Caspase Inhibitors Attenuate Oxyhemoglobin-Induced Apoptosis in Endothelial Cells

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Background and Purpose—Our recent study showed that oxyhemoglobin (OxyHb) induces apoptosis in cultured endothelial cells. Apoptosis requires the action of various classes of proteases, including a family of cysteine proteases known collectively as the caspases. This study was undertaken to investigate the effect of 2 caspase inhibitors, Z-VDVAD-FMK and Z-DEVD-FMK, in the protection of endothelial cells from OxyHb-induced apoptosis.

Methods—Cultured bovine brain microvascular endothelial cells (passages 5 to 9) were exposed to OxyHb (10 μmol/L) for 24 to 72 hours with and without caspase inhibitors. Cell attachment, DNA ladder, Western blotting of poly(ADP-ribose) polymerase (PARP), and caspase activities were measured to confirm the cytotoxic effect of OxyHb and the protective effect of the caspase inhibitors.

Results—(1) OxyHb produced cell detachment in a time-dependent manner. (2) OxyHb increased caspase-2 and -3 activities, produced DNA ladders, and cleaved PARP in endothelial cells. (3) Z-VDVAD-FMK and Z-DEVD-FMK (100 μmol/L) attenuated OxyHb-induced cell detachment, reduced caspase-2 and -3 activities, abolished OxyHb-induced DNA ladders, and prevented OxyHb-induced cleavage of PARP.

Conclusions—OxyHb activates caspase-2 and -3 in cultured brain microvessel endothelial cells. Caspase inhibitors attenuated the cytotoxic effect of OxyHb. (Stroke. 2001;32:561-566.)

Key Words: apoptosis ■ caspase ■ cerebral ischemia, transient ■ endothelium ■ oxyhemoglobins

Endothelium in cerebral arteries contributes not only to the formation of the blood-brain barrier but also to the regulation of vascular tone. Endothelial damage is an important feature of pathological changes documented in the cerebral arteries after subarachnoid hemorrhage (SAH). Formation of craters, blebbing, and vacuolation in endothelial cells have been described in cerebral arteries after SAH, leading to the disruption of tight junctions and the detachment of endothelial cells from the internal elastic lamina and exposing basement membrane and collagen to the blood stream. Metabolic activities, such as prostaglandin metabolism, endothelial-derived relaxation factor production, and endothelin secretion, are altered, leading to changes of endothelial permeability and vascular contractility.

Oxyhemoglobin (OxyHb) is the leading cause of cerebral vasospasm. OxyHb produces vasoconstriction as well as cytotoxicity in smooth muscle cells and endothelial cells. Recently, several reports have documented the ability of hemoglobin to induce apoptosis in brain cell after cerebral hemorrhage. We previously reported that OxyHb induced apoptosis in cultured endothelial cells in a time- and concentration-dependent manner. The cytotoxic effect of OxyHb was not prevented by the voltage-dependent Ca2+ channel blocker nicardipine or by voltage-independent Ca2+ channel blockers econazole or lanthanum.

The protease family of the caspases, the mammalian homologues of the Caenorhabditis elegans death gene, is required for mammalian apoptosis. Different death stimuli may activate different caspases. Among them, caspase-2 and caspase-3 are 2 of the important caspases in mediating apoptosis in a variety of tissues. In this study we investigated the effect of OxyHb on the activities of caspase-2 and -3 and the prophylaxis effect of caspase inhibitors on OxyHb-induced apoptosis in bovine brain microvessel endothelial cells.

Materials and Methods

Cell Lines and Reagents

Bovine brain microvascular endothelial cells were purchased from Cell Systems Corporation. Cells were cultured in CS-C Complete Medium (Cell Systems Corporation) and grown at 37°C in a humidified 5% CO2 incubator. Passages 5 through 9 were used for the experiments. During treatments with caspase inhibitors and OxyHb, the cells were incubated in CS-C Medium (Cell Systems Corporation) without growth factor and with 1% (vol/vol) fetal bovine serum.

Z-VDVAD-FMK (caspase-2 inhibitor I) and Z-DEVD-FMK (caspase-3 inhibitor II) were purchased from Calbiochem. Caspase inhibitor stock solution (20 mmol/L) was prepared in dimethyl sulfoxide (DMSO) and stored at −20°C for <1 month. The final DMSO concentrations of caspase inhibitor treatment groups (10 μmol/L, 100 μmol/L, and combined 100 μmol/L) were 0.05%,
Cells were preincubated with or without each caspase inhibitor (Z-VDVAD-FMK, 10 μmol/L; Z-DEVD-FMK, 10 μmol/L, 100 μmol/L) for 30 minutes at 37°C before final addition of OxyHb (10 μmol/L). The cells with caspase inhibitors and OxyHb were incubated for 24, 48, or 72 hours. After incubation, the number of adherent cells was calculated by using a hemocytometer and expressed as a percentage of the mean number of saline-treated cells. Results are means±SD (from 4 separate experiments). *P<0.05, **P<0.01 compared with cells treated with OxyHb (alone).

**Preparation of OxyHb**

The OxyHb was prepared according to the procedures described by Martin et al. Briefly, human hemoglobin (Sigma) was reduced to OxyHb with 10-fold molar excess of sodium dithionate. The sodium dithionite was later removed by dialysis against 200 volumes of normal saline for 18 hours at 4°C. The normal saline was replaced every 6 hours. The concentration of the OxyHb was determined spectrophotometrically. OxyHb was stored at −80°C before use.

**Cell Density Assay**

Cell viability was determined by counting the number of adherent cells. The counting method was performed as previously described with some modifications. Cells were seeded at a density of 5×10^4 cells per well in 24-well plates. After 2 days, the cells were preincubated with or without caspase-2 or -3 inhibitors for 30 minutes at 37°C before final addition of OxyHb (10 μmol/L). The cells with caspase inhibitors and OxyHb were incubated for various periods (24, 48, or 72 hours). After incubation, nonadherent cells were removed by 2 washes with PBS. Adherent cells were harvested by trypsinization. The number of cells was counted in a hemocytometer. Cell viability was expressed as a percentage of the viable cells in experimental wells compared with that in wells of saline control.

**DNA Fragmentation Analysis (DNA Laddering)**

After incubation, the cells were scraped and collected by centrifugation at 500g for 5 minutes at 4°C. Genomic DNA isolation was performed as specified by the manufacturer’s protocol (Oncogene Research Products). The extracted DNA (each 20 μg) was analyzed in a 1.5% agarose gel electrophoresis fractionation. The gel was then examined under UV light with ethidium bromide staining for visualization and photographed.

**Western Blot Analysis**

Proteolytic cleavage of PARP was detected by Western blot analysis as previously described. After incubation, the cells (10^6) were scraped and suspended in cold PBS twice and were collected by centrifugation at 500g for 5 minutes at 4°C. The cells were then lysed in 1% IGEPAL CA-630, 50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 5 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride for 20 minutes at 4°C; the insoluble materials were removed by centrifugation at 13 000g for 10 minutes at 4°C. Protein content of the lysate was quantified using Bio-Rad DC protein assay, and 30 μg of protein on each lane was applied to 12% SDS-PAGE. After electrophoretic transfer of the separated polypeptides to a nitrocellulose membrane, the membranes were blocked overnight with 5% nonfat dry milk in Tween-PBS (PBS containing 0.1% Tween 20). The membranes were washed with Tween-PBS and incubated with anti-PARP rabbit polyclonal antibody (1:500 dilution) at room temperature for 2.5 hours. The membranes were then washed with Tween-PBS and incubated with anti-rabbit immunoglobulin antibody linked with horseradish peroxidase. The enhanced chemiluminescence system was used for visualization of protein bands.

**Measurement of Caspase Activity**

The caspase-2/ICH-1 and caspase-3/CPP32 activities were measured with the use of a commercially available kit (Medical & Biological Laboratories Co, Ltd) according to the manufacturer’s instructions. Briefly, endothelial cells were treated with 10 μmol/L OxyHb or saline for the indicated period. After incubation, 2×10^5 cells were lysed, and a colorimetrically labeled substrate (final concentration, 200 μmol/L), VDVAD-p-nitroanilide (pNA) or DEVD-pNA, was incubated with cell lysate at 37°C for 60 to 120 minutes. The samples were then read with a spectrophotometer at 405 nm. Comparison of the absorbance of pNA from a treated sample with an untreated control allows determination of the fold increase in caspase activity.

**Data Analysis**

All data are expressed as mean±SD. Statistical significance was assessed by 1-way ANOVA with Scheffé post hoc correction. A level of P<0.05 was considered statistically significant.
Results

Cell Density
Exposing endothelial cells to 10 μmol/L OxyHb caused cell detachment in a time-dependent manner. The cell viability assay showed that 69±3% of cells were attached on the plates after 24 hours of incubation with OxyHb, but only 44±3% of cells were still attached after 72 hours. Caspase inhibitors failed to protect cells at a lower concentration (10 μmol/L) but significantly prevented cell detachment at a higher concentration (100 μmol/L). A combination of both Z-VDVAD-FMK and Z-DEVD-FMK, however, did not enhance the protective effect of each caspase inhibitor (Figure 1).

Caspase Activity
The activity of caspase-2 and -3 was investigated with the use of specific colorimetrically labeled peptides, VDVAD and DEVD, respectively. OxyHb (10 μmol/L), but not saline, significantly enhanced caspase-2 activity after 24 hours. A similar enhancement of caspase-2 activity was observed at 48 hours after exposure to OxyHb. Both Z-VDVAD-FMK and Z-DEVD-FMK attenuated the OxyHb-induced activation of caspase-2 (Figure 2A).

Saline induced 2.1-fold increase of caspase-3 activity after 24 hours of incubation compared with untreated cells. However, OxyHb (10 μmol/L) induced 4.2-fold increase of caspase-3 activity, which was significantly higher than those of either untreated or saline-treated cells. Both Z-VDVAD-FMK and Z-DEVD-FMK inhibitors attenuated the OxyHb-induced activation of caspase-3 (Figure 2B).

Since most cells died and detached 72 hours after exposure to OxyHb, the caspase-2 and caspase-3 activities were not studied. Since red-colored OxyHb might interfere with the spectrophotometric measurement, we have performed additional experiments using cells either untreated or treated with saline for 24 or 48 hours. In these studies, OxyHb was added immediately before caspase measurement so that cells were treated with OxyHb for no more than 2 minutes while OxyHb was in the solution when caspase was measured. We found that OxyHb did not increase the levels of either caspase-2 or caspase-3 (not shown).

DNA Ladder
The DNA ladder is a hallmark of apoptotic changes. OxyHb (10 μmol/L) produced a DNA ladder after a 48-hour incubation with endothelial cells, and this effect was abolished by 100 μmol/L of Z-VDVAD-FMK and Z-DEVD-FMK (Figure 3A). However, OxyHb produced DNA ladders at 72 hours after exposure, and Z-VDVAD-FMK and Z-DEVD-FMK failed to attenuate the effect of OxyHb (Figure 3B).

PARP Cleavage
Proteolytic cleavage of PARP from a 116-kDa polypeptide to an 85-kDa fragment is also a sensitive marker of the onset of apoptosis. OxyHb (10 μmol/L) clearly cleaved the 116- to 85-kDa fragment after 24-hour incubation with endothelial cells. The 116-kDa fragment almost disappeared, and the 85-kDa fragment became clear after 48 hours of exposure (Figure 4A). Z-VDVAD-FMK and Z-DEVD-FMK abolished the effect of OxyHb (Figure 4B).

Discussion
In eukaryotic cells, there are 2 different forms of cell death: necrosis and apoptosis. Necrosis is considered to be a nonphysiological cell death. Necrosis is characterized by an uncoordinated collapse of cellular homeostasis, resulting in early damage of the plasma membrane and consequently the loss of the integrity of the cell. In contrast, apoptosis is a...
vasospasm after SAH.22 Endothelial cells in the spastic middle cerebral artery of a patient who died of cerebral recent observed apoptotic changes in the endothelial cells in an essential component of the cell death pathway.23,24 It is family of cysteine proteases (caspases) appears to represent the cysteine protease protein (CPP)32–like family, which includes caspase-1, caspase-4, and caspase-5; and the ICE and CED-3 homologue (ICH)–like family, which are synthesized as proenzymes (zymogens) and activated by internal cleavage. There are currently >10 known human members of caspase.25 The caspases can be further subdivided into 3 distinct subfamilies on the basis of sequence homology: the interleukin-1β–converting enzyme (ICE)–like family, which includes caspase-1, caspase-4, and caspase-5; the cysteine protease protein (CPP)32–like family, which process of programmed cell death in which unnecessary cells are eliminated from multicellular organism. In apoptotic changes, condensation of the nucleus chromatin and fragmentation of the DNA are manifested. The cell shrinks as a result of cytoplasmic condensation, and organelles preserve their normal ultrastructure. The plasma membrane becomes ruffled and blebbled, which eventually separates the cell into a number of membrane-bound fragments of different sizes. The fragments are known as “apoptotic bodies.”20 Besides its normal role in the development and maintenance of proliferating mature tissue, apoptosis is also involved in abnormal processes such as cancer and degenerative diseases.21 We recently observed apoptotic changes in the endothelial cells in the middle cerebral artery of a patient who died of cerebral vasospasm after SAH.22 Endothelial cells in the spastic arteries demonstrated condensation of the nucleus chromatin in transmission electron microscopy and stained positive to terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) assay.

Morphological changes of apoptosis have been described for decades, and these events are considered the results of complex cellular biochemical pathways.23 In mammals, a family of cysteine proteases (caspases) appears to represent an essential component of the cell death pathway.23,24 It is believed that apoptosis is a result of the proteolysis of various cellular components initiated by activated caspases. Caspases are synthesized as proenzymes (zymogens) and activated by internal cleavage. There are currently >10 known human members of caspase.25 The caspases can be further subdivided into 3 distinct subfamilies on the basis of sequence homology: the interleukin-1β–converting enzyme (ICE)–like family, which includes caspase-1, caspase-4, and caspase-5; the cysteine protease protein (CPP)32–like family, which includes caspase-3, caspase-6, caspase-7, caspase-8, and caspase-10; and the ICE and CED-3 homologue (ICH)–like family, which includes caspase-2 and caspase-9.26,27 Among the caspases, caspase-3 has emerged as a central player in several reports of apoptosis pathways. Caspase-3 is a potent agent of apoptosis triggered via several different pathways in a variety of mammalian cell types.25 Caspase-3 cleaves several substrates, including PARP, lamins, and actins, with resultant chromosomal DNA degradation and cellular morphological changes characteristic of apoptosis. Another important caspase-family protease is a caspase-2. A variety of apoptotic stimuli activated caspase-2 during apoptosis. This activation occurs very early on treatment of cells with apoptotic agents, and the activation of caspase-2 is not seen in cells that are resistant to apoptosis.28 These observations suggest that caspase-2 is also an indispensable agent in the pathway leading to cell death.

The present study showed that OxyHb significantly activated both caspase-2 and caspase-3 in bovine brain microvessel endothelial cells. The irreversible caspase inhibitors Z-VDVAD-FMK (caspase-2 inhibitor) and Z-DEVD-FMK (caspase-3 inhibitor) significantly reduced cell detachment, caspase-2 and -3 activities, DNA ladders, and proteolytic cleavage of PARP. These results strongly suggest that activation of caspase-2 and caspase-3 is essential for OxyHb-induced apoptosis in endothelial cells, and Z-VDVAD-FMK and Z-DEVD-FMK have the potential to protect cells. Since both Z-VDVAD-FMK and Z-DEVD-FMK have almost equal and partial effects, other pathways might be involved in OxyHb-induced cell death.

This speculation is supported by the following observations in our study. First, caspase inhibitors have cross actions: Z-VDVAD-FMK reduced caspase-3 activity, and Z-DEVD-FMK reduced caspase-2 activity. Second, a combination of caspase inhibitors failed to result in a synergistic effect. The relation between caspase-2 and -3 is not clear. Caspase-3 may be an activator of caspase-2 in one study29 and be activated by caspase-2 in another.28 Third, neither of the caspase inhibitors is exclusively selective only for caspase-2 or -3; they also
inhibit other caspases. Thus, even though caspase-2 and -3 activities were increased markedly by OxyHb, we cannot exclude the possible involvement of other caspases.

Most investigators used a moderate concentration of OxyHb (1 to 100 μmol/L) in the cytotoxicity studies. OxyHb (1 to 100 μmol/L) produced detachment and cell death and apoptotic changes in cultured endothelial cells. A higher concentration of OxyHb was used in several studies in either in vitro or in vivo animal models. For example, 780 μmol/L hemoglobin (50% OxyHb) and 190 μmol/L ferrous hemoglobin were used in monkey models of vasospasm. The actual concentration of OxyHb and deoxyhemoglobin in the blood clot on day 7 was approximately 70 μmol/L. However, even though the concentration of OxyHb was high in the extraluminal space, the concentration of OxyHb may have been low in the intraluminal space closed to endothelial cells. It is unlikely that OxyHb from a blood clot can reach endothelial cells at higher levels. We used a moderate concentration of OxyHb (10 μmol/L) but incubated cells for 2 to 3 days in this study to more closely resemble conditions in patients after SAH.

A question that remains to be answered is the mechanism of OxyHb-induced apoptosis in endothelial cells. Two factors most likely responsible for the cytotoxic action of OxyHb are an elevation of intracellular Ca2+ and the generation of free radicals. Many apoptosis producers increase Ca2+ concentration, and the alteration of Ca2+ has been linked to apoptosis in a number of experimental models. Ca2+ regulates several key steps in the apoptotic pathway, from early signaling events to the chromatin cleavage that appears to mark an irreversible commitment to cell death in different cell types. However, our recent study failed to prove that Ca2+ was involved in OxyHb-induced apoptosis in endothelial cells. None of the Ca2+ channel blockers, such as nicardipine, econazol, or lanthanum, prevented apoptosis; on the contrary, econazol and lanthanum enhanced the cytotoxic effect of OxyHb, probably by causing Ca2+ starvation. Another possible route for Ca2+ elevation is release of Ca2+ from internal stores. Future studies are needed to examine this possibility.

Another possible mechanism is free radical generation. The autoxidation of OxyHb to methemoglobin generates active species of oxygen. These include the superoxide ion, the hydroxyl radical, which is formed by the reaction of bivalent iron with hydrogen peroxide, and other active forms of oxygen, such as the ferryl ion, the perfferyl ion, and the peroxylradical. Reactive oxygen species have been implicated as mediators of apoptosis and are considered to be involved in caspase-3 activation. The possible role of free radicals in OxyHb-induced apoptosis in endothelial cells requires further investigation.

Another possible candidate is nitric oxide (NO). NO is produced by NO synthase (NOS) and released either from endothelial cells (eNOS) or from neuronal tissues (nNOS). NO is involved in disparate functions, including vasorelaxation, and plays an important role in regulation of cerebral vascular tone. NO has been found to promote apoptosis in myocytes, pancreatic islet cells, macrophage-like cells, certain tumors, neuronal cells, and vascular smooth muscle cells. In contrast, NO has also been reported to inhibit apoptosis in other cells, including vascular endothelial cells. The mechanisms responsible for these marked differences in the responses to NO have not been clearly defined. However, there are several arguments against a role of NO in OxyHb-induced apoptosis. First, OxyHb binds NO with high affinity, and it may decrease the concentration of NO in the cerebral arterial wall after SAH. Second, the endothelial NOS concentration and the endothelial NOS messenger RNA in cerebral arteries after experimental SAH also tend to decrease during the chronic stage. All these might reduce a potential role of NO in OxyHb-induced apoptosis in vascular endothelial cells.

Apoptosis may play an important role in cerebral vasospasm. Apoptotic endothelial death may destroy the blood-brain barrier and expose smooth muscle cells to vasoconstrictors in the blood flow. The smooth muscle layer might be exposed to neurotransmitters, toxins, and other vasoactive agents in the blood stream through the damaged endothelium, followed by an enhanced response of vessel contraction. Damaged endothelial cells decrease the generation and release of vasodilators, such as NO and prostacyclin, from these cells. Furthermore, damaged endothelial cells may initiate thrombogenesis, cause thrombotic embolic infarction, and worsen the ischemic symptom of cerebral vasospasm. Hence, direct damage of the endothelial cells is considered responsible for the pathogenesis of cerebral vasospasm after SAH. Recent investigations show that apoptosis occurred in global or local cerebral ischemia, and inhibition of caspases prevents apoptosis in neurons and endothelial cells.

We demonstrated that caspase inhibitors prevented OxyHb-induced endothelial apoptosis in cultured bovine brain endothelial cells. Thus, caspase inhibitors may be useful in the treatment of cerebral vasospasm after SAH. In addition, caspase inhibitors may not only prevent apoptosis in endothelial cells but also protect brain tissues against apoptosis caused by cerebral ischemia during cerebral vasospasm.

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