

A study on permeability transition pore opening and cytochrome *c* release from mitochondria, induced by caspase-3 in vitro

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Abstract We recently described that there is a feedback amplification of cytochrome *c* release from mitochondria by caspases. Here we investigated how caspases impact on mitochondria to induce cytochrome *c* release and found that recombinant caspase-3 induced opening of permeability transition pore and reduction of membrane potential in vitro. These events were inhibited by Bcl-xL, cyclosporin A and z-VAD.fmk. Moreover, caspase-3 stimulated the rate of mitochondrial state 4 respiration, superoxide production and NAD(P)H oxidation in a Bcl-xL- and cyclosporin A-inhibitable manner. These results suggest that caspase-3 induces cytochrome *c* release by inducing permeability transition pore opening which is associated with changes in mitochondrial respiration and redox potential. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Apoptosis; Mitochondrion; Caspase-3; Reactive oxygen species; Permeability transition pore; Cytochrome *c*

1. Introduction

Mitochondria play a central role both in the production of ATP and in regulating apoptosis, or programmed cell death [1]. During the process of mitochondrial oxidative phosphorylation, the electron transports along the respiratory chain and generates a proton gradient across the mitochondrial inner membrane to build proton motive force, including mitochondrial membrane potential ($\Delta\Psi_m$) and ΔpH , to drive ATP synthesis by F_1F_0 -ATPase. The maintenance of the proton motive force is critical for the normal function of mitochondria. Perturbation of mitochondrial physiology could lead to increased reactive oxygen production [2], damage to mitochondrial membranes and, consequently, apoptosis [1,3]. Mitochondria could release apoptogenic factors such as cyto-

chrome *c* (cyt *c*) [4], Smac [5], apoptosis-inducing factor (AIF) [6], caspases [7] and endonuclease G [8] to activate the apoptotic cascade. Cyt *c* is a soluble protein located outside the inner mitochondrial membrane and functions as an electron carrier of the mitochondrial respiration chain between complex III and complex IV. Once released, cyt *c* acts as a co-factor in the presence of dATP to stimulate the aggregation of Apaf-1, which can recruit and activate caspase-9 and the downstream caspase cascade [4]. Effector caspases then cleave cellular substrates at a specific tetrapeptide sequence on the carboxyl side of aspartate residues and execute programmed cell death [9]. The molecular mechanisms of the regulation of the release of mitochondrial apoptogenic substances and its relationship with mitochondrial physiology remain elusive.

Elucidating the mechanisms of cyt *c* release is crucial to the understanding of how and why a cell makes a decision to undergo apoptosis. The release of cyt *c* from mitochondria could be the result of breakdown of the outer membrane of mitochondria or opening of the permeability transition pore (PTP) located in the contact site of the inner and outer membrane of the mitochondria [10,11]. Bcl-2 and its related proteins play an important role in regulating PTP, cyt *c* release and caspase activation [12]. The molecular mechanisms of how Bcl-2 and its related proteins regulate these events associated with apoptosis remain a challenge ahead. There is evidence that cyt *c* release could happen with or without PTP opening [13,14]. Also there are reports showing that the release of cyt *c* could be downstream or upstream of caspase activation depending on the systems employed [15]. Recently, we observed that there is a feedback amplification of cyt *c* release and PTP opening by caspases during genotoxic stress-induced apoptosis [16]. We showed that, during the early stage of apoptosis, there is a small amount of cyt *c* release, which is sufficient to activate caspases. Once activated, caspases could induce feedback amplification of cyt *c* release and mitochondrial dysfunction, though the mechanisms are not clear [17,18]. Here, we show that recombinant caspase-3 directly induced mitochondrial PTP opening, resulting in changes in the mitochondrial redox status, mitochondrial dysfunction and cyt *c* release.

2. Materials and methods

2.1. Materials

Sucrose, HEPES and IPTG were purchased from Life Technologies

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Abbreviations: AIF, apoptosis-inducing factor; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CsA, cyclosporin A; cyt *c*, cytochrome *c*; $\Delta\Psi_m$, mitochondrial membrane potential; PTP, permeability transition pore; RCR, respiratory control ratio; Rh123, rhodamine 123; ROS, reactive oxygen species; X, xanthine; XO, xanthine oxidase

(Grand Island, NY, USA). Bovine serum albumin (BSA), ADP, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), cyclosporin A (CsA), rhodamine 123 (Rh123), xanthine (X), xanthine oxidase (XO) and H₂DCFDA were obtained from Sigma (St. Louis, MO, USA). Glutathione-Sepharose 4B was from Pharmacia (Uppsala, Sweden). Ni-NTA agarose was from Qiagen (Hilden, Germany). z-VAD.fmk was obtained from Biomol (Plymouth Meeting, PA, USA). Cyt *c* antibody (clone 7H8.2C12) was from PharMingen (San Diego, CA, USA). Other reagents were of analytical grade from commercial sources in China.

2.2. Isolation of mitochondria and measurement of mitochondrial permeability transition

Mitochondria were isolated from the liver of BALB/c mice. Briefly, the livers were taken after death and homogenized with a glass-Teflon Potter homogenizer. Mitochondria were isolated in 250 mM sucrose, 2 mM HEPES, pH 7.4, 0.1 mM EDTA, 0.1% fatty acid-free BSA. All steps were carried out on ice. Samples were centrifuged at 1000×*g* for 10 min. The supernatant was transferred to another tube and centrifuged at 10 000×*g* for 10 min. Mitochondria were washed twice, then resuspended in the same medium and kept on ice for up to 4 h. Protein content of mitochondria was determined by the microbiuret method using BSA as standard. Mitochondrial swelling was monitored by the decrease of 90° light scatter at 520 nm at 25°C in medium PT-1 containing 250 mM sucrose, 2 mM HEPES, pH 7.4, 0.5 mM KH₂PO₄, 2 μM rotenone and 4.2 mM potassium succinate, using a Jobin Yvon FluoroMax-2 spectrofluorimeter as described [13].

2.3. Expression and purification of recombinant proteins

Human Bcl-xL was expressed as glutathione *S*-transferase fusion proteins in DH5α bacterial cells and purified on a glutathione-Sepharose column [14]. His-tagged pET-CPP32 was expressed in BL21 bacterial cells and purified by Ni-NTA agarose (Qiagen) according to the supplier's protocol. Recombinant caspase-3 protein (1 U=10 pmol/min/mg) was evaluated by cleaving the DEVD-pNA before being used for further experiments.

2.4. Measurement of oxygen consumption and respiratory control ratio (RCR)

Using a Clark oxygen electrode, recombinant caspase-3-induced changes in mitochondrial oxygen consumption rates were monitored at 25°C in medium PT-1 [19]. Statistical analysis was carried out with the one-way ANOVA. Differences were considered statistically significant at *P* < 0.05.

2.5. Determination of mitochondrial membrane potential

After addition of 30 nM Rh123 to a mitochondria suspension as previously described [13], ΔΨ_m was assessed at 25°C in medium PT-1 by measuring the uptake of Rh123 using a spectrofluorimeter (Jobin Yvon FluoroMax-2).

2.6. Assay of reactive oxygen species (ROS) production and determination of the NAD(P)H redox state

Generation of mitochondrial ROS was evaluated in isolated mitochondria in medium PT-1 using DCFH as a probe as described [20]. DCF formation was monitored using a Jobin Yvon FluoroMax-2 spectrofluorimeter. The oxidation of NAD(P)H in the mitochondria was measured at excitation and emission wavelengths of 350 and 450 nm as described [21]. All assays were carried out in triplicate.

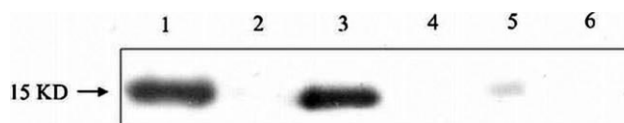


Fig. 1. Recombinant caspase-3 induces cyt *c* release from isolated mitochondria. Isolated mitochondria (1 mg/ml) were treated for 15 min at 25°C with recombinant caspase-3 (6 U/ml) or in the presence of Bcl-xL, CsA or z-VAD.fmk in medium PT-1. Lane 1, pellet of untreated mitochondria. Lane 2, supernatant of untreated mitochondria. Lane 3, supernatant of caspase-3 (6 U/ml)-treated mitochondria. Lanes 4 and 5, supernatant of caspase-3-treated mitochondria in the presence of Bcl-xL (16 μg/ml) and CsA (1 μM). Lane 6, 1 μM z-VAD.fmk was preincubated with caspase-3 for 15 min and then the mixture was added to the mitochondria.

2.7. Western blotting for cyt *c* release

Isolated mitochondria in medium PT-1 were incubated in the presence of recombinant caspase-3 at room temperature. Mitochondrial supernatant was then assayed using cyt *c* monoclonal antibody as described [22].

3. Results

3.1. Recombinant caspase-3 induces cyt *c* release from isolated mitochondria

We previously observed that caspase-3 could induce cyt *c* release from isolated mitochondria from cultured IM-9 cells, a multiple myeloma cell line. Using mitochondria isolated from mouse liver, we confirmed that recombinant caspase-3-induced release of cyt *c* from mitochondria was inhibited by z-VAD.fmk. Moreover, we found that Bcl-xL and CsA, commonly used blockers for PTP, blocked the effects of caspase-3 on cyt *c* release (Fig. 1), suggesting that caspase-3 could induce PTP opening which may be responsible for cyt *c* release.

3.2. Recombinant caspase-3 induces the opening of PTP and loss of mitochondrial membrane potential (ΔΨ_m)

To directly prove that caspase-3 could induce PTP opening, we treated the isolated mitochondria with recombinant caspase-3 and measured mitochondrial swelling, indicative of PTP opening. We found that recombinant caspase-3 induced PTP opening in a dose-dependent manner. Both CsA and Bcl-xL inhibited the mitochondrial swelling induced by caspase-3 (Fig. 2). Loss of ΔΨ_m was a hallmark for mitochondrial apoptosis and another indication of opening of PTP. Thus, we further examined the effect of caspase-3 on ΔΨ_m. Using Rh123 to measure ΔΨ_m, we found that caspase-3 can induce the loss of ΔΨ_m in a dose-dependent manner. High doses of caspase-3 induced almost complete loss of ΔΨ_m as compared with CCCP, a commonly used uncoupler for the dissipation of

Table 1
Effect of recombinant caspase-3 on mitochondrial respiration

Parameter	Control (<i>n</i> = 8)	Caspase-3 (<i>n</i> = 8)	+z-VAD (<i>n</i> = 8)	+Bcl-xL (<i>n</i> = 8)	+CsA (<i>n</i> = 8)
State 4	11.12 ± 3.50	15.01 ± 2.94*	11.22 ± 3.61 [§]	12.20 ± 2.97 [§]	12.15 ± 2.49 [§]
State 3	38.17 ± 16.25	38.15 ± 14.55	37.75 ± 16.24	37.22 ± 13.93	37.96 ± 12.67
RCR	3.33 ± 0.48	2.49 ± 0.61*	3.27 ± 0.41 [§]	2.81 ± 0.46* [§]	2.96 ± 0.46* [§]

Mitochondrial oxygen consumption rates in succinate (4.2 mM)-stimulated state 4 respiration and ADP (200 nM)-stimulated state 3 respiration were monitored at 25°C in medium PT-1 as described. Mitochondria were treated without caspase-3 (control) or with caspase-3 (column 2), or mitochondria were preincubated with z-VAD.fmk (1 μM) (column 3), or in the presence of Bcl-xL (16 μg/ml, column 4) or CsA (1 μM, column 5) before caspase-3 addition. **P* < 0.05 (compared with control group); [§]*P* < 0.05 (compared with caspase-3 group). *P* values (one-way ANOVA): parameters include O₂ consumption with ADP (state 3) and without ADP (state 4), RCR (state 3/state 4). Units for state 3 and state 4 O₂ consumption are μmol O₂/min/g assuming 258 μmol O₂/l at 25°C.

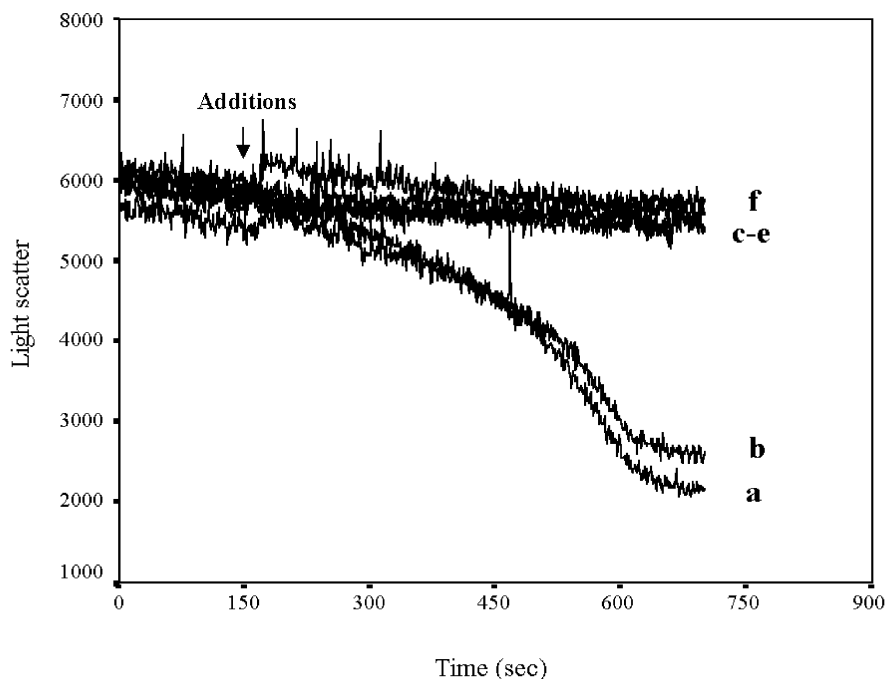


Fig. 2. Recombinant caspase-3 induces mitochondrial swelling inhibited by Bcl-xL and CsA. Mitochondrial swelling was monitored as described in Section 2. Trace a, 6 U/ml was added as indicated. Trace b, X (53.3 μ M) was preincubated with mitochondria first and XO (0.024 unit/ml) was added at 150 s. Trace c, caspase-3 preincubated with z-VAD.fmk then the mixture (caspase-3, 6 U/ml) was added at 150 s. Trace d, mitochondria only without additions. Traces e and f, CsA (1 μ M) and Bcl-xL (16 μ g/ml) were first incubated with the mitochondria, then caspase-3 (6 U/ml) was added as indicated.

the proton gradient across the inner mitochondrial membrane. The caspase-3-induced uncoupling effect of $\Delta\Psi_m$ was prevented by z-VAD.fmk, CsA or Bcl-xL (Fig. 3).

3.3. Caspase-3 induces an increase in state 4 respiration rate without impact on state 3 respiration in isolated mitochondria from mouse liver

As shown in Table 1, incubation of caspase-3 with mitochondria for 3 min induced an increase of 35% in state 4 respiration rate in comparison with the untreated samples, but no effect on state 3 respiration rate. Thus, RCR dropped 25% in comparison with control. When caspase-3 was preincubated with the caspase inhibitor z-VAD.fmk, the stimulating effect on state 4 respiration was completely blocked and RCR was largely restored. CsA and Bcl-xL inhibit the caspase-3-induced increase of state 4 respiration rate without affecting the rates of state 3 respiration.

3.4. Caspase-3 enhances ROS production and NAD(P)H oxidation in mitochondria

Mitochondria are the major places for the production of superoxide and hydrogen peroxide in the cell. We and others have shown that cyt *c* release from mitochondria could result in an increase in production of ROS in the cells, which is associated with the induction of PTP opening. Thus, we examined the effects of recombinant caspase-3 on ROS production in isolated mitochondria. We used DCFH-DA to measure ROS production in isolated mitochondria [20] and found that caspase-3 increased the rate of ROS generation in isolated mitochondria in a dose-dependent manner (Fig. 4). The induction of ROS reached its highest level when caspase-3 was added at a concentration of 6 U/ml. Bcl-xL, CsA and z-VAD.fmk inhibited the increase of ROS generation. The lev-

els of ROS were measured in the presence of succinate, so it is possible that the measured ROS was produced at the site of complex III in the respiratory chain. Exogenously added superoxide dismutase has no effect on recombinant caspase-3-induced ROS increase.

The ratio of the reduced form versus oxidized form of NAD(P)H is an indicator for the redox potential in the mitochondria. We next examined the effects of recombinant caspase-3 on the oxidation of NAD(P)H and found that caspase-3 induces NAD(P)H oxidation in mitochondria. Also, z-VAD.fmk, Bcl-xL and CsA inhibit the oxidation reaction (Fig. 5).

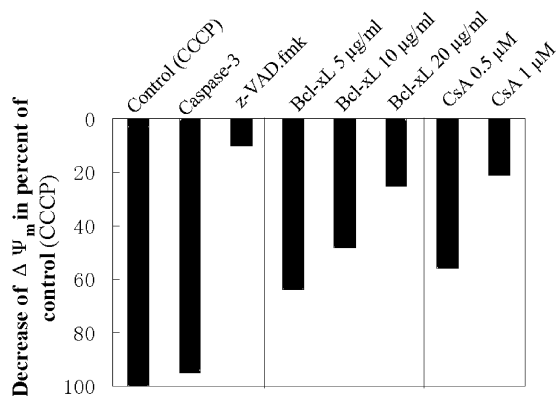


Fig. 3. Recombinant caspase-3-induced loss of membrane potential is inhibited by Bcl-xL and CsA. Mitochondria were incubated in the presence of the caspase-3 (6 U/ml) or caspase-3 pretreated with z-VAD.fmk. Assays were carried out as described in Section 2. 100% decrease of mitochondrial membrane potential was defined using CCCP (10 μ M) as an internal control.

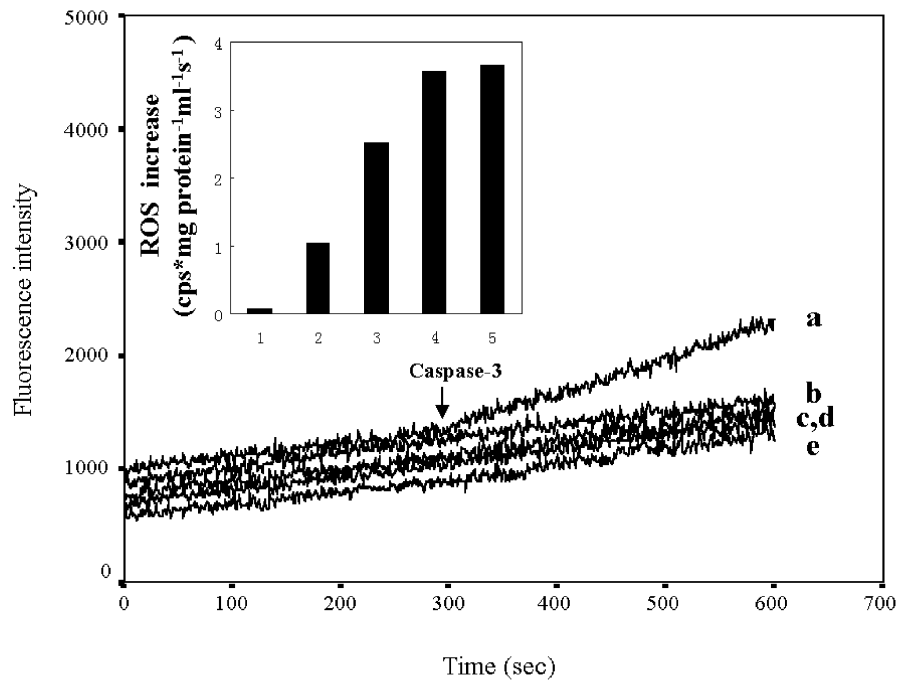


Fig. 4. Recombinant caspase-3 induces an increase of mitochondrial ROS; effects of z-VAD.fmk, Bcl-xL and CsA. Generation of mitochondrial ROS was evaluated as described in Section 2. Trace a, caspase-3 (6 U/ml) was added (arrow). Trace b, caspase-3 (6 U/ml) and z-VAD.fmk (1 μ M) were preincubated before addition at 300 s. Traces c and d, Bcl-xL (16 μ g/ml) or CsA (1 μ M) was preincubated with the mitochondria before caspase-3 (6 U/ml) was added as indicated. Trace e, control. Increase of ROS generation rate was calculated according to the difference of the slope before and after caspase-3 was added. The unit is cps/mg protein/ml/s. Column 1, mitochondria only. Column 2, increase of ROS production rate after addition of caspase-3 (1.5 U/ml). Columns 3–5, addition of caspase-3 reached 3, 4.5 and 6 U/ml, respectively. cps, cycle per second.

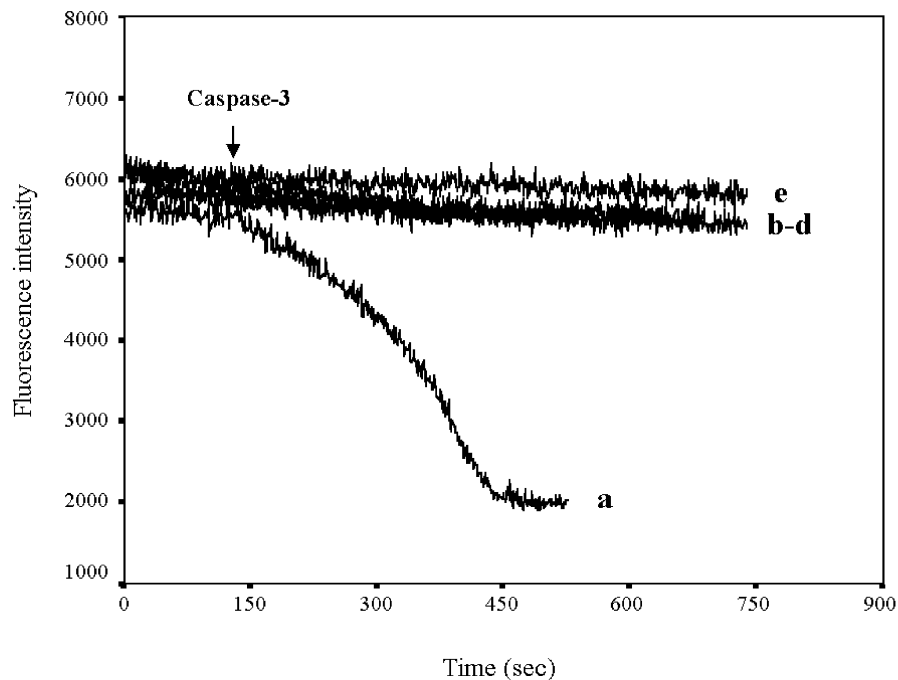


Fig. 5. Caspase-3 induces oxidation of NAD(P)H: effects of z-VAD.fmk, CsA and Bcl-xL. The oxidation of NAD(P)H in the mitochondria was measured as described above. Trace a, caspase-3 (6 U/ml) was added as indicated (arrow). Traces b and c, Bcl-xL (16 μ g/ml) and CsA (1 μ M) was preincubated with mitochondria before caspase-3 (6 U/ml) was added (arrow). Trace d, control, mitochondria only. Trace e, caspase-3 (6 U/ml) was preincubated with z-VAD.fmk (1 μ M) and then the mixture was added to the mitochondrial suspension (1 mg/ml) as indicated (arrow).

4. Discussion

Caspase-3 is a key executioner for the apoptotic phenotype [9,23]. We and others previously showed that recombinant caspase-3 could induce cyt *c* release in a cell-free system [16]. Here we show that caspase-3 directly induces PTP opening and the associated mitochondrial apoptotic phenotype. Thus, we demonstrated that caspase-3 can directly induce the swelling of mitochondria and the collapse of $\Delta\Psi_m$. Our results indicate that the induction of PTP opening and loss of $\Delta\Psi_m$ by caspase-3 is not mediated by cytosolic factors such as tBid as reported, although the target of caspase-induced PTP opening is not known. We and others recently observed that caspase-3 could cleave Bcl-2 and turn Bcl-2 into a Bax-like molecule [16,24]. However, the cleavage of Bcl-2 was detected 2 h after treatment of mitochondria with caspase-3 under identical experimental conditions, while we observed the opening of PTP and loss of $\Delta\Psi_m$ within minutes. Alternatively, caspase-3 could directly target to PTP. Marzo et al. showed that caspase-3 causes disruption of mitochondrial membrane potentials and release of AIF from mitochondria of mouse liver or DiOC₆(3) release from PTP complex-reconstituted liposomes in a dose-dependent fashion and also in a Bcl-2- and Bcl-xL-inhibitable manner [25]. The investigation of the molecular details underlying the action of caspase-3 on mitochondrial PTP in apoptosis is currently under way.

It is of interest to note that in our experiments recombinant caspase-3 could increase mitochondrial respiration rate of state 4 without affecting state 3 respiration. This suggests that caspase-3 does not act on the activity of the mitochondrial respiratory chain per se, but induces mild uncoupling of mitochondrial respiration. We also present evidence showing that caspase-3 induces an increase in ROS production and oxidation of NAD(P)H from mitochondria in a dose-dependent manner. The ratio of NAD(P)H/NAD(P)⁺ is an indicator of the status of redox potential in mitochondria [3]. This is consistent with the results that caspase-3 induces state 4 respiration, which consumes NAD(P)H. These effects were also blocked by z-VAD.fmk, Bcl-xL and CsA, suggesting that the caspase effects on mitochondrial redox are downstream events of mitochondrial PTP opening. However, careful analysis indicates that, upon caspase-3 treatment on mitochondria, NAD(P)H oxidation and increase of ROS happen immediately after caspase-3 addition and NAD(P)H oxidation reaches the highest levels about 4 min after caspase-3 treatment (Figs. 4 and 5). Analysis of the PTP opening reveals that mitochondrial swelling reaches its highest levels at 7 min (Fig. 2). To reconcile these data, we argue that there may be a self-amplifying process of caspase activation, NAD(P)H consumption and PTP opening. It is possible that an increase in ROS generation and increase of state 4 respiration induced by caspase-3 could affect the redox potential in the mitochondria, which then leads to the oxidation of mitochondrial proteins and PTP opening. Indeed, ROS produced by the X/XO system also caused mitochondria swelling with the same kinetics as with caspase-3 (Fig. 2). Redox potential and thiol-disulfide redox state of the ATP/ADP translocator in the inner mitochondrial membrane seems to play a role in controlling PTP opening [26,27]. On the other hand, PTP opening could result in the loss of NAD(P)H from mitochondria.

In summary, we observed that recombinant caspase-3 could

directly induce PTP opening, which is causally related to the loss of mitochondrial membrane potential and cyt *c* release from mitochondria. Furthermore, our data suggested that caspase-3 impacts on the mitochondrial respiration and redox status. These data are consistent with our recent report suggesting that there is a feedback control of cyt *c* release and mitochondrial dysfunction mediated by caspases. Further work is needed to pinpoint targets of caspase-3 on mitochondria.

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