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Biomarkers of Oxidative Stress Study V: Ozone exposure of rats and its effect on lipids, proteins and DNA in plasma and urine

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Abstract

Ozone exposure effect on free radical-catalyzed oxidation products of lipids, proteins and DNA in the plasma and urine of rats was studied as a continuation of the international Biomarker of

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Oxidative Stress Study (BOSS) sponsored by NIEHS/NIH. The goal was to identify a biomarker for ozone-induced oxidative stress and to assess whether inconsistent results often reported in the literature might be due to the limitations of the available methods for measuring the various types of oxidative products. The time and dose-dependent effects of ozone exposure on rat plasma lipid hydroperoxides, malondialdehyde, F₂-isoprostanes, protein carbonyls, methionine oxidation, tyrosine- and phenylalanine oxidation products, as well as urinary malondialdehyde and F₂-isoprostanes were investigated with various techniques. The criterion used to recognize a marker in the model of ozone exposure was that a significant effect could be identified and measured in a biological fluid seen at both doses at more than one time point. No statistically significant differences between the experimental and control groups at either ozone dose and time point studied could be identified in this study. Tissue samples were not included. Despite all the work accomplished in the BOSS study of ozone, no available product of oxidation in biological fluid has yet met the required criteria of being a biomarker. The current negative findings as a consequence of ozone exposure are of great importance, because they document that in complex systems, as the present in vivo experiment, the assays used may not provide meaningful data of ozone oxidation, especially in human studies.

Keywords

biomarkers; oxidative stress; ozone exposure; rats

Introduction

The potential role of oxidative stress in the etiology and pathogenesis of a growing number of human diseases has been recognized in the past two decades and has been reported in various studies and review articles [1-4]. Free radical-mediated oxidative products of lipids, proteins and nucleic acids are thought to be the key biomarkers of oxidative stress in health and disease [5-9]. Although a variety of bio-assays for assessing oxidative stress status now exist [1, 10], the concept of such measuring, recognizing and quantitating of oxidative stress in clinical practice has not been approached as a real possibility because validated and standardized methods for its assessment have not yet been established.

Previous reports have examined several proposed markers of oxidative stress in a model system of CCl₄ administered to rats [11-13]. The criterion used to identify a good marker for the detection and quantitation of oxidative stress was a significant effect seen at two different doses at more than one time point studied. CCl₄-mediated oxidative damage was selected for the initial evaluation of the more general phenomenon of oxidative stress because it is well recognized that CCl₄ causes liver damage via a free radical mechanism [14, 15]. Rodent inhalation exposure to ozone was chosen as the second model of oxidative stress to determine whether exposure to ozone causes oxidative lung damage that can be quantitated by the identification and measurement of the presence of various products of oxidation in blood, plasma or urine. Although the molecular mechanisms of ozone-induced lung cell injury are not fully understood, one hypothesis is that ozone reacts with cell membrane lipids, inducing lipid peroxidation, aldehyde production and generation of lipid ozonation products that are considered to be involved in the early stage of inflammatory

response [16-18]. An earlier publication described the effects of ozone on the concentration of various antioxidants in plasma and in bronchoalveolar lavage fluid of Fisher rats exposed to two doses of ozone with specimen collection at several time points after the exposure [19].

In this international multi-laboratory and comprehensive validation study, the time and dose-dependent effects of 2 h ozone inhalation exposure on the production of oxidation products of lipids, proteins and DNA in biological samples of rats was evaluated. Measurements were performed on blood, plasma and urine collected from rats treated under the experimental protocol previously described [19]. Tissue samples were not included in this study as the criterion used to identify a good marker for the detection and measurement of oxidative stress was that a significant effect could be identified and measured in a biological fluid seen at both doses at more than one time point. It was hypothesized that plasma and/or urine levels of various oxidation products would increase in these biological fluids due to the oxidative stress induced by ozone exposure in a time-and dose-dependent pattern.

Materials and Methods

Chemicals and reagents

All chemicals and reagents used in the study were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Animals and treatment protocol

Male Fisher 344 rats (260-280 g) obtained from Charles River Laboratories (Raleigh, NC) were used in all experiments. The animals were housed three to a solid-bottom polycarbonate cage with a filter top. Animal rooms were maintained at 20-25°C with 35-70% relative humidity with alternating 12-h light and dark cycles. The rats had free access to deionized, reverse-osmosis-treated water and received autoclaved NIH 31 rodent chow (Zeigler Bros., Gardners, PA) *ad libitum*. For all experiments, rats were fasted overnight and then placed in ozone exposure chambers. Control rats received an equal exposure to air. Fasting continued through the experiment. Rats were anesthetized with Nembutal (0.1 ml/100 g body weight) and 5 ml blood was removed from the dorsal aorta proximal to its bifurcation into the common iliac arteries. The studies adhered to the National Institutes of Health Guidelines for the Care and Handling of Experimental Animals. All animal studies were approved by the National Institute of Environmental Health Sciences and the Environmental Protection Agency Review Boards.

Study design

Ozone Inhalation Exposure—Animals from each of the three dose groups (Control, 2 ppm and 5 ppm ozone) were exposed for 2 h and specimen collection was performed at several time points (2 h, 7 h, and 16 hours after the ozone exposure for blood plasma collection). Each group consisted of 5 or 6 rats for each analysis. Animal exposure treatment and sample preparation were described previously [19] and carried out at US EPA, Research Triangle Park (RTP), NC.

Exposures to ozone began no later than 8 am on a Monday. The 8 a.m. start time was in the light cycle for the animals, but it was their dormant period because rats are nocturnal and exercise during the dark cycle. Urine was collected twice daily (7:00 am and 6:00 pm) beginning Monday evening; the last collection was on Friday morning.

Each sample was marked with a code number so that investigators carrying out the corresponding assay measurements were not aware of the treatment type of the animals.

Whole blood and plasma sample preparation—Blood (5 ml) was drawn through single draw vacutainer needles of 21 G into open vacutainer blood collection tubes containing heparin. The contents of the tubes were mixed by gently inverting the tubes for 2-3 times. For plasma specimens blood was centrifuged (2,000 rpm for 10 min at 4°C), and both the blood and plasma were frozen immediately on dry ice and stored at -70°C.

Urine sample preparation—Rats were housed individually in 4400 cm³ (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 ozone, the animals were returned to the metabolism cages, and urine was collected for 4 days (2 times/day, at 7 a.m. and 6 p.m. each day). The temperature of the urine collection tubes was maintained at 4 degrees C by enclosing them in coils made from copper tubing through which cooled ethylene glycol was circulated. After collection, urine samples were centrifuged to remove extraneous debris (400×g, 15 min., 4 degrees C), and the supernatants were removed and stored at - 80°C.

I. Oxidation products of lipids in plasma and urine

I. A. Plasma

I. A. 1. Lipid hydroperoxides

I. A. 1. 1. A Lipid hydroperoxide assay kit: (catalog No 705002) by Cayman Chemical Company (Ann Arbor, MI) was used to measure hydroperoxides directly, utilizing the redox reaction with ferrous ion. Hydroperoxides were extracted into chloroform and reacted with ferrous ions to produce ferric ions. The resulting ferric ions were detected using thiocyanate ion as the chromogen. The hydroperoxide concentration was determined based on the absorption at 504 nm using a Beckman DU-7 spectrophotometer at room temperature. The range of the assay is 0.25-5 nmol hydroperoxide per tube. The experiments were performed at Rush University Medical Center, Chicago, IL.

I. A. 1. 2. Cholesteryl ester hydroperoxides (CE-OOH): Frozen plasma samples were thawed and then extracted with 10 vol cold methanol and 50 vol hexane as described previously [20]. The hexane phase was collected, dried and re-suspended in isopropyl alcohol, and then subjected to HPLC post-column chemiluminescence detection, as described in [21]. The detection limit of the assay was 200 fmol for cholesteryl linoleate hydroperoxide, used as a standard of CE-OOH. Measurements were done at the Heart Research Institute, Camperdown, Australia.

I. A. 2. Malondialdehyde (MDA): Several different assays for measurement of plasma MDA levels were used.

I. A. 2. 1. MDA was assayed using the Bioxytech LPO-586 colorimetric assay kit with 3rd derivative spectroscopy: The Bioxytech LPO-586 colorimetric assay kit assay is based upon the reaction of the chromogenic reagent *N*-methyl-2-phenylindole (NMPI) with MDA at 45°C. One molecule of free MDA combined with 2 molecules of the chromogenic agent to produce a stable chromophore with a maximal absorbance at 586 nm. The concentration of MDA was determined with 3rd derivative spectroscopy. Sensitivity is defined as 80 pmol/ml. The assay was performed at OXIS Laboratories, Inc., Portland, OR.

I. A. 2. 2. MDA measured by gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS): Plasma MDA concentrations were also measured by using GC-MS in the negative chemical ionization mode as described by [22]. The stable isotope internal standard [²H₂] MDA and an antioxidant (2,6-tert-butyl-4-methylphenol) were added to the plasma samples. The samples were then incubated at room temperature in 6.6 mol H₂SO₄ for 10 min to hydrolyze the aldehydes from the proteins. Plasma MDA was converted to a stable pentafluorophenyl hydrazine derivative which was quantified by using the GC-MS in the negative chemical ionization mode. The method detection limit is 5 pg/ml. The assay was performed at Children's Hospital Oakland Research Institute, Oakland, CA.

I. A. 3. 8-iso-PGF_{2α} measured by gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) and radioimmunoassay: Different methods by different laboratories were used to measure the effect of ozone on the plasma levels of F₂-isoprostanes (also known as 8-*epi*-PGF_{2α} and iPF_{2α}-III and 15-F_{2t}-IsoP).

I. A. 3. 1. Free F₂- Isoprostanes (8-iso-PGF_{2α}) measured by radioimmunoassay: The plasma samples from this study were analysed for free 8-iso-PGF_{2α} by a radioimmunoassay developed by Basu [14]. In brief, an antibody was raised in rabbits by immunization with 8-iso-PGF_{2α} coupled to bovine serum albumin (BSA) at the carboxylic acid by 1,1'-carbonyldiimidazole method. The cross-reactivity of the antibody with 8-iso-15-keto-13,14-dihydro-PGF_{2α}, 8-iso-PGF_{2β}, PGF_{2α}, 15-keto-PGF_{2α}, 15-keto-13,14-dihydro-PGF_{2α}, TXB₂, 11β-PGF_{2α}, 9β-PGF_{2α} and 8-iso-PGF_{3α} was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6 %, respectively. The detection limit of the assay is 23 pmol/l. All measurements were performed at Uppsala University, Uppsala, Sweden.

I. A. 3. 2. Free 8-iso-PGF_{2α} concentration: Free 8-iso-PGF_{2α} concentrations in plasma were determined by gas chromatography/negative ion chemical ionization mass spectrometry (GC/NICI-MS) by the method of Morrow *et al.* [3]. Briefly, [²H₄] 8-iso-PGF_{2α} internal standard was added to plasma and F₂-isoprostanes were extracted with a C₁₈ Sep-Pak Cartridge (Waters Associates). The extracted F₂-isoprostanes were converted to pentafluorobenzyl ester and purified by TLC, after which they were converted to a trimethylsilyl ether derivative and subsequently analyzed by GC-MS. Limits of detection of the assay are 2 pg. The assay was developed and performed at Vanderbilt University, Nashville, TN.

I. A. 3. 3. Hydroxyeicosatetraenoic acids (HETE) isomers present in plasma samples:

Negative ion chemical ionization GC-MS analyses of plasma samples obtained from control- and ozone-exposed rats were used to determine HETE levels. The method of Thomas et al [23] was modified and improved as described in detail by Wiswedel et al [24]. Briefly, 12-hydroxyheptadecatrienoic acid (12-HHT) as the internal standard was added to the aliquots of plasma. Samples were immediately hydrogenated with hydrogen gas using PtO_2/H_2 as catalyst. The ester lipids were cleaved by alkaline hydrolysis and the hydroxyl fatty acids were enriched by solid phase extraction on C_{18} -cartridges and derivatized with pentafluorobenzylbromide and bis(trimethylsilyl)trifluoroacetamide. Under our chromatographic conditions only 15-HETE was well resolved and could be identified unambiguously, whereas HETEs with hydroxyl groups in the positions 5-12 partially overlap and were taken together as one fraction (5-12-HETEs). The limit of detection for each HETE isomer of the method described is 5 pg. The assay was developed and performed at Otto-Von-Guericke-University, Magdeburg, Germany.

I. B. Urine

I. B. 1. Urinary MDA analyses by HPLC: To 500 μl urine samples, 25 μl BHT and 750 μl 0.5% TBA solution were added, in that order. Samples were incubated at 60°C for 60 min. After cooling, chloroform (750 μl) was added and mixed well. Following centrifugation, the aqueous layer was removed and used for HPLC analysis (column 250 $\times$ 46 mm; mobile phase, 6:4(v/v) 0.04 M acetate buffer (pH 5.5)/MeOH; detection, 532 nm). Sensitivity is defined as 3.29 standard deviations about zero or 52 pmol/ml. The procedure was developed and performed at OXIS Laboratories, Inc., Portland, OR.

I. B. 2. Urinary 8-iso-PGF $_{2\alpha}$ measured by a radio-immuno-assay. Measurements of urine samples were performed by the method of Basu [14]: The urinary levels of 8-iso-PGF $_{2\alpha}$ were adjusted for creatinine values, which were measured by a commercial kit (ILTM Test by Monarch Instrument, Amherst, NH, USA). The intra-assay coefficient of variation of 8-iso-PGF $_{2\alpha}$ is 14.5% at low and 12.2% at high concentrations. The detection limit is 8 pg/ml. All measurements were performed at Uppsala University, Uppsala, Sweden.

II. Oxidation products of proteins in plasma and urine**II. A. Plasma**

II. A. 1. Protein carbonyls: The level of plasma protein oxidative damage, indicated by the protein carbonyl content [25], was measured according to the method of Levine *et al.* [26], based on derivatization of carbonyls with tritiated borohydride. The isolated protein was counted in a Beckman scintillation counter and calculated using a standard curve. Because there is no absolute standard for the protein carbonyls, the sensitivity of the assay has been roughly ascertained on the basis of intra-assay variability (0.1 nmol/mg protein) and the minimal amount of protein needed for the assay (0.5 mg). The measurements were performed at the University of Southern California, Los Angeles, CA

II. A. 2. Methionine oxidation: Methionine oxidation was measured according to Levine *et al.* [27] using CNBr cleavage. CNBr cleaves peptide bonds on the carboxyl side of methionine but does not cleave methionine sulfoxide. Serum samples were hydrolyzed with

HCl in the presence and absence of CNBr. In the absence of CNBr the amount of methionine detected represents the level of methionine plus methionine sulfoxide present in the sample since the acid hydrolysis converts the methionine sulfoxide to methionine. In the presence of CNBr the methionine is destroyed and the sulfoxide residue is converted to methionine by the acid hydrolysis and represents the methionine sulfoxide level in the protein. The samples were analyzed on a Beckman Model 7300 amino acid analyzer. The lower limits of detection of the amount of methionine sulfoxide are between 1.0 and 1.5% of the total methionine found. The methionine concentrations were measured at Weill Medical College of Cornell University, New York, NY, USA.

II. A. 3. Tyrosine and phenylalanine oxidation products: Oxidation products of protein-bound tyrosine (di-tyrosine, 3,4-dehydroxyphenylalanine) and phenylalanine (ortho- and meta-tyrosine) were quantified in protein hydrolysates by UV and fluorescence spectroscopy after separation by HPLC using a method described previously [28]. Limit of detection dityrosine, DOPA, other hydroxytyrosines is typically ~ 1 pmole with a good fluorescence detector, Ex 280 nm, Em 320 nm. These tyrosine and phenylalanine products were measured at The Heart Research Institute, University of Sydney, Sydney, Australia.

II. A. 4. Protein associated heme in blood: An aliquot of blood was removed from each sample, diluted in pure water to ensure hemolysis before and adjusted to pH 5 for HPLC analysis as described previously [29]. There was no evidence of the extent of oxidative damage to the heme inducing covalent heme globin cross-links. Therefore the fraction of heme eluting associated with protein absorbance was measured and compared (by area) to the fraction eluting as free heme. The method detection limit gives a coefficient of variance in the order of 0.05 or 5%. The measurements were performed at School of Biological Sciences, University of Essex, Colchester, UK.

Lung histopathology and biochemical analyses

Lung histopathology and biochemical analyses were performed and described in more detail previously [19]. In addition, other clinical parameters such as plasma cholesterol and urinary creatinine were determined. Creatinine was determined with Sigma kit 557 (Sigma Diagnostics, St. Louis, MO). Protein was assayed using the Coomassie plus protein assay (Pierce, Rockland, IL). The assays were adapted for analysis on a Cobas Fara II clinical analyzer (Roche Diagnostics, Branchburg, NJ). Biochemical analyses were performed at the EPA, Research Triangle Park, NC.

Statistical Analysis

Statistical comparisons were performed by analysis of variance, using pooled estimates of error. When the variance increased substantially with the mean, data were logarithmically transformed prior to analysis. Where multiple urinary samples were obtained from the same animals, both the assay variance within animals and the variance between animals were taken into account in the analysis. Exposed to ozone groups of rats were compared to controls at the same time point. Values of $p < 0.05$ were considered statistically significant. Statistical analyses were performed at NIEHS, RTP, NC.

Results

Lipid Oxidation Products

Plasma—The relevant plasma concentrations of the lipid peroxidation products determined in this study are presented in Table 1. Lipid hydroperoxides (LOOH), cholesterol ester hydroperoxides (CE-OOH), malondialdehyde (MDA), F₂-isoprostanes and HETE's are major endogenous products derived from oxidation of plasma lipids and therefore were selected as candidates for the identification and quantitation of oxidative stress induced by ozone exposure. Exposure to both doses of ozone produced measurable levels of all these products in plasma samples. However, the increases were not significantly different from those of the controls although CE-OOH data for the 16 h time point appeared to show increase from 0.17 ± 0.06 to 0.32 ± 0.15 and 0.43 ± 0.10 for control, 2 ppm and 5 ppm respectively (Table 1).

Different assays were used to measure F₂-isoprostanes in plasma since these products of lipid peroxidation were identified as a marker of oxidation in the previous study using CCl₄ as a model oxidative stress [12, 13]. The F₂-isoprostane levels in plasma measured by dissimilar assays were not changed by either dose of ozone at any time point studied. HETEs concentrations in plasma were also not different from the controls (Table 1). In general, ozone exposure did not cause an increase of lipid peroxidation products in plasma except the elevation of CE-OOH concentrations in rat plasma only at 16 h after the ozone exposure (Table 1).

Urine—Specimen collection was done for an extended period of time and oxidation products of lipids present in the urine samples were measured at 8 h, 22 h, 32 h, 46 h, 56 h, 70 h and 80 h after the exposure to ozone. Determination of MDA by HPLC with chromatography demonstrated no significant differences between control groups and groups exposed to ozone at any post-exposure time (Table 2).

Measurement of F₂-isoprostanes in urine samples by radioimmunoassay showed no significant differences between controls and groups exposed to ozone at any post-exposure time (Table 2). These data were similar to the results from the plasma samples measurements both by radioimmunoassay and GC/MS.

Protein Oxidation Products in Plasma and Urine—To determine whether ozone exposure is associated with increased protein oxidation in vivo, protein carbonyls, methionine sulfoxidation, protein associated heme, and various tyrosine and phenylalanine oxidation products (*di-tyrosine*, *3,4-dehydroxyphenylalanine*, *ortho-* and *meta-tyrosine*) were measured 2 h, 7 h, and 16 h after the exposure. Ozone exposure did not induce significant changes in the plasma of the rats at any of the doses given at any of the time points studied (Table 1).

Table 1 shows that all of protein oxidation products assessed were close to those of the corresponding control groups. No statistically significant changes or even tendencies could be identified. The data for tyrosine and phenylalanine oxidation products are not shown as

they were either difficult to detect or unchanged. Histopathological evaluation of lungs as a result of the ozone exposure has been described earlier [19].

Discussion

The present ozone exposure study was conducted as a continuation of the multi-laboratory international Biomarker of Oxidative Stress Study (BOSS) with its main goal to identify products of oxidation that can be used for measurement of oxidative stress in biological systems with the ultimate aim to further apply them in humans. Ozone was chosen as the second model of oxidative stress induction after CCl_4 in the BOSS study. Ozone is a strong oxidizing agent, moreover it is known to have a variety of effects *in vivo* including lung inflammation, alveolar epithelial damage, and changes in chemical composition of lung lavage fluids as well as the induction of an inflammatory response that lasts several days after exposure [30-33]. Studies have suggested that at least some of these effects are initiated by free radical reactions which are induced by the decomposition of secondary ozonides [17, 18, 32]. Free radicals have been detected using ESR spin trapping techniques in lipid extracts of lung tissue obtained from rats exposed to ozone [34], [35]. In a previous publication, the role of plasma antioxidants as markers of oxidative stress induced by ozone was investigated and it was concluded that their measurement was not useful for the assessment of oxidative damage induced by ozone *in vivo*. [19]. It is well known that most markers of oxidative stress are assumed to be a consequence of free radical attack on macromolecules resulting in lipid-, protein-, and DNA oxidation [1, 36]. However, in the case of ozone literature data indicate that some of the fastest reactions are for direct reaction with Cys residues (and GSH), with $k \sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$, with the rate constants for reaction with other aminoacid side chains being considerably slower [37]. In contrast, reaction with mono- and polyunsaturated fatty acids and their esters (oleic and linoleic) and α -tocopherol have been reported as being $1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [37]. On the basis of this data it might be expected that major changes would occur in the thiol-disulfide status of the exposed animals, with lesser changes to other targets. Nonetheless our previous study showed that ozone exposure did not change the thiol-disulfide status of the rats [19]. This report examined two concentrations of ozone which represented the higher and lower doses of response for lung toxicity according to prior histopathological findings [19] and the available literature data [38]. Because molecular products formed from the reaction of free radicals with biomolecules are more stable than the free radicals themselves, in the present study, the time- and dose- dependent effects of ozone on the generation of various oxidation products of lipids, proteins, and DNA in rat plasma and urine were examined. The questions examined in this study were the same as in the previous CCl_4 -induced oxidative stress report [12] namely: (1). Can the oxidative products of lipids, proteins or DNA in plasma and urine be measured and identified as valid biomarkers of oxidative stress (2) what are the best techniques to measure them and (3) how do the currently available analytical procedures for their measurement compare?

Effect of ozone exposure on plasma and urine products of lipid peroxidation

Plasma—Products of lipid peroxidation such as 4-hydroxy-2-nonenal and isoprostanes have been detected in the lung from ozone exposed humans and animals [39] [40] [41].

Different mechanisms have been described by which ozone may enhance oxidative damage and a variety of relatively stable decomposition end products of oxidation can be measured in plasma and urine as indirect indexes of oxidative stress [42-44]. Lipid hydroperoxides (LOOH) are well known primary lipid oxidation intermediates of peroxidative reactions. Their measurement is thought to reflect an early response of the target cell to an oxidative stress [45-47]. Because certain cholesteryl-ester hydroperoxides (CE-OOH) are the predominant lipids in lipoproteins and hence plasma, they have been measured very effectively in studies related to the peroxidation of plasma and lipoprotein lipids [45, 48-50]. In the current work a 2 h exposure of rats to ozone at both low and high doses resulted in no evidence for an elevation of plasma LOOH. These data are in agreement with one report [51] but contradictory to another [52]. Some studies and reviews have shown measurement of plasma LOOH to be non-specific, semiquantitative and very often subject to false interpretation due to interference by non-peroxidized compounds [34, 36, 39, 40, 41, 42]. Although a tendency of increase of CE-OOH was found at 16 h, no evidence for ozone exposure triggering an increase in the plasma level of CE-OOH was indicated in the present in vivo study for the earlier time points. In vitro plasma exposure to ozone at levels up to 8 ppm for 2 h did not produce an increase in CE-OOH [53].

Two other products - malondialdehyde and F₂-isoprostanes - have been used extensively as markers of lipid peroxidation and oxidative stress in vivo [42, 43, 54]. The HPLC and GC/MS methodologies used in the present study represent the most reliable analytical techniques for the specific isolation, unequivocal identification and accurate quantification of these products in biological samples. When two different isolation methods for the measurement of MDA (3-d derivative spectrophotometry and GC/MS) and two different methods for the evaluation of F₂-isoprostanes (radioimmuno-assay and GC/MS) in plasma of rats were used, no significant differences between the experimental and control groups at either ozone dose time point studied could be identified. Other authors have reported an enhancing effect of ozone on the formation of MDA and F₂-isoprostanes after acute and chronic ozone exposure of humans [39, 55-57]. Additionally, human data suggest that inhalation to ambient ozone impacts numerous cell types in the lung, elicits a variety of responses including cellular damage, enhanced apoptosis, cytokine production and biologic response to ozone is dependent on a complex interaction with innate immune signaling [58] [59] [44].

Although rats and other species, including humans, vary considerably in their response to ozone, [60] the present data indicate a lack of dose response when examining ozone's effect on primary and secondary products of lipid peroxidation that were utilized in this study. These data further demonstrate that plasma F₂-isoprostanes and MDA, measured by GC/MS or radioimmuno-assay are not useful for the assessment of oxidative stress induced by ozone exposure.

Urine—The levels of lipid peroxidation products MDA and F₂-isoprostanes in the urine of rats exposed to ozone were examined in this study as they can be obtained noninvasively. Urine was collected as spot samples in the present study and the concentrations of both MDA and isoprostanes were adjusted by the creatinine level. Multiple urine samples were collected repeatedly over time in order to obtain the best estimate for the excretion of MDA

and F₂-isoprostanes. The time-course for specimen collection was extended to additional time points up to 80 h. In part this design was utilized as exposure to ozone for a relatively short duration has been shown to result in pulmonary and biochemical changes that are essentially biphasic [52]. Although urinary F₂-isoprostanes may partly come from lipid peroxidation in the kidney, evidence suggests that they originate primarily from free F₂-isoprostanes filtered from the circulation [61, 62]. In this study we demonstrated that both doses of ozone exposure did not produce significant increase. Literature data that show the biochemical and morphological changes of initial decreases versus subsequent increases of metabolic activities to be dependent on ozone concentration, the length of exposure, and the animal species and strains [52, 60]. Studies comparing GC-MS methods and enzyme immunoassays of the same samples demonstrated that the values from the various assays sometimes correlated only in terms of trends [54, 63, 64]. However, use of urinary isoprostane measurements to detect or monitor disease states is problematic because it varies between studies and among different species. For example, false elevations in measured urinary isoprostanes from ex vivo formation has been reported when excessive lipid is present in control urine samples [65]. The available evidence suggests that measurement of F₂-isoprostanes is one of the approaches to identify oxidative stress status in vivo and it is always important to recognize that more than one product of oxidation should be measured when evaluating for the presence of oxidative stress. In the present study MDA concentration in urine was measured as another end product of lipid peroxidation. Urinary MDA levels were measured by HPLC. No significant difference for this biomarker at both doses of ozone and at all seven time points was seen between the experimental and the control groups. Issues of concerns with MDA measurements have been discussed previously where it was pointed MDA can form both from thromboxane synthase and as a result of free radical lipid peroxidation [12]. No literature data examining the effect of ozone exposure on urinary MDA concentrations in either experimental animal models or humans could be found. It must be pointed out, however, that the failure to detect elevated levels of aldehydes in either plasma or urine could be due either to the volatility of these aldehydes or be caused by a reaction of these aldehydes with proteins and therefore not detected using the methodology in this study [53].

Effects of ozone exposure on plasma/urine oxidation products of proteins and DNA

Protein oxidation can be estimated by a number of methods that range from nonspecific indicators of oxidation to highly sensitive assays of specific protein side-chain oxidation products. It is well known that proteins are a major target for oxidants as a result of their abundance in biological systems and their high rate constants for reaction with many (but not all) oxidants [5]. Thus, protein-based carbonyl groups produced as a result of oxidation have been used as an easily measured, relatively stable indication of protein oxidation and potential damage [66]. The present data related to protein carbonyls measured in plasma by HPLC revealed no effect at either dose of ozone or at any of the three time points assessed. In vitro exposure of human plasma to ozone has been shown to produce oxidative damage to plasma proteins [53, 66]. The levels of ozone exposure used in these in vitro studies, however, were much higher (16ppm) than those used in the present in vivo study (2ppm and 5 ppm) moreover, the concentration of ozone reaching the blood is certainly much lower [67, 68]. Likewise, use of measurements of protein carbonyl production and levels measured

in vivo may not be as accurate as these obtained in vitro, as generation of ketones and aldehydes resulting from the metabolism of lipids or sugars may bind to proteins [69].

Methionine oxidation

Highly specific methods have been developed for the detection and assay of the different kinds of protein modification [5, 70, 71]. The sulfur-containing amino acid residue methionine present in a peptides/proteins has been shown to be readily oxidized to methionine sulfoxide by ozone [5, 70, 71]. The specific mechanism by which this sulfoxide is formed is not completely clear [5]. The measurement of methionine sulfoxide levels in plasma proteins in the present study did not differ between experimental and control animals. Although surface-exposed methionine and cysteine residues of proteins are particularly sensitive to oxidation relative to other amino acids, the oxidation of these sulfur-containing amino acid residues is usually reversible with low extents of oxidation as used here (i.e. in the absence of over-oxidation to methionine sulfone for Met, and cystic acid for Cys, which are irreversible lesions) [5, 70, 71].

The data presented showed no obvious change between controls and experimental animals in other amino acid oxidation products (e.g. o-, m-, di-tyrosine) that could be related to either the dose or time after ozone exposure. This is consistent with the slow rate of reaction of these species with ozone discussed above. It needs to be recognized however, that the accumulation of oxidized protein is a complex function of the rate of ROS formation, antioxidant levels, and the ability to proteolytically eliminate oxidized proteins. Therefore in complex systems, as is present in an in vivo experiment the assays used may not provide meaningful data as a result of the oxidative insult [5, 70, 71].

Oxidation of hemoglobin has proven to be a useful biomarker of oxidative damage because it represents 95% of the total protein in erythrocytes and its oxidation is known to produce a spectral shift that can be quantitated to determine the concentration of various oxyhemoglobin products [29]. No evidence of protein-associated heme oxidative damage was seen in the present study. Nevertheless, some oxidation of protein-associated heme was observed (5-7% of total heme) in a few of the samples. The present findings support literature data that ozone neither damages erythrocytes, nor induces clinically significant intracellular hemoglobin [72] and contradicts reports that ozone reacts specifically with the hemoglobin within the red blood cells and selectively occurs at the iron atoms sites within the heme groups of hemoglobin [73].

Concluding Remarks

Ozone exposure of rats was another example of the more general phenomenon of oxidative stress besides the classical model of CCl₄ exposure. The investigation result is important as different oxidative insults may give rise to distinct oxidation products. Accurate methods are essential to identify what roles reactive species and oxidative damage play in health and disease. Currently, a very large number of methods exist by which oxidation of lipids, proteins and DNA can be estimated with different end points. The current ozone exposure study of rodents demonstrated that all products of oxidation measured in blood, plasma and urine did not fill the criterion used to recognize a marker of ozone exposure such that a

significant effect could be identified and measured in a biological fluid seen at both doses at more than one time point.

The current negative findings as a consequence of ozone exposure study are important because they document that identifying a specific biomarker of oxidative stress is complicated, not easy, and many factors need to be taken into consideration. For example, assay sensitivity and specificity may be lacking, free radical mechanism of oxidative damage may not be relevant, or there is always the possibility that the oxidative injury may have occurred and repaired prior to its detection. In addition, the measurement of these various products may suggest that contradictory results may be the result of inter-laboratory variations and artifacts. The emphasis of the current study was to detect and quantitate oxidative products in biological fluids that are easy to obtain and process. Despite all the work accomplished in the BOSS study of ozone, no available product of oxidation measured has yet met the required criteria of being a biomarker, that is sensitive, specific, applicable to stored specimens and, most importantly, relevant to animal and human studies. As in the previous CCl₄ BOSS study, it should be mentioned that quantification of tissue oxidation products were not assessed in the ozone study.

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Highlights

The following oxidized molecules in plasma and urine are not markers of ozone: Lipid hydroperoxides, MDA, F₂-Isoprostanes, Cholesteryl ester hydroperoxides, HETEs Protein Carbonyls, Methionine sulfoxide, Tyrosine Products (nitro-, o/m, di) DNA strand breaks, 8-OHdG

Table 1
Effect of ozone exposure on oxidation products of lipids and proteins in rat plasma

Plasma	2 h			7 h			16 h		
	Control	Ozone 2ppm	Ozone 5ppm	Control	Ozone 2ppm	Ozone 5ppm	Control	Ozone 2ppm	Ozone 5ppm
Dose/Assay									
LOOH (μM) (by LPO assay kit)	1.6 $\pm$ 0.3	2.0 $\pm$ 0.6	1.8 $\pm$ 0.5	2.4 $\pm$ 0.5	1.2 $\pm$ 0.3	2.5 $\pm$ 0.4	3.0 $\pm$ 0.7	1.3 $\pm$ 0.5	2.6 $\pm$ 0.4
CE-OOH (μM) (at 0 h)	0.35 $\pm$ 0.19	0.25 $\pm$ 0.11	0.37 $\pm$ 0.23	0.22 $\pm$ 0.10	0.38 $\pm$ 0.18	0.30 $\pm$ 0.05	0.17 $\pm$ 0.06	0.32 $\pm$ 0.15	0.43 $\pm$ 0.10
MDA (μM) (by TBA 3 rd derivative)	0.092 $\pm$ 0.005	0.125 $\pm$ 0.012	0.104 $\pm$ 0.003	0.095 $\pm$ 0.004	0.099 $\pm$ 0.005	0.105 $\pm$ 0.008	0.106 $\pm$ 0.009	0.105 $\pm$ 0.007	0.098 $\pm$ 0.006
MDA (μM) (by GC-MS)	0.20 $\pm$ 0.01	0.22 $\pm$ 0.02	0.21 $\pm$ 0.02	0.23 $\pm$ 0.04	0.21 $\pm$ 0.02	0.29 $\pm$ 0.03	0.20 $\pm$ 0.01	0.22 $\pm$ 0.02	0.24 $\pm$ 0.03
F ₂ -Isoprostanes (ng/ml) (by radioimmuno - assay)	0.104 $\pm$ 0.007	0.086 $\pm$ 0.008	0.109 $\pm$ 0.019	0.112 $\pm$ 0.036	0.076 $\pm$ 0.013	0.094 $\pm$ 0.021	0.083 $\pm$ 0.004	0.079 $\pm$ 0.005	0.090 $\pm$ 0.015
Free F ₂ -Isoprostanes (ng/ml) (by GC-MS)	0.051 $\pm$ 0.005	0.053 $\pm$ 0.004	0.053 $\pm$ 0.005	0.053 $\pm$ 0.003	0.069 $\pm$ 0.012	0.059 $\pm$ 0.003	0.057 $\pm$ 0.008	0.062 $\pm$ 0.007	0.061 $\pm$ 0.003
15-HETE (nM) (by GC-MS)	93.0 $\pm$ 22	52 $\pm$ 8	83 $\pm$ 19	76 $\pm$ 20	103 $\pm$ 22	97 $\pm$ 12	48 $\pm$ 7	52 $\pm$ 9	79 $\pm$ 30
5-12-HETE (nM) (by GC-MS)	1587 $\pm$ 308	920 $\pm$ 166	1419 $\pm$ 249	1198 $\pm$ 301	1477 $\pm$ 312	1518 $\pm$ 246	887 $\pm$ 109	947 $\pm$ 156	944 $\pm$ 377
Protein Carbonyls (nmol/mg protein)	0.94 $\pm$ 0.06	0.96 $\pm$ 0.05	0.97 $\pm$ 0.06	0.90 $\pm$ 0.05	0.94 $\pm$ 0.05	0.94 $\pm$ 0.05	0.96 $\pm$ 0.04	0.91 $\pm$ 0.03	0.90 $\pm$ 0.03
Methionine sulfoxide (% of total methionine)	6.2 $\pm$ 1.3	7.0 $\pm$ 0.6	7.4 $\pm$ 1.0	7.1 $\pm$ 0.9	7.0 $\pm$ 0.6	7.7 $\pm$ 0.6	6.5 $\pm$ 1.1	7.9 $\pm$ 0.7	7.0 $\pm$ 0.5
Protein-associated heme (% of total heme in vivo)	6.5 $\pm$ 0.2	6.4 $\pm$ 0.2	6.5 $\pm$ 0.2	6.6 $\pm$ 0.2	6.5 $\pm$ 0.2	6.5 $\pm$ 0.2	6.6 $\pm$ 0.3	7.0 $\pm$ 0.4	6.5 $\pm$ 0.3

Entries are Means $\pm$ SE. Sample size is n=5-6/group. Samples were collected 2h, 7h and 16 h after the ozone exposure. Statistical testing methods are described in the methods section.

Table 2
Effect of Ozone Exposure on Oxidation Products of Lipids in Rat Urine

Dose/Assay Time point	MDA (nmol/mg creatinine) (by HPLC and spectrophotometry)			8 β -Isoprostanes(ng/mg creatinine (by radioimmunoassay)		
	Control	Ozone 2 ppm	Ozone 5 ppm	Control	Ozone 2 ppm	Ozone 5 ppm
8 h	3.5 $\pm$ 0.4	3.4 $\pm$ 0.4	3.4 $\pm$ 0.3	0.35 $\pm$ 0.01	0.50 $\pm$ 0.05	0.51 $\pm$ 0.07
22h	2.9 $\pm$ 0.4	3.1 $\pm$ 0.5	3.0 $\pm$ 0.4	0.56 $\pm$ 0.14	0.55 $\pm$ 0.13	0.40 $\pm$ 0.05
32h	2.7 $\pm$ 0.8	2.1 $\pm$ 0.7	4.1 $\pm$ 0.4	0.52 $\pm$ 0.09	0.49 $\pm$ 0.08	0.50 $\pm$ 0.15
46h	2.9 $\pm$ 0.6	2.6 $\pm$ 0.8	2.9 $\pm$ 0.3	0.39 $\pm$ 0.05	0.43 $\pm$ 0.05	0.32 $\pm$ 0.03
56h	3.7 $\pm$ 0.4	5.0 $\pm$ 0.4	4.5 $\pm$ 0.5	0.37 $\pm$ 0.02	0.45 $\pm$ 0.07	0.39 $\pm$ 0.05
70h	2.7 $\pm$ 0.4	3.6 $\pm$ 0.8	3.6 $\pm$ 0.6	0.43 $\pm$ 0.08	0.53 $\pm$ 0.06	0.52 $\pm$ 0.07
80h	4.7 $\pm$ 0.2	5.4 $\pm$ 0.5	4.8 $\pm$ 0.5	0.43 $\pm$ 0.04	0.49 $\pm$ 0.10	0.46 $\pm$ 0.06

MDA and Isoprostanes were measured in urine samples at seven time points after ozone exposure. Entries are Means $\pm$ SE. Sample size is n=5-6/group. Experimental data were not statistically significant (p < 0.05) compared to the control at the same time point; statistical testing methods are described in the methods section.