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Ozone inhalation modifies the rat liver proteome ☆

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ABSTRACT

Ozone (O_3) is a serious public health concern. Recent findings indicate that the damaging health effects of O_3 extend to multiple systemic organ systems. Herein, we hypothesize that O_3 inhalation will cause downstream alterations to the liver. To test this, male Sprague-Dawley rats were exposed to 0.5 ppm O_3 for 8 h/day for 5 days. Plasma liver enzyme measurements showed that 5 day O_3 exposure did not cause liver cell death. Proteomic and mass spectrometry analysis identified 10 proteins in the liver that were significantly altered in abundance following short-term O_3 exposure and these included several stress responsive proteins. Glucose-regulated protein 78 and protein disulfide isomerase increased, whereas glutathione *S*-transferase M1 was significantly decreased by O_3 inhalation. In contrast, no significant changes were detected for the stress response protein heme oxygenase-1 or cytochrome P450 2E1 and 2B in liver of O_3 exposed rats compared to controls. In summary, these results show that an environmentally-relevant exposure to inhaled O_3 can alter the expression of select proteins in the liver. We propose that O_3 inhalation may represent an important unrecognized factor that can modulate hepatic metabolic functions.

Introduction

Approximately 50% of the U.S. population resides in areas where ambient ozone (O_3) concentrations exceed the current 0.075 ppm 8 h time average set by the National Ambient Air Quality Standards (NAAQS) [1]. O_3 is a primary component of photochemical smog and is a serious public health concern especially when air quality is poor. Persons particularly sensitive to O_3 exposure include the elderly, young children, and those with pre-existing pulmonary diseases such as asthma and chronic obstructive pulmonary disease. It is established that exposure to high levels of O_3 decreases lung function, increases pulmonary hyper-reactivity, causes airway epithelial cell damage and remodeling, and increases epithelial permeability [2–4].

In addition to adverse pulmonary effects, emerging evidence shows that O_3 inhalation can cause tissue injury and altered metabolism in other systemic organ systems. For example, studies in nonhuman primates show that postnatal episodic O_3 exposure only during infancy (30 days to 6 months of age) resulted in long-term effects to the systemic innate immune system that were detectable up to 1 yr of age [5]. Recent epidemiologic studies report a positive correlation of air pollution exposure with increased cardiovascular-related morbidity and mortality resulting in increased hospital admissions related to cardiac events [6–9]. Additional studies reported that successive days of high ambient O_3 exposure correlated with increased blood pressure, blood lipids, and decreased glucose tolerance in humans [8]. Ballinger, Postlethwait, and colleagues showed that exposure to 0.5 ppm O_3 induced vascular dysfunction and increased aortic mitochondrial DNA damage in healthy wild-type mice, and increased progression of atherosclerosis in mice genetically predisposed to cardiovascular disease [10]. Moreover, a single 5-day regimen of O_3 also increased mtDNA damage in the abdominal aorta of infant nonhuman primates [10]; a model more closely mimicking human exposures. This work is important because not only does it provide direct evidence that O_3 inhalation damages the cardiovascular system, but also shows that O_3 has the potential to exacerbate cardiovascular disease including atherosclerosis [11].

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O_3 inhalation has also been shown to induce metabolic changes in other peripheral organ systems. For example, pentobarbital-induced sleeping times increased after O_3 exposure, suggesting alterations in hepatic drug metabolism and clearance mechanisms [12,13]. Since this early work, other groups have addressed the effect of inhaled O_3 on the liver. Last and colleagues, using a microarray approach, examined the effects of O_3 (1.0 ppm) on the liver transcriptome and showed that O_3 inhalation significantly decreased the mRNA levels of several xenobiotic, carbohydrate, and fatty acid metabolism genes [14]. Other groups have shown that acute exposures of O_3 (1.0–2.0 ppm) for 3 h increased nitric oxide production in isolated hepatocytes, as well as increased rates of protein synthesis [15]. Herein, our goal was to determine whether inhalation of O_3 at a concentration lower than used in previous studies, alters the hepatic proteome and specific xenobiotic metabolizing enzymes in an animal model without pre-existing disease.

Materials and methods

O_3 exposure protocol

Male Sprague-Dawley rats were purchased from Harlan Laboratories (Barrier 217 VAF, Indianapolis, IN) and were provided standard rat chow and water *ad libitum*. Animals were housed two per cage under barrier conditions and maintained using a standard 12 h light-dark cycle. Rats were exposed to either filtered air (FA) or ozone (O_3) at 0.5 ppm for 8 h/day for 5 days between 9:00 AM to 5:00 PM in the University of Alabama at Birmingham (UAB) Environmental Exposure Facility. Standard laboratory rat chow was removed from cages before the start of exposures to prevent rats from ingesting food that may contain oxidized nutrients (e.g., lipids, thiols, antioxidants) as a consequence of O_3 reaction. Rat chow was also removed from cages of FA exposed rats. Fresh food was returned to cages after exposures and present throughout the entire dark period when rats are active and consume food. The exposure protocol had no effect on body weight (FA: 348 ± 4 g and O_3: 341 ± 15 g, $p=0.69$). Prior to exposures, animals were acclimated to the chambers for at least 72 h before beginning FA and O_3 exposures to minimize stress associated with a novel environment. Importantly, rats remained in their home cage with bedding and water, with cages (stainless steel wire mesh coverings) placed into the exposure chamber. O_3 was generated from 100% O_2 using a model OZ1PCS-V/SW O_3 generator (Ozotech Inc, Yreka, CA), mixed with FA, and flowed into 0.8 m^3 stainless steel chambers (~22 °C, 50% relative humidity; 30 volume changes/hr). O_3 concentrations were continuously monitored using a Thermo-Environmental Model UV Photometric analyzer (Thermo-Environmental, Franklin, MA). Measurements obtained inside the cages demonstrated that O_3 concentrations in the animals breathing zones were equivalent to the chamber bulk phase concentrations. FA controls were housed in separate chambers receiving the same clean air supply at equal air exchange rates throughout the duration of the exposure regimen. Animals were anesthetized with a 50 mg/kg body weight (i.p.) injection of sodium pentobarbital and euthanized via exsanguination. Tissues were harvested within 1 h of cessation of the exposure. All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Bronchoalveolar lavage and cell differential analysis

A cannula was inserted into the trachea via a midline tracheotomy, the chest cavity opened via a midline thoracotomy, and 9 mL of warmed (37 °C) phosphate buffered saline (pH 7.0; 310 mOsm) was gently instilled and withdrawn 3 times to yield bronchoalveolar lavage (BAL) [16]. The BAL was subjected to a standard cytospin procedure (StatSpin Cytofuge, Norwood, MA) to determine cell counts and types. Slides were stained with a modified Wright-Giemsa stain and 300 cells (100 cells per lane) were counted under a light microscope using characteristics unique to each cell type [17]. Total cell counts were averaged for each cell type and percent of total cells calculated. The remaining BAL was centrifuged to generate BAL fluid (BALF) and protein concentrations in the BALF were measured to determine whether altered epithelial permeability [18] was detectable after the 5th period of O_3.

Plasma chemistries for liver enzymes and histology

Blood was collected via the abdominal aorta using a heparin-coated syringe and centrifuged at 4 °C at 2000*g* for 10 min to obtain plasma. Alanine and aspartate aminotransferase (ALT and AST, respectively) activities were measured in plasma using a spectrophotometric assay per manufacturer's directions (Pointe Scientific, Inc, Canton MI). Briefly, NAD^+ oxidation was recorded under constant temperature conditions (37 °C) over a time period of 10 min at 340 nm. The rate was measured and ALT and AST levels reported as international units/liter (IU/L). Liver tissue was fixed in formalin and paraffin embedded. Sections were mounted on slides and stained with hematoxylin and eosin (H&E). Slides were scored for injury, steatosis, and inflammation by a pathologist blinded to the experimental groups.

Two dimension isoelectric focusing/SDS-PAGE (2D IEF/SDS-PAGE)

Livers were excised and homogenized in ice-cold 0.25 M sucrose, 5 mM Tris–HCl, and 1 mM EDTA, pH 7.4, containing protease inhibitors [phenylmethylsulfonyl fluoride (40 μg/mL), leupeptin (5 μg/mL), and pepstatin A (7 μg/mL)] [19]. Protease inhibitors were included to prevent sample degradation prior to proteomic analyses. For proteomic studies there were six FA (control) and six O_3 exposed rats per group. Post-nuclear supernatant fraction was prepared by centrifugation of liver homogenates at 568*g* for 10 min at 4°C. Protein concentrations were determined using the Bradford protein assay and bovine serum albumin (BSA) as a standard [20]. Proteomic analyses were performed by methods as previously described [21]. Post-nuclear supernatant (100 μg) from liver homogenates was added to IEF gel strip rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (w/v) n-dodecyl-ß-D-maltoside, 0.002% (w/v) bromophenol blue, ampholine electrophoresis reagent (Sigma, St. Louis, MO, range pH 3–10), 0.04 M DTT and 2 mM tributylphosphine. Following protein extraction, samples were applied to IEF gel strips (Invitrogen ZOOM Strips, pH 3–10, Carlsbad, CA) and rehydration of IEF strips was done overnight. For SDS-PAGE, IEF gel strips were placed horizontally on top of a 10% resolving gel with 4% stacking gel, and sealed into place using warm agarose (1%, w/v) and gels were run at 100 V for $1\frac{1}{2}$ h. After electrophoresis, gels were stained with Sypro Ruby™ (Invitrogen, Carlsbad, CA) for total protein. Protein stained gels were imaged using a Bio-Rad ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc, Hercules, CA).

2D gel image analyses

Methods for gel analyses are described in our previous work [21]. Differences in protein density were performed using PDQuest Image Analysis software (Bio-Rad, Hercules, CA). Individual protein spots on all twelve 2D gels ($n=6$ for FA and $n=6$ for O_3) were identified using the program software and visually matched for accuracy. A master gel was created to serve as the reference gel for FA and O_3 groups, which the PDQuest software program uses automatically to match the spots across all gels. Manual verification was then used to correct for incorrect spot matching to the reference gel or spots not initially detected by software. In order to