Available online at www.sciencedirect.com
SCIENCE DIRECT
Toxicology 225 (2006) 119–127
TOXICOLOGY
www.elsevier.com/locate/toxicol

Increased susceptibility of the lungs of hyperthyroid rats to oxidant injury: Specificity of effects

L.J. Huffman [a,b], C.M. Beighley [a], D.G. Frazer [a,b], W.G. McKinney [a], D.W. Porter [a,b,*]

[a] *Health Effects Laboratory Division, National Institute for Occupational Safety and Health, M/S 2015, 1095 Willowdale Road, Morgantown, WV 26505, United States*
[b] *Department of Physiology and Pharmacology, West Virginia University School of Medicine, Morgantown, WV 26506, United States*

Received 8 July 2005; received in revised form 28 April 2006; accepted 13 May 2006
Available online 20 May 2006

Abstract

Results from previous studies indicate that hyperthyroidism increases the risk of ozone-induced lung toxicity. This observation raised the possibility that pulmonary damage from other oxidant substances might be greater in a hyperthyroid state. To address this hypothesis, pulmonary responses to crystalline silica, a particulate with oxidant properties, were evaluated in normal or hyperthyroid adult male rats. To induce a hyperthyroid condition, time-release pellets containing thyroxine were implanted subcutaneously; control rats received placebo pellets. After 7 days, the animals were exposed to saline or silica (0.1 mg/100 g BW or 1.0 mg/100 g BW) by intratracheal instillation. Following silica treatment, there was a dose-related increase in bronchoalveolar lavage (BAL) albumin levels and neutrophil numbers. However, the effects of silica were similar in both normal and hyperthyroid rats. These findings were confirmed and contrasted with those regarding ozone (1 ppm, 4 h inhalation) in a subsequent experiment. The results indicated that, although exposure to either ozone or silica resulted in increases in BAL albumin levels and neutrophil numbers, only responses to ozone were enhanced in hyperthyroid rats. These findings suggest that specificity exists in regards to the modulation of oxidant-induced lung damage and inflammation by thyroid hormones.
Published by Elsevier Ireland Ltd.

Keywords: Silica; Ozone; Thyroid hormone; Rat

1. Introduction

Oxidant-induced lung damage can result from exposure of the lung to a number of different substances. For example, exposure to either gases or particulates can result in oxidant lung injury. Ozone is a well-characterized example of an oxidant gas which can cause pulmonary damage in humans, rats, and other animal models (Hatch et al., 1986, 1994). On the other hand, crystalline silica is an example of a particulate which can have pro-oxidant pulmonary effects (Fubini and Hubbard, 2003).

Although oxidants differ in substance, acute pulmonary responses to these materials are generally similar. Specifically, elevations in both bronchoalveolar lavage (BAL) protein levels and neutrophil numbers are observed within the first 24 h period following exposure to either ozone or silica (Bhalla and Gupta, 2000; Blackford et al., 1997; Hatch et al., 1994; Zeidler et al., 2004). This finding suggests that there may be common pathways involved in the initial pulmonary responses to different oxidants. It also raises the possibility that factors affecting the susceptibility of the lung to dam-

* Corresponding author. Tel.: +1 304 285 6320; fax: +1 304 285 5938.
E-mail address: DPorter@cdc.gov (D.W. Porter).

0300-483X/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.tox.2006.05.008

age from one type of oxidant may influence pulmonary responses to other oxidants.

We have previously shown that the risk of ozone-induced lung toxicity is much greater in a hyperthyroid state (Huffman et al., 2001). At 18 h following ozone exposure, hyperthyroid rats had 3- to 6-fold increases in BAL albumin levels and neutrophil numbers compared to values observed in ozone-exposed control rats. Thyroid hormone status also appears to influence the susceptibility of animals to other oxidant gas exposures. For example, thyroid hormone treatment has been associated with more severe pulmonary damage and decreased survival rates of rodents exposed to 100% oxygen relative to those of normal exposed animals (Grossman and Penrod, 1949; Smith et al., 1960; Yam and Roberts, 1979). It has also been demonstrated that the consequences of inhaling nitrogen dioxide are more severe in a hyperthyroid condition (Fairchild and Graham, 1963). Collectively, these results indicate that elevations in thyroid hormone levels generally increase the pulmonary toxicity of oxidant gases.

The finding that the pulmonary toxicity of oxidant gases is greater in a hyperthyroid state suggests that elevated thyroid hormone levels might enhance lung responses to other materials that induce oxidant stress. One objective of the present study was to test this hypothesis. Specifically, pulmonary responses to crystalline silica were evaluated in control or hyperthyroid rats. The results indicate that silica-induced lung damage and inflammation were similar in both a normal and hyperthyroid state. Because of this result, a second objective of this study was to explore factors which might explain why pulmonary responses to an oxidant gas, such as ozone, but not to a particulate with oxidant properties, such as silica, are modulated by thyroid status. Specifically, we focused on how markers of alveolar type II cell and alveolar macrophage activation were affected in normal or hyperthyroid rats following exposure to these substances.

2. Methods

2.1. 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. All animal protocols were approved by the Animal Care and Use Committee of the National Institute for Occupational Safety and Health.

2.2. Subcutaneous implantation of time-release thyroxine pellets to induce hyperthyroidism

To create a hyperthyroid condition, thyroid-intact rats (214–268 g BW) were implanted subcutaneously with time-release pellets containing thyroxine (25 mg; 5 mm diameter; 21-day 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 which 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 BW; Phoenix Scientific, Inc., St. Joseph, MO) and xylazine (1 mg/100 g BW; 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.

2.3. Silica exposures

Normal or hyperthyroid rats were exposed to saline or silica by intratracheal instillation on day 7 following pellet implantation (day 1 = day of pellet implantation). The animals were initially lightly anesthetized with an intraperitoneal injection of 2 mg/100 g BW sodium methohexital (Eli Lilly Co., Indianapolis, IN). Crystalline silica (Min-U-Sil, <5 μm diameter, US Silica Corp., Berkeley Springs, WV) was then instilled intratracheally at a dose of 0.1 mg/100 g BW. Control animals were instilled with endotoxin-free saline (0.9% NaCl; Baxter HealthCare Corp., Deerfield, IL). Each rat received 0.1 ml/100 g BW of solution. The dose of silica used in this study was chosen based on the results of a preliminary study which established a silica dose response curve using silica doses of 0, 0.01, 0.025, 0.05, 0.1 and 1.00 mg silica/100 g BW (data not shown). At 24 h post-IT exposure, no significant pulmonary inflammation or damage at silica doses <0.1 mg silica/100 g BW was observed, whereas silica doses of 0.1 and 1.00 mg silica/100 g BW resulted in significant pulmonary inflammation and damage, as measured by BAL PMNs and BAL fluid albumin levels. In the current study, the 0.1 mg/100 g BW silica dose was used because this silica dose would not result in particle overload, based on silica lung burden values and overload calculations previously reported by our laboratory (Porter et al., 2001).

2.4. Ozone exposures

Normal or hyperthyroid rats were exposed to air or ozone on day 7 following pellet implantation. A whole-body inhalation exposure system was used to expose the animals to ozone (1 ppm for 4 h). The inhalation system has previously been described in detail (Huffman et al., 2001). Control rats were exposed to HEPA-filtered air for 4 h in a whole-body exposure chamber that was similar to the chamber used for ozone exposures. The animals were studied 24 h after the start of the exposures. The ozone exposure in this study (1.0 ppm, 4 h) provided a equivalent total dose to rats exposed for 2.0 ppm for 2 h, the effects of which have been previously reported by our laboratory (Huffman et al., 2001).

2.5. Collection of blood and bronchoalveolar lavage (BAL) fluid and cell samples

The rats were first anesthetized with sodium pentobarbital (>100 mg/kg, i.p.; Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA). Blood was collected from the abdominal vena cava into tubes without anticoagulant. The left renal artery was then cut. A tracheal cannula was inserted and an initial BAL was performed with 6 ml of ice-cold Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; 145 mM NaCl, 5 mM KCl, 9.4 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 5.5 mM 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 of lavage fluid was collected. The initial and subsequent lavage samples were then centrifuged ($500 \times g$, 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.

2.6. Determination of BAL cell counts and cell profiles

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 % alveolar macrophages and leukocytes present on the slides were determined using light microscopy.

2.7. 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).

2.8. Analyses of albumin levels 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 Company, St. Louis, MO).

2.9. Determination of alveolar macrophage reactive oxygen/nitrogen species [R(O/N)S] production

The production of R(O/N)S from alveolar macrophages was determined using luminol-enhanced chemiluminescence following stimulation with unopsonized zymosan as previously described (Porter et al., 2002). It should be noted that the use of unopsonized zymosan as a stimulant selectively permits evaluation of R(O/N)S production from rat alveolar macrophages since these cells, in contrast to neutrophils, respond to this stimulant without the need for opsonization (Castranova et al., 1987).

2.10. Determination of phospholipid and disaturated phosphatidylcholine (DSPC) levels in BAL fluid

Phospholipid levels in initial BAL fluid samples were determined by measuring the phosphorus present in lipid extracts of BAL fluid as previously described in detail (Porter et al., 2001). The total amount of phospholipid and % DSPC content in alveolar lavage material were determined by the method described by Miles et al. (1995).

2.11. Statistical analyses

Multiple two-way analyses of variance were conducted to determine if the factors of hormone treatment and substance exposure would result in significant differences on the measured variables. Separate analyses were performed for saline/silica exposures and air/ozone exposures. 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 and substance exposure using the Tukey–Kramer method to adjust for multiple comparisons. In all cases, two-sided tests were used with p-values ≤ 0.05 used as evidence of findings not attributable to chance.

3. Results

3.1. Serum thyroxine levels

Serum thyroxine levels in normal rats (treated with placebo pellets) or in hyperthyroid rats (treated with thyroxine pellets) are presented in Table 1. The implantation

Table 1
Circulating thyroxine levels (μg/dl) in normal or hyperthyroid rats

Treatments	Exposure			
	Saline (0.1 ml/100g BW)	Silica (0.1 mg/100 g BW)	Air	Ozone (1 ppm, 4 h)
Normal (placebo pellet)	4.2 ± 0.3[a]	3.9 ± 0.2[a]	3.8 ± 0.3[a]	3.6 ± 0.2[a]
Hyperthyroid (thyroxine pellet)	27.0 ± 1.5[b]	30.0 ± 3.2[b]	22.0 ± 2.0[b]	22.8 ± 1.9[b]

Rats were implanted subcutaneously with placebo pellets (normal) or thyroxine pellets (hyperthyroid). After 7 days, the rats were exposed to saline or silica by intratracheal instillation or air or ozone (1 ppm, 4 h). Blood samples were collected 24 h later and the serum was separated for analysis. Serum thyroxine levels were then measured by radioimmunoassay. Values are the means ± S.E. (n = 5–6 rats/group). Values with different superscripts (a and b) within an exposure group are significantly different. Significance was set at $p \leq 0.05$.

of time-release pellets containing thyroxine resulted in an approximately 7-fold 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 rats receiving hormone-containing pellets. In addition, thyroxine levels in normal or hyperthyroid rats were not changed after silica or ozone exposure compared to levels in the saline or air-exposed controls, respectively. Similar circulating levels of this thyroxine have been reported by our laboratory in a previous study, and these rats were determined to be in hyperthyroid state based on increased whole body metabolic rates (Huffman et al., 2001).

3.2. Effect of hyperthyroidism on pulmonary inflammation after exposure to silica and ozone

The numbers of neutrophils harvested by BAL from normal or hyperthyroid rats following exposure to silica or ozone was determined to investigate the effect of hyperthyroidism of pulmonary inflammation. Following silica treatment (Fig. 1A), increases in the numbers of neutrophils in bronchoalveolar spaces were observed in both normal and hyperthyroid rats when compared to saline-exposed controls. Comparison of the number of neutrophils harvested from silica-treated hyperthyroid rats to silica-exposed normal rats showed there was a decrease in BAL neutrophils obtained from hyperthyroid rats, but his decrease was not statistically significant.

For ozone-exposed rats (Fig. 1B), ozone-exposure caused a significant increase in BAL neutrophils for both normal and hyperthyroid rats. Furthermore, there was a statistically significant 9-fold increase the number of neutrophils harvested by BAL from ozone-exposed hyperthyroid rats in comparison to ozone-exposed normal rats.

3.3. Effect of hyperthyroidism on BAL fluid albumin after exposure to silica and ozone

BAL fluid albumin levels from normal or hyperthyroid rats following exposure to silica or ozone was

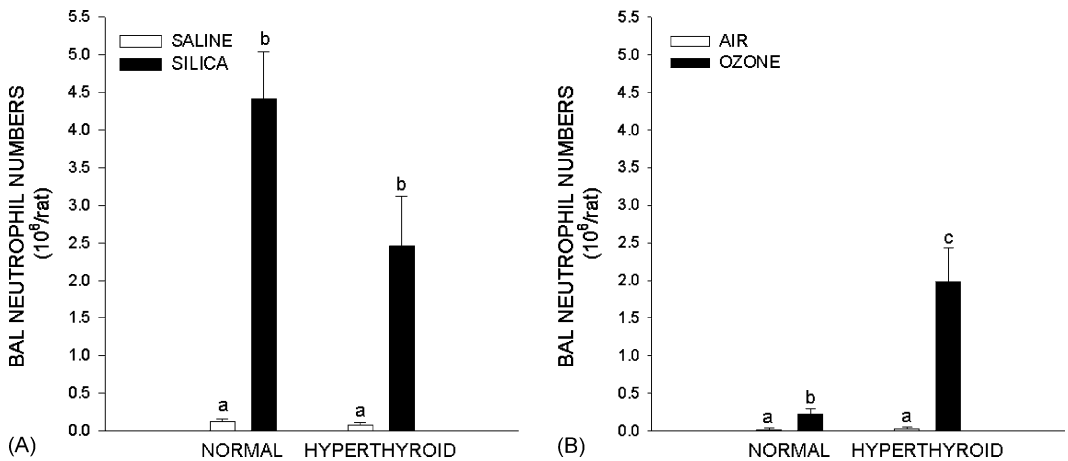

Fig. 1. BAL neutrophil numbers. (A) Normal or hyperthyroid rats were IT exposed to either saline or silica (0.1 mg/100 g BW), and BAL was performed 24 h later. (B) Normal or hyperthyroid rats were exposed to either air or ozone (1 ppm, 4 h), and BAL was performed 24 h later. BAL neutrophil numbers were determined by counting the total number of harvested cells and then using cell differential values to calculate the numbers of neutrophils in the samples. Values are the means ± S.E. (n = 6 rats/saline or silica group; n = 4–6 rats/air or ozone group). Values with different letters are significantly different. Significance was set at $p \leq 0.05$.

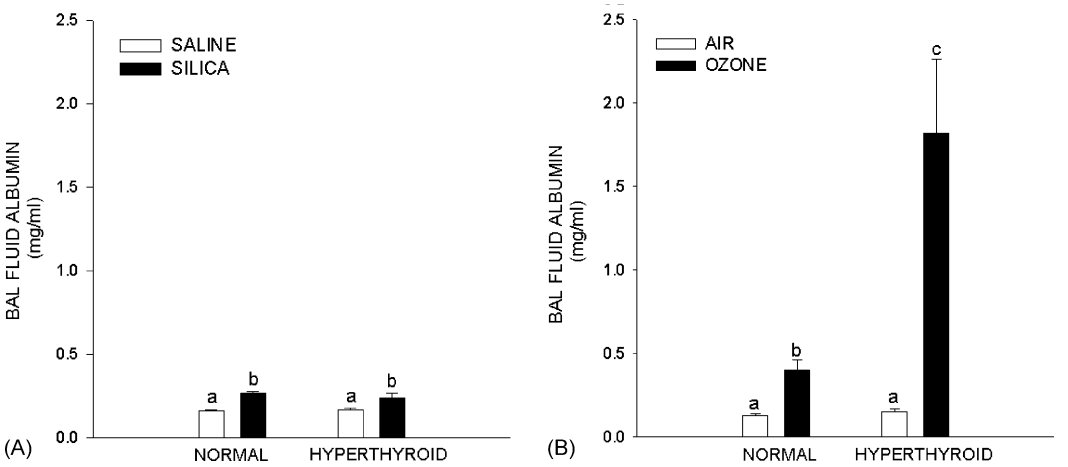

Fig. 2. BAL fluid albumin levels. (A) Normal or hyperthyroid rats were IT exposed to either saline or silica (0.1 mg/100 g BW), and BAL was performed 24 h later. (B) Normal or hyperthyroid rats were exposed to either air or ozone (1 ppm, 4 h), and BAL was performed 24 h later. Albumin levels in initial acellular BAL samples were analyzed using commercially available reagents. Values are the means ± S.E. ($n = 6$ rats/group). Values with different letters are significantly different. Significance was set at $p \leq 0.05$.

determined to investigate the effect of hyperthyroidism on integrity of the blood-pulmonary epithelial cell barrier integrity. Following silica treatment (Fig. 2A), BAL fluid albumin levels were significantly increased in both normal and hyperthyroid rats when compared to saline-exposed controls. Comparison of the BAL fluid albumin levels from silica-treated hyperthyroid rats to silica-exposed normal rats showed there was no significant difference.

For ozone-exposed rats (Fig. 2B), ozone-exposure caused a significant increase in BAL fluid albumin levels for both normal and hyperthyroid rats. Furthermore, BAL fluid albumin level in ozone-exposed hyperthyroid rats was significantly higher than that in ozone-exposed normal rats.

3.4. Effect of hyperthyroidism on chemiluminescence after exposure to silica and ozone

The degree of alveolar macrophage activation was assessed in this experiment by measuring R(O/N)S production by BAL cells following stimulation with unopsonized zymosan. R(O/N)S production was indexed using luminol-enhanced chemiluminescence. Silica exposure resulted in a significant increase in production of R(O/N)S by alveolar macrophages from both normal and hyperthyroid rats compared to that produced by alveolar macrophages from saline-treated rats (Fig. 3A). It was also found that the stimulated R(O/N)S production by alveolar macrophages from silica-treated hyperthyroid rats was significantly less than that produced by alveolar macrophages from silica-treated normal rats. In contrast, exposure to ozone was not associated with alterations in R(O/N)S production by alveolar macrophages from either normal or hyperthyroid rats (Fig. 3B).

3.5. Effect of hyperthyroidism on BAL fluid phospholipids after exposure to silica and ozone

Phospholipid levels in BAL fluid samples were measured as an index of surfactant production in this study. In comparison to saline-exposed controls, silica-exposure did not produce any significant change in phospholipid levels in either normal or hyperthyroid rats (Fig. 4A). Similarly, for rats with normal circulating thyroxine levels, comparison of air- and ozone-exposed rats also determined that ozone exposure did not alter BAL fluid phospholipids levels (Fig. 4B). However, for hyperthyroid rats, comparison of air- and ozone-exposed rats determined a significant increase in phospholipid levels (Fig. 4B).

3.6. Effect of hyperthyroidism on pulmonary surface phospholipids and the percent DSPC content

To evaluate the possibility that there was an overall increase in surfactant production by alveolar type II cells following exposure of hyperthyroid rats to ozone, pulmonary surface phospholipids and the percent DSPC content were measured in normal and hyperthyroid rats exposed to air or ozone (Table 2). The results for lung surface phospholipid levels paralleled those for phospholipid levels in the acellular BAL fluid samples

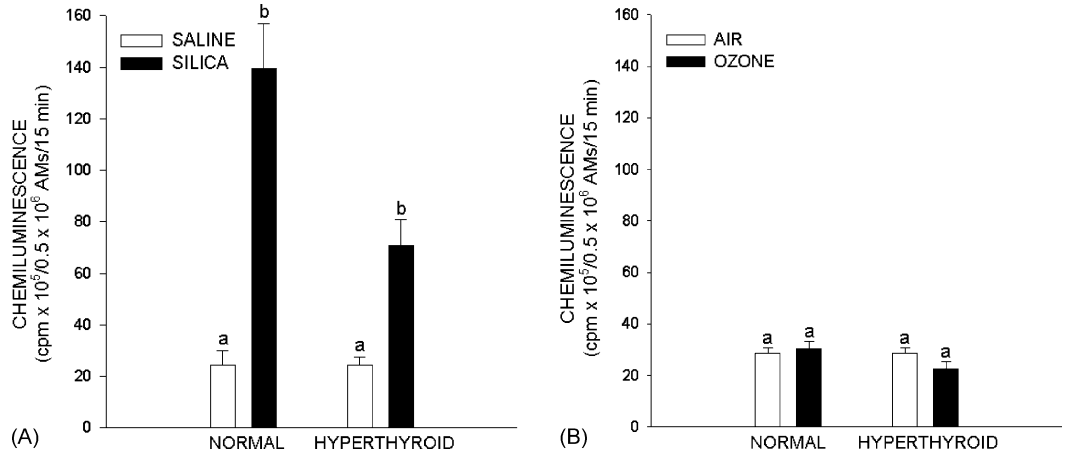

Fig. 3. R(O/N)S production as measured by chemiluminescence. (A) Normal or hyperthyroid rats were IT exposed to either saline or silica (0.1 mg/100 g BW), and BAL was performed 24 h later. (B) Normal or hyperthyroid rats were exposed to either air or ozone (1 ppm, 4 h), and BAL was performed 24 h later. R(O/N)S production was determined by luminol-enhanced chemiluminescence following stimulation of BAL cells with unopsonized zymosan. Values are the means ± S.E. ($n = 6$ rats/group). Values with different letters are significantly different. Significance was set at $p \leq 0.05$.

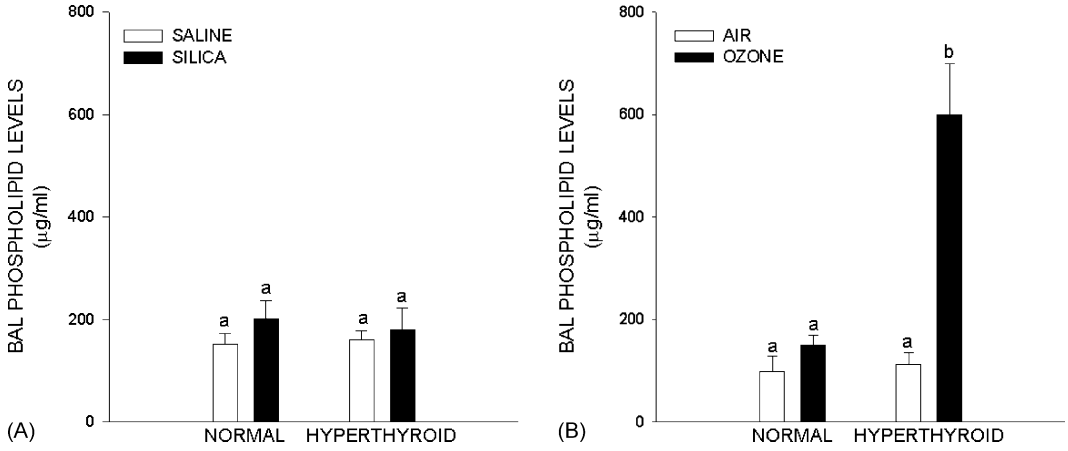

Fig. 4. BAL fluid phospholipid levels. (A) Normal or hyperthyroid rats were IT exposed to either saline or silica (0.1 mg/100 g BW), and BAL was performed 24 h later. (B) Normal or hyperthyroid rats were exposed to either air or ozone (1 ppm, 4 h), and BAL was performed 24 h later. Phospholipids were measured as the phosphorus present in lipid extracts of initial acellular BAL fluid samples. Values are the means ± S.E. ($n = 6$ rats/group). Values with different letters are significantly different. Significance was set at $p \leq 0.05$.

Table 2
Total amount of phospholipid (mg/lung) and % DSPC content in alveolar lavage material from normal or hyperthyroid rats following exposure to air or ozone

Treatments	Phospholipid (mg/lung)		DSPC (%)	
	Air	Ozone (1 ppm, 4 h)	Air	Ozone (1 ppm, 4 h)
Normal (placebo pellet)	1.60 ± 0.10^{a}	1.61 ± 0.09^{a}	46.8 ± 2.0^{a}	46.7 ± 2.0^{a}
Hyperthyroid (thyroxine pellet)	1.83 ± 0.13^{a}	3.36 ± 0.22^{b}	39.2 ± 2.5^{b}	42.0 ± 2.9^{b}

Normal or hyperthyroid rats were exposed to air or ozone. BAL was performed 24 h later. The total amount of phospholipids and the % DSPC content in alveolar lavage material were measured as described in Section 2. Values are the means ± S.E. ($n = 6$/group). Values with different superscripts (a and b) are significantly different. Significance was set at $p \leq 0.05$.

(Fig. 4B) and indicated that these levels were specifically increased in ozone-exposed hyperthyroid rats. Furthermore, it was found that DSPC represented approximately 40–45% of the phospholipid across the groups. This finding indicated that the increase in phospholipid levels in hyperthyroid rats following ozone exposure was due to increased surfactant production by alveolar type II cells.

4. Discussion

The ozone dose used in this study was selected on the basis of two major considerations. First, the ozone dose used should be relevant to human ozone exposures. A previous study used 18-labeled ozone ($^{18}O_3$) to compare both ozone dose and biological responses between humans and rats (Hatch et al., 1994). This study reported that comparison of $^{18}O_3$-exposed rats (2.0 ppm, 2 h) to $^{18}O_3$-exposed exercising humans (0.4 ppm, 2 h) had similar ozone dosages and biological responses. The total ozone dose reported by Hatch et al. was 4 ppm h (2.0 ppm × 2 h). The total ozone dose in the present study is also 4 ppm h (1.0 ppm × 4 h). Thus, the dose used in this study represents a biologically relevant human ozone dose.

Secondly, the ozone dose needed to produce a significant effect versus air-exposed controls, but this response needed to be less than maximal in order to allow hyperthyroidism-induced alterations in responses to be detected. Our laboratory has previously reported the effects of ozone doses ranging from 0.5 to 3.0 ppm ozone for 3 h (total ozone dose range = 1.5–9.0 ppm h) (Huffman et al., 2001). Based on these previously reported findings, we selected the ozone exposure of 1.0 ppm × 4 h (4 ppm h) as a dose that would meet these criteria.

The criteria to select a silica dose was similar to that applied to ozone, i.e., the silica dose used needed to produce a significant effect versus saline-exposed controls, but this response needed to be less than maximal in order to allow hyperthyroidism-induced alterations in responses to be detected. To select the appropriate dose, we conducted a preliminary study (data not shown) which established a silica dose response curve using silica doses of 0, 0.01, 0.025, 0.05, 0.1 and 1.00 mg silica/100 g BW. At 24 h post-IT exposure, BAL fluid LDH, albumin, and PMNs were determined. The data indicated that at silica doses ≤0.1 mg silica/100 g BW, no significant increase in any of these three endpoints occurred. However, silica doses of 0.1 and 1.00 mg silica/100 g BW resulted in significant increases in all three endpoints. The 0.1 mg silica/100 g BW was selected for use in the present study because it increased toxicity markers above saline controls, but did not produce a maximal effect. Furthermore, the 0.1 mg/100 g BW silica dose was used because this silica dose would not result in particle overload, based on silica lung burden values and overload calculations previously reported by our laboratory (Porter et al., 2001).

In this study, elevations in both BAL fluid albumin levels and neutrophil numbers were observed following exposure to ozone or silica. Albumin is normally confined to the intravascular space. However, albumin levels in BAL samples increase when the alveolar-capillary barrier is disrupted. Regarding neutrophils, very few of these cells are normally found in bronchoalveolar areas, but the numbers that can be harvested by BAL increase during acute lung inflammation. Collectively, these findings indicate that exposure to either ozone or silica resulted in increases in the permeability of the alveolar-capillary barrier and a pulmonary inflammatory response. However, despite the fact that both ozone and silica had these similar pulmonary effects, only responses to ozone were enhanced in hyperthyroid rats. This finding suggests that specificity exists in regards to the modulation of oxidant-induced lung damage and inflammation by thyroid hormones.

It is possible that the relative magnitude of the silica-induced lung damage and inflammation contributed to the finding that these responses were not enhanced in a hyperthyroid state. However, this appears to be unlikely. For instance, BAL fluid albumin levels in normal rats treated with silica were either lower or similar to those of ozone-treated normal animals. This finding indicates that the degree of barrier disruption was not maximal in normal rats following the doses of silica used in the present experiment. In regards to the degree of pulmonary inflammation, it was observed that the numbers of inflammatory cells harvested by BAL were greater in normal rats exposed to silica compared to ozone. However, the numbers of neutrophils harvested by BAL following the low dose of silica were 3-fold less than those harvested following the high dose of silica. This finding suggests that BAL neutrophil numbers could have increased in the hyperthyroid rats which were exposed to the lower dose of silica.

In the present experiment, we explored some factors which might explain why pulmonary responses to ozone but not silica were enhanced in a hyperthyroid state. Specifically, markers of AM and type II cell activation were examined. Regarding AM activation, we focused on the release of reactive oxygen/nitrogen species by these cells. This was accomplished by assessing luminol-amplified chemiluminescence following stimulation of BAL cells with unopsonized zymosan. The

use of unopsonized zymosan in the assay measures only AM responses, because unopsonized zymosan stimulates R(O/N)S production by AMs but not neutrophils (Castranova et al., 1987). It was found that AMs from silica-treated rats were primed to generate R(O/N)S. Specifically, the amount of chemiluminescence produced by AMs from silica-treated rats following zymosan stimulation was approximately 3- to 6-fold higher than that produced by AMs from saline-treated rats. These results are consistent with a body of evidence suggesting that R(O/N)S production by AMs play an important role in silica-induced pulmonary responses (Castranova, 1994). Of potential interest was the observation that these responses actually appeared to be down-regulated in AMs from thyroid hormone-treated rats. It has been proposed that free radicals play a role in silica-induced pulmonary inflammatory responses (Vallyathan et al., 1995). Decreases in free-radical production by AMs in thyroid hormone-treated rats exposed to silica could be a factor contributing to the decreased number of neutrophils harvested by BAL in these same animals. In contrast to findings following silica treatment, zymosan-induced chemiluminescence responses by AMs from ozone-exposed rats were similar to those of AMs from rats exposed to air. This observation is consistent with other literature suggesting that, 18–24 h after acute in vivo exposure to ozone, R(O/N)S production by AMs is generally not enhanced and may even be decreased (Esterline et al., 1989; Torres et al., 1996; Voter et al., 2001). In addition, the results indicate that a hyperthyroid state did not affect R(O/N)S production by AMs in ozone-exposed animals. Collectively, these findings suggest that AMs play a much larger role in responses to silica than to ozone 24 h after exposure. Furthermore, AM release of R(O/N)S does not appear to be a factor contributing to enhanced pulmonary responses to ozone in a hyperthyroid state.

In regards to type II cell activation, phospholipid levels in BAL fluid were measured. Increased BAL fluid phospholipid levels are considered, in part, to represent an enhanced production and secretion of surfactant proteins by alveolar type II cells (Crouch et al., 1991; Hook, 1991). We found that after silica treatment, phospholipid levels in normal or hyperthyroid rats were not different from those in the saline-treated controls. Although silica exposure can be associated with significant alveolar phospholipidosis and increased BAL phospholipid levels (Porter et al., 2001), this appears to be a time- and dose-dependent event. It has been reported that rat lung extracellular phospholipids were not elevated 3 days following the intratracheal instillation of silica at a dose of 25 mg/100 g body weight (Dethloff et al., 1987). Since we evaluated BAL phospholipids 24 h after the intratracheal administration of 0.1 mg/100 g body weight silica, it is not surprising that phospholipid levels were not elevated in the silica-treated group of rats compared to the saline-treated controls. In the present study, we also did not observe changes in BAL phospholipid levels in normal rats 24 h following ozone exposure. It has previously been reported that phospholipid pool sizes in BAL are not affected in normal rats following short-term ozone exposures (Putman et al., 1995). However, in hyperthyroid rats exposed to ozone, there was a marked increase in BAL phospholipids. Specifically, BAL phospholipid levels in hyperthyroid rats exposed to ozone were approximately 6-fold higher than those in the air-exposed rats or in normal rats exposed to ozone. The additional observation that 40–45% of the lung surface phospholipid was DSPC across the groups indicated that the observed increase in phospholipid levels in hyperthyroid rats exposed to ozone was due to increased surfactant production by alveolar type II cells. These findings suggest that alveolar type II cell activation is a significant event during initial pulmonary responses to ozone in hyperthyroid rats.

In summary, findings from the present study indicate that a hyperthyroid state is not associated with a generalized increase in pulmonary sensitivity to oxidant-induced injury. Furthermore, observations from the present study are consistent with the following hypothesis concerning thyroid hormone modulation of oxidant responses. If alveolar epithelial cells are a primary target of an oxidant (i.e., ozone), then acute pulmonary responses to the oxidant will be enhanced in a hyperthyroid state. However, if AMs are a primary target of the oxidant (i.e., silica), then pulmonary responses will not be substantially changed in a hyperthyroid state. Lastly, the findings reported here are only for one post-exposure time, and thus it cannot be ruled out that at other post-exposure times a hyperthyroid state may be associated with changes in pulmonary sensitivity to oxidant-induced injury. Further experiments will, of course, be necessary to test these hypotheses.

Acknowledgments

We thank D.J. Prugh, M. Barger, M. Donlin, A. Frazer, and L. Bowman for expert technical assistance.

References

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

Blackford Jr., J.A., Jones, W., Dey, R.D., Castranova, V., 1997. Comparison of inducible nitric oxide synthase gene expression and lung inflammation following intratracheal instillation of silica, coal, carbonyl iron, or titanium dioxide in rats. J. Toxicol. Environ. Health 51, 203–218.

Castranova, V., 1994. Generation of oxygen radicals and mechanisms of injury prevention. Environ. Health Perspect. 102 (Suppl. 10), 65–68.

Castranova, V., Lee, P., Ma, J.Y.C., Weber, K.C., Pailes, W.H., Miles, P.R., 1987. Chemiluminescence from macrophages and monocytes. In: Van Dyke, K., Castranova, V. (Eds.), Cellular Chemiluminescence. CRC Press, Boca Raton, pp. 4–19.

Crouch, E., Persson, A., Chang, D., Parghi, D., 1991. Surfactant protein D Increased accumulation in silica-induced pulmonary lipoproteinosis. Am. J. Pathol. 139, 765–776.

Dethloff, L.A., Gladen, B.C., Gilmore, L.B., Hook, G.E.R., 1987. Quantitation of cellular and extracellular constituents of the pulmonary lining in rats by using bronchoalveolar lavage effects of silica-induced pulmonary inflammation. Am. Rev. Respir. Dis. 136, 899–907.

Esterline, R.L., Bassett, D.J.P., Trush, M.A., 1989. Characterization of the oxidant generation by inflammatory cells lavaged from rat lungs following acute exposure to ozone. Toxicol. Appl. Pharmacol. 99, 229–239.

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

Fubini, B., Hubbard, A., 2003. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. Free Radical Biol. Med. 34, 1507–1516.

Grossman, M.S., 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., 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., Graham, J.A., 1986. Species comparison of acute inhalation toxicity of ozone and phosgene. J. Toxicol. Environ. Health. 19, 43–53.

Hook, G.E., 1991. Alveolar proteinosis and phospholipidoses of the lungs. Toxicol. Pathol. 19, 482–513.

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

Miles, P.R., Bowman, L., Frazer, D.G., 1995. Properties of lavage material from excised lungs ventilated at different temperatures. Respir. Physiol. 101, 99–108.

Porter, D.W., Millecchia, L., Robinson, V.A., Hubbs, A., Willard, P., Pack, D., Ramsey, D., McLaurin, J., Khan, A., Landsittel, D., Teass, A., Castranova, V., 2002. Enhanced nitric oxide and reactive oxygen species production and damage after inhalation of silica. Am. J. Physiol. Lung Cell. Mol. Physiol. 283, L485–L493.

Porter, D.W., Ramsey, D., Hubbs, A.F., Battelli, L., Ma, J., Barger, M., Landsittel, D., Robinson, V.A., McLaurin, J., Khan, A., Jones, W., Teass, A., Castranova, V., 2001. Time course of pulmonary response of rats to inhalation of crystalline silica: histological results and biochemical indices of damage, lipidosis, and fibrosis. J. Environ. Pathol. Toxicol. Oncol. 20 (Suppl. 1), 1–14.

Putman, E., Boere, A.J.F., van Bree, L., Van Golde, L.M.G., Haagsman, H.P., 1995. Pulmonary surfactant subtype metabolism is altered after short-term ozone exposure. Toxicol. Appl. Pharmacol. 134, 132–138.

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

Torres, A., Voter, K.Z., Utell, M.J., Whitin, J.C., Morrow, P.E., Frampton, M.W., 1996. Production of reactive oxygen intermediates following exposure to ozone relative contribution of alveolar macrophages. Chest 109 (Suppl. 3), 8S.

Vallyathan, V., Castranova, V., Pack, D., Leonard, S., Shumaker, J., Hubbs, A.F., Shoemaker, D.A., Ramsey, D.M., Pretty, J.R., McLaurin, J.L., Khan, A., Teass, A., 1995. Freshley fractured quartz inhalation leads to enhanced lung injury and inflammation: potential role of free radicals. Am. J. Respir. Crit. Care Med. 152, 1003–1009.

Voter, K.Z., Whitin, J.C., Torres, A., Morrow, P.E., Cox, C., Tsai, Y., Utell, M.J., Frampton, M.W., 2001. Ozone exposure and the production of reactive oxygen species by bronchoalveolar cells in humans. Inhal. Toxicol. 13, 465–483.

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

Zeidler, P.C., Hubbs, A., Battelli, L., Castranova, V., 2004. Role of inducible nitric oxide synthase-derived nitric oxide in silica-induced pulmonary inflammation and fibrosis. J. Toxicol. Environ. Health 67, 1001–1026.