ORIGINAL ARTICLE

Ozone Exposure Increases Circulating Stress Hormones and Lipid Metabolites in Humans

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Abstract

Rationale: Air pollution has been associated with increased prevalence of type 2 diabetes; however, the mechanisms remain unknown. We have shown that acute ozone exposure in rats induces release of stress hormones, hyperglycemia, leptinemia, and glucose intolerance that are associated with global changes in peripheral glucose, lipid, and amino acid metabolism.

Objectives: To examine ozone-induced metabolic derangement in humans using serum metabolomic assessment, establish human-to-rodent coherence, and identify novel nonprotein biomarkers.

Methods: Serum samples were obtained from a crossover clinical study that included two clinic visits (n = 24 each) where each subject was blindly exposed in the morning to either filtered air or 0.3 parts per million ozone for 2 hours during 15-minute on-off exercise. Serum samples collected within 1 hour after exposure were assessed for changes in metabolites using a metabolomic approach.

Measurements and Main Results: Metabolomic analysis revealed that ozone exposure markedly increased serum cortisol and corticosterone together with increases in monoacylglycerol, glycerol, and medium- and long-chain free fatty acids, reflective of lipid mobilization and catabolism. Additionally, ozone exposure increased serum lysolipids, potentially originating from membrane lipid breakdown. Ozone exposure also increased circulating mitochondrial β-oxidation–derived metabolites, such as acylcarnitines, together with increases in the ketone body 3-hydroxybutyrate. These changes suggested saturation of β-oxidation by ozone in exercising humans.

Conclusions: As in rodents, acute ozone exposure increased stress hormones and globally altered peripheral lipid metabolism in humans, likely through activation of a neurohormonally mediated stress response pathway. The metabolomic assessment revealed new biomarkers and allowed for establishment of rodent-to-human coherence.

Clinical trial registered with www.clinicaltrials.gov (NCT 01492517).

Keywords: air pollution; stress response; lipid mediators; fatty acids

Several epidemiologic studies nationally and internationally have predicted a link between air pollution and prevalence of diabetes (1–6). It is apparent that the conventional risk factors, such as sedentary lifestyle, obesogenic high-caloric diets, and/or genetics, alone do not fully explain the causal relationship. The contribution of stress and environmental factors has been postulated. Near-road air pollution exposure has also been linked to diabetes (7–9) and a recent study has associated

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At a Glance Commentary

Scientific Knowledge on the Subject: The association between air pollution and diabetes has motivated research to understand how pollutants affect metabolic processes. Ozone levels exceed current National Ambient Air Quality standards in many areas and are expected to further increase because of the warming climate trend.

What This Study Adds to the Field: This manuscript provides new evidence of acute systemic metabolic effects of ozone in humans, characterized by stress hormone–mediated release of lipid metabolites into the circulation. The data set shows coherency between rat and humans in their metabolic response to ozone exposure. The global metabolomic approach offers a novel tool to identify nonprotein metabolite biomarkers of acute pollutant exposure.

roadway proximity with hyperglycemia in women (10). Although both particulate matter and gaseous pollutants have been associated with diabetes, in one study this association was presumed to be stronger with gaseous pollutants than particulate matter (11).

Subchronic ambient particulate matter exposure studies using rodent models have shown increased adipose and brain inflammation, and liver insulin resistance (12, 13). It has been postulated that pulmonary injury and inflammation, following inhalation of air pollutants, leads to increased release of cytokines and biologically active mediators causing systemic inflammation and metabolic effects. However, most air pollution studies examining circulating cytokines fail to demonstrate their increases in the blood. More recently, epidemiologic and experimental studies have associated inhaled pollutants with a variety of neural outcomes (14–17). Specifically, exposure to ozone activates the nucleus tractus solitarius and stress responsive regions of the hypothalamus through stimulation of pulmonary vagal C fibers (18). Acute ozone exposure can increase levels of circulating stress hormones, such as epinephrine and corticosterone, in rats (19–21). Ozone exposure also induces cardiac autonomic effects in humans (22); hypothermia and bradycardia in rats (23); and leptinemia, hyperglycemia, glucose intolerance, and global changes in circulating metabolites involved in peripheral glucose, lipid, and amino acid metabolism in rats (20). These changes are reflective of classical stress response–mediated homeostatic alterations involving activation of sympathetic neurons and hypothalamus-pituitary-adrenal (HPA) axis (20).

We have previously performed global metabolomic assessment of serum to characterize the metabolic responses to ozone and to gain mechanistic insights in rats (20). The goal of this exploratory study was to perform a global metabolomic assessment of archived human serum samples from a prior clinical study involving ozone exposure, and determine ozone-induced metabolic changes in humans, establish rodent-to-human coherence, and identify novel nonprotein biomarkers of ozone exposure. We hypothesized that global metabolomic assessment of serum samples collected after air or ozone exposure in humans will reveal metabolic derangements through a neurohormonally mediated stress response, comparable with rats. Because most human ozone exposures are performed during intermittent exercise and daylight cycle, we predicted that there would be some discrepancies in the metabolite profile of the serum between nonexercising rats (nocturnal) exposed during their nonactive circadian cycle and exercising humans. Some of the results of this study have been previously reported in the form of an abstract (24).

Methods

Original Study Design: Study Population and Ozone Exposure

Serum samples for this exploratory study were obtained from Clinical Trial NCT01492517. In this trial, subjects were recruited under an Environmental Protection Agency contract with Westat Corporation (Rockville, MD). Cardiac and pulmonary function, and systemic effects of ozone on coagulation and inflammatory markers have been previously reported from this study (25). A total of 24 volunteers were involved in the study. The protocol and consent forms were approved by the University of North Carolina, School of Medicine Committee on the Protection of the Rights of Human Subjects and the U.S. Environmental Protection Agency's Institutional Review Board. The exposure was conducted in a randomized crossover design where two clinical visits of each individual were separated by at least 2 weeks. During each visit, the subjects were exposed to either 0.3 parts per million ozone or filtered air for 2 hours in the morning time in a blinded manner. During the exposure, subjects alternated between 15 minutes of rest and 15 minutes of exercise on a cycle ergometer (25). Exposures were conducted at the U.S. Environmental Protection Agency, Human Studies Facility on the campus of the University of North Carolina, Chapel Hill. Ozone was generated by a silent electric discharge method (model 502; Meckenheim, Bonn, Germany). The exposure chambers were maintained at 40% relative humidity for all exposures. For more details of the exposure system, *see* online supplement 1 and Devlin and coworkers (25).

Serum Samples and Metabolomic Analysis

Serum samples collected before the start of exposure, immediately postexposure (within 1 h), and during the next day morning follow-up visit were assessed for glucose, triglycerides, and cholesterols (LabCorp Inc., Durham, NC) when the clinical study was performed. In the present study, archived serum samples collected immediately (within 1 h) after air or ozone exposure were used for exploratory metabolomic assessment. These samples were sent to Metabolon, Inc. (Durham, NC) for global metabolomic analysis (*see* Methods section in online supplement 1). Briefly, each sample was accessioned into the Metabolon Laboratory Information Management System and assigned a unique identifier. Samples were prepared using the automated MicroLab STAR system from Hamilton Co. (*see* Methods section in online supplement 1). The liquid chromatography–mass spectrometry portion of the platform was based on a Waters ACQUITY ultraperformance liquid chromatography (Milford, MA) and a Thermo Scientific Q-Exactive high-resolution/accurate mass spectrometer interfaced with a heated electrospray ionization source (Waltham, MA) and Orbitrap mass analyzer (Thermo Fisher Scientific, Waltham, MA) operated at 35,000 mass resolution (26). The gas

chromatography–mass spectrometry portion of the platform used a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer (San Jose, CA) using electron impact ionization. The informatics system consisted of four major components: (*1*) the Laboratory Information Management System, (*2*) the data extraction and peak-identification software, (*3*) data processing tools, and (*4*) data interpretation and visualization tools (*see* METHODS, Tables E1 and E2, and Figures E1 and E2 in online supplement 1).

The hardware and software foundations for these informatics components were the LAN backbone and a database server running Oracle 10.2.0.1 Enterprise Edition (Redwood Stores, CA). Compounds were identified by comparison with library entries of purified standards or recurrent unknown entities. Peaks were quantified using area under the curve. Statistical analysis included log transformation and imputation followed by one-way analysis of variance with repeated measures. An estimate of the false discovery rate (q value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. Significant ($P < 0.05$) pathway enrichment output (cumulative hypergeometric distribution) was assessed for each of the selected contrasts using MetaboLync, version 1.1.2 (Metabolon Inc, Durham, NC) (27) to determine the metabolic processes impacted by ozone.

Serum samples collected immediately post air or ozone exposure were also analyzed for insulin, leptin, IL-6, and tumor necrosis factor-α using human-specific antibody-based electrochemiluminescence assays (Meso Scale Discovery, Gaithersburg, MD) according to manufacturer instructions.

Results

Demographics of Human Subjects

The clinical study from which serum samples were obtained included 24 healthy, young adult participants: 20 males and 4 females (Table 1). The average age of the subjects was 25.6 with a range of 22–30 years. Weights averaged 78.5 kg with a range from 50.7 to 97.3 kg. Eleven subjects had a body mass index between 18.5 and 24.9, and 13 subjects were between 25 and 29.9 (*see* online supplement 2 for details on individual subjects). Most of the subjects never smoked. Only three subjects were previous smokers, all before the year 2006. All subjects participated in weekly exercise: 2 mild level (90–180 min/wk), 9 moderate level (180–360 min/wk), and 13 high level (>360 min/wk). Participants did not have a recent history of prescription medication use, cardiopulmonary disease, or allergies as determined by a detailed medical history and physical examination. Their specific characteristics are summarized in Table 1 and detailed in online supplement 1.

Heart rate, blood pressure, and serum glucose, triglycerides, and cholesterols measured before, immediately after air or ozone exposure, and after a 1-day follow-up visit for each subject are provided as line graphs in online supplement 1 (*see* Figures E3 and E4) and individual subject's data in online supplement 2. There were no marked pre or post air or ozone exposure differences noted in heart rate and blood pressure; however, between-subject variability was noted in the levels of triglycerides and cholesterols. Between-subject glucose levels varied slightly at preexposure time but not after postexposure or next day follow-up visit.

Table 1. Demographics of Study Participants*

Demographics	n or Mean ± SD (*n = 24*)
Sex	
Male	20
Female	4
Race	
White	22
Hispanic	2
Smoking	
Never smoker	21
Former smoker†	3
Medication‡	3
Exercise§	
Mild	2
Moderate	9
Heavy	13
Age, yr	25.6 ± 3.8
Height, cm	177.9 ± 9.3
Weight, kg	78.5 ± 13.9
Body mass index	24.7 ± 3.0
18.5–24.9	11
25–29.9	13
Systolic	121.0 ± 8.4
Diastolic	73.9 ± 6.8
Heart rate, bpm	73.4 ± 14.4

Definition of abbreviation: bpm = beats per minute.
*For details on individual subject demographics, *see* online supplement 1.
†Smoked before the year 2006.
‡Currently on medication (multivitamins and/or Lexapro).
§Exercise levels: mild, 90–180 minutes per week; moderate, 180–360 minutes per week, heavy, >360 minutes per week.

Ozone Exposure Increases Circulating Cortisol but Does Not Change Cytokines or Homeostatic Model Assessment for Insulin Resistance

Cortisol and corticosterone were significantly increased in the serum samples collected after ozone exposure when compared with air (Figure 1). In addition, a 1.62-fold increase in corticosterone metabolite 11-dehydrocorticosterone, but not cortisone (*see* online supplement 2), suggests HPA axis involvement in acute ozone-induced extrapulmonary effects. Serum levels of tumor necrosis factor-α and IL-6 as reported earlier (25) showed no significant difference between air and ozone immediately postexposure (data not shown). Serum leptin and insulin (*see* Figure E5 in online supplement 1 and data in online supplement 2) were not significantly different between air and ozone samples immediately postexposure. High individual sample variability was noted for three to four individuals in the levels of insulin and leptin. Homeostatic model assessment for insulin resistance provided no significant differences between air and ozone exposure (*see* Figure E5 in online supplement 1 for line graph and online supplement 2 for data).

Correlations of Subject Characteristics with Individual Metabolites and Ozone-induced Changes

We attempted to correlate ozone-induced changes (fold-increase) in selected metabolites, including steroid hormones, with individual subject's body mass index, but no significant relationships seemed to occur (*see* Figures E6A–6E in online supplement 1). Although the number of subjects included in the study was small, especially when stratified for their prior exercise habit (as mild, moderate, or heavy) or sex, we have provided the correlation plots and results in Figures E7A–7E and E8A–8E and RESULTS section of online supplement 1. Likewise, circulating levels

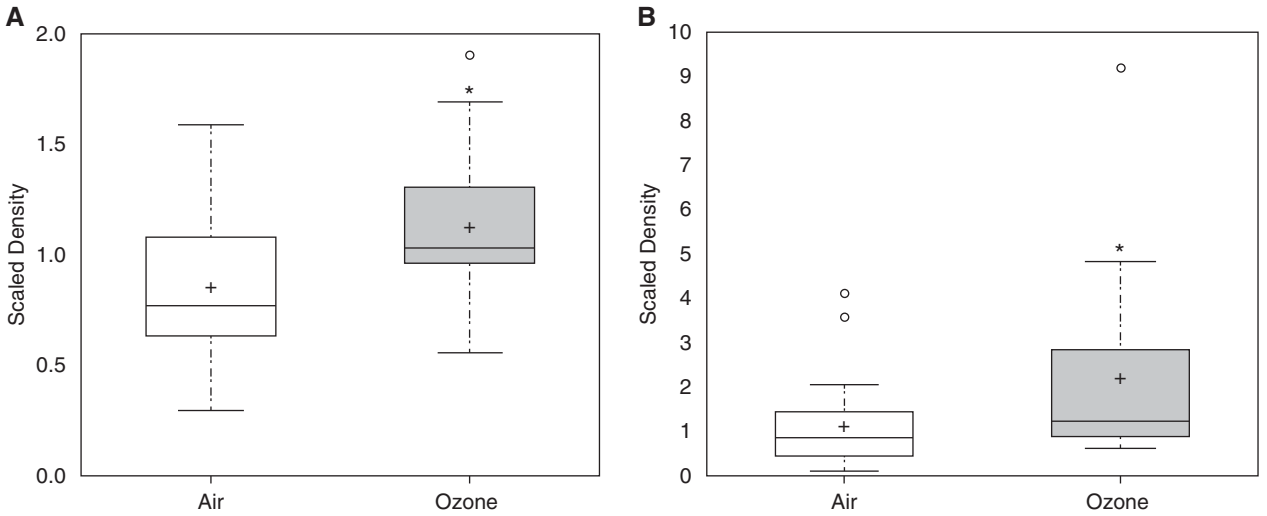

Figure 1. Acute 2-hour ozone exposure increases serum levels of the stress hormones cortisol (*A*) and corticosterone (*B*) in humans. *Box-and-whisker plots* convey the spread of the data with the interquartile range represented by the *box* and the range of the data shown by the *whiskers* (n = 24 per group). Outlier values are defined as those that exceed, in either direction, 1.5 times the interquartile range (shown as *open circles* in each plot). The *solid bar* across the box represents the median value, and + *symbol* represents the mean. *Significantly different from air group ($P < 0.05$). For each biochemical, data are median scaled with the median value across all samples set to 1.0. The *y-axis* thus reflects scaled intensity for each metabolite.

of metabolites (glucose, triglycerides, and cholesterols) after air or after ozone exposure were analyzed for correlations with body mass index, prior exercise habit, and sex, and these plots are provided in Figures E9–E11 in online supplement 1. No significant differences were noted between air and ozone exposure for preexposure normalized glucose, triglycerides, and lipids levels (*see* online supplement 2 for all individual subject's clinical data).

Ozone Exposure Changes the Profile of Circulating Metabolites

There were 663 compounds of known identity detected in the human serum samples (*see* online supplement 2 for all original data, normalized data, heat maps, and scatter plots). Of those, statistically significant differences were found for 121 biochemicals ($P < 0.05$) between air and ozone exposure as determined by analysis of variance. Specifically, 85 were increased and 36 were decreased after ozone exposure compared with air. There were also several biochemicals for which the difference between the air and ozone exposures approached significance ($0.05 < P < 0.10$; 23 increased and 29 decreased). A pathway enrichment analysis using MetaboLync (27) indicated seven biochemical pathways (sphingolipid metabolism, endocannabinoid synthesis, fatty acid metabolism, β-oxidation, dicarboxylic acid metabolism, steroid hormone biosynthesis, and phospholipid metabolism) to be significantly altered by ozone exposure (Figure 2). All of these pathways are associated with lipid metabolism.

Ozone Exposure Increases Circulating Free Fatty Acids and Lysolipids in Humans

One of the most consistent and strongest effects associated with ozone exposure in this study was the pronounced increases in serum free fatty acids (Table 2). There were significant ($P < 0.05$) increases in medium- and long-chain free fatty acids in the serum samples collected after ozone exposure relative to air, along with a significant increase in serum glycerol, a marker of lipolysis (Table 2). Additionally, higher levels of a variety of lysolipids were found in the serum samples collected after ozone exposure relative to air (Table 3), which may indicate membrane phospholipid hydrolysis contributing to fatty acid generation. Finally, increasing trends were also noted in sphinganine ($P = 0.0511$) and sphingosine 1-phosphate ($P < 0.00005$) in combination with elevated long-chain fatty acids in the serum samples obtained after ozone exposure relative to the filtered air.

Ozone Exposure during Intermittent Exercise Increases Fatty Acid Oxidation in Humans

There were significant changes in biochemicals associated with fatty acid β-oxidation (Table 4). These changes included significantly higher levels of octanoylcarnitine, decanoylcarnitine, and cis-4-decanoylcarnitine together with a trend of an increase in acetylcarnitine ($P = 0.079$; a surrogate for the fatty acid oxidation end product acetyl-CoA) in the serum samples collected after ozone relative to air exposure. Ozone exposure was also associated with increases in serum laurylcarnitine, myristoylcarnitine, palmitoylcarnitine, oleoylcarnitine, linoleoylcarnitine, and myristoleoylcarnitine. Long-chain fatty acids (>12 carbons) are conjugated with carnitine to facilitate transport into the mitochondrial matrix where they may undergo fatty acid β-oxidation for energy. These changes were also accompanied by lower levels of carnitine in the serum samples collected after ozone exposure, which may be a marker of increased use for acylcarnitine conjugation.

Moreover, the dicarboxylates, azelate, and 2-hydroxyglutarate were significantly ($P < 0.05$) increased in the serum after ozone exposure when compared with air (Table 4), and may suggest that the

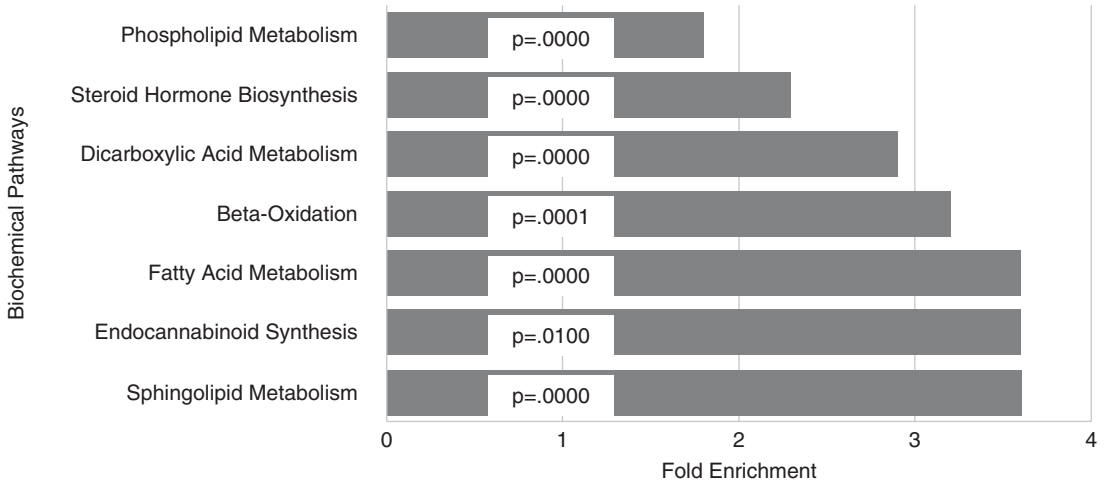

Figure 2. Ozone altered lipid metabolism pathways in humans. Pathway enrichment analysis (using MetaboSync) identified seven specific biochemical pathways (sphingolipid metabolism, endocannabinoid synthesis, fatty acid metabolism, β-oxidation, dicarboxylic acid metabolism, steroid hormone biosynthesis, and phospholipid metabolism) as having a significant ($P < 0.05$) fold enrichment value for ozone exposure compared with air. This analysis was based on several parameters including the total number of detected metabolites in the study and selected pathway, and the abundance of total metabolites associated with each pathway.

β-oxidation process was saturated, thereby shifting to fatty acid ω-oxidation. Taken together, these findings support increased fatty acid β-oxidation for energy generation following the 2-hour ozone exposure during intermittent exercise. Further, indication of increased fatty acid β-oxidation was revealed by the elevation in the ketone body, 3-hydroxybutyrate (Table 4), a marker of hepatic fatty acid β-oxidation, which can accumulate in cases where the capacity of the tricarboxylic acid cycle is overwhelmed by the availability of substrate.

Ozone Exposure Increased Polyunsaturated Fatty Acids in the Serum

In addition to the observed increases in free fatty acids and fatty acid oxidation by-products, n3 and n6 polyunsaturated fatty acids were also significantly ($P < 0.05$) increased in the serum from ozone-exposed subjects relative to air and may contribute to production of inflammatory mediators (Figure 3). These included linolenate alpha or gamma (18:3n3 or n6; Figure 3A), eicosapentaenoate (20:5n3; Figure 3B), docosapentaenoate (n3 22:5n3; Figure 3C), docosahexaeonate (22:6n3; Figure 3D), linoleate (18:2n6; Figure 3E), dihomo-linolenate

Table 2. Acute Ozone Exposure Increases Circulating Free Fatty Acids and Monoacylglycerols in Humans

Free Fatty Acids	Metabolite	Fold Change: Ozone/Air*	*P* Value†	*q* Value
Medium-chain fatty acid	Caproate (6:0)	1.17	0.0041	0.048
	Laurate (12:0)	1.17	0.0649	0.218
	5-Dodecenoate (12:1n7)	1.57	0.0058	0.053
	Myristate (14:0)	1.42	0.0102	0.072
	Myristoleate (14:1n5)	1.80	0.0005	0.025
	Palmitoleate (16:1n7)	1.72	0.0019	0.042
Long-chain fatty acid	Margarate (17:0)	1.30	0.0097	0.070
	10-Heptadecenoate (17:1n7)	1.48	0.0041	0.048
	Oleate (18:1n9)	1.38	0.0014	0.034
	Cis-vaccenate (18:1n7)	1.20	0.0759	0.238
	10-Nonadecenoate (19:1n9)	1.54	0.0042	0.048
	Eicosenoate (20:1n9 or 11)	1.44	0.0082	0.065
Fatty acid, monohydroxy	3-Hydroxyoctanoate	1.48	0.0195	0.115
	3-Hydroxydecanoate	1.78	0.0031	0.045
	3-Hydroxylaurate	1.77	0.0021	0.043
Glycerolipid metabolism	Glycerol	1.34	0.0072	0.059
Monoacylglycerol	1-Stearoylglycerol (1-monostearin)	2.32	0.0046	0.048
	2-Stearoylglycerol (2-monostearin)	1.89	0.0028	0.044
	1-Oleoylglycerol (1-monoolein)	1.56	0.0000	0.004
	2-Oleoylglycerol (2-monoolein)	1.85	0.0047	0.048

*Values indicate relative fold differences for each biochemical between ozone and filtered air samples (n = 24 per group). A fold change >1 indicates an increase.
†When $P < 0.05$, the change is considered significant.

Table 3. Ozone Exposure Elevates Circulating Lysolipid and Sphingolipid Metabolites in Humans

	Metabolite	Fold Change: Ozone/Air*	*P* Value†	*q* Value
Lysolipids	1-Pentadecanoylglycerophosphocholine (15:0)	1.10	0.0901	0.256
	1-Palmitoylglycerophosphocholine (16:0)	1.08	0.0035	0.047
	2-Palmitoylglycerophosphocholine (16:0)	1.06	0.0787	0.239
	1-Palmitoleoylglycerophosphocholine (16:1)	1.11	0.0313	0.155
	1-Margaroylglycerophosphocholine (17:0)	1.24	0.0004	0.023
	1-Stearoylglycerophosphocholine (18:0)	1.16	0.0027	0.044
	2-Stearoylglycerophosphocholine (18:0)	1.19	0.0108	0.075
	1-Oleoylglycerophosphocholine (18:1)	1.18	0.0014	0.034
	2-Oleoylglycerophosphocholine (18:1)	1.11	0.0665	0.219
	1-Linoleoylglycerophosphocholine (18:2n6)	1.08	0.0614	0.218
	2-Linoleoylglycerophosphocholine (18:2n6)	1.14	0.0324	0.158
	1-Linolenoylglycerophosphocholine (18:3n3)	0.82	0.0856	0.250
	2-Linolenoylglycerophosphocholine (18:3n3)	0.85	0.0406	0.176
	1-Nonadecanoylglycerophosphocholine (19:0)	1.29	0.0046	0.048
	1-Dihomo-linoleoylglycerophosphocholine (20:2n6)	1.22	0.0007	0.028
	1-Arachidoylglycerophosphocholine (20:0)	1.44	0.0112	0.076
	1-Eicosenoylglycerophosphocholine (20:1n9)	1.33	0.0029	0.044
	2-Eicosenoylglycerophosphocholine (20:1n9)	1.48	0.0012	0.033
	1-Eicosatrienoylglycerophosphocholine (20:3)	1.22	0.0023	0.044
	2-Eicosatrienoylglycerophosphocholine (20:3)	1.20	0.0009	0.032
	1-Arachidonoylglycerophosphocholine (20:4n6)	1.15	0.0006	0.028
	2-Arachidonoylglycerophosphocholine (20:4n6)	1.16	0.0002	0.014
	1-Eicosapentaenoylglycerophosphocholine (20:5n3)	1.17	0.0378	0.170
Sphingolipid metabolism	Sphingosine 1-phosphate	1.37	0.0000	0.042
	Sphinganine	1.20	0.0511	0.198

*Values indicate relative fold differences for each biochemical between ozone and filtered air samples (n = 24 per group). A fold change >1 indicates an increase; fold change <1 indicates a decrease.
†When $P < 0.05$, the change is considered significant.

(20:3n3 or n6; Figure 3F), and arachidonate (20:4n6; Figure 3G).

Discussion

Epidemiologic studies have associated air pollutants with increased prevalence of type 2 diabetes and changes in markers of insulin resistance (1–9, 28, 29). However, controlled human studies examining systemic metabolic effects and potential mechanisms have not been performed. Several environmental stressors over a long period of time are postulated to be interactively involved in the development of insulin resistance and are linked to lipidemia and high blood glucose. We have recently reported that acute ozone exposure in rodents produces systemic homeostatic changes in circulating metabolites reflective of activation of neuronal stress response

Table 4. Ozone Exposure during Intermittent Exercise Increases Circulating β-Oxidation Metabolites in Humans

Pathway	Metabolite	Fold Change: Ozone/Air*	*P* Value†	*q* Value
Fatty acid metabolism (acylcarnitine)	Acetylcarnitine	1.09	0.0787	0.239
	Hexanoylcarnitine	1.22	0.0827	0.246
	Octanoylcarnitine	1.32	0.0224	0.127
	Decanoylcarnitine	1.42	0.0132	0.085
	Cis-4-decenoylcarnitine	1.27	0.0235	0.132
	Laurylcarnitine	1.79	0.0094	0.069
	Myristoylcarnitine	1.57	0.0360	0.169
	Palmitoylcarnitine	1.25	0.0319	0.157
	Oleoylcarnitine	1.38	0.0022	0.043
	Linoleoylcarnitine	1.31	0.0130	0.085
	Myristoleoylcarnitine	1.81	0.0051	0.048
Fatty acid, dicarboxylate	2-Hydroxyglutarate	1.26	0.0320	0.187
	Azelate	1.55	0.0002	0.004
Carnitine metabolism	Carnitine	0.92	0.0102	0.072
Ketone bodies	3-Hydroxybutyrate	1.82	0.0451	0.187

*Values indicate relative fold differences for each biochemical between ozone and filtered air samples (n = 24 per group). A fold change >1 indicates an increase; fold change <1 indicates a decrease.
†When $P < 0.05$, the change is considered significant.

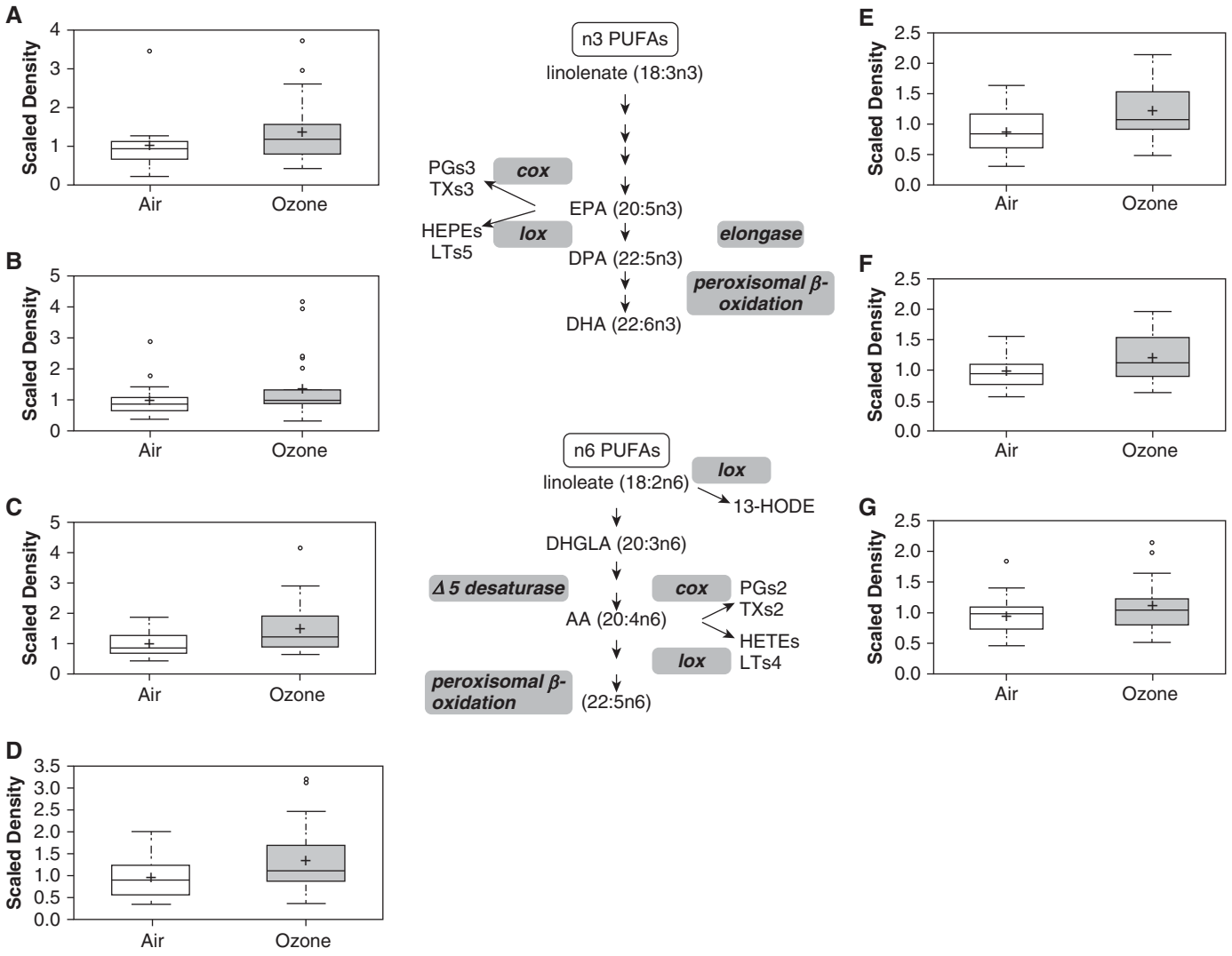

Figure 3. Acute ozone exposure increases circulating polyunsaturated lipids in humans. Polyunsaturated lipids (*A*) linolenate (α or γ; 18:3n3 or n6), (*B*) eicosapentaenoate (EPA; 20:5n3), (*C*) docosapentaenoate (n3 DPA; 22:5n3), (*D*) decosahexaenoate (DHA; 22:6n3), (*E*) linoleate (18:2n6), (*F*) dihomo-linolenate (DHGLA; 20:3n3 or n6), and (*G*) arachidonate (AA; 20:4n6) were increased significantly in the serum after ozone exposure in subjects. The metabolic pathways in which these metabolites were generated are summarized in the *middle panel*. *Box-and-whisker plots* convey the spread of the data with the interquartile range represented by the *box* and the range of the data shown by the *whiskers* (n = 24 per group). Outlier values are defined as those that exceed, in either direction, 1.5 times the interquartile range (shown as *open circle* in each plot). The *solid bar* across the box represents the median value, and the + *symbol* represents the mean. For each biochemical, data are median scaled with the median value across all samples set to 1.0. The *y-axis* thus reflects scaled intensity for each metabolite. Significant difference between air and ozone ($P \leq 0.05$ for *A–F*; $P \leq 0.1$ for *G*). 13-HODE = 13-hydroxy octadecadienoic acid; HETE = hydroxytetraenoic acid; PUFA = polyunsaturated fatty acid.

pathways (19, 20). The goal of the current study was to determine if metabolomic assessment of serum samples obtained from a prior clinical study where humans were acutely exposed to ozone show similar systemic metabolic alterations as rodents.

Serum metabolomic analysis revealed increases in circulating cortisol and corticosterone, but not cortisone, in samples collected after ozone exposure, reflective of an activation of a neurohormonally mediated stress response in humans. Pathway analysis of metabolites significantly ($P < 0.05$) changed in serum after ozone exposure implicated altered lipid metabolic processes. As observed in rodents, increased levels of several circulating free fatty acids and glycerols in the serum from ozone-exposed individuals suggested stimulation of adipose lipolysis of triglyceride stores and their liberation into the circulation. Increased lysolipids, likely released from hydrolysis of cellular and membrane phospholipids, and serum polyunsaturated fatty acids in ozone-exposed humans may be linked to proinflammatory mechanisms. Unlike rodents, samples obtained from humans who exercised intermittently during ozone exposure showed elevated circulating metabolites of β-oxidation, such as ketone bodies, dicarboxylates, and metabolites of ω-oxidation. No ozone effects were noted in homeostatic model assessment for insulin resistance. Overall, this study demonstrates that ozone exposure in humans is associated with

increased release of stress hormones causing lipolysis as in rodents, likely through activation of HPA axis. Chronic elevations of these metabolites in the circulation might contribute to metabolic diseases and systemic inflammation (30, 31).

In nonexercising rats, acute ozone exposure induces glucose intolerance and increases circulating leptin and epinephrine (19, 20). Both leptin and epinephrine changes are associated with a reversible decrease in body temperature (23), suggesting involvement of the sympathetic axis and changes in hypothalamic thermoregulation. Increased corticotropin and cortisol levels after ozone exposure have also been noted in other rodent studies (21, 32). In this study, humans exposed to ozone also presented elevated circulating cortisol and corticosterone, suggesting the activation of the HPA axis, similar to rats. However, unlike rats (19, 20), no differences were noted in leptin levels in serum samples obtained after ozone exposure in humans (*see* Figure E5 in online supplement 1). This might be caused by the differences in the study design and exposure protocol. The present human study did not involve ozone exposure during resting and exposure occurred only for 2 hours. Acute ozone exposure has been shown to activate central stress responsive regions and the nucleus tractus solitarius where pulmonary vagal nerves terminate in the brain (18, 32). Thus, pulmonary vagal C fibers, likely through neurotransmission in the brain, can stimulate sympathetic and/or the HPA axis, leading to the release of stress hormones from sympathetic nerve endings and also from the adrenal gland (33, 34). As a result, these stress hormones can target metabolic organs in a tissue-specific manner and alter glucose and lipid metabolism through activation of cellular glucocorticoids and adrenergic receptors.

Acute ozone exposure in humans increased circulating long- and medium-chain fatty acids as observed in rats (20). This is likely induced through stress hormone–mediated adipose lipolysis. Endogenous and ingested free fatty acids are reesterified in adipose tissue as triacylglycerides for storage (35). During a stress response, circulating epinephrine and cortisol through β-adrenergic and glucocorticoid receptors, respectively, can increase hormone-sensitive lipase-mediated lipolysis of adipose triglycerides into free fatty acids and glycerol (35). Ozone-induced elevation of free fatty acids in the serum could lead to uptake of these lipid metabolites by the peripheral tissues, including liver and muscle, for oxidation. In a previous study, we noted that genes involved in mitochondrial metabolism and biogenesis were markedly altered in the livers of ozone-exposed rats (20). Because the metabolic changes occurring immediately following an ozone exposure in humans are likely reversible after termination of exposure, as noted in rats after a single exposure (20), there is likely to be minimal long-term impact on healthy individuals. However, in those with underlying metabolic impairment or those with defects in homeostatic mechanisms, exposure to ozone might be more impactful and may contribute to metabolic imbalance.

Lysolipids, also known as lysophospholipids, were elevated in serum samples obtained after ozone exposure, indicating likely increased phospholipid turnover and plasma membrane remodeling by phospholipases. Although specific tissue sources cannot be ascertained through serum metabolomic assessment, these lipids through their action on G-protein-coupled lysophospholipid membrane receptors have been shown to impact a variety of cellular responses, such as cytoskeletal integrity/stability, mitogenesis, inflammation, energy production, and lipid metabolism (36).

Ozone exposure also increased circulating polyunsaturated fatty acids (Figure 3). In the lung, ozone exposure has been shown to increase polyunsaturated fatty acids, which are formed by a combination of activation of phospholipases and inhibition of free fatty acid esterification pathways (37, 38). The resulting increases in arachidonic acid metabolites can be involved in homeostatic and acute inflammatory responses. For example, arachidonic acid released by phospholipase A_2 is metabolized through the lipoxygenase or cyclooxygenase pathways to form eicosanoids, including leukotrienes, prostaglandins, and thromboxanes (39). Ozone exposure was associated with significant increases in plasma thromboxane B_2, 6-keto prostaglandin F1 alpha, and prostaglandin E_1 in guinea pigs (40). It is unclear whether the identified mediators shown in Figure 3 of ozone-induced phospholipase activation (41) can exert extrapulmonary effects. Enzymes involved in lipid metabolism were shown to be altered in a primate model after subchronic exposure to 0.3 parts per million ozone (42). Interestingly, it has also been demonstrated that cortisol inhibits phospholipase activity and prostaglandin production (43). Because these lipids were increased in the serum samples obtained after ozone exposure done with intermittent exercise in humans, but not in rats that were exposed during inactivity, the potential mechanism and implication of these findings requires further studies.

Acute ozone exposure might alter mitochondrial β-oxidation involved in catabolism of free fatty acids. Decreases in carnitine and increases in acylcarnitines together with elevated 3-hydroxybutyrate, a marker of hepatic β-oxidation, in serum after ozone exposure relative to air suggests changes in fatty acid oxidation that may be attributed to mitochondrial involvement (44). Additionally, increases in dicarboxylic acids in serum after ozone exposure may relate to increased ω-oxidation, a nicotinamide adenine dinucleotide phosphate– and cytochrome P-450–dependent process where the omega carbon of a fatty acid is oxidized to an alcohol and then to a carboxylic acid, thereby generating dicarboxylic acid. Dicarboxylic acids are produced by peroxisomes when the supply of fatty acids to the liver exceeds the capacity for β-oxidation and reesterification. Because subjects during air and ozone exposure underwent an exercise regimen, and exercise can influence the metabolic status, it is likely that the ozone response was influenced by exercise. Exercise-induced oxidative stress (45) was postulated to contribute to ozone-induced cytogenetic damage to human lymphocytes after ozone exposure (46). Interestingly, these changes were not observed in rats exposed to ozone (17) during inactivity. It is noteworthy that the ozone-induced hypothermia in nonexercising rodents exposed during their inactive (daytime) cycle (23) may lead to diminution of peripheral metabolic processes (muscle), whereas this response may not be apparent in humans exposed during their active cycle (daytime).

There are several limitations of our study. We only performed metabolomic analysis post air or ozone exposure during two clinical visits; thus, this study is not a

complete crossover design except for clinical measures. Ozone effects were not determined in humans during inactivity/resting and thus, the contribution of exercise and interaction with ozone cannot be assessed directly. Because we are attempting to compare the ozone effects in exercising humans with nonexercising rats, only some speculation of the role of exercise can be made. Moreover, human exposures occurred over 2 hours, whereas rats were exposed for 6 hours. Recently, we have noted that as with the hypothermic response to ozone, which is observed within 1 hour (23), metabolic changes in rats are also apparent early, as determined after 2 hours (data not shown).

The discrepancies in effective lung ozone dose could be partially explained by the evidence that exercising humans will inhale a larger ozone dose relative to nonexercising rats (47). We have demonstrated that acute ozone-induced metabolic changes are reversible the following day despite a continued inflammatory response in rodents (20), but we were neither able to analyze the reversibility of these changes in humans nor study the long-term ozone effects. In a previous study involving subchronic episodic exposure of Brown Norway rats, we noted that glucose intolerance still occurred after 12 weekly exposures (19). Because humans are exposed episodically to ozone throughout their lifetime, it is important to determine if persistent episodic metabolic derangement contributes to metabolic diseases.

This study is the first to characterize the global metabolic derangement in humans after ozone exposure using a metabolomic assessment. We show that ozone exposure in humans is associated with marked increases in a variety of fatty acids in the circulation together with increases in cortisol and corticosterone, suggesting activation of the neurohormonal stress response pathway and subsequent changes in peripheral metabolic homeostasis. This study establishes the coherence between humans and rodents in ozone-induced stress hormone increase and metabolic effects. Through this approach, novel biomarkers, such as cortisol, free fatty acids, monoacylglycerols, and lysolipids, for acute pollutant-induced health effects are identified. Further studies are needed to examine if these responses persist during chronic exposure and contribute to metabolic disorders. ■

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