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SPIN TRAPPING AGENT, PHENYL N-*TERT*-BUTYL NITRONE, INHIBITS INDUCTION OF NITRIC OXIDE SYNTHASE IN ENDOTOXIN-INDUCED SHOCK IN MICE

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SUMMARY: Spin trapping agent, phenyl N-*tert*-butyl nitrone (PBN) significantly reduces mortality due to lipopolysaccharide (LPS)-induced shock in Balb/c mice as had previously been shown in rats. We hypothesized that PBN decreases mortality by directly or indirectly inhibiting nitric oxide (NO) generation. Therefore, we determined the effect of PBN administration on LPS-induced NO generation in mice. NO generation was monitored in the mouse liver after administration of LPS by an *in vivo* NO-spin trapping, followed by *ex vivo* EPR measurement. When the mice was treated with PBN 0.5hr before LPS administration, NO generation in the liver was reduced by 80%. However, when PBN was given 3hrs after LPS, NO generation did not change. Both pre- or post-administration of NO synthase (NOS) inhibitor, N^G-monomethyl-L-arginine inhibited the NO generation. Western blotting of inducible NOS (iNOS) in mouse liver cytosol obtained from PBN-pretreated animals demonstrated a decreased expression of iNOS, indicating the reduction in NO generation was caused by the decrease in the amount of enzyme present but not by the inhibition of iNOS enzyme activity *per se*.

Preadministration of the spin trapping agent (spin trap), phenyl N-*tert*-butyl nitrone (PBN) has been shown to reduce the mortality associated with endotoxin shock in the rat (1-3). It has been suggested that the mortality reduction is due to PBN's ability to trap oxygen radical species thus forming spin adducts, *i.e.*, less reactive species. However, the administration of spin traps after endotoxin has no effect in reducing mortality, suggesting that a mechanism other than free radical trapping is occurring. PBN has demonstrated many *in vivo* pharmacological effects. Chronic administration of PBN has been shown to reverse age-related parameters in Mongolian gerbils (4). PBN also has been shown to alleviate ischemia-reperfusion injury in the brain (5) and decrease

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The abbreviations used are: NO, nitric oxide; PBN, phenyl N-*tert*-butylnitrone; LPS, lipopolysaccharide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; NMMA, N^G-momomethyl L-arginine; EPR, electron paramagnetic resonance; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel.

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myocardial infarction size in ischemia-reperfusion in dog heart (6). It virtually removed liver edema in carbon tetrachloride intoxicated rats (7). But whether there exists a common mechanism to these pharmacological effects is not known.

Attempts have been made to utilize PBN-type spin traps for the detection of NO generation in biological systems (8). However, interactions of spin traps with the process involved in NO generation has not been investigated. In this study, based on the two facts: A). dramatic elevation of NO generation in many organs occurs in endotoxin shock (9-12), B). PBN can decrease endotoxin-shock causing mortality (1-3), we hypothesized that PBN may suppress NO over-production resulting in mortality reduction. Therefore, we have determined the effect of PBN on NO generation and on inducible NO synthase (iNOS) expression in the liver of endotoxin-treated mice. Direct NO quantitation was performed using *in vivo* NO-spin trapping technique whereby the amount of NO present is quantitated as the Fe-NO complex. Effects of PBN on the amount of iNOS in the liver were also determined.

MATERIALS and METHODS

Materials and reagents: PBN and NO-spin trap, sodium N-methyl-D-glucamine dithiocarbamate (sodium MGD) was purchased from OMRF Spin Trap Source (Oklahoma City, OK). Lipopolysaccharide (LPS, *E. coli*), N^G-monomethyl-L-arginine (NMMA) and other chemicals were purchased from Sigma Chemical Co. (St.Louis, MO). The NO-spin trap solution was prepared by mixing saline solutions of sodium MGD (100 mM) and $FeSO_4$ (10 mM) under nitrogen atmosphere. The solution was taken into 1 ml syringe and injected into mice without exposing it to air. Balb/c mice were purchased from Harlan (Indianapolis, IN).

Mortality measurement: Since mortality reduction had previously been demonstrated in rats (1-3), we determined the same occurs in mice. Adult male Balb/c mice (average weight 20 g) were administered with an i.p. LPS dose of 1.0 mg/20 g in 0.5 ml saline solution. This dose was selected because it was found to be a lethal dose in 24 hrs. Control group received saline (0.5 ml) 0.5 hr before LPS administration instead of PBN. PBN was administered i.p. 6 mg/20 g (in 0.5 ml saline solution) 0.5 hr before or 3 hrs after LPS administration. Five animals were tested for each group and lethality was judged at 24 hrs after LPS injection.

In vivo NO-spin trapping measurement: *In vivo* NO-spin trapping measurement was carried out according to the method of Lai *et al.*(13) with modification. Adult male Balb/c mice (average weight 20g) were i.p. injected with LPS (1.0 mg/20 g) and in 6hrs, NO-spin trap which consists of MGD-Fe complex solution (MGD:100mM, $FeSO_4$:10mM, volume 0.3ml) was i.p.-administered. In 0.5 hr after the NO-spin trap administration, mice were euthanized and a liver specimen obtained. The liver specimen was immediately placed in an EPR tissue cell (sample volume is 5 x 10 x 0.5 mm, Wilmad, WG-806) and a Bruker ER300E EPR spectrometer (X-band) was used to record the EPR spectra. The liver specimen was loaded to the groove (25 μl) such as to completely fill the volume and pressure was applied to a cover glass. The excess tissue was removed and the sample-cell was covered with a quartz cover glass. The EPR intensity can be directly compared because it was normalized to an equal volume of specimen from each animal. Typical spectrometer settings are as follows: field modulation frequency; 100 kHz, modulation amplitude; 2.0 gauss, microwave power; 20 mW, time constant; 0.1 s, field sweep; 100 gauss/84 s. Saline solution of PBN or NMMA was administered i.p. 0.5 hr before or 3 hr after LPS injection and the signal intensity of the NO adduct was compared.

Determination of iNOS by Western blotting: Western blotting technique was used to quantitate iNOS in the liver of PBN-treated mice according to the method of Laemmli *et al.* (14) Mouse liver was obtained 6 hrs after LPS injection and homogenized in the cell lysis buffer with protease inhibitors. After centrifugation (13,000 x g for 10 min), the supernatant was used for total protein assay and SDS-PAGE analysis. After electrophoresis, band-separated proteins were transferred to a nitrocellulose membrane (MSI, Westboro, MA). A blot was blocked in a blocking buffer and incubated with anti-mouse iNOS antibody (Transduction Lab. Lexington, KY) followed by washing and incubation with alkaline phosphatase conjugated anti-mouse IgG antibody (Sigma). The blot was stained with Sigma *Fast* BCIP/NBT. The total amount of protein in the isolated cytosolic fraction was determined using a protein assay kit (Sigma) according to Lowry's method.
Statistical Analysis: Results are expressed as the mean ± s.e.m. for n separate experiments. Statistical analyses were carried out using ANOVA (analysis of variance: one way) for these mean values; a p value < 0.05 was taken as statistically significant.

RESULTS

Mortality: Mortality rate was measured in mice which had received PBN pre- and post-administration of LPS. As shown in Table 1, the mice which had received PBN 0.5 hr before LPS administration all survived (n=5) more than 24 hrs after a lethal dose of LPS. In contrast, none of the animals which had not received PBN (n=5) survived. One of those that received PBN 3 hrs after LPS administration survived (n=5).

In vivo spin trapping of NO: Intense EPR spectra were obtained from the liver of mice 6 hrs after LPS administration (Fig. 1A). This spectrum is assigned to the mononitrosyl complex of MGD-Fe spin trap (NO-MGD-Fe). The three line indicates the presence of hyperfine interaction between the unpaired electron and the nitrogen nucleus in NO. Deformation of the lowest field line is probably due to immobilization of NO-MGD-Fe in a viscous tissue. The EPR intensity obtained is proportional to the amount of NO present in a finite volume of liver tissue because the volume of the monitored liver specimen was constant (25 µl). No signal was obtained in liver if LPS was not administered (Fig. 1F). The EPR signal became detectable in 2 hrs after LPS injection. The signal

Table 1: Mortality of LPS administered mice determined in 24 hrs and the effect of pre- and post-treatment by PBN

		Control	PBN Pre-treatment	PBN Post-treatment
Treatment at:	*t=-0.5 hr*	saline (0.5ml)	PBN (6mg/20g)	none
	t= 0	LPS (1mg/20g)	LPS (1mg/20g)	LPS (1mg/20g)
	t= 3 hr	none	none	PBN (6mg/20g)
24 hrs survivors / Number of subjects		0/5	5/5	1/5

Mice recieved i.p. injection of LPS or PBN dissolved in 0.5 ml saline.

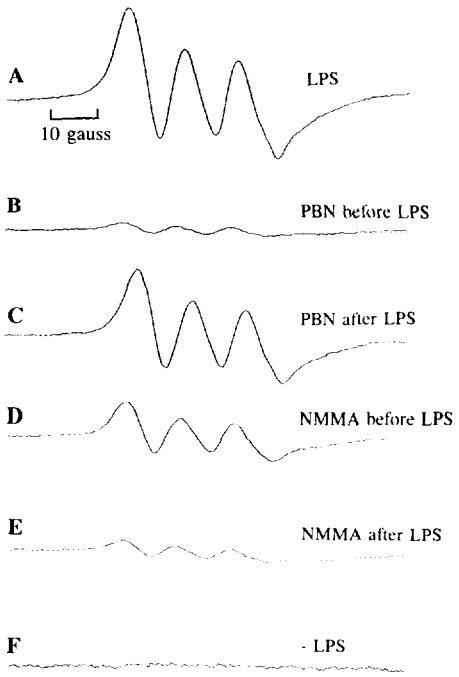

Fig. 1. EPR spectra of NO complex detected by the NO-spin trapping method in the mouse liver. Mice were treated with LPS, PBN or L-NMMA, followed by i.p. injection of NO-spin trap (Fe-MGD) solution. The liver tissue was mounted on an EPR tissue cell (25 μl in volume) and the EPR spectra were recorded. **A:** The mouse received LPS (1.0mg/20g) i.p. and in 5.5hrs, Fe-MGD (100 mM MGD, 10 mM $FeSO_4$ in 0.3 ml saline) was i.p. injected, and the EPR spectrum of the liver specimen was obtained. **B:** The mouse was i.p. injected with PBN (6.0mg/20g in 0.5 ml saline) 0.5hr before LPS administration and followed the same protocol as A. **C:** Same as B except PBN was injected 3hrs after LPS administration. **D:** NMMA (1.0 mg/20g) was administered 0.5hr before LPS. **E:** NMMA (1.0 mg/20g) was administered 3hrs after LPS. **F:** The animal received only Fe-MGD 0.5hr before its liver was examined by EPR (x2 sensitivity).

intensity continued to rise up to 5 hrs (data not shown). When PBN (6 mg/20g) was administered 0.5 hr before LPS (1.0 mg/20g) injection, the signal intensity after 6 hrs was decreased by 80% compared to those not receiving PBN (Fig.1B). Decrease in NO generation by PBN became significant at the dose more than 2mg/20g (data not shown). When PBN was given 3 hrs after LPS, virtually no change in the EPR intensity was obtained compared to the control group (Fig. 1C). The NOS inhibitor, NMMA, reduced the signal intensity when it was injected either before or after LPS administration (Figs. 1D and 1E). Figure 2 summarizes the results.

Determination of amounts of iNOS: Expression of iNOS was determined by Western blotting in the liver obtained from the LPS treated mouse (Fig.3). We detected three bands (40, 90, 130 kDa) (Fig. 3) in LPS-treated mice. Intrinsic iNOS band at 130kDa is thin, however, the lower molecular weight bands (40, 90 kDa) recognized by this antibody most likely due to a truncated and/or degradation

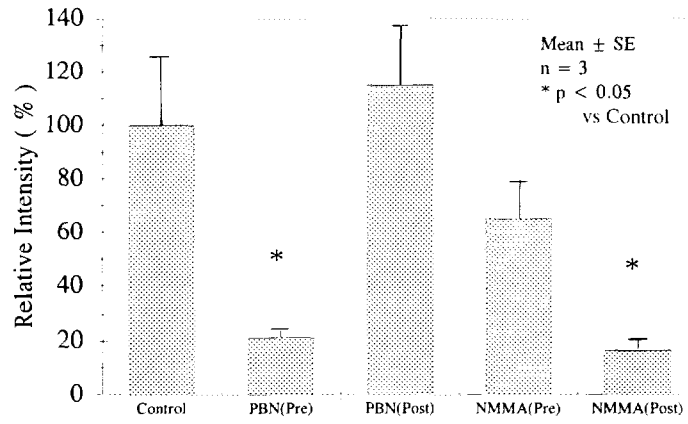

Fig. 2. EPR spectral intensity of NO complex detected by the NO-spin trapping method in the mouse liver. The intesity is proportional to the amount of NO in the liver produced in 6hrs after the animal received LPS. The unit EPR intensity corresponds to approximately 2μM of NO trapped in the liver. Data are expressed as mean±s.e.m. for n=3 and * denotes that the value has $p<0.05$ *vs.* control. **Control:** LPS (1.0mg/20g) was i.p. administered and in 5.5hrs, Fe-MGD (100 mM MGD, 10 mM $FeSO_4$ in 0.3 ml saline) was i.p. injected and the EPR intensity of the liver specimen was measured. **PBN(Pre):** The mouse was i.p. injected with PBN (6.0mg/20g in 0.5ml saline) 0.5hr before LPS administration and followed the same protocol as control. **PBN(Post):** Same as PBN(Pre) except PBN was injected 3hrs after LPS administration. **NMMA(Pre):** L-NMMA (1.0 mg/20g) was i.p. administered 0.5hr before LPS. **NMMA(Post):** L-NMMA (1.0mg/20g) was i.p. administered 3hrs after LPS.

products of iNOS which is in agreement with the findings reported previously (15-17). When PBN was administered 0.5 hr before LPS injection, iNOS expression seen in the density of the blots at 40, 90, and 130 kD was weaker than the PBN non-treatment group (Fig. 3). An iNOS blot from the liver cytosol obtained from mice treated with PBN 3hrs after LPS showed the similar expresiion of iNOS to the LPS treated animals, indicating that post-treatment by PBN is not effective in suppressing the expression of iNOS.

DISCUSSION

PBN has been widely used in free radical research as a spin trapping agent, but it has been shown to have a wide range of pharmacological effects. For example, previous studies have demonstrated that administration of PBN or other similar spin traps to rats before a lethal dose of endotoxin administration significantly reduced the mortality rate (1-3). The present experiments have shown that this is also true for mice. Because PBN as an effective trapping agent can scavenge and transform oxygen radicals to less reactive ones, this trapping capability is postulated to be a common underlying mechanism of its pharmacological actions. However, detailed mechanisms of PBN's pharmacological effects are not understood.

In endotoxin-treated animals, increasing evidence indicates that enhanced NO generation plays a major role in determining the pathological outcome (9-12). Thus, various approaches have

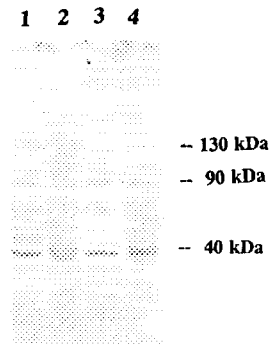

Fig. 3. Western blotting for cytosolic fraction of the liver obtained from mice treated with LPS and PBN. Liver cytosols with normalized amounts of total proteins were seeded on SDS-PAGE, transferred, blocked, and blotted with mouse monoclonal anti-iNOS antibody, and finally treated with alkaline phosphatase-conjugated anti-IgG antibody and then stained with BCIP/NBT. **Lane 1 and 4:** Blots from liver cytosol obtained 6 hrs after the mouse was treated with LPS (1.0 mg/20g, i.p.), showing 130, 90, and 40 kDa bands from iNOS. **Lane 2:** PBN (6.0 mg/20g) was administered 0.5hr before LPS and the liver cytosol was obtained in 6 hrs. **Lane 3:** PBN(6.0mg/20g) was administered 3 hrs after LPS and in 3hrs the liver cytosol was obtained.

been attempted to suppress enhanced NO levels to alleviate symptoms such as septic hypotension. The present study clearly shows that administration of PBN to mice before endotoxin administration can suppress the rise of NO generation in the liver, and that the reduction of NO generation is due to suppression of iNOS induction but not due to the inhibition of the NOS enzyme activity *per se*. In contrast, reduction of NO by NOS inhibitor, L-NMMA was less when it was administered before LPS than that after LPS (Fig. 2). The sequence of events after endotoxin administration has so far revealed that A). endotoxin induces cytokines and interferon (INF) γ from macrophages and T-cells (12), B). these factors induces expression of NOS-mRNA through activation of transcription factors such as, nuclear factor kappa B (NfκB) (18), and C). iNOS is expressed high levels. Usually, these induction steps are finished within a few hrs after endotoxin administration in mice. The fact that administration of PBN 3hrs after LPS was not effective in either reducing mortality nor decreasing the amount of NO as well as iNOS present indicates that PBN interacts with these induction steps to achieve the effect. Pogrebniak *et al.*(19) observed cytokine down-regulation when they treated mice with PBN, 15 min before LPS administration and found concomitant rise in 72-hr survival. Cytokine down-regulation is an effective way of inhibiting NOS induction, supporting the notion that PBN inhibits the iNOS induction. NfκB has been shown to be up-regulated by pro-oxidants (20) and down-regulated by antioxidants (21), suggesting that iNOS down-regulation could be the result of anti-oxidant activity of PBN. However, at present it is not possible to identify the specific induction steps that PBN may alter.

Recently, clinical trials of NOS inhibitors has been performed for the therapy of septic hypotension (22-23). Available NOS inhibitors are usually non-specific to all NOS and its

administration results in contraction of the small blood vessels. Therefore, reagents which will inhibit iNOS but not constitutive NOS (cNOS) are suitable for the improvement of the hypotensive state. Specific inhibition of the induction of iNOS has the same advantage (24). Inhibition of iNOS induction by glucocorticoids (25-28), antibodies (29) and drugs (30) have been reported. However, these drugs have additional pharmacological activity. Because PBN has low toxicity (31) it has the potential to be of therapeutic use.

In conclusion, we have demonstrated that PBN contributes to the mortality reduction in a mouse model of endotoxin induced shock by decreasing NO generation. This decrease of NO generation is associated with the prevention of the expression of iNOS proteins.

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REFERENCES

1. Novelli, G. P., Angiolini, P., Consales, G., Lippi, R. and Tani, R. (1986) In: Oxygen Free Radicals in Shock. (Novelli, G. P.; Ursini, F., eds.) Krager, Basel, pp.119-124.
2. McKechnie, K., Furman, B. L. and Parratt, J. R. (1986) *Circ Shock*, **19**: 429-439.
3. Hamburger, S. A. and McCay, P. B. (1989) *Circ Shock* **29**: 329-334; 1989.
4. Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Landum. R. W., Cheng, M. S., Wu, J. F. and Floyd, R. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**: 3633-3636.
5. Floyd, R. A. (1990) *FASEB J.* **4**: 2587-2597.
6. Bolli, R., Patel, B.S., Jeroudi, M. O., Lai, E. K. and McCay, P. B. (1988) *J. Clin. Invest.* **82**: 476-485.
7. Janzen, E. G., Towner, R. A. and Yamashiro, S. (1990) *Free Rad. Res. Comm.* **6**: 325-335.
8. Pou, S., Keaton, L., Surichamorn, W., Frigillana, P. and Rosen, G. M. (1994) *Biochem. Biophys. Acta* **1201**: 118-124.
9. Joulou-Schaeffer, G., Gray, G. A., Fleming, I., Schott, C., Parratt, J. R. and Stoclet, J-C. (1990) *Am. J. Physiol.* **259**: H1038-1043.
10. Nava, E., Palmer, R. M. and Moncada, S. (1991) *Lancet* **338**: 1555-1557.
11. Kilbourn, R. G., Jubra, A., Gross S. S., Griffith, O. W., Levi, R., Adams, J. and Lodato, R. F. (1990) *Biochem. Biophys. Res. Commun.* **172**: 1132-1138.
12. Szabó, C. (1995) *New Horizon* **3**: 2-32.
13. Lai, C. -S. and Komarov, A. M. (1994) *FEBS Letters* **345**: 120-124.
14. Laemmli, U. K. (1970) *Natrure* **227**: 680-685.
15. Springall, D. R., Riveros-Moreno, V., Buttery, L., Suburo, A., Bishop, A. E., Merrett, M., Moncada, S. and Polak, J. M. (1992) *Histochemistry* **98**: 259-266.
16. Lloyd, R. V., Jin, L., Qian, X., Zhang, S. and Scheithauer, W. (1995) *Am. J. Pathol.* **146**: 86-94.
17. Nakane, M., Pollock, J. S., Klinghofer, V., Basha, F., Marsden, P. A., Hokari, A., Ogura, T., Esumi, H. and Carter, G. W. (1995) *Biochem. Biophys. Res. Commun.* **206**: 511-517.
18. Xie, Q-W., Whisnant, R. and Nathan, C. (1993) *J. Exp. Med.* **177**: 1779-1884.
19. Pogrebniak, H. W., Merino, M. J., Hahn, S. M., Mitchell, J. B. and Pass, H. I. (1992) *Surgery* **112**: 130-139.
20. Schreck, R., Rieber, P. and Baeuerle, P. A. (1991) *EMBO J.* **10** (8): 2247-2258.

21. Schreck, R., Meier, B., Männel, D. N., Dröge, W. and Baeuerle, P. A. (1992) *J. Exp. Med.* **175**: 1181-1194.
22. Petros, A., Bennett, D. and Vallance, P. (1991) *Lancet* **338**: 1557-1558.
23. Schilling, J., Cakmakci, M., Bättig, U. and Geroulanos, S. (1993) *Intensive Care Med.* **19**:227-231.
24. Fukuto, J. M. and Chaudhuri, G. (1995) *Ann. Rev. Pharmacol. Toxicol.* **35**: 165-194.
25. Wadler, C. E., Thiemermann, C. and Vane, J. R. (1991) *Br. J. Pharmacol.* **102**: 967-973.
26. Wright, C. E., Rees, D. D., and Moncada, S. (1992) *Cardiovascular Res.* **26**: 48-57.
27. Rees, D. D., Cellek, S., Palmar, R. M. J. and Moncada, S. (1990) *Biochem. Biophys. Res. Commun.* **173**: 541-547.
28. Momcada, S., Palmar, R. M. J., and Higgs, E.A. (1991) *Pharmacol. Reviews*, **43**: 109-142.
29. Hosford D., Paubert-Braquet, M. and Braquet, P. (1989) *Resuscitation* **18**: 207-218.
30. Palacios, M., Padron, J., Glaria, L., Rojas, A., Delgado, R., Knowles, R. and Moncada, S. (1993) *Biochem. Biophys. Res. Commun.* **196**: 280-286.
31. Janzen, E. G., Poyer, J. L., Schaefer, C. F., Downs, P. E., and DuBose, C. M. *J. Biochem. Biophys. Method.* (in press).