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Thymic Stromal Lymphopoietin in Cigarette Smoke-Exposed Human Airway Smooth Muscle

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Thymic stromal lymphopoietin (TSLP) is a newly identified IL-7–like cytokine known to be expressed in airway biopsies of patients with asthma and chronic obstructive pulmonary disease (COPD) (1, 2). In turn, airway diseases, such as asthma, are additionally characterized by increased responsiveness of airway smooth muscle (ASM) to bronchoconstrictor agonist (airway hyperresponsiveness), thus linking inflammation to contractility (3, 4). The mechanisms by which cigarette smoke results in an inflammatory response and/or airway hyperresponsiveness are still under investigation. In this regard, there is increasing recognition that ASM not only responds to inflammatory signals derived from immune cells or other sources in the airway but can itself produce a host of pro- and anti-inflammatory molecules (5–7). Accordingly, it could be hypothesized that cigarette smoke exposure results in modulation of ASM-derived inflammatory signals, thus altering overall airway inflammation and contractility.

Thymic stromal lymphopoietin (TSLP) is a recently identified IL-7–like cytokine (8, 9) that is known to be produced by epithelial cells in a number of organ systems including lung (10), gut (11), and skin (10). Cigarette smoking and secondhand smoke exposure leads to chronic inflammation and airway diseases including asthma and chronic obstructive pulmonary disease (COPD) (1, 2). In turn, airway diseases, such as asthma, are additionally characterized by increased responsiveness of airway smooth muscle (ASM) to bronchoconstrictor agonist (airway hyperresponsiveness), thus linking inflammation to contractility (3, 4). The mechanisms by which cigarette smoke results in an inflammatory response and/or airway hyperresponsiveness are still under investigation. In this regard, there is increasing recognition that ASM not only responds to inflammatory signals derived from immune cells or other sources in the airway but can itself produce a host of pro- and anti-inflammatory molecules (5–7). Accordingly, it could be hypothesized that cigarette smoke exposure results in modulation of ASM-derived inflammatory signals, thus altering overall airway inflammation and contractility.

In this regard, there is increasing recognition that TSLP is an early player in triggering of airway inflammation, via activation of dendritic cells, which polarize naïve T cells toward the Th2 pathway, stimulating production of cytokines, such as IL-4 and IL-13, as well as TNF-α (9, 10, 12). In the airways of asthmatics, TSLP expression correlates to disease severity (13). In mice, TSLP expression results in spontaneous airway inflammation and asthma phenotype (14), whereas absence of the TSLP receptor (TSLP-R) substantially blunts allergic airway inflammation (15). Thus, TSLP appears to be extremely important for development of airway inflammation. However, less is known about TSLP and airway contractility. Intriguingly, one study found that in addition to airway epithelium, ASM also produces TSLP and that, in vitro, TSLP expression by ASM increases in the presence of proinflammatory cytokines, such as TNF-α (16). Furthermore, TSLP immunoreactivity appears to be greater in ASM samples from patients with COPD, compared with normals (16). In terms of cigarette smoke, a single study found that, in mice, intranasal exposure of cigarette smoke extract (CSE) increases mRNA and protein expression of TSLP (17). Overall, although TSLP expression in ASM is very likely, its functional role in ASM is not known. In this regard, the expression and function of TSLP in ASM has not been determined.

In the current study, we hypothesized that ASM-derived TSLP contributes to increased airway responsiveness resulting from cigarette smoke. To address this issue, we used isolated human ASM cells to determine 1) ASM as a possible source of TSLP following cigarette smoke exposure; and 2) the potential autocrine effect of TSLP on ASM intracellular Ca2+ ([Ca2+]i) regulation, a key determinant of ASM contractility. Mechanisms by which TSLP expression may be regulated as well as those by which TSLP modulates [Ca2+]i were then explored.

Materials and Methods

Chemicals

Reagents for electrophoresis were obtained from Bio-Rad (Hercules, CA), anti-TSLP mAb and anti–TSLP-R polyclonal Ab from R&D Systems (Minneapolis, MN), anti-STAT5 polyclonal Ab, anti–phospho-STAT5
(Tyr694) polyclonal Ab, and anti-GAPDH polyclonal Ab from Cell Signaling Technology (Danvers, MA), nonspecific IgG sheep Ab from Sigma-Aldrich (St. Louis, MO), and DMEM:Ham’s F-12 medium, penicillin/streptomycin Lipofectamine 2000, and fura 2-AM from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma-Aldrich unless otherwise noted.

CSE preparation

The technique for CSE preparation has been described previously (18). Briefly, aqueous CSE was prepared using a modification of the method of Blue and Janoff (19) via a smoking apparatus (50-ml plastic syringe with a three-way stopcock) to which a cigarette (Kentucky 1RF4 cigarettes) and a sterile plastic tip were attached. Via the plastic tip, 35 ml cigarette smoke was slowly bubbled into 30 ml sterile RPMI 1640 at 37˚C for 30 min. One cigarette per 10 ml medium each was used. The CSE solution was then filtered and used immediately. Although a broad range of CSE concentrations could have been used in this study to determine CSE effects on ASM, in pilot studies, we determined the effect of CSE on ASM cell viability. Using trypan blue or propidium iodide exclusion, we found that 5% CSE concentration produced significant cell death. This was exacerbated by longer exposures of 48–72 h. Accordingly, we used lower concentrations (1 or 2%) to determine modulatory effects of CSE. A time period of 24 h was selected based on previous observation of altered protein expression within that time frame in human ASM cells. Nicotine and other metabolite concentrations in CSE were analyzed using liquid chromatography-mass spectroscopy as reported previously (18).

Human ASM cells

The technique for isolation of ASM cells from human lungs has also been described previously (20). Briefly, bronchi (third to sixth order) were excised from surgical lung tissue incidental to thoracic surgery at Mayo Clinic Rochester (approved by the Mayo Clinic Institutional Review Board). Normal lung areas were identified by the pathologist. Tissues were initially placed in HBSS (Invitrogen) with 2.5 mM extracellular Ca2+. The bronchiolies were freed of cartilage, epithelium, and surrounding tissues to excise ASM bundles. Samples were finely minced in ice-cold Ca2+-free HBSS (0” Ca2+ HBSS). For some biochemical work, tissue fragments were homogenized using cell lysis buffer. Following collagenase dissociation and ovomucoid/albumin separation, cell pellets were resuspended in DMEM:Ham’s F-12 medium with 10% FBS, centrifuged, and resuspended to a concentration of ~106 cells/ml. ASM cells were grown in a humidified incubator 95% air/5% CO2 in DMEM:Ham’s F-12 medium supplemented with 10% FBS. All experiments were performed in cells before the third passage to ensure retention of ASM phenotype (verified in sample subsets by immunostaining or Western blot analysis), human ASM expression of TSLP and TSLP-R (Fig. 1A) and TSLP-R expression over that of 1% CSE; however, this trend did not reach statistical significance. To determine whether even higher CSE levels had effects on ASM, in pilot studies, 5 and 10% CSE were used. However, we found that cell viability was <50% with these high concentrations. Accordingly, subsequent studies were limited to 1 or 2% CSE.

Immunoblotting of TSLP and TSLP-R

Tissue homogenates (25 μg protein) were first subjected to SDS-PAGE in 4–15% gels, and the fractionated proteins were transblotted to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked for 1 h with 5% milk in TBS containing 0.1% Tween 20, incubated with appropriate primary Ab overnight at 4˚C, washed with TBS containing 0.1% Tween 20, detected with HRP-conjugated secondary Abs, and developed with Supersignal West Pico Chemiluminescent Substrate (Pierce Chemical Co., Rockford, IL).

[Ca2+]i imaging

The techniques for real-time fluorescence [Ca2+], measurements in ASM have been described previously (20). Briefly, ASM cells plated in 8-well borosilicate coverglass chambers were incubated for 60 min in 5 μM fura-2-AM, washed, and perfused with HBSS. Cells were visualized with a real-time fluorescence imaging system (MetaFluor; Universal Imaging, Downingtown, PA) on a Nikon 300 inverted microscope (Fryer Instruments, Edina, MN) with a Cascade 12-bit digital camera system (Roper Scientific, Tucson, AZ). Images were acquired at 1.33 Hz and results expressed as the ratio of 510 nm emissions following excitation alternately at 340 nm and 380 nm (λ 10-2 filter changer; Sutter Instrument, Novato, CA). Cells were initially perfused with HBSS, and baseline fluorescence established. [Ca2+], responses of 10–15 cells per chamber were obtained using individual, software-defined regions of interest.

Statistical analysis

Experiments were performed using tissues obtained from five different patients, although all protocols were not performed in every sample. TSLP protein expression (measured by densitometry) was compared between groups using unpaired t test. [Ca2+]i responses in ASM cells were compared across groups of cells and interventions using two-way ANOVA with Bonferroni corrections for repeated comparisons. A p value of <0.05 was accepted as significant. All results are expressed as mean ± SE.

Results

CSE induces TSLP and TSLP-R levels in ASM

Using Western blot analysis, human ASM expression of TSLP and TSLP-R was determined (normalized to GAPDH expression). ASM cells were exposed for 24 h to vehicle only (growth medium; control), 1 or 2% CSE. Even in control ASM cells, substantial TSLP (Fig. 1A) and TSLP-R (Fig. 1B) expression was observed. Overnight exposure to CSE significantly increased TSLP and TSLP-R levels in both the 1 and 2% CSE groups (p < 0.05; n = 4 for each group). Exposure to 2% CSE tended to increase TSLP and TSLP-R expression over that of 1% CSE; however, this trend did not reach statistical significance. To determine whether even higher CSE levels had effects on ASM, in pilot studies, 5 and 10% CSE were used. However, we found that cell viability was <50% with these high concentrations. Accordingly, subsequent studies were limited to 1 or 2% CSE.
Although the above protocol determined that TSLP was expressed within ASM cells, it was important to further evaluate whether TSLP was actually secreted into the extracellular medium. In additional experiments, the serum-free extracellular medium of cells exposed to vehicle versus 2% CSE was collected, concentrated (Amicon Centriprep; Millipore, Bedford, MA), and then immunoblotted for TSLP. We detected basal levels of TSLP in the supernatant, with substantial increase in levels following exposure to 2% CSE (p < 0.05 compared with control; Fig. 2).

Two of the major biologically active constituents of cigarette smoke are nicotine and reactive oxygen species (ROS). To isolate the effect of these factors on upregulation of TSLP, human ASM was exposed to 1 mM nicotine or 0.02 mM hydrogen peroxide for 24 h (17, 18). Cells were then lysed, and relative protein levels were determined. Neither nicotine- nor hydrogen peroxide-treated cells increased TSLP substantially, compared with untreated cells (n = 4; Fig. 3). To determine whether CSE-induced increase in TSLP expression was a result of ROS activity, human ASM cells were pretreated to the antioxidant and ROS scavenger N-acetylcysteine (NAC) for 1 h at 10 mM (17) before additional overnight exposure to vehicle only or 2% CSE. Western blot analysis determined that NAC exposure by itself did not significantly alter TSLP expression (compared with untreated controls). NAC pretreatment significantly blunted 2% CSE-induced increase in TSLP expression (p < 0.05; n = 5; Fig. 3).

**CSE enhances [Ca^{2+}], responses in ASM**

In fura 2-loaded control ASM cells (i.e., not exposed to CSE or other agents), baseline [Ca^{2+}] levels ranged from 85 to 115 nM (n = 225, derived from five patient samples, at least 25 cells/sample). Exposure to 10 μM histamine acutely produced the typical transient [Ca^{2+}] elevation, with a higher peak (550 ± 34 nM), followed by a lower plateau (230 ± 29 nM). Overnight exposure of ASM cells to CSE significantly increased peak [Ca^{2+}] responses, with 2% CSE having a greater effect compared with 1% CSE (p < 0.05 for both comparisons; n = 138 for 1%, n = 159 for 2%, derived from five patient samples; Fig. 4).

**TSLP increases [Ca^{2+}], responses in ASM**

To investigate the effects of TSLP protein exposure on [Ca^{2+}] responses, ASM cells were treated with TSLP (20 ng/ml) for 24 h and then loaded with fura 2 for [Ca^{2+}] imaging. Previous studies investigating the effects of TSLP formed the basis for the concentration used in our study (15). Compared with vehicle-exposed control cells, TSLP-exposed cells showed a significantly higher [Ca^{2+}] peak response to histamine (p < 0.05; n = 145 derived from five samples; Fig. 5). Removal of extracellular Ca^{2+} (using “0” Ca HBSS with 5 mM EGTA) substantially blunted the [Ca^{2+}], response to histamine in vehicle-exposed cells as well as in TSLP-exposed cells. However, even in the absence of extracellular Ca^{2+}, TSLP produced significant (albeit smaller) enhancement of [Ca^{2+}] responses to histamine (p < 0.05; n = 74, derived from three samples; Fig. 5).

**CSE activates STAT5 in ASM**

Western blot analysis of ASM samples exposed overnight to 1 or 2% CSE or to TSLP showed significantly higher levels of the phosphorylated form of STAT5 (Tyr694; p < 0.05; n = 5; Fig. 7).
Incubation of ASM cells with TSLP-R Ab for 1 h prior to and during CSE exposure or TSLP treatment significantly decreased STAT5 activation ($p$, 0.05; $n$ = 4; Fig. 7). Values were corrected for total STAT5 levels.

Based on the above results, the overall interactions between CSE, TSLP, and calcium regulation in human ASM are summarized in Fig. 8.

**Discussion**

The IL-7–like cytokine TSLP is key to triggering and modulation of airway inflammation, relevant to diseases associated with cigarette smoking including asthma and COPD (9, 10, 12). In addition to the now well-known expression of TSLP-R in dendritic cells and activated monocytes (9), TSLP-R has been found in heart, skeletal muscle, and kidney (21) and, importantly, in lung. Specifically in the airway, TSLP is expressed not only by airway epithelium (22, 23) but also ASM cells (16). Furthermore, ASM TSLP expression is modulated (at least in vitro) by proinflammatory cytokines (16). Although considerable attention has focused on TSLP effects on inflammation, less is known about TSLP effects on cells other than those of the immune system.

**FIGURE 7.** Effect of CSE on STAT5 phosphorylation. Exposure of ASM to CSE or TSLP significantly increased phosphorylation of the signaling p-STAT5, while total STAT5 levels were unchanged. CSE exposure increased p-STAT5 levels to a greater extent compared with TSLP. Inhibition of TSLP binding to TSLP-R substantially blunted both TSLP and CSE induced STAT5 phosphorylation. Values are means ± SE. *, #, % $p$ < 0.05; significant CSE effect, significant TSLP-R Ab effect, and significant TSLP Ab effect, respectively.
The novel results of the current study in human ASM suggest that not only does ASM produce and secrete TSLP but also expresses TSLP-R, thus making ASM a potential autocrine/paracrine and thus a novel noncanonical target of TSLP. We found that TSLP enhances normal [Ca^{2+}] responses to bronchoconstrictor agonist, thus contributing to increasing contractility. Furthermore, CSE not only enhances TSLP expression and secretion by ASM but also increases TSLP-R expression, thus increasing ASM responsiveness (at least in terms of [Ca^{2+}] to TSLP. Neutralizing TSLP as well as preventing TSLP-R activation (via functional Abs) substantially blunts CSE effects on [Ca^{2+}], responses. Overall, these data suggest that CSE-induced increase in airway contractility (evaluated in this study in terms of [Ca^{2+}]), is mediated, at least in part, by increased TSLP derived from ASM, and its effects on ASM [Ca^{2+}], regulation (Fig. 8).

**TSLP in the airway**

Originally identified as a growth factor for a mouse B cell line (24), TSLP has been found to be highly expressed in epithelial cells and mast cells (10), suggesting an important regulatory role in mucosal immunity. However, TSLP was also found to be highly expressed by tonsillar epithelial cells (25), cultured human bronchial epithelial cells (26, 27), and smooth muscle cells (16), suggesting involvement of other airway cell types in TSLP-mediated immune responses.

TSLP is known to activate dendritic cells, promoting development of proallergic Th2 secreting CD4 T cell differentiation (dependent on upregulation of dendritic cell OX40 ligand [12]). Dendritic cell-induced upregulation of the IL-17 pathway and IL-25R (28) is critical to development and progression of allergic inflammation (29). Accordingly, TSLP is a key player in the development of Th2-type allergic inflammation, especially in diseases, such as asthma, COPD, and other nonlung diseases, for example, atopic dermatitis. In mouse models of Ag-induced asthma, lung TSLP is elevated (14). Separately, overexpression of lung TSLP enhances Th2-mediated airway inflammation and airway hyperresponsiveness (30), whereas deficiency of TSLP antagonizes airway hyperresponsiveness (15). In humans, TSLP mRNA is increased in airways of asthmatics and correlates to disease severity and Th2 profile (13). Although these studies support a key role for TSLP in airway inflammation, what is less established is the normal source of TSLP in the inflamed/asthmatic airway and potential noncanonical roles of TSLP.

ASM cells are now known to be key players in the pathogenesis of diseases, such as asthma (3, 4, 31). In addition to increasing airway contractility, ASM cells can modulate the inflammatory milieu (5–7). Accordingly, TSLP in ASM is topic of recent interest. Only a single study has examined this issue. Zhang et al. (16) found that cultured human ASM cells express (and release) TSLP in vitro and that exposure to Th1 cytokines, such as IL-1β and TNF-α, upregulates TSLP expression (via MAPK pathways). The results of our study on TSLP expression and secretion in human ASM are entirely consistent with this previous finding. However, what remained unknown from the study by Zhang et al. (16) is whether TSLP expression is a consequence of ASM stimulation by other airway cells, and what the functional target of TSLP is: ASM itself versus others, such as epithelium and immune cells.

A novel aspect of the current study is the finding that not only does human ASM express TSLP under normal conditions but also TSLP-R, suggesting that ASM itself is a target of TSLP. Indeed, in normal human ASM cells, exposure to TSLP resulted in increased [Ca^{2+}], response to agonist, an effect prevented by neutralizing TSLP as well as blocking TSLP-R activation. In this regard, the increased [Ca^{2+}], may have downstream genomic effects on ASM that need to be examined further (e.g., does TSLP modulate ASM generation of other cytokines, or ASM proliferation?).

**Cigarette smoke and ASM**

Although it is well recognized that smoking is a risk factor for development of airway inflammation, airway hyperresponsiveness, and COPD, surprisingly little is known concerning the effect of cigarette smoking on ASM contractility. In animal studies, sidestream cigarette smoke exposure increases bronchoconstriction (32), which may involve altered [Ca^{2+}], and force regulation, airway irritability, and/or airway remodeling. In this regard, the results of the current study provide novel evidence that CSE enhances [Ca^{2+}], responses of human ASM cells to bronchoconstrictor agonist.

**TSLP and cigarette smoke**

The previous finding by Zhang et al. (16) that ASM from COPD patients show increased TSLP levels suggests a link between cigarette smoke and TSLP. A causal relationship between the two has been examined only in a single study. Using a mouse model of repeated intranasal exposures to CSE, Nakamura et al. (17) found increased lung TSLP expression. Furthermore, in an OVA-sensitized/challenged mouse model, inhibition of TSLP activity blunted airway inflammation. Although this study establishes the functional role of TSLP in lung and airway inflammation, it remains unclear whether TSLP levels in specific airway components are modulated by cigarette smoke or other factors, such as oxidative stress or inflammatory cytokines induced by smoke. Nakamura et al. (17) found that oxidative stress and TNF-αR activation are important to CSE-induced TSLP production. Thus, these limited data help link TSLP to cigarette smoke-induced changes in the lung.

A novelty of the current study lies in the finding that cigarette smoke, TSLP, and ASM are linked in that CSE enhances TSLP production and secretion by ASM. We also found that nicotine does not mimic the effect of CSE, suggesting involvement of other components of CSE. In this study, oxidative stress is an obvious choice. Interestingly, exposure to hydrogen peroxide did not substantially influence TSLP levels. In contrast, CSE-induced increase in TSLP was blunted by NAC, suggesting at least partial involvement of oxidative stress. Further characterization of the role of oxidative stress in cigarette smoke-induced changes in TSLP signaling in ASM are required.

The finding that CSE increases TSLP-R expression in ASM has several implications. Even if TSLP levels were unchanged by CSE, the potential exists for greater TSLP effects on ASM either in terms
of [Ca²⁺], regulation or downstream genomic effects. This is further supported by the fact that STAT3 phosphorylation (required for TSLP signaling) is actually greater with CSE exposure than with TSLP alone. Considering that TSLP itself enhances [Ca²⁺]i regulation in ASM, and CSE increases TSLP, there is a potentially synergistic effect of the two on [Ca²⁺]i regulation, which may substantially enhance ASM contractility. Such synergistic effects may also occur in terms of cell proliferation and other changes that occur in diseases, such as asthma and COPD.

Overall, the current study demonstrates that human ASM expresses both TSLP and TSLP-R, suggesting that ASM itself is a target of TSLP. In this regard, TSLP may increase [Ca²⁺]i responses to agonist. Both TSLP and its receptor are upregulated by CSE exposure, suggesting a potential mechanism by which cigarette smoke enhances [Ca²⁺]i regulation in ASM.

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Disclosures
The authors have no financial conflicts of interest.

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