ROS-dependent caspase-9 activation in hypoxic cell death

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Abstract Mitochondria are known to play a fundamental role in apoptosis by releasing apoptogenic molecules such as cytochrome *c* into the cytoplasm, thereby sequentially activating initiator caspase-9. However, the mechanisms of cytochrome *c* release or caspase-9 activation in response to hypoxia are unclear. In this report, we show that caspase-9 is activated by reactive oxygen species (ROS) without involvement of cytochrome *c* release in hypoxic injury. In addition, activated caspase-9 induces permeability transition (PT)-independent cytochrome *c* release, suggesting that caspase-9 may disrupt mitochondrial diffusion limit of cytochrome *c* and serve to amplify further release of cytochrome *c*.

Key words: Hypoxia; Caspase-9; Cytochrome *c*; Reactive oxygen species; Apoptosis; Mitochondrion

1. Introduction

Cells undergo numerous changes in gene transcription, enzyme activities, and mitochondrial function in response to hypoxia [1]. On exposure to severe hypoxia beyond cellular adaptive capability, cells are irreversibly injured and die in necrosis or apoptosis. A large body of evidence suggests that mitochondria play crucial roles in hypoxic cell death passively by the inability to synthesize adenosine triphosphate (ATP) or actively by releasing proapoptotic molecules such as cytochrome *c* into the cytoplasm [2,3]. On entry into the cytoplasm, cytochrome *c*, together with dATP and apoptotic protease activating factor-1 (Apaf-1), recruits and cleaves pro-caspase-9 into active caspase-9 [4]. Activated caspase-9, in turn, cleaves effector caspases such as caspase-3, -6, and -7 [5]. Thus, cytochrome *c* release is a key step for activation of pro-caspase-9 in apoptotic cell death. However, even though several models of release have been proposed [6,7], the molecular mechanisms of cytochrome *c* release in response to hypoxia are not clear.

Caspases are principal effectors of apoptosis and can be categorized into initiator and effector on the basis of their positions in the apoptotic cascades [8]. The initiator caspases are activated in the earlier phase of apoptosis and cleave effector caspases into active forms, which are responsible for dismantling cells [5]. Activation cascades of initiator caspases have been delineated into two forms, death receptor-associated pathway and mitochondrial pathway. The first pathway links cell surface receptors such as Fas or tumor necrosis factor receptor (TNFR) to initiator caspase, caspase-8 [9,10]. The second pathway is responsible for apoptosis induced by diverse extra- and intra-cellular stresses such as hypoxia and growth factor withdrawal [11,12]. However, accumulating evidences show that caspase-9, an initiator caspase of mitochondrial pathway, can be activated without involvement of cytochrome *c*. For example caspase-12 or caspase-3 directly cleaves pro-caspase-9 to active form [13,14].

We performed this work to understand the mechanisms of caspase-9 activation and cytochrome *c* release in response to hypoxia. In this report, we show that reactive oxygen species (ROS) activate caspase-9 antecedent to cytochrome *c* release. In addition, activated caspase-9 is required for cytochrome *c* release from mitochondria. Our data propose the possibility that pre-activated cytosolic caspase-9 amplifies cytochrome *c* release cascade from mitochondria by disruption of mitochondrial diffusion barrier.

2. Materials and methods

2.1. Reagents, cell culture and hypoxic condition

Caspase inhibitors, recombinant caspase-9, and caspase-3 assay kit were purchased from Calbiochem (San Diego, CA, USA). All other chemicals were ordered from Sigma unless otherwise specified. SK-N-MC cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in a 37°C, CO_2 incubator, with RPMI 1640 medium (Gibco BRL, CA, USA) containing 10% heat-inactivated fetal bovine serum. For hypoxic condition, cells in degassed serum- and glucose-deficient medium were transferred to a hypoxic chamber with 94.5% N_2/0.5% O_2/5% CO_2 for indicated times.

2.2. Cell fractionation and immunoblot

SK-N-MC cells were treated or untreated with caspase inhibitors and subjected to hypoxic condition for indicated times. The harvested cells were washed and suspended in hypotonic solution (10 mM Hank's balanced salt solution (HBSS), 10 mM $MgCl_2$, 42 mM KCl) for 5 min on ice, and passed through a 30-gage needle. The cytosolic fraction was obtained by centrifugation at $150\,000 \times g$ for 1 h. The proteins obtained from cytosolic fraction or whole cell lysates were separated using 12% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to nitrocellulose membrane. The blots were incubated with anti-cleaved caspase-9, anti-caspase-8, anti-caspase-3 (Cell Signaling Tech., MA, USA), or anti-cytochrome *c* (BD-Pharmingen, CA, USA) antibody, followed by electrochemoluminescent (ECL)-based detection (Amersham Pharmacia Biotech., NJ, USA).

2.3. Isolation of mitochondria

Mitochondria were freshly isolated from SK-N-MC cells by differ-

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Abbreviations: AIF, apoptosis-inducing factor; Apaf-1, apoptotic protease activating factor-1; BA, bonkrekic acid; CsA, cyclosporin A; DCFH-DA, dichlorodihydrofluorescein diacetate; MMP, mitochondrial membrane potential; NAC, *N*-acetylcysteine; PT, permeability transition; PTP, permeability transition pore; ROS, reactive oxygen species

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ential centrifugation, as described previously [15]. The isolated mitochondria were washed and resuspended in respiration buffer (10 mM HEPES-KOH, pH 7.4, 0.3 M mannitol, 5 mM KH_2PO_4, 10 mM sodium succinate, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)) and used within 4 h. Exposure of isolated mitochondria to caspase-9 was performed in PT-1 buffer (2 mM HEPES, pH 7.4, 5 mM KH_2PO_4, 2 μM rotenone, 250 mM sucrose, and 4.2 mM potassium succinate) [16].

2.4. Determination of mitochondrial membrane potential (MMP)

The SK-N-MC cells were cultured in the indicated condition and then Mitotracker Red CMXRos (Molecular Probes, Inc., OR, USA) was added to the media at a final concentration of 400 nM. After 20 min of incubation, the cells were harvested and washed three times with phosphate-buffered saline (PBS). The fluorescent intensity was measured by flow cytometry (FACScaliver, Becton Dickinson, CA, USA) using the CellQuest program.

2.5. Caspase cleavage assay

Caspase-3 activity was measured using the Colorimetric Caspase-3 Assay Kit (Calbiochem) according to the manufacturer's instruction. Caspase-9 activity was measured using colorimetric Ac-LEHD-*p*NA substrate in the presence or absence of caspase-9 inhibitor LEHD-CHO. Briefly, 50 μl cell lysates were obtained from 10^6 cells in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% 3[3-cholamino-propyl diethylammonio]-1-propane sulfonate (CHAPS), 1 mM dithiothreitol (DTT), 0.1 mM ethylenediamine tetraacetic acid (EDTA)), incubated for 5 min on ice, and then centrifuged at $10\,000\times g$ for 10 min at 4°C. The supernatants were incubated with Ac-LEHD-*p*NA substrate in the presence or absence of caspase-9 inhibitor. Absorbance at 405 nm was determined ~6 h following initiation of the reaction. Activity was expressed as fold(s) change over control once corrected for baseline (protein and buffer without colorimetric substrate).

3. Results and discussion

Hypoxia is known to trigger apoptotic or necrotic cell death, depending on type or status of the cells. Initially, in an attempt to delineate the nature of cell death in our hypoxic experimental system, we characterized the cell death based on morphologic changes and caspase cleavage assay. SK-N-MC cells exposed to hypoxic condition for 9 h demonstrated characteristic nuclear condensation with fragmentations (Fig. 1A) and increase of caspase-3 activity (Fig. 1B). These findings indicate that SK-N-MC cells undergo apoptosis in hypoxic condition. Mitochondria play pivotal roles in hypoxia-induced apoptosis by releasing apoptogenic molecules such as cytochrome *c* and apoptosis-inducing factor (AIF) [2,3]. On entry into the cytoplasm, cytochrome *c*, together with dATP and Apaf-1, recruits and cleaves pro-caspase-9 into active caspase-9 [4]. Thus, it is conceivable that mitochondrial perturbation (loss of MMP) with resultant cytochrome *c* release precedes caspase-9 activation. To clarify these sequential events on the base of the time course, we exposed SK-N-MC cells to hypoxia for varying time points and determined apoptotic parameters including MMP, cytochrome *c* release and caspase activation from 1 h of hypoxia. Loss of MMP, determined by potential-dependent Mitotracker, was initially observed after 6 h of hypoxia (Fig. 2A). Interestingly, we found cleaved caspase-9 and cytochrome *c* release from 3 and 4 h of hypoxia, respectively (Fig. 2B). These time points are antecedent to mitochondrial perturbation or activation of death receptor-associated caspase (caspase-8) and effector caspase (caspase-3) (Fig. 2B). Our data contradict to typical mitochondrial apoptotic cascades in which mitochondrial permeability transition (PT) induces cytochrome *c* release and caspase activation. However, we cannot completely discard the possibility

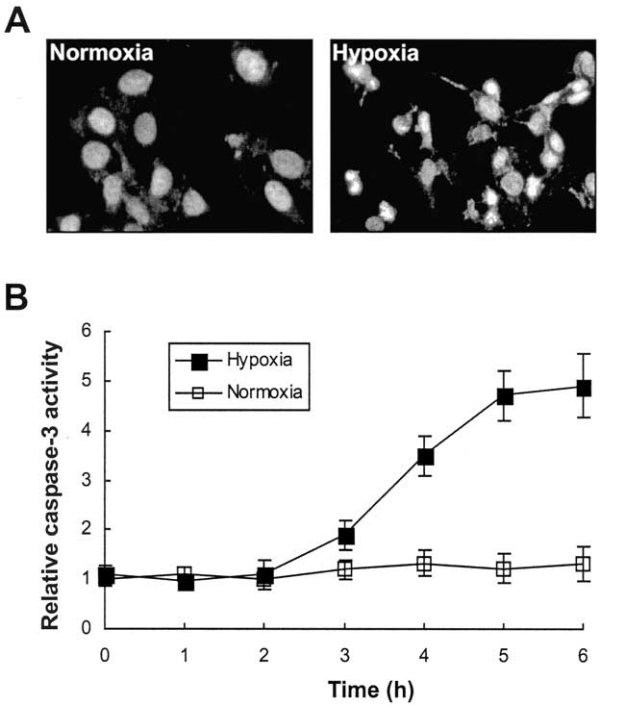

Fig. 1. Hypoxia induces apoptosis in SK-N-MC cells. A: SK-N-MC cells were exposed to hypoxia (0.5% O_2) for 9 h, harvested, and stained with 4,6-diamino-2-phenylindole (DAPI). Morphologic changes were analyzed using fluorescence microscope. B: Cells were exposed to hypoxia for indicated time point and harvested. Caspase-3 cleavage assay using Ac-DEVD-*p*NA was performed according to manufacturer's instruction. Relative enzyme activities to normoxic cells were plotted. Means ± S.E.M. of three independent experiments are shown.

that small amounts of cytochrome *c* under detection limit of immunoblot were released to activate caspase-9. Based on our observation, we hypothesized that (i) cytochrome *c* is released from mitochondria through permeability transition pore (PTP)-independent pathway and (ii) caspase-9 is activated without involvement of cytochrome *c* release in response to hypoxia. To validate this hypothesis, we exposed SK-N-MC cells to hypoxia with PT inhibitor 20 μM cyclosporin A (CsA) or 100 μM bonkrekic acid (BA) and determined apoptotic parameters including MMP, cytochrome *c* release, and caspase activity. PT inhibitors suppressed dissipation of MMP whereas they did not block cytochrome *c* release and caspase-9 activation in hypoxia (Fig. 2C and D, left panels). In comparison, PT inhibitors suppressed all apoptotic parameters in ceramide-treated cells (Fig. 2C and D, right panels). Ceramide is known to facilitate apoptosis via typical mitochondrial cascades, sequentially inducing dissipation of MMP, cytochrome *c* release, and caspase-9 activation [17]. Taken together, our results indicate that hypoxia induced cytochrome *c* release independently of PT. Opening of PTP induces leakages of proapoptotic molecules such as cytochrome *c* and AIF, which is the obligatory step for mitochondrial cascades of apoptotic processes. However, it is controversial that cytochrome *c* releases by proapoptotic proteins are obligatorily related to opening of PTP. For example, some researchers found that Bax induced opening of PTP with resultant cytochrome *c* release [18,19], whereas others discarded this concept because they found that Bax formed large conduct channels on outer mitochondrial membrane independent

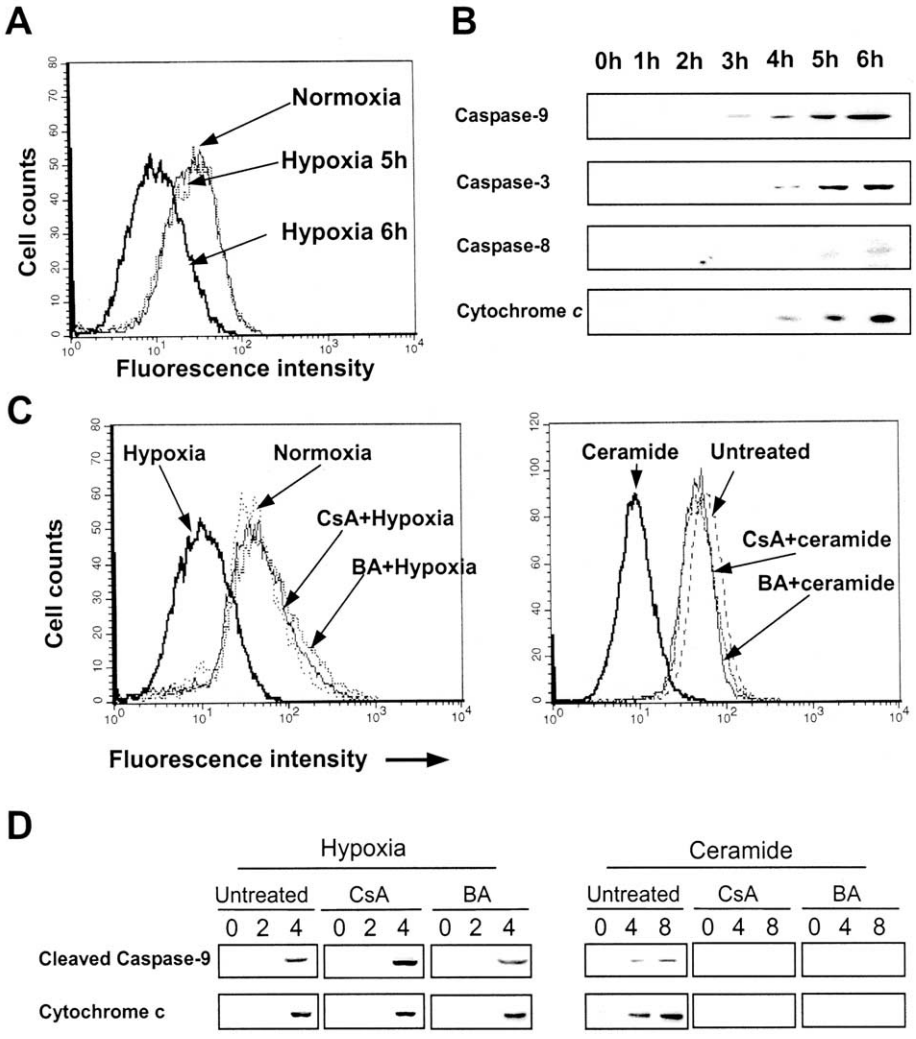

Fig. 2. Hypoxia induces caspase-9 activation prior to perturbation of MMP and cytochrome *c* release in PT-independent pathway. A: SK-N-MC cells were exposed to hypoxia for indicated times and incubated with 400 nM Mitotracker for 20 min. After washing three times, MMP was determined by FACScan using CellQuest program. B: Cells were exposed to hypoxia for indicated times and then cytosolic compartment was fractionated by centrifugation as described in Section 2. Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted with anti-cleaved caspase-9, anti-caspase-3, anti-caspase-8, or anti-cytochrome *c* antibody. Cleaved products of caspase-3 and -8 were shown. C: Cells treated or untreated with 20 μM CsA or 100 μM BA were exposed to hypoxia (left panel) for 6 h or 10 μM ceramide (right panel) for 4 h. MMP was determined as above. D: Cells were treated as in C for indicated time points. Immunoblots for cytosolic cytochrome *c* or cleaved caspase-9 were performed.

of PTP opening [20,21]. In addition, it has been reported that BH3-only members of Bcl-2 family proteins induced cytochrome *c* release without MMP loss [22,23]. Next, we wished to address the possibility of caspase-9 activation prior to cytochrome *c* release. There can be several possible mechanisms for caspase-9 activation without involvement of cytochrome *c* release: (i) the activation of caspase-9 by endoplasmic reticulum (ER)-specific caspase-12 [13], (ii) direct activation by cytosolic caspase-3 [14], (iii) the activation of other cellular proteases such as calpain, which eventually can cleave procaspase-9 [24], or (iv) unknown pathways as can be seen in Sendai virus-infected cells [25]. To elucidate the possibilities of caspase-12 or -3 involvement, we performed Western blots for detection of caspase-12 or -3 cleavage product in hypoxic injury. However, we could not find any evidence of caspase-3 or -12 activation prior to caspase-9 activation (Fig. 2B and data not shown). Hypoxic signals increase generation of ROS, which mediates cell injury. Therefore, we determined changes of ROS levels using ROS-sensitive dichlorodihydrofluorescein diacetate (DCFH-DA) dyes and found increased fluorescent intensities from 2 h of hypoxia as compared to normoxia (Fig. 3A). 500 μM *N*-acetylcysteine (NAC) inhibited increase of ROS levels induced by hypoxia (Fig. 3A). To address the roles of ROS in caspase-9 cleavage, we performed caspase-9 cleavage assay. Caspase-9 activity was detected from 3 h of hypoxia in NAC-untreated cells whereas activity was not detected in NAC-treated cells (Fig. 3B), indicating that hypoxia induced caspase-9 activation in a ROS-dependent manner. To reinforce our results, we treated SK-N-MC cells with 200 μM H_2O_2 and determined time sequential correlation between cytochrome *c* release and caspase-9 activation. Caspase-9 cleavage products were detected from 2 h of oxidative stress, which was antecedent to cytochrome *c* release (Fig. 3C). Taken together, our results demonstrate that ROS is crucial in cytochrome *c*-independent caspase-9 activation in hypoxic injury. Further, we investigated the effects of caspase-9 activation on

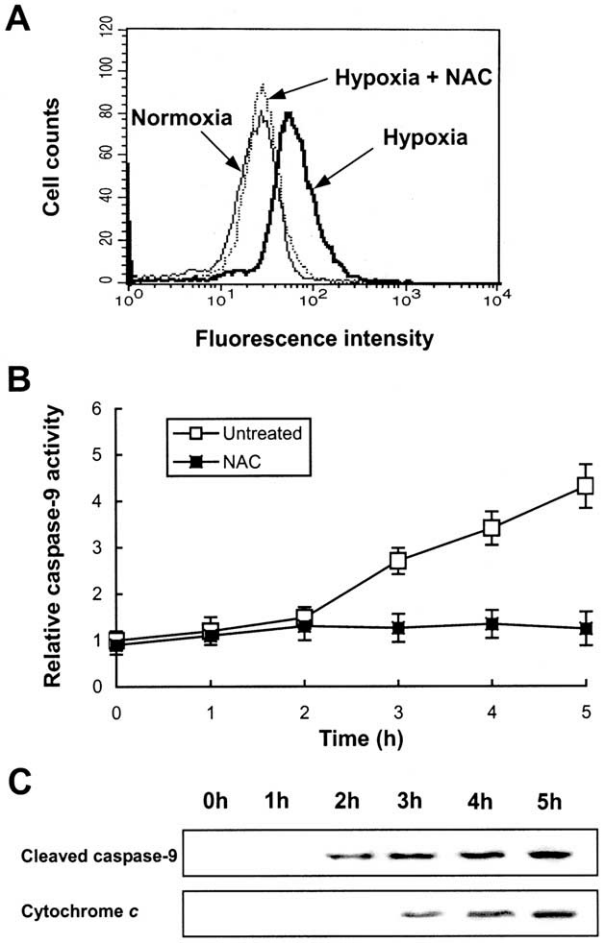

Fig. 3. ROS activates caspase-9 without cytochrome *c* involvement. A: SK-N-MC cells were treated or untreated with 500 μM NAC for 2 h prior to hypoxia, subjected to hypoxia for 2 h, and stained with DCFH-DA. After washing three times, fluorescence intensity was determined by flow cytometry. B: Cells were treated or untreated with 500 μM NAC for 2 h prior to hypoxia, subjected to hypoxia for indicated times, and harvested. Caspase-9 cleavage assay was performed using Ac-LEHD-*p*NA substrate. Enzyme-catalyzed release of *p*NA was measured at 405 nm and fold(s) change was presented. Means ± S.E.M. of three independent experiments are shown. C: Cells were treated with 200 μM H_2O_2 for indicated times and harvested. Immunoblots for cleaved caspase-9 product and cytochrome *c* were performed.

cytochrome *c* release from mitochondria in order to clarify the mechanism of cytochrome *c* release in response to hypoxia. We treated or untreated SK-N-MC cells with caspase inhibitors (20 μM LEHD-CHO for caspase-9, 40 μM DEVD-CHO for caspase-3, and 20 μM Ac-VAD-CHO for pan-caspase inhibitor) prior to hypoxic stress and determined cytochrome *c* translocation and MMP. LEHD-CHO or Ac-VAD-CHO inhibited dissipation of MMP (Fig. 4A, left panel) and cytochrome *c* release (Fig. 4B, left panel) in hypoxia-exposed cells, suggesting that mitochondrial perturbation cascades were downstream to caspase-9 activation in hypoxia. Interestingly, all caspase inhibitors failed to inhibit loss of MMP (Fig. 4A, right panel) and cytochrome *c* release (Fig. 4B, right panel) in ceramide-treated cells. Our data suggest that hypoxia induces apoptosis differently from typical mitochondrial cascades. To further examine whether caspase-9 directly induces cytochrome *c* release from mitochondria, we exposed the isolated mitochondria from SK-N-MC to recombinant caspase-9 in the presence or absence of caspase-9 inhibitor Ac-LEHD-CMK or CsA. After incubation of 15 min, mitochondrial compartment was removed by centrifugation and cytochrome *c* release was determined by Western blot analysis. As shown in Fig. 4C, caspase-9 induced CsA-independent cytochrome *c* release from isolated mitochondria, which was inhibited by caspase-9 inhibitor Ac-LEHD-CMK. Our results indicate that cytochrome *c* was directly released by caspase-9 through PT-independent pathway. Recently it has been reported that in addition to cleavage of subsequent effector caspases, caspase-9 increases the diffusion limit of the nuclear pores, which allows large molecules including caspase-3 that cannot pass through the nuclear pores to enter the nucleus [7]. Considering the roles of caspase-9 in cellular disassembly, it may disrupt mitochondrial barrier to release cytochrome *c* in hypoxic injury or induce opening of cytochrome *c*-specific pores, which remain to be elucidated. To date, it has been reported that recombinant caspase-3 directly induced cytochrome *c* release from isolated mitochondria by PTP opening [16]. However, the molecular mechanism of caspase-9 to induce cytochrome *c* release seems to be distinct from that of caspase-3 since caspase-9 induced in vitro cytochrome *c* release through PTP-independent pathway. In conclusion, we report here that hypoxic signals induce caspase-9 activation prior to loss of MMP and cytochrome *c* release, and ROS is involved in cy-

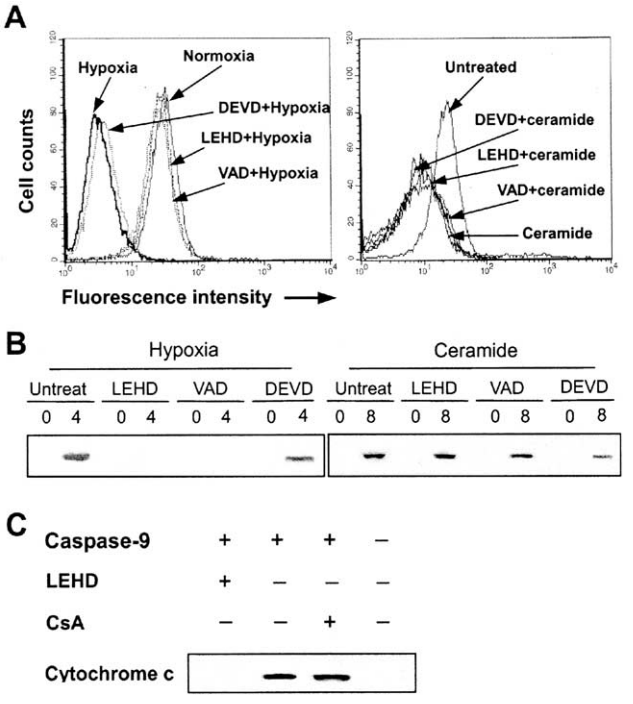

Fig. 4. Caspase-9-dependent cytochrome *c* release in hypoxic injury. A: SK-N-MC cells were exposed to hypoxia (left panel) for 6 h or 10 μM ceramide (right panel) in the presence or absence of caspase inhibitors (20 μM LEHD-CHO, 40 μM DEVD-CHO and 20 μM Ac-VAD-CHO). MMP was determined using 400 nM Mitotracker. B: Cells were exposed to hypoxia or treated with ceramide in the presence or absence of caspase inhibitors as above for indicated times. Immunoblots were performed after cell fractionation using anti-cytochrome *c* antibody. C: Isolated mitochondria (0.5 mg of protein/ml) were exposed to recombinant caspase-9 (2 U/ml) with or without 100 μM Ac-LEHD-CMK or 1 μM CsA for 15 min at 25°C in PT-1 buffer. After centrifugation, the supernatants were immunoblotted using anti-cytochrome *c* antibody.

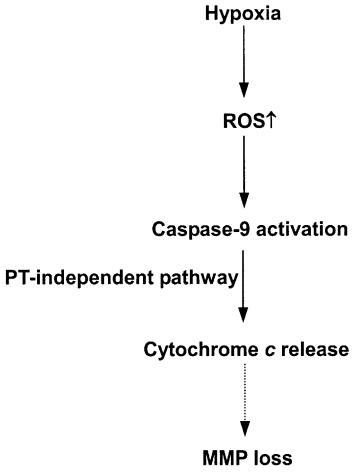

Fig. 5. Schematic diagram of cytochrome *c* release and caspase-9 activation in response to hypoxia. Incontinuous line, putative pathway.

tochrome *c*-independent caspase-9 activation process (Fig. 5). In addition, activated caspase-9 induces cytochrome *c* release from mitochondria via PT-independent pathway. All together, our data suggest that mitochondrial catastrophe is secondary to caspase activation in hypoxia.

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