NOTES

Mechanism of mitochondrial respiratory control in caspase-3 induced positive feedback loop in apoptosis

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Abstract **Caspase-3 plays a central role in the execution of apoptosis. Besides many substrates of caspase-3, mitochondria seem to be one of the candidate targets in the apoptotic process. We evaluated the effects of caspase-3 on the isolated mitochondria in detail, and especially focused on the mechanism involved in mitochondrial functions, which were not fully assessed till now. Our results showed that recombinant caspase-3 induced the increase of superoxide production, the dissipation of mitochondrial membrane potential and rate increasing of mitochondrial state 4 respiration. Caspases inhibitor, z-VAD-fmk can inhibit these effects of caspase-3 on mitochondria. Bcl-xL and cyclosporin A were also shown to be able to inhibit these changes. These results suggested a possible mechanism in caspase-3 induced disruption of mitochondrial membrane barrier which formed a positive feedback loop in apoptosis.**

Keywords: mitochondria, caspase-3, ROS, RCR, membrane potential, PTP.

Now it is believed that mitochondria play a critical role in the control of apoptosis[1]. In apoptotic signal transduction, the mitochondria control apoptosis by releasing apoptogenic factors such as cytochrome c[2], apoptosis-inducing factor (AIF)[3] and Smac/DIABLO[4], a process that can be regulated by targeting Bcl-2 family members to the outer mitochondrial membrane[5]. When cytochrome c is released into the cytoplasm, it binds to Apaf-1, then recruits and activates a major apical caspase, caspase 9. Mature caspase 9 activates various effector caspases, including caspase-3, which processes various cellular proteins to execute apoptotic cell death[6].

Currently, there exist two pathways by which upstream caspases are activated to initiate apoptosis. The extrinsic pathways include delivery of granzyme B and TNFR-1/Fas receptor ligation, which activate caspase 8. Alternatively in the intrinsic pathway, the mitochondria receive death signals and release cytochrome c to activate caspase 9. Both activated initiator caspases converge on the activation of caspase-3. The activated caspase-3 can cleave a wide variety of proteins, including many key substrates in the cell, which kill the cell via apoptosis. In our latest results, we found that in genotoxic stress induced apoptosis caspase-3 could induce cytochrome c release from mitochondria leading to mitochondrial dysfunction[7]. So caspase-3 is the key executioner in apoptosis[8].

How does caspase-3 contribute to the apoptotic events? The overall picture is not fully understood. Besides many substrates of caspase-3, mitochondria seem to be one of the candidate targets in the apoptotic process[9]. Caspase-3 could induce disruption of the mitochondrial membrane potential. Furthermore, pre-incubation of mitochondria with a low dose of recombinant caspase-3 increases the sensitivity of mitochondria to Ca^{2+}-induced permanent disruption of the $\Delta\Psi_m$, which is a sign of mitochondrial permeability transition[9]. Another experiment of the same group shows that $DiOC_6(3)$ induced by caspase-3 is released in a dose-dependent fashion using PTP reconstituted in liposomes whereas they have no effects on protein-free liposomes[10]. From the results above, we can see that caspases may act on PTP to disrupt mitochondria membrane barrier function, which forms a circular self-amplification loop to accelerate the apoptotic process and maybe coordinate the apoptotic response between different mitochondria within the same cell[10].

Stimulated by these findings, we decided to further evaluate the effects of caspase-3 on the isolated mitochondria *in vitro* in detail, and especially focused on mitochondrial superoxide generation, mitochondrial respiration and mitochondrial permeability transition, which were not fully evaluated till now. Our results provided a novel mechanism of caspase-3 induced disruption of mitochondrial membrane barrier function, in which respiratory control was involved.

1 Materials and methods

(ⅰ) Materials. Sucrose and IPTG were purchased from Life Technologies (Grand Island, N.Y.). BSA, ADP, CCCP, cyclosporin A rhodamine 123, rotenone, xanthine, xanthine oxidase and dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Sigma (St. Louis, MO). Glutathione Sepharose 4B was from Amersham Pharmacia Biotech (Uppsala, Sweden). Ni-NTA agarose was from Qiagen (Germany). Caspase inhibitor (z-VAD-fmk) was obtained from Biomol Research Lab. Triton X-100 and Hepes were products of Merck. Others were analytical grade reagents from commercial sources in China.

(ⅱ) Isolation of mitochondria. Mitochondria were

isolated from the liver tissue of Balb/c mice. Briefly, the livers were taken after sacrifice and dounce homogenized with a glass Teflon Potter homogenizer. Mitochondria were isolated in 250 mmol/L sucrose, 2 mmol/L Hepes, pH 7.4, 0.1 mmol/L EDTA, 0.1% fatty acid-free BSA. All steps were carried out at 4℃. The samples were centrifuged at 1000×*g* for 10 min. The supernatant was transferred to another tube and centrifuged at 10000×*g* for 10 min. Mitochondria were washed twice, then resuspended and stored on ice for up to 4 h[11]. Protein content of mitochondria was determined by the biuret method using BSA as standard.

(iii) Expression and purification of recombinant proteins. DH5α bacterial cells containing a pGEX-2TK expression vector with the full-length human Bcl-xL were treated with 0.1 mmol/L isopropyl-1-thio-β-D-galactopyranoside for 4 — 6 h at 37℃. Bacterial cell pellets were lysed in 1 mmol/L DTT, 1% Triton X-100, 1 mmol/L PMSF in PBS by FRENCH pressure cell press at 1300 PSIG at 4℃. Cell lysates were then centrifuged at 10000×*g* for 10 min at 4℃. The supernatant was loaded onto a Glutathione Sepharose 4B column, and then the column washed with PBS. Bound GST-Bcl-xL protein was eluted from the column by 10 mmol/L reduced glutathione in 50 mmol/L Tris, pH 8.0. His-tagged pET-CPP32, encoding recombinant caspase-3 was transformed into *E. coli* BL21 and purified by Ni-NTA agarose (Qiagen) affinity purification according to supplier's protocol. The freshly prepared caspase-3 protein was evaluated by cleaving the DEVD-*p*NA substrate before being used for further experiments. For certain experiments, the purified caspase-3 was first incubated with 1 μmol/L z-VAD-fmk for 10 min at room temperature. All purified proteins finally were suspended in the same control buffer composed of 20 mmol/L Hepes-K^+ (pH 7.4) and 1 mmol/L DTT[12].

(iv) Determination of ROS production. Generation of mitochondrial ROS was evaluated in isolated mitochondria by using DCFH as a probe as previously described[13] with some modification. Briefly, in the basal state, the assay buffer contained 225 mmol/L sucrose, 20 mmol/L KCl, 5 mmol/L $MgCl_2$, 10 mmol/L KH_2PO_4, 2 mmol/L Tris (pH 7.4) and 4.2 mmol/L succinate as substrate. Isolated mitochondria were incubated with 5.0 μmol/L DCFH-DA in the buffer at 37℃ for 15 min to allow DCFH-diacetate to cross mitochondrial membrane. The solution was then centrifuged at 10000×*g* for 8 min, and the supernatant containing excess DCFH-diacetate was discarded. The mitochondrial pellets were resuspended, and 1 mg/mL protein was used for assay. DCF formation was done at the excitation wavelength of 488 nm and emission wavelength of 525 nm for 30 min by using a Jobin-Yvon FluoroMax-2 spectrofluorimeter at 37℃. The rate of DCFH conversion to DCF was linear for at least 60 min, corrected with the autooxidation rate of DCFH without protein. All assays were carried out in triplicates.

(v) Test of mitochondrial membrane potential. Isolated mitochondria (1 mg/mL) were incubated at 25℃ in the medium containing 250 mmol/L sucrose, 2 mmol/L Hepes, pH 7.4, 0.5 mmol/L KH_2PO_4 and 4.2 mmol/L potassium succinate to energize mitochondria. $\Delta\psi_m$ was assessed by measuring the membrane potential dependent uptake of rhodamine 123 (Rh123) using a spectrofluorimeter (Jobin-Yvon FluoroMax-2) with excitation at 505 nm and emission at 534 nm after addition of 30 nmol/L Rh123 to a mitochondria suspension as previously described in ref. [14].

(vi) Oxygen consumption. By using a Clark oxygen electrode (YSI Model 53 Oxygen Monitor, USA), caspase-3 induced changes of mitochondrial oxygen consumption rates in succinate-stimulated state 4 and ADP-stimulated state 3 were monitored at 25℃ in 3 mL medium containing 225 mmol/L sucrose, 20 mmol/L KCl, 5 mmol/L $MgCl_2$, 10 mmol/L KH_2PO_4, 2 mmol/L Tris (pH 7.4) and 2 μmol/L rotenone. The concentration of mitochondria was 1.0 mg/mL in all the experiments unless otherwise stated. The oxygen consumption rate of mitochondria under different respiratory states and RCR was calculated as described in ref. [11].

(vii) Caspase-3 induces NAD(P)H oxidation. The oxidation of NAD(P)H in the mitochondrial suspension (1 mg/mL) was followed in a spectrofluorimeter (Jobin-Yvon FluoroMax-2) operating with a slit width of 1 nm at excitation and emission wavelengths of 350 and 450 nm, respectively as described in ref. [15]. The reaction medium contains 250 mmol/L sucrose, 2 mmol/L Hepes, pH 7.4, 0.5 mmol/L KH_2PO_4 and 4.2 mmol/L potassium succinate at 25℃.

2 Results

(i) Caspase-3 induces increase of state 4 respiration and respiratory control ratio. To elucidate the direct effect of caspase-3 on mitochondrial bioenergetics, the isolated mouse liver mitochondria were incubated with recombinant caspase-3, and the mitochondrial respiratory rate of succinate-stimulated state 4 and ADP-stimulated state 3 was measured as described in sec. 1. After that the respiratory control ratio was calculated. Addition of caspase-3 induced an increase of the rate of state 4 respiration, whereas addition of caspase-3 pre-incubated with

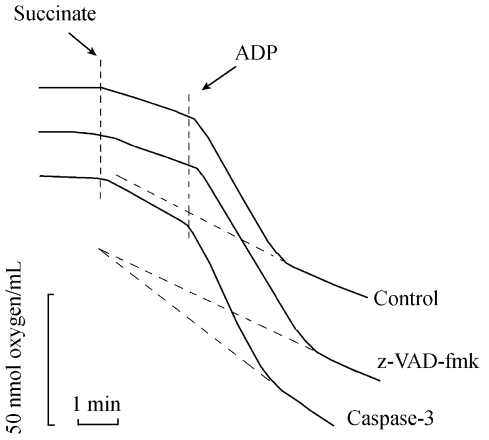

Fig. 1. Caspase-3 increases the rate of state 4 respiration. Trace control, only succinate and ADP were present. Trace z-VAD-fmk, caspase-3 was first preincubated with z-VAD-fmk for 15 min at 25℃, then caspase-3 (6 U/mL) mixture was added to the mitochondrial suspension to test respiration. Trace caspase-3, the protein (6 U/mL) was added to the mitochondria suspension, then oxygen consumption was monitored.

caspase inhibitor z-VAD-fmk inhibited such an increase (fig. 1). Caspase-3 has no effect on the rate of state 3 res piration. These results suggest that the caspase-3 stimulates state 4 respiration, which is probably the result of reduced mitochondrial membrane potential.

(ii) Caspase-3 inducing membrane potential loss is dependent on PTP. Mitochondrial function is usually monitored by cationic fluorescent probes that are accumulated in response to the $\Delta\psi_m$ in the mitochondrial matrix of isolated mitochondria. We used rhodamine 123 to test the possibility that the membrane potential loss in duced by caspase-3 was also a result of PTP. Rhodamine 123 was used to detect the membrane potential changes after caspase-3 was added. All the experiments were monitored in a time-based manner to directly detect the whole process. We found that caspase-3 can induce membrane potential loss, and this process can also be inhibited by caspase inhibitor z-VAD-fmk, permeability transition pore specific inhibitor cyclosporin A and Bcl-xL inhibit this process, indicating that caspase-3 induced membrane potential loss was PTP-dependent (fig. 2).

(iii) Caspase-3 induces an increase of ROS production. Generation of reactive oxygen species (ROS) was evaluated in the isolated mitochondria by using DCFH as a probe as described previously[16]. The DCFH assay is sensitive to reactive oxygen intermediates in mitochondrial matrix. Once defusing across mitochondrial membrane, DCFH-diacetate is cleaved by an esterase, the resultant DCFH can be oxidized to the highly fluorescent dichlorofluorescein (DCF). We used this probe to directly detect the rate of reactive oxygen species production in time-based mode after adding caspase-3 to the isolated mitochondria in the medium. With this method, we found that caspase-3 protein could increase the rate of ROS generation in the isolated mitocho ndria pre-loaded with DCF-DA (fig. 3(a)). The caspase inhibitor z-VAD-fmk can inhibit the increase of ROS generation rate (fig. 3(b)). These results indicate that caspase-3 can induce the increase of ROS generation rate, and this activation is also inhibited by Bcl-xL, an anti-apoptotic protein, which acts on PTP and cyclosporin A, as a specific inhibitor of PTP.

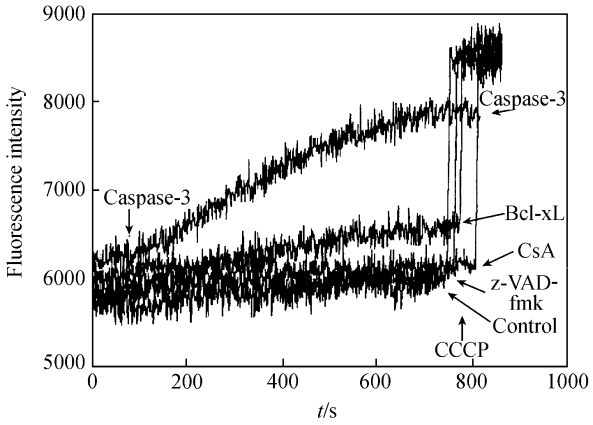

Fig. 2. Caspase-3 induces membrane potential losses in mitochondria and the effects of z-VAD-fmk, Bcl-xL and CsA. Trace caspase-3, protein (6 U/mL) was added (arrow). In trace z-VAD-fmk, caspase-3 (6 U/mL) and z-VAD-fmk (1 μmol/L) were incubated for 15 min first, then the mixture was added at 100 s. Trace Bcl-xL and CsA, the medium was supplemented with Bcl-xL (16 μg/mL) and CsA (1 μmol/L), then caspase-3 (6 U/mL) was added as indicated. Trace control, no additions. CCCP was used as internal control in all the experiments.

(iv) Caspase-3 induces NAD(P)H oxidation. NAD(P)H redox state can manifest the oxidative status in mitochondria. We found that caspase-3 could induce NAD(P)H oxidation, and gave further evidence for the capacity of caspase-3 to induce ROS increase. z-VAD-fmk, Bcl-xL and CsA could inhibit the process. This result shows the protease activity of caspase-3 and mitochondrial permeability transition pore can influence the mitochondrial redox status (fig. 4).

3 Discussion

In this note, we demonstrate that recombinant caspase-3 can increase superoxide generation and can also bring out disruption of membrane barrier function through inducing mitochondrial permeability transition. How can caspase-3 achieve this goal? As we know, the protease function caspase-3 is executed by cleaving many important protein substrates after aspartic acid, which brings out

apoptotic phenotype. So it is reasonable that the superoxide generation increase, respiration rate increase and the following membrane permeabilizing effect of caspase-3 are results of its cleavage effect on specific protein substrates in the outer mitochondrial membranes.

Marzo et al.[10] also found that caspase-3 had substrates located on mitochondria, especially on the permeability transition pore complex, whose cleavage would disrupt mitochondrial membrane integrity. Then what is the mechanism of caspase-3 inducing disruption of mitochondrial membrane barrier? The whole picture is not clear now.

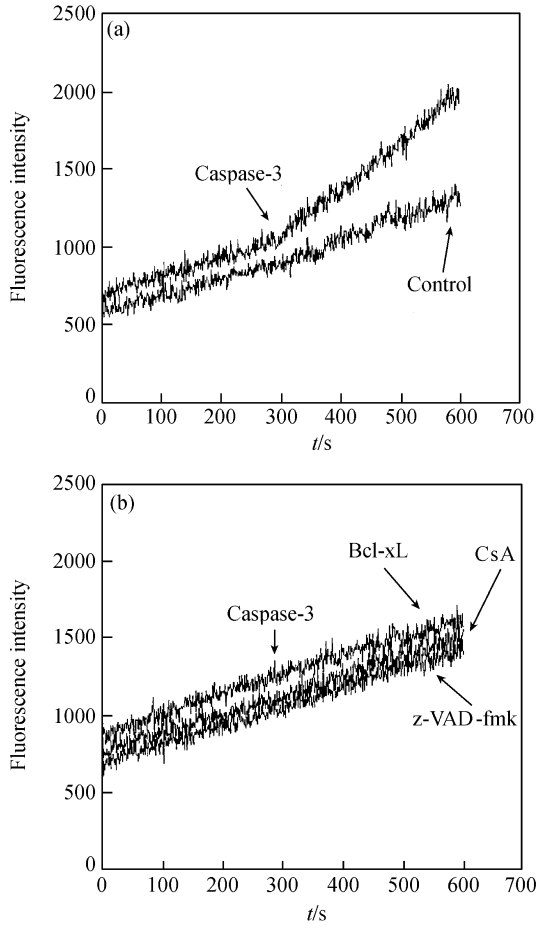

Fig. 3. Caspase-3 induces the increase of mitochondria reactive oxygen species; and the effects of z-VAD-fmk, Bcl-xL and CsA. (a) Caspase-3 (6 U/mL) was added as indicated, other trace was control; (b) trace z-VAD-fmk, caspase-3 (6 U/mL) and z-VAD-fmk (1 μmol/L) were incubated for 15 min first, then added at 300 s. Trace Bcl-xL and CsA, Bcl-xL (16 μg/mL), CsA (1 μmol/L) was first incubated with the mitochondria, then caspase-3 (6 U/mL) was added as indicated.

First, we found that recombinant caspase-3 could increase the rate of mitochondrial state 4 respiration (fig. 1), but had no effect on mitochondrial state 3 respiration, which resulted in a reduced RCR. This result demonstrates that caspase-3 does not act on mitochondrial respiratory chain directly, and it acts on the parts that regulate the mitochondrial respiration. z-VAD-fmk, Bcl-xL and cyclosporin A can inhibit the effect of caspase-3 on mitochondrial state 4 respiration, which completely or partially restore RCR. This result proves that the effect of caspase-3 on mitochondrial respiration depends upon the protease activity and the permeability transition pore, and suggests the relationship between the status of mitochondrial permeability transition pore and the regulation of mitochondrial respiration.

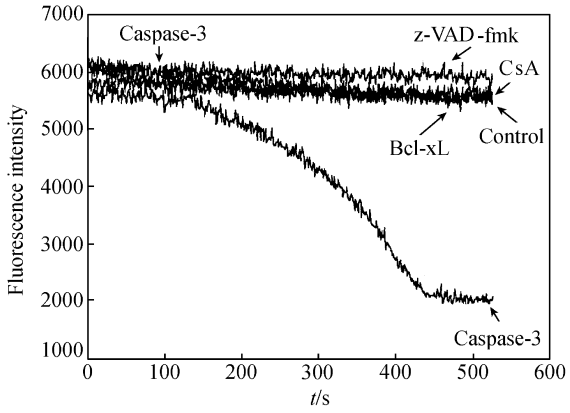

Fig. 4. NAD(P)H oxidation in isolated SD rat mitochondria induced by capase-3. Capase-3 and the inhibitors are added same as fig. 3 with excitation and emission wavelength of 350 nm and 450 nm, respectively.

In fig. 2, we prove that caspase-3 can also induce the mitochondrial membrane potential dissipation. z-VAD-fmk can inhibit the loss of $\Delta\psi_m$ induced by caspase-3. Bcl-xL and cyclosporin A can also inhibit the $\Delta\psi_m$ loss and the inhibitory effects are dose-dependent. As we know, mitochondria state 4 respiration is controlled by mitochondrial $\Delta\psi_m$, and reduction in mitochondria $\Delta\psi_m$ increases the rate of mitochondrial state 4 respiration[17]. This result shows that caspase-3 induces the mitochondrial $\Delta\psi_m$ drop, followed by increase of the rate of mitochondria state 4 respiration. These changes are dependent upon the protease activity of caspase-3, and mediated by mitochondrial PTP.

As we all know, during mitochondrial state 4 respiration, respiratory chain complex Ⅰ and complex Ⅲ produce superoxide and other reactive oxygen species through single electron leakage, and superoxide can induce mitochondrial membrane potential reduction to form a positive feedback loop [18]. So we believe that the stimulation of mitochondria state 4 respiration and mitochon-

drial membrane potential drop by caspase-3 are relevant to mitochondrial ROS production. Fig. 3 shows that caspase-3 indeed induces the increase of ROS generation rate, and the increase is dose-dependent. As above, the stimulation of ROS production is dependent upon the protease activity of caspase-3 and mitochondrial PTP. The result in another assay shows that addition of SOD cannot quench the ROS production (data not shown), while dithionite can quench the ROS production. This result shows that caspase-3 inducing ROS production enhancement is located in the mitochondrial matrix since SOD cannot enter mitochondrial matrix but dithionite can. We showed in the past experiments that the superoxide produced in mitochondrial state 4 respiration or exogenous ROS generated by xanthine/xanthine oxidase could bring out proton leakage, which resulted in mitochondrial $\Delta\psi_m$ reduction[11]. So we inferred that caspase-3 induced stimulation of mitochondrial state 4 respiration, and membrane potential reduction was related to mitochondrial ROS production. The oxidation ratio of NAD(P)H and NAD(P) can manifest the redox status in the mitochondrial matrix. The increased oxidation of NAD(P)H (fig. 4) also demonstrates ROS increasing in mitochondria.

Based upon the above results, we argue that the feedback loop induced by caspase-3 is important for those cells which cannot have a full-blown apoptotic process with relatively little caspase-3. Caspase-3 not only amplifies the apoptotic signal through this feedback loop, but also destroys much more mitochondria to cause redox status changes and energy crisis in the cell, which make apoptosis irreversible. This positive feedback loop of caspase-3 on mitochondria is helpful, sometimes necessary to the cell apoptotic process.

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