Glucose dynamics during ozone exposure measured using radiotelemetry: Stress drivers

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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 used 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 compared 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 from 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 were adrenalectomized and/or treated with glucocorticoid inhibitor. The depletion of cortisol was dampened in humans exposed to ozone during intermittent exercise. **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.

Keywords: stress response, ozone, glucocorticoids, catecholamines, real-time glucose radiotelemetry, pituitary hormones, adrenalectomy

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 deserves further exploration. 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 central nervous system (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] Centrally-mediated release of 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]

95 Thus, the temporal assessment of the stress dynamics is critical to understanding an
96 individual's susceptibility to environmental insults.

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

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

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

125

126 **Materials and Methods**

127 ***Animals***

128 All male Wistar Kyoto rats were purchased from Charles River Laboratories Inc.,
129 Raleigh, NC at 10-12 weeks of age and maintained in our Association for Assessment and
130 Accreditation of Laboratory Animal Care-approved EPA animal facility. Animals were pair-
131 housed in polycarbonate cages with beta chips bedding and EnviroDry enrichment material,
132 except when stated. Animal rooms were maintained on a 12 hr light/dark cycle (6AM-6PM) at
133 ~22°C and 50% relative humidity. They were provided free access to Purina (5001) pellet rat
134 chow (Brentwood, MO) and water ad libitum, unless stated during experimental procedures. All
135 animal protocols were approved by the EPA's Institutional Animal Care and Use Committee
136 prior to starting studies and we followed National Institutes of Health guide for the care and use
137 of rats (NIH Publications No. 8023).

138 ***Glucose telemetry surgeries***

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

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

156 ***Blood glucose concentration data acquisition prior to exposure***

157 After surgery, rats were individually housed in cages with pine shavings bedding. Rats
158 were provided with water and powdered as well as pelleted standard Purina (5001) rat chow
159 (Brentwood, MO) *ad libitum*. Blood glucose levels, core body temperature, and activity level
160 measurements were sent via radio signals and collected using receivers placed under each
161 cage. The recording for each rat began soon after the surgery and continued until the end of the
162 experiment (~ 9 weeks). The cages were placed over receivers (RPC-1, DSI) and data were
163 simultaneously collected in a computer placed in an adjacent isolated room (Figure S1). For
164 verification and calibration purposes, single point calibrations were carried out. As
165 recommended, these calibrations were done twice weekly throughout the study based on the
166 protocol explained by Brockway and collaborators at Data Sciences Inc.[20] Data used for
167 calibration are excluded from analysis and graphs. Interpolation of glucose levels and electric
168 current detected by the sensor was corrected for differences in body temperature versus room
169 temperature, merged and analyzed for each animal with a resolution of 1 minute using
170 Dataquest® software acquisition system (DSI). Dataquest® acquisition system (DSI) included
171 telemetry signal receiver for each animal, a data matrix for analyzing receiver signals and a

172 computer. These telemetry devices allowed continuous sampling of 8 animals that are
173 individually housed in cages with receivers directly underneath. To avoid signal mixing between
174 two animals, the animal racks were equipped with stainless steel dividers. The software allowed
175 device configuration and the protocol for continuous data collection as described previously.[20]

176 ***Timeline of telemetered rat experiments***

177 Two weeks after surgery, rats were exposed in pairs whole body to ozone (0.2, 0.8 or
178 0.4 ppm) or filtered air (0.0 ppm) in Rochester style "Hinners" chambers, using a crossover
179 exposure design such that each rat was exposed to each concentration across the first 4 weeks
180 of testing (Table S1). Briefly, rats for the first exposure were randomized in pairs for 0.0 (clean
181 air), 0.2, 0.4 or 0.8 ppm of ozone exposure for 4 hrs. A different set of receivers were placed in
182 each ozone exposure chamber to acquire data while animals were being exposed. After 1 week
183 of washout period, these exposures were repeated but the targeted ozone exposure
184 concentration for each pair of rats was changed. This experiment was repeated a total of four
185 times over 4 weeks to cover all the targeted ozone concentrations for all eight rats (n=7
186 independent recordings). Rat #6 was discarded from the experiment due to malfunction of the
187 sensor and/or transmitter. During week 5 and 6, these 7 telemetered rats were randomized into
188 two groups, air and 0.8 ppm ozone, and again using crossover design they were exposed for 4
189 hours, once per week for 2 weeks (Table S1) and glucose levels were continuously monitored
190 (n=7 per concentration). Immediately after each exposure, glucose tolerance test (GTT) was
191 performed using a glucometer in addition to continuous recording of glucose through telemetry
192 as described below. Finally, during week 7, animals were divided into air (n=3) and ozone (n=4)
193 and at this time they were exposed to air or 0.8 ppm ozone 4 hours/day for four consecutive
194 days to determine adaptation upon continuous exposure (Table S1). Because animals were
195 exposed for 4 consecutive days during the week, a crossover design was not possible.

196 ***Glucose/temperature telemetry data acquisition and analysis during exposures***

197 During the day of exposure, animals were transported to the nearby exposure room
198 where a control and three ozone exposure chambers were preprepared with a different set of
199 receivers connected to an independent but identical data acquisition system to acquire data
200 during exposure for each rat. Within ~5 minutes of removal from their home cages, animals
201 were immediately placed at designated locations (with receivers placed underneath) in the
202 exposure chambers with wire-mesh bottom dividers and data were collected with the data
203 acquisition system during exposure. Rats were place at two opposite corners of the chamber to
204 avoid signal mixing. All exposures were aligned to minute 0 when 80% of target ozone
205 concentration was reached. For each minute, glucose level and temperature were averaged
206 from exposed rats (n=7/concentration). Immediately following the exposure, animals were
207 likewise transported to their home cages for continued data acquisition. The data collected
208 during non-exposure periods when telemetered rats are in their home cages and during air or
209 ozone exposure periods were temporally aligned to tally continuous recording each day and
210 during the entire experimental period. Glucose and temperature data with a resolution of 1
211 minute was averaged to prepare graphs. As mentioned above, for the adaptation study (7th
212 week), because of the daily exposure for 4 days, crossover design could not be used, and the
213 sample size is thus n = 3 for 0.0 ppm and n = 4 for 0.8 ppm ozone. Multiple hours of data after
214 the exposure stopped were included to follow days of recovery during non-exposure periods.

215 ***Ozone generation and exposure for all rat experiments***

216 Ozone exposure methods have been described in detail in previous publications.[18,21,22(p)]
217 Briefly, ozone was generated from oxygen using a silent arc discharge generator (OREC,
218 Phoenix, Arizona) and measured using mass flow controllers (Coastal Instruments Inc., Burgaw,
219 North Carolina). The target ozone concentrations were recorded by photometric analyzers (API
220 Model 400, Teledyne, San Diego, California). Mean chamber temperature, relative humidity,
221 and air flow were recorded hourly.

222 ***Glucose tolerance tests (GTT) for all rat experiments***

223 For glucose telemetry, gain of function adrenalectomy experiment, and pharmacological
224 inhibitor studies, GTT was performed in rats after air or ozone exposure as previously
225 described.[18,21,23] Because rats underwent air or ozone for 4 hours exposure prior to GTT when
226 no food was provided, this served as fasting for GTT. Immediately after air or ozone exposure,
227 baseline blood glucose concentrations (0 hr) were determined by tail prick using a sterile 23-
228 gauge needle with a Bayer Contour Glucometer (Leverkusen, Germany). For glucose telemetry
229 animals, a different glucometer was used (Nova Biomedical StatStrip® Xpress, DSI). Rats were
230 then injected with 20% D-glucose (20% pharmaceutical grade D-glucose; Covetrus, Dublin, OH;
231 diluted to 2 g/kg/10mL, i.p.) as described for telemetry experiment. Glucose levels were
232 measured by tail prick at 30, 60, 90, and 120 min.

233 ***Time course assessment of hormonal response to ozone in rats***

234 A separate cohort of healthy male 12-13-week-old WKY rats was exposed to clean air or
235 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)
236 and necropsied immediately after each time point (Figure 3A) to collect blood samples for
237 assessment of various pituitary-derived and other hormones. Ozone exposures were performed
238 as described above.

239 ***Necropsy and blood samples collection for hormones in time course study***

240 For hormonal time course study, as reported in previous study,[24] rats were necropsied
241 within 15 minutes after each exposure. Necropsies were performed in a staggered manner.
242 Rats were euthanized with Fatal Plus (sodium pentobarbital, Virbac AH, Inc., Fort Worth, TX;
243 >200 mg/kg, i.p.). Blood samples were collected from the abdominal aorta directly in vacutainer
244 serum separator tubes and EDTA tubes for serum and plasma separation, respectively. Blood
245 samples were spun at 3500 x g for 15 min at 4° C; serum and plasma samples were aliquoted
246 and stored at -80 ^{o}C until analysis.

247 ***Plasma and serum analysis of hormones in time course study***

248 Plasma levels of epinephrine (adrenaline) and corticosterone were quantified using kits
249 from Rocky Mountain Diagnostics (Colorado Springs, CO) and Arbor Assays (Ann Arbor, MI),
250 respectively. These two hormones' data are recently published in a table form along with
251 immune effects.[24] Serum pituitary hormone levels for adrenocorticotropic hormone (ACTH),
252 thyroid stimulating hormone (TSH), prolactin (PRL), luteinizing hormone (LH), growth hormone
253 (GH), brain-derived neurotrophic factor (BDNF), and follicle stimulating hormone (FSH) were
254 determined using MILLIPLEX MAP Rat Pituitary Magnetic Bead Panel following manufacturer's
255 protocol (Merck-Millipore, Burlington, MA). Serum free fatty acids were measured using kits
256 from Cell Biolabs, Inc (San Diego, CA) adapted for use on a Konelab Arena 30 clinical analyzer
257 (Thermo Chemical Lab Systems, Espoo, Finland). Serum insulin and leptin were quantified
258 using Meso Scale (Meso Scale Discovery, Gaithersburg, MD).

259 ***Adrenalectomy (AD) and sham (SH) surgeries, drug treatments, and ozone exposure***

260 GTT was performed in our previous study for which pulmonary data are recently
261 published.[25] In that study, to assess the role of adrenal-derived stress hormones, male WKY
262 rats (12–13 weeks old) that underwent total bilateral adrenalectomy (AD) or control sham (SH)
263 as previously described.[25]Briefly, rats were anesthetized with ketamine (25–50 mg/kg in saline,
264 i.p.), and once anesthetized, injected with the analgesic buprenorphine (0.02 mg/kg/ml in saline,
265 s.c.). During the surgery, anesthesia was maintained by nose-inhalation of vaporized isoflurane
266 (~3%, 1-2 LPM of O_2). Animal surgeons from Charles River Laboratories Inc performed the
267 surgeries. Animals were placed in sternal recumbency, and dorsal incision was made. Adrenals
268 from both sides were removed and the muscle layer was sutured to close the abdominal cavity.
269 The surgical wound clips were used to clip the skin and close the wound. SH surgeries were
270 performed using the same anesthesia and surgical approaches as AD except for the removal of
271 adrenal glands. Rats were recovered on heating pads and assessed for signs of distress and

272 pain. Once awake, meloxicam (0.2 mg/kg in saline; s.c.) and buprenorphine (0.02 mg/ml/kg in
273 saline, s.c. every 8–12h for 2 times) was administered for analgesia. After the surgery, AD rats
274 received water with 0.9% NaCl to maintain adequate salt-water balance in the absence of
275 mineralocorticoids eliminated due to AD along with other adrenal-derived stress hormones. All
276 animals were provided with powdered as well as pelleted food *ad libitum*. The rats were pair
277 housed with EnviroDry enrichment/nesting material and allowed to recover for 4–6 days prior to
278 drug treatment as reported in our previous companion paper[25] and described below. These SH
279 and AD rats, as detailed in our recent publication[25], were randomized by body weight into four
280 groups (vehicle: air, vehicle: ozone, clenbuterol (CLEN; β2AR agonist) plus dexamethasone
281 (DEX; GR agonist):air, and CLEN+DEX: ozone) resulting in 8 total groups (n=8/group). In brief,
282 after 4-6 days of recovery from SH and AD, rats were treated with vehicles (saline 1 mL/kg as
283 control for CLEN, i.p.) and corn oil (1 mL/kg as control for DEX, s.c.) or clenbuterol
284 hydrochloride, a long acting β2AR agonist (CLEN; 0.2 mg/kg in saline, i.p.) and GR agonist,
285 dexamethasone (DEX; 2 mg/ml corn oil/kg, s.c.). Generally, CLEN injections were followed by
286 DEX. The drug treatment began 1 day prior to start of ozone exposure and continued the day of
287 air or ozone exposure in the morning at ~6am. These high doses were selected to restore the
288 depleted activities of epinephrine and corticosterone in AD groups. CLEN and DEX doses are
289 comparable to those used in other controlled experiments using rodents and are sufficient to
290 induce bronchodilation and immunosuppression, respectively.[26,27] These rats were exposed to
291 air or 0.8 ppm ozone for 4 hours and GTT were performed immediately after exposure as
292 described above .

293 ***Treatment of rats with βAR and GR antagonists and ozone exposure***

294 GTT assessment was also performed in three additional independent experiments,
295 which were carried out to evaluate the role of 1) β adrenergic receptor antagonist propranolol
296 (PROP), 2) glucocorticoid receptor antagonist mifepristone (MIFE) individually, or 3) both in

297 combination to determine their influence on ozone pulmonary effects as published previously.[21]
298 For each study, 12-13-week-old male WKY rats were randomized by body weight into four
299 groups (vehicle/air, drug/air, vehicle/ozone, drug/ozone, n = 8/group). In the first experiment,
300 rats were injected with either sterile saline (vehicle; 1 mL/kg, i.p.) or propranolol hydrochloride
301 (PROP, Sigma-Aldrich, St Louis, MO; 10 mg/kg in saline, i.p.). In the second experiment, rats
302 were injected with pharmaceutical grade corn oil (vehicle; 1 mL/kg, s.c.) or mifepristone (MIFE,
303 Cayman Chemical Co., Ann Arbor, MI; 30 mg/kg in corn oil, s.c.). In the third experiment, rats
304 were injected with vehicles, saline (1 ml/kg, i.p.) followed by corn oil (1 ml/kg, s.c.) or drugs,
305 PROP (10 mg/kg, i.p.) followed by MIFE (30 mg/kg, s.c.) (PROP+MIFE). The rationale for drug
306 selection and treatment protocol are explained in our previous companion paper describing
307 pulmonary and immune responses.[21] To assure complete inhibition of βAR and GR receptors,
308 the daily morning treatment began 7 days prior to the air or ozone exposure and was continued
309 the day of exposure. In each study, rats were exposed to filtered air or 0.8 ppm ozone for 4
310 hours as indicated above, and GTT was performed immediately after exposure.

311 ***Assessment of glucose and cortisol in human clinical study samples***

312 Human plasma samples were obtained from a clinical study conducted through the
313 University of North Carolina (Chapel Hill, NC) under IRB# #13-1644. The study involved
314 exposure of young healthy human volunteers to filtered air or 0.3 ppm ozone exposure in a
315 cross-over design where the same subjects were randomly exposed to air or ozone during two
316 distinct visits that were separated by two weeks or longer. Prior consents were obtained from all
317 individuals participating in the study. Blood samples were collected prior to and immediately
318 following 2-hour exposure to air or ozone for each of 34 subjects. Demographic information is
319 provided in Table S2. For human plasma samples, glucose levels were analyzed using Bayer
320 Contour Glucometer and test strips (Leverkusen, Germany). Plasma cortisol levels were
321 analyzed using human cortisol kit from Arbor Assays (Ann Arbor, MI).

322 ***Statistics***

323 For all analyzed endpoints, a threshold of $p < .05$ was used to determine significant
324 effects. Outliers were identified using the boxplot method, defined as above Q3 + 1.5 IQR or
325 below Q1 – 1.5 IQR and discarded. Analysis of glucose telemetry data was done by assessing
326 peak glucose levels and area under the curve (AUC) for set timepoints (i.e. during exposure,
327 during GTT, etc.). AUC was calculated using the trapezoidal method as previously described.[22]
328 Significant effects of exposure were determined using one-way analysis of variance (ANOVA)
329 with a Tukey's post hoc comparison to assess differences between ozone concentrations or a
330 student's t-test when only one ozone concentration was used. To determine the relationship
331 between glucose and animal body temperature, a linear regression was performed. To compare
332 the similarities of glucose telemetry and handheld glucometer, significant effects were examined
333 using a two-way ANOVA (measurement technique, exposure) with a Tukey's post hoc
334 comparison used for pair-wise comparisons. To determine adaptation over time, a two-way
335 repeated measures ANOVA was performed (exposure, day) with Tukey's post-hoc comparison
336 used for pair-wise comparisons. For analysis of the effects of ozone on serum pituitary and
337 adrenal hormones and serum metabolic hormones and free fatty acids, a two-way repeated
338 measures ANOVA (exposure, time) was performed and followed by Tukey's post-hoc
339 comparisons for each ozone concentration. To explore the effects of adrenalectomy and/or
340 pharmaceutical interventions on ozone's glucose-inducing effects, a three-way (exposure, drug,
341 surgery) or two-way (exposure, drug) ANOVA was performed with Tukey's post hoc used for
342 pairwise comparisons. For human data, the percent change in plasma levels of glucose and
343 cortisol pre- and post-exposure were calculated and used for paired-samples t-test. GraphPad
344 Prism 9 (version 9.1.2) was used for statistical analysis and graph generation. Statistical
345 summaries pertaining to all data presented in figures is provided the Supplemental Excel File
346 (Excel Table S1) with a detailed breakdown of all statistical analyses, effect size calculations,
347 and notes on any outliers removed.

348 **RESULTS**

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

350 We used a novel real-time blood glucose telemetry system (Figure 1A and Figure S1)[20]
351 that has not been employed in previous air pollution studies. Using a cross-over design with the
352 7 telemetered rats, we obtained independent readings at each concentration for a weekly 4-hour
353 exposure to air or ozone with 1-week washout (Table S1). We have shown that one week wash-
354 out period after a single 4-hour ozone exposure is sufficient to clear effects from a previous
355 exposure in rats.[28] Continuous monitoring of glucose during 4 hours of ozone exposure at
356 various concentrations and post exposure periods in rats allowed insights in precise timing for a
357 stressor to impact changes in circulating glucose and the longevity of a stress response. It also
358 allowed monitoring of glucose intake-related and diurnal changes. Because glucose telemetry
359 also included the assessment of core body temperature, we were able to show that a drop in
360 core body temperature correlated with glucose changes.

361 Hyperglycemia began to occur at ~90 min into a 4-hour ozone exposure but only in the
362 0.8 ppm group (Figure 1B). In the 0.8 ppm group, both peak glucose and AUC are significantly
363 increased, and glucose plateaued between 2.5 and 3 hours, followed by a second peak
364 between 4-5 hours during the first day of exposure. These changes may reflect fine oscillatory
365 adjustment in homeostatic processes, which might be important for centrally balanced and
366 precisely controlled responses to ozone stress. A third peak of hyperglycemia in the 0.8 ppm
367 ozone group was noted roughly 1 hour after the beginning of dark cycle (and 4 hours post
368 cessation of ozone exposure) when rodents were active and feeding (Figure 1C). The increase
369 in glucose at 90-min was strongly associated with hypothermia in the 0.8 ppm ozone group, but
370 without the fluctuations seen in glucose levels (Figure S2).

371 After completing 4-week exposure using cross over design, animals were assigned air or
372 0.8 ppm ozone group for subsequent weeks. On the 5th and 6th week, rats were exposed to air

373 or 0.8 ppm ozone for 4 hours using crossover design (alternating exposure assignment to air or
374 ozone each week), and GTT was performed immediately following exposure in telemetered rats.
375 These rats exhibited ozone-induced glucose intolerance in addition to hyperglycemia, as
376 evidenced by both increased peak glucose levels and increased AUC (Figure 1D) consistent
377 with our previous studies involving post exposure assessment.[18] The telemetry data for blood
378 glucose after bolus glucose injection matched the data obtained through a handheld glucometer
379 (Figure 1E), with both telemetry and glucometer finding a significant increase in basal glucose
380 (Figure 1F) and AUC (Figure 1G) during the 0.8 ppm exposure and no difference between
381 measurement techniques (glucometer vs telemetry). Combined, these data suggest that
382 glucose monitoring in real-time offers opportunities to concurrently assess effects of dietary
383 glucose and acute environmental stressor exposure. The temporal co-occurrence of ozone-
384 induced hypothermia and hyperglycemia in the 0.8 ppm group suggests that these processes
385 are linked or induced through common upstream events (Figure S3A). This hypothermia did not
386 occur after glucose injection. In general, when glucose levels are high, core body temperature
387 increases along with activity as noted during the dark cycle for rodents (Figure S3B). However,
388 ozone-induced changes in blood glucose and body temperature were in the opposite direction,
389 suggesting stress-induced disturbance in homeostasis to conserve metabolic energy and direct
390 it where needed. Hypothermia, which has been linked to stress-induced glucocorticoid
391 increases in humans[29] was also evident after ozone exposure in our previous study that
392 employed real-time ECG monitoring in rats.[30]

393 **Real-time glucose monitoring during ozone adaptation and diurnal variation**

394 After a single 4-hour 0.8 ppm ozone exposure, hyperglycemia was not noted during
395 subsequent days of no exposure (Figure 2A). The diurnal changes were apparent in all animals
396 showing higher levels of glucose at nighttime when compared to daytime. Repeated daily
397 exposure to ozone has been associated with adaptation/tolerance in mice.[31] However, the

398 mechanism of adaptation remains elusive. With the use of glucose telemetry, we linked ozone
399 adaptation to glucose changes and obtained precise timing of adaptation. On week 7, animals
400 were exposed to air or 0.8 ppm ozone (4 hours each day) for 4 consecutive days to determine if
401 adaptation occurs with regards to glucose and body temperature changes during continued
402 daily exposure. Because of the repeated exposures during week 7 (daily for 4 days), crossover
403 design was not possible and therefore sample sizes are smaller than for all other experiments
404 (air, n=3; ozone, n=4). Ozone at 0.8 ppm 4 hours/day for 4 consecutive days led to significant
405 increases in peak glucose and glucose AUC only on days 1 and 2, with a notable lack of
406 increase in blood glucose during and right after the exposure on the third and fourth days
407 despite continued exposure(Figure 2B). Near complete adaptation to ozone exposure was
408 evident by the 3rd day despite continued exposure on 3rd and 4th day. Thus, continuous glucose
409 monitoring indicated that this adaptation occurs on the 3rd day but not on the 2nd day of ozone
410 exposure, and is associated with attenuation of ozone-induced hyperglycemia (Figure 2B). The
411 adaptation was also noted in hypothermia on the 3rd day (Figure S3C). These ozone-induced
412 changes in circulating glucose reflect the status of glucose metabolic processes in tissues.[28]
413 Thus, real-time glucose monitoring allows one to assess the timings of metabolic alterations and
414 adaptation during ozone exposure.

415 **Temporality of ozone-induced HPA and SAM activation**

416 Because the method for continuous monitoring of corticosterone in animals is still not
417 available, we exposed a separate cohort of naïve rats to air or ozone for variable durations
418 spanning a 4-hour time frame to assess temporal changes in adrenal-derived and other
419 neuroendocrine hormones (Figure 3A). This study followed a similar paradigm to real-time
420 glucose monitoring but used a distinct cohort of rats at each timepoint during 4-hour ozone
421 exposure to assess serum samples for key pituitary, adrenal-derived, and metabolic hormones
422 (Figure 3). A sharp rise in adrenocorticotropic hormone (ACTH) occurred at 30 min into the 0.8

423 ppm ozone exposure and peaked at 1 hour, reflecting the activation of the HPA axis and
424 concomitant ACTH release from the anterior pituitary as early as 30 min (Figure 3B). Upon
425 receiving stress signals in the paraventricular nucleus of the hypothalamus, the secreted
426 corticotrophin releasing hormone traverses to the pituitary through the hypothalamic-pituitary
427 portal system and activates ACTH secretion from the anterior pituitary. ACTH released into the
428 systemic circulation reaches the adrenal cortex to stimulate corticosterone/cortisol synthesis
429 and release involving hypothalamus-pituitary-adrenal (HPA) axis.[10] Consistent with this, we
430 noted that the levels of circulating corticosterone in rats increased starting at 1 hour into ozone
431 exposure (0.8 ppm) prior to the increase in glucose and remained significantly elevated until 4
432 hours of exposure (Figure 3C)[24] despite the restoration of ACTH to baseline levels at this time
433 point. It is important to note that although no significant ACTH increase occurred during the 0.4
434 ppm ozone exposure, the increase in corticosterone was significant at 4 hours, suggesting that
435 the stress response was concentration dependent. The data for corticosterone changes were
436 recently published in a table form.[24]

437 The stress response induced by acute physical and emotional stress also involves the
438 activation of the splanchnic sympathetic nerves via the hypothalamus leading to a release of
439 epinephrine from chromaffin cells of the adrenal medulla.[32] The sympathetically-mediated (SAM
440 axis) epinephrine increase generally precedes the increases in corticosterone during a fight-or-
441 flight response. The data show that after 30 min of 0.8 ppm ozone exposure, the levels of
442 epinephrine were increased as recently reported.[32] Exposure to the lower concentration of
443 ozone (0.4 ppm) also caused an increase in epinephrine, although the time required for this
444 increase was longer, as it was evident only after 4 hours of exposure (Figure 3B).[32] The
445 sustained increases in circulating epinephrine during the 4 hour of ozone exposure (Figure 2B)
446 also corroborated our prior findings involving a single end of exposure measurement.[18,23]

447 **Temporal changes in HPT and HPG hormones during ozone exposure**

448 Because ozone-mediated stimulation of the neuroendocrine system may also influence
449 other hypothalamic stress pathways such as hypothalamic-pituitary-thyroid (HPT) and
450 hypothalamic-pituitary-gonadal (HPG) axes[33], we next assessed temporal effects of ozone
451 exposure on relevant hormones. We demonstrate here that ozone exposure results in time- and
452 concentration-dependent decline in TSH levels, which occurs sooner (1 hour) at 0.8 ppm and is
453 temporally linked to increases in corticosterone and precedes the increased glucose response
454 with 0.8 ppm ozone (Figure 3B).We also assessed pituitary hormones involved in gonadal axis
455 includin follicle stimulating hormone (FSH), prolactin (PRL), and luteinizing hormone (LH)
456 (Figure 3B). We report that ozone exposure depleted not only LH, but also PRL (>95%), with
457 the 0.8 ppm concentration causing a more rapid depletion. This corroborates our recent study
458 where we reported a decrease in circulating PRL assessed once after 4 hour of ozone
459 exposure.[33] Here, the temporal assessment shows depletion of circulating PRL as early as 1
460 hour into exposure concomitant with peak ACTH levels. However, LH levels did not decrease
461 until 2-hour of exposure (Figure 3B) and the levels of FSH were not changed after ozone
462 exposure in male WKY rats (*data not shown*). These data provide insights on how acute ozone
463 inhalation can dynamically and differentially impact various neuroendocrine axes that have
464 major impact on homeostatic physiological processes.

465 Because pituitary-derived growth hormone (GH) is involved in cell growth and
466 regeneration processes,[34] and tied to metabolic changes,[35] we assessed the kinetics of growth
467 hormone changes and noted that a delayed but concentration-dependent increase in GH
468 occurred at 4-hour after ozone exposure suggesting that the anabolic processes are being
469 activated (Figure 3B). A similar temporal pattern was noted for the increase in leptin, which
470 regulates satiety at the level of hypothalamus (Figure 3C). On the other hand, the increase in
471 circulating free fatty acids was noted as early as 1 hour after ozone exposure, suggesting the
472 early activation of lipolytic activity in adipose tissue coinciding with changes in circulating
473 adrenal-derived hormones, but a delayed increase in GH coincides with leptin release from

474 adipose tissue (Figure 3C). Overall, ozone-induced hyperglycemia is reflective of
475 neuroendocrine responses that might involve a wide array of changes in metabolic and cell
476 growth processes.
477

478 **The role of epinephrine and corticosterone in mediating hyperglycemia**

479 Adrenal-derived epinephrine and glucocorticoids are the major regulators of liver
480 metabolic processes during stress,[36] and adrenalectomy diminishes ozone-induced
481 hyperglycemia and glucose intolerance.[22] Therefore, we further assessed the roles of adrenal-
482 derived stress hormones in mediating changes in circulating glucose after ozone exposure.
483 Ozone-induced hyperglycemia and glucose intolerance were nearly eliminated in vehicle-treated
484 AD rats, confirming our earlier findings.[22] Moreover, all animals treated with CLEN+DEX
485 developed marked hyperglycemia and glucose intolerance, in both SH and AD rats exposed to
486 air. Further, this response was exacerbated in rats exposed to ozone demonstrating βAR+GR
487 activation through increased epinephrine and corticosterone mediating hyperglycemia (Figure
488 4B). PROP or MIFE given individually did not reduce ozone-induced hyperglycemia, but
489 PROP+MIFE in combination significantly decreased hyperglycemia severity. Similarly, the
490 blockade of PROP and MIFE individually only partially reversed ozone-induced glucose
491 intolerance, but together, they markedly diminished ozone-induced glucose intolerance,
492 suggesting the involvement of both epinephrine and glucocorticoids in mediating glucose
493 increases during ozone exposure (Figure 4C). These results indicate a causal link between
494 ozone-induced increases in the release of adrenal-derived stress hormones through SAM and
495 HPA activation and resultant glucose metabolic alterations. Together, these data suggest that
496 there are dynamic relationships between ozone-induced changes in neuroendocrine stress
497 pathways, HPA activation and glucose homeostatic changes.

498 **Ozone-induced stress response in humans**

499 Hormonal response to stress is conserved across species; in this study, we wanted to
500 determine if the results seen in rats would extend to humans. We assessed glucose and cortisol
501 levels in plasma samples of human volunteers exposed to air or 0.3 ppm ozone during 2 hours
502 of intermittent exercise in a cross-over design where each volunteer was exposed to air and
503 ozone but separated by at least two weeks interval (IRB# #13-1644; Figure 5A). There were no
504 differences in blood glucose levels between volunteers exposed to air or ozone (Figure 5B),
505 likely because all subjects were exposed in a protocol involving intermittent exercise. In
506 contrast, although exercise resulted in decreased cortisol levels in air exposed individuals, this
507 decrease was significantly attenuated in volunteers exposed to ozone as determined by %
508 change between pre- and postexposure (Figure 5B).

509

510 **DISCUSSION**

511 Prior research on air pollution health effects focused primarily on cardiorespiratory
512 outcomes elicited by local effects in the lung. Recently it has become apparent that inhaled
513 irritant air pollutants might be perceived as stressors by the autonomic nervous system,
514 resulting in stimulation of neuroendocrine axes which mediate acute effects in the lung and
515 periphery.[37] The impairment and/or persistent hyperactivity of stress responses has been linked
516 to chronic psychiatric, neurobehavioral, cardiometabolic, and reproductive health abnormalities.
517 Because stress responses are dynamic and CNS-regulated to induce reversible peripheral
518 changes, its real-time assessment is necessary for in-depth understanding of its impact on
519 health and resiliency. We used real-time glucose telemetry in rats, combined with temporal
520 assessment of neuroendocrine hormones to demonstrate in rodents a contextual relationship
521 between air pollution induced stress response and hyperglycemia. We demonstrated a
522 mechanistic link between hyperglycemia and adrenal-derived stress hormones using
523 adrenalectomy and pharmacological interventions of adrenergic and glucocorticoid receptors
524 (βAR and GR). Further, we showed that exercise-induced depletion of cortisol in humans was

525 significantly attenuated by ozone, supporting the role of HPA axis consistent with our prior
526 human study.[19] Thus, real-time monitoring of glucose and stress hormones may serve as
527 immediate biomarkers of air-pollutant-induced pulmonary stress and the interactive impacts of
528 other environmental contaminants and non-chemical stressors.

529 Hyperglycemia is one of the earliest markers of stress-induced homeostatic changes that
530 is consistently noted after a single ozone exposure[18,22,23] and other irritant exposures.[38] Here, we
531 show that the temporality of this neuroendocrine response induced by ozone can be assessed
532 using real-time glucose telemetry in rats. Upon daily ozone exposure for 3 or more consecutive
533 days, the ozone-induced hyperglycemic and hypothermic response is no longer evident,
534 reflective of stress adaptation. We show that ozone-induced hyperglycemia, which coincides
535 with hypothermia during exposure, is preceded by changes in circulating anterior pituitary-
536 derived hormones and the release of adrenal-derived epinephrine and corticosterone, linking
537 the activation of SAM and HPA axes and the inhibition of HPT and HPG axes to hyperglycemia.
538 Further, eliminating adrenal-derived stress hormones from circulation through adrenalectomy or
539 pharmacologically inhibition of stress hormone receptors diminishes ozone-induced
540 hyperglycemia, and treatment with stress hormone receptors agonists restores ozone-induced
541 hyperglycemia in adrenalectomized rats. Combined, these results highlight the utility of real-time
542 glucose measurement as a sensitive marker that reflects the impacts of air-pollutant exposure,
543 and may be useful in linking source to health outcomes.

544 Acute ozone exposure has been shown to activate stress-responsive regions in the rat
545 brain.[39] The release of stress hormones by activation of SAM and HPA axes is integral in
546 mediating key metabolic processes that channel energy resources where needed by acting on
547 AR and GR.[40] Glucocorticoid feedback regulation on HPA activity, and the roles of
548 mineralocorticoids and catecholamines, have been implicated in stress adaptation and the
549 plasticity of organismal neural responses.[10] However, the full understanding of molecular
550 mechanisms linked to impaired stress adaptation and neuropsychiatric disorders is still lacking.

551 The adaptation from ozone-induced hyperglycemia on the third consecutive day of exposure
552 suggests a neuroendocrine contribution to ozone adaptation. This adaptation response was not
553 evident on the second day of ozone exposure. Moreover, one-week of no exposure washout-
554 period was associated with the loss of adaptation, indicating remarkable plasticity that may be
555 influenced by stressor type, potency, and longevity of exposure. Given the contribution of
556 glucocorticoids in stress adaptation,[11] and the link between changes in circulating cortisol and
557 chronic neurobehavioral and metabolic diseases,[41,42] the temporal assessment of changes in
558 glucose and cortisol is critical for evaluation of health status, resiliency, and longevity of stressor
559 effects. Further, the loss of dynamicity or oscillatory rhythms is an important indicator of chronic
560 health issues,[43] which may provide additional insight from temporal monitoring.

561 Real-time glucose monitoring is now more frequently employed for diabetic individuals
562 who require repeated assessment of blood glucose. In our study, we were able to assess
563 increases in blood glucose during GTT and from food intake during nighttime in rats. Thus, the
564 real-time glucose assessment offers the opportunity to study interactive effects of diet,
565 metabolic disease, and stress from air pollutant exposures. Stress in diabetics may exacerbate
566 preexistent hyperglycemia as reported in diabetic Goto Kakizaki rats[44] and in diabetic patients.[45]
567 Thus, the temporal assessment of glucose could provide insights on influence of stressors on
568 overall physiological status and underlying metabolic health condition.

569 Combined, evidence of temporal and concentration-dependent changes in hormones
570 associated with not only SAM and HPA, but also HPT and HPG axes during acute ozone
571 exposure, indicate that the stress response involves a complex interplay of multiple
572 neuroendocrine pathways. The selective activation of HPA and SAM axes was associated with
573 concurrent inhibition of HPT and HPG axes as observed by changes in respective hormones.
574 These responses are consistent with acute psychosocial stress-induced increases in ACTH and
575 cortisol, which are associated with depletion of gonadal hormones in men and women.[46] This is
576 in contrast, however, with concurrent increases in thyroid hormone and corticosterone after

577 exercise in rats.[47] With regards to HPG hormones, a rapid decline PRL in this study and the
578 reported reversal of its depletion in adrenalectomized rats[33] suggests the possible involvement
579 of glucocorticoid feedback regulation. While circulating glucocorticoids might also inhibit LH
580 secretion after ozone exposure, a role of gonadotropin releasing hormone and gonadotropin
581 inhibitory hormone is likely in ozone-induced inhibition of LH.[48] These findings indicate that
582 stress responses may not be uniform between stressor types and that ozone may impact
583 specific neuroendocrine pathways that involve input from multiple interactive signaling
584 processes in the brain to develop a tailored, integrated and temporally regulated host response.
585 Circulating corticosterone, cytokines, exhausting exercise, caloric deprivation, and even
586 sepsis are linked to depletion of TSH, T4, and T3 in a stress paradigm.[49] Acute exposure to
587 ozone has been shown to deplete circulating thyroxine and THS in rats.[50] We have shown that
588 AD reverses ozone-induced inhibition of TSH release, suggesting a role for circulating adrenal-
589 derived stress hormones.[33] Pituitary TSH release is regulated by hypothalamic thyrotropin
590 releasing hormone (TRH) with feedback controls on HPT at different levels.[51] Thus, stressor
591 specific differences in activation versus inhibition of given hormonal systems may impact
592 downstream physiological responses. The mechanisms by which anabolic pituitary hormones,
593 such as ACTH and GH, increase whereas the catabolic hormones, such as TSH, FSH, LH and
594 PRL, decrease after ozone exposure may involve a precise and selective activation or inhibition
595 of upstream regulators of hormonal responses after ozone exposure, such as the activation of
596 CRH neurons, FK506 binding protein regulation of glucocorticoid feedback, and other
597 neurotropic factors within the stress-responsive regions impacted by ozone.[10,39]
598 We have previously noted that ozone-induces pulmonary and liver transcriptional
599 changes reflective of processes that regulate metabolism, cell cycle and regeneration.[52,53]
600 Because pituitary-derived GH is involved in these processes,[34] and tied to metabolic changes,[35]
601 we assessed kinetics of GH changes and noted a delayed but concentration-dependent
602 increase at 4-hour after ozone exposure, suggesting that anabolic processes are being

603 activated. Similar patterns were noted for increases in leptin, which regulates satiety at the level
604 of the hypothalamus. However, the increase in circulating free fatty acids was noted as early as
605 1 hour, suggesting early activation of lipolytic activity in adipose tissue coinciding with changes
606 in circulating adrenal-derived hormones, but a delayed increase in GH coincides with leptin
607 release from adipose tissue.

608 Our approach focused on establishing a causal role of SAM and HPA axes activation on
609 hyperglycemia response in a gain of function experiment using AD and stress hormone receptor
610 agonists/antagonists.[25] Increased circulating epinephrine and glucocorticoids regulate glucose
611 metabolic processes, in addition to regulating other physiological and immunological processes
612 through their action on multiple tissues, including the liver and pancreas.[54] AD or treatment with
613 pharmacological blockers of βAR plus GR inhibited ozone-induced hyperglycemia and glucose
614 intolerance whereas the combination of βAR and GR agonists amplified ozone-induced
615 hyperglycemia and glucose intolerance. We have previously shown that ozone exposure is
616 associated with increased gluconeogenesis and inhibition of glucose-mediated insulin secretion
617 in rats.[28] Each AR and GR subtype may be selectively influencing different processes of glucose
618 metabolism, such as gluconeogenesis, and β-cell insulin secretion in the liver and pancreas.
619 These changes following acute ozone exposure suggest that adrenal-derived hormones
620 regulate glucose homeostatic changes induced by ozone.

621 Stress response is proportional to stressor severity and duration, and is precisely
622 directed to the affected organ system. Furthermore, this response is reversible upon stress
623 discontinuation and in some cases, even after continued stressor application (habituation).
624 However, these reversible physiological stressor effects in healthy individuals can be impaired
625 in susceptible individuals both at the CNS and peripheral organ levels, contributing to health
626 burdens from environmental exposure.[43] Based on evidence presented in this paper, we
627 surmise that evaluation of the dynamicity of this response through real-time glucose and cortisol
628 monitoring could unravel critical information on the magnitude and persistence of stress from

629 environmental exposures and its impairment in individuals with preexisting diseases including
630 psychosocial and metabolic. Using ozone inhalation as an example, we here demonstrated the
631 utility of such an approach to monitor the dynamics of stressor effects on health that is
632 amenable in humans with currently available technologies.[55]

633 This study assessed responses after exposure to only ozone. Whereas we have shown
634 similar changes in stress hormones and glucose after exposure to other gaseous irritant,
635 acrolein,[38] this response may be linked to irritancy and should not be generalized to all
636 pollutants and pollution mixtures. Similarly, the nature and timing of responses to other
637 stressors may vary leading to differences in the organ being affected and the pathology.
638 Moreover, this study assessed only acute health outcomes in healthy animals after inhaled
639 ozone exposure; however, the mechanisms by which repeated exposures and underlying health
640 conditions increase disease susceptibility can be better studied using models of compromised
641 health status. Finally, variations between humans and rodents in respiratory anatomy and
642 physiology as well the uniqueness of the hypothermic response to ozone in rats are important
643 caveats when translating findings to humans. The influence of physiological status at the time of
644 stress encounter is also critical, for example, an observed lack of increase in glucose, but
645 evidence of cortisol response after ozone exposure in humans during intermittent exercise.

646 In conclusion, we show that health effects of air pollution-induced stress can be
647 monitored in real-time by assessment of blood glucose using telemetry and where possible
648 cortisol/corticosterone in experimental models and in humans. Our data show that exposure to
649 prototypic air pollutant (ozone) rapidly induces anabolic (ACTH, GH) neuroendocrine pathways
650 involving sympathetic activation and adrenal medullary release of epinephrine and HPA-
651 mediated release of glucocorticoids, while inhibiting catabolic (HPT, HPG) pathways. These
652 reversible and dynamic neuroendocrine changes are followed by systemic metabolic alterations
653 and hyperglycemia as well as hypothermia during ozone exposure. The use of real-time glucose
654 telemetry is rats provided insights into the precise temporal relation to changes in stress

655 hormones. We further mechanistically confirm the contribution of adrenal-derived stress
656 hormones based on the evidence that AD and pharmacological inhibitors of stress hormones
657 eliminate, and agonists reverse ozone-induced hyperglycemia and glucose intolerance.
658 Because dynamic changes in circulating stress hormones likely mediate interactive metabolic
659 effects of irritant air pollutants such as ozone, this approach of continuous monitoring of glucose
660 and stress hormones may be useful in assessing health effects and susceptibility variations in
661 epidemiological studies.

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695 **REFERENCES**

696 1. Landrigan PJ, Fuller R, Acosta NJR, et al. The Lancet Commission on pollution and health.
697 *Lancet*. 2018;391(10119):462-512. doi:10.1016/S0140-6736(17)32345-0

698 2. Calderón-Garcidueñas L, Herrera-Soto A, Jury N, et al. Reduced repressive epigenetic
699 marks, increased DNA damage and Alzheimer's disease hallmarks in the brain of humans
700 and mice exposed to particulate urban air pollution. *Environ Res*. 2020;183:109226.
701 doi:10.1016/j.envres.2020.109226

702 3. Greve HJ, Mumaw CL, Messenger EJ, et al. Diesel exhaust impairs TREM2 to dysregulate
703 neuroinflammation. *J Neuroinflammation*. 2020;17(1):351. doi:10.1186/s12974-020-02017-
704 7

705 4. Paul KC, Jerrett M, Ritz B. Type 2 Diabetes Mellitus and Alzheimer's Disease: Overlapping
706 Biologic Mechanisms and Environmental Risk Factors. *Curr Environ Health Rep*.
707 2018;5(1):44-58. doi:10.1007/s40572-018-0176-1

708 5. Berman JD, Burkhardt J, Bayham J, Carter E, Wilson A. Acute Air Pollution Exposure and
709 the Risk of Violent Behavior in the United States. *Epidemiology*. 2019;30(6):799-806.
710 doi:10.1097/EDE.0000000000001085

711 6. Camargo Maluf F, Feder D, Alves de Siqueira Carvalho A. Analysis of the Relationship
712 between Type II Diabetes Mellitus and Parkinson's Disease: A Systematic Review.
713 *Parkinsons Dis*. 2019;2019:4951379. doi:10.1155/2019/4951379

714 7. Norwitz NG, Mota AS, Norwitz SG, Clarke K. Multi-Loop Model of Alzheimer Disease: An
715 Integrated Perspective on the Wnt/GSK3β, α-Synuclein, and Type 3 Diabetes Hypotheses.
716 *Front Aging Neurosci*. 2019;11:184. doi:10.3389/fnagi.2019.00184

717 8. Hajat A, Diez Roux AV, Castro-Diehl C, et al. The Association between Long-Term Air
718 Pollution and Urinary Catecholamines: Evidence from the Multi-Ethnic Study of
719 Atherosclerosis. *Environ Health Perspect*. 2019;127(5):57007. doi:10.1289/EHP3286

720 9. Russell G, Lightman S. The human stress response. *Nat Rev Endocrinol*. 2019;15(9):525-
721 534. doi:10.1038/s41574-019-0228-0

722 10. Herman JP, Nawreen N, Smail MA, Cotella EM. Brain mechanisms of HPA axis regulation:
723 neurocircuitry and feedback in context Richard Kvetnansky lecture. *Stress*.
724 2020;23(6):617-632. doi:10.1080/10253890.2020.1859475

725 11. Biessels GJ, Despa F. Cognitive decline and dementia in diabetes mellitus: mechanisms
726 and clinical implications. *Nat Rev Endocrinol*. 2018;14(10):591-604. doi:10.1038/s41574-
727 018-0048-7

728 12. Danan D, Matar MA, Kaplan Z, Zohar J, Cohen H. Blunted basal corticosterone pulsatility
729 predicts post-exposure susceptibility to PTSD phenotype in rats.
730 *Psychoneuroendocrinology*. 2018;87:35-42. doi:10.1016/j.psyneuen.2017.09.023

731 13. Faulenbach M, Uthoff H, Schwegler K, Spinas GA, Schmid C, Wiesli P. Effect of
732 psychological stress on glucose control in patients with Type 2 diabetes. *Diabet Med*.
733 2012;29(1):128-131. doi:10.1111/j.1464-5491.2011.03431.x

14. Teyin E, Derbent A, Balcioglu T, Cokmez B. The efficacy of caudal morphine or bupivacaine combined with general anesthesia on postoperative pain and neuroendocrine stress response in children. *Paediatr Anaesth*. 2006;16(3):290-296. doi:10.1111/j.1460-9592.2005.01711.x

15. Hásková A, Radovnická L, Petruželková L, et al. Real-time CGM Is Superior to Flash Glucose Monitoring for Glucose Control in Type 1 Diabetes: The CORRIDA Randomized Controlled Trial. *Diabetes Care*. 2020;43(11):2744-2750. doi:10.2337/dc20-0112

16. Baghelani M, Abbasi Z, Daneshmand M, Light PE. Non-invasive continuous-time glucose monitoring system using a chipless printable sensor based on split ring microwave resonators. *Sci Rep*. 2020;10(1):12980. doi:10.1038/s41598-020-69547-1

17. Torrente-Rodríguez RM, Tu J, Yang Y, et al. Investigation of cortisol dynamics in human sweat using a graphene-based wireless mHealth system. *Matter*. 2020;2(4):921-937. doi:10.1016/j.matt.2020.01.021

18. Miller DB, Karoly ED, Jones JC, et al. Inhaled ozone (O3)-induces changes in serum metabolomic and liver transcriptomic profiles in rats. *Toxicol Appl Pharmacol*. 2015;286(2):65-79. doi:10.1016/j.taap.2015.03.025

19. Miller DB, Ghio AJ, Karoly ED, et al. Ozone Exposure Increases Circulating Stress Hormones and Lipid Metabolites in Humans. *Am J Respir Crit Care Med*. 2016;193(12):1382-1391. doi:10.1164/rccm.201508-1599OC

20. Brockway R, Tiesma S, Bogie H, et al. Fully Implantable Arterial Blood Glucose Device for Metabolic Research Applications in Rats for Two Months. *J Diabetes Sci Technol*. 2015;9(4):771-781. doi:10.1177/1932296815586424

21. Henriquez AR, Snow SJ, Schladweiler MC, et al. Adrenergic and glucocorticoid receptor antagonists reduce ozone-induced lung injury and inflammation. *Toxicol Appl Pharmacol*. 2018;339:161-171. doi:10.1016/j.taap.2017.12.006

22. Miller DB, Snow SJ, Schladweiler MC, et al. Acute Ozone-Induced Pulmonary and Systemic Metabolic Effects Are Diminished in Adrenalectomized Rats. *Toxicol Sci*. 2016;150(2):312-322. doi:10.1093/toxsci/kfv331

23. Bass V, Gordon CJ, Jarema KA, et al. Ozone induces glucose intolerance and systemic metabolic effects in young and aged Brown Norway rats. *Toxicol Appl Pharmacol*. 2013;273(3):551-560. doi:10.1016/j.taap.2013.09.029

24. Henriquez AR, Williams W, Snow SJ, et al. The dynamicity of acute ozone-induced systemic leukocyte trafficking and adrenal-derived stress hormones. *Toxicology*. 2021;458:152823. doi:10.1016/j.tox.2021.152823

25. Henriquez AR, Snow SJ, Schladweiler MC, et al. Beta-2 Adrenergic and Glucocorticoid Receptor Agonists Modulate Ozone-Induced Pulmonary Protein Leakage and Inflammation in Healthy and Adrenalectomized Rats. *Toxicol Sci*. 2018;166(2):288-305. doi:10.1093/toxsci/kfy198

26. Griffin ÉW, Yssel JD, O'Neill E, et al. The β2-adrenoceptor agonist clenbuterol reduces the neuroinflammatory response, neutrophil infiltration and apoptosis following intra-striatal IL-

774 1β administration to rats. *Immunopharmacol Immunotoxicol*. 2018;40(2):99-106.
775 doi:10.1080/08923973.2017.1418882

776 27. Jonasson S, Wigenstam E, Koch B, Bucht A. Early treatment of chlorine-induced airway
777 hyperresponsiveness and inflammation with corticosteroids. *Toxicol Appl Pharmacol*.
778 2013;271(2):168-174. doi:10.1016/j.taap.2013.04.037

779 28. Miller DB, Snow SJ, Henriquez A, et al. Systemic metabolic derangement, pulmonary
780 effects, and insulin insufficiency following subchronic ozone exposure in rats. *Toxicol Appl*
781 *Pharmacol*. 2016;306:47-57. doi:10.1016/j.taap.2016.06.027

782 29. Kainuma E, Watanabe M, Tomiyama-Miyaji C, et al. Association of glucocorticoid with
783 stress-induced modulation of body temperature, blood glucose and innate immunity.
784 *Psychoneuroendocrinology*. 2009;34(10):1459-1468. doi:10.1016/j.psyneuen.2009.04.021

785 30. Gordon CJ, Johnstone AF, Aydin C, et al. Episodic ozone exposure in adult and senescent
786 Brown Norway rats: acute and delayed effect on heart rate, core temperature and motor
787 activity. *Inhal Toxicol*. 2014;26(7):380-390. doi:10.3109/08958378.2014.905659

788 31. Hamade AK, Tankersley CG. Interstrain variation in cardiac and respiratory adaptation to
789 repeated ozone and particulate matter exposures. *Am J Physiol Regul Integr Comp*
790 *Physiol*. 2009;296(4):R1202-1215. doi:10.1152/ajpregu.90808.2008

791 32. Okada S, Yamaguchi N. Possible role of adrenoceptors in the hypothalamic
792 paraventricular nucleus in corticotropin-releasing factor-induced sympatho-
793 adrenomedullary outflow in rats. *Auton Neurosci*. 2017;203:74-80.
794 doi:10.1016/j.autneu.2017.01.008

795 33. Henriquez AR, House JS, Snow SJ, et al. Ozone-induced dysregulation of neuroendocrine
796 axes requires adrenal-derived stress hormones. *Toxicol Sci*. Published online August 9,
797 2019:kfz182. doi:10.1093/toxsci/kfz182

798 34. Lim CT, Khoo B. Normal Physiology of ACTH and GH Release in the Hypothalamus and
799 Anterior Pituitary in Man. In: Feingold KR, Anawalt B, Boyce A, et al., eds. *Endotext*.
800 MDText.com, Inc.; 2000. Accessed January 31, 2022.
801 http://www.ncbi.nlm.nih.gov/books/NBK279116/

802 35. Qiu H, Yang JK, Chen C. Influence of insulin on growth hormone secretion, level and
803 growth hormone signalling. *Sheng Li Xue Bao*. 2017;69(5):541-556.

804 36. Napolitano G, Barone D, Di Meo S, Venditti P. Adrenaline induces mitochondrial
805 biogenesis in rat liver. *J Bioenerg Biomembr*. 2018;50(1):11-19. doi:10.1007/s10863-017-
806 9736-6

807 37. Kodavanti UP. Susceptibility Variations in Air Pollution Health Effects: Incorporating
808 Neuroendocrine Activation. *Toxicol Pathol*. 2019;47(8):962-975.
809 doi:10.1177/0192623319878402

810 38. Snow SJ, McGee MA, Henriquez A, et al. Respiratory Effects and Systemic Stress
811 Response Following Acute Acrolein Inhalation in Rats. *Toxicol Sci*. 2017;158(2):454-464.
812 doi:10.1093/toxsci/kfx108

813 39. Gackière F, Saliba L, Baude A, Bosler O, Strube C. Ozone inhalation activates stress-
814 responsive regions of the CNS. *J Neurochem*. 2011;117(6):961-972. doi:10.1111/j.1471-
815 4159.2011.07267.x

816 40. Begg DP, Woods SC. Interactions between the central nervous system and pancreatic islet
817 secretions: a historical perspective. *Adv Physiol Educ*. 2013;37(1):53-60.
818 doi:10.1152/advan.00167.2012

819 41. Morgese MG, Schiavone S, Trabace L. Emerging role of amyloid beta in stress response:
820 Implication for depression and diabetes. *Eur J Pharmacol*. 2017;817:22-29.
821 doi:10.1016/j.ejphar.2017.08.031

822 42. Schatzberg AF, Lindley S. Glucocorticoid antagonists in neuropsychiatric [corrected]
823 disorders. *Eur J Pharmacol*. 2008;583(2-3):358-364. doi:10.1016/j.ejphar.2008.01.001

824 43. Berger M, Sarnyai Z. “More than skin deep”: stress neurobiology and mental health
825 consequences of racial discrimination. *Stress*. 2015;18(1):1-10.
826 doi:10.3109/10253890.2014.989204

827 44. Snow SJ, Henriquez AR, Fisher A, et al. Peripheral metabolic effects of ozone exposure in
828 healthy and diabetic rats on normal or high-cholesterol diet. *Toxicol Appl Pharmacol*.
829 2021;415:115427. doi:10.1016/j.taap.2021.115427

830 45. Goetsch VL, VanDorsten B, Pbert LA, Ullrich IH, Yeater RA. Acute effects of laboratory
831 stress on blood glucose in noninsulin-dependent diabetes. *Psychosom Med*.
832 1993;55(6):492-496. doi:10.1097/00006842-199311000-00004

833 46. Stephens MAC, Mahon PB, McCaul ME, Wand GS. Hypothalamic-pituitary-adrenal axis
834 response to acute psychosocial stress: Effects of biological sex and circulating sex
835 hormones. *Psychoneuroendocrinology*. 2016;66:47-55.
836 doi:10.1016/j.psyneuen.2015.12.021

837 47. Parra-Montes de Oca MA, Gutiérrez-Mariscal M, Salmerón-Jiménez MF, Jaimes-Hoy L,
838 Charli JL, Joseph-Bravo P. Voluntary Exercise-Induced Activation of Thyroid Axis and
839 Reduction of White Fat Depots Is Attenuated by Chronic Stress in a Sex Dimorphic Pattern
840 in Adult Rats. *Front Endocrinol (Lausanne)*. 2019;10:418. doi:10.3389/fendo.2019.00418

841 48. Kirby ED, Geraghty AC, Ubuka T, Bentley GE, Kaufer D. Stress increases putative
842 gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *Proc Natl*
843 *Acad Sci U S A*. 2009;106(27):11324-11329. doi:10.1073/pnas.0901176106

844 49. Chatzitomaris A, Hoermann R, Midgley JE, et al. Thyroid Allostasis-Adaptive Responses of
845 Thyrotropic Feedback Control to Conditions of Strain, Stress, and Developmental
846 Programming. *Front Endocrinol (Lausanne)*. 2017;8:163. doi:10.3389/fendo.2017.00163

847 50. Clemons GK, Wei D. Effect of short-term ozone exposure on exogenous thyroxine levels in
848 thyroidectomized and hypophysectomized rats. *Toxicol Appl Pharmacol*. 1984;74(1):86-90.
849 doi:10.1016/0041-008x(84)90273-4

850 51. Joseph-Bravo P, Jaimes-Hoy L, Uribe RM, Charli JL. 60 YEARS OF
851 NEUROENDOCRINOLOGY: TRH, the first hypophysiotropic releasing hormone isolated:

852 control of the pituitary-thyroid axis. *J Endocrinol*. 2015;226(2):T85-T100. doi:10.1530/JOE-
853 15-0124

854 52. Colonna CH, Henriquez AR, House JS, et al. The Role of Hepatic Vagal Tone in Ozone-
855 Induced Metabolic Dysfunction in the Liver. *Toxicol Sci*. 2021;181(2):229-245.
856 doi:10.1093/toxsci/kfab025

857 53. Henriquez A, House J, Miller DB, et al. Adrenal-derived stress hormones modulate ozone-
858 induced lung injury and inflammation. *Toxicol Appl Pharmacol*. 2017;329:249-258.
859 doi:10.1016/j.taap.2017.06.009

860 54. Kuo T, McQueen A, Chen TC, Wang JC. Regulation of Glucose Homeostasis by
861 Glucocorticoids. *Adv Exp Med Biol*. 2015;872:99-126. doi:10.1007/978-1-4939-2895-8_5

862 55. Patlar Akbulut F, Ikitimur B, Akan A. Wearable sensor-based evaluation of psychosocial
863 stress in patients with metabolic syndrome. *Artif Intell Med*. 2020;104:101824.
864 doi:10.1016/j.artmed.2020.101824

865

866 **Figure Captions**

867 **Figure 1.** Real-time glucose monitoring in freely moving animals reveal dynamicity of ozone-
868 induced changes and response to glucose injection. A) A schema showing surgical implantation
869 of glucose radiotelemetry devices followed by recovery and then ozone exposure at different
870 concentrations. Glucose levels were measured real-time in a weekly cross over design over 4
871 weeks and averages of every minute are plotted as mean ± SEM of n=7/concentration. B) Real-
872 time changes in blood glucose levels during 4 hours of air (0.0 ppm) or ozone exposure (0.2,
873 0.4, and 0.8 ppm). C) Glucose levels during first 24 hours after 4-hour air or ozone exposure
874 (n=7/concentration). D) Glucose levels during 5th and 6th week of cross-over exposure to air or
875 0.8 ppm ozone and during glucose tolerance test performed immediately following exposure
876 (n=7/concentration). E) Comparison of glucose levels measured through tail prick every 30 min
877 and during continuous monitoring through telemetry (n=7/concentration). F) Comparison of
878 baseline glucose levels assessed using tail prick and telemetry following 4-hour air or ozone
879 exposure. G) Comparison of area under the curve assessment of glucose tolerance test
880 showing similarity in the glucose levels assessed using tail prick and those assessed through
881 telemetry.

882 **Figure 2.** Recovery from a single 4-hour ozone exposure induced hyperglycemia, diurnal
883 variation, and adaptation during repeated ozone exposure. A) Glucose levels were measured
884 real-time in a weekly cross over design over 4 weeks and averaged for every minute are plotted
885 as mean of n=7/concentration. The data show ozone-induced hyperglycemia during and
886 following exposure and reversal of this effect in subsequent non-exposure days. Note the higher
887 levels of circulating glucose at nighttime when animals are active and feeding. B) Ozone-
888 induced hyperglycemia during repeated daily 4-hour exposure for 4 consecutive days followed
889 by 3-day non-exposure period showing nearly complete adaptation on day 3 and day 4 where
890 no ozone effect is evident (n=3-4/group). Data used during telemetry calibration were not used
891 in analysis or graphing.

892 **Figure 3.** Temporal changes in anterior pituitary, adrenal-derived, and metabolic hormones, as
893 well as circulating metabolites during 4-hour exposure to air or ozone. A separate cohort of
894 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
895 and necropsies were performed immediately following each exposure (within 15-20 min to
896 collect blood samples). A) experimental design; B) serum pituitary and adrenal-derived stress
897 hormones; and C) metabolic hormones and levels of free fatty acids. Data show mean ± SEM
898 (n=6-8 animals/group). Significant differences ($p<0.05$) are denoted by "*" for 0.8 ppm versus
899 0.0 ppm, "†" for 0.4 ppm versus 0.0 ppm, and "‡" for 0.8 ppm versus 0.4 ppm for time matched
900 groups. ACTH, adrenocorticotropic hormone; TSH, thyroid stimulating hormone, PRL, prolactin;
901 LH, luteinizing hormone; GH, growth hormone. Note that the data for corticosterone and
902 epinephrine are recently published in a table form (Henriquez et al., 2021).

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

916 **Figure 5.** Changes in plasma glucose and cortisol in humans within 1-2 hour following exposure
917 to air or 0.3 ppm ozone. (A) Archived serum samples from a clinical study were obtained where
918 blood samples were collected pre and post exposure during two clinical visits from healthy
919 young volunteers who are exposed to air or 0.3 ppm ozone for 2 hours in a cross-over design
920 separated for at least 2 weeks. During air or ozone exposure volunteers were intermittently
921 exercising. B. There was no significant increase in blood glucose likely due to exercise,
922 however, normal cortisol decreases from exercise were significantly attenuated (paired-samples
923 t-test; n=29; p = 0.02). Samples > 1.5 interquartile range were removed as outliers.

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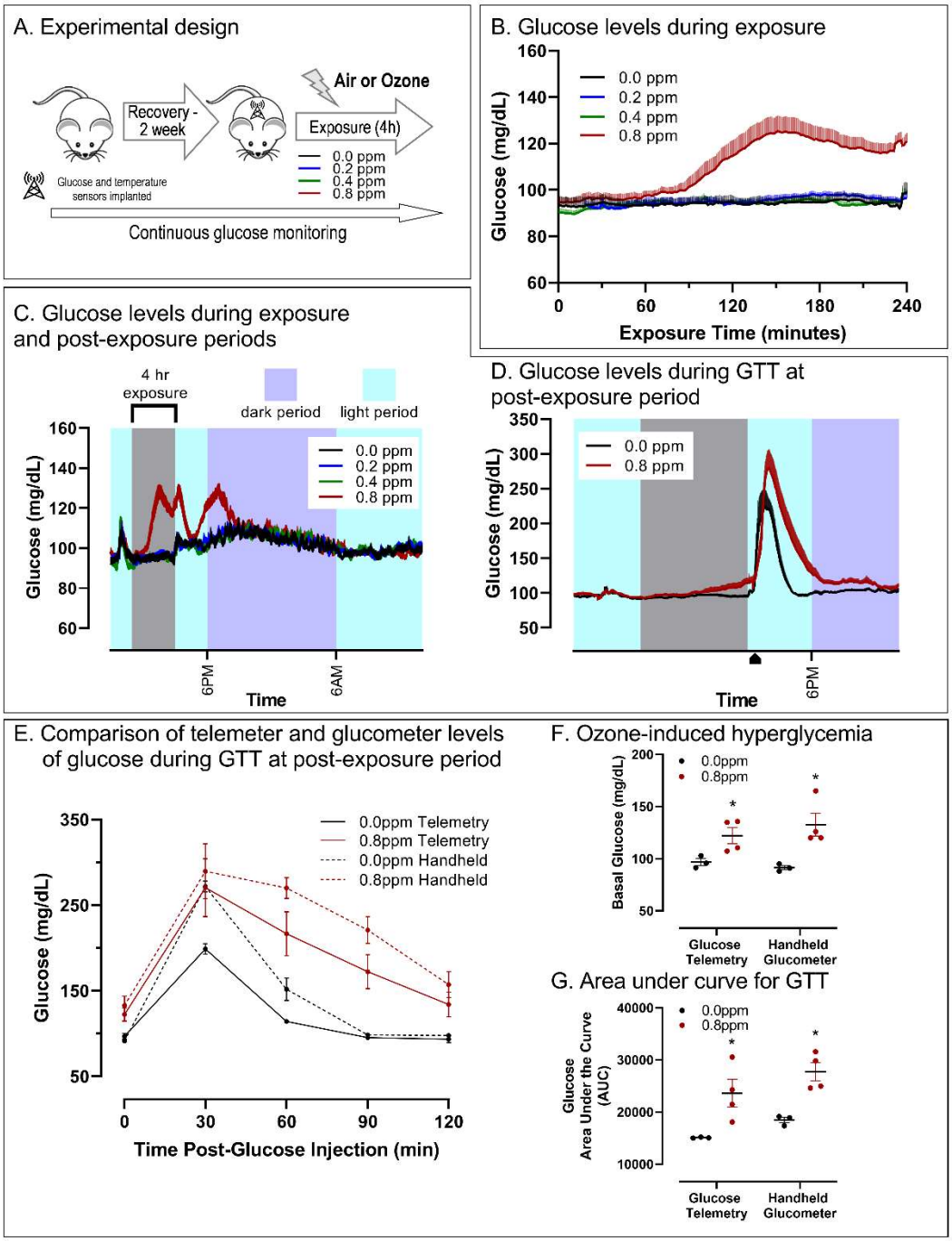
926 Figure 1



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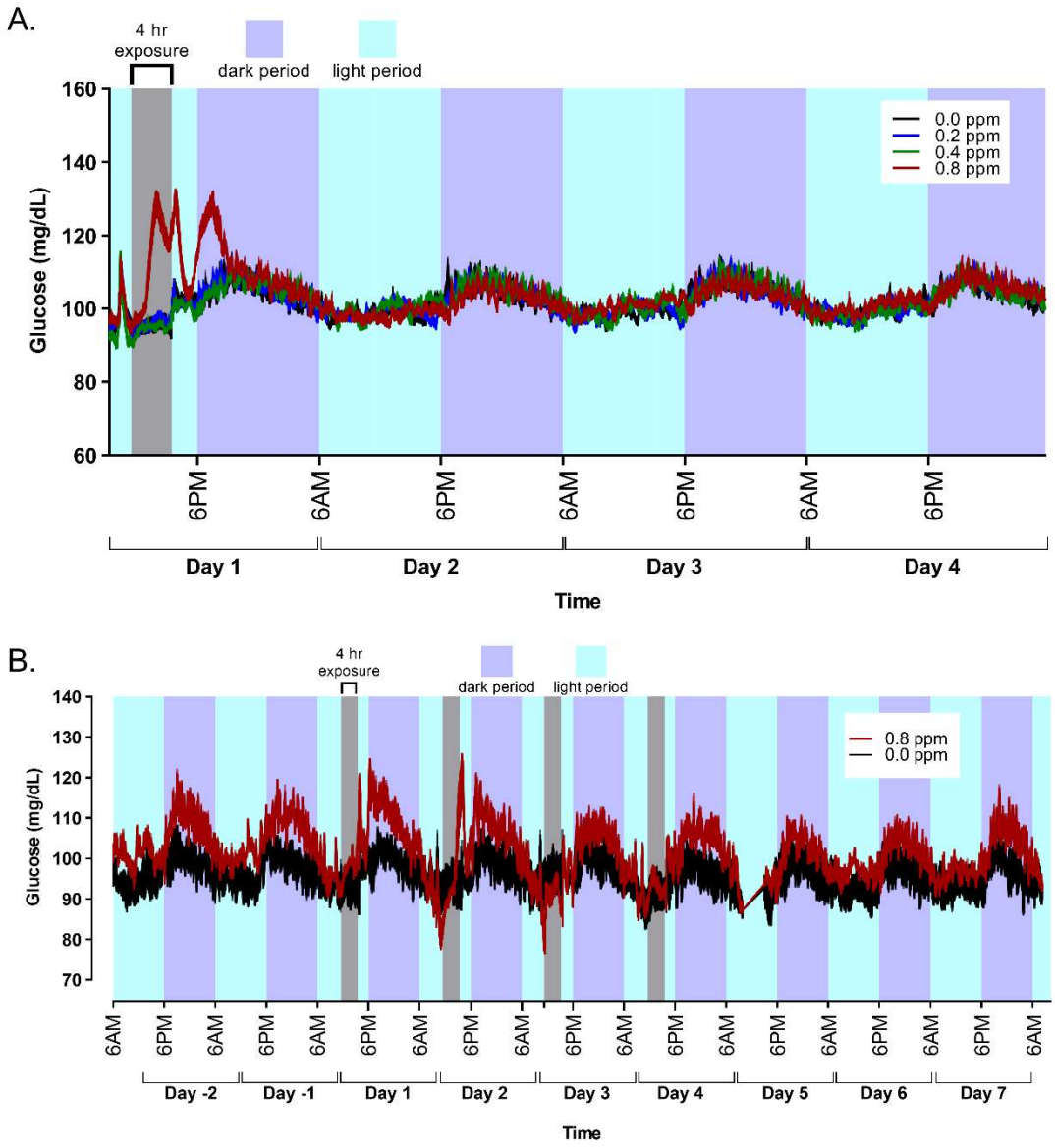
929 Figure 2



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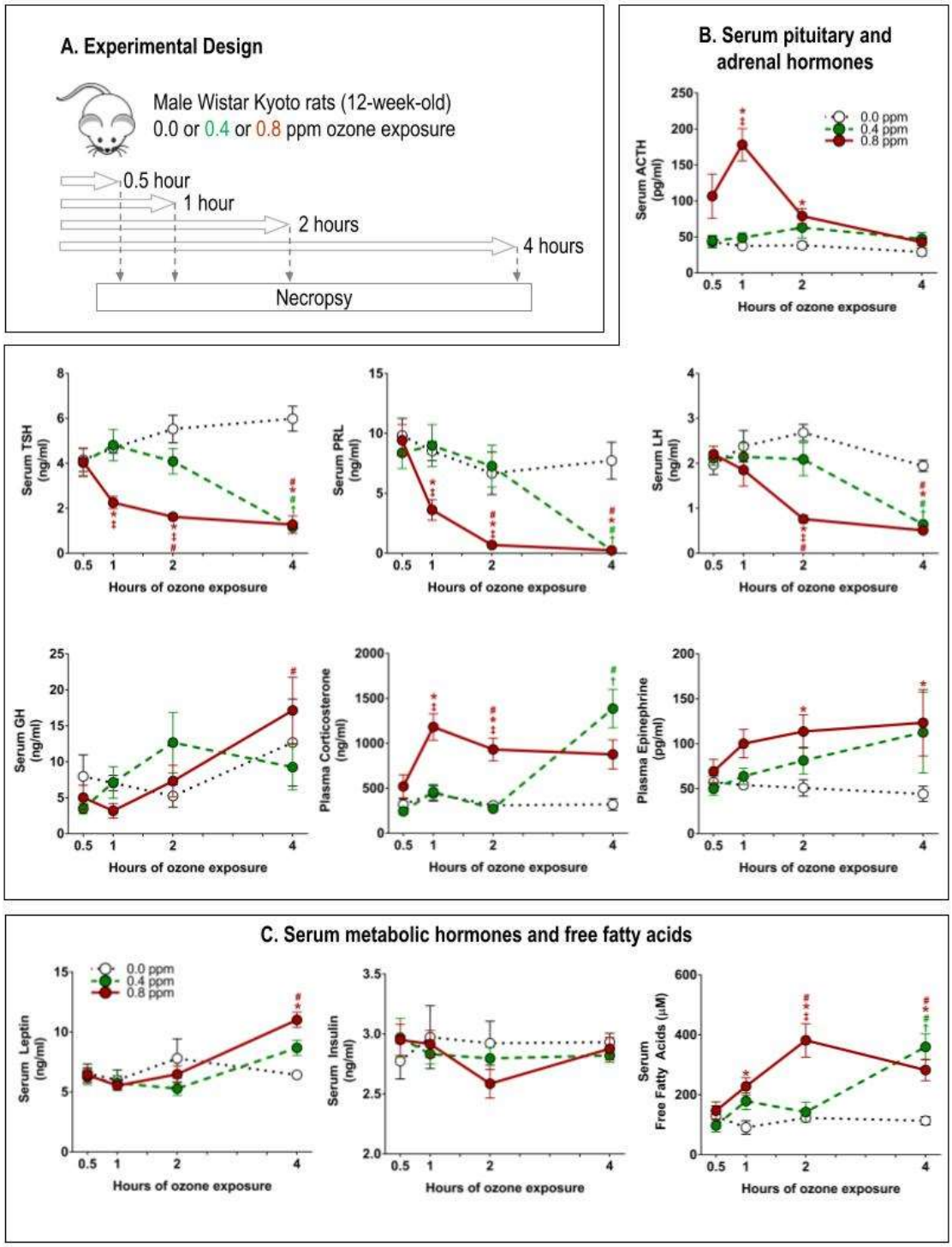
932 Figure 3



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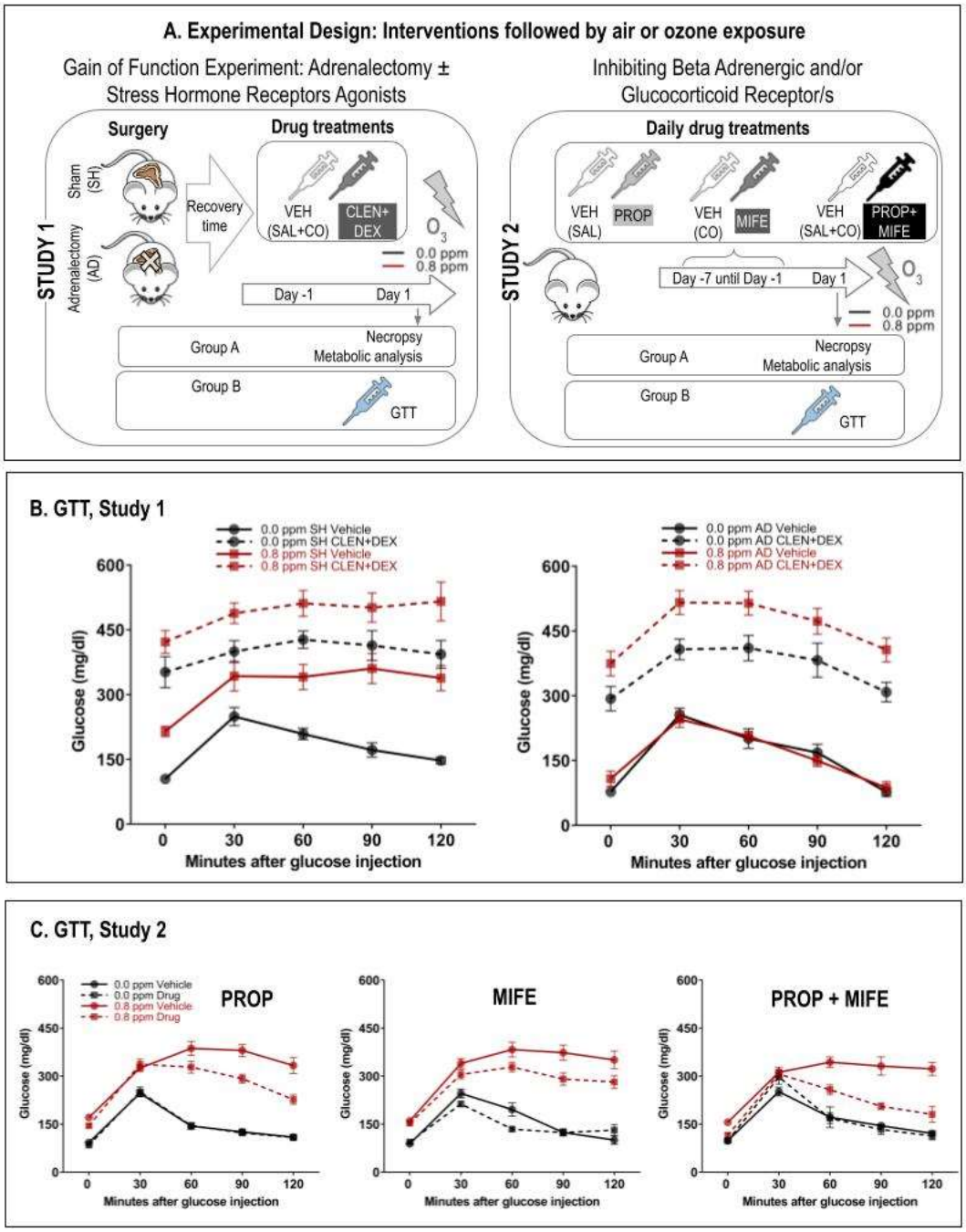

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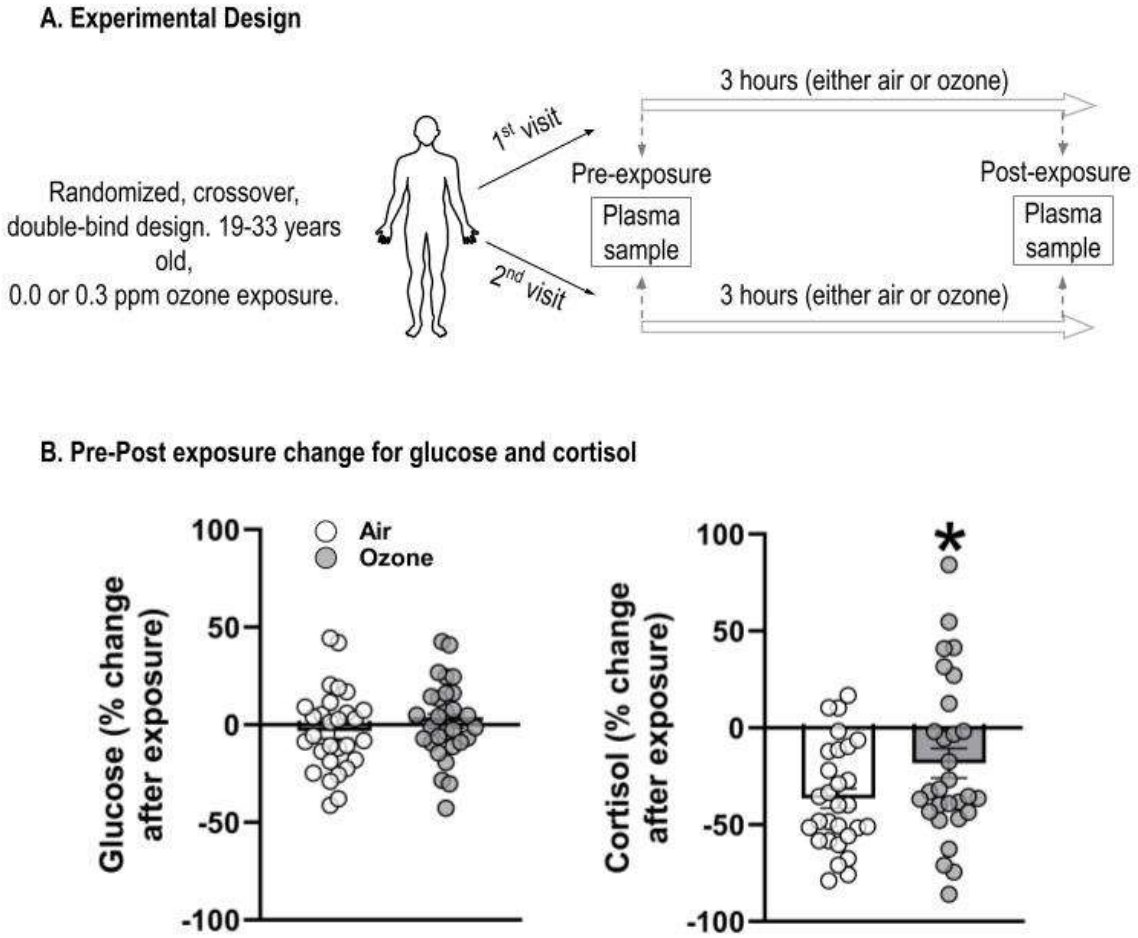
938 Figure 5



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