Protective Effect of Phenyl-*t*-Butylnitrone in Rats with Focal Cerebral Ischemia

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Phenyl-*t*-butylnitrone produces a potent neuroprotective effect in rats with focal cerebral ischemia modeled by distal occlusion of the middle cerebral artery. The infarction area markedly decreased after treatment with phenyl-*t*-butylnitrone. The content of phenyl-*t*-butylnitrone in the brain, liver, and kidneys was measured by the method of electron paramagnetic resonance.

Key Words: *middle cerebral artery occlusion; electron paramagnetic resonance; reactive oxygen species; phenyl-t-butylnitrone; neuroprotection*

Overproduction of short-living highly toxic reactive oxygen species (ROS) caused by disturbances in blood supply to the brain play an important role in the pathogenesis of ischemic brain injury and contributes to the development of oxidative stress [8,13]. ROS-neutralizing substances are used to protect the brain during ischemic and reperfusion stress. These substances include enzymes (superoxide dismutase, catalase, and peroxidase) [2,8,12] and low-molecular-weight ROS-trapping agents (ubiquinone, α-tocopherol, and spin traps) [6-8].

Here we studied the neuroprotective effect of free radical-trapping agent phenyl-*t*-butylnitrone (PBN) in rats with focal cerebral ischemia modeled by distal occlusion of the middle cerebral artery. Butylnitrones discovered 30 years ago are extensively used in analytical chemistry. Recent studies showed that PBN possesses neuroprotective activity [7,9,15] and traps various free radicals, including hydroxyl radical [10], superoxide anion radical, and alkoxyl radical [14]. PBN is a lipophilic compound that crosses the blood-brain barrier and can be detected in brain tissue 20 min after intraperitoneal administration [4,6]. PBN is easily metabolized and eliminated from the organism via the kidneys [4,6].

Ischemic brain injury is a complex and long process associated with overproduction of ROS [8]. Necrotic zone is formed 3 h after the onset of cerebral ischemia [2,13]. It is necessary to measure PBN content in brain tissue 3 h after middle cerebral artery occlusion (MCAO) accompanied by cascade ischemic pathobiochemical reactions.

In the present study PBN content in the brain and other organs was measured by the method of electron paramagnetic resonance (EPR). Paramagnetic spin adducts were detected after initiation of the Fenton's reaction. This method allows identifying noncatabolized PBN that effectively traps ROS in the brain.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 350-400 g. The animals were randomly divided into the control and 2 experimental groups (10 rats per group).

Focal cerebral ischemia was modeled by the method of Chen [5]. The rats was anesthetized with sodium ethaminal (40 mg/kg intraperitoneally). A median incision was made in the skin between the left auricle and left eye (2 cm). The incision was expanded, fibers of the temporal muscle were separated, and

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the surface of the skull was opened. A hole (2 mm) was made below a level of the zygomatic arch using a dental drilling machine. This procedure gave access to the point of intersection of the left middle cerebral artery and inferior cerebral vein. A special metal hook was introduced under the middle cerebral artery. Thermocoagulation of the artery was performed proximal to its bifurcation into the parietal and frontal branches. Termination of blood flow in the coagulated artery distal to the site of complete occlusion was estimated visually. Treatment was followed by the development of ischemia in the left cerebral hemisphere.

Group 1 rats intravenously received PBN in 1.5 ml physiological saline (100 mg/kg) during MCAO. The preparation dissolved in physiological saline was administered in similar doses after 24 and 48 h. Control animals received only physiological saline (1.5 ml) in the same periods.

Group 1 and control rats were decapitated 72 h after MCAO. The brain was removed. The volume of cerebral infarction was estimated by histochemical staining of frontal slices (1 mm) with 2,3,5-triphenyltetrazolium chloride (TTC) [3]. Stained slices were sub jected to bilateral scanning and computer planimetry with Mocha software (Jandel Scientific).

In group 2 rats a microdialyzer was implanted into the left lateral hypothalamus (coordinates 1-4-7) 1 day before MCAO [13]. The dialyzer was perfused with Ringer's solution containing 148 mM NaCl, 4 mM KCl, and 2.2 mM $CaCl_2$ (pH 7.4) at a flow rate of 2.5 μl/min using a CMA-100 perfusion pump. The solution was forced through an inlet silicone capillary (outer diameter 0.25 mm) into a semipermeable polyethylene membrane (pore permeability below 10 kDa) and then removed via an outlet capillary (0.15 mm). The rats received single intravenous injection of PBN before MCAO. The doses of PBN and physiological saline were similar to those in animals receiving single injection of the preparation. Two 20-min samples of the dialysate were collected 60-80 and 120-140 min after MCAO. Group 2 rats were decapitated after 3 h under ether anesthesia. The content of PBN was measured in samples from cerebral hemispheres, liver, and kidneys.

Tissue samples were placed in 100 mM formic acid (100 mg tissue per 1 ml solution), minced, and homogenized in a glass homogenizer for 10 min.

Calibration samples were prepared from minced tissue and solution of PBN in concentrations of 1 and 10 μg. Samples were extracted with 0.1 M formic acid (100 mg tissue per 1 ml formic acid) and homogenized [4].

The Fenton's reaction was initiated with an aqueous solution of $FeSO_4 \times 7H_2O$ and H_2O_2 (final concentrations 100 μM and 80 mM, respectively) before recording of EPR spectra.

EPR spectra were recorded on an E-109E X-range EPR spectrometer (Varian). The power and frequency of microwave radiation were 10 mW and 9.15 GHz, respectively. The amplitude of magnetic field modulation was 0.1 mT.

The data are expressed as means and errors. The results were analyzed by Student's *t* test.

RESULTS

Neuroprotective activity of PBN and its concentration in the brain (ipsilateral and contralateral hemispheres), liver, and kidneys in rats with ischemia were determined under standard experimental conditions.

The volume of infarction zone in control and treated rats was 22.51±3.03 and 12.81±1.74% ($p<0.001$) of the ipsilateral hemisphere volume, respectively. Therefore, PBN produced a neuroprotective effect under these experimental conditions.

EPR spectra for the PBN spin-adduct in physiological saline consisted of 3 pairs of narrow components, which illustrates rapid isotropic rotations of molecules (Fig. 1, *1*). The shape and ratio between intensities of components in EPR spectra of the spin adduct changed with the transition from physiological saline to tissues (Fig. 1, *2*). Probably, the adduct microenvironment in tissue samples became heterogeneous for various fractions. Under these conditions the rate of molecular movement was much lower than that in physiological saline.

The EPR study showed that PBN content in the kidneys (26.22±3.71 μg/g) markedly surpassed that in the contralateral hemisphere (20.68±3.96 μg/g), ipsilateral hemisphere (22.41±1.32 μg/g), and liver (8.80±2.85 μg/g). The main pathway for elimination

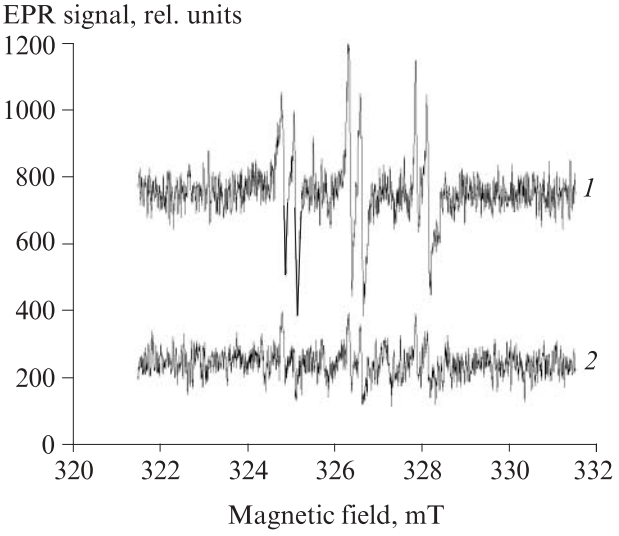

Fig. 1. EPR spectrum for spin PBN adduct in physiological saline (*1*) and brain tissue (*2*) in the presence of $FeSO_4 \times 7H_2O$ and H_2O_2.

of PBN (including non-catabolized substance) from the organism is urinary excretion, which probably contributes to the observed differences [5,6]. PBN undergoes pronounced metabolic transformations in the liver and loses the ability to trap ROS [11]. Therefore, the PBN adduct produced only a weak signal in this organ. It should be emphasized that PBN content in the brain tissue was slightly lower than in the kidneys, but higher than in the liver. Intravenously infused PBN is characterized by high biological accessibility for brain tissue (compared to intraperitoneal treatment). These differences probably contribute to the neuroprotective effect of PBN [9,15]. It is of considerable importance over the first hours of cerebral ischemia. In this period the development of moderate and reversible damage to penumbral neurons can be prevented by therapeutic treatment.

Signals of the PBN spin adduct were undetectable in dialysates collected 1 and 2 h after MCAO. It was probably associated with low content of PBN in the interstitium and cytosol and its fixation in membrane structures of the brain. Our assumption was confirmed by effective extraction of PBN from brain tissue with formic acid. Signals of the PBN spin adduct appeared after this procedure.

Our results show that PBN produces a neuroprotective effect during focal cerebral ischemia. The efficiency of PBN can be explained by its membrane localization, which determines protection of phospholipids from free radical oxidation.

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REFERENCES

1. H. Atamna, A. Paler-Martinez, and B. N. Ames, *J. Biol. Chem*., **275**, 6741-6748 (2001).
2. K. Baker, C. B. Marcus, K. Huffman, *et al*., *J. Pharmacol. Exp. Ther*., **284**, No. 1, 215-221 (1998).
3. J. B. Bederson, L. H. Pitts, S. M. Germano, *et al*., *Stroke*, **17**, 1304-1308 (1986).
4. G. M. Chen, T. M. Bray, E. G. Janzen, *et al*., *Free Radic. Res. Commun*., **9**, No. 3-6, 317-323 (1990).
5. S. T. Chen, C. Y. Hsu, E. L. Hogan, *et al*., *Stroke*, **17**, No. 4, 738-743 (1986).
6. R. A. Floyd, K. Hensley, M. J. Forster, *et al*., *Ann. N. Y. Acad. Sci*., **959**, No. 3, 321-329 (2002).
7. J. Folberqrova, Q. Zhao, K. Katsura, *et al*., *Proc. Natl. Acad. Sci. USA*, **92**, No. 11, 5057-5061 (1995).
8. Y. Gilgun-Sherki, Z. Rosenbaum, E. Melamed, *et al*., *Pharmacol. Rev*., **54**, No. 2, 271-284 (2002).
9. N. Marklund, T. Lewander, F. Clausen, *et al*., *J. Cerebral Blood Flow Metab.*, **21**, 1259-1267 (2001).
10. C. E. Thomas, D. F. Ohlweiler, A. A. Carr, *et al*., *J. Biol. Chem*., **271**, No. 6, 3097-3104 (1996).
11. L. A. Reinke, D. R. Moore, H. Sang, *et al*., *Free Radic. Biol. Med*., **28**, No. 3, 345-350 (2000).
12. K. Shimizu, N. Rajapakse, T. Horiguchi, *et al*., *Brain Res*., **963**, 8-14 (2003).
13. N. J. Solenski, A.-L. Kwan, and H. Yanamoto, *Stroke*, **28**, No. 12, 2545-2552 (1997).
14. N. Vrbjar, S. Zollner, R. F. Haseloff, *et al*., *Mol. Cell Biochem*., **186**, Nos. 1-2, 107-115 (1998).
15. S. Zausinger, E. Hungerhuber, A. Baethmann, *et al*., *Brain Res*., **863**, 94-105 (2000).