Glucose dynamics during ozone exposure measured using radiotelemetry: Stress drivers and human concordance

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Running Head: Dynamic of ozone-induced stress response

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Abstract

Background. Stress-related neurobehavioral and metabolic disorders are associated with altered circulating adrenal-derived hormones and hyperglycemia. Temporal assessment of glucose and these hormones is critical for insights on an individual's health. **Objectives.** Here we use implantable-telemetry in rats to assess real-time changes in circulating glucose during and after exposure to the air pollutant ozone, and link responses to circulating neuroendocrine stress and metabolic hormones. We also proposed to compare rodent glucose and corticosterone (cortisol in humans) responses to humans exposed to ozone. **Methods.** First, using a cross-over design, we monitored glucose levels during single or repeated ozone exposures (0.0, 0.2, 0.4 and 0.8-ppm) and non-exposure periods in male Wistar-Kyoto-rats implanted with glucose-telemeters. A second cohort of un-implanted rats was exposed to ozone (0.0, 0.4 or 0.8-ppm) for 30-min, 1-hour, 2-hour, or 4-hour with hormones measured immediately after exposure. Then we assessed glucose metabolism in sham and adrenalectomized rats with or without pharmacological interventions of adrenergic and glucocorticoid receptors. Finally, we assessed glucose and cortisol in serum samples form a clinical study involving exposure of human volunteers to air or 0.3 ppm ozone. **Results.** Ozone (0.8-ppm) caused hyperglycemia and hypothermia beginning 90-min into exposure, with reversal of effects 4-6 hours post-exposure. Glucose monitoring during four daily 4-hour ozone exposures revealed duration of hyperglycemia, adaptation, and diurnal variations. Ozone-induced hyperglycemia was preceded by increased adrenocorticotropic hormone, corticosterone, and epinephrine, but depletion of thyroid-stimulating, prolactin, and luteinizing hormones. Hyperglycemia was inhibited in rats that are adrenalectomized and/or treated with glucocorticoid inhibitor. There was coherence among rats and humans in ozone-induced corticosterone/cortisol increases. **Discussion.** We demonstrate for the first time the temporality of neuroendocrine-stress-mediated biological sequalae responsible for ozone-induced metabolic dysfunction as exposure occurs. Real-time glucose monitoring with stress hormones assessment may be useful in identifying interactions among pollutants and stress-related illnesses.

INTRODUCTION

Air pollution, climate change, the epidemic of COVID-19, social inequalities, unhealthy dietary habits, and sedentary lifestyle are likely to further escalate mental health crises and metabolic syndrome world-wide. Among many environmental risk factors, air pollution accounts for nearly 70% of all environmental causes of human mortality,[1] and is linked to neurobehavioral and metabolic diseases. Increased incidence of Alzheimer's disease,[2,3] late life cognitive decline,[4] anxiety, and even criminality have been associated with the exposure to air pollutants.[5] Moreover, associations have been found between air pollution and concurrent exacerbation of diabetes and Alzheimer's disease.[4] Those with Alzheimer's and Parkinson's disease also often suffer from diabetes suggesting potential neural contribution to peripheral diseases and mechanistic linkages.[6,7]

With the emerging link between air pollution, stress, and neuro-cognition,[5,8] the role of neuroendocrine system is inevitable. Psychosocial and environmental stressors are the primary contributors to chronic disease susceptibility. The central neuroendocrine system responds to stress-induced autonomic sensory activation and orchestrates a peripheral stress response, while initiating two survival processes, namely metabolism and immune surveillance in an organ-specific manner.[9] In addition to sympathetic neurons innervating the majority of peripheral organs, sympathetic-adrenal-medullary (SAM) and hypothalamic-pituitary-adrenal (HPA) axes-mediated release of catecholamines and corticosteroids produce peripheral cellular response to stress.[9] These are the same hormones that regulate stress adaptation through their action on the CNS centers via feedback controls.[10] Chronic alterations in the levels of circulating stress hormones, especially glucocorticoids have been linked to psychological disorders and cardiometabolic diseases.[11] Release of centrally- mediated stress hormones and peripheral responses to stress are plastic, and are temporally and spatially regulated, such that no adverse effects persist upon discontinuation of stress.[10] However, when the neuroendocrine system is

impaired or overactive, disease may ensue.[12] Thus, the temporal assessment of the stress dynamics is critical to understanding an individual's susceptibility to environmental insults.

Monitoring of stress response through real-time assessment of circulating cortisol and glucose in humans is not common. However, in children recovering from surgery, blood glucose and cortisol are often measured to assess stress.[13] Individuals with type 2 diabetes, when subjected to acute moderate psychological stress (Trier Social Stress Test), have spikes in blood glucose as determined using real-time glucose monitors.[14] Real-time glucose monitoring sensors, which have been developed in early 1990's, are now applied more widely for diabetics.[15] In humans, continuous monitoring of glucose using non-invasive sensor-based electromagnetic coupling is already gaining popularity,[16] providing the opportunity to use such monitors in detecting environmental stressor effects such as air pollutants. Very recently, a wireless graphene-based sweat stress sensing mHealth system has been developed for dynamic and non-invasive assessment of cortisol in sweat.[17] The use of new techniques for dynamic stress assessment will be valuable for determining the health impact of stressors on the body. In particular, this approach in air pollution health effects studies could allow evaluation of short and long-term effects on health and provide diagnostic and mechanistic insights.

We have shown that acute single exposure to ozone induces a classical stress response associated with increases in circulating catecholamines, glucocorticoids and glucose.[18,19] However, the neuroendocrine stress response is temporal and reversible in healthy individuals. In order to link ozone-induced alterations in metabolic processes, and neuroendocrine stress, it is critical to determine the dynamicity of peripheral metabolic effects and how that relates to neuroendocrine changes. The purpose of our study was to determine the temporality of glucose changes during and after ozone exposure in a rat model using implantable radiotelemetry, which has not been previously employed for experimental assessment of air pollution effects. We hypothesized that increased glucose levels during ozone exposure will be secondary to ozone-induced increases in stress hormones. Real-time glucose measures were coupled with

separate measures of stress hormones as well as mechanistic studies to assess the role of stress responses in ozone-induced glucose fluctuations. Moreover, to establish coherence with human responses, we assessed the impacts of ozone exposure on glucose and cortisol levels in young healthy volunteers.

Materials and Methods

Animals

All male Wistar Koto rats 10-12 weeks were purchased from Charles River Laboratories Inc., Raleigh, NC and maintained in our Association for Assessment and Accreditation of Laboratory Animal Care-approved EPA animal facility. Animals were pair housed in polycarbonate cages with beta chips bedding and EnviroDry enrichment material, except when stated. Animal rooms were maintained on a 12 hr light/dark cycle (6AM-6PM) at ~22°C and 50% relative humidity. They were provided free access to Purina (5001) pellet rat chow (Brentwood, MO) and water ad libitum, unless stated during experimental procedures. Animal protocols were approved by the EPA's Institutional Animal Care and Use Committee prior to starting studies and we followed National Institutes of Health guide for the care and use rats (NIH Publications No. 8023).

Glucose telemetry surgeries

Eight male Wistar-Kyoto (WKY) rats (at 13 weeks of age) were implanted with DSI glucose telemeters (HD-XG, St. Paul, MN) using aseptic techniques (Figure S1, Table S1). Anesthesia was induced by vaporized isoflurane inhalation (4%, 1-2 LPM of O_2) and maintained during the surgery (2-3%, 1-2 LPM of O_2). Once anesthetized, analgesic meloxicam (2 mg/kg, in saline s.c.) and artificial tear ointment were provided before surgery. Anesthesia was continuously checked by toe pinch. A trained surgeon implanted the sensor in the descending abdominal aorta and the transmitter subcutaneously. The blood glucose (Nova Biomedical,

Waltham, MA) sensor uses glucose oxidase to convert glucose and oxygen into gluconic acid and hydrogen peroxide. The amount of hydrogen peroxide, which is proportional to the amount of glucose, reacts with a noble metal electrode to transfer electrons and create a current.[20] The glucose telemetry system includes a reference electrode with an electronics/battery (Ag/AgCl) with a separate lead. This was sutured to the inner abdominal wall module into the midline abdominal muscle (Figure S1). During abdominal wall closure, the abdominal cavity was washed with saline (15 mL/kg) and rats were given bupivacaine (1 mg/kg, in saline s.c.). Recovery took place on heating pads under close observation of distress signals and once awake the rats were placed into their home cages and administered with meloxicam (1 mg/kg, in saline s.c.) 24, 48 and 72 hours after the surgery.

Blood glucose concentration data acquisition

After surgery, rats were individually housed in cages with pine shavings bedding. Rats were provided with water and powdered as well as pelleted standard Purina (5001) rat chow (Brentwood, MO) *ad libitum*. Blood glucose levels, core body temperature, and activity level measurements were sent via radio signals and collected using receivers placed under each cage. The recording for each rat began soon after the surgery and continued until the end of the experiment. The cages were placed over receivers (RPC-1, DSI) and data were simultaneously collected in a computer placed in an adjacent isolated room (Figure S1). For verification and calibration purposes, single point calibrations were carried out. While glucose monitoring was going on, glucose tolerance test was performed. Food was removed from cages 6 hours in advance and then baseline glucose readings were obtained through tail prick using a glucometer (Nova Biomedical StatStrip® Xpress, DSI). Rats were then injected with glucose (20% pharmaceutical grade D-glucose; Covetrus, Dublin, OH; 2 g/kg/10mL, i.p.) and data were collected using glucometer via a tail prick every 30 minutes for 2 hours. These readings were then correlated with glucose measurements done using radio telemetry. Interpolation of glucose levels and electric current detected by the sensor was corrected for differences in body

temperature versus room temperature, merged and analyzed for each animal with a resolution of 1 minute using Dataquest® software acquisition system (DSI).

Ozone exposure

Two weeks after surgery, rats were exposed (n=1-2 each) whole body to ozone (0.2, 0.8 or 0.4 ppm) or filtered air (0.0 ppm) in Rochester style "Hinners" chambers, using a crossover exposure design (Table S1). Briefly, rats for first exposure were randomized in pairs for 0.0 (clean air), 0.2, 0.4 or 0.8 ppm of ozone exposure for 4 hrs. A different set of receivers were placed in each ozone exposure chambers to acquire data while animals were being exposed. After 1 week of washout period, these exposures were repeated but changing the targeted ozone exposure concentration for each pair of rats. This experiment was repeated a total of four times to cover all the targeted ozone concentrations for all eight rats. Rat #6 was discarded from the experiment due to mal function of the sensor and/or transmitter. Ozone was generated from oxygen using a silent arc discharge generator (OREC, Phoenix, Arizona) and measured using mass flow controllers (Coastal Instruments Inc., Burgaw, North Carolina) as we have reported in our prior studies.[18,21] The target ozone concentrations were recorded by photometric analyzers (API Model 400, Teledyne, San Diego, California). Mean chamber temperature, relative humidity, and air flow were recorded hourly.

Glucose/temperature telemetry data acquisition and analysis

After 4-5 days of surgery and at regular interval the device was calibrated against intraperitoneal glucose tolerance test (GTT) performed as indicated below based on the protocol explained by Brockway and collaborators at Data Sciences Inc.[20] As recommended, single-point calibrations were done twice weekly throughout the study. Dataquest® acquisition system (DSI) included telemetry signal receiver for each animal, a data matrix for analyzing receiver signals and a computer. This telemetry devices allowed continuous sampling of 8 animals that are individually housed in cages with receivers right underneath. To avoid signal mixing between

two animals the animal racks were equipped with stainless steel dividers. The software allowed us to configure the devices and the protocol for continuous data collection as described previously.[20] Glucose and temperature data with a resolution of 1 minute was averaged to prepare graphs. All exposures were aligned to minute 0 when 80% of target ozone concentration was reached. For each minute glucose levels and temperature was averaged from exposed rats (0.0 ppm, n=6; 0.2 ppm, n=5; 0.4 ppm, n=5; 0.8 ppm, n=5). Based on the animal position in the exposure chamber, occasionally data were not acquired and therefore for telemetry studies number of observations varied between 3-7. Multiple hours of data after the exposure stopped were included to follow days of recovery during non-exposure periods.

Time course assessment of hormonal response in rats

A separate cohort of healthy male 12-13-week-old rats was exposed to clean air or ozone at two concentrations (0.4 and 0.8 ppm) for 30 min, 1 hr, 2 hr or 4 hr (n=6-8 per group) and necropsied immediately after each time point (Figure 2) to collect blood samples as described below. We have shown in our previous studies that ~10% of male WKY rats display spontaneous cardiac hypertrophy,[22] therefore at necropsy heart to body weight ratios were calculated for all animals and those with 20% or greater increases were removed from data analysis as we have done for many studies.

Adrenalectomy and sham surgeries

Rats (12–13 weeks old) underwent total bilateral adrenalectomy (AD) or control sham (SH) aseptic surgeries using protocols established at Charles River Laboratories and previously described in our recently published studies.[23,24] Briefly, rats were anesthetized with ketamine (25–50 mg/kg in saline, i.p.), and once anesthetized, injected with the analgesic buprenorphine (0.02 mg/kg/ml in saline: s.c.). During the surgery, anesthesia was maintained by nose-inhalation of vaporized isoflurane (~3%, 1-2 LPM of O_2). Animal surgeons from Charles River Laboratories Inc performed the surgeries. Animals were placed in sternal recumbency and

dorsal incision was made. Adrenals from both sides were removed and the muscle layer was sutured to close the abdominal cavity. The surgical wound clips were used to clip the skin and close the wound. SH surgeries were performed using the same anesthesia and surgical approaches as AD except for the removal of adrenal glands. Rats were recovered on heating pads and assessed for signs of distress and pain. Once awake, meloxicam (0.2 mg/kg in saline; s.c.) and buprenorphine (0.02 mg/ml/kg in saline, s.c. every 8–12h for 2 times) was administered for analgesia. After the surgery, AD rats received water with 0.9% NaCl to maintain adequate salt-water balance in the absence of mineralocorticoids eliminated due to AD along with other adrenal-derived stress hormones. All animals were provided with powdered as well as pelleted food *ad libitum*. The rats were pair housed with Enviro Dry enrichment/nesting material and allowed to recover for 4–6 days prior to any drug treatment as reported in our previous companion paper.[24]

Clenbuterol (CLEN; β2AR agonist) and dexamethasone (DEX; GR agonist) treatments and ozone exposure

As detailed in our recent publication,[24] SH and AD rats were randomized by body weight into four groups (vehicle: air, vehicle: ozone, CLEN+DEX: air, and CLEN+DEX: ozone) resulting in 8 total groups (n=8/group). The treatment protocol for CLEN and DEX has been explained in the published paper.[24] In brief, after 4-6 days of recovery from SH and AD, rats were treated with vehicles (saline 1 mL/kg as control for CLEN, i.p.) and corn oil (1 mL/kg as control for DEX, s.c.) or clenbuterol hydrochloride, a long acting β2AR agonist (CLEN; 0.2 mg/kg in saline, i.p.) and GR agonist, dexamethasone (DEX; 2 mg/ml corn oil/kg, s.c.). Generally, CLEN injections were followed by DEX. The drug treatment began 1 day prior to start of ozone exposure and continued the day of air or ozone exposure in the morning at ~6am. These high doses were selected to restore the depleted activities of epinephrine and corticosterone in AD groups. CLEN and DEX doses are comparable to those used in other controlled experiments using rodents

and are sufficient to induce bronchodilation and immunosuppression, respectively.[25,26] Rats were exposed to air or 0.8 ppm ozone for 4 hours as described above, and glucose tolerance test (GTT) was performed immediately following exposure.

Ozone exposure in SH and AD rats treated with βAR and GR antagonists

Additional three independent experiments were carried out to evaluate the role of 1) β adrenergic receptor antagonist propranolol (PROP) individually, 2) glucocorticoid receptor antagonist mifepristone (MIFE) individually, and 3) both in combination to determine their influence on ozone effects as published previously.[27] For each study, 12-13-week-old male WKY rats were randomized by body weight into four groups (vehicle/air, drug/air, vehicle/ozone, drug/ozone, n = 8/group). In the first experiment, rats were injected with either sterile saline (vehicle; 1 mL/kg, i.p.) or propranolol hydrochloride (PROP, Sigma-Aldrich, St Louis, MO; 10 mg/kg in saline, i.p.). In the second experiment, rats were injected with pharmaceutical grade corn oil (vehicle; 1 mL/kg, s.c.) or mifepristone (MIFE, Cayman Chemical Co., Ann Arbor, MI; 30 mg/kg in corn oil, s.c.). In the third experiment, rats were injected with vehicles, saline (1 ml/kg, i.p.) followed by corn oil (1 ml/kg, s.c.) or drugs, PROP (10 mg/kg, i.p.) followed by MIFE (30 mg/kg, s.c.) (PROP+MIFE). The rationale for drug selection and treatment protocol are explained in our previous companion study.[27] To assure complete inhibition of βAR and GR receptors, the daily morning treatment began 7 days prior to the air or ozone exposure and was continued the day of exposure. In each study, rats were exposed to air or 0.8 ppm ozone for 4 hours and glucose tolerance test (GTT) was performed immediately after exposure.

Glucose tolerance test for intervention studies

For glucose telemetry, gain of function and pharmacological intervention studies, glucose tolerance test (GTT) was performed in rats after air or ozone exposure as previously described.[18,28] Since rats underwent air or ozone for 4 hours exposure prior to GTT when no food was provided, this served as fasting for GTT. Immediately after air or ozone exposure,

baseline blood glucose concentrations (0 hr) were determined by tail prick using a sterile 23-gauge needle with a Bayer Contour Glucometer (Leverkusen, Germany). Rats were then injected with 20% D-glucose (20% pharmaceutical grade D-glucose; Covetrus, Dublin, OH; diluted to 2 g/kg/10mL, i.p.) as described for telemetry experiment. Glucose levels were measured by tail prick at 30, 60, 90, and 120 min.

Necropsy and blood samples collection for hormones in time course study

For hormonal time course study, rats were necropsied within 15 minutes after each exposure. Necropsies were performed in a staggered manner. Rats were euthanized with Fatal Plus (sodium pentobarbital, Virbac AH, Inc., Fort Worth, TX; >200 mg/kg, i.p.). Blood samples were collected from the abdominal aorta directly in vacutainer serum separator tubes and EDTA tubes for serum and plasma separation, respectively. Blood samples were spun at 3500 x g for 15 min at 4° C, serum and plasma samples were aliquoted and stored at -80 ℃ until analysis. Heart tissues were weighed to monitor spontaneous cardiac hypertrophy in this strain of rats.

Plasma and serum analysis of hormones

Plasma levels of epinephrine (adrenaline) and corticosterone were quantified using kits from Rocky Mountain Diagnostics (Colorado Springs, CO) and Arbor Assays (Ann Arbor, MI). Serum pituitary hormone levels for adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), prolactin (PRL), luteinizing hormone (LH), growth hormone (GH), brain-Derived neurotrophic factor (BDNF), and follicle stimulating hormone (FSH) were determined using MILLIPLEX MAP Rat Pituitary Magnetic Bead Panel following manufacturer's protocol (Merck-Millipore, Burlington, MA). Serum free fatty acids were measured using kits from Cell Biolabs, Inc (San Diego, CA adapted for use on a Konelab Arena 30 clinical analyzer (Thermo Chemical Lab Systems, Espoo, Finland). Serum insulin and leptin were quantified using Mesoscale Discovery® Multi-Spot® assay system following manufacturer's protocol (Rockville, MD).

Statistics for hormones and glucose tolerance assessment data

For all analyzed endpoints a p value less than 0.05 was considered significant and depending on the study, different approaches were taken for data analysis. For hormones and metabolite analysis, ozone concentration response was calculated using non-parametric one-way ANOVAs for each time point (Kruskal Wallis test). Dunn's multiple comparisons post-test was employed to determine the concentration effect for a given time point. The GTT data for all studies were analyzed as stated: In gain of function experiment, first one-way ANOVA (Krustal-Wallis test) was employed to evaluate if ozone or AD effect was significant by analyzing vehicle- and CLEN+DEX-treated groups separately and if CLEN-DEX effect was significant, analyzing SH and AD groups separately. Ozone effect was calculated for matching surgery-drug-treated groups, AD effect was calculated for matching exposure-drug-treated groups, and CLEN+DEX effect was calculated for matching surgery-exposure groups. Dunn's multiple comparisons test was employed to calculate p values for each pair of comparison. For the antagonists, treatment in animals where beta adrenergic and/or glucocorticoid receptors were inhibited, three independent one-way ANOVAs (for PROP, MIFE and PROP+MIFE) were carried out. For each endpoint, one-way ANOVAs (Krustal-Wallis test) were employed to evaluate whether the ozone effect (*) was significant or drug effect (†) was significant. Dunn's multiple comparisons post-test was used to derive p values for each pair of comparison. For glucose tolerance test (GTT), area under the curve (AUC) was calculated using the trapezoidal method as previously described.[23] GraphPad Prism 9 (version 9.1.2) was used for statistical analysis and graph generation.

Assessment of glucose and cortisol in human clinical study samples

Human plasma samples were obtained from a clinical study conducted through the University of North Carolina (Chapel Hill, NC) under IRB# #13-1644. The study involved exposure of young healthy human volunteers to filtered air or 0.3 ppm ozone exposure in a cross-over design where the same subjects were randomly exposed to air or ozone during two distinct visits that were separated by two weeks or longer. Prior consents were obtained from all individuals participating in the study. Blood samples were collected prior to and immediately

following 2-hour exposure to air or ozone for each of 34 subjects. Demographic information is provided in Table S2.

For human plasma samples, glucose levels were analyzed using Bayer Contour Glucometer and test strips (Leverkusen, Germany). Plasma cortisol levels were analyzed using human cortisol kit from Arbor Assays (Ann Arbor, MI). The percent change in human plasma levels of glucose and cortisol pre and post exposure was analyzed using a repeated measures one-way ANOVA. For human data, outliers were identified using boxplot method, defined as those above Q3 + 1.5 IQR or below Q1 – 1.5 IQR and discarded.

RESULTS

Real-time *in vivo* glucose monitoring during and after ozone exposure

We used a novel real-time blood glucose telemetry system (Figure 1A, Figure S1 and Table S2)[20] that has not been employed in previous air pollution studies. Using a cross-over design with the 7 telemetered rats, we obtained independent readings at each concentration for a weekly 4-hour exposure to air or ozone with 1-week washout (Table S1). We have shown that one week wash-out period after a single 4-hour ozone exposure is sufficient to clear effects from a previous exposure in rats.[29] Continuous monitoring of glucose during 4 hours of ozone exposure at various concentrations and post exposure periods in rats allowed insights in precise timing for a stressor to impact changes in circulating glucose and the longevity of a stress response. It also allowed monitoring of glucose intake-related changes and diurnal changes. Since glucose telemetry also included the assessment of core body temperature, we were able to show that a drop in core body temperature was related to glucose changes.

Hyperglycemia began to occur at ~90 min into a 4-hour ozone exposure but only in the 0.8 ppm group (Figure 1B). The significance of a small reduction in this hyperglycemia at about 3 hr and then reoccurrence of a peak between 4-5 hour during first day of exposure is unclear

but may reflect fine oscillatory adjustment in homeostatic processes, which might be important for centrally balanced and precisely controlled responses to ozone stress. A third peak of hyperglycemia in the 0.8 ppm ozone group was noted roughly 1 hour after the beginning of dark cycle (and 4 hours post cessation of ozone exposure) when rodents were active and feeding (Figure 1C). The increase in glucose at 90-min was associated with hypothermia in the 0.8 ppm ozone group, but without the fluctuations seen in glucose levels (Figure S2). We performed a glucose tolerance test (GTT) after ozone exposure in telemetered rats. These rats exhibited ozone-induced glucose intolerance in addition to hyperglycemia (Figure 1D) consistent with our previous studies involving post exposure assessment.[18] The telemetry data for blood glucose after bolus glucose injection matched the data obtained through a handheld glucometer (Figure 1E). Combined, these data suggest that glucose monitoring in real-time offers opportunities to concurrently assess effects dietary glucose and acute environmental stressor effects. The temporal co-occurrence of ozone-induced hypothermia and hyperglycemia in the 0.8 ppm group suggests that these processes are linked or induced through common upstream events (Figure S3A). This hypothermia did not occur after glucose injection. In general, when glucose levels are high, core body temperature increases along with activity as noted during the dark cycle for rodents (Figure S3B). However, ozone-induced changes in blood glucose and body temperature were in the opposite direction, suggesting stress-induced disturbance in homeostasis to conserve metabolic energy and direct it where needed. Hypothermia, which has been linked to stress-induced glucocorticoid increases in humans[30] was also evident after ozone exposure in our previous study that employed real-time ECG monitoring in rats.[31]

Real-time glucose monitoring during ozone adaptation and diurnal variation

Repeated daily exposure to ozone has been associated with adaptation/tolerance in mice.[32] However, the mechanism of adaptation remains elusive. With the use of glucose

telemetry, we linked ozone adaptation to glucose changes and obtained precise timing of adaptation. After a 4-hour 0.8 ppm ozone exposure on the first day, hyperglycemia was not noted during subsequent days of no exposure (Figure 2A). The diurnal changes were apparent in all animals showing higher levels of glucose at nighttime when compared to daytime. After completing 4-week exposure using cross over design, animals were assigned air or 0.8 ppm ozone group for subsequent weeks. On the 5th and 6th week, these rats were exposed to air or 0.8 ppm ozone for 4 hours using crossover design (alternating exposure assignment to air or ozone each week), and GTT was performed immediately following exposure. On week 7, again these animals were crossed over and exposed to air or 0.8 ppm ozone (4 hours each day) for 4 consecutive days to determine if adaptation occurs with regards to glucose and body temperature changes during continued daily exposure. Ozone at 0.8 ppm 4 hours/day for 4 consecutive days led to lack of increase in blood glucose during and right after the exposure on the third and fourth days (Figure 2B) despite continued exposure indicating adaptation. Animals assigned to ozone exhibited higher nighttime glucose levels even prior to beginning 4-day exposure protocol (Figure 2B). Although these elevated levels were small, they may relate to the difference in the characteristics of rats and their prior placement in crossover design. However, near complete adaptation to ozone exposure was evident by the 3rd day despite continued exposure on 3rd and 4th day. Thus, continuous glucose monitoring indicated that this adaptation occurs on the 3rd day but not on the 2nd day of ozone exposure, and this adaptation is associated with attenuation of ozone-induced hyperglycemia noted during ozone exposure on day 1 and day 2 (Figure 2B). The adaptation was also noted in hypothermia on the 3rd day (Figure S3C). These ozone-induced changes in circulating glucose of rats reflect the status of glucose metabolic processes in tissues.[29] Thus, real-time glucose monitoring allows one to assess the timings of metabolic alterations and adaptation during ozone exposure.

Temporality of ozone-induced HPA and SAM activation

Since the method for continuous monitoring of corticosterone in animals is still not available, we next exposed rats to ozone for variable durations spanning a 4-hour time frame to assess temporal changes in adrenal-derived corticosterone and other neuroendocrine hormones (Figure 3A). This study followed a similar paradigm to real-time glucose monitoring but used a distinct cohort of rats at each timepoint during 4-hour ozone exposure to assess serum samples for key pituitary, adrenal-derived, and metabolic hormones (Figure 3). A sharp rise in adrenocorticotropic hormone (ACTH) occurred at 30 min into the 0.8 ppm ozone exposure and peaked at 1 hour, reflecting the activation of the HPA axis and concomitant ACTH release from the anterior pituitary as early as 30 min (Figure 3B). Upon receiving stress signals in the paraventricular nucleus of the hypothalamus, the secreted corticotrophin releasing hormone traverses to the pituitary through the hypothalamic-pituitary portal system and activates ACTH secretion from the anterior pituitary. ACTH released into the systemic circulation reaches the adrenal cortex to stimulate corticosterone/cortisol synthesis and release involving hypothalamus-pituitary-adrenal (HPA) axis.[10] Consistent with this, we noted that the levels of circulating corticosterone in rats increased starting at 1 hour into ozone exposure (0.8 ppm) prior to the increase in glucose and remained significantly elevated until 4 hours of exposure (Figure 3C)[33] despite the restoration of ACTH to baseline levels at this time point. It is important to note that although no significant ACTH increase occurred during the 0.4 ppm ozone exposure, the increase in corticosterone was significant at 4 hours, suggesting that the stress response was concentration dependent. Corticosterone/cortisol is a ligand for glucocorticoid receptors with ubiquitous tissue distribution and complex transcriptional regulation involved in maintaining immune and metabolic homeostatic processes and adaptation.[34]

The stress response induced by acute physical and emotional stress also involves the activation of the splanchnic sympathetic nerve via the hypothalamus leading to a release of epinephrine from chromaffin cells of the adrenal medulla.[35] The sympathetically mediated (SAM axis) epinephrine increase generally precedes the increases in corticosterone during a fight-or-

flight response. The data show that after 30 min of 0.8 ppm ozone exposure, the levels of epinephrine were increased. Exposure to the lower concentration of ozone (0.4 ppm) also caused an increase in epinephrine, although the time required for this increase was longer, as it evident only after 4 hours of exposure (Figure 3B).[33] The mechanism by which neural centers are activated immediately after ozone exposure is poorly understood. The role of the activation of vagal sensory fibers has been postulated in rats after acute ozone exposure.[36] The sustained increases in circulating epinephrine during the 4 hour of ozone exposure (Figure 2B) corroborated our prior findings involving a single end of exposure measurement.[18,28] These hormones, once released, can exert a wide-array of systemic metabolic, vascular, and immunological effects through adrenergic G-protein coupled receptors activation and subsequent cyclic AMP mediated signaling through protein kinase A.[37]

Temporal changes in HPT and HPG hormones during ozone exposure

Since ozone-mediated stimulation of the neuroendocrine system may also influence other hypothalamic stress pathways such as hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-gonadal (HPG) axes, we next assessed temporal effects of ozone exposure on relevant hormones. Recent evidence links exposure to air pollution with impaired thyroid function in newborns.[38] Earlier experimental studies have shown depletion of TSH and thyroxine after a single ozone exposure[39] and increased ozone pulmonary toxicity in rats treated with thyroxine.[40] We find here that ozone exposure results in time- and concentration-dependent decline in TSH levels, which occurs sooner (1 hour) at 0.8 ppm and is temporally linked to increases in corticosterone and precedes the increased glucose response with 0.8 ppm ozone (Figure 3B).

To gain additional insights in how other pituitary hormones involved in gonadal axis may also be impacted following ozone exposure, we next assessed follicle stimulating hormone (FSH), prolactin (PRL), and luteinizing hormone (LH) (Figure 3B). Exposure to air pollution has

448 been associated with poor reproductive performance and sperm quality.[41] However, only limited
449 experimental evidence exists to support neuroendocrine mechanisms. We report that ozone
450 exposure depleted not only LH, but also PRL (>95%), with the 0.8 ppm concentration causing a
451 more rapid depletion. This corroborates our recent study where we reported a decrease in
452 circulating PRL assessed once after 4 hour of ozone exposure.[42] Here, the temporal
453 assessment shows depletion of circulating PRL as early as 1 hour into exposure concomitant
454 with peak ACTH levels. However, LH levels did not decrease until 2-hour of exposure (Figure
455 3B). The mechanism by which ozone may inhibit the release of PRL into the circulation in this
456 study is unclear, however, its rapid decline and the reversal of ozone induced PRL depletion in
457 adrenalectomized rats[42] suggests the possible involvement of glucocorticoid feedback
458 regulation. While circulating glucocorticoids might also inhibit LH secretion after ozone
459 exposure, a role of gonadotropin releasing hormone and gonadotropin inhibitory hormone is
460 likely in ozone-induced inhibition of LH.[43] However, the levels of FSH were not changed after
461 ozone exposure in male WKY rats (*data not shown*). These data provide insights on how acute
462 ozone inhalation can dynamically and differentially impact various neuroendocrine axes that
463 have major impact on homeostatic physiological processes.

464 Previously we noted ozone-induced pulmonary and liver transcriptional changes
465 reflective of processes that regulate cell cycle, growth, and regeneration.[44,45] Since pituitary-
466 derived growth hormone (GH) is involved in these processes,[46] and tied to metabolic changes,[47]
467 we assessed the kinetics of growth hormone changes and noted that a delayed but
468 concentration-dependent increase in GH occurred at 4-hour after ozone exposure suggesting
469 that the anabolic processes are being activated (Figure 3B). A similar temporal pattern was
470 noted for the increase in leptin, which regulates satiety at the level of hypothalamus. On the
471 other hand, the increase in circulating free fatty acids was noted as early as 1 hour after ozone
472 exposure, suggesting the early activation of lipolytic activity in adipose tissue coinciding with
473 changes in circulating adrenal-derived hormones, but a delayed increase in GH coincides with

leptin release from adipose tissue. Overall, ozone-induced hyperglycemia is reflective of neuroendocrine responses that involve a wide array of changes in metabolic and cell growth processes.

The role of epinephrine and corticosterone in mediating hyperglycemia

Adrenal-derived epinephrine and glucocorticoids are the major regulators of liver metabolic processes during stress,[48] and adrenalectomy diminishes ozone-induced hyperglycemia and glucose intolerance.[23] Therefore, we further assessed the roles of adrenal-derived stress hormones in mediating changes in circulating glucose after ozone exposure. Adrenalectomy, in addition to depleting circulating epinephrine and corticosterone also depletes circulating mineralocorticoids. To delineate the contribution of two major stress hormones, epinephrine and corticosterone, without the influence of mineralocorticoids, we conducted GTT in a gain of function experiment, where we treated sham (SH) and adrenalectomized (AD) rats with the β2 adrenergic (β2AR) agonist, clenbuterol (CLEN) plus glucocorticoid receptor (GR) agonist dexamethasone (DEX). Animals were treated with both drugs simultaneously prior to and during ozone exposure as reported.[49] In the second experiment, we inhibited epinephrine and corticosterone receptors, individually or in combination, to determine the role of each receptor type in mediating ozone-induced hyperglycemia and glucose intolerance. Rats were treated with pharmacological antagonist of β-adrenergic receptor (βAR), propranolol (PROP), and/or a GR antagonist mifepristone (MIFE) (Figure 4C) individually or in combination prior to and during ozone exposure as reported earlier.[27]

Ozone-induced hyperglycemia and glucose intolerance were nearly eliminated in vehicle-treated AD rats confirming our earlier findings.[23] Moreover, all animals treated with CLEN+DEX developed marked hyperglycemia and glucose intolerance, in both SH and AD rats exposed to air. Further, this response was exacerbated in rats exposed to ozone demonstrating

βAR+GR activation through increased epinephrine and corticosterone mediating hyperglycemia (Figure 4B). PROP or MIFE given individually did not reduce ozone-induced hyperglycemia, but PROP+MIFE in combination, significantly decreased hyperglycemia severity. In a related manner, the blockade of βAR and GR individually only partially reversed ozone-induced glucose intolerance, but when given together, they markedly diminished ozone-induced glucose intolerance, suggesting the involvement of both epinephrine and glucocorticoids in mediating glucose increases during ozone exposure (Figure4C). These results allowed us to establish a causal link between ozone-induced increases in the release of adrenal-derived stress hormones through SAM and HPA activation and resultant glucose metabolic alterations. Together, these data suggest that real-time monitoring of glucose and cortisol levels may be used as a proxy for environmental pulmonary exposures.

Ozone-induced stress response in humans

Hormonal response to stress is conserved across species, in this study we wanted to confirm if the results seen in rats would extend to humans. We assessed glucose and cortisol levels in plasma samples of human volunteers exposed to air or 0.3 ppm ozone during 2 hours of intermittent exercise in a cross-over design where each volunteer was exposed to air and ozone but separated by at least two weeks interval (IRB# #13-1644; Figure 5A). There were no differences in blood glucose levels between volunteers exposed to air or ozone (Figure 5B), likely since all subjects were exposed in a protocol involving intermittent exercise. In contrast, although exercise resulted in decreased cortisol levels in air exposed individuals, this decrease was significantly attenuated in volunteers exposed to ozone as determined by % change between pre- and postexposure (Figure 5B). Ozone exposure was thus, associated with significant increase in cortisol (Figure 5B), which is in concordance with our earlier observations in humans.[19] The present findings of cortisol increase demonstrate a coherent ozone-induced stress response between humans and rodents. Overall, the temporal monitoring of glucose and

cortisol in studies examining air pollution and other stressors may provide better understanding of the mechanisms of pathogenesis and individual variations in susceptibility for chronic diseases in humans.

DISCUSSION

Prior research on air pollution health effects focused primarily on cardiorespiratory outcomes elicited by local effects in the lung. Recently it has become apparent that inhaled irritant air pollutants might be perceived as stressors by the autonomic nervous system, resulting in stimulation of neuroendocrine axes which mediate acute effects in the lung and periphery.[50] The impairment and/or persistent hyperactivity of stress responses has been linked to chronic psychiatric, neurobehavioral, cardiometabolic, and reproductive health abnormalities. Since stress responses are dynamic and CNS-regulated to induce reversible peripheral changes, its real-time assessment is necessary for in-depth understanding of its impact on health and resiliency. We used real-time glucose telemetry in rats, combined with temporal assessment of corticosterone and other neuroendocrine hormones to demonstrate in rodents a contextual relationship between air pollution induced stress response and hyperglycemia. We demonstrated mechanistic link between hyperglycemia and adrenal-derived stress hormones using adrenalectomy and pharmacological interventions of adrenergic and glucocorticoid receptors (βAR and GR). We followed this up by showing coherency in the human stress response to ozone, providing initial evidence that real-time monitoring of glucose and human stress hormones may serve as immediate biomarkers of air-pollutant-induced pulmonary stress and the interactive impacts of other environmental contaminants, and non-chemical stressors.

Hyperglycemia is one of the earlier markers of stress-induced homeostatic changes that is consistently noted after a single ozone exposure[18,23,28] and other irritant exposures.[51] Here we show that the temporality of this neuroendocrine response induced by ozone can be assessed using real-time glucose telemetry in rats. We show that ozone-induced hyperglycemia, which

coincides with hypothermia during exposure, is preceded by changes in circulating anterior pituitary-derived hormones and the release of adrenal-derived epinephrine and corticosterone, linking the activation of SAM and HPA axes and the inhibition of HPT and HPG axes to hyperglycemia. Further, eliminating adrenal-derived stress hormones from circulation through adrenalectomy or pharmacologically inhibition of stress hormone receptors diminishes ozone-induced hyperglycemia, and treatment with stress hormone receptors agonists restores ozone-induced hyperglycemia in adrenalectomized rats. This glucose increase is transient, discontinuation of ozone exposure reverses hyperglycemia. Moreover, upon daily ozone exposure for 3 or more consecutive days, the ozone-induced hyperglycemic and hypothermia response is no longer evident, reflective of stress adaptation. This adaptation is nearly eliminated if ozone exposure re-occurs after 6 days of recovery, suggesting the adaptation response is also transient in an experimental setting. Combined, these results highlight the utility of real-time glucose measurement as a sensitive marker that reflects the impacts of near-term exposure, and when coupled with exposure assessments may be useful in linking source to outcomes. Further understanding of dynamic changes in circulating glucose and stress hormones could provide insights on real-time health status and individual susceptibility in humans.

The release of stress hormones by activation of SAM and HPA axes is integral in mediating key metabolic processes that channel energy resources where needed by acting on (AR) and glucocorticoid receptors (GR).[52] Here and in previous study[19] we show increased circulating cortisol after ozone exposure in humans demonstrating the conserved nature of stress responses and coherency between rodents and humans. Glucocorticoid feedback regulation on HPA activity, and the roles of mineralocorticoids and catecholamines, have been implicated in stress adaptation and the plasticity of organismal neural responses.[10] However, the full understanding of molecular mechanisms linked to impaired stress adaptation and neuropsychiatric disorders is still lacking. The adaptation from ozone-induced hyperglycemia on

the third consecutive day of exposure suggests a neuroendocrine contribution to ozone adaptation. This adaptation response was not evident on the second day of ozone exposure. Moreover, one-week of no exposure washout-period was associated with the loss of adaptation, indicating remarkable plasticity that may be influenced by stressor type, potency, and longevity of exposure. Given the contribution of glucocorticoids in stress adaptation,[11] and link between changes in circulating cortisol as well as blood glucose and chronic neurobehavioral and metabolic diseases,[53,54] the temporal assessment of changes in glucose and cortisol is critical for evaluation of health status and longevity of stressor effects. Further, the loss of dynamicity or oscillatory rhythms is an important indicator of chronic health issues,[55] which may provide additional insight from temporal monitoring.

Real-time glucose monitoring is now more frequently employed for diabetic individuals who require repeated assessment of blood glucose. In our study, we were able to assess increases in blood glucose during GTT and from food intake during nighttime in rats. Thus, the real-time glucose assessment offers the opportunity to study interactive effects of diet, metabolic disease, and stress from air pollutant exposures. Stress in diabetics may exacerbate preexistent hyperglycemia as reported in diabetic Goto Kakizaki rats[56] and in diabetic patients.[57] We have shown that ozone-induced hyperglycemia results from increased gluconeogenesis and impaired insulin secretion from pancreas.[29]

Combined, evidence of temporal and concentration-dependent changes in hormones associated with not only with SAM and HPA, but also in HPT and HPG axes during acute ozone exposure, indicate that the stress response involves a complex interplay of multiple neuroendocrine pathways. The selective activation of HPA and SAM axes was associated with concurrent inhibition of HPT and HPG axes as observed by changes in respective hormones. These responses are consistent with acute psychosocial stress-induced increases in ACTH and cortisol, which are associated with depletion of gonadal hormones in men and women.[58] This is contrast, however, with concurrent increases in thyroid hormone and corticosterone after

exercise in rats.[59] These findings indicate that stress responses may not be uniform between stressor types and that ozone may impact specific neuroendocrine pathways that involve input from multiple interactive signaling processes in the brain to develop a tailored, integrated and temporally regulated host response. The mechanisms by which anabolic pituitary hormones, ACTH and GH, increase while the catabolic hormones, TSH, FSH, LH and PRL, decrease after ozone exposure may involve a precise and selective activation or inhibition of upstream regulators of hormonal responses after ozone exposure, such as the activation of CRH neurons, FK506 binding protein regulation of glucocorticoid feedback, and other neurotropic factors within the nuclei of hippocampal commissure area, which can differentially influence a number of different pituitary hormonal axes through the paraventricular nucleus of the hypothalamus, as reported in birds.[60] Circulating corticosterone, cytokines, exhausting exercise, caloric deprivation, and even sepsis are linked to depletion of TSH, T4, and T3 in a stress paradigm.[61] We have shown that AD reverses ozone-induced inhibition of TSH release, suggesting a role for circulating adrenal-derived stress hormones.[42] Pituitary TSH release is regulated by hypothalamic thyrotropin releasing hormone (TRH) with feedback controls on HPT at different levels.[62] Thus, stressor specific differences in activation versus inhibition of given hormonal systems may impact downstream physiological responses.

We have previously noted that ozone-induced pulmonary transcriptional changes reflective of processes that regulate cell cycle growth and regeneration.[45] Since pituitary-derived growth hormone is involved in these processes,[46] and tied to metabolic changes,[47] we assessed kinetics of GH changes and noted a delayed but concentration-dependent increase at 4-hour after ozone exposure, suggesting that anabolic processes are being activated. Similar patterns were noted for increases in leptin, which regulates satiety at the level of the hypothalamus. However, the increase in circulating free fatty acids was noted as early as 1 hour, suggesting early activation of lipolytic activity in adipose tissue coinciding with changes in circulating

adrenal-derived hormones, but a delayed increase in GH coincides with leptin release from adipose tissue.

Our approach focused on the causal role of SAM and HPA axes on hyperglycemia response in a gain of function experiment using AD and stress hormone receptor agonists/antagonists.[24] This strategy is useful since AR and GR subtypes not only mediate pulmonary and peripheral metabolic effects induced by stress but also are the key regulators of central feedback mechanisms that govern the duration of stress responses and adaptation.[10] Given that stress hormones are involved in ozone-induced pulmonary and systemic effects, they likely regulate adaptation or tolerance with continued ozone exposure[32,63] and perhaps the suppression of thyroid and gonadal axes. We have recently reported that gene expression changes induced by a single 4-hour ozone exposure in the brain stem and hypothalamus were found to be similar to those induced by glucocorticoids and are markedly reduced in AD rats implying the role of glucocorticoids in feedback regulation of stress response.[42]

Increased circulating epinephrine and glucocorticoids regulate glucose metabolic processes through their action on multiple tissues, including the liver and pancreas.[64] AD or combination treatment with pharmacological blockers of βAR and GR inhibited ozone-induced hyperglycemia and glucose intolerance while each antagonist individually was less effective. The combination of agonists amplified ozone-induced hyperglycemia and glucose intolerance. We have previously shown that ozone exposure is associated with increased gluconeogenesis and inhibition of glucose-mediated insulin secretion in rats.[29] Each AR and GR subtype may be selectively influencing different processes of glucose metabolism, such as gluconeogenesis, and β-cell insulin secretion in the liver and pancreas. These changes following acute ozone exposure suggest that repeated intermittent exposure may exacerbate or initiate metabolic syndrome.[29]

Stress response is proportional to stressor severity, and the duration, and is precisely directed to the affected organ system. This response is reversible upon stress discontinuation

and in some cases, even after continued stressor application (habituation). Because it involves common neuroendocrine pathways and physiological processes,[9] air-pollution induced acute-stress response is considered non-specific. However, all observed acute ozone-induced pathological sequalae, including lung injury and inflammation, and metabolic homeostatic changes are linked to this stress response. Moreover, these reversible stressor effects in healthy individuals can be impaired in susceptible individuals both at the CNS and peripheral organ levels, contributing to health burdens from environmental exposure.[55] Based on evidence presented in this paper, we assert that evaluation of the dynamicity of this response through real-time glucose and cortisol monitoring could unravel critical information on the magnitude and persistence of stress from environmental exposures and its impairment in individuals with preexisting diseases including psychosocial and metabolic. Using ozone inhalation as an example, we demonstrate the utility of such an approach to monitor the dynamics of stressor effects on human health that is amenable with currently available technologies.[65]

This study assessed responses after exposure to only ozone. While we have shown similar changes in stress hormones and glucose after exposure to other gaseous irritant, acrolein,[51] this response may be linked to irritancy and should not be generalized to all pollutants and pollution mixtures. Similarly, the nature and timing of responses to other stressors may vary leading to differences in the organ being affected and the duration of pathogenesis. Moreover, this study assessed only acute health outcomes in healthy animals after inhaled ozone exposure, however, the mechanisms by which repeated exposures and underlying health conditions may lead to increased disease susceptibility can be better studied using models of compromised health status. Finally, species variation among humans and the rodent model in respiratory anatomy and function as well the uniqueness of the hypothermic response to ozone in rats are important caveats when translating findings to humans. The rat model, however, has long been used to successfully predict the adverse cardiorespiratory

response potential of air pollution in humans and we show that humans respond to ozone stress in a similar manner to rodents.

In conclusion, we show that health effects of air pollution-induced stress can be monitored in real-time by assessment of blood glucose using telemetry and where possible cortisol/corticosterone in experimental models and in humans. Our data show that exposure to prototypic air pollutant (ozone) rapidly induces anabolic (ACTH, GH) while inhibiting catabolic (HPT, HPG) neuroendocrine pathways along with sympathetic activation and adrenal medullary release of epinephrine and HPA-mediated release of glucocorticoids. These reversible and dynamic neuroendocrine changes are associated with increases in circulating corticosterone and other neuroendocrine hormones, which is followed by systemic metabolic alterations and hyperglycemia as well as hypothermia during ozone exposure. We further mechanistically confirm the contribution of adrenal-derived stress hormones based on the evidence that AD and pharmacological inhibitors of stress hormones eliminate and agonists reverse ozone-induced metabolic effects. Since dynamic changes in circulating stress hormones likely mediate interactive metabolic effects of chemical stressors such as ozone, as well as non-chemical stressors, this approach may be useful in assessing health effects and susceptibility variations in epidemiological studies.

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Figure Captions

Figure 1. Real-time glucose monitoring in freely moving animals reveal dynamicity of ozone-induced changes and response to glucose injection. A) A schema showing surgical implantation of glucose radiotelemetry devices followed by recovery and then ozone exposure at different concentrations. Glucose levels were measured real-time in a weekly cross over design over 4 weeks and averages of every minute are plotted as mean ± SEM of n=5-6 exposures. B) Real-time changes in blood glucose levels during 4 hours of air (0.0 ppm) or ozone exposure (0.2, 0.4, and 0.8 ppm). C) Glucose levels during first 24 hours after 4-hour air or ozone exposure (n=5-6). D) Glucose levels during 5^{th} and 6^{th} week of cross-over exposure to air or 0.8 ppm ozone and during glucose tolerance test performed immediately following exposure (n=6). E) Comparison of glucose levels measured through tail prick every 30 min and during continuous monitoring through telemetry (n=3-4). F) comparison of baseline glucose levels assessed using tail prick and telemetry following 4-hour air or ozone exposure. G) Comparison of area under the curve assessment of glucose tolerance test showing similarity in the glucose levels assessed using tail prick and those assessed through telemetry.

Figure 2. Recovery from a single 4-hour ozone exposure induced hyperglycemia, diurnal variation, and adaptation during repeated ozone exposure. A) Glucose levels were measured real-time in a weekly cross over design over 4 weeks and averaged for every minute are plotted as mean of n=5-6 exposures. The data show ozone-induced hyperglycemia during and following exposure and reversal of this effect in subsequent non-exposure days. Note the higher levels of circulating glucose at nighttime when animals are active and feeding. B) Ozone-induced hyperglycemia during repeated daily 4-hour exposure for 4 consecutive days followed by 3-day non-exposure period showing nearly complete adaptation on day 3 and day 4 where no ozone effect is evident (n=3-4/group).

Figure 3. Temporal changes in anterior pituitary, adrenal-derived, and metabolic hormones, as well as circulating metabolites during 4-hour exposure to air or ozone. A separate cohort of

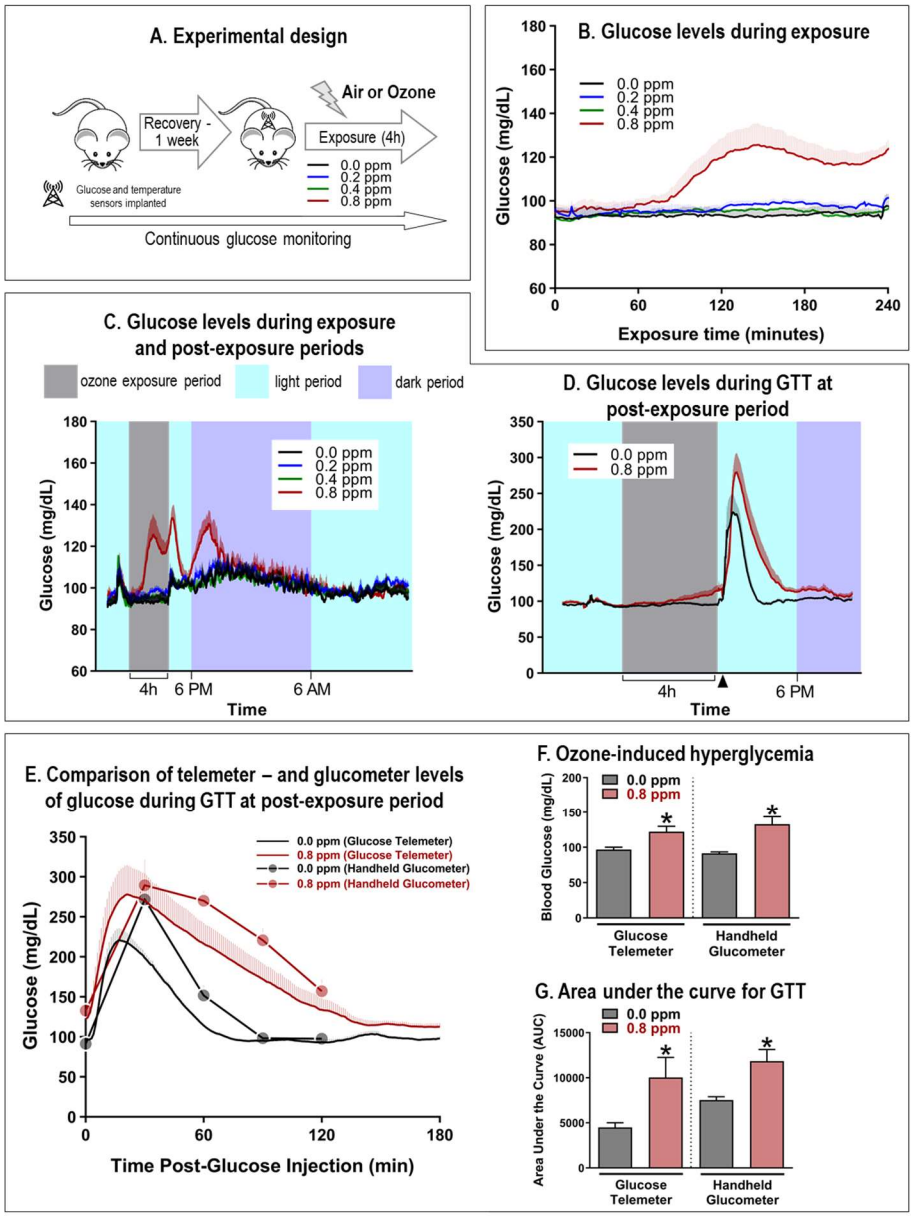
healthy rats were exposed to air or ozone at 0.4 or 0.8 ppm for 30 min, 1 hour, 2 hour or 4 hour and necropsies were performed immediately following each exposure (within 15-20 min to collect blood samples). A) experimental design; B) serum pituitary and adrenal-derived stress hormones; and C) metabolic hormones and levels of free fatty acids. Data show mean ± SEM (n=6-8 animals/group). Significant differences ($p<0.05$) are denoted by "*" for 0.8 ppm versus 0.0 ppm, "†" for 0.4 ppm versus 0.0 ppm, and "‡" for 0.8 ppm versus 0.4 ppm for time matched groups. ACTH, adrenocorticotropic hormone; TSH, thyroid stimulating hormone, PRL, prolactin; LH, luteinizing hormone; GH, growth hormone. Note that the data for corticosterone and epinephrine are recently published in a table form (Henriquez et al., 2021).

Figure 4. Mechanistic link between blood glucose changes and adrenal-derived stress hormones as determined using adrenalectomy (AD), and pharmacological interventions. A. Left panel shows the experimental design involving AD and treatment of rats with vehicle (VEH) or β2AR plus GR agonists (clenbuterol [CLEN] + dexamethasone [DEX]) 1-day prior to and the day of air or ozone exposure (Henriquez et al., 2018a). Right panel shows the treatment of healthy rats with vehicle (VEH) or βAR and/or GR blockers (propranolol [PROP] and mifepristone [MIFE], respectively, followed by air or ozone exposure (Henriquez et al., 2018b). These published papers evaluated pulmonary effects of interventions. To assure effective receptor blockade the treatment began 7 days prior to air or ozone exposure. B and C. Glucose tolerance test (GTT) and the glucose data at baseline and following glucose injection in each study (n=6-8). SH, sham surgery; AD, adrenalectomy surgery; VEH, vehicle; CLEN, clenbuterol; DEX, dexamethasone; PROP, propranolol; MIFE, mifepristone; SAL, saline; CO, corn oil.

Figure 5. Changes in plasma glucose and cortisol in humans within 1-2 hour following exposure to air or 0.3 ppm ozone. (A) Archived serum samples from a clinical study were obtained where blood samples were collected pre and post exposure during two clinical visits from healthy

young volunteers who are exposed to air or 0.3 ppm ozone for 2 hours in a cross-over design separated for at least 2 weeks. During air or ozone exposure volunteers were intermittently exercising. B. There was no significant increase in blood glucose likely due to exercise, however, normal cortisol decreases from exercise were significantly attenuated (one way ANOVA; n=29; p = 0.05). Samples > 1.5 *interquartile range* were removed as outliers.

997 Figure 1



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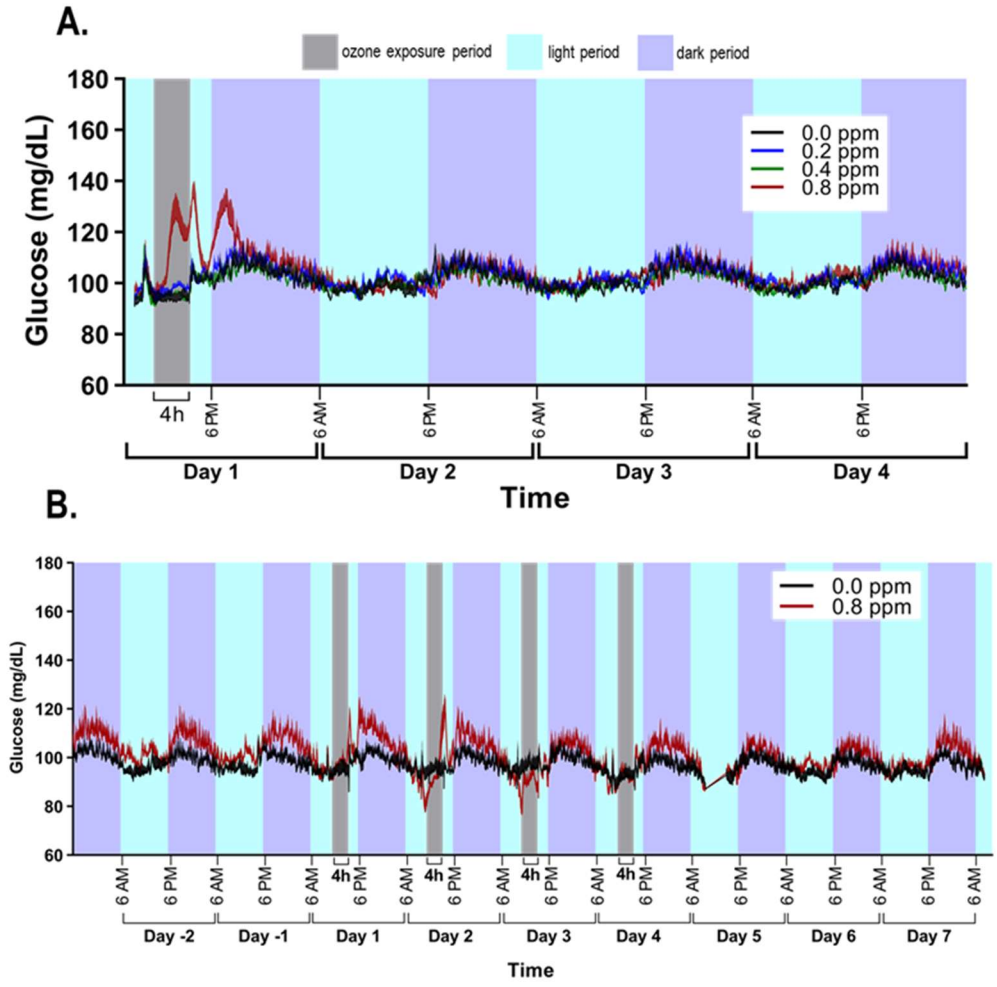
1000 Figure 2



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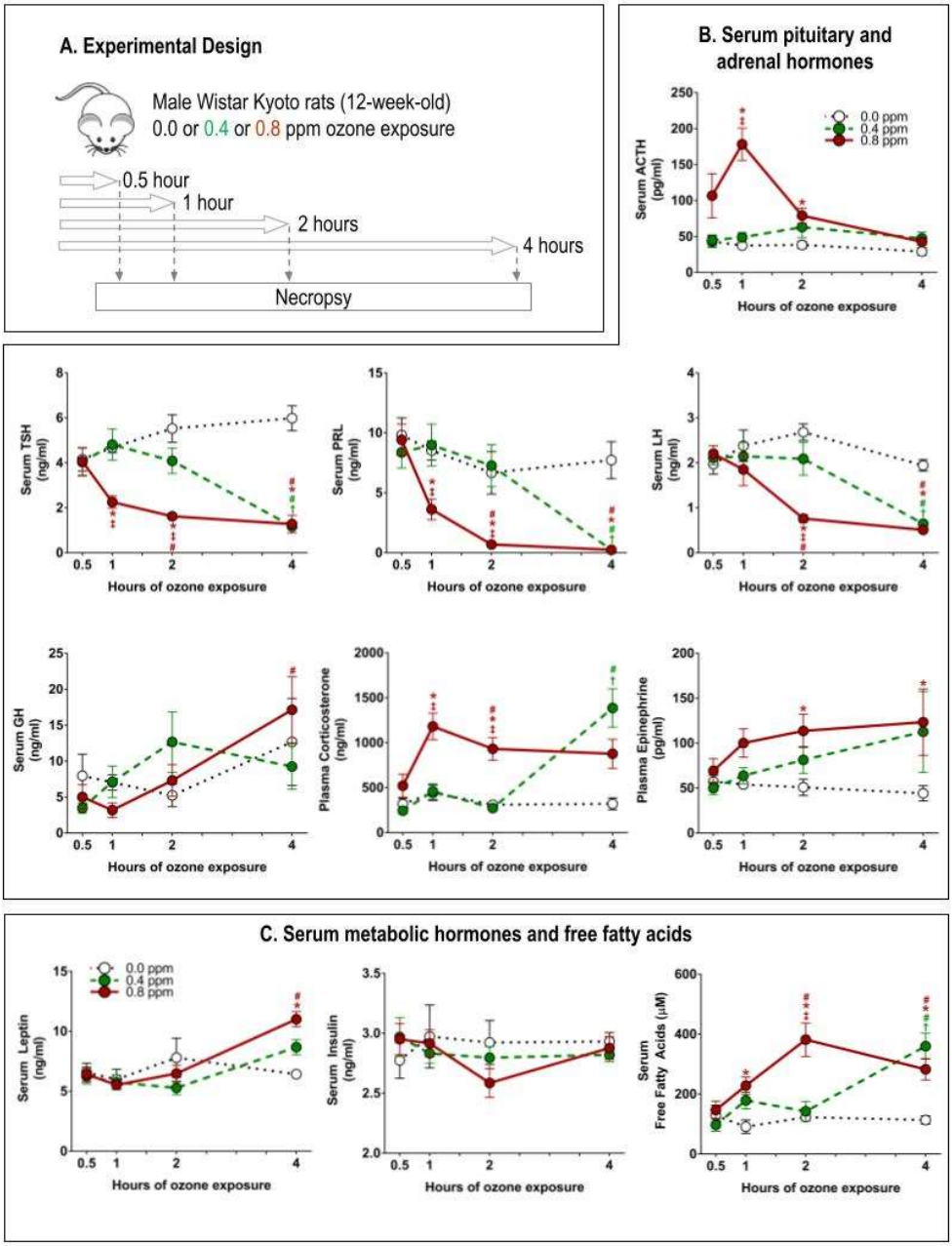

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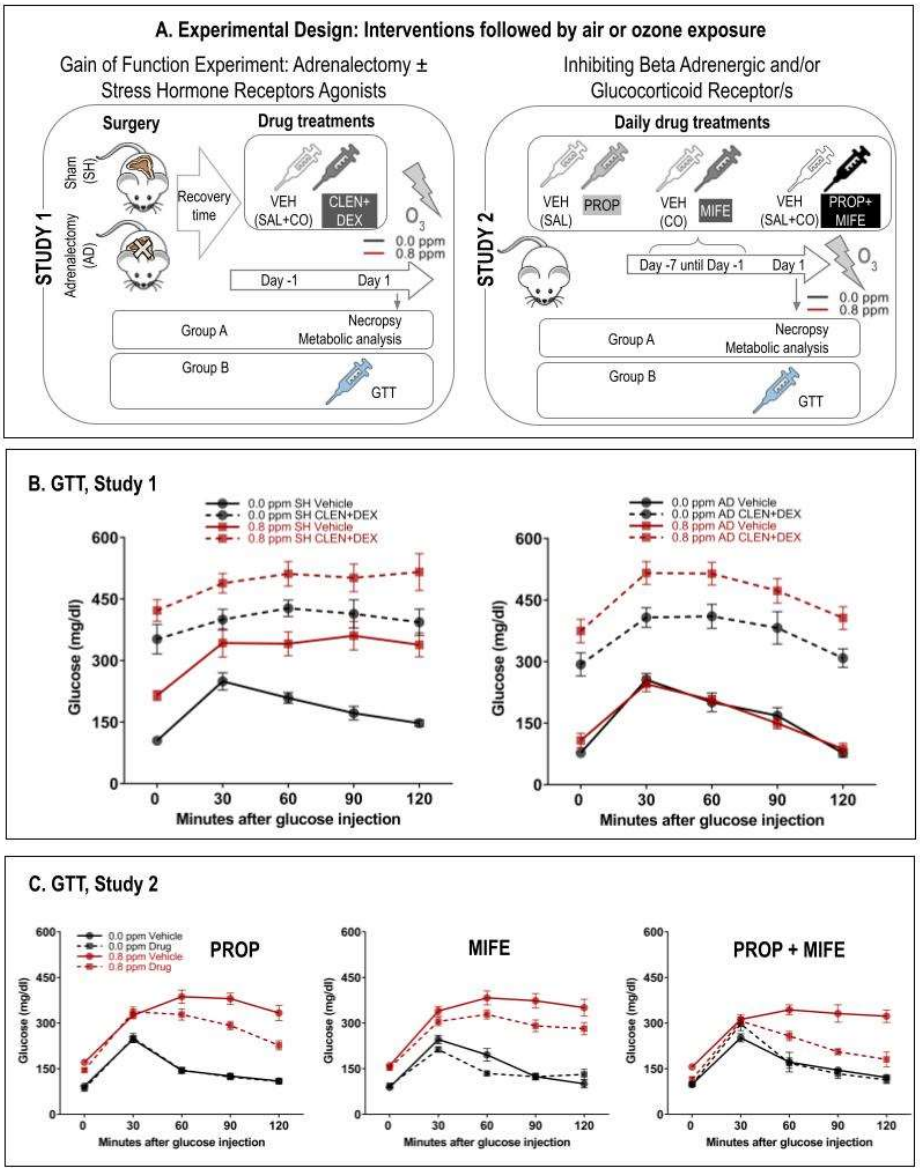

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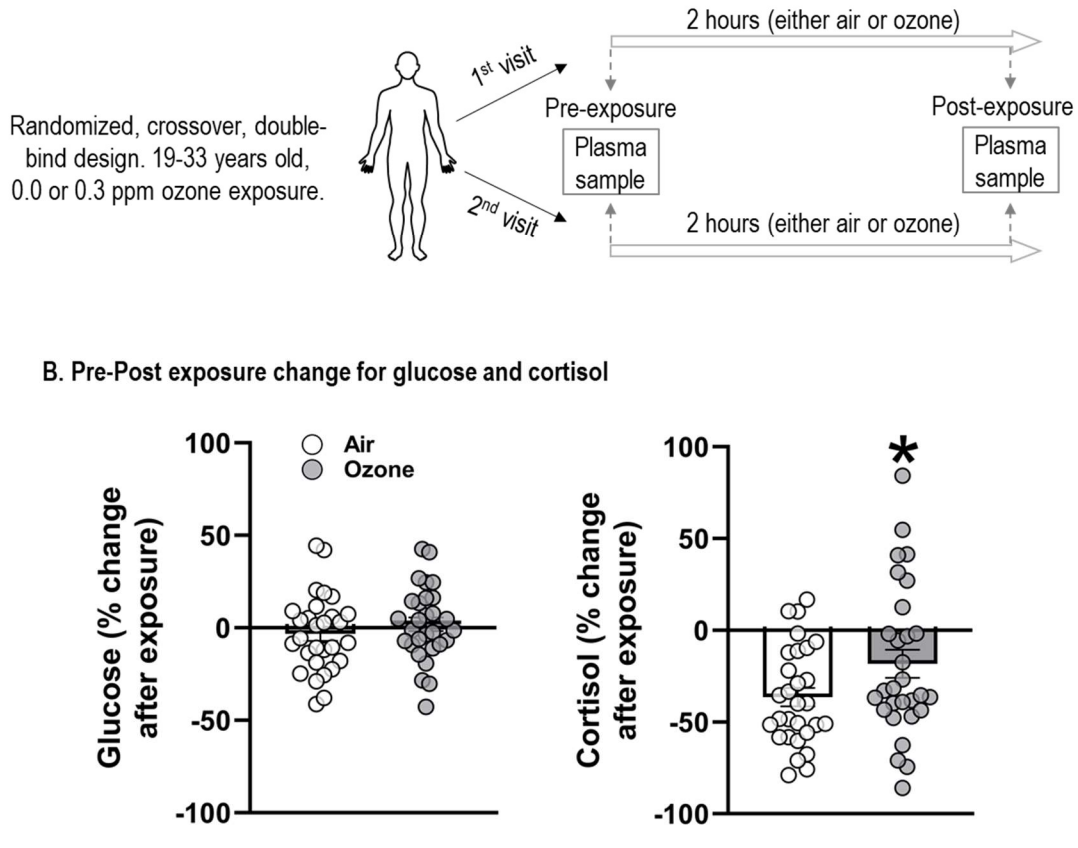
1009 Figure 5



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