Free Rad. Res. Comms., Vol. 16, No. 3, pp. 167-174
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MEASUREMENT OF FREE RADICALS FROM SMOKE INHALATION AND OXYGEN EXPOSURE BY SPIN TRAPPING AND ESR SPECTROSCOPY

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(*Received September 16, 1991; in revised form December 24, 1991*)

Research in smoke inhalation has established that free radicals are produced from gases released during combustion and these species impair lung function. Using spin traps and their adducts in an animal model free radicals were measured. Various hyperbaric oxygen regimens were tested in an attempt to attenuate pulmonary damage caused by free radical reactions. Our data demonstrated that persistent oxygen- and carbon-centered free radicals are detectable in intravascular fluids after smoke inhalation. The smoke inhalation model showed however, clearing of spin trap adducts one hour after smoke exposure. Other researchers have found that when 100% oxygen is given at 1 atmosphere absolute (ATA) for 1 h, free radicals were not detectable. However, oxygen given at 2.5 ATA does produce detectable free radicals. With continued exposure at this pressure, the levels of free radicals increase for up to 60 min. This study suggests that the level of free radical induced oxygen toxicity may be a function of oxygen pressure and duration of oxygen exposure.

KEY WORDS: Smoke, oxygen toxicity, spin trapping, ESR, PBN.

INTRODUCTION

Compounds such as those found in gases produced during combustion or high concentrations of inspired oxygen can cause direct lung damage because of the formation of oxygen- or carbon-centered free radicals.[1-3] These free radicals can readily enter lung cells after inhalation and disrupt normal function when their concentrations rise to toxic levels.[1] Selective toxicity may occur to bronchial or pulmonary epithelial cells from surface contact with gaseous products. Free radical toxins can accumulate against a gradient in lung endothelium and then may produce damage directly or indirectly by activation of lung leucocytes and macrophages.

A number of studies[5-8] have suggested a role of free radicals in smoke inhalation injury but have used methods that do not directly quantify the amount or type of free radicals produced either primarily or secondarily to the inhalation injury process. Reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radicals have been suggested to be present following a smoke inhalation injury and

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play a role in the injury process because various oxygen free radical scavengers have shown some effectiveness in reducing aspects of inhalation injury damage.[5,7] Using a more direct method for the measurement of free radicals would help to overcome some of the difficulties in previous studies and allow for a quantification that could provide a basis for a more rational approach to therapy.

Spin traps, such as N-t butyl alpha phenylnitrone (PBN), are altered in the presence of free radicals and these changes may be measured by electron spin resonance spectroscopy (ESR). In the present study, we measured changes in PBN spectra after smoke inhalation, in an animal model, to determine the amount and type of free radical production. Since oxygen is often used to treat smoke inhalation injury[9] we also measured the amount of free radicals produced by oxygen administration alone and in combination with the smoke inhalation injury.

MATERIALS AND METHODS

In Vitro

To determine the dose of PBN and its suitability for detection of free radicals in smoke and oxygen exposure systems, an *in vitro* system of rabbit blood or lung cells in a culture suspension was used. Suspensions of normal lung cells and blood were equilibrated with a stream of smoke and high pressure oxygen in the presence of the spin trap, PBN. Freshly drawn rabbit blood was added to sterile Eagle's Minimum Essentials Medium (Sigma Chemical Co.) and smoke from cotton combustion[10] was bubbled through the suspension for 4 min.

This smoke was produced by burning absorbent cotton under reduced air flow. The smoke was not filtered but was cooled to room temperature before exposure to the cell suspensions. PBN (Sigma Chemical Co.) at 169 mM was added to the suspension and gently mixed for 5 min. The right lung from an euthanized rabbit was homogenized in a tissue grinder and added to sterile culture medium as described for the blood sample. Smoke was bubbled through the suspension for 4 min and then PBN at 1.69 mM was added and gently mixed for 5 min. The dose of PBN added was that used by Bolli *et al.*[11] in a study of *in vivo* radical production during myocardial ischemia. Control suspensions of lung and blood cultures were also prepared in which the suspensions were handled in the same way as for the smoke exposure except that smoke was not bubbled through them. Additional groups of lung and blood cell cultures were established in which the suspensions were placed in a hyperbaric chamber and pressurized to 2.5 atmospheres absolute (ATA) with 100% oxygen and held for 30 min before returning to normal room pressure. At the end of oxygen exposure, PBN at 300 μg/ml was added and allowed to mix for 5 min.

In all culture experiments, at the end of the PBN mixing, the suspensions were placed into test tubes and centrifuged to obtain the supernatant solution which was frozen and stored immediately at -70°C until extraction, as PBN adducts are known to be stable especially at lower temperatures. Studies by Bolli *et al.*[11] involved PBN adducts in plasma and were frozen at -70°C for subsequent analysis. Hence, extraction and concentration of the PBN were performed by this method. Briefly, this involved adding the supernatant to a chloroform/methanol solution, mixing thoroughly then adding sodium chloride for phase partitioning, recovering the chloroform layer and evaporating it to produce a 100 μl sample for EPR analysis.

In Vivo

New Zealand White rabbits (3–5 kg) were anesthetized with intramuscular injection of ketamine (35 mg/kg) and acepromazine (1 mg/kg) and catheters installed in an artery and vein of the ear. Those animals receiving smoke inhalation ($n = 10$) were intubated with 2.5 to 3.0 mm endotracheal tubes. Stable anesthetized test animals were allowed to spontaneously breathe cooled (22°C) cotton smoke until blood carbon monoxide levels of 60–70% were achieved.[10,12] Arterial carbon monoxide levels were determined on a Radiometer Hemoximeter®. The time of total smoke exposure was two to four minutes. Control animals ($n = 5$) were anesthetized and intubated but not exposed to smoke or hyperoxia. Hyperoxia was accomplished in a hyperbaric chamber supplied with 100% oxygen. One group of anesthetized animals ($n = 5$) was exposed to 100% O_2 at room atmospheric pressure (1 ATA) for 90 min total time. Subsequent groups ($n = 3$ for each time exposure) were pressurized to 2.5 atmospheres absolute (ATA) over a 10 min period, allowed to remain at pressure for 10, 20, 30 or 60 min and then decompressed over 10 min. This regimen of hyperbaric exposure has been shown to produce little overall stress to the animals and no visible toxic effects.[12]

At the end of the smoke or oxygen exposure period, PBN at 300 μg/ml of intravascular blood volume (65 ml blood volume/kg body weight) was injected after being dissolved in 5 ml of 0.9% normal saline. The PBN was slowly injected into an ear vein over a 2 min period. Five minutes after the end of PBN infusion, a 20 ml blood sample was taken from the ear artery in a heparinized syringe. The blood sample was immediately centrifuged and the plasma frozen for later extraction and concentration. Extraction and concentration were as described for *in vitro* samples.

EPR Analysis

Samples in sealed glass capillaries were run on either a Bruker ESP 300 ESR Spectrometer or a Varian E 3 Spectrometer. Spectrometer settings were as follows: microwave power, 19.7 mW; modulation amplitude, 3 G; time constant, 163 ms; scan range, 140 G; field center, 3476 G; sweep time 42 s with 16 averaging. All spectra were recorded at room temperature 23°C.

RESULTS

In vitro studies showed the presence of oxygen and carbon-centered radical adducts in exposures to smoke and hyperoxia as shown in Figure 1. Spectra obtained were characterized by a triplet of doublets with some asymmetry of the central doublet and with coupling constants $a_N = 14.7–15.0$ G and $a_B^H = 2.6–3.0$ G. The spectra are consistent with a nitroxyl-radical adduct resulting from the spin trapping by PBN of either oxygen- or carbon-centered radicals. The spectra obtained in this study are similar to either cellulose spectra[16] or yellow pine wood smoke.[15] Comparisons to tobacco smoke, polyethelene or rubber smoke showed marked differences from the spectra obtained from cotton smoke.[15,16] Controls with PBN alone however, showed no indication of radical adduct formation.

In vivo studies with smoke inhalation showed spectra as seen in Figure 2. As in the *in vitro* study, controls showed nearly flat spectra when analyzed by ESR. Plasma samples from smoke exposed animals exhibited spectra similar to those *in vitro* smoke

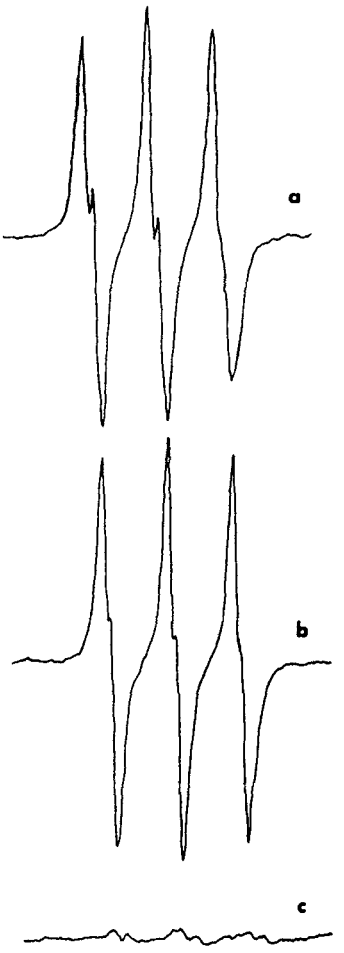

FIGURE 1 EPR spectra of extracts of *in vitro* preparations. EPR settings as described in text, Gain = 4×10^5. (a) PBN adducts from smoke exposure, (b) PBN adducts resulting from hyperbaric oxygen exposure, (c) control exposed only to air.

samples and had a characteristic triplet of doublets. Plasma samples from animals with blood withdrawn up to 15 min after PBN injection showed intense signals while those taken up to 60 min post exposure had nearly flat signals indicating that the radicals produced during smoke inhalation were short-lived. Spectra from animals given hyperoxic exposures are shown in Figure 3. Those animals exposed to 100% oxygen at 1 ATA exhibited nearly flat spectra indicative of little or no radical formation. Animals exposed to hyperbaric oxygen at 2.5 ATA, however, showed spectra indicative of significant radical production. While similar to spectra obtained from smoke exposed animals, it appears that the free radical adducts from oxygen exposures are simpler and may not contain as complex a mix. Increasing the duration of oxygen exposure resulted in strongly increased signals and thus more free radical production as shown in Figure 4. As with smoke exposures, plasma samples removed from

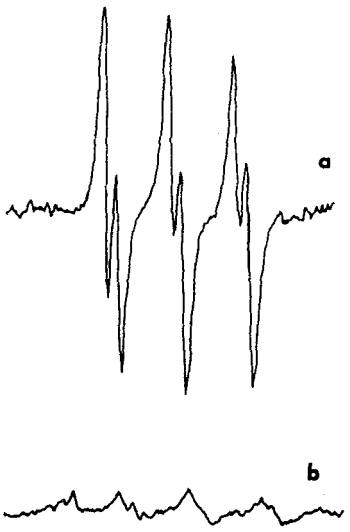

FIGURE 2 EPR spectra from *in vivo* preparations. Gain = 4×10^5. (a) Typical PBN adduct signal from smoke exposed animals, plasma collected within 15 min of cessation of smoke, (b) control plasma sample from animal breathing room air showing PBN signal.

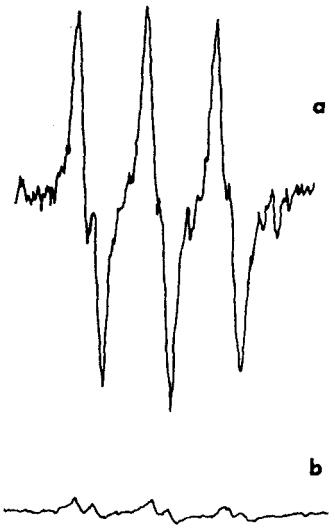

FIGURE 3 EPR spectra from *in vivo* preparations. Gain = 4×10^5. (a) Typical PBN adduct signal from hyperbaric oxygen exposed animals (30 min total exposure), (b) PBN adduct signal from animals exposed to 1 ATA 100% oxygen for 90 min.

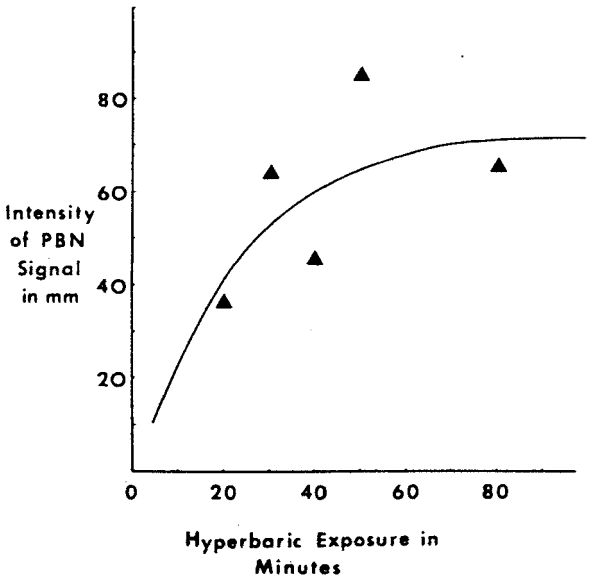

FIGURE 4 Amount of free radical production *in vivo*, expressed as intensity of PBN signal in mm of height of second doublet of EPR spectrum, versus time of exposure to hyperbaric oxygen at 2.5 ATM. For each exposure time, $n = 3$. The curve represents theoretical response.

oxygen exposed animals 60 min after termination of exposure showed weak or flat spectra.

DISCUSSION

Indirect proof of free radical production after smoke inhalation has been previously shown primarily by the reduction of vascular permeability after treatment with free radical scavengers.[5,7] Pulmonary edema, one of the manifestations of smoke inhalation, has been shown to be at least, in part, mediated by free radicals and can be attenuated to some degree by radical scavengers such as superoxide dismutase, dimethylsulfoxide and catalase.[13] Direct damage from smoke may result from the influx of neutrophils to the lungs and their subsequent release of free radicals.[7,14] Free radicals are also known to be present in woodsmoke and smoke from cellulose, in addition to other chemical components.[15,16] These sources may cause damage by direct action as well as through activation of macrophages and neutrophils. In this study we have shown that the spin trap PBN can be used to detect free radicals in both *in vitro* cell suspensions and live animals exposed to cotton smoke. The dose of PBN used was sufficient to obtain ESR signals yet did not produce signals in control animals not exposed to a source of free radicals. This precludes the possibility of a nonspecific toxic effect of PBN and shows that the ESR signal is related to free radical production. Previous work[17] using PBN in rats has shown that its excretion is slow and that it distributes evenly to various tissues. In the present study, PBN concentrations could be expected to have remained stable over the course of time necessary to form adducts with smoke produced free radicals.

In addition to radicals produced by smoke and neutrophils, oxygen at high concentrations is known to cause toxicity related to polymorphonuclear leukocytes or direct contribution of reactive radical species.[18-21] Oxygen is, however, a normal part of supportive care for victims of smoke inhalation[22] and could be a contributing factor in radical production. In this study we have found a significant contribution of free radicals from exposure to hyperbaric oxygen at 2.5 ATA but no evidence of radicals at 1 ATA 100% oxygen. This suggests that oxygen at 1 ATA does not appear to contribute to free radical toxicity for the time exposures used in this study, but when hyperbaric oxygen is used, free radicals are produced. Lengthening the duration of hyperbaric oxygen exposure produced an increase in free radical adducts. One hour after cessation of oxygen exposure, no free radical adducts were recoverable. The direct contribution of free radicals in the hyperbaric oxygen model, as with smoke, seems to be a relatively short-lived phenomenon yet with unknown specific pathological consequences. The contribution of free radicals from a smoke inhalation injury combined with that from hyperbaric oxygen, though, could be expected to have physiological impacts directly affecting the lungs. In this study, the free radical adducts found were similar to those described in an ischemia/reperfusion study using the same spin trapping agent.[23] Smoke inhalation and hyperbaric oxygen exposures could be causing a situation that is at least, in part, similar to ischemia and may lead to further release of free radicals.

Indirect proof of free radical involvement in smoke or oxygen exposure has been previously reported, but this study shows direct evidence of free radical formation using spin trapping and ESR analysis. This technique has implications in further investigation of smoke induced pathophysiology. While free radical scavengers have shown some usefulness in attenuating damage caused by smoke, it remains unknown as to which scavenger is most effective in reducing the free radicals produced or what dosage is required for radical attenuation. The antioxidants of human extracellular fluids could also be very important in the attenuation of free radicals produced by either smoke or oxygen and could be investigated as to their reactivity in this type of system.[24] The release of free radicals associated with polymorphonuclear leukocytes after the initial smoke exposure could be investigated using a direct measure of free radical production such that a strategy for control could be developed. Oxygen exposures for treatment of smoke inhalation or other conditions could similarly be investigated to determine optimal benefits from oxygenation while minimizing possibilities or actual production of free radicals.

Acknowledgement

The authors are grateful for financial support provided by The International Association of Fire Fighters Burn Foundation.

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Accepted by Prof. B. Halliwell