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Protective Effect of Spin Trap Agent, *N-tert*-butyl-α-phenylnitrone on Hyperoxia-induced Oxidative Stress and Its Potential As a Nitric Oxide Donor

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We have previously suggested that the spin trap agent, *N-tert*-butyl-α-phenylnitrone (PBN) can function not only as an antioxidant but also as a nitric oxide (NO) donor. To characterize the pharmacological activities of PBN against oxidative damage, we examined the effect of PBN on NO generation under hyperoxic conditions. The formation of NO in mice exposed to 95% oxygen was determined using a NOx analyzer and electron spin resonance (ESR). Levels of NOx, an oxidative product of NO, increased in the blood of mice under these conditions. However, the increase was returned to a normal level by the NOS (nitric oxide synthase) inhibitor, L-NMMA, indicating that the NO was formed via a biosynthetic pathway. In addition, ESR spectra of the liver and brain of control and experimental mice that were measured using $Fe(DETC)_2$ as an NO trap reagent showed strong ESR signals from NO complexes in the livers of mice exposed to 95% oxygen. When examining the effect of PBN in mice, PBN reduced the NOx formation in the blood under the same hyperoxic conditions. In addition, the ESR intensity of the NO complex was weaker in the PBN-treated mice than in the non-treated mice, showing that PBN possess anti-inflammatory properties. However, under a normal atmosphere, NOx and ESR analyses showed that NO levels increased in PBN-treated mice but not in control mice. These findings suggested that PBN functions as an NO donor under specific physiological conditions. PBN appears to protect against hyperoxia-induced NO toxicity by anti-inflammatory action rather than by serving as an NO donor.

Keywords: *N-tert*-butyl-α-phenylnitrone; Nitric oxide; Oxidative stress; Antioxidant; Hyperoxia

INTRODUCTION

Although *N-tert*-butyl-α-phenylnitrone (PBN), which is frequently applied as a spin trap agent in free radical research, has been shown to have several pharmacological actions against oxidative damage,[1–10] the anti oxidative action of PBN is not well understood. Recent reports have indicated that the action of PBN is related to the expression of various cytokine genes, as well as to the activation of the transcription factors nuclear factor κB (NFκB) and activator protein-1 (AP-1).[27,28]

Our studies have indicated that PBN and other nitrone spin traps can generate nitric oxide and improve physiological functions under oxidative conditions by serving as a nitric oxide donor rather than as a radical scavenger.[11–13] If PBN is indeed a nitric oxide source, this would be in good agreement with the finding that PBN can reverse age-related spatial memory,[5,6] because NO plays an important role in signal transduction in the central nervous system.[14,15] Although PBN is extremely stable in aqueous solution, PBN decomposes to release NO after reacting with free radicals,[11,12] which are known to be involved in cancer, aging and several diseases.[16–19] To understand the mechanism of PBN action, it is thus important to determine whether PBN releases NO under oxidative conditions *in vivo*. Hyperoxia appears to be the most

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fundamental cause of oxidative stress related to aging.[20–22] Gerschman *et al.* demonstrated that the life span of mice decreased after exposure to hyperoxic conditions and proposed that oxygen toxicity might be attributable to the formation of free radicals, which could then lead to destructive oxidation.[20] We have also reported that PBN administered to older animals prolonged the mean and maximum life span, suggesting that PBN improves physiological functions and may alter cell dys-differentiation.[13] These results indicate that aging, which is accelerated by free radicals, might be caused by the same physiological functions as hyperoxia *in vivo*. Thus, PBN may improve physiological functions by releasing NO under a hyperoxia-induced condition of destructive oxidation as well as by functioning as an antioxidant.

This report is a first attempt to detect NO release from PBN in tissues using electron spin resonance (ESR). Additional protective effects of PBN against hyperoxia and its potential to act as an NO donor are also discussed.

MATERIALS AND METHODS

Reagents

Diethyldithiocarbamate (DETC) and PBN were purchased from Sigma Chemical (St Louis, MO, USA). N^G-monomethyl-L-arginine (L-NMMA), citric acid, and iron (II) sulfate were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Pure oxygen gas was obtained from Sumitomo Seika (Tokyo, Japan)

Animals

Female ddY mice (5–6 weeks old) were obtained from SRL (Shizuoka, Japan) and housed five or one per cage, with a stainless steel feeder under standard conditions with a 12 h/light/dark cycle. Animals were allowed free access to water and a standard diet. The experimental protocol was conducted according to the Guidelines for Animal Experiments at the University of Shizuoka.

Oxygen Exposure

Mice were exposed to 95% oxygen at a flow rate of 2 l/min for 3 h or 3 days in plastic chambers with small air holes. Control mice were bred under a normal atmosphere.

Tissue Preparation

Mice were sacrificed by cervical dislocation. Blood, brain, liver and urine were collected 1 h after injecting the NO trap reagent and 0.5 mg of each tissue was immediately frozen in liquid nitrogen.

Spin Trapping of NO and ESR Measurement

To trap NO, 50 ml/25 g of $FeSO_4$ solution (10 mg/ml) containing citric acid (50 mg/ml) and 50 ml/25 g of DETC (100 mg/ml) were injected intraperitoneally and subcutaneously, respectively, 1 h before sacrifice. ESR spectra were measured at the temperature of liquid nitrogen using an X-band ESR (JEOL, FR30) spectrometer, operating at 9.5 GHz with 100 kHz field modulation. The modulation width was 0.25 mT for measuring NO adducts.

Determination of NOx Formation

NOx levels were measured in cardiac blood. NOx (total amount of nitrite and nitrate), an oxidative product of NO, was determined using a NOx analyzer (Eicom Inc., Tokyo, Japan) based on the Griess reaction (nitrate is reduced to nitrite that reacts with sulfanilamide and naphthylethylenediamine dichloride under acidic conditions, and NOx is determined spectrophotometrically at 540 nm).[11] The concentrations of NOx were corrected by reference to a standard curve obtained using sodium nitrite and sodium nitrate.

PBN (50 mg/kg) was injected to mice intraperitoneally 3 h prior to blood collection.

NOS Inhibitor Study

Determination of whether NO was biosynthesized was carried out by intraperitoneally injecting nitric oxide synthase (NOS) inhibitor, L-NMMA (50 mg/kg) 1 h prior to blood collection.

RESULTS

Effect of Hyperoxia on Nitric Oxide Formation in Mice

It is well known that NO is released *in vivo* by cells such as macrophages, endothelial cells and neurons. Therefore, weak ESR signals of NO complex (indicated with asterisks, $g = 2.04$), which were formed with $Fe(DETC)_2$, were seen in the livers of control mice that were bred under a normal atmosphere (Fig. 1A). These signals can be attributed to NO derived from normal biological cells *in vivo*. Using mice that were exposed to 95% oxygen for 3 h, strong triplet signals of the NO complex (indicated with asterisks, $g = 2.04$) were detected from the liver (Fig. 1B), indicating that hyperoxia affects NO generation *in vivo*. In contrast to the liver, NO complex signals were not detected

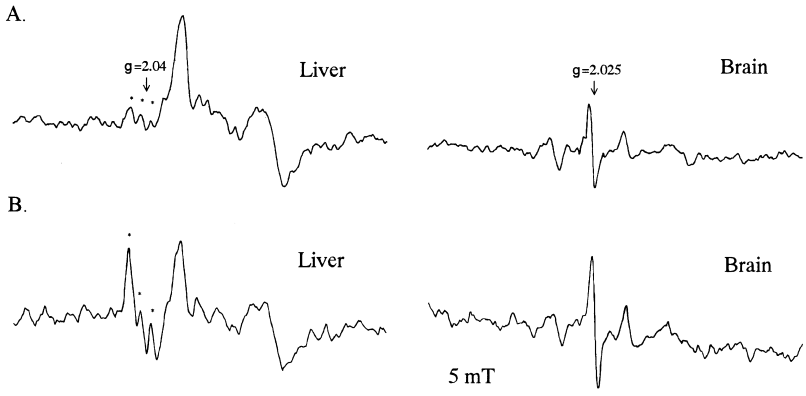

FIGURE 1 Effect of hyperoxia on ESR spectra in liver and brain of mice. (A) Mice were bred under normal atmosphere. (B) Mice were exposed to hypoxia for 3 h (95% oxygen at a flow rate of 2 l/min). An NO trap reagent, $Fe(DETC)_2$ was injected 1 h before sacrifice. Tissues were then collected and ESR spectra were measured at liquid nitrogen temperature with an X-band ESR spectrometer, operating at 9.5 GHz with 100 kHz field modulation of 0.25 mT.

in the brain (Fig. 1A, B). Strong ESR signals from both liver and brain (Fig. 1A, B) were assumed to be caused by $Cu(DETC)_2$ ($g = 2.025$), which is consistent with the literature.[24] The $Cu(DETC)_2$ signal of the brain under 95% oxygen (Fig. 1B) was more intense than that of the normal brain (Fig. 1A), suggesting that hyperoxia may affect reactive oxygen species (ROS) in the brain. The ESR spectra were measured at liquid nitrogen temperature using an X-band ESR spectrometer. The conditions under which the NO trap reagent was employed were as described in "Materials and Methods". We also determined the NOx formation in the blood of mice using a NOx analyzer (Fig. 2). Levels of NOx were higher in the blood of mice exposed to 95% oxygen for 3 h or 3 days than in the blood of controls, indicating that NO formation increased over time following exposure to hyperoxic conditions. These results were in agreement with the NO formation shown in Fig. 1, which was directly detected by ESR spectrometry.

Effect of the NOS Inhibitor, L-NMMA

Figure 3 shows the NOx formation in the blood of mice exposed to 95% oxygen for 3 h after an intraperitoneal injection of the NOS inhibitor, L-NMMA. Control NOx formation was suppressed by 50% in the presence of the NOS inhibitor, whereas the NOx formation in the blood of mice exposed to 95% oxygen was suppressed over 50% compared to the control level, indicating that NO levels were increased through biosynthesis. These data suggest that hyperoxia stimulates NO productive cells, such as macrophages and endothelium cells, and that the increase in NO is a transient reaction that returns to normal.

Effect of PBN on NOx Formation in Mice

To determine the effect of PBN on NO formation under hyperoxia, PBN was administered to mice and NOx concentration of the blood was measured using the NOx analyzer. Under a normal

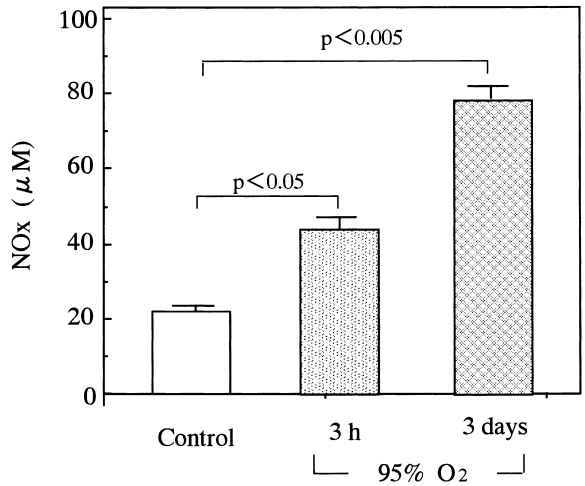

FIGURE 2 Effect of hyperoxia on NOx formation in blood of mice. Concentration of NOx in cardiac blood of mice exposed to hypoxia (95% O_2;21/min) was determined using a NOx analyzer ($n = 5$).

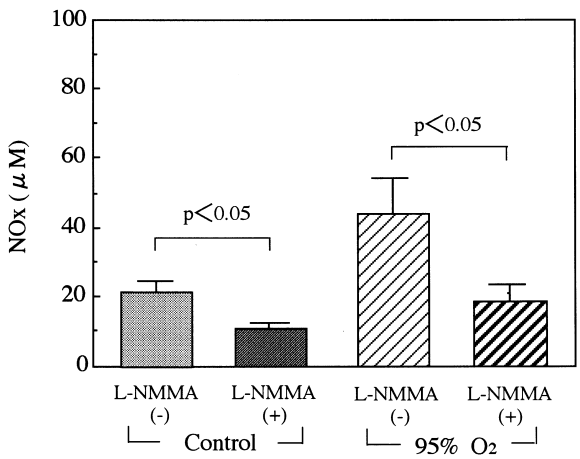

FIGURE 3 Effect of NOS inhibitor on NOx formation in blood of mice exposed to hypoxia for 3 h (95% O_2;21/min). NOx concentration of the blood was determined using a NOx analyzer ($n = 5$).

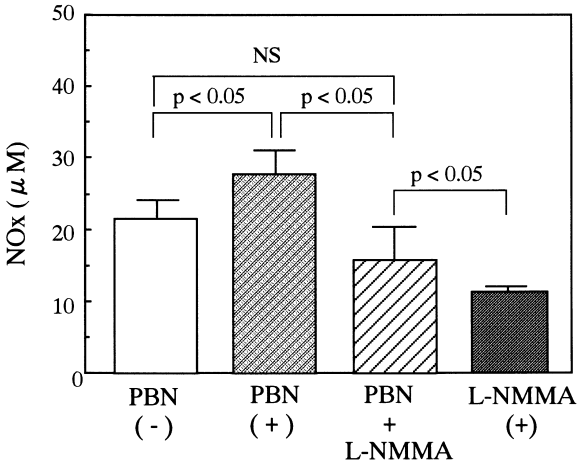

FIGURE 4 NOx formation in blood of mice bred and treated with PBN under a normal atmosphere. PBN (50 mg/kg) and L-NMMA (50 mg/kg) were injected intraperitoneally 3 and 1 h prior to blood collection, respectively. NS, No significance ($n = 5$).

atmosphere, NOx formation in the blood of mice administered with PBN and L-NMMA 3 and 1 h, respectively, before sacrifice is shown in Fig. 4. In the presence of PBN, NO formation in the mouse blood significantly increased. It initially appeared that PBN stimulated macrophages to generate NO for the immune response. However, following administration of L-NMMA, this NO formation did not reduce to the amount of NO in mice given L-NMMA alone, and was not transient. In addition, the significant difference was not seen in mice that were subjected to PBN prior to L-NMMA compared to that in control mice. These results indicate that the increase in NO formation was induced not by biosynthesis but by PBN. It is possible that small amounts of NO are released from PBN and/or that other PBN-activated mechanisms to increase NO formation are functioning. The effects of PBN on NOx formation in mice exposed to 95% oxygen for 3 h are shown in Fig. 5. In the presence of PBN, the increase in NOx formation in the blood was returned to normal

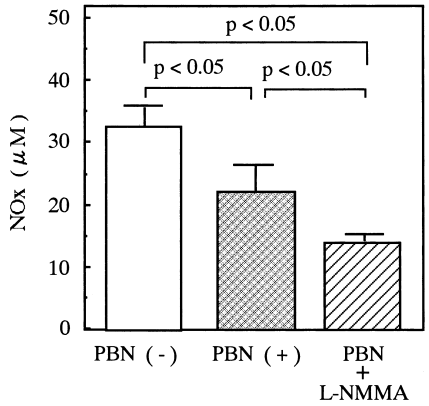

FIGURE 5 NOx formation in blood of mice treated with PBN exposed for 3 h to hypoxia (95% O_2; 2 l/min). PBN (50 mg/kg) and L-NMMA (50 mg/kg) were injected intraperitoneally 3 and 1 h prior to blood collection, respectively ($n = 5$).

levels, indicating that PBN had a protective effect against excess NO which is highly toxic *in vivo*. PBN is reported to reduce NO release from macrophages via NOS activity during endotoxin-induced inflammation due to the anti-oxidant activity of PBN.[25–27] The mechanism of NO generation in hyperoxia is apparently similar during the inflammation, indicating that PBN might act as an anti-oxidant to reduce excess NO *in vivo*.

ESR Spectra of Mice Administered with PBN

The effects of PBN on the ESR spectra of the liver and brain of mice exposed to 95% oxygen for 3 h are shown in Fig. 6. The administration of an NO trap reagent and ESR spectrometry proceeded as described in Fig. 1. Following the administration of PBN 3 h prior to sacrifice, the weakening of the significantly increased ESR signal of the NO complex in the liver indicated that PBN reduces NO generation induced by hyperoxia. This observation is in agreement with the results shown in Fig. 5. In contrast, the effect of PBN administration on the ESR spectra of the brains was insignificant. To examine the effect of PBN in mice under a normal atmosphere, we measured NO formation using ESR spectrometer. Figure 7 shows the ESR spectra of the livers and brains of mice bred under a normal atmosphere. PBN increased the ESR intensity of NO complex in the liver in comparison with controls, indicating that the effect of PBN was the same as that shown in Fig. 4. A small amount of NO complex was detected in the absence of PBN because NO is biosynthesized under normal conditions. However, the level of NO was significantly increased in the presence of PBN, perhaps partly because iron was injected after PBN as a spin trap agent. PBN is catalyzed by iron to release NO,[29] and our previous reports also suggested that PBN was decomposed to release NO by the Fenton reaction ($Fe^{2+} + H_2O_2$) *in vitro*,[11,12] suggesting that PBN releases NO according to specific physiological conditions. Although an ESR signal of the NO complex was not detected in the brains of mice in this experiment, PBN might affect the redox reaction, which would alter the NO function.

DISCUSSION

Gerschman *et al.* first proposed that oxygen toxicity might be caused by free radicals, which could then lead to destructive oxidation.[20] Oxygen-derived free radicals, such as superoxide, hydroxyl radical, as well as peroxyl radicals and oxidative events are implicated in altered cellular functions, and oxygen toxicity is partly a consequence of increased rates of

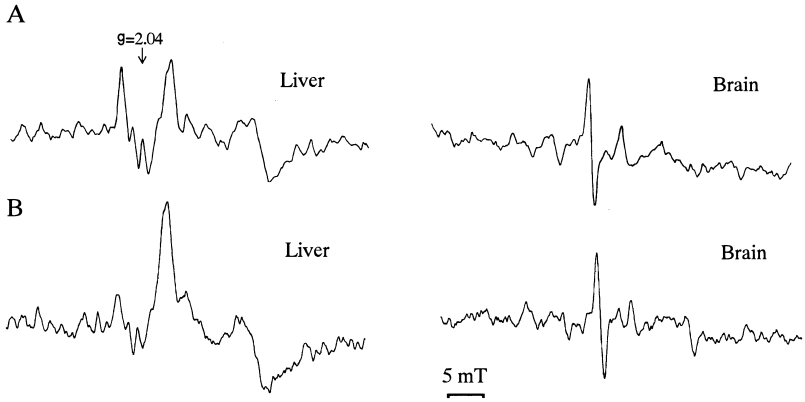

FIGURE 6 Effect of PBN on ESR spectra of the liver and brain of mice exposed to hypoxic condition. (A) PBN not administered; (B) PBN (50 mg/kg) injected intraperitoneally 3 h prior to blood collection. Mice were bred under 95% oxygen at a flow rate of 2 l/min. Administration of NO trap reagent and ESR spectrometry proceeded as described in the legend to Fig. 1.

intracellular superoxide and H_2O_2 production.[22] Thus, hyperoxia causes critical oxidative stress. The initial damage caused by hyperoxia consists of platelet aggregation in the alveoli pulmonis capillaries, which causes hypertension,[23] and stimulates the release of NO to relax the blood vessels. This phenomenon is evident in Fig. 1, indicating that under a hyperoxic condition, NO generation can be directly detected by ESR. Although, the amount of NO formed in the liver of mice was small under a normal atmosphere, NO formation in the liver was remarkably increased when exposed to 95% oxygen, indicating that NO was generated presumably to expand the blood vessels and normalize blood pressure. These physiological phenomena agree with the report by Miralles *et al.*, which described the expression of iNOS protein and mRNA in isolated rat liver that were induced by oxygen tension,[30] and the oxygen regulated expression of iNOS in the rat liver at the transductional level through the production of ROS. In fact, ROS are generated during hyperoxia.[31] Thus, Kupffer cells in the liver, which act like neutrophils, might release NO as a result of damage caused by hyperoxia. In addition, NO formation in the blood of mice exposed to 95% oxygen was inhibited by a NOS inhibitor, indicating that the increase in NO was induced by NOS, which might relate to ROS (Fig. 3). Furthermore, the ESR signal intensity of $Cu(DETC)_2$ in the brains of mice exposed to 95% oxygen was intense compared with that of mice under normal conditions, suggesting that hyperoxia affected the redox or altered functions in the brain (Fig. 1).

Although the pharmacological effects of PBN are typically credited to its radical scavenging activity, Kotake and co-workers recently demonstrated that PBN reduces iNOS mRNA-mediated NO generation when inflammation is induced by endotoxin, and suggested that its mechanism of action is related to a signal transduction factor, for example NFκB, a redox-sensitive transcription factor.[27,28] With respect to the effect of PBN under hyperoxic conditions, the mechanism of NO generation is apparently similar during inflammation, since the atmosphere of 95% oxygen that the mice were exposed to in the present study was highly toxic; NOx levels increased in a time dependent manner (Fig. 2). However, this increase was significantly reduced by administration of PBN (Fig. 5). When NO was measured directly, as shown in Fig. 6, consistent

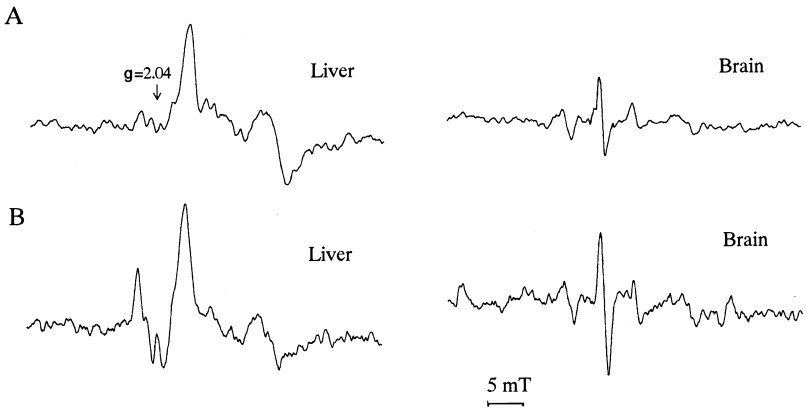

FIGURE 7 Effect of PBN on ESR spectra of the liver and brain of mice bred and exposed to a normal atmosphere. (A) PBN not administered; (B) PBN (50 mg/kg) injected intraperitoneally 3 h prior to blood collection. Administration of NO trap reagent and ESR spectrometry proceeded as described in the legend to Fig. 1.

results were obtained, suggesting that PBN acted as an anti-inflammatory agent. This would be associated with iNOS as well as endotoxin-induced shock, but whether PBN correlates with the expression of NOS mRNA remains unknown. NO generation by endotoxic shock induces cytokine and interferon production by macrophages and T-cells, which leads to NOS-mRNA expression through the activation of transcription factors. The reduction in NO formation might be due to the suppression of iNOS induction but not to the inhibition of NOS enzyme activity, and iNOS down-regulation might be the result of PBN antioxidant activity.[25] In other words, PBN does not act as an inhibitor of iNOS activity in endotoxin-induced shock. The results shown in Fig. 4 support this notion. Administration of PBN increased NOx formation in the blood of mice, even under a normal atmosphere, and this increase was reduced by a NOS inhibitor administered after PBN, suggesting that PBN does not inhibit the activity of NOS. PBN does not release NO without oxidation, since we found that PBN is degraded to release NO under oxidative conditions *in vitro*.[11,12] However, PBN was also seen to increase NOx formation in the rat brain under normal conditions by microdialysis (data not shown), indicating that a physiological function that decomposes PBN to release NO may exist *in vivo*. In addition, PBN might increase NO formation due to its superoxide scavenging action. Superoxide, which is produced in physiological conditions, reacts with NO to form peroxynitrite, and accordingly leads to a decrease in NO.[32] However, PBN can scavenge superoxide because of its ability as a spin trap agent. Inanami and Kuwabara demonstrated that administration of PBN increased the blood flow by inhibiting the breakdown of NO in anesthetized rats, and suggested the possibility of superoxide scavenging, which inactivates NO, by PBN.[33] Thus, the increase in NO observed in mice under a normal atmosphere might be due to the reduction of superoxide by PBN. As a result, it appears that PBN can flexibly regulate NO, and/or PBN may also affect the redox reaction to alter the function under both hyperoxic and normal conditions.

PBN has several pharmacological effects, but the mechanisms of its action remain unknown. Even if PBN has NO-releasing properties *in vivo*, NO released from PBN and NO derived from biological cells cannot be discriminated. To further investigate the mechanism of PBN as an NO donor under oxidative stress induced by conditions such as hyperoxia, NO released from PBN must be identified *in vivo*, and therefore our next objective is to synthesize ^{15}N-PBN for an ESR study that would differentiate between ^{14}NO and ^{15}NO spectra and determine ^{15}NO from PBN *in vivo*.

The results presented here suggest that PBN can regulate NO formation and affect the redox reaction to improve physiological functions under hyperoxic conditions.

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