Postnatal Exposure History and Airways Oxidant Stress Responses in Airway Explants

Shannon R. Murphy¹, Edward S. Schelegle^{2,3}, Patricia C. Edwards¹, Lisa A. Miller^{2,3}, Dallas M. Hyde^{2,3}, and Laura S. Van Winkle^{1,2}

¹Center for Health and the Environment, University of California Davis; ²Vet Med: Department of Anatomy, Physiology and Cell Biology, University of California Davis; and ³California National Primate Research Center, University of California Davis, Davis, California

Postnatally, the lung continues to grow and differentiate while interacting with the environment. Exposure to ozone (O₃) and allergens during postnatal lung development alters structural elements of conducting airways, including innervation and neurokinin abundance. These changes have been linked with development of asthma in a rhesus monkey model. We hypothesized that O₃ exposure resets the ability of the airways to respond to oxidant stress and that this is mediated by changes in the neurokinin-1 receptor (NK-1R). Infant rhesus monkeys received episodic exposure to O₃ biweekly with or without house dust mite antigen (HDMA) from 6 to 12 months of age. Age-matched monkeys were exposed to filtered air (FA). Microdissected airway explants from midlevel airways (intrapulmonary generations 5–8) for four to six animals in each of four groups (FA, O₃, HDMA, and HDMA+O₃) were tested for NK-1R gene responses to acute oxidant stress using exposure to hydrogen peroxide (1.2 mM), a lipid ozonide (10 μM), or sham treatment for 4 hours *in vitro*. Airway responses were measured using real-time quantitative RT-PCR of NK-1R and IL-8 gene expression. Basal NK-1R gene expression levels were not different between the exposure groups. Treatment with ozonide or hydrogen peroxide did not change NK-1R gene expression in animals exposed to FA, HDMA, or HDMA+O₃. However, treatment *in vitro* with lipid ozonide significantly increased NK-1R gene expression in explants from O₃-exposed animals. We conclude that a history of prior O₃ exposure resets the steady state of the airways to increase the NK-1R response to subsequent acute oxidant stresses.

Keywords: bronchial epithelium; lung; Substance P; Nur77

Asthma is a chronic disease affecting more than 9 million children in the United States (1). Asthma symptoms are exacerbated by air pollutants, including ozone (O₃), and children are particularly vulnerable (2). Epidemiologic studies have linked childhood exposure to O₃ with increased incidence of asthma in exercising children (3) and decreased small airways function in adolescents (4). Cyclic exposure to O₃ in an animal model of postnatal development that is similar to humans, the rhesus monkey, demonstrated that O₃ exposure remodels the conducting airways, resulting in hyperplastic airway epithelium and changes in smooth muscle bundle orientation (5). An allergic model of childhood asthma, also in rhesus monkeys, found that exposure to O₃ and house dust mite antigen (HDMA) during early postnatal development led to

CLINICAL RELEVANCE

This work demonstrates that postnatal exposure to ozone alters neurokinin-1 receptor (NK-1R) expression and resets the steady state of the intrapulmonary airways to favor increased NK-1R responses to a short-term oxidant challenge. This response is intrinsic to the airway environment because it is maintained *ex vivo* in nonhuman primate airway explants. This is relevant to air pollution health effects in children, particularly asthma, because exposure to oxidant pollutants exacerbates asthma and the NK-1R changes we describe may be mediators in this process.

altered epithelial afferent and efferent nerve–tissue interactions, including a transient but substantial decrease in nerve density and distribution and an increase in neuroendocrine-like cells (6). This decrease reverses when exposure is followed by several months of recovery in filtered air (FA), resulting in a robust surge in epithelial nerve density and neuroendocrine-like cells relative to control animals (7). This study investigates these changes with respect to the role that neuropeptides play in altering the cycle of injury, inflammation, and repair in an allergic asthma model. A better understanding of how postnatal oxidant and aeroallergen exposure influence neuropeptide mediated mechanisms and cell signaling is crucial to assessing lung injury and repair mechanisms in the context of development. Selgrade and colleagues have suggested that children and adults with asthma react differently to inhalation of allergens and oxidative pollutants as a result of differences in the lung during early developmental versus mature life stages (8), yet we have little understanding of how this occurs, particularly in an atopic individual. Ozone and HDMA are two of the most common inhaled pulmonary irritants. Studying their effects during early postnatal development is relevant to airway remodeling and asthma exacerbations in children.

In the current study, we used a sensitized monkey model of childhood asthma to investigate the relationship of previous *in vivo* episodic inhalation exposure to O₃ and/or HDMA with responses to *in vitro* oxidant challenge in airway explant culture. The asthma/allergic phenotype in this model is characterized by cyclic airway remodeling including injury, inflammation, and repair (9). Air pollution is often associated with respiratory disease. Elevated O₃ levels are a trigger for asthma exacerbations, especially in children (10). Prolonged acute O₃ exposure in atopic individuals has been shown to induce eosinophilic airway inflammation, again linking an oxidant stressor and an antigen-mediated immune response (11). One goal of our current study is to define, in the atopic animal, the relative contribution of O₃ alone and of O₃ combined with allergen to oxidant stress-induced airway responses, specifically the O₃–aeroallergen interaction.

O₃ is a pervasive photochemical oxidant air pollutant. Cyclic epithelial injury and repair after O₃ exposure is associated with necrotic or programmed cell death in Wistar rats (12, 13). Cell

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Correspondence and requests for reprints should be addressed to Laura S. Van Winkle, Ph.D., DABT, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, CA 95616-8732. E-mail: lsvanwinkle@ucdavis.edu

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death can be mediated in part by activation of neurokinin receptor-1 (NK-1R) through binding of its ligand, Substance P (SP) (14). O₃-induced NK-1R activation leads to epithelial expression of intracellular nuclear transcription factor and orphan receptor Nur77 in rats (12). Nur77 is regulated by a mitogen-activated protein kinase (MAPK) pathway, and its activation initiates a cell death cascade. *In vitro* work in rat neurons using MAPK pathway inhibitors that block SP-initiated cell death suggests that NK-1R-mediated nonapoptotic cell death is modulated by Nur77 (15). Independent of programmed cell death, epithelial cell necrosis is commonplace with acute O₃ exposure, particularly in ciliated cells. This death process results in the release of inflammatory mediators that serve a variety of roles, including mast cell and leukocyte recruitment. Chemokines such as IL-8 (CXCL8) attract neutrophils and eosinophils to inflammation sites (16). IL-8, in particular, is a proinflammatory cytokine that, when expressed in the airway epithelium, serves as an indicator of cellular inflammation (17). Chang and colleagues observed that IL-8 is one of the primary chemokines produced in the airway epithelia of adult rhesus monkeys after acute O₃ exposure and facilitates neutrophil influx after O₃-induced epithelial injury (9).

Allergic asthma comprises an elaborate spectrum of symptoms and physiological processes. This continuum coupled with the intricate nature of the O₃-aeroallergen interaction compounds the complexity of how these exposures affect atopic individuals. The central hypothesis of this study is that a history of O₃ exposure resets the ability of the airways to respond to oxidant stress and that this is mediated by changes intrinsic to the airway epithelial mesenchymal trophic unit itself (18). These responses may be mediated by changes in NK-1R. Additionally, we surmise that coexposure with HDMA may antagonize or exacerbate these changes in airway neurokinin gene and protein responses. We tested this hypothesis using a combined *in vivo* and *in vitro* approach by adding oxidants to airway explants *in vitro* and by examination of airway expression of NK-1R in tissue from HDMA- and/or O₃-exposed rhesus monkeys. Our objectives for this study were to determine if a history of *in vivo* oxidant exposure in the presence or absence of HDMA results in increased NK-1R gene expression and decreased proinflammatory IL-8 expression from airway explant cultures subjected to oxidant challenge *in vitro*.

MATERIALS AND METHODS

Animals and Exposure Protocol

Male infant rhesus monkeys (6 mo old) were exposed to cyclic episodes of filtered air, O₃, and/or house dust mite (*Dermatophagoides pteronyssinus*) allergen. Exposures are similar to those previously described (19). At 6 months of age, monkeys were randomly assigned to receive 11 cycles of FA, allergen (HDMA) (2 h/d, 3 d on and 11 days off), O₃ (0.5 ppm for 8 h/d, 5 d on and 9 d off), or HDMA+O₃ (allergen exposure on last 3 d of O₃ exposure) (Figure 1). Animals were killed at 12 months of age (19), and necropsies were performed 3 to 5 days after the last HDMA/HDMA+O₃ exposure. Additional experimental details are provided in the online supplement.

Airway Microdissection and Explant Culture

Lungs were inflated with DMEM (Nutrient Mixture F-12 Ham media; Sigma, St. Louis, MO) and microdissected on ice. Midlevel airway pieces approximately 2 mm thick containing intrapulmonary generations approximately 5 through 8 were removed from the surrounding parenchyma and subjected to oxidant challenge in explant culture. Airway explants from four to six animals per treatment group were placed on 0.4-μm Costar Transwell membrane inserts (Millipore, Billerica, MA) (20). Airways were exposed to 1.2 mM 30% hydrogen peroxide (Fisher Scientific, Waltham, MA) (21), 10 μM lipid ozonide

(1-palmitoyl-2-(9'-oxononanoyl)-sn-glycero-3-phosphocholine [PC-ALD]), (870605P; Avanti Polar Lipids, Alabaster, AL) (22, 23), or sham treatment (vehicle solution: 10% chloroform [vol/vol] + 90% methanol [vol/vol]) for 4 hours *in vitro* at 37°C. Subsets of each treatment group were treated with 10 μM SP (Fisher) (24) in the presence or absence of 10 μM L119 (Sigma) (25), an NK-1R antagonist (Table 1).

RT-PCR

NK-1R and IL-8 gene expression responses were measured using quantitative RT-PCR in microdissected airway pieces as previously described (26). Samples were run in triplicate with RPL13A as the internal reference gene (sequence: 5' primer CACGACGTTGGCTGGAAGT, 3' primer TCTTTCCTCTTCTCTCCAAGGT, and probe CCAGGCA-GTGACAGC) (27). The NK-1R and IL-8 reactions used Taqman inventoried probe/primer assays (CAT# Hs00199894_m1 and Hs00174103_m1, respectively).

Immunohistochemistry and Histologic Staining

Tissue was fixed in 1% paraformaldehyde, embedded in paraffin, and sectioned. Paraffin sections from the left caudal lobe of approximately four animals per treatment group were immunostained for NK-1R (purified goat polyclonal antibody NK-1R [N-19] sc-5218; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or Nur77 (purified goat polyclonal antibody Nur77 [N-19] sc-7014; Santa Cruz Biotechnology, Inc.). An antigen retrieval buffer (AR-10; BioGenex, Fremont, CA) and decloaking chamber (BioCare Medical, Concord, CA) were used for better epitope retrieval. Sections from all groups were run together for each antibody as previously described to minimize run-to-run variability (28).

Statistical Analysis

Data ($n = 4$ –6 per group) are expressed as mean $\pm$ SEM and were analyzed using a split-plot design analysis, with the animal as the experimental unit (whole-animal treatments as the main plot effects and explant treatments as subplot effects). Pairwise *post hoc* comparisons among the least squares means were done and are presented as least significant difference values. Normality and the residual variance assumption were checked using a Wilk-Shapiro test and Levene ANOVA, respectively. Significance is claimed at $P < 0.05$.

RESULTS

Exposure Timeline and Experimental Design

Explant culture treatment combinations are summarized in Table 1. Figure 1 illustrates the *in vivo* exposure protocol for each of the four exposure groups. All animals were housed under FA conditions for the first 6 months of life and then began 11 cycles of 14-day group-specific exposures: FA, O₃, HDMA only, or combined O₃+HDMA (19). All animals were sensitized, and sensitization was confirmed with a positive skin test to HDMA as previously described (29). Allergic sensitization was conducted concurrently with the exposure protocol.

Challenge by Oxidative Stress

After exposure, airway explants from each set of treatment conditions were homogenized, and RNA was isolated to assess changes in NK-1R gene expression via quantitative RT-PCR. NK-1R gene expression trends are summarized in Table 2. Values for each exposure group were analyzed relative to their own sham control. There was no difference in basal NK-1R mRNA expression in the sham controls of any of the four groups (*see* Figure E1A in the online supplement). These values were analyzed relative to FA control sham. Figures 2A and 2B and E1B and E1C demonstrate basal NK-1R expression in the absence of *in vitro* oxidant challenge. Basal levels of NK-1R mRNA expression in explants from animals exposed to FA (Figure 2A) or HDMA alone

Figure 1. Experimental design and exposure timeline. Sensitized animals were randomly assigned to one of four exposure groups and subjected to 11 2-week cycles of filtered air (FA), ozone (0.5 ppm 8 h/d, 5 d on and 9 d off), house dust mite antigen (HDMA) (2 h/d, 3 d on and 11 d off), or ozone (0.5 ppm+HDMA, allergen exposed last 3 days of ozone sequentially).

(Figure E1B) do not change in the presence of NK-1R agonist, but expression in explants from O_3 -exposed animals (Figure 2B) significantly increases when SP is added *in vitro* ($P = 0.0004$). After oxidant challenge with H_2O_2 or lipid ozonide (Figure 2C), NK-1R mRNA expression in explants from FA animals nearly doubles in the presence of NK-1R agonist SP. Concurrent treatment with SP and NK-1R antagonist L119 significantly ablates this response ($P = 0.001$), indicating that this response is mediated by NK-1R. Under the same oxidant treatment conditions, explants from O_3 -exposed animals (Figure 2D) show increased NK-1R mRNA expression when challenged with H_2O_2 or ozonide by approximately 1.5-fold (not significant) and 3-fold ($P = 0.002$), respectively, as compared with sham control. This increase is diminished, though not significantly, in the presence of ozonide combined with agonist or agonist+antagonist and significantly with H_2O_2 in combination with agonist+antagonist ($P = 0.04$). Compared with O_3 -exposed sham control, explants exposed to SP alone show significantly increased NK-1R mRNA expression ($P = 0.0004$). This increase is significantly diminished with the addition of L119 to SP ($P = 0.01$). Explants from animals exposed to HDMA alone (Figure 2E) showed greater NK-1R mRNA expression relative to control when treated with SP in combination with an oxidant. This increase is significantly ablated in the presence of H_2O_2 +SP+L119 or ozonide+SP+L119 ($P = 0.001$ and $P = 0.004$, respectively). Explants from combined exposure animals (Figure 2F) showed increases in NK-1R mRNA expression similar to both the singular (O_3 or HDMA only) exposure groups with respect to SP treatment in the sham and H_2O_2 groups. Though only the sham+SP and H_2O_2 +SP were significant over the matching SP+L119 groups ($P = 0.01$; $P = 0.00004$), the overall impact on NK-1R showed trends similar to both individual exposures, specifically, the sham+SP response in O_3 animals and H_2O_2 +SP response in HDMA-exposed animals.

Paraffin sections used to localize protein expression were from midlevel airway slices not subjected to *in vitro* culture, representing the native state of the tissue at the time of necropsy. Immunohistochemical localization of NK-1R (Figures 3A–3E) and Nur77 (Figures 3F–3J) protein was defined from each of the four exposure groups (Figure 3). The negative control lung slice for each protein assay lacked immunostaining (Figures 3E and 3J, respectively). In FA animals (Figure 3A), NK-1R protein was found in the epithelium, localized to the luminal side of the cell apex. Relative to FA, animals exposed to O_3 (Figure 3B) or HDMA (Figure 3C) have cells in which NK-1R protein expression is more intense, and a greater proportion of the cell contains the receptor. Combined-exposure animals show similar NK-1R expression to FA animals. For Nur77, FA animals (Figure 3F) have a distinct immunostaining pattern in the apex of the epithelium with a moderate amount of positive cells. Relative to FA, O_3 -exposed (Figure 3G) and HDMA-exposed (Figure 3H) animals had increased protein expression. However, the greatest Nur77 protein abundance was in animals exposed to O_3 and allergen (Figure 3I).

Morphology

To assess the morphology of this airway level *in vivo*, representative lung was histostained with hematoxylin and eosin (Figure E2). Relative to FA animals (Figure E2A), O_3 -treated animals (Figure E2B) had marked mucus cell hyperplasia. Antigen-treated animals (Figure E2C) had mucus cell hyperplasia and thickening of the basement membrane zone. Combined-exposure animals (Figure E2D) show effects characteristic of both exposures, including mucus cell hyperplasia and thickening of the basement membrane zone.

Protein Expression of NK-1R

To determine whether the amount of NK-1R protein expressed in the airways is affected by *in vitro* oxidant challenge and if a history of oxidant stress with O_3 alters protein expression, we defined the relative abundance of NK-1R protein (Figure 4) in the midlevel airways of an episodically exposed monkey lung compared with that of an explant from an identically exposed animal that was challenged *in vitro* with lipid ozonide (Figure 4B). Compared with the *in vivo* only exposure, which ended approximately 5 days before necropsy (Figure 4A), the acutely exposed explant, which contains substantially more NK-1R protein/positive airway epithelial cells, demonstrated the ability to up-regulate this expression in response to oxidant challenge. The relative protein expression of other exposure groups was similar to FA control animals and was unchanged by *in vitro* oxidant challenge with H_2O_2 or lipid ozonide (data not shown).

IL-8 Gene Expression

In addition to examining epithelial oxidant stress responses in animals with a history of previous oxidant exposure, we defined mRNA expression of a proinflammatory marker associated with airway epithelial inflammation, the chemokine IL-8. All values were analyzed relative to FA sham control. The presence of agonist or agonist+antagonist in combination with the oxidant H_2O_2 increased basal levels of IL-8 gene expression in explants from animals exposed to FA (Figure 5A) but did not change mRNA expression in explants from O_3 - or HDMA-exposed animals (Figures 5B and 5C). After oxidant exposure with H_2O_2 , IL-8 gene expression in explants from FA-exposed animals

TABLE 1. EXPLANT CULTURE TREATMENT

Base Treatment	+ Agonist	+ Antagonist	+ Agonist, Antagonist
10 μ M ozonide	10 μ M ozonide 10 μ M Substance P	10 μ M ozonide 10 μ M L119	10 μ M ozonide 10 μ M Substance P 10 μ M L119
1.2 mM H_2O_2	1.2 mM H_2O_2 10 μ M Substance P	1.2 mM H_2O_2 10 μ M L119	1.2 mM H_2O_2 10 μ M Substance P 10 μ M L119
Sham control	Sham control 10 μ M Substance P	Sham control 10 μ M L119	Sham control 10 μ M Substance P 10 μ M L119

TABLE 2. SUMMARY OF *IN VITRO* NEUROKININ-1 RECEPTOR GENE EXPRESSION

Oxidant Treatment	Base Treatment Only	10 μ M Substance P	10 μ M Substance P + 10 μ M L119
10 μ M ozonide	$\uparrow$ O ₃ *	$\uparrow$ FA $\uparrow$ O ₃ $\uparrow$ HD	$\downarrow$ FA $\uparrow$ O ₃ $\downarrow$ HD
1.2 mM H ₂ O ₂	$\uparrow$ O ₃	$\uparrow$ FA $\uparrow$ HD $\uparrow$ OH	$\downarrow$ FA $\downarrow$ HD $\downarrow$ OH
Sham control	No change	$\uparrow$ O ₃ * $\uparrow$ OH	No change

* Statistically significant change relative to exposure sham control ($P < 0.05$).

increased slightly in the presence of SP and significantly with concurrent treatment of SP and L119 compared with SP+H₂O₂ ($P = 0.03$) or SP+L119+sham ($P = 0.02$). Combined-exposure animals showed significantly increased mRNA expression in the presence of SP+L119 independent of the presence of H₂O₂ compared with SP alone (sham $P = 0.008$; H₂O₂ $P = 0.007$). Relative to sham control animals, H₂O₂ alone or ozonide alone or with agonist/antagonist had no impact on IL-8 gene expression (data not shown).

DISCUSSION

We investigated the interaction of O₃ and antigen using a novel *in vivo/ex vivo* approach to define the effects of an aeroallergen,

with or without oxidant stress, on airway remodeling in an allergic asthma model. It is well known that O₃ causes inflammation, injury, and repair in the lung and that children are especially susceptible to its negative health effects, some of which may persist for years (4). However, the mechanisms by which aeroallergens interact with oxidative stress are much less well understood. Neurogenic mechanisms are implicated in airway remodeling from O₃ exposure alone, but our findings suggest that allergen exposure alone has little impact on altering the NK-1R cascade. Given the minimal impact of antigen challenge exposure alone and the implications of neural changes in the susceptible but nonallergic rat model work from Oslund and colleagues (12), we attempted to further explore the relationship between O₃, SP, and NK-1R in the intrapulmonary airways.

Figure 2. Neurokinin-1 receptor (NK-1R) gene expression in midlevel airway explants from monkeys exposed to 11 cycles of filtered air (FA), 0.5 ppm ozone (O₃), HDMA, or 0.5 ppm O₃+HDMA *in vivo* and subjected to oxidant exposure (1.2 mM H₂O₂ or 10 μ M lipid ozonide) *in vitro*. Some airways were also cultured *in vitro* with NK-1R agonist (Substance P [Sub P]) or agonist and antagonist (Substance P+L119). All values were analyzed relative to exposure sham control (e.g., FA animals relative to FA sham control). Baseline NK-1R expression in FA-exposed (A) and O₃-exposed (B) animals. NK-1R expression in airway explants from FA- (C), O₃- (D), HDMA- (E), or O₃+HDMA- (F) exposed animals treated with vehicle control, agonist, or agonist+antagonist and with sham, H₂O₂, or ozonide. $n = 4$ to 6 animals. Significance was considered at $P < 0.05$. * $P \leq 0.001$. †Different from matched control group. ‡Different from matched SP+L119 group. §Different from matched sham group. §Different from matched H₂O₂ group.

Figure 3. Neurokinin-1/Nur77 receptor protein expression. Immunohistochemistry for NK-1R or Nur77 orphan receptor in midlevel conducting airway slices of monkeys episodically exposed to FA, 0.5 ppm O_3 , HDMA, or O_3 +HDMA. The airway from a FA-exposed animal shows some NK-1R immunoreactivity in the epithelium (A) compared with no reactivity in the negative control (E). Relative to FA, animals exposed to O_3 (B) and HDMA (C) have increased NK-1R immunostaining. Animals exposed to O_3 +HDMA (D) have slightly less staining than FA. The FA animals (F) show immunoreactivity for Nur77 in the apices of epithelium compared with the negative control (J). Relative to FA, animals exposed to O_3 (G), HDMA (H), or O_3 +HDMA (I) showed increased immunostaining with the greatest reactivity in animals from the combined exposure. Arrows note location of inset image. Scale bar = 50 μ m (inset scale bar = 10 μ m).

Specifically, we determined that a history of O_3 exposure resets the ability of the airways to respond to oxidant stress and that some of these responses are mediated by changes in NK-1R.

We found that microdissection and short-term explant culture of rhesus airways preserves epithelial integrity and allows the study of these interactions. We also found that a history of O_3 exposure results in persistent effects in the airways, resetting the steady state so that NK-1R expression is increased in airways subjected to subsequent oxidant challenge *in vitro*. This response can be mitigated by incubation with NK-1R agonists or antagonists. This indicates that these responses are intrinsic to alterations that occur in the local airway microenvironment. Adding support to the role of previous exposure history in these responses, NK-1R expression is relatively unchanged, even in the presence of oxidants, in animals with no previous O_3 exposure history. We also observed that O_3 exposure, in combination with HDMA, may exhibit antagonistic effects, dampening responses induced by O_3 exposure alone. NK-1R protein expression in midlevel airways exposed to O_3 correlates with the protein expression of Nur77, a downstream receptor of the

activated NK-1R pathway. This indicates possible activation of programmed cell death. Finally, we found that a history of O_3 exposure results in a muted proinflammatory effect upon subsequent oxidant challenge. Proinflammatory chemokine IL-8 gene expression is relatively unaltered in O_3 -exposed animals but is up-regulated in FA animals exposed to a specific oxidant in combination with an NK-1R agonist or agonist+antagonist. This suggests that NK-1R may have a temporary protective, or adaptive, effect in O_3 -exposed animals. In this study, we examined the role of previous exposure history on the ability of sensitized, developing conducting airways to respond to subsequent oxidant stress. Modulation of airway responses based on previous exposure, particularly with respect to NK-1R, suggests that NK-1R may serve a dichotomous role as an indicator of oxidant stress and inflammation and as an adaptive, protective response (30).

The epithelial-mesenchymal trophic unit (EMTU) of the conducting airway, first described by Evans and colleagues, is composed of several key areas that combine to form a highly sensitive network of immunologic, neural, muscular, and cellular interactions (18). The EMTU basal cell-basement membrane zone-fibroblast complex underlies the pseudostratified epithelium and a network of innervation (7, 31). This serves as a signaling interface for the respiratory system that matures in both the pre- and postnatal periods of lung development. The EMTU is susceptible to disruption and, in patients with asthma, is characterized by chronic inflammation and remodeling (32). The origins of this remodeling are thought to lie in childhood, yet animal models of asthma infrequently study the developing lung. When exposed to external pollutants and/or allergens, the EMTU can potentially modulate the afferent (sensory) and efferent (parasympathetic) neural networks within the airway (31), and this is especially true when these networks are still maturing. The complexity of the EMTU allows it to interact with and respond to local and systemic environments. The benefit of this explant culture method is that it preserves the EMTU local structure so that local changes in neurokinin-tissue interactions can be observed and exploited without the complications of the intact *in vivo* system. Though it is imperative to understand these relationships in the context of the whole animal, the conditions of this approach allow selective manipulation of the intact EMTU from

Figure 4. *In vivo* versus *in vitro* NK-1R protein expression. Immunohistochemistry for NK-1R in a midlevel conducting airway slice of a monkey episodically exposed to 0.5 ppm O_3 *in vivo* (A) and then an explant of an identically exposed monkey (B) subjected *in vitro* to 10 μ M lipid ozonide. Explants were maintained in culture for 4 hours at 37°C. NK-1R immunoreactivity in airway epithelium is increased with more protein/positive cell after lipid ozonide oxidant challenge in culture (arrowheads mark similar regions). Scale bar = 50 μ m.

Figure 5. IL-8 gene expression in mid-level airway explants from monkeys exposed to 11 cycles of 0.5 ppm O_3 , HMDA, or 0.5 ppm O_3 +HMDA *in vivo* and subjected to oxidant exposure (1.2 mM H_2O_2) *in vitro*. Airways were cultured *in vitro* with NK-1R agonist (Sub P) or agonist and antagonist (Sub P+L119). All values were analyzed relative to filtered air sham control. IL-8 expression in airway explants from FA (A), O_3 (B), HMDA (C), or combined exposure (D) animals. $n = 4$ to 6 animals. Significance was considered at $P < 0.05$. *Different from matched Sub P group. †Different from matched sham group.

specific regions of the lung and in very young animals where pathology is still being established, thereby facilitating the study of the origins of later disease.

NK-1R is implicated in nerve-mediated cell death mechanisms within the airway. Oslund and colleagues found that after acute O_3 exposure (1 ppm, 8 h), adult Wistar rats pretreated with NK-1R antagonist SR140333 had a significant decrease in epithelial injury and proliferation relative to control animals (12). Additionally, they demonstrated in animals not treated with the antagonist that ethidium homodimer-1-positive cells, indicating necrosis, colocalized with orphan receptor Nur77, further implicating NK-1R's role in mediating targeted, acute O_3 -induced damage. Our current study demonstrates that O_3 has a localized impact on the NK-1R-mediated cascade at the level of airway-nerve interactions. Additionally, we demonstrate that oxidants can induce significant changes in NK-1R expression in airways with prior oxidant exposure history. This occurs in a relatively short time frame via local mechanisms of action, as evidenced by the isolated explant approach in lung tissue from developing animals. However, it is still imperative to interrogate the impact of O_3 on NK-1R-mediated activity in the context of allergic airways. Schelegle and colleagues observed that a cyclic combination exposure of O_3 and HMDA resulted in significant amplification of hallmark allergic airway disease, including eosinophilia, structural remodeling, and airways reactivity (19). Our study suggests that in allergic airways, oxidants induce EMTU level changes in the NK-1R that are modulated by the pollutant exposure history and can ultimately result in an exacerbated state of airway disease, particularly in the developing lung.

The roles of O_3 , NK-1R, and neurokinin pathways, particularly with respect to SP, are further associated with airway inflammatory disease in humans under these episodic exposure conditions in an allergic model. When Hazbun and colleagues acutely exposed (0.25 ppm, 1 h) healthy human adults to O_3 during intense exercise, significant increases of SP and 8-epi-prostaglandin $F_{2\alpha}$, an inflammatory marker, were noted in the airway lavage fluid (33). Similarly, Krishna and colleagues exposed healthy adults to acute O_3 intermittently during intense exercise and noted significant increases in SP in lung tissue after exposure, suggesting that SP, and by default neurokinins, likely play a role

in neutrophil-mediated inflammation in conducting airways (34). If O_3 induces SP production, as reported in these adult human exposures, it is also probable that NK-1 receptors are already activated in episodically exposed airways. Repeated episodic O_3 exposure may induce a microenvironment of injury-repair cycling that results in a state of chronic neurogenic inflammation, facilitated by chronically elevated SP levels that result in more NK-1R desensitization and possible receptor recycling. Treatment with additional SP may have a minimal impact on increasing NK-1R gene expression because the majority of the receptors are occupied, approaching saturation. However, exogenous treatment with 10 μ M SP *in vitro*, though greater than physiological levels, is sufficient to induce neurogenic and immunomodulatory responses similar to those seen in acute oxidant stress (35). Possibly this is due to the levels being high enough to overcome NK-1R desensitization and recycling resulting in greater overall NK-1R expression in oxidant-stressed cells. Our study demonstrates that baseline NK-1R gene expression in midlevel airways of FA-exposed animals remains unchanged regardless of the presence of an NK-1R agonist or antagonist (Figure 2A). We observed a similar pattern in airways from animals episodically exposed to O_3 in the presence of a combined NK-1R agonist and antagonist; however, NK-1R expression deviates from baseline with a significant increase in airways episodically exposed to O_3 and acutely treated with SP alone (Figure 2B). This response is repeated in FA-exposed airways treated with SP in the presence of an oxidant such as H_2O_2 or ozonide (Figure 2C).

The protective versus malicious role of SP is a subject of continued debate regarding neurogenic inflammation and tissue injury (30). The use of a strong NK-1R antagonist (L119) allows us to observe whether SP acts via NK-1R binding or another pathway. In the case of episodic oxidant exposure, it appears that SP-NK-1R binding facilitates neuromediated processes, including cell death (via Nur77). In the case of IL-8, SP likely exerts its effects only partially through NK-1R and acts beyond the NK-1R pathway, as indicated by the increased expression of IL-8 in airway treated with SP in conjunction with antagonist L119. Future studies could determine the activation status of NK-1Rs by detecting the presence of total and phosphorylated ERK1/2 pro-

tein, key downstream kinases phosphorylated upon activation of NK-1R via SP (36).

Conversely, the presence of an oxidant alone further stimulates NK-1R expression in airways "primed" for response by a history of episodic O₃ exposure. This oxidant challenge may inflame the previously stressed airway epithelia, stimulating a surge of NK-1R expression on the epithelial surface. This is particularly evident when O₃ exposed airways are treated with lipid ozonide, a substance that readily interacts with the lipophilic epithelial surface. In the absence of an oxidant, airways with an exposure history show a significant up-regulation in NK-1R expression in the presence of SP. The lack of oxidant challenge would likely mitigate the amount of endogenous SP produced and enhance receptor expression, possibly leading to greater receptor activity and, ultimately, NK-1R-mediated changes. Though NK-1R gene expression in O₃-exposed airways is similar with ozonide or H₂O₂, the more pronounced impact of ozonide may be attributed to the fact that it is more structurally similar to membrane lipid products produced by O₃. Ozonide results in the formation of lipid peroxidation products that facilitate the release of inflammatory mediators, particularly those of the arachidonic acid cascade (23). The lipophilicity of O₃, and similarly its lipid ozonide derivatives, combined with its ability to react highly with unsaturated fatty acids to produce hydroxyhydroperoxides and aldehydes causes O₃'s biochemical effects to reach a greater depth in the epithelial surface (37). Conversely, the use of DMEM/F12 media may have some mitigating effects on the oxidizing capabilities of H₂O₂.

Nur77, also known as nerve growth factor 1B, is a downstream member of the NK-1R cascade and is associated with macrophage-mediated inflammatory responses (38). Additionally, Nur77 modulates nonapoptotic programmed cell death in dysfunctional cells (39). Activation of Nur77 may play a role in determining whether oxidant-stressed cells pursue a programmed cell death versus a necrotic, inflammatory pathway (40, 41). Though Nur77 has not been studied extensively in the lung, oxidant-induced activation of NK-1R establishes the grounds for further study of Nur77 activity in airway epithelium and its influence on cell death responses to oxidant stress and persistent neurogenic inflammation. In the current study, similar to the NK-1R protein expression abundance, Nur77 expression was enhanced by exposure, particularly in the O₃ and combined O₃+HDMA groups. The increased NK-1R-positive immunoreactivity and structural damage of airway epithelia exposed to O₃ and O₃+HDMA suggests that the increase of Nur77 expression may be a response of oxidant-stressed and compromised cells. This may mitigate the activation of necrotic, inflammatory pathways. Reduction of necrotic processes would, in turn, minimize trauma to epithelial structure and the likelihood that unmyelinated, highly sensitive nerve structures such as c-fibers would be exposed and activated, resulting in neurogenic inflammation. Though we did not specifically evaluate receptor activation, the correlative protein expression of NK-1R and Nur77 implies a treatment-induced response that involves the MAPK cascade. Further investigation of Nur77 gene expression and interruption of the NK-1R downstream cascade, perhaps with kinase-specific inhibitors, could be used in future studies to determine the causative relationship of NK-1R to Nur77 from O₃-induced oxidative stress.

Oxidant-stressed cells can activate proinflammatory signaling cascades, which release chemotactic molecules, such as IL-8, that recruit leukocytes to sites of injury (17). Under the conditions of this study, with a 4-hour *in vitro* exposure, only H₂O₂ elicited an increase in IL-8 gene expression, and this response was confined to FA airway explants exposed to SP in the presence of L119 (Figure 5A). Ozonide alone or in the presence of NK-1R agonists

or antagonists failed to generate a treatment response in any group (not shown). The failure of these groups to generate a distinct IL-8 response to ozonide alone or in the presence of NK-1R ligand or antagonist is not irrelevant. This concurs with the data of Kafoury and colleagues, where the release of select proinflammatory mediators such as IL-8 is specific to the type of lipid ozonide used. Under similar exposure conditions, Kafoury and colleagues demonstrated that the ozonide PC-ALD, also used in our study, selectively induced prostaglandin E₂ in human bronchoepithelial cell lines exposed at 10 μ M for 4 hours, whereas another lipid ozonide (HHP-C9) selectively induced IL-8 after 4 hours only at the higher 100 μ M concentration for 1 hour (23). This primary difference was attributed to the selective nature of each lipid ozonide's cell signaling cascade with PC-ALD selective to phospholipase-A₂ and HHP-C9 to phospholipase-C-mediated pathways. Additionally, the HHP-C9 cascade was shown to be protein kinase-C dependent under the conditions of their study and is likely involved in further signaling pathways, such as activation of NF- κ B (22). The presence of H₂O₂ has been shown to cause lipid oxidation and result in NF- κ B activation, which could enhance subsequent IL-8 release from airway epithelia (42). NK-1R expression in airway explants from FA animals challenged with H₂O₂ in the presence of SP is significantly greater than those explants exposed to SP+L119. This up-regulation in NK-1R gene expression correlates inversely with IL-8 expression in identically treated animals. Conversely, the reduced NK-1R expression in H₂O₂-challenged SP+L119 explants inversely correlates with the significant increase in IL-8 gene expression in the same animals. These findings suggest that the up-regulation of NK-1R in the presence of an oxidant such as H₂O₂ may initiate a neurokinin-mediated adaptive effect that mitigates the proinflammatory response, perhaps lessening the overall inflammatory insult to the epithelia.

Our study was designed to investigate the antigen-O₃ interaction. We observed that the coexposure of HDMA with O₃ results in antagonism of O₃-induced responses. Though exposure to oxidant air pollutants like O₃ may exacerbate inflammation during allergic asthma episodes (19), its impact on neurokinin-mediated pathways is altered when exposed in the presence of antigen, perhaps resulting in a different neural-immune response profile than that observed in airways exposed to O₃ alone. In this study, O₃ exposure alone showed the greatest impact on the NK-1R pathway. The long-term sequelae of these early changes is not known. A 1-year-old monkey is roughly equivalent to a 3-year-old child (43). It is not known whether these changes continue to persist and offset each other as the lung continues to grow and mature or if they exacerbate each other. In addition, the overall inflammatory profile that results from these early postnatal exposures must be further studied. The expression and activity of specific neural receptors like NK-1R and their local impact on mediating and perhaps perpetuating neurogenic inflammation in the lung have yet to be fully elucidated.

The monkey model serves as a useful surrogate for the airways of atopic children. This model effectively mimics the clinical manifestations of children episodically exposed to two common and well known respiratory irritants (29) in an airway epithelium with pronounced structural and cellular similarities to humans. Further, in conjunction with an *in vitro* explant approach, the role of local mediators within the EMTU in early childhood airway growth and remodeling can be defined. Many areas of the United States are flooded with atopically prone children who live in areas of high seasonal O₃ levels (44). This, in combination with the well documented rise in childhood asthma, warrants the use of animal models that can effectively model the pattern of postnatal lung development that is affected by continued poor air quality. In this study, we demonstrate that

the explant culture method is a focused approach by which to study multidimensional and complicated aspects of the lung, including specific sites in the intrapulmonary airways and the mediators and receptors localized to these sites.

We conclude that a history of O₃ exposure does reset the ability of the allergic airways to respond to oxidant stress and that some of these changes are likely mediated by NK-1R. Further studies are needed to evaluate the potential for O₃ to affect neurogenic inflammation and cell death processes via the NK-1R and its specific activity within the conducting airways of the lung and specifically in children with allergic asthma.

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