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Biochemical Effects of Ozone on Asthma During Postnatal Development

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Abstract

Background—Ozone exposure during early life has the potential to contribute to the development of asthma as well as to exacerbate underlying allergic asthma.

Scope of Review—Developmentally regulated aspects of sensitivity to ozone exposure and downstream biochemical and cellular responses.

Major Conclusions—Developmental differences in antioxidant defense responses, respiratory physiology, and vulnerabilities to cellular injury during particular developmental stages all contribute to disparities in the health effects of ozone exposure between children and adults.

General Significance—Ozone exposure has the capacity to affect multiple aspects of the “effector arc” of airway hyperresponsiveness, ranging from initial epithelial damage and neural excitation to neural reprogramming during infancy.

Introduction

The rising prevalence of asthma among populations in industrialized societies has provoked many investigations of the contributions of air pollutants like ozone [1]. Since ozone can provoke both airway hyperreactivity and “prime” epithelial inflammatory responses, [2] it is a likely contributor to the overall burden of asthma. The role of ozone exposure in the initiation of asthma pathophysiology is controversial, but it certainly acts as an exacerbating factor and chronic exposure may have durable effects on susceptibility to airway hyperreactivity. This review will focus on the possible molecular pathways by which early life ozone exposure could affect susceptibility to the development of asthma and asthma exacerbations, as well as its potential interactions with perinatal priming events and co-exposures. The details of ozone interactions in simplified model systems are considered in detail elsewhere in this issue.

Developmental Regulation of Ozone Effects: Anti-Oxidant Responses

The impact of ozone exposure in the very young will depend on the actual oxidative molecular damage incurred in alveolar and airway epithelium, the initiation and resolution of the consequent inflammatory/injury response, and the consequences of acute or repetitive events during particular windows of developmental plasticity/vulnerability of the entire

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“effector arc” of airway hyperresponsiveness (AHR). The first events in acute ozone exposure include oxidation of components in airway and alveolar lining fluid. In alveolar lining fluid, ozone reacts with surfactant lipids, producing lipid-ozone reaction products (LOP) which are themselves regulators of transcription factors that govern pro-inflammatory cytokine and chemokine expression, as depicted in Figure 1. These events are reviewed in detail elsewhere in this issue, but it should be pointed out that most studies of acute ozone exposure aimed at disentangling these multiple interacting contributors have been conducted using relatively brief rather than chronic exposures. The actual delivered ozone dose in children is likely to be greater for a given concentration since they have relatively higher minute ventilation and spend more time outdoors. In rats, ozone does not suppress ventilatory response in the 2 week aged juvenile rats as it does in mature animals [3]. However, the relatively greater inducibility of antioxidant enzyme systems, at least in the very young, may partly offset higher oxidative stressor exposure [4] [5]. Two week old juvenile mice exposed to ozone demonstrated less evidence of lung injury than adult counterparts of the same genetic strain [6] and develop less AHR than adult mice [7]. However, the resistance that young mice demonstrate is not necessarily found in other species. In contrast to induction of antioxidant enzymes observed following hyperoxia (see ref# [8] for review), juvenile (postnatal day 21) rats exposed to 0.5 ppm O₃ for 12h/day for one week demonstrated no induction of superoxide dismutase, catalase, or glutathione peroxidase activities, although they suffered greater DNA damage assessed as 8-oxo-7,8-dihydro 2'-deoxyguanosine accumulation [9].

The inducibility of antioxidant defenses in response to oxidative stress in the young has been studied in more detail during the immediate postnatal and neonatal period in a number of species, but much less thoroughly in late infancy and early childhood, the stages of human development during which ozone exposure would be expected to increase. Although recent studies have implicated oxidant/antioxidant gene polymorphisms as ozone susceptibility factors (reviewed by [10]), the developmental regulation of these enzymes has not been studied in detail. Two examples are polymorphisms for glutathione S-transferase mu 1 (GSTM1, EC 2.5.1.18) and NAD(P)H quinone oxidoreductase (NQO1, E.C. 1.6.99.2). The developmental expression of GSTM1 activity in humans differs depending on the organ, [11] and GST isoforms demonstrate heterogeneous expression within the lung [12]. Lack of GSTM1 activity in children in Mexico City was associated with worse ozone-related decrements in pulmonary spirometry that were attenuated in those receiving antioxidant supplementation [13]. NAD(P)H:quinone oxidoreductase (NQO1) has been less well studied, but *NQO1* gene polymorphisms appear to confer some protection against acute ozone effects on epithelial injury and AHR [14]. Adult mice lacking NQO1 (*Nqo1*^{-/-}) are relatively resistant to acute ozone provoked inflammation and AHR [15]. Preliminary data from our laboratory suggests that lack of NQO1 during neonatal ozone exposure also protects against the development of AHR [16]. Further information about age and organ-specific expression and inducibility of these enzyme systems could illuminate the mechanisms underlying developmental vulnerability to ozone exposure.

Ozone exposure & inflammation: infancy→ childhood

Oxidative stress and inflammation interact in response to ozone exposure, and are pivotal to the pathogenesis of asthma. Experimental efforts to estimate the relative contributions of the relevant pathways have employed genetic approaches aimed at candidate pathways. Ozone effects on acute epithelial permeability and on inflammatory responses that follow and affect AHR will hinge on host antioxidant responses and regulation of both initiation and termination of inflammatory responses, both of which are developmentally regulated. The interaction of these pathways to confer susceptibility to short and long-term ozone induced AHR and pulmonary inflammation has been explored indirectly using inbred adult mouse

strains in adult mouse [17], and more recently in newborn mice [6] that demonstrated similar strain-dependence of ozone-induced lung inflammation, with the notable exception that C3HeJ pups—with defective TLR4 signaling—demonstrated greater PMN influx than adults and some other strains. Detailed consideration of ozone exposure and the innate immune system is considered elsewhere in this issue, but even in juvenile mice at postnatal day 4, ozone provoked induction of transcription factors was found to be dependent on TLR4 signaling [18].

Since the mechanistic studies of ozone effects have been conducted in animal models, it is important to take account of the differences in the patterns of pre- and post-natal lung development [19] and specific attributes of lung structure [20] when interpreting the studies. Mice and rats, for example, are born when lungs are at the sacular stage of lung development, whereas macaques and humans are born during the alveolar stage of lung development. Since progenitor cells important to lung homeostasis and repair may also be vulnerable to oxidative stress, ozone exposures at these vulnerable stages may have differing effects on later susceptibility to antigen or pollutant challenge. Inter-species differences in the maturation of immunologic responses are also important to consider when interpreting animal model systems.

In general, ozone exposure induces less lung inflammation in mouse pups (<2 week old) than in adult mice, [21] [7] consistent with generally greater inducibility of anti-oxidant enzymes that oppose formation of lipid-ozone products (LOPs) [22]. These patterns of maturational sensitivity may change with exposures to multiple provocations that are present in the environment.

Despite lower initial inflammatory provocation by acute ozone among neonatal and juvenile animals compared with adults, ozone exposure during early life has been proposed as an agent that can contribute to pulmonary inflammatory responses and the development of asthma [23]. Airborne pollutants have been linked in some epidemiologic studies and experimental studies to skewing of T-lymphocytic responses towards the T-helper 2 (T_H2) pattern associated with allergic asthma [24]. However there are no direct experimental studies that link ozone exposure with initial ‘polarization’ of $T_H1 \rightarrow T_H2$ lymphocytic responses. On the other hand, natural killer T cells appear to be required for semi-acute (5d) ozone-induced AHR in adult mice.

An important aspect of ozone-induced AHR in the context of childhood asthma is the apparent interaction with antigen sensitization. Macaques exposed to ozone and house dust mite antigen during infancy demonstrated increased abundance of activated T-lymphocytes (CD8+/CD25+) in the airway mucosa, with ozone exposure shifting the increased abundance towards more proximal airways [25]. These effects do not appear to be correlated with large changes in resident mast cell populations in macaque airways [26], since antigen exposure increased mast cell abundance, while the addition of ozone exposure to antigen exposure attenuated this response.

In most model systems, ozone seems to serve as an adjuvant, increasing the phenotypic lung mechanical and inflammatory responses during antigen challenge, rather than during antigen presentation [10]. The interactions between ozone-provoked inflammatory responses and subsequent development of asthma phenotypes are complex, and likely depend on highly individualized host responses. Children that have diminished ability to be sensitized, for example those with diminished IgE responses—*e.g.*, Fc ϵ R1 functional polymorphisms—have less frequent wheezing with ozone exposure [27]. Most the information linking ozone exposure with early childhood sensitization in the context of childhood asthma is based on very limited computational estimations of exposure [28].

Oxidative stress interaction with nitric oxide (NO) metabolism and childhood asthma

The mechanisms by which ozone could affect the risk for childhood asthma must also include interactions with NO signaling. Regulation of oxidative stress in the context of ozone exposure may have effects through interaction of ROS with endogenous NO. NO and its reaction products (*e.g.*, S-nitrosothiols) are required for airway smooth muscle relaxation (see review by Erzurum in this issue) and reduced degradation of S-nitrosogluathione ablates AHR in allergen challenged mice [29]. Endogenous NO appears to be linked to multiple mechanisms in asthma pathogenesis, but understanding its contributions has been plagued by inconsistent results in animal model systems [30]. The role of NO in asthma pathogenesis associated with ozone exposure in children will likely depend on the developmental differences in physiology and antioxidant defenses discussed above. There are fewer published studies linking exhaled airway NO (eNO) concentrations with disease states in children than in adults, but in general disease states dominated by impaired epithelial function—*e.g.*, primary ciliary dyskinesia [31]—will exhibit low eNO while inflammation such as asthma exacerbations typically exhibit higher levels [32].

Nevertheless, interest in the interaction of NO and asthma pathophysiology in children remains strong because of a number of NO metabolism gene association studies. For example, children who lack GSTM1 (*GSTM1* $-/-$) and possess inducible NO synthase (*NOS2A* promoter region) polymorphisms are at higher risk to develop asthma [33]. NO production and NOS activity depend on arginine metabolism by arginase [34], and decreased arginine bioavailability, as well as increased arginase activity in serum is associated with asthma [35]. The association of altered arginase activity with asthma risk is also suggested by the prevalence of arginase gene polymorphisms for *ARG1* and *ARG2* with childhood asthma, which appears to be enhanced by ozone exposure [36]. To date, these gene polymorphism studies represent only associations, since functional studies on human gene transcription/translation have not yet been published. Direct inhibition of arginase in murine allergic asthma models reduces AHR and airway inflammation [37], as does L-arginine supplementation [38].

Ozone and childhood asthma: never alone

Airborne pollutant exposures during childhood are predominantly to mixtures. For example, gas-phase co-pollutants can exert their own direct effects on the respiratory tract; however, they may also enhance the deposition fraction for solid-phase air pollutants (fine and coarse particles) and localize and intensify injury to epithelial surfaces [39]. Short term effects of particulate and photochemical air pollution in asthmatic children from representative urban city centers in Europe and North America have been associated with several health outcomes, including asthma attacks, symptoms of nocturnal cough, and changes in lung function [40–45]. In addition, other air quality criteria pollutants, such as carbon monoxide, nitrogen dioxide, and sulfur dioxide have also been linked to asthma aggravation [46–48].

Long-standing efforts to disentangle the relative contributions of individual pollutant species are inevitably fraught with the challenges of balancing the high relevance and low precision of epidemiologic studies with the converse trade off in experimental human and animal studies. The severity (dose), chronicity, and developmental window of vulnerability to ozone exposure, as well as the nature of co-exposures in experimental models will produce differing phenotypes, some of which are summarized in Table 1. The addition of mixed or sequential pollutant exposure to experimental models has become commonplace for *in vitro* studies and acute adult exposures, but has not been as fully developed in the study of the role of ozone and other co-exposures or pre-natal exposures on the development of asthma.

Furthermore, most animal experiments have relied on components of air pollutants as surrogates, since they have been designed as classical toxicologic studies.

As experimental studies have become more sophisticated, threshold effects and synergies have become apparent that were previously unappreciated. For example, the “resistance” of young animals to acute ozone effects on lung inflammation disappears when it follows a priming event such as LPS exposure. The converse was also shown, with ozone serving to augment sensitivity to LPS in newborn mice [49]. Priming events that enhance susceptibility to later environmental challenges may also take place antenatally, such as maternal pulmonary exposure to air pollutants. Fedulov and colleagues found that diesel particles or carbon black instillation in pregnant mice could lower the threshold for ovalbumin sensitization in offspring [50]. Using a similar approach, we found that tracheal instillation of an industrial particulate matter (NIST SRM#1648) to pregnant mice induced inflammatory cytokines in placenta and lung in the fetus, and augmented chronic ozone induced AHR in offspring [51]. Most of the epidemiologic studies linking ozone exposure with childhood asthma have focused on exacerbations or acute effects [1]. However, recent cross-sectional studies suggest that chronic ozone exposure is associated with the development of childhood asthma [52], which has prompted the revision of experimental models accordingly. Recent preliminary studies in our laboratory confirm that maternal exposure to inhaled fresh diesel exhaust [53] worsens chronic ozone exposure induced AHR in offspring.

Ozone Exposure Effects in Early Life: Neural Plasticity

Much of the recent experimental and investigational emphasis has been on the immunologic responses that undergird allergic asthma. However, the “effector arc” for ozone-induced airway hyperresponsiveness (AHR) involves airway sensory nerve responses, possibly via transient receptor potential channels [54]. In newborn rats, early ozone exposure (postnatal days 2–6) but not late exposure (days 19–23) increased airway sensory nerves, suggesting a particular window of neural developmental plasticity [55]. Furthermore, the early exposure increased susceptibility to re-exposure induced AHR during adulthood. Chronic intermittent ozone exposure in early life in animal models increased the abundance of airway sensory nerves in Rhesus monkeys, as well as potentiating the effect of chronic allergen exposure on nerve distribution [56] [57]. Early effects on innervation could thus increase susceptibility to later ozone exposure or to other airway irritants.

Although the majority of studies on the contribution of ozone to neuronal development have been on airway afferent innervation, it should be understood that the central nervous system also plays a role in asthma exacerbations in humans [58] and may contribute to asthma ontogeny. Rats born to dams exposed to chronic ozone administration during pregnancy exhibited neurobehavioral changes, [59] as well as abnormalities of neurotransmitter precursors in the brain measured at birth or during the first postnatal week [60]. Recent studies suggest that prenatal ozone exposure has long-lasting effects on central nervous system plasticity [61]. Rhesus monkeys exposed to chronic ozone during infancy exhibited decreased responsiveness of neurons in the nucleus tractus solitarius [62]. Surprisingly, the effects of postnatal ozone exposure on central nervous system structure and function have not been studied in detail.

The potential effects of ozone on behavior during critical periods must be considered when interpreting both animal and human studies of ozone effects during development. Studies that separate rodent dams from suckling pups have significant effects on the hypothalamic-pituitary axis that could potentially confound airway immunologic interactions important to ozone exposure effects [63]. Indirect effects could also include impairment of postnatal weight gain or decreased maternal-pup transfer of immunologic mediators via suckling

[64,65]. In human studies of chronic ozone exposure among children it is important to consider the indirect effects as well. Human maternal depression, linked with childhood asthma susceptibility in offspring [66], is also influenced by impoverished residential conditions [67] more likely to have proximity to air pollutant-rich environments such as roadways.

Ozone exposure during infancy and structural changes in the lung

Because of the obvious immediate effects of ozone exposure on respiratory function, and the association with asthma exacerbations, chronic ozone exposure would be expected to adversely affect lung structural development. Ozone exposure has been linked with decrements in lung function in children [68], but the pathophysiology in this age group has not been studied in detail.

To address the gap in knowledge about developmental effects, many of the studies in recent years have used rodents as experimental models (Table 1). There are inherent advantages and liabilities of altricial species such as mice as suitable models of human asthma that have been reviewed in detail [69,70]. Mice exposed to chronic intermittent ozone beginning at birth for 1 month did not have significant changes in airway or alveolar structure despite demonstrating increased AHR in response to nebulized methacholine [51], but in our preliminary studies were shown to exhibit long-term AHR at age 8 weeks [53]. Rats exposed as juveniles, near weaning, at postnatal day 20, to acute ozone do not demonstrate significant structural changes [71].

Studies of neonatal and juvenile ozone exposure in precocial species with more extensive alveolar development are more limited. It should be emphasized that the interpretation of the physiological observations in animal studies must be informed by a thorough understanding of the species-related interrelations between structure and function [72]. As with rodents, puppies (6 weeks) [73] and lambs (2 weeks) [74] exposed to < 2 ppm ozone for 5 days do not develop significant structural changes.

A series of detailed morphological analyses using Rhesus macaques exposed during the first six months of life to chronic ozone have revealed a complex of relatively subtle changes that, taken together, could contribute to AHR (see ref# [57] for review). The significant advantages as well as the limitations of non-human primate models are reviewed elsewhere [75]. We have summarized some of the findings in this model system in Table 1. As in the mouse studies, chronic ozone exposure during infancy produced little evidence of airway smooth muscle hypertrophy, and only modest changes in airway muscle fiber arrangement unless macaques were also exposed to house dust mite antigen. As with the majority of chronic experimental exposure studies, ozone exposure of infant macaques at environmentally relevant ozone concentrations augmented the antigen exposure-induced structural changes that contributed to experimental asthma [76].

Ozone exposure during early life and airway homeostasis/repair

Oxidative stress in the late saccular to alveolar stages of lung development can have significant effects on alveolar development and repair responses in adults that could contribute to airway stability. Relatively brief exposures to hyperoxia (postnatal days 1–4) impair alveolar development in adult mice and increase their vulnerability to influenza-induced pneumonitis [77]. This susceptibility has been suggested to depend on loss of alveolar repair capacity, possibly through effects on alveolar progenitor cells. The source of these cells has not been identified but does not appear to include terminal bronchiolar epithelium [78]. Recent studies suggest that airway epithelial progenitor cell populations in adult mice exhibit different proliferation capacities depending on the insult (ozone, SO₂,

naphthalene) [79]. Whether ozone exposure can also have effects on long-term repair capacity in the airway or alveoli is unclear.

Summary

Ozone exposure during postnatal development may serve as a priming event that enhances the toxicity of other pollutants or allergens. Its impact likely hinges on the local antioxidant defenses, which vary depending on age, and which may be modifiable with antioxidant supplementation. Ozone effects on innate and adaptive immunologic pathways relevant to asthma have been observed in animal models, but the relevance to human asthma is not yet clear. The best evidence from non-human primates suggests that eventual development of asthma in children may also be attributable in part to remodeling of afferent airway nerves and subtle effects on airway structure and function. These changes may have implications for life-long vulnerability to other oxidative and allergic challenges that produce clinical asthma.

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Figure 1.

Ozone (O₃) reacts with surfactant phospholipids and cellular phospholipids in alveoli to form lipid ozonation products (LOP) that include lipid peroxides, 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn glycerol-3-phosphocholine that can stimulate IL-8 release [80], 1-Hydroxy-1-hydroperoxynonane, which activates NF-IL-6 and NF-κB upstream of IL-8 in BEAS-2B cells [81], and others that are capable of inducing local injury and inflammation.

Table 1

List comparing results from ozone exposures in selected studies in newborn/juvenile animals.

Reference #	year	species	age	exposure regimen	duration	finding
[69]	1978	rat	P20	0.85 ppm × 3d	acute	minimal morphologic change at terminal airways
[71]	1986	dog	6 wk	1–2 ppm, 4h/d × 5d	semi-acute	small effects on alveolar development
[72]	1986	sheep	2 wk	1 ppm, 4h/day × 5d	semi-acute	delayed involution of mucus cells in trachea
[3]	2000	rat	2–12 wk	2 ppm × 4h	acute	O ₃ reduces ventilation: young < mature
[7]	2004	mouse	2–12 wk	0.3–3.0 ppm × 3h	acute	O ₃ induces AHR: young < mature
[56]	2004	macaque	infant	0.5 ppm × 8h × 5d × 11 cycles	chronic	remodeled neural comp of EMTU
[21]	2004	mouse	P2–P56	2 ppm × 4h	acute	varied responses
[49]	2006	mouse	P4	2.5 × 4h	acute	O ₃ induction of c-jun/c-fos TLR4- dependent
[56]	2006	macaque	infant	0.5 ppm × 8h × 5d × 11 cycles	chronic	abnormal airway structure
[57]	2007	macaque	infant	“ “ “ “ “	chronic	O ₃ +allergen→↑airway sensory nn.
[25]	2009	macaque	infant	“ “ “ “ “	chronic	CD25 cells airway prevalence altered
[51]	2009	mouse	neonatal P1–P28	1 ppm 4h/d, 3d/wk × 4	chronic	maternal PM “primes” neonatal O ₃ -induced AHR
[55]	2010	rat	neonatal v. juvenile	“prime” at P2–6 v. P19–23 + re-challenge P28	acute	neonatal exposure→↑airway sensory nn.
[26]	2010	macaque	infant	0.5 ppm × 8h × 5d × 11 cycles	chronic	O ₃ blocked allergen→↑tracheal mast cells