Cigarette smoke enhances proliferation and extracellular matrix deposition by human fetal airway smooth muscle

Elizabeth R. Vogel,1,2 Sarah K. VanOosten,1 Michelle A. Holman,3 Danielle D. Hohbein,1 Michael A. Thompson,1 Robert Vassallo,4 Hitesh C. Pandya,5 Y. S. Prakash,1,2 and Christina M. Pabelick1,2

1Department of Anesthesiology, Mayo Clinic, Rochester, Minnesota; 2Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota; 3Mayo Medical School, Rochester, Minnesota; 4Department of Pulmonary and Critical Care Medicine, Mayo Clinic, Rochester, Minnesota; and 5Department of Pediatrics, University of Leicester, Leicester, United Kingdom

Submitted 24 April 2014; accepted in final form 23 October 2014

Vogel ER, VanOosten SK, Holman MA, Hohbein DD, Thompson MA, Vassallo R, Pandya HC, Prakash YS, Pabelick CM. Cigarette smoke enhances proliferation and extracellular matrix deposition by human fetal airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 307: L978–L986, 2014. First published October 24, 2014; doi:10.1152/ajplung.00111.2014.—Cigarette smoke is a common environmental insult associated with increased risk of developing airway diseases such as wheezing and asthma in neonates and children. In adults, asthma involves airway remodeling characterized by increased airway smooth muscle (ASM) cell proliferation and increased extracellular matrix (ECM) deposition, as well as airway hyperreactivity. The effects of cigarette smoke on remodeling and contractility in the developing airway are not well-elucidated. In this study, we used canalicular-stage (18–20 wk gestational age) human fetal airway smooth muscle (fASM) cells as an in vitro model of the immature airway. fASM cells were exposed to cigarette smoke extract (CSE; 0.5–1.5% for 24–72 h), and cell proliferation, ECM deposition, and intracellular calcium ([Ca2+]i) responses to agonist (histamine 10 μM) were used to evaluate effects on remodeling and hyperreactivity. CSE significantly increased cell proliferation and deposition of ECM molecules collagen I, collagen III, and fibronectin. In contrast, [Ca2+]i responses were not significantly affected by CSE. Analysis of key signaling pathways demonstrated significant increase in extracellular signal-related kinase (ERK) and p38 activation with CSE. Inhibition of ERK or p38 signaling prevented CSE-mediated changes in proliferation, whereas only ERK inhibition attenuated the CSE-mediated increase in ECM deposition. Overall, these results demonstrate that cigarette smoke may enhance remodeling in developing human ASM through hyperplasia and ECM production, thus contributing to development of neonatal and pediatric airway disease.}

NEONATAL LUNG DISEASE, recurrent wheezing, and asthma are significant causes of morbidity and mortality in the pediatric population. Premature infants are at particularly increased risk because of the immaturity of the pulmonary system at birth and interventions in the hospital setting (2, 4, 14, 21, 27). As with adults, diseases such as asthma in the neonatal/pediatric population likely involve inflammation, and alterations in airway structure and function, including that of epithelium and airway smooth muscle (ASM) (7, 25, 43), leading to airway hyperreactivity and airway remodeling. Recently, increased focus has been placed on perinatal factors that may impact pulmonary development and airway remodeling that contribute to both pediatric and (eventually) adult airway disease (2, 4, 12, 21). Our prior work demonstrated the impact of hyperoxia (a common intervention in premature infants) on fetal airway smooth muscle (fASM) proliferation, apoptosis, and mitochondrial function (22). However, numerous other important factors have been implicated in development of airway hyperreactivity (2, 4, 21). One such environmental exposure is cigarette smoke exposure (4, 15, 21), particularly in the setting of premature infants discharged to homes with smoking adults.

Maternal tobacco use during pregnancy has been shown to have negative effects on neonatal and pediatric pulmonary function that may persist into adult years (16, 26, 31). Premature birth is another known complication of maternal tobacco use (30). This risk can be further increased when infants are exposed to numerous factors in the perinatal period, including prematurity itself, hyperoxia in the intensive care unit, and mechanical ventilation (2, 12, 14, 15, 21). Added to these factors is the further secondhand smoke exposure upon discharge from the hospital that may precipitate respiratory problems and repeated hospital visits. These repeated “hits” are certainly detrimental to lung function and development, but the ultimate cellular effects and mechanisms of cigarette smoke exposure have not been well-studied, particularly in the immature airway.

Key mechanisms of airway hyperreactivity in both adult and pediatric airways involve increased intracellular Ca2+ ([Ca2+]i), increased contractility, and ASM hyperplasia (24, 25, 40). In addition, there is evidence that extracellular matrix (ECM) remodeling and increased ECM protein deposition play roles (8, 28, 44). While environmental cigarette smoke exposure is associated with airway hyperreactivity, the mechanisms by which it produces these effects have not been well elucidated, and the potential impact of cigarette smoke on developing ASM cell proliferation or ECM deposition is unknown.

A number of mechanisms control [Ca2+]i, and cell proliferation in ASM. Prior data from our lab and others demonstrate that plasma membrane invaginations, termed caveolae, play an important role in regulation of ASM contractility through modulation of [Ca2+]i (38, 42). In adults, the key caveolar protein and signaling molecule caveolin-1 is thought to be a mediator of cell proliferation and ECM deposition, with decreased levels of caveolin-1 resulting in increased cellular proliferation and fibrosis (19, 32, 45, 46). Accordingly, we hypothesized that altered caveolar protein expression and signaling is one mechanism through which cigarette smoke ex-
posure exerts an impact on fASM structure and function in the setting of prematurity. We explored cell proliferation, ECM deposition, [Ca$^{2+}$], responses to agonist histamine, and caveolar protein expression.

**MATERIALS AND METHODS**

**Materials.** Tissue culture reagents, including Hanks’ Balanced Salt Solution (HBSS), fetal bovine serum (FBS), and DMEM-F-12 were obtained from Invitrogen (Carlsbad, CA). Sigma (St. Louis, MO) was the source for the remaining chemicals and supplies unless otherwise noted.

**Summary of methods.** fASM cells were exposed to 0.5, 1, or 1.5% cigarette smoke extract (CSE) for 18–72 h. Western blot analysis was used to determine the level of proliferative, caveolar, and mitogen-activated protein (MAP) kinase pathway protein expression. A nuclear dye-based CyQuant NF proliferation assay was used to assess cellular proliferation. Extracellular matrix deposition was analyzed with a modified in-cell Western technique. FASM cells (control and CSE exposed) were additionally loaded with fura 2-AM, a fluorescent Ca$^{2+}$ indicator dye, to investigate the effects of CSE on histamine agonist-induced [Ca$^{2+}$]$_i$ responses. Details of these techniques are expanded below.

**Isolation of human fASM cells.** Human fASM cells from the canalicular stage (18–20 wk gestation) were provided by Dr. Pandya from the University of Leicester, England, or purchased from Novogenix (Los Angeles, CA) through protocols approved by ethics committees in the United Kingdom (considered exempt by the Mayo Institutional Review Board since the cells were cultured and deidentified when received). Cells were obtained through enzymatic dissociation of fetal tracheobronchial tissue using previously described techniques (33, 34). Standard cell culture techniques were used to grow and maintain cells in a 95% air-5% CO$_2$ humidified incubator. All media were phenol red-free DMEM-F-12 with 10% FBS. Cells were serum starved to arrest growth in 0.5% FBS media for a minimum of 24 h before treatments. Smooth muscle phenotype was frequently verified by confirmation of expression of smooth muscle actin, calponin, and acetylcholine receptor via Western blot. Use of these markers to delineate smooth muscle phenotype has been previously described (5, 22).

**Cigarette smoke extract.** The procedure used for CSE preparation is a modification of the Blue and Janoff technique (3, 47). Cigarettes (Kentucky 1RF4 cigarettes) were covered with a sterile tip, and ~35 cm$^3$ of cigarette smoke were collected from one cigarette using a previously described (5, 22).

**Western blot analysis.** Standard techniques were used to separate proteins from whole cell lysates on SDS-PAGE (4–15% gradient gels, Criterion Gel System; Bio-Rad, Hercules, CA) at 120 volts for 60 min. A Bio-Rad Trans-Blot Turbo system (Bio-Rad) was used to transfer proteins to nitrocellulose membranes. Membranes were blocked with Odyssey Blocking buffer (Li-Cor Biosciences, Lincoln, NE) and incubated overnight at 4°C in a 1% bovine serum albumin (BSA) solution. Membranes were washed with TBS-Tween 20 (TBST) before a 60-min incubation in secondary antibodies conjugated to 800CW or 680CW IRdye (Li-Cor Biosciences). Imaging was performed via a Li-Cor Odyssey system (Li-Cor Biosciences) with quantification performed via densitometry. Protein concentrations were normalized to GAPDH unless noted otherwise. All membranes were stripped and reprobed for loading controls.

**Cellular proliferation assay.** In addition to measurement of proliferative protein markers via Western blot, proliferation was assessed using a CyQuant NF fluorescence kit (Invitrogen). Cells were plated in 96-well clear-bottom plates at a density of 5,000 cells/well in serum-free media and incubated overnight. Cells were then treated with vehicle (PBS) or 0.5, 1, or 1.5% CSE in 1% PBS media. Plates were incubated for 48 h after which the media was aspirated and the cells incubated in CyQuant dye in HBSS for 60 min at 37°C. Fluorescence was measured on a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA) at an excitation of 480 nm and emission of 530 nm. Cell proliferation was determined using a standard curve, and proliferation of CSE-treated cells was normalized to vehicle.

**Calcium imaging.** [Ca$^{2+}$]$_i$ imaging techniques used by our laboratory have been previously published (38, 42). In brief, cells were incubated in 5 μM fura 2-AM (Invitrogen) in HBSS at room temperature for 60 min. The plates were then washed, and cells were imaged.
in real time (MetaFluor; Universal Imaging, Downingtown, PA) on a Nikon Diaphot inverted microscope (Nikon Instrument, Melville, NY). Cell responses were based on 20 areas/field and software-defined regions of interest. Agonist stimulation was performed with 10 μM histamine. Amplitudes of responses were calculated as the difference between peak and baseline Ca²⁺.

**Modified In-cell western for ECM proteins.** Cells were grown to 60% confluence in clear-bottom 96-well plates before serum starvation for 24 h. They were then treated with CSE in 0.5% FBS media at final concentrations of 0.5, 1.0, or 1.5% for 72 h. Cells were then lysed and lifted from the plate with 0.016 N NH₄OH. Wells were washed with TBS and blocked with Li-Cor Odyssey Blocking Buffer for 60 min before overnight incubation in primary antibody at 4°C. Primary antibodies were used at a concentration of 10 μg/ml and included collagen I and collagen III from Abcam and fibronectin from Santa Cruz. Wells were washed with TBST before 60 min incubation with IR-dye-conjugated secondary antibodies at a concentration of 5 μg/ml. Control wells (without cells) were exposed to media with or without CSE treatments and incubated with primary and secondary antibodies to control for any background noise. Plates were imaged via a Li-Cor OdysseyXL system with quantification performed via densitometry. Protein concentrations were normalized to well cell count determined by performing a CyQuant NF proliferation assay on each plate before cell lysis.

**MAP kinase inhibitor studies.** Cells were plated in 96-well plates according to the Cyquant NF proliferation protocol or modified in-cell Western protocol described above. Following serum starvation, cells were then treated for 30 min with either CAS 219138-24-6 (p38 MAP kinase inhibitor, Calbiochem) or ERK activation inhibitor peptide I (Calbiochem). Following inhibitor exposure, cells were treated with 1% CSE for 48–72 h, and proliferation and ECM deposition were assessed as described above.

**Statistical analysis.** All experiments included a minimum of four fetal airway samples with a minimum of two replicate experiments per sample. Statistical analysis was performed using a Sigma Plot (SYSTAT, San Jose, CA) software statistical package. Data were analyzed via one-way ANOVA with Student-Newman-Keuls or Dunnet's post hoc analysis with statistical significance set at P < 0.05. Values are means ± SE.

---

Fig. 2. CSE increases extracellular matrix (ECM) deposition by human fASM cells. A: a modified LiCor In-Cell Western technique (semiquantitative immunofluorescence) was used to examine the impact of CSE exposure on ECM deposition of ECM molecules collagen I, collagen III, and fibronectin, all of which were significantly increased by 72 h CSE exposure. B: cells were treated with vehicle or N-acetylcysteine (NAC; 10 mM) before CSE exposure. NAC pretreatment prevented CSE-induced increase in ECM deposition. Values are means ± SE from n = 4 samples with proteins normalized to Cyquant NF kit cell count. *Significance from vehicle control with P < 0.05. #Significant difference from 1% CSE group with P < 0.05.
CSE increases fASM cell proliferation. CyQuant analysis demonstrated a significant increase in fASM cell proliferation with CSE exposure (P < 0.05; Fig. 1A). Western blot analysis demonstrated an associated increase in proliferative markers cyclin E and PCNA following 24 h CSE exposure (0.5 and 1%; P < 0.05; Fig. 1B). The proliferative effects of CSE appeared to decrease with higher levels of exposure (1.5% CSE).

CSE increases ECM deposition of collagens and fibronectin. To assess the impact of CSE on ECM deposition, modified In-Cell Westerns were performed to analyze three key molecules: collagen I, collagen III, and fibronectin. CSE exposure (72 h) resulted in increased expression of all three ECM proteins in a non-dose-dependent manner (P < 0.05; Fig. 2A). In contrast, control wells (no cells but media with and without treatment and all primary and secondary antibodies) were blank. To assess the possible contribution of oxidative stress as a mechanism for this increase, cells were pretreated with 10 mM N-acetylcysteine (NAC) for 60 min before CSE exposure. NAC pretreatment resulted in alleviation of the CSE effect on...

Fig. 3. CSE does not alter intracellular Ca^{2+} ([Ca^{2+}]_i) responses to histamine in human fASM cells. A: in fura 2-AM-loaded fASM cells, CSE exposure did not significantly affect [Ca^{2+}]_i, response to 10 μM histamine. Arrow indicates time of histamine administration in representative tracings. B: summary of average baseline and peak amplitude [Ca^{2+}]_i, responses. Values are means ± SE from n = 4 samples.

Caveolin-1 knockdown does not impact CSE effects on [Ca^{2+}]_i responses. A: fASM cells transfected with caveolin-1 small-interfering RNA (siRNA) demonstrated significantly decreased expression of caveolar proteins compared with controls, indicating adequate transfection efficiency. CSE exposure in these transfected cells did not result in significant alteration of caveolar protein expression. B: in fASM cells transfected with caveolin-1 siRNA and loaded with fura 2, [Ca^{2+}]_i responses to 10 μM histamine were not influenced by caveolin-1 siRNA compared with nontransfected and scrambled siRNA controls. Arrow indicates time of histamine administration in representative tracings. C: calculated average baseline and peak amplitude [Ca^{2+}]_i responses. Values are means ± SE from n = 4 samples. *Significant difference from vehicle control with P < 0.05.
Cigarette smoke exposure significantly increases caveolar protein expression in fetal airway smooth muscle (fASM) cells. Cigarette smoke exposure (CSE) for 24 h resulted in increased expression of caveolin-1 and caveolin-2 but did not significantly impact caveolar protein expression. Western blot analysis revealed increased expression of caveolin-1 and caveolin-2 in fASM cells treated with CSE compared with vehicle-treated controls. Transfection of fASM cells with caveolin-1 small-interfering RNA (siRNA) resulted in a 40–50% decrease in caveolin-1 and cavin-1 protein levels compared with nontransfected and scrambled siRNA controls. However, CSE treatment of such transfected cells did not significantly impact caveolar protein expression. Transfection with caveolin-1 siRNA also did not significantly impact [Ca2+]i responses compared with nontransfected and scrambled siRNA transfected controls.

**Mechanisms of CSE effects in fASM remodeling.** To examine whether CSE exposure resulted in decreased caveolar protein expression that could contribute to the observed changes in proliferation and ECM deposition, caveolar protein expression was examined through Western blot. Exposure of fASM cells to CSE (0.5–1.5%) for 24 h in fact did result in significantly increased expression of caveolar proteins caveolin-1 and caveolin-2 compared with vehicle (P < 0.05; Fig. 5). Cavin-1 did not demonstrate significant change in expression (Fig. 5).

In light of the fact that CSE resulted in increased caveolin protein expression rather than an expected decreased expression (and thus, consistent with the data of Fig. 4, was unlikely to be the contributory mechanism for CSE effect on proliferation), we explored additional mechanisms important to cellular proliferation. We specifically examined MAP kinase pathway induction with focus on JNK, ERK1/2, and MAP kinase (p38). Protein phosphorylation was examined 5, 10, 15, 30, and 60 min after cell treatment with CSE. fASM exposure to CSE resulted in a rapid induction of ERK and p38 phosphorylation with significant increase in p-ERK and phospho-p-38 by 5 min, with peak phosphorylation at 10 min (P < 0.05; Figs. 6, A and B). There was no significant phosphorylation of JNK noted (Fig. 6C).

**MAP kinase pathway inhibition prevents CSE-mediated remodeling.** To further assess the potential role of MAP kinase pathway signaling in fASM proliferation and ECM deposition, cells were treated with either p38 or ERK inhibitors before CSE exposure. ERK inhibition prevented CSE-mediated proliferation and increased deposition of collagen I, collagen III, and fibronectin (P < 0.05; Figs. 7 and 8A). p38 inhibition also resulted in prevention of CSE-mediated proliferation but did not have a significant effect on ECM deposition (P < 0.05; Figs. 7 and 8B).

**DISCUSSION**

Wheezing, asthma, and airway disease remain significant health burdens in the neonatal and pediatric population. Environmental exposures such as allergens, pollution, and cigarette smoke are important risk factors in development and exacerbation of these diseases (4, 6, 16, 21). In this study, we investigated the impact of cigarette smoke exposure on key properties of fASM cells that may contribute to development of reactive airway disease in the neonatal population. CSE exposure resulted in increased fASM proliferation and increased deposition of ECM molecules collagen I, collagen III, and fibronectin. Surprisingly, there was no significant impact of CSE exposure on [Ca2+]i responses to agonist (histamine). Overall, these results imply that cigarette smoke exposure may lead to airway remodeling, which may have long-term consequences on airway structure and function relevant to diseases such as asthma.

Airway wall thickening is a key feature of reactive airway diseases such as asthma and neonatal lung disease (13, 43). Postmortem examinations of infants with chronic lung disease of prematurity and bronchopulmonary dysplasia have demonstrated significant airway wall thickening (29). These wall changes can result from proliferation of ASM cells as well as increased deposition and alteration in composition of the ECM, resulting in thickening and stiffening of the airways (13, 35, 43). Based on our findings, it appears that cigarette smoke...
Exposure may result in altered proliferation of fASM cells, with low levels of exposure leading to increased proliferation. This proliferative trend begins to decrease with higher levels (1.5%) of CSE exposure and may indicate a toxic effect with increased exposure. These changes in fASM proliferation may result in alteration of airway development and decreased airway caliber, potentially contributing to airway thickening and stiffness, particularly in the setting of an otherwise compliant chest wall and lung during postnatal development. Interestingly, our prior investigations demonstrated higher fASM proliferation in cells exposed to hyperoxia (22). Thus, in preterm infants exposed to hyperoxia in the hospital followed by secondhand environmental smoke exposure at home, the issue of airway thickening due to ASM proliferation may be further compounded and the risk of developing airway disease increased.

Extracellular matrix composition has been shown to significantly impact cellular morphology, properties, and cell responses to stress largely through connection of the internal cellular cytoskeleton to the ECM via focal adhesion complexes (13, 35, 37). Matrix metalloproteinases (MMPs) are endopeptidases responsible for breaking down and regulating the components of the ECM, particularly collagens and fibronectin (10, 11). Numerous studies have demonstrated alterations in MMP expression in bronchoalveolar lavage specimens from neonates with bronchopulmonary dysplasia, implying that dysregulation of ECM remodeling contributes to neonatal lung disease (8, 44). Expression of ECM by human fASM cells has not been previously explored. Our findings demonstrate that fASM cells are capable of building and remodeling the extracellular matrix, with collagen I, collagen III, and fibronectin significantly expressed. Furthermore, exposure to cigarette smoke results in increased expression of these molecules, whereas pretreatment with the antioxidant NAC prevents these changes. This implies that oxidative stress plays a role in the CSE-mediated increase of fASM production and remodeling of the ECM. In terms of further potential mechanisms, TGF-β and Smad pathways have been previously demonstrated to be important in ECM remodeling, and it is possible that CSE exposure could impact these signaling pathways in fASM (1, 49).

Although the effects of CSE on developing ASM proliferation and ECM deposition have not been previously studied, similar studies have been performed in mature ASM with...
comparable results. A number of prior papers have demonstrated increased mature ASM proliferation in the setting of CSE exposure (23, 36, 39, 51). The etiology of CSE-mediated ASM proliferation is complex, with multiple pathways implicated in previous investigations. A number of studies have demonstrated an Akt pathway-mediated increase in cyclin D1 expression, leading to ASM proliferation in mature ASM cell lines (23, 51). Other studies in adult human tracheal smooth muscle and mature bovine tracheal smooth muscle have focused on reactive oxygen species-mediated effects of CSE and induction of the ERK1/2 pathway as a cause of ASM proliferation (36, 39). These studies demonstrating ERK-mediated proliferation in mature ASM support our data demonstrating ERK-mediated proliferation in developing ASM. The effects of CSE on ECM deposition are less well elucidated, even in mature ASM. One study in guinea pigs exposed to cigarette smoke demonstrated increased airway wall thickening with increased collagen deposition, consistent with the increased collagen deposition we observed with CSE exposure in developing ASM (50).

In light of the remodeling changes with CSE exposure characterized by increased proliferation and ECM deposition, we evaluated caveolar protein expression as a possible mechanism for these changes. Caveolin-1 has been shown to normally have a suppressive effect on ASM proliferation, at least in adults (17, 18, 20). Additionally, caveolin-1 is an important mediator of fibrosis, with decreased levels of caveolin-1 associated with increased fibrosis and collagen deposition in the mature lung (17, 32, 45, 46, 48). Intriguingly, in this study, both caveolin-1 and caveolin-2 protein levels were increased in...
fASM cells exposed to CSE, and thus the opposite to the expected trend. Whereas we did not specifically explore any potential effect of this increased caveolin-1 on proliferation or remodeling, our data suggest that caveolar proteins may play a less important role at this stage of development or may represent dysfunctional or decreased insertion of caveolins in the cell membrane. Regardless, caveolin-1 may not play a role in ECM modulation per se, at least in the context of remodeling.

To further assess possible mechanisms contributing to the observed fASM remodeling changes, we assessed induction of key MAP kinase pathways, specifically ERK, JNK, and p38. Both ERK and p38 phosphorylation were rapidly and significantly increased with CSE exposure, whereas JNK induction was not affected. A key role for ERK and p38 activation is further supported by the inhibitor data demonstrating decreased fASM proliferation with both p38 and ERK inhibition. ECM changes appear to be more strongly influenced by ERK inhibition, since collagen I, collagen III, and fibronectin deposition were decreased with ERK inhibition but no significant changes were noted with inhibition of p38. These data are consistent with a prior paper in mature bovine ASM demonstrating ERK and p38 induction in the setting of CSE and LPS exposure, although at a higher concentration of CSE (36). It is likely that free radical and oxidant stress induced by CSE exposure contribute to the observed ERK and p38 activation, as has been noted in mature human ASM cells exposed to CSE (9, 36, 39).

The import of oxidant stress as a major mechanism in causing the effects of CSE is further supported by the fact that pre-treatment with the antioxidant NAC prevented increased ECM deposition in fASM, as previously noted. fASM cells have been previously shown to respond to bronchoconstriction agonists such as acetylcholine and histamine, although the responses are smaller in amplitude and delayed when compared with adult ASM cells (22). The mechanisms of calcium regulation in these cells have not been previously closely examined. We have previously demonstrated the importance of caveolar protein caveolin-1 in regulation of [Ca^{2+}]_i in mature ASM (38, 41, 42). In adult ASM, knockdown of caveolin-1 abrogates cytokine-mediated increases in [Ca^{2+}]_i (40, 41). In this study, we hypothesized that similar effects would occur in developing ASM. However, although exposure to CSE increased caveolin-1 protein expression and resulted in increased variability of fASM histamine response, we did not find significant changes in [Ca^{2+}]_i with CSE exposure. Similarly, knockdown of fASM caveolin-1 with siRNA did not result in a statistically significant decrease in [Ca^{2+}]_i response to histamine. These data imply that fASM [Ca^{2+}]_i regulation is not as dependent on caveolar mechanisms and may rely on other mechanisms of calcium regulation at this stage of development. In terms of additional control points, the balance between sarcoplasmic calcium release vs. extracellular calcium influx has not been closely examined in these cells. Whereas fASM cells do respond to histamine, the decreased amplitude of response compared with adult ASM cells noted in prior studies may indicate that the fASM cells are more dependent on other sources for calcium influx.

In summary, our findings demonstrate that cigarette smoke exposure modulates key components of airway remodeling in developing human ASM. Specifically, CSE exposure results in increased proliferation and increased deposition of key ECM molecules, including collagen I, collagen III, and fibronectin. These remodeling changes may lead to development of a thicker more fibrotic airway that can contribute to airway diseases such as wheezing and asthma. These remodeling changes may also result in a particular burden of increased work of breathing in an already vulnerable neonatal and pediatric population. MAP kinase pathway induction, specifically ERK and p38 induction, likely plays a significant role in the etiology of these observed changes, with ERK signaling playing a particularly key role in mediating both proliferation and ECM effects. Antioxidants may represent a potential avenue for amelioration of these effects. Overall, these results indicate that environmental tobacco smoke may significantly contribute to development of airway disease in the neonatal and pediatric population.

GRANTS
This study was funded by Clinical Innovator Grants through the Flight Attendant Medical Research Institute (C. M. Pabelick, R. Vassallo). C. M. Pabelick’s Flight Attendant Medical Research Institute support is through a grant to the American Academy of Pediatrics Julius B. Richmond Center. Further support was provided by the National Heart Lung and Blood Institute of the National Institutes of Health (T3-HL-105355, E. R. Vogel; HL-056470, Y. S. Prakash; HL-090059, C. M. Pabelick).

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

AUTHOR CONTRIBUTIONS

REFERENCES
9. Cheng SE, Luo SF, Jou MJ, Lin CC, Kou YR, Lee IT, Hsieh HL, Yang CM. Cigarette smoke extract induces cytosolic phospholipase A2 expres-


