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Interleukin-1 β -induced brain injury in the neonatal rat can be ameliorated by α -phenyl-*n*-tert-butyl-nitrone

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Abstract

To examine the possible role of inflammatory cytokines in mediating perinatal brain injury, we investigated effects of intracerebral injection of interleukin-1 β (IL-1 β) on brain injury in the neonatal rat and the mechanisms involved. Intracerebral administration of IL-1 β (1 μ g/kg) resulted in acute brain injury, as indicated by enlargement of ventricles bilaterally, apoptotic death of oligodendrocytes (OLs) and loss of OL immunoreactivity in the neonatal rat brain. IL-1 β also induced axonal and neuronal injury in the cerebral cortex as indicated by elevated expression of β -amyloid precursor protein, short beaded axons and dendrites, and loss of tyrosine hydroxylase positive neurons in the substantia nigra and the ventral tegmental areas. Administration of α -phenyl-*n*-tert-butyl-nitrone (PBN, 100 mg/kg i.p.) immediately after the IL-1 β injection protected the brain from IL-1 β -induced injury. Protection of PBN was linked with the attenuated oxidative stress induced by IL-1 β , as indicated by decreased elevation of 8-isoprostane content and by the reduced number of 4-hydroxynonenal or malondialdehyde or nitrotyrosine positive cells following IL-1 β exposure. PBN also attenuated IL-1 β -stimulated inflammatory responses as indicated by the reduced activation of microglia. The finding that IL-1 β induced perinatal brain injury was very similar to that induced by lipopolysaccharide (LPS), as we previously reported and that PBN was capable to attenuate the injury induced by either LPS or IL-1 β suggests that IL-1 β may play a critical role in mediating brain injury associated with perinatal infection/inflammation.

Keywords

α -Phenyl-*n*-tert-butyl-nitrone; Interleukin-1 β ; Brain injury; Microglial activation; Oxidative stress

Introduction

Increasing evidence indicates that maternal or placental infection is a major contributor to perinatal brain injury in addition to hypoxia-ischemia (Hagberg et al., 2002; Rezaie and Dean 2002; Volpe, 2003). Inflammatory cytokines derived from maternal infection have been considered as mediators between maternal infection and periventricular leukomalacia (PVL), a predominant form of brain injury in the premature infant brain (Dammann and Leviton,

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1997; Hagberg et al., 2002; Volpe, 2003). Occurrence of PVL is frequently associated with increased concentrations of inflammatory cytokines such as tumor necrosis factor- α (TNF α), interleukin-1 β (IL-1 β) and IL-6 in the infant brain (Kadhim et al., 2001, 2003; Yoon et al., 1997b), in the cord blood (Yoon et al., 1996) or amniotic fluid (Yoon et al., 1997a). However, the mechanisms relating increased inflammatory cytokines to the white matter and other types of brain injury are still not completely understood.

In previous studies we developed a neonatal rat model to mimic the scenario of infection/inflammation through intracerebral injection of lipopolysaccharide (LPS) in the postnatal day 5 (P5) rat brain. We found that intracerebral injection of LPS in neonatal rats induced great increases in TNF α and IL-1 β concentrations in the brain and resulted in white matter and neuronal injury (Cai et al., 2003; Fan et al., 2005, 2008b, 2008c; Pang et al., 2003). While intracerebral injection of IL-1 β resulted in a similar white matter injury in the neonatal rat (Cai et al., 2004), co-administration of LPS with IL-1 receptor antagonist (IL-1ra) protected the rat brain from LPS-induced white matter injury (Cai et al., 2003). These data indicate that IL-1 β plays an important role in mediating LPS-induced neonatal brain injury. We also found that α -Phenyl-*n*-tert-butyl-nitrone (PBN), a nitron-based antioxidant capable of trapping and stabilizing free radicals in biological systems (Floyd et al., 2002), provided protection against LPS-induced neonatal white matter and neuronal injury (Fan et al., 2008a, 2008b, 2008c). We speculate that PBN should also be able to protect the neonatal brain from injury induced by direct injection of IL-1 β , assuming IL-1 β plays a major role in mediating LPS-induced brain injury. To test this possibility and further explore roles of IL-1 β in mediating LPS-induced neonatal brain injury, we evaluated effects of PBN on IL-1 β -induced brain injury in the neonatal rat.

Materials and methods

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma (St. Louis, MO). Recombinant rat interleukin-1 β (IL-1 β) was purchased from R&D System (Minneapolis, MN). Monoclonal mouse antibodies against late OL progenitor cell marker O4, immature OL marker O1, β -amyloid precursor protein (APP), microtubule-associated protein 2 (MAP2), MAP1, tyrosine hydroxylase (TH) or neuron-specific nuclear protein (NeuN) (biotin conjugated); adenomatus polyposis coli (Clone CC1) (APC-CC1); OX42 (CD11b) or ED1; and IL-1 β were purchased from Chemicon (Temecula, CA), Calbiochem (San Diego, CA), Serotec (Raleigh, NC), and the R&D Systems (Minneapolis, MN), respectively. Polyclonal rabbit antibodies against caspase-3 (active form), or 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. The TUNEL staining kit was purchased from Chemicon.

Animal treatment

Intracerebral injection of IL-1 β to 5-day old Sprague-Dawley rat pups of both sexes was performed as previously described (Cai et al., 2004). Under light anesthesia with isoflurane (1.5%), IL-1 β (1 μ g/kg) in sterile saline containing 0.1% BSA (total volume of 2 μ l) 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 dose of IL-1 β was chosen based on the peak concentrations of IL-1 β achieved in the rat pup brain following LPS administration as reported previously (Cai et al., 2003, 2004; Pang et al., 2003) and on the published data (Holmin and Mathiesen, 2000). The injection site was located at the area just above the left cingulum as shown by the

arrow in Fig. 1B. The control rats were injected with the same volume of sterile saline containing 0.1% BSA. All animals survived the intracerebral injection.

Both IL-1 β - and saline-injected animals were further divided into two groups: one received intraperitoneal (i.p.) injections of α -Phenyl-*n*-tert-butyl-nitrone (PBN) and the other sterile saline. PBN (100 mg/kg) or vehicle alone was administered immediately after the IL-1 β injection. Our previous study showed that this dosing and timing of PBN administration protects the neonatal rat brain from LPS-induced (Fan et al., 2008a, 2008b, 2008c) and hypoxia-ischemia-induced white matter injuries (Lin et al., 2004, 2006). Each dam had an equal litter size (12 pups). One or three days after the surgery (P6 or P8), rat pups were sacrificed by transcardiac perfusion with normal saline followed by 4% paraformaldehyde for brain section preparation. There were 12 animals (6 male and 6 female) in each treatment group for either postnatal day. The twelve rat brains in each treatment group were divided into two sets (3 male and 3 female in each set) for preparation of frozen brain sections and free-floating brain sections, respectively. Some rat pups were sacrificed by decapitation 24 h after the surgery for collection of fresh brain tissues and at least 4 rat brains were included in each treatment group. After removal from animals, the brain (without the cerebellum) was weighed, quickly frozen in liquid nitrogen and stored at -80 °C until use. 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 Nissl staining and immunohistochemistry not involving O4 or O1 staining. Coronal free-floating brain sections at 40 μ m of thickness were prepared in a freezing sliding microtome for OL surface antigen O4 or O1 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: O4 or O1, 1 μ g/ml; 4-HNE, 1:500; caspase-3, MDA, iNOS, OX42 or ED1, 1:200; APP, MAP2, MAP1, TH, NeuN, IL-1 β or NT, 1:100; and APC-CC1, 1:20. 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 IL-1 β -injected rat brain was blocked by NT, but not by aminotyrosine or by phosphotyrosine, indicating the specificity of the antibody for NT.

Detection of cell death

Cell death was detected by TUNEL staining kits. When double-labeling of O4 cells was required, O4 immunostaining was first performed as described above, with rhodamine as the fluorophore. TUNEL staining was then performed in these sections following the manufacturer's instructions. The TUNEL positive cells show a green color and the O4 positive cells show a red color.

Since both necrosis and apoptosis may result in TUNEL-positive staining (Stadelmann and Lassmann, 2000), we further performed caspase-3 and O4 double-labeling to confirm apoptotic cell death of OLs following LPS exposure. Caspase-3 is a key mediator of apoptotic cell death (Stadelmann and Lassmann, 2000). Free-floating brain sections were simultaneously incubated with rabbit antibody against caspase-3 and mouse antibody against O4. FITC-conjugated secondary antibody against rabbit IgG and rhodamine-conjugated secondary antibody against mouse IgM were used to visualize the result.

8-ISO quantification

To validate the 4-HNE and MDA immunostaining data, contents of 8-ISO in the rat brain were determined as a marker of lipid peroxidation by an enzyme immunoassay kit as described previously (Cai et al., 2006; Fan et al., 2008c). Briefly, frozen rat brains (without the cerebella) were thawed in 1 ml of absolute ethanol, homogenized by sonication for 20 s and then placed on ice for 5 min. After centrifugation (15,000 $\times$ g at 4 °C for 10 min), 8 ml of 100 mM phosphate buffer (pH 7.4) was added to supernatants. Samples were then applied on 8-ISO affinity columns (Cayman). After washing with 2 ml of 100 mM phosphate buffer (pH 7.4) and 2 ml of ultra-pure water, lipids were eluted with 2 ml of 95% ethanol. The fraction containing 8-ISO was then vacuum-dried. 8-ISO contents were assessed spectrophotometrically at 410 nm, using a competitive immunoassay kit (Cayman) according to the manufacturer's instruction. The results were expressed as pg 8-ISO per mg of brain tissue.

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., 2005). 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 IL-1 β 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., 2004). 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 midbrain 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 (such as some O4 or O1 positive cells), numbers of DAPI-stained nuclei from the superimposed images were counted as the cell number. In the white matter injury study, three digital microscopic images were randomly captured at the areas where the positive cells were abundant (mainly the cingulum area, unless otherwise stated) for each section. In

the neuronal damage study, cortical neuronal changes were primarily observed in the layer V of the parietal cortex of the forebrain following IL-1 β 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. APP and MAP2 staining were quantified by calculating the percentage area of the whole image or image frame that contains APP or MAP2 immunostaining using NIH image software. This method has been successfully used to quantify the density of cortical serotonin transporter-immunoreactive fiber networks (Maciag et al., 2006) and the density of cortical MAP2 staining (Fan et al., 2008a).

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 or μ m. Quantified data were presented as the mean $\pm$ SEM and analyzed by one-way ANOVA followed by Student-Newman-Keuls test. Results with a $P < 0.05$ were considered statistically significant. In our preliminary studies, contents of 8-ISO in the left and the right hemispheres were measured separately. No significant difference in the above contents between the ipsilateral and the contralateral hemispheres was observed. Therefore, data from the left (ipsilateral to the IL-1 β injection site) hemisphere were presented in the paper.

Results

PBN attenuated IL-1 β -induced ventricle enlargement and injury to oligodendrocytes

As shown in the Nissl stained sections, IL-1 β administration caused bilateral ventricle dilatation (Fig. 1B) and the dilatation on the ipsilateral (the left) ventricle was more prominent than in the contralateral side in both P6 and P8 rat brains (Table 1). The dilated ventricles in the IL-1 β treated rat brain were still surrounded entirely by ependymal cells as shown in the insert in Fig. 1B, although the ependymal cell layer was much thinner than in the control brain (Fig. 1A insert). This might be an indication of the injury inside the ventricles. Treatment with PBN significantly decreased ventricle enlargement and ependymal cell layer impairment induced by IL-1 β in both the P6 and P8 rat brain (Fig 1C and Table 1). Nissl staining also showed that there were pyknotic cells in the cingulum white matter (Fig 1E and Table 1) and the cortical gray matter (Table 2) of the rat brain 24 hr, but not 72 hr, after the IL-1 β injection. The pyknotic cells in the cortical gray matter were primarily localized in the fifth layer of the parietal cortex in the forebrain. PBN treatment significantly reduced the number of pyknotic cells in these areas. No significant differences in the number of pyknotic cells between the ipsilateral and the contralateral brains were found following IL-1 β administration. Therefore, data from the ipsilateral brain are presented here. No gender differences in IL-1 β -induced brain injury were observed in the P6 and P8 rat brain.

Late OL progenitor cells (O4+/O1-) are the main OL population in the P6 rat brain (Fan et al., 2005; Follett et al., 2000). Abundant O4 positive cells, which had positive staining primarily localized at the cell membrane and processes, were observed in the P6 (Fig. 1G and Table 1) and P8 control rat brain (Table 1), mostly at the corpus callosum and subcortical white matter tract. As compared to the control group, IL-1 β significantly reduced the number of O4+ cells (about 37% reduction) in the cingulum area of the P6, but not P8 rat brain and resulted in pyknotic O4+ cells in this area (Fig. 1H, arrows indicated). These pyknotic cells displayed acute degeneration features that included apparent condensation of the cell body and the appearance of O4 immunoreactivity at the whole shrunken cell body (Back et al., 2002, also see insert in Fig. 1H). PBN treatment reduced the number of pyknotic OLs and significantly attenuated the IL-1 β -induced loss of O4+ cells in the P6 rat brain (Fig. 1I and Table 1). IL-1 β injection also significantly reduced the number of O1+ cells in the subcortical white

matter tract as compared with that in the control group 1 or 3 days after the injection. PBN treatment prevented the IL-1 β -induced loss of O1+ cells (Table 1). By P8, mature OLs detected by APC-CC1 antibody, which immunostains the mature OL cell body without labeling processes (Murtie et al., 2005), were mostly observed in the corpus callosum and the subcortical white matter tract at the bregma level and in the internal capsule areas. IL-1 β injection reduced the number of APC-CC1 positive cells by about 25% (Fig. 1K) as compared with that in the control rat brain (Fig. 1J). PBN treatment attenuated the IL-1 β -induced reduction in the number of mature OLs in the P8 rat brain (Fig. 1L).

PBN reduced apoptotic cell death of OLs following IL-1 β

IL-1 β -induced apoptotic OL cell death was demonstrated by the TUNEL and caspase-3 staining. Few TUNEL positive cells were found in the control rat brain (Fig. 2A and Table 1). An increased number of TUNEL positive cells was observed primarily at the periventricular areas 24 h after the IL-1 β injection (Fig. 2B and Table 1). Double-labeling (Figs 2F & 2I) showed that many TUNEL positive (Fig. 2D) or caspase-3 positive cells (Fig. 2G) were also O4 positive (Figs. 2E & 2H), indicating that apoptosis was involved in IL-1 β -induced OL cell death. PBN treatment effectively prevented the IL-1 β -induced increase in the number of TUNEL positive (Fig. 2C and Table 1) or caspase-3 positive O4 cells (Table 1).

PBN decreased IL-1 β -induced axonal and dendritic damage

Accumulation of APP, a marker of axonal injury, was determined by immunostaining in the P6 rat brain. As shown in Fig. 3A, weak expression of APP was barely detectable in the control rat brain. The beaded APP immunostaining was found in the cingulum area connecting with upper cortical layers (Fig. 3B), the parietal cortex and the caudate putamen (Table 2) in the IL-1 β -exposed brain. Strong immunoreactivity of APP was also observed in the lateral ventricle surrounding areas and the dorsal third ventricle surrounding area of the IL-1 β -exposed rat brain (data not shown). APP immunostaining in these brain regions of the PBN treated group (Fig. 3C) was similar to that in the control rat brain, indicating that PBN treatment attenuated the IL-1 β -induced injury to axons.

Neuronal dendritic processes were identified by MAP2 immunostaining. IL-1 β treatment elicited changes in dendrite density within the cingulum area adjacent to the parietal cortex 24 hr after the injection (Fig. 3E and Table 2). PBN treatment prevented the induction of dendritic loss by IL-1 β (Fig. 3F and Table 2). MAP1 immunostaining was used as an additional marker for detection of IL-1 β -induced injury to axons and dendrites. Long descending MAP1 immunoreactive axons were observed in the cortex of the control rat forebrain (Fig. 3G). IL-1 β treatment caused short beaded MAP1 immunostained axons in layer V of the cortex of the P8 rat brain (Fig. 3H and Table 2), an indication of damage to axons and dendrites in the cortex. However, neuronal cell density determined by NeuN immunostaining in the layer V of the somatosensory cortex was not significantly changed by IL-1 β treatment (Table 2). PBN treatment attenuated the IL-1 β -induced injury to axons and dendrites in the cortex (Fig. 3I and Table 2).

PBN reduced losses of tyrosine hydroxylase-immunoreactive neurons following IL-1 β

Positive staining of TH was used to detect dopamine (DA) neurons in the substantia nigra (SN) and ventral tegmental area (VTA). 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. IL-1 β treatment significantly reduced the number of TH positive neurons in both the SN and the VTA regions (Figs. 4B, 4H and Table 2). However, IL-1 β exposure did not significantly

alter the total number of NeuN positive cells in these regions (Fig. 4E and Table 2), suggesting that IL-1 β did not result in actual neuronal cell death in this area. As a consequence of phenotypic suppression of TH expression but not actual neuronal cell death, IL-1 β treatment decreased the ratio between the number of TH positive cells and the number of NeuN positive cells, from $76.4 \pm 1.6\%$ to $63.8 \pm 0.9\%$ in the SN of the rat brain. PBN treatment attenuated IL-1 β -induced reduction in the number of TH positive neurons (Figs. 4C, 4I and Table 2) and increased the ratio between the TH positive and the NeuN positive cells to $72.0 \pm 0.6\%$ (Fig. 4F).

PBN protection was linked with reduction in nitrosative and oxidative damages following IL-1 β

NT, a product of tyrosine residues of proteins that have reacted with peroxynitrite (a highly reactive molecule derived from reactive nitrogen species [RNS]), is a marker of nitrosative stress (Haynes et al., 2003). 4-HNE and MDA, aldehyde products of the lipid component of cellular membranes that have reacted with reactive oxygen species (ROS), are markers of lipid peroxidation (Haynes et al., 2003; Raza and John 2006). An increased number of cells expressing NT (Fig. 5A and Table 1), 4-HNE (Fig. 5D) or MDA (Fig. 5G) was observed in the cingulum and the subventricular areas, as well as in the cortex (Table 2) of the rat brain 24 h after IL-1 β injection. Many of these cells in the cingulum area were also O4+ cells (Figs. 5B, C, E, F, H and I). Positive staining of NT or 4-HNE and MDA was rarely found in the control, the saline+PBN, or the IL-1 β +PBN rat brain (Table 1). Consistent with the immunostaining data, IL-1 β exposure resulted in an almost two-fold increase in 8-ISO content (Fig. 6), another marker of lipid peroxidation, in the rat brain 24 h after injection (7.89 ± 1.39 vs 4.05 ± 0.63 pg/mg tissue). PBN treatment significantly reduced 8-ISO level in the IL-1 β +PBN rat brain (5.30 ± 0.60 pg/mg tissue, $P < 0.05$ vs the IL-1 β group), indicating a reduction in IL-1 β -induced oxidative stress.

Protection by PBN was also associated with suppression of microglial activation and inflammatory responses

Activated microglia are a major source of ROS and RNS (Colton and Gilbert, 1993; Chao et al., 1992), both of which may put OLs at risk of nitrosative and oxidative damage (Thorburne and Juurlink, 1996). Therefore, we examined effects of PBN on microglial activation following IL-1 β exposure. In the control rat brain, few OX42 positive cells were detected and most of those cells were in resting status with a ramified shape (Figs. 7A & 7D, Tables 1 and 2). A significantly increased number of activated microglia showing bright staining, a round shape and blunt processes, were found not only in the corpus callosum and the periventricular area of the rat brain 24 and 72 h (by 2.9- and 1.5-fold, respectively) after IL-1 β injection (Fig. 7B and Table 1), but also in the cortex 24 h after IL-1 β injection (Fig. 7E and Table 2). PBN treatment reduced the number of activated microglia following IL-1 β injection both in the cingulum area (Fig. 7C, Table 1) and cortex (Fig. 7F, Table 2). The OX42 staining data were further confirmed by ED1 staining and lectin histochemistry (Figs. 7G & 7J, Tables 1 & 2). Double-labeling showed that many activated microglia were IL-1 β expressing cells (Figs. 7G~I) and that some activated microglia were iNOS expressing cells (Figs. 7J~L). No iNOS positive cells were detected in the control rat brain.

Discussion

Increasing evidence indicates that maternal infection could stimulate increased production of proinflammatory cytokines, such as IL-1, IL-6, and TNF α in the three relevant maternal/fetal compartments (uterus, fetal circulation, and fetal brain). These cytokines are able to cross boundaries (the placenta and blood-brain barrier) and have access to the fetal brain (Dammann and Leviton, 1997). In the current study, we administer IL-1 β directly into the neonatal brain

to assess the effect of IL-1 β exposure on neonatal brain injury. Consistent with our previous data (Cai et al., 2004) and data reported by other investigators (Holmin and Mathiesen, 2000; Carvey et al., 2005), results from the current study further demonstrate that IL-1 β alone can directly cause apoptotic death of OL progenitor cells and other types of brain injury. The pattern of acute brain injury induced by IL-1 β is very similar to that induced by exposure to LPS as we previously reported (Fan et al., 2005, 2008b, 2008c). The prominent injury following administration of IL-1 β or LPS (Fan et al., 2005, 2008c; Lehnardt et al., 2002) was the reduction of OL progenitor cells in the white matter. Loss of late OL progenitor cells (O4+/O1-) is a major pathological characteristic in the infant brain with PVL (Back et al., 2001). Therefore, our data support the hypothesis that increased IL-1 β concentrations (Deguchi et al., 1996, 1997; Yoon et al., 1997) derived from maternal infection may contribute to the occurrence of PVL in the infant brain. More interestingly, our current data further demonstrated that IL-1 β -induced damage to OL progenitor cells can be attenuated by PBN which was similar to the attenuation by PBN of the damage induced by LPS (Fan et al., 2008c), possibly through similar mechanisms. Our additional experiments further show that PBN in a similar fashion attenuates neonatal IL-1 β -induced brain injury and neurobehavioral deficits in juvenile rats (P21) as it did to LPS-induced brain injury and behavioral dysfunction in our previous study (Fan et al., 2008a) (L.-W. Fan, unpublished data). These observations confirm and support our speculation that IL-1 β may play a major role in mediating LPS-induced brain injury. Use of IL-1 receptor antagonist to block the effects of IL-1 β has been shown to successfully protect the neonatal brain from LPS- or hypoxia-ischemia-induced injury (Cai et al., 2003; Yang et al., 1999). Therefore, targeting the action of inflammatory cytokines might be one of the strategies in developing therapeutic treatments for brain injury associated with perinatal infection/inflammation.

PBN is one of the most widely used in vivo nitron-based spin-trapping compounds to trap and stabilize free radicals (Endoh et al., 2001; Floyd et al., 2002). PBN has been shown to have neuroprotective effects in many in vivo and in vitro animal models of human neurodegenerative diseases, such as Alzheimer's disease (Floyd et al., 2000, 2002). PBN has also been shown to protect against 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 free radicals (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). Results from the current study support the involvement of these factors in the neuroprotective action of PBN. PBN has been reported to inhibit apoptotic features such as activation of caspase-3, up-regulation of Bax and p53, and down-regulation of Bcl-2 in cells upon exposure to ionizing radiation (Lee and Park, 2005) or following focal or global ischemia in vivo (Li et al., 2001). In the present study, PBN reduced the loss of IL-1 β -induced OL immunostaining (Fig. 1 and Table 1) and this effect appeared to be, at least partially, the result of the attenuation of apoptotic OL cell death by PBN (Fig. 2 and Table 1), suggesting that the protection of PBN is linked with its anti-apoptotic properties.

Late OL progenitor cells, which are believed to be the major target in the infant brain with PVL (Back et al., 2001), are specifically vulnerable to nitrosative and oxidative stress (Back et al., 2002, 2005; Haynes et al., 2003). Nitrotyrosine, a marker of nitrosative stress, was found in O4 cells in the infant brain with PVL (Haynes et al., 2003). HNE and MDA (Christen 2000), products of lipid peroxidation which are known to be toxic to premyelinating OLs (Gard et al., 2001; McCracken et al., 2000), have also been found in the infant brain with PVL (Haynes et al., 2003). These findings strongly support the link between the loss of OLs and ROS or RNS.

In the current study, many O4+ OLs in the rat brain have been found to be double-labeled with 4-HNE, NT and MDA (Fig. 5) following IL-1 β exposure. This is an indication that developing OLs might be under oxidative and nitrosative stress following IL-1 β injection and that the death of O4+ OLs might be the result of such a stress. The 8-ISO content data (Fig. 6) from the present study further support this possibility. PBN treatment significantly reduced the number of 4-HNE and MDA expressing O4+ cells and decreased 8-ISO content in the rat brain following IL-1 β injection, suggesting that protection of PBN on developing OLs is through its action as an antioxidant. Our data indicate that the antioxidant effect of PBN is likely associated with its effect on microglial activation following IL-1 β (Fig. 7). In the present study, many activated microglia following IL-1 β injection are IL-1 β expressing cells (Fig. 7). This phenomenon may reflect a positive feedback of inflammatory responses following IL-1 β challenge. A reduction in the number of activated microglia may interrupt this positive feedback. In addition, activated microglia are a major source of ROS and RNS, such as iNOS as shown in Fig. 7 (Chabot and Yong, 2002; Colton and Gilbert, 1993). Attenuation of IL-1 β -induced oxidative stress appears to be a logical consequence of the reduction in the number of activated microglia.

Results from the current study have demonstrated that IL-1 β induces injury to not only developing OLs, but also axons and neurons, especially the DA neurons in the SN, an area that is far away from the site of injection. Intra-parenchymal injection of IL-1 β has been shown to cause death of DA neurons in the adult rat brain (Carvey et al., 2005). Our immunostaining data indicate that following neonatal exposure to a single dose of IL-1 β the number of TH positive neurons in the SN was reduced (Fig. 4) but the number of total neurons in this area was not altered. Since the predominant neuron population in the SN region is that of DA neurons, our results suggest that these DA neurons may become dysfunctional rather than dying following exposure to IL-1 β . At the present time, it is unclear how an injection of IL-1 β at the forebrain causes injury in distant brain regions. Several possibilities could be considered. IL-1 β may diffuse across other brain regions from the site of injection. If this is the case, then there should have been a gradient of IL-1 β concentrations in the rat brain. The increased IL-1 β level in the distant brain regions may subsequently stimulate responses from neural cells in those regions, such as activation of microglia. We have found that the number of activated microglia (OX42+, with a round shape and blunt processes) in the SN was significantly increased 24 hr after IL-1 β injection as compared with that in the control group (128.9 ± 5.1 vs 44.4 ± 4.4 cells/mm²). As discussed previously, the activated microglia are a major source of ROS and RNS, which may cause cell injury or death (Chabot and Yong, 2002; Colton and Gilbert, 1993). In addition to microglia, other neural cells such as astrocytes may also become a source of ROS/RNS when exposed to IL-1 β (Thornton et al., 1998). Alternatively, activated microglia at the site of injection may migrate to distant brain regions (Carbonell et al., 2005) or secondary signaling molecules generated at the IL-1 β injection site may mediate the injury in distant brain regions. All these possibilities need further verifications. Nevertheless, loss of DA neurons in the brain is a hallmark of Parkinson's disease. Recent studies have shown that IL-1 β increases susceptibility of DA neurons to degeneration or exacerbates DA neuron degeneration in animal models of Parkinson's disease (Koprich et al., 2008; Godoy et al., 2008), suggesting IL-1 β may play an important role in the degeneration of DA neurons. Our results suggest even a single exposure to IL-1 β during the perinatal stage may have effects on the DA neuronal system. Although the exact consequences of perinatal IL-1 β exposure on development of neurodegenerative diseases remain unknown, it is worth further investigation.

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Fig. 1.

Representative photomicrographs of Nissl staining (P6, A~F), O4 (P6, G~I) and APC-CC1 (P8, J~L) immunostaining in the rat brain 24 h (P6) or 72 h (P8) after IL-1 β injection. The injection site (the needle tip) is shown by an arrow in B. IL-1 β injection resulted in the ventricle dilation in the forebrain (B) and pyknotic cells in the cingulum area (E) (indicated by arrows). The dilated ventricle in the IL-1 β treated rat brain was still surrounded entirely by ependymal cells. Partial ependymal cell layers surrounding the ventricle in each treatment group (indicated by white boxes in A-C) are magnified and shown in respective inserts. PBN attenuated IL-1 β -induced ventricle dilation (C), and reduced number of pyknotic cells (F). Abundant O4 positive cells, which had positive staining at the cell membrane and processes, were found in

P6 and P8 control rat brains (G). IL-1 β injection resulted in pyknotic O4 cells with condensed cell body and immunoreactivity in the cytoplasm (arrow indicated in H) and reduced the number of normal O4 positive cells. Inserts in G, H and I are magnified parts indicated by the white arrows. PBN attenuated IL-1 β -induced loss of O4 positive cells (I). APC-CC1 positive staining was detected in the P8 control rat brain (J). IL-1 β resulted in loss of APC-CC1 positive staining (K). PBN treatment reduced the loss of APC-CC1 positive staining (L). The scale bar shown in A represents 500 μ m for A~C, or shown in D represents 50 μ m for D~L.

Fig. 2.

Cell death in the rat brain 24 h (P6) after IL-1 β injection. More TUNEL positive cells were observed in the IL-1 β -injected rat brain (B) than in the control (A) or the IL-1 β +PBN rat brain (C). Double-labeling showed that many TUNEL positive cells (D) were O4+ cells (E). F is a merged image of D and E. Double-labeling also showed that many caspase-3 positive cells (G, arrows indicated) in the IL-1 β -injected rat brain were O4+ cells (H, arrows indicated). I is a merged image of G and H. The scale bar in A represents 50 μ m.

Fig. 3.

Representative photomicrographs of APP (P6, A~C), MAP2 (P6, D~F), and MAP1 (P8, D~F) immunostaining in the rat brain 24 h (P6) or 72 h (P8) after IL-1 β injection. Weak APP positive staining was detectable in the control brain (A). The beaded APP immunostaining was found in the cortex adjacent to the cingulum white matter (B) in the IL-1 β -exposed brain. PBN treatment attenuated the IL-1 β -induced injury to axons in the above areas (C). IL-1 β 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 E). IL-1 β injection also caused axonal and dendritic impairment as indicated by the short beaded MAP1 positive staining in layer V of the cortex (H). PBN attenuated the IL-1 β -induced loss

of MAP2 staining (F) and morphological alterations in MAP1 stained axons and dendrites (I). The scale bar shown in A represents 50 μm .

Fig. 4.

Representative photomicrographs of TH staining in the rat brain 24 h (P6) after IL-1 β 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. IL-1 β injection caused a loss of TH positive staining (B & H). PBN attenuated the IL-1 β -induced loss of TH positive staining (C & I). Double labeling with TH and NeuN antibodies (D~F) shows that IL-1 β 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 rat brain 24 h after IL-1 β injection. Double-labeling showed that many NT positive cells (A) in the IL-1 β -injected rat brain were O4+ cells (B). C is a merged image of A and B. Double-labeling also showed that many 4-HNE positive cells (D) and MDA positive cells (G) in the IL-1 β -injected rat brain were O4+ cells (E and H, respectively). F and I are merged images of D and E, and G and H, respectively. The scale bar in A represents 50 μ m.

Fig. 6. Inhibition of IL-1 β -stimulated increases in 8-Isoprostane (8-ISO), a marker of lipid peroxidation, by PBN in the rat brain 24 h after the injection. 8-ISO contents in the rat brain were assayed by an enzyme immunoassay kit as described in methods. Results were expressed as the mean $\pm$ S.E.M. of six animals for each group. IL-1 β treatment induced an almost two-fold increase in 8-ISO content in the rat brain, while PBN significantly attenuated IL-1 β -induced increase in 8-ISO contents. * $P < 0.05$ vs the control group. # $P < 0.05$ vs the IL-1 β group.

Fig. 7.

IL-1 β -stimulated microglial activation in the rat brain. As shown by OX42 immunostaining (A~C, cingulum area; D~F, cortical area), most microglia were at the resting status with a ramified shape in the control group (arrow indicated in A & D). Numerous activated microglia with a round shape and blunt processes were observed in the rat brain 24 h after IL-1 β injection (arrow indicated in B & E). PBN treatment significantly reduced number of activated microglia stimulated by IL-1 β (C & F). Double-labeling showed that lectin positive activated microglia (G) in the IL-1 β -injected rat brain were IL-1 β expressing cells (H). I is a merged image of G and H. Double-labeling also showed that ED1 positive microglia (J) in the IL-1 β -injected rat

brain were iNOS expressing cells (K). L is a merged image of J and K. The scale bar in A represents 50 μm .

Table 1PBN attenuated IL-1 β -induced white matter injury in the rat brain 24 h (P6) and 72 h (P8) following IL-1 β injection

Treatment	Saline	Saline+PBN	IL-1 β	IL-1 β +PBN
P6				
Ventricle size index				
Left ventricle (%)	0.52 $\pm$ 0.04	0.54 $\pm$ 0.06	2.01 $\pm$ 0.25 *	0.51 $\pm$ 0.05 #
Right ventricle (%)	0.48 $\pm$ 0.02	0.50 $\pm$ 0.03	1.19 $\pm$ 0.17 *	0.55 $\pm$ 0.06 #
Pyknotic cells				
Cingulum area	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	246.7 $\pm$ 33.2 *	46.7 $\pm$ 7.5 * #
Oligodendrocytes				
O4+	871.2 $\pm$ 57.3	862.2 $\pm$ 46.7	552.2 $\pm$ 17.8 *	784.4 $\pm$ 38.9 #
O1+	371.1 $\pm$ 19.3	371.2 $\pm$ 23.9	160.0 $\pm$ 14.6 *	340.1 $\pm$ 17.5 #
O4+ cells with apoptotic marker				
TUNEL+	8.9 $\pm$ 4.1	11.1 $\pm$ 4.1	73.3 $\pm$ 8.9 *	13.4 $\pm$ 4.8 #
Caspase-3+	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	44.4 $\pm$ 5.6 *	0.0 $\pm$ 0.0 #
Injured O4+ cells				
NT+	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	120.1 $\pm$ 13.8 *	2.2 $\pm$ 2.2 #
4-HNE+	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	224.4 $\pm$ 26.3 *	11.1 $\pm$ 7.2 #
MDA+	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	77.8 $\pm$ 22.4 *	0.0 $\pm$ 0.0 #
Activated Microglia				
OX42+	197.8 $\pm$ 18.3	211.1 $\pm$ 15.6	566.7 $\pm$ 42.1 *	306.7 $\pm$ 20.7 * #
Lectin+	209.9 $\pm$ 16.4	202.2 $\pm$ 18.9	657.6 $\pm$ 73.3 *	314.3 $\pm$ 29.8 * #
ED1+	88.9 $\pm$ 8.9	86.1 $\pm$ 8.9	251.1 $\pm$ 8.1 *	162.2 $\pm$ 13.2 * #
Lectin+/IL-1 β +	62.2 $\pm$ 4.5	64.4 $\pm$ 8.1	373.3 $\pm$ 30.8 *	144.4 $\pm$ 13.9 * #
ED1+/iNOS+	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	97.8 $\pm$ 11.2 *	13.3 $\pm$ 6.9 #
P8				
Ventricle size index				
Left ventricle (%)	0.59 $\pm$ 0.05	0.57 $\pm$ 0.04	0.89 $\pm$ 0.46 *	0.62 $\pm$ 0.03 #
Right ventricle (%)	0.55 $\pm$ 0.03	0.53 $\pm$ 0.01	0.79 $\pm$ 0.06 *	0.57 $\pm$ 0.03 #
Oligodendrocytes				
O4+	602.2 $\pm$ 18.3	608.9 $\pm$ 18.8	522.2 $\pm$ 36.5	580.0 $\pm$ 28.9
O1+	513.1 $\pm$ 21.4	528.9 $\pm$ 26.8	215.6 $\pm$ 20.5 *	535.6 $\pm$ 32.2 #
APC-CC1+	1226.7 $\pm$ 29.1	1208.9 $\pm$ 25.7	917.8 $\pm$ 25.2 *	1175.6 $\pm$ 43.2 #
Activated Microglia				
OX42+	226.7 $\pm$ 14.2	217.8 $\pm$ 7.4	340.0 $\pm$ 20.6 *	224.5 $\pm$ 18.3 #
Lectin+	188.9 $\pm$ 15.2	175.6 $\pm$ 19.3	326.7 $\pm$ 21.2 *	180.0 $\pm$ 25.2 #
ED1+	95.6 $\pm$ 6.4	97.8 $\pm$ 6.6	211.1 $\pm$ 15.9 *	104.4 $\pm$ 15.9 #

Note. Intracerebral (i.c.) injection of IL-1 β and intraperitoneal (i.p.) PBN treatment were performed as described in the text. The ventricle size index was determined by a densitometer as described in methods. Cell counting of O4+, O1+, APC-CC1+, OX42+, lectin+, or ED1+ cells was performed in the cingulum 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. 7B) were considered as the activated microglia. Data are presented as the mean $\pm$ S.E.M. (cells/mm²) of six animals for each group and 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 IL-1 β group.

Table 2PBN attenuated IL-1 β -induced neuronal injury in the rat brain 24 h (P6) and 72 h (P8) following IL-1 β injection

Treatment	Saline	Saline+PBN	IL-1 β	IL-1 β +PBN
P6				
Pyknotic cells				
Parietal cortex	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	202.2 $\pm$ 30.3 *	71.1 $\pm$ 5.6 *#
APP stained axons				
Cingulum area (%)	0.28 $\pm$ 0.04	0.26 $\pm$ 0.04	4.29 $\pm$ 0.97 *	0.24 $\pm$ 0.03 #
Parietal cortex (%)	0.07 $\pm$ 0.02	0.08 $\pm$ 0.02	2.08 $\pm$ 0.12 *	0.10 $\pm$ 0.04 #
Caudate putamen (%)	0.32 $\pm$ 0.03	0.29 $\pm$ 0.04	3.03 $\pm$ 0.08 *	0.41 $\pm$ 0.08 #
MAP2 stained dendrites				
Cingulum area (%)	22.9 $\pm$ 0.8	21.9 $\pm$ 0.8	12.8 $\pm$ 1.9 *	23.1 $\pm$ 1.0 #
Dopamine Neurons				
TH+ cells in SN	65.1 $\pm$ 2.4	63.9 $\pm$ 2.8	52.9 $\pm$ 1.6 *	61.8 $\pm$ 1.4 #
NeuN+ cells in SN	85.3 $\pm$ 2.8	86.1 $\pm$ 1.9	82.9 $\pm$ 2.3	85.8 $\pm$ 1.8
TH+ cells in VTA	123.1 $\pm$ 3.3	124.7 $\pm$ 3.5	80.4 $\pm$ 7.3 *	118.6 $\pm$ 3.2 #
Activated Microglia in cortex				
OX42+	28.9 $\pm$ 6.4	24.4 $\pm$ 4.1	242.2 $\pm$ 24.7 *	102.2 $\pm$ 12.3 *#
Lectin+	33.3 $\pm$ 5.7	35.6 $\pm$ 5.6	442.2 $\pm$ 28.7 *	235.6 $\pm$ 30.5 *#
ED1+	20.0 $\pm$ 5.7	17.8 $\pm$ 5.6	175.6 $\pm$ 15.9 *	73.3 $\pm$ 8.3 *#
P8				
MAP1 stained axons and dendrites in parietal cortex				
Length (μ m)	158.1 $\pm$ 5.1	156.1 $\pm$ 5.4	44.7 $\pm$ 2.7 *	132.5 $\pm$ 3.8 *#
Neurons in parietal cortex				
Number of NeuN cell	1584.4 $\pm$ 73.2	1626.7 $\pm$ 78.6	1782.2 $\pm$ 77.5	1638.8 $\pm$ 71.6
Activated Microglia in cortex				
OX42+	42.4 $\pm$ 7.2	40.0 $\pm$ 2.9	44.4 $\pm$ 8.9	37.8 $\pm$ 8.0
Lectin+	37.8 $\pm$ 8.1	42.2 $\pm$ 10.6	44.4 $\pm$ 10.7	40.0 $\pm$ 9.7
ED1+	20.0 $\pm$ 5.7	17.7 $\pm$ 6.6	24.4 $\pm$ 4.1	22.2 $\pm$ 4.4

Note. Intracerebral (i.c.) injection of IL-1 β and intraperitoneal (i.p.) PBN treatment were performed as described in the text. Immunostaining (except for TH staining) was performed in sections from the forebrain at the bregma level. APP and MAP2 staining were performed in the cingulum area, parietal cortical area, or caudate putamen in the P6 rat brain. Data are presented as mean $\pm$ SEM (% of area) of six animals for each group. Cell counting of 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. 7B) 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 levels 1/4 and 1/3 rostral from the lambda to the bregma. MAP1 staining was performed in sections from the forebrain at the bregma level. Data are presented as the mean $\pm$ SEM (cells/mm² or μ m) of six animals for each group and 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 IL-1 β group.