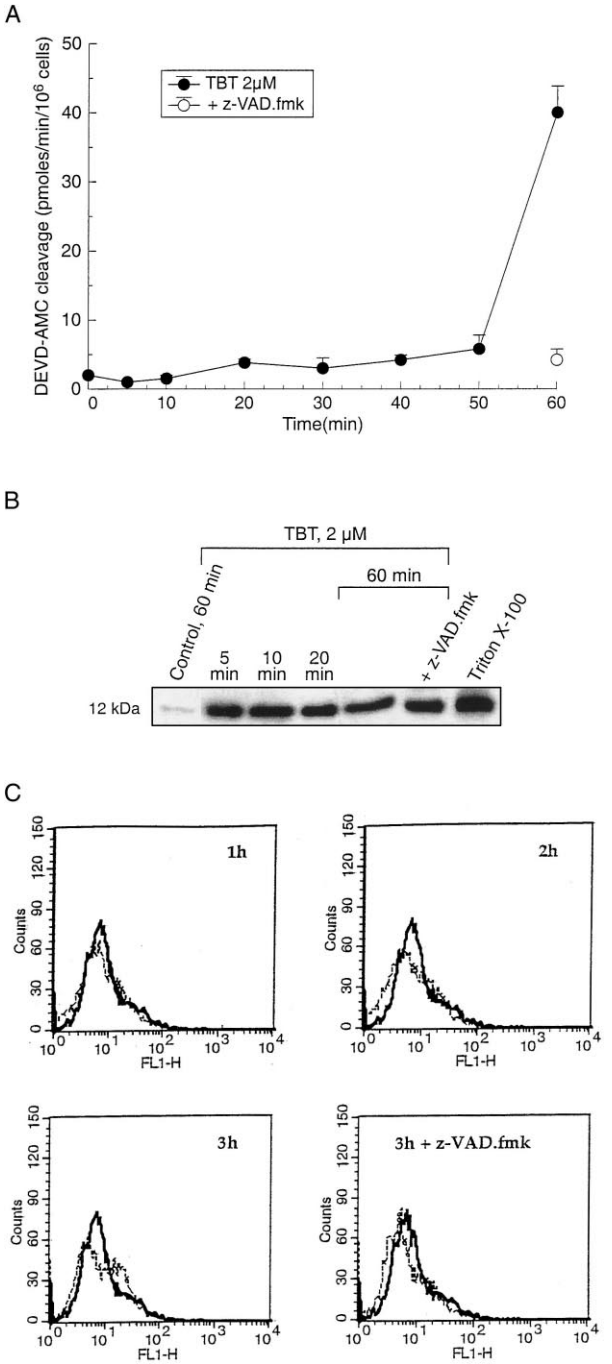


Fig. 2. Time course of caspase activation, cytochrome *c* release and mitochondrial membrane potential in Jurkat cells treated with tributyltin. Experiments were performed as in Fig. 1, except cells were treated with 2 μM tributyltin.

investigate whether a decrease in $\Delta\Psi_m$ could activate cytochrome *c* release in the hydrogen peroxide model, we used the fluorescent dye JC-1 to measure $\Delta\Psi_m$ following treatment. This mitochondrial specific dye forms aggregates at the normal membrane potential, and a loss in $\Delta\Psi_m$ can be monitored by flow cytometry as an increase in JC-1 monomers (FL-1 channel) [18,19]. We detected a loss in the $\Delta\Psi_m$ with hydrogen peroxide, but not until 4 h (Fig. 1C), indicating that it was unlikely to be involved in hydrogen peroxide-mediated cytochrome *c* export.

The second agent investigated was tributyltin, which is known to be an effective inducer of apoptosis [13,20] but the role of cytochrome *c* release and caspase activation has not been determined. Addition of 2 μM tributyltin resulted in caspase activation within 60 min (Fig. 2A), and maximal cytochrome *c* release was detected within 5–10 min (Fig. 2B), i.e. well before caspase activity could be detected. z-VAD-fmk had no effect on cytochrome *c* release by tributyltin, consis-

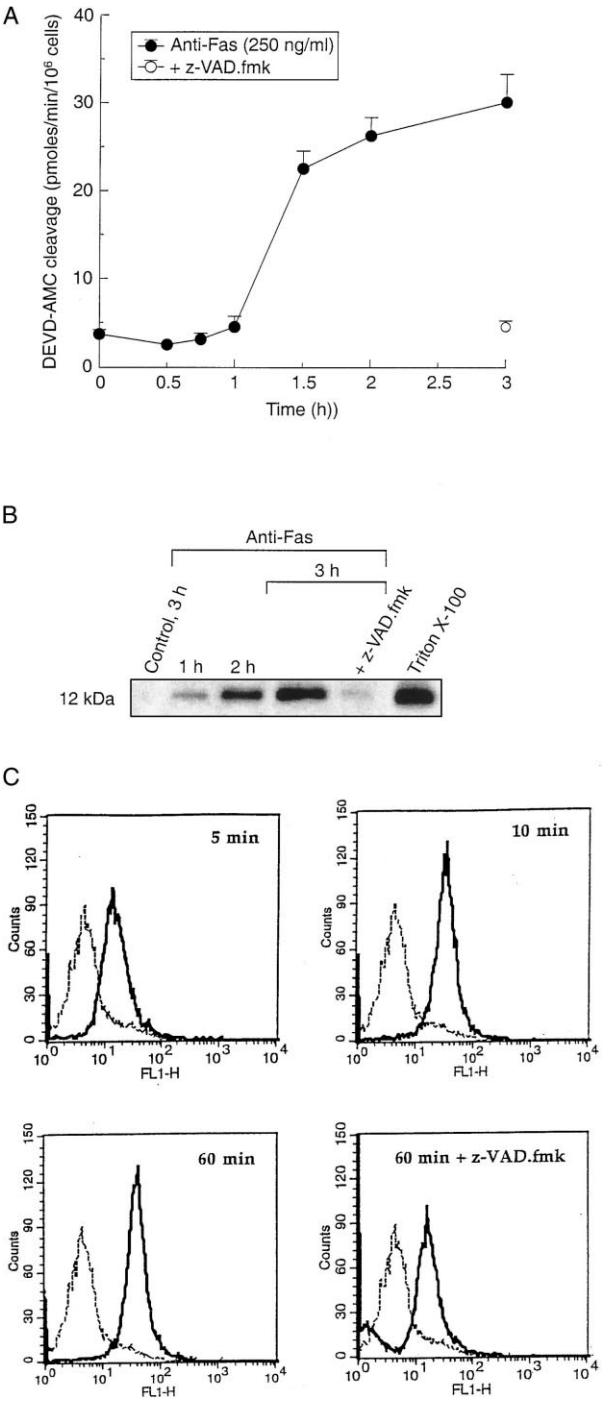


Fig. 3. Time course of caspase activation, cytochrome *c* release and mitochondrial membrane potential in Jurkat cells treated with anti-Fas antibody. Experiments were performed as in Fig. 1, except cells were treated with 250 ng/ml anti-Fas antibody.

tent with this process occurring upstream of caspase activation.

While quantitation from the Western blots is difficult, comparison with the amount of cytochrome *c* released from Triton X-100-lysed mitochondria indicates that tributyltin and hydrogen peroxide caused the release of a large amount of total mitochondrial cytochrome *c* (Figs. 1B and 2B). The amounts released did appear to plateau though, suggesting that either a specific fraction of the hemoprotein was available for export, or that part of the mitochondrial population resisted the cytochrome *c* releasing effects of tributyltin and hydrogen peroxide.

As expected from early toxicological studies, tributyltin induced a rapid dissipation of $\Delta\Psi_m$, making this effect a potential mechanism for cytochrome *c* export (Fig. 2C). However, because of the rapid time course of tributyltin-induced loss of $\Delta\Psi_m$, and cytochrome *c* release, it was not possible to discern which came first in this model.

To compare the results obtained with the two mitochondrial toxicants, we induced apoptosis with an anti-Fas antibody which is thought to occur by a mechanism without early mitochondrial involvement. With this treatment cytochrome *c* release and DEVD-AMC cleavage were measured after 2 h and both were prevented by z-VAD-fmk (Fig. 3B,C), indicating that they were the effects rather than the causes of caspase activation. As with hydrogen peroxide treatment, loss of $\Delta\Psi_m$ was a late event that did not appear to be important in either cytochrome *c* release or caspase activation.

4. Discussion

Mitochondria are thought to play a central role in the activation of apoptosis induced by multiple agents by a mechanism that involves release of cytochrome *c* and its binding to a multiprotein complex that activates caspase-3. This may provide a general mechanism whereby agents that induce mitochondrial dysfunction activate apoptosis. Here we show that hydrogen peroxide and tributyltin, two agents known to disrupt mitochondrial function, can trigger cytochrome *c* release sufficiently early to account for caspase activation. This adds to a growing list of toxic exposures, including etoposide, paclitaxel, UVB, ionizing radiation and MPP^+, where mitochondrial cytochrome *c* release precedes activation of caspase-3. Thus, accumulating data indicate that mitochondrial cytochrome *c* release is an early common event in the induction of apoptosis induced by many toxic conditions.

However, despite this apparently common role of cytochrome *c* in activation of caspases, the mechanisms involved in its release from mitochondria appear to differ for different activating agents. Oxidative stress is known to alter various facets of mitochondrial function including calcium homeostasis and triggering of the permeability transition pore, leading to a dissipation of $\Delta\Psi_m$ [17]. It has been proposed that the transition pore and associated loss in $\Delta\Psi_m$ could be responsible for the release of proapoptotic factors such as cytochrome *c* [21]. However, we could detect no link between the $\Delta\Psi_m$ and cytochrome *c* release with hydrogen peroxide where loss in $\Delta\Psi_m$ was detectable, but only 1–2 h after cytochrome *c* release.

Other evidence also appears to exclude the general involvement of $\Delta\Psi_m$ in cytochrome *c* release. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which rapidly dissipates $\Delta\Psi_m$, was not able to trigger cytochrome *c* release (result not shown). Yang et al. [7] and Kluck et al. [8] could not detect changes in $\Delta\Psi_m$ in their apoptotic models. Also, other reports of a loss in $\Delta\Psi_m$ during apoptosis place it at a similar time [22] or after [23] glutathione loss in apoptotic cells. We have previously characterised the export of glutathione from apoptotic cells to be a mid to late event in apoptotic cells, clearly downstream of caspase activation [24].

In contrast, a loss of $\Delta\Psi_m$ could play a role in the tributyltin model. Tributyltin dissipated $\Delta\Psi_m$ very quickly, with a concomitant release of mitochondrial cytochrome *c*. The effects of organotin compounds on cell function were studied extensively during the 1950's. Trialkyltin compounds act as Cl^-/OH^- exchangers, which could provide an explanation for the rapid dissipation of $\Delta\Psi_m$ and blocking of oxidative phosphorylation [25]. They were also shown to directly inhibit the mitochondrial ATP synthase, and cause mitochondrial swelling [25]. In another study, we attempted to block tributyltin- induced loss of $\Delta\Psi_m$ with bongkrekic acid, an agent that inhibits MPT, but observed no inhibition. Thus, the results suggest that the loss of $\Delta\Psi_m$ induced by tributyltin may involve Cl^-/OH^- exchange or other mechanisms not directly linked to the MPT. Alternatively, bongkrekic acid may not be sufficiently permeable to inhibit MPT in the cells. Further studies are required to determine which, if any, of these effects are involved in tributyltin-mediated cytochrome *c* release.

Mitochondria are not generally implicated in transduction of the Fas signal. The sequence of events following trimerisation of the Fas receptor has been shown to include direct activation of caspase-8 (FLICE/MACH) at the cytosolic site of the receptor [26]. Caspase-8 can cleave and activate the DEVD-cleaving group II caspases such as caspase-3, thereby eliminating the requirement for additional factors in caspase activation. While a mechanism whereby Fas activates caspase-1, which then acts on the mitochondria to cause release of other caspase-activating agents, has been proposed [21], it remains to be shown if these changes occur before activation of the group II caspases. Our present findings exclude cytochrome *c* from being one of these caspase-activating factors as it is not exported in the time frame necessary.

The ability of the non-specific caspase inhibitor z-VAD to prevent Fas-mediated cytochrome *c* release shows, however, that activated caspases can alter mitochondrial function. Babior, Gottlieb and colleagues [27,28] have reported impaired mitochondrial function in Fas-treated Jurkat cells that corresponded with a caspase-dependent loss in cytochrome *c*. Interestingly, however, their effect was observed within 5 min and was attributed to an inactivation of cytochrome *c*, without export from the mitochondria.

A loss of $\Delta\Psi_m$ was observed after cytochrome *c* release in hydrogen peroxide- and Fas-mediated apoptosis. These results indicate that the export of cytochrome *c* does not occur as a consequence of activating the permeability transition pore, which is associated with a loss of $\Delta\Psi_m$. Therefore, specific export mechanisms should be considered. Van der Heiden et al. [29] have recently reported a novel mechanism. They suggest that swelling and rupture of the outer mitochondrial membrane leads to release of cytochrome *c*, without disruption of the inner membrane and $\Delta\Psi_m$. This was observed upon treatment of Jurkat cells with anti-Fas or staurosporine, and interleukin 3 deprivation of a pro B-cell line. The mech-

anism remains to be clarified, but alterations in mitochondrial volume homeostasis were implicated.

In summary, the present studies show that two mitochondrial toxicants induce cytochrome *c* release and caspase activation with a time course appropriate for a role in the signalling of apoptosis. Tributyltin causes a rapid loss of the mitochondrial membrane potential that cannot be temporally separated from cytochrome *c* release. In contrast, the mitochondrial membrane potential is retained in peroxide-treated cells until well after release of cytochrome *c* and caspase activation. Thus, at least for hydrogen peroxide treatment, cytochrome *c* release appears to occur by a more specific mechanism than the permeability transition pore.

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