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The Protective Effects of PBN Against MPTP Toxicity Are Independent of Hydroxyl Radical Trapping

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FERGER, B., P. TEISMANN, C. D. EARL, K. KUSCHINSKY AND W. H. OERTEL. *The protective effects of PBN against MPTP toxicity are independent of hydroxyl radical trapping*. PHARMACOL BIOCHEM BEHAV **65**(3) 425–431, 2000.—To study the mechanism of the protective effect of the spin-trapping agent α-phenyl-*N-tert*-butyl nitrone (PBN) against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) toxicity hydroxyl radicals and functional parameters of neuroprotection were determined. C57BL/6 mice received PBN (100 mg/kg IP) over a time period of 15 days and on day 8 MPTP (40 mg/kg SC). On day 15 striatal levels of dopamine, serotonin, and metabolites were analyzed. For radical determination mice received a single injection of salicylic acid (SA) (100 mg/kg IP) in the time period of 0.5 h before to 72 h after MPTP injection. In vivo maximum hydroxyl radical levels indicated by 2,3-dihydroxybenzoic acid/SA ratios were obtained 4 h after MPTP injection, and were not affected by PBN treatment. However, the MPTP-induced mortality, reduction of locomotor activity, continuous loss of body weight, and striatal dopamine depletion were significantly less pronounced in PBN-treated animals. These results elucidate the time course of hydroxyl free radical formation in MPTP toxicity. PBN improved the functional parameters of neuroprotection against MPTP toxicity, but there is no evidence for hydroxyl radical scavenging properties to this effect. © 2000 Elsevier Science Inc.

Hydroxyl radicals   PBN   MPTP   Neuroprotection   Parkinson's disease   Salicylate trapping
Radical scavenger

ONE important factor contributing to neurodegeneration is that the central nervous system is unable to cope sufficiently with excessive free radicals. Reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide can lead to oxidative stress (17,38). The brain is particularly vulnerable to oxidative stress because of its high oxygen turnover, its relatively low levels of antioxidants (e.g., reduced form of glutathione), its relatively little protective enzyme activity (e.g., catalase, superoxide dismutase (SOD), glutathione peroxidase), and high endogenous ROS generation (e.g., by accumulation of iron and subsequent iron-catalyzed ROS formation) (8,25). These facts seem to be particularly relevant in the degeneration of nigrostriatal dopaminergic neurons, and it has been hypothesized that oxidative stress is implicated in the pathogenesis of Parkinson's disease (PD) (22,31). However, it is still under discussion whether oxidative stress is a major cause of cell death or simply a secondary phenomenon in PD (15).

To study the effects of the spin-trapping agent α-phenyl-*N-tert*-butyl nitrone (PBN) on functional parameters of neuroprotection and hydroxyl radicals, we used the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model, which is one of the most widely used models for PD (14).

Neuroprotective properties of PBN and its sulfophenyl analogue α-2-sulfophenyl-*N-tert*-butyl nitrone (S-PBN) could be shown mainly after acute application in various animal models of ischemia/reperfusion injury, for example, transient global ischemia (32), transient focal ischemia (12,42), permanent focal ischemia (4), and in a model of excitotoxicity (9,28,29,30,35, 36). Only a few studies of chronic PBN treatment have been

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performed. Chronic PBN treatment in old gerbils caused a decrease of oxidized protein levels, and led to an improvement of age-associated errors in a radial arm maze test comparable to results of much younger animals (5). More recently, protective effects against MPTP-induced deficits in motor activity after acute and subchronic PBN administration were shown (13). Most of the studies postulate that the protective effects are related to the radical scavenging ability of the nitrones, although in vivo studies are lacking to demonstrate the direct relationship between neuroprotection and hydroxyl radical scavenging capacity at the site of action. PBN was selected for the in vivo experiments in the MPTP mouse model because of its good blood–brain barrier penetration. We investigated the following parameters of MPTP toxicity: 1) PBN effects on MPTP-induced loss of body weight, reduction of locomotor activity and mortality; 2) PBN effects on striatal dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) levels in MPTP-treated and control animals; 3) time course of MPTP-induced hydroxyl radical formation in vivo; and 4) PBN effects on MPTP-induced hydroxyl radical formation in vivo.

METHOD

Animals

Adult male C57BL/6 mice weighing 20–25 g, approximately 3 months old (Charles River, Sulzfeld, Germany) were housed in groups of 10 to 12 under standardized conditions (temperature 23 ± 2°C, relative humidity 55 ± 5%, 12 L:12 D cycle, lights on at 0700 h), with free access to standard diet Altromin® (Altromin, Lage, Germany) and water.

The experimental protocols were approved by the appropriate institutional governmental agency (Regierungspräsident Gießen, Germany).

Experiment of MPTP-Induced Neurotoxicity

Saline (Sal) or PBN (100 mg/kg IP) were given twice daily (4–5 h apart) over a time period of 15 days. On day 8, MPTP (40 mg/kg SC) or saline was administered immediately after the first injection of PBN or saline. The experimental protocol consisted of four groups: the Sal+Sal group ($n = 13$), the Sal+MPTP group ($n = 19$), the PBN+Sal group ($n = 14$), and the PBN+MPTP group ($n = 13$).

Determination of Body Weight

To determine body weight alterations, animals were weighed every morning before the first injection.

Determination of Locomotor Activity

Locomotor activity measurements were performed in activity cages (50 × 50 × 35 cm) connected to an IBM computer that automatically counted the interruptions of the eight photobeams placed 2 cm above the bottom, with a sampling rate of about 50 Hz. Immediately after injection of saline or PBN, the mice were placed individually in the activity boxes in a randomized manner. After 5 min of habituation the locomotor activity was monitored for 60 min.

Tissue Preparation

Eight days after the subcutaneous MPTP or saline injection, mice were sacrificed by cervical dislocation. The brains were rapidly removed and immediately placed on an ice-cooled metal plate for dissection of the striatum. Immediately after dissection, the tissue was placed in a 2.0-ml tube, which contained 500 μl of 0.4 M ice-cooled perchloric acid, homogenized for 2 min at 20,000 rpm (Ultra Turrax model T5, Bachofer Reutlingen, Germany) and centrifuged 5000 rpm, 15 min, 4°C, Minifuge Heraeus, Germany). Aliquots of the supernatant were directly analyzed or frozen at −70°C.

HPLC Analysis

We developed an HPLC method to simultaneously separate salicylate (SA), 2,3- and 2,5-dihydroxybenzoic acid (DHBA), DA, its metabolites DOPAC, HVA, and well as 5-HT and its metabolite 5-HIAA.

Aliquots were injected by a metal-free injector (Rheodyne model 9125, 20-μl sample loop) into an HPLC system with UV detector (ERC 7210, Alteglofsheim, Germany), 295 nm wavelength, connected to an electrochemical detector (BAS LS 4C, Bioanalytical Systems, West Lafayette, IN). The detector potential was set at +750 mV using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase contained 0.14 g octane sulfonic acid sodium salt as an ion-pair reagent, 0.1 g disodium EDTA, 6 ml triethylamine, 35 ml acetonitrile in 1 liter of millipore Q® water. Concentrated phosphoric acid was used to adjust the pH to 2.8. An on-line degasser (CMA 260, Carnegie Medicin, Sweden) degassed the mobile phase before an inert HPLC-pump (CMA 250, Carnegie Medicin, Sweden) delivered the eluent at a rate of 0.6 ml/min onto a reversed-phase column (125 × 3 mm with precolumn 5 × 3 mm) filled with Nucleosil 120-3 C18 (Knauer, Berlin, Germany). Data were recorded by a two channel integrator (model 1022x, Perkin-Elmer, Norwalk, CT), and calibrated by use of external standards. The concentrations are given as ng/mg wet tissue weight.

Determination of MPTP-Induced Alterations of Hydroxyl Radical Levels In Vivo

MPTP (30 or 40 mg/kg SC) or saline was injected in a total of 61 mice. At 10 time points (0, 0.5, 1, 2, 4, 8, 16, 24, 48, 72 h after MPTP injection) analysis of striatal hydroxyl radical levels was performed. Thirty minutes before dissection of the striata, mice were injected with salicylic acid (100 mg/kg IP) to form 2,3-DHBA with hydroxyl radicals according to the salicylate trapping method (11).

To evaluate the effect of PBN-treatment the treatment schedule of the neurotoxicity experiment as described above was applied. On day 8, PBN (100 mg/kg) ($n = 10$) or saline ($n = 10$) was injected immediately before and 4 h after MPTP (40 mg/kg SC) treatment. Salicylic acid (200 mg/kg) was administered, and 30 min later mice were sacrificed and striata were analyzed for hydroxyl radical levels.

Drugs and Chemicals

MPTP-HCl was purchased from Research Biochemicals International (RBI) Cologne, Germany. PBN, DA, DOPAC, HVA, 5-HT, 5-HIAA, 2,3-DHBA, 2,5-DHBA, SA sodium salt, and octanesulfonic acid sodium salt were obtained from Sigma Chemicals GmbH, Munich, Germany. HPLC grade acetonitrile and perchloric acid were purchased from Merck, Darmstadt, Germany. HPLC standards were dissolved in 0.1 M perchloric acid, PBN was dissolved in saline, and injected in a volume of 10 ml/kg body weight. All doses or concentrations are expressed in terms of the free base or acid.

Statistics

Statistical analysis was performed using Student's *t*-test for pairwise comparisons and ANOVA with post hoc Duncan's test for multiple comparisons. A p-value of 0.05 was considered significant.

RESULTS

Changes in Body Weight

Figure 1 summarizes the results of body weight determination. PBN treatment by itself produced no decrease in body weight in comparison with saline controls over the experimental period (day 1: PBN+Sal 26.6 g, Sal+Sal 27.4 g, day 14: PBN+Sal 26.4 g, Sal+Sal 26.7 g). After a single dose of MPTP on day 8, the body weight of the saline-treated MPTP animals (Sal+MPTP) rapidly and continuously decreased up to the end of the experimental period by 15.78%. The PBN-treated MPTP animals (PBN+MPTP), however, only slightly decreased in body weight on the first day after MPTP injection. On the following days the values nearly recovered to the levels of the non-MPTP–treated groups, and remained stable up to the end of the experiment (Fig. 1).

Locomotor Activity

Figure 2 summarizes the results of a 60-min automatically counted locomotor activity test obtained 1 day after MPTP treatment. The values of the Sal+Sal group were regarded as 100% control levels. One day before MPTP administration no significant differences between the groups were obtained (data not shown). In contrast, 1 day after MPTP treatment the locomotor activity in the Sal+MPTP and PBN+MPTP groups was significantly reduced compared with the corresponding non-MPTP–treated groups. The lowest activity was observed in the Sal+MPTP group. The mice in the PBN+MPTP group showed a significant attenuation of the MPTP-induced reduction of locomotor activity compared with the Sal+MPTP group.

Survival Rates

PBN+MPTP-treated animals showed a marked increase in survival rate (12 of 13) compared to Sal+MPTP treated mice (10 of 19; $p < 0.02$; χ^2-test) over the experimental period of 15 days. All mice in the Sal+Sal group and PBN+Sal group survived (Fig. 3).

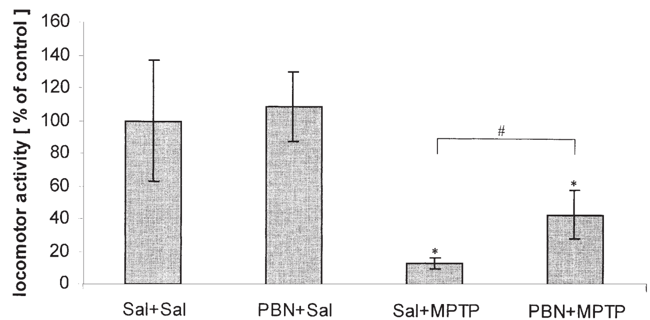

FIG. 2. Effects of saline + saline (Sal+Sal), PBN (100 mg/kg) + saline (PBN+Sal), saline + MPTP (40 mg/kg) (Sal+MPTP), PBN (100 mg/kg) + MPTP (40 mg/kg) (PBN+MPTP) treatment on locomotor activity in a 60-min session 1 day after MPTP or saline injection. The locomotor activity levels of each experimental group obtained 1 day before MPTP or saline treatment were regarded as 100% control levels. Data are mean ± SEM of $n = 10$–14 mice. Statistical analysis was performed using ANOVA and post hoc Duncan's test. *$p < 0.05$ was considered significant comparing the MPTP-treated groups with their corresponding saline-treated controls and #$p < 0.05$ comparing Sal+MPTP with PBN+MPTP.

Neurochemical Analysis

Eight days after MPTP treatment striatal levels of DA, 5-HT, and their metabolites were analyzed. The results are summarized in Fig. 4A–E. The values of the Sal+Sal group were regarded as 100% (dopamine 14.97 ± 1.48, DOPAC 1.19 ± 0.07, HVA 1.39 ± 0.08, 5-HT 0.053 ± 0.03, and 5-HIAA 0.37 ± 0.05 ng/mg). In the Sal+MPTP group, MPTP produced a pronounced reduction in dopamine, DOPAC and HVA levels to 5.67, 22.06, and 37.06% of saline controls, respectively, whereas serotonin and its metabolite 5-HIAA were not affected. In the PBN+MPTP group we obtained a significant attenuation of dopamine depletion to 26.1% compared to the dopamine depletion to 5.67% of the Sal+MPTP group (Fig. 4A), whereas the dopamine metabolites DOPAC and HVA

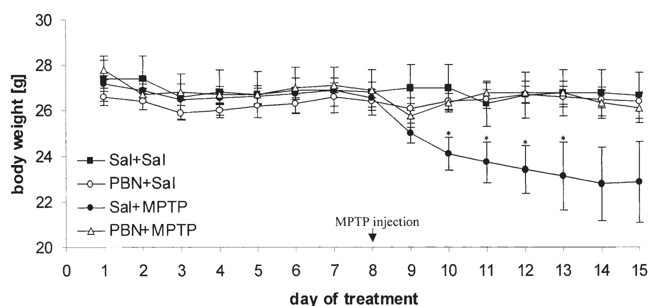

FIG. 1. Effects of saline + saline (Sal+Sal), PBN (100 mg/kg) + saline (PBN+Sal), saline + MPTP (40 mg/kg) Sal+MPTP), PBN (100 mg/kg) + MPTP (40 mg/kg) PBN+MPTP) treatment on the time course of body weight. Data are mean ± SEM of $n = 10$–14 mice. Statistical analysis was performed using ANOVA and post hoc Duncan's test. *$p < 0.05$ was considered significant.

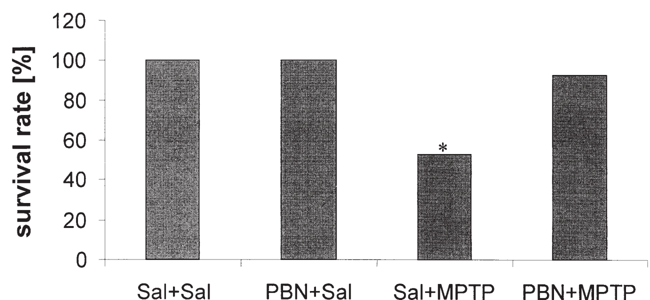

FIG. 3. Effects of saline + saline (Sal+Sal), PBN (100 mg/kg) + saline (PBN+Sal), saline + MPTP (40 mg/kg) (Sal+MPTP), PBN (100 mg/kg) + MPTP (40 mg/kg) (PBN+MPTP) treatment on survival during the experimental period of 15 days. Columns represent the percentage of surviving mice. Statistical analysis was performed using χ^2 -test (*$p < 0.05$).

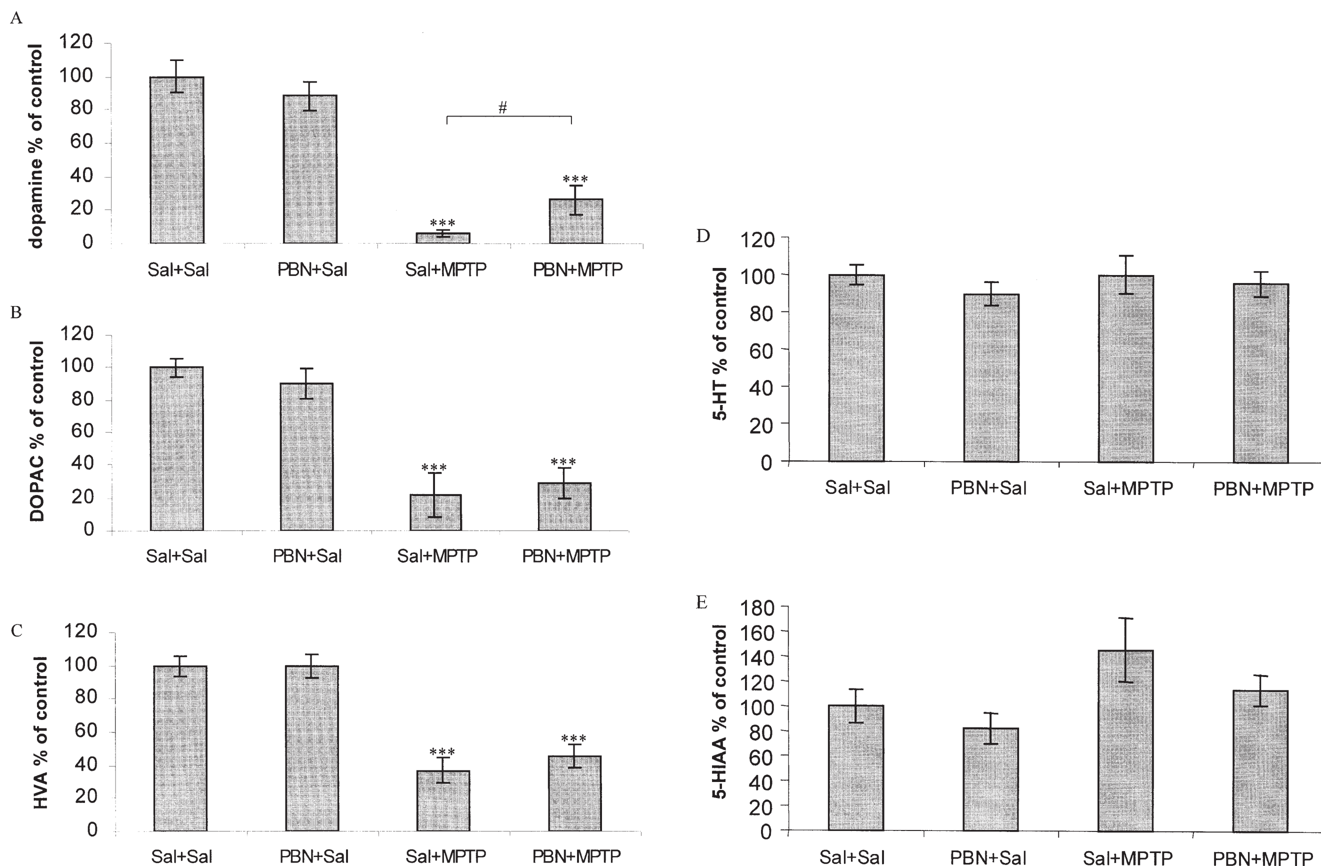

FIG. 4. Effects of saline + saline (Sal+Sal), PBN (100 mg/kg) + saline (PBN+Sal), saline + MPTP (40 mg/kg) (Sal+MPTP), and PBN (100 mg/kg) + MPTP (40 mg/kg) (PBN+MPTP) on striatal dopamine (A), DOPAC (B), HVA (C), 5-HT (D), 5-HIAA (E). Data are mean ± SEM of n = 10–14 mice expressed as percentages of the Sal+Sal control group. Statistical analysis was performed using ANOVA and post hoc Duncan's test. ***$p < 0.001$ comparing the MPTP-treated groups with their corresponding saline-treated controls #$p < 0.05$ was considered significant comparing the PBN-treated groups with their corresponding saline-treated groups.

as well as serotonin and 5-HIAA did not significantly differ compared to the Sal+MPTP group Fig. 4B–E). No differences in dopamine, serotonin, or their metabolite levels were observed between the Sal+Sal and PBN+Sal groups.

MPTP-Induced Alterations of Hydroxyl Radical Levels

The hydroxyl radical marker 2,3-DHBA was measured at different time points after MPTP administration. In non-MPTP–treated mice control values for 2,3-DHBA and SA were 0.054 and 30.37 ng/mg, respectively. Figure 5 shows that the maximum elevation of 2,3-DHBA/SA ratios was found 4 h after a single administration of MPTP (40 mg/kg SC). The 2,3-DHBA/SA ratios in the MPTP group were approximately 20-fold higher (4 h after MPTP) than in control mice. Mice that received a lower dosage of MPTP (30 mg/kg) showed only a nonsignificant tendency (sevenfold higher, 8 h after MPTP) towards an increase of 2,3-DHBA/SA ratio (Fig. 5).

PBN treatment was not able to reduce the MPTP-induced increase of 2,3-DHBA/SA measured 4.5 h after MPTP (40 mg/kg) administration (Fig. 6).

DISCUSSION

In the present study we demonstrate that a single administration of MPTP is able to produce a pronounced elevation of hydroxyl radicals in the striatum 4 to 8 h after MPTP injection, and that PBN leads to an improvement of functional parameters of neuroprotection against MPTP-induced neurotoxicity. Unexpectedly, PBN was not able to reduce MPTP-induced hydroxyl radical formation in vivo.

We observed a delayed radical formation 4–8 h after MPTP treatment, indicated by an increased 2,3-DHBA/SA ratio (Fig. 5). This is partly in line with Smith and Bennett (39), who also found increased 2,3-DHBA levels after a delay of 72 h. The discrepancies in the time course may be caused by a different methodology. They used a different schedule of MPTP administration (4 × 24 mg/kg SC) and only measured 2,3-DHBA but not salicylic acid brain levels to take into consideration potential changes of salicylic acid uptake after MPTP toxicity. In agreement with the present study, a single MPTP injection in mice decreased glutathione levels after 2 h in the striatum and midbrain, and increased 2′,7′-dichlorofluorescein fluorescence, as a marker of ROS, 4 and 8 h after MPTP in the striatum and midbrain (40). Additionally, MPP+ was found to induce NADH-dependent superoxide formation (18), and transgenic mice overexpressing the enzyme copper/zinc superoxide dismutase were resistant to MPTP-induced neurotoxicity (34), which underlines the involvement of oxygen radicals in MPTP toxicity.

Free hydroxyl radicals are extremely reactive, and attack

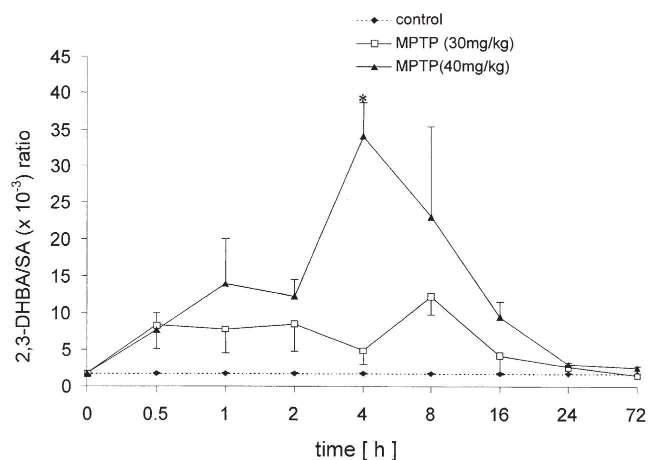

FIG. 5. Effects of a single MPTP administration (30 and 40 mg/kg SC) in mice on 2,3-DHBA/SA ratios as indicator of hydroxyl radicals. Data of different time points after MPTP administration are given as mean ± SEM of $n = 3$–5 mice. The dotted line represents the control values obtained in saline-treated mice. Statistical analysis was performed using ANOVA and post hoc Duncan's test. $*p < 0.05$ was considered significant comparing MPTP-treated mice with control animals.

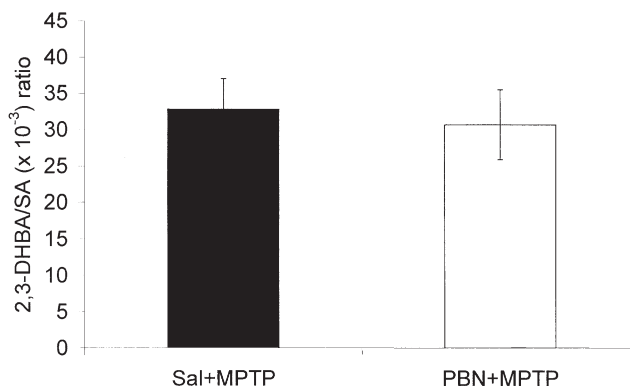

FIG. 6. Effects of PBN or saline on MPTP-induced hydroxyl radical formation. The treatment schedule for the PBN and saline-treated groups was adopted from the neurotoxicity study. Salicylic acid (100 mg/kg) was given immediately after the last PBN injection [4 h after MPTP (40 mg/kg)]. Thirty minutes later all animals were sacrificed, and striatal 2,3-DHBA, and SA levels were analyzed. Data are mean ± SEM of $n = 10$ mice. Statistical analysis was performed using unpaired Students' *t*-test. There was no significant difference between the groups.

nearly every molecule in their neighborhood. One should take into consideration that the hydroxyl radical is the most reactive oxygen species, with a half-life of about 1 ns and rate constants with biological molecules of 10^9 to 10^{10} $M^{-1} \cdot s^{-1}$ (2,23). The competitive reaction of PBN with the attacked biomolecules is mainly dependent on the reaction rate, the stability of the adducts, and the concentration of the reactants. It is postulated that PBN and other nitrone compounds react covalently with reactive paramagnetic radicals to produce more or less stable unreactive nitroxide spin adducts (10,24). Despite its good penetration into brain tissues (6,7), PBN failed to reduce MPTP-induced hydroxyl radical formation (Fig. 6). This does not rule out that PBN possesses a radical scavenging activity in vivo for the following reasons: PBN was coadministered with SA, which obviously trapped hydroxyl radicals in the present study (indicated by increased 2,3-DHBA levels). This might have covered the PBN effect. In addition, SA was found to completely block low-dose MPTP toxicity (1) and antagonized MPTP toxicity to a greater extent than PBN under the same experimental conditions as applied in the present study (data not shown). Furthermore, PBN reacts rapidly with carbon centered radicals (41), such as those generated by lipid peroxidation, and might prevent damage "downstream" of the generation of hydroxyl free radicals. We cannot exclude that PBN might trap radicals inaccessible to salicylate, due to its lipophilicity.

The effects of PBN in the present study and the numerous reports of neuroprotection, mainly in models of cerebral ischemia and neurodegeneration, cannot solely be explained by the radical scavenging activity of PBN. On the contrary, free radial OH spin adducts of PBN are quite unstable in aqueous media (26), and the antioxidant activity of PBN measured in initiated rat liver microsomal peroxidation was 10 to 100 times less compared with hindered phenols (21). To overcome the disadvantages of PBN, molecular modeling was used to create more effective radical traps (41), and the novel free radical spin-trap MDL 101,002, was found to protect against MPTP toxicity and malonate-induced striatal lesions (29). Novel cyclic variants of PBN greatly improved the potency relative to PBN in preventing oxidative injury (29,41), and novel azulenyl nitrone spin traps were found to be superior against MPTP toxicity compared with S-PBN (24).

Although PBN did not show a direct hydroxyl radical scavenging effect, PBN treatment led to a significant functional improvement against MPTP toxicity. This was reflected by a reduced loss of body weight (Fig. 1), an improved motor function (Fig. 2), and an attenuation of striatal dopamine depletion (Fig. 4). The general toxicity of MPTP applied in the dose of 40 mg/kg was found to be in the range of the LD_{50}. This high lethality was rather unexpected. One reason might be that we calculated the MPTP dosage in terms of the free base, so that we administered a higher dose than if the calculation was based on the salt. Alternatively, our mice might be more sensitive to MPTP toxicity, although we applied the usual strain for MPTP studies. A positive aspect of the high lethality seems to be that PBN impressively enhanced survival under the protocol applied. Only 1 out of 13 mice died in the PBN+MPTP group (Fig. 3).

Recently, alternative mechanisms of action were identified for PBN that could contribute to its protective effects against MPTP toxicity. In vitro, PBN showed a potent inhibition of nuclear factor kappa-B (NF-κB) activation and also inhibition of the inflammatory factors inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) (27). Inhibition of NF-κB translocation mediated the protective effect of acetylsalicylic acid and SA against glutamate toxicity in rat primary neuronal cultures and hippocampal slices (16). Inhibition of nNOS is protective against MPTP toxicity as demonstrated by the NOS-inhibitors 7-nitroindazole in MPTP-treated Swiss–Webster mice (37) and C57BL mice (33). Furthermore, mice lacking the neuronal NOS gene were more resistant to MPTP-induced toxicity (33). We cannot exclude the possibility that repeated treatment with PBN might enhance the metabolism of MPTP and by this reduce its toxic effects. Even if this was the case (which seems unlikely), the missing effects on hydroxyl free radicals cannot be explained.

Convincingly, it was suggested that the broad-spectrum antioxidant and antiinflammatory activity of PBN is caused by the inhibition of mitochondrial flavin dehydrogenases upstream from the rotenone binding site of complex I (19). In this context it seems likely that PBN might inhibit electron leakage from MPP+-inhibited complex I activity. In addition, complex I was identified as a major site of hydrogen peroxide formation in brain mitochondria (3,20). PBN may inhibit hydrogen peroxide production via that mechanism (19).

In conclusion, it seems unlikely that the hydroxyl radical scavenging properties of PBN are related to the pronounced effect on functional parameters of neuroprotection against MPTP toxicity in vivo. Alternative explanations such as inhibition of the transcription factor NF-κB, inflammatory factors, modulation of flavin dehydrogenase function—especially in complex I of brain mitochondria—or detoxification of carbon-centered radicals are feasible.

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