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Developmental nicotine exposure results in programming of alveolar simplification and interstitial pulmonary fibrosis in adult male rats

Chiranjib Dasgupta[a,b,*], DaLiao Xiao[b], Zhice Xu[a,b], Shumei Yang[c], Lubo Zhang[a,b]

[a] Institute for Fetal Origin Diseases, First Hospital of Soochow University and Prenatal Biology, Center of Soochow University, Suzhou, China
[b] Center for Perinatal Biology, Division of Pharmacology, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA, USA
[c] Department of Chemistry & Biochemistry, California State University, San Bernardino, CA, USA

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ABSTRACT

Aims: To determine the long-term effect of maternal nicotine intake on the lung development of the offspring in adult life, we analyzed the alveolar structure, protein expression in the adult rat offspring lungs.
Methods: We determined animal body weight (BW), lung weight (LW), lung/body weight ratio (L/BWR), lung volume (LV), radial alveolar count (RAC), alveolar septal thickness (AST) and expression of collagen, AT1R, AT2R, TGF-β1, pSmad3, Smad3 and CTGF proteins.
Results: Male offspring lung showed decreased RAC, thickened alveolar septa, increased collagen, AT1R, TGF-β1, pSmad3 and CTGF proteins. In contrast, female offspring lungs had reduced L/BWR, increased LV, and expression of AT2R, resulting in decreased AT1R to AT2R ratio.
Conclusions: Maternal nicotine use during development programs abnormal lung development in male rats. This finding links maternal nicotine use to increased susceptibility to interstitial pulmonary fibrosis in adult male but not female offspring, indicating sex-dependent effects of developmental nicotine exposure.

1. Introduction

Maternal cigarette smoking is a worldwide health concern. Because inhaled nicotine crosses the fetoplacental barrier and appears in mother's milk, the growing fetus may be affected by nicotine. It has been shown that *in utero* nicotine exposure adversely affects fetal lung development and pulmonary function, causing lower respiratory tract illness in both human and rat fetuses and neonates [1,2]. However, whether and to what extent fetal and early postnatal nicotine exposure has long term effects in giving rise to adverse pulmonary conditions in adult offspring remains unclear. Additionally, it is unknown whether male and female offspring are affected differently. Furthermore, the potential mechanisms of nicotine-induced fetal programming of aberrant lung development are unknown.

Both transforming growth factor (TGF)-β1 and the receptor linked Smads, in particular Smad3 are critically required for rat lung and alveolar development [3]. It has been shown that alveolar development is inhibited in Smad3 [4,5] null mice. Also, disruption of the plasminogen activator inhibitor-1 (PAI-I) gene in mouse results increases in alveolar size and damaged alveolar structure [6]. Both Smad3 and PAI-1 are downstream mediators of TGF-β signaling that support the process of alveolarization. Surprisingly, the overexpression of biologically active TGF-β1 also inhibits the alveolarization process in newborn rat lungs [7]. Hence the magnitude of TGF-β signaling that regulates the alveolar development is critically controlled throughout lung development in rodents as well as humans [8]. Tight control of the TGF-β1 signaling is crucial because an overexpression of TGF-β1 invokes fibrosis, inflammatory cells and induces transdifferentiation of the rat alveolar epithelial cells (AEC) to a mesenchymal phenotype [7]. Transdifferentiation of AEC to a mesenchymal cell type could trigger a fibroproliferative response in the lung. An upstream signal that regulates the TGF-β1 pathway is angiotensin II (Ang II) *via* Ang II-type 1 receptor (AT1R) in human lungs in idiopathic pulmonary fibrosis (IPF) [9]. It has been demonstrated that compared to normal lungs, IPF lungs produce more Ang II and increase the synthesis of the biologically active TGF-β1 protein and TGF-β1 mRNA, resulting in lung fibrotic response that is blocked by ACE inhibitors or AT1R antagonists [9]. Thus, the present study tests the hypothesis that maternal nicotine administration during gestation, and thru postnatal day 10 (PND10) causes an altered programming of Ang II receptor expression

Abbreviations: ACE, angiotensin converting enzyme; AEC, alveolar epithelial cells; Ang II, angiotensin II; AT1R, Ang II-type 1 subtype receptor; AT2R, Ang II-type 2 subtype receptor; CTGF, connective tissue growth factor; EMT, epithelial-mesenchymal transitions; IPF, idiopathic pulmonary fibrosis (IPF); PMSF, phenyl methyl sulfonyl fluoride; PND, postnatal day; TGF, transforming growth factor.

* Corresponding author. Tel.: +1 909 558 4325; fax: +1 909 558 4029.
E-mail address: cdasgupta@llu.edu (C. Dasgupta).

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and TGF-β/Smad3 signaling pathways, resulting in the aberrant alveolar development and interstitial pulmonary fibrosis in adult male rats in a gender-dependent manner.

2. Materials and methods

2.1. Animal protocols

Time-dated pregnant Sprague–Dawley rats (Charles River Laboratories) were used in the present study. Nicotine was administrated through osmotic minipumps implanted *s.c.* as described previously [10]. Briefly, on the 4th day of pregnancy, rats were anesthetized with ketamine and xylazine, and an incision was made on the back to insert osmotic minipumps. The incision was closed with four sutures. Half of the pregnant rats were implanted with minipumps containing nicotine at a concentration of 102 mg/ml, and the other half were implanted with minipumps containing only sterile normal saline, which served as the vehicle control. The minipumps delivered nicotine at a dose of 4 μg/kg/min with blood concentrations closely resembles those occurring in moderate to heavy human smokers [11]. According to the manufacturer's specifications, the delivery period for the pumps is 28 days, so delivery continued after birth until postnatal day 10. Nicotine treatment did not affect the length of gestation, and all of the pregnancies reached their full term. Pups born to the dams were kept with their mothers until weaning. After that time, male and female pups were separated and transferred to cages where they were housed in groups of two. All male and female offspring were killed at 3 months of age, and lungs were isolated for histological and protein studies. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Measurement of body weight, lung weight and volume

For this study, five control and five nicotine exposed adult offspring belonging to both genders were used ($n = 5$), and their body weight determined. Next, from these animals, lungs with all lobes were isolated; trachea and bronchial tubes were removed. Lung wet weight was determined. Lung volume was measured following the method described by Scherle [12] with rat liver. Briefly, fixed lungs were submerged in a beaker filled with normal saline up to a preset mark. The organ was then removed. The lung volume was determined by the volume of liquid that had to be replaced until the beaker was filled back to the preset mark, and was expressed as cubic centimeter (cm^3, *syn* ml).

2.3. Preparation of lung tissue for histology

Formaldehyde was used to fix the lung tissue, as described recently by the Joint ATS/ERS task force in the lung tissue preparation for light microscopy and diagnostic pathology [13]. Lungs were perfused through the trachea with 10% formalin–phosphate buffered saline (PBS) solution at 20 cm H_2O pressure, as previously described in mouse and rat lungs [7,14]. After 24 h formalin fixation, lungs were changed to 70% ethanol to avoid overfixation followed by washes with two quick changes of cold PBS to remove external debris. Lungs were then transferred to filtered sterile PBS/30% sucrose (w/v) solution (changed frequently) and stored at 4 °C until the lung is fully equilibrated, and sinks at the bottom of the solution container. Lungs were next paraffin-embedded by the standard protocol, and 5 μm serial transverse sections were cut, and laid on Superfrost microscope glass slides.

2.4. Lung morphometry

For this study, four control and four nicotine exposed adult offspring belonging to both genders were used ($n = 4$). For histological and morphometric analyses, lung tissue from each animal were fixed, and sectioned as described above (Section 2.3). Lung sections were dewaxed by heating at 55 °C in a convection oven for 10 min as per recommendations from BD Biosciences, and then de-paraffinized in xylene. Sections were sequentially rehydrated in alcohol and stained in hematoxylin followed by eosin (H&E staining), then dehydrated again to be mounted. Stereologic analysis of lung morphometry was performed by examining nine serial sections obtained from the base, middle, and apex portions, respectively, in each lung. The extent of alveolarization was determined by the radial alveolar count (RAC) method as described previously with human lungs [15]. The number of alveolar septa that were transected by a perpendicular line drawn from the terminal bronchiole to the nearest connective tissue septum was counted. No counts were made if the respiratory bronchiole was nearer to the edge of the slide than to the nearest connective tissue septum. Alveolar septal thickness (AST) was determined near the center of septae from 30 to 40 different alveoli in each section with the Image-Pro Plus 6.0 image analysis software, as described recently in mouse lungs [16]. At least three different sections from each lung were used for these measurements, and for collagen staining (Section 2.5).

2.5. Collagen staining

Collagen specific picrosirius red staining was performed to reveal collagen deposition in lungs between the control and nicotine treated groups in both genders. Lung paraffin sections were dewaxed, deparaffinized by xylene and rehydrated sequentially in ethanol. Tissue sections were then hematoxylin stained and washed for 10 min in water. Next the sections were stained in 0.1% picrosirius red (Sirius Red F3B: 1 gm in 1000 ml of saturated picric acid solution) for 1 h and then washed in two changes of acidified water (5 ml glacial acetic acid in 1 liter of water). Tissue sections were then dehydrated again by increasing concentration of ethanol up to 100%, followed by wash in xylene and mounted. Picrosirius red staining of lung sections was examined with a Zeiss microscope and images were captured with an attached SPOT digital camera. The relative pixel intensities of picrosirius red staining in control and nicotine-treated samples were measured using Gel Logic 112 (Kodak) software, and were expressed as fold of control.

2.6. Protein extraction and Western blotting

For this study, five control and five nicotine exposed adult offspring lungs belonging to both genders were used ($n = 5$). Lung tissues were homogenized with a tissue grinder in RIPA buffer containing 1 mM EDTA and EGTA (Boston Bioproducts, MA), supplemented with 1 mM phenyl methyl sulfonyl fluoride (PMSF), complete proteinase inhibitor cocktail (Roche Diagnostics), and phosphatase inhibitor cocktail 2 (Sigma–Aldrich). Samples with equal amounts of protein were loaded onto 10% polyacrylamide gel with 0.1% SDS and separated by electrophoresis at 100 V for 90 min. Proteins were then transferred onto 0.45 μm PVDF membrane. Nonspecific binding sites was blocked for 1 h at room temperature in TBS-Tween-20 (TBST) plus 5% milk. The membranes were then probed with primary antibodies against collagen 1 (1:400), collagen 3 (1:400), AT1R (1:400), angiotensin II type 2 receptor (AT2R) (1:400), TGF-β1 (1:400), connective tissue growth factor (CTGF) (1:400) (Santa Cruz Biotechnology), pSMAD3 (1:400) and Smad3 (1:400) (Cell signaling) in TBST + 5% milk overnight at 4 °C. This was followed by incubation with anti-rabbit secondary antibody at 1:2000 dilution. Proteins were visualized with ECL western blotting detection reagents (GE Healthcare) on autorad film. The results were analyzed with the Kodak ID image analysis software. To ensure equal loading, band intensities were normalized to β-actin on the respective blots.

2.7. Statistical analysis

Data are expressed as mean ± SEM. Experimental number (n) represents offspring from different dams. Statistical significance ($P < 0.05$) between control and nicotine-treated animals were determined by two-way ANOVA followed by Neuman–Keul's *post hoc* analysis. Where applicable Student's *t*-test was used.

3. Results

3.1. Body weight, lung weight, and lung volume

The litter size was the same between the control and nicotine-treated groups. As shown in Fig. 1, while body weight (BW) and lung weight (LW) were significantly higher in male offspring than those in females, the LW/BW ratio was significantly lower in males than females in control animals. Maternal nicotine administration had no significant effect on BW in either gender groups (Fig. 1A). In contrast, the nicotine treatment selectively lowered the LW in adult female offspring in a gender-dependent manner, resulting in significantly reduced LW/BW ratio in females (Fig. 1B and C). In the nicotine-treated animals there was no significant difference in the LW/BW ratio between male and female offspring (Fig. 1C). Compared to the difference in the lung weight, males have disproportionally larger lung volume than that of females (Fig. 1D). Antenatal nicotine had no significant effect on the lung volume in males, but significantly increased it in females (Fig. 1D).

3.2. Lung morphology and morphometry

Fig. 2 shows representative H&E stained lung sections in control and antenatal nicotine-treated male and female offspring. In female lungs, there appear to be no significant changes in the size, number of alveoli and alveolar sacs between control and nicotine-treated animals. Compared with female lungs, male lungs appear to have more alveoli (Fig. 2). Unlike in female lungs,