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Environmental Health Insights

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ORIGINAL RESEARCH

# Fate of Pathologically Bound Oxygen Resulting from Inhalation of Labeled Ozone in Rats

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**Abstract:** Inhaled ozone ($O_3$) reacts chemically with respiratory tract biomolecules where it forms covalently bound oxygen adducts. We investigated the fate of these adducts following inhalation exposure of rats to labeled ozone ($^{18}O_3$, 2 ppm, 6 hr or 5 ppm, 2 hr). Increased $^{18}O$ was detected in blood plasma at 7 hr post exposure and was continuously present in urine for 4 days. Total $^{18}O$ excreted was ~53% of the estimated amount of $^{18}O_3$ retained by the rats during $^{18}O_3$ exposure suggesting that only moderate recycling of the adduct material occurs. The time course of excretion, as well as properties of the excreted $^{18}O$ were determined to provide guidance to future searches for urinary oxidative stress markers. These results lend plausibility to published findings that $O_3$ inhalation could exert influences outside the lung, such as enhancement of atherosclerotic plaques.

**Keywords:** ozone, oxidative stress, biomarkers, adducts, excretion

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## Introduction

Ozone ($O_3$) pollution of ambient air affects a worldwide population where exposure to $O_3$ has been shown to be associated with a variety of cardiopulmonary health impairments.[1–4] Due to its low water solubility and high chemical reactivity, $O_3$ is able to pass through the nose into the lung where it causes injury and forms stable adducts and reactive intermediates such as peroxides, aldehydes and carbonyls.[5,6] These intermediates are believed to be responsible for the reported oxidation of molecules in the blood[7,8] since $O_3$ itself is so reactive that it is not expected to penetrate beyond the respiratory tract surface.[9,10] Animal studies have shown that $O_3$ can affect extrapulmonary sites such as enhancement of atherosclerotic plaques and vascular injury in susceptible animals,[11,12] however, the mechanisms responsible for these effects are unknown. $O_3$ has been considered to be a good model oxidant for the elucidation of clinical markers of in vivo oxidative stress. A published series of studies in rats showed that some traditional measures of oxidative stress (isoprostanes and malondialdehyde in blood plasma and urine) that were effective markers of $CCl_4$—induced oxidative stress to the liver, were not effective following inhalation of $O_3$.[13–15]

We embarked on the present study with the goal of tracing the fate of $O_3$ reaction products that might enter the circulation and be excreted. Our previous studies showed that exposure to $^{18}O$-labeled ozone ($^{18}O_3$) results in measurable $^{18}O$ in nasal and bronchoalveolar lavage fluid (BALF), and that the concentration of $^{18}O$ in BALF is related to the level of injury.[16–18] We hoped to elucidate methods for detection of oxidative stress clinically as well as shed light on the mechanism by which $O_3$ induces extrapulmonary effects. Relatively high exposure concentrations of $^{18}O_3$ were employed (4–12 fold higher than maximal ambient levels) because we were searching for chemicals in blood and urine after a large dilution from their pulmonary concentration. $O_3$ at these concentrations induces pulmonary edema in the rats which is detectable as increased total protein in BALF.[16]

## Methods

### Animals

Male 60 day old Fischer 344 rats, (Charles River Laboratories, Raleigh, NC) were housed in temperature and humidity controlled rooms (20 °C–25 °C, 35%–70% relative humidity) with a 12 hr light/dark cycle (light period = 06:00 to 18:00). Standard rat chow (ProLab, Brentwood, MO) and water were provided *ad libitum*. The rats had free access to deionized, reverse-osmosis-treated water and received autoclaved NIH 31 rodent chow (Zeigler Bros., Gardners, PA). All experiments were performed according to the United States Environmental Protection Agency Guidelines for the Care and Handling of Experimental Animals.

### $^{18}O_3$ and $O_3$ exposures

Rats were exposed to $^{18}O_3$ or $O_3$ in individual stainless steel wire mesh cages inside a 135 liter Rochester chamber at an airflow rate of 1.6 $m^3$/hr. Control rats were exposed to filtered room air. $^{18}O_3$ was generated from $^{18}O_2$ using a corona discharge unit from a commercial NOx monitor (Bendix Corp., Louisburg, WV). Efficiency of conversion from $^{18}O_2$ was approximately 1.5%. This resulted in an excess $^{18}O_2$ concentration of 130 ppm over a natural abundance background of 400 ppm $^{18}O_2$ (ambient air contains 0.2% $^{18}O_2$). We have shown previously that this small increase in abundance of $^{18}O_2$ does not result in an appreciable increase in $^{18}O$ in tissues.[16] Chamber $O_3$ concentration was monitored with a Dasibi model 1003 AH $O_3$ monitor (Dasibi Environmental, Glendale, CA). Pre-exposures to unlabeled $O_3$ were performed similarly.

### Experimental design

Table 1 shows a summary of the five experiments reported here. Experiment 1 employed a lower $^{18}O_3$ concentration for a longer time than subsequent experiments. Urine collection times were 07:00–08:00 and 17:00–18:00 for 4 days post $^{18}O_3$ exposure on all experiments.

In experiment 2, the $^{18}O_3$ exposed rats were divided into two groups and half of the rats were bathed in detergent to remove $^{18}O$ that could have been present as a reaction product with lipids or proteins on the fur and licked off during the urine collection period. Bathing was done immediately post $^{18}O_3$ exposure by immersion of rats briefly anesthetized with 5% halothane (Aldrich, Milwaukee, WI) in 0.4 liters of 0.1% sodium dodecyl sulfate. They were then rinsed in warm tap water and dried. A sample of the washing solution was lyophilized and the $^{18}O$ content of the residue determined. In experiment 3, rats were



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**Table 1.** Summary of experiments performed in the present study.

| Expt. | Purpose | Exposure | Groups | Comments |
|---|---|---|---|---|
| 1 | Demonstrate feasibility of detecting $^{18}O_3$ reaction products in urine and their molecular weight. | 2 ppm $^{18}O_3$, 6 hr and air at 09:00–15:00. | Urine was collected twice daily for 4 days (n = 6 rats/group). | A sample of urine from each rat was dialyzed to retain the MW > 500 fraction which was analyzed separately for $^{18}O$ content. Urease treatment was also done. |
| 2 | Demonstrate that $^{18}O_3$ reaction products are of respiratory origin rather than licked off the fur. | 5 ppm $^{18}O_3$, 2 hr and air at 08:00–10:00. | Urine was collected twice daily for 4 days. A separate group of rats were bathed in detergent solution immediately post $^{18}O_3$ exposure (n = 4 rats/group). | Other measurements made included urine dry weight protein, albumin, urea, creatinine, and stability to heat. |
| 3 | Demonstrate the effect of pre-exposure to unlabeled $O_3$ one week before the $^{18}O_3$ exposure. | Pre-exposure to air, 2 or 5 ppm unlabeled $O_3$ for 2 hr was followed one week later by exposure of all rats to 5 ppm $^{18}O_3$ for 2 hr. | Urine was collected daily for 4 days from air, 2 and 5 ppm pre-exposed groups (n = 6 rats/group). | Other measurements made included urine volume, dry weight, urea and creatinine. |
| 4 | Quantify $^{18}O$ label in bronchoalveolar lavage fluid (BALF) and in blood following $^{18}O_3$ exposure. | 5 ppm $^{18}O_3$, 2 hr and air. | Blood was drawn and BALF cells and supernatants collected from separate groups of rats at 2, 7 and 16 hr post exposure (n = 6 rats/group). | BALF protein was also measured as an indicator of $^{18}O_3$ induced alveolar injury which resulted from loss of the blood-air barrier of the lung. |
| 5 | Examine the time course of appearance of $^{18}O$ in urine following intratracheal instillation of BSA or PC pre-labeled in vitro with $^{18}O_3$. | Instilled 30 ug of $^{18}O$/rat in 14 mg/rat of BSA or PC. | Urine was collected for 4 days from saline, $^{18}O$-BSA and $^{18}O$-PC rats (n = 5 rats/group). | Other measurements made included urine volume, dry weight, urea and creatinine. |



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pre-exposed a week prior to unlabeled $O_3$ to determine whether the pre-exposure would affect the subsequent elimination of $^{18}O$ in the urine. The $^{18}O_3$ exposure involved three groups of rats with differing pre-exposure to unlabeled $O_3$.

Experiment 4 examined the quantities of $^{18}O$ present in blood plasma and BALF. Rats were exposed similar to the urine studies and at 2, 7 and 16 hr post exposure they were anesthetized with pentobarbital (50 mg/kg body weight) and 5 mL of blood was removed from the dorsal aorta proximal to its bifurcation into the common iliac arteries. Immediately following exsanguination, lungs were lavaged with 37 °C saline (30 mL/kg body wt.) as previously described.[19] BALF was centrifuged (400 × g, 15 min, 4 °C) and cell pellets and supernatants assayed for $^{18}O$ and total protein.

Experiment 5 examined the time course of appearance of $^{18}O$ in urine following intratracheal instillation of BSA or PC pre-labeled *in vitro* with $^{18}O_3$ (see details below).

## Urine collection and preparation

Rats were housed individually in 4.4 liter volume (20 cm diam. × 14 cm high) plastic metabolism cages (Nalge Nunc, Rochester, NY) for seven days prior to exposure for acclimation to the cages. Following exposures to $^{18}O_3$, rats were returned to the metabolism cages and urine was collected for 4 days. The temperature of the urine collection tubes was maintained at 4 °C by enclosing them in copper tubing through which cooled ethylene glycol was circulated. Urine samples were centrifuged to remove extraneous debris (400 × g, 15 min, 4 °C), volumes were recorded and the supernatants were removed and stored at −80 °C. In the first two studies, the mg dry weight excreted per hour of urine collection was determined.

## $^{18}O$ determination

The main purpose of the study was to examine $^{18}O$ concentrations in urine and related tissues of the rats after exposure to $^{18}O_3$. All samples for $^{18}O$ analysis were stored frozen (−80 °C) until lyophilization and then at 4 °C for a maximum of two months. Analysis for $^{18}O$ content was performed on the dried material as previously described.[16] Briefly, ~0.5 mg of each sample was weighed into a silver cup and subjected to elemental analysis for oxygen content in a Carlo Erba elemental analyzer (model 1106, Fisions Inc., Danvers, MA and Elemental Microanalysis, Manchester, MA). This analyzer converted all oxygen in the sample to carbon monoxide which exited the analyzer in a helium stream. The effluent of the analyzer was directed by continuous flow through columns where the sample was further oxidized to $CO_2$ (140 °C, $I_2O_5$ granules) and a cold trap (−57 °C) to remove formed $I_2$. A small capillary stream of the resulting gas was pulled into the vacuum of an isotope-ratio mass spectrometer (model SIRA 10, VG Isogas, Cheshire, UK). The $^{18}O/^{16}O$ ratio of unknown samples was determined by reference to standards included in each sample run.

## Preparation and intratracheal instillation of $^{18}O$-labeled lipid and protein solutions

Egg phosphatidylcholine (PC, Avanti Polar Lipids, Alabaster, AL) was dissolved in chloroform, dried under a stream of $N_2$ gas, then suspended by sonication in distilled water to achieve a concentration of 100 mg/mL. Bovine serum albumin (BSA) (Sigma, St. Louis, MO) was dissolved in distilled water to 100 mg/mL. Each solution was then exposed to 26 ppm $^{18}O_3$ for 1 hr in 125 mL flasks. $^{18}O_3$ was allowed to flow through a glass tube directly into each solution at a flow rate of 3.9 mL/min. After labeling, samples of each solution were lyophilized and stored at −20 °C. This dry material had stable concentrations of $^{18}O$ over a ~1 year period. BSA was labeled to achieve 2.33 mg $^{18}O$/g dry wt., and PC was labeled to a value of 2.14 mg $^{18}O$/g dry wt.

Intratracheal instillations were performed on rats anesthetized with 5% halothane. A 16 gauge cannula was inserted into the trachea after which an 18 gauge cannula attached to a syringe containing the solution to be instilled (0.3 mL of 45 mg/mL BSA or PC) was injected through the 18 gauge cannula into the lungs. This resulted in 31.4 µg $^{18}O$ (1.7 umoles) per rat for $^{18}O$-BSA and 28.9 µg $^{18}O$ (1.6 umoles) per rat for $^{18}O$-PC (see results of instillations in Table 2). The instillations had no noticeable toxic effect on the rats.

## Dialysis and heat stability of $^{18}O$ in urine

Dialysis of urine in Expt. 1 was performed by adding a 2.0 mL aliquot of each urine sample to 500 MW cutoff SpectroPor DispoDialyzer® dialysis tubes (Spectrum, Laguna Hills, CA). Urine was dialyzed against 8 liters of distilled water for 24 hr at 4 °C. To determine the



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**Table 2.** The levels of retained or instilled $^{18}O$ (per rat) following inhalation of $^{18}O_3$ or intratracheal instillation of $^{18}O$ labeled protein or lipid: percentage of $^{18}O$ retained in different tissue pools relative to exposure levels.

| Treatment | $^{18}O$ retained by or instilled into the whole animal | | Measured $^{18}O$ in tissue pool | | |
|---|---|---|---|---|---|
| | Method of prediction of retained $^{18}O$ | umoles $^{18}O$/rat | Tissue pool | umole/ rat | Retained/ measured, % |
| $^{18}O_3$, 5 ppm 2 hr | Allometric equation and % uptake calculations | 4.0 | Urine | 2.1 | 53 |
| $^{18}O_3$, 5 ppm 2 hr | Allometric equation and % uptake calculations | 4.0 | BAL fluid | 0.8 | 20 |
| $^{18}O$ labeled bovine serum albumin | Amount instilled intratracheally | 1.8 | Urine | 1.0 | 54 |
| $^{18}O$ labeled phosphatidyl choline | Amount instilled intratracheally | 1.6 | Urine | 0.2 | 12 |

heat lability of the $^{18}O$ label, dried samples of the urine from $^{18}O_3$-exposed rats were heated for 30 min in a ceramic radiant heater (Omega Engineering, Inc., Stamford, CT) controlled by a rheostat (Superior Electric Co., Bristol, CT) and monitored by thermocouple (Omega type K). After cooling, the samples were again weighed and $^{18}O$ contents determined.

## Biochemical analyses

Urinary creatinine and urea were determined by coupled enzyme reaction (Sigma Diagnostics, St. Louis, MO). Total protein was analyzed by a Coomassie blue colorimetric method (Pierce Rockford IL). Albumin was analyzed using an immunoprecipitation based kit (DiaSorin, Stillwater, MN). These assays were adapted for use on a Cobas Fara II clinical analyzer (Roche Diagnostics, Branchburg, NJ). Some samples of urine were treated to convert the urea to $CO_2$ and $NH_3$ using urease (Sigma Type III from Jack Beans, final concentration of 1.7 U/mL). Samples were incubated 18 hr at 4 °C.

## Data analysis

$^{18}O/^{16}O$ ratios were derived from the mass spectrometer as 'delta values' relative to high and low standards containing known $^{18}O/^{16}O$ ratios included in each sample run. Delta values of $^{18}O_3$ exposed and air exposed samples were compared to determine whether the $^{18}O_3$ exposed samples were elevated (*t*-test, *P* value $\leq 0.05$) above the air exposed samples. In all but experiment 5, $^{18}O_3$ treated samples were significantly elevated above natural abundance samples. Levels of $^{18}O$ enrichment due to the $^{18}O_3$ exposures were determined by subtracting the mean natural abundance of $^{18}O$ (~0.2 atom%) from all samples. The natural abundance $^{18}O$ concentration was obtained from analysis of the same type of samples from air-exposed rats. Units of $^{18}O$ enrichment were converted from umoles $^{18}O$/mole of total oxygen to ug $^{18}O$/gram dry weight by use of the mean percentage of oxygen in the dry samples (obtained as output from the elemental analyzer). The 'excess $^{18}O$ in samples resulting from $^{18}O_3$ exposure' will hereafter be termed simply '$^{18}O$ incorporation' or '$^{18}O$.'

### Elemental oxygen and nitrogen content of urine

Since the elemental oxygen percentage (%O) of the samples was used in the calculations of $^{18}O$ incorporation per gram dry weight (see Methods), we report that %O of the lyophilized urine pooled across collection times was 21.3% ± 0.3% (n = 42). The %N was also measured and averaged 25.4% ± 0.4% (n = 42). Exposure to $^{18}O_3$ did not significantly affect these percentages. Following dialysis, the urinary % N was reduced to 10.5% ± 0.9%, while the %O was not changed. Following urease treatment of urine %N was reduced by ~43%.

# Results

The following results suggest that $^{18}O_3$ reaction products leave the respiratory tract, pass through the blood and are excreted in the urine. The time course of appearance of the $^{18}O$ label in blood and urine, as well as the properties of the labeled material are reported.

### Urinary $^{18}O$ following $^{18}O_3$ exposure

Experiment 1 results showed that urinary $^{18}O$ concentration (per gram of dry weight) was significantly elevated on all 4 days following $^{18}O_3$ exposure (Fig. 1), decreasing from 16 to 7 µg $^{18}O$/g dry during this period. Following dialysis to remove material



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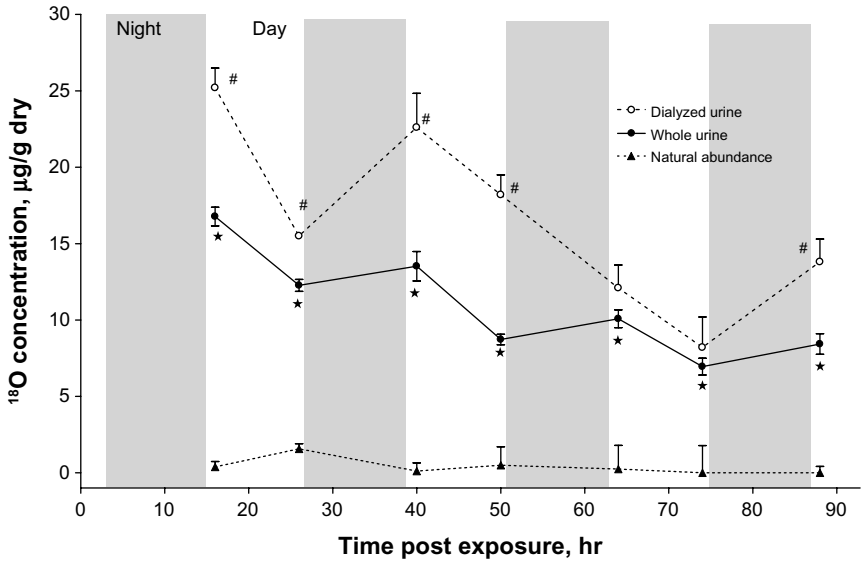



**Figure 1.** The time course of excretion of $^{18}$O in the urine of F344 rats following exposure to $^{18}O_3$ (2 ppm, 6 hr), and the effect of removal molecules <500 MW by dialysis. Excess $^{18}$O was easily detectable in all urine samples for 4 days following the $^{18}O_3$ exposure. Darkened bars represent periods of night time (18:00 to 06:00) in this and subsequent figures.
**Notes:** *Significantly elevated above natural abundance samples ($P < 0.05$, n = 6 per group); #significantly elevated above non-dialyzed urine ($P < 0.05$, n = 6).

smaller than 500 Daltons, the urine dry weight was reduced to about one fifth of its original value and the $^{18}$O concentration was increased ~60%. Urease treatment caused a ~50% higher $^{18}$O/gram dry weight in the first urine collection sample, however, all later samples showed insignificant changes in $^{18}$O content.

## Effect of washing the fur

Washing the fur of the rats immediately after the $^{18}O_3$ exposure did not appear to alter the urinary $^{18}$O (Fig. 2). The dried washing solution contained ~230 ug $^{18}$O (13 umoles of $^{18}$O)/rat or about 5 times the amount recovered in urine.

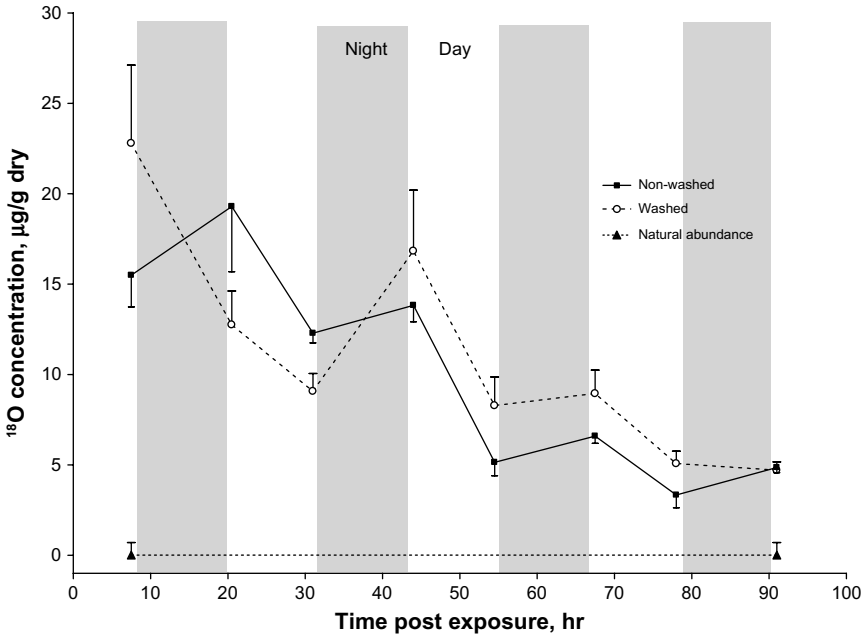



**Figure 2.** Time course similar to that shown in Figure 1, but following a higher exposure level for a shorter time (5 ppm, 2 hr) and showing the effect of washing the fur of half of the rats to remove the possible influence of licking $^{18}$O from the fur. Note that the $^{18}$O appeared to be unaffected by the washing step, meaning that the $^{18}$O appears to be of respiratory origin. The amount of $^{18}$O recoverable in the dried wash fluid was ~13 umole/rat or about 5 times the amount excreted into the urine.



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## Night versus day excretion

In the first three experiments there was a tendency for urine to be more concentrated at the morning collection time than at the evening collection. This created a sawtooth appearance of the time course of $^{18}O$ disappearance. The possibility that the rate of $^{18}O$ excreted per hour might also be higher during the night was investigated by obtaining the product of the $^{18}O$ concentration (per gram dry) and the grams dry weight excreted per hour at the different collection periods. Figure 3 shows the rate of excretion of urine dry weight (mg dry weight per hour) during the times preceding each urine collection period versus the urine collection time. These data seemed to explain the sawtooth pattern of $^{18}O$ excretion since the excretion rate of dry material was 1.6 to 3.9 fold higher during the night than during the day. There was a visual tendency for higher rates of dry weight excretion at the later times of urine collection in all exposures suggesting that at the early times there was a stress-induced reduction of excretion rates. This reduction in rate was observed in the air exposed and $^{18}O_3$ exposed, however, it was more prolonged after $^{18}O_3$ exposure (Fig. 3). Thus, both the rate of $^{18}O$ excretion and urine dry weight excretion appeared to be higher during the night than during the day.

## Effect of pre-exposure to $O_3$

The possibility that pre-exposure to $O_3$ might induce an adaptive response measurable by altered excretion of $^{18}O_3$ products was addressed in the third experiment which first exposed rats to air, 2 or 5 ppm $O_3$ (2 hr) then followed up with a second exposure a week later of all rats to 5 ppm $^{18}O_3$. We analyzed the $^{18}O$ disappearance curve following $^{18}O_3$ exposure by selecting only the data for the urine collected in the morning. This led to smooth logarithmic washout curves with high R values (Fig. S1). Equations shown on the figures describe the fitted trend lines for the logarithmic decline in $^{18}O$ over time. Rats pre-exposed to $O_3$ had a slightly steeper slope and higher Y intercept than the air pre-exposed group, however, these changes were small (<17%) and of questionable biological significance.

## Excreted $^{18}O$ compared to inhaled and retained $^{18}O_3$

The total amount of $^{18}O$ found in urine of rats exposed to $^{18}O_3$ over the four day collection period was 2.1 µmols (Table 2), and the calculated amount of $^{18}O$ that should have been retained per rat was 4.0 µmoles (see Supplement A for calculations). Thus, 53% of the amount of $^{18}O$ retained by each rat

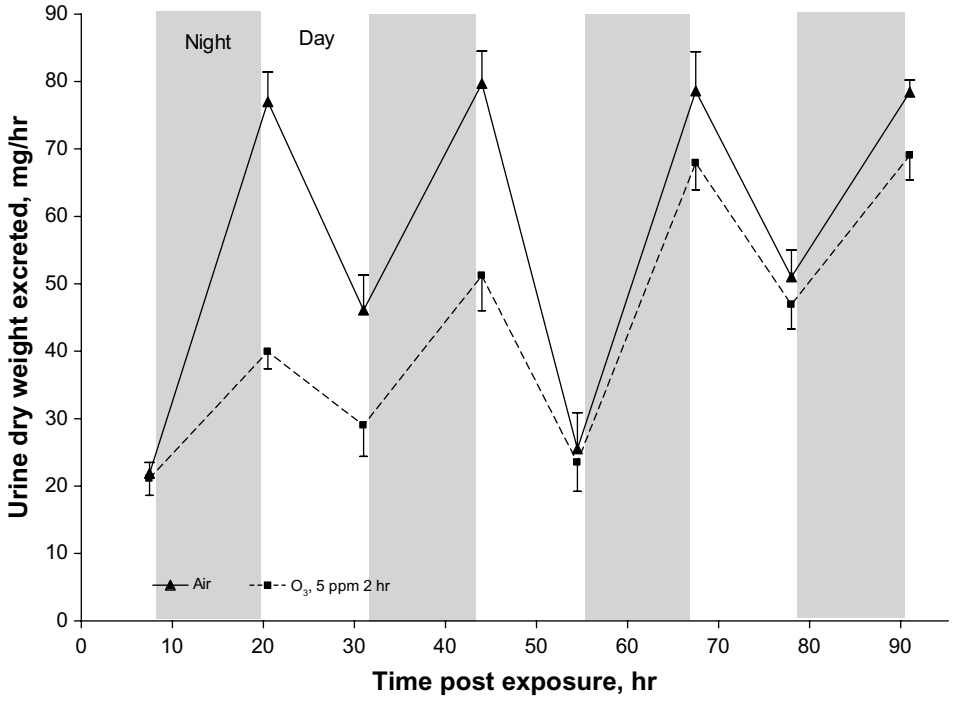



**Figure 3.** Rate of urine dry weight excreted (per hour per rat) following exposure to 5 ppm $^{18}O_3$ for 2 hr. Note that the excretion rate was higher during nighttime periods compared to daytime periods and that $^{18}O_3$ exposure appeared to lower the dry weight excretion at the early sampling times.



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following the $^{18}O_3$ exposure appeared to be excreted into the urine over the four post exposure days (Table 2).

### $^{18}O$ in bronchoalveolar lavage fluid

We compared the quantities of $^{18}O$ detected in the BALF supernatants of rats after $^{18}O_3$ exposure with the amounts excreted in urine by performing an experiment in which BALF was collected after exposure to $^{18}O_3$. Rats exposed to 5 ppm $^{18}O_3$ (2 hr) showed high levels of BALF extracellular protein (~3 mg/mL) compared to normal BALF protein levels (~0.1 mg/mL, Fig. 4). Protein levels decreased ~30% over 16 hr. $^{18}O$ concentration in the BALF supernatant was ~150 ug $^{18}O$/g dry and decreased ~74% over 16 hours (Fig. 4). We estimate that the increase in BALF protein corresponds to about 0.3 mL of plasma leakage into the air spaces of the lung at the 2 hr post exposure time (Supplement E).

### $^{18}O$ in blood

The loss over time of $^{18}O$ in BALF suggested that there should be a corresponding appearance of $^{18}O$ in the blood. We estimated that if all of the $^{18}O$ present in BALF at 2 hr post exposure (13.8 ug/rat—see Supplement A) were immediately added to the blood plasma, the level of $^{18}O$ would be ~24 ug/g dry which is much higher than our measured concentration of ~1.8 ug/g dry at 7 hr post exposure but not at other times (Fig. 5 and Supplement D). We did not find detectable $^{18}O$ in red blood cells following $^{18}O_3$ exposure.

### $^{18}O$ in urine of rats intratracheally instilled with $^{18}O$-PC and $^{18}O$-BSA

We investigated the possibility that simple transport of ozonized lipids or proteins from the pulmonary airways might account for the appearance of $^{18}O$ in blood and urine following $^{18}O_3$ exposure by intratracheally instilling $^{18}O$ PC or $^{18}O$-BSA generated by $^{18}O_3$ exposure *in vitro*. Amounts of $^{18}O$ instilled were targeted to be similar to what was achieved following inhalation exposures to $^{18}O_3$ (see Table 2). A small increase in urinary $^{18}O$ was observed in all $^{18}O$-BSA and $^{18}O$-PC instilled rats (Fig. S2). Due to

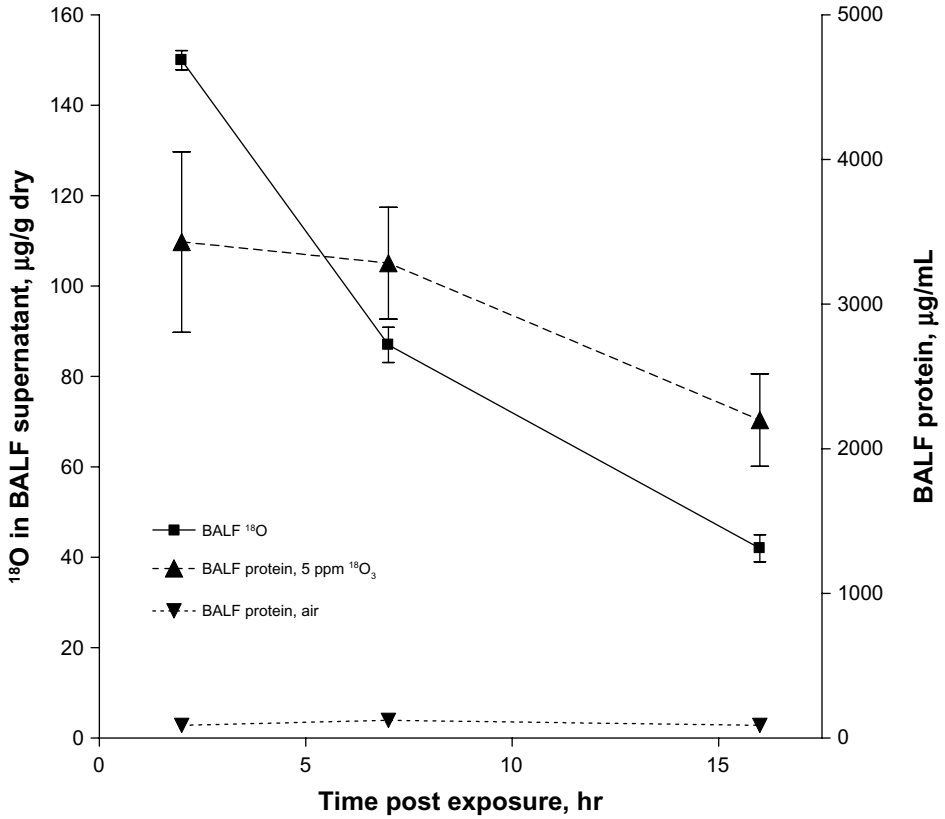



**Figure 4.** Disappearance of $^{18}O$ and total protein from bronchoalveolar lavage fluid (BALF) low speed supernatants following exposure to $^{18}O_3$ (5 ppm, 2 hr). Normal protein concentrations in BALF are ~0.1 mg/mL and are elevated by $^{18}O_3$ exposure. The amount of $^{18}O$ present in the BALF at 2 hr post exposure was about 20% of the amount of $^{18}O_3$ calculated to be removed from respired air (see Table 2 and Supplement C).



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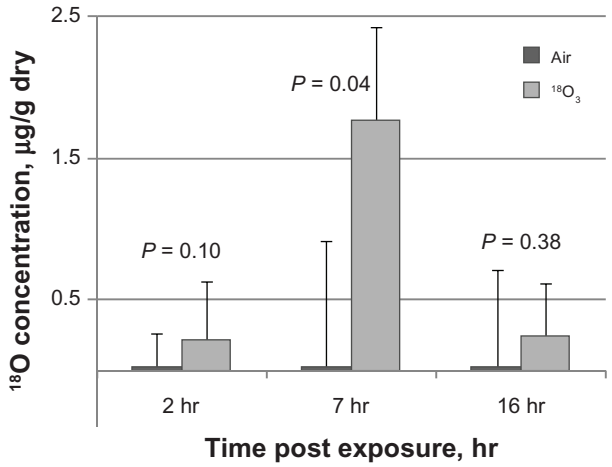



**Figure 5.** Concentration of excess $^{18}$O in blood plasma from rats breathing 5 ppm $^{18}O_3$ for 2 hours at 2, 7 and 16 hr post exposure. Significantly elevated plasma $^{18}$O was observed at 7 hr post exposure.

problems encountered with the pyrolysis of urine samples, and also to relatively low levels of $^{18}$O detected in these experiments, it was not possible to obtain the desired statistical rigor or to define a clear washout behavior for $^{18}$O PC or $^{18}$O-BSA. We estimate that ~12.3% and ~54% of the instilled $^{18}$O-PC or $^{18}$O-BSA, respectively, was excreted into the urine over the four days of collection (Fig. S2 and Table 2).

### Biochemical measurements made on urine

In addition to the dry weight of the urine samples, we measured urinary volume, creatinine, urea, total protein and albumin as potential denominators for expressing the $^{18}$O found in the urine. We found that urine volumes and dry weights (per day per rat) were correlated and that dry weights were always ~100 mg/mL of urine. Urinary albumin concentrations were always very low (<6 ug/mL). Urinary urea showed values of 1000–2500 mg/dL (Fig. S3) and were elevated in both the air and $^{18}O_3$ exposed rat urine at the early collection times. Urinary creatinine ranged from ~30–150 mg/dL and showed a similar increase at the first collection times for both air and $^{18}O_3$ exposed rats. The $^{18}O_3$ exposed rats had a more prolonged elevation of creatinine and urea levels than the air exposed rats (Fig. S4). Intratracheal instillation of $^{18}$O-BSA, $^{18}$O-PC and sham saline increased urinary urea and creatinine for the first two days of collection similar to the inhalation exposures to air and $^{18}O_3$. There was no difference between the three treatments (data not shown).

### Heat stability of $^{18}$O in urine

Samples of lyophilized urine from $^{18}O_3$ exposed rats were heated from 200 °C to 500 °C and remaining weights and $^{18}$O contents graphed (Fig. S5). Whereas the sample weights fell off rapidly to ~40% of the original dry weight as heat was increased to 200 °C, the $^{18}$O concentration was unaffected. As temperatures were further raised to 400 °C, $^{18}$O concentration fell to ~40% of unheated samples while sample weights did not show a further decrease. At 500 °C, both sample weight and $^{18}$O concentration were decreased to ~20% of unheated values.

## Discussion

We report here that the use of $^{18}O_3$ enabled quantification of the generalized product of $^{18}O_3$ reactions originating in the respiratory tract in urine and blood. These findings appear to be the first proof that $O_3$ reaction products leave the respiratory tract, pass through the blood, and are excreted in the urine. They also appear to be the first application of $^{18}$O technology to measuring products of oxidative stress in urine. The time course of appearance of $^{18}$O in blood and urine, as well as properties of the labeled material provide insights that may be useful in explaining extrapulmonary effects of $O_3$. For example, atherosclerotic plaque formation has been shown to be enhanced by $O_3$ exposure. It is possible that oxidized proteins and lipids leaving the lung through the pulmonary veins could deposit in the walls of arteries leaving the heart. The detection of $^{18}$O in blood plasma and urine proves that the reaction products of $^{18}O_3$ pass through the blood; however, the lower-than-expected $^{18}$O levels in blood plasma may suggest significant binding of $^{18}$O-labeled products to vascular endothelium. Our previous attempts to measure excess $^{18}$O in red blood cells have not been successful. The percentage of $^{18}$O label excreted over 4 days relative to the amount deposited through inhalation was high (53%) suggesting that little recycling of $^{18}O_3$ reaction product occurs. It also implies that the oxygen addition reactions induced by $^{18}O_3$ are irreversible, damaging, and must be removed. Our finding that pre-exposure of the rats to $O_3$ one week prior to the $^{18}O_3$ exposure did not appear to alter the urinary disappearance curve of $^{18}$O suggests that adaptation to the oxidative stress of $^{18}O_3$ does not involve altering the rate of adduct removal.



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Our observation that $^{18}O$ was relatively heat stable and also enriched in the high molecular weight fraction of the urine might guide future efforts to focus on specific chemical biomarkers. Details about the rates and times of excretion of $^{18}O$ might simplify and give direction to future urine collection for biomarker measurement. A published series of studies showed that some traditional measures of oxidative stress (isoprostanes and malondialdehyde in blood plasma and urine) that were effective following $CCl_4$—induced oxidative stress to the liver, were not effective following inhalation of $O_3$ in the rat at the same level of exposure as that employed here.[13–15] Our quantitation of $^{18}O$ in the blood plasma and urine suggests the possibility of finding other biomarkers that could be more effective in the future.

The $^{18}O$ label in urine could have originated from injured cells in the lung or vasculature that were replaced by proliferative repair or from simple transport of extracellular $^{18}O$-containing adducts of proteins and lipids. If simple transport of the labeled proteins in BALF were to occur, we would have expected to recover in urine about the same amount of $^{18}O$ that was present in the BALF supernatant fraction. We detected about 1/5th as much $^{18}O$ in BALF as the estimated $^{18}O_3$ retained by the rat. This percentage was lower than the percentage of $^{18}O$ that was excreted into urine (see Table 2 and Supplement C). Most of the $^{18}O$ present in the BALF was associated with plasma proteins that had leaked into the injured airway lumen during the $^{18}O_3$ exposure because of damage to the air-blood barrier of the lung. Our experiments with intratracheally instilled serum albumin or phosphatidyl choline pre-labeled in vitro by exposure to $^{18}O_3$ showed that ozonated proteins and surfactant lipid can leave the lung and appear in the urine, however, concentrations detected were lower than expected. Only about 12% of the instilled $^{18}O$-PC and 54% of $^{18}O$-BSA appeared to be recoverable in the 4 days of urine collection post exposure (Table 2). A previous study instilled $^{125}$Iodine labeled serum albumin into the alveoli and reported transport into the blood minutes following its instillation.[20] Previous studies of vascular injections of radiolabeled precursors of surfactant proteins and lipids showed that turnover of surfactant occurs rapidly—on the order of hours—rather than days as we observed here with $^{18}O_3$ reaction products. A high level of recycling of labeled surfactant lipids was also reported in normal rats[21] in contradiction to the present study where $^{18}O_3$ reaction products appeared to be in large part excreted. It appears, therefore, that the injured lung may release $^{18}O_3$ reaction products slowly (over days) in comparison to the normal turnover of proteins and surfactant lipids (over hours). The slow transport of labeled material through the blood or possibly sequestration and slow release of label from the vascular endothelium may explain why it was difficult to detect $^{18}O$ in blood plasma even though the quantities of $^{18}O$ passing through the blood are significant (see Supplement D).

It appeared that the elevated levels of urea and creatinine we observed following exposure or intratracheal instillations could have been due to the reduced urinary volumes and dry weights at the early times after exposures. Reduced water and food consumption, along with a concomitant decrease in urine volume excretion often occurs due to stress. The wire mesh exposure cages appear to induce a stress response even in control rats that is manifest as hyperthermia that lasts about 2 hr.[22]

The present study is limited due its exploratory and descriptive nature. The calculations of percentages of recovered $^{18}O$ in urine could be affected by estimates of $^{18}O_3$ inhaled and retained by the rats that are based on inexact allometric equations. $^{18}O$ measurement of tissue samples suffer from four sources of error: (1) preparatory column conditioning, (2) instrument drift, (3) sample memory effects, and (4) dependence on accurate background $^{18}O$ measurements included in each sample run.[23] It might be difficult to perform the present study at lower (and less injurious) exposure concentrations of $^{18}O_3$ because our sensitivity of detection of $^{18}O$ in tissues was at the lower limit. Urine samples showed more variability and difficulties due to column conditioning (possibly related to the presence of inorganics) than plasma samples. Tracing the fate of oxygen using a stable isotope such as $^{18}O$, is necessary because radioactive forms of oxygen have extremely short half lives ($<$134 sec).

In summary, we have shown that $^{18}O_3$ exposure of rats results in pathologically bound oxygen that is excreted into urine over a period of 4 or more days. Our findings suggest that new biomarker molecules specific to ozonized lung tissue could be identified in the future. The demonstrated transport of reaction products of $O_3$ formed in the lung or in the blood



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passing through the lung during exposure lends plausibility to published findings that $O_3$ inhalation could exert influences outside the lung. Future studies should search for $O_3$ reaction products in the vascular endothelium and investigate the chemical structures of oxidized biomolecules in urine.

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## Author Contributions
Conceived and designed the experiments: GEH. Analysed the data: GEH and RS. Wrote the first draft of the manuscript: GEH and RS. Contributed to the writing of the manuscript: RS. Agree with manuscript results and conclusions: GEH, RS and JM. Jointly developed the structure and arguments for the paper: GEH and RS. Made critical revisions and approved final version: GEH, RS and JM. All authors reviewed and approved of the final manuscript.

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## Competing Interests
Author(s) disclose no potential conflicts of interest.

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## Supplementary Data

### A. Determining the expected amount of $^{18}O$ taken up per rat based on $^{18}O_3$ gas uptake

In order to estimate the fraction of the inhaled $^{18}O_3$ that was detectable in urine, we calculated the expected umoles of $^{18}O$ retained per rat from breathing parameters. Stahl[1] derived the following allometric relationship to estimate minute ventilation (Ve) across several animal species: $379 * M^{0.8}$ where M = mass in kg and Ve has the units of mL/min. In the present study, the average mass of the rats was 0.224 kg; therefore, $Ve = 379\ (0.224^{0.8}) = 115$ mL/min. The fractional uptake of $O_3$ by rats has been reported as 47%.[2] Multiplying 5 mL of gaseous $^{18}O_3/10^6$ mL (5 ppm $^{18}O_3$) by the Ve of 115 mL/min/rat, and by 120 minutes/exposure, and by the fractional retention of $O_3$ by the rat of 0.47 gives the value of 0.032 mL of pure gaseous $^{18}O_3$ taken up per rat which equals a molar value 1.3 umoles of $^{18}O_3$ (using 41 umoles/mL of any gas at 25 °C). This molar quantity of $^{18}O_3$ yields $1.3 * 3 = 4.0$ moles of $^{18}O$ retained per rat. Note: some of our studies show that wire mesh exposure chambers induce a higher (+18%–27%) Ve than what is estimated by the Stahl, 1967 equation.[3]

### B. Determining the total amount of $^{18}O$ excreted in urine per rat in four days

We multiplied the micrograms of $^{18}O$ per gram dry weight of urine solids by the grams dry weight of urine solids per rat in each sampling period. The amounts of $^{18}O$ per rat that were present in the urine in each sampling period were added together to yield the total per rat assuming that each voided quantity was independent of the previous one.

### C. Determining the amount of $^{18}O$ per rat in BALF following $^{18}O_3$ exposure

The sample of BALF taken at 2 hr post exposure was assumed to contain the entire protein and $^{18}O$ label of the rat BALF, with subsequent sampling times irrelevant because they were derived from the same initial quantity. We multiplied the micrograms of $^{18}O$ per gram dry weight of BALF solids by the grams dry weight of BALF per rat to obtain the micrograms of $^{18}O$ per rat. To obtain the grams dry weight of BALF solids we added the saline used for BALF (8.5 mg NaCl/mL) to the BALF protein (~3 mg/mL) which gives 11.5 mg of dry weight per mL (we ignored the mg of lipid and of cells in the BALF because their contribution was small (<0.2 mg/mL). Multiplying the dry weight/mL by 8 mL instilled, we obtain 92 mg of dry weight in BALF per rat. At 150 ug $^{18}O$/gram dry weight in the BALF supernatant (see Fig. 4), we would have 13.8 ug of $^{18}O$ (or 0.8 umoles of $^{18}O$)/rat or ~20% of that retained by the rat (see above and Table 2).

### D. Determining the plasma concentration of $^{18}O$ if all of the BALF or intratracheally instilled $^{18}O$ was suddenly added to it

The blood volume per rat would be ~14.4 mL based on the formula $65.6\ M^{0.98}$ [1] and a body weight of 0.224 kg. Blood plasma volume is about half the blood volume or 7.2 mL. Blood plasma is ~8% dry weight. Thus, dry blood plasma/rat would be ~0.58 g. $^{18}O$/rat in BALF supernatant (see above) if added to blood plasma would result in 13.8 ug $^{18}O$/0.58 g dry or 23.8 ug $^{18}O$/g dry plasma. This value is much higher than the measured value at 7 hr post exposure of ~1.8 ug/g dry. In a similar manner, the rapid addition of $^{18}O$-BSA or $^{18}O$-PC into blood plasma should result in 32.4 ug $^{18}O$/0.58 g dry or ~56 ug/g dry or 28.8 ug $^{18}O$/0.58 g dry or ~50 ug $^{18}O$/g dry—much higher than the measured value of ~2–4 ug $^{18}O$/g dry (see Fig. S2).

### E. Determining the volume of blood plasma leaked into the pulmonary airways by $^{18}O_3$ exposure

Rats achieved a BALF protein level of ~3 mg/mL after 2 hr of 5 ppm $^{18}O_3$ exposure which compares to a normal background level of 0.1 mg/mL. Thus, BALF contains an excess of 2.9 mg/mL × 8 mL of instilled saline/rat. Rat blood plasma contains about 6% protein (60 mg/mL). Protein/rat leaked would be $2.9 \times 8 = 23.2$ mg, and volume of plasma leaked would be $23.2/60 = 0.39$ mL of blood plasma.



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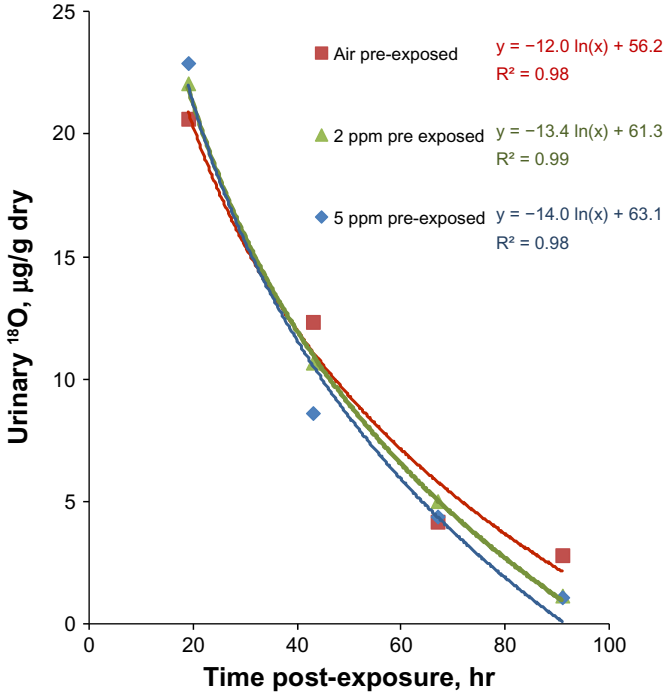



**Figure S1.** Effect of a pre-exposure to $O_3$ (5 ppm, 2 hr) one week previous to an exposure to $^{18}O_3$ (5 ppm, 2 hr). Mean values of $^{18}O$ concentration taken at the morning time were plotted along with their respective equations and R values of logarithmic trend lines. Note that the pre-exposure had a minimal effect on the washout curve of $^{18}O$ in the urine.

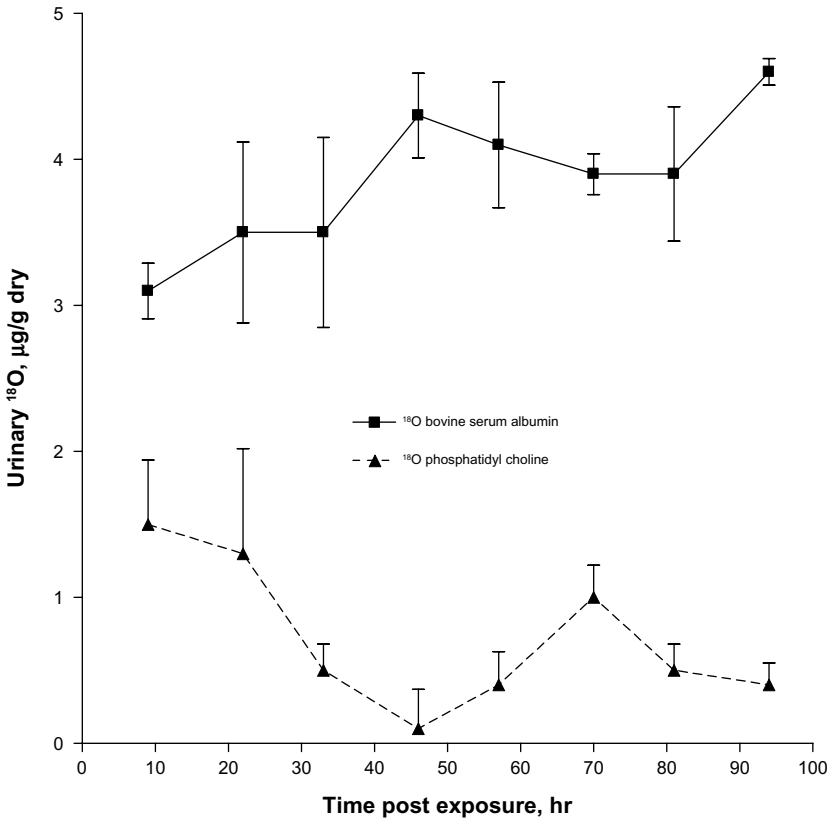



**Figure S2.** Levels of $^{18}O$ measured in urine following intratracheal instillation of bovine serum albumin or phosphatidyl choline that had been pre-labeled with $^{18}O$ by in vitro bubbling of $^{18}O_3$ through the solution. See Table 2 for estimation of $^{18}O$ recovery in urine. We were unable to perform the usual statistical analysis of the delta values on these samples because of drift encountered in the natural abundance samples. Therefore, the enrichments were calculated from the single most relevant natural abundance measurement.



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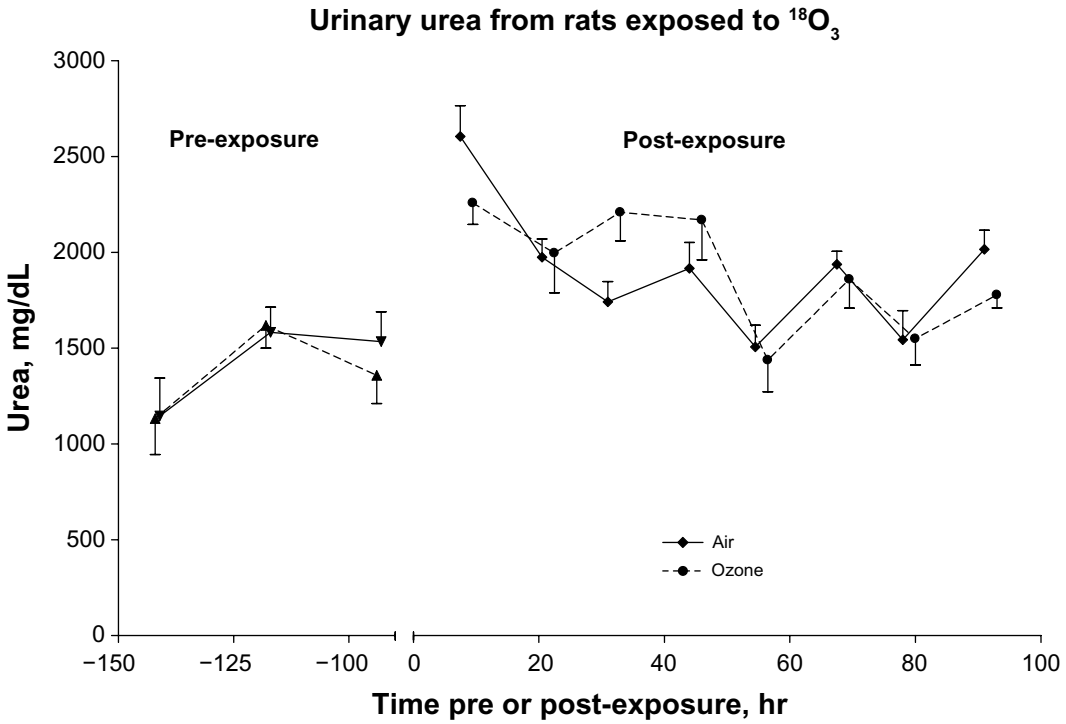



**Figure S3.** Urinary urea concentrations of rats pre- and post exposure to $^{18}O_3$, 5 ppm, 2 hr. Exposure to both air and $^{18}O_3$ resulted in more concentrated urine due apparently to stress induced by individual housing in wire mesh exposure cages.

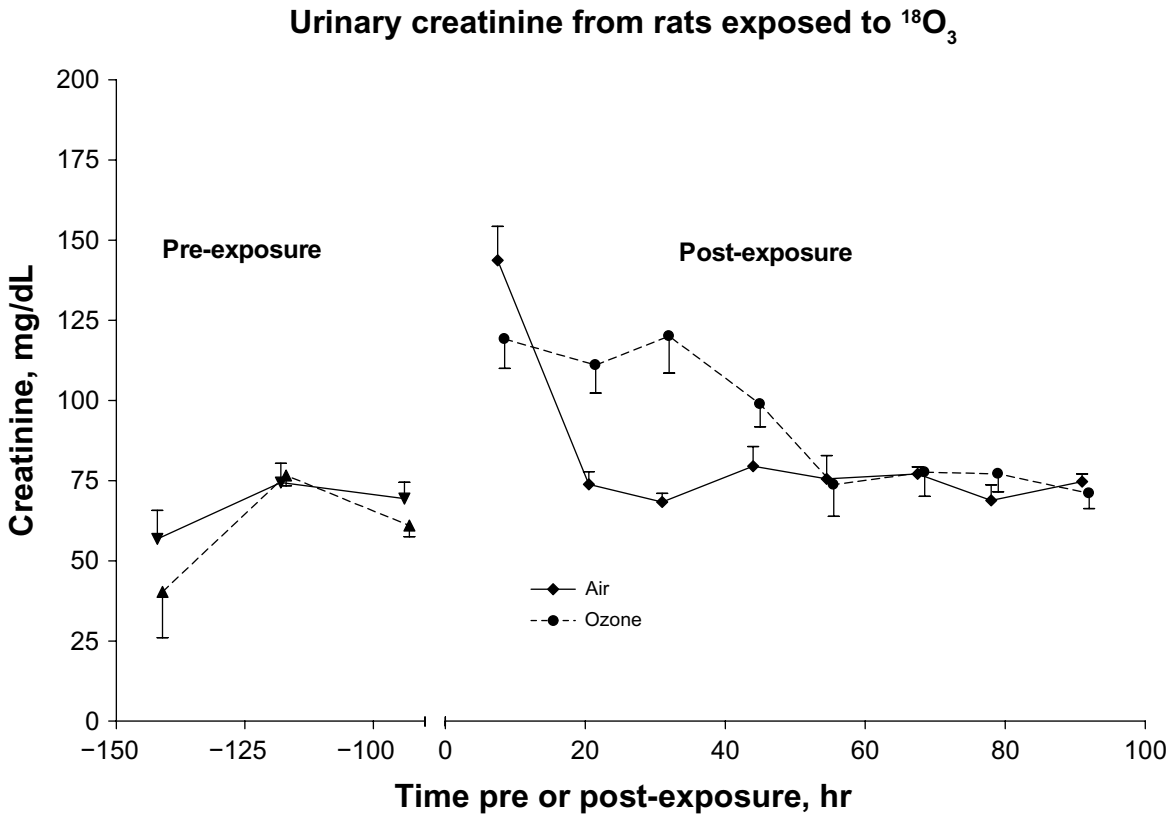



**Figure S4.** Urinary creatinine concentrations of rats pre- and post exposure to $^{18}O_3$, 5 ppm, 2 hr. Effects are similar to those seen with urea in the previous figure.



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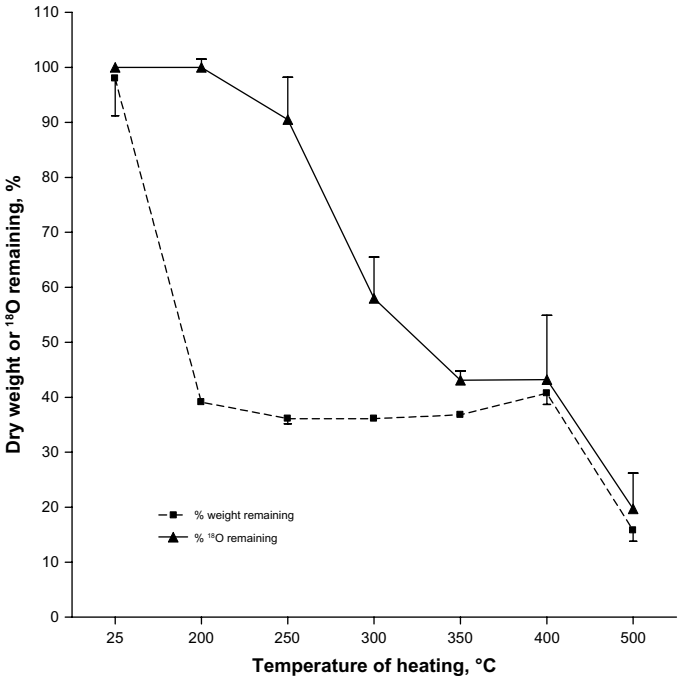



**Figure S5.** The effect of heating dried urine samples to temperatures up to 500 °C. Note that dry weight was decreased at lower temperatures than $^{18}O$ content. The appearance of the residues was as follows: 250 °C, black, 300 °C–400 °C, light gray turning to brown after cooling, 500 °C, white.

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