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ORIGINAL ARTICLE

Dual Role of Intrauterine Immune Challenge on Neonatal and Adult Brain Vulnerability to Hypoxia-Ischemia

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Abstract

Epidemiologic evidence has underlined the impact of prenatal inflammation on the development of postnatal hypoxia-ischemia (HI) brain injury. To study to what extent prenatal inflammation affects CNS vulnerability later during development, C57BL/6 mice were subjected to intrauterine injection of lipopolysaccharide (LPS) at gestational day 15. At postnatal day (PND) 5, 9, and 70, the offspring were subjected to HI. It was found that, in neonatal mice, LPS-exposed brains showed markedly enhanced brain injury after HI, whereas in adult mice, LPS exposure resulted in a significant reduction in tissue loss after HI. Reduced myelin in subcortical white matter was noticed after HI in the LPS-exposed brains at PND14 and PND75. Increased activities of nuclear factor-κB and caspase-3 were obtained in fetal/neonatal brain after LPS administration. Conclusions were that 1) a prenatal low dose of LPS sensitized to HI-induced brain injury in neonates but confers protection in adulthood, 2) reduced myelination is seen after prenatal LPS exposure and HI in both neonatal and adult mice despite the fact that LPS reduced total tissue loss in adult mice; and 3) nuclear factor-κB and caspase-3 activation early after LPS exposure may play a role in the sensitization/protection (preconditioning) effects.

Key Words: Brain, Hypoxia-ischemia, Inflammation, Precondition, Sensitization.

INTRODUCTION

Accumulating epidemiologic evidence suggests that the majority of cerebral palsy-related brain injury occurs during the antepartum period and only approximately 15% may be attributed to perinatal events (e.g. birth asphyxia) (1–3). Among all the risk factors, inflammation/infection alone or in combination with asphyxia is emerging as the most important contributor (4–6). Specifically, the combined exposure to antenatal infection and asphyxia was found to dramatically increase the risk of cerebral palsy (7–10). In line with epidemiologic studies, experimental data suggest that intrauterine administration of bacterial lipopolysaccharide (LPS), including intracervical, intra-amnion/chorion, or intra-amniotic exposure, may cause rapid cytokine elevation and white matter injury in the brain (11). Furthermore, neonatal exposure to inflammation may not by itself induce observable CNS lesions but may affect cerebral vulnerability (so-called sensitization or preconditioning) and thereby act in concert with other insults, such as hypoxia-ischemia (HI), later in development (12–15). However, up to now, few data on the cerebral effects of antenatal inflammation/immune challenges in combination with secondary HI insults after birth are available. Furthermore, to what extent early exposure to inflammation affects CNS vulnerability later in life and what mechanisms may be involved remain unknown. Our hypothesis is that antenatal inflammation causes a fetal brain inflammatory reaction, which will alter CNS vulnerability in response to secondary insults later in life. Therefore, the aims of this study were 1) to establish an animal model that reflects the clinical situation of intrauterine infection in combination with postnatal HI and 2) to investigate the effect of intrauterine LPS on HI-induced brain injury at different postnatal developmental ages.

MATERIALS AND METHODS

Animals

Time-mated pregnant C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Mice were housed with a 12-hour light/dark cycle. Free access to a standard laboratory chow diet (B&K, Solna, Sweden) and deionized drinking water was provided. All animal experimentation was approved by the Ethical Committee of Göteborg (no. 314-2005). The C57BL/6 strain was chosen because previous publications suggest that different mouse strains show differences in the degree of brain injury and mortality after HI (16) and have different reactions to LPS (17, 18). Compared with C57BL/6 mice, other mouse strains are more resistant to HI injury (19) whereas the

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C57BL/6 mice not only develop reproducible brain injuries after HI but also have reasonably low mortality (20) and reproducible reactions to LPS according to our previous work (21).

Intrauterine LPS Injection

LPS (*Escherichia coli* 055:B5; Sigma, Stockholm, Sweden) was injected into the uteri of pregnant mice at gestational day (G) 15 (C57BL/6 mice normally deliver pups on day 19 to 20 of gestation) by using a method modified from previous publications (21, 22). As pilot experiments showed that doses of 4, 2, or 0.5 mg/kg resulted in either preterm birth (21) or fetal death (a dramatic increase in fetal death was found at doses greater than 0.1 mg/kg, consistent with the study in rats [23]), we chose LPS doses of 0.1 and 0.01 mg/kg in the present study. Briefly, animals were anesthetized with isoflurane (5% for induction and 3.5% for maintenance) in nitrous oxide/oxygen (1:1) in an induction chamber. Laparotomy was performed in the mouse lower abdomen. The mouse uterus was exposed, the number of fetuses in each horn was counted, and LPS (0.1 mg/kg or 0.01 mg/kg in 100 μL of saline/dam) or saline (100 μL/dam) was injected into the space between the top 2 gestational sacs in both of the 2 uterine horns between the amniotic and chorionic membranes (50 μL/horn). The reason for choosing these 2 injection sites was that in our previous study of preterm birth (21) and in pilot experiments we found that injecting LPS into the right uterine horn at a site between the lower 2 gestational sacs most proximal to the cervix resulted in an obvious "distance effect" on mortality, so that the fetuses furthest away from the LPS injected site were the ones most likely to be alive at 48 hours after administration of LPS. By modifying the original method and injecting a lower dose of LPS (0.1 and 0.01 mg/kg) between the top 2 gestational sacs in both of the 2 uterine horns, we found a low mortality, whereas almost all fetuses showed a reddish reaction (as an indication of the inflammatory reaction) at 2 hours after LPS injection. Therefore, this modification of the injection site allows for an even distribution of LPS while minimizing the disturbance to the pregnancy. After the uterus was returned to the abdomen, the fascia and the skin were closed, and animals recovered in individual cages until normal delivery. After birth, offspring were kept together with the dam in individual cages. For adult mouse experiments, offspring were separated into individual cages according to gender on postnatal day (PND) 21.

Hypoxia-Ischemia Procedure

HI was induced with an adaptation of the Rice-Vannucci HI model (24). At PND 5, 9, and 70 (brain maturity corresponding to that of human preterm infants, term infants, and adults [25–27]), mice offspring of both sexes were anesthetized with halothane (3.0% for induction and 1.0%–1.5% for maintenance) in a mixture of nitrous oxide and oxygen (1:1). The left common carotid artery was doubly ligated with Prolene sutures. After surgery and ligation, mice were returned to the cage, recovered for 1 hour, and were subsequently placed in an incubator perfused with a humidified gas mixture (10.00% ± 0.01% oxygen in nitrogen) for 50 minutes (at PND5), 40 minutes (at PND9), and 35 minutes (at PND70), respectively. The temperature in the incubator and the temperature of the water used to humidify the gas mixture were kept at 36°C. After hypoxic exposure, the pups were returned to their dam until death. Different hypoxia times were chosen based on the fact that animals have different reactions to this HI model according to their age, as previously shown (28–30). Therefore, the duration of hypoxia must be varied for individual ages to achieve an adequate, consistent degree of brain damage with minimal mortality. As investigated in our previous work (29) and according to pilot experiments, durations of 50 minutes of hypoxia at PND5, 40 minutes at PND9, and 35 minutes of hypoxia at PND70 produced comparable degrees of injury in the 3 age groups with a low mortality.

Primary Antibodies

The following primary antibodies were used in the study: microtubule-associated protein-2 (MAP-2, clone HM-2; Sigma, Stockholm, Sweden), mouse monoclonal antibody myelin basic protein (MBP, SMI 94; Sternberger Monoclonals, Lutherville, MD), and anti-2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNPase, MS-349-P; Lab Vision, Fremont, CA), antiphosphorylated neurofilament (NF, SMI 312; Sternberger Monoclonals), nuclear factor (NF)-κB p65 (sc109; Santa Cruz Biotechnology, Inc., Santa, Cruz, CA), and anti-actin (A2066; Sigma).

Assessment of Brain Damage and Immunohistochemistry Staining

At PND14 or PND75, 9 or 5 days after HI, pups were killed by decapitation. Brains were removed and processed for thionin/acid fuchsin and immunohistochemistry staining. Parallel coronal sections were used for various staining protocols. Immunohistochemical staining were performed as described previously (31). Control experiments were done by omission of the primary antibodies with substitution by mouse IgG (for MAP-2, NF, and MBP). In the brain injury assessment, the MAP2-negative volumes reflected infarcts, whereas the MAP2-positive volume in the contralateral hemisphere minus the MAP2-positive volume in the ipsilateral hemisphere reflected total tissue loss. White matter injury was analyzed by quantitative measurements (at 10× magnification) of the immune-positive staining area for MBP, CNPase, and NF using Micro Image, version 4.0 (Micro-Macro AB, Göteborg, Sweden). The infarct volume/tissue loss measurement, the neuropathologic scoring and the subcortical white matter area measurement were performed as described previously (32–34) by an operator blinded to the treatment groups. The neuropathologic scoring was evaluated using a semiquantitative neuropathologic scoring system as described previously (20, 32). Briefly, sections were stained with thionin/acid fuchsin and scored by an observer blinded to the treatment of the animals. The cortical injury was graded from 0 to 4, with 0 being no observable injury and 4 being confluent infarction encompassing most of the hemisphere. The damage in

hippocampus, striatum, and thalamus was assessed both with respect to hypotrophy (shrinkage) (0–3) and observable cell injury/infarction (0–3), resulting in a neuropathologic score for each brain region (0–6). The total score (0–22) was the sum for all 4 regions.

Caspase Activity Assays

On G15, at 2, 6, and 24 hours and on PND1 after intrauterine injection of saline (controls) or LPS (0.01 mg/kg), fetal or PND1 neonatal brains were rapidly dissected out and snap-frozen in liquid nitrogen. The tissue was homogenized in 1,000 μl of ice-cold 50 mM Tris-HCl (pH 7.3) containing 2 mM EDTA, and the protein concentration was determined according to Whitaker and Granum (35), adapted for microplates, aliquoted, and stored at −80°C. Caspase-3 and -1 activities were measured as described previously (36). Cleavages of Ac-DEVD-AMC (for caspase-3-like activity; Peptide Institute, Osaka, Japan) and Ac-WEHD-AMC (for caspase-1 like activity; Alexis Corp., San Diego, CA) were measured at 37°C using a Spectramax Gemini microplate fluorometer (Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm and expressed as pmol of 7-amino-4-methylcoumarin released per mg of protein per minute.

Immunoblotting

The brain homogenates were centrifuged at 800 × *g* at 4°C for 10 minutes. The pellets were washed in homogenizing buffer and recentrifuged, producing a nuclear pellet. The supernatant was further centrifuged at 15,600 × *g* for 15 minutes at 4°C, producing a cytosolic fraction in the supernatant. Immunoblotting for NF-κB was performed by using a previously described method (20, 37). Briefly, after blocking with 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 containing 5% fat-free milk powder for 1 hour at room temperature, the membranes were incubated with primary antibodies: NF-κB p65 or anti-actin at room temperature for 1 hour followed by an appropriate peroxidase-labeled, goat-anti-rabbit secondary antibody (Vector, Burlingame, CA) for 30 minutes at room temperature. Immunoreative species were visualized using the Super Signal Western Dura substrate (Pierce Chemical, Rockford, IL) and a LAS 1000-cooled CCD camera (Fujifilm, Tokyo, Japan). Immunoreative bands were quantified using Image Gauge software (Fujifilm).

Statistics

Student unpaired *t*-test was used for the brain infarct volume, tissue loss, and immune reaction-positive area quantification. Mann-Whitney U test was used to compare the neuropathology score differences between the LPS-exposed and saline-exposed mice. Analysis of variance followed by the Fisher protected least significant difference post hoc test was used for comparing different activity data. $p < 0.05$ was considered statistically significant. Data are expressed as means ± SEM.

RESULTS

Intrauterine Lipopolysaccharide Exposure Sensitized Postnatal Hypoxia-Ischemia Brain Injury in Neonates

Intrauterine Lipopolysaccharide (0.1 mg/kg) Followed by 50 Minutes of Hypoxia-Ischemia at Postnatal Day 5

After intrauterine LPS (0.1 mg/kg), 50 minutes of HI was induced at PND5, a time that corresponds to human preterm infants. At 9 days post-HI (PND14), a time when the white matter is partly myelinated in mice, pups were killed, and brain injury was evaluated. The total tissue loss was increased from 8.7 ± 3.5 mm^3 in saline controls to 28.0 ± 3.3 mm^3 in LPS pre-exposed mice (68.9% increase, $p < 0.001$) (Fig. 1A). The neuropathologic total score was significantly increased from 5.6 ± 1.8 in saline pre-exposed mice to 10.4 ± 1.3 in LPS pre-exposed mice ($p < 0.05$) (Fig. 1B). The neuropathologic score was increased in all brain regions, including cortex (0.8 ± 0.3 vs 1.9 ± 0.3), thalamus (0.9 ± 0.3 vs 2.4 ± 0.4), striatum (1.1 ± 0.3 vs 2.0 ± 0.4), and

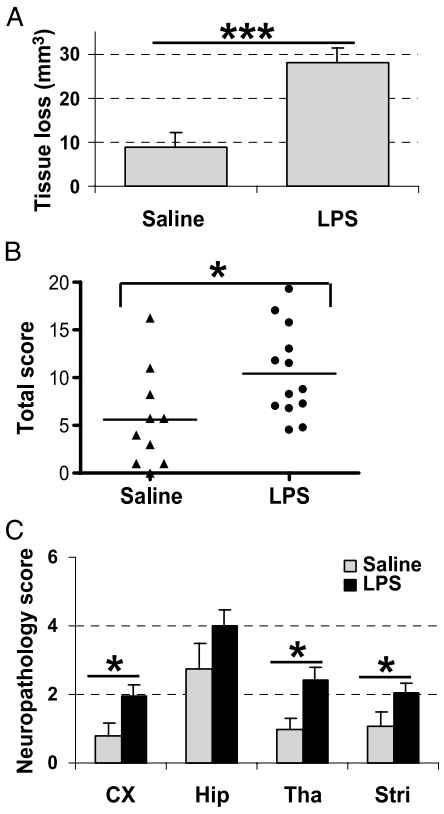

FIGURE 1. Hypoxia-ischemia (HI) brain injury in postnatal day (PND) 5 mice is increased after intrauterine injection of lipopolysaccharide (LPS). Shown are brain total tissue loss volume **(A)**, neuropathologic total score **(B)**, and neuropathologic score in multiple brain areas **(C)** with the combination of LPS (0.1 mg/kg) (n = 14) or saline (n = 10) at gestation age 15 days and HI for 50 minutes at PND5. *, $p < 0.05$; ***, $p < 0.001$. Cx, cortex; Hip, hippocampus; Stri, striatum; Tha, thalamus.

hippocampus (2.8 ± 0.7 vs 4.0 ± 0.4), in saline versus LPS pre-exposed mice (Fig. 1C).

Intrauterine Lipopolysaccharide (0.01 mg/kg) Followed by 40 Minutes of Hypoxia-Ischemia at Postnatal Day 9

To examine the effect of prenatal LPS in neonatal mice at a time that corresponds to human term infants, we next tested the combination of LPS pre-exposure at G15 and HI at PND9 with a reduced dose of LPS (0.01 mg/kg) as the higher dose of 0.1 mg/kg resulted in 33.3% mortality in the offspring. At PND14, it was found that the total tissue loss was increased from 19.6 ± 1.9 mm^3 in saline to 39.0 ± 1.6 mm^3 in LPS pre-exposed mice (49.7% increase, $p < 0.0001$) (Fig. 2A). The neuropathologic total score was significantly increased from 8.2 ± 0.8 in saline pre-exposed mice to 15.6 ± 0.6 in LPS pre-exposed mice ($p < 0.0001$), and the total score for each animal is shown in Figure 2B. The neuropathologic score was significantly higher in LPS compared with saline pre-exposed mice for all brain regions evaluated, being most marked in the cerebral cortex (Fig. 2C).

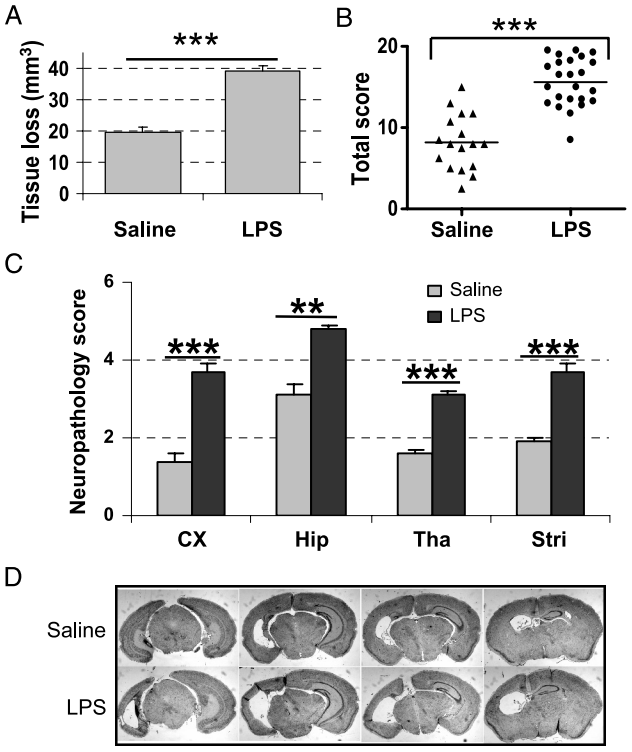

FIGURE 2. Hypoxia-ischemia (HI) brain injury in postnatal day (PND) 9 mice is increased after intrauterine injection of lipopolysaccharide (LPS). Shown are brain total tissue loss volume **(A)**, neuropathologic total score **(B)**, and neuropathologic score in multiple brain areas **(C)** with the combination of LPS (0.01 mg/kg) (n = 24) or saline (n = 17) at gestation age 15 days and HI for 40 minutes at PND9. **(D)** Representative pictures of brain injury in saline and LPS pre-exposed brain sections stained with thionin/acid fuchsin at PND14. **, $p < 0.01$; ***, $p < 0.001$. Cx, cortex; Hip, hippocampus; Stri, striatum; Tha, thalamus.

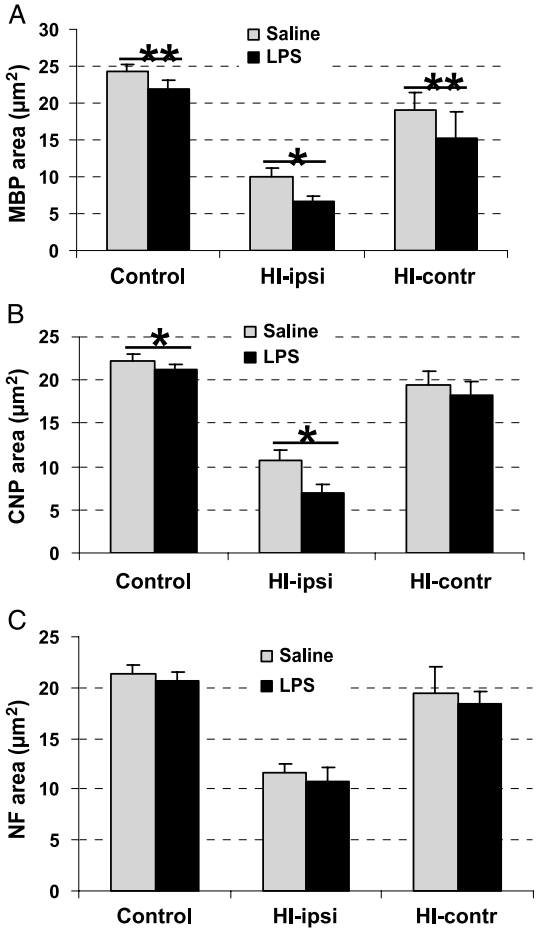

FIGURE 3. White matter components are reduced in neonatal mice after intrauterine injection of lipopolysaccharide (LPS) in combination with postnatal hypoxia-ischemia (HI). Quantification of myelin basic protein (MBP) **(A)**, 2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNPase) **(B)**, and neurofilament (NF) **(C)** immunostaining in subcortical white matter in the saline and LPS pre-exposed brains in control animals (control), and 5 days after HI (ipsilateral hemisphere [HI-ipsi] and contralateral hemisphere [HI-contr]) at postnatal day 14. *, $p < 0.05$; **, $p < 0.01$.

Intrauterine Lipopolysaccharide Exposure Caused White Matter Injury Alone or in Combination With Hypoxia-Ischemia in Neonatal Mice

To investigate the effect of LPS pre-exposure on the white matter with and without HI, immunostaining for oligodendrocyte and myelin markers MBP and CNPase was used. It was found that prenatal LPS (0.01 mg/kg) exposure caused an obvious decrease in myelination on PND14 in the subcortical white matter demonstrated by both MBP and CNPase immunostaining (Fig. 3A, B). HI at PND9 caused a marked decrease of myelination in the subcortical area in both the saline and LPS pre-exposed animals that was more pronounced in the LPS group (Fig. 3). The same tendency was observed in the PND5 HI mice (data not shown). HI-induced white matter demyelination was observed in both hemispheres (Fig. 3A), but it was more

obvious in the ipsilateral hemisphere (Fig. 3A, B). Quantification of immunostaining of the axonal marker NF did not show any differences between saline and LPS pre-exposed brains (Fig. 3C).

Intrauterine Lipopolysaccharide Exposure Reduced Total Lesion Size but Selectively Increased White Matter Vulnerability to Hypoxia-Ischemia in Adult Mice

Intrauterine Lipopolysaccharide Exposure Conferred Neuroprotection to Hypoxia-Ischemia in Adult

To investigate very long-term interactions between prenatal LPS exposure and postnatal HI, we next examined the effect of intrauterine LPS exposure on brain HI in adulthood. LPS (0.01 mg/kg) was injected into the uterus at G15 and mice were subjected to HI for 35 minutes at PND70. Brain injury was evaluated at 5 days after HI. Contrary to the situation in neonates, the total tissue loss was reduced from 21.6 ± 6.2 mm^3 in saline pre-exposed mice to 4.8 ± 1.7 mm^3 in LPS pre-exposed mice (77.8% reduction, $p < 0.01$) (Fig. 4A). The neuropathologic total score was significantly reduced from 5.5 ± 1.0 in saline pre-exposed

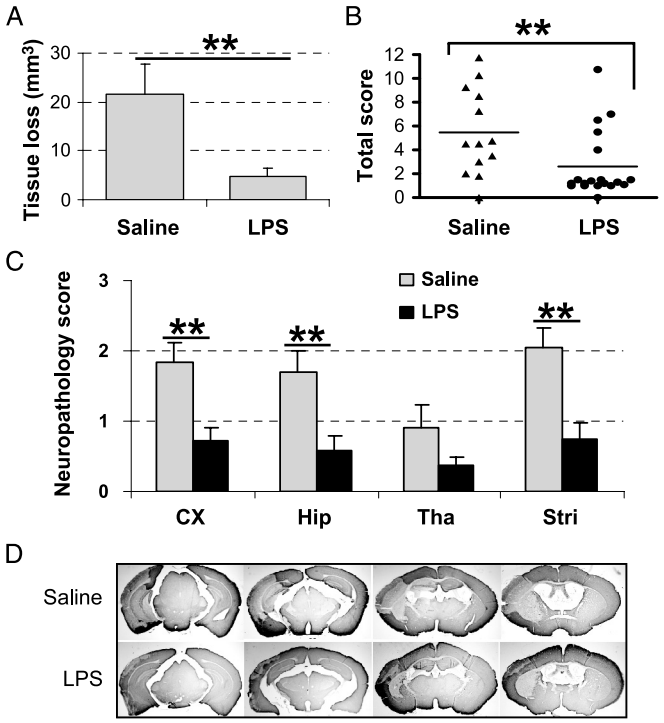

FIGURE 4. Grey matter injury is reduced in adult mice after intrauterine injection of lipopolysaccharide (LPS) in combination with hypoxia-ischemia at postnatal day (PND) 70. Brain total tissue volume loss **(A)**, neuropathologic total score **(B)**, and neuropathologic score **(C)** in the cortex (Cx), hippocampus (Hip), thalamus (Tha) and striatum (Stri) at PND75. Saline group, n = 13; LPS group, n = 19. **(D)** Representative picture of saline and LPS pre-exposed brain sections with microtubule-associated protein-2 (MAP-2) staining. **, $p < 0.01$.

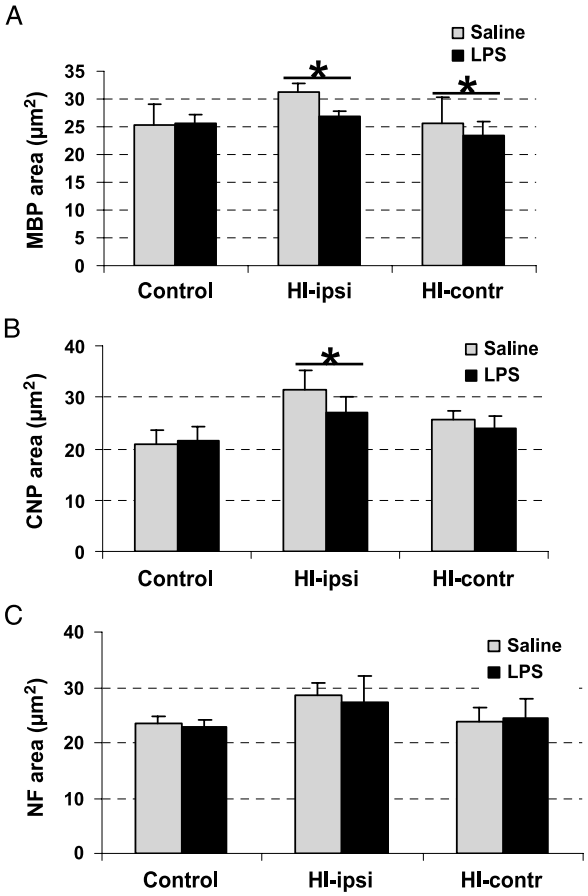

FIGURE 5. White matter components are reduced in adult mice after intrauterine injection of lipopolysaccharide (LPS) in combination with postnatal day (PND) 70 hypoxia-ischemia (HI). Quantification of myelin basic protein (MBP) **(A)**, 2′,3′-cyclic nucleotide 3′ phosphodiesterase (CNPase) **(B)**, and neurofilament (NF) **(C)** immunostaining in subcortical white matter in the saline and LPS pre-exposed brains in control animals (control), and 5 days after HI (ipsilateral hemisphere [HI-ipsi] and contralateral hemisphere [HI-contr]) at PND75. *, $p < 0.05$.

mice to 2.3 ± 0.5 in LPS pre-exposed mice (57.5% reduction, $p < 0.01$), and the total score for each animal is shown in Figure 4B. The neuropathologic score was significantly decreased in the cortex (1.8 ± 0.3 vs 0.7 ± 0.2), hippocampus (1.6 ± 0.3 vs 0.6 ± 0.2), and striatum (2.0 ± 0.3 vs 0.7 ± 0.3; 63.1%) ($p < 0.01$ in all above areas) but not in the thalamus (0.9 ± 0.3 vs 0.4 ± 0.1) in saline versus LPS pre-exposed mice (Fig. 4C).

Intrauterine Lipopolysaccharide Exposure Increased White Matter Vulnerability to Hypoxia-Ischemia in Adult

As shown by both the MBP and CNPase immunostaining quantification, the LPS-pre exposure alone did not result in reduced myelination in adulthood at PND75 (Fig. 5). However, when combined with HI, LPS pre-exposed brains displayed a limited but significantly decreased myelination in subcortical white matter shown

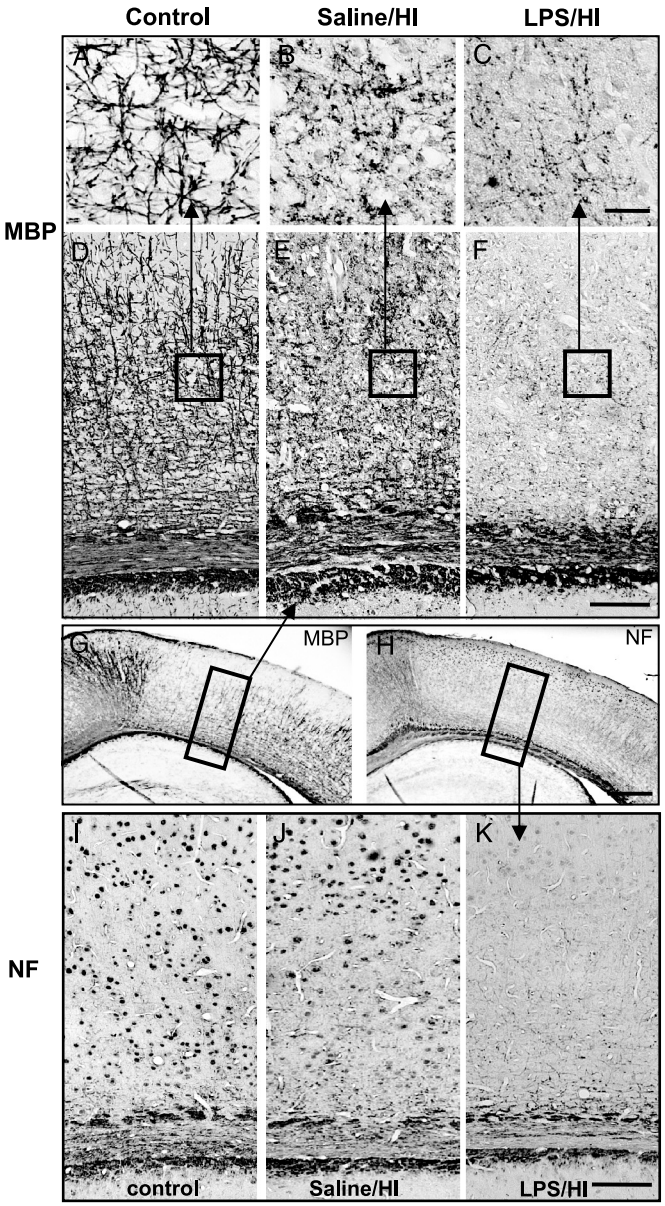

FIGURE 6. Representative pictures of reduced myelin basic protein (MBP) and neurofilament (NF) immunoreactivity in adult mice after intrauterine injection of lipopolysaccharide (LPS) in combination with postnatal day (PND) 70 hypoxia-ischemia (HI). Pictures show MBP **(A–G)** and NF **(H–K)** immunohistochemistry staining at 5 days after HI at PND75 in the ipsilateral hemisphere cortical and subcortical areas of normal control (control, **A**, **D**, **I**), intrauterine saline exposure in combination with PND70 HI (saline/HI, **B**, **E**, **G**, **J**) and intrauterine LPS exposure in combination with PND70 HI (LPS/HI, **C**, **F**, **H**, **K**). **(A–C)** Higher magnification for MBP. **(G)** Representative picture of MBP staining in the ipsilateral hemisphere after saline/HI. **(H)** Representative picture of NF staining in the ipsilateral hemisphere after LPS/HI. Scale bars = **(A–C)** 20 μm; **(D–F, I–K)** 100 μm; **(G, H)** 50 μm.

by both MBP (Fig. 5A) and CNPase (Fig. 5B) immunostaining compared with saline pre-exposed brain at 5 days after HI at PND75, despite the fact that LPS pre-exposure resulted in significantly reduced gray matter damage after HI at this age. In addition, in contrast to the orderly radial distribution of nerve fibers in normal control animals, there was an obvious distorted placement/orientation of MBP processes and more pronounced progressive loss of MBP nerve fibers interspersed in the gray matter in the LPS pre-exposed brains after HI compared with normal control and saline-exposed brains after HI (Fig. 6A–F).

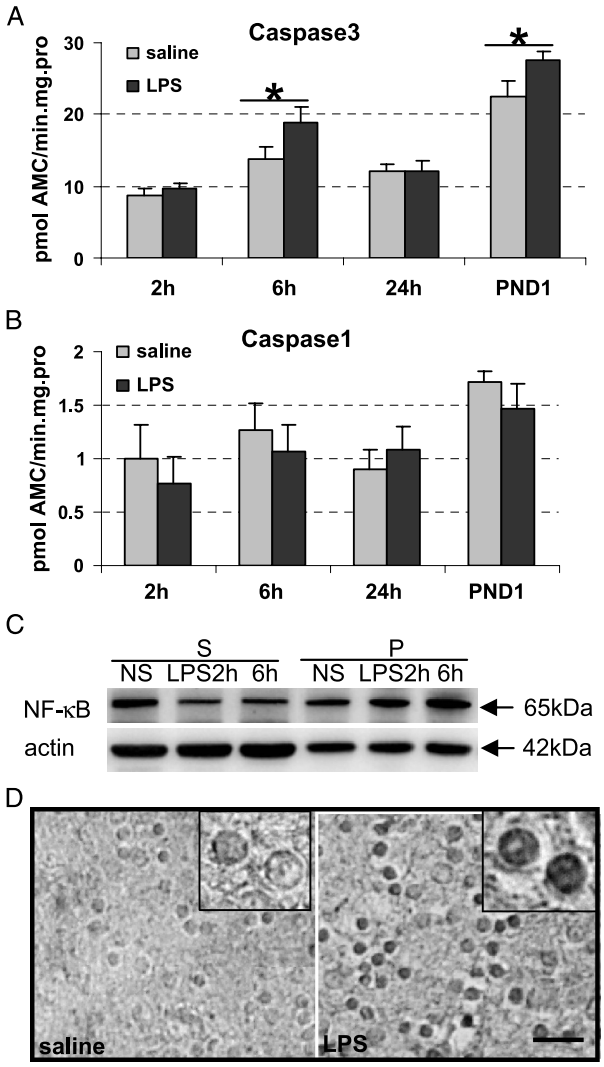

FIGURE 7. Increased caspase-3 and nuclear factor (NF)-κB activation after intrauterine lipopolysaccharide (LPS) injection in the fetal/neonatal brain. Caspase-3 **(A)** and caspase-1 **(B)** activation after LPS administration on G15 compared with saline pre-exposed fetal/neonatal brains. *, $p < 0.05$. **(C)** Representative picture of NF-κB Western blot in cytosolic (S) and nuclear (P) fractions of G15 fetal brain in saline pre-exposed brain (NS) and at 2 hours (LPS2h) and 6 hours (LPS6h) after LPS exposure. The actin Western blot shows equal loading of protein. **(D)** Representative pictures of NF-κB immunohistochemistry staining in saline- and LPS-exposed brain at 6 hours after the injection. AMC, 7-amino-4-methylcoumarin. Scale bar = 30 μm.

Axonal development, as shown by the quantification of NF immunoactivity, was not affected by the prenatal low dose of LPS alone or in combination with postnatal HI (Fig. 5C). However, abnormal morphologic expression of neurofilament proteins in dysmyelinating axons was noticed after HI in the ipsilateral hemisphere and the structure of NF-immunoreactive fibers in the LPS exposed brain demonstrated a generalized disruption and diminution of NF staining in the subcortical and cortical areas compared with normal control and saline-exposed brains after HI (Fig. 6I–K).

Intrauterine Lipopolysaccharide Exposure Triggered Caspase-3 and Nuclear Factor-κB Activation in the Fetal/Neonatal Brain

Lipopolysaccharide Exposure Increased the Caspase-3 Activation in Fetal/Neonatal Brain

Capase-3 and -1 activities in fetal/neonatal brain homogenate were measured at 2, 6, and 24 hours after LPS administration on G15 and PND1. It was found that caspase-3 activity (Fig. 7A) was significantly increased at 6 hours and at PND1 after LPS administration at G15, but caspase-1 activity (Fig. 7B) showed no significant changes compared with the saline-exposed fetal brains.

Lipopolysaccharide Exposure Induced Nuclear Factor-κB Activation in Fetal Brain

To further confirm the LPS-related mechanisms in the brain after intrauterine LPS exposure, NF-κB Western blot analysis in the cytosolic and nuclear fractions and immunohistochemistry was performed. Intrauterine LPS exposure triggered the transcription of NF-κB from the cytosol to the nucleus and resulted in increased NF-κB activation in fetal brain at 2 and 6 hours after LPS administration at G15 compared with saline pre-exposure as shown by both Western blot (Fig. 7C) and NF-κB immunostaining (Fig. 7D).

DISCUSSION

In the present study, we have established a novel animal model of intrauterine infection in combination with postnatal HI. We believe that the model is clinically relevant as it is more and more widely accepted that a significant proportion of neonatal encephalopathy is preceded by antenatal exposure to infections. The model in this study presents 2 advantages: LPS was administered in a low dose that resulted in minimal maternal illness and fetal mortality, allowing for neonatal follow-up; and LPS was administered between the chorionic and amniotic membranes, mimicking where intrauterine inflammation supposedly is initiated in humans.

We found that a low dose of intrauterine LPS, applied between the chorionic and amniotic membranes, sensitized to HI-induced grey matter injury in neonatal mice but conferred protection to HI in the adult brain. Previously, depending on the specific stimulus and the time interval between events, both increased vulnerability of the brain to subsequent insults (sensitization) and protection/tolerance have been noted during the first 3 days after LPS exposure (12, 14). A recent study showed that LPS injected intraperitoneally to pregnant rats on G17 exacerbated the extent of brain lesions induced by postnatal HI at PND1 (37), which agrees with our present findings. Taken together, these findings suggest that early life immune challenges influence white matter development and CNS vulnerability later in life.

In addition to the effects during the neonatal period, early life immune challenges have also been suggested to induce long-term consequences in the adult, including programming of neuroendocrine regulation (38), abnormal behavioral and pharmacologic responses (39), and sensorimotor gating deficits (40), whereas the predominant response reported in adults is a protective effect of LPS on middle cerebral artery occlusion-induced brain damage (41, 42). Recently, neonatal LPS exposure was found to alter behavioral and temperature responses to cerebral ischemia in the adult rat, which was associated with increased neuronal loss in the amygdala (43). In the present study, we found that intrauterine LPS pre-exposure markedly reduced grey matter lesions after HI in adult mice, suggesting that there may be different responses to cerebral ischemia in the adult depending on whether the early immune challenge occurred before or after birth or there may be species differences. The opposite consequences of prenatal LPS on neonatal and adult HI in this study also emphasizes the fact that the effects of prenatal LPS priming are complex. The delayed long-lasting neuroprotection may involve changes in gene expression, resulting in suppression of the inflammatory reaction and apoptotic cascade. Indeed, previously we have shown that several hundreds of genes associated with processes such as protein metabolism, immune and inflammatory responses, neurogenesis, and cell death/survival are regulated in the neonatal brain after LPS exposure (44), indicating a complex cerebral response to peripheral LPS exposure. There are also data suggesting that endogenous corticosterone plays a critical role for acquiring endotoxin-induced HI tolerance in neonatal rats (15). Therefore, rather than a single alteration in the inflammatory response, a more complex concerted action of different pathways might be postulated, which affect a diversity of pathophysiologic mechanisms.

LPS is mediated through the interaction with toll-like receptor 4 and myeloid differentiation factor 88 to activate downstream signaling processes including the activation and the nuclear translocation of the nuclear transcription factor NF-κB (45) and further transcribes proinflammatory cytokines such as interleukin-1β, interleukin-6, and tumor necrosis factor-α, prostaglandins and a variety of adhesion molecules and acute-phase proteins (46–48). NF-κB has been found in various cell populations in the brain including microglia, astrocytes, and neurons (49–51). In the present study we found that NF-κB was upregulated at an early stage after the LPS administration in the fetal brain, agreeing with earlier studies in fetal sheep (52). The time of NF-κB elevation fits with the cytokine changes that occurred after intrauterine LPS administration found in previous studies (53). These results indicate that among the numerous altered

genes/molecules, NF-κB may play a critical role in LPS sensitization/preconditioning effects on HI. Indeed, NF-κB has been found to be involved in preconditioning-induced neuroprotection (54, 55). However, the role of NF-κB in LPS sensitization/preconditioning need to be further investigated because NF-κB not only contributes to the mounting of an effective immune response but is also involved in the regulation of cell proliferation, development, and apoptosis (56).

In the present study, we also find that immediately after LPS administration, protein levels of caspase-3 activation were upregulated, agreeing with earlier studies in pregnant and neonatal rats (44, 57, 58). It is well known that the caspase family plays a crucial role in apoptotic cell death and that caspase-3 is an especially important executioner molecule for apoptosis induction. However, recently a role for caspase-3 in a multitude of nonapoptotic functions, including its role in the immune system has been noted. For example, it has been suggested that caspase-3 may be involved in B cell cycling and proliferation (59, 60). Furthermore, caspase-3 was found to be involved in the neuroprotection in a preconditioning paradigm (61). More interestingly, it has been indicated that both the initiator caspases (e.g. caspase-2 and -8) and executioner caspases can initiate NF-κB activation (62–64). Caspase-3 may apparently act at the NF-κB transactivation level after DNA binding through a poly(ADP-ribose) polymerase-1-mediated mechanism (64). Caspase 3 may also affect NF-κB activation through cleavage of mitogen-activated protein kinase kinase kinase-1 (65, 66) offering further support for a close cross-talk between NF-κB and caspases. In this study we found that caspase-3 activation was increased after LPS administration and that the first peak occurred at the same time as NF-κB was activated. Speculatively, this may indicate that NF-κB and caspase-3 act synergistically to induce sensitization/precondition. In contrast to previous findings in adult rats, in which LPS injection increases caspase-1 activity and interleukin-1β expression in hippocampus and entorhinal cortex (67), we did not find changes in caspase-1 after administration of LPS. Similarly, LPS administration in neonatal rats did not evoke any changes of caspase-1 mRNA (44). Given the nature of caspase-1 and the role of caspase-1 in inflammation (68, 69), it would be interesting to further explore the role of caspase-1 in the LPS induced inflammatory response.

It is known that the premyelinating oligodendrocyte is the predominate cell type in periventricular white matter regions during the window of increased vulnerability to periventricular leukomalacia (24–34 postconceptional weeks, approximately 60%–85% of pregnancy) (70, 71) and the time period from mid-gestation to postnatal ages is critical for axonal development, particularly with regard to periventricular leukomalacia (72, 73). As a confirmation of previous studies (57, 74–76), when LPS was administered at the time of 75% of pregnancy, it was found that prenatal LPS exposure resulted in hypomyelination at PND14 compared with saline-treated animals. However, in the adult mouse brain, this effect had diminished, suggesting that the LPS-induced loss of myelin is transient and indicating that adult animals probably can compensate for the early damage, which is in agreement with other findings (77, 78). Together, these findings indicate that the hypomyelination found at PND14 may be due to delayed development rather than damage to the oligodendrocytes, as white matter volumes seem to have recovered by adulthood as also shown by a previous study (78). However, although the adult animal has the ability to compensate for earlier attained white matter damage, it is still highly vulnerable when exposed to a second insult. This increased vulnerability in adulthood appears specific for the white matter, as the gray matter was actually protected in the adult mice. Furthermore, LPS pre-exposed brains displayed obvious abnormal structure and distorted placement/orientation of nerve processes in the cortical and subcortical area after HI as shown by both MBP and NF immunostaining. The disturbed NF immunostaining in the white matter after LPS alone or in combination with HI insult indicates that prenatal exposure of LPS impairs not only the oligodendrocytes, but also the axons. These results clearly show that the white and gray matter respond differently to subthreshold LPS exposures and suggest that the mechanisms of white and gray matter injury after HI might be different, which has to be taken into consideration when cerebroprotective strategies are developed. To support this difference, it was shown that intraperitoneal injections of LPS given postnatally caused increased permeability of the blood-brain barrier to proteins, and these plasma proteins were detected in the brain only within white matter tracts, whereas only selective permeability to small molecules (sucrose and insulin) was found in the adult animal brain (78). This finding further indicates that the same early life inflammatory stimulation can give different reactions in the neonatal and adult white matter and gray matter.

Taken together, our results illustrate the critical influence of prenatal immune events upon neonatal and adult CNS vulnerability and susceptibility to a secondary insult and provide the first experimental evidence that endotoxin may already operate in prenatal life to modulate the development of HI-induced brain injury in the offspring, both neonatally and in adulthood.

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