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α -PHENYL-*N*-*TERT*-BUTYL-NITRONE ATTENUATES LIPOPOLYSACCHARIDE- INDUCED NEURONAL INJURY IN THE NEONATAL RAT BRAIN

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Abstract

Although white matter damage is a fundamental neuropathological feature of periventricular leukomalacia (PVL), the motor and cognitive deficits observed later in infants with PVL indicate the possible involvement of cerebral neuronal dysfunction. Using a previously developed rat model of white matter injury induced by cerebral lipopolysaccharide (LPS) injection, we investigated whether LPS exposure also results in neuronal injury in the neonatal brain and whether α -phenyl-*n*-*tert*-butyl-nitron (PBN), an antioxidant, offers protection against LPS-induced neuronal injury. A stereotactic intracerebral injection of LPS (1 mg/kg) was performed in Sprague-Dawley rats (postnatal day 5) and control rats were injected with sterile saline. LPS exposure resulted in axonal and neuronal injury in the cerebral cortex as indicated by elevated expression of β -amyloid precursor protein, altered axonal length and width, and increased size of cortical neuronal nuclei. LPS exposure also caused loss of tyrosine hydroxylase positive neurons in the substantia nigra and the ventral tegmental areas of the rat brain. Treatments with PBN (100 mg/kg) significantly reduced LPS-induced neuronal and axonal damage. The protection of PBN was associated with an attenuation of oxidative stress induced by LPS as indicated by the reduced number of 4-hydroxynonenal, malondialdehyde or nitrotyrosine positive cells in the cortical area following LPS exposure, and with the reduction in microglial activation stimulated by LPS. The finding that an inflammatory environment may cause both white matter and neuronal injury in the neonatal brain supports the possible anatomical correlate for the intellectual deficits and the other cortical and deep gray neuronal dysfunctions associated with PVL. The protection of PBN may indicate the potential usefulness of antioxidants for treatment of these neuronal dysfunctions.

Keywords

neuronal injury; tyrosine hydroxylase; microglial activation; oxidative stress; antioxidant

Periventricular leukomalacia (PVL), the dominant form of brain injury in the preterm infant, is frequently associated with subsequent adverse neurological outcomes such as cerebral palsy and mental retardation (Rezaie and Dean, 2002; Volpe, 2003). 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 precise nature

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of the relationship between the white matter lesion and the subsequent neuronal disorders is unclear. Swollen axons have been found during the progression of PVL (Rezaie and Dean 2002). With the use of a quantitative volumetric three-dimensional MRI technique it was reported that PVL is associated with a marked reduction in cortical gray matter (Inder et al., 1999). Increased expression of inflammatory cytokines in cortical and subcortical neurons in the infant brain with PVL has also recently been reported (Kadhim et al., 2003). It is possible that the insults causing white matter damage may also cause injury to neurons or that PVL has a deleterious effect on subsequent cerebral cortical neuronal development.

Increasing evidence indicates that maternal or placental infection is a major contributor to PVL in addition to hypoxia-ischemia (Hagberg et al., 2002; Rezaie and Dean 2002; Volpe, 2003). In our previous work we used an intracerebral injection of lipopolysaccharide (LPS) to create a similar scenario of infection/inflammation in the postnatal day 5 (P5) rat brain (Cai et al., 2003; Fan et al., 2005a; Pang et al., 2003). In this model we found that LPS resulted in preferential white matter injury in the neonatal rat brain and the injury was closely associated with increased oxidative stress following LPS exposure (Fan et al., 2005a). Although we did not find any cortical neuron death in hematoxylin and eosin stained brain sections following LPS exposure, we did observe that LPS injection caused alterations in dopamine neurons in the substantia nigra (SN) by tyrosine hydroxylase (TH) immunostaining and that LPS injection resulted in neurological dysfunction, suggesting possible neuronal involvement in this neonatal rat model of infection/inflammation-induced white matter injury (Fan et al., 2005b).

α -Phenyl-*n*-tert-butyl-nitrone (PBN) is one of the most widely used nitron-based antioxidants to trap and stabilize free radicals in biological systems (Floyd et al., 2002). The neuroprotective action of PBN has been observed in the LPS-mediated septic shock model, hypoxia-ischemia model, and other neuronal degenerative diseases through its ability to trap free radicals and inhibit oxidative damage or through cessation of enhanced signal transduction processes associated with neuroinflammation (Endoh et al., 2001; Floyd et al., 2000, 2002; Sang et al., 1999). In our previous study we found that PBN provided protection to neonatal white matter following hypoxia-ischemia insult (Lin et al., 2004, 2006). The present study has a two-fold objective: to investigate whether LPS exposure also induces neuronal injury in our neonatal rat model of white matter injury and whether PBN also offers protection against LPS-induced neuronal and axonal damage in the neonatal rat brain.

EXPERIMENTAL PROCEDURES

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO). Monoclonal mouse antibodies against β -amyloid precursor protein (APP), microtubule-associated protein 1 (MAP1), tyrosine hydroxylase (TH) or neuron-specific nuclear protein (NeuN) (biotin conjugated); OX42 (CD11b) or ED1; and interleukin-1 β (IL-1 β) were purchased from Chemicon (Temecula, CA), Serotec (Raleigh, NC), and the R&D Systems (Minneapolis, MN), respectively. Polyclonal rabbit antibodies against nitrotyrosine (NT), 4-hydroxynonenal (4-HNE) or inducible nitric oxide synthase (iNOS), and malondialdehyde (MDA) were obtained from Chemicon, Alexis (San Diego, CA), and Abcam (Cambridge, MA), respectively.

Animal model

Intracerebral injection of LPS to 5-day old Sprague-Dawley rat pups of both sexes was performed as previously described (Fan et al., 2005a; Pang et al., 2003). Under light anesthesia with isoflurane (1.5%), LPS (1 mg/kg, from *Escherichia coli*, serotype 055: B5) in sterile saline (total volume of 2 μ l) was administered to the rat brain at the location of 1.0 mm posterior and

1.0 mm left to the bregma, and 2.0 mm deep to the skull surface in a stereotaxic apparatus with a neonatal rat adapter. The dose of LPS was chosen based on our previously reported results which produced preferential white matter injury (Fan et al., 2005a; Pang et al., 2003). The injection site was located at the area just above the left cingulum. The control rats were injected with the same volume of sterile saline. All animals survived the intracerebral injection.

Both LPS- and saline-injected animals were further divided into two groups: one received intraperitoneal (i.p.) injections of PBN and the other sterile saline. PBN (100 mg/kg) or vehicle alone was administered immediately after the LPS injection. Our previous study showed that this dose of PBN protects the neonatal rat brain from hypoxia-ischemia-induced white matter injury (Lin et al., 2004,2006). Each dam had an equal litter size (12 pups). One or three days after the injection (P6 or P8), rat pups were sacrificed by transcardiac perfusion with normal saline followed by 4% paraformaldehyde for brain section preparation. There were six animals (3 male and 3 female) in each treatment group. The experimental procedure was approved by the Institutional Animal Use and Care Committee at the University of Mississippi Medical Center and was in accordance with the guidelines of the National Institutes of Health on the care and use of animals. Every effort was made to minimize the number of animals used and their suffering. Coronal frozen brain sections at 10 μ m of thickness were prepared in a cryostat for APP, NeuN and TH immunostaining. Coronal free-floating brain sections at 40 μ m of thickness were prepared in a sliding microtome for other immunostaining.

Immunohistochemistry

Brain injury was estimated based on the results of Nissl staining and immunohistochemistry in consecutive brain sections prepared from rats sacrificed 1 or 3 days (P6 and P8) after the intracerebral injection. For immunohistochemistry staining, primary antibodies were used in the following dilution: 4-HNE, 1:500; MDA, iNOS, OX42 or ED1, 1:200; and APP, MAP1, TH, NeuN, IL-1 β or NT, 1:100. Microglia were detected using lectin histochemistry (10 μ g/ml biotin-conjugated tomato lectin, Sigma), as well as by OX42 immunostaining, which recognizes both the resting and the activated microglia, and by ED1 immunostaining, which detects the activated microglia or macrophages. Sections were incubated with primary antibodies at 4 °C overnight and further incubated with secondary antibodies conjugated with fluorescent dyes (FITC or rhodamine) for 1 h in the dark at room temperature. DAPI (100 ng/ml) was used simultaneously to identify nuclei in the final visualization. Sections incubated in the absence of primary antibody were used as negative controls. When double-labeling was required, primary antibodies from different hosts were used in combination with appropriate secondary antibodies, which were against the immunoglobulin from the corresponding hosts. For dual labeling studies using TH antibody in combination with biotin conjugated NeuN antibody, sections were first incubated with mouse TH antibody followed by rhodamine-labeled anti-mouse secondary antibody. Prior to a further incubation with biotin conjugated NeuN antibody, sections were incubated with 1% mouse serum for 1 h to block any excessive anti-mouse IgG. After incubation with biotin conjugated NeuN antibody, sections were further incubated with fluorescent-labeled avidin for 1 h in the dark at room temperature. The resulting sections were examined under a fluorescent microscope at appropriate wavelengths. Our preliminary study demonstrated that this procedure does not cause interference between TH and NeuN staining. To confirm the specificity of NT immunostaining in our preliminary studies, prior to addition of the NT antibody to brain sections, NT antibody was incubated with 10 mM of NT, 10 mM of aminotyrosine, or 10 mM of phosphotyrosine as reported by Kooy et al. (1997). The results showed that the NT positive staining in the LPS-injected rat brain was blocked by NT, but not by aminotyrosine or by phosphotyrosine, indicating the specificity of the antibody for NT.

Estimate of ventricle size

To compare the size of lateral ventricles, Nissl stained sections at the bregma level were scanned by a densitometer (Bio-Rad, Hercules, CA) and areas of the left and right ventricles as well as that of the whole brain section were measured (Fan et al., 2005a). 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.

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 white matter tract of the forebrain (Cai et al., 2003; Fan et al., 2005a; Pang et al., 2003). In the present study, therefore, brain sections at the bregma level and the middle dorsal hippocampus level were used for determination of the most pathological changes. TH+ cell counting was performed in the mid-brain sections at a level 1/3 rostral from the lambda to the bregma. Most immunostaining data were quantified by counting of positively stained cells. 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, cortical neuronal changes were primarily observed in the fifth layer of the parietal cortex of the forebrain following LPS exposure. Therefore, unless otherwise stated, three digital microscopic images were randomly captured at the fifth layer of the parietal cortical areas or at the substantia nigra (SN) and ventral tegmental area (VTA) where the TH+ cells were most abundant for each section. The number of positively stained cells in the three images was averaged. Three sections at each of the two section levels were examined by an observer blind to the treatment and the mean value of cell counting was used to represent one single brain. For convenience of comparing results among the treatment groups, results were standardized as the average number of cells/mm². Quantified data were presented as the means $\pm$ SEM and analyzed by one-way ANOVA followed by Student-Newman-Keuls test. Results with a $p < 0.05$ were considered statistically significant.

RESULTS

PBN attenuated LPS-induced ventricle enlargement

Pyknotic cells as shown by Nissl staining were observed in both the cingulum white matter (Fig 1E) and the cortical gray matter in the rat brain 24 hr, but not 72 hr, after the LPS injection (Fig 1H and Table 1). The pyknotic cells in the cortical gray matter were primarily localized in the fifth layer of the parietal cortex in the forebrain. Our preliminary study showed that no significant differences in the number of pyknotic cells were found between the ipsilateral and the contralateral brains. Therefore, data from the ipsilateral brain are presented here. As reported previously (Fan et al., 2005a; Pang et al., 2003), LPS exposure resulted in dilatation of bilateral ventricles in the current study (Fig 1B). The dilatation in the ipsilateral (left) ventricle was more prominent than in the contralateral side in both the P6 and P8 rat brain (Table 1). Treatment with PBN significantly reduced the number of pyknotic cells and ventricle enlargement induced by LPS (Fig 1C, F, I and Table 1).

PBN decreased LPS-induced axonal and dendritic damage

Up-regulation of APP, a marker of axonal injury, was determined by immunostaining in P6 rat brain. As shown in Fig. 2A and 2D, weak expression of APP was barely detectable in the control rat brain. The beaded APP immunostaining was found in the cingulum area along axons connecting with upper cortical layers (Fig. 2B) and in the parietal cortex (Fig. 2E) in the LPS-exposed brain. Strong immunoreactivity of APP was also observed in the lateral ventricle surrounding areas and the dorsal third ventricle surrounding area in the LPS-exposed rat brain (data not shown). PBN treatment attenuated the LPS-induced injury to axons in the above areas,

as indicated by the weak APP immunostaining similar to that observed in the control rat brain (Fig. 2C and 2F).

MAP1 immunostaining was used as an additional marker for detection of LPS-induced injury to axons and dendrites. Long descending MAP1 immunoreactive axons were observed in the cortex of the control rat forebrain (Fig 3A). The LPS treatment induced damage to axons and dendrites in the cortex, as indicated by the reduced length and the increased width of the MAP1 immunostained axons in the fifth cortical layer of the P8 rat brain (Fig 3B and Table 1). PBN treatment attenuated the LPS-induced injury to axons and dendrites in the cortex (Fig. 3C and Table 1).

PBN decreased LPS-induced neuronal nuclear alteration

The positive staining of neuron-specific nuclear protein (NeuN) is primarily localized in the nuclei of neurons with slightly staining in the cytoplasm. The average diameter of NeuN-immunoreactive neurons in the fifth layer of the somatosensory cortex was increased by LPS treatment, but the cell density of neurons in this area was not significantly changed (Fig. 3E and Table 1). PBN treatment prevented the LPS-induced enlargement of the nucleus of NeuN-immunoreactive neurons in the cortex (Fig. 3F and Table 1).

PBN reduced losses of tyrosine hydroxylase-immunoreactive neurons following LPS

Positive staining of TH was used to detect dopamine neurons in the substantia nigra (SN) and ventral tegmental area (VTA). Our previous studies and data reported by other investigators indicate that perinatal LPS exposure reduces the number of TH positive neurons in these areas (Fan et al., 2005b; Ling et al., 2002), but it is unknown whether the reduction in the number of TH positive cells is a consequence of phenotypic suppression of TH expression or actual neuronal cell death. To answer this question, double labeling with TH and NeuN antibodies was performed and all TH positive and NeuN positive cells in the three midbrain sections for each animal (at a level 1/3 rostral from the lambda to the bregma) were counted bilaterally. In the P6 control rat brain, TH positive cells were more predominant in the compact and the lateral regions of the substantia nigra (Fig. 4A) and the VTA (Fig. 4G) in the ventral midbrain. LPS treatment significantly reduced the number of TH positive neurons in both the SN and the VTA regions in the rat brain (Fig. 4B, 4H and Table 1). However, LPS exposure did not significantly alter the total number of NeuN positive cells in these regions (Fig 4E), suggesting that LPS did not result in actual neuronal cell death in this area. Therefore, LPS treatment decreased the ratio between the number of TH positive cells and the number of NeuN positive cells, from $76.1 \pm 1.7\%$ to $61.9 \pm 1.4\%$ in the SN of the rat brain. PBN treatment attenuated the LPS-induced reduction in the number of TH positive neurons (Fig. 4C, 4I and Table 1) and increased the ratio between the TH positive and the NeuN positive cells to $71.1 \pm 2.1\%$ (Fig. 4F).

PBN protection was linked with attenuation of LPS-induced oxidative and nitrosative damage and with suppression of microglial activation

Positive staining of NT or 4-HNE and MDA, markers for metabolites of reactive nitrogen species and lipid peroxidation, respectively, was rarely found in the control or the saline+PBN rat brain (Fig. 5A, 5D, 5G, and Table 1). An increased number of cells expressing NT (Fig. 5B and Table 1), 4-HNE (Fig. 5E) or MDA (Fig. 5H) was observed not only in the cingulum and the subventricular areas, but also in the cortex of the rat brain 24 h after LPS injection. These NT+, 4-HNE+, or MDA+ cells were clustered and irregularly distributed in the cortex of the forebrain. PBN treatment significantly decreased LPS-induced expression of NT, 4-HNE and MDA in the rat brain (Fig. 5C, 5F, 5I, and Table 1).

Activated microglia are an important source of reactive oxygen and nitrogen species. Therefore, we investigated effects of PBN on microglial activation. LPS treatment stimulated

activation of microglia in the rat brain as indicated by OX42 immunostaining (Fig. 6B, Table 1). In the control rat brain, few OX42 positive cells were detected and most of those cells were in resting status with a ramified shape (Fig. 6A, Table 1). Significantly increased numbers of activated microglia showing bright staining, a round shape and blunt processes were found not only in the white matter and the periventricular area, but also in the cortex of the rat brain 24 and 72 h after LPS injection (Fig. 6B, Table 1). PBN treatment reduced the number of activated microglia following LPS injection (Fig. 6C, Table 1). The OX42 staining data were further confirmed by lectin histochemistry and ED1 immunostaining (Table 1). Double-labeling showed that many of the activated microglia were IL-1 β expressing cells (Figs. 6D~6F) and that some activated microglia were iNOS expressing cells (Figs. 6G~6I). No iNOS positive cells were detected in the control rat brain.

DISCUSSION

Although early routine neuropathological studies did not generally show evidence of apparent cortical neuronal injury in PVL (Banker and Larroche, 1962; DeReuck et al., 1972), the frequently observed cognitive and movement disorders, classically attributable to neuronal dysfunction, suggest an impaired development of cortical and deeper neurons in brains with PVL. Axonal swelling and injury around the foci of white matter necrosis and the neuronal damage of the adjacent cortex, predominantly in the pyramidal neurons of the cortical layer 5, have been found in the prenatal-onset PVL patients (Deguchi et al., 1999; Meng et al., 1997). The marked reduction in cortical gray matter found in the infant brain with PVL (Inder et al., 1999) and the increased expression of inflammatory cytokines in cortical and subcortical neurons in the PVL brain (Kadhim et al., 2003) have provided further evidence for an impaired development of cortical and deeper neurons in brains with PVL. In the present study, the beaded APP immunostaining was found in the white matter and adjacent cortical areas in the rat brain 24 h after LPS exposure (Fig 2B and 2E). APP, a membrane spanning glycoprotein, is normally produced in the neuronal cell body and quickly carried along the axon through fast anterograde axonal transport (Deguchi et al., 1999; Meng et al., 1997; Sheriff et al., 1994). The amount of APP in normal axons and neurons is not enough to be detected, but the accumulation of APP can be visualized in human and animal brain with lesions (Deguchi et al., 1999; Meng et al., 1997; Uehara et al., 1999). APP has been detected as an early sign of axonal and neuronal lesions in prenatal-onset PVL, but is not detectable in the late stage of prenatal PVL (Deguchi et al., 1999; Meng et al., 1997; Uehara et al., 1999). LPS exposure also resulted in the reduced length and the increased width of the MAP1 immunostained axons in the fifth layer of parietal cortex in the P8 rat brain (Fig 3B) and the enlarged nuclear size of neurons in this area (Fig 3E). These findings suggest that an inflammatory environment may cause not only white matter injury as we reported previously (Fan et al., 2005a), but also neuronal and axonal injury in this neonatal rat model, which is well correlated with the impaired physical development and neurobehavioral performance including locomotor function and cognitive ability in P21 rats following LPS exposure on P5 (Fan et al., 2005b). Our results support a possible anatomical correlate for the intellectual deficits and the other cortical and deep gray neuronal dysfunctions associated with PVL. It is interesting to notice that although pyknotic cells were found in the fifth layer of the parietal cortex and the size of neuronal nuclei in this area was increased, the number of neuronal cells in this area was not significantly changed after LPS exposure as indicated by the NeuN staining data (Table 1). It is possible that LPS does not cause death of cortical neurons in this area but causes functional alterations in these neurons.

Intrauterine exposure to LPS has been shown to cause reduced TH immunoreactivity and loss of dopamine neurons in the SN (Ling et al., 2002). Our previous results also showed LPS administration in the forebrain may cause injury to dopamine neurons as indicated by the reduction in the number of TH positive neurons in the rat brain 16 days after LPS injection; however, it is not known whether this is due to neuronal loss or to a functional abnormality

(Fan et al., 2005b). In the present study LPS treatment significantly reduced the number of TH positive neurons in both SN and VTA regions in the rat brain (Fig. 4B, 4H and Table 1) but the number of NeuN positive cells remained unchanged (Fig. 4E) suggesting that LPS exposure caused a consequence of phenotypic suppression of TH expression rather than actual neuronal cell loss in the P6 rat brain.

PBN is one of the nitron-based spin-trapping compounds to trap and stabilize free radicals (Endoh et al., 2001; Floyd et al., 2002). PBN has been shown to be neuroprotective in many neurodegenerative diseases, such as Alzheimer's disease (Floyd et al., 2002; Floyd and Hensley, 2000), LPS-mediated septic shock (Sang et al., 1999), hypoxia-ischemia (Lin et al., 2004, 2006), and stroke (Green et al., 2003). The neuroprotective action of PBN has been attributed to many factors, including formation of a spin adduct with the free radical (Green et al., 2003; Lee and Park, 2005), suppression of ROS production from mitochondria (Floyd and Hensley, 2000), inhibition of the induction of pro-inflammatory cytokines and iNOS (Kotake et al., 1998; Lin et al., 2006; Sang et al., 1999), inhibition of nuclear factor-kappa B (NF- κ B, a transcription factor for a wide variety of pro-inflammatory cytokine genes) (Kotake et al., 1998; Sang et al., 1999), and its anti-apoptosis properties (Lee and Park, 2005; Li et al., 2001). We have shown in our previous study that developing OLs were under oxidative and nitrosative stress following LPS injection and the death of O4 OLs was a result of such a stress (Fan et al., 2005a). In the current study, LPS-induced oxidative and nitrosative stress was also evidenced in the cortex as indicated by increased NT (Fig. 5B) or 4-HNE (Fig. 5E) and MDA (Fig. 5H) positive cells in the cortex following LPS exposure. The LPS-induced cortical neuronal damage observed in the present study might be the result of such a stress. Activated microglia are a major source of reactive oxygen and nitrogen species (Colton and Gilbert, 1993; Chao et al., 1992) and the surrounding area may be affected by the oxidative and nitrosative damage (Haynes et al., 2003, 2005; Thorburne and Juurlink, 1996). Recent studies have demonstrated the role of NADPH oxidase as a major factor for production of reactive oxygen species in both neurons and glial cells. NADPH oxidase can reduce oxygen to superoxide radical (O_2^-), which in turn gives rise to the production of other secondary reactive oxidants (Babior, 1999). LPS-induced neurotoxicity in a mice model of Parkinson's disease is mediated by microglial NADPH oxidase (Qin et al., 2004). Inhibition of microglial NADPH oxidase and microglial activation has been shown to be neuroprotective and has been proposed as a new avenue of anti-inflammatory and neuroprotective therapy (Choi et al., 2005; Qin et al., 2005). In the current study, therefore, NADPH oxidase associated with LPS-activated microglia may contribute to the oxidative stress. Astrocytes are also an important source of iNOS and inflammatory cytokines upon stimulation by endotoxin (Abramov et al., 2004; Kopnisky et al., 1997). In the present study, we found that the number of GFAP+ astrocytes in the cortex was significantly increased in the LPS group 24 and 72 h after injection (275.7 ± 21.7 and 257.8 ± 20.3 cell/mm², respectively), as compared to that in the control rat brain (<40 cells/mm²). The treatment of PBN decreased the LPS-induced elevation in the number of GFAP+ astrocyte (80.0 ± 12.4 and 122.2 ± 12.6 cell/mm², respectively). However, these GFAP+ astrocytes were not double-labeled with iNOS or IL-1 β . Contributions of the activated astrocytes to LPS-induced oxidative stress in the current study requires further investigation. Results from the present study show that PBN is capable of reducing LPS-stimulated microglial activation and attenuating LPS-induced oxidative stresses, as indicated by the reduced NT, 4-HNE and MDA positive staining. However, it is unclear whether the reduction in LPS-stimulated microglial activation and oxidative stresses is due to the direct effect of PBN on microglia or on NADPH oxidase, or due to the attenuated injury secondary to the free radical scavenging effects of PBN. Further investigations are needed. Nevertheless, the protective effects of PBN on LPS-induced neuronal injury in the neonatal brain as observed in the present study may provide insight into the potential use of antioxidants for treatment of neuronal dysfunctions associated with PVL.

Acknowledgements

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Abbreviations

APP, β -amyloid precursor protein
 4-HNE, 4-hydroxynonenal
 IL1- β , interleukin 1- β
 iNOS, inducible nitric oxide synthase
 LPS, lipopolysaccharide
 MAP1, microtubule-associated protein 1
 MDA, malondialdehyde
 NT, nitrotyrosine
 OL, oligodendrocyte
 PBN, α -Phenyl-*n*-tert-butyl-nitron
 P5, postnatal day 5
 PVL, periventricular leukomalacia
 SN, substantia nigra
 TH, tyrosine hydroxylase
 VTA, ventral tegmental area

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Fig. 1. Representative photomicrographs of Nissl staining in the rat brain 24 h (P6) after LPS injection. LPS injection resulted in the ventricle dilation in the forebrain (B) and pyknotic cells in the cingulum (E) and the fifth layer of the parietal cortical area (H) (indicated by arrows). PBN attenuated LPS-induced ventricle dilation (C), and reduced number of pyknotic cells (F & I). The scale bar shown in A represents 500 μ m for A~C, or shown in D represents 25 μ m for D~I.

Fig. 2.

Representative photomicrographs of APP immunostaining in the rat brain 24 h (P6) after LPS injection. Weak APP positive staining was detectable in the control brain (A&D). The beaded APP immunostaining was found in the cortex adjacent to the cingulum white matter (B) and in the parietal cortical area (E) in the LPS-exposed brain. PBN treatment attenuated the LPS-induced injury to axons in the above areas (C&F). The scale bar shown in A represents 50 μm .

Fig. 3.

Representative photomicrographs of MAP1 and NeuN immunostaining in the rat brain 72 h (P8) after LPS injection. LPS injection caused axonal and dendritic impairment as indicated by the reduced length and the increased width of MAP1 positive staining in the fifth layer of the parietal cortex (B). PBN attenuated the LPS-induced beaded or shortening of MAP1 staining (C). LPS injection affected the nucleus of neurons primarily localized in the fifth layer of the somatosensory cortex, as indicated by the increased diameter of NeuN positive staining, (arrows indicated in E). PBN attenuated the LPS-induced enlargement of neuronal nuclei (F). The scale bar shown in A represents 50 μm .

Fig. 4.

Representative photomicrographs of TH staining in the rat brain 24 h (P6) after LPS injection. TH positive staining was detected in the SN (A) and the VTA (G) areas of the midbrain sections at a level 1/3 rostral from the lambda to the bregma in the control rat brain. LPS injection caused a loss of TH positive staining (B&H). PBN attenuated the LPS-induced loss of TH positive staining (C&I). Double labeling with TH and NeuN antibodies (D-F) shows that LPS injection caused a reduction in TH positive cells (red), but not in the number of NeuN (green) positive cells (E) (D, E and F are magnified part indicated by the white box in A, B and C, respectively). The scale bars shown in A&G represent 200 μ m for A~C&G~I, or shown in D represents 50 μ m for D~F.

Fig. 5.

Representative photomicrographs of NT, 4-HNE or MDA staining in the cortex of rat brain 24 h after LPS injection. LPS injection caused increases in NT (B), 4-HNE (E) and MDA positive staining (H) in the cortex of the rat brain. No positive staining for these antibodies was detected in the cortex of the control rat brain (A, D and G, respectively). PBN attenuated the LPS-induced NT (C), 4-HNE (F) and MDA expressing (I) in the cortical area. The scale bar in A represents 50 μ m.

Fig. 6. LPS-stimulated microglial activation in the rat brain 24 h after the injection. As shown by OX42 immunostaining (A~C), most microglia were at the resting status with a ramified shape in the control group (arrow indicated in A). Numerous activated microglia with a round shape and blunt processes were observed in the cortex of the rat brain 24 h after LPS injection. PBN treatment significantly reduced number of activated microglia stimulated by LPS (C). Double-labeling showed that many lectin positive activated microglia in the cortex (D) of the LPS-injected rat brain were IL-1 β expressing cells (E). F is a merged image of D and E. Double-labeling also showed that ED1 positive microglia (G) in the LPS-injected rat brain were iNOS expressing cells (H). I is a merged image of G and H. The scale bar shown in A represents 50 μ m.

Table 1

PBN attenuated LPS-induced neuronal injury and microglial activation in the rat brain 24 h (P6) and 72 h (P8) following LPS injection

Treatment	Saline	Saline+PBN	LPS	LPS+PBN
P6				
Ventricle size index				
Left ventricle (%)	0.53±0.03	0.55±0.03	2.32±0.17 [*]	0.72±0.07 [#]
Right ventricle (%)	0.50±0.02	0.54±0.02	1.69±0.14 [*]	0.65±0.06 [#]
Pyknotic cells				
Cingulum area	0.0±0.0	0.0±0.0	553.3±77.7 [*]	62.2±10.1 [#]
Parietal cortex	0.0±0.0	0.0±0.0	351.1±57.3 [*]	91.1±8.0 [#]
Dopamine neurons				
TH+ cells in SN	64.1±1.3	65.9±1.7	49.9±1.8 [*]	61.4±1.5 [#]
NeuN+ cells in SN	84.3±2.2	87.5±1.8	80.4±1.9	86.4±0.9
TH+ cells in VTA	129.2±4.8	127.6±4.6	76.8±7.7 [*]	114.2±3.7 [#]
Markers of oxidative stress in cortex				
NT+	0.0±0.0	0.0±0.0	224.4±25.6 [*]	55.6±18.0 [#]
4-HNE+	0.0±0.0	0.0±0.0	206.7±31.3 [*]	37.8±11.6 [#]
MDA+	0.0±0.0	0.0±0.0	162.2±17.4 [*]	37.8±15.9 [#]
Activated microglia in cortex				
OX42+	26.7±6.9	24.4±6.4	586.7±32.5 [*]	148.9±21.6 [#]
Lectin+	31.1±6.6	28.9±7.2	731.1±57.6 [*]	273.3±18.5 [#]
ED1+	15.6±5.4	20.0±7.5	251.1±24.2 [*]	91.1±9.4 [#]
P8				
Ventricle size index				
Left ventricle (%)	0.57±0.03	0.56±0.02	4.41±0.33 [*]	1.02±0.08 [#]
Right ventricle (%)	0.53±0.02	0.52±0.02	2.44±0.20 [*]	0.75±0.03 [#]
MAP1-stained axons and dendrites in parietal cortex				
Length (μm)	160.1±6.1	163.4±4.2	37.9±1.8 [*]	111.8±8.6 [#]
Width (μm)	1.6±0.2	1.5±0.2	5.0±0.5 [*]	2.0±0.3 [#]
Neurons in parietal cortex				
Number of NeuN cell	1622.2±69.4	1602.2±51.6	1771.1±109.6	1684.4±82.5
Diameter of NeuN cell (μm)	11.5±0.3	11.7±0.1	16.5±0.2 [*]	12.2±0.3 [#]
Activated microglia in cortex				
OX42+	37.8±9.4	31.1±7.4	311.1±29.2 [*]	66.7±16.5 [#]
Lectin+	46.7±8.3	40.0±8.4	362.2±26.9 [*]	77.8±18.4 [#]
ED1+	15.6±6.4	17.8±5.6	193.3±11.8 [*]	37.8±12.6 [#]

Intracerebral injection of LPS and PBN treatment were performed as described in the text. The ventricle size index was determined by a densitometer as described in methods. Immunostaining (except for TH staining) was performed in sections from the forebrain at the bregma level. Cell counting of NT+, 4-HNE+, MDA+, OX42+, lectin+, or ED1+ cells was performed in the parietal cortical area of the forebrain sections at the bregma level. OX42 or lectin positive cells with a round shape and blunt processes (as indicated in Fig. 6B) were considered as the activated microglia. TH+ cell counting was performed in the SN or the VTA and NeuN+ cell counting was performed in the SN of the midbrain sections at a level 1/3 rostral from the lambda to the bregma.

Data are presented as the mean±S.E.M. (cells/mm²) of six animals for each group. Data are presented as the mean±S.E.M. (μm) 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.