Perinatal Nicotine-Induced Transgenerational Asthma

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Running Title: Transgenerational Transmission of Asthma

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ABSTRACT
Asthma is a major public health hazard world-wide. Its transgenerational inheritance has been inferred from epidemiologic studies. More recently, using nicotine as a proxy for maternal smoking, we have demonstrated that an asthma-like phenotype can be inherited by rat offspring for up to two generations, i.e., multigenerationally, after the initial intrauterine exposure. We hypothesized that asthma transmission to offspring following perinatal nicotine exposure is not restricted up to F2 generation, but it also extends to subsequent generations. To test this hypothesis, using a well-established rat model of nicotine exposure-induced childhood asthma, we determined if perinatal nicotine exposure of F0 gestating dams would transmit asthma transgenerationally to F3 offspring. We now extend our findings to third generation offspring, including abnormal pulmonary function, particularly as it relates to the occurrence in the upper airway exclusively in males, and to its effects on molecular functional markers (fibronectin and PPARγ), previously shown to be consistent with the asthma phenotype, herein expressed in fibroblasts isolated from the lung. These data, for the first time, demonstrate the transgenerational transmission of the asthma phenotype to F3 offspring following perinatal nicotine exposure of F0 dams.

Key Words: Asthma, Transgenerational, PPARγ, Nicotine, Maternal smoking, Pulmonary function
INTRODUCTION

Asthma is a major public health problem, with an estimated 300 million people affected worldwide, and this number is projected to increase to 400 million by 2025 (1,25). Although a multitude of causes contribute to childhood asthma, maternal smoking during pregnancy is a well-established contributor (6,7,9,10,23,30), being a major modifiable risk factor, the elimination of which could significantly reduce the prevalence of childhood asthma. The recognition that childhood asthma is induced by smoke exposure during pregnancy is particularly important since there is emerging evidence that, following in utero exposure to maternal smoke, asthma can be transmitted multigenerationally (18). Yet, up until our recent demonstration of the multigenerational transmission of asthma in an animal model (35), there was neither experimental evidence nor any mechanistic explanation for this phenomenon. Using a well-established rat model of in utero nicotine exposure for childhood asthma (12,13,19,20,24,34), we recently demonstrated that following perinatal exposure to nicotine during F0 pregnancy (17,35), asthma can be inherited by rat offspring up to the F2 generation, i.e., multigenerationally. Now, we hypothesize that asthma transmission to offspring following perinatal nicotine exposure is not restricted up to F2 generation, but it also extends to subsequent generations. Using our rat model of perinatal nicotine exposure-induced childhood asthma [16,19, 20, 34, 35 ], in this study we aimed to determine if perinatal nicotine exposure of F0 gestating dams would transmit asthma transgenerationally to F3 offspring.
For clarification, it is important to point out the difference between multigenerational vs. transgenerational transmission of an acquired trait. Transmission of a trait from the gestating F0 dam up to F2 generation offspring, in response to an environmental exposure, for example, asthma in the case of in utero nicotine exposure, constitutes multigenerational inheritance; however, such inheritance is not “transgenerational” since the transmission of the acquired trait is likely to be due to the direct exposure of the F2 generation germline to the environmental challenge during the F0 gestation. In contrast, transmission of an induced trait following exposure to an environmental challenge during the F0 generation out to the F3 generation, the generation that’s not directly exposed to the environmental challenge, constitutes “transgenerational” inheritance such as that reported in this manuscript.

METHODS

Materials- Nicotine bitartrate was acquired from Sigma-Aldrich (St. Louis, MO, USA). All plastic ware and culture media were purchased from Corning (Corning, NY, USA) and Invitrogen, Inc. (San Diego, CA, USA).

The Animal Model: Time-mated, first-time pregnant, pair-fed Sprague Dawley rat dams (F0) received either placebo (diluent) or nicotine [1 mg/kg, subcutaneously], in 100 µl volumes daily from embryonic day (e) 6 of gestation to postnatal day (PND) 21. Following delivery at term, the F1 pups were allowed to breast feed ad libitum. At PND21, the pups were subjected to pulmonary function tests (PFTs), tracheal tension
and airway contractility studies, and mesenchymal differentiation mRNA and protein levels [fibronectin and peroxisome proliferator-activated receptor γ (PPARγ)] determinations, as described below. Some F1 male and female littermates were weaned at PND21, and maintained in separate cages as breeders to generate F2 rats, but without any subsequent exposure to nicotine in the F1 or F2 pregnancies. F3 pups were similarly obtained, using F2 offspring as breeders. At PND21, F3 pups underwent studies similar to those performed on F1 rats.

**Pulmonary Function Testing:** Measurement of respiratory function was performed using plethysmography for restrained animals (Buxco Inc, Troy, NY) as described by us previously (19,35). Briefly, the pups were deeply anesthetized and sedated with ketamine (70 mg/kg, Bioniche Teoranta Inverin, Co., Galway, Ireland) and xylazine (7 mg/kg, Akorn, Inc., Decatur, IL), tracheostomized and ventilated. Then the pups were exposed to increasing concentrations of aerosolized methacholine (0, 1.25, 2.5, 5, 10, and 20 mg/ml) over a period of 3 minutes each, and lung resistance (Rrs) and dynamic compliance (Cdyn) were subsequently measured, and plotted as a function of the methacholine concentration administered.

**Tracheal tension studies.** The whole trachea was excised immediately after sacrifice, and dissected free of connective tissue in ice-cold modified Krebs-Ringer bicarbonate buffer (expressed as mM concentrations: 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 11.1 glucose). Approximately a 6 mm tracheal ring was resected from the mid-section of each trachea, and suspended in an organ chamber
containing 10 ml of modified Krebs-Ringer bicarbonate buffer solution maintained at 37 ± 0.5°C, and aerated with 95% O₂-5% CO₂ (pH 7.4). Each ring was suspended via two stirrups, each passed through the lumen: one stirrup was anchored to the bottom of the organ chamber, and the other stirrup was connected to a strain gauge (model FT03C, Grass Instrument, Quincy, MA) for the measurement of isometric force, as described previously (5).

For tracheal tension measurements, each tracheal ring was initially stretched to its optimal resting tension, which was achieved by step-wise stretching in 0.1-g increments, until the contractile response to 100 mM KCl reached a plateau. Each tracheal ring was allowed to equilibrate for one hour, after which the effect of acetylcholine was determined at least 30 min after the administration of nitro-L-arginine (1×10⁻⁴ M, an inhibitor of nitric oxide synthase). In all experiments, indomethacin (1×10⁻⁵ M) was added to the bath to prevent possible interference by prostanoids.

Lung fibroblast isolation. PND21 rat lung fibroblasts from both F1 and F3 generation pups were cultured using slight modifications of our previously described methods (40,41). Briefly, the lungs were trimmed to remove major airways, and rinsed with calcium- and magnesium-free Hanks' balanced salt solution (HBSS). Lung tissue was minced into 1 to 2-mm³ pieces, and was suspended in pre-warmed (37°C) digestion buffer containing 2.5 ml of heat-inactivated chicken serum (2.5 ml), Hepes (1.25 ml at 500 mM, pH 7.4), collagenase I (12.5 mg, Sigma Chemical, St.Louis, MO), Collagenase IA (12.5 mg, Sigma Chemical, St.Louis, MO) in Waymouth’s medium (in a final
volume of 25 ml). The tissue was tritured 100 times with a 10 ml pipette, 100 times
with a 5 ml pipette, and 100 times with a 9” Pasteur pipette. The tissue was further
dissociated in a 37°C water bath using a Teflon™ stirring bar to disrupt the tissue
mechanically. Once the tissue was dispersed into a unicellular suspension, the cells were
pelleted at 500 x g for 10 min at room temperature in a 50 ml polystyrene centrifuge
tube. The supernatant was decanted, and the pellet was resuspended in Minimal
Essential Medium (MEM) containing 20% fetal bovine serum (FBS) to yield a mixed
cell suspension of ca. 3 x 10^8 cells, as determined with a Coulter particle counter
(Beckman-Coulter, Hayaleah, FL). The cell suspension was then added to tissue culture
flasks (75 cm²) for 30-60 min to allow for differential adherence of the lung fibroblasts.
These cells are greater than 95% pure fibroblasts based upon their morphologic
appearance when viewed at the light microscopic level, and by immunohistochemical
staining for vimentin.

Western analysis. Western analysis on protein lysates from cultured lung fibroblasts
from F1 and F3 generation pups was performed according to previously described
methods (37). The protein concentration of the supernatant was measured by the
Bradford method, using bovine serum albumin as the standard. Aliquots of the
supernatant, each containing 30 μg of protein, were separated by SDS-PAGE gel, and
electrically transferred to nitrocellulose membranes. Nonspecific binding sites were
blocked by incubation with Tris-buffered saline (TBS) containing 5% nonfat dry
powdered milk (w/v) for 1h at room temperature. After a brief rinse with TBS
containing 0.1% tween 20 (TBST), the protein blots were incubated in primary antibody
(PPAR\textsubscript{γ}, 1:500, Santa Cruz, Cat #: sc-7196; fibronectin, 1:1,000, BD Biosciences, Cat. N#: 610078; or GAPDH, 1:4,000, MILLIPORE, Cat. #: MAB374), overnight at 4°C followed by incubation with an appropriate secondary antibody for 1h at room temperature. After three more washes in TBST, the blots were exposed to X-ray film using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL), and developed. The relative densities of the protein bands were determined with UN-SCAN-IT software (Silk Scientific Inc, Orem, Utah), and normalized to that of GAPDH.

**Real Time RT-PCR.** In brief, total RNA was isolated using an RNAqueous-4PCR kit (Ambion), and was DNase-treated and quantitated by light absorbance using a Nanodrop™ spectrophotometer (Nanodrop Instruments, Wilmington, DE). The structural integrity of the RNA was assessed based on the visual appearance of the ethidium bromide-stained ribosomal bands following fractionation on a 1.2% (wt/vol) agarose-formaldehyde gel, and quantitated by light absorbance at 260 nm. 1 \( \mu \)g of total RNA was reverse-transcribed into single-stranded cDNA using a TaqMan Gold™ RT-PCR Kit at 50°C for 30 min in a total volume of 20 \( \mu l \). The PCR reaction mix consisted of 1 \( \mu l \) of 10-fold diluted cDNA, PCR Gold™ DNA polymerase reagent mix, and optimized for forward and reverse gene specific primers (900 nMs each) with a gene-specific probe (250 nM, FAM dye label). Primer sets were purchased pre-designed (TaqMan Gene Expression™ Assays, Applied Biosystems). Real-Time PCR reactions were run in triplicate on 96 well plates using an ABI PRISM 7900 HT Sequence Detection System™ (Applied Biosystems, Foster City, CA). Reactions proceeded by
activation of DNA polymerase at 95°C for 10 min, followed by 38 PCR denaturing cycles at 95°C for 15 sec, and annealing/extension at 60°C for 1 min. Data were normalized to 18S ribosomal RNA using an RNA TaqMan Gene Expression™ Assay, and were analyzed to select a threshold level of fluorescence that was in the linear phase for PCR product accumulation [the threshold cycle (C_T) for that reaction]. The C_T value for 18S ribosomal RNA was subtracted from the C_T value for the gene of interest to obtain a delta C_T (ΔCT) value. The relative fold-change for each gene was calculated using the ΔΔCT method. Results are expressed as the mean ± SE, and considered significant at p < 0.05. RT-PCR probes used included rat PPARγ: F-5′-CCAAGTGACTCTGCTCAAGTATGG-3′ and R-5′-CATGAATCCTTGTCCCTCTGATATG-3′ (106bp); rat fibronectin: F-5′-AGCACACCCGTTTTCATCCA-3′ and R-5′-TTTCACGTCGGTCACTTCCA; and rat 18s: 5′ TTAAAGCCATGCATGTCTAAGTAC and 3′ TGTTATTTTTGCTCAGCTACCTCC.

**Immunofluorescence staining:** Rat lungs were inflation fixed *in situ* with 4% paraformaldehyde in phosphate buffer at a standard inflation pressure of 5 cm of H₂O. Fibronectin and PPARγ protein expression were assessed by double immunofluorescence staining. Briefly, 5μm sections were incubated with a mouse monoclonal antibody against fibronectin (1:500 dilution, BD Biosciences, San Jose, CA, catalog # 610078) and a rabbit polyclonal antibody against PPARγ (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, catalog # SC-7196) at 4°C overnight and then incubated with Alexa Fluor 594 goat anti-mouse IgG (1:500 dilution, Invitrogen, Carlsbad, CA, catalog # A31624 for fibronectin) and Alexa Fluor 488 goat...
anti-rabbit IgG (1:50 dilution, Invitrogen, Carlsbad, CA, catalog # A31628 for PPARγ) was applied to the sections for 1h at room temperature. The sections were washed with phosphate buffered saline, and then mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA) for visualization under a fluorescence microscope.

Statistics. The data for analysis were obtained from at least three independent sets of experiments. Analysis of variance (ANOVA) for multiple comparisons with Bonferroni post-hoc analysis and Student’s t-test were used as indicated, and $p < 0.05$ was considered to indicate statistically significant differences among the experimental groups.

RESULTS

We initially determined the effects of perinatal nicotine exposure on total respiratory system resistance (Rrs) and compliance (Cdyn) in F3 generation rats at postnatal day 21 following a methacholine challenge, and compared these data with our previously published data in F1 generation rats [please note that F1 generation data included here for comparison with F3 generation data have been published previously (35)]. Similar to the F1 generation data, compared to the control group, with perinatal nicotine exposure only to F0 dams, there was a significant increase in Rrs, and a decrease in Cdyn of the total respiratory system following the methacholine challenge in F3 rats, even though the F3 rats were not exposed to any nicotine either during gestation or postnatally (Fig. 1). After establishing this experimental evidence for the transgenerational (TG) transmission of perinatal nicotine exposure-induced asthma, we then examined whether
this TG transmission of asthma was gender-specific. We found that, compared to controls, with perinatal nicotine exposure only to F0 dams, the total airway Rrs increased, and the total airway Cdyn decreased in both the males and females in both the F1 and F3 generations, but these changes were significantly greater in the males than the females in both generations (Fig. 2). Since we previously found that in the F1 generation the effect of perinatal nicotine exposure on the tracheal constriction response was gender-specific, i.e., seen exclusively in males (19), we next determined if the same was true for the F3 generation, and found a similar differential gender effect in the F3 generation as well (Fig. 3). It is important to point out that the gender composition and male:female ratios of the F1 and F3 animals studied were similar (Table 1). To determine the potential mechanism of airway hyperresponsiveness to the methacholine challenge, we next determined the expression of airway contractility and differentiation markers by the lung fibroblasts isolated from the F3 generation rats. Similar to our findings for the changes in the levels of these markers in F1 rat lungs, as determined by Real Time RT-PCR and Western analysis (19,20), both fibronectin mRNA and protein levels were increased, and PPARγ mRNA and protein levels were decreased in cultured fibroblasts from F3 rats (Fig. 4A and 4B), providing further evidence for the nicotine-induced TG transmission of the lung cellular/molecular phenotype from the F1 through the F3 generation. The up-regulation of fibronectin and down-regulation of PPARγ protein levels in both F1 and F3 generation rat lungs was also corroborated by immunofluorescence staining of lung sections for these same proteins (Fig. 4C and 4D).
DISCUSSION

We have previously observed significant effects of nicotine treatment on lung function in generations F1 and F2, constituting ‘multigenerational’ inheritance (17,35). In the present study, using the same experimental approach, we now document a transgenerational effect of perinatal nicotine exposure on lung function for the first time, i.e. F1-F3. As in the previous study, we have observed significant effects of nicotine treatment on lung function in the F3 generation, affecting both the male and female offspring, nicotine treatment only affecting the tracheal contractility of the male offspring. These functional effects of nicotine were again accompanied by increased expression of the myogenic protein fibronectin, but decreased expression of PPARγ in the isolated lung fibroblasts, consistent with the effect of nicotine on myofibroblast differentiation (42,43).

Our previously-reported study of the multigenerational effect of maternal nicotine treatment on the asthma phenotype was groundbreaking in our understanding of the putative mechanisms involved in the transmission of epigenetic human disease, which to date has only been speculated, albeit based on strong epidemiologic grounds (3). The fact that in this instance the phenotypic effect was on an asthma-like phenotype, a well-recognized epidemiologic example for the epigenetic transmission of the cause of a public health epidemic, makes this series of experiments all the more significant and compelling, heralding a new and rational way of more effectively coping with the rising asthma epidemic (1,25,28,39,45). Moreover, since we had found that in our previous study of F1 and F2 generation pups most of the nicotine-induced lung and gonadal
epigenetic changes were normalized by treatment with the PPARγ agonist rosiglitazone, and since we have observed decreased PPARγ expression in the F3 generation, we predict that its up-regulation will normalize the asthma-like phenotype here too. Furthermore, in line with our previous data in F1 offspring, there was a gender-specific tracheal contractility response in F3 offspring as well, with increased nicotine exposure-induced contractility seen only in the males. Though the mechanism underlying this phenomenon remains to be determined, we speculate that it might reflect differential up-regulation of Wnt signaling in the male upper airway, as has been previously observed in F1 generation offspring (19).

The evidence for fetal programming as a mode of TG transmission of phenotypic traits in humans is limited- for example, mothers from the Dutch Hunger Winter who were exposed to famine as fetuses delivered offspring of lower birth weight than those with no fetal exposure to famine (21). There is also evidence for increased morbidity and mortality associated with parental and grandparental nutritional status, suggesting a role for fetal programming, possibly via epigenetic mechanisms, accounting for the TG effects (2,14,32).

In contrast to such limited human data, in a variety of animal models gestational exposure to carcinogens, endocrine disruptors, or other toxins has been claimed to have TG effects; however, most of these studies have not determined such effects beyond the 2nd generation (22,27,36,46). Some examples are the multigenerational effect of overfeeding causing the diabetic phenotype (33), prenatal exposure to allergens (31) and
hepatotoxicity of carbon tetrachloride (47). It is noteworthy that the latter model strongly parallels our findings for the generational effects of nicotine, since hyperactive myofibroblasts are implicated in the pathobiology of both asthma and liver fibrosis. However the only valid examples of TG inheritance in the literature are those of an altered stress response (4), and multiple structural and functional alterations (22) in F3 rat offspring exposed to either vinclozolin (38), or plastic-derived endocrine disruptors such as bisphenol-A, bis (2-ethylhexyl) phthalate, or dibutyl phthalate (DBP) to gestating F0 females (22), respectively.

The compelling data presented herein provide the first experimental evidence for TG transmission of an asthma-like phenotype following in utero exposure to maternal smoking, potentially shifting the current paradigm for our understanding of childhood asthma. These data pave the way for determining the epigenetic mechanisms, such as DNA methylation, histone modifications, and non-coding RNA production, likely underlying in utero smoke exposure-induced TG transmission of asthma (8,11,26,29,44).
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Figure Legends

Fig.1. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F1 and F3 rats. Compared to the control group, with nicotine administration to rat dams there was a significant increase in total airway resistance (Rrs) and a decrease in total compliance (Cdyn) following methacholine (Mch) challenge in both F1 (left panel) and F3 (right panel) rat offspring of mixed gender, even though the F3 rats were not exposed to any nicotine during the F 1 and 2 gestations. Values are means ± SE. n = 10 to 12 for each group. *P < 0.05, **P < 0.01, versus control.

Fig.2. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F1 and F3 male and female rats. Compared to the control group, nicotine treatment significantly increased the resistance (Rrs) and decreased the compliance (Cdyn) of the lung following methacholine (Mch) challenge in F1 (left panel) and F3 (right panel) male (open diamonds) and female (closed diamonds) rats even though the F1 and F2 pups were not exposed to nicotine during gestation. Values are means ± SE. n = 5 to 6 for each group. *P < 0.05, **P < 0.01 versus control.
Fig. 3. Effect of perinatal nicotine exposure on tracheal constriction response to acetylcholine in F1 and F3 male and female rats. Compared to the control group, with perinatal nicotine administration there was a significant increase in tracheal constriction in response to acetylcholine only in males (upper panel), not in females (lower panel) in both F1 (left panel) and F3 (right panel) rats. Values are means±SE; *p<0.05, **p<0.01 vs. control group; n=4.

Fig. 4. Effect of perinatal nicotine exposure in F0 generation on the levels of mesenchymal markers of airway differentiation in lung fibroblasts derived from F1 and F3 rats. Compared to controls, with nicotine exposure to F0 dams, mRNA (Fig. 4A) and protein (Fig. 4B) levels of fibronectin increased, whereas that of PPARγ decreased in fibroblasts isolated from F1 and F3 rat lungs. Values are means±SE. N=3; *p<0.05 vs. control. The up-regulation of fibronectin (red staining, white arrows) and down-regulation of PPARγ (green staining, yellow arrows) protein levels in both F1 and F3 generation rat lungs was corroborated by immunofluorescence staining lung sections for these proteins (Fig. 4C). The mean fluorescence intensity for fibronectin and PPARγ staining of 6 comparable lung fields from each group, quantified using ImageJ software, is shown (Fig. 4D). Values are means±SE; **p<0.01 vs. control group; n=3.
Fig. 1

**Fig. 1**

**F1**

- Rrs (Arbitrary Unit)
- Control
- Nicotine
- Methacholine (mg/ml)

**F3**

- Rrs (Arbitrary Unit)
- Control
- Nicotine
- Methacholine (mg/ml)

**Fig. 1**

**F1**

- Cdyn (Arbitrary Unit)
- Control
- Nicotine
- Methacholine (mg/ml)

**F3**

- Cdyn (Arbitrary Unit)
- Control
- Nicotine
- Methacholine (mg/ml)
Fig. 2

**F1**

- **Rrs (Arbitrary Unit)**
- **Methacholine (mg/ml)**
- **Male**
- **Female**

**F3**

- **Rrs (Arbitrary Unit)**
- **Methacholine (mg/ml)**
- **Male**
- **Female**
Fig. 3

F1

Male

F3

Female

Control
Nicotine

Tension (% of KCl)

Ach (-Log M)

**

*
**Fig. 4A**

**F1**

![Bar chart showing relative fibronectin mRNA level in F1.](chart1)

**F3**

![Bar chart showing relative fibronectin mRNA level in F3.](chart2)

**Nicotine**

![Bar chart showing relative PPARγ mRNA level in F1.](chart3)

![Bar chart showing relative PPARγ mRNA level in F3.](chart4)

*P<0.05
Fig. 4B

G1

Fibronectin

PPAR\(\gamma\)

GAPDH

205KD

68KD

37KD

C     Nicotine              C     Nicotine

0

0.2

0.4

0.6

0.8

1

1.2

1.4

1.6

C     Nicotine            C     Nicotine

Fibronectin/GAPDH

PPAR\(\gamma\)/GAPDH
Fig. 4D

**Mean Fluorescence Intensity (Arbitrary Unit)**

- **F-1**
  - Fibronectin: **Control**, **Nicotine**
  - PPARγ: **Control**, **Nicotine**

- **F-3**
  - Fibronectin: **Control**, **Nicotine**
  - PPARγ: **Control**, **Nicotine**

**Significance Levels**: **P < 0.01**
Table 1. Gender Composition in Figure 1.

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