Respiratory Physiology & Neurobiology 223 (2016) 1–8

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

# Side-stream tobacco smoke-induced airway hyperresponsiveness in early postnatal period is involved nerve growth factor

Z-X. Wu*, D.D. Hunter, T.P. Batchelor, R.D. Dey

*Department of Neurobiology and Anatomy, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506, United States*

ARTICLE INFO

*Article history:*
Received 1 September 2015
Received in revised form
11 November 2015
Accepted 12 November 2015
Available online 2 December 2015

*Keyword:*
Airway innervation
Asthma
Neurokinin receptor
Substance P

ABSTRACT

Epidemiological studies have shown that children are more susceptible to adverse respiratory effects of passive smoking than adults. The goal of this study is to elucidate the possible neural mechanism induced by exposure to passive smoking during early life. Postnatal day (PD) 2 and PD 21mice were exposed to side-stream tobacco smoke (SS), a surrogate to secondhand smoke, or filtered air (FA) for 10 consecutive days. Pulmonary function, substance P (SP) airway innervation, neurotrophin gene expression in lung and nerve growth factor (NGF) release in bronchoalveolar lavage (BAL) fluid were measured at different times after the last SS or FA exposure. Exposure to SS significantly altered pulmonary function in PD2, accompanied with an enhanced SP innervation in airway. However, exposure to SS during the later developmental period (PD21) did not appear to affect pulmonary function and SP innervation of the airways. Interestingly, SS exposure in PD2 group significantly induced an increased gene expression on NGF, and decreased NGF receptor P75 in lung; parallel with high levels of NGF protein in BAL. Furthermore, pretreatment with NGF antibody significantly diminished SS-induced airway hyperresponsivenss and the increased SP airway innervation in the PD2 group. These findings suggest that enhanced NGF released in the lung contributes to SS-enhanced SP tracheal innervation and airway responsiveness in early life.



## 1. Introduction

Epidemiological studies have shown that children are more susceptible to adverse respiratory effects of passive smoking than adults (Gilliland et al., 2003; Haberg et al., 2007; Lebowitz et al., 1992; Raherison et al., 2007; Wang et al., 2008a; Weitzman et al., 1990). Susceptibility to respiratory diseases throughout life can occur after environmental exposure during early postnatal life (Frischer et al., 1992; Gilliland et al., 2003; Gilliland et al., 2000; Haberg et al., 2007; Martinez et al., 1992; Neuman et al., 2012; Raherison et al., 2007; Ronchetti et al., 1992; Wang and Pinkerton, 2008; Weiss, 1994; Weiss et al., 1999; Yu et al., 2008). Our recent study showed that exposure to SS during early postnatal period increases the incidence of airway hyperreactivity later in life (Wu et al., 2009), suggesting that adult asthma may be traced back to exposures that occur during early postnatal development. However, the mechanism responsible for early postnatal susceptibility to passive smoking exposure that affects lung function later in life remains undefined.

Human lung development begins during the gestational period and continues well into adolescence. In parallel with the development of the lungs, airway innervation develops rapidly during fetal and early postnatal life (Dey et al., 1998; Sparrow et al., 2004). The nervous system, including the nerves supplying the airways, is highly susceptible to environmental influences during development (Wang et al., 2008b). The lungs and airways are extensively innervated by sensory nerves and stimulation is known to elicit the release of substance P (SP) with potent effects on airway smooth muscle tone, vascular permeability and mucus secretion (Barnes et al., 1991; Lundberg et al., 1984, 1983a). SP plays an important role in irritant-induced AHR and asthma (Lundberg et al., 1983b; Ollerenshaw et al., 1991; Wu and Lee, 1999; Wu et al., 1997, 2003). Thus we hypothesized that SS exposure during the early postnatal period alters SP airway innervation and that could be responsible for increased susceptibility to asthma in later life. Therefore, the first goal of the experiment is to determine the persistent changes in airway sensory nerves and lung function after SS exposure during early prenatal periods.

*Abbreviations:* AHR, airways hyperresponsiveness; BALF, bronchoalveolar lavage fluid; $C_{dyn}$, dynamic pulmonary compliance; ELISA, enzyme-Linked Immunosorbent Assay; ETS, environmental tobacco smoke; FA, filtered air; MCh, methacholine; NGF, nerve growth factor; PD, postnatal days; $R_L$, pulmonary resistance; SS, side-stream tobacco smoke; SP, substance P.

* Corresponding author at: Department of Neurobiology and Anatomy, P.O. Box 9128, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506, United States. Fax: +1 304 293 8159.
*E-mail address:* zwu@hsc.wvu.edu (Z-X. Wu).

http://dx.doi.org/10.1016/j.resp.2015.11.009
1569-9048/

Nerve growth factor (NGF) is a neurotrophic factor that promotes and maintains growth of the central and peripheral nervous systems, including those that innervate the airways (Levi-Montalcini, 1987b). Disruption of normal synthesis and release of NGF and resulting changes in airway innervation after inhalation of toxic material is well documented and leads to disease-related abnormalities in respiratory system (Hu et al., 2002; Hunter et al., 2010a; Wilfong and Dey, 2005). Our recent studies have shown that NGF is produced in response to irritant exposures and mediates changes in the phenotype and distribution of SP-containing neurons in the airways (Hunter et al., 2010a, 2011; Wilfong and Dey, 2004, 2005). SS exposure during the early postnatal period alters SP airway innervation, which is possibly mediated by NGF. The second goal of the experiment will characterize the role of NGF in SS exposure during early postnatal period.

## 2. Methods

ICR mice (Harlan, Indianapolis, IN) were housed with access to food and water ad libitum in a Food and Drug Administration-approved facility. All procedures were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, and the protocols were approved by the West Virginia University Animal Care and Use Committee (#05#1#0503). The animals were treated humanely and with regard for alleviation of suffering.

### 2.1. SS exposure

Postnatal day (PD) 2 and PD 21 mice were used to investigate the effects of SS exposure. Based on our recent study (Wu et al., 2009), we utilized a design where mice were exposed to either SS or filtered air (FA) six hours per day for ten consecutive days beginning on PD2 or PD21. All measurements were conducted 1 day or 3 days after the end of the last exposure. The SS exposure protocol and methods in this study have been described in our recent publication (Wu et al., 2009). Briefly, mice were randomly placed in an exposure chamber (BioClean, DuoFlo, model H 5500, Lab Products Inc) that measured $1.92 \times 1.92 \times 0.97$ m (3.58 $m^3$). The mice were housed in separate cages located inside the exposure chamber. Side-stream smoke from Marlboro filter cigarettes (Phillip Morris, Richmond, VA) was introduced into the exposure chamber at a rate of four cigarettes every 15 min for 6 h per day for 10 days using a smoking machine (RM 1/G, Heinr Borgwald GmbH, Hamburg, Germany). At the end of the 6-h exposure period, an exhaust fan on the BioClean unit was turned on to rapidly lower the level of SS in the exposure chamber. Mice exposed to filtered air (FA) under identical conditions in the same chambers except without smoke generation served as controls. The mice were then transported to the animal facilities overnight. The concentrations of carbon monoxide in the exposure chamber were monitored and kept to an average of about 50 parts per million (ppm), relative humidity was about 50%, temperature was about 23 °C. Total suspended particulate concentration was about 1.1 mg/$m^3$, similar to exposure levels used by others to approximate the cloud of particulates surrounding a person during active smoking (Yu et al., 2008). The level of cotinine in blood was measured at the end of the daily CS exposure on PD3, PD6 and PD10 in PD2 group, and on PD22, PD25 and PD29 in PD21 group from exposed mice by cotinine Elisa Kit (Sigma, St. Louis, MO). The average cotinine levels of these total measurement are approximately $50.98 \pm 5.14$ ($n = 10$) in PD2 SS exposure group, $48.01 \pm 6.30$ ($n = 10$) PD21 SS exposure group which was similar to nicotine levels typically found in daily human light smokers (10–100 ng/mL) (Benowitz and Jacob, 1984; World Health, 2003). $0.19 \pm 0.08$ ($n = 10$) in PD2 FA exposure group and $0.20 \pm 0.09$ ($n = 10$) in PD21 FA exposure group.

### 2.2. In vivo NGF-antibody (Ab) or immunoglobulin G (IgG) treatment

Previous studies have shown that NGF antibody inhibited airway innervation in infected lung (Cardenas et al., 2010; Hu et al., 2002). To test the role of NGF on SS-induced changes in pulmonary function and SP innervation, mice were treated with an NGF antibody (NGF-Ab) to reduce smoking-enhanced SP production. A single dose of NGF antibody has been shown to inhibit NGF activity (Cardenas et al., 2010; Hu et al., 2002). Thus, the mice were injected intraperitoneally with 0.2 ml of 1:2000 rabbit anti-mouse NGF antibody or rabbit IgG as a control (both 3 μg protein/ml; Sigma–Aldrich, St. Louis, MO) at 60 min before exposure. Then, 30 min later after every intraperitoneal injection, the same mice were exposed to 1 ml of an aerosolized solution containing anti-NGF or IgG diluted 1:2000. The combined subcutaneous and aerosol strategies to inhibit NGF have proven effective in assessing neuropeptide Y (NPY) expression after cigarette smoke exposure in fetal mouse (Wu et al., 2012). The aerosol exposures were done in a plexiglass chamber ($15 \times 15 \times 10$ cm), which was connected to a mini ultrasonic nebulizer with an output rate of 0.1 ml/min for 10 min.

### 2.3. Measurements of pulmonary function

Lung function was determined by measuring changes of pulmonary resistance and dynamic compliance 1 day or 3 days after last SS or FA exposure using a modification of our previously described protocol (Wu et al., 2009). Briefly, mice were anesthetized with pentobarbital (70 mg/kg, intraperitoneally) after exposure. The trachea was cannulated just below the larynx via a tracheotomy and a four-way connector was attached to the tracheotomy tube. Two ports were connected to the inspiratory and expiratory tubes of a respirator (Harvard model 683; South Natick, MA). The mice were ventilated at a constant rate of 200 breaths/minute and a tidal volume of approximately 0.2 ml. Aerosolized MCh (Sigma, St. Louis, MO) was administered for 30 s in increasing concentrations (0, 6.25, 12.5, 25, and 50 mg/ml). For 5 min before and after each MCh challenge, total $R_L$ and $C_{dyn}$ were analyzed by computer on a breath-by-breath basis.

### 2.4. Neurotrophin gene expression

Lungs were obtained 1 day after the last SS exposure and were immediately flash frozen in liquid nitrogen and stored in a freezer at −80 °C. The frozen specimens were placed in lysis buffer and homogenized using a conventional rotor-stator homogenizer. To determine neurotrophins and their receptors, a portion (2 μg) of total RNA isolated from lung was treated with Turbo DNase (Turbo DNA-free kit; Ambion) and reverse-transcribed into cDNA with the use of the $RT^2$ first strand kit (Qiagen Inc, Valencia CA) following the manufacturer's instructions. Mouse neurotrophin and receptors $RT^2$ Profiler™ PCR Array was selected for the present study. PCR Array was set up following the manufacturer's instructions and was performed according to the manufacturer's protocol. An experimental cocktail was prepared for each plate made up of the processed cDNA and 2× instrument-specific and ready-to-use array $RT^2$ qPCR master mix, containing SYBR Green and a reference dye. A portion (25 μl) of the experimental cocktail was placed into each well of the PCR array plate containing the pre-dispensed gene-specific primer sets, and PCR was performed on the ABI Prism 7500 Sequence Detection System. A two-step cycling program was used (10 min at 95 °C to activate the HotStart DNA polymerase,