Comparative Metabolism of *N-tert*-Butyl-*N*-[1-(1-oxy-pyridin-4-yl)-ethyl]- and *N-tert*-Butyl-*N*-(1-phenyl-ethyl)-nitroxide by the Cytochrome P450 Monooxygenase System

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The use of spin-trapping agents for a direct ESR detection of ·OH in biological systems is limited by the low stability of the hydroxyl radical-derived nitroxides. Among the various probes used for trapping of ·OH, DMSO has proven to be highly efficient. The reaction between ·OH and DMSO yields methyl radical (CH_3·), which can react with *N-tert*-butyl-α-phenylnitrone (PBN) and α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (POBN) to form stable, ESR-detectable nitroxides. The latter approach has been successfully used in in vivo experiments for analysis of ·OH; in these experiments, POBN/·CH_3 and PBN/·CH_3 were detected in the bile duct and the urine of the treated animals. However, the sites of generation of ·OH produced in vivo are unknown. Currently, no ESR data is available for the formation of ·OH in liver of animals subjected to oxidative stress. Since nitroxides containing aromatic rings are likely to be substrates of cytochrome P450, experiments were carried out for assessing the ability of the cytochrome P450 monooxygenase system to metabolize PBN/·CH_3 and POBN/·CH_3, respectively. In the presence of NADPH, rat liver microsomes catalyzed the reduction of POBN/·CH_3 to the corresponding hydroxylamine (POBN/CH_3), while PBN/·CH_3 was metabolized without accumulation of its hydroxylamine form (PBN/CH_3). The metabolism of PBN/·CH_3 was inhibited by 4-methylpyrazole and ketoconasole, suggesting that cytochrome P450-catalysis was required for the consumption of this nitroxide. Under anaerobic conditions, both the nitroxide and hydroxylamine forms of PBN/CH_3 were metabolized, implying that these adducts may undergo reductive cytochrome P450-catalyzed biotransformation. On the basis of the susceptibility of PBN/·CH_3 to undergo irreversible metabolic transformation, it is discussed that POBN may prove to be a more efficient spin-trapping agent for the in vivo detection of ·OH.

Introduction

Superoxide ion ($O_2^{\bullet-}$) is an abundant metabolic product that can cause substantial cellular damage. Although $O_2^{\bullet-}$ is a rather inert radical [i.e., a poor H-abstractor (*1*)], its reaction with transition metal complexes, or its self-termination to H_2O_2 followed by iron-catalyzed generation of hydroxyl radical [·OH; Fenton reaction (*2*)] can potentiate its toxicological impact. The Fenton reaction has been implied in the pathogenesis of numerous disease states (*3*). Direct detection of ·OH has proven to be difficult as this species is highly reactive; ·OH interacts indiscriminately with low molecular weight compounds, lipids, and proteins present in cells in a diffusion-controlled manner. The methods for detection of ·OH in biological systems are based on the introduction of a molecular probe that successfully competes with the cellular molecules for ·OH, forms stable hydroxyl radical-derived analytes, and is relatively nontoxic (reviewed in refs *4* and *5*).

The ESR spin-trapping technique is the most direct method for analysis of radical intermediates. While many spin-trapping agents (e.g., POBN and PBN)[1] are relatively nontoxic, their use for a direct detection of ·OH in biological systems is limited by the low stability of the hydroxyl radical-derived nitroxides (*6*, *7*). Among the various scavengers used for trapping of ·OH, dimethyl sulfoxide (DMSO) has proven to be highly efficient. DMSO can be tolerated by living systems in up to 1 M concentrations (*8*) and has an appreciable rate of interaction with hydroxyl radical [$k = 7 \times 10^9$ M^{-1} s^{-1} (*9*)]. The reaction between ·OH and DMSO yields methyl radical, CH_3· (*9*), which can react with PBN and POBN to form stable, ESR detectable nitroxides (PBN/·CH_3 and POBN/·CH_3, respectively; Scheme 1).

Recently, Burkitt and Mason reported that the bile duct of rats treated with iron, DMSO and PBN contained PBN/·CH_3 (*10*, *11*). This observation suggests that the hepatic metabolism of iron could be paralleled by the generation of ·OH. However, direct ESR evidence for the formation of PBN/·CH_3 in the liver of animals subjected to iron overload has not been presented yet. This may be due to the reduction of the corresponding spin adducts to ESR-silent hydroxylamines (Scheme 1) and/or their biotransformation to secondary metabolites. Hence, experiments were carried out to evaluate the NADPH-dependent rat liver microsomal metabolism of POBN/·

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[1] Abbreviations: PBN, *N-tert*-butyl-α-phenylnitrone; POBN, α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone; POBN/·CH_3, *N-tert*-butyl-*N*-[1-(1-oxy-pyridin-4-yl)-ethyl] nitroxide; PBN/·CH_3, *N-tert*-butyl-*N*-(1-phenyl-ethyl) nitroxide; POBN/CH_3, *N-tert*-butyl-*N*-[1-(1-oxy-pyridin-4-yl)-ethyl] hydroxylamine; PBN/CH_3, *N-tert*-butyl-*N*-(1-phenyl-ethyl) hydroxylamine; ESR, electron spin resonance; EC, electrochemical.

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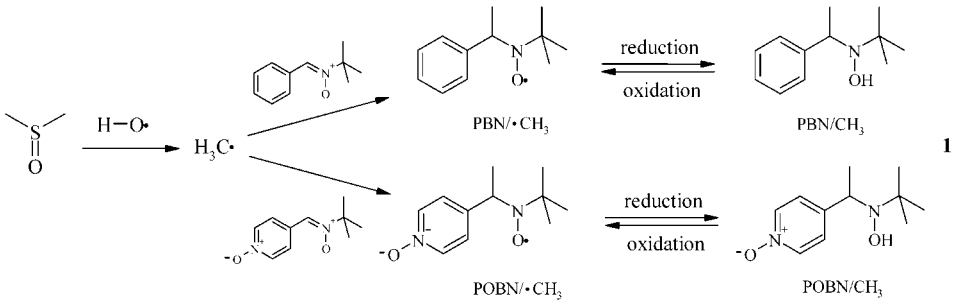
Scheme 1



CH_3 and PBN/·CH_3, respectively. These experiments aimed to optimize spin-trapping methodology for the detection of ·OH in biological systems.

Materials and Methods

Reagents. All reagents used were purchased from Sigma Chem. Co. (St. Louis, MO). The solutions used in the experiments were prepared in deionized and Chelex-100 treated water or potassium phosphate buffer. Standard rat liver microsomes, as well as microsomes enriched with cytochrome P450 1A and 3A were purchased from InVitro Technologies, Inc. (Baltimore, MD). Human P450 reductase microsomes that do not contain cytochrome P450 and microsomes enriched with cytochrome P450 2E1 were purchased from Gentest Corporation (Woburn, MA). Product characterization tables for the microsomes used could be accessed at http://www.invitrotech.com and http://www.gentest.com, respectively.

ESR Measurements. ESR measurements were performed on a Bruker ECS106 spectrometer with 50 kHz magnetic field modulation at room temperature (25 °C). ESR spectrometer settings were modulation amplitude 0.7 G, scan time 40 s, time constant 0.64 s, microwave power 20 mW, and receiver gain 1×10^5.

Preparation of POBN/CH_3 and PBN/CH_3 Adducts. POBN/·CH_3 and PBN/·CH_3 were prepared via ·OH-dependent oxidation of DMSO to ·CH_3 in the presence of POBN and PBN, respectively (*12*). The nitroxide formed after 5 consecutive additions of 0.02 mL of $Fe(NH_4)(SO_4)_2$ (0.05 M) into a 1 mL solution of a spin-trapping reagent (0.02 M), H_2O_2 (2 mM), EDTA (5 mM), and DMSO (2%) in 0.1 M phosphate buffer (pH 7.4) was extracted with 2 × 1 mL of ethyl acetate (POBN/CH_3) or 2 × 1 mL of hexane (PBN/CH_3). The ethyl acetate (hexane) phase was separated and the crystals formed after evaporation of the solvent (stream of nitrogen, 25 °C) were redissolved in 1 mL of double-distilled water (POBN/CH_3) or 1 mL of ethanol. The solutions of POBN/·CH_3 and PBN/·CH_3 in phosphate buffer (0.1 M, pH 7.4) exhibited ESR spectra with a triplet of doublets. The hyperfine structure of the ESR spectra was consistent with the results obtained by Augusto et al. and Saprin and Piette [(a_N = 16.10 G; a_H = 2.77 G) and (a_N = 16.46 G; a_H = 3.36 G, respectively (*13*, *14*)] and allows the assignment of the nitroxides as that formed by addition of methyl radical to POBN and PBN, respectively. Reduction of both POBN/·CH_3 and PBN/·CH_3 to their hydroxylamine derivatives (POBN/CH_3 and PBN/CH_3, respectively) was performed by incubation of the corresponding nitroxide with ascorbate (5 mM) in 0.1 M phosphate buffer (pH 7.4; 10−40 min of incubation at 25°). Subsequently, the reaction solution was extracted with ethyl acetate and the crystals formed after evaporation of the organic solvent (stream of nitrogen; 25 °C) were redissolved in ethanol. The ethanolic solutions obtained did not exhibit the typical ESR spectra of POBN/·CH_3 and PBN/·CH_3, which were observed before the incubation with ascorbate. The POBN/CH_3 and PBN/CH_3 adducts were further purified by semipreparative HPLC and stored in ethanol at −80° C.

Alternatively, PBN/·CH_3 and PBN/CH_3 were prepared as described in ref *15*. Briefly, PBN/·CH_3 and PBN/CH_3 were obtained via direct coupling of PBN (0.08 M) with CH_3MgBr (0.3 M) in helium-deaerated, dry diethyl ether (10 mL). After an incubation of 10 min (20 °C), the excess of the Grignard reagent was carefully decomposed with 50% ethanol (v/v; 2 mL), the organic layer was separated, dried over Na_2SO_4, and rotor-evaporated (25 °C). The dry residue containing both the nitroxide and hydroxylamine forms of the methylated PBN was redissolved in methanol and subjected to HPLC purification.

HPLC Analysis. HPLC was performed with a Waters liquid chromatograph (Milford, MA). Separation was achieved with a C-18 reverse phase column (Microsorb, 4.6 mm × 25 cm, 5 μm, 100 A Rainin Instrument Company, Inc., Emeryville, CA). The mobile phase was saturated with helium and contained 10 mM lithium perchlorate and either water with 30% (v/v) methanol for analysis of POBN/CH_3, or 70% methanol for analysis of PBN/CH_3. All HPLC analyses were conducted at a flow rate of 1 mL/min. Electrochemical detection of the POBN/CH_3 and PBN/CH_3 was carried out at +0.8 V with a LC-4C/CC5 amperometric system (Bioanalytical Systems, West Lafayette, IN) equipped with glassy carbon electrode and a Ag/AgCl reference electrode (*12*).

Purification of the POBN/CH_3 and PBN/CH_3 adducts was carried out with a semipreparative column (C18; 10 mm × 25 cm; Waters, Milford, MA) at a flow rate of 3 mL/min. The mobile phase was the same as this used for the analytical separations, except that it did not contain lithium perchlorate. UV detection was carried out within the wavelength range 200−400 nm using a SPD-M10VP Shimadzu diode array detector (Kyoto, Japan). Injection loops of 0.02 mL and 0.2 mL were used for the analytical and preparative separation, respectively. The methanol from the fractions containing the individual POBN and PBN adducts was rotor-evaporated at room temperature. The resulting water solutions of the adducts were diluted with equal volume of 0.2 M phosphate buffer (pH 7.4) and used within 1−2 h.

Metabolism of POBN/CH_3 and PBN/CH_3 Adducts. Liver microsomes (2 mg of protein/1 mL of 0.1 M phosphate buffer; pH 7.4) were incubated at 37° C in a shaking water bath with NADPH (1 mM) and the corresponding PBN or POBN derivative. After initiation of the reaction with NADPH, at various time points a 0.1 mL aliquot of the reaction suspension was mixed with 0.1 mL of acetonitrile. The resulting suspension was centrifuged to remove precipitated proteins (5 min × 5000*g*), and the supernatant was subjected to HPLC-EC analysis. In selected experiments, anaerobiosis was achieved via deoxygenation of all reaction solutions with a stream of nitrogen for 15 min at 20 °C. Subsequent additions were made under nitrogen, the tubes containing the final reaction suspensions were sealed, and the reactions carried out as described in the Results.

Results

ESR and HPLC-EC Monitored Metabolism of POBN/CH_3 and PBN/CH_3 Nitroxides. The disappearance of the ESR signal of nitroxides subjected to reduction appears to be a major obstacle in the bio-application of the ESR spin-trapping technique. In the presence of

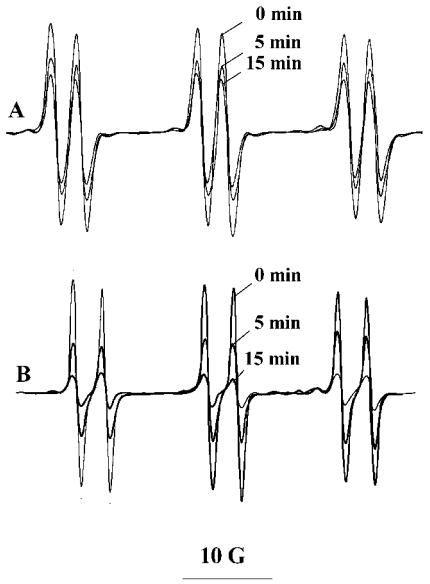


Figure 1. ESR-monitored metabolism of PBN/·CH_3 and POBN/·CH_3 by liver microsomes. All reactions were carried out at 25 °C in 0.1 M phosphate buffer (pH = 7.4) containing rat liver microsomes (2 mg of protein/mL), NADPH (1 mM) and either POBN/·CH_3 (0.014 mM; A) or PBN/·CH_3 (0.01 mM; B).

rat liver microsomes, most nitroxides undergo NADH- and NADPH-dependent reduction to ESR-silent hydroxylamines ($R_2N-O\cdot \rightarrow R_2N-OH$). Iannone et al. proposed that the latter reaction is catalyzed by cytochrome *c* reductases without a direct involvement of cytochrome P450 (*16*). The corresponding hydroxylamine derivatives could be reoxidized back to ESR active nitroxides (Scheme 1) with either potassium ferricyanide or O_2 in alkaline solutions (*12*, *17*, *18*). However, nitroxides containing aromatic rings are likely to be substrates of cytochrome P450. Therefore, experiments were carried out for assessing the ability of the cytochrome P450 monooxygenase system to metabolize PBN/·CH_3 and POBN/·CH_3, respectively. The biotransformation of these compounds is of particular interests as they are used as ESR and HPLC-EC markers of hydroxyl radical.

In the presence of rat liver microsomes and NADPH, the ESR spectra of both PBN/·CH_3 and POBN/·CH_3 decreased in a time-dependent manner (Figure 1), suggesting the occurrence of reactions with the participation of the cytochrome P450 monooxygenase system. No spectral changes were observed in the absence of NADPH (data not shown). Figure 2A depicts the HPLC-EC profile of a suspension containing PBN/·CH_3 and rat liver microsomes (chromatogram 1, peak I); in the presence of NADPH, PBN/·CH_3 was consumed in a time-dependent manner without any detectable accumulation of PBN/CH_3 (Figure 2A; chromatogram 2; Figure 3A, open circles). In the absence of NADPH, PBN/·CH_3 was relatively stable; no consumption of PBN/·CH_3 was observed after an incubation of 20 min with rat liver microsomes at 37 °C (Figure 3A). A cytochrome P450-dependent consumption of PBN/·CH_3 is suggested by the formation of PBN/CH_3 when the reaction was carried out with human P450 reductase microsomes that do not contain cytochrome P450. In these experiments, NADPH triggered a semiquantitative conversion of PBN/·CH_3 to the corresponding hydroxylamine (Figure 2A; chromatogram 3, peak II). It should be noted that PBN/CH_3 is less efficiently oxidized in the electrochemical cell than PBN/·CH_3. Therefore, the ratio of peaks I and II (Figure 2A, chromatogram 3) does not reflect the stoichiometry of the studied analytes (*12*).

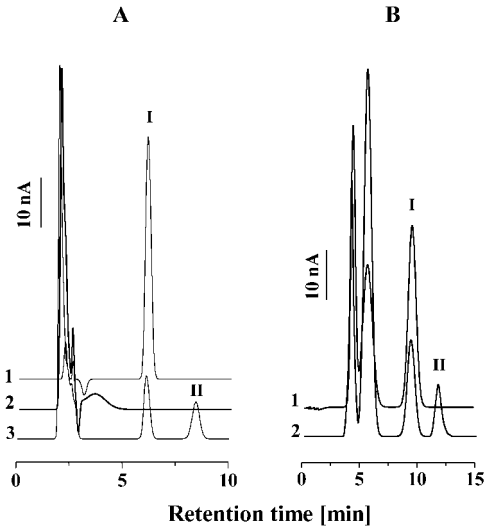


Figure 2. HPLC-EC-monitored metabolism of PBN/·CH_3 and POBN/·CH_3 by liver microsomes. All experiments were carried out for 20 min at 37 °C in 0.1 M phosphate buffer (pH 7.4). (A) Chromatogram 1, PBN/·CH_3 (3 μM) and microsomes (2 mg of protein/mL); chromatogram 2, PBN/·CH_3, microsomes, and NADPH (1 mM); chromatogram 3, PBN/·CH_3, P450 reductase microsomes lacking cytochrome P450 (2 mg of protein/mL), and NADPH (1 mM). Peaks I and II reflect the elution of PBN/·CH_3 and PBN/CH_3, respectively. (B) Chromatogram 1, POBN/·CH_3 (3.4 μM) and microsomes (2 mg of protein/mL); chromatogram 2, POBN/·CH_3, microsomes, and NADPH (1 mM). Peaks I and II reflect the elution of POBN/·CH_3 and POBN/CH_3, respectively.

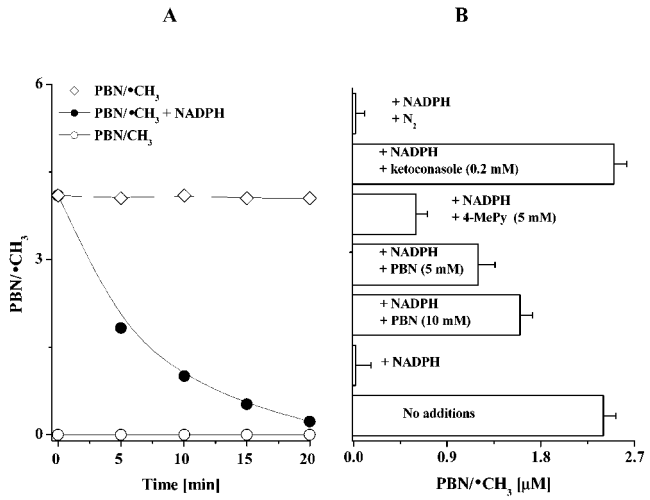


Figure 3. Effects of PBN, 4-methylpyrazole and ketoconasole on the NADPH-dependent microsomal metabolism of PBN/·CH_3. All reactions were carried out for 20 min at 37 °C in 0.1 M phosphate buffer (pH 7.4). The experiments under anaerobic conditions were performed as outlined in the Materials and Methods. Quantitation of PBN/·CH_3 was carried out by HPLC-EC. (A) Consumption of PBN/·CH_3 (4.1 μM) by rat liver microsomes (2 mg of protein/mL) in the presence of NADPH (1 mM). In these experiments, the metabolism of PBN/·CH_3 was not paralleled by formation of PBN/CH_3 (open circles). (B) Effects of cytochrome P450 inhibitors on the metabolism of PBN/·CH_3 (2.6 μM) catalyzed by rat liver microsomes (2 mg of protein/mL) in the presence of NADPH (1 mM). The data presented are the mean ± SEM of three independent experiments (n = 3).

In contrast to PBN/·CH_3, rat liver microsomes plus NADPH metabolized POBN/·CH_3 (Figure 2B; chromatogram 1, peak I) to the corresponding hydroxylamine

Table 1. Rates of PBN/·CH_3 Metabolism by Microsomes Enriched with Cytochrome P450, Types 1A, 3A, and 2E1[a]

cytochrome P450	rate of PBN/·CH_3 metabolism [nmol of nitroxide/min/nmol of P450]
1A	0.38 ± 0.04
3A	0.52 ± 0.05
2E1	0.29 ± 0.03

[a] All reactions were carried out for 20 min at 37 °C in phosphate buffer (0.1 M; pH 7.4) containing NADPH (1 mM) and liver microsomes enriched with cytochrome P450. The consumption of PBN/·CH_3 was monitored by HPLC-EC. The data presented are the mean ± SEM of three independent experiments ($n = 3$).

(POBN/CH_3; Figure 2B; chromatogram 2, peak II). In the absence of NADPH, consumption of POBN/·CH_3 was not observed. P450 reductase microsomes that do not contain cytochrome P450 were as efficient in reducing PBN/·CH_3 as the control microsomes. The latter suggests that POBN/·CH_3 was not metabolized by cytochrome P450 to any significant extent (data not shown).

Effects of Cytochrome P450 Inhibitors on the Rat Liver Microsomal Metabolism of PBN/CH_3. The NADPH-dependent rat liver microsomal metabolism of PBN/·CH_3 was inhibited by 4-methylpyrazole and ketoconasole (Figure 3B). The latter further supports the notion that cytochrome P450-catalysis is required for the metabolism of PBN/·CH_3; at the concentrations used, both 4-methylpyrazole and ketoconasole are known to inhibit the activity of most cytochrome P450 isozymes (*19*). Recently, Reinke et al. reported that PBN undergoes a cytochrome P-450 metabolism to 4-hydroxyPBN (*20*), suggesting that this nitrone may interfere with the cytochrome P450-dependent metabolism of PBN/·CH_3. Indeed, PBN inhibited the metabolism of both PBN/·CH_3 and PBN/CH_3 in a concentration-dependent manner (Figures 3B and 4, respectively). However, this effect was pronounced at concentrations of PBN that were considerably higher than its LD_{50} (*21*). Under anaerobic conditions, both PBN/·CH_3 and PBN/CH_3 were metabolized by rat liver microsomes plus NADPH (Figures 3B and 4, respectively), suggesting that these adducts may undergo a reductive cytochrome P450-catalyzed biotransformation. Cytochrome P450 2E1, 1A, and 3A exhibited similar activity in metabolizing PBN/·CH_3 (Table 1). The consumption of PBN/·CH_3 by microsomes enriched with cytochrome P450 2E1, 1A, and 3A was not affected by superoxide dismutase, catalase, and desferrioxamine, suggesting that $O_2^{-\bullet}$, H_2O_2 and metal ions were not involved in the overall reaction mechanism (data not shown).

Discussion

The results of this work provide evidence that the ESR markers of hydroxyl radical, PBN/·CH_3, and POBN/·CH_3 could be metabolized by the cytochrome P450 monooxygenase system. In the presence of NADPH, liver microsomes reduced POBN/·CH_3 to POBN/CH_3, whereas PBN/·CH_3 was metabolized to ESR-silent product(s) without a detectable formation of its hydroxylamine form.

Studies of oxidative stress have attracted considerable interest and have been the focus of much research in recent years. Cumulative oxidative damage to tissues has been implicated in a number of disease states, e.g. the aging process, cancer, and ischemia reperfusion. In the last two decades, the understanding of the molecular mechanisms of bio-oxidative damage was strongly enhanced with the advent of ESR spectrometry and the later development of the spin-trapping technique. The spin-trapping technique is based on the high rates of interaction of nitrones or nitroso compounds with radical species. The paramagnetic nitroxides formed as a result of the latter interaction exhibit ESR spectra that are "structural fingerprints" of the parent radical species. However, direct ESR detection of ·OH in biological systems has proven to be difficult as the ·OH-derived nitroxides are relatively unstable compounds. The latter experimental difficulty could be solved with the introduction of DMSO as a primary molecular probe for the interception of ·OH. The reaction between ·OH and DMSO yields methanesulfinic acid and CH_3·. Methanesulfinic acid can be quantified after derivatization with diazonium salts (*22*), while CH_3· can alkylate PBN and POBN via 1,3-addition to remarkably stable nitroxides. The latter approach has been successfully used in in vivo experiments for detection of cyclosporin A-, iron-, and copper-dependent generation of ·OH; in these experiments, POBN/·CH_3 and PBN/·CH_3 were detected in the bile duct and the urine of the treated animals (*11*, *23*, *24*). However, no ESR spin-trapping data is available for the formation of POBN/·CH_3 and PBN/·CH_3 in liver of animals subjected to oxidative stress. The reason for this could be, at least in part, that these nitroxides are metabolized by the cytochrome P450 monooxygenase system. To test this hypothesis, we have carried out experiments for evaluating the potential of liver microsomes to catalyze the biotransformation of PBN/·CH_3 and POBN/·CH_3, respectively.

Liver microsomes plus NADPH mediated the conversion of PBN/·CH_3 to ESR-silent products without a detectable formation of PBN/CH_3. However, human P450 reductase microsomes that did not contain cytochrome P450 reduced PBN/·CH_3 to PBN/CH_3, suggesting that both cytochrome P450 and the cytochrome P450 reductase can metabolize this nitroxide. The microsomal metabolism of PBN/·CH_3 was inhibited by 4-methylpyrazole and ketoconasole, which further supports the notion for a preponderant cytochrome P450 catalysis. The consumption of PBN/·CH_3 by microsomes enriched with cytochrome P450 2E1, 1A, or 3A was not affected by superoxide dismutase, catalase, and desferrioxamine, suggesting that $O_2^{-\bullet}$, H_2O_2, and ·OH-like species were not involved in the overall reaction mechanism (*25*). Under anaerobic conditions, NADPH triggered microsomal consumption of both PBN/·CH_3 and PBN/CH_3, which implies that these adducts may undergo a reductive cytochrome P450-catalyzed biotransformation. Since the metabolism of PBN/·CH_3 was not paralleled by changes in its ESR spectrum, it could be speculated that cytochrome P450 modified the side chain of the nitroxide rather than hydroxylated its aromatic ring; mechanistically, the latter reaction may proceed via a reductive cleavage of the N-C_α function of PBN/·CH_3 (Scheme 2).

Contrary to PBN/·CH_3, liver microsomes plus NADPH catalyzed the reduction of POBN/·CH_3 to its ESR-silent hydroxylamine form. This reduction was most likely mediated by the microsomal cytochrome P450 reductase and can be reversed; POBN/CH_3 could be reoxidized back to POBN/·CH_3 with potassium ferricyanide, or with O_2 in alkaline solutions (Scheme 1). Alternatively, both POBN/·CH_3 and POBN/CH_3 could be directly quantified by HPLC-UV/EC.

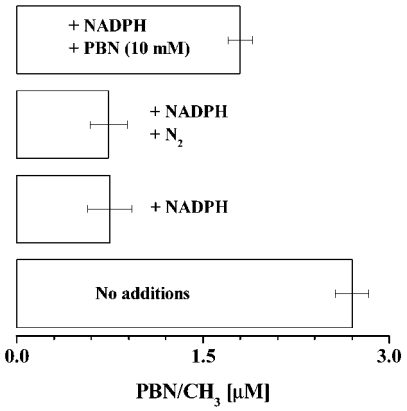
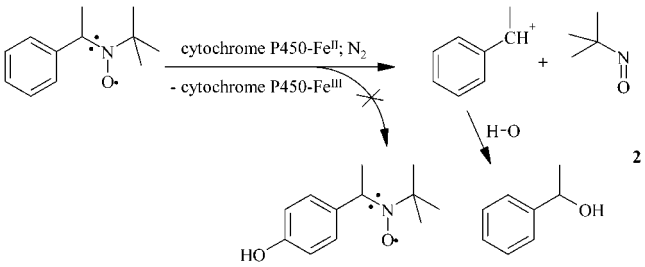


Figure 4. HPLC-EC-monitored metabolism of PBN/CH_3 by rat liver microsomes. All reactions were carried out as described in Figure 3. The data presented are the mean ± SEM of three independent experiments ($n = 3$).

Scheme 2



These results suggest that the cytochrome P450 monooxygenase system may interfere with the ESR analysis of PBN/·CH_3 and POBN/·CH_3, respectively. PBN is often used for the in vivo detection of ·OH. However, the ESR detection of PBN/·CH_3 could be successful if its rate of generation is higher that that of its metabolism. Hence, the susceptibility of PBN/·CH_3 to undergo an irreversible metabolic transformation should be taken in consideration when spin-trapping experiments with PBN are to be carried out. In contrast, POBN/·CH_3 can undergo a reductive microsomal metabolism to POBN/CH_3, whereas the structural information of the parent analyte is preserved. The latter suggests that POBN may prove to be a more efficient spin-trapping agent than PBN for the in vivo analysis of ·OH.

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References

(1) Golden, D. M., Bierbaum, V. M., and Howard, C. J. (1990) Reevaluation of the Bond-Dissociation Energies (Delta-Hdbe) for H-OH, H-OOH, H-OO-, H-O., H-OO-, and H-OO. *J. Phys. Chem.* **94**, 5413−5415.

(2) Koppenol, W. H. (1993) The centennial of the Fenton reaction. *Free Radical Biol. Med.* **15**, 645−651.

(3) Halliwell, B., and Gutteridge, J. M. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.* **186**, 1−85.

(4) Halliwell, B., Grootveld, M., and Gutteridge, J. M. (1988) Methods for the measurement of hydroxyl radicals in biomedical systems: deoxyribose degradation and aromatic hydroxylation. *Methods Biochem. Anal.* **33**, 59−90.

(5) Mason, R. P., Hanna, P. M., Burkitt, M. J., and Kadiiska, M. B. (1994) Detection of oxygen-derived radicals in biological systems using electron spin resonance. *Environ. Health. Perspect.* **102** (Suppl. 10), 33−36.

(6) Janzen, E. G., Kotake, Y., and Hinton, R. D. (1992) Stabilities of hydroxyl radical spin adducts of PBN-type spin traps. *Free Radical Biol. Med.* **12**, 169−173.

(7) Towell, J., and Kalyanaraman, B. (1991) Detection of radical adducts of 5,5-dimethyl-1-pyrroline n-oxide by the combined use of high-performance liquid chromatography with electrochemical detection and electron spin resonance. *Anal. Biochem.* **196**, 111−119.

(8) Babbs, C. F., and Steiner, M. G. (1990) Detection and quantitation of hydroxyl radical using dimethyl sulfoxide as molecular probe. *Methods Enzymol.* **186**, 137−147.

(9) Klein, S. M., Cohen, G., and Cederbaum, A. I. (1981) Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical generating systems. *Biochemistry* **20**, 6006−6012.

(10) Kadiiska, M. B., Burkitt, M. J., Xiang, Q. H., and Mason, R. P. (1995) Iron supplementation generates hydroxyl radical in vivo. An ESR spin-trapping investigation. *J. Clin. Invest.* **96**, 1653−1657.

(11) Burkitt, M. J., and Mason, R. P. (1991) Direct evidence for in vivo hydroxyl-radical generation in experimental iron overload: an ESR spin-trapping investigation. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8440−8444.

(12) Stoyanovsky, D. A., Melnikov, Z., and Cederbaum, A. I. (1999) ESR and HPLC-EC analysis of the interaction of hydroxyl radical with DMSO: rapid reduction and quantification of POBN and PBN nitroxides. *Anal. Chem.* **71**, 715−721.

(13) Augusto, O., Du Plessis, L. R., and Weingrill, C. L. (1985) Spin-trapping of methyl radical in the oxidative metabolism of 1,2-dimethylhydrazine. *Biochem. Biophys. Res. Commun.* **126**, 853−858.

(14) Saprin, A. N., and Piette, L. H. (1977) Spin trapping and its application in the study of lipid peroxidation and free radical production with liver microsomes. *Arch. Biochem. Biophys.* **180**, 480−492.

(15) Novakov, C. P., Feierman, D., Cederbaum, A. I., and Stoyanovsky, D. A. (2001) An esr and hplc-ec assay for the detection of alkyl radicals. *Chem. Res. Toxicol.* **14**, 1239−1246.

(16) Iannone, A., Tomasi, A., Vannini, V., and Swartz, H. M. (1990) Metabolism of nitroxide spin labels in subcellular fraction of rat liver. I. Reduction by microsomes. *Biochim. Biophys. Acta* **1034**, 285−289.

(17) Stoyanovsky, D. A., and Cederbaum, A. I. (1998) ESR and HPLC-EC analysis of ethanol oxidation to 1-hydroxyethyl radical: rapid reduction and quantification of POBN and PBN nitroxides. *Free Radical Biol. Med.* **25**, 536−545.

(18) Sentjurc, M., and Mason, R. P. (1992) Inhibition of radical adduct reduction and reoxidation of the corresponding hydroxylamines in in vivo spin trapping of carbon tetrachloride-derived radicals. *Free Radical Biol. Med.* **13**, 151−160.

(19) Newton, D. J., Wang, R. W., and Lu, A. Y. (1995) Cytochrome P450 inhibitors. Evaluation of specificities in the in vitrometabolism of therapeutic agents by human liver microsomes. *Drug Metab. Dispos.* **23**, 154−158.

(20) Reinke, L. A., Moore, D. R., Sang, H., Janzen, E. G., and Kotake, Y. (2000) Aromatic hydroxylation in PBN spin trapping by hydroxyl radicals and cytochrome P-450. *Free Radical Biol. Med.* **28**, 345−350.

(21) Janzen, E. G., Poyer, J. L., Schaefer, C. F., Downs, P. E., and DuBose, C. M. (1995) Biological spin trapping. II. Toxicity of nitrone spin traps: dose-ranging in the rat. *J. Biochem. Biophys. Methods* **30**, 239−247.

(22) Babbs, C. F., and Gale, M. J. (1987) Colorimetric assay for methanesulfinic acid in biological samples. *Anal. Biochem.* **163**, 67−73.

(23) Kadiiska, M. B., Hanna, P. M., Hernandez, L., and Mason, R. P. (1992) In vivo evidence of hydroxyl radical formation after acute copper and ascorbic acid intake: electron spin resonance spin-trapping investigation. *Mol. Pharmacol.* **42**, 723−729.

(24) Zhong, Z., Connor, H. D., Yin, M., Moss, N., Mason, R. P., Bunzendahl, H., Forman, D. T., and Thurman, R. G. (1999) Dietary glycine and renal denervation prevents cyclosporin A-induced hydroxyl radical production in rat kidney. *Mol. Pharmacol.* **56**, 455−463.

(25) Caro, A. A., Cederbaum, A. I., and Stoyanovsky, D. A. (2001) Oxidation of the ketoxime acetoxime to nitric oxide by oxygen radical-generating systems. *Nitric Oxide* **5**, 413−424.

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