Toxicology and Applied Pharmacology **173,** 18–26 (2001)
doi:10.1006/taap.2001.9174, available online at http://www.idealibrary.com on IDEAL®

Hyperthyroidism Increases the Risk of Ozone-Induced Lung Toxicity in Rats

L. J. Huffman,*,† D. J. Judy,* K. Brumbaugh,* D. G. Frazer,*,† J. S. Reynolds,* W. G. McKinney,* and W. T. Goldsmith*

**Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505; and †Department of Physiology, West Virginia University School of Medicine, Morgantown, West Virginia 26506*

Received December 6, 2000; accepted March 9, 2001

Hyperthyroidism Increases the Risk of Ozone-Induced Lung Toxicity in Rats. Huffman, L. J., Judy, D. J., Brumbaugh, K., Frazer, D. J., Reynolds, J. S., McKinney, W. G., and Goldsmith, W. T. (2001). *Toxicol. Appl. Pharmacol.* **173, 18–26.**

The risk of lung injury from ozone exposure has been well documented. It is also known that various factors may significantly influence the susceptibility of animals to the toxic effects of ozone. In the present study, we investigated the possibility that hyperthyroidism might be associated with increases in ozone-induced pulmonary toxicity. To create a hyperthyroid condition, mature male Sprague–Dawley rats were given injections of thyroxine (dose range: 0.1 to 1 mg/kg body wt daily for 7 days). Control rats received vehicle injections. The animals were then exposed to air or ozone (dose range: 0.5 to 3 ppm for 3 h). At 18 h postexposure, bronchoalveolar lavage fluid and cells were harvested. In hyperthyroid animals, ozone exposure was associated with three- to sixfold increases in bronchoalveolar lavage fluid lactate dehydrogenase activities and albumin levels as well as the number of polymorphonuclear leukocytes harvested by bronchoalveolar lavage above levels observed in ozone-exposed control rats. Additional results from the present study suggest that these thyroid hormone-linked effects cannot be fully explained by differences in whole-body metabolic rate or changes in the inhaled dose of ozone. These findings indicate that the risk of ozone-induced lung toxicity is substantially increased in a hyperthyroid state and suggest that the susceptibility of the lung to damage from ozone exposure may be significantly influenced by individual thyroid hormone status.

Key Words: **thyroxine; rat; pulmonary edema.**

Ozone is a photochemical oxidant that is found in ambient air and exposure to this gas poses a health hazard. Upon inhalation, ozone triggers a spectrum of effects that can result in respiratory toxicity. For instance, the inhalation of high concentrations of ozone is associated with severe, often fatal pulmonary edema (Jaffe, 1967). Exposure to lower concentrations of ozone can also have respiratory effects. Humans who are exposed to near-ambient levels of ozone exhibit cellular and biochemical changes in the lung that are indicative of injury and inflammation (Devlin *et al.,* 1991; Koren *et al.,* 1989; Seltzer *et al.,* 1986). These changes include increases in bronchoalveolar lavage fluid lactate dehydrogenase activities, protein levels, and the number of polymorphonuclear leukocytes that are harvested by bronchoalveolar lavage. Exposure of animals to ozone is also associated with lung injury and inflammatory responses that are dose dependent (Hatch *et al.,* 1986). In general, the pulmonary effects of ozone in various animal models parallel those seen in humans (Hatch *et al.,* 1986, 1994).

There is growing awareness that various factors may affect individual susceptibility to ozone toxicity. For instance, studies in mice suggest that genetic factors play a significant role in the degree of ozone-induced acute lung injury (Kleeberger *et al.,* 1997; Prows *et al.,* 1999). In addition, physical factors and dietary components, such as age, exercise, and vitamin E intake, appear to affect the susceptibility of animals to the adverse effects of ozone (Fukase *et al.,* 1978; Menzel, 1979; Nambu and Yokoyama, 1981). There is also some evidence that hormonal factors may influence ozone-induced toxicity. Specifically, thyroid hormone status may determine the severity of response. Chemical or surgical thyroidectomy resulted in increased survival rates of mice and rats exposed to otherwise lethal doses of ozone (Fairchild and Graham, 1963). Conversely, survival rates following ozone exposure were decreased when mice received thyroid hormone preparations. However, how changes in thyroid hormone status might impact pulmonary responses to ozone has not been fully explored. Therefore, the purpose of this investigation was to more completely examine relationships between hyperthyroidism and ozone-induced lung toxicity. Specifically, we assessed how increases in circulating thyroid hormone levels affect lung cell damage, the permeability of the alveolar capillary barrier, and the influx of polymorphonuclear leukocytes into alveolar regions following ozone exposure in a rat animal model. In addition, we evaluated whether the effects of a hyperthyroid state on pulmonary responses to ozone could be explained by alterations in whole-body metabolic rate or by changes in the inhaled dose of this oxidant.

0041-008X/01

Methods

Animal treatments to alter circulating thyroid hormone levels. Specific pathogen-free male Sprague–Dawley rats (Hilltop, Scottdale, PA) were used. The animals were maintained on standard laboratory rat chow and tap water *ad libitum* and were housed in laminar flow hoods under controlled light (12 h light/12 h dark) and temperature (22–24°C) conditions. The research protocol was approved by the institutional Animal Care and Use Committee. To create a hyperthyroid condition, thyroid-intact rats (37–40 days of age; 180–210 g body wt) 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 doses ranging from 0.1 to 1 mg/kg BW. 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 (PBS; 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 wt sc) for 7 days. Inhalation exposures (see below) for thyroid hormone-treated or vehicle-treated control rats occurred on day 7.

Inhalation exposures. A whole-body inhalation exposure system was used to expose the rats to ozone. In these experiments, the doses of ozone that were given ranged from 0.5 to 3 ppm and the inhalation exposures lasted 3 h. The ozone generation system employed feedback control to maintain a constant, user-defined ozone concentration in the exposure chamber. An Enmet Ozone Generator was adjusted to produce up to 10 ppm of ozone as dry HEPA-filtered air flowed through it at a constant rate of 1.2 L/min. The generator utilized a low-pressure mercury vapor lamp to produce ozone by UV radiation. A computer controlled stepper motor turned a needle valve that regulated the amount of dried filtered diluent air added to the system. The diluent air was then mixed with the air–ozone blend produced by the generator and introduced into the exposure chamber. An ozone analyzer (API, model 400a) continuously monitored the ozone concentration within the chamber. The feedback control program used the analyzer output to estimate chamber concentration and adjust the amount of diluent air added to the ozone to achieve the desired exposure concentration. When the ozone levels exceeded the desired value, the amount of diluent air was increased, and when the ozone concentration fell below the desired value, the diluent air was decreased. The mixture of ozone gas and air that flowed from the exposure chamber passed through a charcoal filter prior to entering the laboratory exhaust. This system was used to expose individually housed animals for 3 h to ozone concentrations between 0.5 and 3 ppm. Prior to experiments, the ozone analyzer was calibrated with a self-contained ozone calibration system. Control rats were exposed to HEPA-filtered air in a whole-body exposure chamber that was similar to the chamber used for ozone exposures.

The effects of inhalation exposures on indices of lung cell damage, the permeability of the alveolar capillary barrier, and the influx of polymorphonuclear leukocytes into alveolar regions were evaluated 18 h after the end of the exposure period. The decision to study the animals at the 18-h postexposure point was based upon reports indicating that ozone-induced pulmonary damage and pro-inflammatory effects can be observed across a number of animals species and in human subjects at this time (Hatch *et al.*, 1986, 1994).

Collection of blood and bronchoalveolar lavage fluid and cell samples and lung wet/dry weight measurements. The rats were first anesthetized with sodium pentobarbital (65 mg 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. In one experiment, the lungs were removed from the animals and placed in tared metal pans and weighed before being placed in an oven (65°C) to dry. An initial dry weight was taken after 3 days of drying and then all lungs were weighed each day for 4 more days. We found that a constant value had been reached after the initial 3-day drying period and this value was used to calculate the wet/dry weight lung ratios. In other experiments, a tracheal cannula was inserted and an initial bronchoalveolar lavage was performed with 6 ml of cold Ca^{2+}/Mg^{2+}-free PBS (145 mM NaCl, 5 mM KCl, 9.4 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 5.5 mM 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 of PBS each until a total volume of 80 ml of 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 and albumin levels. The cell pellets from the initial and subsequent lavages were combined and resuspended in 5 ml of 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 mM NaCl, 5 mM KCl, 10 mM Hepes, 1 mM $CaCl_2$, and 5.5 mM dextrose, pH 7.4).

Determination of bronchoalveolar lavage cell counts and cell profiles. Total counts of phagocytes (alveolar macrophages and polymorphonuclear leukocytes) 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, England) and stained with a Wright stain (Volu-Sol, Henderson, NV). The percentages of alveolar macrophages and leukocytes present on the slides were determined using light microscopy. Greater than 99% of these cells were either alveolar macrophages or neutrophils.

Analysis of circulating thyroid hormone levels. Blood samples collected from the rats were centrifuged (1010*g*, 10 min, 4°C). The serum was separated from the blood and stored at −20°C prior to analysis. Serum thyroxine levels were measured using a commercially available radioimmunoassay kit (Diagnostic Products Corp., Los Angeles, CA). The results are expressed as μg/dl.

Analyses of LDH activities and albumin levels in bronchoalveolar lavage fluid samples. LDH activities in supernatants from initial bronchoalveolar lavage fluid samples were analyzed using the Roche Reagent for LDH using a COBAS FARA II chemistry system (Roche Diagnostic Systems, Nutley, NJ). The results are expressed as U/L. Albumin levels in supernatants from initial bronchoalveolar lavage fluid samples were measured using a dye-binding assay procedure (Sigma Diagnostic Procedure 631; Sigma) on a COBAS FARA II chemistry system (Roche Diagnostic Systems). The results are expressed as mg/ml.

Whole-body metabolic rate measurements. Metabolic rates were determined just prior to inhalation exposures by measuring heat release from the rats. A calorimeter box (SEC-A-1201; Thermonetics, San Diego, CA) measured radiative heat losses that were conducted through the box. Temperature–humidity probe (HMP35E; Vaisala, Boston, MA) measurements on the air input and output were used to calculate the sensible and latent heat added to the ventilation air. The sum of the radiative, sensible, and latent heat was equivalent to the heat release of the animal. Heat release was normalized with respect to the surface area of each animal. Surface area was estimated using the following relationship:

$$\text{Area } (m^2) = \frac{9.0 \times \sqrt[3]{[\text{body weight (g)}]^2}}{10{,}000}$$

(Evans *et al.*, 1958). Sensor readings were continuously acquired with a computer and the average metabolic output was recalculated at 5-s intervals. Animals were placed in the calorimeter box until equilibration. Equilibration time was defined as the time when the metabolic output was level ($<\pm 0.5$ Cal/h/m^2 variation) for 1 min, after an initial waiting period of 10 min. The average heat release at the equilibration time was equivalent to the metabolic rate of the rat.

Ventilation measurements. Breathing rate, tidal volume, and minute ventilation measurements were made just prior to the inhalation exposures and just after the end of the exposure period. The animals were placed in a head-out plethysmograph. A latex seal separated the animal's head from the thorax. Flow produced by movement of the animal's thorax was measured by the

pressure drop across a pneumotach (Fleisch #000) with a pressure transducer (Model E239, ±0.25 in H_2O; Setra Systems, Inc., Boxborough, MA). The pressure signal was digitized at 1000 Hz using a National Instruments DAQ board in a PC running a custom Labview program. The pneumotach was calibrated using a set of steady flows measured by a DryCal DC-Lite Flow Primary Meter (Model DCLT 12K; Bios International, Pompton Plains, NJ). Three 6-s segments of data were collected for each animal at each time point. From these data, breathing rate, tidal volume, and minute ventilation were calculated.

Breathing frequency was calculated using a spectrum-based method. The power spectral density of each segment was calculated using a sliding window of 1024 samples zero padded to a length 8192 with an overlap of 67%. The frequency at which the peak of the spectrum occurred was then taken as the breathing frequency for each segment. A flow zero-crossing based method was used to validate the results of the spectrum method.

Tidal volume was found by integration of the flow signal. Each data segment was first separated into inhalation/exhalation pairs. Extraneous signal at the beginning and end of each of the three flow segments was eliminated. The total time span from first inhalation until last exhalation was noted. The area (volume) under each inhalation curve was calculated by numerical integration. The average of all the areas was then taken as tidal volume. Minute ventilation was calculated by dividing the sum of all the inhalation volumes by the total time span. Multiplication of tidal volume and breathing frequency was used as a quality control check of the calculations.

Estimation of the inhaled dose of ozone. The minute ventilation value for a given rat was used to estimate the inhaled dose of ozone for that animal. Standard conditions of temperature and pressure were first used to convert the ozone concentration from ppm to mg/m^3. The total inhaled dose of ozone was then calculated using the formula dose (mg) = ozone concentration × minute ventilation × minutes exposed × ($m^3/10^6$ ml).

Statistical analyses. Using SAS, two separate 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 (1) thyroxine levels and (2) lung weight ratios (Figs. 1 and 2).

The differences between thyroid hormone-treated and control groups at each level of ozone exposure were tested using appropriate contrasts in a two-way analysis of variance model. To determine if there was a significant increase in LDH, albumin, and polymorphonuclear leukocytes levels above the initial thyroid effect, a linear regression model was fit with interactions between the hormone treatment and each ozone exposure level. Using linear regression to model increased levels allowed the analysis to adjust for trends in the control group over ozone levels (Figs. 3 and 4).

A one-way analysis of variance was conducted to determine if different doses of thyroid hormone would result in significant differences in thyroxine levels. The Dunnett's Procedure was used to compare each thyroid dose level with the zero dose (Fig. 5).

A one-way analysis of variance was conducted to determine if different doses of thyroid hormone would result in significant differences in LDH, albumin, and polymorphonuclear leukocytes levels. The Tukey–Kramer method was used to adjust for multiple comparisons (Figs. 6 and 7).

Log transformations of all response variables were used to satisfy assumptions of normality and homogeneity of variance in conducting analysis of variance tests. Means and standard errors were reported in the original units. The significance level was set at $p \leq 0.05$.

Linear regression analyses were used to determine possible relationships between whole-body metabolic rate and bronchoalveolar lavage fluid levels of LDH activity, albumin levels, or the numbers of polymorphonuclear leukocytes harvested by bronchoalveolar lavage. The square of the correlation coefficient for each of these analyses was then calculated and reflected the proportion of variance in these pulmonary responses that could be attributed to differences in metabolic rate.

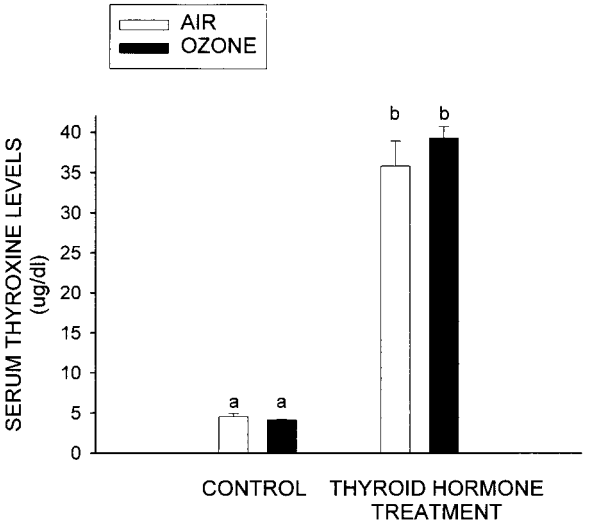

FIG. 1. Effect of thyroid hormone treatment on circulating thyroxine levels. Animals were treated with thyroid hormone (1 mg thyroxine/kg body wt sc daily for 7 days) or vehicle injections (control) and then exposed to air or ozone (3 ppm; 3 h) by inhalation as described under Methods. Blood samples were collected 18 h after the end of the inhalation exposures. Blood was obtained by venipuncture and the serum was separated for analyses. Serum thyroxine levels were then measured by radioimmunoassay. Values are the means ± SE for five or six determinations in each group. Different letters are significantly different, $p \leq 0.05$.

RESULTS

Effect of thyroid hormone treatment on circulating thyroxine levels and lung edema following ozone exposure. The effect of thyroid hormone treatment on serum thyroxine levels and lung wet/dry weights under basal conditions and following ozone exposure were initially determined. Administration of thyroid hormone (1 mg thyroxine/kg BW sc daily for 7 days) elevated circulating thyroxine levels in treated rats approximately eightfold above values in control rats (Fig. 1). Exposure to ozone (3 ppm, 3 h), did not significantly affect serum thyroxine levels when these levels were measured 18 h following the end of the exposure.

The effect of thyroid hormone treatment on lung edema formation is shown in Fig. 2. A marked increase in lung wet/dry weights occurred only in animals that had been treated with thyroid hormones and then exposed to ozone (3 ppm, 3-h exposure ending 18 h before the time of study). These results indicate that, if thyroid hormone levels are elevated, then there is a more severe pulmonary reaction to subsequent ozone exposure.

Effect of thyroid hormone treatment on lung cell damage, permeability of the alveolar–capillary barrier, and polymorphonuclear leukocyte recruitment following exposure to varying doses of ozone. We next determined what effect thyroid hormone treatment might have on indices of lung cell damage, the permeability of the alveolar–capillary barrier, and the recruitment of polymorphonuclear leukocytes into bronchoal-

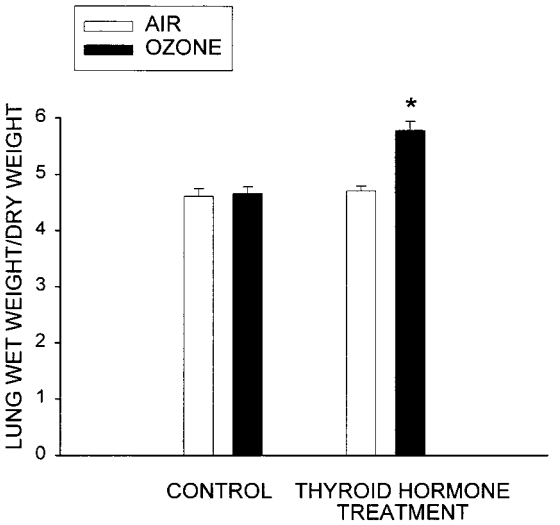

FIG. 2. Effect of thyroid hormone treatment on lung wet/dry weight ratios. Animals were treated with thyroid hormone (1 mg thyroxine/kg body wt sc daily for 7 days) or vehicle injections (control) and then exposed to air or ozone (3 ppm; 3 h) by inhalation as described under Methods. Lungs were collected 18 h after the inhalation exposures and both wet and dry weights measured. Values are the means ± SE for five or six determinations in each group. $*p \leq 0.05$ vs control/air.

veolar areas following exposure to ozone. In these experiments, the effects of varying doses of ozone were also assessed. Thyroid hormone treatment (1 mg thyroxine/kg BW sc daily for 7 days) again resulted in approximately eightfold increases in serum thyroxine levels compared with values from control rats (data not shown). Significant increases in whole-body metabolic rates also occurred in rats receiving thyroid hormone treatment (83.1 ± 1.1 cal/h/m^2) relative to values from vehicle-injected controls (63.5 ± 1.9 cal/h/m^2).

The effects of thyroid hormone treatment on LDH activities and albumin levels in bronchoalveolar lavage fluid samples are shown in Fig. 3. LDH is an intracellular enzyme and its presence in bronchoalveolar lavage fluid 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 absence of ozone exposure, a slight significant increase in bronchoalveolar lavage fluid LDH activity was observed in animals that had been treated with thyroid hormone compared to control animals (Fig. 3, top). Following ozone exposure, LDH activities and albumin levels in bronchoalveolar lavage fluid samples from control rats were only slightly elevated at the highest ozone concentrations studied (2.5–3 ppm, 3-h exposure ending 18 h before the time of study). In contrast, pulmonary damage in animals that had been treated with thyroid hormone was much greater following ozone exposure and, in these animals, LDH activities and albumin levels in bronchoalveolar lavage fluid were markedly increased after exposure to ozone concentrations of 1.5–3 ppm.

The effect of thyroid hormone treatment on the number of polymorphonuclear leukocytes harvested by bronchoalveolar lavage was also examined in this experiment. Polymorphonuclear leukocytes are not normally found in bronchoalveolar areas, but the numbers of these cells that can be harvested by bronchoalveolar lavage increase during acute lung inflammation. Thyroid hormone treatment was associated with marked increases in the number of polymorphonuclear leukocytes harvested by bronchoalveolar lavage from rats exposed to ozone compared to the number harvested from vehicle-treated controls (Fig. 4). The pattern of this response was similar to that

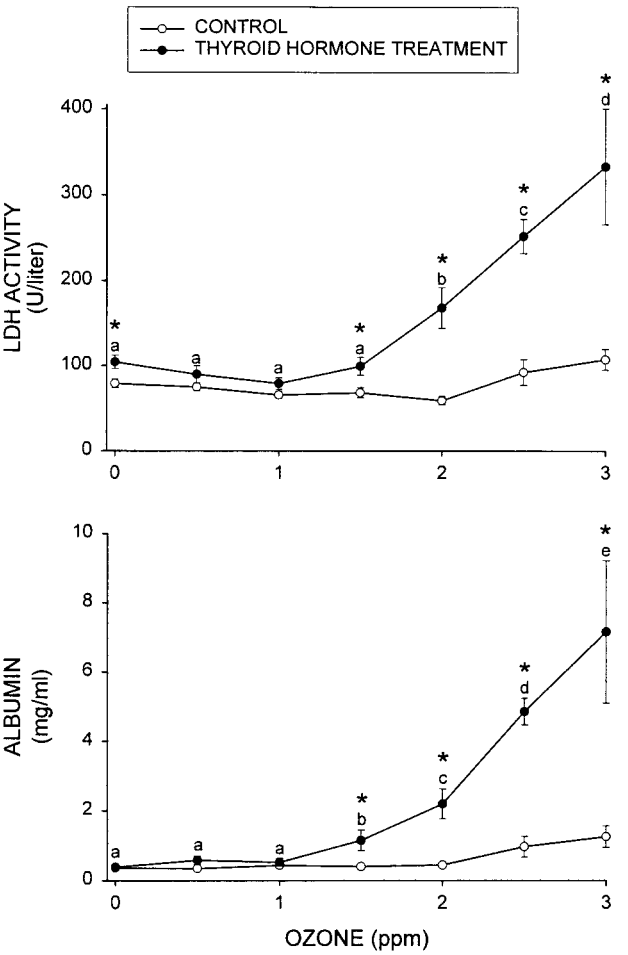

FIG. 3. Effect of thyroid hormone treatment on LDH activity (top) and albumin levels (bottom) in bronchoalveolar lavage fluid following exposure to varying doses of ozone. Animals were treated with thyroid hormone (1 mg thyroxine/kg body wt sc daily for 7 days) or vehicle injections (control) and then exposed to air or ozone by inhalation as described under Methods. Bronchoalveolar lavage fluid samples were obtained 18 h after the inhalation exposures. LDH activities and albumin levels in initial acellular bronchoalveolar lavage fluid samples were analyzed using commercially available reagents. Values are the means ± SE for four to six determinations in each group, except for the group receiving thyroid hormone treatment and 3 ppm ozone ($N = 2$). In this experiment, some of the hormone-treated rats exposed to 3 ppm ozone died overnight. $*p \leq 0.05$, hormone-treated vs control rats at a given ozone exposure. For the hormone-treated group, ozone levels with different letters are different from each other, $p \leq 0.05$.

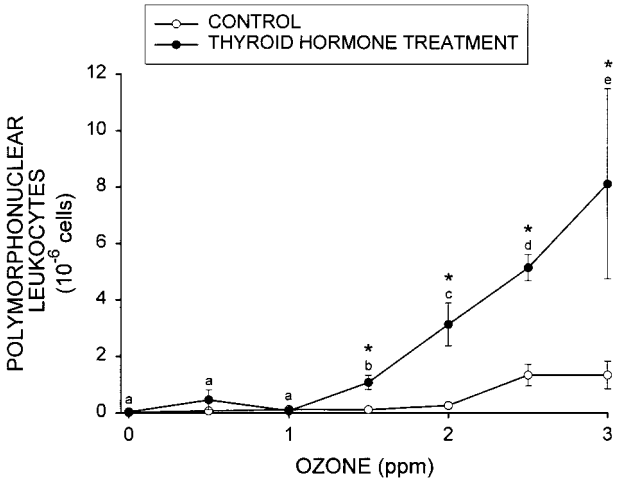

FIG. 4. Effect of thyroid hormone treatment on the number of polymorphonuclear leukocytes harvested by bronchoalveolar lavage following exposure to varying doses of ozone. Animals were treated with thyroid hormone (1 mg thyroxine/kg body wt sc daily for 7 days) or vehicle injections (control) and then exposed to air or ozone by inhalation as described under Methods. Bronchoalveolar lavagable cells were harvested 18 h after the inhalation exposures. The numbers of total cells that were harvested by bronchoalveolar lavage were determined using an electron cell counter equipped with a cell-sizing attachment. Values obtained from differential analysis of the percentages of alveolar macrophages and leukocytes present in the cell populations were used to then calculate the number of polymorphonuclear leukocytes. Values are the means ± SE for four to six determinations in each group, except for the group receiving thyroid hormone treatment and 3 ppm ozone ($N = 2$). In this experiment, some of the hormone-treated rats exposed to 3 ppm ozone died overnight. *$p \leq 0.05$, hormone-treated vs control rats at a given ozone exposure. For the hormone-treated group, ozone levels with different letters are different from each other, $p \leq 0.05$.

observed for LDH activity and albumin levels, as described above. In contrast, numbers of alveolar macrophages were not significantly altered by thyroid hormone treatment or ozone relative to the number obtained from control rats ($12.8 \pm 2.4 \times 10^6$ alveolar macrophages; mean ± SE).

Collectively, these results demonstrate that greater pulmonary epithelial barrier disruption and increased numbers of lung inflammatory cells occur following exposure of hyperthyroid rats to relatively moderate doses of ozone.

Effect of treatment with varying doses of thyroid hormone on lung responses to ozone exposure. In the previous experiments, the dose of thyroid hormone that was administered resulted in marked elevations in circulating thyroxine levels. We next gave varying doses of thyroid hormones in order to determine how more modest alterations in circulating thyroid hormone levels might affect pulmonary responses to ozone. The effect of treatment with thyroid hormone at doses ranging from 0.1 to 1 mg thyroxine/kg BW (sc daily for 7 days) on circulating thyroxine levels is shown in Fig. 5. Dose-related increases in circulating thyroxine levels were observed, ranging from an approximately twofold increase for the lowest amount of thyroid hormone given to an approximately eightfold increase for the highest amount given, relative to values in vehicle-injected animals. It should be noted that the circulating thyroxine levels presented in Fig. 5 were measured in rats that had been exposed to ozone (2 ppm, 3-h exposure ending 18 h before the time of study). However, based upon results presented from initial experiments, this exposure and sampling regime appears to have no effect on circulating thyroxine levels.

The effect of the different thyroid hormone treatment doses on LDH activities and albumin levels in bronchoalveolar lavage fluid samples following ozone exposure (2 ppm, 3-h exposure ending 18 h before the time of study) are shown in Fig. 6. Pulmonary damage, as assessed by elevations in bronchoalveolar lavage fluid LDH activity, and albumin levels, was greater in all animals that had been treated with thyroid hormones compared to animals that received only vehicle injections. In addition, the number of polymorphonuclear leukocytes harvested by bronchoalveolar lavage was increased in all thyroid hormone-treated rats (Fig. 7). Alveolar macrophage numbers were not affected by thyroid hormone treatment (data not presented).

These results indicate that even modest elevations in circulating thyroxine levels are associated with greater pulmonary damage and inflammatory responses to ozone.

Effect of thyroid hormone treatment on breathing rate, tidal volume, minute ventilation, and the inhaled dose of ozone. One mechanism by which thyroid hormone treatment could result in more severe pulmonary reactions to ozone might be by

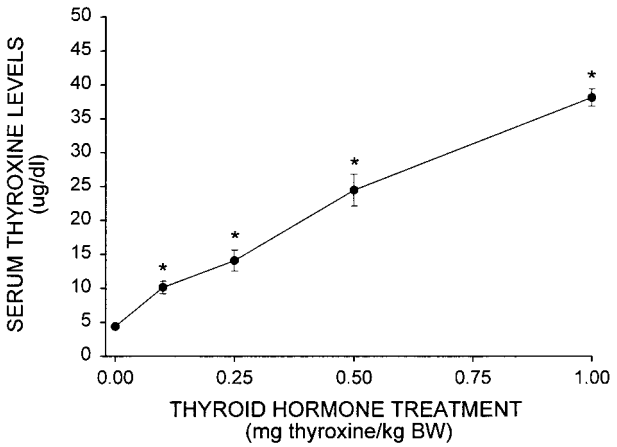

FIG. 5. Effect of treatment with varying doses of thyroid hormone on circulating thyroxine levels. Animals were treated with varying doses of thyroid hormone (thyroxine sc daily for 7 days) or vehicle injections (control) and then exposed to ozone (2 ppm; 3 h) by inhalation as described under Methods. Blood samples were collected 18 h after the end of the inhalation exposures. Blood was obtained by venipuncture and the serum was separated for analyses. Serum thyroxine levels were then measured by radioimmunoassay. Values are the means ± SE for five or six determinations in each group, except for the group receiving 1 mg/kg thyroxine and ozone ($N = 3$). In this latter group, some of the rats died overnight. In this experiment, serum thyroxine levels in control rats not exposed to ozone were 4.1 ± 0.3 μg/dl ($N = 6$). *$p \leq 0.05$ vs 0 mg thyroxine/kg body wt group.

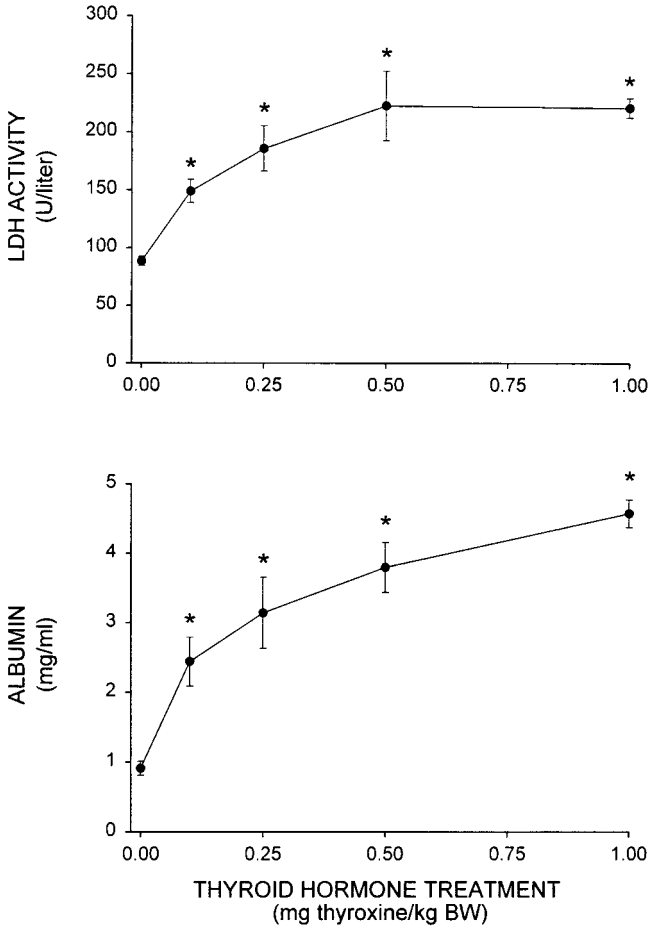

FIG. 6. Effect of treatment with varying doses of thyroid hormone on LDH activity (top) and albumin levels (bottom) in bronchoalveolar lavage fluid following ozone exposure. Animals were treated with varying doses of thyroid hormone (thyroxine sc daily for 7 days) or vehicle injections (control) and then exposed to air or ozone (2 ppm; 3 h) by inhalation as described under Methods. Bronchoalveolar lavage fluid samples were obtained 18 h after the inhalation exposures. LDH activities and albumin levels in initial acellular bronchoalveolar lavage fluid samples were analyzed using commercially available reagents. Values are the means ± SE for six determinations in each group, except for the group receiving 1 mg/kg thyroxine and ozone ($N = 3$). In this latter group, some of the rats died overnight. In this experiment, LDH activities and albumin levels in initial acellular bronchoalveolar lavage fluid samples from control rats not exposed to ozone were 77 ± 10 U/L and 0.25 ± 0.03 mg/ml, respectively ($N = 6$). *$p \leq 0.05$ vs 0 mg thyroxine/kg body wt group.

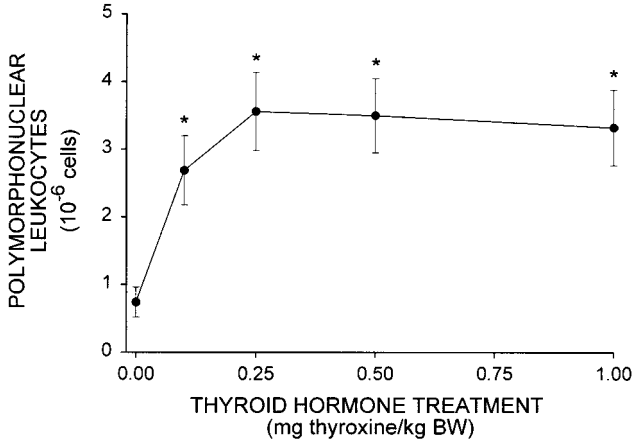

FIG. 7. Effect of treatment with varying doses of thyroid hormone on the number of polymorphonuclear leukocytes harvested by bronchoalveolar lavage following ozone exposure. Animals were treated with varying doses of thyroid hormone (thyroxine sc daily for 7 days) or vehicle injections (control) and then exposed to ozone (2 ppm; 3 h) by inhalation as described under Methods. Cells were harvested from the lung by bronchoalveolar lavage 18 h after the inhalation exposures. The total numbers of cells that were harvested were determined using an electron cell counter equipped with a cell-sizing attachment. Subsequently, values obtained from differential analysis of the percentages of alveolar macrophages and leukocytes present in the cell populations were used to calculate the number of polymorphonuclear leukocytes. Values are the means ± SE for five or six determinations in each group, except for the group receiving 1.0 mg/kg thyroxine and ozone ($N = 3$). In this latter group, some of the rats died overnight. In this experiment, no polymorphonuclear leukocytes were detected in the number counted from control rats not exposed to ozone ($N = 6$). *$p \leq 0.05$ vs 0 mg thyroxine/kg body wt group.

altering the inhaled dose of ozone. In order to assess this possibility, breathing rate and tidal volume were measured and these values were then used to calculate minute ventilation. These measurements were made on rats that had received thyroid hormone treatment (1 mg thyroxine/kg BW sc daily for 7 days) or vehicle injections and the measurements were made prior to exposure to varying doses of ozone. The results for breathing rate, tidal volume, and minute ventilation for the control or thyroid hormone-treated rats are shown in Table 1. Thyroid hormone treatment was associated with an increase in tidal volume and, as a consequence, minute ventilation was increased in these animals relative to values of vehicle-treated control rats.

We then determined how changes in minute ventilation might be related to changes in the total inhaled dose of ozone. To accomplish this, the inhaled dose of ozone was estimated for the groups of vehicle or thyroid hormone-treated animals exposed to varying concentrations of ozone. These results were then plotted against the values obtained for bronchoalveolar

TABLE 1
Effect of Thyroid Hormone Treatment on Breathing Rate, Tidal Volume, and Minute Ventilation

Measurement	Control	Thyroid hormone treatment
Breathing rate (breaths/min)	203 ± 6	198 ± 5
Tidal volume (ml/breath)	1.73 ± 0.09	2.09 ± 0.12*
Minute ventilation (ml/min)	349 ± 18	408 ± 19*

Note. Animals were treated with thyroid hormone (1 mg/kg body wt sc daily for 7 days) or vehicle injections (control) as described under Methods. Breathing rates and tidal volumes were measured and these values were then used to calculate minute ventilation. These measurements were made just before exposure to air or varying doses of ozone. Values are the means ± SE for 19 or 20 determinations in each group.

* $p \leq 0.05$ vs control.

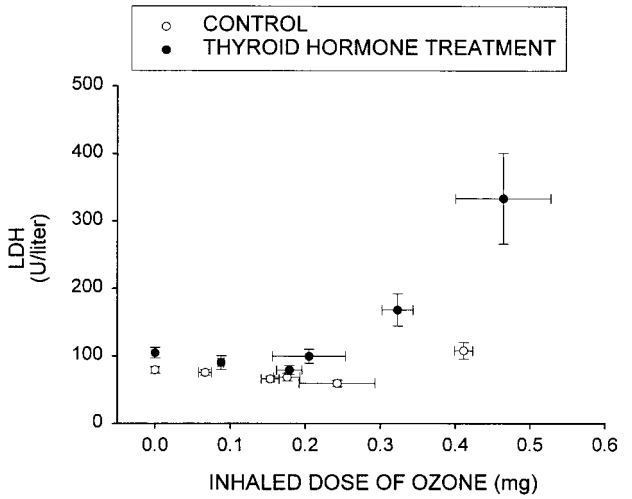

FIG. 8. Relationship between the inhaled dose of ozone and LDH activity in bronchoalveolar lavage fluid from thyroid hormone-treated and control rats. The total inhaled dose of ozone was estimated for animals that had been treated with thyroid hormone (1 mg thyroxine/kg body wt sc daily for 7 days) or vehicle injections (control) and that were then exposed to air or varying amounts of ozone as described under Methods. The values shown are the means ± SE for three or four determinations in each group, except for the groups receiving 1.5 ppm ozone and the control group receiving 3 ppm ozone ($N = 2$).

lavage fluid LDH activities (Fig. 3) and are presented in Fig. 8. As can be seen, LDH activity was still increased in thyroid hormone-treated rats relative to vehicle-treated controls after normalization for the total inhaled dose. Similar results were observed for bronchoalveolar lavage fluid albumin levels and numbers of polymorphonuclear leukocyte harvested by bronchoalveolar lavage (data not shown). These results suggest that the increased pulmonary damage and inflammatory response that is observed in thyroid hormone-treated rats following ozone exposure is not primarily a consequence of the delivery of greater amounts of ozone to the lung.

Relationship between whole-body metabolic rate and pulmonary responses to ozone exposure. As noted above, thyroid hormone treatment was associated with increases in whole-body metabolic rate. In order to evaluate possible relationships between whole-body metabolic rate and pulmonary responses to ozone exposure, metabolic rate measurements were made on rats that had received varying doses of thyroid hormones (0.1 to 1 mg thyroxine/kg BW sc daily for 7 days) or vehicle injections. These measurements were made prior to exposure to ozone. These results were then plotted against the values obtained for bronchoalveolar lavage fluid LDH activities after ozone exposure and are presented in Fig. 9. Linear regression analysis indicates that only 28% of the variance in LDH activities can be attributed to differences in metabolic rate. Similar estimates for bronchoalveolar lavage fluid albumin levels and numbers of polymorphonuclear leukocytes harvested by bronchoalveolar lavage were 53 and 23%, respectively. These results suggest that the increased pulmonary damage and inflammatory responses that are observed in thyroid hormone-treated rats following ozone exposure are not solely linked to changes in whole-body metabolic rate.

DISCUSSION

This study demonstrates that hyperthyroidism substantially increases the risk of ozone-induced lung toxicity. This was evidenced by increases in lung cell damage, the permeability of the alveolar–capillary barrier, and the number of polymorphonuclear leukocytes in bronchoalveolar areas in thyroid hormone-treated rats relative to that in euthyroid controls following the inhalation of ozone. In these experiments, we also explored dose–response relationships between a hyperthyroid state and ozone-induced lung toxicity. We observed that even a twofold rise in circulating thyroid hormone levels was associated with enhanced pulmonary toxicity to the short-term inhalation of 2 ppm ozone using the rat as an animal model. This finding is of potential significance in evaluating the overall relevance of this phenomenon. Two- to threefold increases in circulating thyroid hormone levels are routinely observed in hyperthyroid humans (Kung *et al.*, 1992; Philippou *et al.*, 1992). In addition, the biologic effects of a short-term exposure to 2 ppm ozone in rats appear to be relatively equivalent to exposure levels of 0.4 ppm ozone in exercising human subjects (Hatch *et al.*, 1994). These observations suggest that the susceptibility of the lung to ozone toxicity may be significantly influenced by individual thyroid hormone status.

Our results provide some pathophysiological basis for the

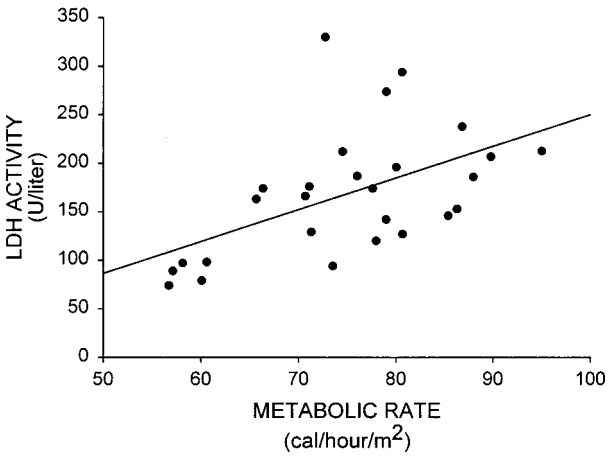

FIG. 9. Relationship between whole-body metabolic rate and LDH activity in bronchoalveolar lavage fluid from thyroid hormone-treated and control rats. Animals were treated with varying doses of thyroid hormone (thyroxine sc daily for 7 days) or vehicle injections (control). Metabolic rates were determined prior to ozone exposure (2 ppm; 3 h) as described under Methods. LDH activities in bronchoalveolar lavage fluid samples were measured on the same animals 18 h after the end of the inhalation exposure. The line depicts the best-fit relationship between these two variables using linear regression analysis.

observation of increased mortality in groups of mice that had been treated with thyroid hormone preparations prior to ozone exposure (Fairchild and Graham, 1963). In fact, we noted that some rats that had received combinations of the highest doses of thyroid hormone and ozone died during the night following the ozone exposure period and is the reason for the low number of animals in some of the experimental groups in the present study. Our findings concerning thyroid hormone effects on ozone-induced pulmonary toxicity and edema also complement those of Sen and colleagues (1993). In that study, an isolated lung perfusion system was used. They observed that pulmonary vascular perfusion pressure was significantly increased in lungs from rats that had been previously treated with thyroid hormone and exposed *in vitro* to 1 ppm ozone for 24 h relative to that of lungs from ozone-exposed controls and was associated with visual evidence of pulmonary edema.

In the present study, we investigated two mechanisms whereby thyroid hormone treatment might have resulted in more severe pulmonary reactions to ozone. Specifically, we assessed whether alterations in either whole-body metabolic rate or the total inhaled dose of this gas could account for the observed increases in ozone-induced lung toxicity following thyroid hormone treatment. It is well known that thyroid hormones exert a calorigenic effect and that elevated circulating thyroid hormone levels are associated with increases in whole-body metabolic rate in mammalian species, including the rat (Gemmill and Browning, 1965). In the present study, possible relationships between changes in whole-body metabolic rate induced by thyroid hormone treatment and the consequent pulmonary effects of ozone were explored using regression analyses. While our results suggest that a proportion of the pulmonary effects of ozone might be related to alterations in whole-body metabolic rate, it is evident that this phenomenon cannot fully account for the increased pulmonary damage and inflammatory response to ozone following thyroid hormone treatment. These findings are similar to those of Fairchild and Graham (1963), who observed that the induction of a hypermetabolic state per se using a metabolic stimulator, 2,4-dinitrophenol, was not associated with an increase in ozone-associated mortality.

We also investigated whether alterations in ventilation may have occurred in a hyperthyroid state and, as a consequence, resulted in increases in the total inhaled dose of ozone. A tendency for tidal volume and minute ventilation to be elevated following thyroid hormone treatment in the rat has been noted by other researchers (Ianuzzo *et al.*, 1984). In our study, we found that treatment with the highest dose regime of thyroid hormone (1 mg thyroxine/kg BW sc daily for 7 days) was associated with significant increases in both tidal volume and minute ventilation. However, our results indicate that indices of ozone-induced lung toxicity were still increased in thyroid hormone-treated groups even after normalization for the total inhaled dose of ozone. These observations suggest that the increased pulmonary damage and inflammatory response that are observed following ozone exposure in thyroid hormone-treated rats cannot be explained solely as a consequence of the delivery of greater amounts of ozone to the lung. It should be noted that the ventilation measurements used in our evaluations were made just prior to exposure to ozone. It has been reported that significant decreases in minute ventilation in normal rats can occur during ozone exposure (Mautz and Bufalino, 1989; Wiester *et al.*, 1987). In order to assess how alterations in ventilation during the course of exposure might impact the conclusions drawn in our study, ventilation measurements were also made at the end of the exposure period. These values were then used to provide separate estimates of the inhaled dose of ozone for both the control and thyroid hormone-treated groups of rats. Results from these estimates also suggest that the increase in ozone-induced pulmonary toxicity in thyroid hormone-treated rats was not caused by the delivery of greater amounts of ozone to the lung (unpublished observations). It should be noted that the ventilation measurements in the present study do not provide information concerning changes in specific respiratory patterns that might have occurred in hyperthyroid or control rats during the period of ozone exposure. In this regard, it has been shown that, at equivalent inspired doses of ozone, a rapid, shallow breathing pattern during the exposure period is associated with less lung damage than that seen with a slow, deep breathing pattern (Joad *et al.*, 2000). In addition, our measurements do not provide information concerning possible differences in the pulmonary distribution of the inhaled ozone between hyperthyroid and control rats. It is known significant site-specific differences in acute epithelial injury can be associated with exposure to ozone (Plopper *et al.*, 1998). Exploration of both ventilatory patterns during ozone exposure and the specific pattern of distribution of ozone within the lung in hyperthyroid animals would provide important additional information.

It is possible that direct cellular effects of thyroid hormones may underlie the observed potentiation of ozone-induced pulmonary toxicity. For instance, thyroxine has been shown to substantially enhance the osmotic fragility of erythrocytes exposed to ozone *in vitro* (Wong and Hochstein, 1981). This was attributed to modifications of membrane proteins as a result of thyroid hormone deiodination within the erythrocyte membrane in the presence of ozone. If such effects also occur at lung cells, then this could contribute to the increased pulmonary damage that is seen following ozone in hyperthyroid animals. In addition, thyroxine has been reported to directly stimulate the production of superoxide anion by human alveolar neutrophils and macrophages (Nishizawa *et al.*, 1998). Such prooxidant effects of thyroid hormones at these, or other, specific lung cell types could also contribute to the amplification of ozone-induced lung toxicity. Investigation of these possible mechanisms warrants attention in future studies.

It is of interest to note that thyroid hormone status also appears to influence the susceptibility of animals to prolonged exposure to 100% oxygen. For instance, thyroid hormone

treatment was associated with markedly decreased survival rates of rats exposed to high oxygen levels relative to those of exposed controls (Grossman and Penrod, 1949; Smith *et al.*, 1960; Yam and Roberts, 1979). The lung appears to be a primarily target organ in that gross pathological examination at autopsy revealed more severe pulmonary damage and pulmonary edema, as measured by lung wet weight to body weight ratios and the amount of pleural fluid, in thyroid-treated animals. The finding that thyroid hormone treatment increases both ozone and oxygen-induced lung toxicities suggests that pulmonary sensitivity to oxidants, in general, may be influenced by thyroid hormone status. If this is the case, then pulmonary damage from oxidant stressors derived from diverse sources, such as drugs or inhaled particulates, and surgical procedures, e.g., cardiopulmonary bypass and lung transplantation, may also be negatively influenced by hyperthyroidism.

ACKNOWLEDGMENTS

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

REFERENCES

Devlin, R. B., McDonnell, W. F., Mann, R., Becker, S., House, D. E., Schreinemachers, D., and Koren, H. S. (1991). Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am. J. Respir. Cell Mol. Biol.* **4,** 72–81.

Evans, E. S., Simpson, M. E., and Evans, H. M. (1958). The role of growth hormone in calorigenesis and thyroid function. *Endocrinology* **63,** 836–852.

Fairchild, E. J., and Graham, S. L. (1963). Thyroid influence on the toxicity of respiratory irritant gases, ozone and nitrogen dioxide. *J. Pharmacol. Exp. Ther.* **139,** 177–184.

Fukase, O., Watanabe, H., and Isomura, K. (1978). Effects of exercise on mice exposed to ozone. *Arch. Environ. Health* **33,** 198–201.

Gemmill, C. L., and Browning, K. M. (1965). The metabolism, pulse rate and body temperature of normal, thyroidectomized and hypermetabolic rats. *Lab. Anim. Care* **15,** 329–335.

Grossman, M. S., and Penrod, K. E. (1949). The thyroid and high oxygen poisoning in rats. *Am. J. Physiol.* **156,** 182–184.

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.

Hatch, G. E., Slade, R., Stead, A. G., and Graham, J. A. (1986). Species comparison of acute inhalation toxicity of ozone and phosgene. *J. Toxicol. Environ. Health* **19,** 43–53.

Ianuzzo, C. D., Chen, V., O'Brien, P., and Keens, T. G. (1984). Effect of experimental dysthyroidism on the enzymatic character of the diaphragm. *J. Appl. Physiol.* **56,** 117–121.

Jaffe, L. S. (1967). The biological effects of ozone on man and animals. *Am. Ind. Hyg. Assoc. J.* **28,** 267–277.

Joad, J. P., Bric, J. M., Weir, A. J., Putney, L., Hyde, D. M., Postlethwait, E. M., and Plopper, C. G. (2000). Effect of respiratory pattern on ozone injury to the airways of isolated rat lungs. *Toxicol. Appl. Pharmacol.* **169,** 26–32.

Kleeberger, S. R., Levitt, R. C., Zhang, L. Y., Longphre, M., Harkema, J., Jedlicka, A., Eleff, S. M., DiSilvestre, D., and Holroyd, K. J. (1997). Linkage analysis of susceptibility to ozone-induced lung inflammation in inbred mice. *Nat. Genet.* **17,** 475–478.

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.

Kung, A. W. C., Hui, W. M., and Ng, E. S. K. (1992). Serum and plasma epidermal growth factor in thyroid disorders. *Acta Endocrinol.* **127,** 52–57.

Mautz, W. J., and Bufalino, C. (1989). Breathing pattern and metabolic rate responses of rats exposed to ozone. *Respir. Physiol.* **76,** 69–78.

Menzel, D. B. (1979). Nutritional needs in environmental intoxication: Vitamin E and air pollution, an example. *Environ. Health Perspect.* **29,** 105–114.

Nambu, Z., and Yokoyama, E. (1981). The effect of age on the ozone-induced pulmonary edema and tolerance in rats. *Jpn. J. Ind. Health* **23,** 146–150.

Nishizawa, Y., Fushiki, S., Amakata, Y., and Nishizawa, Y. (1998). Thyroxine-induced production of superoxide anion by human alveolar neutrophils and macrophages: A possible mechanism for the exacerbation of bronchial asthma with the development of hyperthyroidism. *In Vivo* **12,** 253–258.

Philippou, G., Koutras, D. A., Piperingos, G., Souvatzoglou, A., and Moulopoulos, S. D. (1992). The effect of iodide on serum thyroid hormone levels in normal persons, in hyperthyroid patients, and in hypothyroid patients on thyroxine replacement. *Clin. Endocrinol.* **36,** 573–578.

Plopper, C. G., Hatch, G. E., Wong, V., Duan, X., Weir, A. J., Tarkington, B. K., Devlin, R. B., Becker, S., and Buckpitt, A. R. (1998). Relationship of inhaled ozone concentration to acute tracheobronchial epithelial injury, site-specific ozone dose, and glutathione depletion in rhesus monkeys. *Am. J. Respir. Cell Mol. Biol.* **19,** 387–399.

Prows, D. R., Daly, M. J., Shertzer, H. G., and Leikauf, G. D. (1999). Ozone-induced acute lung injury: Genetic analysis of F_2 mice generated from A/J and C57BL/6J strains. *Am. J. Physiol.* **277,** L372–L380.

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.

Sen, S., Dulchavsky, S. A., and Dutta, S. (1993). Effects of triiodothyronine (T3) supplementation upon ozone-induced lung injury. *Free Radical Res. Commun.* **18,** 299–308.

Smith, C. W., Bean, J. W., and Bauer, R. (1960). Thyroid influence in reactions to O_2 at atmospheric pressure. *Am. J. Physiol.* **199,** 883–888.

Wiester, M. J., Williams, T. B., King, M. E., Menache, M. G., and Miller, F. J. (1987). Ozone uptake in awake Sprague–Dawley rats. *Toxicol. Appl. Pharmacol.* **89,** 429–437.

Wong, D. S., and Hochstein, P. (1981). The potentiation of ozone toxicity by thyroxine. *Res. Commun. Chem. Pathol. Pharmacol.* **31,** 483–492.

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