The ly-6 protein, lynx1, is an endogenous inhibitor of nicotinic signaling in airway epithelium

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Fu XW, Rekow SS, Spindel ER. The ly-6 protein, lynx1, is an endogenous inhibitor of nicotinic signaling in airway epithelium. Am J Physiol Lung Cell Mol Physiol 303: L661–L668, 2012. First published August 24, 2012; doi:10.1152/ajplung.00075.2012.—Our laboratory has previously shown that lynx1 is highly expressed in airway epithelial cells, it would seem likely that lynx1 agonists or mimetics are a potentially important therapeutic target to develop new therapies for smoking-related diseases characterized by increased mucin expression.

Potential negative regulators of airway nicotinic signaling have important implications for chronic lung disease because so much of lung disease is linked to smoking and excess mucin production. Smoking is intimately related to asthma, chronic bronchitis, and chronic obstructive pulmonary disease (COPD). Greater than 40% of smokers develop chronic bronchitis (31), and 25% of smokers will develop COPD, which combines chronic mucus production with decreases in lung function. Similarly, smoking both increases risk of asthma and the severity of asthma (32). In COPD, increased mucus secretion is continuous and a major component of the disease; in asthma, mucus secretion is also increased, and mucus plugs are associated with the most severe exacerbations and fatalities (12, 16). Thus negative modulators of nicotinic activity would have the potential to decrease mucin expression.

The goal of this study was to further characterize the neurotransmitter cascade in BEC to determine whether lynx1 regulates cholinergic activation of GABAergic signaling and mucin expression and hence provides a new therapeutic target for smoking-induced lung disease.

MATERIALS AND METHODS

Epithelial cell culture. All animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Utilization Committee. Bronchial epithelial cell cultures were...
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established from lungs obtained from rhesus macaques (newborn to 2 yr old) undergoing necropsy from protocols not expected to alter lung function as described by Wu et al. (33, 48) with modifications as previously described by Fu et al. (14). All drugs used for the study were obtained from Sigma (St. Louis, MO).

Immunofluorescence for lynxl and α7 nAChR receptors. The primary antibodies used were rabbit polyclonal antiserum raised against: anti-mock lynxl (antibody R4, rabbit, 1:300) (39) and anti-α7 nAChR (antibody 306, mouse, 1:1,000, Sigma). Secondary antibodies were FITC-conjugated horse anti-mouse (1:400 dilution) and Cy3-conjugated goat anti-rabbit (1:400 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Mouse brain sections were used for positive controls. Primary cell cultures were established then incubated in serum-free bronchial epithelium culture media (Cell Application, Carlsbad, CA) for 7–10 days and then fixed with 4% paraformaldehyde for immunofluorescence. For negative controls, the primary antisera were omitted. Samples were viewed on a confocal microscope (Leica TCS SP, Jena, Germany). Unless otherwise specified, antibodies used were against the human protein, but the high homology between rhesus and human proteins ensures in our experience almost 100% cross-reactivity.

RT-PCR and real-time PCR. Total RNA was extracted from monkey brain, lung, and cultured BEC (5 × 10⁶ cells) using Trizol (Invitrogen, Carlsbad, CA), and real-time PCR was performed as described previously (38). Primers and probes not previously described were as follows: lynxl: 5'-GGGCTTCTACGACATCGAG-3'; GGTCCACGAACGGAACTTCA, 3'-AATACGCTCAGGTGGGCTGAT-5'. Secondary antibodies were run in duplex with primers and a probe for 18S (internal standard set from Life Technologies), which was used for standardization. Real-time conditions were 50°C for 15 s, 95°C for 10 min, 95°C for 15 s, 60°C for 1 min with the last two steps repeated 40 times.

siRNA knockdown. ON-TARGET plus siRNAs for lynxl, α7 nAChR, α4 nAChR, and negative control siRNA were purchased from Dharmacon (Lafayette, CO). siRNAs were transfected at a concentration 100–150 nM with DharmaFECT 1 according to the manufacturer’s instruction. Forty-eight hours after transfection, cells were harvested for real-time PCR or for Western blotting. The lynxl siRNA (TCAGCAACATCGGAAACTTT) was chosen to be distinct from the other transcripts of the lynxl gene and was 100% homologous to NM_0012666622.

Immunoprecipitation and Western blotting. Western blots were performed as described previously (42). For immunoprecipitation, solubilized BEC cellular extracts (400–600 μg of protein) were incubated in the presence of anti-α7 (antibody 306, mouse, 1:800) or IgG (1–2 μg) for 4 h at 4°C, followed by the addition of 20 μl of protein A/G agarose for 12 h. Pellets were washed, boiled in SDS sample buffer, subjected to SDS-PAGE, transferred to a PVDF membrane (Millipore, Billerica, MA) and incubated with anti-lynxl antibody (rabbit, 1:100) and or anti-β-actin (mouse, 1:20,000, Sigma) overnight at 4°C. Blots were visualized using Immobilon Western Chemiluminescent horseradish peroxidase (HRP) substrate (Millipore) using HRP-anti-goat or anti-rabbit (1:800; Promega, Madison, WI) and analyzed using a FluorchemHD2 and Fluorchem software (Alphalnnotech, Santa Clara, CA). All experiments were repeated at least three times. Antibodies used for Western blotting were anti-GABAₐR β2(3 (rabbit, 1:1,500, Millipore no. 05–474); anti-GABAₐR α5 (rabbit, 1:500, Millipore no. AB9678), anti-c-Src (mouse, no. sc-130124; Santa Cruz Biotechnology, Santa Cruz, CA) and p-Src (mouse, Santa Cruz no. sc-81521).

Statistics. All data are given as means ± SE, and statistical comparisons were made using ANOVA followed by Fisher’s post hoc multiple comparisons or unpaired, two-tailed Student’s t-tests.

RESULTS

Lynxl and α7 nAChR expression in rhesus lung and BEC. Lynxl colocalizes with α7 nAChR. Lynxl immunostaining in monkey lung was strongest in airway epithelium as previously reported (Fig. 1A) (39). Dual immunofluorescence for lynxl and α7 nAChR showed colocalization in epithelium. In large airway epithelium, lynxl and α7 staining were both primarily apical with α7 even more apical (Fig. 1A). To further prove colocalization, primary cultures of rhesus BEC were established and stained for lynxl and α7. As shown in Fig. 1B, BEC clearly expressed both α7 and lynxl. These experiments establish that lynxl is colocalized with α7 nAChR in monkey BEC. Next immunoprecipitation was performed to determine whether lynxl and α7 forms a complex in BEC as has been reported for neurons and HEK cells (20, 27). As shown in Fig. 1C, Western blot analysis showed the presence of α7 and lynxl in BEC total extracts. Immunoprecipitation of BEC with anti-α7 showed that α7 and lynxl form a complex in BEC just as occurs in neurons.

Lynxl is a negative regulator of nicotine-induced nAChR expression. We and others have previously reported that chronic nicotine increases nAChR expression at the mRNA and protein level (1, 13, 35, 50). To determine whether lynxl plays a role in the ability of nicotine to upregulate nAChR mRNA levels, cultured BEC were treated with 1 μM nicotine for 48 h in the presence of control siRNA or lynxl siRNA, and nAChR levels of nAChR were measured by real-time PCR. The siRNA used was chosen to target only lynxl and not either of the forms of slurp-2 that are also transcribed from the lynxl gene (2). The lynxl siRNA caused a 75% decrease in lynxl siRNA levels. As shown in Fig. 2A, the combination of nicotine and lynxl knockdown significantly increased levels of α4, α7, α9, and β4 nAChR subunit mRNAs (Fig. 2A). As shown in Fig. 2, B and C, changes in levels of lynxl and α7 proteins followed the same trends as the changes in levels of their respective mRNAs. Thus these data show that lynxl acts as a negative modulator of the ability of nicotine to upregulate nAChR mRNA and protein levels.

Lynxl negatively modulates the downstream effects of α7 nAChR signaling in BEC. We previously reported that activation of nAChR in BEC by nicotine or ACh leads to increased levels of GAD, GABAₐR, and mucin expression by BEC (14). Thus the ability of nicotine to upregulate GABA signaling in BEC provides a good readout of nAChR signaling. Consistent with our previous report (14) treatment of cultured BEC with nicotine (1 μM × 48 h) significantly increased mRNA levels for GABAₐR α5 compared with control (Fig. 3A) and similar effects were seen on GABAₐR protein levels (Fig. 3, C and D). Importantly, knockdown of lynxl enhanced the ability of nicotine to increase levels of GABAₐR α5 and GABAₐR β2 (Fig. 3, A and B). This effect appeared to be mediated by α7 nAChR, as the effect of the lynxl knockdown on the nicotine-induced increase in GABA was abolished by siRNA knock-
down of α7 nAChR (70% knockdown of mRNA for α7 nAChR) (Fig. 3, E and F). The α7 nAChR-specific antagonist methyllycaconitine (30 nM) similarly blocked the ability of the lynx1 siRNA to increase the effects of nicotine on GABAAR expression (data not shown). These results suggest that lynx1 serves as a negative modulator of the α7 signaling as shown by the effect of lynx1 knockdown on the α7 nAChR-mediated effect of nicotine on GABAergic expression in BEC.

**Lynx1 modulation of nicotine-induced activation of GABA signaling is dependent on Src phosphorylation.** The protein tyrosine kinase Src has been shown to mediate downstream effects of nicotinic activation of α7 nAChR lung cancer cells (7, 9). We thus examined whether Src activation might also be involved in lynx1 modulation of α7 signaling in BEC. First, as shown in Fig. 4A, knockdown of lynx1 increased basal and nicotine-stimulated levels of Src and p-Src in BEC. Knockdown of also α7 prevented the increase in Src and p-Src caused by the lynx1 knockdown. These results suggest that lynx1 acts to limit activation of Src signaling by α7 nAChR and thereby modulates GABAAR levels. As shown in Fig. 5, the increases in levels of p-Src reflected both increases in total Src as well as increases in the p-Src/Src ratio.

Next, the role of Src in mediating the effects of nicotine and lynx1 on GABA expression was confirmed by the use of inhibitors. As shown in Fig. 5A, 1 μM PP2, a potent inhibitor of Src family kinases, blocked the ability of nicotine and lynx1 knockdown to increase BEC GABAAR α5 mRNA expression. By contrast, the PKC inhibitor GF 109203X had no effect (Fig. 5A). This suggests that nicotine increases GABAAR expression through a Src-dependent mechanism that is inhibited by lynx1.

**Lynx1 modulates MUC5AC mRNA expression.** Mucus overproduction characterizes most smoking-associated lung dis-
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Fig. 2. Lynx1 knockdown potentiated the increase in nAChR mRNA and protein caused by nicotine. A: effect of lynx1 siRNA knockdown on the ability of nicotine (1 μM for 48 h) to increase levels of nAChR subunits. Values are expressed as relative fold change of each condition vs. control with error bars showing SE (*P < 0.05 compared with control by Fisher’s multiple-comparison tests after 1-way ANOVA). All control RNA levels were normalized to 1, and 18S RNA levels were used as an internal standard (n = 5–9 per group). B: representative Western blot analysis showing the lynx1 knockdown-induced decrease in lynx1 protein and the resulting increase in nicotine-induced α7 nAChR protein. C: quantification of protein changes shown in B (n = 4 per group).

The present study shows that lynx1 colocalizes and forms a complex with α7 nAChR in BEC and serves as a negative regulator of α7 nAChR signaling. Knockdown of lynx1 increased the ability of nicotine to sequentially activate nicotinic and GABAergic signaling by BEC, leading to increased nicotine-stimulated MUC5AC RNA expression. This finding has implications both for the general physiology of transmitter signaling cascades in airway epithelium and specifically for potential new ways to target airway diseases characterized by the overproduction of mucus.

Airway epithelial cells express multiple classical neurotransmitters and their receptors including ACh, serotonin, GABA, glycine, and glutamate (14). We have recently reported that these systems communicate within BEC similarly to neuronal networks to modulate physiological function, as evidenced by the ability of nicotinic signaling to upregulate GABA signaling (14). In this study, we extend the parallels of BEC signaling to neuronal networks by showing that BECs similarly use transmitter receptor-negative allosteric modulators to regulate signaling. In 1999, Miwa et al. (20, 27) isolated lynx1 based on its homology to the α7 nAChR antagonist αBGT and showed that lynx1 functioned as a negative allosteric modulator of nAChR function to play critical roles in neuronal survival and plasticity (29). We have previously reported that high levels of lynx1 are expressed in BEC and that levels of lynx1 are affected by chronic nicotine exposure (39), thus suggesting that lynx1 likely affects nicotinic signaling in lung as in brain. Consistent with this, as shown in Fig. 1, lynx1 specifically colocalizes with α7 nAChR in BEC both in vivo and in primary cultures of BEC. Furthermore, lynx1 forms a complex with α7 nAChR in BEC as shown by immunoprecipitation (Fig. 2). This suggests that lynx1 serves to modify α7 signaling in BEC. Although immunohistochemistry for nicotinic receptors can be nonspecific (30), specificity of the staining reported here is supported by our previous validation of α7 expression in lung by DNA sequencing and ligand binding (37) and lynx1 expression by DNA sequencing and in situ hybridization (39).

Reflecting the high expression of nAChR in airways, multiple reports of nicotinic functions in lung have been reported, including regulation of chloride secretion (18), chemosensation (22), differentiation (23), and cell growth (34, 47). Our laboratory and others have also reported direct results of nicotinic activation in lung, including upregulation of nAChR expression (1, 13, 35, 50) and upregulation of GABA signaling and mucin expression (14). This would suggest that, if lynx1 is a negative regulator of nicotinic signaling in BEC, then these specific results of nicotinic signaling in BEC should be modified by lynx1. This is indeed the case. First, as shown in Fig. 2, siRNA knockdown of lynx1 significantly increased the ability of nicotine to increase nAChR mRNA levels. No effect was seen with control siRNAs, whereas siRNA knockdown of lynx1 significantly increased the ability of nicotine to increase levels of α4, α7, α9, and β4 nAChR mRNAs. Similarly, knockdown of lynx1 increased levels of GABA_A_R (Fig. 3) in an α7 nAChR-dependent manner. Thus lynx1 serves as a negative regulator of nicotinic signaling and also modulates the ability of nicotinic signaling to activate GABA signaling. These effects of lynx1 appear to be mediated by α7 nAChR because both α7 knockdown and α7 antagonists block the ability of lynx1 knockdown to increase levels of both nAChR and GABA_A_R mRNAs (Figs. 2 and 3). The role of α7 is further supported by its coimmunoprecipitation with lynx1 as shown in Fig. 1.

DISCUSSION

The present study shows that lynx1 colocalizes and forms a complex with α7 nAChR in BEC and serves as a negative regulator of α7 nAChR signaling. Knockdown of lynx1 increased the ability of nicotine to sequentially activate nicotinic and GABAergic signaling by BEC, leading to increased nicotine-stimulated MUC5AC RNA expression. This finding has implications both for the general physiology of transmitter signaling cascades in airway epithelium and specifically for potential new ways to target airway diseases characterized by the overproduction of mucus.
Dasgupta et al. (7) have shown that the Src signaling pathway mediates the effects of α7 activation on lung cancer proliferation. In A549 lung adenocarcinoma cells, nicotine induces the formation of an oligomeric complex, involving arrestin-β1 (ARRB1), Src, and α7 nAChR that results in the activation of Src (7–9). This in turn leads to activation of Ras, Raf, and Erk and increased cell proliferation (7, 8). Our study shows that Src similarly appears to mediate the ability of nicotine and lynx1 knockdown to increase levels of nAChR and GABAAR in normal BEC. As shown in Fig. 4, nicotine increases Src and Src phosphorylation, and this increase is further enhanced by lynx1 knockdown. The Src activation caused by nicotine and lynx1 knockdown is dependent on α7, as α7 knockdown prevents both increased Src activation and increased GABAAR expression. This suggests that one way in which lynx1 modulates nicotinic signaling is through inhibition of Src activation. This is supported by the ability of PP2, an inhibitor of Src kinase to block the nicotine and lynx1 knockdown-induced increase in and GABAAR mRNA levels (Fig. 5A). The mechanism of lynx1 regulation of activation of α7 signaling by lynx1 remains to be determined but may involve displacement of lynx1 from α7 and a resulting conformational shift in α7.

Dasgupta et al. (8) have demonstrated that nicotinic activation of α7 nAChR leads to transcriptional activation through ARRB1 translocating into the nucleus and forming a complex with E2F. Thus a similar mechanism may underlie the mechanism by which nicotine activation of α7 nAChR in BEC increases levels of nAChR and GABAAR mRNAs although this clearly requires further investigation. Transcriptional activation may also explain the increase in total levels of Src protein (Fig. 4) although this too requires further investigation. The increases in levels of total Src caused by nicotine thus have potential to create a positive feedback loop.
Xiang et al. (49) have demonstrated that increased GABA signaling in BEC leads to increased mucus production. We recently reported that one mechanism by which smoking increases mucus production is nicotine activating \( \alpha 7 \) nAChR signaling, which in turn leads to increased GABA signaling (14). Thus, if lynx1 acts as a negative modulator of \( \alpha 7 \) signaling, then lynx1 should also modulate the ability of nicotine to cause increased mucin expression. As shown in Fig. 5B, this is indeed the case; knockdown of lynx1 significantly increases the ability of nicotine to upregulate Muc5ac mRNA levels and, like the effects on GABA\(_A\)R mRNA levels, is Src dependent.

Although this study is focused on the role of lynx1 in normal lung, this also has important implications for lung cancer.

**Fig. 4.** Lynx1 modulates the ability of nicotine to activate Src signaling. A: representative Western blot showing knockdown of lynx1 increased basal and nicotine-stimulated (2 \( \mu \) M nicotine \( \times 48 \) h) levels of Src and p-Src. Knockdown of \( \alpha 7 \) prevented the increase in Src and p-Src caused by the lynx1 knockdown. As shown in B and C, increases in p-Src primarily reflect increases in total Src, as levels of both c-Src and p-Src are increased. Images are representative from 4–5 similar experiments that were pooled for the densitometric analyses. *P < 0.05 compared with corresponding control by Fisher’s multiple-comparison tests after 1-way ANOVA.

**Fig. 5.** Src mediates regulation of GABA\(_A\)R and mucin mRNA expression by nicotine and lynx1. A: Src inhibitor PP2 (1 \( \mu \) M) decreased the ability of nicotine (1 \( \mu \) M) and lynx1 knockdown to increase GABA\(_A\)R \( \alpha 5 \) mRNA levels in cultured BEC. By comparison, the protein kinase C inhibitor GF 109203X (GF) had no effect. *P < 0.05 for nicotine + siRNA-treated group compared with groups shown by Fisher’s multiple-comparison tests after 1-way ANOVA. B: nicotine-induced increase in mucin mRNA was blocked by lynx1 knockdown. *P < 0.05 for nicotine + siRNA-treated group compared with groups shown by Fisher’s multiple-comparison tests after 1-way ANOVA (n = 5 per group). Drugs and siRNAs were added to cultures 48 h before harvesting of cells.
Given the increased expression of nicotinic receptors in lung cancer and the ability of nicotinic activation to stimulate lung cancer growth (7, 36, 41, 46), this suggests that levels of lynx1 expression might also modulate lung cancer growth. This is indeed the case, as we have recently reported in abstract form (40) and have further studies in progress.

The finding that lynx1 can modulate the nicotine-induced increase in mucin mRNA expression is potentially important given the role that mucus hypersecretion plays in COPD and asthma. COPD is almost always associated with smoking and is characterized by increased mucus production, and the degree of mucin expression correlates with the degree of airflow obstruction (5, 5, 11, 31). In asthma, mucus plugs occlude small airways (4, 10, 11, 17, 28, 32), and overproduction of mucus is associated with mortality in severe asthma attacks (4).

Although, however, mucin is a key component of many common respiratory diseases, there are few effective and specific treatments for excess mucin production. Thus lynx1 presents a new therapeutic target for asthma and COPD, and lynx1 agonists may serve to limit both basal and smoking-induced mucus production. Whether lynx1 levels are changed in diseases characterized by excess mucus production remains to be determined.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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