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Recruitment of GABA_A Receptors in Chemoreceptor Pulmonary Neuroepithelial Bodies by Prenatal Nicotine Exposure in Monkey Lung

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Abstract

Pulmonary neuroepithelial bodies (NEB) act as airway oxygen sensors and produce serotonin, a variety of neuropeptides and are involved in autonomic nervous system control of breathing, especially during the neonatal period. We now report that NEB cells also express a GABAergic signaling loop that is increased by prenatal nicotine exposure. In this study, cultured monkey NEB cells show hypoxia-evoked spikes and hypoxia-sensitive K⁺ current. As shown by both immunofluorescence and RT-PCR, monkey NEB cells synthesize and contain serotonin. The monkey NEB cells express the β 2 and β 3 GABA_A receptor subunits, GAD and also express α 7, α 4 and β 4 nicotinic receptor (nAChR) subunits. The α 7 nAChR is co-expressed with GAD in NEB. The numbers of NEB and β 3 GABA_A receptor subunits expressed in NEB cells in lungs from control newborn monkeys were compared to lungs from animals that received nicotine during gestation. Prenatal nicotine exposure increased the numbers of NEB by 46% in lung and the numbers of NEB cells expressing GAD and GABA_A β 3 receptors increased by 67% and 66%, respectively. This study suggests that prenatal nicotine exposure can modulate NEB function by increasing the numbers of NEB cells and by increasing both GAD expression and β 3 GABA_A receptor subunit expression. The interaction of the intrinsic GABAergic system in the lung with nicotinic receptors in PNEC/NEB may provide a mechanism to explain the link between smoking during pregnancy and SIDS.

1. INTRODUCTION

Pulmonary neuroepithelial bodies (NEB) form innervated cell clusters in airways and express voltage-activated hypoxia-sensitive K⁺ channels and act as airway chemoreceptors (Youngson et al, 1993). Our previous studies showed that NEB cells in hamster express functional heteromeric α 3 β 2, α 4 β 2 and homomeric α 7 nicotinic acetylcholine receptors (nAChR) (Fu et al, 2003). Nicotine has previously been shown to regulate the function of the oxygen-sensitive A type K⁺ channels in rabbit NEB cells (Fu et al, 2007). Increased numbers of PNEC/NEB cells have been observed in several smoking-associated pediatric lung disorders such as bronchopulmonary dysplasia, cystic fibrosis, sudden infant death syndrome (SIDS), and asthma (Adgent et al, 2006; Plummer et al, 2000). Deficiency of NEBs' oxygen-sensor function under nicotine and tobacco-specific toxicants may be the one of factors that causes SIDS. It has recently been reported that both GABA_A receptors and GAD are expressed in human bronchial epithelial cells (Xiang et al, 2007), but little is yet known about GABAergic expression in NEB. Little is also yet known about NEB function in nonhuman primates which provide an animal model closest to humans. In the present study, we report that monkey NEB cells show hypoxia-evoked spikes and hypoxia-sensitive K⁺ current and that monkey NEB express a GABAergic signaling loop that is increased by prenatal nicotine exposure.

2. METHODS

2.1 Monkey neuroepithelial cell and bronchial epithelial cell culture

Lungs were obtained from rhesus monkeys (fetal to 1 year old) sacrificed as part of ongoing protocols. All protocols were approved by the Oregon Health & Science University Animal Care and Use Committee. Whole lungs were dissected and immersed in ice cold MEM medium. The major bronchi were crudely dissected and then incubated overnight at 4° C in 0.1% Protease type 14 (Sigma) in MEM. The cells were washed from mucosal surface of the airway using MEM with 5% fetal calf serum, centrifuged, resuspended, and plated in bronchial epithelium culture medium containing 2% fetal calf serum (Wu et al 2000). For patch clamp analyses, the cells were plated on Thermanox plastic cover slips (NUNC, Rochester, NY). For immunofluorescence analyses, cells were plated on glass cover slips.

2.2 Patch clamp recording of whole-cell voltage and current configurations

Action potential and K⁺ currents were recorded from cultured monkey neuroepithelial cells using standard whole cell patch-clamp techniques. Identity of cells as neuroepithelial cells was confirmed by staining for vital dye neutral red (0.02mg/ml) for 10 min at room temperature. The external Krebs solution bathing the NEB had the following composition (in mM): 130 NaCl, 3 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 NaHCO₃, 10 Hepes, and 10 glucose, pH 7.35 ~7.4. To isolate outward K⁺ currents, an internal pipette solution with following composition was used (mM): 30 KCl, 100 potassium gluconate, 1 MgCl₂, 10 EGTA, 10 Hepes, 4 Mg-ATP; pH adjusted to 7.2 with CsOH. The pipette resistance was 3 to 5 MΩ. The access resistance was ≤ 15 MΩ. The chamber, which had a volume of 1 ml, was perfused continuously with Krebs solution at a rate of 3-4 ml/min. Drugs were applied to the perfusate, and their delivery to the cells was controlled by separate valves. A multiClamp700B (Axon Instruments, Foster, CA, USA) amplifier was used to record for whole-cell currents (voltage clamp) or membrane potential (current clamp). The data were filtered at 5 KHz. Voltage and current clamp protocols, data acquisition and analysis were performed using pClamp9 software and DigiData 1322A interface (Axon Instruments). All data values are given as mean ± S.E.M.

2.3 Immunohistochemistry and fluorescence

Lung samples were obtained from timed-pregnant rhesus monkeys administered nicotine (1-1.5 mg/kg/d, subcutaneously) or water using osmotic minipumps from days 26-134 or 26-160 of gestation (full term=165d). Lungs were obtained at necropsy and fixed with 4% paraformaldehyde. Fluorescent staining was performed on monkey lung tissue following cryostat sectioning (5μM). The primary antibodies used were as follows: polyclonal rabbit anti-glutamate decarboxylase 65/67 (GAD 65/67) (1:100, Chemicon, Billerica, MA); polyclonal rabbit anti-GABA_A receptor β3 (1:100, Chemicon); mouse monoclonal α7 nAChR (306, 1:200, Sigma,); monoclonal rat anti-serotonin (1:100, Medicorp, Montreal, Canada). The secondary antibodies used were FITC-conjugated horse anti-mouse IgG, anti-rabbit and rat IgG (all 1:400, Jackson ImmunoResearch Laboratories, West Grove, PA). For negative controls, the primary antisera were omitted. Samples were viewed under an Axioskop 2 and Axiovision 4.2 was used for image acquisition (Zeiss, Germany).

3. RESULTS

3.1 Hypoxia sensitive action potential and outward K⁺ currents in cultured monkey NEB cells

Neuroepithelial bodies (NEB) in primary cultures of bronchial epithelial cells were visualized by vital dye neutral red (0.2mg ml⁻¹, Fig. 1 A). To test whether cultured NEB cells were sensitive to hypoxia, current clamp was used to record membrane potential. NEB cells did not exhibit action potentials when cells were perfused with normoxic Krebs solution. By contrast,

hypoxic solution (bubbling 95% N₂, PO₂ = 20 mmHg) evoked action potentials from NEB cells (Fig. 1 B). The mean membrane potential of cultured NEB cells was -50 ± 8 mV (n=11). Using whole-cell voltage clamp, depolarizing voltage steps from holding potential of -60 mV to $+50$ mV activated a delayed rectifier outward K⁺ current. The activation threshold of this K⁺ current was around -30 mV. The current amplitudes at test potential of 50 mV were -367 ± 10.5 pA (n=6). Upon exposure to hypoxia, outward K⁺ current was reversibly reduced by 30% (Fig. 1 C and D). The *I-V* relationship curve shows that the oxygen sensitive K⁺ current is voltage dependent (Fig. 1 D). These results suggest that monkey NEB cells express oxygen-sensitive K⁺ channels. Thus hypoxia inhibition of K⁺ channels plays a role in inducing action potentials in NEB cells.

3.2 Recruitment of GAD and GABA_A β3 receptors in NEB cells by prenatal nicotine exposure

The expression of GAD and GABA_A β3 receptors in NEB cells was examined using antisera specific for GAD65/67 and β3, respectively. Serotonin staining was used as a marker for NEB cells. The numbers of NEB was increased 46% in prenatal nicotine exposed-lung compared to control lung (Fig. 2A-a,b). In NEB cells, both GAD and GABA_A β3 immunoreactivity were expressed on the plasma membrane and submembrane location (Fig. 2A-a,c). The numbers of NEB cells expressing GABA_A β3 receptor subunits from control newborn monkeys were compared to lungs from animals that received prenatal nicotine exposure. Prenatal nicotine exposure increased the numbers of NEB cells expressing GAD and GABA_A β3 receptors by 67% and 66%, respectively (Fig. 2A-b,d and 2B). The α7 nAChR subunit (Fig 2A-e) is co-expressed with GAD in NEB (Fig. 2A-f).

4. DISCUSSION

Our study shows that NEB cells cultured from monkey lung express oxygen sensors as previously reported for rabbit, hamster and mouse. Because lung development in monkeys is highly similar to lung development in humans this finding provides an important new model to study developmental influences on oxygen sensing by NEB. The oxygen-sensing properties of NEB cells is very important during the neonatal period because NEB cells function as auxiliary chemoreceptors while the function of the carotid bodies is still immature. NEB appear to have a dual function; first through secretion of their amine and peptide products, NEBs may modulate lung growth and differentiation; second, both before and after birth, innervated NEB cells could play an important role as airway chemoreceptors (Linnoila et al, 2006). Consistent with the first function, NEB cells are associated with stem cell niches in both the proximal and distal airways. One hypothesis to explain the link between maternal smoking during pregnancy and sudden infant death syndrome (SIDS) is that prenatal nicotine exposure alters the oxygen-sensing properties of NEB cells and hence the ability of NEB to respond to hypoxia.

The potential importance of GABAergic signaling in lung has recently been highlighted by Xiang et al (2007) who reported that GAD and GABA_A receptors are expressed in airway epithelial cells of human and mouse and are increased in rodent models of asthma. Thus GABAergic signaling may play a similarly important role in NEB function. In this study we show that NEB cells express a GABAergic signaling loop. Thus our study suggests that nicotine may modulate the function of NEB cells both by increasing the numbers of NEB cells and also by increasing GAD and β3 GABA_A receptor subunit expressions in NEB cells. Interestingly, our study also shows that the α7 nAChR subunit is co-expressed with GAD in the same NEB cells. This suggests there may be direct protein-protein interaction between cholinergic and GABAergic systems in NEB cells. In neurons, two proteins, Plc-1 and the GABA_A receptor associated protein (GABARAP) have been shown to regulate GABA_A receptors. Plc-1, an Ubiquitin-like protein, regulates GABA_A receptor α and β subunits (Fiona et al, 2001). GABA_A receptor associated protein has been shown to regulate expression of the γ1, γ2s and

γ 2L GABA receptor subunits (Chen et al, 2007). Cultured monkey NEB cells express both Plic-1 and GABA_A associated protein. Thus nicotine may modulate function of GABA_A receptors in NEB via Plic-1 and the GABA_A associated protein. Further studies are needed to clarify this.

Acknowledgments

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A

C

B

D

Figure 1.

Effect of hypoxia on action potential and outward K^+ current in cultured NEB cells. **A.** Staining of cultured NEB cells using neutral red. **B.** Hypoxic solution induced a spike in a single NEB cell. **C.** Outward K^+ current evoked by depolarization steps from -60 mV to 50 mV in control Krebs solution and hypoxic solution. Outward K^+ current was reduced by hypoxia and a recovery K^+ current by washout of the hypoxic solution. **D.** I-V relationship for the current in the control solution (●) and in the hypoxic solution (○) are plotted together with recovery K^+ current (▽) against holding voltage. Asterisks indicate significant differences from control ($P < 0.05$).

A

a

b

c

d

B

Figure 2.

Prenatal nicotine exposure increased GAD and GABA_A β3 receptor expression in NEB cells. Serotonin immunostaining was used as an NEB cell marker. (a) Double staining for 5HT and GAD in NEB cells. (c) Double staining for 5HT and GABA_A β3 receptor in NEB cells. In lungs from animals which received prenatal nicotine exposure, nicotine increased GAD and GABA β3 receptor expression in NEB cells (panels b and d). (e) nAChR α7 subunit is expressed in NEB cells. (f) Double staining of α7 nAChR with GAD in NEB cells. **B.** Summary graph shows the effects of nicotine on the numbers of NEB, and the numbers of NEB cells expressing GAD and GABA_A β3 receptors. Serotonin-positive cells, and cells double stained for 5-HT and GAD or GABA_A β3 receptor cells were counted in fields obtained with a 20X objective. A total of 20 fields were counted per data point. The calibration bar is 30μm in panels a -d and 50 μm in panels e and f.