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Neonatal rat age, sex and strain modify acute antioxidant response to ozone.

JA Dye^a, EA Gibbs-Flournoy^b, JH Richards^a, J Norwood^a, K Kraft^c, GE Hatch^a

^aNational Health and Environmental Research Laboratory (NHEERL), U.S. Environmental Protection Agency, Research Triangle Park, NC, USA.

^bORISE, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA.

^cChevron Energy Tech. Co, Houston, TX, USA.

Abstract

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the US and its impact continues to increase in women. Oxidant insults during critical periods of early life appear to increase risk of COPD through-out the life course. To better understand susceptibility to early life exposure to oxidant air pollutants we used Fisher (F344), Sprague-Dawley (SD) and Wistar (WIS) male and female neonatal rat pups to assess: (A) if strain (i.e. genetics), sex, or stage of early life development affected baseline lung antioxidant or redox enzyme levels and (B) if these same factors modulated antioxidant responsiveness to acute ozone exposure (1 ppm × 2 h) on post-natal day (PND) 14, 21, or 28. In air-exposed pups from PND14–28, some parameters were unchanged (e.g. uric acid), some decreased (e.g. superoxide dismutase), while others increased (e.g. glutathione recycling enzymes) especially post-weaning. Lung total glutathione levels decreased in F344 and SD pups, but were relatively unchanged in WIS pups. Post-ozone exposure, data suggest that: (1) the youngest (PND14) pups were the most adversely affected; (2) neonatal SD and WIS pups, especially females, were more prone to ozone effects than males of the same age and (3) F344 neonates (females and males) were less susceptible to oxidative lung insult, not unlike F344 adults. Differences in antioxidant levels and responsiveness between sexes and strains and at different periods of development may provide a basis for assessing later life health outcomes – with implications for humans with analogous genetic or dietary-based lung antioxidant deficits.

Keywords

Antioxidants; lung; neonatal rats; ozone

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Introduction

Early life exposure to an adverse environment during critical or sensitive periods of respiratory and immune development may increase risk of developing chronic diseases of various organ systems, including respiratory conditions, throughout the life course (Duijts et al., 2014; Martinez, 2016; Miller & Marty, 2010; Postma et al., 2015). Currently, chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the US, hence more people die from COPD than any other condition except cardiovascular disease and cancer (Blanchette et al., 2012; Xu et al., 2016). COPD is also the third leading cause of death globally — in large part due to exposure to household air pollution generated by rudimentary stoves used for cooking and heating (Burney et al., 2015). Stove emissions disproportionately impact women and children (Gordon et al., 2014).

In developed countries, cigarette smoking remains the primary known cause of COPD (Blanchette et al., 2012). In the US, the impact of COPD in women has continued to increase such that the annual number of COPD-related deaths in females now exceeds that of males (Mehari & Gillum, 2015), with the trend stronger in specific states (Edwards et al., 2005). In females, smoking appears to result in greater lung function decline compared to males and even after adjusting for the amount of smoking, females exhibit greater risk of hospitalization for COPD (Prescott et al., 1997). Females also appear to be at increased risk of developing adult-onset asthma, developing more severe asthma (Melgert et al., 2007) and are more likely to be hospitalized for asthma compared to men (Lin et al., 2013).

Of note, the proportion of females with COPD who lack a history of smoking is 3–4 fold higher than the proportion of males (Terzikhan et al., 2016). Thus, other inhaled exposures such as air pollution may contribute to these trends (Eisner et al., 2010; Liu et al., 2016; Schikowski et al., 2014; To et al., 2016). For example, heavy exposure to traffic-related air pollution is associated with lower forced expiratory lung capacity, especially in women (Carlsen et al., 2015). Increased emergency room visits (Malig et al., 2016) and hospital admissions (Ghanbari Ghazikali et al. 2016) for COPD are associated with chronic exposure to higher concentrations of the oxidant pollutant, ozone. Additional factors contributing to sex differences in COPD prevalence, progression and mortality include genetic predisposition (Raghavan & Jain, 2016), sex steroid signaling (Sathish et al., 2015) and sociocultural factors (Pinkerton et al., 2015).

A large body of literature suggests that the pathogenesis of COPD involves systemic and distal airspace oxidant/anti-oxidant imbalances (Bernardo et al., 2015; Maury et al., 2015; Zinellu et al., 2016) which in some manner accelerate aging processes within the lung (Choudhury & MacNee, 2016). In healthy people, age and sex influence pro/antioxidant status (Kowalska & Milnerowicz, 2016). In the elderly, greater risk of developing COPD is associated with reduced vitamin C (ascorbic acid) and vitamin E (alpha-tocopherol) serum levels (Rodriguez-Rodriguez et al., 2016). Maury et al. (2015) reported that nearly 90% of COPD patients have a systemic antioxidant imbalance based on decreases in serum ascorbic acid, glutathione and glutathione peroxidase — while female COPD patients also show evidence of increased lipid peroxidation.

Definitive linkage of early life insults to later life lung disease is lacking despite plausible biological mechanisms and good evidence that air pollution affects lung development in childhood (Gauderman et al., 2007) as well as COPD exacerbations in adults (Malig et al., 2016). This may be in part due to the intervening 20–40 year period before the maladaptive lung changes (emphysema, chronic bronchitis and bronchiolitis) associated with COPD culminate into clinically apparent disease. Supportive evidence may be obtained from experimental studies in which laboratory animals are exposed to environmental agents throughout their life course. A limited number of chronic inhalation studies exist, including studies in rats utilizing a worse-case urban ozone exposure profile (Costa et al., 1995) and other exposure paradigms (Chang et al., 1992; Harkema & Mauderly 1994; Mattie et al., 1991, 2010; Tepper et al., 1989, 1991; Wiester et al., 1995, 1996). Results indicate that near life-long ozone exposure in rodents results in functional lung stiffening without overt fibrosis (Costa et al., 1995). The limited degree of resultant lung dysfunction is thought to be due to lung functional (e.g. breathing frequency and tidal volume) and biochemical (e.g. glutathione and ascorbic acid) adaption during repeated or more chronic ozone exposure protocols (Tepper et al., 1989; Wiester et al., 1995, 1996).

It should be noted, however, that the majority of these studies did not begin exposures until animals were adults and most studies used only male, Fisher 344 (F344) rats — similar to the National Toxicology Program which used this strain for nearly 50 years as a cancer bioassay model (Maronpot et al., 2016).

To date a paucity of inhalation toxicity studies directly compare effects of ozone across rat strains and fewer still compare effects across sexes. Limited studies in male rats suggest that the F344 strain may be less susceptible to oxidant insults (e.g. following acute exposure to ozone (Bassett et al., 2000; Dye et al., 1999) or to asbestos (Shannahan et al., 2012)). In chronic ozone-exposed F344 rats, lung volume decrements were somewhat more pronounced in females (Harkema & Mauderly, 1994). In male rats of non-F344 strains, differences in lung and airway lining fluid antioxidant levels appeared to modulate ozone-induced damage — with strains having the least antioxidant reserve incurring the greatest lung injury (Dye et al., 2015; Hatch et al., 2015). Lacking are chronic air pollution inhalation studies incorporating relatively susceptible versus resilient rat strains, with both sexes, to better define key factors by which early life insults may promote lung disease later in life.

To this end, the present study examined several of the aforementioned factors using both male and female neonatal rat pups of the F344, SD and WIS strains to assess: (A) if rat strain (i.e. genetics), sex, or stage of early life development affected baseline lung antioxidant and redox-related enzyme levels and (B) if any of these factors modulated antioxidant responsiveness to early life ozone exposure. Immediately post-exposure, changes in lung antioxidants (e.g. total glutathione, ascorbic acid, uric acid, alpha-tocopherol), superoxide dismutase (SOD) and enzyme content/activity related to glutathione recycling were assessed. We hypothesized that F344 pups, like F344 adults, would be relatively resistant to acute ozone exposure. We further postulated that the most immature pups would be the most sensitive, but that sex-based differences would be negligible because these pups had yet to reach sexual maturity.

Materials and methods

Animals

Timed-pregnant female specific pathogen-free Fischer 344 (CDF[F-344]CrIbr), Sprague-Dawley (CrI:CD[SD]Br) and Wistar (CRL:Wistar[WI]Br) rats were purchased from Charles River Laboratories (Raleigh, NC). Animals were housed individually in plastic cages with pine shavings in AAALAC accredited facilities at the USEPA (Research Triangle Park, NC). After birth, neonates were housed with dams until weaning at PND21. Litters were culled and cross-fostered to normalize pup numbers to $\cong 10$ per dam. Due to even lower fecundity of the F344 dams than anticipated, group sizes for exposure of F344 pups were reduced and the PND28 exposure was not performed. All animals were maintained in temperature and humidity controlled rooms with a 12 h light/dark cycle. Dams were fed Purina rodent chow and water ad libitum.

Exposure protocol, equipment and facility

F344, SD and WIS neonatal pups were exposed to either air or 1.0 ppm ozone ($\times 2$ h without their dams) at post-natal day 14 (PND14, pre-weaning), PND21 (weaning), or PND28 (post-weaning). PND14 and PND21 pups were exposed in sets of 10–12 animals per exposure group. Older animals (PND 28) were exposed in sets of 6–8 animals for each exposure. Sets of PND14, PND21 or PND28 animals were randomly derived from multiple litters for each exposure. Exposures for the three age comparisons were performed between the hours of 8 am and 12 pm, Monday–Friday, on a revolving schedule to control for potential diurnal effects. Pups were exposed without dams present, in separated compartments in wire cages which were free of bedding materials. Exposure protocols were preapproved by the US EPA Institutional Animal Care and Use Committee. On each day of exposure, for each strain, pups were selected to allow for nearly equivalent numbers of male and female animals. Prior to the exposures, animals were identified with two tail markings to indicate sex and exposure (air or ozone).

Exposures were carried out at the USEPA's Acute Exposure Facility. Exposures were performed in identical 1.3 m³ stainless steel dynamic exposure chambers with computer interfaced mechanical monitoring and control of chamber airflow rate, temperature, humidity and ozone concentrations. Animals were randomly distributed throughout clean, stainless steel holding cages. Due to their size, PND14 and PND21 animals were exposed in holding cages designed for exposures of mice (holding cage measurements for young rat exposures were 5 $\times$ 5 $\times$ 10 cm). The PND28 animals were exposed in standard wire mesh cages designed for adult rats (11 $\times$ 11 $\times$ 30 cm). Ozone was generated from oxygen using a silent arc discharge ozone generator (model 3V1, Or Research Equipment Co., Phoenix, AZ). The concentrations of ozone were monitored continuously by an ozone monitor (model 8002, Combustion Engineering Inc., Lewisburg, WV). The monitors were calibrated against a Dasibi transfer standard that was referenced on a quarterly basis to a primary ultraviolet ozone standard. Chamber flow rate was 28 liters/minute. The ozone concentration range around the 1 ppm target was $\pm 3\%$. The chamber temperature ranged from 69 to 73 °F and the relative humidity was 40–60% during pre-exposure and exposure periods.

Tissue collection and preparation for analysis

Immediately post-exposure, pups were weighed, anesthetized by intraperitoneal sodium pentobarbital (Abbott Laboratories, Abbott Park, IL) and euthanized by exsanguination (via severance of the abdominal aorta). All lung lobes were removed from the thoracic cavity along with the heart. Individual lung lobes were then dissected and weighed. The right cranial and middle lobes were used to assess SOD and enzymes related to glutathione and energy recycling (i.e. glutathione reductase, glutathione peroxidase, glutathione transferase and glucose-6-phosphate dehydrogenase (G-6-PDH)). The right caudal and accessory lobes were used to assess ascorbic acid, uric acid and total glutathione (representing the combination of both reduced and disulfide forms). The left lung lobe was used to assess alpha-tocopherol.

Lung tissues were homogenized with a Kinematica tissue homogenizer (model PT 10–35, Brinkmann Instruments, Westbury, NY) in 1.15% KCl-50 mM Tris buffer, pH 7.6 (for enzymatic analysis) or in 3.0% perchloric acid (for antioxidant analysis). The homogenates for antioxidant analysis were clarified by centrifugation at 20,000 g for 30 min at 4 °C and supernatants were stored frozen at –80 °C until analysis. For alpha-tocopherol, lung tissues were homogenized in 80% ethanol and extracted using concentrated heptane (Vandewoude et al., 1984).

Enzymatic analysis

After being thawed, lung aliquots used for enzymatic activity determinations were clarified by centrifugation at 12,000 × g for 20 min. Glutathione peroxidase activity was determined from the consumption of NADPH in the presence of tert-butyl hydroperoxide and glutathione reductase activity from the reduction of di-thio (bis) nitrobenzoic acid (Jaskot et al., 1983). Glutathione S-transferase activity was measured as the amount of glutathione conjugate of the glutathione transferase substrate 1-chloro-2, 4-dinitrobenzene, as described previously (Habig et al., 1974; Jaskot et al., 1983). G-6-PDH activity was determined by previously published methods of Lohr & Waller (1974). SOD activity was determined by inhibition of the reduction of pyrogallol (Minami & Yoshikawa, 1979). All enzymatic analysis were adapted for and performed on a COBAS FARA clinical analyzer (Roche Diagnostics, Indianapolis, IN) and all concentrations were normalized to lung wet weight.

Antioxidant analysis

Clarified supernatants of lung tissue homogenates were assayed for ascorbic and uric acid by high-performance liquid chromatography (HPLC; C-18 Bondpack column, Millipore Waters Chromatography, Milford, MA) using amperometric electrochemical detection (Bioanalytical Systems, West Lafayette, IN) (Kutnink et al., 1987). Total glutathione consisting of reduced glutathione + glutathione disulfide, was measured using 5,5'-dithiobis-(2-nitrobenzoic) acid-glutathione disulfide reductase recycling assay (Sigma-Aldrich) on the COBAS FARA clinical analyzer (Anderson, 1985). Lowest detectable levels were estimated using standards and were 0.2 mM for ascorbic and uric acid and 1.0 mM for glutathione. Tissue alpha-tocopherol concentrations were determined by HPLC analysis of the concentrated heptane extract (Vandewoude et al., 1984). Again, end points were normalized to lung wet weight.

Statistical analysis

In air-exposed controls, a one-way analysis of variance (ANOVA) was used to assess group differences across the three strains or across three ages using Holm-Sidak's multiple comparisons test. Where Brown-Forsythe statistics indicated non-homogeneity of variances, data was reanalyzed by Kruskal-Wallis nonparametric ANOVA with Dunn's multiple comparisons test. For comparison of males versus females or air- versus ozone-exposed animals within the same strain, a two-tailed unpaired Student's t test was utilized. If the data were not normally distributed, a two-tailed Mann-Whitney U test was used (GraphPad Prism, version 6.07). Results are depicted as the mean $\pm$ SEM and significance of $p < .05$ is indicated within the figures.

Results

Neonatal rat body and lung weights

During the stages of early life development examined herein, neonatal rats were undergoing rapid growth not only in terms of body weight gain but also increasing activity and corresponding increases in lung capacity, especially post-weaning. Pup weight increased significantly from PND14 to PND28 (≈ 60 gm) with body weights nearly tripling (Figure 1(A)). At the corresponding age, pups of the outbred strains (i.e. SD and WIS) were consistently larger than the inbred F344 strain. The only sex-based difference in body weight occurred in the oldest (PND28) WIS pups, with males (M) being larger than females (F) (Figure 1(A)). Over this same period, owing to increasing lung alveolarization, the increases in lung wet weight increased only 0.4 gm (Figure 1(B)). Hence, lung wet weight (expressed as a % of body weight) decreased significantly (Figure 1(C)). By comparison, in adult female WIS rats weighing 200 gm this parameter value would be only ~ 0.65 (Peters & Boyd, 1966).

Lung antioxidant and enzyme levels in air-exposed neonatal rats

Changes in lung antioxidant concentrations and activity of select enzymes related to glutathione recycling for the air-exposed control pups are depicted by strain (Figure 2). Lung concentrations of ascorbic acid and alpha-tocopherol were highly variable, often below detection and when detectable, showed no consistent trend across age, sex, or strain (data not shown). Whereas lung uric acid concentrations remained relatively constant in all strains (Figure 2(A)), SOD activity decreased from pre-weaning to weaning (32–53%), and then appeared to stabilize post-weaning. Notably, F344 rats had significantly greater SOD activity (34–44% at PND14 and 20% at PND21) than SD or WIS pups (Figure 2(B)). In F344 and SD pups, lung total glutathione decreased similarly to SOD, however in WIS pups, glutathione levels remained constant or mildly increased (20%) at PND28 (Figure 2(C)). The higher glutathione levels in PND28 WIS pups may have reflected the greater increases observed in glutathione recycling enzymes (e.g. glutathione peroxidase, glutathione reductase and G-6-PDH), especially post-weaning (Figure 2(D–F)). The only sex-based differences noted were in PND14 SD pups, with females having a slightly greater glutathione peroxidase and uric acid lung content than SD males (Figure 3 and 4).

Lung antioxidant responses to acute ozone-exposure in neonatal rats

Immediately post-exposure to 1 ppm ozone ($\times 2$ h without their dams), no significant decreases in body weight were observed relative to the air-exposed controls of the corresponding age and strain (data not shown). However, in PND14 pups, the lung wet weight (expressed as % of body weight) of ozone-exposed females appeared to increase (9–15%) over that air-exposed females. SD pups (both female and male) had statistically significant increases (Figure 3(A,B)), consistent with development of pulmonary edema.

Ozone-exposed PND14 SD females also had lower uric acid levels, although levels in air-exposed PND14 females were somewhat higher than air-exposed SD males or other air-exposed females of this age group (Figure 3(C,D)). Furthermore, ozone-exposed SD female and SD male pups showed significant reduction in lung SOD activity, as did WIS females (Figure 3(E,F)). No significant differences were observed for these parameters in ozone-exposed F344 or WIS males (Figure 3(B,D,F)). Likewise, in Figure 4, we show that PND14 ozone-exposed females exhibited 15–27% decreases in total lung glutathione — with significant depletion detected in the WIS females (Figure 4(A)). Total glutathione levels in males were relatively unaffected (Figure 4(B)). These observations corresponded to lower levels of lung glutathione peroxidase (Figure 4(C)), glutathione reductase (Figure 4(E)) and glutathione transferase (data not shown) in SD or WIS females; meanwhile F344 females and males of all strains failed to show significant changes in these parameters (Figure 4(D,F)). Lung G-6-PDH was quite low at PND14 in the F344 and WIS pups (Figure 4(G,H)). Although levels were somewhat more detectable in SD pups, both sexes showed G-6-PDH depletion after ozone exposure. Conversely, levels in F344 females increased post-ozone; while F344 and WIS males were unaffected (Figure 4(G)).

At PND21, all pups were weaned. Post-ozone exposure, no significant increase in % lung/body weight were observed, except in SD female pups (Figure 5(A)). Findings are again suggestive of ozone-induced lung edema in this group. By PND21, ozone exposure was without effect on lung uric acid (Figure 5(C)), SOD (Figure 5(E)), or other indices (data not shown) in the SD females, WIS females, or SD males. By contrast, the F344 females as well as F344 and WIS males exhibited significant increases in lung uric acid levels (Figure 5(C,D)), consistent with development of an acute antioxidant response to ozone. Ozone exposure had no effect on total glutathione or the other parameters evaluated (data not shown).

In PND28, only SD and WIS strain comparisons were possible. There was no evidence that ozone exposure resulted in lung edema in either sex of either strain (data not shown). However, strain-based differences were readily apparent. For example, male and female air-exposed SD pups had lower baseline lung glutathione (Figure 6(A,B)) and glutathione peroxidase levels (Figure 6(C,D)) and post-ozone exposure SD pups were able to increase lung glutathione 33–38%, consistent with the capacity to mount an acute antioxidant response to ozone exposure. By contrast, total glutathione levels in air-exposed WIS controls were seemingly 36% higher (compared to air-exposed SD pups). However, WIS pups did not appear to exhibit an antioxidant response to ozone (Figure 6(A–D)).

Discussion

There is considerable and growing interest in understanding how exposure to an adverse environment during sensitive periods in early life may increase risk of developing chronic lung conditions throughout the life course (Duijts et al., 2014; Martinez, 2016; Postma et al. 2015). The present study was an initial step to investigate whether rat strain differences in early life lung antioxidant response to ozone, a ubiquitous oxidant air pollutant, could be used to investigate differential risk of developing chronic lung disease upon repeated oxidant insult. By comparing neonatal rats of both sexes across three rat strains we show for the first time the relative influences of sex, strain and stage of early life development on acute antioxidant and redox-associated enzyme responses to ozone exposure.

Consistent with their pre-pubertal stage, baseline sex differences in air-controls were minimal. Puberty occurs around PND30 (depending on the strain) as evidenced by vaginal opening in females and preputial separation in males. Strain-based (i.e. genetic) differences in baseline concentrations of several antioxidant parameters were more readily apparent. Notably PND14 F344 pups had significantly greater lung SOD activity and total glutathione content than PND14 SD or WIS pups. Likewise, PND21 F344 neonates showed greater SOD activity than SD or WIS pups. By PND28, the WIS pups not only showed the greatest body weight gain, the male pups were becoming larger than the females. Correspondingly, there were remarkable increases in glutathione recycling enzymes in WIS pups at PND28 and they had greater lung total glutathione levels than SD pups. Our findings in air-exposed control animals are consistent with earlier studies of lung enzymes in SD male rats reporting that physical and biochemical measures of the lung increased with age until around day 60, at which time they tend to stabilize (Elsayed et al., 1982; Mustafa et al., 1985). Likewise, Tyson et al. (1982) reported that glutathione reductase, glutathione peroxidase and G-6-PDH increase in direct relation to age until rats are approximately 60 days of age.

Herein, pups of all strains were exposed at the same PND age in order to compare effects at the same developmental stage. However, WIS pups appeared somewhat more mature than SD pups and clearly SD and WIS neonates were more physically developed than F344 pups at a given PND. Consequently, any strain-based differences observed likely reflect a combination of genetic and developmental (i.e. maturation) influences. From PND14 to 28, lung volumes in these pups would increase in proportion to body weight increases (nearly 3-fold) reflecting increasing alveolarization of the maturing lung. In children, not unlike these young rats, lungs grow partly by neoalveolarization throughout childhood and adolescence (Narayanan et al., 2012). Secondary septation, together with architectural changes to the vascular structure of the alveolar region are key objectives of late lung development (Madurga et al., 2013). In neonatal mice exposed to hyperoxia, mitochondrial oxidative stress resulted in decreased alveolarization and septation (Datta et al., 2015). In infant rhesus monkeys exposed to ozone, lung maturation (i.e. alveolar septation) was delayed (Avdalovic et al., 2012). These findings have important implications, suggesting that developing lungs may have the potential to recover from early life insults. Alternatively, repeated environmental exposures affecting alveolarization processes may affect lung development throughout childhood, and thus potentially throughout the remaining life course.

Lung development occurs in the context of a highly integrated system of glutathione recycling enzymes and co-factors (in concert with enzymes like SOD) that act to protect the lung against oxidant injury (Mustafa, 1990). Maintaining redox balance is a very dynamic process, in particular as ventilation increases to accommodate increases in body size and metabolism (Mustafa et al., 1985; Zinellu et al., 2016). Glutathione is notably the most ubiquitous intracellular antioxidant and serves as a critical redox sensor (Aquilano et al. 2014). The numerous enzymes contributing to maintaining appropriate balance between reduced glutathione (GSH) and its oxidized disulfide form (GSSG) are depicted in the Figure 7 schematic. G-6-PDH, for example, catalyzes the rate-limiting step in the pentose phosphate pathway and produces NADPH to fuel glutathione recycling (Tian et al., 1998). Other water-soluble antioxidants like uric acid and ascorbic acid are potent reducing agents (i.e. electron donors), with over half the antioxidant capacity of human plasma due to uric acid content (Sevanian et al., 1991). By contrast, the fat-soluble antioxidant, alpha-tocopherol, primarily co-locates within cell membranes and lipid-rich molecules including surfactant (Elsayed & Mustafa, 1982). Alpha-tocopherol is relatively unstable and is easily oxidized (Mortensen et al., 2001), which may have contributed to the variable levels detected in the neonatal lungs herein. Ascorbic acid fundamentally acts to regenerate alpha-tocopherol from its pro-oxidant form (generated after interacting with radical species ($\bullet\text{ROO}$)) back to its reduced form (Niki, 1987). Alpha-tocopherol dietary deficiency or reduction in its regeneration, owing to decreased GSH and/or ascorbic acid, can result in diminished ability to scavenge lipid peroxides (Choudhury & MacNee, 2016). Relatedly, based on reduced serum ascorbic acid, alpha-tocopherol and glutathione levels, most COPD patients appear to have systemic antioxidant imbalance — and female COPD patients have evidence of increased lipid peroxidation (Maury et al., 2015; Rodriguez-Rodriguez et al., 2016).

Ozone inhalation may elicit lung injury through oxidation and peroxidation of biomolecules both directly and via radical-based chain reactions (Ciencewicki et al., 2008). Antioxidants like alpha-tocopherol serve to limit cellular injury by breaking the chain reactions. Enzymatic mechanisms (i.e. GSH redox cycle) serve to further eliminate reactive by-products (Mustafa, 1990). As summarized in Table 1, our data showed that following acute exposure to ozone, pre-weanling female SD and/or WIS pups appeared to be depleted of key lung antioxidants and glutathione recycling enzymes, while levels in F344 females and male pups were largely unaffected (although SD males showed variable responses). Sex-related differences in PND14 and PND21 pups (e.g. depletion of glutathione peroxidase) were somewhat unexpected in light of their pre-pubertal status. At weaning, males and F344 females appeared able to generate an antioxidant response (defined as an increase in concentration or enzyme activity); whereas SD and WIS females did not, and again SD males showed variable responses. Post-weaning, male and female SD pups showed similar responses, as did male and female WIS pups. Although PND28 male and female SD pups had lower baseline glutathione levels, they exhibited significant antioxidant responses to ozone exposure. Our results match an earlier report showing that ozone exposure for three consecutive days in immature SD pups (PND 7 and 12) resulted in decreased lung enzyme levels, whereas ozone-exposure in older (PND24) pups and 90 day SD adults resulted in increased levels (Elsayed et al., 1982).

We acknowledge that this initial study had a number of limitations. Firstly, levels of important antioxidants like ascorbic acid or alpha-tocopherol in the neonates were seemingly so low that we were not able to consistently assess changes related to development or oxidant exposure. Secondly, we only evaluated total lung glutathione and therefore were unable to assess alterations in the ratio of GSH to GSSG — potentially a more sensitive indicator of redox change and signaling (Aquilano et al., 2014). Thirdly, comparisons across rat strains with differing fecundity, maternal behavior and susceptibility to stress (Pritchett-Corning et al., 2013; Uchida et al., 2010) proved difficult such that we were unable to assess the PND28 F344 pups within this same study cohort. Nevertheless, prior to air or ozone exposures, equivalent litter sizes were maintained. Fourthly, although our data revealed differential antioxidant response to ozone, we have not provided definitive evidence that the antioxidant responses observed were beneficial in terms of reducing ozone-induced lung injury or inflammation. Supportive evidence comes from the finding that antioxidant/enzyme decreases were primarily observed in pups with evidence of pulmonary edema (i.e. increased relative lung wet weight) in which fluid and/or protein flux occur owing to ozone-induced deep lung injury and increased permeability (Parker & Townsley, 2004). Moreover, owing to fragility of the still developing lungs, in an earlier pilot study we observed that bronchoalveolar lavage fluid (BALF) protein concentrations in air-exposed PND14 WIS neonates was moderately elevated (relative to 60-day-old WIS rats), thus precluding use of BALF protein levels as a metric to assess additional ozone-induced increases (unpublished observations by K. Kraft). However, we did find that relative to 60-day-old WIS rats, ozone-exposed PND14–28 WIS neonates had greater lung cellular toxicity based on increased recovery of sloughed epithelial cells in BALF and decreased viability of recovered alveolar macrophages (unpublished observations by K. Kraft). These preliminary observations are consistent with the report submitted by Vancza et al. (2009) in which several mouse strains (but not all) showed greater numbers of sloughed epithelial cells in lung lavage fluid of neonates compared to adults of the same strain.

Lastly, we acknowledge that ozone dosimetry between adult and neonatal rats may be different owing to differences in metabolism and respiratory rates in younger animals. These pups appeared to incur significant hypothermia (data not shown) during the acute exposure; hence, reduced ventilation. This type of hypothermic response is typical of rodents after acute exposure to toxic xenobiotic agents and is characterized by profound depression of physiological functions such as core temperature, heart rate and metabolism (Watkinson et al., 2003). These observations were supported by a pilot lung labeling study with heavy (18 O-labeled) ozone which indicated that 18 O reaction product concentrations in the lung were four-fold higher in 60-day-old WIS adults compared to WIS PND14 neonates (unpublished observations by Hatch). Taken together, data suggest that despite one-fourth the overall ozone lung dosimetry, neonatal rats incurred disproportionately greater lung injury, consistent with an underdeveloped or immature antioxidant defense early in life. By analogy, it may be relevant that children living near a major roadway that have polymorphisms in glutathione-S-transferases (i.e. GSTT1 null carriers) were reportedly at increased risk of wheeze or asthma in adolescence (Bowatte et al., 2016). GSTM1-null status in combination with other genotype variants (NQO1) and GSTP1 polymorphisms have been linked to decreased lung function in association with chronic exposure to ozone and risk appeared to

be modified by sex-specific factors (Chen et al., 2007). Moreover, individuals exposed to higher levels of air pollution reportedly had three-fold greater odds of developing asthma-chronic obstructive pulmonary disease overlap syndrome (ACOS), a condition associated with poorer quality of life and more rapid lung function decline than either asthma or COPD alone (To et al., 2016).

Conclusions

Antioxidant deficiencies, whether genetic or dietary, may predispose the lung to damage upon exposure to air pollutants and other oxidative stressors. In part because of limitations assessing neonatal humans and because of the long pre-patent period for developing COPD symptoms, it is difficult to disentangle which early life intrinsic factors (e.g. genetics, sex, or life stage) versus environmental influences (e.g. nutritional deficits) and exposures (e.g. oxidant or roadway air pollutants, secondhand smoke/cigarette smoking, aeroallergens) contribute most to later life disease (Brunst et al., 2012). To date, risk assessments on cumulative effects of ozone exposure have been based to some extent on toxicology studies investigating chronic, near-lifelong ozone exposure in adult male F344 rats. Much discussion has since occurred as to whether inbred F344 rats are (or are not) the most appropriate model for toxicology studies compared to outbred Sprague Dawley (SD) or Wistar (WIS) strains (Costa & Kodavanti, 2003; Weber et al., 2011) or disease-prone strains (Dye et al., 2015). The few available studies suggest that F344 rats may be more resistant to oxidant stressors than other strains. Additionally, there is increasing emphasis for animal-based research to incorporate both sexes similar to National Institutes of Health-funded human clinical trials where just over half of the participants are now women (Clayton & Collins, 2014). Importantly, our current findings suggest that strain- and sex-based differences in antioxidant response to ozone are already present early in life; with F344 pups being comparatively resilient and SD and WIS pups (especially females) more susceptible, to ozone. We propose that by comparing effects of repeated or chronic exposure to oxidant air pollutants in male and female rats of resilient and susceptible strains — beginning in early life — we might better define risk in terms of windows of increased vulnerability, sex/hormonal and genetic/epigenetic influences on lifelong pulmonary function decline and find opportunities for more effective dietary or nutraceutical interventions.

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Figure 1.

Trends in (A) body weight, (B) lung wet weight and (C) % lung weight/body weight across early life (PND14, 21 and 28) for the three strains of air-exposed control rat pups (pooled sexes) ($n = 7-16$ pups/group; Mean $\pm$ SE). *Indicates the group is significantly different than F344 pups of the same age. #Indicates WIS pups are significantly different than SD pups of the same age. Line indicates significant difference between age groups of the same strain, or sex differences for the same age and strain. NA: not available.

Figure 2.

Trends in lung antioxidants, enzymes and related indices across early life (PND14, 21 and 28) for three strains of air-exposed control rat pups (pooled sexes). (n = 7–16 pups/group; Mean $\pm$ SE). *Indicates the group is significantly different than F344 pups of the same age. #Indicates pups are significantly different than SD pups of the same age. Lines indicate significant difference between age groups of the same strain. NA: not available.

Figure 3.

Acutely after PND14 (pre-weaning) pups were exposed to air or 1 ppm ozone ($\times 2$ h), differences by sex across three rat strains are depicted (F344 $n = 3-4$ pups/group, SD and WIS $n = 7-8$ pups/group; Mean $\pm$ SE). *Indicates the group is significantly different than air-exposed F344. *With line indicates significant difference between air- and ozone-exposed groups of the same strain. F > M indicates that levels in female pups were significantly greater than males.

Figure 4.

Acutely after PND14 pups were exposed to air or 1 ppm ozone ($\times 2$ h), differences by sex across three rat strains are depicted (F344 $n = 3-4$ pups/group, SD and WIS $n = 7-8$ pups/group; Mean $\pm$ SE). *Indicates the group is significantly different than air-exposed F344. #Indicates that air-exposed WIS pups were significantly different than air-exposed SD pups. *With line indicates significant difference between air- and ozone-exposed groups of the same strain. F > M indicates that levels in female pups were significantly greater than males.

Figure 5.

Acute after PND21 pups (at weaning) were exposed to air or 1 ppm ozone (×2 h), differences by sex across three rat strains are depicted (F344 n = 6–8 pups/group; SD n = 7–8 pups/group; WIS n = 6 pups/group; Mean ± SE). *Indicates the group is significantly different than air-exposed F344. *With line indicates significant difference between air- and ozone-exposed groups of the same strain.

Figure 6.

Acutely after PND28 (post-weaning) pups were exposed to air or 1 ppm ozone ($\times 2$ h), differences by sex across three rat strains are depicted ($n = 6-8$ pups/group; Mean $\pm$ SE). #Indicates air-exposed WIS pups were significantly different than air-exposed SD pups. *With line indicates significant difference between air- and ozone-exposed groups of the same strain. NA: not available.

Figure 7.
Glutathione recycling pathways.

Table 1.

Summary of directional alterations in lung antioxidants and related enzymes in neonatal rats acutely after ozone (1 ppm $\times$ 2 h) exposure at three ages of early life development.

Age	Life stage	Females			Males		
		F344	SD	WIS	F344	SD	WIS
PND14	Pre-weaning	$\Leftrightarrow$	$\Downarrow$	$\Downarrow$	$\Leftrightarrow$	$\Leftrightarrow$ or $\Downarrow$	$\Leftrightarrow$
PND21	Weanlings	$\Uparrow$	$\Leftrightarrow$	$\Leftrightarrow$	$\Uparrow$	$\Leftrightarrow$ or $\Uparrow$	$\Uparrow$
PND28	Post-weaning	NA	$\Uparrow$	$\Leftrightarrow$	NA	$\Uparrow$	$\Leftrightarrow$