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ROLE OF NICOTINE ACETYLCHOLINE RECEPTORS, NEUROPEPTIDES
AND FIBRONECTIN EXPRESSION IN LUNG

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14. ABSTRACT We hypothesize that prenatal exposure to nicotine, a major component of tobacco that transverses the placenta, is largely responsible for the development of asthma in children born of mothers who smoke. Specifically, we hypothesize that nicotine is recognized by specific cellular proteins called nicotinic acetylcholine receptors (nAChRs) that are expressed by lung cells termed fibroblasts and pulmonary neuroendocrine cells (PNEC). In fibroblasts, this interaction triggers the exaggerated expression of a connective tissue protein called fibronectin. In PNECs, nicotine stimulates cell growth and the excessive secretion of neuropeptides that affect airway formation and lung growth, and that stimulate smooth muscle cells to contract. In this fashion, nicotine can affect airways development and promote disease during childhood. This proposal will test the hypothesis in animal models of lung development and hyperreactive airways.					
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INTRODUCTION

Asthma is a chronic lung disease characterized by airway dysfunction. Of the many factors implicated in the pathogenesis of asthma, a strong association exists between prenatal and postnatal exposure to environmental tobacco smoke (ETS) (1). This is particularly true in infancy and in childhood where ETS exposure is associated with a higher incidence or prevalence of asthma, and with measures of decreased flow in the airways, bronchial hyperresponsiveness, and increased respiratory infections (2). It has been speculated that the relationship between ETS and asthma is secondary to reduced airway flow caused by tobacco-induced prenatal alterations in airway architecture and/or bronchial reactivity (3,4). However, the exact mechanisms by which prenatal ETS promotes airway dysfunction in children remain unelucidated.

We hypothesize that prenatal exposure to nicotine, a major component of tobacco that transverses the placenta, is largely responsible for childhood asthma in the setting of exposure to ETS. Specifically, we hypothesize that nicotine is recognized by nicotinic acetylcholine receptors (nAChRs) expressed by fibroblasts and pulmonary neuroendocrine cells (PNECs), among other embryonic lung cells. In fibroblasts, this interaction triggers an intracellular signaling cascade that promotes the exaggerated expression and aberrant deposition of fibronectin, a matrix glycoprotein that is highly expressed in developing and injured tissues, and that is found deposited in asthmatic airways (5). The excessive deposition of fibronectin in fetal lungs stimulates cleft formation and the development of an increased number of primitive airway tubules with small caliber in the setting of increased cell proliferation. In PNECs, nicotine stimulates the production of neuropeptides like bombesin (in mice) and gastrin-releasing peptide (GRP; the human counterpart of bombesin) that also stimulate lung branching and cellular proliferation, and that have constrictive effects on bronchial smooth muscle cells. Thus, nicotine induces the excessive deposition of fibronectin and the hyperplasia/hypersecretion of PNECs in developing lungs. These effects are manifested structurally by airway wall remodeling and an increase in the number of small-caliber airways. Functionally, these effects are manifested by airflow limitation and hyperactivity. Together, these events prepare the stage for childhood asthma which is formally established/perpetuated by inflammation induced by continued exposure to ETS and infection in the postnatal period, among other factors. The hypothesis will be tested in specific aims designed to:

- Aim I. Elucidate the mechanisms by which nicotine affects murine lung development using cultured embryonic lung explants.
- Aim II. Examine the effects of prenatal nicotine exposure on postnatal airway structure and function *in vivo*, and study how this relates to fibronectin overexpression and PNEC hyperfunction.

BODY

The following discussion summarizes our recent findings related to Aims I and II:

Aim I. Elucidate the mechanisms by which nicotine affects murine lung development using cultured embryonic lung explants.

We believe that nicotine affects lung development by acting on specific nAChRs. Initial studies supported by this grant revealed that nicotine stimulated branching morphogenesis in embryonic murine lung explants (Figure 1A below). We also found that this stimulatory effect was inhibited by reagents (e.g., α -bungarotoxin) capable of blocking the activation of $\alpha 7$

nAChRs, which are highly expressed in many lung cells including fibroblasts (Figure 1B). Furthermore, we examined the distribution of these receptors in developing lungs and found that they are most prominent in lungs during the pseudoglandular stage of lung development, the stage in which branching morphogenesis takes place (not shown). We also found that nicotine stimulates the growth of lung explants (Figure 1C), but blockers of $\alpha 7$ nAChR were not as effective suggesting a role for other nAChRs. This work was well-received at last year's International American Thoracic Society Meeting in San Diego, California (oral presentation in symposium titled: "Best of ATS 2006").

In order to confirm the role of $\alpha 7$ nAChRs in nicotine-induced lung branching, we treated lung explants with an agonist of $\alpha 7$ nAChRs (GTS-21) and found that it, like nicotine, stimulated lung branching (Figure 1D). Furthermore, we obtained genetically engineered animals with knockout mutations in $\alpha 7$ nAChRs (Figure 1D, Chrna7). These animals appear normal at baseline and undergo normal lung branching morphogenesis. However, lung explants harvested from $\alpha 7$ nAChR knockout animals did not show alterations when exposed to nicotine (Figure 1D, red arrow).

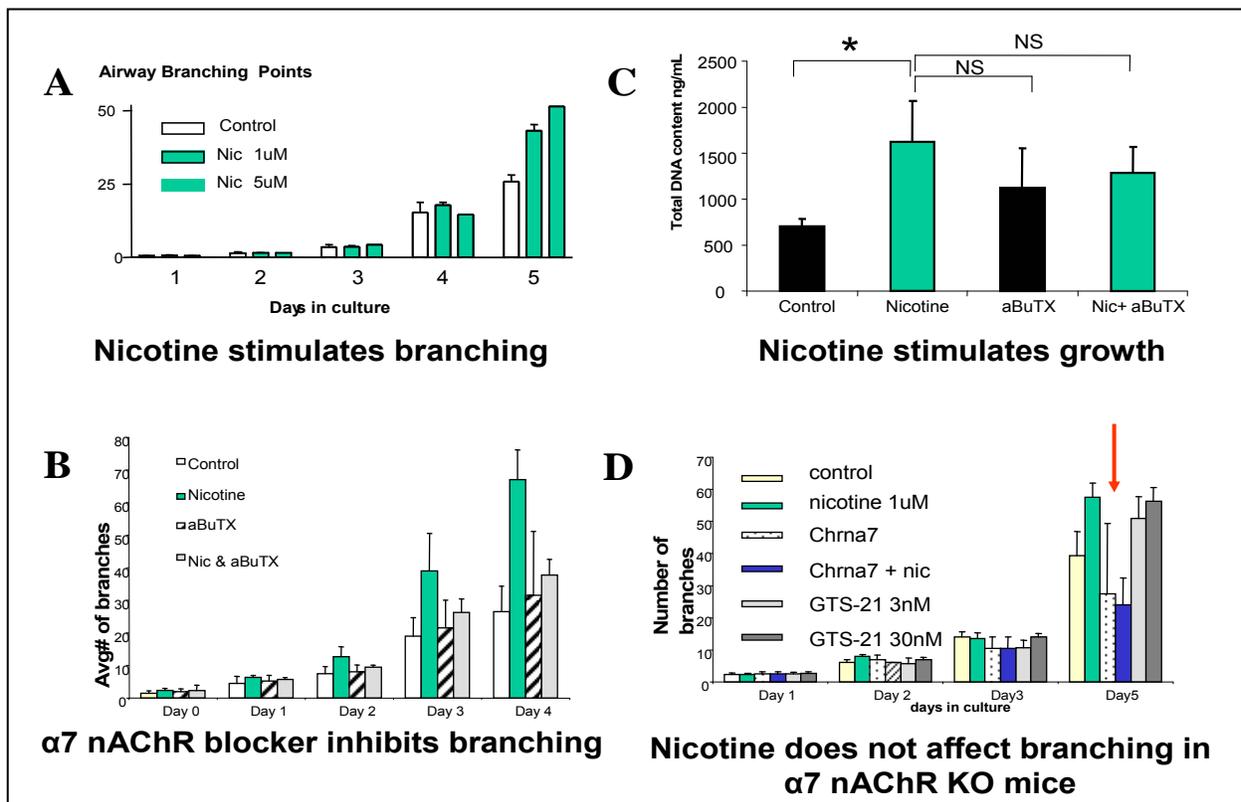


Figure 1. Effects of nicotine, nicotine antagonists, and nicotine agonists on lung branching in wildtype and $\alpha 7$ nAChR knockout mice. **A, Nicotine stimulates lung branching.** Lung explants harvested at day 11 of gestation were cultured in the presence of nicotine and evaluated for number of branches every day. **B, Branching is inhibited by the $\alpha 7$ nAChR blocker.** Explants cultured with nicotine in the presence or absence of α -bungarotoxin were evaluated for branching. **C, Nicotine stimulates lung growth.** Lung explants cultured for 4 days with nicotine (in the presence or absence of α -bungarotoxin) were harvested and cell number estimated by measuring DNA via DNA fluorometry. **D, Nicotine does not affect branching in KO mice.** Lung explants were harvested from wild type and $\alpha 7$ nAChR KO (Chrna7) and cultured to evaluate branching. Some explants were treated with the $\alpha 7$ nAChR agonist GTS021.

The observations discussed above suggest that $\alpha 7$ nAChRs might mediate some of the effects of nicotine in lung, whereas other receptors mediate other effects. The use of knockout animals represents a good strategy to identify the specific receptors involved in lung development and we will continue such strategy. However, the use of siRNA knock-down technology has also proven useful. To establish such technology, we have designed and generated several control and $\alpha 7$ nAChR siRNAs to be used in the lung explant model. To date, we have found that one such siRNA inhibits the response of fibroblasts to nicotine and we will begin experiments with this reagent soon (not shown).

Aim II. Examine the effects of prenatal nicotine exposure on postnatal airway structure and function *in vivo*, and study how this relates to fibronectin overexpression and PNEC hyperfunction.

Although the above studies strongly suggest that nicotine stimulates lung branching morphogenesis, we need to emphasize that they were performed in cultured lung explants treated with nicotine *ex vivo*. Thus, formal proof of our hypothesis requires evaluating the effects of nicotine *in vivo*. To this end, we sent one of our collaborators to the laboratory of our consultant, Dr. Dallas Hyde (National Primate Center, UC Davis, CA), to learn how to engage in morphometric analysis of embryonic tissues. Armed with this new knowledge, we exposed animals to nicotine in the drinking water (100 mg/ml) and bred them at our institution's animal facility. The offspring were kept on nicotine after birth until sacrifice. Then, the lungs were harvested, fixed, and processed carefully for morphometric analysis directed at evaluating lung branching. Following the recommendations of Dr. Hyde, we focused on measuring total airway length which would reflect total number of airways in the lung. To date, we have carefully analyzed 5 lungs obtained from the nicotine-treated group and 5 lungs from the control group. These studies revealed that lungs harvested from the offspring of nicotine-treated animals show increased total airway length providing further evidence in support of our hypothesis.

Another original goal of the grant was to study the physiology of lungs harvested from control and nicotine-treated animals. This year, we purchased and established a full lung physiology equipment that is currently being used in several studies. With regards to this project, we have begun by testing lung physiology in control- and nicotine-treated wild type and $\alpha 7$ nAChR knockout mice. Preliminary studies suggest that nicotine might enhance airways hyperactivity in the absence of obvious inflammation. If this finding were to be corroborated, it would open a new area of investigation dealing with the development of hyperactive airways disease (e.g., asthma) through non-inflammatory pathways. Finally, we have gained an interest in the process of apoptosis, how it might be affected by nicotine, and how it might be involved in lung development. This idea was based on observations generated by this group showing that apoptotic cells are most prominent in pseudoglandular stage lungs (Figure 2, left image). Furthermore, we recently found that agents capable of inhibiting apoptosis (zinc and auticarboxylic acid) inhibit lung branching morphogenesis (Figure 2, right image). This effect appears to be modulated by extracellular matrix components like fibronectin and type I collagen. Fibronectin is a matrix glycoprotein highly expressed by lung fibroblasts and other cells during the pseudoglandular stage of lung development. Fibronectin and its receptors have been implicated in lung branching morphogenesis and we are

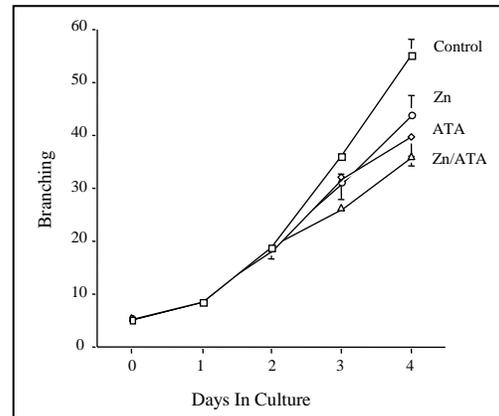
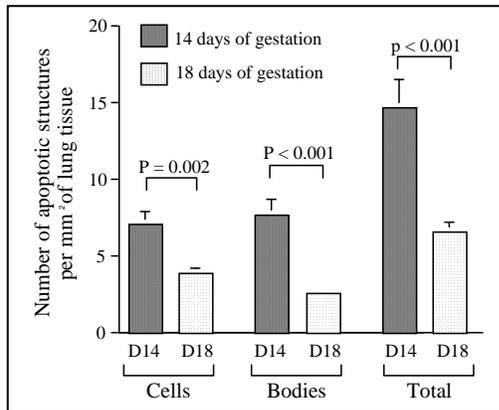


Figure 2. Apoptosis is increased during the pseudoglandular stage of lung development and inhibitors of apoptosis (zinc and ATA) reduce branching. Left image, Apoptosis is increased in pseudoglandular stage lungs. Lung were obtained at 14 and 18 days of gestation and submitted to TUNEL analysis. Afterwards, apoptotic structures were quantified and found to be highest in pseudoglandular (14 days) stage lungs. **Right image, Zinc and ATS inhibit branching.** Lung explants were obtained at day 11 of gestation and cultured for 4 days with the presence of Zinc and ATA. Note that both agents diminished branching when compared to control. These effects were not related to cytotoxicity.

KEY RESEARCH ACCOMPLISHMENTS

- Our studies show that nicotine stimulated branching, and that this effect was mediated through $\alpha 7$ nAChRs as demonstrated in by the fact that antagonists of nAChRs inhibit the effect and so did the use of $\alpha 7$ nAChR knockout mice.
- We have designed and generated control and $\alpha 7$ nAChR siRNA that will be used to test the role of $\alpha 7$ nAChR versus other nAChRs in cells and in *ex vivo* lung explants.
- In an attempt to test our hypothesis *in vivo*, we examined lungs harvested from animals born from control and nicotine-treated mice. The lungs were dissected, fixed and processed for morphometric analysis. Preliminary results reveal that the total airway length is increased in nicotine-treated lungs confirming that nicotine stimulates lung branching *in vivo*.
- We have purchased and established a lung physiology system in our laboratory that is being used to evaluate lung function in control- and nicotine-treated wildtype and $\alpha 7$ nAChR animals. Preliminary results reveal that nicotine stimulates airway hyperactivity.
- The ability of nicotine to affect both lung branching and growth in lung explants suggests that nicotine might cause *dysanaptic lung growth*. Dysanaptic growth refers to the disproportionate growth between conducting airway and alveolar parenchyma first described to explain variability in expiratory flow volume curves. The abnormal lung function associated with prenatal nicotine exposure may be a consequence of dysanaptic growth by changes in branching morphogenesis without an equal change in growth.

REPORTABLE OUTCOMES

- The data described above was presented in an oral presentation during a mini-symposium titled: *Best Science at ATS* that was held during the 2006 International American Thoracic Society Meeting in San Diego, CA. Another abstract has been accepted for presentation at the 2007 meeting to be held in May in San Francisco, CA.
- A manuscript summarizing data related to nicotine and nAChRs and their role in lung branching is under revision at the *Am J Respir Cell Mol Biology*.
- A manuscript summarizing our work related to apoptosis and mammalian lung development is being considered for publication in *Exp Lung Res*.

CONCLUSIONS

Nicotine can affect the development of the primitive airways as well as the growth of the lung. In this fashion, nicotine could promote 'dysanaptic lung growth' which may, in turn, promote airway dysfunction after birth alone or after exposure to inhaled stimulants. Further work is necessary to determine the implications of these events in the clinical setting.

REFERENCES

1. U.S. EPA. Respiratory health effects of passive smoking: Lung cancer and other disorders. EPA/600/6-90/0067. Washington, DC:U.S. Environmental Protection Agency, 1992.
2. Gold DR. Environmental tobacco smoke, indoor allergens, and childhood asthma. *Environ Health Perspect* 108:643-651, 2000.
3. Hanrahan JP. Antenatal interventions in childhood asthma. *Eur Respir J* 12:46s-51s, 1998.
4. Collins MH, Moessinger AC, Kleinerman J, Bassi J, Rosso P, Collings AM, James LS, Blanc WA. Fetal lung hypoplasia associated with maternal smoking: a morphometric analysis. *Pediatr Res* 19:408-412, 1985.
5. Roman, J. and McDonald, J. A. Fibronectin and fibronectin receptors in lung development, injury and repair. In Crystal, West, Barnes, Cherniak, Weibel (eds.), *The Lung: Scientific Foundations* (second edition). NY: Raven Press, 1998.

APPENDICES None