CHOP Silencing Reduces Acute Brain Injury in the Rat Model of Subarachnoid Hemorrhage

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Background and Purpose—Endoplasmic reticulum stress triggers apoptotic cascades in neurons of the central nervous system after subarachnoid hemorrhage. The aim of this work was to study the mechanism of neuroprotection conferred by targeting cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) in the acute brain injury following subarachnoid hemorrhage.

Methods—A total of 172 rats were used. Endovascular perforation induced subarachnoid hemorrhage. Two small interfering RNAs for CHOP were injected 24 hours before hemorrhage induction. At 24 or 72 hours, rats were neurologically evaluated and euthanized. The brains were recovered for molecular biology and histology studies.

Results—Western blot analysis revealed effective silencing of CHOP associated with suppression of Bim-Caspase-3 apoptotic pathway. Moreover, the antiapoptotic Bcl2 was found upregulated with CHOP siRNA treatment. A reduced number of TUNEL-positive cells in the subcortex and in the hippocampus reflected histological protection. CHOP siRNA treatment ameliorated intracranial sequelae of and improved functional performance.

Conclusions—We conclude that CHOP silencing alleviates early brain injury following subarachnoid hemorrhage via inhibiting apoptosis and that CHOP siRNA treatment has a clinical potential for patients with this type of hemorrhagic stroke. (Stroke. 2012;43:484-490.)

Key Words: subarachnoid hemorrhage ■ sirna ■ chop ■ bim ■ bcl2 ■ apoptosis

Extensive protein damage occurs after subarachnoid hemorrhage (SAH), which leads to overloading endoplasmic reticulum (ER) with aberrant and unfolded proteins. This phenomenon may trigger unfolded protein response as an adaptive cellular mechanism, which, when overwhelmed, leads to ER stress and activation of apoptotic mechanisms. All major ER stress pathways (mediated by inositol-requiring enzyme-1, PKR-like ER kinase, and activating transcription factor 6) converge on 1 transcription factor named C/EBP homologous protein (CHOP alias DDIT3/GADD153). PKR-like ER kinase phosphorylates eIF-2, which, in turn, activates activating transcription factor 4. The cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) gene promoter contains a binding site for activating transcription factor 4. IRE1 may activate CHOP at the post-transcriptional level through p38 MAPK. The contribution of CHOP to neuronal death via apoptosis has been evidenced in the experimental cerebral ischemia. In response to severe ER stress, CHOP activates the expression of Bim, leading to Caspase-3 cleavage and apoptosis; however, the contribution of this pathway to the acute brain injury after SAH remains unknown. Meanwhile, the components of extravasated blood and the impact of generalized acute brain ischemia are capable of inducing ER stress in SAH. Therefore we sought to determine whether silencing CHOP confers neuroprotection in the hemorrhagic brain. To this end, we used 2 distinct CHOP siRNAs in the rat perforation model of SAH.

Methods

Animal Groups and End Point Measures
A total of 172 male Sprague-Dawley rats (Loma Linda University, Loma Linda, CA) were randomly assigned to the following groups: sham surgery, SAH untreated (vehicle group), and groups subjected to SAH and before intracerebroventricular injection (24 hours before SAH) of either of 2 sequences of siRNA for CHOP or scrambled RNA. SAH was performed by endovascular perforation with 4 to 0 nylon monofilament sutures. SAH was performed by endovascular perforation with 4 to 0 nylon monofilament sutures. At 24 and 72 hours following SAH, the rats were euthanized under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia and perfused transcardially, with ice-cold phosphate-buffered sodium alone for molecular biology, followed by...
10% buffered formalin for histology. At 72 hours, only brain water content and neurobehavioral tests were performed. The Institutional Animal Care and Use Committee at Loma Linda University approved this study.

SAH Severity and Neurological Scoring Systems
In order to confirm the equivalent level of SAH severity across groups, we applied an SAH severity classification system developed by Sugawara and colleagues, as described. We used a modified Garcia score system for neurological testing in a blinded fashion. The maximum neurological score was 24, indicating a healthy rat. Seventy-two-hour mortality was calculated by dividing the number of dead animals by the number of total used animals.

Evans Blue Dye Extravasation
The permeability of blood–brain barrier (BBB) was evaluated on the basis of Evans Blue extravasation, as described previously. The brain level of Evans Blue was determined by spectrofluorophotometry at excitation wavelength 610 nm, emission wavelength of 680 nm, and a bandwidth of 10 nm.

Brain Water Content
Brain water content was determined at 24 and 72 hours after SAH by weighing then-drying brain tissue for 24 hours at 105°C in the oven. The water content was calculated according to the following formula: wet weight to dry weight/wet weight×100%.

CHOP Silencing
CHOP silencing experiments used intracerebroventricular infusion of 2 CHOP siRNAs with the following coordinates: 1.5 mm posterior, 1.0 mm lateral, and 3.2 mm below the horizontal plane of bregma. The sequence of the first siRNA for CHOP was sense, 5′ GGAAAGACUAGAAACGGA; antisense, 5′ UCCGUGUUC- CUAGUUCUCC. The second siRNA sequence was sense, 5′ CUGGGAAAACGCAGCAUGAA; antisense, 5′ UUCAUGGCCGU- GUUUCCACT. The irrelevant-scrambled RNA served as a control (Dharmacon/Thermo Fisher Scientific, Lafayette, CO). RNAs were injected in the sterile PBS at a rate of 0.5 μmol/L/min at 24 hours prior to SAH surgery. Sham animals received a burr hole; however, they were not subjected to intracerebroventricular injections.

Western Blot Analysis
Cerebral tissue-facing blood clots were homogenized in the RIPA buffer containing protease/phosphatase inhibitors, as described. The aliquots of 30 μg of total protein were run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred onto the nitrocellulose membranes. The membranes were probed at 4°C overnight with the following primary antibodies (Millipore): CHOP, Bim, Bcl2, Caspase3, and β-actin, then incubated for 2 hours at room temperature with respective secondary antibodies diluted 1:200. The chemiluminescence signal was developed with electrochemiluminescence substrate (ECL) kit reagents (Amersham) and detected by exposure of Kodak radiographic films. The densitometry analysis was conducted with Image J software (NIH). The results were expressed relative to β-actin bands and normalized with regard to sham.

Triple Fluorescence Staining
Brain specimens were fixed in formalin, then processed as described to obtain 10 μmol/L frozen sections on glass slides. The antigens were retrieved by microwaving in 0.1 μmol/L sodium citrate at pH 6 for 10 minutes. This was followed by incubation with blocking serum for 1 hour at room temperature and overnight incubation with CHOP and NeuN primary antibodies (Millipore) (diluted 1:100), followed by secondary antibody (2 hours at room temperature). TUNEL was done with a kit. Histological preparations were coverslipped with an antifade reagent (Millipore) and looked at under an Olympus BX51 microscope. The photomicrographs for AMCA, Texas Red, and fluorescein stains were taken with computer camera and merged with MagnaFire software (Systat). A negative staining procedure was conducted with the omission of primary antibodies.

Quantitative Cell Count
TUNEL-positive cells were counted in a blind manner; numbers of TUNEL-positive cells were calculated, and subcortical regions in the hippocampus were averaged from 8 photomicrographs from each animal. Four rats per group were used for quantitative histology analysis.

Statistical Analysis
Data are expressed as means±standard error of mean. Statistical significance was verified with analysis of variance, followed by Tukey test for multiple comparisons. The probability level P<0.05 was considered statistically significant. The analysis of mortality was done with χ2 test. Nonparametric analysis of variance was used for categorical variables.
Results

SAH Severity Scores
Mean SAH severity score measured 24 hours after the bleeding was within the range from 14 to 15 points (Figure 1A). There was no significant difference in the severity scores across groups (ANOVA).

Mortality Rates
No mortality was recorded in the sham-operated rats (Figure 1B). Eighteen percent mortality was recorded in the vehicle group, while 19.5% mortality occurred in the scramble RNA group. Treatments with siRNA1 and siRNA2 reduced mortality rates to 7% and 11.6%, respectively. Our study, however, was not powered enough to detect the statistical difference in the mortality across groups ($P<0.05$, ANOVA).

Neurological Scores
The score plunged from 22 to 13.5 points in the vehicle group and to 13.7 points in the scrambled RNA group (Figure 2A). Then there was a higher score in the CHOP siRNA-treated rats in comparison with untreated controls (17.3 points for siRNA1 and 16.7 points for siRNA2). At 72 hours, the scores in the vehicle and scrambled group were 16.3 and 16 points, respectively. Small difference in neurological scores (not exceeding 1.5 points) between the siRNA-treated group and sham proved to be insignificant.

BBB Permeability
The Evans Blue level increased in cerebral tissues in the vehicle group compared with sham at 24 hours after surgery; 4.6-fold in the left hemisphere, 6.51-fold in the right hemisphere, 3.34-fold in the cerebellum, and 6.76-fold in the brain stem (Figure 2B). The increase in similar magnitude were recorded in rats injected with the scrambled RNA. CHOP siRNA reduced brain permeability for Evans Blue in all examined brain regions, as compared with vehicle and scramble RNA-treated controls. As compared with the vehicle group, the reduction in Evans Blue level was 46.06% and 43.60% in the left hemisphere; 63.66% and 63.51% in the right hemisphere, 34.84% and 36.10% in the cerebellum, and 61.40% and 61.22% in the brain stem, with siRNA1 and siRNA2 treatments, respectively ($P<0.05$ versus sham).

Brain Water Content
Results are shown in Figure 2. At 24 hours after SAH, the brain water content increased by 0.78% in the left hemisphere, 0.74% in the right hemisphere, 0.43% in the cerebellum, while no significant change was found in the brain stem (Figure 2C). At 72 hours after SAH, the increase in brain water was 0.64% in the left hemisphere and 0.61% in the right hemisphere while the cerebellum and brain stem showed no increase in water content (Figure 2D). With both siRNA
treatments, the brain water content was nearly normalized at 24 hours and 72 hours after SAH.

The Protein Expression of CHOP and Its Downstream Targets

CHOP silencing reduced the CHOP protein level in the cerebral tissue, as determined by Western Blot analysis (Figure 3A). The increase in CHOP in vehicle-treated SAH was 2.74-fold (2.96-fold in the scramble RNA group) respective to sham, indicative of ER stress response activated after the hemorrhage; however, in the siRNA-treated rats, the increase in CHOP proved to be only 33.78% and 32.45% of the increase in the vehicle group, with siRNA1 and siRNA2 treatment, respectively. The increase in Bim level was 3.57-fold in the SAH group compared with sham (Figure 3B). Silencing CHOP with 2 different siRNAs reduced expression of Bim in the cerebral tissue, to 31.18% (siRNA1) and 35.22% (siRNA2) of increase noted in the vehicle-treated group. Scramble RNA had no preventive effect on the SAH-induced Bim upregulation (3.83-fold increase with regard to sham). The Bcl2 level at 24 hours after SAH dropped to 27.33% of the basal level. In the scramble RNA-injected rats, the Bcl2 level was still depressed after SAH, at 25.11% of control; however, following CHOP siRNA treatment, the levels of Bcl2 determined in the cerebral tissues were 2.27 and 2.87 times higher than those found in the vehicle group (Figure 3C). The level of cleaved Caspase-3 in the hemorrhagic brain increased in the untreated and scramble RNA-treated rats, respectively, up to 365% and 347% of sham control levels (Figure 3D); however, these increases in cleaved Caspase-3 levels were reduced with the siRNA and siRNA2 treatments, by 69.85% and 72.89%, respectively.

Apoptotic Labeling Colocalizes With Neuronal Expression of CHOP After SAH

Triple fluorescence staining revealed increased CHOP immunoreactivity in the hippocampus and in the subcortical region and at 24 hours after SAH (Figure 4i and 4v). Merged images revealed that CHOP immunoreactivity colocalized with TUNEL in the neuronal cells (Figure 4iv and 4viii).

The Effect of CHOP siRNA on Apoptotic Cell Count After SAH

The quantitative analysis of cells with apoptotic labeling (TUNEL) in the posthemorrhagic brain are demonstrated in Figure 5. Numerous TUNEL-positive cells were found in the subcortical brain region after the SAH was treated with vehicle and scramble-RNA (Figure 5Ai and 5Aii), in contrast with weak TUNEL positivity in the sham-operated animals (Figure 5Ai inset). Cell count showed 70±6 and 72±4 TUNEL-positive cells per magnification field in subcortex after the SAH was treated with vehicle and scramble RNA, respectively (Figure 5B). Treatment with siRNA for CHOP grossly reduced numbers of TUNEL-positive cells, although it did not eliminate apoptotic cells completely (Figure 5Aiii).
and Aiv). In the subcortical region, percent reduction in TUNEL-positive cells was 63.2% for CHOP siRNA1 and 53.9% for siRNA2 as compared with vehicle (Figure 5B). The number of TUNEL-positive cells after vehicle-treated SAH amounted $62 \pm 16$ in the hippocampus (Figure 5Av and 5C) while there was no hippocampal TUNEL positivity in the sham group (Figure 5Av inset). As shown by the graph (Figure 5C), the numbers of TUNEL-positive hippocampal cells were significantly reduced, by 63.2% and 58.9% with siRNA1 and siRNA2 treatment, respectively.

**Discussion**

The results of this study indicate that CHOP siRNA treatment provides neuroprotection in the rat model of subarachnoid hemorrhage. Suppression of the major mediator of apoptotic pathway after SAH resulted in the significant upregulation of antiapoptotic protein Bcl2, while the expression of proapoptotic molecules, including the executioner Caspase-3, was attenuated. Thus silencing CHOP, a major mediation of ER stress, resulted in the inhibition of apoptosis. Furthermore, the observed reduced BBB disruption brought significant reduction in the brain edema. Histological and molecular neuroprotection was accompanied by neurological improvement in the treated rats. The tendency toward reduced mortality rates with the treatment further emphasizes translational significance of the proposed siRNA-based therapy.

Our study has demonstrated that SAH induces ER stress and CHOP overexpression with activation of apoptotic cascades in the pyramidal cells of the hippocampus and subcortical neurons of the brain. This effect can be mediated by ischemic stimulation but also by the action of hemoglobin degradation products including iron. Previous studies revealed that CHOP mediates apoptosis after cerebral ischemia. It has been also proven that ER stress initiates widespread pathological apoptosis, which underlies injury of the brain and other organs. The neurological deficits after SAH have been ascribed to this excessive apoptosis. There-
fore, reduced apoptosis with CHOP siRNA treatment could produce the beneficial effect of CHOP inhibition on the functional performance in the treatment group. Interestingly, widespread apoptosis in this present study was detected in neurons, which is consistent with impaired neurological status after SAH. There was only little innoreactivity of CHOP seen outside neurons. Indeed, the role of CHOP in the astrocyte death is debatable.15,16

Studies have documented that ER stress triggers apoptosis through activation of Bim.17 The role of Bim in early brain injury after SAH has been hypothesized by earlier authors although never thoroughly studied.18 Bim is upregulated by ER stress through direct CHOP transcriptional induction.17 Subsequent permeability transition pore activation leads to cytochrome c release, apoptosome formation, and Caspase-3 activation.19 As a result of CHOP knockdown, the upregulation of Bim after SAH was prevented, which led to suppression of the downstream apoptosis pathway.20 Consequently, cleaved Caspase-3 was attenuated with the siRNA treatment.

Moreover, by blocking CHOP, we were able to prevent SAH-induced Bcl2-suppression as the Bcl2 level was not significantly different from sham with either of the treatments. Bcl2-suppression via activation of transcriptional repressors is an established mechanism of CHOP-induced apoptosis.4 Thus, the inhibition of CHOP could reduce apoptosis via prevention of Bcl2 downregulation. ER-restricted Bcl2 can inhibit apoptosis induced by a variety of stimuli.20 Bcl2 can antagonize apotropic effect via sequestrating Bim, required for mitochondrial permeabilization mediated by Bax.4

Interestingly, in primary neuronal cultures, CHOP appears to protect against neuronal death56, showing that the role of this factor varies across different experimental settings and may depend on different cell-type interactions. These conflicting results further support a need to investigate the role of CHOP in distinct models of cerebrovascular diseases.

Our results, showing a robust protection of BBB with siRNA treatment may point toward inhibition of CHOP-mediated apoptosis in the endothelial cell compartment. CHOP inhibition reduced vasogenic brain edema, which occurs as a consequence of BBB disruption after SAH.5 The role of ER stress in the endothelial apoptosis is well-documented and occurs in response to a variety of stimuli, including atherosclerosis-promoting factors and immunosuppressants.22,23 Although we have shown that CHOP inhibition could reduce BBB damage further, studies are warranted to gain mechanistic insight on this effect.

We used 2 siRNAs targeting CHOP mRNA in individual transfections rather than via a pooling approach. Thereby we wanted to verify the specificity of the protective phenotype derived from gene-silencing before SAH and to minimize the cumulative effect of off-target genes that often occurs in the pooled experiments24; however, for the sake of clinical relevance, a rapid post-SAH siRNA administration is desired. In order to work toward this requirement, further investigations may employ intranasal delivery, which bypasses BBB and promotes fast onset of action for siRNA-based therapeutics.25

Conclusions
In summary, the results of the present study confirm the involvement of CHOP in ER stress as a major inducer of apoptosis after SAH. CHOP silencing leads to reduction of apoptosis, preservation of BBB, and improved neurological function. This may suggest that antiapoptosis as a therapeutic approach in acute SAH may not be fully effective without targeting the ER component. To our knowledge, neuroprotective effects of targeting CHOP in SAH have been demonstrated for the first time in this present study. Our findings also reveal translational potential of siRNA-based therapeutics targeting apoptotic mechanisms after SAH.

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