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# EFFECTS OF TRIIODOTHYRONINE (T3) SUPPLEMENTATION UPON OZONE-INDUCED LUNG INJURY

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Ozone exposure results in an acute decrease in the serum levels of thyroid hormones; the physiologic sequelae of this are unclear. Whereas thyroid hormone supplementation appears to benefit pulmonary function in septic, oxyradical models of injury, thyroid hormone increases ozone toxicity. We demonstrated an increase in metabolic rate and pulmonary injury in lungs from ozone exposed, $T_3$ treated animals. This was evidenced by an increase in pulmonary weight gain, vascular perfusion pressure, and decrease in compliance in the supplemented animals. However, an increase in alkane generation, as an index of lipid peroxidation, was not seen in the ozone exposed, hormonally treated animals. This suggests that although thyroid hormone supplementation increases metabolic rate and ozone toxicity, an increased rate of lipid peroxidation plays a minimal role.

KEY WORDS: Ozone, oxidative stress, sick euthyroid syndrome, triiodothyronine, glutathione



## INTRODUCTION

Ozone, a photochemical oxidant, remains one of the three most important air pollutants worldwide.[1] Exposure to ozone results in a dose dependent respiratory compromise involving a cascade of events including activation and release of oxygen derived free radicals, oxidative stress on the glutathione defence system, epithelial cell damage by peroxidation of membrane lipid and/or protein macromolecules and, finally, breakdown of cationic homeostasis leading to increased cell volume and osmotic fragility.[2-4] If the exposure is of sufficient duration or concentration, respiratory failure and death secondary to pulmonary edema may ensue.

Acute decreases in the serum concentrations of the thyroid hormones $T_3$ and $T_4$ are noted following a variety of insults including sepsis, haemorrhagic and septic shock, and following exposure to ozone.[5-8] The physiological significance of these alterations in thyroid economy are not clear. Fairchild and Graham demonstrated a survival advantage in thyroidectomized rodents exposed to ozone or $NO_3$.[9] Animals supplemented with thyroid hormones showed increased susceptibility to oxidant damage. Conversely, Baue et al correlated mortality with the induced nadir in thyroid

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hormone in a septic model.[10] We recently corroborated this study by demonstrating an increased survival and pulmonary function in rats supplemented with $T_3$ following a septic, oxyradical challenge.[7]

The purpose of this study was to evaluate the relationship between ozone induced thyroid hormone depletion and oxidative stress mediated pulmonary dysfunction. Specifically, this study addresses: 1) the concentration and duration of ozone exposure required to produce plasma thyroid hormone depletion as well as pulmonary oedema, 2) the effect of such exposure on functional parameters and indicators of oxidative stress mediated injury in a lung perfusion model, and 3) the effect of $T_3$ supplementation on the above parameters.

## MATERIALS AND METHOD

The procedures described herein conform to the NIH Guidelines for the Care of Laboratory Animals. The role of ozone lung injury and thyroid hormonal status was evaluated in 3–4 month old male Sprague Dawley rats (Holtzman strain). The animals were acclimated to the animal care facility for one week prior to the experiment and fed rat Purina chow ad libitum.

To determine the impact of thyroid hormonal augmentation, a subgroup of animals was supplemented with $T_3$ (Cytomel, SmithKline Beecham, Philadelphia, PA) prior to experimentation. The hormone was administered via an Alzet osmotic infusion pump placed subcutaneously 6 hours prior to the exposure. The pump was cooled (25°F) prior to operative placement to allow slow onset of drug delivery at the approximate beginning of the exposure as the pump warmed in the animal. The drug was delivered at 3 ng/hr; this dosage has been previously shown to correct the stress induced decrease in free $T_3$ and maintain hormonal levels in the euthyroid range.[7]

### *Ozone Exposure*

To ensure uniform experimental parameters and ozone exposure, paired experiments were conducted. The rats with or without hormonal augmentation, were placed in parallel airtight glass chambers through which filtered room air was passed at 6 L/min. One animal was then randomly exposed to 1.0 ppm ozone by exposing the prechamber room air to ultraviolet light in a stable ozone generator (SOG-2, Ultra-Violet Products, San Gabriel, CA). The level of ozone generated was maintained by altering the exposed surface of the light source and was monitored with a Dasibi Ozone Analyzer (Model 1003-PC, Dasibi Environment Corporation, Glendale, CA). After 24 hours of room air or ozone exposure in the chambers, the animals were quickly anesthetized with intraperitoneal pentobarbital (40 mg/kg) and heparinized (1000 U/kg). A blood sample was obtained and frozen at − 20°C for later free $T_3$ or $T_4$ analysis by radioimmunoassay. The lungs were then subjected to isolated lung perfusion as described by Dutta.[4]

### *Lung Isolation*

The chest was rapidly opened by median sternotomy and the pulmonary artery was cannulated to deliver HEPES buffered solution (HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid 0.196 g/L, $MgCl_2$-$6H_20$ 0.244 g/L, NaCl

8.123 g/L, $CaCl_2$ 0.184 g/L, glucose 1.180 g/L adjusted to pH 7.40, T 37°C) bubbled with 100% $O_2$. The perfusate was delivered via a Rainin perfusion pump (Rainin Corporation, Braintree, MA) at 5 ml/min and recirculated with a total volume of 150 ml. The low flow rate was used to circumvent the use of albumin in the perfusate which interferes with measurement of glutathione.[11] Following washout of blood in the pulmonary vascular tree, the isolated lungs were placed in a closed perfusion chamber. The isolation chamber was flushed with ultrapure nitrogen to allow later determination of headspace alkane accumulation. The chamber was maintained isobaric by a teflon balloon filled with balance $N_2$. Static compliance was determined at time 0 and 95 minutes by insufflation of the tracheal catheter with 5 ml of $N_2$ and recording pressure during inspiratory hold.

Continuous monitoring of pulmonary artery vascular pressure was accomplished by a pressure transducer connected to a CODAS digital to analog converter to a 386SX computer (CODAS, DATAQ Instruments Inc., Columbus, OH). Continuous monitoring of post perfusion $O_2$ tension in the perfusate was done via an in-line Orion $O_2$ electrode (model 97-08-99, Orion Research Inc., Boston, MA). Oxygen consumption was calculated from the difference in oxygen concentration between the perfusate and the effluent, flow rate (5 $ml/min^{-1}$), solubility of $O_2$ in HEPES at 37°C ($3.25 \times 10^{-5}$ ml $O_2$/ml solution/mm Hg $O_2$) and is expressed as $\mu$l/min/gram dry wt. Headspace ethane determinations were made at intervals of 5, 35, 65, and 95 minutes by withdrawing 10 ml of gas from the chamber in a teflon, gastight syringe. At similar intervals, 1 ml of perfusate was obtained for assay of the glutathione and LDH content.

### *Headspace Alkane Analysis*

Gas chromatographic analysis of headspace ethane content followed the method of Dutta.[4] Ten ml of accumulated headspace gas was collected in a gastight syringe and immediately injected into a 10 ml sample loop on a Hewlett-Packard (Model 5890 Series IIA) gas chromatograph. The chromatographic conditions included: Poracil C column, isothermia at 80°C, $N_2$ carrier gas at 60 ml/min, FID temperature 200°C, elution period 10 min. Alkane peaks were compared with known standards by an HP 3396 Integrator with PEAK96 software and are expressed as pmol/gm dry weight.

### *Glutathione and LDH Determinations*

Total glutathione (GSSG + 2 GSH) and LDH activity in the effluent sample were determined spectrophotometrically using kinetic enzymatic methods.[12,13] An aliquot of the sample is added to a test solution containing NADPH, glutathione reductase and dithiobisnitrobenzoic acid (DTNB) in a quartz cuvette. The reaction is monitored by a Hitachi U-2000 spectrophotometer at 412 nm for 2 mins. The glutathione levels calculated were then expressed as nmol/gram dry weight.

To measure LDH activity, an aliquot of sample was added to a test solution containing 0.15 mM NADH and the reaction started by adding 1.5 mM pyruvate. The reaction mixture was monitored by following the concentration of NADH spectrophotometrically at 340 nm for 2 mins. The LDH level was expressed in U/gram dry wt.

TABLE 1

Serum hormonal levels were determined by radioimmunoassay after 24 hours of ozone exposure or control and are expressed as ng/dl. Ozone exposure significantly reduced T3 values; supplementation maintained serum euthyroidism. Values for T4 are not given in the hormonally supplemented animals as cross-specificity of the assay yielded falsely high values

| Group | Serum $T_3$ | Serum $T_4$ |
|---|---|---|
| Control | 58.0 ± 4.1 | 3.43 ± 0.18 |
| Ozone | 40.0 ± 3.3* | 2.4 ± 0.23* |
| Ozone + $T_3$ | 51.0 ± 5.0 | NA |
| Control + $T_3$ | 88 ± 14.0 | NA |

*$p < 0.05$ Compared to Control or $T_3$ Supplemented

### *Statistical Analysis*

The results for time intervals were compared between any of the four groups using an analysis of variance (ANOVA) with Bonferroni correction and unpaired *t* test where appropriate with the use of an IBM Instat program. Significance was inferred when the level of confidence exceeded 95%. Results are expressed as mean +/− standard error of the mean.

## RESULTS

### *Serum $T_3$ and Lung Oedema Formation*

All of the animals survived the treatment and ozone exposure without incident. Chamber ozone concentration remained at 1 ppm ± 0.05 ppm for the experimental period. Control animals had $T_3$ and $T_4$ values in the normal range; exposure of the rats to 1 ppm ozone for 24 hours resulted in a significant decline in serum concentrations of $T_3$ and $T_4$ (Table 1). Treatment with $T_3$ increased serum hormonal levels in the control animals and maintained more normal $T_3$ levels in the ozone exposed animals. A high variability in the measured values of $T_4$ was seen in the hormonally treated animals and is not reported; this has previously been noted and is most likely secondary to non-specificity of the $T_4$ assay.

### *Glutathione Efflux*

Perfusion glutathione efflux (GSH + GSSG) slowly increased over the duration of isolated lung perfusion in control animals in agreement with our earlier studies; $T_3$ augmentation did not significantly alter glutathione efflux during any experimental period (Table 2). Ozone exposure significantly increased early and late perfusion glutathione efflux. Concurrent hormonal treatment with ozone exposure significantly decreased glutathione efflux when compared with untreated, ozone exposed animals (Table 2).

### *LDH Leakage*

Perfusate lactate dehydrogenase slowly increased in the control and $T_3$ treated control animals (Table 3) over the experimental period. Lung cellular injury, as evidenced by LDH leakage, was significantly increased during every time period by

TABLE 2

Lungs were removed following control room air or ozone exposure with or without T3 supplementation and perfused in a closed isolation chamber for 95 minutes. Perfusate glutathione levels (GSH + 2 GSSG) were determined enzymatically and are indexed to dry lung weight. The results of each time period are expressed as nmol per gram of dry lung tissue. An average glutathione efflux per minute was calculated and demonstrates a significantly higher glutathione efflux in both ozone exposed groups compared to control.

GLUTATHIONE (mnol/gm dry wt)

| Group | 5 | 35 | 65 | 95 | Average (per min) |
|---|---|---|---|---|---|
| Control | 188 ± 53* | 319 ± 79* | 482 ± 112* | 628 ± 144* | 2.4 |
| Ozone | 892 ± 156 | 1611 ± 262 | 2380 ± 518 | 3370 ± 578 | 26.0 |
| Ozone + $T_3$ | 1065 ± 157 | 1462 ± 204 | 1869 ± 158 | 2078 ± 99# | 10.7 |
| Control + $T_3$ | 165 ± 97* | 278 ± 102* | 341 ± 106* | 391 ± 130* | 4.6 |

*$p < 0.05$ Compared to Ozone or Ozone + T3
#$p < 0.05$ Compared to Ozone

TABLE 3

Perfusate was collected every 30 minutes from the isolated, perfused lungs to determine cellular viability following ozone exposure and treatment and is expressed as U per gram of dry lung weight. Ozone exposure significantly increased LDH efflux compared to control animals; T3 treatment did not affect this.

LDH (U/gm dry wt)

| Group | 5 | 35 | 65 | 95 | Average (per min) |
|---|---|---|---|---|---|
| Control | 37 ± 13* | 60 ± 15* | 73 ± 18* | 96 ± 24* | 0.6 |
| Ozone | 152 ± 26 | 232 ± 37 | 274 ± 39 | 404 ± 58 | 2.6 |
| Ozone + $T_3$ | 162 ± 34 | 252 ± 19 | 352 ± 36 | 447 ± 2 | 3.0 |
| Control + $T_3$ | 32 ± 12* | 49 ± 19* | 56 ± 21* | 69 ± 21* | 0.4 |

*$p < 0.05$ Compared to Ozone or Ozone + $T_3$

ozone exposure. Thyroid hormone supplementation did not significantly alter LDH release in ozone exposed animals when compared to non-treated exposed animals.

### *Alkane Production*

Headspace ethane production, as an index of lipid peroxidation, slowly increased in control, perfused lungs and averaged 0.40 pmol/gm dry weight/min (Table 4). Ozone exposure significantly increased cumulative headspace ethane accumulation at the end of perfusion when compared with control animals. $T_3$ treatment did not significantly alter ethane production rate in control or ozone exposed animals.

### *Vascular Perfusion Pressure*

Pulmonary vascular perfusion pressure remained low in control and control/$T_3$ animals throughout the experimental protocol (Figure 1). Ozone exposure caused a gradual rise in pulmonary vascular perfusion pressure during late perfusion however this increase was not significantly different from control. In contrast, animals treated with thyroid hormone and exposed to ozone had a significant and sustained rise in pulmonary vascular pressure during early and late perfusion (Figure 1). This rise in perfusion pressure corresponded to visual evidence of pulmonary edema including fluid in the tracheal carrula, translucency in the lung parenchyma and increase in lung size at the end of the perfusion.

TABLE 4

The lungs were perfused in a $N_2$-flushed, closed isolation chamber to allow determination of lung surface ethane generation. A 10 ml sample of headspace gas was obtained every 30 minutes to determine headspace alkane accumulation as an indicator of lipid peroxidation. Ethane content was measured by gas chromatography and is expressed as pmol ethane/lung dry weight/minute. Ozone exposure significantly increased lung ethane production, but to a lesser extent than model oxidant agents. T3 treatment did not alter lung ethane generation.

ETHANE (pmol/gm dry wt/min)

| Group | 5 | 35 | 65 | 95 | Average (per min) |
|---|---|---|---|---|---|
| Control | 0 | 17.4 ± 5.2 | 30.9 ± 2.4 | 38.4 ± 1.8* | 0.40 |
| Ozone | 0 | 18.6 ± 4.6 | 37.4 ± 5.8 | 62.7 ± 8.1 | 0.66 |
| Ozone + $T_3$ | 0 | 13.9 ± 6.6 | 36.2 ± 4.2 | 65.1 ± 5.0 | 0.69 |
| Control + $T_3$ | 0 | 11.1 ± 3.8 | 29.2 ± 1.6 | 40.4 ± 1.1* | 0.42 |

*$p < 0.05$ Compared to Ozone or Ozone + $T_3$

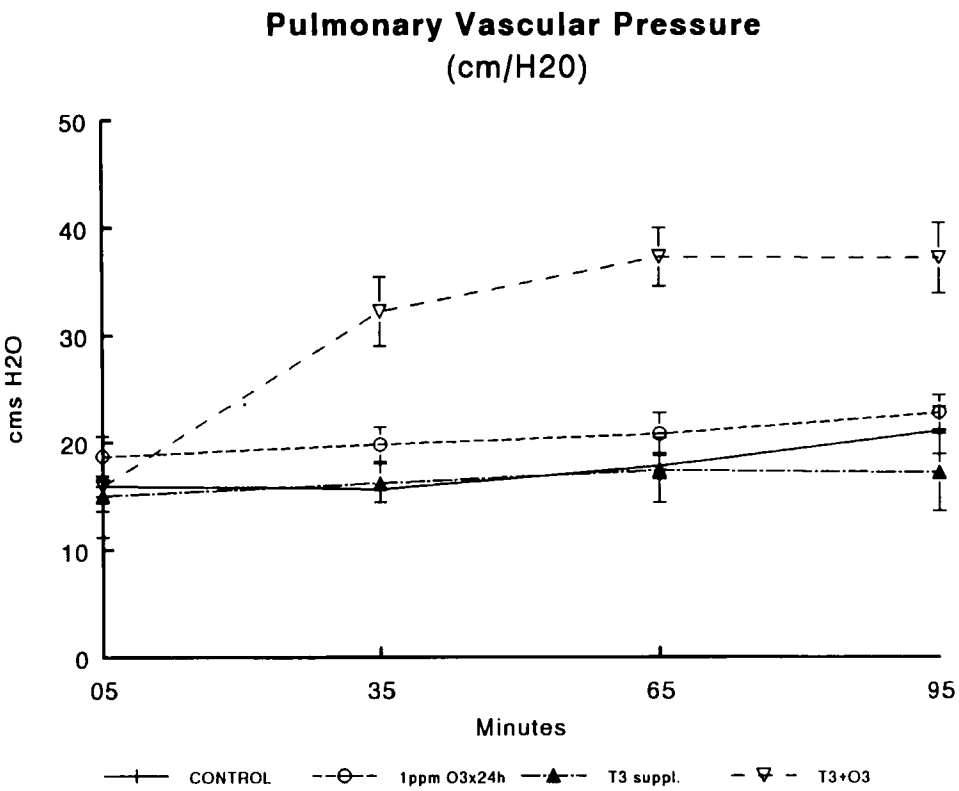


FIGURE 1 Average oxygen consumption of the isolated perfused lung was calculated by the decrement in oxygen concentration of the perfusate and is indexed to dry lung weight. Thyroid hormone supplementation significantly increased oxygen consumption of the isolated perfused lung. Although ozone exposure did not significantly alter lung oxygen consumption, a decrement in late oxygen consumption, possible due to cellular dysfunction, was noted in the T3 treated, ozone exposed lungs.

TABLE 5
Isolated, perfused lung static compliance was determined by static inflation of the lung at the beginning and end of perfusion. The deterioration in lung compliance is expressed as the percent decrement in original compliance and is significantly lower in the ozone exposed animals.
Pulmonary Static Compliance

| Group | Time o | 95 min | % Change |
|---|---|---|---|
| Control | 0.37 ± 0.06 | 0.31 ± 0.04 | 15.0 |
| Ozone | 0.33 ± 0.04 | 0.19 ± 0.03 | 41.1* |
| Ozone + $T_3$ | 0.28 ± 0.05 | 0.17 ± 0.01 | 38.2* |
| Control + $T_3$ | 0.38 ± 0.05 | 0.31 ± 0.04 | 16.4 |

*$p < 0.05$ Compared to Control or Control + $T_3$

### *Static Lung Compliance*

Although initial static lung compliance as determined by sequential lung inflation was decreased in both ozone exposure groups, the results did not reach statistical significance (Table 5). Treatment with thyroid hormone did not significantly alter pre-perfusion compliance. End-perfusion compliance was significantly decreased in ozone exposed animals with or without $T_3$ treatment when compared to control or pre-perfusion compliance corresponding to an increase in pulmonary perfusion pressure and visual edema in the $T_3$ supplemented animals.

### *Oxygen Consumption*

Isolated perfused, non-working lung oxygen consumption did not vary significantly between the control and the ozone exposed groups at any time period (Figure 2). Thyroid hormonal supplementation significantly increased early and late oxygen consumption at all time periods when compared to control in both ozone exposed and control animals. A gradual decline in late oxygen consumption was noted in the ozone exposed, $T_3$ treated animals.

## DISCUSSION

Ozone continues to be an important environmental oxidant that exerts toxicity to biologic systems through a complex mechanism. Ozone is a powerful oxidant and can directly injure cell lipid and protein structures. The exact pathophysiology of ozone toxicity and the cellular response to this are not fully defined. Furthermore, the role of thyroid hormone status on ozone toxicity is not clear.

The experiments described in this paper demonstrate an acute decrease in circulating $T_3$ and $T_4$ levels with ozone exposure. This corroborates earlier work by Clemons and Garcia who demonstrated a profound depression of the thyroid axis with a decreased TSH, $T_3$ and $T_4$ concentration in a similar rat model of 1 ppm ozone exposure.[14,15] This report also shows that subcutaneous thyroid hormonal augmentation at a rate of 3 ng/hr provides an appropriate replacement of $T_3$ in this altered hormonal economy to maintain serum euthyroidism. The physiological consequences of these ozone-induced hormonal alterations are not clear. Fairchild and Graham examined the role of thyroid hormonal status on ozone toxicity in a rat model.[9] Their studies demonstrated a protective effect of hormonal blockade and a

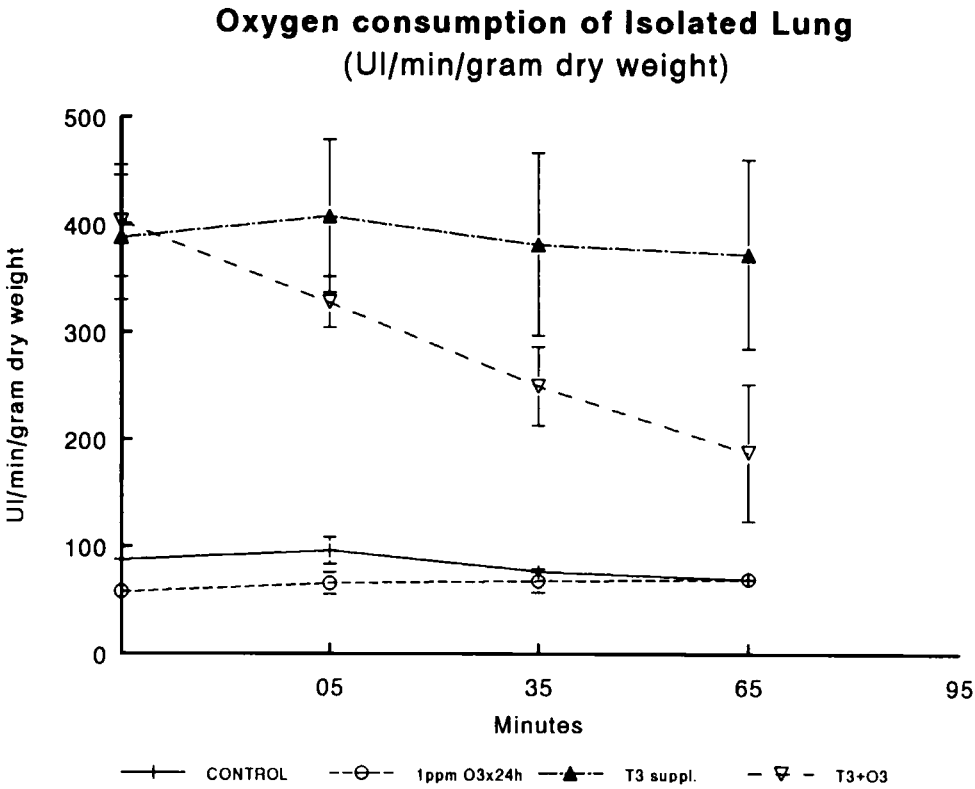


FIGURE 2 Average oxygen consumption of the isolated perfused lung was calculated by the decrement in oxygen concentration of the perfusate and is indexed to dry lung weight. Thyroid hormone supplementation significantly increased oxygen consumption of the isolated perfused lung. Although ozone exposure did not significantly alter lung oxygen consumption, a decrement in late oxygen consumption, possible due to cellular dysfunction, was noted in the $T_3$ treated, ozone exposed lungs.

heightened ozone toxicity with hormonal supplementation. They hypothesized that a heightened metabolic response as well as a hormonally dependent decrease in -SH group availability was responsible for the alteration in oxidant toxicity. Alternatively, it is reasonable to speculate that in vivo, inhaled ozone increases the metabolic destruction of thyroid hormone causing iodination of cellular surface proteins and their inactivation. This may partially explain the deleterious pulmonary and distant cellular effects of ozone exposure. In support of this, Wong and Hochstein[16], demonstrated an increase in ozone induced erythrocyte osmotic fragility if thyroxine was added to the media. Erythrocytes not "primed" with thyroid hormone were able to withstand significantly greater ozone exposure concentrations in their experiments. They proposed that in the presence of ozone, the thyroid hormones undergo deiodination within the plasma membrane leading to modification of membrane proteins. These altered proteins are then responsible for the altered cation permeability and osmotic fragility in the exposed cells. If this hypothesis is correct, ozone-induced hypothyroidism may reflect usage of thyroid hormone for facilitation of ozone toxicity and serve as a marker of the severity of exposure.

Lung antioxidants, particularly GSH, provide the primary cellular defence against

pulmonary oxidant injury caused by inhaled gases, oxygen derived free radicals and peroxide induced injury. Cellular -SH metabolism, and glutathione oxidant status, are dependent on the thyroid hormonal milieu.[17] A chronic decrease in available thyroid hormone leads to an increase in reduced glutathione and -SH availability.[21] This should theoretically lead to an increased cellular tolerance to an oxidant challenge. The anti-oxidant effects of an acute decrease in circulating levels of $T_3$ and $T_4$ as seen in ozone exposure have not been previously reported. We did not see any alterations in total glutathione efflux in control animals with $T_3$ supplementation. This may be in part due to the low dosage of hormone used in the animals. Also, the effects of acute hyperthyroidism on glutathione availability and efflux are not known and may be considerably less than the previously reported effects of chronic hypothyroidism. The low level of glutathione release without a corresponding increase in LDH leakage in the control animals would suggest that the perfusate glutathione content is due to lung cellular release and not the result of cellular death. Ozone exposure significantly increased glutathione efflux during the ex-vivo study. This is in contrast to our earlier studies which demonstrated a modest increase in glutathione production in isolated, perfused lungs exposed ex vivo to ozone during the experimental period and may be due to the prolonged duration and method of ozone exposure used in this study. Animals supplemented with thyroid hormone had significantly less glutathione efflux than non treated ozone exposed animals. This is in agreement with earlier studies demonstrating a decreased glutathione availability with thyroid hormonal augmentation and would suggest that glutathione oxidase activity is dynamically related to thyroid hormonal alterations.[17,21] Nishiki *et al.* demonstrated similar correlation between antioxidant status and glutathione efflux in oxygen (100%) exposed animals.[18] In his study, a tocopherol-deficient diet, vis-a-vis reduced antioxidant potential, was necessary to show the maximal glutathione efflux.

The mechanism for cellular injury during ozone exposure has been suggested to include lipid peroxidation, protein macromolecular degradation or inactivation, and lung lining fluid alterations. Although each of these mechanisms may contribute to the overall physiologic alterations noted during ozone exposure, the relative contributions of each are unknown.

The isolated perfused lungs of ozone exposed animals continued to produce ethane at a higher rate than control animals indicating that lipid peroxidation may play a role in ozone toxicity during in vivo exposure. Thyroid hormonal supplementation did not significantly alter the ethane production rate, suggesting that the heightened ozone toxicity seen with hormone supplementation is not due to lipid peroxidation. Although the increase in lipid peroxidation in the ozone exposed animals was significantly greater than in control animals, it is much less than that seen with a model peroxidizing agent such as t-Butyl hydroperoxide.[4] Alternatively, the limited extent of lipid peroxidation may reflect the discontinuation of the oxidant challenge with isolated perfusion although we have not demonstrated a greater effect with continuous ethane exposure. Furthermore, these findings are in agreement with Cross et al who did not see an increase in lipid hydroperoxides, alkanes, or lipoprotein electrophoretic mobility during ex-vivo exposure of plasma to high levels of ozone.[20] This study and others would suggest that peroxidation of poly-unsaturated fatty acids plays a limited role in ozone toxicity.

Thyroid hormone supplementation increased lung metabolic rate, as revealed by oxygen consumption in this model. The increase in pneumocyte metabolic rate occurs in the face of a decrease in glutathione scavenging capacity and may result in a heightened pulmonary injury during stress. In support of this, there was an increased

LDH efflux and pulmonary vascular pressure and a decreased pulmonary compliance in the hormonally supplemented, ozone exposed animals. The decrease in compliance could be due to accumulation of edema fluid or alteration of lung surfactant or both.[19] Furthermore, there was a gradual decline in oxygen consumption in the $T_3$ treated, ozone exposed lungs during late perfusion. This decline in oxygen consumption may be a premorbid alteration in pulmonary metabolism heralding greater cellular injury.

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