

Perinatal nicotine-induced transgenerational asthma

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Rehan VK, Liu J, Sakurai R, Torday JS. Perinatal nicotine-induced transgenerational asthma. *Am J Physiol Lung Cell Mol Physiol* 305: L501–L507, 2013. First published August 2, 2013; doi:10.1152/ajplung.00078.2013.—Asthma is a major public health hazard worldwide. Its transgenerational inheritance has been inferred from epidemiological 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 peroxisome proliferator-activated receptor γ), 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.

peroxisome proliferator-activated receptor γ ; maternal smoking; pulmonary function

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 (TG) 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 TG inheritance such as that reported in this manuscript.

METHODS

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

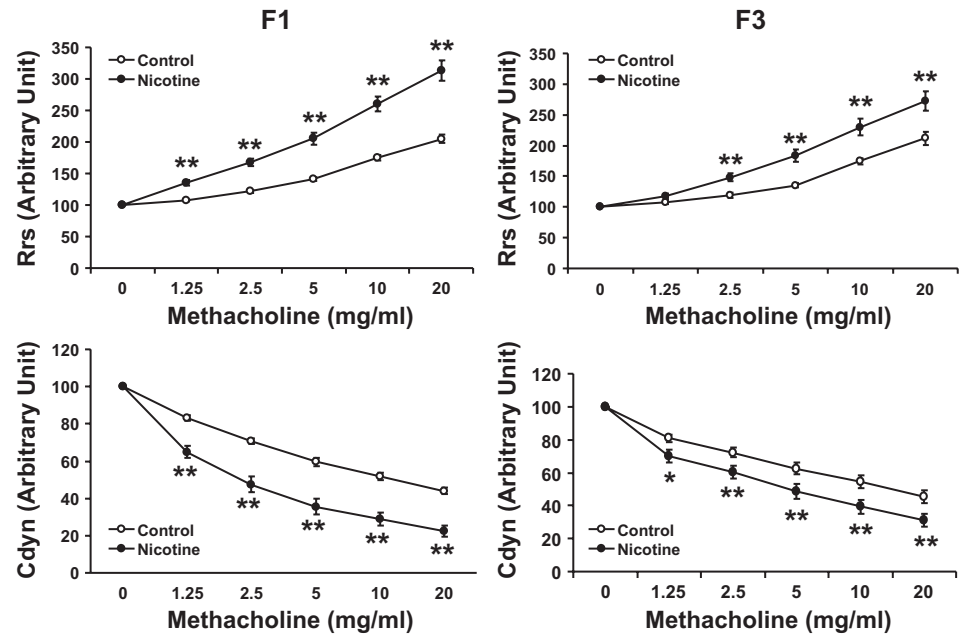
The animal model. Time-mated, first-time pregnant, pair-fed Sprague Dawley rat dams (F₀) received either placebo (diluent) or nicotine (1 mg/kg sc) in 100- μ l volumes daily from *embryonic day 6* of gestation to *postnatal day* (PND) 21. Following delivery at term, the F₁ pups were allowed to breast feed ad libitum. At PND21, the pups were subjected to pulmonary function tests, tracheal tension and airway contractility studies, and mesenchymal differentiation mRNA and protein level [fibronectin and peroxisome proliferator-activated receptor γ (PPAR γ)] 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, 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, Galway, Ireland) and xylazine (7 mg/kg; Akorn, Decatur, IL), tracheostomized, and ventilated. Next, 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 min each, and lung resistance (Rrs) and dynamic compliance (C_{dyn}) were subsequently measured and plotted as a function of the methacholine concentration administered.

Tracheal tension studies. The whole trachea was excised immediately after death 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,

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Fig. 1. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F_1 and F_3 rats. Compared with 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 F_1 (left) and F_3 (right) rat offspring of mixed gender, even though the F_3 rats were not exposed to any nicotine during the F_1 and F_2 gestations. Values are means $\pm$ SE; $n = 10$ –12 rats for each group. * $P < 0.05$ and ** $P < 0.01$ vs. control.



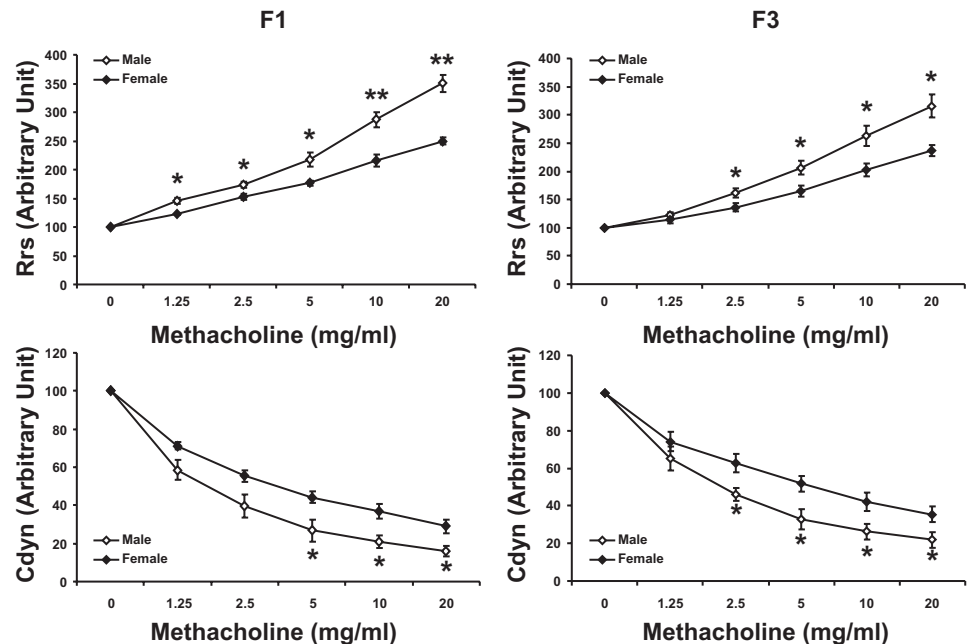
25.0 NaHCO_3 , and 11.1 glucose). Approximately a 6-mm tracheal ring was resected from the midsection of each trachea and suspended in an organ chamber containing 10 ml of modified Krebs-Ringer bicarbonate buffer solution maintained at $37 \pm 0.5^\circ\text{C}$ and aerated with 95% O_2 -5% CO_2 (pH 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 stepwise 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 1 h, 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 prostanooids.

Lung fibroblast isolation. PND21 rat lung fibroblasts from both F_1 and F_3 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. Lung tissue was minced into 1- to 2-mm³ pieces and was suspended in prewarmed (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), and collagenase 1A (12.5 mg; Sigma Chemical) in Waymouth's medium (in a final volume of 25 ml). The tissue was triturated 100 times with a 10-ml pipette, 100 times with a 5-ml pipette, and 100 times with a 9-in. Pasteur pipette. The tissue was

Fig. 2. Effect of perinatal nicotine exposure on total respiratory system resistance and compliance in F_1 and F_3 male and female rats. Compared with the control group, nicotine treatment significantly increased Rrs and decreased Cdyn of the lung following methacholine (Mch) challenge in F_1 (left) and F_3 (right) male (open diamonds) and female (closed diamonds) rats even though the F_1 and F_2 pups were not exposed to nicotine during gestation. Values are means $\pm$ SE; $n = 5$ –6 for each group. * $P < 0.05$ and ** $P < 0.01$ vs. control.



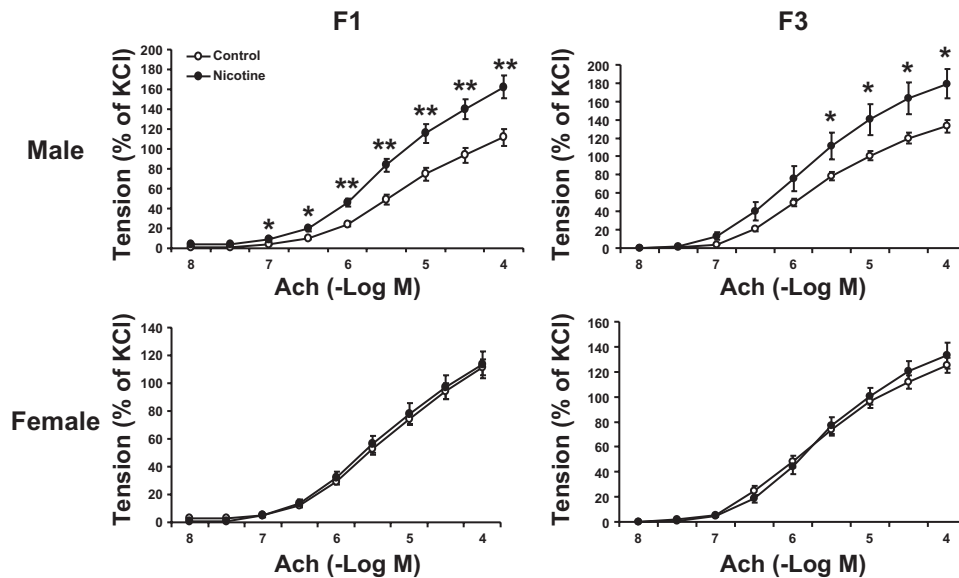


Fig. 3. Effect of perinatal nicotine exposure on tracheal constriction response to acetylcholine in F₁ and F₃ male and female rats. Compared with the control group, with perinatal nicotine administration there was a significant increase in tracheal constriction in response to acetylcholine only in males (top) and not in females (bottom) in both F₁ (left) and F₃ (right) rats. Values are means $\pm$ SE; * P < 0.05 and ** P < 0.01 vs. the control group; n = 4.

further dissociated in a 37°C water bath using a Teflon stirring bar to disrupt the tissue mechanically. Once the tissue was dispersed in a unicellular suspension, the cells were pelleted at 500 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 containing 20% fetal bovine serum to yield a mixed cell suspension of ca. 3×10^8 cells, as determined with a Coulter particle counter (Beckman-Coulter, Hialeah, 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 morphological 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 μ g 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 (wt/vol) for 1 h at room temperature. After a brief rinse with TBS containing 0.1% Tween 20 (TBST), the protein blots were incubated in primary antibody (PPAR γ , 1:500, Santa Cruz, catalog no. sc-7196; fibronectin, 1:1,000, BD Biosciences, catalog no. 610078; or GAPDH, 1:4,000, Millipore, catalog no. MAB374) overnight at 4°C followed by incubation with an appropriate secondary antibody for 1 h 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, Orem, UT) and normalized to that of GAPDH.

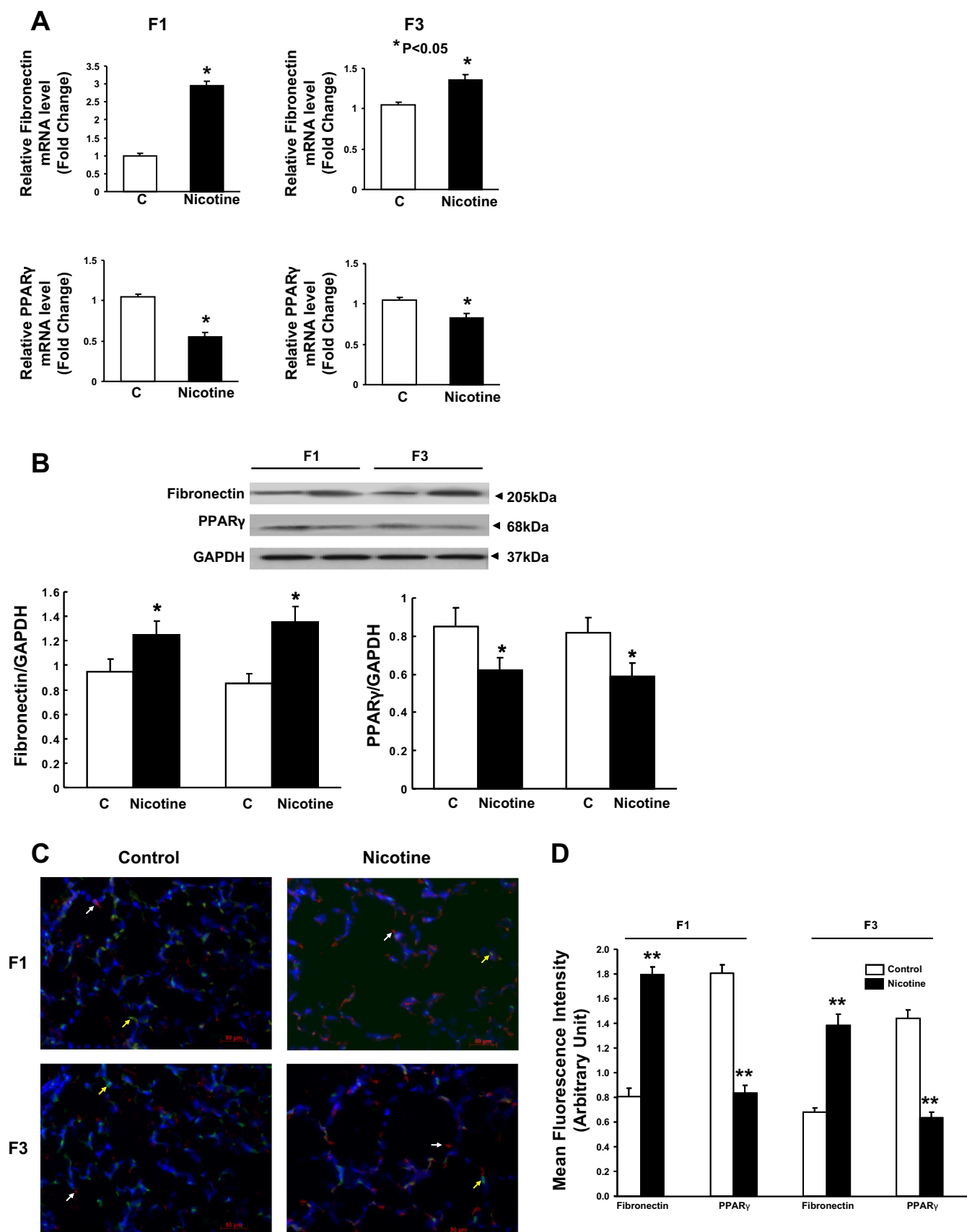
Real-time RT-PCR. In brief, total RNA was isolated using an RNAqueous-4PCR kit (Ambion) and was DNase-treated and quantitated by light absorbance using a Nanodrop spectrophotometer (Nanodrop Instruments, Wilmington, DE). The structural integrity of the RNA was assessed based on the visual appearance 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. Total RNA (1 μ g) 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 μ l. The PCR reaction mix consisted of 1 μ l of 10-fold diluted cDNA and PCR Gold DNA polymerase reagent mix, and optimized for forward and reverse gene-specific primers (900 nM each) with a gene-specific probe (250 nM, FAM dye label). Primer sets were purchased predesigned (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 s 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 (ΔC_T) value. The relative fold-change for each gene was calculated using the $\Delta\Delta C_T$ method. Results are expressed as means $\pm$ SE and considered significant at P < 0.05. RT-PCR probes used included rat PPAR γ : forward-5'-CCAAGTGACTCTGCTCAAGTATGG-3' and reverse-5'-CATGAATCCTTGTCCCTCTGATATG-3' (106 bp); rat fibronectin: forward-5'-AGCACACCCGTTTTCATCCA-3' and reverse-5'-TTTCACGTCGGTCACTTCCA; and rat 18s: 5'-TTA-AGCCATGCATGTCTAAGTAC and 3'-TGTTATTTTCGTCAC-TACCTCC.

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 PPAR γ protein expression were assessed by double-immunofluorescence staining. Briefly, 5- μ m sections were incubated with a mouse monoclonal antibody against fibronectin (1:500 dilution, catalog no. 610078; BD Biosciences, San Jose, CA) and a rabbit polyclonal antibody against PPAR γ (1:50 dilution, catalog no. SC-7196; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight and then incubated with Alexa Fluor 594 goat

Table 1. Gender composition in Figure 1

Generation	Group	Male	Female	Total No.
F ₁	Control	6	6	12
	Nicotine	6	6	12
F ₃	Control	7	6	13
	Nicotine	7	6	13



anti-mouse IgG (1:500 dilution, catalog no. A31624 for fibronectin; Invitrogen, Carlsbad, CA), and Alexa Fluor 488 goat anti-rabbit IgG (1:50 dilution, catalog no. A31628 for PPAR γ ; Invitrogen) was applied to the sections for 1 h at room temperature. The sections were washed with phosphate-buffered saline and then mounted with Pro-Long Gold antifade reagent with DAPI (Invitrogen) for visualization under a fluorescence microscope.

Statistics. The data for analysis were obtained from at least three independent sets of experiments. Analysis of variance 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 Rrs and Cdyn in F₃ generation rats at PND21 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 with 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 TG transmission of perinatal nicotine exposure-induced asthma, we then examined whether this TG transmission of asthma was gender specific. We found that, compared with 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). Because 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-to-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 PPAR γ mRNA and protein levels were decreased in cultured fibroblasts from F₃ rats (Fig. 4, A and B), providing further evidence for the nicotine-induced TG transmission of the lung

cellular/molecular phenotype from the F₁ through the F₃ generation. The upregulation of fibronectin and downregulation of PPAR γ protein levels in both F₁ and F₃ generation rat lungs was also corroborated by immunofluorescence staining of lung sections for these same proteins (Fig. 4, C and D).

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 TG 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 and 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 PPAR γ 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 epidemiological grounds (3). The fact that in this instance the phenotypic effect was on an asthma-like phenotype, a well-recognized epidemiological 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 PPAR γ agonist rosiglitazone, and since we have observed decreased PPAR γ expression in the F₃ generation, we predict that its upregulation 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. Although the mechanism underlying this phenomenon remains to be determined, we speculate that it might reflect differential upregulation 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

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 with controls, with nicotine exposure to F₀ dams, mRNA (A) and protein (B) levels of fibronectin increased, whereas that of peroxisome proliferator-activated receptor γ (PPAR γ) decreased in fibroblasts isolated from F₁ and F₃ rat lungs. Values are means $\pm$ SE; $n = 3$. * $P < 0.05$ vs. control. The upregulation of fibronectin (red staining, white arrows) and downregulation of PPAR γ (green staining, yellow arrows) protein levels in both F₁ and F₃ generation rat lungs were corroborated by immunofluorescence staining of lung sections for these proteins (C). The mean fluorescence intensity for fibronectin and PPAR γ staining of 6 comparable lung fields from each group, quantified using ImageJ software, is shown (D). Values are means $\pm$ SE. ** $P < 0.01$ vs. the control group; $n = 3$.

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 second 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 (4), or plastic-derived endocrine disruptors such as bisphenol-A, bis(2-ethylhexyl)phthalate, or dibutyl phthalate 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 noncoding RNA production, likely underlying in utero smoke exposure-induced TG transmission of asthma (8, 11, 26, 29, 44).

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: V.K.R. conception and design of research; V.K.R. and J.L. analyzed data; V.K.R. and J.S.T. interpreted results of experiments; V.K.R. drafted manuscript; V.K.R. and J.S.T. edited and revised manuscript; V.K.R., J.L., R.S., and J.S.T. approved final version of manuscript; J.L. and R.S. performed experiments; J.L. and R.S. prepared figures.

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