Inhalation Toxicology
International Forum for Respiratory Research

ISSN: 0895-8378 (Print) 1091-7691 (Online) Journal homepage: https://www.tandfonline.com/loi/iiht20

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Inhalation Toxicology, 14:1161–1174, 2002

0895–8378/02 $12.00 + .00
DOI: 10.1080/0895837029008484 5

Taylor & Francis healthsciences

INFLUENCE OF HYPERTHYROIDISM ON RAT LUNG CYTOKINE PRODUCTION AND NUCLEAR FACTOR-κB ACTIVATION FOLLOWING OZONE EXPOSURE

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Results from previous studies indicate that hyperthyroidism increases the risk of ozone-induced lung toxicity. To better understand the processes that might contribute to the increased pulmonary inflammatory response to ozone in hyperthyroidism, we evaluated bronchoalveolar lavage fluid levels of selected cytokines in control and hyperthyroid rats after exposure to air or ozone. In addition, we assessed whether there is a relative increase in nuclear factor-kappa B (NF-κB) binding activity in cells harvested by bronchoalveolar lavage from hyperthyroid rats following the inhalation of ozone. A hyperthyroid condition was induced by the administration of thyroxine (0.5 mg/kg body weight) for 7 days. Control rats received vehicle injections. The animals were then exposed by inhalation to air or ozone (2 ppm for 3 h) and studied 18 h following the exposure. Bronchoalveolar lavage levels of MIP-2 and MCP-1 were increased in both control and hyperthyroid rats by ozone exposure. However, the increases in hyperthyroid rats were much greater, MIP-2 1.5-fold and MCP-1 11-fold, when compared to levels in controls following ozone. These changes appeared to be relatively specific; bronchoalveolar lavage fluid levels of interleukin (IL)-6, IL-4, and IL-10 were generally low or nondetectable across all of the studied groups at the 18-h postexposure time point. We also found that NF-κB binding activity was increased at both 4 and 18 h following ozone exposure in bronchoalveolar lavage cell extracts from hyperthyroid rats relative to the activity in control samples. Collectively, these results suggest that mechanisms contributing to the enhanced pulmonary inflammatory response to ozone in a hyperthyroid state include an increase in NF-κB activation and an upregulation of chemokine production.

Ozone is a highly reactive oxidant gas and is a pollutant present in ambient air (U.S. Environmental Protection Agency, 1996). It has been well documented that an acute exposure to ozone can result in biochemical and cellular changes in the lungs of a variety of animal species, including humans (Gustafsson & Cotgreave, 1996; Hatch et al., 1986; Koren et al.,

Received 20 February 2002; sent for revision 13 March 2002; revision received 16 April 2002; accepted 19 April 2002.

We thank R. E. Goldsmith, S. Tomblyn, D. Shahan, and W. G. McKinney for expert assistance with the inhalation exposures.

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1989). The major consequence of acute ozone exposure is the initiation of a pulmonary inflammatory response during the first 24-h period following exposure. These proinflammatory effects of ozone are associated with increased protein levels in the air spaces and an influx of leukocytes into alveolar regions (Alpert et al., 1971).

There is growing awareness that there are specific factors that can influence the magnitude of pulmonary responses to ozone. Recent results from our laboratory indicate that thyroid hormone status is one such factor. Specifically, the acute proinflammatory effects of ozone at 18 h following exposure are markedly increased in a hyperthyroid state in a rat animal model (Huffman et al., 2001). Exposure of hyperthyroid rats to ozone was associated with three- to sixfold increases in the levels of albumin and the numbers of neutrophils in bronchoalveolar lavage samples compared to values from euthyroid, control animals. In addition, we found that these effects occurred when the percent increases in circulating thyroid hormone levels in hyperthyroid rats were similar to those routinely observed in hyperthyroid humans. Furthermore, these responses occurred at doses of ozone that have previously been used to link ozone dose-and-effect relationships between rats and humans (Hatch et al., 1994). The mechanisms contributing to the enhancement of ozone-induced lung toxicity in a hyperthyroid state have not been fully defined. However, our previous findings suggest that changes in the inhaled dose of ozone or differences in whole-body metabolic rate cannot fully explain the thyroid hormone-linked effects (Huffman et al., 2001).

Recent studies suggest that chemotactic cytokines may play an important role in mediating lung responses to ozone exposure (Bhalla, 1999). In particular, macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1) are two chemotactic cytokines that appear to participate in ozone-induced inflammatory responses in rodent animal models. For instance, it has been shown that the inhalation of ozone is associated with increases in lung levels of MIP-2 and MCP-1 mRNAs in both rats and mice (Driscoll et al., 1993b; Zhao et al., 1998). Furthermore, it is well known that MIP-2 is a potent neutrophil chemotactic factor in both rats and mice and that, following ozone exposure, there is a temporal correlation between increases in MIP-2 mRNA levels and the recruitment of neutrophils into alveolar regions (Driscoll et al., 1993b). MCP-1 appears to play an important role as a monocyte chemoattractant after ozone exposure, since ozone-induced increases in monocyte chemotactic activity in bronchoalveolar lavage fluid samples can be inhibited by MCP-1 antibodies (Zhao et al., 1998).

These changes in lung cell cytokine expression in response to ozone appear to be linked to the activation of transcription factors, such as nuclear factor (NF)-κB. NF-κB is a DNA-binding factor that stimulates the transcription of many cytokines that are considered to be important in acute lung inflammation (Rahman & MacNee, 1998). A specific role for NF-κB in ozone-induced cytokine expression is suggested by the observation that NF-κB activity in bronchoalveolar lavage cell populations peaks at the same time as MCP-1 gene expression following ozone exposure (Zhao et al., 1998).

In this study, we tested the hypothesis that hyperthyroidism augments ozone- induced lung toxicity by increasing the production of known mediators of this process. Specifically, we examined bronchoalveolar lavage fluid levels of MIP-2 and MCP-1 in control and hyperthyroid rats 18 h after exposure to air or ozone. The specificity of responses was assessed by evaluating bronchoalveolar lavage fluid levels of interleukin-6 (IL-6), interleukin-4 (IL-4), and interleukin-10 (IL-10) in these same animals. In addition, we determined whether there is an increase in the DNA binding activity of NF-κB transcription factor in bronchoalveolar lavage cells from hyperthyroid animals following ozone exposure.

METHODS

Animal Treatments to Create a Hyperthyroid State

Specific-pathogen-free male Sprague-Dawley rats (Hilltop, Scottdale, PA) were used. To create a hyperthyroid condition, thyroid-intact rats (37–40 days of age; 180–210 g body weight) were shipped to our animal facility and received daily injections of thyroxine for 7 days (start of injections = day 1). In these experiments, thyroxine was administered at 0.5 mg/kg body weight, sc. To facilitate solubility, thyroxine was initially dissolved in 0.01 *N* NaOH. The thyroxine solution was neutralized with an equal volume of Dulbecco's phosphate-buffered saline (pH 6.8; 10× solution; Sigma, St. Louis, MO) just before injection. Age-matched control rats received daily injections of neutralized NaOH (0.2 ml/100 g body weight; sc) for 7 days. The animals were maintained in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. The rats were provided standard laboratory rat feed and tap water ad libitum, and were housed in HEPA-filtered ventilated cages under controlled light (12 h of light, 12 h of darkness) and temperature (22–24°C) conditions. All animal protocols were reviewed and approved by the local animal studies committee.

The establishment of a hyperthyroid state was assessed by measuring serum thyroxine levels on the day following the last injection. Serum thyroxine levels were measured using a commercially available radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA). The administration of thyroid hormone (0.5 mg thyroxine/kg body weight; sc; daily for 7 days) resulted in a hyperthyroid condition in which circulating levels of thyroxine were elevated fourfold compared to levels in vehicle-treated controls (19.8 ± 2.8 vs. 4.9 ± 0.1 μg/dl).

Inhalation Exposures

A whole-body inhalation exposure system was used to expose individually housed rats to ozone (2 ppm for 3 h). This system has previously been described in detail (Huffman et al., 2001). Control rats were exposed to

HEPA-filtered air in a whole-body exposure chamber that was similar to the chamber used for ozone exposures.

Time of Sample Collection

The effects of inhalation exposures on indices of pulmonary inflammation and bronchoalveolar lavage fluid levels of cytokines were evaluated 18 h after the end of the exposure period. This time point was chosen since our previous work indicated that proinflammatory effects of ozone in vehicle-treated or hyperthyroid rats are observed at this time (Huffman et al., 2001). NF-κB activation was measured at 4 and 18 h postexposure. These times were chosen based on reports that increased NF-κB activity in rat lung cells can be observed at times ranging from 2 to 24 h following ozone exposure (Haddad et al., 1996; Laskin et al., 1998; Zhao et al., 1998), and it is known that increases in NF-κB activity often precede changes in cytokine expression (Blackwell & Christman, 1997).

Collection of Blood and Bronchoalveolar Lavage Fluid and Cell Samples

The rats were first anesthetized with sodium pentobarbital (65 mg/kg, ip; Butler, Columbus, OH). Following anesthesia, blood was collected from the abdominal vein into a syringe and placed in a glass tube without anticoagulant. The left renal artery was then cut. A tracheal cannula was inserted and an initial bronchoalveolar lavage was performed with 6 ml of 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* dextrose, pH 7.4). This lavage solution was introduced into and withdrawn from the lungs for a total of three times. The total return of the initial lavage averaged 4 ml per rat. Subsequent bronchoalveolar lavages 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 supernatants from the initial lavage were processed for analyses of lactate dehydrogenase (LDH) activities, serum albumin levels, and aliquots were frozen (–80°C) for subsequent cytokine level measurements. The cell pellets from the initial and subsequent lavages were 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 a total of three times. Following the final wash, the cells were resuspended in HEPES-buffered medium (145 m*M* NaCl, 5 m*M* KCl, 10 m*M* HEPES, 1 m*M* $CaCl_2$, and 5.5 m*M* dextrose, pH 7.4). Blood samples were centrifuged (1000 × g, 10 min, 4°C). The serum was separated from the blood and stored at –20°C prior to analysis of serum thyroxine levels (described earlier).

Determination of Bronchoalveolar Lavage Cell Counts and Cell Profiles

Total counts of cells greater than 5 μm in the bronchoalveolar lavage cell suspensions 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 by counting 100 cells using light microscopy. Greater than 99% of the counted cells were either alveolar macrophages or neutrophils.

Analyses of Lactate Dehydrogenase Activities and Serum Albumin Levels in Bronchoalveolar Lavage Fluid Samples

Lactate dehydrogenase (LDH) activities and serum albumin levels were measured as previously described (Huffman et al., 2001).

Analyses of Cytokine Levels in Bronchoalveolar Lavage Fluid Samples

Levels of MIP-2, MCP-1, IL-6, IL-4, and IL-10 were determined in bronchoalveolar lavage fluid samples using ELISA kits from Biosource International, Inc. (Camarillo, CA) according to the manufacturer's instructions. Results are expressed as picograms per milliliter.

Analysis of DNA Binding Activity of NF-κB in Nuclear Extracts of Bronchoalveolar Lavage Cell Samples Using an Electrophoretic Mobility Shift Assay

Nuclear proteins were extracted from bronchoalveolar lavage cells as previously described (Ye et al., 1999). Briefly, the bronchoalveolar lavage cell populations from individual rats were treated with 500 μl lysis buffer (50 m*M* KCl, 0.5% NP-40, 25 m*M* HEPES, pH 7.8, 1 m*M* PMSF, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 100 μ*M* DTT) on ice for 4 min. After 1 min of centrifugation at 14,000 rpm, the pellets of nuclei were washed once with 500 μl of the same buffer without NP-40 and then suspended in 300 μl of extraction buffer (500 mM KCl, 10% glycerol with the same concentrations of HEPES, PMSF, leupeptin, aprotinin, and DTT as in the lysis buffer). The mixture was centrifuged at 14,000 rpm for 5 min and the supernatant was collected as the nuclear protein extract and stored at –70°C. The protein concentration was determined using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

A double-stranded oligonucleotide encoding the NF-κB binding sequence in the human IL-6 gene promoter (-74 TGGGATTTTCCCATGAGTCT-54) was labeled with ^{32}P-dCTP (Amersham, Arlington Heights, IL) using a Klenow fragment (BRL, Gaithersburg, MD). The DNA-NF-κB binding reaction was conducted in a 24-μl reaction mixture containing 1 μg poly dI-dC (Sigma, St. Louis, MO), 10 μg nuclear protein extract, 3 μg BSA, 12% glycerol, 12 m*M* HEPES, pH 7.9, 4 m*M* Tris-HCl, pH 7.9, 1 m*M* ethylenediamine tetraacetic acid (EDTA), and 1 m*M* DTT. This mixture was incubated on ice for 10 min and then 1 μl of ^{32}P-labeled oligonucleotide probe (~4×10^4 cpm) was added. The mixture was incubated for another 20 min at room temperature and the DNA–protein complexes were resolved on a 5% polyacrylamide gel using 0.5× Tris borate–EDTA running buffer. The retarded band, representing DNA–protein complexes, was detected by autoradiography.

Statistical Analyses

Using SAS, two-way analysis of variances were conducted to determine if the factors of hormone treatment and ozone exposure would result in a significant difference on LDH activities and serum albumin levels, total cells, the percentages of neutrophils, and MIP-2 and MCP-1 levels in bronchoalveolar lavage samples. Logarithmic and arcsine transformations were used to satisfy assumptions of normality and homogeneity of variance in conducting analysis of variance tests. Means and standard errors are reported in original units. Linear regression analysis was used to determine the possible relationship between MIP-2 levels and the percentages of neutrophils harvested by bronchoalveolar lavage in ozone-treated rats. The significance level was set at $p \leq .05$.

RESULTS

Lung Cell Damage, Permeability of the Alveolar–Capillary Barrier, and Neutrophil Recruitment in Control or Hyperthyroid Rats Exposed to Air or Ozone

The effects of a normal or hyperthyroid state on LDH activities, albumin levels, total cells, and percentages of neutrophils in bronchoalveolar lavage fluid samples at 18 h following air or ozone exposure are shown in Table 1. LDH is an intracellular enzyme and its presence in bronchoalveolar lavage fluid samples is an indicator of lung cell damage. Albumin is normally confined to the intravascular space. However, albumin levels in bronchoalveolar lavage samples increase when the alveolar–capillary barrier is disrupted. In the present study, ozone exposure was associated with increases in albumin levels, total cell numbers, and the percentages of neutrophils in bron-

TABLE 1. LDH activities, albumin levels, and percentages of neutrophils in bronchoalveolar lavage samples from control or hyperthyroid rats exposed to air or ozone

Treatment	LDH (U/L)	Albumin (mg/ml)	Total cells (10^{-6})	Neutrophils (%)
Control + air	81.0 ± 5.7	0.21 ± 0.02	8.1 ± 0.5	0.2 ± 0.2
Control + ozone	100.0 ± 6.1	1.00 ± 0.08[a]	10.3 ± 1.0[d]	10.2 ± 0.8[a]
Hyperthyroid + air	106.0 ± 6.2[c]	0.27 ± 0.05	11.2 ± 1.8[e]	0.5 ± 0.3
Hyperthyroid + ozone	179.2 ± 7.6[b]	3.75 ± 0.56[b]	14.9 ± 1.6[d,e]	25.2 ± 5.4[b]

Note. Animals were treated with vehicle injections or thyroid hormone (0.5 mg thyroxine/kg body weight; sc; daily for 7 days) and then exposed to air or ozone (2 ppm; 3 h) by inhalation as described in the Methods. Bronchoalveolar lavage samples were obtained 18 h after the inhalation exposures. Values are the means ± SE (n = 4).

[a]Significant at $p \leq .05$ vs. control + air group.

[b]Significant at $p \leq .05$ vs. hyperthyroid + air and control + ozone groups.

[c]Significant at $p \leq .05$ vs. control + air group.

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

[e]Significant at $p \leq .05$, main effect of hyperthyroid vs. control.

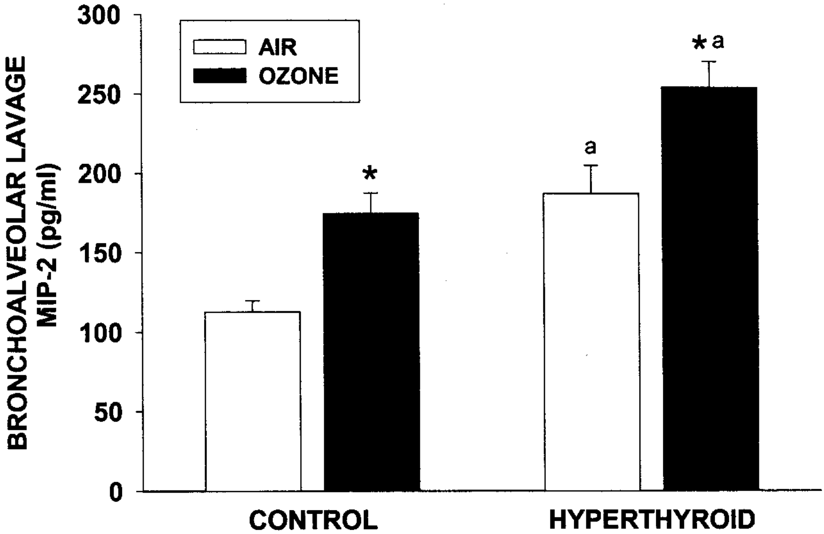

FIGURE 1. MIP-2 levels in bronchoalveolar lavage fluid samples from control or hyperthyroid rats exposed to air or ozone. Animals were treated with vehicle or thyroid hormone by injection (0.5 mg thyroxine/kg body weight, sc; daily for 7 days) and then exposed to air or ozone (2 ppm for 3 h) by inhalation as described in the Methods. Bronchoalveolar lavage samples were obtained 18 h after the end of the inhalation exposures. MIP-2 levels were measured using ELISA. Values are the means ± SE (n = 4/group). Asterisk indicates significant at $p \leq .05$ vs. the respective air group; a, significant at $p \leq .05$ vs. the respective control group.

choalveolar lavage fluid samples in both normal and hyperthyroid rats. LDH activities in bronchoalveolar lavage fluid samples also tended to be greater following ozone exposure (p = .06). However, hyperthyroid rats were much more responsive than control rats to the pulmonary effects of ozone. For instance, LDH activities in samples from hyperthyroid rats were 1.8 times greater than those from vehicle-injected controls exposed to ozone. Ozone exposure was also associated with a 14-fold increase in bronchoalveolar lavage albumin levels in hyperthyroid rats, compared to a 5-fold increase in control rats above values in the respective air-exposed groups. In addition, the percentage of neutrophils in bronchoalveolar lavage samples from hyperthyroid rats was 2.5-fold greater than that in samples from control rats following ozone exposure.

Cytokine Levels in Bronchoalveolar Lavage Fluid Samples from Control or Hyperthyroid Rats Exposed to Air or Ozone

The effects of a normal or hyperthyroid state on MIP-2 levels in bronchoalveolar lavage fluid samples at 18 h following air or ozone exposure are shown in Figure 1. Both control and hyperthyroid rats responded to ozone with increases in bronchoalveolar lavage MIP-2 levels. We also found that MIP-2 levels were increased in hyperthyroid animals relative to levels in control animals, and that this effect was observed in both air- and ozone-exposed groups of animals. Furthermore, following ozone, MIP-2 levels were highly correlated with the percentages of neutrophils harvested by bronchoalveolar lavage from the individual rats ($r = .90$; Figure 2).

Bronchoalveolar lavage MCP-1 levels in normal and hyperthyroid rats 18 h following exposure to air or ozone are shown in Figure 3. Bronchoalveolar lavage levels of MCP-1 were generally low in the air-exposed groups. Ozone exposure was associated with an increase in bronchoalveolar lavage MCP-1 levels in both normal and hyperthyroid rats, but this increase was much greater in hyperthyroid rats relative to that in control animals. Hyperthyroid rats responded to ozone with an 11-fold increase in MCP-1 levels above those observed in controls exposed to ozone. The alterations in bronchoalveolar lavage levels of MIP-2 and MCP-1 that were observed appeared to be relatively specific in that bronchoalveolar lavage fluid levels of IL-6, IL-4, or IL-10 were generally low or nondectectable across all of the studied groups of rats (data not presented).

DNA Binding Activity of NF-κB in Nuclear Extracts of Bronchoalveolar Lavage Cell Samples from Control or Hyperthyroid Rats Exposed to Ozone

The effects of a normal or hyperthyroid state on DNA binding activity of NF-κB in bronchoalveolar lavage cell samples following ozone exposure are

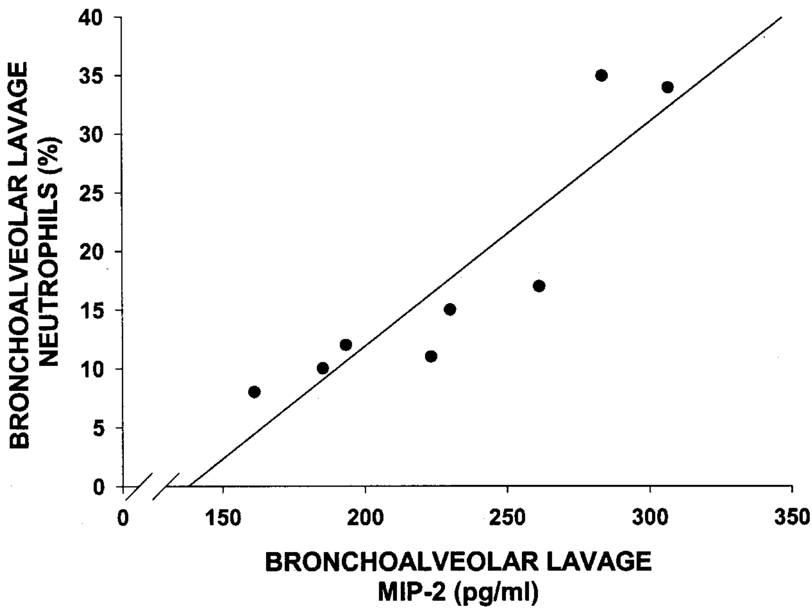

FIGURE 2. Relationship between bronchoalveolar lavage fluid MIP-2 levels and the percentages of neutrophils harvested by bronchoalveolar lavage from individual rats following ozone exposure. Animals were treated as described in the legend to Figure 1. Bronchoalveolar lavage samples were obtained 18 h after the end of the inhalation exposure. The line depicts the best-fit relationship between these two variables using linear regression analysis.

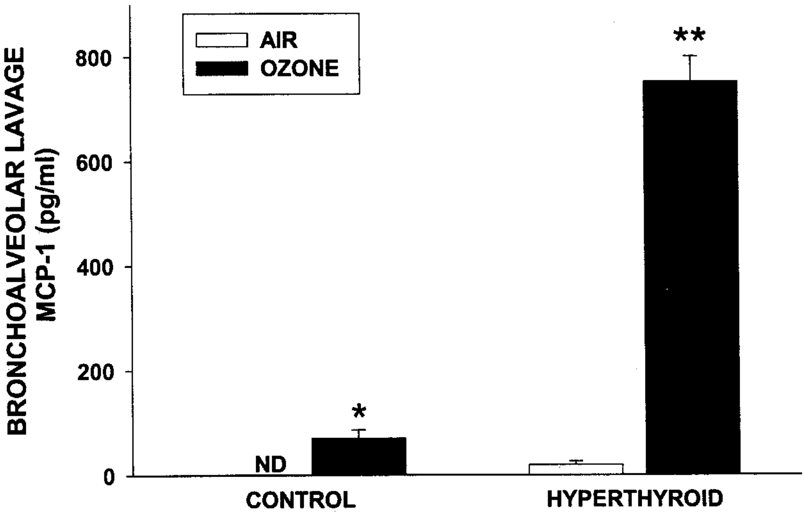

FIGURE 3. MCP-1 levels in bronchoalveolar lavage fluid samples from control or hyperthyroid rats exposed to air or ozone. Animals were treated as described in the legend to Figure 1. Bronchoalveolar lavage samples were obtained 18 h after the end of the inhalation exposures. MCP-1 levels were measured using ELISA. Values are the means ± SE (n = 4/group). Asterisk indicates significant at $p \leq .05$ vs. the control + air group; double asterisk, at $p \leq .05$ vs. the hyperthyroid + air and control + ozone groups.

shown in Figure 4. NF-κB binding activity was increased in nuclear extracts of bronchoalveolar lavage cells from control rats at 18 h following ozone exposure. In hyperthyroid rats, increases in NF-κB binding activity were observed at both 4 and 18 h following ozone exposure in bronchoalveolar lavage cell extracts and were more marked than those in control samples. At 4 h following ozone, the percentages of neutrophils in bronchoalveolar lavage samples from these control and hyperthyroid rats averaged 9.5 ± 0.5% and 48.5 ± 19.5%, respectively. At 18 h following ozone exposure, these values were 18.5 ± 1.5% and 41.5 ± 5.5%, respectively.

DISCUSSION

The results from this study confirm our previous finding that hyperthyroidism substantially increases the risk of ozone-induced lung toxicity in a rat animal model (Huffman et al., 2001). This was manifested by increases in lung cell damage, the permeability of the alveolar–capillary barrier, and an influx of neutrophils into bronchoalveolar areas in hyperthyroid rats relative to that in euthyroid controls following the inhalation of ozone. Our results also suggest that an upregulation of cytokine production by lung cells may mediate some of these effects. This is based upon the finding that bronchoalveolar lavage fluid levels of both MIP-2 and MCP-1 were relatively higher in ozone-exposed hyperthyroid rats. In addition, changes in the DNA-binding activity of NF-κB in cells lining airspace surfaces may contribute to enhanced pulmonary inflammatory responses to ozone in a hyperthyroid state.

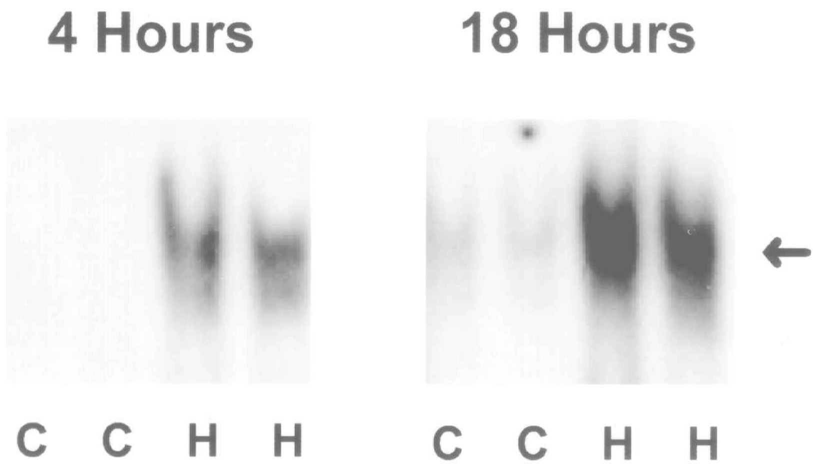

FIGURE 4. DNA binding activity of NF-κB in nuclear extracts of bronchoalveolar lavage cells from control or hyperthyroid rats exposed to ozone. Animals were treated as described in the legend to Figure 1. Bronchoalveolar lavage cell samples were obtained at 4 or 18 h after the end of the inhalation exposure from 2 rats in each group (C = control; H = hyperthyroid). The DNA binding activity of NF-κB in nuclear extracts of bronchoalveolar lavage cells was determined using an electrophoretic mobility shift assay and is indicated by the arrow in the figure.

The recruitment and accumulation of inflammatory cells in the lung following ozone exposure has been well documented and appears to depend on the local synthesis and release of specific chemokines. In particular, it has been suggested that an increase in MIP-2, a chemotactic protein, is responsible in part for ozone-induced increases in lung neutrophils in rodent animal models. This is based on observations that there is a temporal relationship between elevations in steady state MIP-2 mRNA levels and the appearance of increased neutrophils in lung lavage fluid from mice following ozone exposure (Driscoll et al., 1993b). In addition, suppression of ozone-induced MIP-2 gene expression is associated with decreased neutrophil accumulation in rat lungs (Haddad et al., 1995). In the present study, we observed that the inhalation of ozone resulted in increased levels of MIP-2 in lung lavage fluid in both control and hyperthyroid rats and that these levels were highly correlated with the percentages of neutrophils harvested by bronchoalveolar lavage. In addition, the absolute levels of MIP-2 were higher in hyperthyroid animals, suggesting that increased production of this chemokine may be one factor contributing to enhanced lung neutrophil recruitment in a hyperthyroid state following ozone exposure.

MCP-1 is also a chemokine that can be expressed by lung cells following ozone exposure (Zhao et al., 1998). In this case, MCP-1 appears to function as a monocyte chemoattractant. This is based on the observation that ozone-induced increases in monocyte chemotactic activity in bronchoalveolar fluid samples can be inhibited by an antibody directed against MCP-1 (Zhao et al., 1998). In the present study, we observed that the inhalation of ozone was associated with an increase in MCP-1 levels in bronchoalveolar lavage fluid samples from both control and hyperthyroid rats. The magnitude of this increase was much greater, however, in hyperthyroid animals, suggesting that an upregulation of the production of MCP-1, as well as MIP-2, is a feature of the enhanced pulmonary response to ozone that occurs in hyperthyroidism.

In the present study, we observed much more marked changes in bronchoalveolar fluid levels of MCP-1 relative to those of MIP-2 in hyperthyroid versus control rats exposed to ozone. This may be a reflection of the time of study. It is known that monocyte accumulation in the lung is delayed relative to that of neutrophils following ozone exposure, and it has been documented that there appears to be a sequential expression of the chemokines that mediate these events. In the present investigation, bronchoalveolar lavage samples were obtained at 18 h postexposure. At this time, it appears that ozone-induced increases in MIP-2 expression may be declining while that of MCP-1 is reaching a peak in rodents (Zhao et al., 1998). It would be of interest in future investigations to more fully define the time course of changes in the expression of these chemokines; nevertheless, the results from our study suggest that increases in both MIP-2 and MCP-1 expression contribute to the enhanced pulmonary inflammatory response of hyperthyroid rats to ozone.

In contrast to increases in bronchoalveolar lavage fluid levels of MIP-2 and MCP-1 in hyperthyroid rats relative to controls following ozone exposure, levels of IL-6, IL-4, and IL-10 were generally low or nondetectable across the studied groups at the 18 h postexposure time-point. These findings suggest that there may be a relatively specific upregulation of the production of certain lung cytokines in hyperthyroid rats exposed to ozone. However, as discussed earlier, levels of these cytokines were assessed only at one time point. Increases in bronchoalveolar lavage fluid levels of IL-6 in rats have been reported following ozone, with the highest levels observed immediately following the exposure and a subsequent decline to control levels by 15 h postexposure (McKinney et al., 1998).

In the present study, cytokine levels were measured in bronchoalveolar lavage fluid samples and the observed changes in chemokine levels could reflect production from a number of lung cell types. For instance, it is known that alveolar macrophages, type II epithelial cells, and neutrophils from rodent species all have the potential to produce both MIP-2 and MCP-1 (Barrett et al., 1998; Brieland et al., 1992; Driscoll et al., 1993a, 1996; Nygardas et al., 2000; Shenkar & Abraham, 1999). Further investigation will be necessary to determine the specific cell type(s) contributing to the relative increase in chemokine levels in hyperthyroid rats following ozone exposure. However, results from the present investigation suggest that a specific activation of alveolar macrophages and/or recruited neutrophils contributes to the enhanced pulmonary inflammatory responses to ozone in a hyperthyroid state. This is based on the observation that increases in NF-κB binding activity in bronchoalveolar lavage cell populations were more marked in hyperthyroid rats than in controls following ozone exposure.

NF-κB is a member of a novel family of transcription factors and is involved in regulating the transcription of many genes during inflammatory processes (Blackwell & Christman, 1997). NF-κB-binding motifs have been described in the promoter regions of cytokine genes, such as MIP-2, tumor necrosis factor (TNF)-α, and IL-6, and NF-κB appears to be involved in regulating the transcription of these cytokines (Kuprash et al., 1999; Vanden Berghe et al., 2000; Widmer et al., 1993). In addition, transcriptional regulation of MCP-1 has also been linked to NF-κB activation (Goebeler et al., 2001). Our present findings concerning NF-κB binding activity as well as MIP-2 and MCP-1 levels are consistent with the hypothesis that NF-κB may act as an upstream transcriptional regulator of these chemokines and that the specific activation of chemokine production by alveolar macrophage and/or neutrophil cell populations within the airspaces may be a mechanism contributing to the enhanced pulmonary inflammatory response to ozone in a hyperthyroid state. Our results also suggest that future studies addressing this hypothesis utilizing inhibitors of NF-κB, such as antioxidants, are warranted.

The exact triggering mechanisms that lead to enhanced pulmonary NF-κB activation, chemokine production, and inflammation in hyperthyroid rats following ozone exposure remain to be determined. Our previous findings suggest that changes in the inhaled dose of ozone or differences in whole-body metabolic rate cannot fully explain the thyroid hormone-linked effects (Huffman et al., 2001). It is possible that direct effects of thyroid hormones on lung cells may underlie the observed potentiation of ozone-induced pulmonary responses. In this regard, thyroid hormones have been shown to increase the osmotic fragility of erythrocytes exposed to ozone in vitro (Wong & Hochstein, 1981). This was attributed to a modification of specific membrane proteins as a result of thyroid hormone deiodination within the erythrocyte membrane in the presence of ozone. This process may also occur in the lungs. If so, the resulting modification of lung cell membrane proteins in hyperthyroid rats during ozone exposure could significantly affect initial intracellular signaling processes and thereby modulate the subsequent magnitude of inflammatory responses to ozone. In addition, it is known that the administration of thyroid hormones increases the oxygen consumption of lung cell slices (Joyner et al., 1976). Thyroid hormones have also been reported to directly stimulate the production of superoxide anion by human alveolar neutrophils and macrophages (Nishizawa et al., 1998). Any resulting changes in the overall oxidant/antioxidant balance in the lungs as a consequence of these thyroid hormone-linked effects could also significantly modulate pulmonary responses to ozone. Investigation of these possible mechanisms would be of definite interest in future studies.

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