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Caspase 3 is Activated through Caspase 8 instead of Caspase 9 during H_2O_2-induced Apoptosis in HeLa Cells

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Key Words

Caspase • Hydrogen peroxide • Bax • Apoptosis

Abstract

Oxidative stress is known to be involved in a variety of pathological processes including atherosclerosis, diabetes, and neurodegenerative diseases. Understanding how intracellular signaling pathways respond to oxidative stress will have a significant implication in the therapy of these diseases. In this study, we applied hydrogen peroxide (H_2O_2) to trigger apoptosis and investigated the dynamic activation of various caspases using a FRET technique. We measured the activation dynamics of caspase 3 and caspase 9 based on two reporter systems, SCAT 3 and SCAT 9. We found that caspase 3 activation was earlier than that of caspase 9 following H_2O_2 treatment. Caspase 3 was activated rapidly, reaching a maximum in 12±3 min, while the average duration of caspase 9 activation was 21±3 min. When cells were pretreated with Z-LEHD-fmk, a caspase 9 specific inhibitor, caspase 3 activation and apoptosis by H_2O_2 treatment were little affected, although the caspase 9 activation was completely inhibited. When cells were pretreated with Z-DEVD-fmk, a caspase 3 specific inhibitor, the activation of both caspase 3 and caspase 9, as well as apoptosis, were inhibited. When cells were pretreated with Z-IETD-fmk, a caspase 8 specific inhibitor, the activation of caspase 3 and caspase 9 were significantly delayed. Finally, we found that Bax did not translocate from the cytosol to the mitochondrial membrane during H_2O_2-induced apoptosis. Our results suggest that, during H_2O_2-induced apoptosis, caspase 3 is activated directly through caspase 8 and is not through the mitochondria-dependent caspase 9 activation.

Introduction

Reactive oxygen species (ROS) produced through a variety of cellular processes or derived from exogenous sources play an important role in the regulation cell proliferation, survival, senescence, and apoptotic cell death [1]. Unchecked and excessive production of ROS, recognized as oxidative insult or stress, could result in severe damage to cellular components including DNA, proteins, and lipids. Oxidative stress has been implicated in a variety of human diseases including atherosclerosis, diabetes, arthritis, cancer, and neurodegenerative disorders [1]. Thus, understanding how cellular signaling pathways respond to oxidative insults such as hydrogen peroxide

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(H_2O_2) may lead to novel strategies for therapeutic interventions.

Reactive oxygen species (ROS) are known to induce a wide range of responses dependent on cell type and the levels of ROS within the cell [2, 3]. At low concentration, it acts as a second messenger for several signaling pathways [4], whereas high level of ROS is shown to induce apoptotic cell death. The mechanisms of cell death induced by ROS, especially by H_2O_2, are not yet fully known. One possible mechanism of H_2O_2-induced apoptosis is through the activation of ASK1-JNK [5]. Several studies have provided accumulative evidence that caspases play a pivotal role in H_2O_2-induced apoptosis [6, 7].

There are 14 members of the caspase family in mammals [8], seven in *Drosophila melanogaster* [9], and three in *Caenorhabditis elegans* [10]. During the extrinsic and intrinsic apoptotic pathways, caspase 8, 9 and 3 are the most important components. In the extrinsic apoptotic pathway, caspase 8 is first activated by the death receptors and it then activates caspase 3. In the intrinsic pathway, caspase 9 is first activated by mitochondria-released cytochrome c and it then activates caspase 3. Interestingly, caspase 9 is also reported to be a substrate of caspase 3 during apoptosis [11]. To date, the activation pathway of these caspases in H_2O_2-induced apoptosis is still remaining to be elucidated.

In this study, we used the fluorescence resonance energy transfer (FRET) technique to directly measure the temporal relationship between the activation of caspase 3 and caspase 9. FRET is a process that transfer of energy occurs from a donor to an acceptor fluorophore molecule in close proximity [12]. By construction of a reporter protein with a donor and an acceptor site, this technique has enabled researchers to monitor caspase activation at the single-cell level in real time [13, 14]. Recently, two reporter proteins named SCAT3 and SCAT9 were developed to observe the dynamics of caspase 3 and caspase 9 activation [15]. They consist of cyan fluorescent protein (CFP) as a donor and a variant of EYFP protein (Venus) as an acceptor linked with the DEVD sequence (SCAT3) or the LEHD sequence (SCAT9), which can be cleaved by activated caspase 3 or caspase 9. Using the SCAT3 and SCAT9 FRET probes, we examined the dynamic activation of caspase 3 and caspase 9 in HeLa cells under different conditions. We found that caspase 3 is activated before caspase 9, suggesting that caspase 8 is the initiator caspase for caspase 3 activation during H_2O_2-induced apoptosis. Our findings suggest that the mitochondria-dependent intrinsic pathway is not the major apoptotic pathway involved in the H_2O_2-induced apoptosis in HeLa cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, USA). Z-IETD-fmk (caspase 8 inhibitor), Z-DEVD-fmk (caspase 3 inhibitor) and Z-LEHD-fmk (caspase 9 specific inhibitor) were purchased from BioVision (Mountain View, USA). Lipofectamine reagent was purchased from Invitrogen (Carlsbad, USA). DNA extraction kit was purchased from Qiagen (Valencia, USA). Hydrogen peroxide was purchased from Sigma. SCAT 3 and SCAT 9 were kindly supplied by Dr. Masayuki Miura [15]. pCFP-Bax was kindly supplied by Richard J.Youle [16]. pDsRed-Mit was kindly supplied by Dr. Y. Gotoh [17].Other chemicals were mainly from Sigma (St. Louis, USA).

Cell culture and treatment

HeLa cells were cultured in DMEM supplemented with 15% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 mg/ml) with 5% CO2 at 37 ºC in a humidified incubator. Transfection was performed with Lipofectamine reagent according to the manufacturer's protocol. The medium was replaced with fresh culture medium after 5 h. Cells were examined 24-48 h after transfection. For the generation of stable cell lines, transfected cells were selected in the presence of G418 (1 mg/ml) for 2 weeks and fluorescent clones were enriched. Cells were pretreated with Z-IETD-fmk (10 μM), Z-DEVD-fmk (40 μM) and Z-LEHD-fmk (40 μM) for 1 h respectively before H_2O_2 treatment. The inhibitors were kept in the medium throughout the experimental process.

FRET analysis

For analysis of caspase 3 and caspase 9 activation in HeLa cells stably expressing SCAT 3 and SCAT 9, we detected the dynamics of caspases activation at the single cell level by FRET analysis. FRET was performed on a commercial laser scanning microscope (LSM 510/ConfoCor2) combination system (Zeiss, Jena, Germany). For excitation, the 458 nm line of an Ar-ion laser was attenuated with an acousto-optical tunable filter, reflected by a dichroic mirror (main beam splitter HFT 458), and focused through a Zeiss Plan-Neofluar 40x/1.3 NA oil DIC objective onto the sample. CFP and Venus (FRET-acceptor) emission was collected through 470–500 nm and 535–595 nm barrier filters, respectively. The quantitative analysis of the fluorescence images was performed using Zeiss Rel 3.2 image processing software (Zeiss). After background subtraction, the average fluorescence intensity per pixel was calculated. The onset of caspase 3 and caspase 9 activation were defined as the time point at which the Venus/CFP emission ratio irreversibly declined. During control experiments, bleaching of the probe was negligible.

CFP-Bax translocation assay

To observe the translocation of Bax, HeLa cells were co-transfected with CFP-Bax and DsRed-Mit. Using Zeiss LSM 510 confocal microscope, we imaged both the distribution pattern of CFP-Bax and that of DsRed-Mit simultaneously during UV and H_2O_2-induced apoptosis. Confocal images of CFP-Bax fluorescence from the cells were obtained using a 458 nm excitation light from an argon laser and a 470-500 nm band-pass filter. Images of DsRed-Mit fluorescence from the cells were obtained using a 543 nm excitation light from a He-Ne laser and a 600-650 nm band-pass filter. Cells were maintained at 37 °C using the temperature regulator in the whole experiment process. Temperature elevations of culture liquid were less than 0.2 °C throughout the experimental process. Bax redistribution was assessed by the matching fluorescence of CFP-Bax and DsRed-Mit emission. The cells exhibiting strong punctate staining of CFP, which overlapped with the distribution of DsRed, were counted as the cells with mitochondrially localized Bax.

Caspase assays

Cells were washed twice with ice-cold PBS and lysed for about 30 min at 4 °C with gentle rotation in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate) in the presence of a cocktail of protease inhibitors. Cell lysates were clarified by centrifugation at 4 °C for 15 min at 16,100 g, and a Bradford protein assay was performed on the supernatant. 50 ug of total protein was mixed with 2×caspase assay buffer: 25 mM HEPES-NaOH, pH 7.4, 5 mM DTT and a 100 uM concentration of one of the following caspase fluorogenic substrates: Ac-DEVD-AFC (for caspase 3), Ac-IETD-AFC (for caspase 8), and Ac-LEHD-AFC (for caspase 9). After incubation at 37 °C for 3 h, the fluorometric detection of cleaved AFC product was performed on a CytoFluor Multi-Well Plate Reader Series 4000 (PerSeptive Biosystems) using a 400 nm excitation filter and a 530 nm emission filter. For preparation of the AFC calibration curve, 80 uM free AFC was diluted in the caspase assay buffer without substrate to give 1.6, 3.2, and 4.8 umol of free AFC, and fluorescence was measured on the fluorometer.

Confirmation of cell apoptosis

HeLa cell lines were plated into 96-well plates at $3.0x10^3$ cells/well for 24 h. The cells were then divided into five groups and were treated with 0 (control), 75, 150, 300, 600, 1200 uM H_2O_2 for 8 h respectively. 4 h before the termination of simulation, 20 µl 3-(4,5-dimethythiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) solution was added to each well. Then, the cells were washed and solubilized with 150µl dimethyl sulphoxide (DMSO). The absorbance was measured on a spectrometer, using a test wavelength of 570 nm and a reference wavelength of 655 nm.

Flow cytometry

For quantification of cells in early and late apoptosis, the indicated cells were collected and washed with ice-cold PBS before being fixed with 75% ethanol. The fixed cells were incubated with annexin-V-FITC/PI for 10-15 min at room temperature, according to the manufacturer's protocol (Pharmingen) and analyzed by flow cytometry. Cells (10^4) were analyzed by a FACScan cell sorter (Becton-Dickinson, Franklin Lakes, NJ, USA). Dot plots were prepared using FlowJo software (Tree Star, San Carlos, CA, USA).

Results

H_2O_2-induced apoptosis in HeLa cells

In order to study H_2O_2-induced apoptosis, we treated HeLa cells with various concentration of H_2O_2 for 8 h and examined the cell numbers by an MTT assay The results indicated that the cell number (measured by A_{570} value) was decreased when the H_2O_2 concentration was increased (Fig. 1A), suggesting that H_2O_2 treatment induced apoptosis in HeLa cells. This effect was obviously in a dose-dependent manner. The time-dependence of H_2O_2-induced cell death was also examined by treating cells with 600 uM of H_2O_2 for different time lengths (Fig. 1B, open columns).

To determine whether caspase 9, 3 and 8 are involved in H_2O_2-induced apoptosis, we treated the cells with Z-LEHD-fmk (a caspase 9 specific inhibitor), Z-DEVD-fmk (a caspase 3 inhibitor), or Z-IETD-fmk (a caspase 8 inhibitor), for 1 h before H_2O_2 treatment. Our MTT assay results showed that Z-LEHD-fmk had little effect on the H_2O_2-induced cell number decrease while Z-DEVD-fmk had a very strong inhibitory role on the H_2O_2 treatment (Fig. 1B, black columns). We observed a moderate inhibitory role of Z-IETD-fmk on the H_2O_2-induced cell number decrease (Fig. 1B). Consistently, a flow cytometry assay with annexin staining to detect apoptosis indicated that many cells were undergoing apoptosis after H_2O_2 treatment (36.8 %) and caspase 3 inhibitor Z-DEVD-fmk blocked H_2O_2-induced apoptosis (8.3%) (Fig. 1C-D). These results indicated that the H_2O_2-induced apoptosis may depend on the caspase 3 activity but not on the activity of caspase 9. This result was somewhat surprising since caspase 9 is known to be an upstream mediator for caspase 3 activation during the process of mitochondia-dependent apoptosis [18].

Real-time monitoring of caspase 9 activation during H_2O_2-induced apoptosis in living HeLa cells

To observe the dynamics of caspase 9 activation during H_2O_2-induced apoptosis in living cells, we performed a real-time FRET measurement in HeLa cells stably expressing the SCAT 9 reporter. Typical time-dependent images of the SCAT 9 reporter activation are shown in Fig. 2A. We observed that the fluorescence

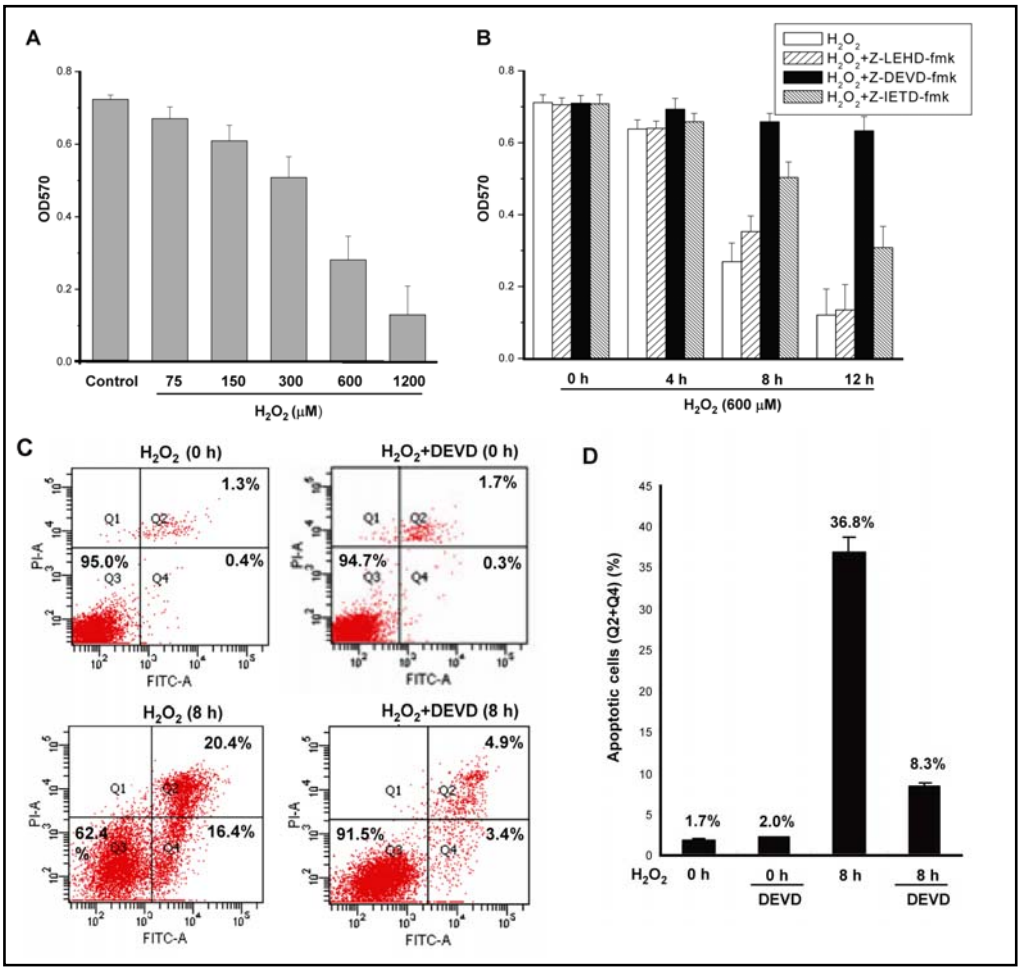


Fig. 1. H_2O_2 induces apoptosis in HeLa cells. (A) H_2O_2 induces apoptosis in HeLa cells in a dose dependent manner. Cells viability was analyzed by a MTT assay at 8 h after various concentration of H_2O_2. (B) H_2O_2 induced apoptosis is inhibited by caspase 3 inhibitors, delayed by caspase 8 inhibitor, little affected by caspase 9 inhibitor. Cell viability was assessed by a MTT assay at 0 (control), 4, 8, and 12 h after 600 uM H_2O_2 treatment in the presence or absence of 10μM Z-IETD-fmk, 40μM Z-DEVD-fmk or 40μM Z-LEHD-fmk. Error bars are calculated from four independent experiments. (C-D) H_2O_2 induced apoptosis is blocked by caspase 3 inhibitors. Flow cytometry experiment (C) was performed using HeLa cells under the indicated conditions and a quantitative presentation of apoptotic cell population is shown (D).

intensity ratio between Venus and CFP decreased abruptly at about 405 min after H_2O_2 treatment and reached a stable level in 20 min (Fig. 2B). This result implied that caspase 9 was activated at that time. To determine the average initiation time and duration of caspase 9 activation, we compared the dynamics of caspase 9 activation in three individual cells treated with H_2O_2. The average initiation time of caspase 9 activation was 405±10 min after H_2O_2 treatment, and the duration of caspase 9 activation was 21±3 min (Fig. 2C).

The dynamics of caspase-3 activation during H_2O_2-induced apoptosis in living HeLa cells

To measure the activation of caspase 3 within a single cell during H_2O_2-induced apoptosis, we examined the dynamics of caspase 3 activation in HeLa cells stably expressing the SCAT 3 reporter. Typical time-dependent images of the SCAT 3 reporter activation are shown in Fig. 3A. We found that the FRET ratio between Venus and CFP decreased abruptly at about 390 min after H_2O_2 treatment, implying that caspase 3 activation was initiated at 390 min after H_2O_2 treatment and reached its maximum activation in 10 min (Fig. 3B). The average initiation and duration of caspase 3 activation after H_2O_2 treatment were determined to be 390±10 min and 12±3 min, respectively (Fig. 3C).

Since there was a 15 min gap (405 min via 390 min) between the activation initiation times of caspase 9 and caspase 3 following the H_2O_2 treatment, the activation of caspase 3 appears to occur earlier than that of caspase 9. This result suggests that caspase 3 acts upstream of caspase 9 and thus caspase 9 could not be the initiator of caspase 3 activation during H_2O_2-induced apoptosis.

Comparing the activation of caspases 9, 3 and 8 after the H_2O_2 treatment

To analyze the relationship among caspase 9, 3 and 8 activation during H_2O_2-induced apoptosis, we treated HeLa cells with different caspase inhibitors for 1 h before addition of H_2O_2. The FRET analysis results showed that the FRET ratio of SCAT 9 remained unchanged after H_2O_2 treatment in the presence of either Z-LEHD-fmk or Z-DEVD-fmk but decreased at about 635 min in the presence of Z-IETD-fmk (Fig 4A).

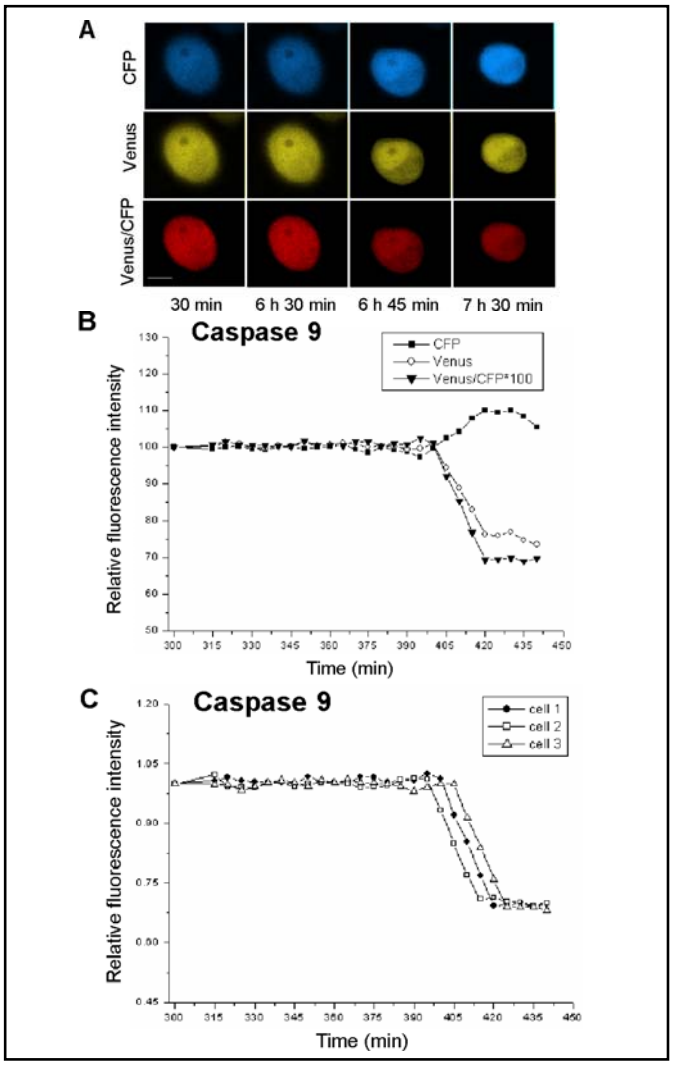


Fig. 2. Dynamics of caspase 9 activation during H_2O_2-induced apoptosis in living cells. (A) The typical time-course images of SCAT 9 during H_2O_2 –induced apoptosis. HeLa cells stably expressing SCAT 9 were treated with H_2O_2 (600 uM). The fluorescence images were acquired by confocal microscopy. Scale bar=10 μm. (B) The intensities of CFP emission, YFP emission and YFP/CFP ratio correspond to the data in (A). (C) The dynamics of caspase 9 activation (YFP/CFP ratio) in three individual cells during H_2O_2-induced apoptosis. Similar results were obtained from four separate experiments.

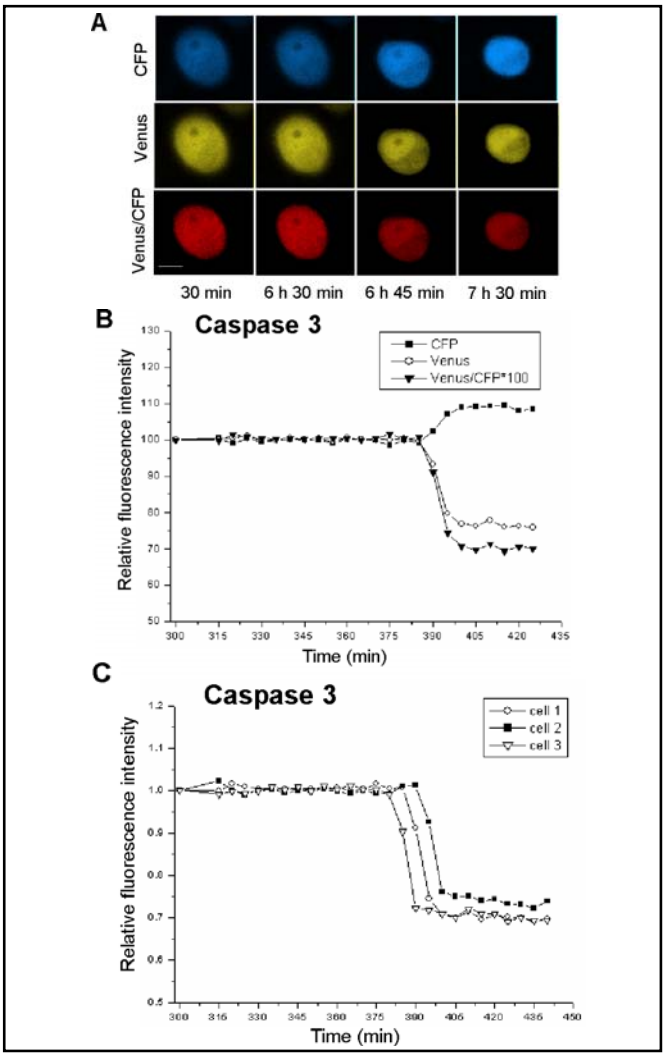


Fig. 3. Real-time examination of caspase 3 activation during H_2O_2-induced apoptosis in living cells. (A) The typical time-course images of SCAT 3 during H_2O_2 -induced apoptosis. HeLa cells stably expressing SCAT 3 were treated with H_2O_2 (600 uM). The fluorescence images were acquired by confocal microscopy. Scale bar=10 μm. (B) The intensities of CFP emission, YFP emission and YFP/CFP ratio correspond to the data in (A). (C) The dynamics of caspase 3 activation (YFP/CFP ratio) in three individual cells during H_2O_2-induced apoptosis. Similar results were obtained from four separate experiments.

This result indicated that caspase 9 activation was fully inhibited by Z-LEHD-fmk, an inhibitor of caspase 9, and Z-DEVD-fmk, an inhibitor of caspase 3, and delayed by Z-IETD-fmk, an inhibitor of caspase 8. Our data suggested that the activation of caspase 3 and 8 occurred upstream of caspase 9 activation during H_2O_2-induced apoptosis. Apparently, activation of caspase 3 was absolutely required for the activation of caspase 9 in this process.

To analyze the activation of caspase 3 during H_2O_2-induced apoptosis, we examined the FRET ratio in the HeLa cells with the SCAT 3 reporter. Our results showed that the caspase 9 inhibitor Z-LEHD-fmk had no effect on the activation of caspase 3 (Fig. 4B), again suggesting that activation of caspase 3 is upstream of caspase 9. Interestingly, we observed that the caspase 8 inhibitor Z-IETD-fmk delayed the activation of caspase 3 (Fig. 4B), suggesting that activation of caspase 8 is upstream of caspase 3 activation.

To further confirm the FRET experiment results, we used a caspase activity assay kit to directly examine the activities of caspase 9, 3 and 8 under the H_2O_2

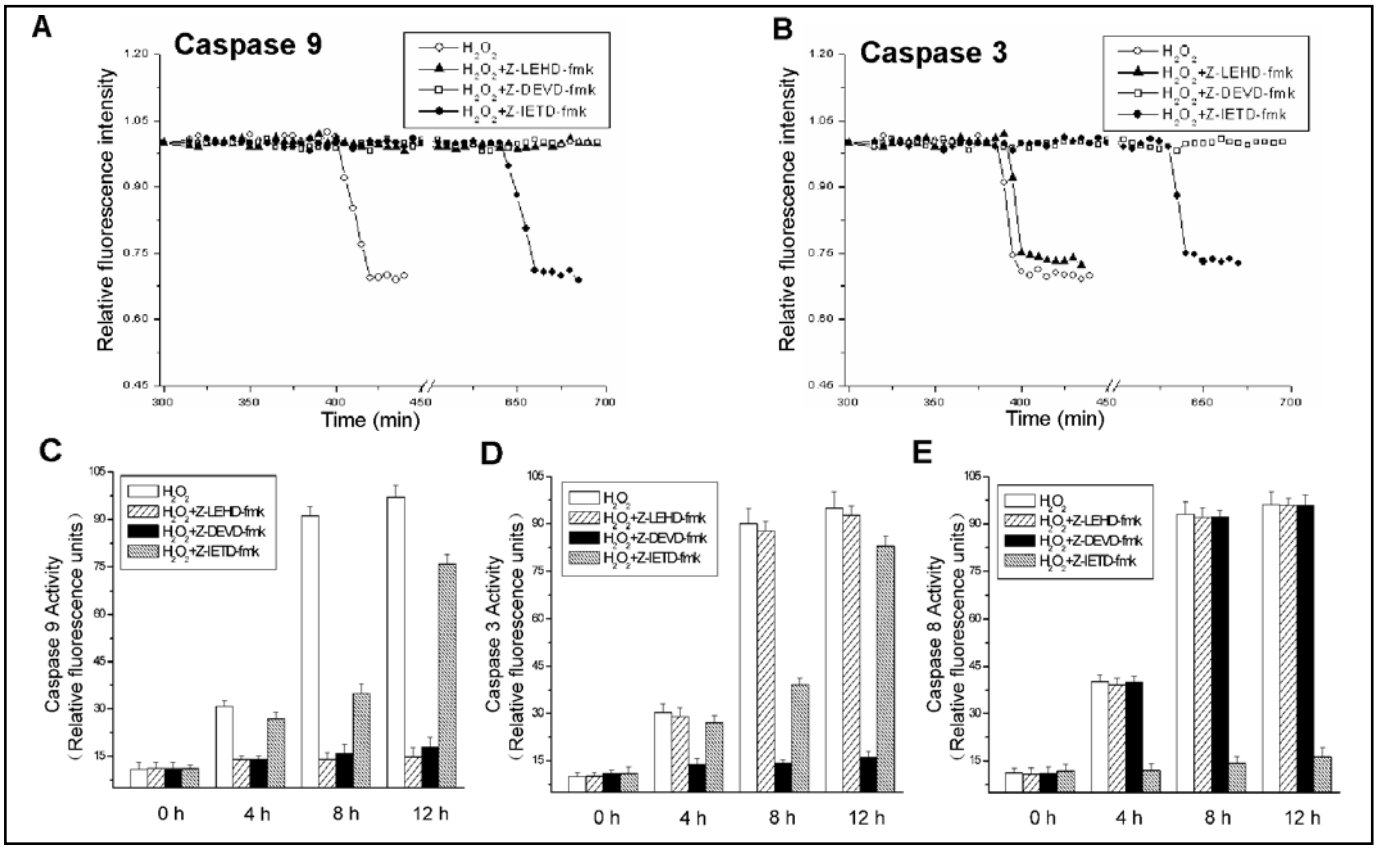


Fig. 4. The relationship among caspase 9, 3 and 8 activation by H_2O_2 treatment (A,B) FRET analysis of caspase 9 (SCAT 9) (A) or caspase 3 (SCAT 3) (B) activation by H_2O_2 treatment. HeLa cells stably expressing SCAT 9 or SCAT 3 were pretreated with the indicate inhibitor 1 h before addition of H_2O_2 (600 uM). The fluorescence images were acquired by a confocal microscopy. YFP/CFP ratio was performed using Zeiss Rel 3.2 image processing software. (C-E) The analysis of caspase 9 (C), caspase 3 (D) or caspase 8 (E) activation during H_2O_2-induced apoptosis. HeLa cells were pretreated with the indicate inhibitor 1 h before addition of H_2O_2 (600 uM), and the caspase activation was assessed by caspase assay at 0 (control), 4, 8, and 12 h after 600 uM H_2O_2 treatment. Similar results were obtained from four separate experiments.

treatment. These results were consistent with those obtained from the FRET experiments (Fig. 4C, 4D). Furthermore, when we examined the caspase 8 activity after H_2O_2 treatment, we found that both the caspase 3 and 9 inhibitors Z-DEVD-fmk and Z-LEHD-fmk failed to inhibit the activation of caspase 8 while Z-IETD-fmk inhibited the caspase 8 activity strongly (Fig. 4E). Taken together, these data suggested that caspase 8 is an initiator caspase for caspase 3 during H_2O_2-induced apoptosis.

Bax did not translocate to mitochondria during H_2O_2-induced apoptosis

The intrinsic apoptotic pathway is known to involve translocation of pro-apoptotic members of the Bcl-2 protein family (such as Bax or truncated Bid) from cytosol to the mitochondrial membrane [19, 20], which triggers the release of cytochrome c into the cytosol. Cytochrome c binds with the apoptosis-activating factor 1 (APAF-1), and induces APEF-1 to assemble into a multimeric caspase activation platform termed 'apoptosome', which recruits and activates caspase 9 and subsequently trigger the activation of the downstream effector caspase 3 [21]. Since our study indicated that caspase 3 was activated upstream of caspase 9 during H_2O_2–induced apoptosis, we would like to examine whether the mitochondria-dependent pathway is involved in the activation of this process.

Thus, we tested whether Bax was translocated during H_2O_2-induced apoptosis by transient co-transfection of CFP-Bax and DsRed-Mit, a marker for mitochondria, in HeLa cells. As a positive control, image measurement using a LSM microscope showed that CFP-Bax was translocated into mitochondria in 5 h and 45 min after UV irradiation (Fig. 5A). When cells were treated with 600 uM H_2O_2, we found that CFP-Bax did not translocate to mitochondria for as long as 7 h and 45 min (Fig. 5B). A statistic analysis showed that Bax translocation occurred in more than 90% of the cells treated with UV irradiation, while it

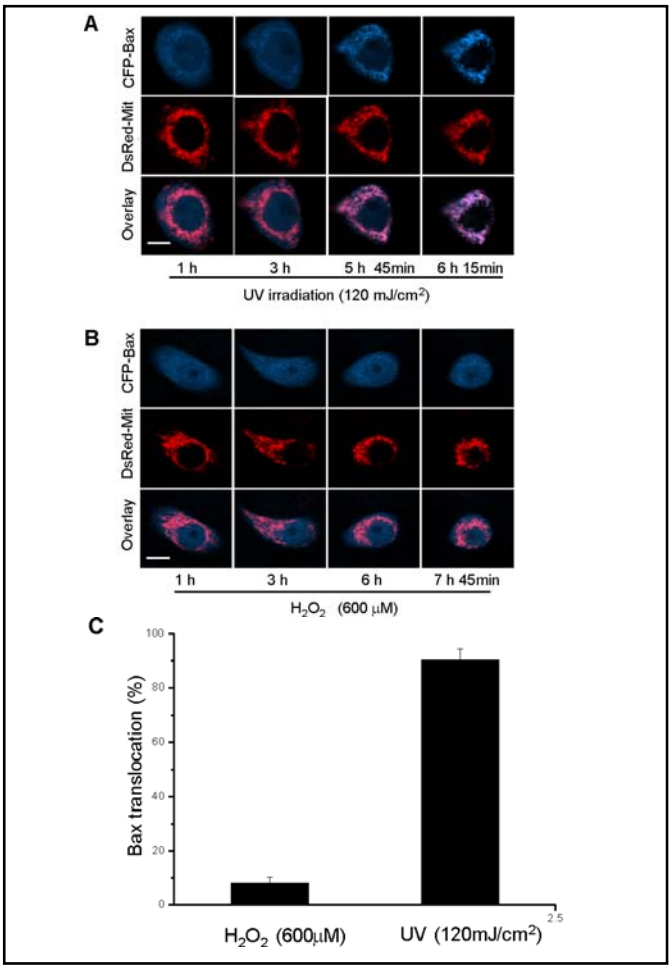
Fig. 5. Bax is not involved in H_2O_2-induced apoptosis. (A) Bax translocates to mitochondria during UV-induced apoptosis. HeLa cells transiently co-expressing CFP-Bax and DsRed-Mit. The image was captured by a laser fluorescence confocal microscopy. (B) Bax is not redistributed to mitochondrial in the apoptosis induced by H_2O_2 treatment. Scale bar=10 μm. (C) A statistic analysis of Bax translocation under H_2O_2 treatment and UV irradiation. Data are representative of three independent experiments.

occurred in less than 10% of cells with H_2O_2 treatment (Fig. 5C). Considering that caspase 3 was activated at 6 h and 30 min after applying H_2O_2, the activation of caspase 3 apparently was not dependent on Bax translocation. Hence, we conclude that the mitochondria pathway is not involved in the H_2O_2-induced apoptosis.

Discussion

Hydrogen peroxide induced apoptosis has been widely studied. The major effect of H_2O_2 is the generation of reactive oxygen species (ROS), which is also an important event for many chemotherapeutic agents. In most of the reports, ROS mediated apoptosis is linked to the intrinsic pathway of apoptosis [22]. The first step for the ROS mediated apoptosis is the activation of Bax, which aggregates into mitochondria and results in the release of cytochrome c. This event leads to the activation of caspase 9 and then caspase 3. In this study, we found that hydrogen peroxide did not activate Bax translocation from cytosol to mitochondria. Furthermore, it activated caspase 3 upstream of caspase 9. During H_2O_2-induced apoptosis, caspase 9 activation was inhibited by a caspase 3 inhibitor, while caspase 3 activation was not affected by the caspase 9 inhibitor. On the other hand, activation of both caspase 3 and caspase 9 was significantly delayed by inhibiting the activation of caspase 8. Based on these observations, we concluded that H_2O_2 must activate capspase 8, which acts as an initiator caspase for caspase 3 activation (Fig. 6). Since caspase 3 is known to have a broad spectrum of substrates, including caspase 9, after the activation of caspase 3, caspase 9 could also be activated through cleavage of its pro-domain [11]. The activation of caspase 9 thus is a side effect and does not appear to play a significant role in the H_2O_2-induced apoptotic pathway.

It is known that caspase 8 can activate caspase 3 either directly or through the truncation of Bid, which subsequently activates the mitochondria-dependent pathway. Our data suggest that in H_2O_2-induced apoptosis,



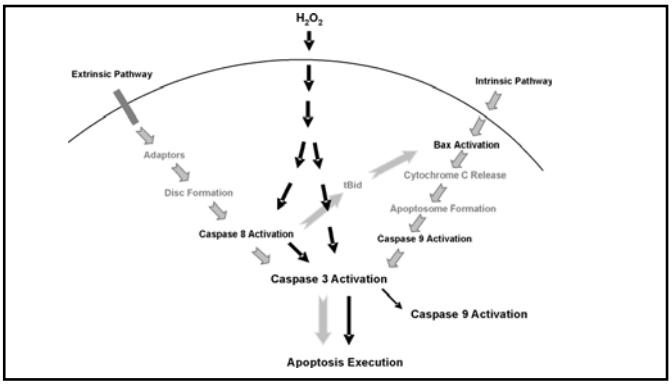


Fig. 6. A model of H_2O_2-induced apoptosis. In this model, the dark lines indicate the pathways of H_2O_2-induced apoptosis. Briefly, H_2O_2 activates caspase 8, which then activates caspase 3. H_2O_2 also activates caspase 3 independent of caspase 8. Caspase 3 activates caspase 9 during H_2O_2-induced apoptosis. The light lines demonstrate the intrinsic and extrinsic pathways of apoptosis induced by different stimuli.

caspase 8 must activate caspase 3 directly (Fig. 6), since activation of caspase 3 was not dependent on caspase 9. On the other hand, we observed that inhibiting caspase 8 could not entirely prevent the activation of caspase 3, but just delayed it. It seems possible that hydrogen

peroxide might trigger additional unknown pathways to activate caspase 3. We still do not know the exact mechanisms for H_2O_2 to activate caspase 8 or caspase 3 but it is speculated that another candidates exit during the activation of caspsase 3 by H_2O_2. It is well known that H_2O_2 generates ROS in the cells and ROS could stimulate a variety of cell signaling cascades including PI3K/AKT and MEK/ERK pathways. ROS could also act as secondary messengers [23, 24] or exerts a non-specific damaging role on DNA and proteins [25, 26]. Therefore, ROS mediated apoptosis could recruit a variety of diverse mechanisms. The detailed molecular mechanism on how ROS activates capspase 8 or caspase 3 needs further investigation.

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