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Spin Trapping Agent, Phenyl *N-tert*-butylnitrone, Reduces Nitric Oxide Production in the Rat Brain During Experimental Meningitis

HIROKAZU ENDOH[a,b], NAOKI KATO[a,b], SATOSHI FUJII[a], YASUHIRO SUZUKI[a,c], SHINYA SATO[b], TAKAMASA KAYAMA[b], YASHIGE KOTAKE[d] and TETSUHIKO YOSHIMURA[a,*]

[a]*Institute for Life Support Technology, Yamagata Public Corporation for the Development of Industry, 2-2-1 Matsuei, Yamagata 990-2473, Japan;* [b]*Department of Neurosurgery, Yamagata University School of Medicine, Yamagata, Japan;* [c]*Department of Neurosurgery, Tohoku University School of Medicine, Sendai, Japan;* [d]*Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, USA*

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Phenyl *N-tert*-butylnitrone (PBN) is a spin trapping agent previously shown to exert a neuroprotective effect in infant rat brain during bacterial meningitis. In the present study, we investigated the effect of systemic PBN administration on nitric oxide (NO) production in a rat model of experimental meningitis induced by lipopolysaccharide (LPS). We assessed the NO concentration in rat brain tissues with an electron paramagnetic resonance (EPR) NO trapping technique. In this model, rats receiving intracisternal LPS administration showed symptoms of meningitis and cerebrospinal fluid (CSF) pleocytosis. The time course study indicated that the concentration of NO in the brain reached the maximum level 8.5 h after injection of LPS, and returned to the control level 24 h after the injection. When various doses of PBN (125–400 mg/kg) were injected intraperitoneally 30 min prior to LPS, NO production in the brain was reduced with increasing PBN dose (250 mg/kg suppressed 80% at 8.5 h after LPS injection), and white blood cells (WBC) in CSF were significantly decreased. We concluded that reduction of NO generation during bacterial meningitis contributes to the neuroprotective effect of PBN in addition to its possible direct scavenging of reactive oxygen intermediate (ROI).

Keywords: Nitric oxide; Meningitis; Phenyl *N-tert*-butylnitrone; Electron paramagnetic resonance; Spin trapping; Lipopolysaccharide

INTRODUCTION

It is now recognized that nitric oxide (NO), which is produced form L-arginine by NO synthases (NOSs), plays pivotal roles in the physiology and

*Corresponding author. Tel.: +81-23-647-3133. Fax: +81-23-647-3138. E-mail: yoshi@ymgt-tcchno.or.jp

pathophysiology of central nervous system (CNS).[1] In the brain, constitutively expressed isoforms of NOS (cNOS) are found in vascular endothelial cells (eNOS) and in neutral cells (nNOS). NO produced from eNOS mediates the regulation of cerebral blood flow and vascular tone, and that from nNOS is a signaling molecule for synaptic potentiation and plasticity.[2–5] The other isoform of NOS, inducible isoform (iNOS) is expressed through inflammatory mediators such as cytokines and lipopolysaccharide (LPS) in a variety of cells including macrophages, monocytes, endothelial cells and parenchymal cells.[6] NO from iNOS is often over-produced in inflammation, and in brain, this excessive NO synthesis is considered to cause symptoms in various neurodegenerative diseases.

Bacterial meningitis results in serious CNS damage.[7–9] Depending on the type of bacterial pathogen and the age of patients, the fatality rate still ranges as high as 25 and up to 50% of survivors are left with long-term neurological sequelae.[7] There is increasing evidence that excessive NO synthesis is involved in various phases during bacterial meningitis.[10–15] Indeed, an elevated concentration of nitrite, a stable metabolite of NO, was detected in the cerebrospinal fluid (CSF) of both pediatric patients[14] and animal models[10] of bacterial meningitis. In animal models of bacterial meningitis, the administration of NOS inhibitors has been shown to protect from pathophysiological alterations such as increase in regional cerebral blood flow and intracranial pressure; disruption of the blood–brain barrier (BBB) and blood–CSF barrier.[10] Recently, we reported the direct evidence of *in vivo* NO production and iNOS mRNA expression in rat brain during experimental bacterial meningitis by using electron paramagnetic resonance (EPR) spectroscopy and reverse transcriptase–polymerase chain reaction (RT-PCR), respectively.[16] Therefore, it seems very likely that the excess NO production from iNOS contributes to the pathophysiology of meningitis.

During the last decade, phenyl *N-tert*-butylnitrone (PBN), which is widely used as a spin trapping agent, has been shown to have beneficial pharmacologic effects on oxidative injury in animal models.[17] Preadministration of PBN protects rodents against tissue damage from ischemia/reperfusion, traumatic, and excitotoxic shock, and reduces the mortality associated with endotoxin shock.[18] It has been suggested that the protection mechanism of PBN is detoxification of reactive oxygen intermediates (ROI) by trapping them, that is, forming less reactive species. However, solid evidence for this notion has been obtained. Pogrebniak *et al.* demonstrated that PBN protection from endotoxin-mediated death in mice was associated with down-regulation of some cytokine genes, and suggested that the PBN's protective mechanism is dual; ROI scavenging and cytokine down-regulation.[19] We have shown that PBN inhibited iNOS expression but not iNOS enzyme activity,[20,21] suggesting that the decrease in iNOS expression is essential for improving survival. Leib *et al.* reported that PBN prevents cerebral ischemia and necrotic and apoptotic neuronal injury in an infant rat model of bacterial meningitis, and suggested that ROI is a major contributor to those injuries in their model.[22] However, whether PBN affects NO production in the CNS during bacterial meningitis has not been investigated. In other studies using a rodent model of global brain ischemia, the administration of a high dose PBN exerted neuroprotective effects, but at the same time, caused systemic hypothermia lasting for long time.[23–25] These studies claim that the beneficial effect of PBN are, in part, attributable to the indirect effect from low body temperature.[23–26]

The objective of this study was to investigate the effects of PBN on NO production in the rat brain during experimental bacterial meningitis. We directly measured NO production in brain using an *in vivo* NO trapping technique with iron–diethyldithiocarbamate (Fe–DETC) complex as an NO trapping agent. We also

investigated the dependence of the time and dosage of PBN administration on brain NO production, following induction of bacterial meningitis by intracisternal injection of LPS.

MATERIALS AND METHODS

Chemicals

Sources of an NO trapping agent, iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), sodium *N,N*-diethyldithiocarbamate trihydrate ($DETC \cdot Na \cdot 3H_2O$), and sodium citrate were purchased from Wako Pure Chemicals (Osaka, Japan). PBN was obtained from OMRF Spin Trap Source (Oklahoma City OK). *Escherichia coli* LPS (Serotype 055:B5) and N^G-monomethyl-L-arginine (NMMA) were purchased from Sigma Chemical (St Louis, MO). Other chemicals were of the highest grade commercially available and were used without further purification.

Induction of Experimental Meningitis

Female Spraguc–Dawley rats weighing about 200 g were anesthetized briefly with sodium pentobarbital and positioned within a stereotaxic head frame. Experimental meningitis was induced by intracisternal (i.c.) injection of 200 μg LPS in 2 μl of sterile artificial cerebrospinal fluid (aCSF; composition: 120 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.67 mM NaH_2PO_4, 0.3 mM Na_2HPO_4; pH 7.4). LPS or aCSF in 10 μl Hamilton syringe was administered by stereotaxic puncture of the atlanto-occipital membrane covering the cisterna magna. Rats were allowed to recover in their cages with free access to food and water. All animal-use procedures described herein were performed according to the criteria outlined in the guideline for animal experimentation by Japanese Association for Laboratory Animal Science, 1987.

NO Trapping and EPR Measurements

NO produced in the rat brain was measured by an NO trapping technique with X-band (~9.4 GHz) electron paramagnetic resonance (EPR) spectroscopy.[27–29] A trapping agent, Fe–DETC complex [composed of DETC. Na $3H_2O$ solution (400 mg/kg) and an Fe-citrate mixture (40 mg/ml of $FeSO_4 \cdot 7H_2O$ and 200 mg/ml of sodium citrate)] was used to detect endogenously produced NO. The solutions of DETC and Fe-citrate were injected intraperitoneally (i.p.) and subcutaneously, respectively, 30 min prior to brain sampling. The NO trap was administered at 0, 3.5, 6.5, 8.5, 12.5 and 24.5 h after i.c. LPS administration. 30 min after the injection of the NO trap, rats were decapitated under deep anesthesia. The right cerebral hemisphere was immediately excised and placed in a 5 ml plastic syringe attached to an infusion tube. The sample was extruded into a glass capillary tube (75 mm in length, 46 μl inner volume) and then inserted into EPR quartz tube (o.d. 5 mm). The EPR signal height, which is proportional to the amount of NO generated, was obtained from the peak-to-peak height of lower field side signal in the three-line EPR spectrum (Fig. 1). The NO adduct concentration of Fe–DETC complex was calculated using the EPR signal height obtained from an aqueous solution of authentic NO–Fe–DETC complex.[30] EPR spectra were recorded at ambient temperature with an EPR spectrometer (~9.5 GHz; TE-200, JEOL, Tokyo). The instrument settings were: field scan range, 10 mT; sweep time, 4 min; time constant, 0.3 s; modulation amplitude, 0.32 mT; modulation frequency, 100 kHz; microwave power, 60 mW.

Effect of PBN

Rats were injected i.p. with saline solutions of PBN, following the procedures reported previously.[20] PBN was administered at specified time before or after i.c. injection of LPS, and after the specified time lapsed, NO trapping

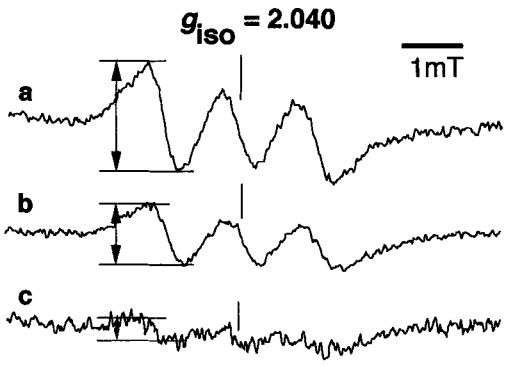

FIGURE 1 Representative X-band EPR spectra of the NO–iron–*N,N*-diethyldithiocarbamate (NO–Fe–DETC) complex observed at ambient temperature in the rat brain tissues during experimental meningitis, which was induced by the i.c. injection of LPS (*E. coli*). (a) EPR spectrum was recorded at 8.5 h after the administration of 200 μg LPS in 2 μl of sterile artificial cerebrospinal fluid (aCSF). The NO trapping reagent (Fe–DETC complex) was injected 30 min before taking measurements. (b) and (c) Rat was injected i.p. with PBN (125, 250 mg/kg, respectively) 30 min prior to LPS administration and followed the same protocol as (a). The signal height, which is proportional to the amount of NO generated was obtained by measuring the peak-to-peak height of lower field side signal (arrows) in the three-line spectrum. The instrument settings were as follows: microwave frequency, ~9.5 GHz; field scan, 10 mT; sweep time, 4 min; time constant, 0.3 s; modulation amplitude, 0.32 mT; modulation frequency, 100 kHz; microwave power, 60 mW.

agent (iron and DETC) was administered, and 0.5 h later the brain sample was subjected to EPR measurement. To investigate the dependence of brain NO production on PBN dose, we employed three different i.p. doses of PBN, (125, 250, 400 mg/kg) in saline. In these experiments, brain NO was determined 8 h after LPS injection.

Time dependence of PBN injection on NO generation was also studied. PBN was administered i.p. 0.5 h before or 3 h after i.c. LPS injection. The dose of PBN for these experiments was selected as 250 mg/kg based on the result of dose dependence study. DETC solution and Fe-citrate mixture were administered at 8 h after LPS injection. For comparison, NMMA (50 mg/kg), an NOS inhibitor, instead of PBN, was administered i.p. 0.5 h before or 3 h after LPS injection.

Determination of CSF White Blood Cells, Blood Pressure, and Body Temperature

The CSF was aspirated from the cisterna magna *via* puncture of the atlanto-occipital membrane 8.5 h after i.c. LPS administration (200 μg). White blood cell count (WBC) was determined with a hemocytometer in an aliquot of the CSF stained with Samson's Reagent Solution. Mean arterial pressure (MAP) was monitored in the left femoral artery after cannulation. The arterial cannula containing heparinized saline was connected to a pressure transducer (P23XL, Spectra Med, USA). MAP was monitored 0, 4, and 8.5 h after i.c. injection of LPS.

Body temperatures in treated rats were measured using a Bollman restriction cage after the recovery from anesthesia. The rectal temperature was monitored with an animal temperature controller (ATB-1100, Nihon–Koden, Tokyo), and the temporalis muscle temperature was measured with a needle microprobe (N454, YSI, Ohio). In some experiments, the rectal temperature was maintained between 37 and 37.5°C using a feed-back controlled heating lamp over the animal (ATB-1100, Nihon–Koden, Tokyo).

Statistical Analysis

Data are expressed as mean±SD. Statistical analyses were carried out using one way of variance (ANOVA). The *t*-test with two-tailed Student's *t*-distribution was employed as a post test. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Physiological Variables

After i.c. inoculation of LPS (200 μg), rats exhibited tufted fur, lethargy and somnolence: in contrast, aCSF-treated (i.c.) rats were alert and

active and showed typical rodent behaviors (e.g. cage exploration and grooming). WBC penetration into CSF (a hallmark of bacterial meningitis[9]) was found (842±83 cells/μl; $n = 3$) 8.5 h after LPS administration. However, when PBN (250 mg/kg) was injected i.p. 30 min prior to LPS, the number of WBCs in CSF significantly reduced (494±69 cells/μl; $n = 3$). No WBC was detected in CSF of control rat receiving aCSF. These observations suggest that the bacterial meningitis was clearly induced in rats treated i.c. with LPS; and that pretreatment with PBN significantly improved CSF pleocytosis.

Intracisternally administered LPS induced a slight but insignificant decrease in MAP during the experiment (baseline, 108±9 mmHg, $n = 6$; 8.5 h after LPS injection, 106±6 mmHg, $n = 6$). The MAP was not affected by PBN preadministration (baseline, 109±6 mmHg, $n = 6$; 8.5 h after the injection of LPS, 107±4 mm Hg, $n = 6$).

Both rectal and temporalis muscle temperatures decreased by 2°C 8–9 h after the injection of PBN alone (250 mg/kg, $n = 3$) and maximal decrease was about 3.5°C, 2–3 h after the injection. This drop in temperature was in agreement with the previous results obtained in rats or mice treated with PBN.[23,26] It has been reported that hypothermia induced by a high dose of PBN lasted for more than 7 h.[23–26] The body temperature in animals treated with LPS combined with the preadministration of PBN (250 mg/kg) showed the same temperature drop as in the animals treated with PBN alone, suggesting that hypothermia is an essential function of PBN.

NO Production During Bacterial Meningitis

To detect NO production during bacterial meningitis, we employed the NO spin trapping technique with EPR spectroscopy. Fe–DETC complex, used as the NO trapping agent, forms a stable NO adduct (NO–Fe–DETC) to show a characteristic three-line EPR signal at room temperature.[27] The signal height of NO–Fe–DETC complex is proportional to the amount of NO generated in the brain.[31,32] The sequential changes in brain NO production during bacterial meningitis after i.c. injection of LPS (200 μg) were followed by the signal of NO–Fe–DETC complex formed in the brain (Fig. 1a).

The signal intensities, which are presented as mean±SD ($n = 6$), gradually increased after LPS administration, and reached a peak at 8.5 h, and then decreased to the basal levels within 24 h after i.c. LPS administration (open circles in Fig. 2). This time course of brain NO formation is similar to that observed in a rat LPS-induced sepsis[32] and a rat meningitis model induced by intraventricular administration of LPS plus interferon-γ (IFN-γ).[16] The signal intensity at 8.5 h corresponds to 16 nmole/g tissue/30 min of NO–Fe–DETC complex.

Effects of PBN on NO Generation

The effect of PBN on NO generation was examined at 8.5 h after LPS injection, at that time the NO production reached maximum level (Fig. 2). Preadministration of PBN indeed reduced the NO production, as indicated by the reduced EPR spectral intensity (Fig. 1b,c) and this reduction was dose dependent. As shown in Fig. 3, the dose of 125, 250, and 400 mg/kg of PBN decreased NO production by 51, 80, and 80%, respectively ($n = 6$ each) at 8.5 h after LPS injection. At 6.5 h, preadministration of PBN (250 mg/kg) reduced 82% of NO production (closed circle in Fig. 2). When rectal temperature was maintained at 37–37.5°C, the reduction of NO production went down to 69% at 8.5 h.

The effect of postadministration of PBN was also examined. When PBN (250 mg/kg) was administered to rats 3 h after LPS,[20] the amount of NO at 8.5 h after LPS was the same level as that of control rats receiving no PBN (Fig. 4). These

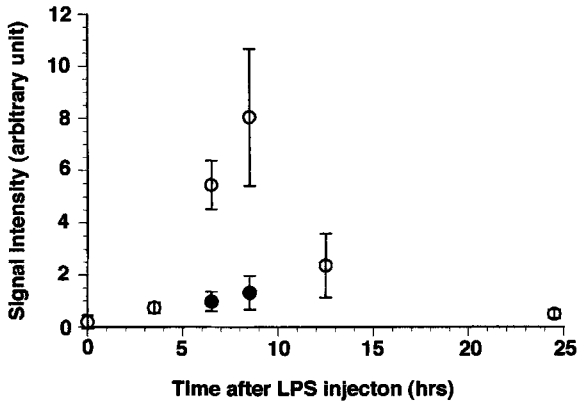

FIGURE 2 Sequential changes of NO amount in the rat brain tissues during experimental meningitis induced by the injection of LPS (*E. coli*). Open circles: X-band EPR spectra were recorded at 0, 3.5, 6.5, 8.5, 10.5, 12.5 and 24.5 h after the i.c. injection of LPS (200 μg/rat) ($n = 6$, mean±SD). The NO trapping reagent) (Fe–DETC complex) was injected 30 min before taking measurements. Closed circles: Rat was injected i.p. with PBN (250 mg/kg) 30 min prior to LPS administration. EPR spectra were recorded at 6.5 and 8.5 h after the injection of LPS ($n = 6$, mean±SD). The signal was not observed in the brain tissue of sham operated group ($n = 3$) and aCSF-treated control group ($n = 3$).

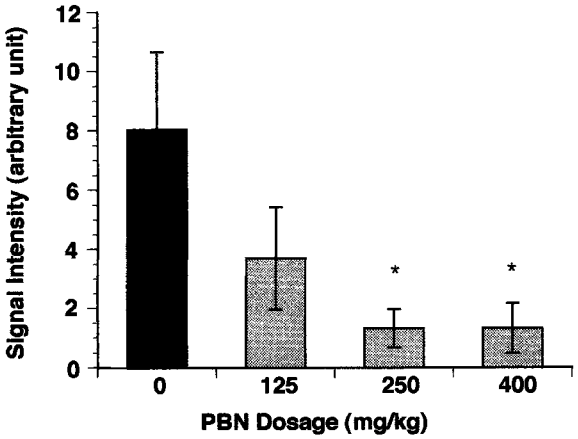

FIGURE 3 The effect of PBN dosage on NO amount in the rat brain tissues during experimental meningitis induced by the injection of LPS (*E. coli*). Three different doses of PBN, i.e. 125, 250 and 400 mg/kg in saline were administered i.p. 0.5 h before LPS (200 μg/rat) injection. The EPR spectra were recorded at 8.5 h after LPS injection. The NO trapping reagent (Fe–DETC complex) was injected 30 min before taking measurements. Data are expressed as mean±SD for $n = 6$ and * denotes that the value has $p<0.05$ vs. LPS-treated control.

inhibition effect are different from those of the typical NOS inhibitor, NMMA as shown in Fig. 4 ($n = 6$). Preadministration of NMMA (50 mg/kg) 0.5 h prior to LPS showed scarcely any inhibition of NO generation, whereas postadministration of NMMA 3 h after LPS resulted in 63% inhibition.

DISCUSSION

In summarizing the present experiments, we have shown that PBN systemic preadministration significantly decreased NO level in the brain of rats which received i.c. injection of LPS. Brain NO level reached at the maximum level 8.5 h after administration. The degree of NO down-regulation was augmented as the PBN dose was raised, and we obtained the maximum reduction at the PBN dose of 250 mg/kg. PBN was most effective when it was administered 0.5 h before LPS injection. We also observed that LPS-mediated increase of WBC counts in CSF was suppressed by PBN, and confirmed the previous observation of PBN's inductibility of hypothermia.

Although the pathophysiological roles of NO in CNS still appear ambiguous, accumulating evidence supports the premise that NO is involved in the pathophysiology of bacterial meningitis such as: (1) pial arteriolar dilation,[10] (2) increases in cerebral blood flow,[10,33] intracranial pressure and brain water content,[10] and (3) disruption of BBB and B-CSF barriers.[12,34] Excessive NO production in the brain has been shown in several models of bacterial meningitis, and implicated to the pathophysiology.[10–16] It seems reasonable to speculate that the source of brain NO in bacterial meningitis is iNOS. Recently, we showed the direct evidence of brain NO production in a rat meningitis model, using *in vivo* EPR spectroscopy, and observed increased iNOS mRNA expression with RT-PCR.[16] In addition, we have previously reported that PBN inhibits the expression of iNOS gene and protein, resulting in the reduction of systemic NO level.[19,20] The requirements of

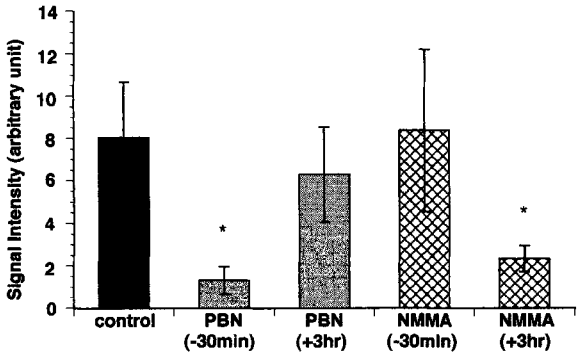

FIGURE 4 The comparison of the effect of the NO inhibition between PBN and NMMA in the rat brain tissues during experimental meningitis induced by the injection of LPS (*E. coli*). Control: LPS (200 μg/rat) was injected i.c. The EPR spectra were recorded at 8.5 h after LPS injection. The NO trapping reagent (Fe–DETC complex) was injected 30 min before taking measurements. PBN (−30 min) and PBN (+3 h): the rat was injected i.p. with PBN (250 mg/kg) 30 min before or 3 h after LPS (200 μg/rat) administration and followed the same protocol as Control. NMMA (−30 min) and NMMA (+3 hr): the rat was injected i.p. with NMMA (50 mg/kg) 30 min before or 3 h after LPS (200 μg/rat) administration and followed the same protocol as Control. Data are expressed as mean ± SD for $n = 6$ and * denotes that the value has $p < 0.05$ vs LPS-treated control.

PBN preadministration and NMMA postadministration to decrease NO level in both brain and liver, strongly support that NO in the brain in the present meningitis model is produced from iNOS.

For many years, PBN has been widely used for the detection of ROI in chemical and biological systems. However, since the finding of beneficial effects of PBN on oxidative injury in animal models, pharmacological effects of PBN have been vigorously investigated.[17] One of the most striking effects may be that preadministration of PBN can reduce the endotoxin-induced mortality in rodents. The protective effects from endotoxin-induced lethality seen in PBN have been suggested due to the reduction of ROI-induced lipid peroxidation and tissue damage. Recent studies also suggest that PBN interferes with nuclear factor kappaB (NF-κB) activation and cytokine synthesis,[35] and induces hypothermia.[23–26] In addition to brain NO determination, we demonstrated here that PBN-preadministration reduced WBC migration into the CSF (i.e. pleocytosis), and induced systemic hypothermia. The decrease in WBC counts is considered to be typical function of anti-inflammatory drugs. In other experiments, we demonstrated that PBN-mediated decrease of brain NO was reversed by physically keeping the body temperature normal, suggesting that PBN-induced hypothermia is a contributing factor for the reduction of brain NO.

Although the mechanism by which systemic PBN reduces iNOS expression in brain is not yet understood, the fact that systemic PBN can pass BBB and enter into the brain[34] could give a clue for the understanding. Because PBN is capable of suppressing iNOS expression in macrophages, it is likely that PBN inhibits iNOS expression in the brain macrophage, microglial cells. As we suggested previously, the reduced expression of iNOS protein is likely to be the result of PBN's inhibition of the activation of NF-κB[21] which is essential for the transcription of iNOS mRNA. NF-κB has been repeatedly shown to be down-regulated by antioxidants such as dithiocarbamates and thiols,[36,37] therefore, it is suggested that ROI are involved in NF-κB activation.[36] It is tempting to speculate that PBN scavenges ROI and inhibits the activation of NF-κB. However, this assumption has not been proven. Leib *et al.* reported that the protective effects of PBN on neuronal injury, and concluded that PBN trapping of ROI and consequent decrease in oxidative damages to tissues or membranes could be a major cause of the protection.[22] Because excessive NO has damaging effects to brain cells, we propose that the PBN-mediated reduction of iNOS protein and subsequent decrease of NO generation also pays a significant role in the neuroprotective effect.

In conclusion, we have demonstrated that PBN reduces NO production in brain during LPS-induced experimental bacterial meningitis in rats. The decrease of NO production may be largely associated with the inhibitory effect of PBN on iNOS mRNA expression.

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