In utero nicotine exposure alters fetal rat lung alveolar type II cell proliferation, differentiation, and metabolism

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Submitted 24 February 2006; accepted in final form 23 September 2006

Rehan VK, Wang Y, Sugano S, Santos J, Patel S, Sakurai R, Boros LW, Lee W-P, Torday JS. In utero nicotine exposure alters fetal rat lung alveolar type II (ATII) cell proliferation, differentiation, and metabolism. Am J Physiol Lung Cell Mol Physiol 292: L323–L333, 2007; doi:10.1152/ajplung.00071.2006.—We recently suggested that alveolar interstitial fibroblast-to-myofibroblast transdifferentiation may be a key mechanism underlying in utero nicotine-induced lung injury. However, the effects of in utero nicotine exposure on fetal alveolar type II (ATII) cells have not been fully determined. Placebo, nicotine (1 mg/kg), or nicotine (1 mg/kg) + the peroxisome proliferator-activated receptor (PPAR)-γ agonist prostaglandin J2 (PGJ2, 0.3 mg/kg) was administered intraperitoneally once daily to time-mated pregnant Sprague-Dawley rats from embryonic day 6 until their death on embryonic day 20. Fetal ATII cells were isolated, and ATII cell proliferation, differentiation (surfactant synthesis), and metabolism were determined after nicotine exposure in utero or in vitro. In utero nicotine exposure significantly stimulated ATII cell proliferation, differentiation, and metabolism. Although the effects on ATII cell proliferation and metabolism were almost completely prevented by concomitant treatment with PGJ2, the effects on surfactant synthesis were not. On the basis of in utero and in vitro data, we conclude that surfactant synthesis is stimulated by nicotine’s direct effect on ATII cells, whereas cell proliferation and metabolism are affected via a paracrine mechanism(s) secondary to its effects on the adepithelial fibroblasts. These data provide evidence for direct and indirect effects of in utero nicotine exposure on fetal ATII cells that could permanently alter the “developmental program” of the developing lung. More importantly, concomitant administration of PPAR-γ agonists can effectively attenuate many of the effects of in utero exposure to nicotine on ATII cells.

chronic lung disease; smoking; surfactant; fibroblast; peroxisome proliferator-activated receptor-γ

There is compelling evidence to suggest that although maternal smoking during pregnancy causes accelerated alveolar type II (ATII) cell differentiation at birth, there are significant long-term deleterious effects on pulmonary outcome (5–7, 9, 10, 15, 35). However, the mechanism(s) underlying these paradoxical pulmonary effects remain(s) largely unknown (23, 24, 28). ATII cell growth and differentiation and, hence, alveolar integrity are regulated by a number of autocrine, paracrine, and endocrine factors. In particular, mesenchymal-epithelial interactions are critically important for normal lung development and injury/repair (29–31, 33). We recently implicated the disruption of a specific epithelial-mesenchymal signaling pathway that specifically downregulates peroxisome proliferator-activated receptor (PPAR)-γ expression by alveolar interstitial fibroblasts (AIFs), resulting in AIF-to-myofibroblast (MYF) transdifferentiation in utero nicotine exposure-induced lung injury (25). It was demonstrated that augmentation of PPAR-γ in AIFs can prevent nicotine-induced AIF-to-MYF transdifferentiation. We have suggested that this AIF-to-MYF transdifferentiation might be a key mechanism underlying the alterations in lung development following in utero nicotine exposure, explaining its long-term detrimental effects on pulmonary outcome (25). However, the effects of in utero nicotine exposure on the pulmonary ATII cell, which secretes surfactant and is critical to the maintenance of alveolar homeostasis, remain to be fully elucidated. We tested the hypothesis that in utero nicotine exposure significantly affects ATII cell proliferation and differentiation and that augmentation of PPAR-γ expression would reduce or prevent the nicotine-mediated alterations in ATII cell proliferation and differentiation. Here, we describe the effects of in utero nicotine exposure on ATII cell proliferation, differentiation, and metabolism. Our data, for the first time, help explain the mechanisms underlying the paradoxical short-term acceleration in pulmonary differentiation but a poor long-term pulmonary outcome in infants born to mothers who smoke during pregnancy. These data provide a rationale and a plausible molecular intervention strategy that is likely to attenuate the in utero nicotine exposure-associated effects on pulmonary outcomes.

MATERIALS AND METHODS

Animals

Pathogen-free timed (embryonic day 0 = day of mating) pregnant Sprague-Dawley rats (200–250 g body wt) were obtained from Charles River (Hollister, CA) at embryonic day 3 and allowed to acclimatize until embryonic day 6. Dams were randomized into control (placebo), nicotine, and nicotine + PPAR-γ agonist groups. Dams received placebo (diluent, normal saline), nicotine tartrate (1 mg/kg) alone, or nicotine tartrate (1 mg/kg) + the PPAR-γ agonist prostaglandin J2 (PGJ2, 0.3 mg/kg) intraperitoneally in 100-μl volumes once daily from embryonic day 6 until they were killed with an overdose of pentobarbital sodium (200 mg/kg) on embryonic day 20. The fetuses were extracted by cesarean section, and the lungs were snap frozen for later analysis or processed for ATII cell or fibroblast culture. To determine whether nicotine-induced effects on fetal lung...
development specifically involved PPAR-γ-mediated mesenchymal-epithelial paracrine pathways, some animals in the nicotine + PGJ2 group were pretreated with a specific PPAR-γ antagonist, GW-9662 (Sigma, St. Louis, MO; 0.25 mg/kg). All studies were approved by the Los Angeles Biomedical Research Institute Institutional Review Board and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The dose (1 mg/kg) chosen for the nicotine treatment in this study has previously been shown in a number of studies to result in a specific lung phenotype characterized by changes in ATII cell proliferation and differentiation (18). This dose of nicotine (0.16–1.8 mg·kg body wt⁻¹·day⁻¹) is comparable to the dose to which habitual smokers are exposed (18). Food and water were provided to the dams ad libitum, and a 12:12-h light-dark cycle was maintained. Three animals were used for each condition per experiment, and each experiment was repeated at least three times.

**Isolation of Fetal Rat Lung ATII Cells**

ATII cells were isolated using differential adherence in monolayer culture, as described previously (2). Briefly, three to five dams were used per preparation. The fetuses were delivered via cesarean section, and fetal lungs were placed in Hank's balanced salt solution without calcium and magnesium. The lungs were chopped into small pieces with sterile scissors, the Hank's balanced salt solution was decanted, and 5 vol of 0.05% trypsin were added. A Teflon stirring bar was used to further dissociate the lungs by mechanical disruption of the tissue during incubation in a 37°C water bath. After the tissue was dispersed into 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 DMEM containing 20% FBS to yield a mixed-cell suspension of ~3 × 10⁸ cells, as determined by Coulter particle counter (Beckman-Coulter, Hialeah, FL). The cell suspension was then added to 75-cm² (T-75) culture flasks for 30–60 min to allow for differential adherence of lung fibroblasts. The unattached cells were then transferred to another T-75 culture flask for an additional 60 min. After this second culture period, the medium and nonadherent cells were removed from the flask and diluted with 1 vol of culture medium. This diluted suspension was cultured overnight in a T-75 culture flask at 37°C in a CO₂ incubator to allow the ATII cells to adhere. The ATII cells were identified by their appearance in culture under phase contrast microscopy, lamellar body content, cytokeratin staining, and microvillar processes. All cell cultures contained >95% ATII cells.

**Cell Culture**

Isolated ATII cells were cultured in DMEM + 10% FBS in 6- and 96-well plates, 100-mm dishes, and T-75 flasks, as needed, and maintained at 37°C in a humidified incubator containing 5% CO₂-95% air. At 80–90% confluence, the cells were processed for cell proliferation, differentiation, and metabolic studies (see below).

**Lung Explant Culture**

Explants derived from three to five litters of rats were used for each experiment during the course of the studies. Lungs were harvested from fetal rats under sterile conditions. The lung tissue was chopped into ~1-mm cubes and incubated in 0.5 ml of Waymouth's MB-252/1 medium containing penicillin (100 U/ml)-streptomycin (100 U/ml) and Fungizone (2.5 µg/ml) in six-well plates while rocking on an oscillating platform (3 cycles/min) in 5% CO₂-95% air at 37°C. The explants were allowed to attach for ~1-2 h.

**Cell Proliferation**

*In vivo ATII cell proliferation assay.* The EnVision double-stain system (DakoCytomation, Carpentaria, CA) was used for immuno-histochemical determination of in vivo ATII cell proliferation by double labeling with a cell proliferation-specific marker, proliferating cell nuclear antigen (PCNA), and an ATII cell-specific marker, surfactant protein (SP) C (SP-C). Briefly, dams were killed by cesarean section, and fetal lung tissue was fixed in 4% paraformaldehyde for 4 h. After fixation, the tissue was suspended in 30% sucrose overnight, washed in PBS, and then embedded in tissue-embedding medium (OCT Tissue-Tek, Sakura). Sections (8 µm) were cut using a cryostome (Leica). Endogenous peroxidase was blocked, and the sections were processed with 10 mM citrate buffer (pH 6.0) in a microwave oven for 5 min at high power. Subsequently, sections were incubated with the first primary antibody, mouse monoclonal proliferating cell nuclear antigen (PCNA) antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature; then the secondary antibody conjugated to horseradish peroxidase was added to the sections for another 30 min at room temperature. Vector SG (Vector Laboratories, Burlingame, CA) was used as a chromogen, and blue-gray nuclear staining was considered positive. After the slides were washed, they were incubated with double-stain block, and the second primary rabbit polyclonal antibody SP-C (1:200 dilution; Chemicon, Temecula, CA) was applied to the sections at room temperature for 30 min and then the secondary antibody labeled with alkaline phosphatase was applied for another 30 min. The immunoreaction was visualized with Vector Red (Vector Laboratories), and red cytoplasmic staining was considered positive. After dehydration, the slides were mounted with permanent mounting medium (VectaMount, Vector Laboratories). As negative controls, sections were incubated with normal serum in the absence of primary antibody. The slides were examined at ×40 magnification, and ATII cells in 10 randomly selected areas (grid size 40,000 µm²) per slide (2 slides/animal) were counted for the purposes of statistical analysis.

*Ex vivo ATII cell proliferation assay.* After in utero nicotine treatment, ATII cells were isolated as described above, and 5,000 cells were plated per well in 96-well plates. According to the manufacturer’s protocol, cell proliferation was determined by the tetrazolium dye assay, which is based on the conversion of a tetrazolium salt to a red formazan product by living cells (Cell Proliferation Assay, Promega).

*In vitro cell proliferation assay.* Cultured ATII cells or explants were treated with nicotine under various experimental conditions (see below), and cell proliferation was determined by Cell Proliferation Assay (Promega) or dual PCNA and SP-C labeling, respectively (see above).

**Measurement of Phospholipid Synthesis**

Incorporation of [methyl-³H]choline chloride (NEN Dupont) into saturated phosphatidylcholine was determined in monolayers of cultured explants and ATII cells. Briefly, subconfluent monolayer cultures of ATII cells in DMEM + 0.1% FBS that had been treated with nicotine or freshly isolated lung explant cultures in Waymouth’s medium in six-well plates were incubated with [methyl-³H]choline chloride (1 µCi/ml) for 4 h. After incubation, explants and cells were washed three times with ice-cold PBS. The explants and the scraped cells were thoroughly homogenized, and the cellular lipids were extracted with chloroform-methanol (2:1) (3). The organic phase was dried under a stream of nitrogen at 60°C, resuspended in 0.5 ml of carbon tetrachloride containing 3.5 mg of osmium tetroxide, and left at room temperature for 15 min. The reaction mixture was redried under nitrogen and resuspended in 70 µl of chloroform-methanol (9:1, vol/vol). The lipid extracts were transferred to silica gel plates (Kodak, Rochester, NY) and developed in a chloroform-methanol-water (65:25:4) solvent system. Pure dipalmitoyl phosphatidylcholine was used as the chromatographic standard. The developed plates were stained with bromothymol blue, blotted, and vacuum dried for 5 min at 90°C. Chromatogram spots corresponding to the migration of saturated phosphatidylcholine were scraped from the plates and...
counted by liquid scintillation spectrometry. The amounts of [methyl-3H]choline chloride incorporated into saturated phosphatidylcholine were expressed as disintegrations per minute per milligram of protein.

Western Analysis

Protein extraction and Western blot analysis for SP-B and choline phosphate cytidyltransferase-α (CCT-α) were performed using standard methods. Briefly, cells were homogenized in 10 mM Tris (pH 7.5), 0.25 M sucrose, 1 mM EDTA, 5 mM benzamidase, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of pepstatin A, aprotinin, and leupeptin and centrifuged at 14,000 rpm for 10 min at 4°C. Equal amounts of the protein from the supernatant were dissolved in electrophoresis sample buffer and subjected to SDS-polyacrylamide (4–12% gradient) gel electrophoresis followed by electrophoretic transfer to a nitrocellulose membrane. The membrane was blocked with 5% milk in 1× Tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with rabbit anti-human SP-B polyclonal antibody (1:1,000 dilution; Chemicon) overnight at 4°C. The antibody for CCT-α (1:2,000 dilution) was a kind gift from Dr. Mallampalli (University of Iowa, Iowa City, IA). Subsequently, the membrane was washed with 1× Tris-buffered saline + 0.1% Tween 20 and incubated with a 1:3,000 dilution of anti-rabbit horseradish peroxidase-linked whole antibody immunoglobulin G (Amersham, Arlington Heights, IL) for 1 h at room temperature, washed again, and developed with a chemiluminescent substrate (ECL, Amersham) following the manufacturer’s protocol. The density of the SP-B and CCT-α bands was quantified using a scanning densitometer (Eagle Eye, Stratagene).

Stable Isotope Labeling of Intracellular Glucose Metabolites

Stable isotope labeling of intracellular glucose metabolites was performed according to previously described methods (13). Briefly, [1,2,3-13C2]glucose was purchased with >99% enrichment for the specified carbon positions from Isotec (Miamisburg, OH). Lung ATII cells were isolated from embryonic day 20 pups from specified treatment groups and cultured in T-75 tissue culture flasks. At near confluence, cells were incubated in the presence of DMEM containing 180 mg/dl [1,2,3-13C2]glucose (50% isotope-enriched glucose) for 72 h to determine the changes in carbon flux under various treatment conditions. The [13C]glucose label is readily incorporated into various metabolites in mammalian cells, including ribonucleic acid (through ribose synthesis), lactate (through glycolysis), glutamate (through the tricarboxylic acid cycle), and palmitate (through the formation of acetyl-CoA). As the molecular weight (atomic mass unit) of these molecules increases on incorporation of the heavier 13C atoms derived from [1,2,3-13C2]glucose, they can be separated and quantitatively analyzed by gas chromatography-mass spectrometry (MS) on the basis of changes in their mass-to-charge ratios (m/z). This method allows simultaneous estimation of the relative synthetic rates of macromolecules in response to various treatments using a common precursor.

Recovery of Glucose Metabolites From Lung ATII Cells

Media glucose and lactate levels were directly measured using a Cobas Mira chemical analyzer (Roche Diagnostics). Glucose oxidation by fibroblasts was determined on the basis of the media 13C to 12C ratios in released CO2 by a Finnegan Delta-S isotope ratio mass spectroscope. The rate of 13CO2 release was measured to estimate the rate of glucose carbon oxidation by the cells, expressed as the atom percent excess, which is the proportion of 13C produced by the cultured cells above background in calibration standard samples.

RNA ribose was isolated by acid hydrolysis (2 N HCl for 2 h) of cellular RNA after TRIzol extraction of cell pellets. Hydroxylamine in pyridine and acetic anhydride was used to derivatize ribose isolated from RNA to its aldonic acid form. We monitored the ion clusters around m/z 256 (carbons 1–5 of ribose, chemical ionization), m/z 217 (carbons 3–5 of ribose), and m/z 242 (carbons 1–4 of ribose, electron impact ionization) to detect the molar enrichment for, and the positional distribution of, the 13C label in ribose.

Lactate in the cell culture medium (0.2 ml) was extracted with ethyl acetate after acidification with HCl. Lactate was derivatized to its propylene-glycolfluorobutyric anhydride form, and the m/z 328 ion cluster (carbons 1–3 of lactate, chemical ionization) was monitored for the detection of M1 (recycled lactate through the pentose cycle) and M2 (lactate produced by the Embden-Meyerhof-Parnas pathway) to estimate pentose cycle activity.

Fatty acids in the cell culture medium were extracted by saponification of the TRIzol cell extract after removal of the RNA-containing supernatant. Cell debris was treated with 30% KOH and 100% ethanol overnight, and petroleum ether was used to extract lipid. Methanolic HCl (0.5 N) was used to convert fatty acids to their methylated derivatives. Palmitate was monitored at ion cluster m/z 270. The enrichment of acetyl units and the de novo synthesis of the lipid fraction were determined using mass isotopomer distribution analysis for different isotopomers of palmitate.

Gas Chromatography-MS

Mass spectral data were obtained on an HP5973 mass-selective detector connected to an HP6890 gas chromatograph (GC). The settings were as follows: GC inlet 230°C, transfer line 280°C, MS source 230°C, MS quad 150°C. An HP-5 capillary column (30 m long, 250 μm diameter, 0.25 μm film thickness) was used for glucose, ribose, and lactate analyses. A Bps70 column (25 m long, 220 μm diameter, 0.25 μm film thickness; SGE, Austin, TX) was used for fatty acid analysis, with specific temperature programming for each compound studied.

Data Analysis and Statistical Methods

In vitro experiments were carried out using three cultures for each treatment regimen, and the experiments were repeated one to three times. Mass spectral analyses were carried out by three independent, automated injections of a 1-μl sample and accepted only if the sample standard deviation was <1% of the normalized peak intensity. Statistical analyses were performed using ANOVA. P < 0.05 was considered to indicate statistically significant differences among different treatment conditions.

RESULTS

Effect of Nicotine on ATII Cell Proliferation

In vivo assessment. Immunohistochemical analysis by double PCNA and SP-C staining demonstrated an almost twofold increase in ATII cell proliferation in the nicotine-exposed group (P < 0.05, n = 4; Fig. 1, A and B), which was completely blocked by the concomitant administration of the PPAR-γ agonist PGJ2. A specific PPAR-γ antagonist, GW-9662, almost completely blocked the PGJ2 effect.

Ex vivo assessment. Similar to the in vivo data, ex vivo assessment by the tetrazolium dye assay demonstrated an almost twofold increase in cell proliferation in the nicotine-exposed group (P < 0.05, n = 6) vs. the control group (Fig. 1C). Concomitant treatment with PGJ2 almost completely blocked the nicotine-induced increase in ATII proliferation. Similar to the in vivo data, GW-9662 blocked the PGJ2 effect.

In vitro assessment. Similar to the in vivo results, in lung explants in culture, 24 h of nicotine treatment significantly increased ATII cell proliferation (P < 0.05, n = 4), which was...
Fig. 1. Effect of nicotine on fetal alveolar type II (ATII) cell proliferation in vivo. In vivo fetal pulmonary ATII cell proliferation was determined immunohistochemically by double labeling with a cell proliferation-specific marker, proliferating cell nuclear antigen (PCNA), and an ATII cell-specific marker, surfactant protein (SP)-C (A and B), or assessed ex vivo by tetrazolium dye assay (C) after in utero nicotine administration to the dam (1 mg/kg ip) once daily from embryonic day 6 to 20. Cell proliferation was increased ~2-fold in the nicotine-exposed group vs. the control group. This increase was completely blocked by concomitant administration of a peroxisome proliferator-activated receptor (PPAR)-γ agonist, PGJ2. A specific PPAR-γ antagonist, GW-9662, almost completely blocked the PGJ2 effect on the nicotine-induced increase in ATII cell proliferation. In lung explants in culture treated with nicotine for 24 h, similar to the in vivo data, there was a significant increase in ATII cell proliferation, which was blocked by PGJ2; again, GW-9662 blocked the PGJ2 effect (D and E). Slides were examined at ×40 magnification; black arrows in A and D show ATII cells labeled with PCNA (blue-gray nuclear stain) and SP-C (red cytoplasmic stain). ATII cells in 10 randomly selected areas (grid size 40,000 μm²) per slide (2 slides/animal) were counted. F: no effect on cell proliferation in ATII cells directly stimulated in vitro with nicotine for 24 h.

Effect of Nicotine on Phospholipid Synthesis by ATII Cells

Effect of nicotine on [3H]choline incorporation into disaturated phosphatidylcholine: ex vivo assessment. Surfactant phospholipid synthesis, as measured by [3H]choline incorporation into saturated phosphatidylcholine, by the cultured ATII cells from different experimental conditions was significantly increased in the nicotine-exposed group vs. the control group (P < 0.05, n = 6; Fig. 2A). However, in contrast to the proliferation data, concomitant treatment with the PPAR-γ agonist PGJ2, alone or in combination with the PPAR-γ antagonist GW-9662, had no effect on the nicotine-induced increase in phospholipid synthesis.

Effect of nicotine on [3H]choline incorporation into disaturated phosphatidylcholine: in vitro assessment. Direct stimulation of ATII cells in vitro with nicotine for 24 h also significantly increased choline incorporation (Fig. 2B). Similar to the in vivo data, concomitant treatment with PGJ2 alone or in combination with the PPAR-γ antagonist GW-9662 had no effect on the nicotine-induced increase in phospholipid synthesis.

Effect of Nicotine on CTP:CCT-α expression. We also determined the protein expression of CCT-α, the rate-limiting enzyme regulating surfactant phospholipid synthesis. Matching the increase in surfactant phospholipid synthesis, we observed a significant increase in CCT-α protein expression after in vivo and in vitro nicotine exposures (Fig. 3). Here again, similar to the choline incorporation data, treatment with PGJ2, alone or in combination with GW-9662, had no effect on the nicotine-induced increase in phospholipid synthesis in vivo or in vitro.

Effect of Nicotine on SP-B Synthesis by ATII Cells

In vivo. Compared with the control group, in utero nicotine exposure significantly increased the steady-state SP-B protein level in the cultured ATII cells, as determined by Western analysis (P < 0.05, n = 3; Fig. 4A). Neither PPAR-γ agonist (PGJ2) nor antagonist (GW-9662) treatment had a significant effect on this nicotine-induced increase in SP-B expression.

In vitro. Similar to the in vivo data, direct treatment of cultured ATII cells with nicotine resulted in a significant increase in SP-B expression that was unaffected by PPAR-γ agonist (PGJ2) or antagonist (GW-9662) treatment (Fig. 4B).
Effect of In Utero Nicotine Exposure on ATII Cell Metabolism

Along with the nicotine-induced increase in ATII cell proliferation and differentiation, there were metabolic changes that indicated significant effects of in utero nicotine exposure on the metabolic profile of the ATII cells. Most significantly, there were changes in the pentose cycle metabolism affecting ribonucleic acid synthesis and lipid metabolism that may have implications for surfactant synthesis.

Ribose synthesis. In vivo nicotine exposure significantly altered the pentose cycle metabolism in such a way that there was a significant increase in ribose synthesis via the oxidative glucose-6-phosphate dehydrogenase pathway, while there was a significant decrease in ribose synthesis via the nonoxidative transketolase pathway (Fig. 5, A and B). These metabolic alterations were completely blocked by concomitant administration of the PPAR-γ agonist PGJ2. In contrast, direct in vitro treatment of cultured ATII cells with nicotine did not alter ribose synthesis via the oxidative or the nonoxidative pathway with or without the PPAR-γ agonist PGJ2 (Fig. 5, C and D).

De novo palmitate synthesis. De novo palmitate synthesis, as a function of the total palmitate in the ATII cells, almost doubled on in utero exposure to nicotine (Fig. 6A). This was accompanied by a modest increase in the [13C]glucose labeling of the acetyl-CoA pool (Fig. 6B). Both of these changes were also completely prevented by the concomitant administration of the PPAR-γ agonist PGJ2. Again, in contrast to the in vivo data, in vitro stimulation of ATII cells with nicotine for up to 72 h did not result in a significant change in de novo palmitate synthesis or [13C]glucose labeling of the acetyl-CoA with or without the PPAR-γ agonist PGJ2 (Fig. 6, C and D).

DISCUSSION

The pulmonary effects of in utero nicotine exposure on the fetus are extremely complex. On the one hand, there is evidence of enhanced functional pulmonary maturity at birth, possibly contributing to a decrease in the incidence of respiratory distress syndrome (7, 9, 15, 35). On the other hand, significant reduction in prenatal and postnatal lung growth has been reported in children of women who smoke (5, 6, 10). Significant suppression of alveolarization, functional residual capacity, and tidal flow volumes has been demonstrated in the offspring of nicotine-exposed pregnancies. Although the mechanisms underlying the general effects of maternal smoking on fetal viability and growth are generally thought to be due to fetal hypoxia, the mechanisms underlying the seemingly par-
adoxical acute and chronic pulmonary effects are far more complex and are just beginning to be elucidated (23–25, 28).

The direct effects of maternal smoke on prenatal lung growth are restricted to only those components of maternal smoke that are transferred across the placenta. Nicotine is the major smoke constituent that crosses the placenta and is concentrated in the fetus and, in animal studies, has been shown to adversely affect fetal lung growth and development (1, 5, 16–19, 22, 27, 28). Therefore, in the human fetus as well, nicotine is likely to be the major constituent causing pulmonary effects. We recently demonstrated that in vitro nicotine exposure specifically disrupts parathyroid hormone-related protein-driven alveolar epithelial-mesenchymal signaling, resulting in AIF-to-MYF transdifferentiation (25). We have also suggested that augmentation of PPAR-γ signaling, the key downstream mesenchymal target of parathyroid hormone-related protein signaling, might be a plausible intervention for prevention of nicotine-induced in utero lung damage.

Because in vitro nicotine exposure disrupts specific alveolar epithelial-mesenchymal interactions, we hypothesized that exposure to nicotine in utero, in addition to affecting ATII cell function through its direct effects on ATII cells, would also affect it indirectly via its effects on the AIFs. Therefore, in this study, we have specifically examined the effects of nicotine exposure on ATII cell proliferation, differentiation, and metabolism in vivo as well as in vitro; furthermore, the effect of concomitant administration of the PPAR-γ agonist PGJ2 was also examined.

We found that in utero fetal exposure to nicotine through parenteral administration to the mother significantly increased ATII cell proliferation and surfactant synthesis and altered glucose and lipid metabolism. In vivo and in vitro data suggest that surfactant synthesis is stimulated via nicotine’s direct effects on ATII cells, whereas cell proliferation and metabolism are affected via the paracrine mechanism secondary to its effects on the adepithelial fibroblasts, suggesting direct and indirect effects of in utero nicotine exposure on fetal pulmonary ATII cells. These paracrine effects were almost completely prevented by the concomitant administration of the PPAR-γ agonist PGJ2.

Our observation of increased ATII cell proliferation in response to in utero nicotine exposure is consistent with the observations of Maritz and Thomas (18). We have extended their observations by demonstrating that ATII cell proliferation increased with in vivo nicotine exposure, but not with direct in vitro nicotine stimulation of ATII cells, suggesting a likely paracrine mechanism underlying this response. Furthermore, the in vivo nicotine-induced ATII cell proliferation was, to a

Fig. 2. Effect of nicotine on surfactant phosphatidylcholine synthesis in vivo and in vitro. Surfactant phospholipid synthesis measured by [3H]choline incorporation (disintegrations/min (dpm) per mg protein) into saturated phosphatidylcholine by cultured ATII cells after in utero (1 mg/kg ip administered once daily pregnant dam from embryonic day 6 to 20; A) or in vitro (1 × 10−9 M for 24 h; B) nicotine treatment was significantly increased in the nicotine-exposed group vs. the control group. Concomitant treatment with the PPAR-γ agonist PGJ2 or antagonist GW-9662 had no effect on the nicotine-induced increase in phospholipid synthesis in utero or in vitro.
large extent, prevented by the concomitant administration of the PPAR-γ agonist PGJ2. The possibility that the nuclear transcription factor PPAR-γ may be playing a role in this response is further demonstrated by the observation that the PPAR-γ agonist-mediated prevention of the increase in ATII cell proliferation was completely blocked by the PPAR-γ-specific antagonist GW-9662. This finding is consistent with the antimitogenic role of PPAR-γ in other systems, where it has been shown that PPAR-γ can inhibit cell proliferation by regulating the activation of cyclins and cyclin-dependent kinases (8, 14, 34). Our recent work has clearly demonstrated that AIFs that are located adjacent to ATII cells express PPAR-γ (26) and nicotine treatment downregulates PPAR-γ expression by these fibroblasts (25), which, in turn, may disturb the balance of fibroblast-derived epithelial cell growth-stimulatory and -inhibitory paracrine mediators, resulting in ATII cell proliferation.

With regard to nicotine’s effect on surfactant synthesis, although a large body of work has been generated on the effects of cigarette smoke on the surfactant system in the adult, there is very limited information on the effects of nicotine exposure in the developing lung in utero. Lieberman et al. (15) reported higher amniotic fluid-saturated phosphatidylcholine contents in human fetuses exposed to intrauterine smoke. A related study reported an increase (18) in the lamellar body content of pulmonary ATII cells after intrauterine nicotine exposure. Recently, however, Chen et al. (4) did not find a significant difference in the saturated phosphatidylcholine contents in the lung tissue of nicotine-exposed vs. nonexposed rat pups on postnatal day 1. In contrast, after in utero nicotine exposure, we found an increase in saturated phosphatidylcholine synthesis, as measured by choline incorporation ex vivo by the fetal rat lung explants. Similarly, there is conflicting information on the effects of nicotine on SP expression by the developing lung. Chen et al. (4) did not find any effect of in utero nicotine exposure (from day 3 to day 21 of gestation) on the lung mRNA expression of SP-A, -B, -C, and -D in the newborn rat. However, Wuenschell et al. (35) reported significant increases in the expression of SP-A and -C mRNAs in a murine developing lung explant model. Hermans et al. (11) did not find any significant differences in amniotic fluid SP-A levels at full term in smoke-exposed vs. non-smoke-exposed pregnancies. The conflicting results in these studies are likely to be related to differences in the models used (species and stage of lung development), duration of nicotine exposure (acute vs. chronic), whether smoke or nicotine was used as a challenge, and the end points, for example, whether phospholipid synthesis, secretion, or total pool size was examined.

**Fig. 3.** Effect of nicotine on CTP:choline phosphate cytidylyltransferase-α (CCT-α) protein expression in ATII cells. CCT-α protein expression by ATII cells increased significantly after in utero (1 mg/kg ip administered to pregnant dam once daily from embryonic day 6 to 20; A) or in vitro (1×10⁻⁹ M for 24 h, B) nicotine treatment. Concomitant treatment with PGJ2 or GW-9662 had no effect on the nicotine-induced increase in CCT-α protein expression in utero or in vitro. Representative Western blot and density histograms are shown.
The effects of in utero nicotine exposure on glucose metabolism in fetal rat ATII cells have been only sparingly studied. In the whole lung of 14-day-old suckling pups, after nicotine exposure during pregnancy and lactation, glucose turnover was increased, and glycolysis and glycogenolysis were decreased (12). This was attributed to an inhibition of the activity of phosphofructokinase. We found that although in vivo nicotine exposure significantly increased ribose synthesis via the oxidative glucose-6-phosphate dehydrogenase pathway and decreased it via the nonoxidative transketolase pathway, direct in vitro treatment of cultured ATII cells with nicotine did not alter ribose synthesis via the oxidative or the nonoxidative pathway. This discrepancy between in vitro and in vivo effects of nicotine on ATII cell glucose metabolism is similar to the previously reported discrepancy in the effect of nicotine exposure on phosphofructokinase activity in adult lung tissue under in vivo and in vitro conditions (12). Since the nicotine-induced in vivo alterations in ATII cell glucose and lipid metabolisms were blocked by the concomitant administration of the PPAR-γ agonist PGJ2, downregulation of PPAR-γ in AIFs is very likely the key modulator for these metabolic changes.

Taken together, these data imply that in utero nicotine exposure significantly affects ATII cell proliferation, differentiation, and metabolism via direct and indirect effects of nicotine on ATII cells. The stimulation of ATII cell proliferation and surfactant synthesis after in utero nicotine exposure likely explains the decrease in the incidence of respiratory distress syndrome in infants of mothers who smoke during pregnancy (7, 9, 15, 35). However, since nicotine also disrupts the homeostatic alveolar epithelial-mesenchymal interactions, resulting in AIF-to-MYF transdifferentiation (25), the stimulatory effect of in utero nicotine exposure on ATII surfactant synthesis ultimately fails. This probably also explains why Chen et al. (4) observed a significant decrease in the saturated phosphatidylcholine synthesis (32), the loss of lipogenic potential of these AIFs in response to in vivo nicotine exposure might be a trigger for the increase in de novo palmitate synthesis by the ATII cells under in vivo conditions. Since the nicotine-induced in vivo alterations in ATII cell glucose and lipid metabolisms were blocked by the concomitant administration of the PPAR-γ agonist PGJ2, downregulation of PPAR-γ in AIFs is very likely the key modulator for these metabolic changes.

Fig. 4. Effect of nicotine on SP-B expression in ATII cells. SP-B protein expression increased significantly after in utero (1 mg/kg ip administered to pregnant dam once daily from embryonic day 6 to 20; A) or in vitro (1 × 10⁻⁹ M for 24 h; B) nicotine treatment. Concomitant treatment with PGJ2 or GW-9662 had no effect on the nicotine-induced increase in SP-B protein expression in utero or in vitro. Representative Western blot and density histograms are shown.
content of the lung tissue in nicotine-exposed vs. nonexposed rat pups on postnatal days 35 and 42, even when they found no differences between the two groups on postnatal day 1. Therefore, it is likely that, after in utero nicotine exposure, the combination of a nicotine-induced AIF-to-MYF transdifferentiation and a decrease in surfactant synthesis, in the long run, impacts lung function adversely. Although the mechanisms underlying the effects of in utero nicotine exposure on ATII cell proliferation and differentiation remain to be fully elucidated, it seems that surfactant synthesis

Fig. 5. Effect of in utero nicotine exposure on ATII cell metabolism. After in utero nicotine exposure (1 mg/kg ip administered to pregnant dam once daily from embryonic day 6 to 20), ribose synthesis, as measured by [13C]glucose labeling, increased significantly via the oxidative glucose-6-phosphate dehydrogenase (G6PD) pathway (A) and decreased significantly via the nonoxidative transketolase pathway (B). Concomitant administration of the PPAR-γ agonist PGJ2 completely blocked these changes. Direct in vitro treatment of cultured ATII cells with nicotine did not alter ribose synthesis via the oxidative or nonoxidative pathway with or without PGJ2 (C and D).

Fig. 6. Effect of nicotine on ATII cell de novo palmitate synthesis. After in utero nicotine exposure (1 mg/kg ip administered to pregnant dam once daily from embryonic day 6 to 20), de novo palmitate synthesis, as a function of total palmitate in ATII cells (A) and [13C]glucose labeling of acetyl-CoA pool (B), increased significantly. Concomitant administration of the PPAR-γ agonist PGJ2 completely blocked these changes. Direct in vitro treatment of cultured ATII cells with nicotine did not alter de novo palmitate synthesis or [13C]glucose labeling of the acetyl-CoA pool with or without PGJ2 (C and D).
is stimulated via nicotine’s direct effects on ATII cells, whereas cell proliferation and metabolism are affected via a paracrine mechanism secondary to its effects on the adiphe-
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In summary, in addition to previously proposed mechanisms for in utero nicotine-induced lung effects, our data, for the first
time, provide evidence for direct and indirect effects of in utero
nicotine exposure on fetal pulmonary ATII cells that could
permanently alter the “developmental program” of the develop-
ing lung. Although the safety and pharmacology of PPAR-γ agonists need to be assessed before they can be considered for human use during gestation, this strategy could, at least par-
tially, ameliorate the complex nicotine-induced lung injury in utero. In this regard, it is important to note that to more
effectively prevent the maternal nicotine exposure-induced effects on the offspring’s ATII cell proliferation, differentia-
tion, and metabolism, PPAR-γ agonist intervention might be
needed not only during gestation but also during lactation.

ACKNOWLEDGMENTS

This study was presented in part at the Pediatric Academic Societies

GRANTS

This study was supported by Philip Morris USA and Philip Morris Inter-
national Grant 11108-02, Tobacco-Related Disease Research Program Grants
14RT-0073 and 15IT-0250, National Heart, Lung, and Blood Institute Grants
HR9253

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