Taming Neonatal Hypoxic–Ischemic Brain Injury by Intranasal Delivery of Plasminogen Activator Inhibitor-1

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Background and Purpose—Plasminogen activator inhibitor-I (PAI-1), a ≈50-kDa serine protease inhibitor, markedly reduces the extravascular toxicity of tissue-type plasminogen activator in experimental hypoxic–ischemic (HI) brain injury of newborns. However, the current treatment with PAI-1 requires intracerebroventricle injection to cross the blood–brain barrier, which is an invasive procedure of limited clinical potential. Thus, we tested whether intranasal administration of PAI-1 can bypass blood–brain barrier and mitigate neonatal HI brain injury.

Methods—Rat pups were subjected to HI, with or without lipopolysaccharide pre-exposure, followed by intranasal delivery of a stable-mutant form of PAI-1 (CPAI).

Results—Immunoblotting showed that CPAI sequentially entered the olfactory bulbs and cerebral cortex after intranasal delivery and reduced ≈75% of brain atrophy in HI or lipopolysaccharide-sensitized HI injury. Mechanistically, CPAI attenuated HI-induced plasminogen activators and lipopolysaccharide/HI-induced nuclear factor-kB signaling, neuroinflammation, and blood–brain barrier permeability.

Conclusions—Intranasal delivery of CPAI is an effective treatment of experimental HI brain injury of newborns. Clinical application of this experimental therapy merits further investigation.

Key Words: chorioamnionitis ■ intrauterine infection ■ hypothermia ■ hypoxic-ischemic encephalopathy ■ neonatal encephalopathy ■ tissue-type plasminogen activator

High concentrations of tissue-type plasminogen activator (tPA) in the brain parenchyma have multiple detrimental effects, ranging from hemorrhagic transformation during thrombolysis therapy to tissue proteolysis in neonatal hypoxia–ischemia (HI).1–3 Intracerebroventricle injection of CPAI, a stable-mutant form of PA inhibitor-I (PAI-1) with a 72-fold longer half-life against tPA, reduces HI and lipopolysaccharide-sensitized HI brain injury in neonatal rats.3,4 As such, CPAI is a potential therapeutics of neonatal encephalopathy. However, current administration of CPAI relies on intracerebroventricle injection to bypass blood–brain barrier (BBB) and avoid the adverse effect of antifibrinolysis in the blood. This invasive procedure is unsuited for broad clinical application. To overcome this obstacle, we tested whether CPAI can enter the brain by intranasal delivery and oppose neonatal HI injury. Intranasal delivery is a powerful method to deliver chemicals and peptides to the brain, but whether it supports larger protein across BBB remains unpredictable.5–9 Our results showed that intranasally administered CPAI enters the brain and mitigates HI injury with and without lipopolysaccharide sensitization. These results suggest a novel therapy of neonatal encephalopathy and possible other conditions of tPA toxicity.

Materials and Methods

Animal Surgery and Intranasal Delivery

The Rice-Vannucci model of neonatal HI, with or without lipopolysaccharide sensitization, was performed in 7-day-old Wistar rats of both sexes in an approximately equal ratio, as previously described.1,4 Intranasal delivery of CPAI (Molecular Innovations; Novi, MI) was performed at 30 or 120 minutes after HI as described.10 Briefly, rat pups were anesthetized and put on their backs on a heating pad (38°C). A total of 2-μL CPAI (0.24 μg/μL) dissolved in PBS (with 4.2% endotoxin-free dimethyl sulfoxide; Sigma D2650) was applied to both nares, alternating at 2-minute intervals for a total 12 μL, via a Hamilton syringe connected with PE10 polyethylene tubing. Male and female pups showed no difference in response to HI injury or the CPAI treatment, and, therefore, the data were combined. Experimental procedures were approved by the Institutional Animal Care and Use Committee and in compliance with the ARRIVE guidelines (Animals in Research: Reporting In vivo Experiments).

Biochemistry

Electrophoretic mobility shift assay for nuclear factor-kB was performed using a commercial kit (Lighshift chemiluminescent kit; Thermo Scientific, Waltham, MA). Plasminogen and gelatin zymographs were performed as described.14

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Real-Time Quantitative Polymerase Chain Reaction
The mRNA extraction, cDNA preparation, and quantitative polymerase chain reaction were performed as described. The quantitative polymerase chain reaction of rat IL-6, Ccl-2, and Tspo cDNAs were detected on the basis of SYBR green using the following primers after normalization to the level of β-actin:

IL-6: 5'-GGAGAGGAGACCTCACAGAGAT-3', 5'-AGTGC ATCATCGCTGTCCATAC-3';
Ccl-2: 5'-ACCACATATGCGAGTCTCTGTCAC-3', 5'-GCTGC TGTGATTCTCTTTGTAGT-3';
Tspo: 5'-CATATGGTTCCCTTGGGTCTCTAC-3', 5'-AGGCCA GGTAAGGATACAGCAAG-3'.

2,3,5-Triphenyltetrazolium Chloride Stain and BBB Permeability Assay
In vivo triphenyltetrazolium chloride stain was performed as described. The BBB permeability was determined on the basis of extravasation of 5% sodium fluorescein as described.

Statistical Analysis
Values are represented as mean±SD or mean±SEM when n>9. Quantitative data were compared using 2-sample (unpaired) t test assuming equal variance.

Results
Intranasally Applied CPAI Enters the Brain Within 2 Hours
To assess the kinetics of brain delivery, CPAI was applied intranasally to 7-day-old rats, with and without HI, or 30-day-old mice. Animals were euthanized 10 to 180 minutes later for immunoblot detection of CPAI in the olfactory bulbs and cerebral cortex. In uninjured P7 rats, the presence of CPAI peaked around 60 minutes in the olfactory bulbs and 120 minutes in the cerebral cortex. In HI-injured P7 rats, the delivery of CPAI to the cerebral cortex was accelerated for an hour. P30 mice showed an even faster transport of CPAI to the olfactory bulbs (peak at 30 minutes) and cerebral cortex (starting at 30 minutes; Figure 1A). Importantly, CPAI was undetectable (<12 pg/mL) in the blood after intranasal application. These results confirmed brain distribution of CPAI by intranasal delivery and suggested that this drug delivery method would not compromise the fibrinolytic capacity in blood.

Intranasal Delivery of CPAI Inhibits PAs and HI Brain Damage
Next, we examined the effects of intranasal delivery of 2.85-μg CPAI in the Rice-Vannucci HI model in P7 rats. Compared with the saline (PBS with 4.2% dimethyl sulfoxide) treatment, CPAI significantly decreased tPA and the urinary-type PA activities in the carotid-ligated hemisphere (the right side, R*) at 4 hours and blocked the induction of matrix metalloproteinase-9 at 24-hour recovery (Figure 1B and 1C; n=6 for each). Similarly, triphenyltetrazolium chloride stain showed a larger infarcted area in animals receiving PBS treatment than those that received CPAI treatment at 24-hour recovery (Figure 1D; n=4). By 7-day post-HI, the PBS-treated rats developed 45% tissue loss in the cerebral cortex, 49% in the striatum, and 54% in the hippocampus (Figure 1D; n=18). When intranasal delivery of CPAI was initiated within 30 minutes after recovery, the extent of tissue loss dropped to 16% in the cerebral cortex, 19% in the striatum, and 22% in the hippocampus (all P<0.01; n=9). If intranasal CPAI was delivered at 2-hours after-HI, tissue loss was 31% in the cerebral cortex (P<0.01), 31% in the striatum (P<0.01), and 45% in the hippocampus (P<0.08; n=9). These results showed that intranasal delivery of CPAI mitigates HI-induced damage in the cerebral cortex and striatum with a 2-hour therapeutic window.

Intranasally Applied CPAI Blocks Lipopolysaccharide/HI-Induced Neuroinflammation and Brain Atrophy
Previous studies have shown that 4-hour pre-exposure to low-dose lipopolysaccharide (0.3 mg/kg, IP) attenuates HI-induced PAs but enhances nuclear factor-xB signaling and microglia activation. We found that intranasal delivery of 2.85-μg CPAI inhibited the lipopolysaccharide/HI-induced nuclear factor-xB activity at 4 hours (Figure 2A; n=6) and IL-6, Ccl-2 (also called Mcp-1), and Tspo (translocator protein of 18 kDa, a marker of activated microglia) transcripts at 24 hours (Figure 2B; n=7-8). These results suggested a strong antineuroinflammatory effect by intranasal delivery of CPAI. Similarly, PBS-treated rats showed increased BBB permeability, as shown by extravasation of sodium fluorescein, in the lipopolysaccharide/HI-injured hemisphere at 24-hour recovery, whereas animals receiving intranasal CPAI delivery exhibited much less sodium fluorescence (Figure 2C; n=5).

Finally, we tested the effect of intranasal delivery of CPAI on lipopolysaccharide/HI-induced brain atrophy. At 7-day recovery, saline-treated rats developed 45% tissue loss in the cerebral cortex, 46% in the hippocampus, and 36% in the striatum. In contrast, intranasal delivery of 2.85-μg CPAI within 30 minutes after lipopolysaccharide/HI insult resulted in reduced tissue loss to 12% in the cerebral cortex, 10% in the striatum, and 11% in hippocampus, indicating 75% reduction of brain atrophy (Figure 2D; all P<0.001; n=14-15).

Discussion
Hypoxic–ischemic encephalopathy (HIE) is an important neurological disorder of neonates. Although therapeutic hypothermia is beneficial, there remains a need for better therapies of severe HIE and infection-sensitized HIE. In preclinical study, HI with pre-exposure to low-dose lipopolysaccharide mimics infection-sensitized HIE and produces greater brain damage. The stable-mutant form of PAI-1 (CPAI) is one of the few therapeutics that reduce both HI and lipopolysaccharide/HI brain injury. Furthermore, it inhibits multiple mechanisms of tPA neurotoxicity, ranging from tissue proteolysis to microglia activation. With such diverse mechanisms, CPAI is an attractive therapeutics of HIE and possibly other tPA-related pathologies.

Clinical application of CPAI in neurological disorders, however, is hampered by the need of intracerebroventricle or subarachnoid injection in current delivery strategies. Because of its large molecular weight (43 kDa), CPAI fails to cross the BBB but would decrease the fibrinolytic capacity in blood after intravenous injection. In the present study, we showed that intranasal delivery of CPAI overcomes this obstacle and
protects neonatal HI and lipopolysaccharide/HI injuries similar to the effect of intracerebroventricle injection. The efficient nose-to-brain drug delivery method, mediated in part by the continuous open channels formed by olfactory ensheathing cells that facilitate protein diffusion into the cerebrospinal fluid space, has been used to apply insulin to the brain in patients with Alzheimer disease. In this study, we confirmed the entry of CPAI in the brain after intranasal delivery by immunoblots. Furthermore, we showed that intranasal delivery of CPAI blocks HI-induced PAs, lipopolysaccharide/HI-induced nuclear factor-κB signaling, proinflammatory cytokine, and BBB disruption. Altogether, this therapeutic strategy confers ≤75% reduction of brain atrophy. These results suggest that intranasal delivery of CPAI is an effective, noninvasive therapy of neonatal HI brain injury with and without infection sensitization.
Figure 2. Intranasal delivery of CPAI mitigates lipopolysaccharide (LPS)-sensitized hypoxia–ischemia (HI) brain injury of newborns. A, Electrophoretic mobility shift assay showed that intranasal delivery of CPAI blocked the acute nuclear nuclear factor (NF)-κB activity at 4h after LPS/HI injury. B, Real-time quantitative polymerase chain reaction showed significant reduction of IL-6, Ccl-2 (Mcp-1), and Tspo mRNAs at 24-h recovery after intranasal delivery of CPAI (n=7–8). *P<0.05, **P<0.01, ***P<0.001 compared with the unchallenged group. C, Left, Representative brain photographs of LPS/HI-injured rat pups receiving the indicated therapy and intraperitoneal injection of sodium fluorescein (NaF) followed by saline perfusion at 24-h recovery. Right, Quantification showed significant reduction of NaF fluorescence, an indicator of blood–brain barrier permeability, by intranasal delivery of CPAI (n=5). D, Left, Representative brain specimens at 7 days after LPS/HI insult in animals receiving intranasal delivery of saline (PBS) or CPAI. Right, Quantification of regional brain atrophy showed significant reduction by intranasal application of CPAI over PBS (n=14–15 for each group). Mut indicates mutant nuclear factor (NF)-κB probe; R*, the HI-injured (right) hemisphere; L, the uninjured (left) hemisphere; and UN, untouched animals.
Finally, limited by the scope of a brief report, our study only used 7-day histopathology as the end point for analysis. Future studies on the dose–response, extended therapeutic window, and long-term behavioral outcomes are warranted to assess clinical potential of this novel anti-tPA strategy.

Disclosures
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References
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