Hemoglobin-Induced Cytotoxicity in Rat Cerebral Cortical Neurons
Caspase Activation and Oxidative Stress
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Background and Purpose—Apoptotic-like pathways may contribute to brain cell death after intracerebral hemorrhage. In this study, we used a simplified in vitro model of hemoglobin neurotoxicity to map the caspase cascades involved and to document the role of oxidative stress.

Methods—Primary neuronal cultures were obtained from rat cerebral cortex and exposed to hemoglobin to induce cell death. Cytotoxicity was assessed via measurements of mitochondrial viability (MTT assay) and lactate dehydrogenase (LDH assay). Activation of caspase-3, -8, and -9 was measured by Western blot and enzyme activity assays. Various caspase inhibitors (zVADfmk, zDEVDfmk, zIETDfmk, and zLEHDfmk) were tested for neuroprotective efficacy. The role of oxidative stress was assessed with the use of U83836E as a potent scavenger of free radicals.

Results—Exposure of primary cortical neurons to hemoglobin induced a dose- and time-dependent cytotoxicity. Western blots showed upregulation of cleaved caspase-3. Enzyme assays showed an increase in caspase-9– and caspase-3–like activity. However, caspase inhibition did not result in neuroprotection. In contrast, the free radical scavenger U83836E significantly reduced hemoglobin-induced neuronal death. Combination treatment with both U83836E and the broad spectrum caspase inhibitor zVADfmk did not yield additional protection.

Conclusions—Upstream and downstream caspases were upregulated after hemoglobin-induced neurotoxicity in vitro, but only an antioxidant approach with a potent free radical scavenger significantly improved neuronal survival. These data suggest that in addition to the activation of caspase cascades, parallel pathways of oxidative stress may predominate in this model of hemoglobin neurotoxicity. (Stroke. 2002;33:1882-1888.)

Key Words: apoptosis • cerebral hemorrhage • free radicals • neuroprotection • stroke • rats

Stroke can be broadly categorized into ischemic or hemorrhagic events. Approximately 15% of all strokes are hemorrhagic in nature, and up to 30% of all ischemic strokes will undergo hemorrhagic transformation. Furthermore, thrombolysis of ischemic stroke can trigger intracerebral hemorrhage in some patients. Yet, compared with investigation regarding ischemia, relatively little experimental investigation has been devoted to studying the pathophysiology of intracerebral hemorrhage.

Emerging data in experimental models now suggest that after hemorrhage, pathways of brain cell death may involve apoptotic-like events. Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)-positive neurons and astrocytes have been detected within and around the hematoma, and hemorrhagic brain tissue has shown DNA laddering. In a previous study using collagenase-induced hemorrhage in rat striatum, we demonstrated biochemically and pharmacologically that caspases are involved. The prototypical substrate gelsolin was cleaved into caspase-specific fragments, and the broad spectrum caspase inhibitor zVADfmk reduced TUNEL-positive cell density around the hemorrhage. However, the precise caspase cascades involved remained to be fully defined. Because it is often difficult to dissect signaling pathways in vivo, we now turn to an in vitro model to examine the caspase cascades that may be involved in hemorrhagic brain injury.

A major component of blood is hemoglobin. After cerebral hemorrhage, the brain may be exposed to high levels of hemoglobin as erythrocytes are degraded over time. It has been shown that hemoglobin may be an important mediator of neurotoxicity when injected into the brain in vivo or added to cultured neurons in vitro. Importantly, hemoglobin-induced cytotoxicity in cultured endothelial cells shows evidence of apoptotic-like pathways. Therefore, hemoglobin-induced neurotoxicity may provide a useful, albeit simplified, in vitro approach for dissecting the caspase cascades involved in cerebral hemorrhage. In the present study, primary cortical rat neurons were exposed to hemo-
globin, and dose- and time-dependent cytotoxicity was measured. Profiles of enzyme activation were examined for upstream and downstream caspases. Cultures were treated with broad-spectrum and specific caspase inhibitors to assess neuroprotective efficacy. And finally, to help judge the specificity of these findings, a free radical scavenger was also used to elucidate the overall role of oxidative stress. This antioxidant approach was relevant because there are extensive interactions between caspase pathways and free radical signaling.20–22

**Methods and Materials**

All experiments were performed by following an institutionally approved protocol in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

**Cell Culture and Reagents**

Cortical neurons were prepared from 16-day-old Sprague-Dawley rat (Charles River Laboratories, Worcester, Mass) embryonic cortex according to standard procedures. Cell suspensions were seeded at 1000 cells/cm² onto poly-d-lysine precoated 6-well plates, 24-well plates, and slide chambers by dilution with neurobasal medium (NBMM, Life Technologies) supplemented with 25 μmol/L glutamic acid, 0.5 mmol/L glutamine, 1% antibiotic-antimycotic solution, and 2% B27 supplement (Life Technologies). Cytosine (10 μmol/L) was added on day 3. Neuron cultures were fed every 4 days with replacement of half of NBMM containing 0.5 mmol/L glutamine, 1% antibiotic-antimycotic solution, and 2% B27 supplement. All cultures were incubated at 37°C in a humid atmosphere with 5% CO₂. Cultures were used at 15 to 17 days in vitro and verified to be >95% neurofilament positive by immunostaining. Before hemoglobin exposure, the medium was changed with NBMM supplemented with 0.1% B27 for 1 day and thereafter was maintained in this medium for the duration of all experiments. In experiments designed to assess the roles for caspase and oxidative stress, inhibitors or scavengers were added to cultures 60 minutes before hemoglobin exposure. These agents were dissolved in 0.2% dimethyl sulfoxide. Normal or untreated culture medium also contained 0.2% dimethyl sulfoxide, which has previously been shown to be nontoxic in our cortical neuronal system. Purified human hemoglobin was obtained from Hemosol Inc. This hemoglobin is oxygenated before storage at −80°C by the manufacturer. But because we did not ourselves quantify ratios of oxyhemoglobin versus deoxyhemoglobin, we uniformly refer to this reagent as hemoglobin. Caspase inhibitors (zVADfmk, zDEVDfmk, zIETDfmk, and zLEHDfmk) were obtained from Enzyme Systems. The potent free radical scavenger U83836E was obtained from Calbiochem.

**Assessment of Cytotoxicity**

For most experiments, hemoglobin-induced cytotoxicity was quantified by a standard measurement of lactate dehydrogenase (LDH) release with the use of the LDH assay kit (Boehringer-Mannheim). Percent cytotoxicity was calculated by subtracting LDH content in remaining viable cells from total LDH in undamaged controls.21 In addition, cytotoxicity was also quantified by measurement of the reduction of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT) to produce a dark blue formazan product. This assay assesses the integrity of mitochondrial function. MTT was added to each culture well at indicated time points after hemoglobin exposure at a final concentration of 0.5% MTT solution (wt/vol). After incubation for 4 hours at room temperature, the medium was moved, and cells were dissolved in 0.4N HCl in 99% isopropanol for 1 hour. The formation of formazan was measured by reading absorbance at a wavelength of 570 nm with a reference setting of 630 nm on a microplate reader (model FL600, Bio-Tek Instruments, Inc.). Both LDH and MTT reduction assays were used to ensure that similar data were obtained after hemoglobin-induced cytotoxicity.

**Western Blot Analysis**

Activation of caspase-3 was assessed by Western blot analysis. Culture medium was removed and washed twice with chilled (4°C) PBS. The cells were quickly scraped and collected by centrifugation and then stored at −80°C until they were assayed. The cell samples were homogenized at 4°C with lysis buffer containing 30 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% polyethoxylated, 0.1% SDS, 0.5% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 2 mmol/L MgCl₂, 1 mmol/L Na₃VO₄, 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Lysates were clarified by centrifugation at 14 000g for 10 minutes. Protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad). Samples were heated with 50 mmol/L dithiothreitol at 95°C for 5 minutes before gel loading. Each sample (25 μg per lane) was loaded onto 4% to 20% Tris-glycine gels with equal volumes of SDS sample buffer (NoveX). After electrophoresis and transferring to polyvinylidene difluoride membranes (NoveX), the membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 and 0.2% 1-block (Tropix) for 60 minutes at room temperature. Membranes were then incubated overnight at 4°C with monoclonal anti-cleaved caspase-3 antibody (1:1000, Cell Signaling Technology, New England Biolabs) and an anti-actin monoclonal antibody (1:3000, Sigma) after incubation with peroxidase-conjugated secondary antibodies and visualization by an enhanced chemiluminescence detection system (Amersham).

**Measurement of Caspase Activity**

Caspase-3, caspase-8, and caspase-9/6–like activities were measured by use of the ApoAlert Caspase Fluorescent Assay Kit (Clontech Laboratories, Inc) according to the manufacturer’s instructions, with minor modifications. After hemoglobin exposure, cells from 3 wells (in 6-well plates) were quickly washed 3 times with chilled PBS and centrifuged, and the cell pellets were stored at −70°C until they were assayed. Cells were resuspended in 50 mL chilled cell lysis buffer (Clontech Laboratories, Inc) for 10 minutes at 4°C. Cell lysates were centrifuged in a microcentrifuge at maximum speed for 5 minutes at 4°C; protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad). Fifty milliliters of 2 Reaction Buffer/DTT Mix (Clontech Laboratories, Inc) was added to each 50-mL supernatant sample (containing 50 mg protein). Five milliliters of 1 mmol/L substrates of DEVD-AFC (for caspase-3), IETD-AFC (for caspase-8), and LEHD-AMC (for caspase-9/6) were added to each reaction tube. After incubation at 37°C for 60 to 120 minutes, samples were transferred to a 96-well plate, and the fluorescence intensities were measured in a fluorescence plate reader (model FL600, Bio-Tek Instruments, Inc.) with 400/ 500-nm filters for caspase-3 and caspase-8 and 380/460-nm filters for caspase-9/6.

**Statistical Analysis**

Quantitative cytotoxicity data were analyzed by using ANOVA followed by Tukey’s honestly significant difference tests. For multiple group studies, a 2-factor ANOVA was used. For time-dependent studies, a repeated-measures ANOVA was used. Data are expressed as mean ± SEM. A value of P < 0.05 was considered significant.

**Results**

**Hemoglobin Induces Dose- and Time-Dependent Neurotoxicity**

Exposure of primary rat cortical neurons to hemoglobin for 24 hours produced cytotoxicity, as assessed via LDH measurements. There was a clear dose-dependent response for hemoglobin concentrations ranging from 6 to 100 μmol/L (Figure 1a). To exclude the possibility that more subtle cell metabolic dysfunction may be missed, an assay for mitochondrial viability (MTT assay) was also performed. An intermediate dose of hemoglobin (25 μmol/L) was selected, and both
MTT and LDH outcomes were followed over a course of 48 hours. A time-dependent cytotoxicity was observed. There were no differences between MTT and LDH assays of cytotoxicity (Figure 1b).

Caspases Are Activated After Hemoglobin Exposure

Western blots showed that control undamaged neurons exhibited low-level baseline expression of cleaved/active caspase-3, consistent with the fact that these were primary cultures derived from embryonic rat brain (Figure 2a). After exposure to hemoglobin, the levels of cleaved caspase-3 increased. Bands at 17 and 19 kDa were detected as early as 6 hours and increased up to 24 hours after hemoglobin exposure (Figure 2a).

To quantitatively assess profiles of downstream and upstream caspases, enzyme assays were performed to measure caspase-3, caspase-8, and caspase-9 activity after hemoglobin exposure. Mean levels of caspase-8 activity appeared to slightly increase, but these elevations did not reach statistical significance (Figure 2b). However, caspase-9 activity was significantly increased at 3 and 6 hours, and caspase-3 activity was significantly increased at 12 hours after hemoglobin exposure (Figure 2c).

Caspase Inhibitors Do Not Reduce Hemoglobin Neurotoxicity

Therapeutic efficacy was tested for a wide range of caspase inhibitors. These included the broad-spectrum inhibitor...
zVADfmk, as well as relatively more specific inhibitors targeted against caspase-3 (zDEVDfmk), caspase-8 (zIETDfmk), and caspase-9 (zLEHDfmk). To ensure that the inhibitors were biochemically effective in our model system, cultures were pretreated with 40 μmol/L of each inhibitor for 1 hour, and then caspase enzyme activities were measured at 6 hours after 25 μmol/L hemoglobin exposure. zVADfmk and zDEVDfmk both significantly reduced hemoglobin-induced caspase-3 activation (Figure 3a). zIETDfmk and zLEHDfmk significantly reduced activation of caspase-8 and caspase-9, respectively (Figure 3a). However, cytotoxicity measured at 24 hours after hemoglobin-induced injury showed no neuroprotection for any of the caspase inhibitors tested (Figure 3b). No toxic side effects of the various inhibitors in normal control cultures were observed at the 40-μmol/L doses used in the present study (data not shown).

Figure 3. a, Inhibitory efficacy of caspase inhibitors on Hb-induced increase of caspase activity measured with enzyme activity assays. The following caspase inhibitors were tested: zDEVDfmk (DEVD) for caspase-3, zIETDfmk (IEDT) for caspase-8, zLEHDfmk (LEHD) for caspase-9, and zVADfmk (ZVAD) as a broad-spectrum inhibitor. Inhibitors (40 μmol/L) were preincubated for 1 hour before exposure to 25 μmol/L Hb, and caspase activities were measured at 6 hours. The targeted caspase activities were all significantly inhibited, as expected (*P<0.05 vs untreated cultures). All data were averaged from 4 independent experiments (n=3 culture wells per experiment). b, Effects of caspase inhibition on Hb cytotoxicity measured at 24 hours with an LDH assay. No protective effects were found for any caspase inhibitor (40 μmol/L). All data were averaged from 4 independent experiments (n=3 culture wells per experiment).

Antioxidants Reduce Hemoglobin Neurotoxicity and Attenuate Caspase-3 Activation

In light of the negative data with the wide range of caspase inhibitors tested, an antioxidant approach was also used. In part, these experiments served as a positive control. Cultures were pretreated with the potent free radical scavenger U83836E for 1 hour and then exposed to 25 μmol/L hemoglobin, and cytotoxicity was assessed at 24 hours. U83836E showed a dose-dependent protection at 1 and 5 μmol/L concentrations (*P<0.05). The broad-spectrum caspase inhibitor ZVADfmk (Z) alone had no protective effect. When ZVADfmk was combined with U83836E, no additional or synergistic neuroprotection was observed. All data were averaged from 4 independent experiments (n=3 culture wells per experiment). Pretreatment with 5 μmol/L U83836E also decreased cleaved caspase-3 levels. The combination of both ZVADfmk plus U83836E completely suppressed the cleavage of caspase-3 down to baseline levels. The results of the analysis for actin is shown as an internal control (bottom blot).

Figure 4. a, Neuroprotection against Hb (H) toxicity with the free radical scavenger U83836E (U). Cytotoxicity was measured at 24 hours after 25 mol/L Hb exposure with a standard LDH assay. U83836E showed dose-dependent efficacy at 1 and 5 μmol/L concentrations (*P<0.05). The broad-spectrum caspase inhibitor ZVADfmk (Z) alone had no protective effect. When ZVADfmk was combined with U83836E, no additional or synergistic neuroprotection was observed. All data were averaged from 4 independent experiments (n=3 culture wells per experiment). b, Expression of cleaved caspase-3 at 12 hours after exposure to 25 μmol/L Hb, as assessed by Western blot analysis (top blot). A slight cleaved band of caspase-3 (19 kDa) was detectable in normal controls, as expected for cultures derived from embryonic rat cortex. Cleaved caspase-3 (17 and 19 kDa) was increased at 12 hours after Hb exposure. Pretreatment with 40 μmol/L ZVADfmk suppressed the activation of caspase-3. Pretreatment with 5 μmol/L U83836E also decreased cleaved caspase-3 levels. The combination of both ZVADfmk plus U83836E completely suppressed the cleavage of caspase-3 down to baseline levels. The results of the analysis for actin is shown as an internal control (bottom blot).
hours after hemoglobin exposure. As expected, zVADfmk reduced the hemoglobin-induced increase in cleaved caspase-3 (Figure 4b). However, U83836E also slightly decreased the levels of cleaved caspase-3 (Figure 4b). When zVADfmk and U83836E were used in combination, cleaved caspase-3 levels were suppressed to almost baseline levels (Figure 4b).

**Discussion**

The mechanisms of brain injury after intracerebral hemorrhage are complex. Nevertheless, these multifactorial pathways can be broadly divided into 2 main categories. The first is mechanical and involves the mass effect of a hemorrhage within the closed cranial vault. This mass effect would lead to mechanical compression and trauma to adjacent brain tissue. Additionally, the mass effect may also increase intracranial pressure with concomitant edema, compression of the cerebrovasculature, and further secondary ischemia. A second major pathway of injury may involve the extravasation of blood components into brain parenchyma. Many components in the blood can be neurotoxic. These include glutamate, thrombin, hemoglobin, and a host of activated cytokines and inflammatory agents that respond to vascular injury. Together, these mechanical, ischemic, and potentially neurotoxic components constitute a complex insult after cerebral hemorrhage.

Emerging data in experimental models suggest that apoptotic-like pathways may ultimately mediate cell death after cerebral hemorrhage. Our laboratory previously explored a rat model of collagenase-induced striatal hemorrhage. Gel electrophoresis showed DNA laddering. TUNEL labeling showed in situ DNA fragmentation in neurons and astrocytes, caspase substrates were cleaved, and a broad-spectrum caspase inhibitor, zVADfmk, significantly reduced the density of TUNEL-positive cells. Taken together, these data provided biochemical and pharmacological evidence in support of apoptotic-like events. However, the specific pathways involved remain unclear, and it can be difficult to elucidate these mechanisms in vivo. In the present study, we used a simplified model of hemoglobin neurotoxicity to dissect these mechanisms in vitro. Induction of neuronal death after hemorrhage most likely involves multifactorial cascades. Nevertheless, hemoglobin is a major component of extravasated blood and a potent oxidant. It has been previously shown that hemoglobin, most likely via heme and/or iron moieties, can trigger cytotoxic responses in neurons. After hemorrhage in vivo, the brain is exposed to elevated levels of hemoglobin as erythrocytes are degraded over time. An experimental study of cerebral hemorrhage in monkeys showed that hemoglobin reached levels between 20 and 100 μmol/L from 1 to 12 days after hemorrhage. The 25 μmol/L hemoglobin concentrations used in the present study should be within range. Overall, hemoglobin neurotoxicity may be a reasonable in vitro model system for dissecting the role of apoptotic-like mechanisms involved in hemorrhage.

In the present study, hemoglobin induced a dose- and time-dependent toxicity in primary cultured neurons. Toxicity involved mitochondrial dysfunction, as assessed via the MTT assay, and cell membrane disruption, as evidenced by LDH leakage. The major finding was that a well-defined caspase cascade was triggered after hemoglobin exposure. As expected, the downstream caspase-3 was upregulated. Interestingly, the upstream signals appeared specific. Caspase-9 showed a suggestive temporal profile, in which it was upregulated before the downstream caspase-3. Mean levels of the other major upstream caspase, caspase-8, were somewhat increased but did not reach statistical significance. On the basis of these in vitro data and our previous in vivo findings of caspase substrate cleavage after hemorrhage, we tested a broad range of caspase inhibitors for neuroprotective efficacy. These included the broad-spectrum inhibitor zVADfmk as well as inhibitors that were relatively specific for caspase-3 (zDEVDfmk), caspase-8 (zIETDfmk), and caspase-9 (zLEHDfmk). Surprisingly, none of the inhibitors were able to reduce hemoglobin neurotoxicity even though enzyme activity measurements showed that the targeted caspases were effectively inhibited.

There may be several reasons for the lack of neuroprotection. First, it is possible that caspases act at a relatively late stage in the cell death cascade, so that inhibition of caspases alone cannot prevent the cell from eventually succumbing to the cumulative effects of the preceding metabolic dysfunction. Others have shown that Bax-, Fas-, staurosporine-, and H$_2$O$_2$-induced cell death can all proceed in the face of broad-spectrum caspase inhibition. Indeed, one study has shown that caspase inhibition was protective against hemin neurotoxicity at early but not at later time points. In the present study, it is possible that our caspase inhibitors were protective early on, but prolonged exposure to hemoglobin ultimately led to cell death. Additionally, even if caspase inhibition can successfully prevent the morphological aspects of cell death, functional recovery in these remaining cells remains uncertain. In a model of global cerebral ischemia, caspase inhibitors prevented the development of morphological markers of CA1 neuronal damage, but long-term potentiation in these neurons was still impaired. It is interesting to note that our data differ from published findings reporting that caspase inhibitors significantly protect against hemoglobin cytotoxicity in endothelial cells. Clearly, cell-specific responses are different, and it will be important for future studies to carefully examine the acute as well as chronic responses of multiple cell types in the brain, including neurons, astrocytes, oligodendrocytes, and vascular cells, such as endothelial and smooth muscle cells.

A second related reason for our failure to detect neuroprotection against hemoglobin is the fact that other pathways may be involved. In the present study, we explored the possibility that in spite of the biochemical evidence for caspase activation, other parallel and potentially predominant pathways may mediate neuronal death in our model system. Caspase signaling is known to have extensive interactions with oxidative stress pathways. Hence, we also tested the efficacy of the potent free radical scavenger U83836E. This lazaroed compound is a potent antioxidant that inhibits lipid peroxidation in many models of oxidative stress. Our data showed that U83836E significantly reduced hemoglobin neurotoxicity in a dose-dependent manner. Combination therapy using both U83836E and the broad-spectrum caspase
Oxidative Stress and Caspase in Hb Neurotoxicity

Figure 5. Schematic diagram showing hypothesized relationship between oxidative stress and activation of caspases after hemoglobin neurotoxicity in vitro. Hemoglobin induces oxidative stress. Activation of upstream caspase-9 and downstream caspase-3 subsequently occurs, which ultimately leads to caspase-mediated cell death. Parallel to this pathway, oxidative stress can also directly damage neurons, leading to cell death independent of caspases.

Inhibitor zVADfmk did not yield any additional protection. Interestingly, however, U83836E decreased the levels of cleaved/active caspase-3. Taken together, these data suggest that oxidative stress may participate in pathways upstream from and parallel to caspase activation (Figure 5). In terms of therapeutic targets, oxidative stress appears to predominate in this model system of hemoglobin neurotoxicity in vitro.

There are a few caveats associated with the present study. First, this model system of hemoglobin-induced neuronal injury is highly simplified. After intracerebral hemorrhage in vivo, there will be a multitude of factors in extravasated blood that can trigger brain cell death. Nevertheless, hemoglobin remains a major component of blood and is highly neurotoxic. Others have also used this model as an in vitro system to investigate pathways that are difficult to dissect in vivo.\textsuperscript{15,16,31} Clearly, these in vitro findings will have to be carefully extended into in vivo systems before data can be translated into a clinical context. A second caveat involves the fact that the hemoglobin model cannot replicate the vascular trauma and tissue ischemia that take place after hemorrhage in vivo. These interactions may be critical, inasmuch as it has been shown that hemoglobin neurotoxicity in vitro. These interactions may be critical, inasmuch as it has been shown that hemoglobin neurotoxicity in vitro. Hemoglobin induces oxidative stress and hemorrhage in vivo. These interactions may be critical, inasmuch as it has been shown that hemoglobin neurotoxicity in vitro. Hemoglobin induces oxidative stress.

In conclusion, the present study used a simplified model of hemoglobin neurotoxicity to dissect the mechanisms of injury in vitro. The major finding was that although caspase cascades were upregulated in a temporally specific sequence, caspase inhibitors did not offer neuroprotection. Alternatively, an antioxidant approach using the potent radical scavenger U83836E significantly reduced cell death and also decreased the levels of caspase upregulation. These data suggest that hemoglobin can upregulate caspase signals but that upstream and parallel pathways of oxidative stress may predominate. Therapeutic strategies against intracerebral hemorrhage should target multiple components of injury for maximal gain.

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