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Assessment of oxidative stress in lungs from sheep after inhalation of wood smoke

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Abstract

To elucidate potential dose-dependent mechanisms associated with wood smoke inhalation injury, the present study evaluated antioxidant status and the extent of pulmonary injury in sheep after graded exposure to smoke. Adult, male sheep ($n = 4$–5 per group) were anesthetized and received 0, 5, 10 or 16 units of cooled western pine bark smoke, corresponding to 0, 175, 350 and 560 s, respectively, of smoke dwell time in the airways and lung. Smoke was mixed at a 1:1 ratio with 100% O_2 to minimize hypoxia. Plasma and expired breath samples were collected pre-smoke, and 6, 12, 18, 24, 36 and 48 h after smoke exposure. Sheep were euthanatized 48 h after smoke exposure and lung and airway sections were evaluated histologically for injury and biochemically for indices of oxidative stress. Plasma thiobarbituric acid reactive substances (TBARS) were 66 and 69% higher than controls after moderate and severe smoke exposure at 48 h, whereas total antioxidant potential was not statistically different among groups at any time after exposure. Lung TBARS showed a dose-dependent response to smoke inhalation and were approximately 2-, 3- and 4-fold higher, respectively, than controls after exposure to 5, 10 and 16 units of smoke. Lung myeloperoxidase (MPO) activity was also higher in smoke-exposed animals than controls, and MPO activity was markedly elevated (19- and 22-fold higher than controls in right apical and medial lobes) in response to severe smoke exposure. Smoke exposure also induced a dose-dependent injury to tracheobronchial epithelium and lung parenchyma. Taken together these data show that few indices of oxidative stress responded in a dose-dependent manner to graded doses of smoke inhalation, although most of the indices measured in lung were affected by the highest dose of smoke. Additional time course studies are necessary to determine whether these oxidants are a cause or a consequence of the airway and lung injury associated with exposure to wood smoke.
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Keywords Wood smoke; Oxidant stress; Lipid peroxidation; Myeloperoxidase; Sheep

1. Introduction

Smoke inhalation injury is a major comorbid factor in patients with thermal injury, and has been reported to occur in about 30% of patients with major burns (Herndon et al., 1985). Such patients with

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combined smoke inhalation and thermal injuries tend to be hemodynamically unstable and generally require about 50% more initial crystalloid fluid for resuscitation than estimated for the burn surface area alone (Navar et al., 1985; Thompson et al., 1986; Demling et al., 1995). In addition, inhalation injury reportedly accounts for 20–84% of the mortality in burned individuals, and is associated with higher mortality rates for every age and burn size category (Herndon et al., 1985; Thompson et al., 1986; Shirani et al., 1987).

Smoke associated with structural fires, arises from the combustion or pyrolysis of wood and the various natural and synthetic fabrics, plastics and other common materials available today (Wong et al., 1984; Alarie, 2002). Such acute lung injury resulting from inhalation of these products involves complex pathophysiologic processes that may not manifest into clinically significant symptoms for 24–72 h (Ruddy, 1994). Impairment of mucocilliary function, inflammatory responses such as interstitial edema, neutrophil infiltration, generation of oxygen free radicals and pseudomembrane formation have all been reported as potentially important consequences and mechanisms of smoke inhalation injury (Hubbard et al., 1991; Youn et al., 1992; Fitzpatrick and Cioffi, 1994, Ruddy, 1994; Lalonde et al., 1995). Direct alveolar damage, including alveolar edema, may also occur depending on the dose of smoke, the toxicity of the chemicals present in the smoke, and/or as a consequence of surfactant denaturation (Herndon et al., 1985; Fitzpatrick and Cioffi, 1994; Nieman et al., 1995).

A number of studies have linked antioxidants, both endogenous and dietary, as playing important roles in preventing lung injury from exposure to various environmental toxicants (Cross et al., 2002; Ho, 2002). In our further characterization of the effects of wood smoke inhalation on antioxidant status, the present study evaluated whether indices of an oxidant stress in plasma and lung from sheep exposed to graded doses of smoke followed a dose-dependent pattern. Sheep have long been employed to investigate smoke inhalation injury. This injury is reproducible, dose dependent (Hubbard et al., 1988; Kimura et al., 1988; Shimazu et al., 1996) and mimics that observed in humans (Traber et al., 1985; Hubbard et al., 1991).

2. Materials and methods

2.1. Animal preparation

Twenty 1- to 2-year-old neutered male sheep (*Ovis aeries*, Rambouillet X), weighing 24 to 33 kg, and free of antibodies to *Coxiella burnetti*, were obtained from a commercial source and used in the study. Animals were observed for 1 week in temperature-controlled, indoor runs, were treated for potential parasites (1% ivermectin, 1 ml/75 lb) and were fed commercial chow and water ad libitum. They were fasted for 24 h before tracheostomy and instrumentation. All animals were maintained in a facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care International.

The day before smoke or sham-smoke exposure, sheep were sedated with ketamine (5 mg/kg i.v.) and midazolam (0.275 mg/kg i.v.) and orotracheally intubated with a 7 mm i.d. cuffed tube. A chest radiograph was taken to exclude animals with active lung disease. Under isoflurane anesthesia (2–4%), a femoral artery was catheterized with a Silastic cannula; an external jugular vein was catheterized with an introducer sheath (8.5 Fr, American Edwards Laboratories Inc., Irvine, CA), followed by an 8-Fr balloon-tipped pulmonary-artery catheter; and open cystostomy and insertion of a urinary catheter were performed. A tracheostomy was then performed, and a 9-mm, low-pressure, cuffed tracheostomy tube (Shiley, Mallinckrodt Medical TPI, Irvine, CA) was inserted. The animals were recovered overnight in an animal intensive-care unit (ICU).

2.2. Smoke inhalation

The following day, animals were anesthetized with sodium pentobarbital (25 mg/kg i.v.). Once general anesthesia was confirmed, a neuromuscular blocking agent was given (pancuronium bromide 0.03–0.04 mg/kg i.v.). These drugs were redosed, and an intravenous analgesic (buprenorphine 0.005 mg/kg) was administered, whenever necessary. All animals remained anesthetized and mechanically ventilated, using a pressure control mode, for the remainder of the study (Siemens 900C ventilator, Siemens-Elema, Solna, Sweden). The animals were fasted, and maintenance intravenous fluid (5% dextrose in lactated

Ringer's solution at 2 ml/kg h) was given throughout the 48-h experimental period.

On the day of injury, each animal was randomized to one of four groups described below. Smoke inhalation injury was induced using the technique employed at our Institute over the past 10 years (Ogura et al., 1994; Harrington et al., 2001; Tasaki et al., 2002). Fifty grams of pine wood bark (Decorative Western Bark, Far West Forests Inc., Tempe, AZ) were cut into 1 cm^2 chips and were placed in a crucible furnace (Lindberg/BlueM laboratory furnace, model 56622, Asheville, NC). The firing chamber of the furnace was heated to 400 °C. The chamber was supplied with desiccated air at 6.0 l/min, to support combustion and to facilitate flow of the smoke from the chamber into a 5.5 l Plexiglas mixing box and reservoir. This reservoir also received 100% oxygen at 6 l/min, at a ratio of 1:1, to permit the smoke to cool to ambient temperature (approximately 25.5 °C) to eliminate thermal injury to the airway, to facilitate mixing, and to minimize hypoxia in the animal. The temperature of the smoke was measured just proximal to the tracheostomy tube using a thermistor-tipped pulmonary-artery catheter. Three minutes after initiation of pyrolysis, the smoke was drawn from the reservoir into a hand-operated piston and was delivered to the animal via its tracheostomy using modified ventilator circuit tubing with a manual breath-hold valve. The tidal volume of each smoke breath was controlled by an adjustable stop on the piston set at 30 ml/kg (Shimazu et al., 1987). One unit of smoke was defined as 5 such breaths; each breath included a 7-s inspiratory hold as previously described (Harrington et al., 2001). Group I (control, $n = 5$) received no smoke, group II (mild, $n = 5$) received 5 units of smoke, group III (moderate, $n = 5$) received 10 units, and group IV (severe, $n = 5$) received 16 units. All animals received the same number of piston-driven tidal volumes regardless of the smoke dose over a period of 17 min. Based on this regimen, the mild, moderate and severe exposure groups were subjected to 175, 350 and 560 s of smoke dwell time in the airways and lungs, respectively. The control animals received the same number of breaths via the same apparatus, but without smoke.

Immediately following smoke exposure, arterial-blood-gas analysis was performed and the carboxyhemoglobin (COHb) level was measured by cooximetry. Animals were ventilated with 100% O_2 for 2 h after smoke exposure, similar to other studies of smoke inhalation in sheep (Sakurai et al., 1999; Tasaki et al., 2002), and were moved back to the animal ICU, where they were housed in individual metabolic cages. Arterial blood pressure, heart rate, and peripheral oxygen saturation were continuously monitored. A rumen tube was placed for the rest of the study.

2.3. Serum oxidant stress markers

Blood samples were drawn at preinjury, 6, 12, 18, 24, 36 and 48 h after smoke exposure into heparinized tubes. The blood was centrifuged in a clinical centrifuge, and resultant plasma samples were stored at −70 °C for less than 3 months until analyzed. Thiobarbituric acid reactive substances (TBARS), expressed as nanomoles of malondialdehyde per milliliter of plasma, were determined in the butanol phase as described by Naito et al. (1993). Ferric reducing ability of plasma (FRAP), as an index of its antioxidant status, was determined spectrophotometrically by the method of Benzie and Strain (1996).

2.4. Lung tissue analysis

Sheep were euthanatized 48 h after smoke exposure using a standard veterinary euthanasia solution that provided an overdose of pentobarbital, and lung parenchyma from right and left apical, medial and diaphragmatic lobes were collected and stored at −70 °C for later assays. Oxidized and reduced glutathione were determined spectrophotometrically using the enzymatic assay described by Anderson (1985). Briefly, total glutathione concentration in 5-sulfosalicylic acid-precipitated supernatants was determined by a 5,5′-dithiobis(2-nitrobenzoic acid)-glutathione reductase recycling assay. Oxidized glutathione was determined by the same assay after reduced glutathione was derivatized by incubating the 5-sulfosalicylic acid-precipitated supernatants with 2-vinylpyridine for 1 h at room temperature. Reduced glutathione concentrations were determined by subtracting the concentration of oxidized glutathione from the total concentration (Anderson, 1985). TBARS and FRAP in lung tissue were determined as described above. Myeloperoxidase activity was determined by a modification of the method of Trush et al. (1994). Briefly, tissues were homogenized in 50 mM potassium

phosphate buffer pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide. The homogenates then underwent three freeze–thaw cycles and sonification, followed by incubation at 60 °C in a water bath for 2 h to extract myeloperoxidase and reduce interfering substances. Samples were centrifuged at 10,000 × g for 30 min at 4 °C. Myeloperoxidase activity was determined in the resultant supernatant using *o*-dianisidine as substrate. Protein concentrations were determined with a commercial kit (BioRad Laboratories, Richmond, CA).

2.5. Exhaled breath analysis

Before injury and 6, 12, 18, 24, 36, and 48 h after injury, exhaled nitric oxide (NO) was assayed by a chemiluminescence method using a Sievers model NOA 280 nitric oxide analyzer (Sievers Instruments, Boulder, CO). Basically, exhaled gas was sampled via a sidestream port according to the manufacturer's standard protocol. Two-point calibration of the analyzer was performed daily. Because NO was present in variable concentrations in inspired air, animals were ventilated before and during NO collection with a mixture of nitrogen and oxygen known to be free of NO.

At these same timepoints, exhaled breath condensate was collected for the measurement of H_2O_2. A segment of sterile ventilator tubing was connected to the existing ventilator circuit, and was submerged in bucket of dry ice for 40 min. The ice precipitate in the tubing was then collected, placed in a sterile vial, and stored at −70 °C until analysis. Measurement of H_2O_2 in expired air condensate was determined spectrophotometrically as described by Gallati and Pracht (1985), using horseradish peroxidase (HRPP)-catalyzed oxidation of tetramethylbenzidine. Briefly, 100 μl of 420 μM of tetramethylbenzidine and 10 μl of HRPP were mixed with 100 μl of expired air condensate. The reaction product was measured spectrophotometrically (Abs 450 nm) using an automated plate reader. The absorbance is directly proportional to the amount of H_2O_2 with a detection limit of approximately 0.1 μM H_2O_2.

2.6. Histology

At the time of euthanasia, sections of lung and trachea were excised for H&E staining and light microscopic examination. Histologic grading of tracheal, bronchial and pulmonary injury was performed using the previously described scoring system (Tasaki et al., 1997). The tracheal/bronchial injury score is as follows:

Injury score	Description
0	Normal
1	Some loss of cilia, and/or apical respiratory epithelium
2	Marked attenuation of pseudostratified epithelium or a single layer of epithelium
3	<50% segmental/focal ulceration of epithelium
4	>50% ulceration of epithelium

Parenchymal damage was evaluated at the apical, medial and diaphragmatic lobes of both lungs. The pulmonary injury score is as follows:

Injury score	Description
0	Normal
1	Minimal to mildly thickened alveolar septae, a few inflammatory cells or a small, single focus of inflammatory cells
2	Thickened alveolar septae with multifocal areas of increased inflammatory cells
3	Diffuse inflammation and/or edema that affects <50% of the section
4	Diffuse inflammation and/or edema that affects >50% of the section

Parenchymal samples from right and left lung were also excised for the determination of blood-free wet-to-dry lung ratio using the method described previously (Drake et al., 1980) as modified by Ogura et al. (1994). Bilateral apical, medial and diaphragmatic tissue samples were weighed and homogenized with an identical weight of distilled water. Duplicate samples of the homogenate and venous blood were weighed and dried at 80 °C for 48 h. Dry weights were measured and the wet-to-dry ratios of the homogenate and blood were calculated. To determine the hemoglobin levels in the homogenate and blood, 20 μl of the homogenate supernatant or the diluted blood was added to 2.5 ml of Drabkin's solution and the absorbance of both solutions was measured

spectrophotometrically at 540 nm. From these data, the fraction of blood in the wet and dry lung sections could be calculated and subtracted from the total wet and dry weights. Blood-free wet-to-dry weight ratio was then calculated as described (Drake et al., 1980).

2.7. Statistical analysis

Data analysis employed SPSS v. 10.1 (SPSS, Inc., Chicago, IL). As appropriate, one-way or repeated-measures analysis of variance was performed, with severity of injury as the between-groups factor. Post-hoc tests corrected for multiple comparisons were performed to compare each injured group to the control group at various time points. Correlations were run within a lobe to determine Pearson correlation coefficients among the doses of smoke and the variables of oxidative stress. A $P < 0.05$ was considered statistically significant.

3. Results

Two animals in the severe group were euthanatized at 29 and 36 h, because of terminal pulmonary failure (inability to maintain a PaO2 of 60 mmHg, despite $FiO_2 = 100\%$ and PEEP = 15 cmH_2O). One animal in the mild group died at 36 h, because of airway obstruction. The rest of the animals lived until the completion of the 48-h study.

Table 1
Effects of smoke inhalation in sheep on blood carboxyhemoglobin and lung wet:dry weights[a]

	Carboxyhemoglobin concentrations (%)	Lung wet:dry weight ratio
Control	6.3 ± 0.64	4.5 ± 0.2
Mild	42.3 ± 5.1*	5.1 ± 0.3
Moderate	78.0 ± 8.8*	5.5 ± 0.4
Severe	91.9 ± 1.3*	6.9 ± 0.3*

[a] Data expressed as mean ± S.E. for four to five animals per group. See text for definitions of smoke doses.
* $P < 0.05$ from control.

Arterial COHb levels measured immediately after smoke inhalation corresponded in stepwise fashion to the dose of smoke, showing a significant difference among groups ($P < 0.001$) and between each injured group and the control group ($P < 0.001$) (Table 1). In addition, lung wet:dry weight ratios were correlated to the dose of smoke, but only the highest smoke dose resulted in a wet:dry weight ratio significantly higher than controls (Table 1).

Plasma protein concentrations were significantly lower in the severe smoke exposure group at 24, 36

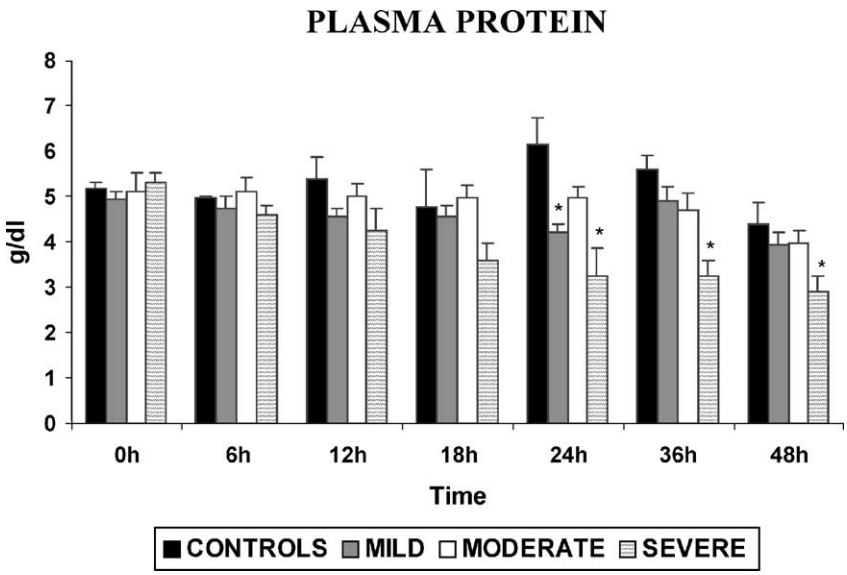

Fig. 1. Plasma protein concentrations in smoke-exposed sheep. Data represent mean ± S.E. from four to five animals per group. (*) $P < 0.05$ from controls.

and 48 h compared with controls (Fig. 1). Plasma protein concentrations in the mild and moderate smoke-exposed animals generally remained similar to control levels throughout the experimental period.

3.1. Serum, breath and tissue oxidative stress markers

Plasma TBARS appeared to rise in animals exposed to smoke compared with controls at 6 h after exposure, but the differences were not statistically significant (Fig. 2). Generally, these values remained elevated throughout the 48-h experimental period, particularly in the moderate smoke-exposed group. Overall, the response to smoke inhalation was variable and no dose-dependent effect of smoke exposure on plasma TBARS concentrations was observed (Fig. 2). At 48 h after smoke exposure, plasma TBARS were 66% and 69% higher in the moderate and severe smoke exposed groups than controls. In contrast to TBARS

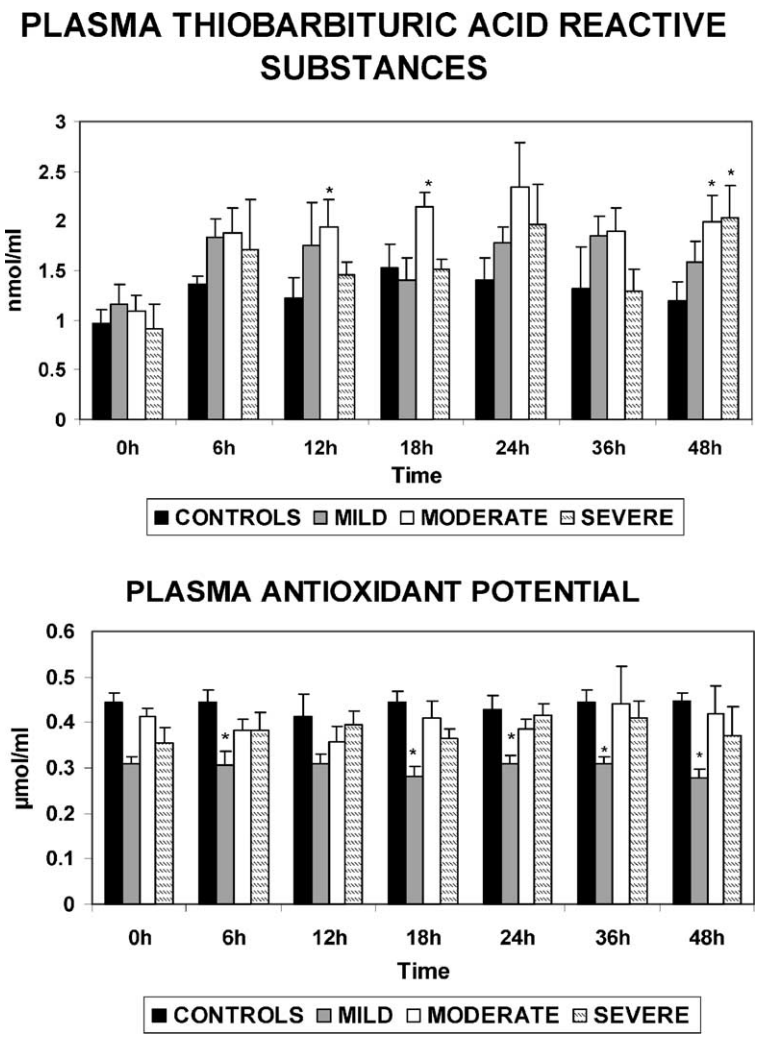

Fig. 2. Top- Plasma Thiobarbituric acid reactive substances (TBARSs) concentrations in smoke-exposed sheep. Bottom- Total antioxidant potential in plasma from smoke-exposed sheep. Data represent mean ± S.E. from four to five animals per group. (∗) $P < 0.05$ from controls.

levels, plasma antioxidant potential was not significantly affected by smoke exposure in any group (Fig. 2). Although, plasma antioxidant potential was consistently lower in the mild smoke exposure group compared with the other groups, the baseline level was also low, suggesting that smoke exposure induced no significant effect in this group.

In addition, expired hydrogen peroxide or NO levels measured in expired air were not significantly affected by smoke exposure at any time during the experimental period (data not shown).

In lung tissue specimens collected 48 h after smoke exposure, a dose-dependent increase in TBARS concentrations were observed in both left and right lung lobes (Fig. 3). In all lobes from moderate and severe smoke exposed animals, TBARS were significantly higher than controls, with levels about 3- and 4-fold higher than controls (Fig. 3). Lung antioxidant potential appeared to be generally affected by all doses of smoke, but only the severe group achieved statistical significance compared with controls, with values 20–25% lower than controls (Fig. 4).

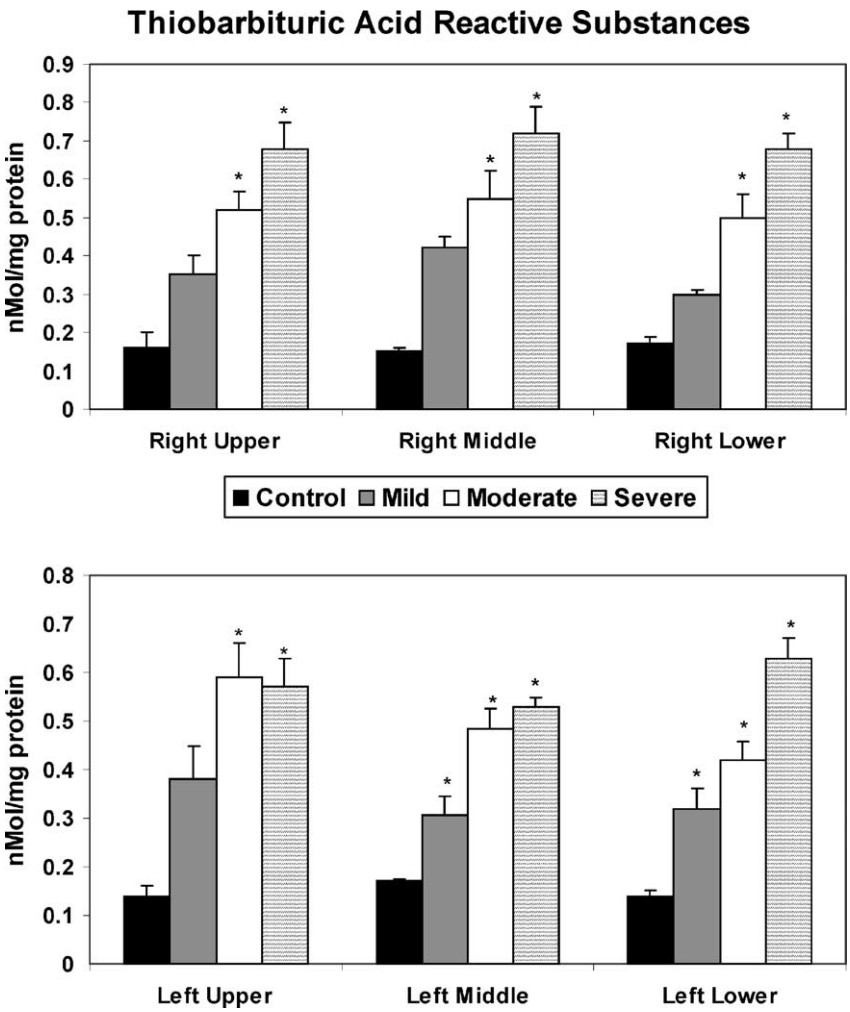

Fig. 3. TBARS concentrations in lung lobes (apical (upper), medial (middle) and diaphragmatic (lower)) from smoke-exposed sheep. Data represent mean ± S.E. from four to five animals per group. (∗) $P < 0.05$ from controls.

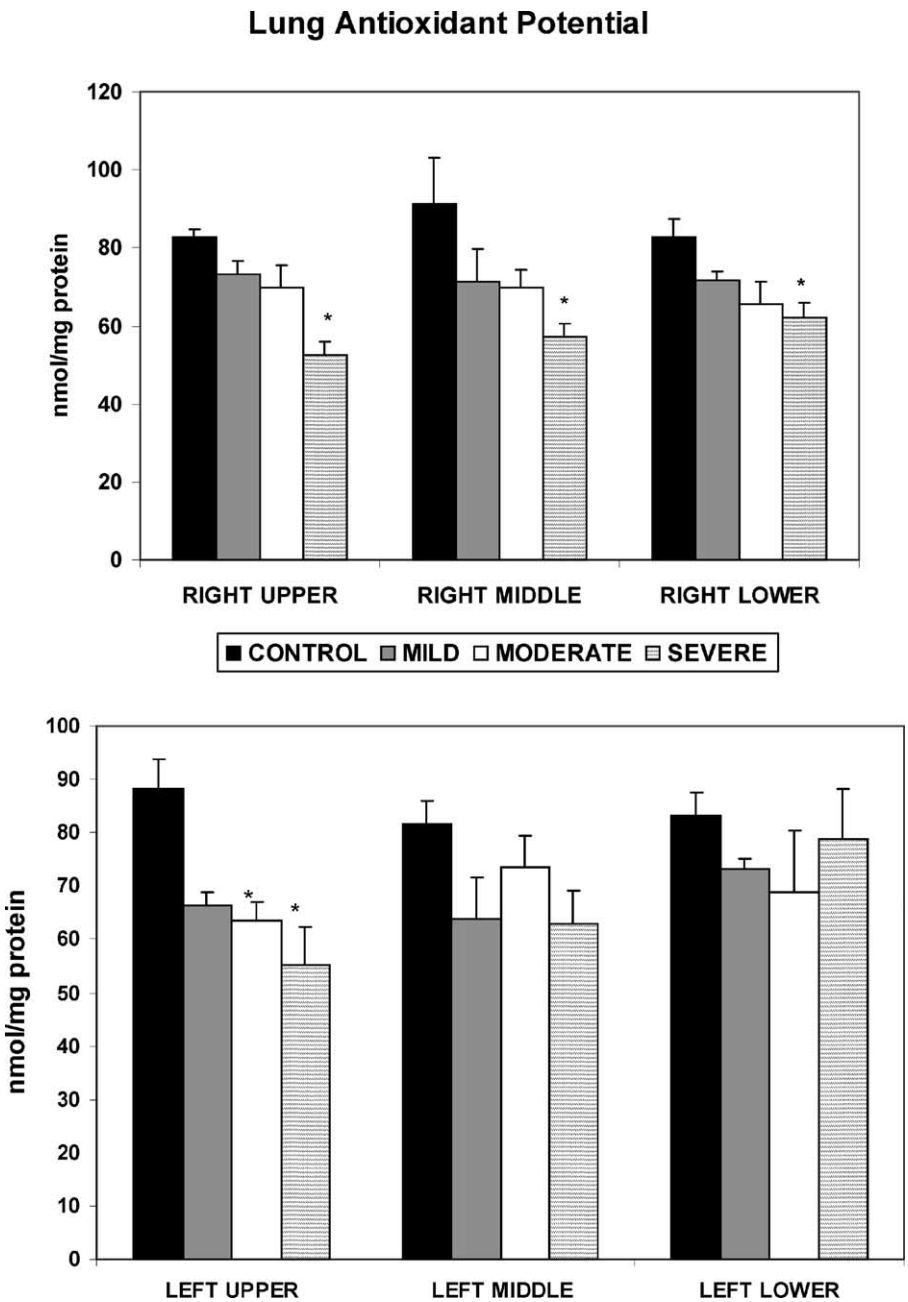

Fig. 4. Total antioxidant potential in lung lobes from smoke-exposed sheep. Data represent mean ± S.E. from four to five animals per group. (∗) $P < 0.05$ from controls.

Lung GSH concentrations were not consistently different from controls in any smoke exposed group (Table 2). Only left medial (middle) GSH concentrations were significantly lower than controls after moderate and severe smoke exposure. Severe smoke exposure resulted in 53% higher GSSG levels than controls in right diaphragmatic (lower) lung lobes (Table 2). Moderate smoke exposure resulted in higher

Table 2
Effects of smoke inhalation in sheep on lung reduced (GSH) and oxidized (GSSG) glutathione concentrations[a]

	Right apical	Right medial	Right diaphragmatic	Left apical	Left medial	Left diaphragmatic
GSH						
Control	10.8 ± 1.7	12.8 ± 1.1	12.2 ± 2.2	12.2 ± 1.5	14.1 ± 0.9	9.5 ± 1
Mild	11.8 ± 1.7	11.6 ± 1.1	9.3 ± 0.7	13.2 ± 2	13.9 ± 1.5	14.2 ± 1.2*
Moderate	13.9 ± 1.7	10.1 ± 1.5	11 ± 0.99	14.8 ± 1.4	9.2 ± 0.9*	7.6 ± 0.4
Severe	7.8 ± 0.7	8.6 ± 1.8	10.9 ± 1.54	13.4 ± 1.1	9.8 ± 0.4*	10.8 ± 1
GSSG						
Control	0.63 ± 0.04	0.60 ± 0.16	0.51 ± 0.02	0.60 ± 0.07	0.70 ± 0.05	0.75 ± 0.02
Mild	0.74 ± 0.05	0.58 ± 0.09	0.49 ± 0.03	0.78 ± 0.14	0.98 ± 0.14	0.74 ± 0.11
Moderate	0.91 ± 0.05*	0.67 ± 0.12	0.90 ± 0.12*	1.08 ± 0.15*	0.82 ± 0.11	0.85 ± 0.07
Severe	0.73 ± 0.05	0.95 ± 0.09	1.03 ± 0.11*	1.00 ± 0.13	0.83 ± 0.08	0.91 ± 0.12

[a] Data expressed as mean ± S.E. nmol/mg protein for four to five animals per group.
* $P < 0.05$ from control.

GSSG levels in right diaphragmatic and left apical (upper) lobes compared with controls (Table 2).

Mild and moderate smoke exposure resulted in slightly higher MPO activity in most lung lobes compared with controls, although few data achieved statistical significance due to variability in the data (Fig. 5). In contrast, severe smoke exposure elevated MPO activity in apical and medial lung lobes compared with controls (Fig. 5). For example, MPO activity in the right apical and medial lobes was 19- and 22-fold higher, respectively, than controls. In addition, MPO activity in the right upper lobe was significantly correlated with the dose of smoke.

3.2. Histology

Based on the criteria described in the Materials and Methods section, a dose-dependent increase in tracheal injury score was observed in response to smoke inhalation, as confirmed by correlation analysis (Fig. 6). The predominant observations following moderate or severe smoke exposure in trachea, were loss of cilia, epithelial attenuation, ulceration of respiratory mucosa and serocellular/mucocellular cast formation. Although correlation analysis also noted that lung parenchymal injury scores were dose related, only the severe smoke exposure produced statistically higher injury scores compared with controls (Fig. 6). Severe wood smoke exposure resulted in the largest number of diffusely distributed inflammatory cells, and edema in lung, compared with the other groups.

4. Discussion

The smoke generated from the pyrolysis of wood is known to contain over 200 compounds, including carbon monoxide, nitrogen oxides, sulfur oxides, aldehydes, polycyclic aromatic hydrocarbons and respirable particulate matter (Pierson et al., 1989; Larson and Koenig, 1994; Zelikoff et al., 2002). In addition, wood smoke can generate stable carbon-centered radicals and hydroxyl radicals (Lachocki et al., 1989; Pryor, 1992; Kou et al., 1997; Leonard et al., 2000). This information has generated much interest in determining the health effects of subchronic and chronic exposure to individuals exposed to smoke generated from woodburning stoves (Tesfaigzi et al., 2002; Zelikoff et al., 2002). The present study, however, focused on the acute effects of wood smoke inhalation that might occur in a structural fire (Wong et al., 1984; Barillo et al., 1994).

In the present study a dose-related rise in blood carboxyhemoglobin (COHb) concentrations was observed immediately after smoke exposure. It is well recognized that carbon monoxide is one of the major toxicants of smoke in fires (Alarie, 2002). For example, average blood COHb levels exceeding 50%, and sometimes as high as 60–80% have been observed in victims of fires and blood COHb levels exceeding 50% have often been cited as evidence for carbon monoxide related deaths in humans (Fitzpatrick and Cioffi, 1994; Alarie, 2002). As noted, however, a number of factors can contribute to fire-associated deaths, and blood COHb levels can be higher or lower than

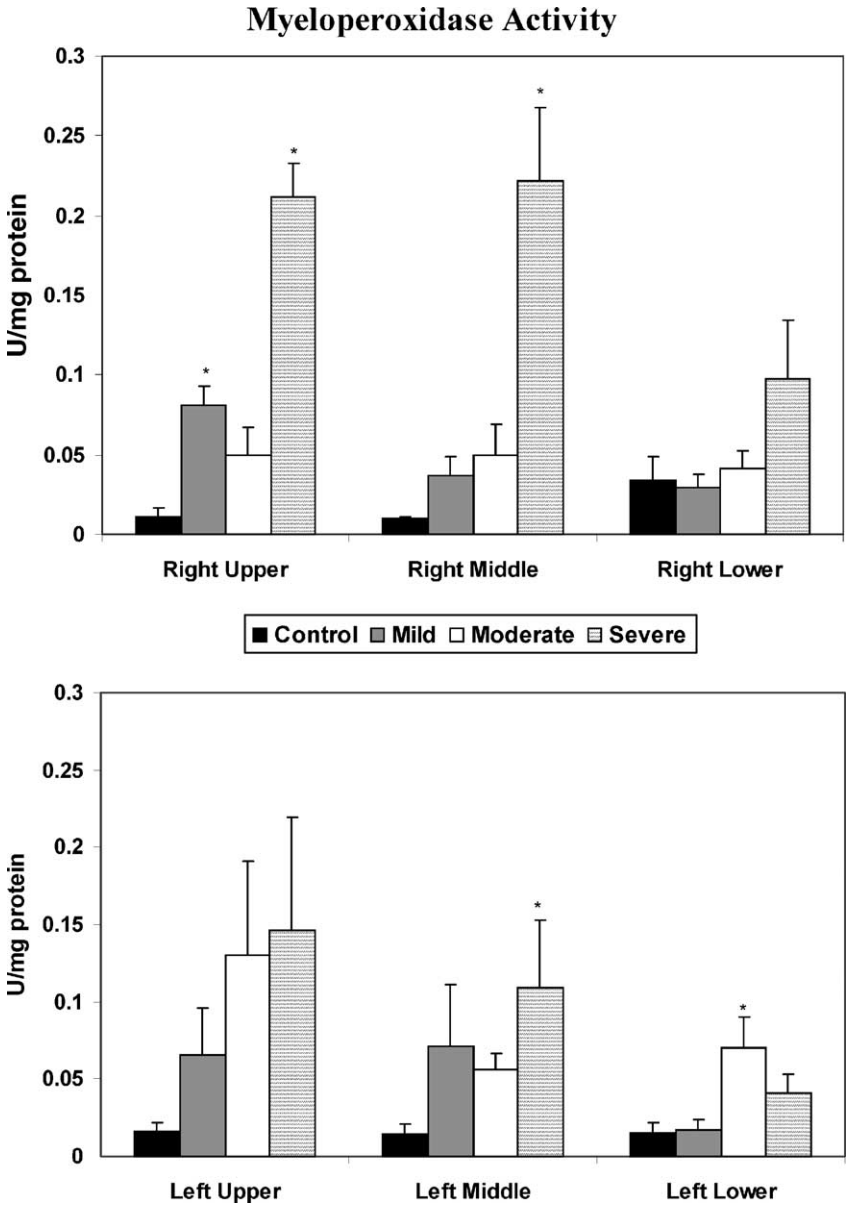

Fig. 5. Myeloperoxidase activity in lung lobes from smoke-exposed sheep. Data represent mean ± S.E. from four to five animals per group. (∗) $P < 0.05$ from controls.

expected depending on circumstances; results that have been confirmed in animal experiments (Alarie, 2002). Thus, it is difficult to extrapolate blood COHb levels in sheep to those observed in human fire victims. Consequently, in previous studies of smoke inhalation injury in sheep at our Institute, blood COHb levels have generally been used to assess the degree of exposure to smoke. For example, in sheep exposed to mild to moderate doses of smoke reported COHb levels of 30–36% (Ogura et al., 1994; Demling et al., 1995), while severe smoke exposure resulted in COHb levels as high as 90% in the sheep models (Kimura

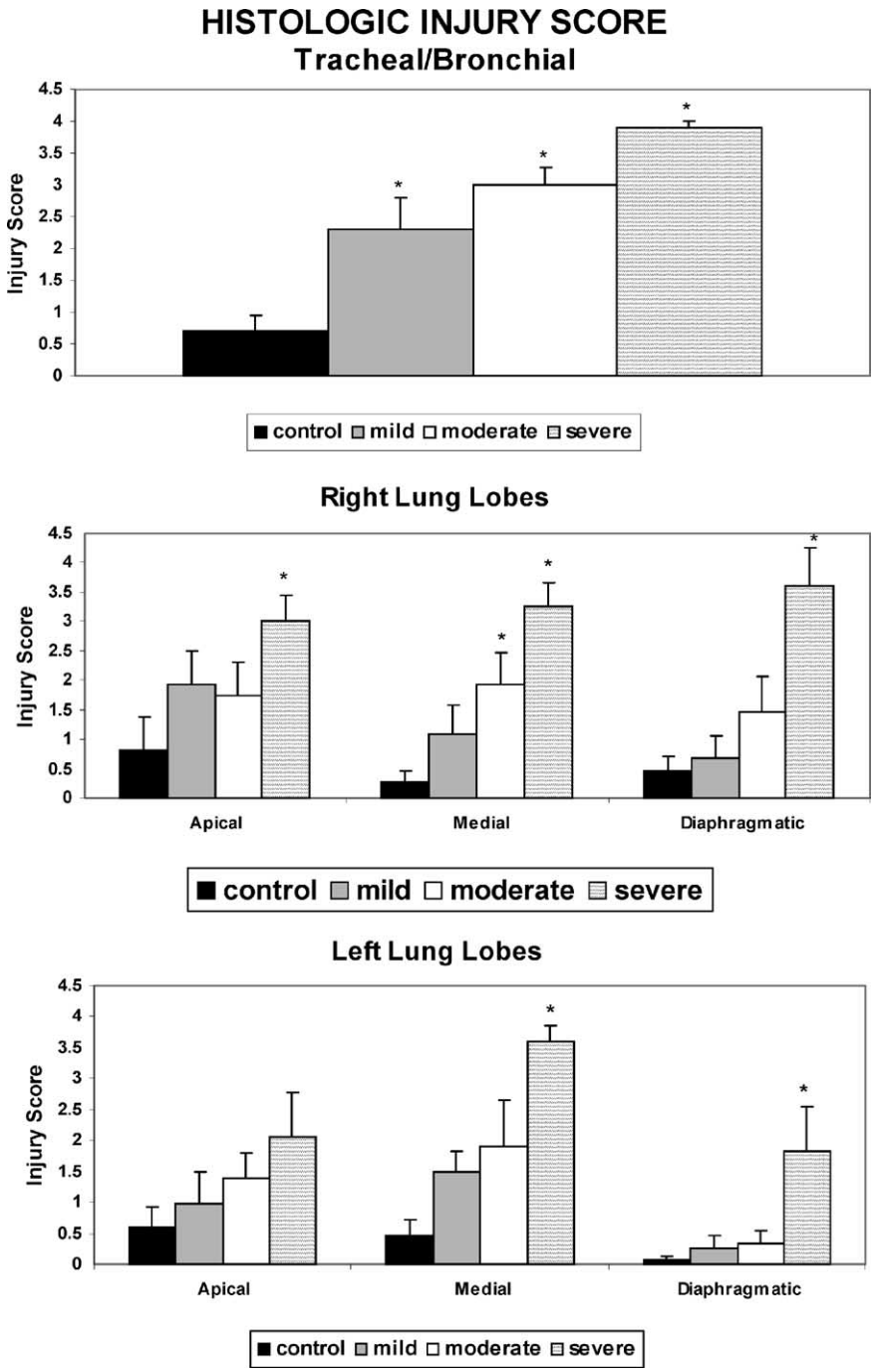

Fig. 6. Histologic injury score in trachea and lung parenchyma. Injury scores were based on the tables presented in the Materials and Methods Section. Data represent mean ± S.E. from four to five animals per group. (∗) $P < 0.05$ from controls.

et al., 1988; Sugi et al., 1990; Takaki et al., 1998). Thus, the COHb measured in the present study falls within the range reported previously in similar sheep models, and further confirms that in sheep, COHb, measured immediately post-exposure, may be useful to document the degree of smoke exposure among groups. It has been proposed that the discrepancy between the survivability from smoke exposure observed between humans and sheep, based on COHb levels, is most likely related to the half-life of COHb in sheep being about 50% of that in humans (Kimura et al., 1988; Shimazu et al., 1990). Thus, it has been suggested that acute exposures to high concentrations of CO may differ in toxicity than prolonged exposure to low levels of CO, even when the peak COHb levels are the same (Shimazu et al., 1990). Another explanation for the low toxicity of COHb in the present study could relate to the animals being ventilated with 100% oxygen for the first 2 h after smoke exposure, which might further reduce the half life of COHb. Nevertheless, additional studies in this area appear warranted.

Inhalation injury has been described to involve both the tracheobronchial tree as well as the lung parenchyma, depending on severity (Hubbard et al., 1991; Fitzpatrick and Cioffi, 1994). The injury scores of the trachea and lung in the present study, agree with these observations. It should be noted that structurally there are few differences between the sheep and human lung. Linares et al. (1989) reported that the sheep had a longer trachea and a distinctive right apical lobe, but that the subgross anatomy of both lungs was similar and could be compared satisfactorily for the responses to wood smoke inhalation. In addition, inhalation of the highest dose of smoke in the present study, resulted in a hypoproteinemia and increase in the blood free lung wet-to-dry weight ratio. Hypoproteinemia has been a common observation following severe smoke inhalation injury (Tasaki et al., 1997, 2002) and together with the elevated wet-to-dry weight ratio, further signifies the severity of the insult. Although the exact mechanism for hypoproteinemia is not fully known, it may relate to smoke inhalation being associated with early sloughing of bronchoepithelial cells into airways (Abdi et al., 1990) and development of proteinaceous casts in the airways (Tasaki et al., 2002). The significantly higher lung wet:dry weight ratios in the severe smoke-exposed sheep are in agreement with the greater degree of pulmonary edema observed in these animals.

In the present study, elevated lung MPO activity, as an index of neutrophil infiltration, was observed after moderate, and more significantly, after severe smoke inhalation. Although the results were somewhat variable, this increased MPO activity was associated with higher TBARS levels in lung parenchyma. Others have reported that infiltration of neutrophils into airways was an early event in the inflammatory process associated with smoke inhalation and activation of neutrophils appears to be involved in injury to the lung parenchyma after smoke inhalation (Traber et al., 1986; Linares et al., 1989; Hubbard et al., 1991). Related, at least in part, to neutrophil infiltration, is the observation of higher levels of indices of lipid peroxidation and oxidative stress in cotton or wood smoke-exposed animals (Demling and Lalonde, 1990; Youn et al., 1992; Demling et al., 1994; Lalonde et al., 1994; Tasaki et al., 1997; Dubick et al., 2002). These data are consistent with our preliminary studies which showed a 46% reduction in ascorbic acid levels compared with controls in lavage fluid collected from rats 1 h after wood smoke exposure (Dubick et al., 1998). In addition, other studies have shown that antioxidants or iron chelators were able to reduce evidence of lipid peroxidation following wood smoke exposure in experimental animals (Zhao, 1990; Nguyen et al., 1995; Demling et al., 1996). The results of the present study are in general agreement with these other studies.

In the present study, only minor elevations in plasma TBARS was observed, suggesting the possibility of some systemic effects related to the inhalation of the higher doses of smoke. Others have reported effects of smoke inhalation on antioxidants in liver and kidney (Demling and Lalonde, 1990; Demling et al., 1994; Lalonde et al., 1994; Dubick et al., 2002), but such studies were beyond the scope of the present investigation.

In addition, the present study evaluated 2 non-invasive indices of oxidant stress, measurement of exhaled nitric oxide (NO) and exhaled hydrogen peroxide (H_2O_2) levels.

Exhaled nitric oxide levels, in our study, did not differ among groups and did not appear to vary over time in a consistent fashion. However, several studies indicate that nitric oxide (NO) production is increased following inhalation injury. For example, inhalation

of ozone caused expression of inducible NO synthase (iNOS) mRNA, and increased nitric oxide production by rat type II pneumocytes (Punjabi et al., 1994). Similarly, sheep receiving a 40% burn and severe smoke inhalation injury demonstrated an increase in iNOS mRNA, iNOS protein, lung nitrotyrosine, and circulating nitrite and nitrate levels at 24 h after injury (Soejima et al., 2001).

Exhaled NO likely originates from several locations and cell types within the airways and pulmonary parenchyma (Barnes and Kharitonov, 1996). Elevated exhaled NO levels have most consistently been demonstrated in patients with certain inflammatory conditions of the airways, including asthma and bronchiectasis (Yates et al., 1995) or in ventilated patients with pneumonia (Adrie et al., 2001). In contrast, exhaled NO levels were reduced in patients with acute respiratory disease syndrome ARDS (Brett and Evans, 1998). Thus, the potential utility of exhaled NO as a biomarker of inhalation injury remains to be explored.

Hydrogen peroxide (H_2O_2) levels in exhaled breath have also been measured as an index of oxidative stress in patients with acute lung failure (Baldwin et al., 1986; Sznajder et al., 1989). In the present study exhaled H_2O_2 levels were not consistently increased following smoke inhalation. Nevertheless, other studies suggest that H_2O_2 likely may play a role in the pathophysiology of inhalation injury. In sheep, for example, plasma levels of H_2O_2 were increased in conjunction with decreased plasma levels of antioxidants (glutathione, catalase, vitamin E), and increased lipid peroxidation in lung and liver (Lalonde et al., 1997). In addition, exhaled H_2O_2 is increased in patients with diseases of the airways such as chronic obstructive pulmonary disease and asthma. In both groups, levels are further increased during acute exacerbations (Dohlman et al., 1993; Dekhuijzen et al., 1996; Emelyanov et al., 2001). Exhaled H_2O_2 levels were also elevated in mechanically ventilated patients with ARDS, in comparison with those without ARDS (Baldwin et al., 1986). To our knowledge, exhaled H_2O_2 levels have not been measured previously in the exhaled breath of patients or animals with smoke inhalation injury.

In addition to exhaled NO and H_2O_2, there is now evidence that carbon monoxide and 8-isoprostane (an arachidonic acid product and marker of lipid peroxidation) may be useful as exhaled breath tests for systemic and pulmonary inflammatory disease (Montuschi et al., 2000; Zegdi et al., 2002). Further studies are necessary to investigate these agents as biomarkers of inhalation injury.

It should be mentioned that in the majority of studies that evaluate antioxidants biochemically, it is a common practice to homogenize a piece of lung and report the results as representative of the whole lung. Although in most cases, investigators have little alternative in the methods available for analyzing tissue, this study offered the unique opportunity to evaluate indices of lipid peroxidation in individual lung lobes after smoke inhalation. In the assays performed, in most cases there were no statistically significant differences among the lobes for each variable measured. Only MPO activity seemed higher in the lung lobes most proximal to the bronchi than the distal diaphragmatic lobe after severe smoke exposure. This may reflect the anatomy of the sheep lung and penetration of the smoke, but again due to variability in the data, the differences were not statistically significant. Nevertheless, sufficient lobe-to-lobe differences in results were observed regarding many of the variables measured to warrant caution to investigators against analyzing random samples from the lung. The present data suggest that at least in evaluating antioxidant status in response to smoke inhalation, for the best comparisons among controls and experimental groups, samples should be obtained from the same lung lobe, at a minimum, if samples from multiple lung lobes are not available.

In summary, the present data are in agreement with previous studies that smoke inhalation induces an oxidative stress, and the relative degree of injury is represented in all lung lobes, but not to the same degree. Although injury to the airways and lung parenchyma 48 h after smoke inhalation was dose-dependent, only TBARS concentrations in the different lung lobes followed this dose-dependent relationship. Changes in the other indices of oxidative stress were more variable and significant differences were generally only observed in the group exposed to the most smoke, where lung injury scores were significantly greater than scores in the control group. Although the data suggest that oxidant production and neutrophil infiltration are associated with wood smoke-induced lung injury, additional studies are required to determine the contribution of the different sources of oxidants

after smoke inhalation. We have previously shown that the wood smoke used in the present study generates hydroxyl and stable carbon radicals (Leonard et al., 2000) and others have reported the presence of stable radicals in wood smoke (Pryor, 1992). Additional time course studies are required to determine whether oxidant generation after wood smoke exposure is a cause or a consequence of the tracheal and lung parenchymal injury observed.

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