Taylor & Francis
Taylor & Francis Group

Journal of Toxicology and Environmental Health, Part A
Current Issues

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: https://www.tandfonline.com/loi/uteh20

Increased Susceptibility of Hyperthyroid Rats to Ozone: Early Events and Mechanisms

L. J. Huffman , C. M. Beighley , D. G. Frazer , W. G. McKinney & D. W. Porter

To cite this article: L. J. Huffman , C. M. Beighley , D. G. Frazer , W. G. McKinney & D. W. Porter (2006) Increased Susceptibility of Hyperthyroid Rats to Ozone: Early Events and Mechanisms, Journal of Toxicology and Environmental Health, Part A, 69:6, 465-479, DOI: 10.1080/15287390500247017

To link to this article: https://doi.org/10.1080/15287390500247017

Journal of Toxicology and Environmental Health, Part A, 69:465–479, 2006

ISSN: 1528–7394 print / 1087–2620 online
DOI: 10.1080/15287390500247017

INCREASED SUSCEPTIBILITY OF HYPERTHYROID RATS TO OZONE: EARLY EVENTS AND MECHANISMS

L. J. Huffman[1,2], C. M. Beighley[1], D. G. Frazer[1,2], W. G. McKinney[1], D. W. Porter[1,2]

[1]Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia
[2]Department of Physiology and Pharmacology, West Virginia University School of Medicine, Morgantown, West Virginia, USA

Previous studies demonstrated that ozone-induced lung damage and inflammation are much greater in hyperthyroid rats, compared to normal rats, at 18 h postexposure. The purpose of the present investigation was to study early events and mechanisms underlying the increased sensitivity to ozone in a hyperthyroid state. Specifically, the degree of lung epithelial cell barrier disruption, the antioxidant status of the extracellular lining fluid, and the release of inflammatory mediators were examined. To induce a hyperthyroid state, mature male Sprague-Dawley rats were implanted with time-release pellets containing thyroxine; control rats received placebo pellets. After 7 d, the animals were exposed to air or ozone (2 ppm, 3 h). Immediately following the end of the exposure, bronchoalveolar lavage (BAL) fluid and cells were harvested. BAL fluid albumin levels and total antioxidant status were examined. In addition, levels of prostaglandin E_2 (PGE_2), macrophage inflammatory protein (MIP)-2, MCP-1, and tumor necrosis factor (TNF)-α were determined in BAL fluid and in media samples following ex vivo culture of BAL cells harvested after in vivo inhalation exposures. The results of this study are consistent with the following hypotheses: (1) A marked increase in the permeability of the alveolar–capillary barrier is an early event following ozone exposure in a hyperthyroid state; however this does not appear to be due to overall changes in BAL fluid antioxidant potential. (2) Early increases in MIP-2, but not PGE_2, are involved in the enhanced lung response to ozone in a hyperthyroid state. (3) Inflammatory mediator production (i.e., PGE_2, MIP-2, MCP-1, and TNF-α) by alveolar macrophages plays a minimal role in the initial responses to ozone in a hyperthyroid state.

It has been well established that the inhalation of ozone can result in pulmonary damage and inflammation (Bhalla, 1999; Krishna et al., 1998a). Biomarkers of lung damage and inflammation following ozone exposure include elevated protein levels and increased numbers of inflammatory cells in bronchoalveolar spaces. For instance, a short-term inhalation exposure to ozone is associated with increases in both bronchoalveolar lavage (BAL) protein levels and neutrophil numbers in humans and rats (Hatch et al.,

Received 17 December 2005; accepted 21 March 2005.
We thank D. J. Prugh, M. Donlin, A. Frazer, and D. Shahan for expert technical assistance.
Address correspondence to Linda Huffman, PhD, M/S 2015, NIOSH, 1095 Willowdale Road, Morgantown, WV 26505, USA. E-mail: ljh3@cdc.gov

1994; Koren et al., 1989). These effects are time dependent and are observed primarily during the first 24-h period following an acute ozone exposure (Bhalla & Hoffman, 1997; Schelegle et al., 1991; Young & Bhalla, 1992).

One of the first events that occurs following the inhalation of ozone is damage to lung epithelial cell integrity and disruption of the pulmonary alveolar–capillary barrier (Bhalla & Gupta, 2000; Kehrl et al., 1987). In part, this is thought to be due to the reaction of ozone with components of the epithelial lining fluid, a decrease in extracellular antioxidant defense mechanisms, and the generation of free-radical-derived ozonation products, which result in cellular damage (Kelly et al., 1995). This cellular damage is associated with increases in airway permeability, the exudation of serum proteins into bronchoalveolar spaces, and increases in BAL fluid protein levels (Bhalla & Crocker, 1987; Guth et al., 1986; Plopper et al., 1973).

Ozone exposure also results in the prompt release of inflammatory mediators, such as prostanoids and cytokines, from lung cells. For example, levels of prostaglandin E_2 (PGE_2) are elevated in lung lavage fluid samples within the first few hours following ozone exposure (Gunnison et al., 1992; Seltzer et al., 1986). Ozone also induces the pulmonary production of macrophage inflammatory protein-2 (MIP-2) in rodents (Bhalla & Gupta, 2000; Driscoll et al., 1993; Zhao et al., 1998). MIP-2 and its human homologue, interleukin-8, are chemokines that have been implicated in playing important roles in the initial recruitment of inflammatory cells into the lung following ozone exposure (Driscoll et al., 1993; Haddad et al., 1995; Krishna et al., 1998b).

Our laboratory has shown a relationship between thyroid status and pulmonary responses to ozone. Specifically, it was found that ozone-induced lung damage and inflammation are much greater in hyperthyroid rats at 18 h postexposure (Huffman et al., 2001). At that time, hyperthyroid animals had three- to sixfold increases in BAL albumin levels and neutrophil numbers compared to values observed in ozone-exposed control rats. The mechanisms underlying the increased sensitivity to ozone in a hyperthyroid state have not been fully defined. However, these effects cannot be explained simply by alterations in whole-body metabolic rate or by changes in the inhaled dose of ozone (Huffman et al., 2001). As already indicated, exposure to ozone results in the prompt initiation of a series of cellular and biochemical events in the lung. Therefore, it is very possible that the increased pulmonary damage and inflammation in hyperthyroid rats following ozone exposure are linked to modulation of these early events. This study was designed to test the hypothesis that both alterations in extracellular lining fluid antioxidant status and changes in the release of PGE_2, MIP-2, MCP-1, and tumor necrosis factor (TNF)-α are early events linked to the increased sensitivity of the lung to ozone in a hyperthyroid state.

METHODS

Animals

Male Sprague-Dawley [Hla:(SD)CVF] rats (Hilltop, Scottdale, PA) were used. The animals were housed in an AAALAC-accredited, specific-pathogen-free facility. The rats were monitored to be free of endogenous viral pathogens, parasites, mycoplasms, *Helicobacter*, and CAR bacillus. Animals were housed in ventilated cages that were provided with HEPA-filtered air. Alpha-Dri virgin-cellulose chips and hardwood Beta-chips were used as bedding. The rats were provided Prolab RMH rodent diet and tap water ad libitum and housed under controlled light (12 h light, 12 h darkness) and temperature (22–24 °C) conditions.

To create a hyperthyroid condition, thyroid-intact rats (6 wk of age; 211 to 244 g body weight) were implanted subcutaneously with time-release pellets containing thyroxine (25 mg; 5 mm diameter; 21-d release pellet; Innovative Research of America, Sarasota, FL). Control rats received placebo pellets. Time-release pellets were used to provide a continuous release of thyroxine over the course of the study. This method of administration avoided the "peak and valley" levels of thyroxine that would have been produced by single, daily injections of this hormone.

Procedures for pellet implantation were performed in a sterile surgery suite. Prior to pellet implantation, the rats were anesthetized intraperitoneally with a mixture of ketamine hydrochloride (7 mg/100 g body weight; Phoenix Scientific, Inc., St. Joseph, MO) and xylazine (1 mg/100 g body weight; Phoenix Scientific, Inc.). An incision was then made in the skin on the lateral side of the neck and a small subcutaneous pocket was formed. The pellet was then placed in the pocket and the incision site was closed with wound clips. Following recovery from the anesthesia, the rats were returned to the animal housing facility.

Inhalation Exposures

Rats were exposed to air or ozone on d 7 following pellet implantation (d 1 = day of pellet implantation). A whole-body inhalation exposure system was used to expose the animals to ozone (2 ppm for 3 h). This dose of ozone was chosen because the biologic effects of a short-term exposure to 2 ppm ozone in rats appear to be relatively similar to those following exposure to 0.4 ppm ozone in exercising humans (Hatch et al., 1994). The inhalation system has previously been described in detail (Huffman et al., 2001). Control rats were exposed to HEPA-filtered air for 3 h in a whole-body exposure chamber that was similar to the chamber used for ozone exposures. The animals were studied immediately after the end of the exposure period.

Collection of Blood and Bronchoalveolar Lavage Fluid and Cell Samples

The rats were first anesthetized with sodium pentobarbital (>100 mg/kg, ip; Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA). Blood was collected

from the abdominal vena cava into tubes with or without anticoagulant (sodium ethylenediamine tetraacetate). The left renal artery was then cut. A tracheal cannula was inserted and an initial BAL was performed with 6 ml ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; 145 m*M* NaCl, 5 m*M* KCl, 9.4 m*M* Na_2HPO_4, 1.9 m*M* NaH_2PO_4, and 5.5 m*M* d-glucose, pH 7.4). This lavage solution was introduced into and withdrawn from the lungs three times. Subsequent BALs were performed with 8 ml PBS each until a total volume of 80 ml lavage fluid was collected. The initial and subsequent lavage samples were then centrifuged (500 × g, 5 min, 4°C). The acellular supernatants from the initial lavage were processed for subsequent analyses. The cell pellets from the initial and subsequent lavages were then combined and resuspended in 5 ml PBS. The samples were centrifuged to pellet the cells and the supernatants were aspirated to waste. This wash procedure was performed three times. Following the final wash, the cells were resuspended in 1 ml PBS.

Determination of BAL Cell Counts, Cell Profiles, and Cell Viabilities

BAL cell counts were determined using an electronic cell counter equipped with a cell-sizing attachment (Coulter Multisizer II, Coulter Electronics, Hialeah, FL). Portions of the harvested cells were then deposited on slides using a cytocentrifuge (Shandon Scientific, London) and stained with a modified Wright–Giemsa stain (Hema-Tek 2000, Bayer Corp., Elkhart, IN). The percentages of alveolar macrophages and leukocytes present on the slides were determined using light microscopy. Viability of the cell population for each rat was assessed using a trypan blue exclusion test. The percentages of viable cells were then determined by placing aliquots of the treated cells in a hemocytometer and scoring 100 cells for either the absence (viable cells) or the presence (dead cells) of blue staining.

Blood Analyses

The serum was separated from coagulated blood and stored at –20°C. Serum thyroxine levels were measured using a commercially available radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA). Blood cell differentials were determined on samples containing anticoagulant using a Cell-Dyne 3500R hematology cell counter (Abbott Diagnostics, Abbott Park, IL).

Analyses of Albumin Levels, Lactate Dehydrogenase Activities, and Total Antioxidant Potential in BAL Fluid Samples

BAL fluid albumin concentrations were determined as an indicator of the integrity of the blood–pulmonary epithelial cell barrier. Serum albumin was assessed colorimetrically at 628 nm based on albumin binding to bromcresol green, using a commercial assay kit (Sigma Chemical Copany, St. Louis, MO). BAL fluid lactate dehydrogenase (LDH) activities were measured as a marker of cytotoxicity. LDH activities were determined by monitoring the LDH-catalyzed oxidation of lactate to pyruvate coupled with the reduction of NAD^+ at 340 nm, using a commercial assay kit (Roche Diagnostics Corp., Indianapolis,

IN). Measurements of albumin levels and LDH activities were performed using a COBAS MIRA auto-analyzer (Roche Diagnostics Corp.).

The total antioxidant potential was measured in BAL fluid samples using a commercial assay kit (BIOXYTECH AOP-490; OxisResearch, Portland, OR). This assay is based on the reduction of Cu^{2+} to Cu^{+} by antioxidants present in the sample, followed by the reaction of a chromogenic agent with Cu^{+}, which was detected colorimetrically at 490 nm.

Assessment of the Production of Inflammatory Mediators by BAL Cells

The production of inflammatory mediators by BAL cells was assessed following ex vivo culture of cells. BAL cells were suspended in culture medium (X-VIVO 15, BioWhittaker, Walkersville, MD) containing penicillin (100 U/ml; Sigma Chemical Co., St. Louis, MO) and streptomycin (100 μg/ml; Sigma Chemical Co). This culture medium is a defined medium that does not contain thyroid hormones. The cells were then placed into wells of 24-well tissue culture plates (Costar Corp., Cambridge, MA). Each well contained 1×10^6 viable cells per ml culture medium. The cells were incubated at 37°C in an incubator with a humidified atmosphere (relative humidity 90%) of 95% air–5% CO_2. After an incubation period of 4 or 18 h, the tissue culture plates were centrifuged (500 × g, 10 min, 4°C). The cell-free supernatant media samples were stored at −80°C prior to analysis.

Analysis of Inflammatory Mediators in BAL Fluid and Media Samples

PGE_2, MIP-2, MCP-1, and TNF-α were measured in both BAL fluid and media samples. Radioimmunoassay was used to measure PGE_2 levels (NEN Life Science Products, Inc., Boston). PGE_2 was first extracted from BAL lavage fluid samples using C-18 reverse-phase cartridges (Purfication Protocol for PGE_2, Cayman Chemical, Ann Arbor, MI). PGE_2 levels in media samples were analyzed directly. Levels of MIP-2, MCP-1, and TNF-α were determined in BAL lavage fluid and media samples using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Biosource International, Inc., Camarillo, CA).

Statistics

Multiple two-way analyses of variance were conducted to determine if the factors of hormone treatment and ozone exposure would result in a significant difference on measured variables. Repeated measures analysis of variances, with time as the repeated within-subject effect, were conducted to determine if the factors of hormone treatment, ozone exposure, and time would result in a significant difference in the amounts of PGE_2, MIP-2, TNF-α, and MCP-1 levels produced by BAL cells after 4 and 18 h. In all cases, tests were performed for homogeneity of variances. In cases of unequal variances, analyses accounted for the heterogeneous variances enabling efficient inferences to be made. Pairwise comparisons were performed for each main effect of hormone treatment, ozone exposure, and time where applicable using the

Tukey–Kramer method to adjust for multiple comparisons. Pairwise comparisons were also performed between each hormone treatment and ozone exposure combination using Fisher's exact test to determine if the number of rats with ≤99% AM and the number of rats with ≥1% neutrophils differed between the groups. All calculated measures are reported as mean values and standard errors. In all cases, two-sided tests were used with p values ≤.05 used as evidence of findings not attributable to chance.

RESULTS

Circulating Thyroxine Levels in Normal or Hyperthyroid Rats Following Air or Ozone Exposure

Serum thyroxine levels in normal rats treated with placebo pellets or in hyperthyroid rats implanted with thyroxine-containing pellets are shown in Figure 1. The subcutaneous implantation of time-release pellets containing

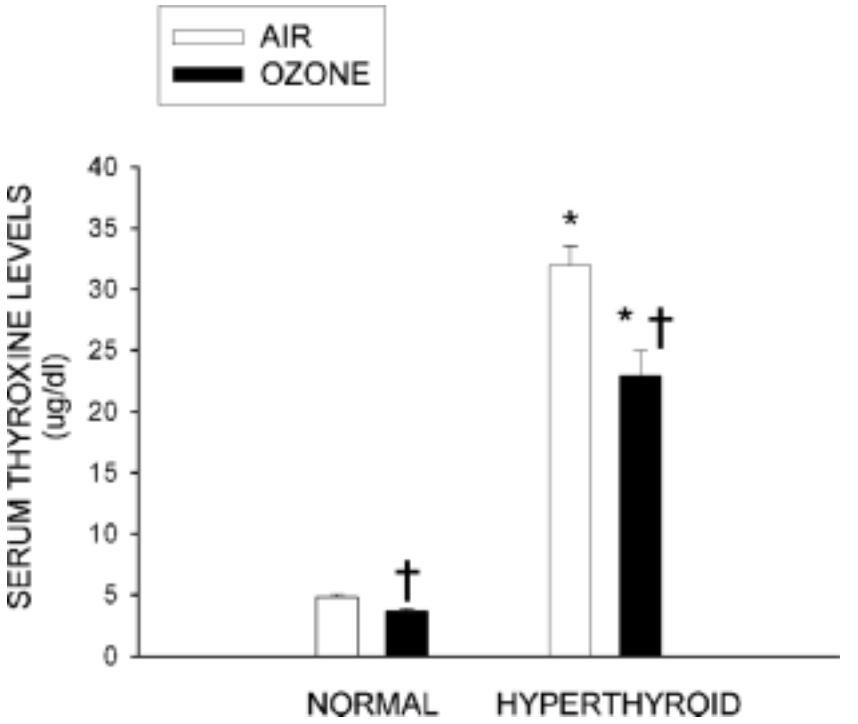

FIGURE 1. Circulating thyroxine levels in normal or hyperthyroid rats following air or ozone exposure. Normal or hyperthyroid male rats were exposed to air or ozone (2 ppm) by inhalation for 3 h as described in the Methods section. Blood samples were collected immediately after the end of the inhalation exposures and the serum was separated for analysis. Serum thyroxine levels were then measured by radioimmunoassay. Values are the means ± SE (n = 11–12 rats/group). Asterisk indicates significant at p = .05 versus normal groups exposed to air or ozone; dagger, significant at p = .05 versus the respective air-exposed group.

thyroxine resulted in an approximately sixfold increase in circulating levels of this hormone compared to levels in rats implanted with placebo pellets. These results indicate that a hyperthyroid condition was established in the groups of rats implanted with thyroxine-containing pellets. Interestingly, ozone exposure was associated with significant reductions in serum thyroxine levels in rats implanted with either placebo or thyroxine pellets, relative to circulating thyroxine levels in the respective air-exposed groups.

Permeability of the Alveolar–Capillary Barrier, Lung Cell Damage, and BAL Cell Profiles in Normal or Hyperthyroid Rats Following Exposure to Air or Ozone

The effects of a normal or hyperthyroid state on albumin levels in BAL fluid samples immediately after air or ozone exposure are shown in Figure 2. Albumin is normally confined to the intravascular space. However, serum albumin levels rise when the permeability of the pulmonary alveolar–capillary barrier increases. Ozone exposure was associated with increases in BAL fluid albumin levels in both normal and hyperthyroid rats. However, hyperthyroid rats were much more susceptible than control rats to the early effects of ozone on the

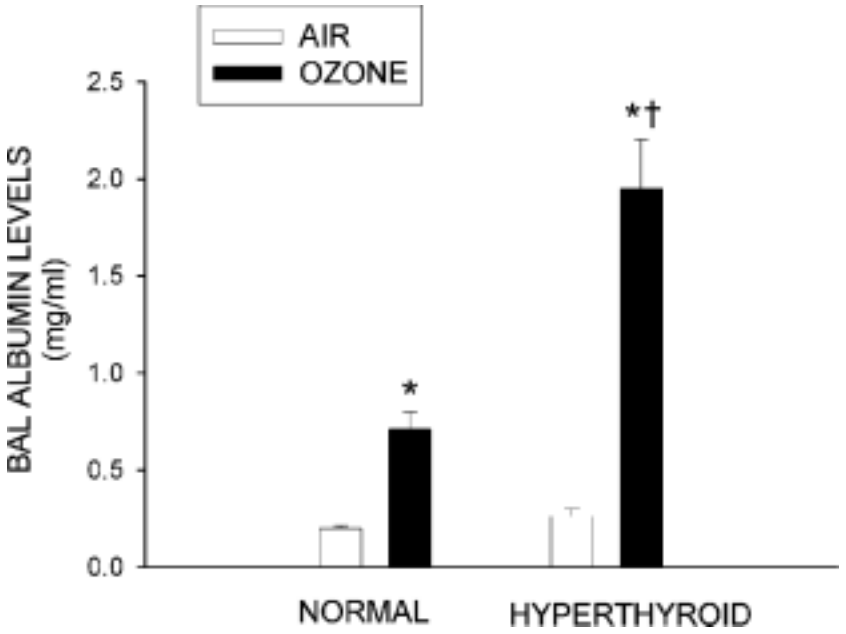

FIGURE 2. BAL fluid serum albumin levels in normal or hyperthyroid rats following air or ozone exposure. Normal or hyperthyroid male rats were exposed to air or ozone (2 ppm) by inhalation for 3 h as described in the Methods section. BAL fluid was collected immediately after the end of the inhalation exposures. Albumin levels in the initial acellular BAL samples were analyzed using commercially available reagents. Values are the means ± SE (n = 11–12 rats/group). Asterisk indicates significant at $p = .05$ versus groups exposed to air; dagger, significant at $p = .05$ versus normal/ozone-exposed group.

pulmonary alveolar–capillary barrier. Ozone exposure was associated with a 7.5-fold increase in BAL albumin levels in hyperthyroid rats, compared to a 3.5-fold increase in normal rats, relative to values in the air-exposed groups.

BAL LDH activities, cell numbers, and cell profiles are presented in Table 1. Following ozone, there was a significant increase in LDH activities in BAL fluid samples from both normal and hyperthyroid rats, but the degree of overt cytotoxicity immediately following ozone exposure was similar in both a normal and a hyperthyroid state. Ozone exposure was also associated with changes in the total number of cells harvested by BAL. In this case, a decrease in the number of cells harvested by BAL was noted that was similar in both normal and hyperthyroid rats. The majority of BAL cells harvested from all rats were alveolar macrophage (AMs), and therefore the decrease in the total numbers of cells harvested by BAL immediately following ozone exposure was a consequence of decreased numbers of harvested AMs. Additional data indicated that the percentage of circulating blood neutrophils was increased in hyperthyroid rats immediately following exposure to ozone (31 ± 4%) compared to the percentage of in normal rats exposed to ozone (16 ± 2%) or normal and hyperthyroid rats exposed to air (11 ± 1% and 15 ± 2%, respectively).

Extracellular Lining Fluid Antioxidant Status in Normal or Hyperthyroid Rats Following Air or Ozone Exposure

The effects of a normal or hyperthyroid state on the total antioxidant potential in BAL fluid samples just following air or ozone exposure are presented in Figure 3. Following ozone exposure, decreases in the total antioxidant potential of BAL fluid samples were noted in both normal and

TABLE 1. BAL LDH Activities, Cell Numbers, and Cell Profiles in Normal or Hyperthyroid Rats Following Air or Ozone Exposure

	Normal		Hyperthyroid	
Parameter	Air	Ozone	Air	Ozone
LDH (U/L)	73.0 ± 5.0	168.1 ± 20.4[b]	82.1 ± 2.9	203.5 ± 13.1[b]
Cell number ($\times 10^{-6}$)	10.64 ± 1.09	6.86 ± 0.62[b]	11.25 ± 0.96	7.99 ± 0.62[b]
AM (%)	99.9 ± 0.1	98.9 ± 0.6	99.8 ± 0.2	96.0 ± 0.7
Rats with ≤ 99% AM[a]	1/12	3/11	2/12	10/12[c]
Neutrophils (%)	0.1 ± 0.1	1.1 ± 0.6	0.1 ± 0.1	3.7 ± 0.8
Rats with ≥1% neutrophils[a]	1/12	3/11	1/12	9/12[c]

Note. Normal or hyperthyroid male rats were exposed to air or ozone (2 ppm) by inhalation for 3 h. BAL fluid and cells were harvested immediately after the inhalation exposure. Values for LDH, cell number, and percent alveolar macrophages (AM) and neutrophils are the means ± SE (n = 11–12 rats/group).

[a] The number of rats/total number of rats per group with ≤ 99% AM and ≥ 1% neutrophils is indicated. The presence of eosinophils in BAL cell differentials resulted in slight differences in the number of rats having ≥1% neutrophils versus the number of rats having ≤ 99% AM in some groups.

[b] Significant at $p \leq .05$, main effect of ozone versus air.

[c] Significant at $p \leq .05$ compared to all other groups.

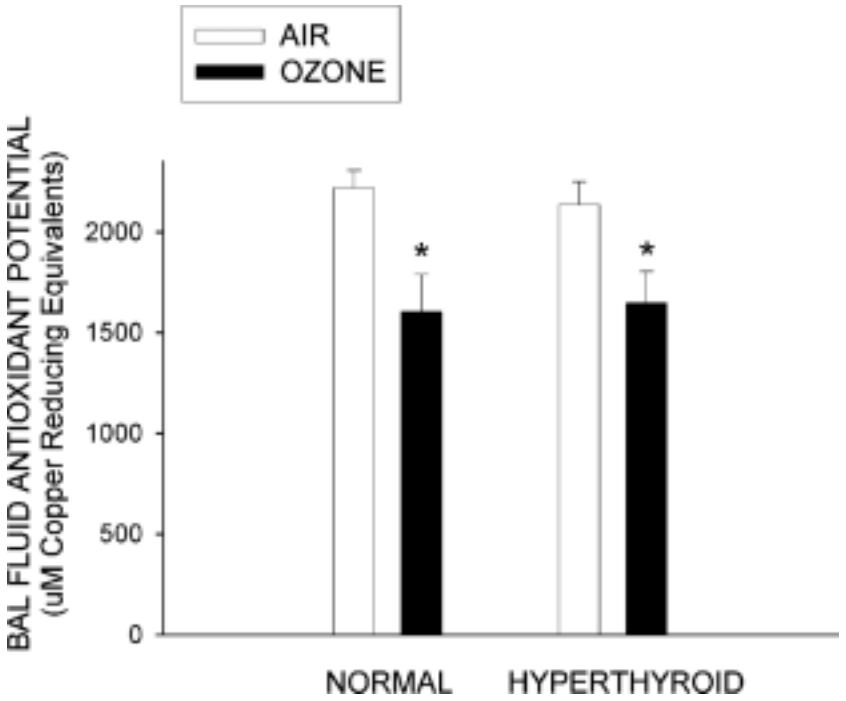

FIGURE 3. BAL fluid total antioxidant potential levels in normal or hyperthyroid rats following air or ozone exposure. Normal or hyperthyroid male rats were exposed to air or ozone (2 ppm) by inhalation for 3 h as described in the Methods section. BAL fluid was collected immediately after the end of the inhalation exposures. Total antioxidant potential levels in the initial acellular BAL samples were analyzed using commercially available reagents. Values are the means ± SE ($n = 6$ rats/group). Asterisk indicates significant at $p \leq .05$ versus groups exposed to air.

hyperthyroid rats. However, the magnitude of this effect was similar in both a normal and hyperthyroid state.

BAL Fluid Levels of Inflammatory Mediators in Normal or Hyperthyroid Rats Following Air or Ozone Exposure

Following ozone exposure, levels of both PGE_2 and MIP-2 were elevated in BAL fluid samples from normal or hyperthyroid rats (Figure 4, left and right panels, respectively). In the case of PGE_2, similar increases were noted in both ozone-exposed normal and hyperthyroid animals. However, in the case of MIP-2, BAL fluid levels of this chemokine were significantly higher in ozone-exposed hyperthyroid rats compared to levels in normal rats exposed to ozone.

BAL fluid levels of MCP-1 and TNF-α are presented in Table 2. MCP-1 was not detectable in BAL fluid samples from air-exposed animals; however, following ozone, detectable levels of MCP-1 were noted in samples from 3 of 5 normal and 4 of 6 hyperthyroid rats. TNF-α levels were not altered in normal

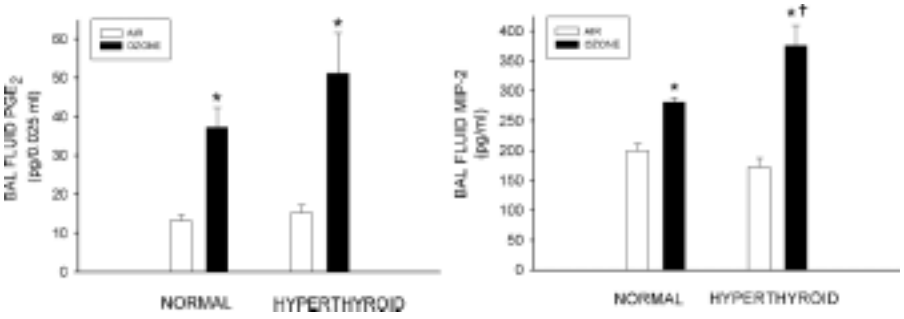

FIGURE 4. BAL fluid levels of PGE_2 and MIP-2 in normal or hyperthyroid rats following air or ozone exposure. Normal or hyperthyroid male rats were exposed to air or ozone (2 ppm) by inhalation for 3 h as described in the Methods section. BAL fluid was collected immediately after the end of the inhalation exposures. Initial acellular BAL fluid samples were analyzed for PGE_2 (left panel) by radioimmunoassay and MIP-2 (right panel) by ELISA. Values are the means ± SE (n = 5–6 rats/group except n = 4 for PGE_2 levels in the normal/ozone group). Asterisk indicates significant at $p \leq .05$ versus groups exposed to air; dagger, significant at $p \leq .05$ versus normal/ozone-exposed group.

TABLE 2. BAL Fluid Levels of MCP-1 and TNF-α in Normal or Hyperthyroid Rats Following Air or Ozone Exposure

	Normal		Hyperthyroid	
Parameter	Air	Ozone	Air	Ozone
MCP-1 (pg/ml)	ND	22.0 ± 14.2	ND	33.4 ± 15.0
TNF-α (pg/ml)	19.7 ± 3.9	29.6 ± 10.6	34.7 ± 3.6	4.2 ± 4.2[a]

Note. Normal or hyperthyroid male rats were exposed to air or ozone (2 ppm) by inhalation for 3 h. BAL fluid was harvested immediately after the inhalation exposure and cytokine levels were measured by ELISA. Values are the means ± SE (n = 5–6 rats/group). ND, nondetectable.

[a] Significant at $p \leq .05$ versus normal/ozone-exposed and hyperthyroid/air-exposed groups; five of six values ND in this group.

animals following ozone exposure, but were substantially lower in ozone-exposed hyperthyroid rats.

Production of Inflammatory Mediators by BAL Cells From Normal or Hyperthyroid Rats After Exposure to Air or Ozone

The amounts of PGE_2 and MIP-2 produced by BAL cells after 4 h in culture are shown in Figure 5. Following ozone exposure, BAL cells from both normal and hyperthyroid rats produced more PGE_2 than cells from the air-exposed groups (Figure 5, left panel). In contrast to PGE_2, the amounts of MIP-2 produced by BAL cells at the 4-h time point were similar across all groups (Figure 5, right panel). No differences in the amounts of PGE_2 and MIP-2 produced by BAL cells across the groups were noted after 18 h of culture (data not shown).

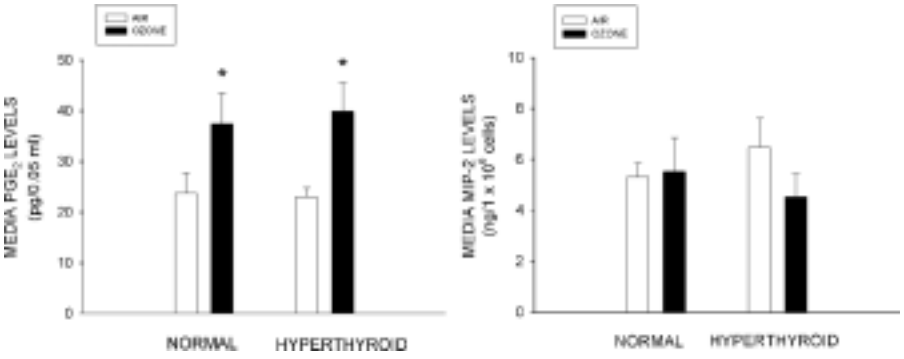

FIGURE 5. PGE_2 and MIP-2 production by BAL cells harvested from normal or hyperthyroid rats following air or ozone exposure. Normal or hyperthyroid male rats were exposed to air or ozone (2 ppm) by inhalation for 3 h as described in the Methods section. BAL cells were collected immediately after the end of the inhalation exposures. The cells were then cultured for 4 h and media samples were analyzed for PGE_2 (left panel) by radioimmunoassay and MIP-2 (right panel) by ELISA. Values are the means ± SE (n = 5–6 rats/group). Asterisk indicates significant at p = .05 ozone-exposed versus air-exposed groups.

In addition, the production of MCP-1 and TNF-α by BAL cells did not differ across the groups at the two time points studied (data not presented).

DISCUSSION

The results from this study indicate that a marked increase in the permeability of the lung alveolar–capillary barrier is an early event following exposure of hyperthyroid rats to ozone. This was reflected in a greater elevation in BAL fluid serum albumin levels in hyperthyroid rats exposed to ozone, compared with levels in ozone-exposed normal rats. In this study, the hypothesis that the increased susceptibility of the alveolar–capillary barrier to ozone in a hyperthyroid state might be due to changes in extracellular lining fluid antioxidant status was addressed. It has been suggested that antioxidants within the pulmonary epithelial lining fluid serve as an important first line of defense against inhaled toxicants and that the consumption of extracellular antioxidants, such as uric acid, ascorbic acid, and glutathione, serves to limit ozone toxicity (Kelly et al., 1996; Mudway & Kelly, 1998). Therefore, if extracellular fluid antioxidant status is lower in a hyperthyroid state, this could result in an increased sensitivity of the lung to the toxic effects of ozone. However, this does not appear to be the case. The antioxidant potential of BAL fluid in air-exposed normal and hyperthyroid rats was the same. Furthermore, while it was observed that the total antioxidant potential of BAL fluid decreased immediately after ozone exposure, these decreases were similar in both normal and hyperthyroid rats. Although studies did not measure specific extracellular antioxidant pools, preliminary results from our laboratory suggest that BAL fluid levels of total glutathione also do not differ between normal and hyperthyroid

rats. Collectively, these results suggest that overall changes in extracellular antioxidant status are not a causal factor underlying the increased sensitivity of hyperthyroid rats to ozone.

It should be noted that possible changes in intracellular antioxidant status were not explored in the current study. In this regard, it has been reported that a hyperthyroid state, induced by daily injections of thyroxine for 10 d, was associated with decreases in lung levels of reduced glutathione (Yam & Roberts, 1979). Furthermore, those hyperthyroid rats were more susceptible to oxidant-induced lung toxicity as assessed by an accelerated development of pulmonary oxygen toxicity. The potential importance of a specific role for intracellular glutathione in the modulation of oxygen-induced lung injury in a hyperthyroid state was supported by the finding that levels of superoxide dismutase and catalase activities in the lungs from those hyperthyroid rats were not different from levels in control animals (Yam & Roberts, 1979). Further investigation of a possible link between decreased intracellular antioxidant levels and the increased susceptibility to ozone in hyperthyroidism would therefore appear to be warranted.

The early involvement of inflammatory mediators in modulating the increased susceptibility of hyperthyroid rats to ozone was also investigated in the present study. Specifically, levels of PGE_2, MIP-2, MCP-1, and TNF-α were examined in BAL fluid samples from normal or hyperthyroid rats immediately after exposure to air or ozone. In addition, the potential contribution of BAL cells to any observed changes in the BAL fluid levels of these inflammatory mediators was assessed. This was accomplished by determining levels of these substances in media following ex vivo culture of BAL cells. Since the majority of BAL cells harvested immediately after air or ozone exposure were AMs, any changes in the cellular production of these mediators should reflect a significant contribution by this cell type. Of course, some caution should be exercised when relating in vitro results to in vivo situations. It is possible that the recovered BAL cell population may not reflect the total population of AMs in bronchoalveolar spaces. Furthermore, in vitro assays present some limitations because cellular responses are often the result of complex interactions among different cell types within the lung.

Regarding PGE_2, it was found that BAL fluid levels of this prostanoid were increased in both normal and hyperthyroid animals following exposure to ozone, relative to levels in air-exposed groups. Our results are in agreement with other reports demonstrating increased PGE_2 levels in BAL fluid and/or pleural fluids in both rats and humans following ozone exposure (Giri et al., 1980; Gunnison et al., 1992; Koren et al., 1989; Seltzer et al., 1986). In addition, it appeared that AMs contributed to the elevations in BAL PGE_2 levels observed in the present study, since increased PGE_2 levels were found in media samples from BAL cells from both normal and hyperthyroid ozone-exposed rats. However, the elevations in the levels of this prostanoid were similar in both normal and hyperthyroid rats following ozone, suggesting that PGE_2 may not play an important role in the hyperthyroid-linked increases in the susceptibility of the lung to ozone.

In contrast, early elevations in MIP-2 may be significantly involved in the enhanced lung response to ozone in hyperthyroidism. This is based on the observation that the increases in BAL levels of this chemokine immediately after ozone exposure were greater in hyperthyroid rats, compared to levels in normal rats. Furthermore, the greater elevation in BAL MIP-2 levels in hyperthyroid rats was associated with a statistically significant increase in the percentage of neutrophils harvested by BAL in these rats as well an increase in the percentage of circulating blood neutrophils. It is generally accepted that MIP-2 plays an important role in ozone-induced lung neutrophil recruitment in rodent animal models (Driscoll et al., 1993; Haddad et al., 1995). Previously, studies reported that BAL MIP-2 levels were greater in hyperthyroid rats than in normal rats 18 h after ozone exposure and that BAL MIP-2 levels were correlated with the percentage of neutrophils harvested by BAL at the 18 h postexposure time point (Huffman et al., 2002). Collectively, these results suggest that an early increase and sustained elevation in BAL fluid MIP-2 levels comprise an important mechanism contributing to the increased lung neutrophil influx and inflammation in hyperthyroid rats exposed to ozone compared to that observed in ozone-exposed normal rats. Although it is known that AMs can produce MIP-2 in response to oxidant exposure (Driscoll et al., 1993), the present results from the ex vivo cell culture determinations suggest that this cell type does not contribute to the early rise in BAL fluid MIP-2 levels following ozone exposure. This finding raises the possibility that other lung cell types, such as alveolar epithelial Type II cells, may be the source of the early increases in BAL MIP-2 levels which were observed in the present study.

MCP-1 levels in BAL samples were nondetectable in air-exposed rats and were very low following ozone exposure. In addition, the ex vivo production of MCP-1 by alveolar macrophages did not differ across the different groups. These results are not surprising, since MCP-1 production was assessed immediately following ozone exposure in this study and it is known that ozone-linked increases in MCP-1 mRNA levels peak approximately 24 h after exposure in rodent animal models (Zhao et al., 1998).

In this study, the potential role of TNF-α in ozone-induced responses in hyperthyroid rats was also investigated. Data showed that BAL fluid TNF-α levels were substantially decreased in hyperthyroid rats exposed to ozone. These alterations in BAL fluid TNF-α levels were not paralleled by a similar pattern in AM TNF-α release following cell culture, suggesting that the changes in BAL fluid TNF-α levels were not due to changes in TNF-α production by AMs. The importance of TNF-α in modulating ozone-induced pulmonary responses is not fully understood. Bhalla and colleagues (2002) have recently shown that the systemic administration of an antibody to TNF-α to rats significantly attenuates the acute epithelial injury and lung neutrophil recruitment induced by subsequent ozone exposure and suggested that these effects are mediated through the modulation of other cytokines. This would suggest that decreases in TNF-α levels might be associated with a downregulation of pulmonary responses to ozone. In the context of the present study, the observation of low

BAL fluid TNF-α levels in hyperthyroid rats following ozone exposure may reflect an acute decrease in the pulmonary production of this cytokine. Potentially, this mechanism could play an adaptive, feedback role and serve to limit the disruptive effects of ozone in a hyperthyroid state. However, this proposal is very speculative and requires further investigation.

The possibility that thyroid hormone-linked adaptive mechanisms may operate to limit ozone-induced lung damage has previously been raised by Clemons and Wei (1984). In that study, short-term exposure to ozone was associated with significant reductions in circulating thyroid hormone levels in both normal and thyroid hormone-treated rats, presumably as a consequence of peripheral changes in plasma binding proteins. This was hypothesized to function as a protective mechanism to limit ozone-induced injury. A similar systemic phenomenon appears to be occurring in the present study, since serum thyroxine levels were decreased in both placebo and thyroid hormone-treated rats exposed to ozone compared to circulating levels in rats exposed to air. It should be noted that effects of ozone on circulating thyroxine levels may be transient. In both the present study and the study by Clemons and Wei (1984), thyroxine levels were measured immediately following ozone exposure. However, previous results indicate that thyroxine levels did not differ between air- and ozone-exposed rats 18 h after the end of the exposure (Huffman et al., 2001).

In summary, the results of the present study are consistent with the following hypotheses: (1) The increased permeability of the alveolar–capillary barrier following ozone exposure in a hyperthyroid state is not a consequence of an overall decrease in extracellular pulmonary antioxidant status but may be related to intracellular antioxidant levels. (2) An early increase in the production of MIP-2, perhaps by alveolar Type II epithelial cells, contributes to the enhanced lung neutrophil recruitment following ozone exposure in hyperthyroid rats. (3) Inflammatory mediator production by AMs plays a minimal role in initial responses to ozone in a hyperthyroid state. Based on these findings, further focus on the roles that intracellular pulmonary antioxidant status and alveolar Type II epithelial cells may play in the increased susceptibility to ozone in a hyperthyroid state would be of value.

REFERENCES

Bhalla, D. K. 1999. Ozone-induced lung inflammation and mucosal barrier disruption: Toxicology, mechanisms, and implications. *J. Toxicol. Environ. Health B.* 2:31–86.

Bhalla, D. K., and Crocker, T. T. 1987. Pulmonary epithelial permeability in rats exposed to O_3. *J. Toxicol. Environ. Health* 21:73–87.

Bhalla, D. K., and Gupta, S. K. 2000. Lung injury, inflammation, and inflammatory stimuli in rats exposed to ozone. *J. Toxicol. Environ. Health* 59:211–228.

Bhalla, D. K., and Hoffman, L. 1997. Time course of airway epithelial and inflammatory changes in rats exposed to moderate levels of ozone. *Inhal. Toxicol.* 9:829–842.

Bhalla, D. K., Reinhart, P. G., Bai, C., and Gupta, S. K. 2002. Amelioration of ozone-induced lung injury by anti-tumor necrosis factor-α. *Toxicol. Sci.* 69:400–408.

Clemons, G. K., and Wei, D. 1984. Effect of short-term ozone exposure on exogenous thyroxine levels in thyroidectomized and hypophysectomized rats. *Toxicol. Appl. Pharmacol.* 74:86–90.

Driscoll, K. E., Simpson, L., Carter, J., Hassenbein, D., and Leikauf, G. D. 1993. Ozone inhalation stimulates expression of a neutrophil chemotactic protein, macrophage inflammatory protein 2. *Toxicol. Appl. Pharmacol.* 119:306–309.

Giri, S. N., Hollinger, M. A., and Schiedt, M. J. 1980. The effects of ozone and paraquat on $PGF_{2\alpha}$ and PGE_2 levels in plasma and combined pleural effusion and lung lavage of rats. *Environ. Res.* 21:467–476.

Gunnison, A. F., Weideman, P. A., Sobo, M., Koenig, K. L., and Chen, L. C. 1992. Age-dependence of responses to acute ozone exposure in rats. *Fundam. Appl. Toxicol.* 18:360–369.

Guth, D. J., Warren, D. L., and Last, J. A. 1986. Comparative sensitivity of measurements of lung damage made by bronchoalveolar lavage after short-term exposure of rats to ozone. *Toxicology* 40:131–143.

Haddad, E.-B., Salmon, M., Sun, J., Liu, S., Das, A., Adcock, I., Barnes, P. J., and Chung, K. F. 1995. Dexamethasone inhibits ozone-induced gene expression of macrophage inflammatory protein-2 in rat lung. *FEBS Lett* 363:285–288.

Hatch, G. E., Slade, R., Harris, L. P., McDonnell, W. F., Devlin, R. B., Koren, H. S., Costa, D. L., and McKee, J. 1994. Ozone dose and effect in humans and rats. *Am. J. Respir. Crit. Care Med.* 150:676–683.

Huffman, L. J., Judy, D. J., Brumbaugh, K., Frazer, D. G., Reynolds, J. S., McKinney, W. G., and Goldsmith, W. T. 2001. Hyperthyroidism increases the risk of ozone-induced lung toxicity in rats. *Toxicol. Appl. Pharmacol.* 173:18–26.

Huffman, L. J., Prugh, D. J., Brumbaugh, K., and Ding, M. 2002. Influence of hyperthyroidism on rat lung cytokine production and nuclear factor-κB activation following ozone exposure. *Inhal. Toxicol.* 14:1161–1174.

Kehrl, H. R., Vincent, L. M., Kowalsky, R. J., Horstman, D. H., O'Neil, J. J., McCartney, W. H., and Bromberg, P. A. 1987. Ozone exposure increases respiratory epithelial permeability in humans. *Am. Rev. Respir. Dis.* 135:1124–1128.

Kelly, F. J., Mudway, I., Krishna, M. T., and Holgate, S. T. 1995. The free radical basis of air pollution: Focus on ozone. *Respir. Med.* 89:647–656.

Kelly, F. J., Cotgrove, M., and Mudway, I. S. 1996. Respiratory tract lining fluid antioxidants: The first line of defence against gaseous pollutants. *Central Eur. J. Public Health.* 4 (suppl.):11–14.

Koren, H. S., Devlin, R. B., Graham, D. E., Mann, R., McGee, M. P., Horstman, D. H., Kozumbo, W. J., Becker, S., House, D. E., McDonnell, W. F., and Bromberg, P. A. 1989. Ozone-induced inflammation in the lower airways of human subjects. *Am. Rev. Respir. Dis.* 139:407–415.

Krishna, M. T., Chauhan, A. J., Frew, A. J., and Holgate, S. T. 1998a. Toxicological mechanisms underlying oxidant pollutant-induced airway injury. *Rev. Environ. Health.* 13:59–71.

Krishna, M. T., Madden, J., Teran, L. M., Biscione, G. L., Lau, L. C.K., Withers, N. J., Sandstrom, T., Mudway, I., Kelly, F. J., Walls, A., Frew, A. J., and Holgate, S. T. 1998b. Effects of 0.2 ppm ozone on biomarkers of inflammation in bronchoalveolar lavage fluid and bronchial mucosa of healthy subjects. *Eur. Respir. J.* 11:1294–1300.

Mudway, I. S., and Kelly, F. J. 1998. Modeling the interactions of ozone with pulmonary epithelial lining fluid antioxidants. *Toxicol. Appl. Pharmacol.* 148:91–100.

Plopper, C. G., Dungworth, D. L., and Tyler, W. S. 1973. Pulmonary lesions in rats exposed to ozone. *Am. J. Pathol.* 71:375–394.

Schelegle, E. S., Siefkin, A. D., and McDonald, R. J. 1991. Time course of ozone-induced neutrophilia in normal humans. *Am. Rev. Respir. Dis.* 143:1353–1358.

Seltzer, J., Bigby, B. G., Stulbarg, M., Holtzman, M. J., Nadel, J. A., Ueki, I. F., Leikauf, G. D., Goetzl, E. J., and Boushey, H. A. 1986. O_3-induced change in bronchial reactivity to methacholine and airway inflammation in humans. *J. Appl. Physiol.* 60:1321–1326.

Yam, J., and Roberts, R. J. 1979. Pharmacological alteration of oxygen-induced lung toxicity. *Toxicol. Appl. Pharmacol.* 47:367–375.

Young, C., and Bhalla, D. K. 1992. Time course of permeability changes and PMN flux in rat trachea following O_3 exposure. *Fundam. Appl. Toxicol.* 18:175–180.

Zhao, Q., Simpson, L. G., Driscoll, K. E., and Leikauf, G. D. 1998. Chemokine regulation of ozone-induced neutrophil and monocyte inflammation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 274:L39–L46.