Early Gestational Exposure to Inhaled Ozone Impairs Maternal Uterine Artery and Cardiac Function

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ABSTRACT

Exposure to air pollutants such as ozone (O₃) is associated with adverse pregnancy outcomes, including higher incidence of gestational hypertension, preeclampsia, and peripartum cardiomyopathy; however, the underlying mechanisms of this association remain unclear. We hypothesized that O₃ exposures during early placental formation would lead to more adverse cardiovascular effects at term for exposed dams, as compared with late-term exposures. Pregnant Sprague Dawley rats were exposed (4 h) to either filtered air (FA) or O₃ (0.3 or 1.0 ppm) at either gestational day (GD)10 or GD20, with longitudinal functional assessments and molecular endpoints conducted at term. Exposure at GD10 led to placental transcriptional changes at term that were consistent with markers in human preeclampsia, including reduced *mmp10* and increased *cd36*, *fzd1*, and *col1a1*. O₃ exposure, at both early and late gestation, induced a significant increase in maternal circulating soluble FMS-like tyrosine kinase-1 (sFlt-1), a known driver of preeclampsia. Otherwise, exposure to 0.3 ppm O₃ at GD10 led to several late-stage cardiovascular outcomes in dams that were not evident in GD20-exposed dams, including elevated uterine artery resistance index and reduced cardiac output and stroke volume. GD10 O₃ exposure proteomic profile in maternal hearts characterized by a reduction in proteins with essential roles in metabolism and mitochondrial function, whereas phosphoproteomic changes were consistent with pathways involved in cardiomyopathic responses. Thus, the developing placenta is an indirect target of inhaled O₃ and systemic maternal cardiovascular abnormalities may be induced by O₃ exposure at a specific window of gestation.

Key words: cardiovascular; intrauterine; maternal; placenta; ozone; gestational hypertension; preeclampsia; cardiomyopathy.

Cardiomyopathic and hypertensive disorders complicate 5%–10% of pregnancies worldwide and are one of the leading causes of maternal and perinatal mortality and morbidity (Endeshaw and Berhan, 2015; Roberts et al., 2003; Say et al.,

2014). Women with a history of hypertensive disorders have an increased risk of developing cardiovascular and cerebrovascular disease (Arany and Elkayam, 2016; Maynard et al., 2003; Patten et al., 2012). Numerous epidemiological studies have noted

relationships between ambient concentrations of air pollutants and hypertensive disorders of pregnancy (Choe et al., 2018; Dadvand et al., 2014; Pedersen et al., 2014; Wang et al., 2018; Xu et al., 2014; Zhu et al., 2017), however, the periods of vulnerability and mechanisms underlying this association remain unclear. A growing body of research has implicated the placenta as a target organ and potential mediator of adverse birth outcomes related to the common ground pollutant ozone (O_3) (Geer et al., 2012; Lee et al., 2012; Miller et al., 2017; Olsson et al., 2013; Salam et al., 2005; Tu et al., 2016). Although some inconsistency exists, epidemiological studies tend to observe the greatest risk between adverse maternal or gestational outcomes and pollution exposures in the first or second trimester (Geer et al., 2012; Lee et al., 2012; Olsson et al., 2013; Salam et al., 2005).

Placentation begins around week 13 of gestation in humans but continues to develop throughout pregnancy to match the increasing metabolic/nutritional demands of the fetus (Sones and Davisson, 2016). Incomplete or compromised placental development can promote hypertensive syndromes in mothers, including preeclampsia (Sones and Davisson, 2016). Inadequate angiogenesis during placentation leads to a poorly perfused placenta, which, in response to increasing metabolic demands of the growing fetus, releases proangiogenic and proinflammatory factors (Gouloupoulou, 2017). Among these, soluble fms-like tyrosine kinase-1 (sFLT-1), also known as vascular endothelial growth factor receptor 1 (VEGFR1), is a potent vasoconstrictor and key driver of hypertensive crises in pregnancy (Maynard et al., 2003). Although the placental role in preeclampsia and cardiovascular/hypertensive disorders of pregnancy is well understood, the role of environmental factors as initiators of placental insufficiency—and contributors to gestational hypertension risk—is unclear.

Air pollution has a well-reported deleterious role on vascular function, an outcome that is consistent across a variety of inhaled pollutants and different vascular beds, as reported from numerous laboratories (Aragon et al., 2017; Chuang et al., 2009; Paffett et al., 2015; Peretz et al., 2008; Tamagawa et al., 2008). Data from our laboratory suggest that the vascular endothelium throughout the body is a target of inflammatory “spillover” from lungs following inhalation exposures (Mostovenko et al., 2019; Paffett et al., 2015; Robertson et al., 2013). A demonstrable “bioactivity” in serum/plasma that inhibits vasodilation and impairs endothelial growth arises in animals and humans shortly after inhalation exposures to either particles or gaseous pollutants (Channell et al., 2012; Paffett et al., 2015; Robertson et al., 2013; Schisler et al., 2015). Peptidomic analysis of serum following exposure to nanomaterials reveals hundreds of cleaved peptides from endogenous proteins, especially extracellular matrix proteins. One specific peptide fragment derived from thrombospondin can recapitulate a portion of this bioactivity, especially endothelial regrowth in a wound healing assay (Aragon et al., 2017; Mostovenko et al., 2019). Recently, the placenta has gained recognition as a potential target organ and mediator of adverse birth outcomes related to ambient air pollution (Geer et al., 2012; Lee et al., 2012; Miller et al., 2017; Olsson et al., 2013; Salam et al., 2005; Tu et al., 2016). As the placenta can be a driver of maternal hypertensive syndromes, negative impacts of inhaled pollution on normal growth, and physiology of the placenta could potentially promote a maternal cardiovascular pathology.

We hypothesized that exposure to air pollution in the early stages of placental development would predispose mothers to late-stage cardiovascular impairments as a result of dysregulation of normal angiogenesis due to the serum bioactivity. To

test this hypothesis, we examined the maternal cardiovascular effects of early (gestational day 10; GD10) and late (GD20) exposures to inhaled O_3 . Placentation is just beginning at GD10 in rats, and the effects of an O_3 exposure on serum-borne factors would be predicted to last for at least 24 h, if not longer, thus compromising the earliest period of placental angiogenesis. On the other hand, a late-stage exposure would be likely to have less of an impact on a fully formed placenta.

MATERIALS AND METHODS

Animals and exposure paradigm. Pregnant Sprague Dawley Rats (10–12 weeks of age at the beginning of the study, arriving at GD4–GD6) were obtained from Taconic Biosciences (Rensselaer, New York). Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility at the University of New Mexico Health Sciences Center. Animals were maintained at constant temperature (20–24°C), relative humidity (30%–60%), and on a 12-h light/dark cycle throughout the study. Animals were provided with normal rat chow and water *ad libitum*.

Dams were exposed to either filtered air (FA) or O_3 for a single 4-h period via whole body inhalation at either GD10 or GD20. O_3 was generated using an OREC silent arc discharge generator (Osmonics, Phoenix, Arizona) as previously described (Robertson et al., 2013). Water was available for dams during the exposure, but food was withheld. Exposures took place in standard shoebox cages without bedding (to prevent O_3 scavenging). Dams exposed at GD10 (cohort 1) were exposed to 0.3 ppm O_3 , whereas dams exposed on GD20 (cohort 2) were exposed to O_3 at concentrations of 0.3 and 1.0 ppm (Figure 1); 0.3 ppm O_3 is a relatively high concentration, but major metropolitan regions around the world can approach this level for short periods (Borja-Aburto et al., 1997; Calderon-Garciduenas et al., 2016; Maji et al., 2019). Because we hypothesized (largely based on findings from Miller et al., 2017) that the early gestational exposures would have a larger impact, we also included a greater exposure stressor (1.0 ppm) at the later gestational period (GD20) to “push the system.” All animal handling and procedures were conducted humanely and approved by the Institutional Animal Care and Use Committee at the University of New Mexico.

Plasma and tissue collection. Dams exposed at GD10 were euthanized by exsanguination at term (GD20) under isoflurane anesthesia. Dams exposed to O_3 on GD20 were similarly euthanized 24 h later on GD21. Blood was collected from both cohorts via cardiac puncture, placed on ice for 30 min, and then centrifuged (1000 × g, 10 min, 4°C) to separate serum. Serum was stored at -80°C. Additionally, placenta and heart were dissected, weighed, and frozen in liquid nitrogen. Tissue was stored at -80°C until used for assays.

Uterine artery myography. Uterine artery ring segments (2 mm) were isolated from FA- and O_3 -exposed Sprague Dawley dams at term, cleaned of connective tissue and adventitial fluid. 40 μ M wires were passed through the lumen and rings were mounted on a 4-chamber multiwire myograph system (610M; Danish Myo Technology A/A, Aarhus, Denmark). Rings were then submerged in physiological salt solution (PSS): 119.0 mM NaCl, 25 mM $NaHCO_3$, 5.5 mM glucose, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 , 0.027 mM EDTA, and 2.5 mM $CaCl_2$. Carbogen was bubbled into the wells at 37°C with 21% O_2 –5% CO_2 balance N_2 and uterine artery rings were left to equilibrate at 2 g of tension for 30 min. Tension was gradually increased in

Figure 1. O₃ exposure paradigm.

0.75 mN increments to an optimal passive tension of 5 mN. Vessel viability was confirmed using a contractile response to the addition of high concentration potassium chloride PSS (KPSS): 64.9 mM NaCl, 25.0 mM NaHCO₃, 5.5 mM glucose, 58.9 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 0.027 mM EDTA, and 2.5 mM CaCl₂. Validation was repeated twice to constrict the vessel. Afterwards, all vessels were washed 3 times with PSS and allowed to equilibrate for 30 min. All uterine artery vessel segments were subjected to cumulative concentrations of phenylephrine (1–100 μ M) to contract rings. Uterine vessels were then incubated in PSS with 1% (vol/vol) serum obtained from rats previously exposed to either FA or O₃ (0.3 ppm). Cumulative concentration-response curves to acetylcholine (ACh) an endothelium-dependent vasorelaxant, were produced after the force response to serum had plateaued. ACh concentrations (10^{-9} – 10^{-5}) were added to the wells every 5 min in increasing concentrations. Data were obtained through LabChart software (AD Instruments) and calculated as a percentage of serum-induced contraction with baseline tension subtracted.

Carotid artery myography. To test endothelial effects of serum components after exposures, carotid artery ring segments (2–3 mm) were isolated from naïve (ie, not exposed to O₃) female Sprague Dawley rats, cleaned of connective tissue, mounted in a 4-chamber multiwire myograph system (610M; Danish Myo Technology A/A, Aarhus, Denmark), and submerged in PSS. Carbogen was bubbled into the wells at 37°C with 21% O₂–5% CO₂ balance N₂ and carotid artery rings were left to equilibrate at 2 g of tension for 30 min. Tension was gradually increased in 2 mN increments to an optimal passive tension of 10 mN. Vessel viability was confirmed using a contractile response to the addition of high concentration KPSS. Validation was repeated twice to constrict the vessel. Afterwards, all vessels were washed 3 times with PSS and allowed to equilibrate for 30 min. Naïve vessels were then incubated in PSS with 1% (vol/vol) serum obtained from dams previously exposed to either FA or O₃ (0.3 ppm). Cumulative concentration-response curves to ACh were produced after the force response to serum had plateaued. ACh concentrations (10^{-9} – 10^{-5}) were added to the wells every 5 min in increasing concentrations. Data were obtained through LabChart software (AD Instruments) and

calculated as a percentage of serum-induced contraction with baseline tension subtracted.

Western blot analysis of serum. Serum from dams exposed to FA and O₃ (0.3 ppm GD10 or 0.3 ppm and 1.0 ppm GD20) were collected and a Bradford Assay was performed to determine protein concentration. Samples were then diluted at a 1:10 concentration in lysis buffer. Fifteen microliters per well of protein was loaded on 4%–12% Bis-Tris Plus gels in MES buffer (Bio-Rad, Hercules, California), separated by electrophoresis and transferred to 0.45 μ m nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk in TBST (20 mM Tris, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) overnight at 4°C. Soluble Flt-1 (sFlt-1) was detected using a recombinant mouse VEGFR1/Flt-1 polyclonal goat IgG antibody (AF471, R&D systems) at 1:1000 followed by a rabbit anti-goat IgG-HRP antibody (sc-2768, Santa Cruz Biotechnology) at 1:1000, both in 1% milk in TBS-T. Membranes were developed using SuperSignal West Femto Substrate (ThermoFisher), and imaged on a Protein Simple FluorChem R system (ProteinSimple, Santa Jose, California). Band intensity was quantitated using ImageJ.

Transthoracic echocardiographic measurements. Echocardiographic measurements were performed on dams at GD 8, 12, 16, and at term (GD20 for GD10 exposure rats; GD21 for GD20 exposed rats) under isoflurane in O₂ anesthesia (Vevo 3100, VisualSonics, Toronto, Canada). A 30 MHz linear array transducer was used to record uterine artery flow and transthoracic 2D M-mode echocardiogram. The uterine artery was visualized primarily off of the femoral artery or off of the aorta cranial to the renal artery. Flow was recorded and a uterine artery resistance index was calculated as [(peak systolic velocity – end diastolic velocity)/peak systolic velocity] (Miller et al., 2017). Software (Vevo LAB) from the ultrasound instrument was used to measure left ventricular posterior wall (LVPW) thickness, pulse wave measurements, and the internal diameter of the left ventricle in end-diastole (LVED) and end-systole (LVES), which was then used to calculate the percentage of fractional shortening using the formula [(LVED – LVES)/LVED] $\times$ 100. Measurements for each parameter (uterine flow, thickness of the LVPW, pulse wave measurements, and the internal diameter of the left ventricle in

end-diastole and end-systole) were obtained from at least 3 consecutive heart beats from 3 separate images of the M-Mode cardiac view or uterine artery view, taken for each animal and averaged. Averages for each rat then were combined with those for animals in the same treatment group/time point. Image collection and analysis were conducted without knowledge of exposure groupings.

RNA isolation and quantitative PCR. Assessment of mRNA expression levels was determined using previously published methods. In brief, total RNA from placental tissues stored at -80°C was isolated using an RNeasy Fibrous Tissue Mini Kit (Qiagen, 74704) and purified using GlycoBlue Coprecipitant (Thermo Fisher Scientific, AM9515). RNA (500 ng by concentration) was reverse-transcribed with random primers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) in accordance with the recommendations of the manufacturer. To quantify the gene expression, 50 ng cDNA was amplified for various gene targets by qPCR using a LightCycler 480 Instrument II (Roche, 05015278001). All primers and probes used were purchased as predesigned TaqMan Gene Expression Assays. Placental genes of interest (GOI) included: *Mmp10* (Rn00591678_m1), *Col1a1* (Rn01463848_m1), *Cd36* (Rn00580728_m1), and *Fzd1* (Rn01755425_s1). Relative expression levels of the GOI in each sample were determined by the comparative threshold method ($2^{-\Delta\Delta\text{CT}}$) and normalized to TATA-binding protein (Rn01455646_m1). Data are expressed as fold change relative to that of the FA-control group.

Left ventricular proteomics. Roughly 20 mg sections of left ventricle were isolated for proteomic analysis from 5 dams per group in GD10 (FA and 0.3 ppm O_3) and GD20 (FA and 0.3 ppm O_3) exposed dams. Purified proteins were reduced, alkylated, and digested using filter-aided sample preparation (Wiśniewski et al., 2009). Tryptic peptides were labeled using a tandem mass tag (TMT) 11-plex isobaric label reagent set (Thermo) in 2 multiplex sample groups with a common reference sample. Labeled peptide multiplexes were enriched using a High-Select TiO_2 phosphopeptide enrichment kit (Thermo) following the manufacturer's instructions. Both enriched and unenriched labeled peptides were separated into 36 fractions on a 100×1.0 mm Acquity BEH C18 column (Waters) using an UltiMate 3000 UHPLC system (Thermo) with a 40 min gradient from 99:1 to 60:40 buffer A:B ratio (Buffer A = 0.5% acetonitrile; Buffer B = 99.9% acetonitrile) under basic conditions (buffers adjusted to pH 10 with ammonium hydroxide), and then consolidated into 12 super-fractions. Each superfraction was then further separated by reverse phase XSelect CSH C18 2.5 μm resin (Waters) on an in-line 150×0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Peptides were eluted using a 60 min gradient from 97:3 to 60:40 buffer A:B ratio under acidic conditions (buffers adjusted to pH 2 with 0.1% formic acid). Eluted peptides were ionized by electrospray (2.15 kV) followed by mass spectrometric analysis on an Orbitrap Fusion Lumos mass spectrometer (Thermo) using multistage MS3 parameters. Mass spectrometry (MS) data were acquired using the FTMS analyzer in top-speed profile mode at a resolution of 120 000 over a range of 375 – 1500 m/z. Following CID activation with normalized collision energy of 35%, MS/MS data were acquired using the ion trap analyzer in centroid mode and normal mass range. Using synchronous precursor selection, up to 10 MS/MS precursors were selected for HCD activation with a normalized collision energy of 65%, followed by acquisition of MS3 reporter ion data

using the FTMS analyzer in profile mode at a resolution of 50 000 over a range of 100 – 500 m/z.

Resultant TMT data were processed using MaxQuant (v1.6.7, Max Planck Institute) to resolve reporter ion quantities and identify peptide sequences. Data were searched against a UniprotKB database specific to *Rattus Norvegicus* with a parent, fragment, and reporter ion tolerances of 3 ppm, 0.5 Da, and 0.001 Da, respectively. Search parameters included trypsin specificity with 2 missed cleavages, fixed carbamidomethyl cysteine modification, variable methionine oxidation and N-terminal acetylation, and variable phosphorylation on serine/threonine/tyrosine. Protein identifies were accepted up to a 1% false identification rate with a minimum of 2 peptides, with protein probabilities assigned by the Protein Prophet algorithm. TMT batch effects were removed using ComBAT in order to correct for technical variation due to sample multiplexing. The distributions of corrected data were median centered across all replicates to adjust for load and sensitivity variation. Where group-consistent values fell below the limit of quantification (across all replicates), a random set of imputation values were imputed having the mean of the limit of quantification and a coefficient of variation equal to the median of the dataset (Fuller et al., 2012).

Statistics. All data were tested for normal distributions (Shapiro-Wilk test) and were confirmed prior to statistical testing. GraphPad Prism Software (v6.0) was utilized for the analysis of cardiac and vascular analysis (GraphPad Prism, La Jolla, California). Terminal endpoint data were analyzed using a Student's t-test for the comparison of 2 groups and analysis of variance (ANOVA) for more than 2 groups. Longitudinal data were assessed by a 2-way ANOVA. *p*-Values $< .05$ were considered significant. For proteomics, results were analyzed by ANOVA and adjusted for multiple measures using the Benjamini and Hochberg procedure (Hochberg and Benjamini, 1990); a false discovery rate (FDR) of 5% was applied. Data were median centered across all replicates and imputed for the limit of quantification. Data points were Log2 transformed to achieve normality. Network analysis using STRING DB was performed on all proteins that were upregulated or downregulated and had a *p*-value $< .05$ and fold change > 1.25 (ie, more inclusive of smaller changes in concentration).

RESULTS

Exposures to Ozone at GD10 and GD20 Did Not Impact General Birth Outcomes

Maternal weight, maternal cardiac weight, litter size, and placental weights were not affected by O_3 exposure at either GD10 or GD20 (Supplementary Figs. S1 and S2).

Alterations in Placental mRNA and Circulating Markers Are Consistent With Preeclampsia

In placentas at term from dams exposed to 0.3 ppm O_3 at GD10, significant changes in transcription of *Mmp10* (down), *Cd36* (up), and *Fzd1* (up) were observed, consistent with findings in clinical placenta samples of preeclampsia patients; *Col1a1* also show an increasing, but nonsignificant trend (Figs. 2A–D). Placentas at term from dams exposed at GD20, however, did not manifest significant changes in these transcripts, even at 1.0 ppm O_3 (Figs. 2E–H).

We also measured soluble Flt-1 in the serum of exposed dams by immunoblotting. After exposure to 0.3 ppm O_3 at GD10, serum obtained at term showed a significant sFlt-1 elevation of

Figure 2. Prenatal O₃ exposure induces transcriptional markers of preeclampsia in placentas and increases circulating sFLT-1. A–D, Transcriptional changes in placentas from dams exposed to filtered air (FA) or 0.3 ppm O₃ at GD10, with placentas harvested at term. E–H, Transcriptional changes in placentas from dams exposed to FA, 0.3 ppm, or 1.0 ppm O₃ at GD20, with placentas harvested 24 h later at term (n = 4–6/group). I, Soluble Flt-1 is significantly elevated in the serum of dams 10 days after exposure to 0.3 ppm O₃ at GD10 (n = 6 dams/group). J, Serum sFlt-1 was elevated 24 h after exposures to 0.3 and 1.0 ppm O₃ at GD20 compared with controls. Data are presented as mean ± SEM. Asterisks indicate significant difference compared with FA controls by Student's t-test (GD10), or 1-way ANOVA (GD20) with Bonferroni post hoc test (*p < .05; **p < .01).

about 50% over control sFLT-1 levels (Figure 2I). Serum from dams exposed at GD20 showed even more substantial increases in sFlt-1 over control (approximately 250%; Figure 2J) at term. As these immunoblots were conducted on serum, Coomassie staining was used as a loading control comparison, which showed no significant differences between groups.

Ozone-Induced Alterations in Uterine Artery Function

Uterine artery resistance index was assessed by doppler ultrasound, comparing the ratio of systolic to diastolic flow as previously described (Figure 3A; Miller et al., 2017). In a subset of GD10-exposed dams, pre-exposure data were collected (GD8) along with postexposure (GD20), which revealed an anticipated overall reduction in uterine artery resistance index during pregnancy for both control and exposed dams (Figure 3B). However, in GD10 O₃-exposed dams, uterine artery resistance index

exhibited a dampened reduction compared with FA control dams. A more complete collection of cross-sectional data at term reveals a consistent pattern of elevated uterine artery resistance after O₃ exposure relative to control dams (Figure 3C). Dams exposed at GD20, however, exhibited no alterations in uterine artery resistance compared with controls (Figure 3C).

To further assess whether impairments to uterine artery tone were caused by circulating factors, we conducted myographic assays on carotids excised from naïve (unexposed) rats and treated them with serum from GD10-exposed dams that was collected at term. Although carotids incubated with 1% serum (vol/vol) in the physiological saline bath exhibited no differences in vasorelaxation responses to ACh (Figure 4A), it was noted that the overall initial contraction response to the added serum was significantly greater for serum obtained from dams exposed to 0.3 ppm O₃ 10 days prior, as compared with serum

Figure 3. Acute prenatal O₃ exposure impairs uterine artery resistance index. A, Uterine artery index resistance was derived based on peak systolic and trough diastolic flow ratios. B, Uterine artery resistance index decreased in all dams from GD8 to GD20, but this change was diminished in dams exposed to 0.3 ppm O₃ at GD10 ($n = 5$ /group). C, Examining uterine artery resistance cross-sectionally in all dams from the collected GD10 and GD20 exposure studies also revealed increased resistance in GD10-exposed dams compared with all other groups. Data are presented as mean $\pm$ SEM. Asterisks indicate significant difference between FA controls by 2-way repeated measures ANOVA (B) or 1-way ANOVA with a 2-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, which reveals that GD10 O₃-exposed dams have higher resistance index values compared with each other group (C).

from FA-exposed dams (Figure 4B). Uterine arteries from dams exposed to O₃ at GD20, when harvested at term, exhibited enhanced contractile response to phenylephrine (Figure 4C). Lastly, serum from GD20 O₃-exposed dams, obtained at term, led to impairments of vasorelaxation in uterine arteries compared with serum from FA-exposed dams (Figure 4D).

GD10, But Not GD20, Ozone Exposure Alters Cardiac Function

Echocardiography was conducted on dams at GD8, 12, 16, and at term under isoflurane anesthesia. During this procedure, heart rates were monitored and maintained above 350 beats/min by adjusting isoflurane anesthesia (Supplementary Figure S3). Expectedly, cardiac function is dynamic throughout pregnancy, with increasing cardiac output over the course of gestation (Figure 5). With early gestational exposures (GD10), the increase in cardiac output and stroke volume appeared compromised 2 days after exposure and did not fully recover. By GD20, dams exhibited significant reductions in both cardiac output and stroke volume compared with FA controls.

Proteomics conducted on hearts from dams exposed to O₃ at GD10 appeared to confirm a cardiac impact of early-stage gestational exposure. With application of a 5% FDR, numerous significant changes were noted (Figure 6). Volcano plots identified significantly altered left ventricular proteins within the GD10-

exposure dams (77 up and 17 down; Figure 6A). To better explore proteomic networks, we included a broader suite of significantly ($p < .05$) but modestly altered (fold change >0.25). With this approach, we see a pattern of reduced mitochondrial proteins and upregulation of factors potentially involved in response to cardiac impairment (Figs. 6C and 6D). A complete list of affected proteins are provided in Supplementary Table 1.

In contrast, the acute exposure scenario in late gestation (GD20) led to negligible cardiac function impacts compared with control dams. Pregnancy-related trends for ejection fraction, stroke volume, and overall cardiac output were observed similarly in both control and exposed groups (Figure 7). No overall effects of ozone exposure were seen with any echocardiographic endpoint. Consistent with a lack of functional effects, proteomic findings in the left ventricle of dams were less substantive than what was observed in GD10-exposed rats (Figure 8; Supplementary Table 2). A volcano plot (Figure 8A) identified significant left ventricular proteins within the GD20-exposed dams (6 up and 6 down). Even when reducing the fold change limit to 1.25, network analysis revealed very few known protein-protein interactions (Figs. 8C and 8D).

Lastly, phosphoproteomic changes in left ventricular samples were assessed in GD10 and GD20-exposed dams. The data reveal an even more striking distinction between GD10 and

Figure 4. Serum from exposed dams exhibits vasoactivity. A, Serum from GD10 O₃-exposed dams, collected at term, had no effect on carotid vascular dilation to acetylcholine compared with serum from rats exposed to filtered air (FA). B, Direct application of the serum from GD10 O₃-exposed dams, collected at term, induced a significantly greater constriction in carotids than did serum from rats exposed to FA. C, Uterine arteries from GD20-exposed dams exhibited potentiated contractile responses to phenylephrine compared with uterine arteries from FA-exposed dams. D, Serum from GD20 O₃-exposed rats (collected at GD21) impaired vasorelaxation in uterine arteries excised from unexposed dams.

GD20 exposures, with O₃ exposure at GD10 leading to increased phosphorylation of over 70 proteins (Figure 9A; Supplementary Table 3) and only 3 dysregulated phosphoproteins in the GD20 exposure (Figure 9B). Network analysis of significant candidate phosphoproteins revealed a striking alignment with cardiomyopathic and metabolic alterations (Figs. 9C and 9D). Affected phosphoproteins included phospholamban, myosin heavy chain, tropomyosin, and cardiac troponin I. Findings are suggestive of early-stage derangements that may prelude long-term pathology.

DISCUSSION

Air pollution has been associated with incidence of preeclampsia in several epidemiological studies (Dadvand et al., 2013, 2014; Lee et al., 2012; Malmqvist et al., 2013; Wang et al., 2018). In the present study, we provide clear evidence of maternal cardiac impact of midgestational O₃ exposure, which when considered in the context of similar models, suggests that O₃ exposure may impair normal development of the placenta. Using O₃ at

environmentally achievable levels, we found effects on the maternal cardiovascular systems after exposure during placentation (GD10) and manifesting 10 days later at term. Exposures just prior to delivery, at GD20, induced relatively modest maternal cardiovascular effects at term. Based on these findings, we postulate that inhaled O₃ negatively impacts early placental development, leading to placental insufficiency and release of factors such as sFlt-1.

Direct comparisons with recent gestational O₃ exposure studies by Miller et al. (2017, 2019) must be noted, due to the similar design of the O₃ exposures. In those studies, O₃ levels were slightly different than the present study (0.4 and 0.8 ppm vs 0.3 and 1.0 ppm), as was the gestational timing of exposures (GD6 vs GD10 and GD20). However, both studies identified increases in uterine artery resistance index, possibly suggesting some deleterious response from the placentas. In a subsequent O₃ study, Miller et al. (2019) also noted that serum from GD6-exposed dams could inhibit blastocyst implantation using an in vitro model. Circulating factors arising from the lung after inhalation exposures of various pollutants can negatively impact

Figure 5. Maternal cardiac function was altered following O_3 exposure at GD10. Echocardiographic data from various stages of gestation reveal significantly decreased cardiac output and stroke volume in GD10 O_3 -exposed rats. Data are presented as mean $\pm$ SEM. Asterisks indicate significant difference between FA controls by 2-way repeated measures ANOVA for both time and exposure factors, along with post hoc test differences at GD20 ($n = 5 - 6$ /group).

vascular endothelial cells, both in terms of vasodilation and barrier integrity (Aragon *et al.*, 2017; Christophersen *et al.*, 2016; Liberda *et al.*, 2014; Mostovenko *et al.*, 2019; Robertson *et al.*, 2013; Thompson *et al.*, 2019). Pulmonary nanotube exposure revealed the potential complexity of serum molecular modifications, with hundreds of biologically active modified/fragmented peptide sequences arising as cleavage products from endogenous proteins (Mostovenko *et al.*, 2019). The findings from the Kodavanti laboratory and the present study suggest that circulating factors arising from inhaled O_3 may negatively impact multiple key aspects of gestation. Importantly, unlike particulate matter air pollution which may have limited systemic uptake and a potential for direct effects beyond the lung, O_3 is widely understood to be highly reactive and penetrate no further than $0.1 \mu m$ into the lung epithelial lining fluid before reacting with antioxidants (urate, glutathione), phospholipids, and proteins (Postlethwait *et al.*, 1990, 1994; Pryor, 1992). Thus, O_3 has no potential for direct access to the maternal circulation or placental tissues.

Vascular effects of O_3 appeared consistent with previous reports, (Chuang *et al.*, 2009; Paffett *et al.*, 2015; Robertson *et al.*, 2013) especially for the GD20 exposure, where myography was conducted within 24 h of exposure and revealed a hyperconstrictive phenotype and potentially evidence of impaired endothelium-dependent vasodilation. Serum from mice exposed to O_3 at GD10, however, still retained a persistent

proconstrictive effect on carotid arteries from nonexposed rats, despite being collected 10 days after the exposure. Interestingly, this serum did not inhibit endothelium-dependent vasodilation, a common finding for various pollutants in the acute setting. We postulate that the reason for this effect may be that elevated levels of sFlt-1 and potentially other constrictive factors arise from the impacted placenta, rather than the general spillover of acute vasoactive factors from the lung. Several O_3 inhalation studies have previously revealed antidiatory factors carried in the circulation 24 h after exposure in male mice and rats (Paffett *et al.*, 2015; Robertson *et al.*, 2013). The CD36 receptor mediates impairment of endothelium-dependent vasodilation from circulating factors arising following O_3 exposure (Robertson *et al.*, 2013). This persistent vasoconstrictive effect 10 days postexposure was unexpected. We postulate that, rather than spillover from lung response, this circulating vasoconstrictive factor after GD10 exposures is of a placental origin and relates to the multi-organ pathology and potential pathological feedback loop with a placental insufficiency.

Overt cardiac effects of O_3 in dams were modest, with no changes in weight and only slight but significant reductions in cardiac output and stroke volume seen in the late-stage period (GD20) following only the GD10 exposure to 0.3 ppm O_3 . The magnitude and direction of cardiac output and stroke volume changes closely mirrored trends reported for other rat models of preeclampsia, including an angiotensin-infusion model, and

Figure 6. Left ventricular proteomic changes following GD10 ozone (O_3) exposures. A and B, Volcano plot and heatmap for left ventricular proteomic changes in dams exposed to 0.3 ppm O_3 at GD10 compared with FA dams. Samples were obtained 10 days after exposure. C, Protein interaction maps for downregulated proteins in the left ventricular myocardium obtained at GD20, based on proteomic findings of $p < .05$ and considering Fold Change of >1.25 , comparing between dams exposed to 0.3 ppm O_3 and filtered air control at GD10. Blue and red-colored protein symbols indicate downregulated proteins with relevance to mitochondrion (UniProt term: KW-0496; FDR = 0.0234) and mechanotransduction in heart failure (PMID: 25858069; FDR = 0.0276) in the cardiac tissue, respectively. D, Protein interaction maps for upregulated proteins in the left ventricular myocardium, again being more inclusive with $p < .05$ and Fold Change >1.25 . Blue and red-colored protein symbols indicate upregulated proteins with relevance to oxidation-reduction processes (GO: 0055114; FDR = 0.0046) and metabolic pathways (KEGG rno01100; FDR = 0.0082) in the cardiac tissue, respectively.

also for human preeclampsia (Kuźniar et al., 1982). Left ventricular proteomic data from GD10-exposed dams offered an interesting glimpse into what may be considered a “preclinical” response in the myocardium, with induction of numerous proteins related to mitochondrial and metabolic processes. The lack of statistical strength for any individual protein change is tempered by the clear interconnectedness of the protein network, which suggests that although there may be many falsely identified proteins, the broader impact on mitochondrial function—especially in light of echocardiographic changes—may be functionally relevant. Notably, those dams exposed to 0.3 ppm O_3 at GD20 manifested very few changes in myocardial protein

expression 24 h post, and those few proteins revealed limited connectivity in terms of a network. Again, we suspect the GD10 exposures set the stage for a late-term placental response that impairs maternal cardiovascular function, whereas later exposures are unable to meaningfully perturb the fully developed placenta.

On the other hand, phosphoproteomic assessment of cardiac tissue from dams provided a striking picture of the early steps of cardiac response to early gestational O_3 exposure. Phosphorylation of proteins such as phospholamban, cardiac troponin I, tropomyosin, and myosin heavy chain are potentially indicators of a pathological remodeling response.

Figure 7. Maternal cardiac function was not altered following O_3 exposure at GD20. Echocardiographic data from various stages of gestation reveal no significant change in GD20 O_3 -exposed rats. Data are presented as mean $\pm$ SEM. No significant exposure-related effects were identified by a 2-way repeated measures ANOVA ($n = 5 - 6$ /group).

Polymorphisms to several of these proteins (Myh7, Tnni3, Tpm1, Myoz2) are associated with familial cardiomyopathy (Ji *et al.*, 2017). Pathway analysis noted that cardiac adrenergic stimulation may be a component of this response, which is consistent with recent studies showing adrenalectomization can blunt O_3 -induced pulmonary and systemic toxicity (Henriquez *et al.*, 2018). Again, the robust responses seen in hearts from GD10-exposed dams compared with GD20-exposed dams suggest that some indirect pathology underlies this phenomenon. At present, however, it is essential to appreciate the limitations of these findings, as we have not yet explored the functional ramifications for the specific phosphorylation sites indicated by the phosphoproteomic analysis. Repeated studies and greater depth of analysis into this dataset will be essential to fully understand the intracellular alterations in response to early gestational O_3 exposure.

Preeclampsia in humans occurs in the later stages of pregnancy and is typically “cured” by delivery and removal of the culprit placenta (Sones and Davisson, 2016; Than *et al.*, 2008, 2018). Depending on the severity and duration, undetected maternal hypertension can promote a myocardial response that leads to (or exacerbates) hypertrophy and potentially

cardiomyopathy (Parikh and Blauwet, 2018; Sones and Davisson, 2016; Than *et al.*, 2008). Indeed, gestational hypertension is a major risk factor for cardiac abnormalities in women postpartum. The rat model for pregnancy-related cardiovascular outcomes in dams may have substantive weaknesses in terms of human disease. For one, there is limited time for the development/occurrence of hypertension and resultant hypertension-related sequelae, such as myocardial hypertrophy. Specifically, following exposures there would be only 10 days at maximum (assuming a consistent and instantaneous rise in blood pressure), but the surge in sFLT-1 and other factors may not occur until later in gestation. Although we did not examine many interim pathophysiological outcomes between GD10 and GD20, echocardiographic data suggested no thickening of ventricular walls nor major changes to function and maternal heart weights were unchanged. Measures of blood pressure were not conducted in this study. Secondly, there may be a “second hit” required for full expression of gestational hypertension or preeclampsia and inhalation of O_3 is insufficient to cause this as a single factor. Lastly, the large litter sizes of rats may serve as a buffer to impairments of blood flow or placental perfusion. Although we did not see a difference in litter

Figure 8. Left ventricular proteomic changes following GD20 ozone (O₃) exposures. **A** and **B**, Volcano plot and heatmap for left ventricular proteomic changes in dams exposed to 0.3 ppm O₃ at GD20 compared with FA dams. Samples were obtained 24 h after exposure. **C**, Protein interaction maps for downregulated proteins in the left ventricular myocardium obtained at GD20, based on proteomic findings of $p < .05$ and considering Fold Change of >1.25 , comparing between dams exposed to 0.3 ppm O₃ and filtered air (FA) control at GD20. Blue and red-colored protein symbols highlight downregulated proteins with relevance to ubiquitin: Proteasome inhibition (PMID: 21468303; FDR = 0.0073) and collagen formation (reactome pathways, RNO-1474290; FDR = 0.0060) in the cardiac tissue, respectively. **D**, Protein interaction maps for downregulated (left) and upregulated (right) proteins in the left ventricular myocardium obtained at GD21, based on proteomic findings of $p < .05$ and a Fold Change >1.25 , comparing between dams exposed to 0.3 ppm O₃ and FA control at GD20. Blue and red-colored protein symbols in the downregulated figure highlight proteins with relevance to mitochondrion (GO: 0005739, FDR = 0.0019) or oxidation-reduction pathways (GO: 0055114; FDR = 0.0101). Note that for GD20 data, networks represented are not significant and should be interpreted as such.

numbers or pup weights between control and exposed groups, fetuses internally positioned around the uterus could be more readily resorbed owing to circulatory steal from the fetuses positioned closer to the origins of the uterine artery. As such, the pathological window between a healthy placenta, insufficient placenta, and a reabsorbed placenta may be quite narrow compared with humans. Arguably the best regarded model for pre-eclampsia in a rat or mouse is a surgical approach that reduces blood flow through both uterine arteries (Alexander et al., 2001; Intapad et al., 2014). Ozone, we predict, leads to pulmonary release of circulating factors that would similarly impact all placentas, but the time frame of release postexposure or potency of those factors may be insufficient to sustain the growth inhibition.

We chose a relatively low exposure paradigm for rats for both the GD10 and GD20 exposures. Additionally, in the GD20 exposure we included a higher concentration (1.0 ppm) to determine if a more potent maternal cardiovascular effect could be elicited. Importantly, O₃ levels in urban regions around the world can reach 0.3 ppm for brief periods of time—consistent with a 4-h exposure period. Although such excursions may only occur a few times a year in major cities such as Beijing or Mexico City, the number of potentially exposed pregnant women is expected to be quite high (Borja-Aburto et al., 1997; Calderon-Garciduenas et al., 2016; Maji et al., 2019). Rats are also well-recognized as being roughly 3 times less vulnerable to O₃ health effects than primates (human and nonhuman), owing to the greater scrubbing of this oxidant gas in the more complex

Figure 9. Phosphoproteomics reveals significant changes in left ventricular cardiomyocytes in GD10-exposed dams. A, Volcano plot highlighting increases in ventricular phosphorylated proteins in dams at term, 10 days following 0.3 ppm O₃ exposure at GD10 relative to FA-exposed dams (only significant values shown). B, Volcano plot highlighting paucity of ventricular phosphorylation changes in dams at term, 24 h following 0.3 ppm O₃ exposure at GD20 relative to FA-exposed rats (only significant values shown). C, Network analysis of significantly phosphorylated proteins in hearts from GD10-exposed dam reveals known phosphopeptides (blue) as well as proteins involved in cardiomyopathic remodeling (red) and in cardiomyocyte adrenergic signaling (green). D, KEGG pathways from the GD10 phosphoprotein data set reveal cardiomyopathic and metabolic responses.

nares (Hatch et al., 1994; Hotchkiss et al., 1989; Postlethwait et al., 1994; Rao et al., 1985). This would suggest that the maternal outcomes seen in rats might be occurring at levels of O₃ approaching the U.S. National Ambient Air Quality Standard and, as such, would help corroborate epidemiological associations between air pollution and gestational hypertension/preeclampsia. Notably, it is estimated that 107 million individuals in the U.S. inhabit regions that are out of compliance for the current 8-h O₃ (2008) National Ambient Air Quality Standard (EPA Current as of November 30, 2019).

CONCLUSIONS

Exposure to a low level of O₃ in pregnant rats led to maternal circulatory and cardiovascular effects that are consistent with several outcomes in preeclampsia, including increased uterine artery resistance index, placental transcriptional changes, reduced maternal cardiac output, and maternal cardiac phosphoproteomic alterations. Early gestational exposures, at approximately the time of placentation, induced such outcomes, whereas later stage exposure did not, suggesting a vulnerability related to the developing placenta. Further research

will be essential to understanding how such environmental exposures can contribute to the incidence of gestational hypertension and preeclampsia and which subpopulations may be at greatest risk.

COMPLIANCE WITH ETHICAL STANDARDS

Studies were conducted with full approval by the Institutional Animal Care and Use Committees of both the University of New Mexico and Virginia Commonwealth University.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

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DECLARATION OF CONFLICTING OF INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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