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Hypoxia induces apoptosis in human neuroblastoma SK-N-MC cells by caspase activation accompanying cytochrome *c* release from mitochondria

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Abstract We have attempted to elucidate the mechanism of apoptotic cell death induced by hypoxia (very low oxygen conditions) in neuronal cells. Human neuroblastoma SK-N-MC cells under hypoxic conditions resulted in apoptosis in a time-dependent manner estimated by DNA fragmentation assay and nuclear morphology stained with fluorescent chromatin dye. Pretreatment with Z-Asp-CH_2-DCB, a caspase inhibitor, suppressed the DNA ladder in response to hypoxia in a concentration-dependent manner. An increase in caspase-3-like protease (DEVDase) activity was observed during apoptosis, but no caspase-1 activity (YVADase) was detected. To confirm the involvement of caspase-3 during apoptosis, Western blot analysis was performed using anti-caspase-3 antibody. The 20- and 17-kDa proteins, corresponding to the active products of caspase-3, were generated in hypoxia-challenged lysates in which processing of the full length form of caspase-3 was evident. With a time course similar to this caspase-3 activation, hypoxic stress caused the cleavage of PARP, yielding an 85-kDa fragment typical of caspase activity. In addition, caspase-2 was also activated by hypoxia, and the stress elicited the release of cytochrome *c* into the cytosol during apoptosis. These results suggest that caspase activation and cytochrome *c* release play roles in hypoxia-induced neuronal apoptosis.

Key words: Neuronal cell; Hypoxia; Apoptosis; Caspase; Cytochrome *c*

1. Introduction

There are two distinct essential morphological manifestations of cell death; one is apoptosis and another is necrosis [1]. Apoptosis is characterized by the condensation of chromatin and cytoplasm, membrane blebbing and apoptotic bodies, whereas necrosis features plasma membrane disruption and mitochondrial swelling associated with formation of large vacuoles. It is still not fully understood whether or not neuronal death in vivo is apoptosis or necrosis. Following transient ischemia in gerbils and rats, however, it has been demonstrated that at least some cell death is attributed to apoptosis, determined by in situ nick-end labeling of biotinylated dUTP by terminal deoxytransferase (TUNEL staining) [2,3].

Brain ischemia brings about hypoxic (or near-anoxic) and hypoglycemic insults. Hypoxia is one of the major pathological factors inducing neuronal injury. In vivo, hypoxic stress leads to increased utilization of endogenous substrates, ATP depletion, membrane depolarization, excessive release and reduced re-uptake of glutamate or aspartate, a loss of neuronal ion homeostasis and finally, irreversible brain damage [4–6]. On the other hand, hypoxia itself causes apoptotic cell death in human fibroblast cell line GM701, human lymphoid cell line SKW6.4, murine pro-B cell line BAF3, rat hepatoma cell line 7316A, and rat pheochromocytoma cell line PC12 [7,8] in vitro. Furthermore, it has been reported that apoptotic cell death induced by ischemia or low level of oxygen is prevented by Bcl-2 and Bcl-X_L [9–13]. Caspase inhibitors have been shown to significantly block cell death, suggesting that some caspases are involved in hypoxia-induced apoptosis in a Bcl-2-dependent manner [14]. Indeed, Shimizu et al. [15] reported that activation of both caspase-1-like and caspase-3-like proteases (YVADase and DEVDase) was observed during hypoxic conditions in rat primary hepatocytes. More recently, Yoshimura et al. [16] demonstrated that ceramide formation during hypoxia is linked to caspase-3-like protease (DEVDase) activation in PC12 cells. However, the precise mechanism of hypoxia-induced apoptosis is still unclear.

In the present study, we attempted to clarify the mechanism of hypoxia-induced apoptosis using human neuroblastoma cell line SK-N-MC. Exposure to hypoxia elicited apoptotic cell death in a time-dependent manner in SK-N-MC cells. The cell death induced by hypoxia was blocked by treatment with caspase inhibitor. Furthermore, we report that hypoxia stimulated caspase-3 activity, but not caspase-1. This may be linked to apoptosis accompanied by the release of cytochrome *c* from mitochondria.

2. Materials and methods

2.1. Cell culture

Human SK-N-MC cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37°C in humidified 5% CO_2/95% air.

2.2. Exposure to hypoxic environment

When SK-N-MC cells achieved 80% confluence, the cells were cultured in a mixture of 5% CO_2 and the balance N_2 in a humidified 37°C incubator (ANX-1, Hirasawa, Tokyo, Japan) that was contained in a sealed, anaerobic glove cabinet fitted with a catalyst to scavenge free oxygen. Oxygen tension in the chamber and in the medium were measured using a portable trace oxygen analyzer (Teledyne, USA) and a blood gas analyzer (ABL-2, Radiometer, Sweden), respectively.

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Abbreviations: Z-Asp-CH_2-DCB, benzyloxycarbonyl-Asp-CH_2OC(O)-2,6-dichlorobenzene; CPP32, cysteine protease p32; DEVD-MCA, *N*-acetyl-Asp-Glu-Val-Asp-4-methylcoumaryl-7-amide; YVAD-MCA, *N*-acetyl-Tyr-Val-Ala-Asp-4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarin; ICE, interleukin-1β-converting enzyme; ICH-1, ICE and CED-3 homolog 1; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride

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The oxygen concentration in the chamber remained below 10 p.p.m. throughout the incubation period. On the other hand, oxygen tension in the culture medium at 15 min, 30 min, 1 h, 6 h, and 48 h after transfer into the hypoxic chamber fell to 4.3, 3.0, 2.2, 2.0, and 2.0 ± 0.2%, respectively.

2.3. DNA fragmentation

The cells were washed with PBS and lysed (lysis buffer: 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.5% Triton X-100) for 20 min at 4°C. The samples were centrifuged at 27 000 × *g* for 15 min at 4°C. The DNA in the supernatant was extracted in three steps with equal volumes of phenol, phenol/chloroform (1:1, v/v), and then chloroform. Then the DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol. The DNA was resuspended in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and treated with 40 μg/ml RNase A for 1 h at 37°C. The DNA concentration was determined by absorbance at 260 nm. Twenty mg of DNA from the samples was subjected to agarose gel electrophoresis on a 1.5% gel in TAE buffer (40 mM Tris-HCl (pH 8.5), 2 mM EDTA). Then the gel was stained with 0.5 mg/ml ethidium bromide for 15 min, and the fragmented DNA was visualized under UV light and photographed [17].

2.4. Assessment of nuclear morphology

Chromosomal condensation and DNA fragmentation were assessed using the chromatin dye Hoechst 33258 as described [18]. Cells were fixed for 30 min in PBS containing 1% glutaraldehyde. After fixation at room temperature, the cells were washed twice with PBS and then exposed to 5 μg/ml Hoechst 33258 in PBS for 30 min at room temperature. After washing, all samples were mounted with glycerol/PBS (1:1, v/v) and observed using a non-confocal fluorescence microscope.

2.5. Assay for caspase activity

Cells in 60-mm plates were cultured in the low oxygen chamber for the indicated periods. At the appropriate time, the medium was aspirated and the cells were washed with PBS and 50 μl of lysis buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 mM digitonin) was added. The cells were incubated for 10 min at 37°C. After incubation, the supernatant was centrifuged at 15 000 rpm for 10 min. Next, the separated lysate (50 μg protein) was incubated at 37°C with 50 μM Ac-DEVD-MCA for 30 min or Ac-YVAD-MCA for 60 min. The amounts of 7-amino-4-methylcoumarin (AMC) released were measured using a spectrofluorometer (Hitachi F-2000 Fluorescence Spectrophotometer) with excitation at 380 nm and emission at 460 nm. One unit was defined as the amount of enzyme required to release 1 pmol of AMC per min at 37°C.

2.6. Western blot analysis

The cells (5×10^5) were washed twice with ice-cold PBS and then 500 μl of buffer A (50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 2 mg/ml leupeptin, 1 mM PMSF, and 1 mg/ml pepstatin) was added. The cells were disrupted with 10 strokes of a Dounce homogenizer. The homogenates were separated as cytosol and membrane fractions by ultracentrifugation. Then, the lysates and membranes were boiled for 5 min with SDS sample buffer. Equal amounts of each sample (20 μg) were subjected to 12% SDS-PAGE at 100 V for 1 h at 4°C, followed by transfer to a nitrocellulose filter. The filters were then blocked with TBST (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature. Human anti-Ich-1 (caspase-2, Santa Cruz), anti-caspase-3 (PharMingen), and anti-PARP (Clontech) were used as primary antibodies, and horseradish peroxidase-labeled goat or mouse Ig was used as a secondary antibody. The antibody-reactive bands were revealed by chemiluminescence (ECL Western detection kit). Cytochrome *c* protein was detected by Western blot analysis as described [19]. Equal amounts of protein (10 μg) were subjected to Western blot analysis using mouse monoclonal anti-cytochrome *c* antibody (7H8.2C12, PharMingen).

3. Results

3.1. Hypoxia induced apoptotic cell death in SK-N-MC cells

To address the ability of hypoxia to induce cell death, we first investigated the effect of hypoxia on cell viability using an LDH leakage assay. Approximately 10% of the total LDH activity spontaneously leaked to the culture medium in control cells (Fig. 1A). A small loss of viability was observed within 18 h of a hypoxia challenge. Loss of viability occurred following treatment with hypoxia in a time-dependent manner. At 24 h the leakage increased to 30% compared to that of control cultures, and by 48 h LDH leakage increased to 70%. We employed two methods to investigate whether the loss of viability caused by hypoxia correlates with a biochemical feature that discriminates between apoptosis and necrosis. In order to characterize apoptosis, internucleosomal DNA fragmentation and nuclear morphology using chromatin dye were examined.

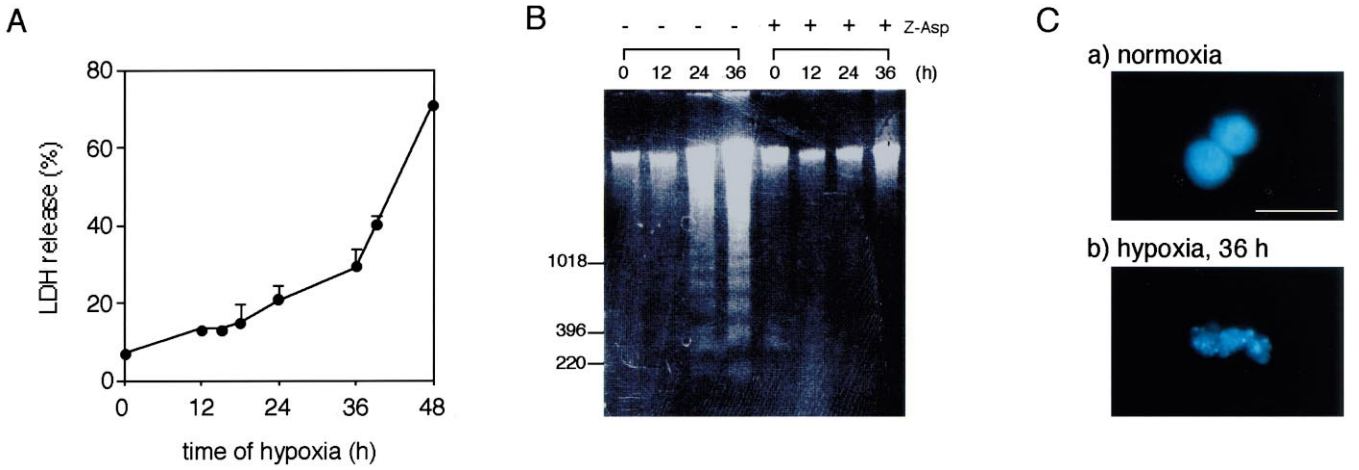

Fig. 1. Exposure to hypoxia resulted in apoptosis in SK-N-MC cells. A: Time course of LDH leakage during hypoxia. The cells were exposed to hypoxia for the indicated periods (0–48 h) and then LDH activity was measured as described in Section 2. Data are means ± S.E.M. of triplicate reactions run in parallel. B: Time course of DNA fragmentation in response to hypoxia and effect of Z-Asp-CH_2-DCB on hypoxia-induced DNA fragmentation. SK-N-MC cells were treated or not treated with 100 mM of Z-Asp-CH_2-DCB (Z-Asp). Then cells were incubated in a hypoxic environment for various periods (0–48 h) and subjected to DNA fragmentation assay as described in Section 2. C: Hypoxia-induced nuclear DNA condensation and fragmentation in SK-N-MC cells. Fluorescence micrographs were taken of cells stained with Hoechst 33258. Cultures incubated with normoxia (a), hypoxia (b) for 36 h are shown. Very few apoptotic nuclei were observed in control SK-N-MC cells (a). Exposure to hypoxia for 36 h induced nuclear DNA condensation and fragmentation (b). In a and b, bar = 50 mm.

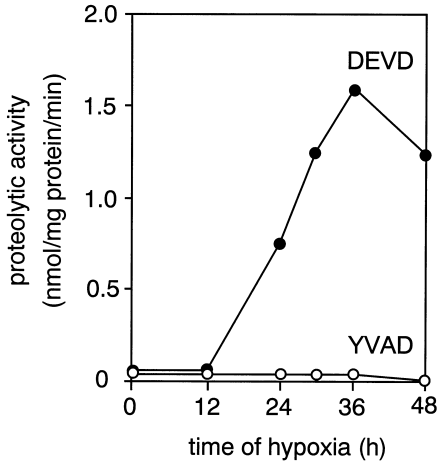

Fig. 2. Hypoxia stimulated caspase activation in SK-N-MC cells. Time course for the activation of Ac-DEVD-MCA cleaving activity in the cell extracts after hypoxia challenge. Activation of Ac-DEVD-MCA (●), but not Ac-YVAD-MCA (○), was observed after hypoxia treatment. Data are the mean of triplicate reactions run in parallel and are expressed as units of fluorescence charge per mg protein, as described in Section 2.

DNA from hypoxia-treated cells was examined for oligonucleosomal fragmentation by agarose gel electrophoresis. No internucleosomal DNA fragmentation was observed in control cultures. Low molecular weight DNA extracted from SK-N-MC cells challenged with hypoxia produced an oligosomal DNA ladder in a time-dependent manner on agarose gels stained with ethidium bromide in parallel with the appearance of significant LDH leakage (Fig. 1B). Further, nuclear staining with Hoechst 33258 chromatin dye was utilized to confirm whether the loss of viability was attributed to apoptosis. Minimal nuclear condensation was observed in non-treated cells (Fig. 1C). In cells incubated with hypoxia for 36 h, condensation and fragmentation of the chromatin, and shrinkage and fragmentation of the nuclei as apoptotic bodies were evident (Fig. 1C).

3.2. *Hypoxia induced apoptotic cell death via caspase activation*

To determine whether caspases contribute to hypoxia-induced apoptosis, the presence of a DNA ladder in SK-N-MC cells following treatment with hypoxia was examined in the presence or absence of Z-Asp-CH_2-DCB, a cell-permeable non-specific caspase inhibitor. Formation of a DNA ladder in cells 24 and 36 h after hypoxia challenge was suppressed by simultaneous treatment with Z-Asp-CH_2-DCB (Fig. 1B).

Having established a role for caspases in cell death induced by hypoxia, we analyzed the process of caspase activation in this system. Detergent extracts prepared from cells at various stages after treatment with hypoxia were tested for cleaving action on two fluorogenic peptide substrates (Ac-DEVD-MCA and Ac-YVAD-MCA). Ac-DEVD-MCA is a fluorogenic, tetrapeptide substrate that is cleaved by caspase-3 and -7, but may also be cleaved by other caspases. The second peptide, Ac-YVAD-MCA, is cleaved by caspase-1 [20,21]. Ac-DEVD-MCA cleaving activity was clearly elevated and sustained with hypoxia treatment. Maximal Ac-DEVD-MCA cleaving activity was detected 36 h after the hypoxia challenge. In contrast, SK-N-MC cells challenged with hypoxia showed only a low level of Ac-YVAD-MCA cleaving activity at any of the times examined in this study (Fig. 2).

Caspase-2 and -3 are synthesized as precursor molecules, and are approximately 48 kDa and 32 kDa in size, respectively. During processing, caspase-2 and -3 are proteolytically cleaved to produce a mature enzyme composed of 18- plus 12-kDa and 20- plus 10-kDa subunits, respectively. First, to investigate whether or not hypoxia activates caspase-3, Western blot analysis was performed using anti-caspase-3 antibody, which can detect the proform and active 20- or 17-kDa fragment of caspase-3. In lysates prepared from control cells, a band at 32 kDa corresponding to the full-length form of caspase-3 protein was evident. As shown in Fig. 3A, hypoxia induced the processing of caspase-3. Furthermore, new bands corresponding to p20 and p17 of caspase-3 were detected at 36 h after hypoxic challenge in samples in which processing of pro-caspase-3 was evident. We investigated the cleavage of PARP by treatment with hypoxia in SK-N-MC cells. PARP is believed to be an endogenous substrate for caspases (caspase-3, -6, -7, -8, and -9). To ascertain whether apoptosis induced by hypoxia in SK-N-MC cells is accompanied by the activation of caspases, we examined the cleavage of PARP. As shown in Fig. 3C, treatment with hypoxia resulted in PARP cleavage, yielding an 85-kDa fragment typical of caspase activity, in a time-dependent manner. The 85-kDa PARP fragment, which is indicative of proteolytic holoenzyme digestion, is visible after an 18-h incubation period with hypoxia. Furthermore, the full-length form of PARP disappeared after a 36-h exposure to hypoxia. PARP cleavage activity coincided with the appearance of DNA fragmentation (Fig. 1B). Next, we examined caspase-2 using anti-C-terminal caspase-2 antibody, which recognizes the full-length form and

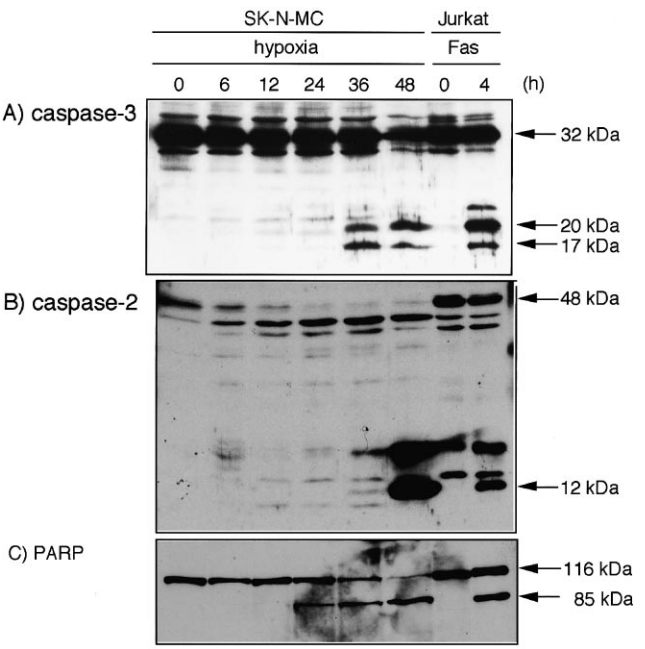

Fig. 3. Decrease in pro-forms of caspase-2 and -3, and PARP in response to hypoxia in SK-N-MC cells. The cells were incubated for various periods in hypoxic environment. Total cell lysates were prepared and then subjected to Western blot analysis using specific antibodies for caspase-2 (C-20, Santa Cruz), caspase-3 (PharMingen), and PARP (C-2-10, Clontech). Kinetic analyses of the levels of (A) caspase-3, (B) caspase-2, and (C) PARP are shown.

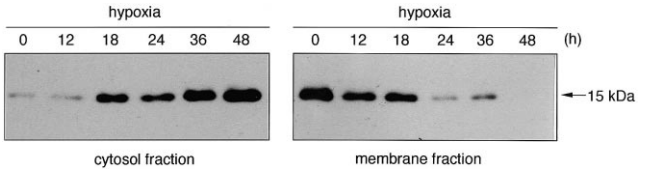

Fig. 4. Induction of cytochrome *c* release into the cytosol by hypoxia. The cells were challenged with hypoxia for various periods. The cytosol fractions were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Cytochrome *c* was detected by Western blot analysis using a monoclonal antibody (7H8.2C12) against cytochrome *c* (PharMingen). These are typical results from three independent experiments.

active p12 subunit of caspase-2 [22,23]. Immunoblot analysis revealed that reduction of the band corresponding to the caspase-2 pro-form occurred as early as 12 h. However, a band at approximately p12, corresponding to the carboxyl-terminal portion of caspase-2, was only easily detected in cell lysates after a 36-h exposure to hypoxia (Fig. 3B). The absence of easily detectable active fragments of caspase-3 and -2 in immunoblots may be because these active bands only react weakly with the caspase-3 and -2 antibodies used in this study.

3.3. Cytochrome c released from the mitochondria during hypoxia-induced apoptosis

The amounts of cytochrome *c* in the membrane and cytosol fractions were then measured by Western blot analysis. Without hypoxia challenge, most of the detectable cytochrome *c* was found in the membrane fraction (data not shown). The amount of cytochrome *c* in the cytosol fraction increased significantly after an 18-h challenge with hypoxia, and the level continued to increase for up to 48 h. The amounts of cytochrome *c* in the membrane fraction showed a corresponding decrease in a time-dependent manner (Fig. 4).

4. Discussion

The aim of this study was to elucidate the mechanism of hypoxia-induced cell death in human SK-N-MC neuroblastoma cells. We showed by studying internucleosomal DNA fragmentation, chromatin condensation and LDH leakage that exposure to hypoxia elicits apoptotic cell death. We demonstrated that caspases (at least caspase-3 and caspase-2) are activated by hypoxia during apoptosis. Caspase activation and apoptosis triggered by hypoxia were inhibited by treatment with a caspase inhibitor. Furthermore, hypoxia resulted in the release of cytochrome *c* into the cytosol from the mitochondria. These findings suggest that activation of caspases accompanied by cytochrome *c* release in response to hypoxia may be involved in apoptotic cell death in this cell line.

Hypoxic conditions induced DNA fragmentation in a time-dependent manner in SK-N-MC cells (Fig. 1B). We examined whether caspases, known to be death proteases, are involved in hypoxia-induced apoptosis. Treatment with caspase inhibitory peptide (Z-Asp-CH_2-DCB) significantly inhibited the formation of a hypoxia-induced DNA ladder (Fig. 1B). Enzymatic activity of a caspase-3-like protease was definitely detected in the cytosol extracts using a fluorescent peptide substrate (DEVD-MCA), following treatment with hypoxia (Fig. 2). This result suggests that hypoxia induces apoptotic cell death through caspase activation. Moreover, we found that caspase-3, -2, and PARP also degrade during the apoptotic process induced by hypoxia, with a time course similar to that of the DNA ladder formation. Hence, we demonstrate conclusively that hypoxia elicits apoptosis in neuronal cells via caspase activation.

In contrast, we could not detect caspase-1 activity in response to hypoxia using another fluorescent peptide substrate (YVAD-MCA) (Fig. 2). Similar results in published reports found that caspase-1 activity is not observed during apoptosis accompanying treatment with etoposide [24], staurosporine [25], or cytokine deprivation [26]. In SK-N-MC cells, the mRNA of caspase-1 was not detected by the RT-PCR method (data not shown). These results indicate that hypoxia may activate a caspase-3-like protease through as yet unidentified pathways, which differ from those seen when cells are challenged with anti-Fas antibody.

Caspase-2 was demonstrated to be most probably activated by caspase-3-like activity rather than by a self-activation mechanism. This indicates that caspase-2 is activated by another member of the caspase family, a caspase-3-like protease, when cells are induced to undergo apoptosis by staurosporine and anti-Fas antibody [23]. There is sequential activation of caspase-1 and caspase-3 when cells are induced to undergo apoptosis by anti-Fas antibody. After treatment with anti-Fas antibody, a caspase-1-like protease is activated first, followed by activation of a caspase-3-like protease, which may be mediated by the caspase-1-like activity, and finally a caspase-3-like protease activates caspase-2. It is unlikely that caspase-1 is activated by hypoxia, because neither caspase-1 mRNA nor caspase-1 activity on a substrate peptide was detected in SK-N-MC cells. We showed evidence for the first time that procaspase-2 is cleaved in response to hypoxia. However, there remains the interesting problem of how caspase-2 is activated by hypoxia in neuronal cells.

Recently, it has been revealed that the release of apoptogenic proteins, such as cytochrome *c* and apoptosis-inducing factor (AIF), from the mitochondria to the cytosol is involved in protease activation linked to apoptosis [27–29]. Cytochrome *c* is an essential component of the mitochondrial respiratory chain and is also released from the mitochondria in response to various stimuli that also lead to apoptosis. These include UV irradiation, etoposide, staurosporine, actinomycin D, H_2O_2, and Ara-C [30,31]. Zhivotovsky et al. [32] showed that injected cytochrome *c* induces apoptosis in several different types of cells. Furthermore, it has been demonstrated that only the oxidized form of cytochrome *c* is able to activate caspases [33]. More recently, it was reported that cytosolic cytochrome *c* activates caspase-9, and subsequently activated caspase-9 cleaves and activates caspase-3 [34]. In this cell line, we examined whether cytochrome *c* is released into the cytosol in response to hypoxia. Western blot analysis showed that significant amounts of cytochrome *c* are released 18 h after a challenge with hypoxia, and the amount continues to increase for up to 48 h. The amount of cytochrome *c* in the membrane fraction including the mitochondria showed a corresponding decrease in a time-dependent manner. The appearance of cytosolic cytochrome *c* correlated with the activation of caspase-3-like protease (DEVDase), and a decrease in the amount of the proform of caspase-2 and -3.

In conclusion, these findings indicate that hypoxia can trigger apoptosis via caspase activation in SK-N-MC neuroblastoma cells. Our data show that hypoxia triggered caspase

activation and internucleosomal DNA fragmentation. In particular, we have demonstrated that hypoxia can stimulate the release of cytochrome *c* from mitochondria. Although we have shown that hypoxia induces apoptosis via the caspase activation accompanying cytochrome *c* release, the detailed mechanism of the hypoxia-induced caspase activation linked to apoptosis remains a subject for further investigation.

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