Transcription Factor Nrf2 Protects the Brain From Damage Produced by Intracerebral Hemorrhage

Xiurong Zhao, MD; Guanhua Sun, MD; Jie Zhang, BS; Roger Strong, MS; Pramod K. Dash, PhD; Yuet Wai Kan, MD; James C. Grotta, MD; Jaroslaw Aronowski, PhD

Background and Purpose—Intracerebral hemorrhage (ICH) remains a major medical problem for which there is no effective treatment. Oxidative and cytotoxic damage plays an important role in ICH pathogenesis and may represent a target for treatment of ICH. Recent studies have suggested that nuclear factor–erythroid 2–related factor 2 (Nrf2), a pleiotropic transcription factor, may play a key role in protecting cells from cytotoxic/oxidative damage. This study evaluated the role of Nrf2 in protecting the brain from ICH-mediated damage.

Methods—Sprague-Dawley rats and Nrf2-deficient or control mice received intracerebral injection of autologous blood to mimic ICH. Sulforaphane was used to activate Nrf2. Oxidative stress, the presence of myeloperoxidase-positive cells (neutrophils) in ICH-affected brains, and behavioral dysfunction were assessed to determine the extent of ICH-mediated damage.

Results—Sulforaphane activated Nrf2 in ICH-affected brain tissue and reduced neutrophil count, oxidative damage, and behavioral deficits caused by ICH. Nrf2-deficient mice demonstrated more severe neurologic deficits after ICH and did not benefit from the protective effect of sulforaphane.

Conclusions—Nrf2 may represent a strategic target for ICH therapies. (Stroke. 2007;38:3280-3286.)

Key Words: inflammation ■ intracerebral hemorrhage ■ neuroprotection ■ oxidative stress

Spontaneous intracerebral hemorrhage (ICH) accounts for 10% to 20% of all strokes in the United States and two thirds of hemorrhagic strokes.1,2 ICH affects an estimated 37 000 to 52 000 people in the United States annually.2 The mortality rate from ICH is 35% to 52% within 30 days after ICH.1,2 No medical or surgical therapy to date has been shown to reduce morbidity or mortality after ICH.3 The current management protocol for ICH is limited to supportive medical care and surgery for a select group of patients. New paradigms to modify the course of this disease are urgently needed.

Intraparenchymal blood deposited during ICH elicits a host of biologic responses, including the generation of cytotoxic byproducts of blood and the overproduction of free radicals and proinflammatory molecules, ultimately leading to neuronal death.4–8 To affect outcome after ICH, one needs to combat this complex multifactorial brain pathology.

Multiple lines of evidence indicate that through targeting a single transcription factor, numerous pathologic cascades can be impeded. For instance, by inhibiting nuclear factor-κB (NF-κB), it is possible to inhibit a broad range of NF-κB–regulated gene products.9–11 Because NF-κB is the master regulator of expression of many proinflammatory genes, its inhibition may ultimately lead to attenuation of many facets of inflammation.

Recently, another transcription factor, NF-erythroid 2–related factor 2 (Nrf2), was shown to play a critical role in orchestrating the expression of the phase II detoxification, hemoglobin-handling, and antioxidant genes that would be suitable in combating the pathogenic events associated with ICH.12 We and others have recently demonstrated that Nrf2 could represent an important factor in protecting the brain from the damage produced by ischemic stroke13,14 and kainate toxicity.15 Therefore, our hypothesis here is that Nrf2 activators may be capable of protecting the brain from damage caused by ICH.

Materials and Methods

ICH Model in the Rat and Mouse

The ICH model in the rat and mouse was induced by intrastriatal injection of autologous blood, as we described previously.16–19 In brief, Sprague-Dawley rats (250 to 350 g) or Nrf2−/− or control Nrf2+/+ mice20 (C57BL/6 background; 25 to 30 g) were immobilized by 0.35 g/kg chloral hydrate anesthesia onto a stereotaxic frame. A 1-mm-diameter burr hole was drilled in the skull, and a 26-gauge stainless steel cannula was inserted for blood infusion (collected from the femoral artery; 15 μL/5 minutes for mice or 45 μL/5

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minutes for rats) into the left brain striatum. Core body temperature was maintained at 37±0.5°C. Nrf2+/− mice were generated and characterized by Chan et al.20

All animal studies conformed to the guidelines outlined in Guide for the Care and Use of Laboratory Animals from the National Institutes of Health and were approved by the University of Texas-Houston Animal Welfare Committee.

Administration of SF

The Nrf2-activating agent sulforaphane (SF, LKT Laboratories, Inc) at 5 mg/kg in corn oil was injected intraperitoneally at 30 minutes after ICH. Corn oil (10% corn oil in phosphate-buffered saline, 100 μL) served as the vehicle control.

Analysis of NDS

All behavioral tests in rats and mice were conducted in a quiet, low-lit room by an experimenter blinded with respect to treatment group. Pretests were done to exclude abnormal animals. Only animals with <20% footfault and normal forelimb placing were subjected to ICH. A combination score from a battery of behavioral tests (footfault, forelimb placing, postural reflex, circling, and circling tests; neurologic deficit score [NDS]) was used as a comprehensive measure of neurologic functional deficits, as we reported previously.16,19

Animal Perfusion and Tissue Dissection

The animals were anesthetized with chloral hydrate (0.5g/kg IP) and perfused with ice-cold phosphate-buffered saline. For biochemical analyses, the brains were removed and subdivided; for immunohistochemistry, the brains were quickly harvested. The tissue was snap-frozen in −80°C 2-methylbutane and stored in a −80°C freezer before processing for RNA isolation, protein extraction, or cryosectioning.

Nuclear Protein Extraction

Nuclear protein extraction was performed as we described previously.19 In brief, the tissue was homogenized in ice-cold hypotonic lysis buffer (10 mmol/L HEPES, pH 7.9; 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, and 1 mmol/L dithiothreitol) containing proteinase inhibitors (0.5 mmol/L phenylmethylsulfonylfluoride, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 0.5 mg/mL benzamidine, 5 mmol/L NaCl, 2 mmol/L sodium pyrophosphate, and 1 mmol/L sodium orthovanadate) and centrifuged at 4°C. The pellets were washed with lysis buffer and resuspended in an ice-cold, hypertonic, nuclear extract buffer (20 mmol/L HEPES, pH 7.9; 420 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L dithiothreitol containing proteinase inhibitors). After incubation on ice and centrifugation, the protein concentration of the nuclear extracts was determined by the Bradford method.

EMSA for Nrf2

For the electrophoresis mobility shift assay (EMSA), 10 μg of nuclear protein was incubated with 32P-labeled, double-stranded oligonucleotides (2 pmol) containing the antioxidant-responsive element sequence (modeled after the heme oxygenase-1 promoter; 5′-GATCTTTTATGCTGTGTCATGGTTT-3′ and used previously to characterize Nrf2).21 Binding reactions were prepared in a final volume of 20 μL (2 μg poly[dI-dC]; 25 mmol/L HEPES, pH 7.9; 0.5 mmol/L EDTA; 0.5 mmol/L dithiothreitol; 1% NP-40; 5% glycerol; and 50 mmol/L NaCl) and incubated for 30 minutes at 37°C. The nucleoprotein-oligonucleotide complexes were resolved electrophoretically on a 7% nondenaturing polyacrylamide gel in 0.5× TrisborateEDTA (TBE) buffer. The gel was autoradiographed. Optical density was determined with the Kodak Analysis 290 (EDAS) system.

Western Blotting

For protein electrophoresis and immunoblotting, we followed the methods as described previously.17 Samples (50 μg protein) were resolved by 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes and stained with Ponceau red to confirm equal loading. Blots were blocked in 3% nonfat milk in TBST (20 mmol/L Tris, 0.15 mol/L NaCl, and 0.05% Tween-20) and then probed with rabbit anti-Nrf2 antibody (1:2000, Abcam, Cambridge, Mass) in 1% bovine serum albumin (BSA)–TBST. The blot was visualized by enhanced chemiluminescence (ECL; Pierce, Rockford, Ill) after incubation in goat anti-rabbit-horseradish peroxidase (HRP; 1:5000). Semiquantification of immunoreactive bands on x-ray film was achieved by analyses of optical density with the EDAS system.

Dot Blotting

To measure oxidative stress to proteins and lipids after ICH, we performed dot blot analyses with anti-nitrotyrosine (1 μg/mL 3′-NT; Upstate Biotechnology) and anti–4-hydroxyisynonenal (1 μg/mL 4-HNE; Chemicon) antibodies, respectively. Ten micrograms of protein from the ICH-affected striatum was loaded onto a nitrocellulose membrane. The membranes were blocked and developed with the same procedures as that for Western blowering.

Reverse Transcription–Polymerase Chain Reaction

mRNA levels were semiquantified by reverse transcription–polymerase chain reaction (RT-PCR).16,19 We used the rat glycerolaldehyde 3-phosphate dehydrogenase (GAPDH) gene as an internal standard. Each set of PCRs included control samples run without RNA, in which the RT step was omitted, to ensure that PCR products resulted from amplification of the purified mRNA rather than genomic DNA. The sequences of primers are listed in the Table. The cycle numbers corresponding to the exponential amplification phase were determined for each gene and each preparation by the time–product curve. The cycle number accepted for quantitation was 25, 23, 28, and 27 for catalase, CuZn superoxide dismutase (SOD), NAD(P)H dehydrogenase, quinone-1 (NQO1), and glutathione S-transferase (GST), respectively. The PCR products were run on 1% to 2% agarose gels and stained with ethidium bromide. The images were digitized and the optical density of each band was quantified with the EDAS system. The data (optical density) were first normalized against the internal standard (GAPDH) and then expressed as percent change from the vehicle control.

Immunohistochemistry

Immunostaining was done as described previously.19 In brief, coronal cryosections (10 μm thick) were postfixed with 4% paraformaldehyde, blocked in 3% normal goat serum, and incubated with rabbit anti-myeloperoxidase (MPO, Dako), rabbit anti-NT (Upstate Biotechnology), or rabbit anti–4-HNE (Chemicon) antibodies in 3% albumin (BSA)–TBST. The blot was visualized by enhanced chemiluminescence (ECL; Pierce, Rockford, Ill) after incubation in goat anti-rabbit-horseradish peroxidase (HRP; 1:5000). Semiquantification of immunoreactive bands on x-ray film was achieved by analyses of optical density with the EDAS system.

Image Capture and Cell Counting

A Zeiss Axioskop-2 microscope equipped with a CCD camera and operated by MetaMorph 6.2 software was used for image acquisition. Four SF-treated and 4 vehicle-treated rats were evaluated. Coronal sections (10 μm thick) at the level of needle insertion (blood injection site) were used in the analyses. Five images from each section were acquired using magnification with a 20× objective. Each image represented a 448×335-μm area and was captured at the locus representing the border zone of the hematoma. The assignment of loci was the same for each slide. The number of MPO-positive cells in each section was calculated. Additionally, the same sections plus sections at 0.5 mm rostral to the needle insertion locus were
evaluated for the number of MPO-positive cells by automated (unbiased) cell counting. Specifically, we counted all MPO-positive cells within the ICH-affected striatum (4.9 ± 0.7 mm²). We used a motorized microscope stage, fluorescence image stitching, and a computer-aided image package (MetaMorph), similar to the procedure we described previously.18

Statistical Analysis
The data were analyzed with GraphPad and InStat programs. One-way ANOVA with a Newman-Keuls posttest was used for multiple comparisons. An unpaired t test was used when 2 groups were compared. Statistical significance was considered at P ≤ 0.05. Data were expressed as mean ± SEM.

Results
SF Reduces Neurologic Deficits After ICH in Rats
We used a well-characterized,17–19 intrastratial autologous blood injection model of ICH in rats. This model for ICH causes measurable NDS.16,19 We found that intraperitoneal administration of the Nrf2 activator, SF, at 5 mg/kg 30 minutes after the onset of ICH in rats reduced the NDS by 53.1% (P ≤ 0.05), as determined 10 days after ICH (Figure 1).

SF Induces Nuclear Translocation and Activation of Nrf2
To test whether SF under the experimental conditions used in this study was capable of activating Nrf2, we measured Nrf2 protein in the nuclear extracts prepared from the ipsilateral striatum (hematoma-affected tissue). Figure 2A shows that SF administration after ICH significantly enhanced Nrf2 protein in the nuclear fraction.

Binding to the antioxidant-responsive element represents a mandatory step in the transcriptional activation by Nrf2. Therefore, we measured DNA binding activity by EMSA with an Nrf2-specific, double-stranded oligonucleotide probe. Intraperitoneal injection of SF, at both 5 and 10 mg/kg (30 minutes after ICH), reproducibly increased the Nrf2 DNA binding activity in the hematoma-affected striatum (Figure 2B). The cold probe efficiently competed with complex formation, suggesting antioxidant-responsive element–Nrf2 interaction specificity (data not included).

SF Reduces Neutrophil Count in the Hematoma Area After ICH
To determine whether SF affects inflammation, we treated the animals with SF and counted the neutrophils (MPO-positive cells) around the hematoma 3 days after ICH. In agreement with our previous results,5,19 ICH produced a robust increase in the number of MPO-positive cells in the hematoma-affected striatum, primarily in the areas bordering the hematoma. Treatment with SF markedly reduced the number of neutrophils, as determined by counting MPO-positive cells in two locations: the perihematoma and affected striatum. Perihematoma neutrophil count was reduced by 61.6% (P ≤ 0.05, Figure 3), whereas the total ICH-affected striatum neutrophil count was reduced by 33% (1962 ± 355 vs 2905 ± 454).

SF Increases Expression of Antioxidative and Detoxifying Enzymes After ICH
SF has been reported to increase the expression of antioxidative and detoxifying enzymes.22 We found that the dose of

Table. Primer List for RT-PCR (Rats)

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<td>R</td>
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</table>
F indicates forward; R, reverse.
SF that reduced neurologic dysfunction upregulated mRNA expression of Nrf2-regulated genes encoding important antioxidative enzymes, including catalase, SOD, NAD(P)H dehydrogenase, quinone-1, and glutathione S-transferase, in the ipsilateral striatum at 3.5 hours after ICH (3 hours after SF administration; Figure 4). The SF-mediated increase in gene expression occurred in addition to the effect of ICH alone. mRNA expression for catalase, SOD, NQO1, and GST was increased by 1.6-, 1.3-, 1.3-, and 2.1-fold, respectively, in the ICH group compared with naive controls (data not shown).

**SF Reduces Oxidative Damage After ICH**

Reactive oxygen–induced damage to lipids and proteins may play an important role in ICH pathogenesis.4,23 Because SF upregulated the expression of antioxidative and detoxifying enzymes, we further tested the level of oxidation-mediated changes in proteins (3'-NT) and lipids (4-HNE) in the hematoma-affected striatum 24 hours after ICH by immunohistochemistry and dot immunoblotting (Figure 5). Immunohistochemistry for 3'-NT and 4-HNE indicated that the oxidative stress after ICH was primarily confined to the perihematoma area (Figures 5A and 5B). Administration of SF 30 minutes after ICH significantly reduced the immunoreactivity for both 3'-NT and 4-HNE (Figures 5C and 5D) in the ICH-affected striatum, as determined 24 hours after ICH.

**Nrf2-Knockout Mice Display More Severe Functional Deficits and Do Not Respond to SF After ICH**

To clarify the beneficial role of Nrf2 in ICH, we subjected Nrf2-knockout mice (Nrf2-/-) and control mice (Nrf2+/+) to ICH in the presence or absence of SF and measured the NDSs 7 days after ICH. Consistent with our prediction, Nrf2 deficiency significantly worsened the neurologic deficit caused by ICH and also obliterated the protective effect of SF, which was seen in the Nrf2+/+ mice (Figure 6).

**Discussion**

Intraparenchymal blood components and local cell debris have a strong cytotoxic effect in the brain parenchyma after ICH. The cytotoxic response occurs within minutes from the onset of ICH and encompasses oxidative stress, proinflammatory responses, cell death, and neurologic damage.5,24–27 Activation of Nrf2 with SF results in upregulation of a battery...
of antioxidative and detoxifying enzymes and reduction of oxidative damage and inflammation in the brain areas endangered by the intraparenchymal hematoma. Therefore, activation of Nrf2 after ICH may represent a potential target for combating damage produced by ICH.

Although the majority of hemolysis of extravasated erythrocytes occurs several days after ICH, lysis of red blood cells has also been documented to occur within <24 hours after ICH.28,29 The hemoglobin release and breakdown into heme, iron, and other toxic molecules promotes the oxidative damage to neurons and other brain cells.6,7,28–31 The prooxidative environment after ICH leads to irreversible damage to proteins, nucleic acids, carbohydrates, and lipids, causing cellular dysfunction and cell death. Direct evidence for the deleterious role of oxidative stress in ICH was generated by demonstrating that treatment with antioxidative drugs, including dimethylthiourea, α-phenyl-N-tert-butyl nitrone, NXY-059, or chelating iron with deferoxamine,7,28,32 significantly reduced brain edema and neurologic deficit caused by ICH. Another important contributor to brain injury after ICH is the proinflammatory responses that encompass microglia activation,33 proinflammatory cytokine generation,5,34,35 and neutrophil infiltration.19,36,37 The deleterious effect of inflammation involves the generation and release of various cytotoxic substances, including free radicals,5 that may further contribute to the oxidative stress and damage to brain cells proximal to the locus of inflammation. In agreement with this scenario, we found that the most profound oxidative stress responses were present within brain areas representing the border zone of the hematoma. The perihematoma oxidative rim extended ∼50 to 150 μm into the parenchyma and virtually encapsulated the entire hematoma. In our previous work, we reported that the same area, identified here to be affected by oxidative damage, represents the location of progressive neuronal loss.18

Nrf2 is a master regulator of antioxidative defense responses and is an important protective-survival factor for central nervous system tissue. Because of the important role of oxidative stress in the pathogenesis of ICH, in this study we investigated the role of Nrf2 in ICH. Existing data have demonstrated that Nrf2-deficient mice are more vulnerable to oxidative stress and that this vulnerability is reduced when these cells are transduced with a functional Nrf2 construct.38,39 In agreement with these findings, we have now demonstrated that Nrf2-deficient mice demonstrated more severe neurologic dysfunction compared with control mice, suggesting that Nrf2 plays an important role in protecting the brain from the damage caused by ICH. In addition, pharmacological activation of Nrf2 with SF significantly reduced the damage produced by ICH in Nrf2-proficient mice but not in Nrf2-deficient mice, suggesting that enhancement of Nrf2 activity may represent a potential target for ICH therapy. This observation is consistent with the existing data that Nrf2 activation contributes to amelioration of brain damage caused by focal ischemia.13,14 In addition to its effect on oxidative stress, Nrf2 has been documented to possess anti-inflammatory properties.40,41 The anti-inflammatory effect of Nrf2 is likely mediated through inhibition of NF-κB,41 the master regulator of many proinflammatory genes, as evi-
dened by the fact that NF-κB activation by lipopolysaccharide and tumor necrosis factor-α was greater in Nrf2-deficient mice.\textsuperscript{41} Inhibition of NF-κB by Nrf2 after treatment with SF could explain the reduced number of neutrophils in ICH-affected striatum. This is evidenced by the expression of monocyte chemoattractant protein-1 and vascular cell adhesion molecule-1 (VCAM), two NF-κB gene targets involved in regulation of neutrophil infiltration, which are blocked in endothelial cells overexpressing Nrf2.\textsuperscript{40} We are currently exploring the role of Nrf2 as a regulator of NF-κB and inflammation after ICH.

SF is a sulfur-containing isothiocyanate derivative that was demonstrated to activate Nrf2.\textsuperscript{22} The dose of SF used in the present study was based on the efficacy of SF established in ischemia and traumatic brain injury models in rats\textsuperscript{13,42} and also on a study of neurons in culture while assessing for cytoprotective effects (data not included). SF promotes expression of the known Nrf2 target genes and mediates phenotypic changes (eg, neuroprotection during mitochondrial stress\textsuperscript{43}) in Nrf2\textsuperscript{-/-} mice\textsuperscript{13,14,22,39,42–44} suggesting a considerable level of specificity in activating Nrf2. Because SF was originally purified from edible plants in the Cruciferaeae family, we believe that it could have a favorable safe profile, allowing for immediate clinical application.

The approach to stimulate Nrf2 as a target to achieve a multifaceted, antioxidative status could represent a strategy that is superior to the use of compounds that directly scavenge/trap free radicals, such as 21-aminosteroids, edaravone, ethe-selen, or derivatives of phenylbutynitrone.\textsuperscript{45} Activation of Nrf2 with SF in our study was associated with increased expression of several antioxidative enzymes recognized to play important roles in combating oxidative stress, including catalase, SOD, NAD(P)H dehydrogenase, quinone-1, and glutathione S-transferase. We and others have suggested that upregulation of catalase and SOD may protect neurons from the damage caused by ICH or ischemia,\textsuperscript{19,46–47} further supporting our hypothesis that recruitment of the antioxidative defense system with SF/Nrf2 could represent one of the mechanisms underlying protection against ICH-induced damage.

Disclosures

None.

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24. Chan K, Lu R, Chang JC, Kan YW. Nrf2, a member of the nfe2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. \textit{Proe Natl Acad Sci U S A.} 1996;93:13943–13948.


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