Ceramide Formation Leads to Caspase-3 Activation during Hypoxic PC12 Cell Death

INHIBITORY EFFECTS OF Bcl-2 ON CERAMIDE FORMATION AND CASPASE-3 ACTIVATION*

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PC12 cells undergo apoptosis as well as necrosis following exposure to hypoxia. Following a 6-h hypoxic treatment, a time-dependent increase in intracellular ceramide level was observed with a concurrent decrease in sphingomyelin. It was also shown that the hypoxia-induced ceramide accumulation resulted from activation of neutral magnesium-dependent sphingomyelinase. Comparative kinetic analyses of the neutral sphingomyelinase in the cells under normoxia and hypoxia showed that hypoxia increased $V_{max}$ but did not affect $K_{m}$ of the enzyme. In PC12 cells overexpressing Bcl-2 which show strong resistance to hypoxia, sphingomyelin hydrolysis was decreased and activation of neutral sphingomyelinase was reduced. Addition of exogenous C2-ceramide induced cell death and activated caspase-3 as markedly as the hypoxia treatment. On the other hand, in PC12 cells overexpressing Bcl-2, significant decreases in cell death and inhibition of caspase-3 activation were observed after exogenous addition of C2-ceramide. The inhibitors of caspase-3 prevented cell death by either hypoxia or C2-ceramide. These results suggest that ceramide generated by activation of neutral magnesium-dependent sphingomyelinase mediates hypoxic cell death and that Bcl-2 has inhibitory effects on ceramide formation and caspase activation.

Cell death due to hypoxia is a major concern in various clinical entities such as cerebral ischemia and other diseases. However, the mechanisms of hypoxic cell death have not yet been fully understood. Cell death by hypoxia has long been generally believed to be represented as necrosis (1, 2), based on various ultrastructural findings in hypoxic cells. In contrast, recent biochemical observations have suggested the possibility of hypoxia-induced cell death including apoptosis (3, 4). These findings are supported by recent reports that hypoxic or ischemic cell death is prevented by the anti-apoptotic proteins, Bcl-2 (5–11), Bcl-XL (12), and by the inhibitors of the interleukin-1β converting enzyme and CPP32 caspases (13, 14). It has also been described that Bcl-2 functions upstream of interleukin-1β converting enzyme-like caspases during hypoxic death of PC12 cells (13). The family of cysteine proteases has been thought to be implicated in apoptotic cell death on the basis of observations that their inhibitors or negative mutants inhibit apoptosis induced by various stimuli (15–21). Ten proteins homologous to interleukin-1β converting enzyme (caspase-1) have been identified in mammals and are classified into three subfamilies, caspase-1, caspase-2 (NEDD2/ICH-1), and caspase-3 (CPP32/Yama/apopain)-like caspases, based on their structures (22). Among these caspases, caspase-3 has been deemed an attractive candidate as a putative mediator of apoptosis.

On the other hand, recently the sphingomyelin (SM) cycle, with regulated conversion of SM to ceramide by sphingomyelinase (SMase), has been extensively studied as a key pathway involved in apoptosis as well as differentiation (23–25). Ceramide is postulated to be a second messenger of apoptosis, because it appears to induce typical morphological changes of apoptosis following the inhibition of cell growth in many types of cells, including neuronal cells (26–28). SM hydrolysis is shown to be induced not only by a variety of cytokines, including tumor necrosis factor-α, interferon-γ (29, 30), interleukin-1β (31), nerve growth factor (32), and cross-linking of Fas (33), but also by physical stresses such as radiation, heat shock, and chemotherapeutic drugs (26, 34, 35).

In the current study, to elucidate the involvement of SM metabolism in hypoxia-induced cell death, we have examined the intracellular levels of ceramide and SM in vector- and Bcl-2-transfected PC12 cells after exposure to hypoxia. N-Acetylsphingosine (C2-ceramide), a membrane-permeable analog, which is known to induce apoptosis in many types of cells including neuronal cells (28, 36, 37), was also used to determine direct action of ceramide. We show here that hypoxia induces ceramide formation in PC12 cells through activation of neutral magnesium-dependent SMase, which was inhibited by highly overexpressed Bcl-2. These results suggest that ceramide formation contributes to hypoxic death of PC12 cells and that Bcl-2 has inhibitory effects on ceramide formation and caspase activation.

EXPERIMENTAL PROCEDURES

Materials—The tetrapeptide substrate for caspase-3, Ac-DEVD-MCA, was purchased from Peptide Institute (Osaka, Japan). The relatively nonselective inhibitor of caspase-1-like proteases, z-VAD.FMK, the selective inhibitor of caspase-1, z-YVAD.AFC, and the selective inhibitor of caspase-3, z-DEVD.FMK, were obtained from Enzyme Sys-
Ceramide Formation during Hypoxic Cell Death

Ceramides were incubated in 0.1M KOH in chloroform:methanol (1:2, v/v) at 37 °C for 30 min, and then washed three times with phosphate-buffered saline, and then suspended in buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, and 20 mpM sodium acetate buffer (pH 5.5) for 10 min and analyzed under a non-confocal fluorometer (Hitachi F-3000 and F-2000) with excitation at 380 nm and emission at 460 nm. Excitation and emission slit width were adjusted to 10 and 20 nm, respectively. One unit was defined as the amount of enzyme required to release 0.22 nmol of 7-amino-4-methylcoumarin per min at 37 °C.

RESULTS

Induction of Cell Death by Hypoxia—Cytotoxicity was assayed by the morphological features of the cells using double staining with Hoechst 33258 and PI under fluorescence microscopy as described by Shimizu et al. (13). Viable, necrotic, early apoptotic, terminal apoptotic cells under hypoxia could be easily distinguished and quantified by this fluorescence microscope analysis with Hoechst 33258 (blue) which stains all nucleii and PI (red) which stains only nuclei in cells with disrupted membrane integrity. Nuclei of viable, necrotic, and apoptotic cells were observed as blue intact nuclei, red round nuclei, and fragmented (or condensed) nuclei, respectively, under a fluorescence microscope.

LDH Assay—Extent of cell death was assessed using a kit to measure released LDH activity from dead cells, because loss of cell membrane integrity was observed in both necrotic and apoptotic cells (10). LDH activity in the culture medium was determined using a commercially available kit (Kyokuto, Tokyo) exactly as described by the manufacturer.

Measurement of Cellular Ceramide Level by Diacylglycerol Kinase Assay—Cellular ceramide level was measured according to the previously described method (43) with a slight modification. Lipids were extracted by a Folch partition (44), dried, and incubated in 0.1 M KOH in chloroform:methanol (1:2, v/v) at 37 °C for 10 min. Ceramide was converted to ceramide 1-[32P]phosphate by E. coli diacylglycerol kinase in the presence of [γ-32P]ATP. Labeled lipids (ceramide-1-phosphate) were separated by high performance thin-layer chromatography in chloroform:acetone:methanol:acetic acid:water (50:20:15:10:5, v/v). Following autoradiography, spots were scraped and the radioactivity was counted in a scintillation counter (Beckman LS 5000). The changes in ceramide content were normalized based on total protein.

SMase Assay—Membrane and cytosolic fractions were prepared from the cells after exposure to hypoxia. Cells were washed in cold phosphate-buffered saline and homogenized in lysis buffer (25 mM Tris/HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml E-64) using 20 strokes in a homogenizer (Iuchiiseido, Tokyo, Japan) with a Teflon pestle. The post-nuclear homogenate was centrifuged at 100,000 × g for 1 h, and the pellet was resuspended in lysis buffer.

The activities of both neutral and acid SMases were determined using a mixed micelle assay system as described (24, 46, 47). For determining neutral SMase activity, membrane and cytosolic fractions (20 μg) were mixed with [14C]-SM (40,000 cpm in 1 nmol of bovine brain SM in 0.25% Triton X-100 solubilized by sonication). The activities of magnesium-dependent and -independent neutral SMase were measured separately using 0.1 M Tris/HCl buffer (pH 7.4) with or without 6 mM MgCl2, respectively. After incubation for 30 min at 37 °C, the reaction was stopped by the addition of 1.5 ml of chloroform:methanol (2:1, v/v) followed by 0.2 ml of H2O. After phase separation, a portion of the upper phase was transferred to scintillation vials and the radioactivity was determined by liquid scintillation counting. Negative controls containing no enzyme was run concomitantly. Acid SMase activity in membrane and cytosol was measured as above except that 0.1 μM sodium acetate buffer (pH 5.5) containing 5 mM EDTA replaced the Tris/HCl buffer.

Activity of Caspase-3(-like) Proteases—Cells were harvested after exposure to hypoxia or ceramide for the indicated periods of time and washed three times with phosphate-buffered saline, and then suspended in buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA. After addition of 10 μg digitonin, cells were incubated at 37 °C for 10 min. Complete cell lysis was verified by the amount of released LDH activity. Lysates were centrifuged at 900 × g for 3 min, and the resulting supernatant (40 μl of protein) was mixed with [14C]-SM (40,000 cpm in 1 nmol of bovine brain SM in 0.25% Triton X-100 solubilized by sonication) and PI (10 μM) for 10 min and analyzed under a non-confocal fluorometer (Hitachi F-3000 and F-2000) with excitation at 380 nm and emission at 460 nm. Excitation and emission slit width were adjusted to 10 and 20 nm, respectively. One unit was defined as the amount of enzyme required to release 0.22 nmol of 7-aminomethyl-4-methylcoumarin per min at 37 °C.

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Vector-transfected PC12 cells (designated as PC12-V) were used as control cells. PC12-V cells underwent a time-dependent decrease in cell viability including apoptosis and necrosis after exposure to 1% hypoxia (Fig. 1A and B). On the other hand, PC12 cells with highly overexpressed Bcl-2 using the pUC-CAGGS system (designated as PC12-Bcl-2) showed strong resistance to hypoxia throughout the time course (Fig. 1A and B).
To confirm cell viability during hypoxia, the LDH assay was performed because loss of cell membrane integrity was observed in both necrotic and apoptotic PC12 cells after exposure to hypoxia (13). The LDH assay in each group was well correlated with the morphological changes under fluorescent microscopy (data not shown).

Ceramide Production with Concomitant Decrease in SM during Hypoxic Cell Death—Changes in the levels of ceramide and SM during hypoxia were measured by the enzymatic analysis with \textit{E. coli} diacylglycerol kinase and also by the metabolic labeling of cells with $[^{14}C]$serine. Up to 3 h after exposure to hypoxia, no significant increase in ceramide level was observed in PC12-V nor PC12-Bcl-2 cells by the diacylglycerol kinase assay. However, a marked increase in the ceramide content was observed at 6 h in PC12-V cells (Fig. 2A).

To confirm the possibility that ceramide formation could mediate hypoxic cell death, we used an inhibitor of sphinganine N-acyltransferase, FB1, which is known to inhibit \textit{de novo} synthesis of ceramide (12, 48, 49). Preincubation of PC12-V cells for 1 h with 100 \textmu M FB1 before hypoxia was unable to prevent cell death (data not shown). In contrast, prolonged (for 96 h) incubation of cells with 100 \textmu M FB1 decreased the intracellular level of both ceramide and SM, and decreased hypoxic cell death by about 40\% compared with the control culture (data not shown). These results suggest that ceramide is produced from SM via SMase during hypoxia, but not from \textit{de novo} synthesis, and that FB1 decreased intracellular ceramide and SM by prolonged incubation, which prevented hypoxic cell death. Thus, these findings also support the hypothesis that ceramide mediates cell death caused by hypoxia.

\textit{Activation of Magnesium-dependent Neutral SMase in...}
**PC12-V Cells but Not in PC12-Bcl-2 Cells Exposed to Hypoxia—** Recently neutral and acid SMases were shown to be implicated in the production of ceramide in response to apoptosis inducers (for review, see Ref. 25). Therefore, we have measured activities of three types of SMases (neutral magnesium-dependent, neutral magnesium-independent, and acid) in both membrane and cytosol fractions. It was observed that hypoxia treatment of PC12-V cells for 24 h produced an increase in membrane-bound neutral magnesium-dependent SMase activity (pH 7.5) (Fig. 3A). Interestingly, such an increase was not observed in PC12-Bcl-2 cells. No significant changes were observed in neutral magnesium-independent and acid (pH 5.5) SMase activities by hypoxia treatment, although the activity of acid SMase was higher than that of neutral SMase in both PC12-V and PC12-Bcl-2 cell membranes (the ratio of neutral to acid SMase is approximately 1:3). Activities of neutral and acid SMases in cytosol were very low and no significant changes were observed during hypoxia (data not shown). A kinetic analysis was performed to know whether hypoxia affects V_{max} or K_{m} of the magnesium-dependent membrane-bound neutral SMase. Double-reciprocal plots showed that hypoxic treatment did not significantly affect K_{m}, but increased V_{max} (1.5-fold increase over the control level) of the neutral SMase in PC12 cells cultured for 24 h under hypoxic condition (Fig. 3B).

**Activation of Caspase-3(-like) Proteases by Hypoxia and Exogenous C_{2}-Ceramide—** The above results suggest that ceramide produced during hypoxia is closely involved in hypoxic death signaling. Recently, caspases, especially caspase-3, are regarded as important regulators of apoptosis. However, the relationship between ceramide and caspases remains unknown. If the caspase-3 acts downstream of ceramide because both Bcl-2 and caspase-3 overexpression of Bcl-2 markedly inhibited activation of caspase-3(-like) proteases during both hypoxia and ceramide treatment.

**Prevention of Cell Death Induced by Hypoxia or Ceramide by Bcl-2 and Caspase-3 Inhibitors—** To further assess the involvement of caspases in cell death induced by hypoxia and C_{2}-ceramide, the cell viability was assessed by the LDH assay after hypoxia or the ceramide treatment in the presence of a caspase-3 inhibitor, z-DEVD.FMK, and a relatively nonselective caspase inhibitor, z-VAD.FMK, in PC12-V cells. The cell death induced by hypoxia at 48 h was found to be less than 20% in the cells treated with 100 μM z-VAD.FMK or z-DEVD.FMK, whereas cell death was approximately 40% in the untreated cells (Fig. 5A). In PC12-Bcl-2 cells, the percentage of cell death was about 15 and 25% at 48 and 72 h, respectively. To know the relationship between activation of caspase-3(-like) proteases and ceramide formation, the cytotoxicity was also measured in the cells treated with exogenous ceramide. The cell death induced by C_{2}-ceramide at 24 h was less than 50% in the cells treated with 100 μM z-VAD.FMK or 100 μM z-DEVD.FMK, whereas the percentage of the non-treated cells underwent cell death 24 h after addition of 20 μM C_{2}-ceramide (Fig. 5B). Dihydro-C_{2}-ceramide, an inactive analog of ceramide, did not induce cell death (data not shown). In PC12-Bcl-2 cells, the percentage of dead cells after 24 h was approximately 25%. These data imply that caspases-3(-like) proteases and Bcl-2 act downstream of ceramide because both Bcl-2 and caspase-3 inhibitors effectively inhibited the cell death induced by exogenous ceramide.

**Effects of Caspase Inhibitors on Ceramide Production during Hypoxia—** Recently, it was reported that ceramide generation in response to tumor necrosis factor-α or Fas activation was inhibited by an inhibitor of caspase-1, YVAD.CMK, or CrmA (50, 51). The results obtained in the present study suggest that activation of neutral SMase is diminished by Bcl-2. Therefore, certain caspases may also act upstream of neutral SMase. To verify this possibility, the ceramide levels during hypoxia were measured in cells treated with various caspase inhibitors (z-VAD.FMK, z-YVAD.FMK, and z-DEVD.FMK). Although these inhibitors effectively decreased cell death induced by hypoxia as shown in Fig. 5A, the level of ceramide was not changed (Table 1). Thus, formation of ceramide via SMase during hypoxia was thought to be independent of caspase-1 and caspase-3.
DISCUSSION

Ceramide, a product of SM hydrolysis by SMase, is postulated as a second messenger of apoptosis in many types of cells including neuronal cells. A number of extracellular agents and insults such as tumor necrosis factor, Fas ligands, and chemotherapeutic agents cause the activation of SMase which acts on membrane SM and generates ceramide. Ischemia can be caused by a decrease in tissue perfusion that results in an inadequate supply of oxygen, glucose, and other metabolites. Prolonged ischemia results in the death of neurons or other cells. Recent biochemical observations have suggested that the hypoxia- or ischemia-induced cell death includes apoptosis (10, 52). Interestingly, it is reported that the level of SM changes in rat cerebral cortex during focal ischemia (53), postulating that SM hydrolysis occurs in vivo. However, the injury caused by ischemia is multifactorial, including severe hypoxia, substrate deprivation, and failure to remove toxic metabolic products. It is not clear whether SM hydrolysis is a cause or a result of ischemic damage.

**Fig. 3.** Activity of neutral magnesium-dependent sphingomyelinase in PC12-V cells and PC12-Bcl-2 cells exposed to hypoxia. A, PC12-V and PC12-Bcl-2 cells were exposed to hypoxia for 24 h, washed in cold phosphate-buffered saline, and pelleted in microcentrifuge tubes. The cellular pellet was homogenized in lysis buffer. The post-nuclear homogenate was centrifuged at 100,000 g for 1 h, and the resulting pellet was dissolved in lysis buffer. The activities of neutral magnesium-dependent, magnesium-independent, and acid SMases were determined using a mixed micelle assay system with [methyl-14C]sphingomyelin at pH 7.5 and 5.5 as described under “Experimental Procedures.” Data are means ± S.D. from two independent experiments, each performed in triplicate. B, membrane fractions were prepared from cells cultured under normoxia or hypoxia for 24 h and incubated with various concentrations of N-[methyl-14C]sphingomyelin (25, 50, 100, 200, and 600 μM). Enzyme activities were determined and results were plotted double reciprocally. K_m and V_max values were calculated according to Lineweaver-Burk. Each plot is a representation of at least two experiments.

**Fig. 4.** Activation of caspase-3(-like) proteases by hypoxia or C2-ceramide. The PC12-V and PC12-Bcl-2 cells were harvested after exposure to hypoxia (A) or 20 μM C2-ceramide and dihydro-C2-ceramide (B) for the indicated periods and washed. After incubation with 10 μM digitonin, the lysates were clarified by centrifugation and the supernatants (40 μg of protein) were incubated with 50 μM of substrate Ac-DEVD-MCA at 37 °C for 1 h. Levels of released 7-amino-4-methylcoumarin were measured using a spectrofluorometer as described under “Experimental Procedures.” Data are means ± S.D. from three independent experiments, each performed in triplicate.

**Fig. 5.** Inhibition of cell death induced by Bcl-2 and caspase inhibitors. The PC12-V and PC12-Bcl-2 cells were preincubated for 1 h with medium containing 100 μM z-VAD.FMK or z-DEVD.FMK, and then exposed to hypoxia (A) or 20 μM C2-ceramide (B) for the indicated periods. Cell death was assessed by LDH release as described under “Experimental Procedures.” Data are means ± S.D. from three independent experiments, each performed in triplicate.

**Table I**

| Ceramide Formation during Hypoxic Cell Death |  |
|---|---|
| Hypoxia | Untreated | z-VAD | z-YVAD | z-DEVD |
| h | pmol/mg protein | pmol/mg protein | pmol/mg protein | pmol/mg protein |
| 0 | 272.3 ± 12.2 | 285.3 ± 27.3 | 281.3 ± 12.2 | 277.2 ± 46.4 |
| 6 | 560.4 ± 99.2 | 551.0 ± 22.3 | 572.1 ± 92.2 | 570.8 ± 101.2 |
| 12 | 659.6 ± 82.7 | 660.3 ± 62.7 | 680.6 ± 52.7 | 677.8 ± 92.7 |
| 24 | 964.0 ± 13.6 | 943.7 ± 24.1 | 973.7 ± 16.7 | 981.1 ± 42.3 |
To our knowledge, this study is the first demonstration that exposure of PC12 cells to hypoxia induced a time-dependent increase in the intracellular ceramide with a concurrent decrease in SM. It was also shown that ceramide accumulation was due to activation of neutral magnesium-dependent SMase. No significant changes were observed in neutral magnesium-independent or acid SMase activities, although activity of acid SMase is higher than that of neutral ones. This may imply that hypoxia causes activation of neutral magnesium-dependent SMase, leading to ceramide formation. Moreover, comparative kinetic analyses of neutral SMase in the cells under normoxia and hypoxia showed that hypoxia increased $V_{\text{max}}$ but did not affect $K_m$ of the enzyme. This may imply that neutral SMase is up-regulated during hypoxia by increasing activating factor(s) or the enzyme protein itself, or by decreasing inhibitory factor(s).

Although many laboratories have shown that ceramide (synthetic and natural) is able to induce apoptosis (for review, see Ref. 25), it has not been clear whether ceramide accumulates nonspecifically in dying cells or acts as a messenger for cell death. Several observations presented in this study provide possible evidence for an important role of ceramide as a mediator for hypoxic cell death. First, we have modulated the intracellular ceramide level by use of FB1, an inhibitor of de novo synthesis of ceramide. Preincubation with FB1 for 1 h did not affect cell death by hypoxia, suggesting that ceramide is derived from SM hydrolysis, but not de novo synthesis. This finding is in accordance with other reports (47, 54). Second, to know whether ceramide formation is directly implicated in hypoxic cell death, the cells were incubated with 100 μM FB1 for 96 h. It has been reported that FB1 treatment for 96 h decreased SM and ceramide by about 50% in rat sympathetic neurons (49). Under the same condition, hypoxic death of PC12 cells was suppressed by about 40% compared with the control culture. Third, exogenously added C$_2$-ceramide caused apoptotic cell death, whereas dihydro-C$_2$-ceramide was unable to cause cell death. Thus, it is likely that ceramide acts as an important mediator in hypoxic cell death.

It is of interest to note that little changes in the levels of ceramide, SM and SMase activity were seen in PC12-Bcl-2 during hypoxia. Zhang et al. (55) reported that ceramide formation in response to chemotherapeutic drugs is not changed by overexpression of Bcl-2 in Mol-4 cells, indicating that Bcl-2 does not affect ceramide formation. Differences in the extent of Bcl-2 expression, cell death inducer, and cell type may explain this discrepancy in ceramide production, since it is reported that Bcl-2 acts in a dose-dependent manner (13). The alternate explanation may be that certain caspases which are inhibitable up-regulated during hypoxia by increasing activating factor(s) or the enzyme protein itself, or by decreasing inhibitory factor(s).

In summary, the results obtained in the present study led us to propose a hypothetical scheme for ceramide signaling of hypoxic cell death in PC12 cells (Fig. 6). Neutral SMase is activated during hypoxia, which causes SM hydrolysis and production of ceramide. Increased ceramide activates caspase-3(-like) proteases, thereby leading to cell death. Bcl-2 is considered to function not only downstream of ceramide, leading to inhibition of caspase-3(-like) proteases, but also upstream of SMase to prevent ceramide production.

![Ceramide Formation during Hypoxic Cell Death](image)

**Fig. 6. A hypothetical scheme of ceramide signaling in hypoxic death of PC12 cells.** Neutral SMase is activated during hypoxia, which causes SM hydrolysis and production of ceramide. Increased ceramide activates caspase-3(-like) proteases, thereby leading to cell death. Bcl-2 is considered to function not only downstream of ceramide, leading to inhibition of caspase-3(-like) proteases, but also upstream of SMase to prevent ceramide production.

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leading to inhibition of caspase-3-like proteases, but also up-stream of SMase to prevent ceramide production. Better understanding of signal transduction pathways leading to cell death induced by hypoxia in this system may provide a basis for development of the new therapeutic approach to control hypoxic neuronal cell death.

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