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α -Phenyl-*n*-tert-butyl-nitron Attenuates Lipopolysaccharide-induced Brain Injury and Improves Neurological Reflexes and Early Sensorimotor Behavioral Performance in Juvenile Rats

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Abstract

Our previous study showed that treatment with α -phenyl-*n*-tert-butyl-nitron (PBN) after exposure to lipopolysaccharide (LPS) reduced LPS-induced white matter injury in the neonatal rat brain. The object of the current study was to further examine whether PBN has long-lasting protective effects and ameliorates LPS-induced neurological dysfunction. Intracerebral (i.c.) injection of LPS (1 mg/kg) was performed in postnatal day (P) 5 Sprague Dawley rat pups and PBN (100 mg/kg) or saline was administered intraperitoneally 5 min after LPS injection. The control rats were injected (i.c.) with sterile saline. Neurobehavioral tests were carried out from P3 to P21, and brain injury was examined after these tests. LPS exposure resulted in severe brain damage, including enlargement of ventricles bilaterally, loss of mature oligodendrocytes, impaired myelination as indicated by the decrease in myelin basic protein immunostaining, and alterations in dendritic processes in the cortical gray matter of the parietal cortex. Electron microscopic examination showed that LPS exposure caused impaired myelination as indicated by the disintegrated myelin sheaths in the juvenile rat brain. LPS administration also significantly affected neurobehavioral functions such as performance in righting reflex, wire hanging maneuver, cliff avoidance, negative geotaxis, vibrissa-elicited forelimb-placing test, beam walking, and gait test. Treatment with PBN, a free radical scavenger and antioxidant, provided protection against LPS-induced brain injury and associated neurological dysfunction in juvenile rats, suggesting that antioxidation might be an effective approach for therapeutic treatment of neonatal brain injury induced by infection/inflammation.

Keywords

white matter injury; lipopolysaccharide; impaired myelination; neurobehavioral performance; antioxidant

Maternal or placental infection plays an important role in the pathogenesis of periventricular leukomalacia (PVL), a form of white matter disease closely associated with cerebral palsy (Hagberg et al., 2002; Rezaie and Dean, 2002; Volpe, 2003). Increasing evidence implicates

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damage to developing oligodendrocytes (OLs) during a specific prenatal window of vulnerability (24–32 weeks' gestation) as a significant underlying factor in the pathogenesis of PVL is usually accompanied by the development of several types of lesions within the central nervous system in the infant brain (Back et al., 2001, 2002; Rezaie and Dean, 2002). Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, is responsible for most of the inflammatory effects of infection from these bacteria. LPS has been detected in amniotic fluid of patients with gram-negative intra-amniotic infection (Romero et al., 1987). It is possible the LPS may reach the fetal brain during maternal infection. Therefore, LPS has been extensively used to study the possible link between inflammation and perinatal brain injury with different ways of administration including maternal intravenous or intrauterine administration, and neonatal systemic or intracerebral (i.c.) injection (Wang et al., 2006). We and others have developed a neonatal rat model to mimic a similar scenario as that during maternal infection through i.c. injection of LPS in neonatal rats to study brain injury associated with the infection/inflammation (Lehnardt et al., 2002; Cai et al., 2003; Pang et al., 2003; Fan et al., 2005a, 2005b, 2008). This neonatal rat model of LPS exposure produces brain injury with similar neuropathological and behavioral features as those seen in infants with PVL.

In this model, we found that LPS resulted in preferential white matter injury in the neonatal rat brain and that the injury was closely associated with increased oxidative stress after LPS exposure (Fan et al., 2005a, 2008). Free radical injury has been shown to affect premyelinating OLs, and this is supported by evidence of oxidative and nitrative stress as indicated by markers of lipid peroxidation and protein nitration in the infant brain with PVL (Haynes et al., 2003, 2005). Therefore, we proposed that an antioxidant treatment approach might provide protection against LPS-induced brain injury. α -Phenyl-*n*-tert-butyl-nitron (PBN) is a nitron-based spin-trapping compound and an antioxidant (Floyd et al., 2002), and has a high degree of blood–brain barrier penetration (Chen et al., 1990; Floyd and Hensley, 2000). PBN has been shown to provide protection in hypoxic–ischemic brain injury and other models of neuronal degenerative disease (Sang et al., 1999; Floyd and Hensley, 2000; Floyd et al., 2000, 2002; Endoh et al., 2001; Lin et al., 2004, 2006). In our previous study, treatment with PBN (100 mg/kg) significantly reduced LPS (1 mg/kg)-induced brain damages, as indicated by the reduction in damage to OLs, axons, and dendrites in the neonatal rat brain 24 hr after LPS exposure (Fan et al., 2008). PVL and other white matter injuries in newborn infants have long-term effects on physical, visual, motor, sensory, cognitive, and social development in human infants (Hagberg et al., 2002). It is unknown whether PBN offers long-lasting protection in our neonatal rat model. The objective of the present study was to determine whether PBN has long-lasting protective effects on LPS-induced brain injury. An additional aim was to evaluate whether PBN also improves LPS-induced deficits in neurobehavioral performance during neurological reflex testing and when engaged in early sensorimotor and locomotor activities.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO). Monoclonal mouse antibodies against adenomatous polyposis coli (clone CC1) (APC-CC1), and myelin basic protein (MBP) or micro-tubule-associated protein 2 (MAP2) were purchased from Calbiochem (San Diego, CA) and Chemicon (Temecula, CA), respectively.

Animals

Timed pregnant Sprague Dawley rats arrived in the laboratory on day 19 of gestation. Animals were maintained in an animal room on a 12-hr light/dark cycle and at constant temperature ($22 \pm 2^\circ\text{C}$). The day of birth was defined as P0. After birth, the litter size was adjusted to 12 pups per litter to minimize the effect of litter size on body weight and brain size. All procedures for animal care were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. Every effort was made to minimize the number of animals used and their suffering.

Surgical Procedures and Animal Treatment

To minimize possible systemic effects of intraperitoneally administered LPS (Wang et al., 2006), i.c. injection of LPS to 5-day-old Sprague Dawley rat pups of both sexes was performed as previously described (Fan et al., 2005a, 2005b; Pang et al., 2003). Under light anesthesia with isoflurane (1.5%), LPS (1 mg/kg, from *Escherichia coli*, serotype O55:B5) in sterile saline (total volume of 2 μL) was administered to the rat brain at the location of 1.0 mm posterior and 1.0 mm lateral to the bregma, and 2.0 mm deep to the scalp at the left hemisphere in a stereotaxic apparatus with a neonatal rat adapter. The control rats were injected with the same volume of sterile saline. After the i.c. injection, the rat pups were placed on a thermal pad with the temperature maintained at $35 \pm 0.5^\circ\text{C}$ before returning to the dams. Our preliminary study has shown that rat pups tolerated this injection well. There was a decrease in body temperature in animals in both the LPS- and the saline-injected groups immediately after the injection (from 36 to 33°C); the body temperature then increased to 37°C within 0.5 hr and returned to the preinjection level within 2 hr. There were no significant differences in body temperature changes and brain lactate and adenosine triphosphate levels (determined 1 hr after injection) between the LPS- and the saline-injected groups (unpublished data). Although the body weight in the LPS-treated group was lower than that in the control group after the injection, the growth pattern was similar in the two groups (as indicated by the parallel growth curves; Fig. 1 in Results). This is an indication that i.c. injection of LPS did not have severe systemic effects and cause deteriorated conditions in the LPS-treated group. The dose of LPS was chosen on the basis of previously reported protocols that produced preferential white matter injury (Pang et al., 2003; Fan et al., 2005a, 2008). Although we found in our later studies that LPS at a dose of 200 $\mu\text{g}/\text{kg}$ resulted in similar injury to developing OLs as did the current dose of LPS, we chose to use the same dose of 1 mg/kg in the current study to be consistent with that used in our previous study on the short-term protection of PBN in this model (Fan et al., 2008). The injection site was located at the area just above the left cingulum. We and others (Lehnardt et al., 2002) found that injection of LPS at this site caused significant damage to periventricular late OL progenitor cells, which are the major cellular target of human perinatal white matter injury (Back et al., 2001). All animals survived the i.c. injection.

Both LPS- and saline-injected animals were further divided into two groups: one received intraperitoneal injections of PBN and the other sterile saline. PBN (100 mg/kg) or vehicle alone was administered 5 min after the LPS injection. Our previous study showed that this dosing of PBN protected the neonatal rat brain from hypoxia-ischemia-induced white matter injury (Lin et al., 2004, 2006). This dose of PBN has no significant effects on body temperature (Christensen et al., 2003; Lin et al., 2004). Each dam had an equal litter size (12 pups). Sixteen days after the injection (P21), rat pups were killed by transcardiac perfusion with normal saline followed by 4% paraformaldehyde for brain section preparation. Equal numbers of rat pups (six male and six female pups) for each treatment group were from 12 different litters. There were 12 pups in each group for behavioral tests. Coronal frozen brain

sections at 10 μ m of thickness from six pups (three male and three female) of each group were prepared in a cryostat for immunohistochemical staining. Another six pups (three male and three female pups; P21) of each group were prepared for electron microscopic (EM) study.

Behavioral Testing

Behavioral tests were performed as described by Fan et al. (2005b) with modification. The developmental test battery that was used was based on previously documented tests for neurobehavioral toxicity (Altman and Sudarshan, 1975; Hermans et al., 1992). Behavioral tests including the righting reflex, wire hanging maneuver, cliff avoidance test, negative geotaxis, and beam walking test were performed for all rat pups from P3 to P21 (Fan et al., 2005b). The vibrissa-elicited forelimb-placing test and gait analysis were performed in all rat pups on P21. The body weight of rat pups was recorded daily. The time of eye opening was recorded also.

Righting reflex—This test is believed to be a reflection of muscle strength and subcortical maturation (Altman et al., 1971; Hermans et al., 1992). Pups were placed on their backs, and the time required to turn over on all four feet and touch the platform was measured. The cutoff time was 60 sec (Altman et al., 1971).

Wire hanging maneuver—This maneuver tests neuromuscular and locomotor development (Altman and Sudarshan, 1975; Hermans et al., 1992). Pups suspended by their forelimbs from a horizontal rod (5×5 mm² area, 35 cm long, between two poles 50 cm high) tend to support themselves with their hind limbs, preventing them from falling and aiding in progression along the rod. A sawdust-filled box at the base served as protection for the falling pups. Suspension latencies were recorded. The cutoff time was 120 sec.

Cliff avoidance test—This test is used to assess the integration of exteroceptive input (vibrissae) and locomotor output (Altman and Sudarshan, 1975; Shen et al., 1991; Hermans et al., 1992). Pups placed on the edge of a platform ($20 \times 20 \times 20$ cm) with forepaws and chest extending over the edge tend to move away by backing up with forepaws or their body and head turning sideways. Avoidance was scored by reflex latency, the time between edge placement and body repositioning. If the pup did not make any response within 60 sec or fell off the platform, it was recorded as 60 sec (Shen et al., 1991).

Negative geotaxis—This test is believed to test reflex development, motor skills and vestibular labyrinth, and cerebellar integration (Altman and Sudarshan, 1975; Hermans et al., 1992). Rats placed on a 15° incline with their head pointing down the slope turn to face upward and begin to crawl up the slope. Each pup was given three trials a day, and the time spent for a turn of 180° upward was recorded. The cutoff time was 60 sec.

Vibrissa-elicited forelimb-placing test—This test uses stimulation of the rat's vibrissae to trigger a placing response to measure forelimb-placing deficit (De Ryck et al., 1992; Schallert and Woodlee, 2005; Frost et al., 2006). Rats use their vibrissae to gain bilateral information about the proximal environment, and this information is integrated between the hemispheres. All animals were tested on P21. The animal was gently held by its torso, allowing the forelimbs to hang free. Independent testing of each forelimb was induced by gently brushing vibrissae of the corresponding side on the edge of a tabletop once per trial for 10 trials. The percentage of trials in which the rat successfully placed its forepaw onto the tabletop was recorded for each side. Intact animals placed the forelimbs of both sides quickly onto the countertop, with 100% success in all variants of this test. If an animal struggled during testing, the data were not included in the overall analysis.

Beam walking test—Motor coordination and balance were assessed by measuring the ability of the animals to traverse a graded series of narrow beam (different size) to reach an enclosed safety platform (Altman and Sudarshan, 1975; Carter et al., 1999; Karl et al., 2003). The test consisted of elevated platforms connected by a 60-cm-long square wood beam with a width of 3 or 1.2 cm. The graded difficulty of motor coordination and balance can be measured by different beam widths. The beam was placed horizontally, 50 cm above the bench surface, with one end mounted on a narrow support and the other end attached to a goal box with a litter of pups. A sawdust-filled box at the base served as protection for the falling pups. A light (60 W) was positioned above and to one side of the start of the beam. A litter of pups was placed on the goal box, and one pup at a time was removed and placed on the start platform. Each pup was given three trials a day. The time spent on each beam was recorded for pups that were able to traverse the beam and join their littermates. The cutoff time was 60 sec.

Gait analysis—This test is used to assess integrity of the cerebellum and muscle tone/equilibrium (Hermans et al., 1992; Franco-Pons et al., 2007). On P21, the hind paws of each rat were smeared with ink. The animal was allowed to walk up a runway (80 × 10 cm) that was covered with white paper and had a darkened region at the end. For motivational purposes, a white light (60 W) was placed at the beginning of the ramp. Six variables from the footprint pattern were measured, including: 1) foot length (the length of hind foot), 2) spreading of toes 1–5 (the length between toes 1–5 of hind foot), 3) spreading of toes 2–4 (the length between the toes 2–4 of hind foot), 4) stride length (distance between the successive placement of the same hind foot), 5) stride width (the vertical distance between one hind foot and the succeeding print of the opposite hind foot), and 6) step angle (the angle between the line of the successive placement of the same hind foot and the line of one hind foot and the succeeding print of the opposite hind foot). Each variable was analyzed as other neurobehavioral measurements by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

EM Procedures and Data Collection

For the EM study, the pups were perfused with saline followed by 3.5% paraformaldehyde plus 0.5% glutaraldehyde in 0.1 M phosphate-buffered saline. The brains were postfixed in the same fixative overnight at 4°C. Brains were then cut coronally into 50–100 µm sections with a vibratome (Lancer). To identify OLs, some of the sections were processed with antibodies against APC-CC1 (1:20 dilution) and then subsequently linked with ABC Vector kit. Sections were then reacted according to the standard DAB (3-3'-diaminobenzidine tetrahydrochloride) method. This antibody binds to adenomatous polyposis coli, a tumor suppressor and a protein that plays important roles in regulation of cell division. Mature OLs are rich in APC content (Bhat et al., 1996). APC-CC1 antibody identifies mature OL cell bodies without labeling of OL processes and astrocytes in the brain (Murtie et al., 2005). A small area/block (~1 mm × 1 mm) in the region of the cingulum/white matter was dissected out with a No. 10 blade. Typically, two to three blocks were dissected from each pup. Each was processed by standard EM osmication with en bloc staining procedures. They were flat embedded in epon, attached to beam capsules, trimmed and cut into ultrathin sections. Tissue was collected onto grids coated with formvar, and then further stained with lead citrate and uranyl acetate. Materials were examined and photographed with a Leo Biological transmission electron microscope. To obtain semiquantitative measurements, profiles of interest depicting myelin sheath formations and/or OL somata were randomly photographed and then analyzed.

Immunohistochemistry

Brain injury was estimated on the basis of the results of hematoxylin and eosin (H&E) staining and immunohistochemistry in consecutive brain sections prepared from rats killed 16 days (P21) after the i.c. injection. For immunohistochemistry, the final concentrations of the primary antibodies were diluted as follows: APC-CC1 (1:20), MBP (1:100), and MAP2 (1:100). Anti-MBP staining was used as a marker of myelination. Anti-MAP2 provides selective staining of neuronal dendritic processes. Sections were incubated with primary antibodies at 4°C overnight. They were then reacted with secondary antibodies that were conjugated to fluorescent dyes (fluorescein isothiocyanate or rhodamine) or biotin (using the avidin–horseradish peroxidase system for final visualization) for an additional 1 hr in the dark at room temperature. DAPI (4',6-diamidino-2-phenylindole; 100 ng/mL) was used simultaneously on the same tissue to identify nuclei in the final visualization. Sections incubated in the absence of primary antibody were used as negative controls. The resulting sections were examined under a fluorescent microscope with the appropriate wavelengths.

Estimate of Ventricle Size

To compare the size of the lateral ventricles, H&E-stained sections at the bregma level were scanned by a densitometer (Bio-Rad) and areas of the left and right ventricles as well as that of the whole brain section were measured (Fan et al., 2005a, 2005b). The ratio between the area of the left or the right ventricle and that of the whole brain section was calculated as the ventricle size index. The ventricle size index for a single brain presented here is an average of measures from three brain sections taken at the bregma level, with each one from every five consecutive sections.

Quantification of Immunostaining Data and Statistics

Our previous studies indicate that in this neonatal rat model LPS injection produces preferential white matter injury primarily in the corpus callosum, the periventricular area, and the fiber tract of the forebrain (Cai et al., 2003; Pang et al., 2003; Fan et al., 2005a). In the present study, therefore, brain sections at the bregma level and the middle dorsal hippocampus level were used for determination of pathological changes. To compare cell numbers among treatment groups, positively stained cells were counted. When the cellular boundary was not clearly separated, numbers of DAPI-stained nuclei from the superimposed images were counted as the cell number. In the present study, dendritic changes were primarily observed in the cingulum and adjacent layer V of the parietal cortex after LPS exposure. Therefore, unless otherwise stated, three digital microscopic images were randomly captured in the cingulum (APC-CC1-positive OLs) or in layer V of parietal cortex where alterations in dendrites were observed. Positively stained OLs in these images were counted. MAP2 staining was quantified by calculating the percentage area of the image frame that contains MAP2 immunostaining by National Institutes of Health (NIH) image software. This method has been successfully used to quantify the density of cortical serotonin transporter-immunoreactive fiber networks (Maciag et al., 2006). For measurement of subcortical MBP staining at the bregma level, MBP stained sections were scanned by a densitometer (Bio-Rad). MBP-positive staining in the corpus callosum and the subcortical white matter tract was outlined over the whole brain section and the area and mean optical density (OD) of MBP staining were determined by NIH software. The OD of the area outside the outlined region (MBP negative) was measured for background. This value was subtracted from the signal OD. The corrected OD was used to represent the intensity of MBP-positive staining. The number, area, or OD of positively stained cells or areas in the three images was averaged. Three sections at each of the two cortical levels were examined by an observer blind to treatment, and the mean value of cell counting or other measurements was used to represent a single brain. For the convenience of comparing results among treatment groups, results were standardized as the average number of cells/

mm², mm², or OD. Immunostaining and the neurobehavioral performance data were presented as mean $\pm$ standard error of the mean (SEM) and analyzed by one-way ANOVA followed by the Student-Newman-Keuls test. The frequency of abnormal myelin sheath (see Results) in the EM study was analyzed by the χ^2 test. Results with a $P < 0.05$ were considered statistically significant.

RESULTS

PBN Improved Neurobehavioral Deficits Induced by LPS Exposure

Physical development—After LPS and PBN treatments, no apparent behavioral changes were observed in these groups. Compared with the control group, LPS-injection on P5 resulted in a lower body weight from P6 to P21 (Fig. 1A), but after the initial weight loss the LPS-treated rats continued to grow with a pattern similar to other groups as indicated by a growth curve almost parallel to those for other groups. PBN treatment significantly reduced the LPS-induced weight reduction in rats. LPS exposure also delayed the day of eye opening. The day of eye opening for the saline and saline + PBN groups was $P15.6 \pm 0.1$ and $P15.5 \pm 0.1$, respectively. LPS exposure delayed the day of eye opening ($P16.9 \pm 0.2$, $P < 0.05$). PBN treatment significantly prevented the LPS-induced delay in eye opening ($P15.5 \pm 0.1$, $P < 0.05$).

Righting reflex—The LPS-injected group exhibited significantly longer mean latency times as compared with the control group from P6 to P10 (Fig. 1B). At day 6, for example, control response on average was 3.2 sec vs. 10.5 sec in the LPS-treated group. Recovery from this impairment started on P11. PBN treatment significantly shortened the LPS-induced increase in righting reflex latency. By P7, there was no difference in righting reflex between the control and the LPS + PBN groups.

Wire hanging maneuver—The wire hanging ability of the rat increased with age. The mean latency time of the LPS-injected group was significantly less than that of the control group from P6 to P13 (Fig. 1C). The reduction in wire hanging latency in the LPS + PBN group was much less prominent than in the LPS group. It was not until P14 that the latency time in the LPS-injected group increased to the level of the control group, whereas that in the LPS + PBN group reached the control level by P9.

Cliff avoidance test—All pups from the control group succeeded in the cliff avoidance test by P11 and the avoidance latency decreased with age (Fig. 1D). It was not until P13 that pups from the LPS-injected group had cliff avoidance responses with a success rate of 66%, whereas PBN treatment advanced cliff avoidance responses of all pups from the LPS + PBN group by 2 days (P11). The avoidance response latency in the LPS-injected group reached the level of the control group by P16, while that in the LPS + PBN group reached the level of the control group by P13.

Negative geotaxis—As shown in Figure 2A, the LPS-injected group exhibited significantly longer mean latency times for negative geotaxis along a 15° incline as compared with the control group from P6 to P12. PBN treatment significantly shortened the duration of the LPS-induced increase in negative geotaxis latency. By P8, there was no difference in negative geotaxis between the control and the LPS + PBN groups.

Vibrissa-elicited forelimb-placing test—All pups from the control group succeeded in the vibrissa-elicited forelimb-placing test (100%) on P21 (Fig. 2B). The LPS-injected group's rate of success was significantly less ($23 \pm 1.3\%$) in the vibrissa-elicited forelimb-

placing test as compared with the control group ($P < 0.05$). The LPS + PBN group exhibited the same performance level as the control group (100%) on P21.

Beam walking test—All pups from the control group succeeded in the beam walking test by P14 ($3 \times 3 \text{ cm}^2$ beam) or P15 ($1.2 \times 1.2 \text{ cm}^2$ beam) and beam walking latency decreased with age (Fig. 2C,D). It was not until P16 ($3 \times 3 \text{ cm}^2$ beam) or P17 ($1.2 \times 1.2 \text{ cm}^2$ beam) that pups from the LPS-injected group succeeded in the beam walking test, whereas PBN treatment advanced successful beam walking for all pups in the LPS + PBN group by 1 day ($3 \times 3 \text{ cm}^2$, P15; $1.2 \times 1.2 \text{ cm}^2$, P16). Beam walking latency in the LPS-injected group did not reach control group value by P21, although the LPS + PBN group did match the performance of the control group by P19 ($3 \times 3 \text{ cm}^2$) or P20 ($1.2 \times 1.2 \text{ cm}^2$).

Gait analysis—The performance of all groups in gait analysis at P21 is summarized in Table I. LPS administration resulted in increased foot length, stride width and step angle. LPS treatment decreased total spreading (spreading of toes 1–5), intermediary spreading (spreading of toes 2–4) and stride length, indicating a generally weak muscle tone/equilibrium. PBN treatment improved LPS-induced impaired gait performance.

PBN Attenuated LPS-induced Ventricle Enlargement

Consistent with our results reported previously (Fan et al., 2005b), LPS administration caused dilatation of the ventricles bilaterally. As shown before, the dilatation of the ipsilateral (left) side was more prominent than the contra-lateral side for P21 rat brains (Fig. 3B and Table II). Treatment with PBN significantly decreased the ventricle enlargement induced by LPS (Fig. 3C and Table II).

PBN Ameliorated LPS-induced Loss of Mature OL and Hypomyelination

Mature OLs were identified with APC-CC1. APC-CC1-positive cells were primarily observed in the cingulum area (Fig. 4A–C), corpus callosum and the subcortical white matter tract at the bregma level and in the internal capsule area. LPS exposure significantly reduced the number of APC-CC1-positive cells (Fig. 4B and Table II), as compared with that in the saline or saline + PBN rat brain (Table II). PBN treatment attenuated the LPS-induced reduction in mature OLs in the P21 rat brain (Fig. 4C and Table II).

OLs are myelin-producing cells and reduction in OLs may result in hypomyelination. MBP staining was used as a marker of myelination in the P21 rat brain. MBP-positive staining was clearly detected in the P21 control rat brain, primarily in the corpus callosum (Fig. 4D), the subcortical white matter tract, and the internal capsule areas. The area and mean OD of MBP staining in the cingulum and adjacent subcortical white matter tract ventral to the forelimb region of the somatosensory cortex were measured for comparison (Table II). LPS injection significantly reduced both the area and mean OD of MBP staining in this region (Fig. 4E and Table II). PBN treatment significantly ameliorated LPS-induced decreases in MBP staining as indicated by the increased area and mean OD of MBP-positive staining (Fig. 4F and Table II). This finding is consistent with the APC-CC1+ cell counting data.

PBN Reduces LPS-induced Myelin Impairment

To further investigate LPS-induced injury to OLs and associated myelin sheaths, EM comparisons of the cingulum/white matter were undertaken in pups (P21) treated with saline, LPS, or LPS + PBN. In order to determine whether LPS triggers any morphological abnormalities in OLs, a sample of randomly selected OL cell bodies (25–40 cells per treatment subgroup) were photographed. In saline treated pups, the OLs exhibited a typical normal morphological appearance (Fig. 5A). This has been described previously by Peters et al. (1991). The normal OLs usually have a round, oval or irregular nucleus and the nuclear

chromatin has a generally homogeneous appearance. The nucleus is often surrounded by only a thin rim of clear cytoplasm, in which mitochondria, endoplasmic reticulum and Golgi apparatus, but no other foreign inclusions, may be present. In contrast, the OLs of LPS treated pups were often observed to possess dual nuclei (Fig. 5C) and/or inclusions of foreign substances, such as myelinated axons (Fig. 5C,D). Interestingly, the OLs of LPS + PBN treated pups (Fig. 5B) tended to have rather a normal morphology as compared with the saline treated pups. Very rarely, was an abnormal OL morphology observed in samples from this group.

To further investigate the ability of OLs to properly insulate axons, myelinated axons from the cingulum/white matter were randomly selected and photo-graphed for our later semi-quantitative analysis. We observed abnormalities of myelin sheath that ranged from hypomyelination (thinner myelin sheath) to gross structural distortion (disintegrated or compartmentalized myelin sheath as shown in Fig. 6D) and lysis. Hence, any of these changes were used as criteria to characterize myelin disruption, i.e., abnormal myelination. In the saline treated pups (Fig. 6A), we noted that 29 of a sample of 737 myelinated axons (3.9%) exhibited an abnormality. In LPS treated pups, approximately 20% (146 of 728) of the axons exhibited an abnormal myelin sheath ($P < 0.001$ vs. the saline-treated group). Two representative examples of such distorted axons are shown in Figure 6C and D. To further investigate whether LPS + PBN treatment (Fig. 6B) offers any protection, we collected data that revealed 7.9% (115 of 1,454) of axons had an abnormal myelin formation. Interestingly, although frequency of abnormally myelinated axons in the LPS + PBN treatment groups was still more than that in the control group, it is significantly less than that in the LPS-treated group ($P < 0.001$). Hence, our data suggest that PBN treatment ameliorated LPS-induced myelin malformation.

PBN Decreases LPS-induced Damage to Dendrites

Axonal and dendritic damage is recognized as a consequence of demyelination. Neuronal dendritic processes were identified by MAP2 immunostaining. As shown in Figure 7B, LPS treatment elicited changes in dendrite density within the cingular area adjacent to the parietal cortex. Apical dendrites within the parietal cortex were also affected, as indicated by the reduction and abnormality (twisted and beaded appearance) of MAP2 immunostained apical dendrites in layer V of the cortex in the P21 rat brain (Fig. 7E and Table II). PBN treatment prevented the induction of dendritic loss by LPS (Fig. 7C,F and Table II).

DISCUSSION

PVL, a form of white matter disease, is a dominant form of brain injury in premature infants and is the precursor in the neuropathology of cerebral palsy (Hagberg et al., 2002; Rezaie and Dean, 2002; Volpe, 2003). PVL is likely the major cause of long-term neurological disability and has been linked to the subsequent development of sensory, motor and cognitive disturbances (Back et al., 2001, 2002; Rezaie and Dean, 2002). Consistent with our previously reported results (Fan et al., 2005b), the neurobehavioral deficits observed in the present study reproducibly demonstrate that neonatal exposure to LPS may result in such disturbances, effecting the development of sensory, motor and cognitive function in this model. Current data indicate that some impairments of neurobehavioral performance, such as impaired righting reflex, wire hanging maneuver, cliff avoidance and negative geotaxis, appear to be temporary and reversible, suggesting that LPS only delays the development of these functions. However, there are neurobehavioral deficits that are still observable at P21, such as performance in the vibrissa-elicited forelimb-placing test (sensorimotor), beam walking (motor coordination and balance) and gait test (assessing integrity of the cerebellum and muscle tone/equilibrium). This indicates that some damaging effects of LPS in this model are long-lasting and might not be eventually overcome by compensatory mechanisms.

The body weight in the LPS-treated rats was lower than that in the control group and this could be the result of the high dose of LPS used in the current study. It is possible that the poor performance in the LPS group was associated with their weaker muscle strength as a result of the lower body weight. However, the LPS-treated rats gained weight in a similar manner as the control rats 48 hr after LPS injection. Our previous study has shown that the LPS-treated and the control animals has similar locomotor activities by P21 (Fan et al., 2005b). Some neurobehavioral deficits in the LPS-treated group are still observable by P49-P56 when the body weight of these animals catches up with that in the control rat (unpublished data). Therefore, the neurobehavioral deficits observed in the LPS-treated rats are, at least partially, associated with the damaging effects of LPS on the central nervous system. Nevertheless, to avoid systemic effects of LPS as much as possible, use of a low dose of LPS will certainly be beneficial in future studies.

Data from the present study further demonstrate that PBN, an antioxidant, can attenuate the delay in the development of neurobehavioral function or even prevent neurological dysfunction after exposure to LPS. The neuroprotective action of PBN has been attributed to many factors, including formation of a spin adduct with free radicals, such as hydroxyl radical in rat striatum produced by local application of glutamate (Ferber et al., 1998), suppression of reactive oxygen species production from mitochondria (Floyd and Hensley, 2000), inhibition of the induction of pro-inflammatory cytokines and inducible nitric oxide synthase (Kotake et al., 1998; Sang et al., 1999; Lin et al., 2006), inhibition of nuclear factor kappa B (a transcription factor for a wide variety of pro-inflammatory cytokine genes) (Kotake et al., 1998; Sang et al., 1999), and its antiapoptotic properties (Li et al., 2001; Lee and Park, 2005). In our short-term effect study, treatment with PBN significantly reduced LPS-induced oxidative and nitrosative stress, as indicated by the reduction in expression of markers of lipid peroxidation (4-hydroxynonenal, malondialdehyde and 8-isoprostane) and protein nitration (nitrotyrosine) (Fan et al., 2008). In addition, PBN also reduced the microglia activation and expression of inflammatory cytokines after LPS exposure in neonatal rats (Fan et al., 2008). Although the detailed mechanisms underlying the protection of PBN on neurobehavioral functions observed in the current study require further investigation, the protection is likely to be closely associated with the attenuation in LPS-induced brain injury as a result of scavenging free radicals or secondary to the anti-inflammatory properties of PBN.

In the present study, the improved neurobehavioral performance in LPS-treated juvenile rats after PBN treatment was associated with the attenuation of injuries in certain brain regions. One of the examples, PBN treatment prevented LPS-induced reduction in MBP-positive staining in the corpus callosum. The corpus callosum, the largest white matter tract in the human brain, has been broadly characterized as playing an integral role in communication between hemispheres, with increasingly complex cognitive tasks requiring greater inter-hemispheric integration (Silveri et al., 2006; Luders et al., 2007). Although a cause-effect relationship between a neurobehavioral deficit and injury in a specific brain area can not be established on the basis of the present results, our data may provide important information regarding the link between LPS-induced brain injury to specific areas and the altered neurobehavioral performance for further development of testing battery.

Late OL progenitors (O4+/O1-) have been considered a major cellular target in PVL (Back et al., 2001, 2002). OLs are myelin-producing cells, and loss of O4+ cells and mature OLs (APC-CC1+) may result in impaired cell proliferation and myelination (Osterhout et al., 2002; Rezaie and Dean, 2002). Therefore, delayed or disturbed myelination is an important pathological feature of PVL (Iida et al., 1995; Carmody et al., 2004). It has been shown that prenatal LPS-exposure alters the capacity of OLs to produce myelin in the ewe fetus and affects conduction velocity and neural function (Loeliger et al., 2007). Destruction of the

myelin sheath in the central nervous system is prominent in the pathophysiology of many clinically relevant disorders and causes many behavioral deficits, including poor sensorimotor and neuromuscular activity, muscle tone/equilibrium, and motor coordination (Kerschensteiner et al., 2004; Frost et al., 2006; Franco-Pons et al., 2007). Improved performance in the beam-walking test has been linked with remyelination after OL progenitor cell transplantation after demyelinating lesions were experimentally induced in a preparation of rat spinal cord (Jeffery et al., 1999). In the current study, LPS-induced impairment of myelination was evidenced by light microscopic immunohistochemistry as well as EM analysis. To our best knowledge, the present EM study is the very first to examine LPS-induced abnormalities of OLs and myelin formation in the cerebral white matter of rodent pups. An intriguing finding of the present study is the occurrence of di- or trinucleated OLs in LPS treated animals. Multi-nucleated OLs (Fig. 5C) have, in fact, been observed previously in cerebral white matter after ischemia (Pantoni et al., 1996). The authors further suggested that this phenomenon is due to swollen OLs. With regard to the inclusions (i.e., myelinated axons) that were found within OL somata after LPS treatment, similar findings have been reported after acute ischemic injury. Abnormal morphologies were detected during the early myelination of the white matter (Wilke et al., 2004), and also in the response of neonatal optic nerves to acute LPS mediated injury (Shermin and Fern, 2005). In addition, our present EM data suggest that PBN-treated rats in the LPS group exhibit a rather normal OL morphology and myelination pattern. It is important to note that the attenuated impairment of myelination in the PBN-treated LPS group was accompanied by improved neurobehavioral performance. As such, these results suggest that LPS-induced disturbances in myelination substantially contribute to deficits in neurobehavioral function observed in the current study.

Although white matter damage is a fundamental neuropathological feature of PVL, the motor and cognitive deficits observed later in these infants indicate possible cerebral cortical neuronal dysfunction. The neuronal damages, predominantly in pyramidal neurons of cortical layer V, have been found in the infant brain with PVL (Meng et al., 1997; Deguchi et al., 1999). In the current study, LPS exposure resulted in abnormal dendritic branching as indicated by 1) the reduced MAP2 immunostaining of dendrites in the area between the white matter and adjacent cortical gray matter (Fig. 7B) and 2) the alterations of MAP2 immunostained apical dendrites in layer V of the parietal cortex in the P21 rat brain (Fig. 7E). It has been shown that normal neurobehavioral performance depends directly on the degree of dendritic arborization and synaptic connectivity (Ba and Seri, 1995). Therefore, LPS-induced abnormalities in the connection of cortical neurons may also contribute to the deficits observed in the present study. These include changes in neurological reflexes, as well as decrements in early sensorimotor and locomotor neurobehavioral performance.

Taken together, neonatal exposure to LPS has long-lasting adverse effects on brain development and neurobehavioral functions in this rat model. LPS-induced abnormalities in myelination and dendritic branching may contribute to deficits in early reflexes and in sensorimotor and locomotor performance. The protective effects of PBN on LPS-induced brain injury and neurobehavioral dysfunction in juvenile rats indicate that antioxidant administration might be an effective approach for therapeutic treatment of neonatal brain injury induced by infection/inflammation. PBN was administered 5 min after the LPS exposure in the current study. For therapeutic treatment in a clinical setting, this timing of PBN administration is not realistic, and further studies are required to investigate the precise therapeutic window and potential uses of a combination with other neuroprotective agents.

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Fig. 1.

PBN attenuated LPS-induced body weight loss (A), elongation of mean latency times in righting reflex (B), reduction of mean latency times in the wire hanging maneuver (C), and deficits in cliff avoidance (D) in the rat. Results are expressed as the mean $\pm$ SEM of 12 animals in each group. * $P < 0.05$ represents a significant difference for the LPS group or LPS + PBN group compared with the saline group. # $P < 0.05$ represents a significant difference for the LPS + PBN group compared with the LPS group. (A) Markers of * below curves are for the LPS group compared with the saline group and markers of * and # above curves are for the LPS + PBN group compared with the saline group and LPS group, respectively.

Fig. 2. PBN attenuated LPS-induced elongation of mean latency times in negative geotaxis (**A**), deficits in vibrissa-elicited forelimb-placing (**B**), and deficits in beam walking tests (**C**, 3×3 cm² beam; **D**, 1.2×1.2 cm² beam) in the rat. Results are expressed as the mean $\pm$ SEM of 12 animals in each group. * $P < 0.05$ represents a significant difference for the LPS group or LPS + PBN group compared with the saline group. # $P < 0.05$ represents a significant difference for the LPS + PBN group compared with LPS group.

Fig. 3. PBN attenuated LPS-stimulated dilatation of the lateral ventricles bilaterally 16 days (P21) after the LPS injection. **A:** H&E-stained brain section showing the normal appearance of a brain sample taken from the control group. **B:** H&E-stained brain section through the forebrain showing bilateral enlargement of the lateral ventricles after LPS treatment. **C:** PBN-attenuated LPS-induced enlargement of the lateral cerebral ventricles. Scale bar = 500 μm (A).

Fig. 4. Representative photomicrographs of APC-CC1 staining (**A–C**) and MBP staining (**D–F**) in the P21 rat brain. LPS administration resulted in loss of APC-CC1+ OLs (**B**) and a reduction in MBP-positive staining (**E**), compared with control group (**A** and **D**, respectively). PBN attenuated LPS-induced loss of APC-CC1+ cells and MBP-positive staining (**C,F**). Scale bar = 50 μ m (**A–C**) and 200 μ m (**D–F**).

Fig. 5. Representative examples of electron photomicrographs of OLs under different treatments. Note the typical morphology of OLs in the saline-treated (**A**) and the LPS + PBN-treated (**B**) animals. In contrast, abnormal morphological characteristics such as dual nucleus (**C**) and inclusions (myelinated axons) were often observed in LPS-treated animals (**C,D**). Arrows in C,D point to the inclusions such as myelinated axons. Scale bar = 5 μ m.

Fig. 6. Representative examples of the overall morphology of axons in animals treated with saline (**A**), LPS + PBN (**B**), and LPS (**C**). The short arrow in (**C**) points to an example of a hypomyelinated axon and the long arrow points to an extensively distorted myelinated axon. **D**: Distorted and compartmentalized axon from LPS-treated animals. Of the axons sampled, abnormal profiles were observed in 3.9% (29 of 737), 20% (146 of 728), and 7.9% (115 of 1,454) axons in the saline, LPS, and LPS + PBN group, respectively. Scale bar = 5 μ m (**A**–**C**) and 1 μ m (**D**).

Fig. 7.

Representative photomicrographs of MAP2 immunostaining in the rat brain 16 days (P21) after LPS injection. Differences in MAP2-positive staining were detected in the cingular area (**A–C**), as well as in layer V of parietal cortex (**D–F**). These differences were observed in the P21 rat brain between treatment groups. LPS caused dendritic impairment as indicated by the loss of MAP2 immunostaining (measured by the percentage area of image that contained MAP2 immunoreactivity in the frame shown in **A–C** or in the whole image in **D–F**) and the presence of short beaded MAP2-positive dendrites (**E**). The control group (**A,D**) is shown for comparison. PBN attenuated the LPS-induced loss of MAP2 staining (**C**) and morphological alterations in MAP2-stained dendrites (**F**). Scale bar = 50 μ m (**A**).

TABLE I

PBN Attenuated LPS-induced Deficits in Gait Analysis Performed 16 Days (P21) after the LPS Injection[†]

Treatment	Saline	Saline+PBN	LPS	LPS+PBN
Foot Length	1.23 ± 0.03	1.25 ± 0.03	1.80 ± 0.06★	1.35 ± 0.04 [#]
Total Spreading (1–5)	1.18 ± 0.02	1.18 ± 0.02	1.00 ± 0.02★	1.15 ± 0.02 [#]
Intermediary Toes (2–4)	0.58 ± 0.01	0.58 ± 0.02	0.46 ± 0.01★	0.58 ± 0.01 [#]
Stride Width	2.92 ± 0.04	2.90 ± 0.05	3.47 ± 0.07★	2.93 ± 0.06 [#]
Stride Length	8.99 ± 0.07	8.92 ± 0.07	7.34 ± 0.17★	8.72 ± 0.06 [#]
Step Angle (°)	32.3 ± 0.5	32.6 ± 0.6	43.2 ± 0.8★	34.3 ± 0.5 [#]

[†] Intracerebral injection of LPS and PBN treatment were performed as described in the text. Data are presented as mean±SEM cm of twelve animals. Data were analyzed by one-way ANOVA followed by Student-Newman-Keuls test.

★ $P < 0.05$ compared with the saline group.

[#] $P < 0.05$ compared with the LPS group.

TABLE II

PBN Attenuated LPS-induced Brain Injury in the Rat Brain 16 Days (P21) after the LPS Injection[†]

Treatment	Saline	Saline+PBN	LPS	LPS+PBN
Ventricle size index				
Left ventricle (%)	0.26 ± 0.05	0.27 ± 0.05	2.04 ± 0.23★	0.82 ± 0.12★,#
Right ventricle (%)	0.15 ± 0.03	0.19 ± 0.03	1.36 ± 0.07★	0.51 ± 0.08★,#
MBP staining				
Area (mm ²)	2.57 ± 0.07	2.61 ± 0.14	1.93 ± 0.09★	2.79 ± 0.07#
Density (OD)	0.164 ± 0.005	0.163 ± 0.004	0.129 ± 0.003★	0.152 ± 0.005#
Number of OLs in cingulum area				
APC-CC11 (cells/mm ²)	1213.3 ± 41.7	1204.4 ± 57.5	706.7 ± 42.0★	1104.4 ± 67.81#
MAP2 stained dendrites in cingulum area				
% of area	38.4 ± 1.5	35.8 ± 1.2	19.3 ± 1.3★	30.0 ± 0.9★,#
MAP2 stained dendrites in parietal cortex				
% of area	7.1 ± 0.3	6.8 ± 0.3	2.8 ± 0.3★	5.2 ± 0.2★,#

[†] Intracerebral injection of LPS and PBN treatment were performed as described in the text. LPS or saline was administered to the rat brain at the location of 1.0 mm posterior and 1.0 mm lateral to the bregma, and 2.0 mm deep to the scalp at the left hemisphere in a stereotaxic apparatus with a neonatal rat adapter. The ventricle size index and the area and OD of MBP staining were determined by a densitometer as described in methods. Immunostaining was performed in sections from the forebrain at the bregma level. Cell counting of APC-CC1+ cells was performed in the cingulum area. Data are presented as the mean±SEM (cells/mm²) of six animals for each group. MAP2 staining was quantified by the measurement of percentage of areas stained with MAP2 antibody within the cingulum region or in the adjacent parietal cortex. The MAP2 staining results are presented as the mean ± S.E.M. (% of area) of six animals for each group. Data were analyzed by one-way ANOVA followed by Student-Newman-Keuls test.

★ $P < 0.05$ compared with the saline group.

$P < 0.05$ compared with the LPS group.