

Oxidative modification of caspase-9 facilitates its activation via disulfide-mediated interaction with Apaf-1

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Intracellular reactive oxygen species (ROS) are known to regulate apoptosis. Activation of caspase-9, the initial caspase in the mitochondrial apoptotic cascade, is closely associated with ROS, but it is unclear whether ROS regulate caspase-9 via direct oxidative modification. The present study aims to elucidate the molecular mechanisms by which ROS mediate caspase-9 activation. Our results show that the cellular oxidative state facilitates caspase-9 activation. Hydrogen peroxide treatment causes the activation of caspase-9 and apoptosis, and promotes an interaction between caspase-9 and apoptotic protease-activating factor 1 (Apaf-1) via disulfide formation. In addition, in an *in vitro* mitochondria-free system, the thiol-oxidant diamide promotes auto-cleavage of caspase-9 and the caspase-9/Apaf-1 interaction by facilitating the formation of disulfide-linked complexes. Finally, a point mutation at C403 of caspase-9 impairs both H₂O₂-promoted caspase-9 activation and interaction with Apaf-1 through the abolition of disulfide formation. The association between cytochrome *c* and the C403S mutant is significantly weaker than that between cytochrome *c* and wild-type caspase-9, indicating that oxidative modification of caspase-9 contributes to apoptosome formation under oxidative stress. Taken together, oxidative modification of caspase-9 by ROS can mediate its interaction with Apaf-1, and can thus promote its auto-cleavage and activation. This mechanism may facilitate apoptosome formation and caspase-9 activation under oxidative stress.

Keywords: oxidative modification, ROS, caspase-9, apoptosis, disulfide

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Introduction

Aerobic cells unavoidably produce reactive oxygen species (ROS) as a result of their metabolic activities and are consequently vulnerable to oxidative stress originating from the intra- or extracellular environments. Oxidative

stress is usually defined as a deteriorative process in which ROS modify numerous cellular components, including proteins, nucleic acids and lipids [1]. Although cells can counter and adapt to oxidative stress by activating diverse mechanisms that detoxify ROS and repair cellular damage, the major outcome of oxidative stress is cell death; modestly increased ROS levels induce apoptosis, whereas severe oxidative damage leads to necrosis [2]. Caspase-9 is an initial caspase in the mitochondrial apoptotic cascade that is activated in response to the insult of oxidative stress [3]. However, the precise mechanism by which caspase-9 molecules respond to increased ROS, specifically, the nature of the association between oxidative stress and caspase-9 activation, remains controversial.

Caspases are named after their properties of acting as cysteine proteases that specifically cleave substrates after aspartic acid residues [4]. Because cysteines provide thiol groups to proteins and allow them to be reversibly modified by oxidants and reductants, the fundamental position of cysteine in the caspase family implies that these proteins may be redox-sensitive. The cysteines present in

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Abbreviations: β -ME (beta mercaptoethanol); As₂O₃ (arsenic trioxide); DTT (dithiothreitol); F5M (fluorescein-5-maleimide); FITC (fluorescein isothiocyanate); H₂O₂ (hydrogen peroxide); IP (immunoprecipitation); IB (immunoblot); Mn-SOD (manganese superoxide dismutase); MW (molecular weight); NAC (*N*-acetyl-L-cysteine); OSC cells (oral squamous carcinoma cells); OSC-SA (OSC SOD-antisense); OSC-WT (OSC wild type); PI (propidium iodide); ROS (reactive oxygen species); TNF- α (tumor necrosis factor- α)

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the catalytic sites of caspases are nucleophilic groups that usually must be kept in their reduced form to maintain enzyme activity. S-nitrosylation of these critical cysteine residues can inhibit caspase activity [5-7], and apoptotic stimuli, such as the Fas ligand, induce denitrosylation of the cysteine in the catalytic site of caspase-3, leading to its activation [6]. These data suggest that caspase activity requires catalytic cysteines to be in their reduced form [8].

On the other hand, growing evidence demonstrates that ROS serve as an important mediator in caspase-9-dependent apoptotic processes [9-12]. Not only can intra- or extracellular oxidants directly induce apoptosis in which caspase-9 is inevitably activated, but also various drugs and cytokines can kill cells via the mitochondrial apoptotic pathway accompanied by ROS generation, and blockage of ROS rescues cells from caspase-9-dependent apoptosis [10-12]. Because many proteins undergo oxidative modifications during apoptotic events in which cysteines are oxidized to form disulfide bonds [9, 13], it has been suggested that caspase-9 may be activated through oxidative modification. We found in our previous studies that the enzymatic activity of caspase-9 was promoted by an intracellular ROS producer, emodin [14, 15]. These observations seem to conflict with the notion that the cysteines in the caspase catalytic site must be kept in a reduced form.

Caspase-9 activation is initiated by the formation of apoptosomes. During apoptosis, cytochrome *c* is released from mitochondria into the cytoplasm, in which it binds to apoptotic protease-activating factor 1 (Apaf-1) and, in the presence of dATP or ATP, these components assemble into a complex called the apoptosome [16, 17]. A recent study demonstrated caspase-9 dimer formation in response to oxidative stress inside of the mitochondria, during which initiation of the intra-mitochondrial auto-cleavage of caspase-9 preceded the onset of the caspase cascade in the cytosol [9]. This work has pointed out the essential role of oxidative modification in initiating caspase activation. In addition, it has been found that ROS promote apoptosome formation by oxidizing Apaf-1, consequently promoting activation of caspase-9 and -3 [10]. These findings indicate that ROS may trigger caspase-9 activation by pathways other than the stimulation of cytochrome *c* release from mitochondria.

In the present study, we investigate the direct effects of ROS and the thiol-oxidizing agent diamide on caspase-9 molecules in an attempt to elucidate the molecular mechanisms by which oxidative stress induces caspase-9 activation in the cytosol, regardless of actions derived from the mitochondria. The results demonstrate that H₂O₂ and diamide can oxidatively modify caspase-9, which facilitates the interaction of caspase-9 and Apaf-1 through

the formation of disulfide bond within a complex and subsequent cleavage of caspase-9, ultimately promoting caspase-9 activation. This finding explicates one of the fundamental mechanisms by which oxidative stress initiates cell apoptosis.

Results

ROS facilitates caspase-9 activation

As₂O₃ has been reported to induce cell apoptosis through an ROS-dependent mechanism. As shown in Figure 1A, As₂O₃ treatment increased caspase-9 activity. The increase could be prevented by *N*-acetyl-L-cysteine (NAC) pre-treatment, indicating that ROS was required for As₂O₃-induced apoptosis. Interestingly, pre-treatment with NAC could also abolish caspase-9 activity induced by TNF- α , an extrinsic pro-apoptotic reagent. To further determine the role of ROS in apoptosis, we compared the caspase-9 activity in TNF- α -treated OSC-WT and OSC-SA cells, two cell lines with different ROS levels. OSC-SA cells exhibited decreased superoxide dismutase (SOD) expression and increased ROS levels relative to OSC-WT cells (Figure 1B, upper panel). TNF- α -induced caspase-9 activity was higher in the OSC-SA cells than in the OSC-WT cells. Similarly, NAC pre-treatment could abolish the activation of caspase-9 induced by TNF- α in both of these cell lines (Figure 1B, lower panel). These

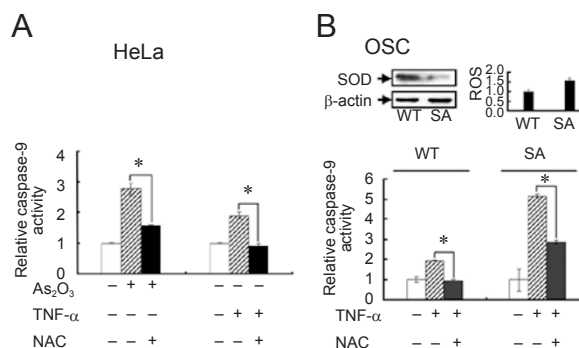


Figure 1 ROS facilitated caspase-9 activation induced by arsenic trioxide and TNF- α . **(A)** HeLa cells were stimulated by 0.5 μ M arsenic trioxide (As₂O₃) for 10 h or 10 ng/ml TNF- α for 20 h. Cells were pre-incubated with 1.5 mM NAC for 4 h. Caspase-9 activity was determined by the intensity of cleaved fluorescence-LEHD. **(B)** Oral squamous carcinoma (OSC) cells stably expressing antisense transcripts of manganese superoxide dismutase (Mn-SOD) were named OSC-SOD-antisense (OSC-SA), whereas the parental OSC strain were named OSC-WT. Expression of SOD and ROS levels in OSC-SA cells and OSC-WT cells were analyzed by immunoblot and DCFH flow cytometry (upper panel). OSC cells were treated with 10 ng/ml TNF- α for 20 h after pre-incubation with 3 mM NAC for 4 h. Caspase-9 activity was determined and samples were duplicated. The values are representative of three independent experiments. **P* < 0.05.

results suggest that ROS could facilitate caspase-9 activation.

H₂O₂ induces the activation of caspase-9

To directly show the role of ROS in caspase-9 activation, HeLa cells were treated with different concentrations of H₂O₂, and the auto-cleavage and activity of caspase-9 were subsequently examined. As shown in Figure 2A, levels of the 35 kDa cleaved fragment and caspase-9 activity increased in H₂O₂-treated cells in a dose-dependent manner. We also determined the level of apoptosis in these cells using Annexin-V/PI flow cytometry and Hoechst 33342/PI double staining, revealing that the H₂O₂ treatment did induce apoptosis (Figure 2B).

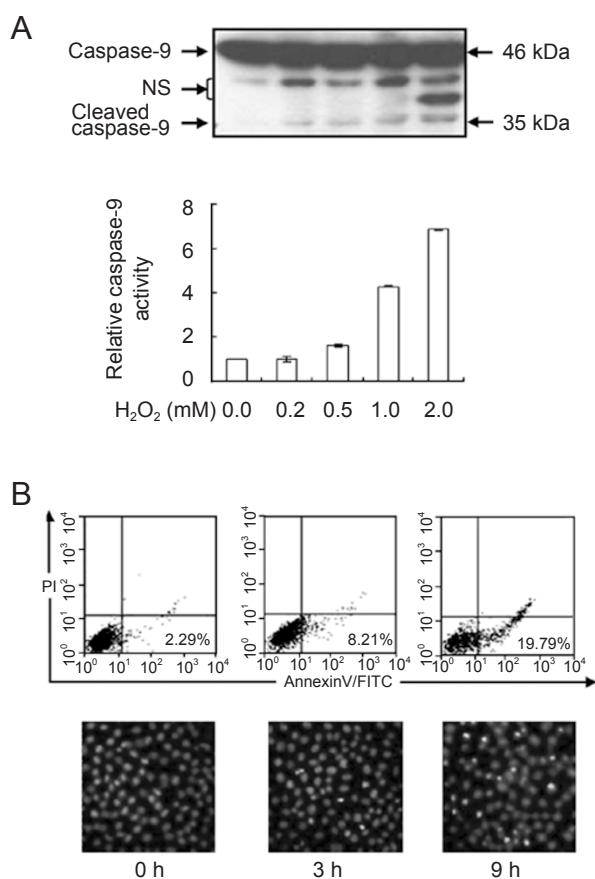


Figure 2 H₂O₂ treatment caused auto-cleavage and activation of caspase-9 and, consequently, cell apoptosis. **(A)** HeLa cells were treated with H₂O₂ at various concentrations for 5 h. Auto-cleavage fragment (35 kDa) and caspase-9 activity were determined. NS indicates non-specific bands. Samples were duplicated in each experiment. The values are representative of three independent experiments. **(B)** HeLa cells were stimulated by 2 mM H₂O₂ for various times. Apoptosis rates were monitored by AnnexinV-FITC/PI flow cytometry and Hoechst 33342/PI cell staining. The figures are representative of three experiments.

H₂O₂ promotes the disulfide-mediated interaction between caspase-9 and Apaf-1

The caspase-9 protein contains cysteines that can be targets for oxidative modification by ROS. To determine whether oxidative modification of caspase-9 occurred in H₂O₂-treated cells, F5M staining was performed to examine the oxidative status of caspase-9. As shown in Figure 3A, the fluorescence of caspase-9 was reduced significantly in H₂O₂-treated cells, indicating that the H₂O₂ treatment could induce oxidation of caspase-9.

During the apoptotic process, Apaf-1, caspase-9 and other components form a complex and together activate caspase-9 activity [16, 18]. We reasoned that oxidative modifications of caspase-9 might affect complex formation. The interaction of caspase-9 and Apaf-1 was thus examined in H₂O₂-treated cells. The oxidative effects of H₂O₂ are likely to manifest in the form of disulfide bridges, which may facilitate inter-protein binding. To determine the disulfide-mediated interaction of caspase-9 and Apaf-1 in H₂O₂-treated cells, co-immunoprecipitation (co-IP) was performed. Cell lysates were immunoprecipitated with an antibody against caspase-9 and subjected to electrophoresis under either non-reduced or reduced conditions. As shown in Figure 3B, H₂O₂ treatment promoted the formation of a complex that contained both caspase-9 and Apaf-1, which could be seen in the non-reduced condition. In the reduced condition, the complex disappeared and the amount of Apaf-1 monomers increased, indicating that the integrity of the complex was maintained by disulfide bridges. A redox diagonal electrophoresis method was also used to detect the formation of intermolecular disulfides (Supplementary information, Figure S1). Immunoblotting for Apaf-1 was performed after the caspase-9 blots were stripped. As shown in Figure 3C, a small fraction of both caspase-9 and Apaf-1 appeared at a molecular weight of greater than 175 kDa in the first dimension, whereas their monomeric positions were revealed in the second dimension, at 46 kDa for caspase-9 and at 135 kDa for Apaf-1, indicating that caspase-9 and Apaf-1 were present in a disulfide-linked complex in H₂O₂-treated cells.

Diamide promotes the disulfide-mediated interaction of caspase-9 with Apaf-1 and induces activation of caspase-9 in a mitochondria-free system

ROS can also induce cytochrome *c* release from mitochondria, which can then bind to Apaf-1 to initiate caspase-9 activation [12, 19]. It was thus possible that cytochrome *c* release induced by H₂O₂ might be a mechanism to explain the enhanced interaction of caspase-9 and Apaf-1. To determine the direct effects of oxidative modification on the activation of caspase-9,

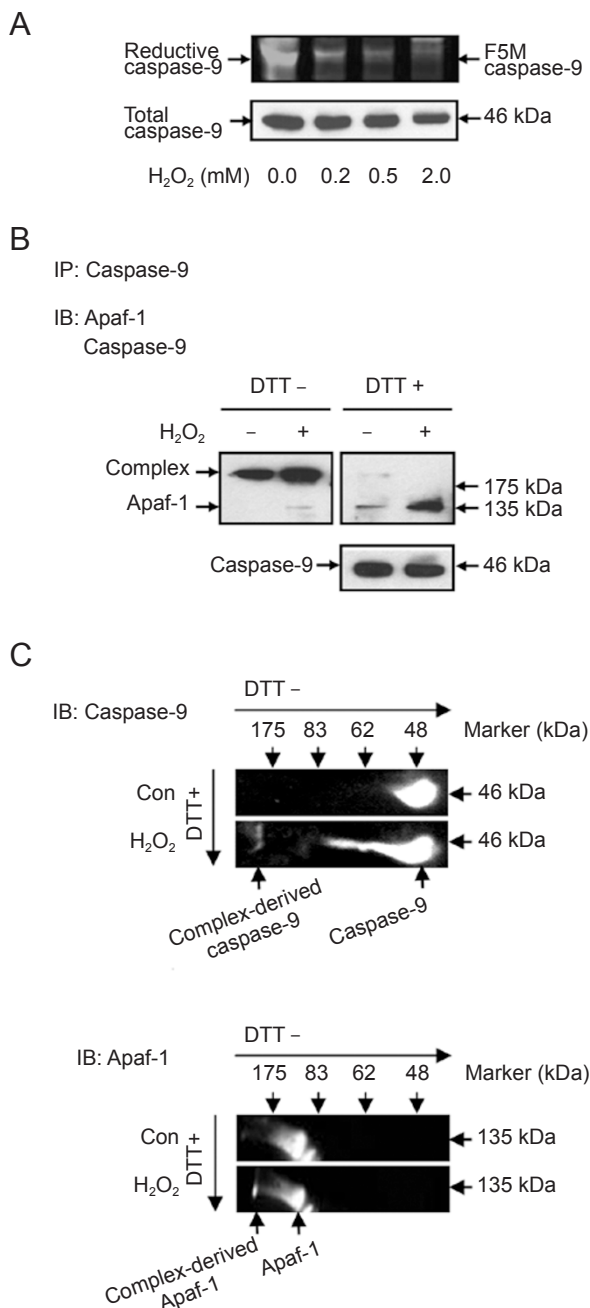


Figure 3 H_2O_2 induced oxidation of thiols in caspase-9 and promoted the caspase-9/Apaf-1 interaction by the formation of disulfides in a protein complex. **(A)** HeLa cells were treated with H_2O_2 at various concentrations for 1 h before incubation with reduced thiol-specific conjugating fluorescence F5M (see Materials and Methods). **(B)** HeLa cells were exposed to 2 mM H_2O_2 for 4 h. The interaction between caspase-9 and Apaf-1 was analyzed by co-immunoprecipitation and electrophoresis in non-reducing or reducing conditions before immunoblot. The figures are representative of three experiments. **(C)** Disulfide-mediated interaction of caspase-9 and Apaf-1 in a protein complex was determined by redox diagonal electrophoresis. Immunoblotting for caspase-9 and Apaf-1 was performed on the same membrane, probing for Apaf-1 after the caspase-9 antibody was stripped. The figures are representative of two experiments.

we used a mitochondria-free system (Figure 4A). The mitochondria-free cytoplasm was treated with diamide, a thiol oxidant. Caspase-9 activation as represented by the level of the 35 kDa cleaved fragments could be induced dose-dependently by diamide in these mitochondria-free extracts, which contained equal concentrations of cytochrome *c* (Figure 4B). The redox diagonal electrophoresis assay (Figure 4C) showed that diamide treatment resulted in caspase-9 (upper) and Apaf-1 (lower) interaction through disulfide bridges. These results suggest that oxidative modifications of caspase-9 could underlie its ROS-induced activation, which did not depend on increase of cytochrome *c*.

Cysteine 403 is responsible for oxidative modification of caspase-9

Caspase-9 contains 13 cysteine residues [16]. To map the oxidation-sensitive sites responsible for H_2O_2 -induced activation of caspase-9, we individually mutated 12 of these cysteines to serines, excluding C287, which is required for activation. These caspase-9 mutants were transfected into cells and followed by H_2O_2 treatment. The cell lysates were analyzed by routine and redox diagonal SDS-PAGE electrophoresis followed by IB with an antibody against the His tag. As shown in Figure 5A, the cleaved fragment of caspase-9 was markedly decreased in the C403S mutant in comparison with its wild-type counterpart. Consistent with this result, redox diagonal electrophoresis showed no interaction between the C403S mutant caspase-9 and Apaf-1 in the H_2O_2 -treated cells (Figure 5B), indicating that C403 of caspase-9 was required for the disulfide-based interaction of caspase-9 with Apaf-1. The other cysteine mutants of caspase-9 displayed similar levels of cleavage and Apaf-1 interactions compared with the wild-type protein (data not shown).

To further address the inter-relationship of the disulfide-linked complex with classical apoptosomes, we examined whether the C403S mutation affected the interaction of caspase-9 with cytochrome *c* or the formation of apoptosomes. After cells were transfected with the wild-type or mutated caspase-9 constructs and exposed to H_2O_2 , the exogenous tagged caspase-9 molecules were pulled down with Ni-IDA columns and IB was performed to detect Apaf-1 and cytochrome *c* in these extracts. Apoptosome formation was indicated by the presence of Apaf-1 and cytochrome *c* in the wild-type caspase-9 co-pull-down complex. Significantly less Apaf-1 and cytochrome *c* were detected in the C403S mutant caspase-9 pull-down samples (Figure 5C). These results suggest that oxidative modification of C403 promotes the disulfide-mediated interaction of caspase-9 with Apaf-1,

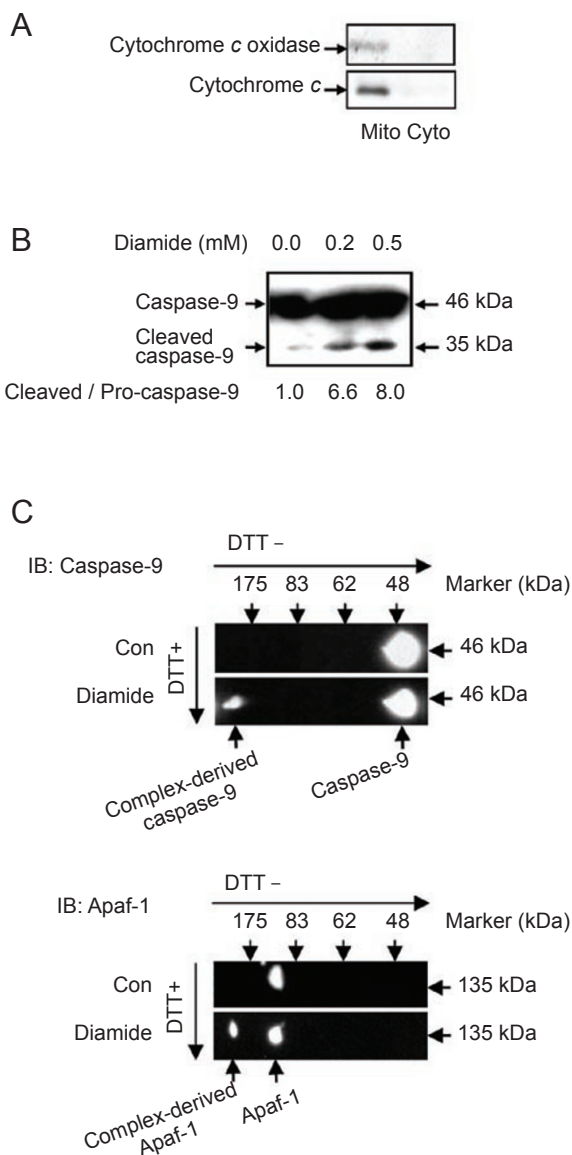


Figure 4 Diamide induced auto-cleavage of caspase-9 and disulfide-mediated interaction of caspase-9/Apaf-1 in an *in vitro* mitochondria-free system. HeLa cell mitochondria were removed from the cytoplasm to establish mitochondria-free systems. Cytosolic fractions were pre-incubated with thiol-specific oxidant diamide at indicated concentrations at 16 °C for 30 min, followed by 37 °C for 4 h in the presence of 2 μg/ml cytochrome *c* and 1 mM dATP. **(A)** Mitochondrial and cytosolic fractions were identified by detection of cytochrome *c* and cytochrome *c* oxidase. **(B)** Auto-cleavage fragments of caspase-9 (35 kDa) were detected and proteins were quantified by densitometry. The extent of caspase-9 activation is represented as the ratio between cleaved-caspase-9 and intact caspase-9. **(C)** Disulfide-mediated caspase-9/Apaf-1 interactions were detected with redox diagonal electrophoresis. Immunoblotting for caspase-9 and Apaf-1 was performed on the same membrane, probing for Apaf-1 after stripping the caspase-9 antibody. The figures are representative of three experiments.

and that this interaction contributes to the formation of the apoptosome that results in caspase-9 activation.

Discussion

Several plausible target(s) at which ROS promote apoptosis have been proposed. Voltage-dependent anion channel and cardiolipin can be oxidized [19, 20], which may promote cytochrome *c* release and collapse of the mitochondrial transmembrane potential. Because ROS may have multiple targets, signaling events that control the balance between pro- and anti-apoptotic factors may also be modulated by oxidative modification of thiols [21, 22].

Sato and colleagues have also studied the mechanisms by which ROS promote caspase-9 activation [10]. They showed that ROS positively regulate the apoptotic events downstream of the mitochondrial permeability transition in Jurkat cells; ROS promote apoptosome formation by oxidizing Apaf-1, which in turn helps to activate caspases-9 and -3. They emphasize that oxidation of Apaf-1, not caspase-9, is responsible for the enhancement of apoptosome formation stimulated by ROS. It is notable, however, that caspase-9 is at least not inactivated by ROS in their experiments, because the simple mixture of dATP, cytochrome *c* and naturally oxidized Apaf-1 and caspase-9 could form the apoptosome.

Despite the fact that ROS can promote or induce caspase-9 activation by modulating many different factors, as demonstrated by aforementioned studies, the results of the present study indicate that activation of caspase-9 by ROS largely depends on the disulfide-mediated caspase-9/Apaf-1 interaction. These results imply that oxidative modification of caspase-9 at the C403 residue can enhance its interaction with Apaf-1. This effect significantly facilitates apoptosome formation and caspase-9 activation.

Recent studies indicate the existence of cytochrome *c*-independent mechanism(s) for caspase-9 activation [23, 24]. Our finding that activity of caspase-9 can be enhanced by oxidative modification, independently from the increased release of cytochrome *c*, may provide an explanation for caspase-9-related apoptosis in such situations. Nevertheless, the question of whether oxidative modification of caspase-9 can activate it in the absence of cytochrome *c* requires further study.

The oxidative sensitivity of specific cysteine residues may impart specificity to ROS-mediated cellular signaling [25], meaning that upon oxidative stress, some proteins will be activated by oxidation, whereas others are inactivated. We postulate that under conditions of oxidative stress, cysteines located at different positions throughout the caspase-9 protein might show different

levels of responsiveness. Although the cysteine in the enzymatic active site needs to be maintained in the reduced form for full protease activity, peripheral cysteines, especially those that might be involved in interactions with other proteins, may require oxidation; the activated state would result from the coordinated response.

Sequence analysis shows that there are two cysteines, C12 and C76, in the caspase recruitment domain (CARD) of caspase-9 that interact with the CARD of Apaf-1. However, these two cysteines are not involved in the interaction between the two CARD domains [26]. Our

predictions based on structure simulations have excluded the possibility of intramolecular disulfide bond formation between these two cysteines, and indeed, mutations at these sites yield no obvious effects. Out of our expectation, C403 that does not locate in CARD is responsive to oxidative modification and appears to mediate a disulfide bond upon oxidative modification. Furthermore, the results of redox diagonal electrophoresis indicate that this bond is inter-molecular rather than intramolecular. We therefore postulate that this disulfide bond might bridge C403 of caspase-9 with a cysteine either in another caspase-9 molecule or in Apaf-1, or perhaps in other proteins that coexist within the same complex, thus promoting the interaction between caspase-9 and Apaf-1, directly or indirectly. The precise spatial relationship between C403 in caspase-9 and its disulfide partner, as well as the identity of the latter, will be addressed in our future work.

Disulfide bridges have traditionally been thought to be inert covalent linkages between cysteine residues that are formed during protein folding. However, an emerging concept identifies two types of disulfide bonds, a structural group and a redox-sensitive regulatory group, with the latter having direct functional consequences for the cell [27, 28]. After bacterial transcription factor OxyR was found to be directly activated by thiol oxidation [29], increasing numbers of reports have recently shown that covalent modification of cysteines by disulfide formation can activate proteins in mammalian cells [30-32] and that disulfides are rapidly formed on cell surfaces in response to changes in the cellular redox state [28]. In agreement with these novel concepts, our finding that oxidative modification of caspase-9 can promote its activation has provided a previously undescribed perspective of how oxidative stress induces apoptosis; in addition to the

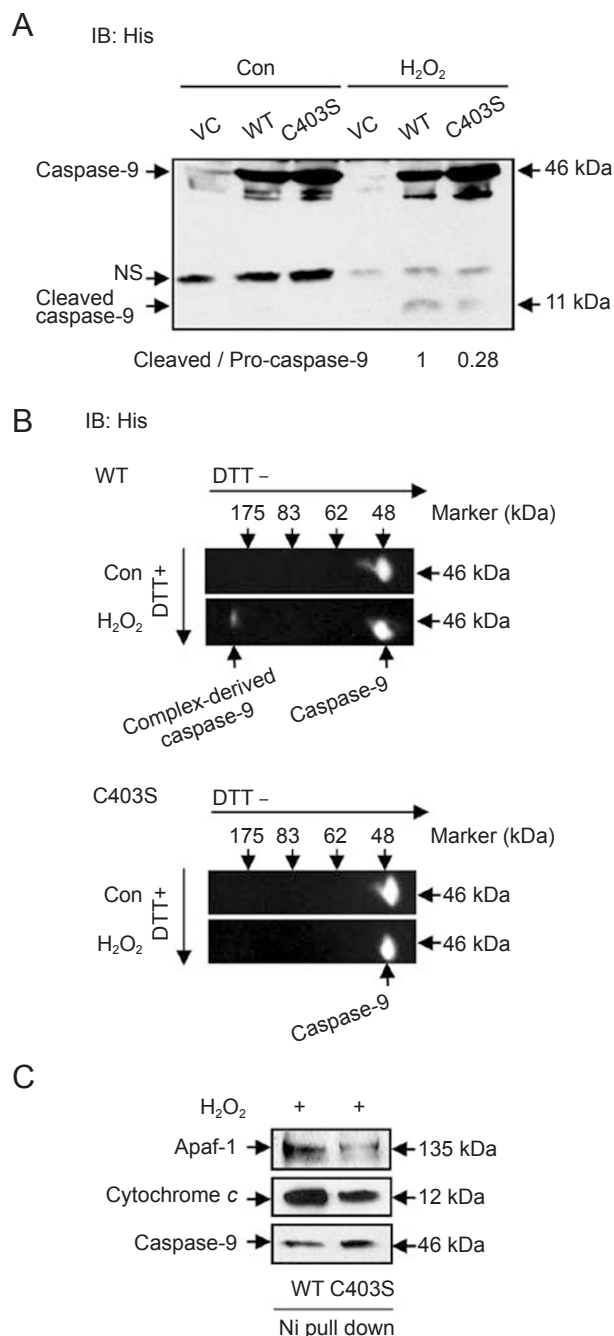


Figure 5 Cys403 mutation blunted the sensitivity of caspase-9 to H₂O₂-induced auto-cleavage and decreased the formation of disulfide-dependent complex and apoptosomes. HeLa cells were transfected with His-caspase-9 (WT) or His-C403S-caspase-9 (C403S). **(A)** Twenty-four hours after transfection, HeLa cells were treated with 2 mM H₂O₂ for 4 h. Auto-cleavage fragments of His-caspase-9 (11 kDa) were detected. The ratio of cleaved-caspase-9 to intact caspase-9 is indicated. VC refers to vector control and NS refers to non-specific bands. **(B)** Twenty-four hours after transfection, HeLa cells were treated with 5 mM H₂O₂ for 2 h. The disulfide-dependent complex containing His-caspase-9 was detected with redox diagonal electrophoresis and immunoblot. **(C)** HEK293T cells were transfected with His-caspase-9 (WT) or His-C403S-caspase-9 (C403S). Twenty-four hours post transfection, cells were treated with 2 mM H₂O₂ for 5 h. Cell lysates were subjected to Ni-IDA pull-down and immunoblot analysis with anti-Apaf-1, anti-cytochrome c and anti-His antibodies. The figures are representative of three experiments.

ROS-triggered mitochondrial cytochrome *c* release that activates caspase-9 on the platform of the apoptosome, a direct thiol modification of caspase-9 significantly facilitates its activation in apoptosomes.

Materials and Methods

Cell culture and reagents

HeLa and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, USA) supplemented with antibiotics and 10% newborn calf serum. Wild-type oral squamous carcinoma cells (OSC-WT) and OSC cells that stably express antisense manganese superoxide dismutase transcripts (OSC-SA) were gifts from Dr T Osaki and T Yamamoto (Kochi University Medical School, Japan) [33]. OSC cells were cultured in 1640 medium (Gibco-BRL). Arsenic trioxide (As_2O_3) and diamide were purchased from Sigma Chemical Co. (St Louis, MO, USA). TNF- α was purchased from Promega (Madison, USA). Hydrogen peroxide, cytochrome *c* and dATP were purchased from Sangon (Shanghai, China). F5M, obtained from Molecular Probes (Eugene, USA), was dissolved in dimethylformide to a final concentration of 250 mM and stored at $-20^\circ C$ until use. Caspase-9 inhibitor LEHD-CHO was purchased from BioVision (Mountain View, USA).

Assay of caspase-9 activity

The caspase-9 activity detection kit was purchased from Calbiochem (San Diego, USA). Red-LEHD-FMK, a fluorescent marker, binds irreversibly to activated caspase-9 in living cells, such that caspase-9 activity can be approximated by the fluorescence intensity (excitation ~ 540 nm; emission ~ 570 nm) detected by flow cytometry. Cells were trypsinized and washed once with complete medium after exposure to different stimuli. Following the addition of 1 ml of Red-LEHD-FMK into 300 ml of cell suspension and incubation for 30 min to 1 h in a $37^\circ C$ incubator with 5% CO_2 , cells were washed twice and analyzed by flow cytometry (Becton Dickinson, USA).

Annexin-V/propidium iodide (PI) flow cytometry and Hoechst 33342/PI staining for apoptosis assay

The Annexin-V/PI assay was performed with a flow cytometer (Becton Dickinson) using the FITC-labeled Annexin-V and PI kit (Calbiochem) according to the manufacturer's instructions, as described previously [15]. For cell staining, cells were incubated in complete medium with 5 $\mu g/ml$ Hoechst 33342 at $37^\circ C$ in the dark for 10 min. PI was co-incubated at $4^\circ C$ in the dark for an additional 10 min. Nuclear condensation was visualized using a fluorescence microscope equipped with a UV-2A filter, and apoptotic cells were identified.

Immunoprecipitation (IP), co-immunoprecipitation (Co-IP) and Ni-IDA pull-down

IP was performed to extract caspase-9 from cells. Cells were washed once with cold PBS and lysed with cold $1\times$ IP buffer (1% Triton X-100, 400 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA (pH 8.0), 0.2 mM sodium ortho-vanadate, 1 mM PMSE, 0.5% protease inhibitor cocktail, 0.5% IGEPAL CA-630) at $4^\circ C$ for 30 min, and then lysates were passed several times

through a 26-gauge needle and centrifuged at $12\ 000\times g$ for 15 min at $4^\circ C$.

To determine the interaction between caspase-9 and Apaf-1, co-IP was performed. The samples were carefully prepared so as not to disrupt mitochondria before their removal (as described below for the mitochondria-free system). The supernatants without mitochondria were then used for IP. The supernatants were incubated with the rabbit polyclonal antibody against caspase-9 (1:100, Neomarker, USA) or the rat monoclonal antibody against Apaf-1 (1:200, Chemicon, USA) overnight at $4^\circ C$, followed by incubation with protein A- or protein G-coated agarose beads, respectively (Santa Cruz Biotechnology, USA), for another 4 h at $4^\circ C$. After samples were washed four times with ice-cold PBS and supernatants were removed by centrifugation at $12\ 000\times g$ for 1 min, caspase-9 and Apaf-1 proteins were precipitated individually or co-precipitated if the two molecules were directly or indirectly bound together. The proteins were then separated from the beads using 0.1 M glycine (pH 2.5) for 15 min on ice. The supernatants were collected and the pH was neutralized by the addition of 1/10 volume of 1 M Tris (pH 8.0).

To examine the effect of the caspase-9 mutation, Ni-IDA pull-down was performed after $6\times$ His-tagged constructs were transfected into HEK293 cells using the His Bind purification kit (Novagen, USA) according to the manufacturer's protocol.

Immunoblot

Immunoblot was performed for various purposes within these experiments and was accordingly adjusted for different protein samples. To determine the amounts of caspase-9 and its auto-cleaved fragments, whole cell lysates and mitochondria-free fractions, respectively, were used. To determine the formation of the caspase-9/Apaf-1 interactive complex in the whole cell system, proteins extracted by co-IP were used. To detect the reactivity of the cysteine-mutant caspase-9 to apoptotic stimuli, caspase-9 wild-type or caspase-9 mutant plasmids with His tags were transfected into HeLa cells and Apaf-1, cytochrome *c*, caspase-9 and its cleavage products were detected. Proteins were loaded onto 10% or 12% SDS-PAGE gels, transferred to nitrocellulose membranes, and then detected by the proper primary and secondary antibodies before visualization by enhanced chemiluminescence (Pierce Biotechnology, USA). Various primary antibodies were used, including anti-SOD (1:200, Santa Cruz), anti-procaspase-9 (1:1 000, Chemicon), anti-pancaspase-9 (targeting the 46 kDa procaspase-9 and the cleaved 35 kDa version, 1:1 000, Neomarker), anti-Apaf-1 (1:1 000, Chemicon), anti-cytochrome *c* oxidase (1:1 000, Santa Cruz), anti-cytochrome *c* (1:2 000, Promega), and anti-His (1:1 000, Univ, China).

Assessment of reduced thiols

To label reduced thiols of protein samples, thiol-specific conjugating fluorescence fluorescein-5-maleimide (F5M) was used according to the previously described method [34]. Caspase-9 within the whole cell system was labeled by F5M by incubating the cells in fresh medium containing 10 mM F5M for 5 min at $37^\circ C$ after a 1 h H_2O_2 treatment. Labeled caspase-9 was retrieved by immunoprecipitation, using the IP buffer containing 1 mM F5M before being subjected to SDS-PAGE. After electrophoresis, the F5M fluorescence of the procaspase-9 bands on the gel was visualized and photographed using the FLA-5000 Science Imaging System (Fu-

jifilm, Japan) at an excitation wavelength of 472 nm. Immunoblot was performed using the same gel to determine the total caspase-9.

Sequential two-dimensional non-reducing/reducing SDS-PAGE

This method has been referred to as diagonal electrophoresis [35]. Samples were prepared in 1× SDS sample buffer without any reducing agent (i.e., neither β-ME nor DTT) and loaded onto 10% SDS-PAGE gels. After the first dimension, non-reducing electrophoresis, the entire lane containing the separated proteins was excised and incubated in SDS sample buffer containing 100 mM DTT (to reduce any disulfide bonds present between proteins or within proteins) for 20 min. The lane was then rotated 90° and laid horizontally on top of a large-format 1.5-mm-thick 12% acrylamide gel. This electrophoresis in the second reducing dimension would produce a series of dots along a diagonal line. Under these conditions, proteins that were linked by a disulfide bond were separated by DTT treatment and then resolved from one another, migrating further down the gel at their true weight, lower than the diagonal line. Thus, proteins that were linked together via disulfide bonds would be present as a complex at a single position in the first dimension, but would separate and move to their monomeric positions in the second dimension. Finally, immunoblot was performed to display the dots containing Apaf-1 and/or caspase-9 (Supplementary information, Figure S1), and the pictures were shown in reverse as black-to-white.

In vitro mitochondria-free system

Mitochondria were separated from the cytoplasm by a routine method. Briefly, 5×10^7 cells were collected, washed twice in cold PBS and resuspended in cold RSB (10 mM NaCl, 2.5 mM MgCl₂ and 10 mM Tris-HCl, pH 7.5) containing 1 mM PMSF and 0.5% protease inhibitor cocktail for 10 min to dilate cells. Samples were then homogenized by 15 passages through a 26-gauge needle, followed immediately by the addition of 2/3 volume 2.5× MS buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl and 2.5 mM EDTA, pH 7.5). Mitochondria were removed by centrifugation of samples at $18\,000 \times g$ at 4 °C for 30 min; supernatants represented mitochondria-free fractions. The cytosol and mitochondria were probed by immunoblot for cytochrome *c* and cytochrome *c* oxidase; the absence of these two proteins in the cytosolic fraction ensured the purity of the mitochondria-free system.

Site-directed mutagenesis of caspase-9

To construct a wild-type caspase-9 eukaryotic expression vector, caspase-9 cDNA containing 6 × His tag was amplified by PCR from the pET23b-caspase9 vector (kindly provided by Drs GS Salvesen and JC Reed, Burnham Institute) using the sense primer 5'-ATG AAT TCT AAG AAG GAG ATA TAC CAT GGA-3' and the antisense primer 5'-TAG GTA CCG TGC TCG CTA GAT TAG TG-3'. The caspase-9 cDNA fragment was subcloned into the *EcoRI* and *KpnI* sites of the pFLAG-CMV4 vector. All single or double cysteine mutations of caspase-9 were generated using the QuikChange site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer's protocol. All mutations were confirmed by DNA sequencing.

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