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Interleukin-13 stimulates production of nitric oxide in cultured human nasal epithelium

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Abstract

The diversity and extent of signaling functions of nitric oxide (NO) in cell physiology as well as its presence and influence as a common component of ambient air pollution and tobacco smoke are gaining increasing research attention relative to both health and disease. While cellular NO production is typically associated with inflammatory cells and processes, the airway epithelium particularly of the paranasal sinuses, has been documented to be a rich source of excreted NO. Inasmuch as excreted NO derives from both mucosal and inflammatory cell sources, distinguishing the individual contribution of these compartments to total excreted cellular NO is potentially problematic. We simulated an inflammatory mucosal environment by stimulating human nasal epithelial cultures with interleukin-13 (IL-13), a mediator produced by eosinophils in asthma, allergic rhinitis, and sinusitis. While a consistent baseline of NO excretion in control cultures was documented, widely variable individual responses to IL-13 exposure were observed in companion cultures maintained under identical conditions and tested at the same time. These studies suggest that cellular NO excretion by the healthy epithelial mucosa is subject to considerable individual variability and may be significantly elevated among some individuals in the presence of IL-13 stimulation.

Keywords

Airway; Allergy; Asthma; Cytokine; Epithelium

Compliance with ethical standards

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Late in the twentieth century the discovery of nitric oxide (NO) as an endothelium-derived relaxing factor led to a surge of research and clinical interest in NO signaling and metabolism in health and disease that persists to the present. The development of a sensitive technique for instantaneous measurement and monitoring of NO concentration in human airways spurred clinical studies in which NO excretion into the nasal and lower airways was related to a variety of diseases as well as to therapeutic interventions. NO flux measurements have been extensively studied for their potential clinical utility in the management of asthma (Taylor et al. 2006; Pavord and Shaw 2008) or as a potential diagnostic marker of rare airways diseases such as cystic fibrosis (CF) (Barnes et al. 2010) and primary ciliary dyskinesia (PCD)(Leigh et al. 2016). The upper and lower conducting airways share the histologic similarity of a ciliated pseudostratified columnar epithelium expressing all three nitric oxide synthase (NOS) isoforms (Ricciardolo et al. 2004) although the NO flux into the airway lumina as well as regional NO concentrations may vary dramatically (Kramer et al. 2010). In vivo, this difference in NO excretory flux may be due in part to the contribution of NO entering the nasal passages from the paranasal sinuses (Maniscalco et al. 2003). Further, the NOS2 enzyme has been documented as being highly constitutively expressed in the apical region of normal paranasal sinus epithelium even in the absence of inflammation (Lundberg et al. 1995) pointing to its potential for rapidly upregulating ciliary function (Jain et al. 1993; Zhou et al. 2009) and maintaining sinus sterility via innate immunity (Bogdan et al. 2000).

Interleukin-13 (IL-13) is a Th2 cytokine that is associated with the manifestation of an asthma phenotype in airway epithelium in vivo (Wills-Karp et al. 1998) and in vitro (Laoukili et al. 2001). These studies have demonstrated that IL-13 may impact mucociliary differentiation, eosinophilic infiltration, and airway hyperresponsiveness. Because non-resident cell types such as eosinophils express inflammatory mediators, specifically IL-13 (Schmid-Grendelmeier et al. 2002), elaborate NO (Yates 2001), and may be heavily represented in the asthmatic or allergic airway, achieving an understanding of the contribution of the airway mucosa itself to NO dynamics in conditions such as asthma and chronic rhinosinusitis has been problematic. In the present study, we have generated air-liquid interface (ALI) cultures of human nasal airway epithelium from healthy non-smoking subjects propagated in the presence of IL-13 and assessed differences in excreted NO relative to control cultures from the same subjects. These studies performed in the absence of other NO excreting cell types have provided a new perspective on the dynamics and range of NO excretion in the isolated airway epithelial compartment in an inflammatory state.

Four healthy, non-smoking human research subjects were recruited and consented for this study using a protocol approved by the UNC institutional review board. Subjects were documented as non-smoking by urine cotinine tests. All subjects were screened at the time of enrollment and questioned regarding overall respiratory health and specifically queried as to whether they had ever been diagnosed with asthma by a physician. At every visit, each volunteer also responded to a brief questionnaire regarding recent respiratory illnesses, exposures, or symptoms and excluded from sampling if any were positively documented. Differentiated nasal epithelium was obtained non-invasively by gentle curettage of the inferior nasal turbinates. The tissue was placed in transport medium and delivered to the laboratory for culture.

Biopsies were transitioned to a culture environment for mitotic expansion, and cells subsequently seeded onto clear transwell culture inserts and transitioned to air-liquid interface (ALI) culture conditions (Müller et al. 2013). Briefly, ALI cultures were established from nasal epithelial cells harvested from the inferior surfaces of inferior nasal turbinates by gentle curettage using a sterile, disposable, thermoplastic curette. Culture medium was supplemented with the following antibiotics: 100 U/ml of penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 50 µg/ml of gentamicin. Technical maneuvers subsequent to nasal sampling further insured sterile conditions including quarantine of nasal epithelial cultures to dedicated incubators, frequent pipette tip changes, and ultraviolet light sterilization and alcohol swabbing of work surfaces in a biological laminar flow hood prior to working with cultures. Initially, pelleted cells from the biopsy were transferred to a conditioned substrate in 12-well plates. Cultures were monitored for substrate adherence with continued feeding and re-seeding of non-adherent cells for up to 7 d. When the cells approached confluency in the 12-well plates, they were transitioned to cell culture flasks to accommodate mitotic expansion. During this phase, the medium was changed every 2–3 d until reaching or approaching confluency at which time, cells were lifted from the flask and seeded onto uncoated membrane transwells positioned in 12-well plates. Cells were maintained in this condition under total immersion with medium for 2–6 d and allowed to reach total confluency. The airliquid interface condition was established upon confluence by first supplementing both apical and basolateral medium with 500 nM retinoic acid (RA) for 48 h. Subsequently, all medium was removed from both surfaces and replenished only on the basolateral side with RA-supplemented medium. This approach to cell culture optimizes the formation of a pseudostratified columnar epithelial layer on the transwell inserts in which the distribution of cell types is comparable to that in the airways in vivo (Figs. 1A, B). Upon transitioning to ALI, the culture medium in half of the cultures from each isolate was supplemented with 10 ng/ml human recombinant IL-13. The cultures were maintained for at least 1 mo under ALI conditions in order to achieve optimal phenotypic differentiation before NO determinations were performed.

Cellular NO production in paired control and IL-13-exposed cultures from the same subject was determined by positioning three transwells in a vessel (Cytometric Sciences LLC, Chapel Hill, NC) designed for cellular NO measurement (Ostrowski et al. 2012). The vessels were incubated for 6 h at 35°C after which NO concentration in the vessel was determined using a Sievers 270B NO analyzer at a sampling flow rate of 500 ml/min. Following NO measurements, total protein was determined in the cultures used for each experiment (Bio-Rad, Atlanta, GA). At the completion of the experiments, a calculation was made of [NO] (ppb)/total protein (mg)/h for each experimental vessel.

Six-paired experiments performed under identical conditions using cultures from four different subjects revealed noteworthy increases in NO concentration among five experiments and a modest decrease in one (Fig. 2). The paired *t* test procedure was used to analyze the data. [NO], [NO]/total protein, and [NO]/total protein/h were set as responses with subsequent tests performed to determine significance relative to control and IL-13-supplemented cultures. Results of the statistical analyses shown in Table 1 indicate that the *p* values are all < 0.05 thus inferring that IL-13 is significant in the added production of NO under ALI conditions.

The constitution of the epithelial mucosa of allergic and asthmatic airways, as well as other chronic airways diseases, exists within a milieu of inflammatory cells and mediators. This situation confers a phenotype that distinguishes it structurally and functionally from the normal state. The presence of eosinophilia is historically considered an integral component of the pathology of the asthmatic airway. Moreover, the finding of IL-13 as well as other interleukins (Woodruff et al. 2007) and the elevated concentrations of nasal nitric oxide (Takeno et al. 2001; Ricciardolo et al. 2004) observed in asthma and allergic disease have typically been attributed to eosinophilic inflammation. Inasmuch as all nitric oxide synthases have been identified in the airway epithelium (Kobzik et al. 1993; Lundberg et al. 1995; Furukawa et al. 1996; Watkins et al. 1997; Norford et al. 1998; Kawamoto et al. 1998; Ricciardolo et al. 2004; Krasteva et al. 2007;), we sought to assess the complement of excreted nitric oxide in human nasal epithelium propagated in the presence of IL-13 and in the absence of other contributory sources of NO such as eosinophils.

Measurement of NO excreted into the airways in vivo is readily quantifiable and commonly used to assess airway inflammation. Previous studies from this laboratory have demonstrated that smokers exhibit higher levels of nasal NO than non-smokers (Zhou et al. 2011). It has been more challenging to characterize NO production and its impact on function at the cell molecular level. Indeed, little is known apart from immunohistochemical and biochemical studies of nitrate/ nitrite production aimed at characterizing the expression of the three known nitric oxide synthase (NOS) isoforms. The ALI culture system used in this study provides a useful experimental platform whereby human cells can be propagated having the same histologic organization and genetic/epigenetic background as the donor tissue and in which the differentiation state of the cultures can be controlled and monitored with ease. Cultures appropriated for such studies can be readily manipulated experimentally and multiplexed for replicate or extended studies. In the present study, we detected in vitro concentrations of NO in control cultures that were consistent with other studies performed in this laboratory (Carson et al. 2017). Moreover, our studies indicated that cohort cultures propagated in the presence of IL-13, and the absence of an eosinophilic compartment were sufficiently stimulated to excrete an average of greater than tenfold the NO levels seen in control cultures. Perhaps most importantly, this feature facilitates the direct measurement of small concentrations of NO at the cell molecular level relative to cell phenotype, function, and experimental manipulation. This approach makes possible the development of profiles of NO concentration associated with disease states such as asthma, chronic bronchitis, and chronic obstructive pulmonary disease (COPD). Moreover, this experimental approach facilitates the calculation of a biologically relevant metric of NO excretion as a function of cell mass per unit time. The application of the ALI airway epithelial culture system for studies such as this provides an optimal experimental stage to perform analyses under highly controlled conditions that emulate the in vivo environment. The physical organization of the ALI culture provides a unique platform capable of facilitating multiple investigational paths. Moreover, as new technical and genetic discoveries are forthcoming, the ALI system represents a means to create expanded populations of cells from specific individuals and disease conditions and to facilitate customized, precisioned approaches for the evaluation of pathology at the cell and molecular levels. While endocrine, immunological, and nutritional

factors are diminished by in vitro conditions, pathologic changes that may be subtle in vivo are magnified in vitro and may be more readily detected and analyzed.

Airway epithelial ALI culture technology provides a particularly attractive model for investigation of specific airway diseases such as asthma, chronic obstructive pulmonary disease (COPD) (Mertens et al. 2017), and primary ciliary dyskinesia (Hirst et al. 2010). Here, genetically homogeneous primary cultures can be multiplexed to provide sufficient quantities of cultures for multiple types of studies as well as providing an adequate number of samples to support statistical rigor. The airway epithelium itself is a pseudostratified columnar epithelium, and the ability to generate an in vitro reproduction of this histologic scaffold in the ALI setting readily lends itself to physiological and morphological investigation of airway epithelial barrier function, phenotypic variability, ciliary function, and regenerative capacities associated with health and disease conditions.

Previous studies from this laboratory (Carson et al. 2010) have demonstrated that nasal biopsies cultured from smokers exhibit persistence of ciliary beat acceleration relative to that of fresh biopsies as well as a reduced complement of ciliated cells, suggesting that similar cellular mechanisms affected by tobacco smoke can be reliably reproduced within the in vitro environment. We have previously demonstrated that active smokers exhibit significant increases in nasal NO levels relative to both non-smokers and nonsmokers exposed to second-hand smoke with the increase most likely deriving from underlying inflammation of the airways (Zhou et al. 2011). In the present study, we offer an approach to actually quantify NO production as a function of cell mass cultured in the presence of IL-13. Although these data reveal considerable variability in NO production among cultures from different individuals; nevertheless, increases in NO production among the majority of subjects was notable.

These studies have experimentally demonstrated the potential of IL-13 to stimulate human nasal epithelial cells to produce substantive levels of NO above baseline in genetically homogeneous tissues. Although individual measurements may vary widely, these results demonstrate the potential of the airway mucosa to contribute a significant complement of excreted NO in chronic inflammatory conditions common to asthma, allergic rhinitis, and chronic obstructive pulmonary disease.

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Figure 1.

(A) Scanning electron micrograph illustrating prominent beds of cilia on the superficial surface of an ALI culture propagated from normal human nasal epithelium. Also present in this normal configuration is a normal complement of non-ciliated cells representing other functional cell types present in the epithelial layer (original plate magnification $\times 1000$). (B) Light micrograph illustrating the histologic organization of an ALI culture propagated from normal human nasal epithelium. Also, in this profile cytochemical localization of secretory products (*white arrows*) by AB-PAS staining is evident as well. Ciliary beds also are revealed by immunohistochemical localization of acetylated alpha-tubulin, a major component of eukaryotic cilia (original plate *magnification* $\times 300$).

Figure 2.
Comparative NO production in control and IL-13-supplemented ALI nasal epithelial cultures.

Table 1.Paired *t* test analysis of the data set

Response	<i>p</i> value	Mean difference
NO	0.0204	98.0
NO/TP	0.0439	149.3
NO/TP/HR	0.0439	24.9

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