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α-PHENYL N-TERT-BUTYL NITRONE (PBN) INCREASES THE CORTICAL CEREBRAL BLOOD FLOW BY INHIBITING THE BREAKDOWN OF NITRIC OXIDE IN ANESTHETIZED RATS

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The effects of intravenous administration of α-phenyl N-*tert*-butyl nitrone (PBN) on cortical cerebral blood flow (CBF) were examined in Wistar rats under pentobarbital anesthesia and artificial ventilation. The cortical CBF in parietal cortex was measured by laser Doppler flowmetry. Intravenous administrations of 2 mg/kg and 20 mg/kg of PBN dose-dependently produced significant increases in cortical CBF and decreases in systemic blood pressure (BP). To examine whether these increased responses in cortical CBF produced by PBN were associated with the vasodilatation system of nitric oxide (NO), the NO synthase inhibitor L-N^G-nitroarginine (L-NOArg), which is an analog of L-arginine, was used to inhibit the NO-related-vasodilatative system. Since the PBN-induced responses in the cortical CBF were much attenuated in L-NOArg-treated rats (30 mg/kg, iv.), it was inferred that NO-related vasodilatation was strongly associated with the PBN-induced increase in cortical CBF.

KEY WORDS: Cortical cerebral blood flow; L-N^G-nitroarginine; nitric oxide; α-phenyl N-*tert*-butyl nitrone; superoxide; vasodilatation.

INTRODUCTION

NO, a short-lived free radical formed from the terminal guanido nitrogen atoms of L-arginine by NO synthase, has been identified as the endothelium-derived relaxing factor (EDRF) and is a potent activator of guanylate cyclase.[1–3] It is involved in various vasodilatative systems *in vivo*, such as cerebral cortical vessels,[4,5] coronary arteries,[6] and renal vessels.[7] In experiments using vascular strips, it was reported that the relaxing response of NO was enhanced by the presence of superoxide dismutase (SOD), which could convert superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) by a disproportionation reaction.[3,8–10] This fact indicates that O_2^- produced in physiological conditions reacts with NO and inactivates it. It is generally accepted that O_2^- is produced not only in various pathological conditions but also in physiological conditions.[11] Rosen and Freeman[12] showed that endothelial cells have

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the ability to produce O_2^-. These phenomena lead us to assume that O_2^- and NO are factors regulating vascular tone *in vivo*.

Recently, Carney *et al.*[13] reported that the administration of PBN, a spin-trapping reagent, improved the abilities of temporal and spatial memory in aged gerbils. This phenomenon was accounted for by the fact that PBN protected biologically important molecules from oxidative damage by efficiently trapping oxygen radicals, including O_2^-. However, if one takes into consideration the fact that the reaction between O_2^- and NO may occur *in vivo* as described above, it is also likely that the scavenging of O_2^-, which inactivates NO, by PBN activates the NO-related-vasodilatative system, resulting in the improvement of cerebral circulation and thereby attenuates the deterioration of the central nervous system with aging. In this experiment, to clarify whether the interaction between oxygen free radicals and NO was involved in the regulation of cerebral circulation *in vivo*, the effects of intravenous administration of PBN on cortical cerebral CBF were examined in anesthetized rats with or without the NO synthase inhibitor L-N^G-nitroarginine (L-NOArg).

MATERIALS AND METHODS

Experiments were performed using healthy adult Wistar rats (9–12 months old, 380–420 g) anesthetized with pentobarbital (50 mg/kg, ip.). The trachea was cannulated and respiration was maintained by an artificial respirator (Harvard pump 681, USA). The right femoral vein and artery were catheterized for injection of drugs and measurement of systemic blood pressure, respectively. Rectal temperature was maintained at 37–38°C by means of a lamp and a heating pad. Systemic BP was monitored via a cannula inserted into the right femoral artery and connected to a pressure transducer. The animal was in a prone position on a stereotaxic instrument (SR-5, Narishige, Japan).

According to previous papers,[4,14] cortical CBF can be measured by laser Doppler flowmetry. After craniotomy, the recording probe (outer diameter: 1.0 mm) of a laser Doppler flowmeter (ALF1100, Advance, Japan) was placed on the primary somatosensory area in the parietal cortex, approximately 0.2 mm caudal from the bregma and 5.0 mm lateral from the midline according to Paxinos and Watson's atlas (1986).[15] This method allowed us to measure local CBF within a depth of approximately 1 mm just below the recording probe.[16]

PBN and L-NOArg were purchased from Aldrich Chemical Company, Inc. (Milwaukee). These drugs were dissolved in physiological saline.

RESULTS

Figure 1 shows the responses of cortical CBF in the parietal cortex and systemic BP to the intravenous administration of PBN. The cortical CBF increased after administration of 2 and 20 mg/kg of PBN, though it changed little after injection of 0.2 mg/kg of PBN. The CBF began to increase within 10–15 s after injection of 20 mg/kg and reached the maximum response from 1 to 2 min later. It then slowly returned to the control level within 60 min. In contrast, systemic BP decreased dose-dependently after administration of 2 and 20 mg/kg of PBN and began to decrease within 10–15 s after injection, a response that was a mirror image of that of cortical

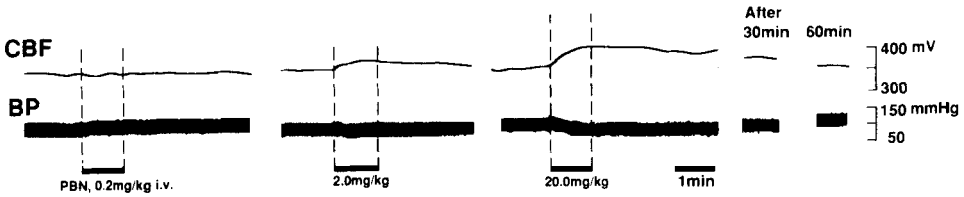

FIGURE 1 Recordings of rat cortical CBF (upper part) and systemic BF (lower part) following intravenous administration of PBN.

CBF. This then slowly recovered to the control level within 60 min. The amplitudes at the maximum responses of the cortical CBF and mean BP were expressed as a percentage of the control levels prior to the PBN injection.

Figure 2 summarizes the responses of cortical CBF (white column) and mean BP (hatched column) in 5 rats. The injection of 0.2 mg/kg of PBN caused little change in the cortical CBF or the mean BP. However, the injection of 2 and 20 mg/kg of PBN caused significant increases in cortical CBF, $+5.6 \pm 0.7\%$ and $+16.5 \pm 1.8\%$, respectively, and significant decreases in mean BP, $-8.2 \pm 1.2\%$ and $-17.6 \pm 2.9\%$, respectively. $PaCO_2$ of the arterial blood in preadministration and postadministration of PBN were 33.5 ± 4 mmHg and 32.7 ± 6 mmHg, respectively, and there was no significant difference between two groups. These facts indicated that PBN induced activation of the vasodilatative system.

The relationship between the NO-related vasodilatative system and the PBN-induced increase in cortical CBF was examined by employing the NO synthase inhibitor L-NOArg. In brain, it is known that NO is formed from L-arginine by cytosolic Ca^{2+}/calmoduline- and NADPH-dependent NO synthase,[17] and the N^G-nitro and N^G-monomethyl derivatives of L-arginine block the NO-synthesis by

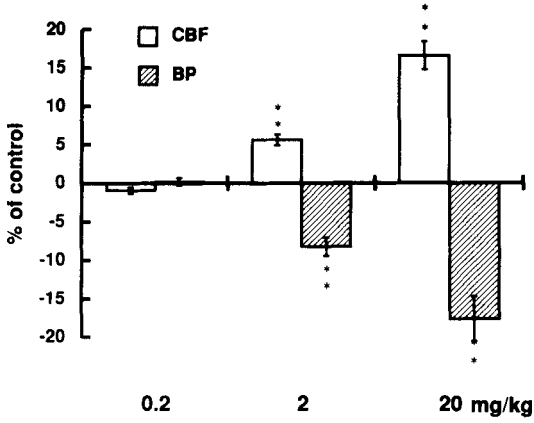

FIGURE 2 Percentages of increase in response of cortical CBF (white column) and decrease in response of mean BP (hatched column) following intravenous administration of PBN. The maximum values of CBF and the minimum values of BP after injection of PBN were taken to express the percentage of the preadministration control value. Columns indicate the mean response of all animal and bars give the SEM; ** indicates a significant difference with $p < 0.01$ in comparison with the preadministration control value examined by paired t-test.

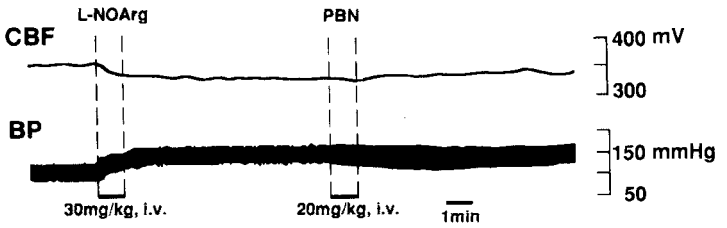

FIGURE 3 Recordings of cortical CBF (upper part) and systemic BP (lower part) following intravenous administration of PBN in L-NOArg-pretreated rats.

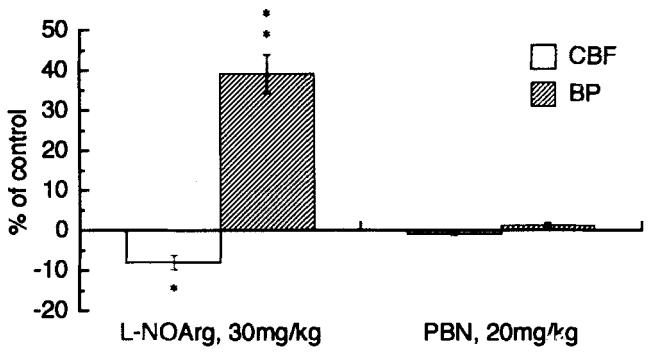

FIGURE 4 Responses of cortical CBF (white column) and mean BP (hatched column) to administrations of L-NOArg and PBN. The maximum values of CBF and the minimum values of BP after injection of L-NOArg or PBN were taken to express percentages of the preadministration control values. Columns indicate the mean response of each animal and bars gives the SEM; * and ** indicate significant differences with $p < 0.05$ and $p < 0.01$, respectively, in comparison with the pre-administration control value examined by paired t-test.

inhibiting the conversion reaction of L-arginine to L-citrulline. In the present study, the intravenous administration of 30 mg/kg of L-N^G-nitroarginine (L-NOArg) was used to inhibit the NO-vasodilatative system in brain, since this dose was sufficient to inhibit the NO-related vasodilatation in the parietal cortex evoked by the electrical stimulation of intrinsic cholinergic nerve fibers originating from the nucleus basalis of Meynert.[4] Five minutes prior to the administration of 20 mg/kg of PBN, 30 mg/kg of L-NOArg was intravenously injected into rats. The responses of cortical CBF and systemic BP to L-NO Arg before the administration of PBN are shown in Figure 3. The intravenous administration of L-NOArg induced a decrease in cortical CBF and an increase in systemic BP. This was interpreted as suggesting that the vasoconstrictive systems became dominant after the NO-related-vasodilatative system was inhibited. Similar responses have been reported in anesthetized rats[5] and rabbits.[18] The results obtained from the subsequent intravenous administration of PBN are also shown in Figure 3. The intravenous administration of 20 mg/kg of PBN caused no changes in cortical CBF and systemic BP.

The responses of cortical CBF (white column) and mean BP (hatched column) obtained from four rats are summarized in Figure 4. The injection of 30 mg/kg of

L-NOArg caused a significant decrease in cortical CBF, $-8.0 \pm 1.8\%$, and a significant increase in mean BP, $+39 \pm 4.8\%$. However, the injection of 20 mg/kg of PBN caused no changes in cortical CBF and BP in L-NOArg-pretreated rats.

DISCUSSION

It was reported that PBN ip. injected into rats penetrated the blood-brain barrier, that its biological half-life was about 134 min,[19] and that the ESR spectrum originating from the spin-adducts between PBN and lipid radicals in brain was observed after γ irradiation of PBN-administered rats.[20] Furthermore, Carney *et al.*[13] reported that chronic treatment with PBN caused a decrease in the level of oxidized proteins in aged gerbils and improved temporal and spatial memory.[13] These results suggested that systemically-injected PBN entered the brain and produced some beneficial effects in the central nervous system.

In our experiments, we demonstrated that intravenous administration of PBN dose-dependently produced significant increases in cortical CBF and decreases in systemic BP, and that PBN did not cause an increase in $PaCO_2$ which normally produces an increase in the cerebral blood flow. It was suggested that NO-related vasodilatation was associated with the PBN-induced increase in cortical CBF, since the PBN-induced responses in the CBF were abolished by pretreatment of L-NOArg. NO is known to be inactivated by the reaction with O_2^-.[2] Moreover, isolated endothelial cells and blood vessels spontaneously produce O_2^-.[3,8-10,12] Therefore, from the present experiments it was concluded that PBN trapped the spontaneously-produced O_2^-[21] and helped NO to survive, and, as a result, the relaxing action of NO became dominant in the circulation system. As for PBN, this is a typical nitrone spin-trapping reagent having the possibility to react with both free radicals O_2^- and NO. However, the reactivity of PBN to NO is assumed to be relatively low. A similar nitrone spin-trapping reagent, 5,5-dimethyl-l-pyrroline N-oxide (DMPO), did not prevent the aggregation of human platelets in which the NO metabolic pathway participated, whereas the nitroso compounds dibromo-4-nitrosobenzene-sulphonate (DBNBS) and 2-methyl-2-nitroso-propane (MNP), which are other types of spin-trapping reagents, inhibited the NO-related aggregation activity of platelets.[22] On the other hand, judging from the hydrophilic properties of spin-trapping reagents, it can be assumed that PBN is more likely to gain access to intracellular spaces and therefore is more easily able to scavenge O_2^- than DMPO. Therefore, it is proposed that PBN improves the cerebral circulation by inhibiting the removal of NO due to O_2^-. This should lead to a deceleration in deterioration of the central nervous system with aging by supplying nutrition to the brain.

Li *et al.*[23] demonstrated that infusion with PBN in open-chest dogs produced increased responses of the blood flow in the coronary artery. Recently, Konorev *et al.*[24] also reported the vasodilatative responses of the coronary artery by the infusion of PBN, POBN, MNP, MNP-OH and DMPO in the isolated rat heart model. As to the mechanism of vasodilative responses induced by PBN, Anderson *et al.*[25] reported that PBN could block calcium channels and induce vasodilation independent of free radicals such as NO or O_2^-. However, contrary to this mechanism of vasodilatation proposed by using peripheral vessels, our result indicated that NO-related vasodilation was associated with the PBN-induced increase in cortical CBF, because the preadministration of a NO-synthase inhibitor much attenuated its effect.

This contradiction of the mechanism of PBN-induced vasodilatation may be explained by the difference of the mechanism of NO-regulated vasodilatation between the cerebral vessels and the peripheral vessels. In the cerebral vessels *in vivo*, it was proposed that NO is produced from not only endothelial cells but also from nitroxidergic nerve fibers and glial cells existing outside the cerebral vessels and that this is related to vasodilative responses in the brain.[1] In view of our results, it can be assumed that the extent of the inactivation of NO by other free radicals such as O_2^- in the brain is much greater than that in the peripheral tissues. The source of NO and O_2^- in the central nervous system will be investigated further.

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