Neuroprotection Against Hypoxic-Ischemic Brain Injury by Inhibiting the Apoptotic Protease Activating Factor-1 Pathway

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Background and Purpose—Emerging evidence suggests that mitochondrial damage–mediated neuronal apoptosis is a major contributor to neonatal hypoxic-ischemic (H-I) brain injury. This study was performed to determine whether targeted inhibition of the apoptotic protease activating factor-1 (Apaf-1) signaling pathway downstream of mitochondrial damage confers neuroprotection in rodent models of neonatal H-I.

Methods—H-I was induced in 7-day-old (P7) transgenic mice overexpressing the specific Apaf-1–inhibitory protein AIP. Apaf-1 inhibition was also achieved in P7 rats by protein transduction–enhanced delivery of recombinant AIP. Pups were euthanized 6 to 24 hours after H-I for assessing caspase activation and mitochondrial release of cytochrome c and AIF, and 7 days after H-I for analyzing brain tissue damage. Sensorimotor functions were assessed in rats up to 4 weeks after H-I.

Results—Transgenic overexpression of AIP protected against H-I brain injury, resulting in attenuated activation of caspase-9 and caspase-3, and attenuated brain tissue loss. In neonatal H-I rats, intraperitoneal injection of TAT-AIP, but not the control proteins TAT-GFP or AIP, decreased caspase activation and brain damage and improved neurological functions. Neuroprotection conferred by AIP was also associated with significantly reduced release of cytochrome c and AIF from mitochondria.

Conclusion—The Apaf-1 signaling pathway, which transmits cell death signals after mitochondrial damage to effector caspases, may be a legitimate therapeutic target for the treatment of neonatal H-I brain injury. (Stroke. 2010;41:00-00.)

Key Words: apoptosis ■ brain ischemia ■ neuroprotection

Hypoxic-ischemic (H-I) injury in the developing brain contributes considerably to morbidity and mortality in children suffering from periventricular leukomalacia or cerebral palsy. In animal models, a mixture of apoptosis and necrosis constitutes the primary mode of cell death following neonatal H-I.1,2 Emerging evidence suggests that apoptosis has a more predominant role in neonatal H-I than in adult brain ischemia,3,4 and indeed it may contribute to the prolonged progression of neurodegeneration and cerebral dysfunction after neonatal H-I.1 Thus, targeted inhibition of proapoptotic pathways represents an attractive and legitimate strategy for therapeutic intervention in neonatal H-I brain injury.

Apoptosis generally consists of 2 main signaling steps: the apoptosis-initiation cascade and the apoptosis-execution pathway. The death signals are initially induced by various proapoptotic stimuli such as oxidative stress, DNA damage, and ER stress. These pathways eventually converge into a mitochondrial-dependent mechanism, the so-called intrinsic pathway, which activates the death–execution terminal caspases.5 An essential step in this pathway is the formation of a multimeric protein complex, the apoptosome,6 in which cytosolic cytochrome c facilitates the oligomerization of apoptotic protease activating factor-1 (Apaf-1). Once Apaf-1 is activated, procaspase-9 is recruited to the complex, which in turn activates terminal caspases such as caspase-3, -6, and -7. This mechanism of apoptosis is evolutionarily conserved and likely plays an important role in mediating neuronal death after cerebral ischemia7,8 and, possibly, in neonatal H-I as well.9

The present study aimed to test the hypothesis that targeted inhibition of the Apaf-1 signaling pathway may confer neuroprotection against neonatal H-I brain injury. Inhibition of the Apaf-1 pathway was achieved by transgenic overexpression or protein transduction–mediated delivery of Apaf-1–interacting protein (AIP). AIP is a recently cloned gene

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product that interrupts the formation of apoptosome by directly binding to Apaf-1.8

Methods

Creation of AIP Transgenic Mice

The chimeric transgene used to create the transgenic mice contains rat AIP cDNA7 under the control of cytomegalovirus enhancer and a chicken β-actin promoter with the first intron. A hemagglutinin (HA) tag was added before the AIP coding region. The microinjection and embryonic stem cells screening were done using standard procedures. Founders were used to establish independent transgenic lines by breeding to wild-type F1 hybrid mice. In all experiments, wild-type mice backcrossed from the same transgenic colonies on the C57/B6 background were used as controls.

Construction, Production, and Administration of TAT-AIP Fusion Protein

The AIP fusion protein containing the TAT protein transduction domain and the HA tag was produced as described previously.10 The purified proteins were verified by Coomassie Blue staining and Western blotting and then stored in 10% glycerol/PBS at −80°C until used. TAT-AIP or TAT-GFP in 180 µL of PBS containing 10% glycerol was injected intraperitoneally into the pups at the completion of H-I and again at 6 and 24 hours after H-I. The animals were randomly assigned to experimental groups.

Models of H-I

Animal protocols were approved by the institutional Animal Care and Use Committee. H-I brain injury was induced in postnatal day 7 (P7) mice or rats based on the Levine method with modifications.9 Pups were anesthetized with 2.0% to 2.5% isoflurane mixed with ambient air under spontaneous inhalation, and the left common carotid artery was permanently ligated. After a 1.0-hour recovery period, the pups were placed in a plastic chamber containing a humidified atmosphere of 8.0% oxygen/92% nitrogen, and submersed in a 37.5°C water bath to maintain normothermia. After 2.5 hours (for rats) or 0.5 hours (for mice) of hypoxia, the pups were returned to their dams for the time indicated in the experiments.

Assessment of Brain Damage

At 7 days after H-I, the brains were removed, paraffin-embedded, and cut into 4-µm-thick coronal sections. Sections through the corpus callosum and dorsal hippocampus were stained with hematoxylin and eosin (HE). The extent of tissue damage was determined as described previously.11 For mouse pups, brain damage was also assessed in 8 predetermined brain regions using the brain damage scoring system described previously. Briefly, each region of interest was scored 0 to 3, in which 0 = no detectable cell damage; 1 = small focal area of neuronal cell damage; 2 = columnar damage; 3 = cystic infarction and gliosis. The score of each region was summed to create a full-range score of 0 to 24 for each animal. All measurements were made by an investigator who was blinded to the experimental conditions.

Neurological Evaluation

To evaluate sensorimotor neurological function, foot fault tests were performed at 1, 2, 3, and 4 weeks after H-I as described previously.11 Overall motor behavior was evaluated using grid walking to determine total steps taken over 1 minute. Gait testing was performed 2, 4, 6, and 7 days after H-I, and data were expressed as the first day that the animal successfully moved off a circle within 30 seconds.11

Results

Activation of the Apaf-1 Pathway After HI

To test the hypothesis that the Apaf-1–dependent intrinsic pathway is activated after H-I, we examined Apaf-1 oligomerization at 6 hours after H-I, at the time caspase-9 activation reached its peak (Figure 1A and 1B). After H-I, a portion of Apaf-1 shifted to large protein complexes of ≈700 kDa (Figure 1C), consistent with the sizes of active apopto-
Moreover, coimmunoprecipitation revealed increased interaction between Apaf-1 and caspase-9 after H-I (Figure 1D). These results confirmed that Apaf-1 entered an active state after H-I.

Transgenic Overexpression of AIP Attenuates Brain Injury

We created transgenic mice overexpressing the Apaf-1 inhibitory protein AIP. Tg-AIP mice express 10-fold AIP in brain over their wild-type (Wt) littermates (supplemental Figure I, available online at http://stroke.ahajournals.org). Double-label immunofluorescence revealed that AIP transgenic expression is localized primarily in neurons and, to a lesser extent, in astrocytes. In the in vitro assays, brain extracts from Tg-AIP mice were incompetent for cytochrome c/dATP-induced caspase-9 or caspase-3 activation (supplemental Figure I). Determined 3 to 24 hours after H-I, caspase-9 and caspase-3 activation was markedly attenuated in Tg-AIP mice compared to Wt mice (Figure 2A and 3C). Tg-AIP mice exhibited significantly decreased brain damage compared to Wt mice 7 days after HI (Figure 2D and 2E).

Brain surface vascular anatomy appeared unaffected by AIP transgenic overexpression. Furthermore, regional blood flow in H-I brains, as determined in 5 Wt and 6 Tg-AIP mice using laser Doppler flowmetry (probe location: 2 mm posterior to the bregma and 2 mm to the midline), was also not significantly different between groups (data not shown). These results suggest that the reduced brain injury observed in AIP transgenic mice was not the result of a diminished H-I insult.

Administration of TAT-AIP Attenuates Brain Injury

To explore the possibility that exogenous delivery of AIP can be a therapeutic strategy protecting against HI, we generated recombinant AIP containing the TAT sequence.9 The TAT-AIP fusion protein was purified to near homogeneity and was first tested for its effects in primary neuron cultures. TAT-AIP conferred significant neuroprotection against cell death induced by bleomycin or oxygen-glucose deprivation (supplemental Figure II).

For in vivo studies, TAT-AIP was injected intraperitoneally into the pups at the completion of H-I. Protein transduction into brain was detected at 2 to 24 hour after injections (Figure 3A and 3B). TAT-AIP administration ameliorated brain damage dose-dependently at 7 days after H-I (Figure 3C and 3D). At the dose of 12 mg/kg (×3 times), TAT-AIP decreased cerebral tissue loss by ~38% (P<0.05 versus controls), ~30% (P<0.05 versus controls), and ~52% (P<0.01 versus controls) in the cortex, striatum, and hippocampus, respectively.

We investigated the effect of TAT-AIP administration on activation of caspase-9 and caspase-3 after H-I. TAT-AIP treatment (12 mg/kg×3 times) robustly decreased the activation of both caspase-3 and caspase-9 after H-I, as determined using Western blot analysis, caspase activity assays, and immunohistochemistry (Figure 4).
Effects of Apaf-1 Inhibition on Cytochrome C and AIF Release After H-I

Two recent studies suggest that under certain conditions, caspase activation may cause secondary release of cytochrome c or AIF from mitochondria.13,14 In this study we examined the effect of TAT-AIP on cytochrome c and AIF release after H-I. Compared to TAT-GFP-treated brains, TAT-AIP treatment significantly attenuated cytochrome c release at 24 hours after H-I (Figure 5A), and significantly decreased AIF nuclear translocation at 6 and 24 hours after H-I (Figure 5B).

Cytochrome c and AIF were also examined in Tg-AIP and Wt mice at 24 hours after H-I. H-I–induced cytochrome c release and AIF translocation were significantly reduced in Tg-AIP mice compared to Wt mice (Figure 5C and 5D).

Previous studies identified tBid and Bax as inducers of cytochrome c release from brain mitochondria after ischemia,15 while activated calpain I was found to be a direct trigger for AIF release.16,17 In this study, we investigated the potential direct effect of TAT-AIP on cytochrome c or AIF release from isolated mitochondria. As shown (Figure 5E and 5F), TAT-AIP failed to inhibit either tBid/Bax-induced cytochrome c release or calpain I–induced AIF release in isolated brain mitochondria, suggesting that the inhibitory effect of TAT-AIP on cytochrome c and AIF release after H-I is secondary instead of primary. This notion was supported by the observation that TAT-AIP treatment prevented H-I–induced degradation of the endogenous calpain inhibitor calpastatin (Figure 5G), which is a substrate for caspase-3.

TAT-AIP Treatment Improves Neurological Outcomes After H-I

Contralateral limb faults were significantly increased in H-I animals compared to sham controls (Figure 6A and 6B). There was no significant difference in total number of steps between groups, indicating that the differences in fault steps were not attributable to alterations in overall motor activity (Figure 6C). Similarly, directed movement (as evidenced by gait testing) was significantly delayed in H-I animals (Figure 6D). The neurological deficiencies were attenuated by TAT-AIP treatment (Figure 6A, 6B, and 6D). Thus, TAT-AIP treatment is effective at improving neurological outcomes after H-I.

Discussion

Emerging evidence suggests that molecular inhibition of neuronal apoptosis is a legitimate therapeutic strategy against neonatal H-I brain injury. Cytochrome c–initiated activation of apoptotic protease activating factor-1 (Apaf-1) is believed to be a key step in the intrinsic signaling pathway for the activation of death-executing caspases in neuronal apoptosis. The current study provides novel evidence that directly targeting this signaling pathway by transgenic overexpression or protein delivery of AIP, a specific Apaf-1 inhibitor, confers robust neuroprotection in models of neonatal H-I injury.

Recently cloned from a rat brain cDNA library, AIP exhibits potent antiapoptotic effects by interfering with the Apaf-1–dependent apoptotic cascade.8 AIP contains an
N-terminal caspase-recruiting domain (CARD), allowing direct binding to Apaf-1 on its activation by cytosolic cytochrome c and disrupting the formation of apoptosome and subsequent activation of caspase-9 and caspase-3. AIP has potent cell death-suppressing activity against various apoptosis-inducing stimuli. In this study, we created transgenic mice overexpressing AIP and found that the Tg-AIP mice were highly protected against H-I brain injury. Moreover, we successfully delivered the recombinant AIP (TAT-AIP) in a rat model of neonatal H-I, and this treatment attenuated H-I–induced activation of caspase-9 and caspase-3. Correlating to this caspase-suppressing effect, administration of TAT-AIP conferred significant neuroprotection against H-I brain injury. These results thus highlight the essential role of the Apaf-1–dependent intrinsic pathway in neonatal ischemic cell death.

Several approaches that target caspase-dependent apoptosis have shown neuroprotective effects in models of neonatal H-I. The magnitude of neuroprotection demonstrated by TAT-AIP in the current study is compatible to that conferred by small-peptide caspase inhibitors in a similar H-I model. However, the use of AIP may be advantageous over the conventional caspase inhibitors, as AIP inhibits specifically the cytochrome c-activated Apaf-1 signaling pathway without interfering with the basal caspase activities in neurons. Recent studies suggest that caspase proteases may have important physiological roles in a variety of normal nondeath-related cellular functions in the nervous system. Interestingly, the Tg-AIP mice are viable and do not show developmental brain abnormality, contrasting to the Apaf-1–null mice which are embryonic lethal and exhibit severe brain malformations. These observations are consistent with the notion that AIP binds to Apaf-1 and inhibits the caspase-9 pathway only when Apaf-1 is activated by cytochrome c, thus AIP is not an inhibitor for developmental neuronal apoptosis.

The mitochondrial signaling pathway induced by ischemia activates both caspase-dependent and caspase-independent death-execution cascades. Whereas cytochrome c release is essential for caspase-dependent apoptosis via activating Apaf-1, AIF release is thought to be independent of caspase activities. In this study, we investigated the effect of Apaf-1 inhibition on cytochrome c and on AIF release after H-I. Interestingly, H-I–induced cytochrome c and AIF release were attenuated in brains overexpressing AIP or receiving TAT-AIP treatment. Although TAT-AIP was effective in reducing cytochrome c release only at 24 hour after H-I, it inhibited AIF release at both 6 and 24 hours after H-I. These results suggest that the release of cytochrome c and AIF after H-I may be mediated by different pathways, but that both may involve secondary mechanisms requiring caspase activation. Considering that the Apaf-1/caspase-9 signaling pathway is downstream of cytochrome c release, it can be speculated that caspase-3 and caspase-7 activation through the Apaf-1–signaling pathway causes secondary mitochondrial damage, thus facilitating cytochrome c release. A recent study also reported that caspase inhibition abrogated cytochrome c release in a model of traumatic brain injury. This action of terminal caspases may not be attributable to the direct attacks of caspase-3 or caspase-7 on mitochondrial membranes, but via the
cleavage-mediated activation of another intermediate proapoptotic molecule, such as an initiator caspase (caspase-2, caspase-8, or caspase-10) or Bid, which in turn targets the mitochondria. Further studies examining these potential intermediate proteins may reveal a secondary mechanism underlying cytochrome c release after H-I.

Recently, calpain I has been found to trigger AIF release from mitochondria in ischemic neurons by truncating AIF at its N terminus. Calpain I is normally scavenged by the endogenous calpain inhibitor, calpastatin, which determines the threshold for calpain I activation by calcium. However, calpastatin is a specific substrate for caspase-3 and caspase-7; it has been suggested that the proteolytic degradation of calpastatin by caspase-3 facilitates calpain activation during neuronal apoptosis. Thus, Apaf-1–mediated activation of caspase-3 may enhance calpain-mediated AIF release by degrading calpastatin. This notion is supported by our data showing that H-I–induced calpastatin degradation was attenuated in TAT-AIP–treated brains. An alternative potential mechanism is that caspase-3 may facilitate AIF release after H-I injury by activating other intermediate proapoptotic proteins, such as caspase-8 and Bid. Taken together, these results suggest that overexpression of AIP confers neuroprotection against H-I not only by preventing the Apaf-1–dependent activation of terminal caspases, but also by interrupting a caspase-mediated feed-forward loop that causes secondary release of cytochrome c and AIF.

In summary, the results suggest that the Apaf-1 apoptotic pathway is a potential therapeutic target for neonatal H-I brain injury. It should be noted, however, that the neuroprotection conferred by targeting Apaf-1 is incomplete. Other

**Figure 5.** TAT-AIP treatment attenuates HI-induced cytochrome c and AIF release in rats. A and B, Western blots of subcellular fractions from cerebral cortices show that cytosolic (cyto) cytochrome c and nuclear (nuc) AIF accumulation is attenuated by TAT-AIP treatment after HI. The graphs in the right panel illustrate the semiquantitative results (n=8/group). *P<0.05 vs TAT-GFP. C and D, Western blots show attenuated cytochrome c and AIF release in Tg-AIP mice at 24 hours after H-I. *P<0.01 vs WT (n=6/group). E and F, Cell-free assay using isolated brain mitochondria (50 μg). Recombinant tBid (±3; +30 ng)/Bax (±10; +100 ng) proteins induce cytochrome c release; calpain I (0.3 or 1.0 U) induces AIF release. Immunoblotting was done using both pellets (mitochondria) and supernatant fractions. The addition of bcl-xL (500 ng) or calpastatin (300 ng) inhibited cytochrome c and AIF release, respectively, whereas the addition of TAT-AIP (1 μg) had no effect on either cytochrome c or AIF release. G, Western blots (representative of 3 sets) show that caspase-dependent loss of calpastatin after H-I was markedly reduced in rat brains treated with TAT-AIP.

**Figure 6.** TAT-AIP improves neurological outcomes after H/I. A and B, Foot fault testing from contralateral forelimb and hindlimb of sham control (n=12) or H-I rats treated with TAT-GFP (n=14) or TAT-AIP (n=15). C, Quantification of total steps among groups. D, Assessment of gait determined by the first day that pups successfully moved off of a circle during the testing period. *P<0.05; **P<0.01 vs the indicated group. #P<0.05 vs sham controls.
mechanisms, including necrosis, inflammation, and autophagy all may contribute to H-I–induced neurodegeneration.26,27 Thus, future studies should evaluate therapeutic strategies that would simultaneously alleviate the multi-injurious factors involved in H-I. Targeting the Apaf-1 pathway by AIP may be a legitimate component of such strategies.

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Disclosures
None.

References


Figure I. Overexpression of AIP in the brain of transgenic mice. A, PCR genotyping of wild-type and AIP transgenic mice. Primers were designed to allow the detection of AIP transgene and endogenous AIP at 386 and 531 bp, respectively. B, Western blots show AIP overexpression in the brains from Tg-AIP mice. Immunoblotting was performed using either the anti-HA antibody (for the HA-tagged AIP) or the anti-AIP antibody (for both endogenous and transgenic AIP). C, Double-label immunofluorescent staining for HA (red) and NeuN (green) in the cortex of wild-type and Tg-AIP mice, respectively. Double-label immunofluorescent staining for HA and GFAP also reveals transgenic overexpression of AIP in astrocytes (D–E).

Figure II. The death-suppressing effect of TAT-AIP in primary cortical neurons. A, Western blots show that the purified proteins could be detected using the anti-HA antibody. B, Western blots show that TAT-GFP and TAT-AIP can be delivered into neurons, but neither GFP nor AIP without the TAT sequence can. Fusion proteins were added to cultures at the concentration of 10 µg/ml for 30 min, and then cells were washed 4 times before collection for protein extraction. C, Cortical cultures were treated with vehicle or fusion proteins at the indicated concentrations for 30 min, and then exposed to bleomycin (10 µM) or 60 min of OGD. Cell viability was determined 24 hr after OGD or bleomycin exposure by quantitative Alamar blue. D–E, Neurons were assessed for apoptosis using Hoechst nuclear staining 24 hr after OGD or bleomycin exposure. Arrows indicate the condensed nuclei under neuronal death conditions. The graph (E) summarizes the quantitative results from 4 independent experiments. *P<0.05; **P<0.01 vs vehicle controls.
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