Free Rad. Res., Vol. 25, No. 6, pp. 475–488
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Spin Trapping Study in the Lungs and Liver of F344 Rats after Exposure to Ozone

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Accepted by Dr B. Kalyanaraman

(Received 10 February 1996; In revised form 26 April 1996)

Fischer 344 rats were injected with the spin traps C-phenyl N-*tert*-butyl nitrone (PBN, 150 mg/kg bw, *ip*) or 4-pyridine-N-oxide N-*tert*-butyl nitrone (POBN, 775 mg/kg bw, *ip*), and exposed to clean air or 2 ppm ozone for two hours. The presence of spin adducts was determined by electron paramagnetic resonance (EPR) spectroscopy of chloroform extracts of lung and liver homogenates. No significant levels of adducts were detected in the lungs of air control animals. Benzoyl N-*tert*-butyl aminoxyl, attributed to direct reaction of ozone with PBN, and *tert*-butyl hydroaminoxyl, the scission product of the hydroxyl adduct of PBN, were detected in the lungs of ozone exposed rats. EPR signals for carbon-centred alkoxyl and alkyl adducts were also detected with PBN in the lungs and liver of animals exposed to ozone. With POBN, only carbon-centred alkyl radicals were detected. Senescent, 24 months old rats were found to retain about twice more ^{14}C-PBN in blood, heart and lungs by comparison to juvenile, 2 months old animals. Accordingly, the EPR signals were generally stronger in the lungs of the senescent rats by comparison to juvenile rats. Together, the observations were consistent with the previously proposed notion that a significant flux of hydrogen peroxide produced from the reaction of ozone with lipids of the extracellular lining, or from activated macrophages in the lungs could be a source of biologically relevant amounts of hydroxyl radical.

Keywords: Ozone, spin trapping, PBN, POBN, free radicals, hydroxyl radical

INTRODUCTION

Ozonolysis of unsaturated fatty acids and phosphatidylcholine has been shown to generate hydrogen peroxide and aldehydes in *in vitro* model systems.[1] While ozone is highly reactive and is probably consumed within molecular distances from the sites of penetration through the extracellular lining of airways and alveolar ducts,[2,3] hydrogen peroxide in principle can diffuse into the epithelial cell layer. This was proposed as a pathway of toxicity of ozone, since hydrogen peroxide can be reduced by single electron transfer from copper or iron in a Fenton type reaction to produce the hydroxyl radical.[1] Given assumptions on the stoichiometry of the reactions of ozone with lipids *in vivo*[1] and the rate of incorporation of ozone in alveolar tissue

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of the central acinus,[4] the flow of hydrogen peroxide through alveolar septal cells during inhalation exposure of rats to 1 ppm ozone could be increased by as much as 15–20 fold above normal.[5] Even if only a fraction of the excess hydrogen peroxide effectively penetrates the glutathione peroxidase and catalase barriers, sufficient amounts of hydroxyl radical may conceivably be formed from scission of the unmetabolized hydrogen peroxide to injure the cells. Inhalation exposure to 1 ppm ozone for two hours was recently shown to result in a measurable increase of 2,3-dihydroxybenzoic acid in the lungs and plasma of rats injected with salicylic acid, consistent with the notion of *in vivo* generation of the hydroxyl radical.[5]

In addition to aldehydes, hydrogen peroxide and hydroxyl radical, spin-trappable free alkyl radicals are produced *in vitro*[6] as well as *in vivo*.[7] In a study using the spin trap 4-(pyridine-N-oxide) N-*tert*-butyl nitrone (POBN), Kennedy *et al.*[7] found, in chloroform lipid extracts of lung homogenates from animals exposed to ozone, an EPR spectrum consisting of a triplet of doublets with parameters a_N = 15.02 G, a_β^H = 3.27 G. The signal intensity of this triplet of doublets increased with the inhaled concentration of ozone. However, the control signal measured in POBN treated, air exposed rats was of significant intensity and had the same EPR parameters as those found after ozone exposure. In this study, ozone uptake by the animals was stimulated by concomitant inhalation of carbon dioxide, and samples were deep frozen until EPR analysis. Under these conditions, it is conceivable that decay of sensitive spin adducts could occur. Exposure of POBN to ozone *in vitro* resulted in an EPR spectrum with two components: a_N = 12.78 G, a_β^H = 2.72 G; and a_N = 16.08 G. In similar studies, no spin adduct signals were detected when C-phenyl N-*tert*-butyl nitrone (PBN) was used; when other spin traps were investigated, spectra were considered artifactual.[8]

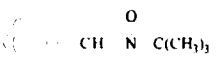
The spin adducts of the PBN-type of spin traps are relatively persistent for alkoxyl and alkyl radicals, but not for hydroxyl or peroxyl. However, degradation of the hydroxyl-PBN spin adduct yields *tert*-butyl hydroaminoxyl (TBHA), which could be viewed as an *in vivo* indicator of the hydroxyl radical.[9,10] Degra-dation of peroxyl-PBN spin adducts will produce benzoyl N-*tert*-butyl aminoxyl (PBNOX). Both TBHA and PBNOX are detectable by EPR. The spin traps PBN and POBN should then allow the survey of a variety of reactive intermediates produced from oxidation of macromolecules during ozone exposure. In the present report, we describe our comparisons of the EPR spectra obtained from the use of PBN and POBN in Fischer 344 male rats acutely exposed to ozone. Groups of three animals were injected with PBN or POBN, and then exposed to clean air or 2 ppm ozone for two hours. The adducts were analyzed in chloroform extracts. For exploratory assessments, we have used experimental animal groups that were heterogeneous in term of age: one animal per treatment group was 2 months of age (juvenile), one was 9 months of age (adult), and one was 24 months old (senescent). Although we have not detected increased hydroxylation of salicylate to 2,3-DHBA with age in response to ozone in previous studies,[5] potential age-dependent shifts in the profile of spin adducts in the lungs have not been previously investigated. In order to extend previous data on the pharmacokinetics of PBN,[11,12] the distribution of ^{14}C-PBN was also characterized in various organs of the aging animals.

MATERIALS AND METHODS

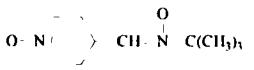
Chemicals

C-Phenyl N-*tert*-butyl nitrone (PBN) and C-(4-pyridyl N-oxide) N-*tert*-butyl nitrone (POBN) were purchased from OMRF Spin Trap Source (Oklahoma, OK). C-Phenyl-[^{14}C] N-*tert*-butyl nitrone (^{14}C-PBN) was custom synthesized by Amersham (Arlington Heights, IL). The specific activity of ^{14}C-PBN was 2.7 mCi/mmol, with radiopurity of 98.15%. Tissue solubilizer,

Protosol, and scintillation counting fluid, Atomlight, were obtained from New England Nuclear (Boston, MA).



PBN
C-phenyl N-*tert*-butyl nitrone



POBN
4-(pyridine-N-oxide)
N-*tert*-butyl nitrone

Animals

Specific pathogen-free, barrier-raised Fischer 344 male rats (Harlan Sprague Dawley, Minneapolis, MN) at 2 months (185–200 g), 9 months (395–410 g), and 24 months (440–460 g) of age were used. Rats were received in filter cages at least one week before the experiments, and were provided food and water *ad libitum*. The protocols were reviewed by the Animal Care Committees of the Health Protection Branch, Health Canada, and of the University of Guelph. The use of experimental animals was in accordance with the guidelines of the Canadian Council on Animal Care.

Distribution of PBN

^{14}C-PBN in saline was injected at a dose of 75 mg/kg *ip* (3 μCi/kg). At 15, 30, 60, 120, and 240 min after injection, the animals were anesthetized with sodium pentobarbital (65 mg/kg), and blood samples were withdrawn immediately from the left ventricle in heparinized tubes. The lungs, heart, liver, kidneys, and spleen were excised, rinsed in saline, blotted dry, and weighed. Tissue samples (1 g) were solubilized in 1 ml Protosol. The radioactivity was determined in 10 ml of scintillation fluid by liquid scintillation spectrophotometry. Each data point reflects the mean value for two animals.

Ozone

Ozone exposures were conducted as described elsewhere.[5] In brief, ozone was generated by flowing pure oxygen in a silent arc generator, mixed with dilution air, and distributed to an eight-port nose-only exposure teflon manifold. Constant inlet and exhaust flows were regulated with mass flow controllers. The ozone concentration was regulated with a mass flow controller linked by a feedback loop to a Dasibi ozone analyzer. Ozone concentration at the ports was verified by sampling through impingers containing a boric acid buffered potassium iodide solution. Flows were adjusted to provide a nominal concentration of 2 ppm ozone. Stability of the ozone concentration was within ±50 ppb.

Spin Trapping by Electron Paramagnetic Resonance (EPR)

Animals were administered PBN (150 mg/kg i.p.; 0.85 mmol/kg) or POBN (775 mg/kg i.p.; 4 mmol/kg) in saline, and immediately exposed to ozone for two hours. Air control animals were introduced in nose-only inhalation cones but exposed to room air for the same period. Animals were sacrificed and the lungs and liver were excised, homogenized and extracted with chloroform for PBN adducts, or Folch solution for POBN adducts. After clarification of the extracts by centrifugation (600 × g), the solvents were evaporated under a stream of nitrogen at room temperature, and the extracts were then dissolved in 0.4 ml of chloroform. Dissolved oxygen was removed by nitrogen bubbling, and the EPR spectra of the samples were immediately recorded at room temperature in an ST-EPR cavity with a Bruker ER-200 X band spectrometer. The instrument settings were: gain, 5×10^5; accumulation, 5–10 scans; microwave power, 20.5 milliwatts; modulation amplitude, 1 Gauss; time constant, 50 milliseconds; scan range, 100 Gauss; sweep time, 50 seconds.

RESULTS

Distribution of PBN

The distribution of α-^{14}C-PBN was measured in several tissues of the Fischer 344 rats over a period of four hours after a load of 75 mg/kg of

body weight. The following generalizations can be made. PBN reached maximal concentrations in plasma (Fig. 1A), heart (Fig. 1B) and lungs (Fig. 1C) within 30 minutes in all age groups, and was cleared thereafter at constant rates, on the order of 0.05–0.15 h^{-1}. Tissue levels of PBN were generally 40%–100% higher in the 24 months old animals by comparison to 2 months old animals. Rapid uptake of PBN in spleen (Fig. 2A), liver (Fig. 2B) and kidneys (Fig. 2C) of 24 months old animals measured at 15 min may be due to a slower penetration of the compound and more residual PBN in the peritoneal cavity. Distribution of the radioactivity to the kidney was delayed in the senescent rats (Fig. 2C), indicating an overall higher retention, possibly due to partition in a greater body fat compartment and/or slower metabolism. Elimination of PBN from plasma, lungs and liver was also slower in 24 months animals (0.075h^{-1}; 0.105 h^{-1}; 0.047 h^{-1}) by comparison to 2 months animals (0.093 h^{-1}; 0.150 h^{-1}; 0.156 h^{-1}). The distribution and clearance of POBN were not examined.

Control EPR Signal

The control EPR spectra at high gain revealed a pair of peaks with about 60–70 G separation. These two peaks come from the inner two lines of a paramagnetic sulphur liganded Cu (II) complex which is extractable in chloroform. Occasional background signal is obtained from the ascorbate anion radical consisting of two very narrow lines separated by a small spacing, but this pattern was not routinely observed in this study. While treatment with PBN did not give EPR spectra in the lungs of air control animals (Fig. 3, left panel), a trace control spectrum in the liver (e.g. 2 months old rat) was due to a 1:1:1 triplet with an $a_N \approx 14.7$ G and a broad triplet with the same N-HFS, namely $a_N = 14.7$ G where perhaps a 3 G doublet could have been hidden in the line width (Fig. 3, right panel). The parameters for the latter could indicate a carbon-centred spin adduct of PBN. These products are attributed to metabolism of PBN in the liver. The pattern was probably due to two aminoxyl triplets. The sharp-lined triplet is attributed to di-*tert*-butyl aminoxyl (DTBA), and the broad-lined triplet to a PBN spin adduct. However, the signal intensity was too weak to assign to a specific structure.

EPR traces from air-control rats injected with POBN revealed an EPR signal in the lungs of the 9 months old rat (Fig. 4, left panel). The pattern is attributed to two components, but the major triplet of doublets has hyperfine splitting (HFS) consistent with a carbon-centred radical adduct: $a_N = 14.75$ G, $a_\beta^H = 3.37$ G in chloroform. This result was surprising in nature and intensity, and could not be explained. In contrast, sizable control spin adduct signals consisting of triplets of doublets were obtained from POBN in liver extracts, with intensity of the signal decreasing with age (Fig. 4, right panel). Again, the patterns are interpreted as two components with the increase in β-H HFS indicating possibly a change in contribution from two different carbon-centred spin adducts (simulated values in parentheses): 2 months, $a_N = 14.55$ G, $a_\beta^H = 1.99$ G ($a_N = 14.55$ G, $a_\beta^H = 2.15$ G); 9 months, $a_N = 14.58$ G, $a_\beta^H = 2.30$ G ($a_N = 14.58$ G, $a_\beta^H = 2.30$ G); 24 months, $a_N = 14.72$ G, $a_\beta^H = 2.71$ G ($a_N = 14.72$ G, $a_\beta^H = 2.71$ G). In the PBN and POBN type of spin traps, the larger HFS's are generally associated with carbon-centred spin adducts. Within the family of carbon-centred adducts, the more bulky radicals give smaller β-H HFS, while the methyl radical gives the largest. Any values above $a_\beta^H = 3$ G are indicative of methyl or small straight chain alkyl radicals.

Spin Adducts of PBN After Inhalation of Ozone

Lungs

EPR spectra obtained from the lungs of 2 months and 9 months old rats treated with PBN and exposed to ozone were similar, although of dif-

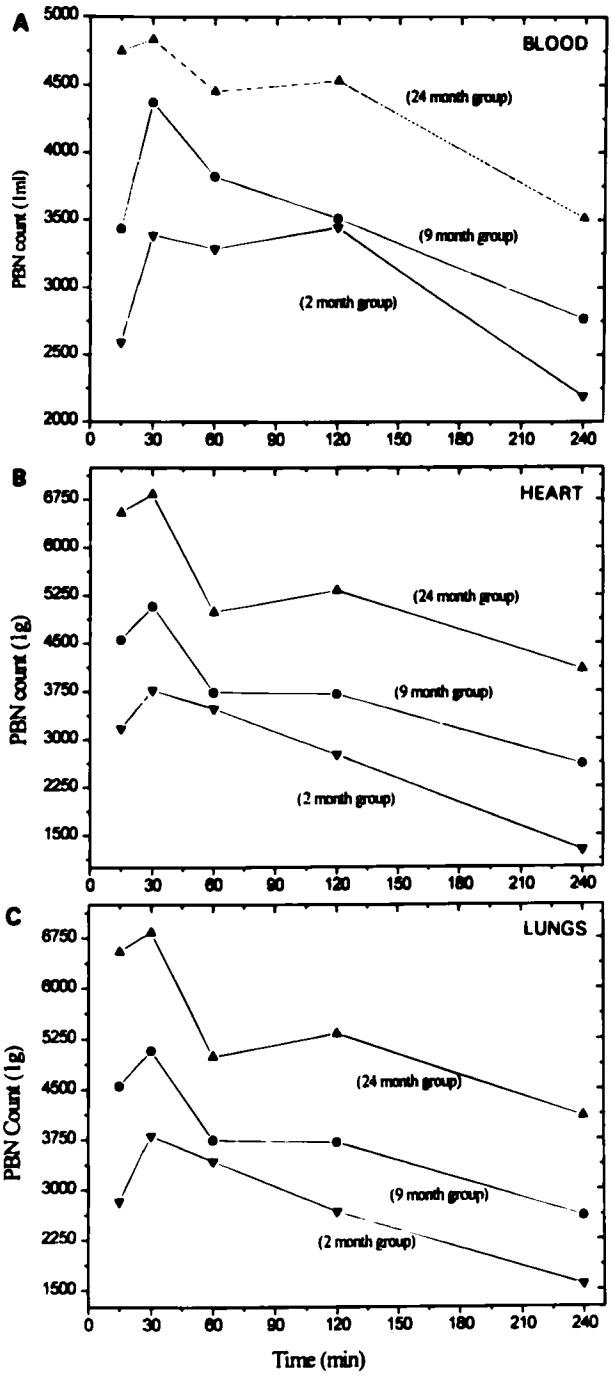


FIGURE 1 Distribution of α-^{14}C-PBN in blood (A), heart (B) and lungs (C) of 2 months (▼), 9 months (●) and 24 months old (▲) Fischer 344 male rats after i.p injection (75 mg/kg, 3 μCi/kg). Each point is the mean from two animals.

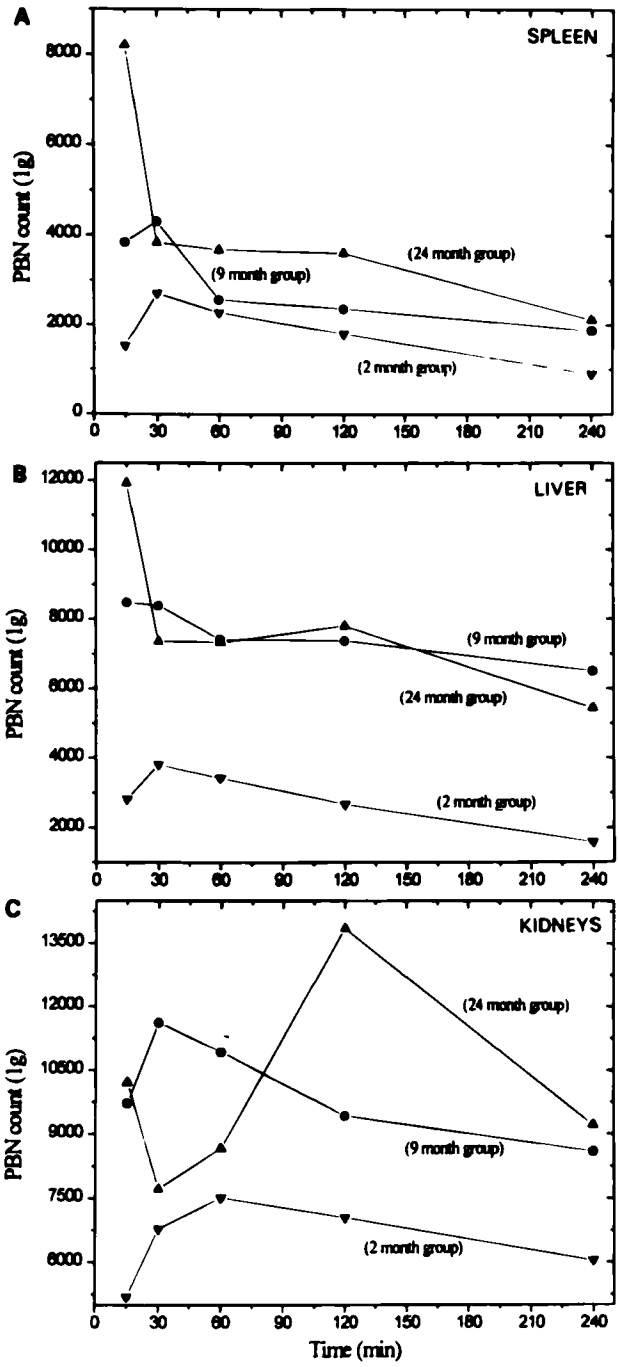


FIGURE 2 Distribution of α-^{14}C-PBN in spleen (A), liver (B) and kidneys (C) of 2 months (▼), 9 months (●) and 24 months old (▲) Fischer 344 male rats after i.p injection (75 mg/kg, 3 μCi/kg). Each point is the mean from two animals.

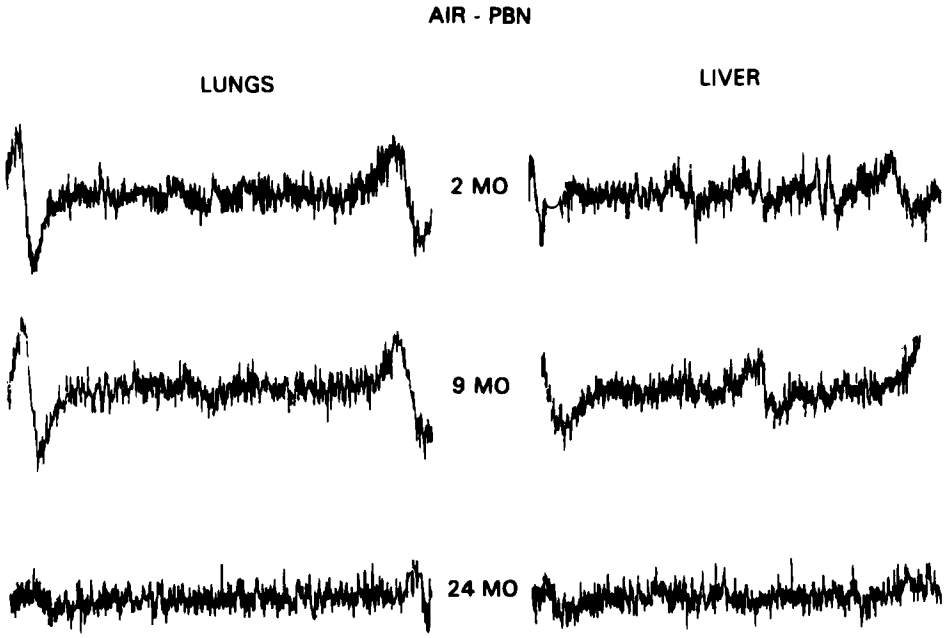


FIGURE 3 EPR spectra of chloroform extracts from the lungs (left) and liver (right) homogenates of air control rats injected with PBN. Note the two background peaks attributed to a Cu(II) sulphur complex.

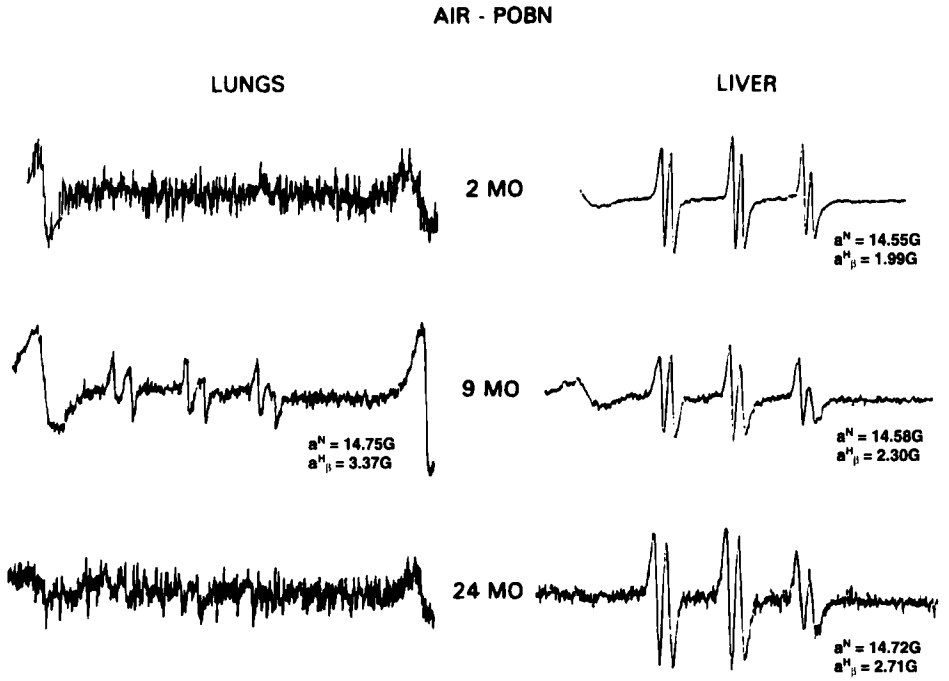


FIGURE 4 EPR spectra of chloroform extracts from the lungs (left) and liver (right) homogenates of air control rats injected with POBN. Note the two background peaks attributed to a Cu(II) sulphur complex.

ferent intensities (Fig. 5, left panel). The 24 month rat gave the strongest EPR signal. There were at least three components in the two spectra from 2 months and 9 months animals: the strongest, a_N = 7.98 G known as PBNOX (three peaks); equally strong, a_N = 14.99 G, a_H = 17.59 G attributed to TBHA (six peaks; two peaks hiding in the outer two lines of PBNOX); the weakest, a_N = 14.38 G, a^H_β = 2.64 G, a spin adduct (six peaks). From the 24 month old rat, there were two components: the strongest, a_N = 14.72 G, a^H_β = 2.71 G, a spin adduct; the weakest, a_N = 7.96 G, PBNOX. The values for the spin adducts indicate carbon-centred radical spin adducts, i.e. typically a_N = 14.4–15.0 G, a^H_β = 2.6–3.3 G, but further assignment is not possible. Absence of a TBHA signal in the 24 months old animal may be due to the known instability of this species.

Liver

The major difference between lung and liver extracts of animals treated with PBN and exposed to ozone is the absence of PBNOX and TBHA in liver (Fig. 5, right panel). A very small trace of a pair of lines for each species is nevertheless visible from the 9 months old rat in the inner portion of the pattern. Only a sizable triplet of doublets is detected at levels above those detected in air control animals: 2 months, a_N = 14.68 G, a^H_β = 2.75 G; 9 months, a_N = 14.68 G, a^H_β = 2.98 G; 24 months, a_N = 14.79 G, a^H_β = 2.85 G. The assignment is to carbon-centred spin adducts. The HFS were all similar, although inspection of shapes and doublet intensities indicates that two components could be present. The values were somewhat larger for the liver extracts than for the

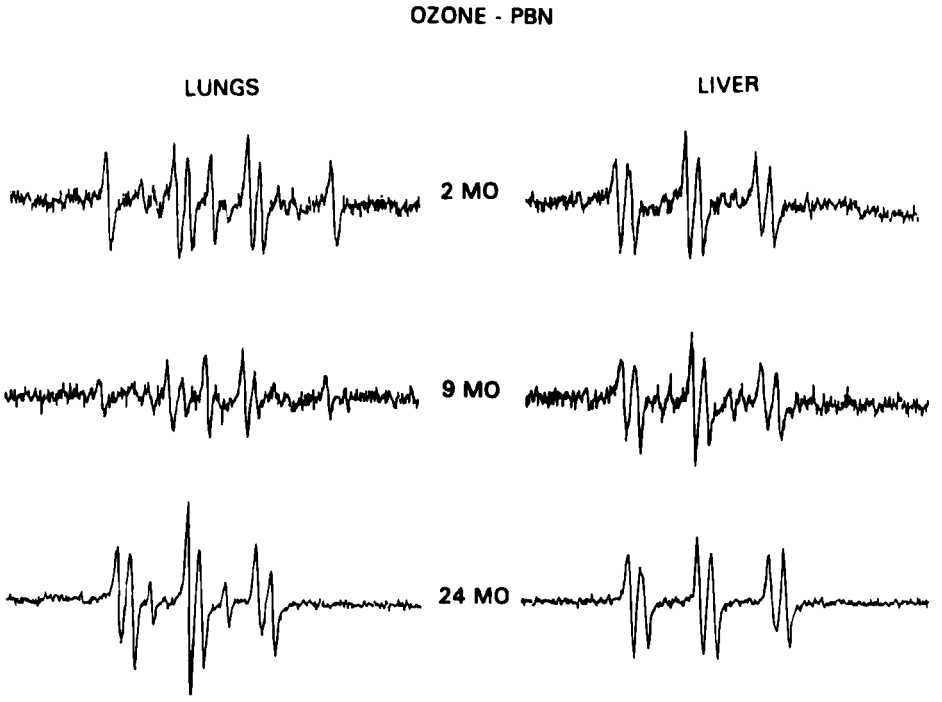


FIGURE 5 EPR spectra of chloroform extracts from the lungs (left) and liver (right) homogenates of ozone exposed rats injected with PBN. In liver, note small peaks between the first and centre doublet, and last and centre doublet assigned to weak PBNOX lines (inner two), and two lines of TBHA (outer two).

lung extracts, which could suggest a shorter chain alkyl radical. While HFS between age groups were similar, the intensity of the EPR signal increased with age, which correlated with intensity of signal in the lungs.

Spin Adducts of POBN After Inhalation of Ozo ne

Lungs

The EPR spectra obtained from the lungs of ozone treated rats using POBN clearly showed the presence of a triplet of doublets (Fig. 6, left panel). Equivalent spectra were obtained in rats of different age, but with the intensity increasing with age (relative intensity in parentheses): 2 months, a_N = 14.72 G, a_β^H = 2.49 G (1X); 9 months, a_N = 14.65 G, a_β^H = 2.40 G (2.25X); 24 months, a_N = 14.62 G, a_β^H = 2.53 G (2.65X); average, a_N = 14.66 G, a_β^H = 2.47 G. Although the spectra were well resolved, closer inspection suggested that each triplet of doublets was due to a mixture of spin adducts with small differences in HFS and g values. It should be noted that in a 9 months air-control animal, only a weak POBN spectra was found, with a different spacing; the doublet spacing, a_β^H = 3.37 G, was larger than for the average spectra in ozone exposed animals, a_β^H = 2.47 G.

Liver

Samples from liver of animals injected with POBN and exposed to ozone also gave well resolved triplets of doublets (Fig. 6, right panel), but the intensities seemed to decrease with age (relative intensity in parentheses): 2 months, a_N =

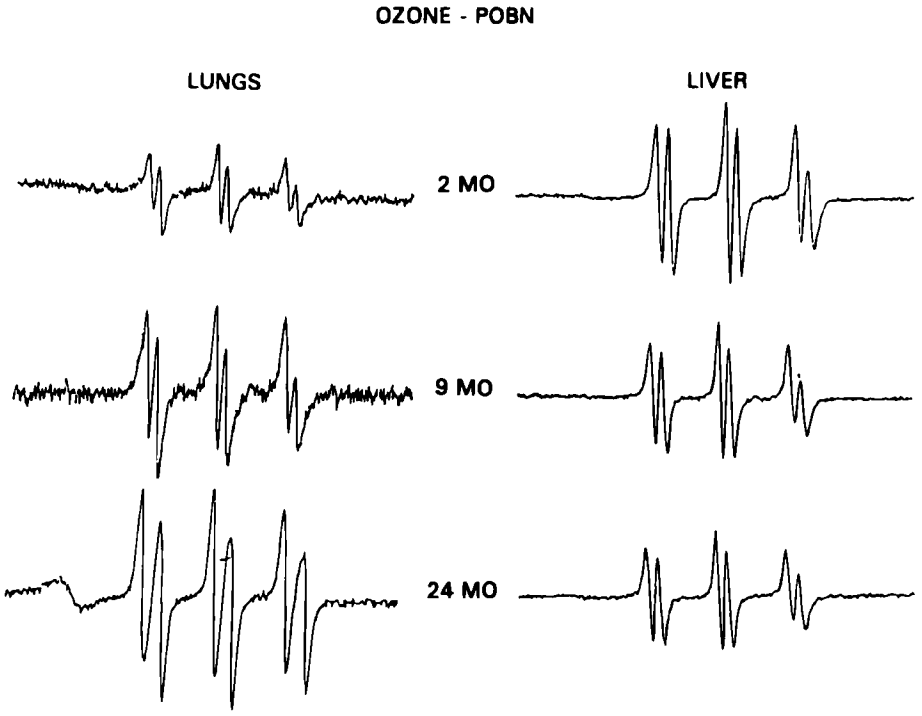


FIGURE 6 EPR spectra of chloroform extracts from the lungs (left) and liver (right) homogenates of ozone exposed rats injected with POBN.

14.45 G, a_{β}^{H} = 2.31 G (1X); 9 months, a_N = 14.55 G, a_{β}^{H} = 2.26 G (0.74X); 24 months, a_N = 14.58 G, a_{β}^{H} = 2.44 G (0.64X). Some distortion in line shapes and peak intensities indicated detection of a mixture of spin adducts. The average values for these three sets of HFS (average, a_N = 14.53 G, a_{β}^{H} = 2.34 G) were slightly smaller than the average values obtained for the lungs (average, a_N = 14.66 G, a_{β}^{H} = 2.47 G) or from liver of air control animals (average, a_N = 14.61 G, a_{β}^{H} = 2.39 G). The differences between the HFS values suggests that different spin adducts were detected between control and ozone-treated animals, and between lungs and liver of ozone-exposed animals.

Effect of Solvent on Hyperfine Splitting Constant of POBN

The literature has relatively few tabulations of POBN spin adducts as a function of the nature of group which was the radical and demonstrating the effect of solvent on the HFS's. A number of organic radicals were trapped by POBN and the EPR spectra recorded in various solvents. This data should provide some indication as to the possible assignment of structure of the spin adducts detected in the present study (Table 1). The parameters obtained from four carbon-centred radicals (methyl, 2-cyanopropyl, hydroxymethyl, and phenyl), two oxygen-centred radicals (2-cyanopropyloxyl and *tert*-butoxyl), and the hydrogen atom show interesting trends. First, the nitrogen HFS's all increase with increase in polarity of the solvent. However, the increase is less with more polar groups in the radical which is trapped. Second, the β-hydrogen HFS also increases in magnitude with increase in solvent polarity, with the exception of hydroxymethyl. For this radical adduct, the β-H HFS decreases with increasing solvent polarity; this effect has been reported before and was attributed to intra-molecular H-bonding within the spin adduct between the hydroxyl proton and the aminoxyl function.[13] This effect is only significant in relatively non-polar solvents such as benzene and chloroform. Finally, both the N- and β-H HFS's are smaller when the adduct is an oxyl adduct as compared to a carbon-centred adduct. The effect of polar groups in the radical have a cumulative effect; e.g. an oxyl adduct with a cyano group produces a smaller set of parameters than an oxyl group by itself.

DISCUSSION

The results of our study in Fischer 344 male rats exposed acutely to a high concentration of ozone bring the following novel observations. *i)* The lungs of animals injected with PBN had detectable levels of benzoyl N-*tert*-butyl

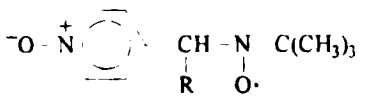


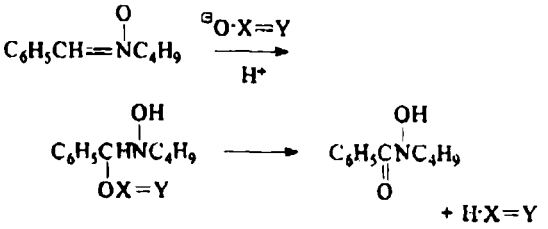
TABLE 1 EPR Hyperfine Splitting Constants for Radical Adducts of 4-POBN in Different Solvents

		Radicals, a_N,[a]						
Solvent	$E_{(T30)}$[b]	· H	· CH_3	· $C(CH_3)_2CN$	· CH_2OH	· C_6H_6	· $OC(CH_3)_3$	· $OC(CH_3)_2CN$[c]
Benzene	34.5		14.68,2.53	14.21,2.76	14.69,3.71	14.38,2.03	14.21,1.80	13.77,1.90;13.76,1.73 13.73,1.76
Chloroform	39.1		14.96,2.32		14.82,3.57	14.52,2.17		13.97,1.76
Methanol	55.5		15.05,2.35		14.81,2.44	14.58,2.62		13.96,1.76
Water	63.1	16.20,10.20	16.12,2.77	15.16,3.07	15.50,2.61	15.60,3.35		14.79,2.03

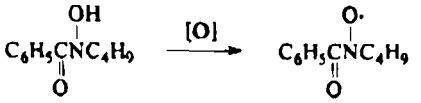
[a]The EPR hyperfine splitting constants are listed in G and were calibrated against Fremy's salt (Aldrich) in aqueous $NaHCO_3$.
[b]$E_{(T30)}$ is "energy" of transition (visible absorption) of an indicator used to establish a solvent polarity scale.
[c]The values in benzene listed are typical "reproducible" results by different EPR operators.

aminoxyl, PBNOX, attributed to direct reaction of ozone with PBN, and *tert*-butyl hydroaminoxyl, TBHA, the scission product of the hydroxyl adduct of PBN. *ii)* Carbon-centred spin adducts of PBN (e.g. a_N = 14.4–15.0 G, a_β^H = 2.6–3.3 G) were detected in both the lungs and liver of ozone exposed rats, but not in air control rats, which could indicate redistribution of spin adducts of PBN from the lungs to the liver. *iii)* TBHA and a nitroxide equivalent to PBNOX were not detected in the lungs of animals injected with POBN and exposed to ozone, suggesting some possible differences with PBN in term of microanatomical distribution. *iv)* Carbon-centred spin adducts of POBN were increased in lungs and liver of ozone exposed animals. However, while the EPR signal was stronger with age in the lungs, it was decreased with age in the liver.

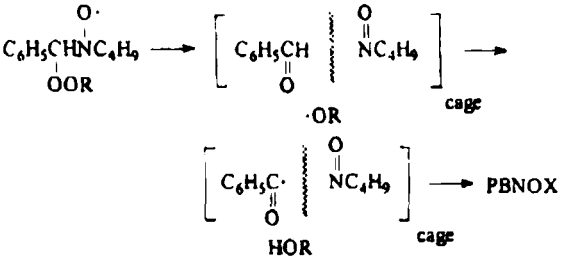
The production of PBNOX is well known in oxidizing systems.[14] A number of radical and non-radical species similar to ozone react with PBN as follows:



If ozone is taken as an example of OXY, then PBNOX could be obtained from the simple one electron oxidation of the hydroxamic acid product:

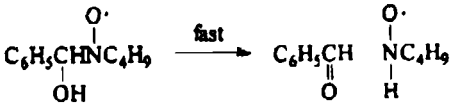


The formation of PBNOX is also possible in the presence of peroxyl radicals since it has been shown that peroxyl radical spin adducts undergo scission to produce PBNOX, possibly by a solvent cage reaction/recombination process:[15]

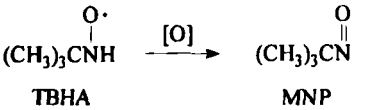


In our study, production of PBNOX is assumed to be the only reaction product from ozone and the spin trap. PBNOX was detected in the lungs, but not in liver. This was consistent with the notion that ozone reacts with material of the extracellular lining and does not reach the liver as unreacted species.[2,3]

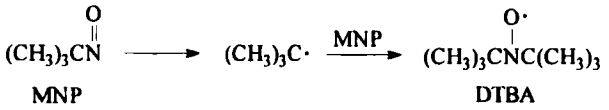
Of particular interest was the detection of TBHA in the lungs of ozone exposed rats. The detection of TBHA as a major nitroxide product can be rationalized in terms of a significant amount of hydroxyl radical adduct to PBN being formed, because the decay of this spin adduct in polar aqueous systems is known to be quantitative and fast:[9,10,16]



Aside from *in vivo* generation of hydroxyl radical, the other possible route for hydroxyl-PBN is hydrolysis of PBN followed by oxidation. However, this seems unlikely since no trace of TBHA was observed in air-exposed, PBN-treated animals. Consequently, we interpret the presence of TBHA as the scission product of the hydroxyl radical adduct to PBN. This reduced form of 2-methyl-2-nitrosopropane (MNP) is rarely seen in oxidizing systems since oxidation of TBHA should be fast to produce MNP:



The presence of MNP as a precursor of di-*tert*-butyl aminoxyl (DTBA) is likely:



It is not clear if the highly active monooxygenase system of the liver will accelerate conversion of TBHA to MNP. Conceivably, the hydroaminoxyl would have a longer half life in the lungs. If this overall scheme is correct, then quite sizable amounts of hydroxyl radical must be produced from reaction of ozone in the lungs *in vivo*. This conclusion is in line with our observation that ozone inhalation can increase lung and plasma 2,3-dihydroxybenzoic acid in rats injected with salicylate, an efficient probe for hydroxyl radical.[5] The detection of hydroxyl radical in the lungs can be rationalized from the reaction of ozone with unsaturated lipids and the production of hydrogen peroxide, which is reduced to hydroxyl radical in a Fenton reaction.[1,6] Conceivably, activated macrophages on the alveolar side of the septum and neutrophils on the capillary side could also produce significant amounts of superoxide anion, hydrogen peroxide, and hydroxyl radical.

The observation that POBN did not produce measurable amounts of TBHA in the lungs of ozone treated animals in spite of the fact that the hydroxyl radical adduct of POBN will decompose to give TBHA is puzzling. Also, no POBN nitroxide equivalent to PBNOX was detected. Furthermore, we have not detected a 12-line spectrum indicative of a POBN/*POBN adduct (a^H = 1.8 G, a^N_1 = 14.9 G, a^N_2 = 1.85 G) which might be expected from activated macrophages or neutrophils.[17] The partition coefficients of PBN and POBN are different, and thus PBN may be sampling a more lipophilic region than POBN in the lungs, such as the surfactant compartment. Alternatively, TBHA is unstable and failure to detect this product from POBN treated animals may be due to rapid degradation. At any rate, the data suggest that elucidation of the microanatomical distribution and mechanisms of clearance of the spin traps within the pulmonary compartment could possibly provide additional clues as to the sites and sequence of reactions leading to tissue injury.

A general observation in our study was the higher levels of spin adducts of PBN and POBN in the lungs with increasing age, which we attribute to higher tissue levels of the spin traps due to age-related differences in their pharmacokinetics. This is supported by measurements of the distribution of ^{14}C-PBN, although we have not investigated the distribution of POBN. In a quantitative comparison of the EPR signals between age groups, equivalent tissue concentrations of the traps would permit the assumption of first-order kinetics with respect to free radical flux; that is the concentration levels of spin adducts would be sensitive mainly to the amount of free radicals produced. In a separate set of studies, we have not observed significant differences between 2 months, 9 months and 24 months old Fischer 344 rats in the rates of incorporation of ^{18}O-ozone to the extracellular lining of the lungs, or in the general status of antioxidant defense.[18] Therefore, it is unlikely that differences in EPR signal intensities in the lungs were dominated by differences in ozone dose or in the efficiency of scavenging of early oxidation products. Our assessments using salicylate as a probe did not suggest quantitative differences between juvenile, adult and senescent Fischer 344 rats in the ozone-induced levels of hydroxyl radical.[5]

It is noteworthy that the intensity of the EPR signal for PBN induced by inhalation of ozone increased with age of the animals for both the lungs and the liver; while higher signal with age is attributed to higher levels of the trap in the lungs, this may not hold true for the liver. We propose that spin adducts of PBN formed in the

lungs were redistributed to the liver. If this interpretation is correct, then some activated species generated in the lungs during ozone exposure must reach other sites in the body. This may have toxicological relevance and explain some of the known extrapulmonary responses to ozone exposure.[19] If the EPR signal for POBN in liver of air control rats was due to metabolism, decrease of this signal with age could imply decrease of hepatic P450 activity in older rats. Those adducts generated in the liver apparently did not produce a background EPR signal in the lungs. Therefore, the strong EPR signal for POBN in the lungs of animals exposed to ozone can be attributed to a site-specific generation of spin adducts. The higher EPR signal in the liver after ozone can be rationalized by a redistribution of adducts of POBN from the lungs to the liver. The present results do not confirm nor refute the possibility that senescent animals could have higher amounts of free radicals produced upon inhalation of ozone. Shifts in the patterns of adducts with age (e.g. differences in HFS) could theoretically reveal subtle differences in the initial cascade of free radical reactions during ozone exposure, even if the overall intensity of these reactions were not significantly different in aging animals by comparison to juvenile animals. However, our preliminary data bring a reality check and indicate that significant age-related differences in the distribution, metabolism and clearance of the spin traps, as well as of the spin adducts, could complicate a quantitative interpretation of results.

Very little is known about the structure of the spin adducts and further work should be performed to define the *in vivo* chemistry of ozone toxicity. Isotopic EPR signal enhancement from the use of deuterated PBN or POBN may be possible, and the utilization of carbon-13 in the case of PBN should also be feasible. Specific traps for stable hydroxyl radical adduct formation would allow a more direct assessment of this species. At the moment, the best structural assignment for the adducts in the lungs of ozone exposed animals is to carbon-centred spin adducts with perhaps some electronegative group attached. For example, the observed EPR parameters did not fit a methyl group (in the case of POBN), but matched well with other possible carbon-centred radical groups, such as ethyl, n-pentyl, or a secondary alkyl radical. The EPR parameters definitely do not fit an alkoxyl type of radical. However, it is believed that the source of alkyl radicals, such as ethyl or n-pentyl, could be an alkoxyl radical. Rapid β-scission may be occurring to give spin adducts due to alkyl radicals.

Acknowledgements

This work was supported by Health Canada (project K281232), the MRI Facility core funding of the University of Guelph, and the Natural Sciences and Engineering Research Council of Canada. We are also grateful to Dr. Yong-Kang Zhang who obtained some of the EPR parameters for POBN spin adducts, and to Ms. Luci White for her help in the preparation of the manuscript.

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