Brain-Derived Neurotrophic Factor in Cigarette Smoke–Induced Airway Hyperreactivity

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Enhanced airway smooth muscle (ASM) contractility contributes to increased resistance to airflow in diseases such as bronchitis and asthma that occur in passive smokers exposed to secondhand smoke. Little information exists on the cellular mechanisms underlying such airway hyperreactivity. Sputum samples of patients with chronic sinusitis, bronchitis, and asthma show increased concentrations of growth factors called neurotrophins, including brain-derived growth factor (BDNF), but their physiological significance remains unknown. In human ASM, we tested the hypothesis that BDNF contributes to increased contractility with cigarette smoke exposure. The exposure of ASM to 1% or 2% cigarette smoke extract (CSE) for 24 hours increased intracellular calcium ($[Ca^{2+}]_i$) responses to histamine, and further potentiated the enhancing effects of a range of BDNF concentrations on such histamine responses. CSE exposure increased the expression of the both high-affinity and low-affinity neurotrophin receptors tropomyosin-related kinase (Trk)–B and p75 pan-neurotrophin receptor, respectively. Quantitative ELISA showed that CSE increased BDNF secretion by human ASM cells. BDNF small interfering (si)RNA and/or the chelation of extracellular BDNF, using TrkB-fragment crystallizable, blunted the effects of CSE on $[Ca^{2+}]_i$ responses as well as the CSE enhancement of cell proliferation, whereas TrkB siRNA blunted the effects of CSE on ASM contractility. These data suggest that cigarette smoke is a potent inducer of BDNF and TrkB expression and signaling in ASM, which then contribute to cigarette smoke–induced airway hyperresponsiveness.

Keywords: neurotrophin; asthma; TrkB; environmental tobacco exposure; secondhand smoke

Airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) can be triggered or exacerbated by cigarette smoke and secondhand smoke exposure (1–4). Furthermore, in people with preexisting airway diseases such as asthma, acute exposure to cigarette smoke can produce a bronchospastic response. Considerable evidence already exists that airway inflammation occurs with cigarette smoke exposure, and contributes to airway disease even in passive smokers. Regardless of the underlying causes for airway inflammation (of which there are many), increased airway smooth muscle (ASM) contractility is certainly a key factor contributing to the pathological airway narrowing and increased airflow resistance observed in asthma and bronchitis. Although smoke exposure is recognized to increase airway contractility, the mechanisms by which cigarette smoke enhances ASM contractility remain under investigation.

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CLINICAL RELEVANCE

We report novel data on the role of the growth factor brain-derived neurotrophic factor (BDNF) in mediating the effects of cigarette smoke on human airway smooth muscle contractility and proliferation. The basic science described here of local factors such as BDNF secreted by the airway with resultant autocrine/paracrine function is highly relevant to understanding the mechanisms by which smoking induces airway hyperreactivity. These findings provide insights into novel avenues for targeting the detrimental effects of cigarette smoke on the airway.

In the nervous system, growth factors called neurotrophins (NTs) are well recognized for their role in the generation, growth, and maintenance of different neuronal populations (5–9). Both short-term (seconds or minutes) and long-term (hours or days) effects of NTs on nuclear and cytosolic signals have been recognized as favoring increased synaptic transmission and plasticity, cell proliferation, and survival. NTs and their receptors have been identified in several non-neuronal systems, and we and others (10–12) have shown that human ASM expresses several NTs as well as their high-affinity receptors (e.g., tropomyosin-related kinase; Trk) and low-affinity receptors (e.g., pan-neurotrophin receptor p75NTR, a member of the TNF receptor superfamily (13)). Specifically, brain-derived neurotrophin factor (BDNF), which activates the high-affinity TrkB receptor (14), is expressed (10) and released (12) by human ASM, and appears to play a role in the regulation of intracellular calcium ($[Ca^{2+}]_i$), and to force regulation under normal circumstances (10) as well as in the presence of inflammation (15). Furthermore, BDNF enhances ASM cell proliferation, potentiating the effects of proinflammatory cytokines (16). These effects were shown to be at least partly mediated via the activation of the full-length TrkB (TrkB-FL) receptor, which leads to tyrosine phosphorylation and the recruitment of several downstream signaling enzymes and adaptor proteins (16–18).

Given the likelihood that limited airflow and increased sputum production comprise innate responses to cigarette smoke exposure, several studies focused on the immune responses and inflammatory pathways activated by smoke exposure (19–21). Interestingly, sputum samples from patients with asthma, allergic rhinitis, and chronic cough all showed significantly elevated NT concentrations, as reviewed by Prakash and colleagues (22). However, the source of such NTs and their physiological relevance remain under investigation. Based on our previous data in ASM, if neurotrophins contribute to increased airway contractility, then the correlative findings of increased neurotrophin concentrations in sputum samples may serve as a functionally relevant diagnostic marker in airway diseases caused or exacerbated by cigarette smoke. Therefore, in this study, we hypothesized that cigarette smoke exposure increases both NT

expression and signaling, priming the airway for cigarette smoke-induced airway hyperresponsiveness.

MATERIALS AND METHODS

Materials

All reagents and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless specified otherwise. Reagents and chemicals from other suppliers included Dulbecco's Modified Eagle's Medium F/12, FBS, and Hanks' balanced salt solution from Invitrogen (Carlsbad, CA); recombinant human BDNF, TrkB-fragment crystallizable (TrkB-Fc), and the BDNF ELISA kit from R&D Systems (Minneapolis, MN); IgG-Fc from MP Biomedicals (Santa Ana, CA); TrkB and BDNF antibodies from Abcam (Cambridge, MA); p75NTR antibody from Millipore (Billerica, MA); and all other antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation of Human ASM Cells and Bronchial Rings

Under a protocol approved by our Institutional Review Board, third to sixth generation bronchi were excised from lung specimens, incidental to nonsmoking patients undergoing thoracic surgery at Mayo Clinic. Bronchial rings from normal lung areas (identified by a pathologist) were denuded of epithelium by gentle abrasion, while ASM cells were isolated as described previously (10). Cells were maintained under standard conditions in 96-well or 8-well clear-bottom imaging plates.

Preparation of Cigarette Smoke Extract

A modification of the technique by Blue and Janoff was used to prepare aqueous cigarette smoke extract (CSE) (24) at 100% concentration from one Kentucky 1RF4 cigarette per 10 ml medium. We previously found that greater than or equal to 5% CSE substantially reduces cell viability (25). Therefore, we used 1–2%.

Western Blot Analysis

Standard techniques were used, including SDS-PAGE (4–15% gradient, Criterion Gel System; Bio-Rad, Hercules, CA), nitrocellulose membrane transfer, an infrared dye secondary antibody (Li-Cor, Lincoln, NE), and gel densitometry (Odyssey Imaging System; Li-Cor).

[Ca²⁺]_i Imaging

The techniques for the [Ca²⁺]_i imaging of ASM cells were previously described (10, 15). ASM cells were incubated with 5 μM fura-2/AM (Invitrogen), and calibrated real-time fluorescence measurements were obtained (20–30 cells/protocol).

siRNA Knockdown

Cultures at 60% confluence were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) alone, or TrkB small interfering (si)RNA (GGUAGAAAUCAUAACGAtt) from Ambion (Carlsbad, CA), p75NTR siRNA (GGGACUAGGAGCACUGUAGtt) and BDNF siRNA (GUAUGUACAUUGACCAUUA) from Dharmacon (Waltham, MA), or negative control (GCGCGCUUUGUAGGAUUCG-dTdT) from Invitrogen.

Real-Time PCR

Standard techniques were used to isolate total RNA and to synthesize and amplify cDNA optimized for an LC480 LightCycler (ABI, Carlsbad, CA). Primers are listed in Table 1. Real-time PCR was performed in duplicates per cDNA template. mRNA expression was calculated by the normalization of cycle threshold [C(t)] values of target gene to reference gene (ribosomal S16), using the comparative C(t) method.

BDNF ELISA

Sandwich ELISA of cell supernatants was performed using standard manufacturer protocols with colorimetric quantification (FlexStation3 reader at 450 nm; Molecular Devices, Sunnyvale, CA).

TABLE 1. PRIMERS FOR REAL-TIME PCR

Primer	Forward	Reverse
BDNF	5'AACCACGATGTGACTCC3'	5'CATTACAGCTCTCCAG3'
TrkB-FL	5'ACTACTACAGGGTCGG3'	5'CCCTAGCCTAGAATGTC3'
TrkB-T1	5'CCACTGGATGGGTAGC3'	5'CCTGAGAGTTACCTCTGC3'
p75NTR	5'CACATAGACTCCTTTACCCA3'	5'GCATCGGTGTCTCGAA3'
S16	5'ATCAAGGTGAACGGG 3'	5'ACGATGGGCTTATCGG 3'

Definition of abbreviations: BDNF, brain-derived neurotrophic factor; TrkB, tropomyosin-related kinase-B; FL, full-length; T1, truncated isoform; p75NTR, low-affinity pan-neurotrophin receptor.

Force Measurements

Epithelium-denuded bronchial rings in physiological salt solution (37°C; 95% air/5% CO₂), suspended at optimal length in organ baths, were contracted with 1 μM acetylcholine (ACh) or 10 μM histamine. To introduce siRNA, previously described reversible permeabilization techniques were used (26) that involve brief membrane permeabilization, siRNA transfection, membrane resealing, and maintenance at 4°C for 18 hours, followed by slow reheating to 37°C. CSE was introduced only after siRNA transfection. Subsequently, medium only or 1 nM BDNF was added for 1 hour, and agonist responses were reevaluated. Maximum force was evaluated using 80 mM KCl.

Cell Proliferation Assay

Previously described techniques were used, based on the DNA binding of fluorescent CyQuant NF dye (Invitrogen), with relative fluorescence converted to cell numbers using empirical standard curves (16).

Statistical Analysis

Bronchial samples from five patients were used. Each protocol repeated at least four times. Effects were compared using two-way ANOVA with the Bonferroni correction. Statistical significance was established at $P < 0.05$. All values are expressed as mean ± SE.

RESULTS

CSE Potentiates Effects of BDNF on [Ca²⁺]_i Response to Agonist

In human ASM cells, 24-hour exposure to 1% or 2% CSE significantly increased [Ca²⁺]_i responses to 10 μM histamine, compared with vehicle control for CSE ($P < 0.05$; Figure 1). In general, baseline [Ca²⁺]_i concentrations were elevated after 2% CSE exposure, whereas 1% CSE exerted no effect for the most part (representative tracings in Figure 1A). Separately, brief (20-minute) exposure of ASM cells to BDNF concentrations of 1 nM or greater also significantly increased [Ca²⁺]_i responses to the agonist ($P < 0.05$). Previous exposure to CSE significantly potentiated the enhancing effect of BDNF at 1 nM or greater on [Ca²⁺]_i responses ($P < 0.05$; Figure 1). In these experiments, separate sets of cells were used for each BDNF or CSE combination. Generally, the effects of 2% CSE were greater than those of 1% CSE. However, for either CSE concentration, the enhancing effects of BDNF reached a ceiling.

CSE Increases BDNF Receptor Expression in Human ASM

Western blot analysis of human ASM cells exposed for 24 hours to either 1% CSE or 2% CSE showed substantial increases in the expression of both high-affinity TrkB-FL and low-affinity p75NTR receptors ($P < 0.05$, compared with vehicle control; Figure 2A). Many cells, including ASM, express both full-length (TrkB-FL) and truncated (TrkB-T1) isoforms of the BDNF receptor. Previous studies demonstrated that TrkB functionality is dependent on the expression of the membrane-bound, full-length

Figure 1. Effects of cigarette smoke extract (CSE) and brain-derived neurotrophic factor (BDNF) on intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) responses to agonist stimulation in human airway smooth muscle (ASM) cells. (A) Twenty-four-hour exposure to 1% or 2% CSE significantly increased $[\text{Ca}^{2+}]_i$ responses to 10 μM histamine in human ASM cells, with baseline $[\text{Ca}^{2+}]_i$ increased for 2% CSE. (B) Concentrations from 1 nM upward of BDNF (20-minute exposure) alone also significantly increased $[\text{Ca}^{2+}]_i$ responses to histamine. Both 1% and 2% CSE exposure potentiated the enhancing effect of BDNF on $[\text{Ca}^{2+}]_i$ responses to histamine. Values represent the means $\pm$ SE ($n = 5$). Veh, vehicle. *Significant effect of BDNF ($P < 0.05$). #Significant effect of CSE ($P < 0.05$).

isoform (5, 27), whereas the role of the intracellular truncated isoform (T1) remains under investigation. CSE exposure substantially increased the ratio of TrkB-FL to TrkB-T1 (Figure 2A), suggesting overall increased functional TrkB expression that would allow for enhanced effects of BDNF. No significant difference was evident between 1% and 2% CSE in terms of their effects on TrkB isoforms or p75NTR. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control for these studies. Changes in TrkB-FL or p75NTR protein concentrations induced by CSE were matched by substantial increases of the mRNA concentrations in ASM for both receptors ($P < 0.05$ for either CSE concentration; Figure 2B).

The role of oxidant stress in the effects of cigarette smoke is generally recognized (28). To determine the potential involvement of reactive oxygen species in CSE-induced changes of TrkB expression, we used two different compounds, namely, 10 mM of the general antioxidant *N*-acetylcysteine (NAC), or 10 mM of the superoxide scavenger 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt, pyrocatechol-3,5-disulfonic acid (Tiron). The pre-exposure of ASM cells to NAC or Tiron blunted subsequent CSE-induced changes in TrkB-FL protein expression ($P < 0.05$; Figure 2C), with comparable effects between the two antioxidants.

Role of TrkB Receptor in CSE-Mediated Effects on $[\text{Ca}^{2+}]_i$

The suppression of TrkB expression using human TrkB siRNA did not significantly alter the $[\text{Ca}^{2+}]_i$ responses to 10 μM histamine *per se* in cells not exposed to BDNF or CSE. However, as

expected, the enhancing effects of 1 nM BDNF on $[\text{Ca}^{2+}]_i$ responses were significantly blunted ($P < 0.05$; Figure 3A), confirming a role for TrkB in mediating the effects of BDNF on human ASM cells (10, 15). More importantly, TrkB siRNA suppressed the enhancing effect of CSE on $[\text{Ca}^{2+}]_i$, with or without additional BDNF ($P < 0.05$; Figure 3A). In contrast, p75NTR siRNA exerted only a small effect on either the BDNF or CSE enhancement of $[\text{Ca}^{2+}]_i$ responses (Figure 3B). For both TrkB and p75NTR, the efficacy of siRNA was verified using Western blot analyses (Figure 3C). Based on the data showing a predominant role for TrkB rather than p75NTR, subsequent studies focused on the high-affinity receptor.

Role of BDNF in CSE Enhancement of ASM Contractility

Epithelium-denuded human bronchial rings, stretched to optimal length, produced characteristic sustained force responses when exposed to 1 μM ACh (Figure 4). The exposure of samples for 1 hour to 1 nM BDNF significantly enhanced the force response to ACh (Figure 4A; $P < 0.05$). In separate samples, exposure to histamine also produced robust force responses (not shown). In time control samples exposed only to medium overnight, force responses were not significantly different between the two ACh or histamine exposures 24 hours apart. The exposure of rings for 24 hours to CSE significantly enhanced force responses to subsequent ACh or histamine exposure (Figure 4B; $P < 0.05$). In bronchial rings exposed to 24-hour CSE, subsequent exposure for 1 hour to 1 nM BDNF significantly potentiated the force responses to ACh or histamine compared with BDNF alone-treated and CSE alone-treated groups (Figure 4B; $P < 0.05$).

Given the $[\text{Ca}^{2+}]_i$ data suggesting a role for TrkB, we investigated the effects of TrkB siRNA, using the reversible permeabilization technique. The suppression of TrkB expression blunted ACh-induced force production even without CSE exposure ($P < 0.05$; Figure 4C). Importantly, TrkB siRNA significantly blunted the enhancing effects of CSE or BDNF on force production ($P < 0.05$; Figure 4C).

Role of Endogenous BDNF Release in the Effects of CSE

Based on the results showing that TrkB siRNA suppressed the effects of CSE on $[\text{Ca}^{2+}]_i$ and force, we examined whether ASM cells were the source of BDNF, and whether CSE affected BDNF secretion by ASM, thus exploring an autocrine role for ASM-derived BDNF. In ASM cells exposed to 1% or 2% CSE, BDNF mRNA concentrations were increased, compared with vehicle control ($P < 0.05$; Figure 5A). In supernatants from cells exposed overnight to 1% or 2% CSE, a substantial increase was evident in secreted BDNF, compared with vehicle control ($P < 0.05$; Figure 5B). As with the effects of CSE on TrkB, the antioxidants NAC and Tiron were equally efficacious in suppressing the CSE enhancement of BDNF release ($P < 0.05$; Figure 5B).

To determine the functional significance of the CSE-induced release of BDNF, we used BDNF siRNA in one set of experiments, and found that the CSE-induced enhancement of TrkB-FL expression was suppressed ($P < 0.05$; Figure 5D), suggesting an autocrine regulation of TrkB expression by its ligand. In a second set of experiments, we used the chimeric protein TrkB-Fc that chelates BDNF extracellularly, thus competing with cell-surface TrkB-FL receptors. The pre-exposure of ASM cells to 1 $\mu\text{g}/\text{ml}$ TrkB-Fc for 1 hour before and during subsequent 2% CSE for 24 hours significantly reduced CSE-induced increases in $[\text{Ca}^{2+}]_i$ responses to histamine ($P < 0.05$; Figures 6A and 6B). In the absence of CSE, the effects of TrkB-Fc on $[\text{Ca}^{2+}]_i$ responses to histamine were slightly reduced,

Figure 2. Effects of CSE exposure on the expression of neurotrophin receptors in human ASM cells. (A) Human ASM cells exposed for 24 hours to either 1% CSE or 2% CSE showed significantly increased protein expression of the full-length isoform of tropomyosin-related kinase (TrkB-FL, the high-affinity receptor for BDNF), as well as the low-affinity pan-neurotrophin receptor p75NTR, as confirmed by Western blot analysis. (B) A similar pattern of CSE-induced increase in TrkB-FL and p75NTR mRNA was also observed. (C) The effects of CSE on either receptor were mediated, at least in part, by oxidative stress, suggested by the blunting effects of the antioxidants *N*-acetylcysteine (NAC) and 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt, pyrocatechol-3,5-disulfonic acid (Tiron). Values represent the means $\pm$ SE ($n = 5$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase. *Significant difference from control samples ($P < 0.05$). #Significant antioxidant effects ($P < 0.05$).

suggesting a baseline BDNF release with an autocrine effect. Control experiments using IgG-Fc instead of TrkB-Fc showed no changes in $[Ca^{2+}]_i$ responses, with or without CSE (Figure 6B).

CSE Increases Cell Proliferation via BDNF Signaling

ASM cells treated for 24 hours with 2% CSE showed significant increases in proliferation compared with vehicle control ($P < 0.05$; Figure 6C). When pre-exposed to TrkB-Fc before treatment with 2% CSE, proliferation was reduced to that observed with vehicle control ($P < 0.05$). In parallel, the proliferation marker proteins proliferating cell nuclear antigen (PCNA) and cyclin E also exhibited increased expression upon CSE exposure. These effects were blunted with TrkB-Fc (Figure 6D; $P < 0.05$).

DISCUSSION

In the present study, we demonstrate that the effects of cigarette smoke on the enhancement of $[Ca^{2+}]_i$ responses of human ASM cells to agonist stimulation involve the neurotrophin BDNF, which acts via the high-affinity TrkB receptor. BDNF appears to be a key component in the effects of CSE in the airway, and thus represents a novel mechanism of action for cigarette smoke. Indeed, neurotrophins may represent a unique nexus between cigarette smoke and airway irritability.

Cigarette smoke exposure is known to increase airway reactivity simply by being an inhaled airway irritant, leading to an enhanced production of endogenous mediators (29, 30). Direct evidence exists to clarify the mechanism of neurogenic inflammation and the effects of cigarette smoke exposure in the pathogenesis of smoke-induced diseases, including chronic obstructive pulmonary disease (COPD) (29–31). Thus the effects of cigarette smoke on the neurogenic regulation of airway tone are relevant, as are the effects of smoke on other cell types within the airway. In this regard, growing evidence, including our own, maintains that both low-affinity and high-affinity neurotrophin receptors are widely distributed in non-neuronal tissues that are not necessarily associated with innervation (recently reviewed by Prakash and colleagues in relation

to the lung) (22). Our novel results now suggest that cigarette smoke exposure increases the expression of the neurotrophin receptors TrkB and p75NTR. In addition to this receptor expression, CSE exposure increases BDNF secretion from human ASM tissue. This leads to a doubly detrimental situation where the up-regulation of secreted BDNF acts on the increased expression of full-length TrkB receptors, which potentiate (and mediate) the effects of CSE on $[Ca^{2+}]_i$ and the force response to agonists, as well as cell proliferation (i.e., the major mechanisms for increased airway contractility) (Figure 7). In this regard, the novel finding that BDNF can itself regulate TrkB-FL expression suggests a feed-forward system wherein the CSE-induced enhancement of BDNF secretion would exert autocrine effects of increasing $[Ca^{2+}]_i$, force, and proliferation, as well as priming ASM for enhanced responsiveness to neurotrophins.

Cigarette Smoke Exposure and the Airway

Secondhand smoke exposure is clearly associated with an increased prevalence of airway disease. On a physiological level, chronic inflammation of the airways manifests as exaggerated airway narrowing, airflow limitation, and accompanying dyspnea, which lead to the remodeling and destruction of the airway, as in COPD (19–21). However, for the symptoms of restricted airflow to occur, cigarette smoke exposure must affect ASM contractility. Smoke challenge studies in both human and animal models (e.g., ovalbumin-sensitized/challenged mice or guinea pigs) (29, 32, 33) demonstrated that cigarette smoke exposure induces airway hyperreactivity. One mechanism would involve cigarette smoke increasing the production of bronchoconstricting agents (e.g., increased ACh, endothelin-1, or substance P release from airway nerves) (29, 32, 34). Alternatively, smoke-induced cytokines may up-regulate $[Ca^{2+}]_i$ and force regulatory mechanisms, as exemplified in diseases such as asthma (35, 36). The limited data on the direct effects on ASM contractility suggest that chronic cigarette smoking can down-regulate the levels of potassium channels and augment Ca^{2+} sensitivity, a possible contributor to airway hyperresponsiveness (37, 38).

Figure 3. Role of neurotrophin receptors in the effects of CSE on $[Ca^{2+}]_i$ regulation. (A) The transfection of human ASM cells with TrkB siRNA did not significantly affect $[Ca^{2+}]_i$ responses to 10 μ M histamine. However, TrkB small interfering (si)RNA blunted the enhancing effects of acute 1 nM BDNF exposure, as well as the effects of CSE (with or without BDNF) on $[Ca^{2+}]_i$ responses to histamine. (B) In contrast, the transfection of cells with p75NTR siRNA did not significantly alter $[Ca^{2+}]_i$ responses in the presence of BDNF or CSE alone, and exerted only a small effect on the combined enhancement of responses by CSE and BDNF. (C) The efficacy of siRNAs was verified using Western blot analysis. Values are means $\pm$ SE ($n = 4$). *Significant effect of BDNF ($P < 0.05$). \$Significant effect of CSE ($P < 0.05$). #Significant effect of siRNA ($P < 0.05$).

Neurotrophins and ASM

Neurotrophins are well-known in the nervous system not only as growth factors involved in the genomic regulation of neuronal development and function (5–9), but also for their acute, non-genomic effects such as enhanced $[Ca^{2+}]_i$ and synaptic transmission (39, 40). Acting via high-affinity Trk or low-affinity p75NTR receptors, neurotrophins may activate several intracellular signaling cascades such as phospholipase C, phosphatidylinositol 3-kinase, and mitogen-activated protein kinases (41–43), which are also known to be involved in mediating the effects of inflammatory cytokines such as TNF- α , relevant to airway disease. In this regard, evidence is increasing that neurotrophins and their receptors are expressed in a range of non-neuronal tissues, including the lung, as recently reviewed by Prakash and colleagues (22). We previously demonstrated,

Figure 4. Effects of BDNF and CSE on force responses of human bronchial rings to bronchoconstrictor agonists. Epithelium-denuded bronchial rings, stretched to optimal length, demonstrated stable force responses to 1 μ M acetylcholine (ACh) and 10 μ M histamine (ACh responses are shown in A). AU, arbitrary units. Overnight exposure to 2% CSE significantly enhanced force responses to ACh (A and B) and histamine (B), compared with pre-exposure values, as well as to time control samples. Acute exposure to BDNF also enhanced force responses to ACh, and potentiated the effect of overnight CSE. (C) The suppression of TrkB expression by siRNA prevented the CSE enhancement of contractility and the effects of BDNF. Values represent the means $\pm$ SE ($n = 4$). *Significant effect of CSE ($P < 0.05$). #Significant effect of BDNF ($P < 0.05$). %Significant effect of TrkB siRNA ($P < 0.05$).

specific to ASM, that BDNF, TrkB, and p75NTR are all expressed by human ASM cells (10, 15), and that BDNF non-genomically enhances $[Ca^{2+}]_i$ responses to the agonist (10) and potentiates the effects of proinflammatory cytokines (15). The

Figure 5. Role of BDNF in effects of CSE on $[Ca^{2+}]_i$ responses. (A) Exposure of ASM cells to 1% or 2% CSE increased BDNF mRNA concentrations. (B) Supernatant serum-free medium from ASM samples exposed to vehicle or 2% CSE were analyzed for BDNF by ELISA, and showed that 2% CSE substantially increased BDNF release compared with vehicle controls. BDNF in this context exerted autocrine effects, in that the suppression of BDNF expression by siRNA (verified by Western blotting in C) prevented CSE-induced increases in TrkB-FL (D). Values represent the means $\pm$ SE ($n = 4$). $\Delta\Delta C_t$, comparative cycle threshold method. *Significant effect of BDNF ($P < 0.05$). #Significant antioxidant effect ($P < 0.05$). %Significant effect of BDNF siRNA ($P < 0.05$).

expression (11) and release (12) of BDNF were also demonstrated by other groups. More recently, we showed that BDNF also exhibits the genomic effects of enhancing cellular proliferation in human ASM (16) via many of the already mentioned signaling cascades. Several studies have also shown that airway cells (i.e., epithelium, ASM, immune cells, and nerves) are capable of producing BDNF (22, 44–46). The relevance of such effects lies in the recent recognition that circulating and local BDNF concentrations, as well as receptor expression, are increased in asthma (22, 44). Furthermore, animal studies suggest a role for BDNF in airway inflammation, remodeling, and hyperreactivity (47, 48).

Given that BDNF can be produced by airway epithelium, sensory innervation, ASM itself, and a host of immune cells (22, 44, 46), ASM becomes both a potential source as well as a target for BDNF. Clinical findings have established increased neurotrophin concentrations in both sputum samples and the bronchoalveolar lavage of patients with chronic inflammatory airway diseases associated with smoking and with secondhand smoke exposure (49, 50). Accordingly, the enhancement of BDNF expression and/or signaling can easily contribute to increased airway reactivity. Neurotrophins may be derived from airway nerves (as suggested by models of neurogenic asthma) or immune cells (44, 51), and thus may be up-regulated by

Figure 6. Effect of released BDNF. (A and B) Pre-exposure of ASM cells to TrkB-fragment crystallizable (Fc), a chimeric receptor peptide that chelates extracellular BDNF, significantly reduced CSE-mediated increases in $[Ca^{2+}]_i$ responses to histamine, whereas control IgG-Fc exerted no effect. (C) Separately, ASM cells exposed to CSE showed significant increases in proliferation, as determined by a fluorescent CyQuant assay (see MATERIALS AND METHODS). Pre-exposure to TrkB-Fc partly prevented the effects of CSE on proliferation. (D) Proliferation protein markers such as proliferating cell nuclear antigen (PCNA) or cyclin E expression showed significant increases with 2% CSE exposure. These effects were also suppressed by TrkB-Fc. Values represent the means $\pm$ SE ($n = 4$). *Significant effect of CSE ($P < 0.05$). #Significant effect of TrkB-Fc ($P < 0.05$). %Significant effect of PDGF ($P < 0.05$).

Figure 7. Proposed model for BDNF in effects of CSE on ASM. Exposure of ASM to cigarette smoke enhances BDNF release and the expression of the TrkB receptor. Released BDNF acts via TrkB-FL in potentiating the effects of cigarette smoke on $[Ca^{2+}]_i$ and cell proliferation, which contribute to enhanced airway contractility.

environmental factors including cigarette smoke exposure. Cytokines (e.g., TNF- α) that are produced in response to cigarette smoke exposure may indirectly up-regulate neurotrophin expression and signaling in the lung. We have previously shown that TNF- α does enhance BDNF and TrkB expression in human ASM (15). These findings, in addition to the results of the present study, suggest that neurotrophins contribute to airway hyperresponsiveness and the eventual remodeling that can occur in smoking-related lung diseases.

The present study contains an important and novel finding regarding the mechanistic link between cigarette smoke and neurotrophins in mediating the effects of cigarette smoke on ASM. CSE increases TrkB and p75NTR expression as well as BDNF secretion by ASM cells. Here, we report the important observation that the full-length isoform TrkB-FL is increased by CSE. As shown previously (5, 27), TrkB functionality is dependent on the expression of this membrane-bound isoform, which contains downstream kinase signaling units, as opposed to the intracellular truncated isoforms such as TrkB-T1. Although the function of truncated isoforms remains under investigation, they may chelate intracellular BDNF. Accordingly, the ratio of TrkB-FL/TrkB-T1 becomes relevant when discussing the effects of extracellular BDNF (e.g., the BDNF released by ASM itself). In this regard, the increased ratio of TrkB-FL/TrkB-T1 by CSE suggests that regardless of changes in BDNF concentrations, cigarette smoke primes the ASM for greater responsiveness to neurotrophins, and in turn, elevated TrkB-FL mediates the effects of CSE when BDNF is present.

Although CSE enhances the expression of both TrkB (especially full-length TrkB, indicating increased BDNF functionality) and p75NTR, as in our previous study (15), we found that the effects of BDNF on $[Ca^{2+}]_i$ are receptor-specific. The targeted siRNA knockdown of TrkB abrogates the BDNF/CSE enhancement of $[Ca^{2+}]_i$, but the suppression of p75NTR exerts no effect. Furthermore, we previously found that the effect of BDNF on cellular proliferation in human ASM also largely (but not exclusively) involves TrkB (16). The present study found that CSE induces significant cellular proliferation in human ASM cells, as evidenced by the proliferation markers PCNA and cyclin E and the measurement of the DNA content, using CyQuant Proliferation Assays (Invitrogen). The CSE-induced secretion of BDNF appears to be critical for enhanced ASM cell proliferation.

This discussion suggests that TrkB is important in the effects of CSE on ASM. However, our data also show that ASM is a source of BDNF and responds to cigarette smoke exposure by enhancing its secretion. We and others previously demonstrated BDNF expression and/or release by human ASM (10, 12). Such enhanced BDNF secretion itself appears to be

important, as suggested by the blunting of CSE effects on $[Ca^{2+}]_i$ or even TrkB expression when BDNF expression is suppressed by siRNA, or more importantly, when extracellular/secreted BDNF is chelated with TrkB-Fc. We also emphasize that the secreted concentrations of BDNF are in the physiological range. For example, our findings of approximately 40 pg/mg (or alternatively, 20–50 pg/ml) of BDNF are consistent with reports of anywhere from 50–300 pg/mg of BDNF in the nervous system. In terms of picograms/milliliters, circulating concentrations of BDNF have been reported in the range of 10–50 pg/ml. Although CSE may act through multiple mechanisms to enhance BDNF secretion, the effects of NAC and Tiron suggest at least the partial involvement of oxidative stress. Previous studies in lung tissue found that CSE can activate pathways such as cAMP response element-binding protein or NF- κ B (21), both of which can alter BDNF gene expression and thus its production. These alternative scenarios remain to be explored.

Overall, the results of our study show that BDNF exerts both short-term and long-term effects on ASM after cigarette smoke exposure. The relevance of these findings lies in the pathophysiological role for secreted neurotrophins in mediating the effects of smoke in the airways. Indeed, we hypothesize that the smoke-induced enhancement of neurotrophin release and signaling likely occurs in other cell types (e.g., epithelium or immune cells), contributing to the increased circulating and local concentrations noted in patients. Enhanced neurotrophins may exert a number of other effects within the airway, including airway irritability via the targeting of airway nerves, as well as immunomodulation. Here, it is important to consider the possibility that not only could BDNF be important, but other neurotrophins, particularly nerve growth factor (NGF), which is known to be released by airway nerves (52), and neurotrophin-3 (NT-3), which is also produced by ASM (10, 12), may be relevant in enhanced airway irritability caused by cigarette smoke, or in the presence of inflammation. In this regard, various Trk receptors such as TrkA (activated by NGF) and TrkC (activated by NT-3) may play differential roles in modulating the overall influence of cigarette smoke on the airway. Thus, neurotrophins may represent a novel therapeutic target in combating the detrimental effects of cigarette smoke exposure in the airway.

Author disclosures are available with the text of this article at www.atsjournals.org.

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