Intrauterine infection exacerabtes neonatal hypoxic–ischemic (HI) brain injury and impairs the development of cerebral cortex. Here we used low-dose lipopolysaccharide (LPS) pre-exposure followed by unilateral cerebral HI insult in 7-day-old rats to study the pathogenic mechanisms. We found that LPS pre-exposure blocked the HI-induced proteolytic activity of tissue-type plasminogen activator (tPA), but significantly enhanced NF-κB signaling, microglia activation, and the production of pro-inflammatory cytokines in newborn brains. Remarkably, these pathogenic responses were all blocked by intracerebroventricular injection of a stable-mutant form of plasminogen activator protein-1 called CPAI. Similarly, LPS pre-exposure amplified, while CPAI therapy mitigated HI-induced blood-brain-barrier damage and the brain tissue loss with a therapeutic window at 4 h after the LPS/HI insult. The CPAI also blocks microglia activation following a brain injection of LPS, which requires the contribution by tPA, but not the urinary-type plasminogen activator (uPA), as shown by experiments in tPA-null and uPA-null mice. These results implicate the nonproteolytic tPA activity in LPS/HI-induced brain damage and microglia activation. Finally, the CPAI treatment protects near-normal motor and white matter development despite neonatal LPS/HI insult. Together, because CPAI blocks both proteolytic and nonproteolytic tPA neurotoxicity, it is a promising therapeutic strategy for neonatal HI injury either with or without infection.

Keywords: diffusion tensor imaging (DTI), neonatal hypoxia–ischemia, plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator (tPA), white matter injury

Introduction

Perinatal hypoxic–ischemic (HI) insult is a major threat to the development of cerebral cortex (Volpe 2003; Khwaja and Volpe 2008). Moreover, perinatal infection amplifies the HI brain injury, often resulting in neonatal mortality or permanent neurological deficits, such as cerebral palsy, epilepsy, white matter (WM) reduction, and cognition impairment (Perlman 1998; Adams-Chapman and Stoll 2006; Allin et al. 2011). Hence, the development of effective therapies of infection-sensitized HI brain injury is an important issue in modern neonatal care.

Studies in animal models suggested that prenatal infection (chorioamnionitis) and the ensuing fetal inflammatory response amplify brain damage via innate immunity and microglia activation (Lehnardt et al. 2003; Eklind et al. 2005). These discoveries, however, have not led to effective therapies, because systemic immune suppression in infants will increase the risk of severe infection, which already accounts for up to 25% of neonatal death (Lawn et al. 2005). Thus, targeted inhibition of the brain microglia activation is a safer and perhaps more effective therapy for neonatal infection/HI injury (Ransohoff and Perry 2009).

Tissue-type plasminogen activator (tPA) is a potential target to suppress microglia activation in the newborn brain. tPA has multiple functional domains, including a C-terminal protease domain, 2 kringle domains that interact with the N-methyl-D-aspartate receptor subunit, an epidermal growth factor-like domain, and an N-terminal finger domain that mediates microglia activation independent of the protease domain (Rogove et al. 1999; Siao and Tsirka 2002; Zhang et al. 2007, 2009; Yepes et al. 2009). We recently reported that anti-tPA treatment with a stable-mutant form of plasminogen activator inhibitor-1 (PAI-1) called CPAI markedly reduced HI-triggered tPA proteolytic activity and destruction of newborn brains (Berkenpas et al. 1995; Adhami et al. 2008; Yang et al. 2009). These findings suggest that hyper-stimulation of the parenchymal tPA activity is an important mechanism of neonatal HI brain injury, but whether CPAI protects against infection-sensitized HI injury is unknown.

Another unanswered question is whether infection merely upregulates the severity of neonatal HI insult or fundamentally changes the nature of brain response to HI. The notion of an infection-altering response to HI warrants consideration, because in pure-HI brain injury microglia are activated secondary to tissue damage in a "sterile inflammation" manner (Chen and Nunez 2010), but they are pre-activated by prenatal infection and may thereby create a different milieu for the subsequent HI response. Consistent with this notion, while the mouse pups lacking myeloid differentiation primary response gene 88 (MyD88)—a critical downstream mediator of innate immunity—had high resistance to lipopolysaccharide (LPS)-sensitized HI insults, they were equally sensitive to pure-HI insult like wild-type animals (Wang et al. 2007). The intriguing differential responses raised the possibility that pure- and infection-sensitized HI may trigger divergent pathogenic mechanisms in immature brains. Key switch of the differential responses, however, is yet to be determined.

To investigate these issues, we compared the response with pure-HI and dual LPS/HI insults and the therapeutic effect of CPAI in neonatal rat brains. Our results indicated that LPS pre-exposure significantly decreased the HI-induced tPA proteolytic activity but amplified the NF-κB signaling pathway, thus in effect altering the brain response to HI. Interestingly, CPAI therapy not only reduced HI insults, but also mitigated microglia activation and LPS/HI-induced neuroinflammation and brain injury. Together, these results suggest that CPAI is a promising therapeutics of pure- and infection-sensitized HI injury in newborns.
Materials and Methods

**Animal Surgery**

The experimental model of LPS-sensitized neonatal hypoxia was performed as previously described (Eklind et al. 2005; Yang et al. 2009). Briefly, 0.3-mg/kg LPS was injected intraperitoneally to 7-day-old Wistar rat pups at 4 h before the induction of unilateral cerebral HI with the Rice-Vannucci model. This low-dose LPS was used to mimic subclinical infection as previously described (Lehnardt et al. 2003; Eklind et al. 2005; Wang et al. 2007), and no apparent impairment of animals was observed. The pups were anesthetized by 3% isoflurane mixed with pure oxygen while the right common carotid artery was ligated with a 4-0 silk thread at -10 min for 15 min and then removed. The brains were removed and photographed using a fluorescence stereomicroscope. The cerebral cortex was then cut into halves and each hemisphere was homogenized with 500 μL trichloroacetic acid (80%) and centrifuged for 10 min at 13,600 × g. An aliquot of 150 μL supernatant added with 50 μL of 5 M NaOH were mixed in triplicates in a 96-well microplate and measured by a spectrophotometer with the absorption wave length at 490 nm.

**Plasminogen Activator Zymogram**

The plasminogen activator zymography was performed as previously described (Adhami et al. 2008; Yang et al. 2009). Briefly, plasminogen and casein were added to the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel. Protein samples were extracted using the radioimmunoprecipitation assay and mixed with 2× sample buffer (0.5 M Tris–HCl, pH 6.8, 100% glycerol, 0.05% bromophenol blue, 10% SDS) without boiling before electrophoresis carried out using a low voltage for up to 5 h. Recombinant human tPA (Activase, Genetech, San Francisco, CA, USA) was used as positive controls. After electrophoresis, the gel was rinsed twice in 2.5% Triton-X-100 at room temperature for 30 min each, followed by incubation in glycine buffer (0.1 M glycine, pH 8.0) at 37°C overnight. The gel was stained by Coomassie blue solution and destained to reveal the lytic zones of protease activity. The zymogram gel was photographed and presented in a black-and-white inverted image. Quantification was performed using the NIH Image J software to determine the size of the lytic zones.

**Matrix Metalloproteinase Zymogram**

The matrix metalloproteinase (MMP) zymography was performed as previously described (Adhami et al. 2008; Yang et al. 2009). Briefly, 0.15% porcine skin gelatin was added to the SDS–PAGE gel. Protein samples were extracted and run in the same condition as for plasminogen activator zymogram. After electrophoresis, gels were washed twice with 2.5% Triton-X-100, incubated in reaction buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl2) at 37°C overnight, then stained and de-stained with Coomassie blue solution.

**In vitro tPA Activity Assay**

In vitro tPA activity was measured using the Cromolizyme™ tPA assay kit (Catalog No. 1105, Trinity Biotech, Inc., Wicklow, Ireland) following the manufacturer’s instructions. Briefly, 100 μL of tPA standards (0.5, 1.0, 1.5, and 2.0 U/mL) and samples were added to a 96-well plate. After mixing and washing for 20 min, 50 μL substrate reagent was added to each well using the repeating pipette, followed by the addition of 50 μL plasminogen reagent. The reaction was carried out at ambient temperature with gentle shaking for 90 min. The reaction activity was measured using a 96-well spectrophotometer with the absorbance at 405 nm.

**Cytokine Array and Enzyme-linked Immunosorbent Assay**

Cytokine array was performed as previously described using the ChemiArray antibody array system (Millipore, Billerica, MA, USA) and following the manufacturer’s instructions (Yin et al. 2007). Rat MCP-1 was measured using a commercial Enzyme-linked Immunosorbent Assay (ELISA) kit (# R0608001C, RayBiotech, Norcross, GA, USA). Mouse MCP-1 was measured using specific antibodies (# 506002, Biolegend, San Diego, CA, USA). Rat and mouse tumor necrosis factor alpha (TNFα) was measured using a cross-species-reactive antibody (AF-410-NA, R&D Systems, Minneapolis, MN, USA).

**NF-κB Electrophoresis Mobility Shift Assay**

NF-κB electrophoresis mobility shift assay (EMSA) was carried out using the Lightshift Chemiluminescent kit (Catalog No. 20148, Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 5 μL nuclear extract from brains, 2 μL binding buffer, 1 μL poly-dl-dC, 1 μL biotin-
labeled oligonucleotide probe and 1 μL super-pure water were mixed and spin down to the bottom of the tube and incubated at room temperature for 30 min. The sample was then loaded to nondenaturing 5% polyacrylamide gel and run for 2.5 h in 0.5× Tris/Borate/EDTA buffer. The binding reactions were electrophoretically transferred to nylon membrane under 300 mA for 45 min and UV cross-linked. The positions of biotin-labeled probe were detected using streptavidin-conjugated chemiluminescence and X-ray film. The sequences of oligonucleotide probes containing normal or mutant NF-κB enhancer-binding sites were identical to those previously described (Zhang et al. 2007; Nijboer et al. 2008).

**Immunoblot Analysis**

Brain samples for immunoblots were homogenized in 1× TLB (1% Triton X-100, 20 mM Tris, pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate, 25 mM Na-pyrophosphate, 2 mM EDTA, 1 mM Na$_3$VO$_4$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.7% protease inhibitor cocktail). The proteins were separated by standard SDS–PAGE procedures, electro-transferred onto a polyvinylidene fluoride microporous membrane (Bio-Rad, Hercules, CA, USA) and detected with designated antibodies followed by the enhanced chemiluminescence detection (Amersham Biosciences). The antibodies used are: anti-ZO1 (Zymed), anti-PAI-1 (Molecular Innovations), anti-IκBα (Cell Signaling Technology), and anti-β-actin (Sigma).

**Behavioral Testing**

Rats were placed on the elevated accelerating rotating rod (3 cm in diameter) beginning at 4 rpm/min for 4 trials per day, during which time the rotating rod underwent acceleration from 4 to 40 rpm over 3 min and then remained at the maximum speed for another 3 min. Animals were scored for their latency (in microseconds) to fall in each
Histology
All immunohistochemistry was performed on 20-μm-thick frozen sections using standard procedures. The following antibodies were used: a rabbit anti-Iba1 (Wako) and a mouse anti-CD11b/c (OX42) (DAKO) antibody. Secondary antibody was conjugated to Alexa Fluor488 or Alexa Fluor 94 (Molecular Probes).

Statistical Analysis
Values are represented as mean ± SD or SEM as indicated. Quantitative statistical analysis was performed using one-way ANOVA or 2-sample (unpaired) t-test assuming an equal variance.

Results
LPS Pre-Exposure Increases HI-Induced BBB Damage and MMP-9 Activation, and CPAI Treatment Protects Against Combined LPS/Hi Insults
To simulate subclinical infection, we injected a single, low-dose LPS (0.3 mg/kg) to P7 rat pups intraperitoneally at 3 h before unilateral ligation of the common carotid artery, and thus 4 h before the induction of 80 min hypoxia (10% oxygen). This relatively short duration of hypoxia was chosen to test whether LPS pre-exposure sensitizes HI brain injury. In initial experiment, we measured the brain permeability to NaF and the MMP-9 activity at 24 h recovery as the indication of BBB damage. This experiment showed that LPS injection alone had very little effect on the BBB permeability to NaF, but when combined with a mild HI insult, it markedly increased NaF extravasation in the carotid-ligated hemisphere (Fig. 1A and B; n = 4–5 for each group). Similarly, although IP injection of LPS produced no obvious MMP-9 activity, dual LPS/Hi insult induced higher MMP-9 activity (Fig. 1C, P < 0.05; n = 4 for each group) and NaF extravasation than HI alone (Fig. 1B, P < 0.001). In addition, either LPS/Hi or pure-HI insult, but not LPS injection per se, reduced the amount of a tight-junction protein ZO-1 in the newborn rodent brain (Fig. 1D, n = 3). Of note, HI and LPS/Hi insults caused mild NaF extravasation on the contralateral hemisphere where no MMP-9 activation or histological damage was detected. The discrepancy is likely because NaF (376 Da) permeates BBB through a paracellular pathway, which does not require extensive MMP activation (Hawkins and Egleton 2008).

Interestingly, intracerebroventricular injection of 1.9 μg CPAI—a dose that reduced pure-HI insults in rat pups (Yang et al. 2009)—almost completely prevented LPS/Hi-induced NaF extravasation (Fig. 1A(A5), B, P < 0.01 by t-test), MMP-9 activation (Fig. 1C, P < 0.01), and ZO-1 reduction (Fig. 1D, P < 0.05 compared with saline-treated animals; CPAI was injected at 10 min post-hypoxia in all experiments unless mentioned otherwise). These data suggest that while exposure to low-dose LPS per se causes no apparent injury in immature brains, its addition to HI greatly magnifies BBB damage, but therapeutic administration of CPAI may protect against LPS-sensitized neonatal HI injury.

LPS/Hi Induces NF-κB Signaling at the Expense of Proteolytic tPA Activity, but Is Inhibited by the CPAI Therapy
To investigate the mechanism of infection/LPS-sensitized HI injury and CPAI-mediated protection, we first examined the distribution and clearance of ICV-injected CPAI (Fig. 2A; arrow indicates the injected right hemisphere, R). Immunoblot analysis with an anti-human/rat PAI-1 antibody showed no detectable PAI-1 expression in the brain of unchallenged animals. The exogenous CPAI was evenly distributed in both hemispheres at 1 h after ICV injection and almost completely disappeared in the brain by 2 h (Fig. 2A).

Next, we tested whether LPS magnifies nonvascular tPA activity to impose greater tissue proteolysis in newborn brains. Surprisingly, biochemical analysis showed that, while LPS exposure by itself had little effect, its addition to a secondary HI insult significantly reduced tPA activity on the HI-stressed hemisphere at 4 h post-hypoxia without changing its protein level, similar to the effect of ICV–CPAI injection (Fig. 2B, the right, R, hemisphere is the carotid-ligated side in all displays throughout the article). Quantification showed that LPS/Hi and CPAI-injection reduces the tPA proteolytic activity to 54.7% and 59% of the basal level, respectively, while even brief 80-min HI challenge increases the tPA activity to 134%; n = 5). At 24 h recovery, the LPS/Hi-challenged hemisphere showed a normal level of tPA proteolytic activity, an increased uPA activity—a general indicator of neural stress or brain injury—similar to those subjected to pure-HI insult (Fig. 2E, 376% and 404%, respectively, n = 5), and expression of the endogenous PAI-1 that was absent in either LPS or pure-HI challenge or the contralateral hemisphere of LPS/Hi-injured brains (Fig. 2C). Interestingly, immunoblotting analysis showed that the ICV-injected CPAI had a delayed clearance in the carotid-ligated hemisphere up to 4 h (R in Fig. 2B), which could inhibit the tPA proteolytic activity by forming a tighter tPA–CPAI complex than endogenous PAI-1 that was induced at 24 h recovery. The mechanism of tPA inhibition in the acute phase following LPS/Hi insult remains unclear and warrants future investigation.

Next, because it has been reported that IP injection of LPS triggers innate immune responses in newborn brains, we examined the effects of LPS/Hi and CPAI injection on the downstream NF-κB signaling pathway (Lehnardt et al. 2003; Wang et al. 2009). Biochemical analysis showed that dual LPS/Hi insult diminished the amount of cytoplasmic IκBα (Fig. 2F), and markedly increased the nuclear NF-κB DNA-binding activity at 4 h post-hypoxia (Fig. 2G). Remarkably, ICV injection of CPAI after LPS/Hi almost completely prevented IκBα depletion (Fig. 2F, n = 3) and nuclear NF-κB activity in neonatal brains (Fig. 2G, n > 6 sets).

These results suggest that LPS pre-exposure changes the pathogenic response to HI in neonatal brains, rendering a shift from tPA proteolysis to escalation of NF-κB signaling in the acute phase to produce greater tissue destruction.

CPAI Attenuates LPS/Hi-Induced Monocyte Chemoattractant Protein-1 Synthesis and Monocyte Entry in the Newborn Brain
The NF-κB signaling pathway regulates the synthesis of pro-inflammatory cytokines (Nijboer et al. 2008). To test
whether the induction of NF-κB activity and its suppression by CPAI in the acute phase of LPS/HI insult translates into changes in the brain cytokine level at 24 h recovery, we performed a multiplex antibody array and found that LPS/HI significantly increased the brain monocyte chemotactic protein-1 (MCP-1) to 9.75-fold of the baseline level (n = 4), which was reduced to 3.13-fold by CPAI (n = 4) (Fig. 3A). Further analysis by ELISA confirmed a greater increase in the brain MCP-1 level at 24 h after LPS/HI insult than pure-HI, as well as a significant attenuation by the CPAI treatment (Fig. 3B, n = 4, P < 0.05 by t-test). In contrast, LPS, pure-HI, and dual LPS/HI challenge all elevated the blood MCP-1 level to a similar degree, which was not diminished by the CPAI treatment (Fig. 3C).
The inability to suppress the blood MCP-1 level by CPAI injection may attribute to a high baseline value in unchallenged animals. Alternatively, brain-specific reduction of MCP-1 by the CPAI treatment may be sufficient to prevent LPS/HI-induced microglia activation and the infiltration of immune-response cells (Ivacko et al. 1997; Galasso et al. 2000; Deng et al. 2009). To test this possibility, we examined the morphology and distribution of Iba1+ and OX42/CD11b+ microglia/macrophages at 16 h recovery in the brains of pure-HI (80 min)-challenged pups and those receiving post-LPS/HI intraventricular injection of saline or CPAI (Fig. 3-A). This analysis showed a swollen corpus callosum filled with numerous large, round Iba1+ cells within the corpus callosum (50 ± 11 per visual field). In contrast, LPS/HI-CPAI-treated animals contained fewer Iba1+ cells within the corpus callosum (50 ± 11 per visual field) that exhibited ramified cytoplasmic processes and did not express OX42. Also note that the swelling of CC was milder in pure-HI-injured animal brains and there was no obvious OX42-immunoreactivity. (J) The tensor trace map from rat brain at 24 h after LPS/HI insult. Note the expansion/swelling of CC/external capsule on the HI-injured hemisphere (indicated by arrows).

Figure 3. CPAI prevents LPS/HI-induced chemokine production and monocyte recruitment. (A) Representative results of a cytokine array using brain extracts at 24 h following indicated conditions (n = 2 for unchallenged and LPS-treated animals; n = 4 for LPS/HI-PBS or LPS/HI-CPAI treatments). The duplicated spots of MCP-1 and the quantification results were indicated. LPS/HI increased the brain MCP-1 level to 9.75 ± 0.54 (mean ± SD) fold of the baseline level, while CPAI treatment attenuated this induction. (B and C) ELISA analysis of the MCP-1 levels from brains (B) or the plasma (C) at 24 h after the indicated conditions (n = 4 for each). The MCP-1 level in the unchallenged brain was 4.4 ± 0.85 pg/mL extracts (mean ± SD), which was increased by LPS/HI insults to 50.3 ± 4.4 pg/mL (asterisk, P < 0.01) and significantly attenuated by the CPAI treatment (P < 0.05 by unpaired t-test). In contrast, the basal plasma MCP-1 level was already high in unchallenged animals (116.7 ± 3.3 pg/mL), and upregulated by various stimuli to a similar degree. (D-I) Immunofluorescence detection of Iba1 (D, E, and F) and OX42/CD11b (G, H, and I) in coronal sections at the fornix-decussation level of brains collected at 16 h after pure-HI, LPS/HI-PBS, or LPS/HI-CPAI treatments (n > 5). White lines mark the boundaries of corpus callosum (CC). LV, lateral ventricle. Scale bar: 50 μm. Note that the CC in LPS/HI-PBS-treated rats was 2 times wider than that of LPS/HI-CPAI-treated rats and filled with numerous large, round Iba1/OX42 double-positive cells (248 ± 24 per visual field). In contrast, LPS/HI-CPAI-treated animals contained fewer Iba1+ cells within the corpus callosum (50 ± 11 per visual field) that exhibited ramified cytoplasmic processes and did not express OX42. Also note that the swelling of CC was milder in pure-HI-injured animal brains and there was no obvious OX42-immunoreactivity. (J) The tensor trace map from rat brain at 24 h after LPS/HI insult. Note the expansion/swelling of CC/external capsule on the HI-injured hemisphere (indicated by arrows).

The CPAI therapy mitigates LPS/HI-induced microglia activation and MCP-1 expression, leading to decreased monocyte infiltration as part of the neuroprotection mechanism.

CPAI Inhibits LPS-Induced and tPA-Dependent Microglia Activation
Growing evidence suggests that microglia have a critical role in infection-sensitized neonatal HI brain injury (Khwaja and Volpe 2008). Because tPA directly interacts with the cell surface receptors on microglia to activate them, and since CPAI sequesters tPA in a tight complex, it seems likely that CPAI may have a direct inhibitory effect on microglia activation (Berkenpas et al. 1995; Siao and Tsirka 2002). To test this possibility, we used an established paradigm of microglia activation and signiﬁcantly diminished LPS-induced MCP-1 expression as part of the neuroprotection mechanism.
issue, we applied ICV–LPS injection in P10 tPA-null, uPA-null, or wild-type mouse pups and compared the brain levels of TNFα and MCP-1 24 h later (n = 5 for each group). This analysis showed that only tPA, but not uPA, deficiency significantly attenuated LPS-induced cytokine production (Fig. 4C and D, P < 0.01 by t-test). These results support the notion of tPA predominance over uPA in microglia activation (Tsirka et al. 1997). Yet, because tPA-null mice have a very high mortality rate in acute HI insult due to thrombosis (Adhami et al. 2008), they cannot be used to test the requirement of tPA for LPS/HI-induced neuroinflammatory responses directly.

**CPAI Decreases LPS/HI-Induced Brain Damage with an Effective Window at 4 h After Insult**

Next, we examined the effect of CPAI treatment in preserving brain tissue following LPS/HI insults. In this experiment, a single-dose 1.9 μg CPAI or PBS was ICV injected into P7 rat pups at 10 min, 2 h, or 4 h after hypoxia (n = 17–20 for each group as indicated in Fig. 5C). The brains of challenged animals were collected at P14, photographed (Fig. 5A and B), serially sectioned, and Nissl stained to quantify the tissue loss in the cerebral cortex, hippocampus, and striatum compared with counterparts on the contralateral hemisphere.

This analysis showed that the majority of PBS-injected pups developed severe brain atrophy in the carotid-ligated hemisphere (arrows in Fig. 5A), while those receiving acute CPAI treatment were mostly free of destruction (Fig. 5B). Quantification showed that, in PBS-injected animals (n = 19), there was 43 ± 2.5% tissue loss (mean ± SEM) in the cerebral cortex, 44.2 ± 3.9% in the hippocampus, and 29.1 ± 3.3% in the striatum. In contrast, in pups receiving CPAI injection at 10 min after hypoxia (n = 20), the amount of tissue loss dropped to 4.7 ± 2.5% (mean ± SEM) in the cerebral cortex, 9 ± 3.2% in the hippocampus, and 9.1 ± 2.8% in the striatum (P < 0.001 by t-test) (Fig. 5C).

To determine the therapeutic window of CPAI treatment in this experimental model, we examined the effect of delayed CPAI treatment after LPS/HI insult. When injected at 2 h post-hypoxia, the CPAI treatment decreased the tissue loss to 15 ± 2.6% in the cerebral cortex, 23.6 ± 4.6% in the hippocampus, and 9.1 ± 2.8% in the striatum (n = 17). When injected at 4 h post-hypoxia, CPAI decreased the extent of tissue loss to 15.2 ± 4% in the cerebral cortex (P < 0.01 compared with PBS-injection by t-test), 28 ± 6.1% in the hippocampus (P < 0.05), and 9.1 ± 2.8% in the striatum (P = 0.07) (n = 18) (Fig. 5C). These results suggested that a single-dose post-hypoxia injection of CPAI is sufficient to reduce infection-sensitized neonatal HI injury. Furthermore, except for the striatum (the core of infarction in this experimental model), CPAI has at least 4-h therapeutic window after LPS/HI insult for brain protection.

**The CPAI Treatment of Neonatal Infection/HI Protects Near-Normal Motor Function and WM Development**

Neonatal cerebral HI causes abnormal WM development and delayed neural network degeneration that correlates or contributes to motor and cognition impairment (Adams-Chapman and Stoll 2006; Stone et al. 2008; Miller and Ferriero 2009; Allin et al. 2011). Therefore, it is important to determine whether CPAI therapy protects motor function and WM development in neonatal infection/HI insult.

To this end, we conducted LPS/HI injury and PBS-versus-CPAI injection in P7 rat pups, and monitored their body weight and rotarod performance until 6 weeks of age (n = 9 for PBS injection, n = 8 for CPAI treatment, n = 4 for unchallenged controls). This analysis showed that PBS-injected pups initially grew slower than unchallenged animals, but reached a similar body weight after P28 (Fig. 5D, asterisk: P < 0.05 by t-test). Nevertheless, saline-treated animals...
showed a significantly shorter latency on rotarods than unchallenged littermates at P30 or P42, suggesting deficits in motor coordination (Fig. 5E). In contrast, the LPS/HI-challenged and CPAI-treated pups manifested a growth curve and rotarod performance indistinguishable from that in unchallenged animals at P42 (Fig. 5D and E).
Next, we used ex vivo DTI to examine WM development in unchallenged ($n=2$) and saline- or CPAI-treated rat pups ($n=3$ for each) at 2 months of age. The rationale for using ex vivo DTI, a method that was also used in a previous study (Stone et al. 2008), is to provide a higher signal-to-noise ratio. The nerve fiber tracts were clearly visualized in directionally encoded color (DEC) maps (Fig. 6a). DTI parameters, including fraction anisotropy (FA), longitudinal/axial diffusivity ($\lambda_1$), and transverse/radial diffusivity ($\lambda_2$), were calculated in the external capsule (ec), the internal capsule (ic), and the fimbria (fm). To adjust for differences in water diffusion among fixed specimens in ex vivo DTI, we used the ratio of FA, $\lambda_0$, and $\lambda_\perp$ between the injured hemisphere (right) and the contralateral side (left) in each animal for comparison (Fig. 6b and c).

This analysis showed that neonatal LPS/HI insult caused severe truncation of external capsule and distortion of internal capsule in PBS-injected rat pups at 2 months of age (Fig. 6a). Hence, we focussed DTI analysis in the hippocampal fm, which retained a relatively normal trajectory in PBS-treated rats. This analysis showed that PBS-treated rats had a 30% reduction of FA and a 2-fold increase of transverse/radial diffusivity in the fm (Fig. 6b), a pattern consistent with demyelination of axonal tracts (Beaulieu 2002). In contrast, both CPAI-treated and unchallenged animals showed bilateral symmetry in DEC maps, and a close-to-1 R/L ratio of FA, $\lambda_0$, and $\lambda_\perp$ in ec, ic, and fm at 2 months (Fig. 6b and c).

Together, these results suggested that CPAI treatment after LPS/HI not only prevents brain atrophy, but also provides near-normal development of WM and motor functions.

**Discussion**

Growing evidences indicate that intrauterine infection activates fetal inflammatory response and causes greater HI brain injury in infants, but effective therapies of this condition remain unavailable for several reasons. First, infection/HI often occurs in premature neonates who are excluded from hypothermia therapy due to side effects. Secondly, systemic suppression of immune function in infants will probably increase the risk of severe infection, which already accounts for >25% of neonatal death (Lawn et al. 2005). Thirdly, intrauterine infection may alter the pathogenic responses to HI. Hence, not all therapies of pure-HI are effective in dual infection/HI insults. For example, while many therapeutics have demonstrated that tPA has a nonproteolytic cytokine function for microglia activation in neonatal HI based on our findings and those in previous studies (see below).

**tPA Has Critical Functions for Microglia Activation in Infection-Sensitized HI Injury**

Microglia, the first-line immune-response cells in the brain, have been suggested to be a convergence point for upstream infection-HI insults and downstream pathogenic mechanisms in perinatal brain injury (Khwaja and Volpe 2008; Ransohoff and Perry 2009). Once activated, microglia release cytokines and free radicals to attack neurons and oligodendrocytes (Lehnardt et al. 2003; Li et al. 2005). Moreover, many microglia-released cytokines, such as MCP-1, mediate the migration of ameboid microglial cells into the periventricular WM to induce greater neuroinflammatory response to HI, thus forming a vicious cycle of brain injury (Ivacko et al. 1997; Galasso et al. 2000). Here, we suggest that tPA has a critical role for microglia activation in neonatal HI based on our findings and those in previous studies (see below).

Briefly, it has been shown that tPA-deficient microglia had an attenuated response to LPS in cultures, and supplement of either wild-type or proteolytically inactive tPA restored the LPS response (Rogove et al. 1999). This interesting finding demonstrated that tPA has a nonproteolytic cytokine function for microglia activation. Further, by reconstituting various mutant tPA proteins, the region responsible for microglia activation was mapped to the N-terminal finger domain, distant from the C-terminal protease activity domain of tPA (Siao and Tsirka 2002). Later studies using pharmacological and genetic
tools suggested that tPA binds to the low-density lipoprotein receptor-related protein (LRP) on the cell surface of microglia (Zhang et al. 2007, 2009). The blockage of tPA–LRP interaction in microglia mitigates NF-κB signaling activation and reduces the infarct volume after focal ischemia in adult animals (Zhang et al. 2007, 2009).

In the present study, we demonstrated that 1) CPAI prevents LPS/HI-induced NF-κB activation (Fig. 2); 2) CPAI therapy decreases the brain MCP-1 level and the number of WM microglia/monocytes after LPS/HI insult (Fig. 3); 3) CPAI prevents microglia activation induced by direct ICV injection of LPS (Fig. 4); 4) Only tPA, but not uPA, deficiency blocks LPS-induced microglia activation (Fig. 4). Together, these results suggest that CPAI exerts protection against infection/LPS-sensitized HI injury at least in part through its inhibition of tPA-dependent microglia activation. Furthermore, the proteolytic tPA/plasmin activity can synergize with activated microglia to form a vicious cycle to further damage the immature brain (Sheehan et al. 2007; Yao and Tsirka 2011).

**CPAI Mitigates Both Pure-HI and Infection-Sensitized HI Brain Injury**

We recently reported that stimulation of nonvascular tPA activity plays a pivotal role for pure-HI injury in neonatal brains (Adhami et al. 2008; Yang et al. 2009). Our findings are in accord with a classical report of elevated tPA activity in fetal human brains (Gilles et al. 1971) and consistent with a large body of literature on tPA neurotoxicity (Kaur et al. 2004; Yepes et al. 2009). While a physiological-level nonvascular tPA activity may promote synaptic plasticity (Yepes et al. 2002; Samson and Medcalf 2006), excessive tPA activity in the parenchyma could elicit a multitude of harmful effects, including induction of plasmin- and MMP-mediated tissue proteolysis, escalation of glutamate excitotoxicity, microglia
Anti-tPA Therapy in Neonatal Infection/Hypoxia-Ischemia

Summary of how infection/LPS alters the pathogenic mechanisms of HI brain injury and how CPAI provides protection in both pure-HI and infection-sensitized HI insults through inhibition of tPA activities.

In conclusion, the present study suggests a mechanism by which infection alters the response to HI in newborn brains to bypass the BBB due to its large molecular weight (~45 kDa), but may impair hemostasis in the blood, its route of drug delivery requires careful consideration. Appropriate routes include intraventricular or intranasal administration, both of which have been used to deliver large protein therapeutics in newborn brains to bypass the BBB (Whitelaw et al. 2007; Hanson and Frey 2008). Future research is warranted to examine the safety and efficacy of CPAI therapy in these routes in large-animal models of perinatal infection/HI brain injury.

In conclusion, the present study suggests a mechanism by which infection alters the response to HI in newborn brains and a powerful CPAI-based therapy of infection-sensitized HI injury in the preclinical model. These findings may provide the basis for a new therapeutic strategy of perinatal brain injury.

Notes
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References
Adams-Chapman I, Stoll BJ. 2006. Neonatal infection and long-term neurodevelopmental outcome in the preterm infant. Curr Opin Infect Dis. 19:290–297.
Adhami F, Yu D, Yin W, Schloemer A, Burns KA, Liao G, Degen JL, Chen J, Kuan CY. 2008. Detrimental effects of plasminogen activators in neonatal cerebral hypoxia-ischemia. Am J Pathol. 172:1704–1716.
Allin MP, Kontis D, Walshe M, Wyatt J, Barker GJ, Kanaan RA, McGuire P, Rfkin L, Murray RM, Nosarti C. 2011. White matter and cognition in adults who were born preterm. PLoS One. 6:e24525.
Beaulieu C. 2002. The basis of anisotropic water diffusion in the nervous system – a technical review. NMR Biomed. 15:435–455.
Berkenpas MB, Lawrence DA, Ginsburg D. 1995. Molecular evolution of plasminogen activator inhibitor-1 functional stability. EMBO J. 14:2969–2977.
Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord J, Collen D, Mulligan RC. 1994. Physiological consequences of loss of plasminogen activator gene function in mice. Nature. 368:419–424.
Chen GY, Nunez G. 2010. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol. 10:826–837.
Deng YY, Lu J, Ling EA, Kaur C. 2009. Monocyte chemoattractant protein-1 (MCP-1) produced via NF-kappaB signaling pathway mediates migration of amoeboid microglia in the periventricular white matter in hypoxic neonatal rats. Glia. 57:604–621.
Ekland S, Mallard C, Arvidsson P, Hagberg H. 2005. Lipopolysaccharide induces both a primary and a secondary phase of sensitization in the developing rat brain. Pediatr Res. 58:112–116.
Galasso JM, Liu Y, Szafarski J, Warren JS, Silverstein FS. 2000. Monocyte chemoattractant protein-1 is a mediator of acute excitotoxic injury in neonatal rat brain. Neuroscience. 101:737–744.
Gilles FH, Price RA, Kevy SV, Berenberg W. 1971. Fibrinolytic activity in the ganglionic eminence of the premature human brain. Biol Neonate. 18:426–432.
Hanson LR, Frey WH, II 2008. Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease. BMC Neurosci. 9 (Suppl 3):S5.
Hawkins BT, Egleton RD. 2008. Pathophysiology of the blood-brain barrier: animal models and methods. Curr Top Dev Biol. 80:277–309.
Ivacko J, Szafarski J, Malinak C, Flory C, Warren JS, Silverstein FS. 1997. Hypoxic-ischemic injury induces monocyte chemoattractant protein-1 expression in neonatal rat brain. J Cereb Blood Flow Metab. 17:759–770.
Kaur J, Zhao Z, Klein GM, Lo EH, Buchan AM. 2004. The neurotoxicity of tissue plasminogen activator? J Cereb Blood Flow Metab. 24:945–963.
Khwaja O, Volpe JJ 2008. Pathogenesis of cerebral white matter injury of prematurity. Arch Dis Child Fetal Neonatal Ed. 93:F153–F161.
Lawn JE, Coussens S, Zupan J. 2005. 4 Million neonatal deaths: When? Where? Why? Lancet. 365:891–900.
Lehnardt S, Massillon L, Follett P, Jensen FE, Ratan R, Rosenberg PA, Volpe JJ, Vartanian T. 2003. Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proc Natl Acad Sci USA. 100:8514–8519.
Lenzser G, Kis B, Snipes JA, Gaspar T, Sandor P, Komjati K, Szabo C, Bussja DW. 2007. Contribution of poly(ADP-ribose) polymerase to postischemic blood-brain barrier damage in rats. J Cereb Blood Flow Metab. 27:1318–1326.
