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TITLE: PRENATAL EXPOSURE TO NICOTINE AND CHILDHOOD ASTHMA: ROLE OF NICOTINE ACETYLCHOLINE RECEPTORS, NEUROPEPTIDES AND FIBRONECTIN EXPRESSION IN LUNG

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14. ABSTRACT We hypothesized 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 hypothesized 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. 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. Studies were performed in primary lung fibroblasts, embryonic murine lungs, and in rodents to test the hypothesis. This work led to the discovery that nicotine stimulates lung branching morphogenesis thereby causing dysanatic lung growth. Of note, these events were found to be mediated by the α7 nAChRs as demonstrated using inhibitors and agonists of the receptor as well as genetically-engineered animals. In animals exposed to nicotine during the prenatal period, lung branching was enhanced based on morphometric analysis of tissues, and this was associated with airway hyperreactivity to methacholine in physiological studies. These studies led to two published original articles and 1 review article.

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asthma, lung development, matrix, receptors

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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 (6). 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.

Below, I summarize our accomplishments. Note that no figures are provided when discussing published data. However, unpublished data are included.

BODY

The following discussion summarizes our 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 hypothesized 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. 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. 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, but blockers of $\alpha 7$ nAChR were not as effective suggesting a role for other nAChRs. This work was well-received at the 2005 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. Furthermore, we obtained genetically engineered animals with knockout mutations in $\alpha 7$ nAChRs. 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.

In search for potential 'morphogens' that could mediate the effects of nicotine on lung development, we tested for fibronectin, a matrix glycoprotein implicated in regulation of lung branching. As we have reported for primary lung fibroblasts, nicotine was found to stimulate fibronectin expression in embryonic lung explants. **This work was published in the *American Journal of Physiology.***

The observations discussed above suggest that $\alpha 7$ nAChRs 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. In preliminary experiments, this reagent has been shown to inhibit lung fibroblast proliferation (not shown).

More recently, we began to explore the effect of neuropeptides on the expression of neuropeptides and how this might affect the development of the neuronal system in the embryonic lung. We began by testing nerve growth factor or NGF, a neuropeptide implicated in the development of the nervous system. Murine lung explants were exposed to nicotine *ex vivo* as described above followed by harvesting of embryonic animals and dissection of the rudimentary lungs. These studies revealed that nicotine exposure was associated with increased NGF mRNA expression as determined by RT-PCR. More interestingly, histological evaluation of these lungs revealed larger neuronal structures as determined with a pan neuronal marker (PGP9.5) (Figure 1). This work was submitted for presentation to the 2008 American Thoracic Society International Conference.

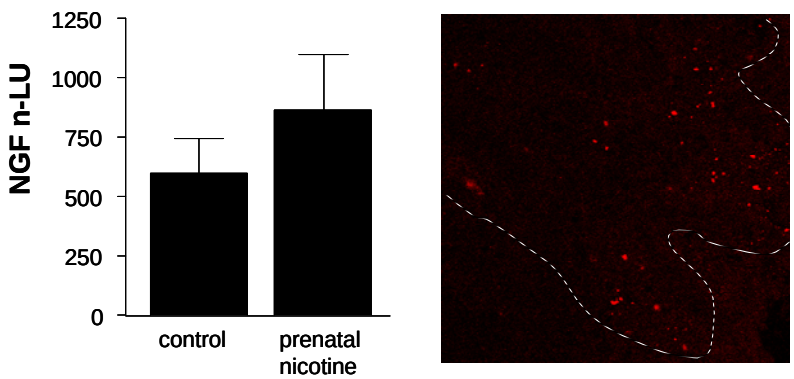


Figure 1. Nicotine stimulates NGF expression and is associated with larger neural structures in developing lungs. Lung explants treated with nicotine for 5 days were evaluated for NGF mRNA expression via RT-PCR (Left image). Also, the lungs were harvested and processed for immunofluorescense with a pan-neuronal marker for neuronal structures (PGP9.5)

<u>Aim II</u>. 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* using morphometric analysis. 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 our consultant, we focused on measuring total airway length which reflects total number of airways in the lung. To date, we have carefully analyzed lungs obtained from the nicotine-treated group and 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 (Figure 2). These studies were presented at the 2006 International American Thoracic Society Conference and a manuscript describing our findings is under preparation.

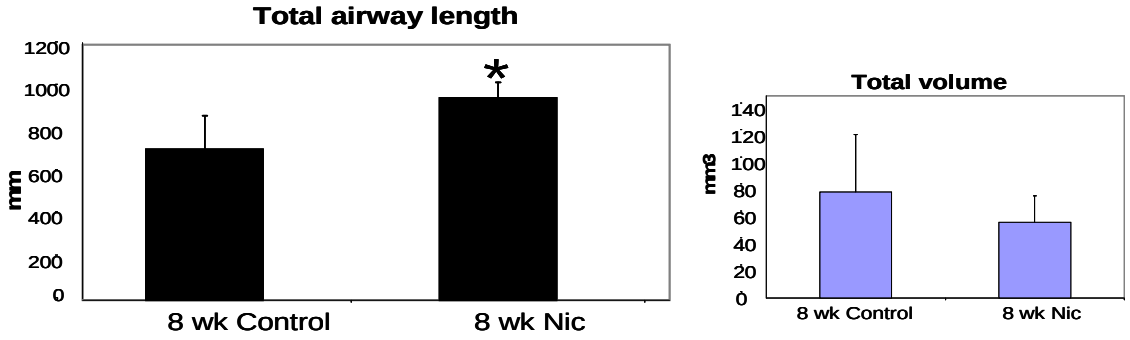
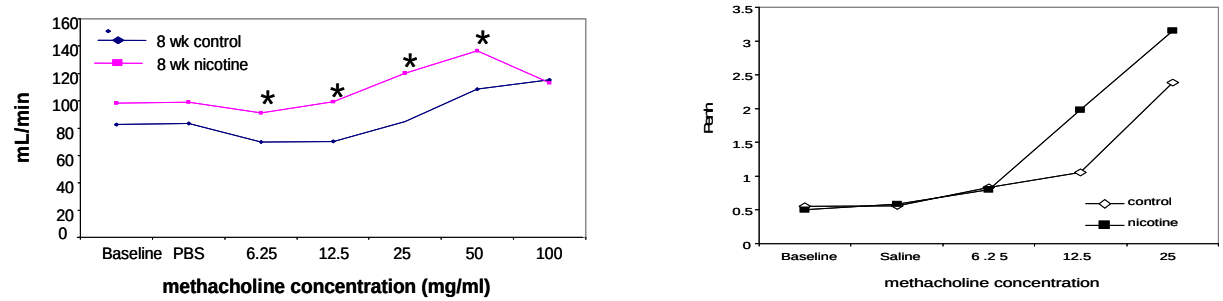


Figure 2. Prenatal nicotine exposure increases total airway length as an adult. In 8 week old mice born to mothers given 100 µg/mL nicotine in the drinking water, total airway length (left bar graph) was significantly increased compared to mice born to mothers drinking water alone. Total volume by Cavalieri's method was not found to be significantly different (right bar graph).

We have also performed experiments designed to evaluate the effects of nicotine on airway physiology. For this, mice were exposed to nicotine and then submitted to evaluation using body plethysmography, which allows us to evaluate several parameters including respiratory rate, tidal volume, and airway resistance, among others. These studies are not complete, but the preliminary data suggest that animals exposed to nicotine show increased minute ventilation and higher airflow resistance when exposed to a bronchoconstrictor like methacholine (Fig 3). In general, we are adding more measurements to this study to strengthen the data.



Legend to Figure 3. Eight-week old mice exposed to prenatal nicotine have higher minute ventilation in response to methacholine challenge. Methacholine challenge was performed in live, unrestrained 8 week old mice born to mothers given nicotine in the drinking water. These mice had

increased minute ventilation requirements during the challenge compared to a control group (left image). They also showed higher resistance to airflow (as determined by measuring PenH) than their control counterparts.

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, the period of coinciding with branching morphogenesis. Furthermore, we recently found that agents capable of inhibiting apoptosis (zinc and autocarboxylic acid) inhibit lung branching morphogenesis. This effect appears to be modulated by extracellular matrix components like fibronectin and type I collagen. **This work was published in *Experimental Lung Research*.**

KEY RESEARCH ACCOMPLISHMENTS

- Our studies showed that nicotine stimulates lung branching, and that this effect is mediated through $\alpha 7$ nAChRs as demonstrated by the fact that antagonists of nAChRs inhibit the effect and so did the use of $\alpha 7$ nAChR knockout mice. The nicotine effect was also associated with upregulation of fibronectin.

- 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.

- We have found that prenatal nicotine exposure results in increased expression of NGF and larger neuronal structures in embryonic lungs. This suggests that nicotine may affect the inervation of the developing airways which, in turn, could affect airway function.

- 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. The results reveal that the total airway length is increased in nicotine-treated lungs confirming that nicotine stimulates lung branching *in vivo.*

- We have evaluated lung physiology in control- and nicotine-treated wildtype and $\alpha 7$ nAChR animals using body plethysmography. Observations made using this system shows that nicotine stimulates airway hyperactivity.

- In an attempt to better understand the mechanisms that control lung growth, we studied the process of cell apoptosis in lung development. We discovered that apoptosis is most prominent in pseudoglandular-stage lungs coinciding with the time period of branching morphogenesis. Interestingly, agents that inhibit apoptosis enhanced branching suggesting an important role for apoptosis in regulation of lung branching. We are now exploring how these events are affected by nicotine.

- 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. Alterations in neuronal innervations of airways might also contribute.

REPORTABLE OUTCOMES

- **Abstracts:** 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 (ATS) Meeting in San Diego, CA. Another abstract was presented at the 2007 ATS meeting in San Francisco, CA. A third abstract was presented at the 2008 ATS meeting held in Montreal, Canada. A fourth abstract is to be presented at the 2009 ATS meeting in San Diego:

  Abstract #1: Wongtrakool C, Aguayo SM, **Roman J**. Prenatal nicotine exposure promotes airway dysfunction by altering lung development. AJRCCM, A525, 2005. Presented during 2005 ATS Meeting, San Diego, CA.

  Abstract #2: Wongtrakool C, Hyde D, Roser-Page S, **Roman J**. Prenatal nicotine exposure results in greater post-natal total airway length by stimulating branching morphogenesis. Am J Respir Crit Care Med, 175:A89, 2007.

  Abstract #3: Wongtrakool W, Roser-Page S, **Roman J**. Prenatal nicotine exposure alters neuronal growth pattern in the developing lung. Submitted to 2008 American Thoracic Society Meeting, Montreal, Canada.

  Abstract #4: Wongtrakook C, Rivera HN, Roser-Page S, **Roman J**. Nicotine-induced nerve growth factor expression is mediated via a7 nAChRs. To be presented during 2009 American Thoracic Society Meeting, San Diego, May 2009.

- **Manuscripts:**

  A manuscript summarizing data related to nicotine and nAChRs and their role in lung branching was published (Wongtrakool et al., Am J Physiol, 293:L611-L618, 2007).

  A manuscript summarizing our work related to apoptosis and mammalian lung development was published (Wongtrakool C and Roman J. Exp Lung Research, 34:481-499, 2008).

  A review article describing this and other work related to tobacco-related lung disease and how tobacco and nicotine exposure may affect the developing lung and predispose the patient to asthma was published (Wongtrakool C and Roman J. Current Respiratory Reviews, in press).

CONCLUSIONS

Nicotine can affect the development of the primitive airways as well as the growth of the lung. Nicotine can also affect the expression of neuropeptides and the development of neuronal structures in embryonic lungs. In this fashion, nicotine could promote 'dysanaptic lung growth' and alterations in airway innervation 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.

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APPENDICES

Wongtrakool C, Roser-Page S, Rivera HN, Roman J. Nicotine alters lung branching morphogenesis through the $\alpha 7$ nicotinic acetylcholine receptor. Am J Physiol, 293:L611-L618, 2007.

Wongtrakool C and Roman J. Apoptosis of mesenchymal cells during the pseudoglandular stage of lung development affects branching morphogenesis. Exp Lung Research, 34:481-499, 2008).

Wongtrakool C and Roman J. Tobacco smoke exposure, nicotine, and the embryologic origins of asthma. Current Respiratory Reviews, 2009, in press.