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Cigarette smoke causes acute airway disease and exacerbates chronic obstructive lung disease in neonatal mice

Jie Jia,1,* Thomas M. Conlon,1,* Carolina Ballester Lopez,1 Michael Seimetz,2,3 Mariola Bednorz,2,3 Zhe Zhou-Suckow,4 Norbert Weissmann,2,3 Oliver Eickelberg,1,5 Marcus A. Mall,4,* and Ali Önder Yildirim1,6

1Comprehensive Pneumology Center (CPC), Institute of Lung Biology and Disease, Helmholtz Zentrum München, Member of the German Center for Lung Research (DZL), Munich, Germany; 2Excellence Cluster Cardio-Pulmonary System (ECCPS), Justus-Liebig-University, Giessen, Germany; 3Department of Internal Medicine, Universities of Giessen and Marburg Lung Center (UGMLC), Member of the German Center for Lung Research (DZL), Giessen, Germany; 4Department of Translational Pulmonology, Translational Lung Research Center Heidelberg (TLRC), University of Heidelberg, Member of the German Center for Lung Research (DZL), Heidelberg, Germany; and 5University Hospital of the Ludwig Maximilians University (LMU), Munich, Germany

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Jia J, Conlon TM, Ballester Lopez C, Seimetz M, Bednorz M, Zhou-Suckow Z, Weissmann N, Eickelberg O, Mall MA, Yildirim AO. Cigarette smoke causes acute airway disease and exacerbates chronic obstructive lung disease in neonatal mice. Am J Physiol Lung Cell Mol Physiol 311: L602–L610, 2016. First published July 22, 2016; doi:10.1152/ajplung.00124.2016.—Epidemiological evidence demonstrates a strong link between postnatal cigarette smoke (CS) exposure and increased respiratory morbidity in young children. However, how CS induces early onset airway disease in young children, and how it interacts with endogenous risk factors, remains poorly understood. We, therefore, exposed 10-day-old neonatal wild-type and β-epithelial sodium ion channel (β-ENaC)-transgenic mice with cystic fibrosis-like lung disease to CS for 4 days. Neonatal wild-type mice exposed to CS demonstrated increased numbers of macrophages and neutrophils in the bronchoalveolar lavage fluid (BALF), which was accompanied by increased levels of Mmp12 and Cxcl1. BALF from β-ENaC-transgenic mice contained greater numbers of macrophages, which did not increase following acute CS exposure; however, there was significant increase in airway neutrophilia compared with filtered air transgenic and CS-exposed wild-type controls. Interestingly, wild-type and β-ENaC-transgenic mice demonstrated epithelial airway and vascular remodeling following CS exposure. Morphometric analysis of lung sections revealed that CS exposure caused increased mucus accumulation in the airway lumen of neonatal β-ENaC-transgenic mice compared with wild-type controls, which was accompanied by an increase in the number of goblet cells and Muc5ac upregulation. We conclude that short-term CS exposure 1) induces acute airway disease with airway epithelial and vascular remodeling in neonatal wild-type mice; and 2) exacerbates airway inflammation, mucus hypersecretion, and mucus plugging in neonatal β-ENaC-transgenic mice with chronic lung disease. Our results in neonatal mice suggest that young children may be highly susceptible to develop airway disease in response to tobacco smoke exposure, and that adverse effects may be aggravated in children with underlying chronic lung diseases.

*J. Jia and T. M. Conlon contributed equally to the work. M. A. Mall and A. Ö. Yildirim contributed equally as senior authors.

Address for reprint requests and other correspondence: A. Ö. Yildirim, Comprehensive Pneumology Center, Institute of Lung Biology and Disease, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85764 Neuherberg, Germany (e-mail: oender.yildirim@helmholtz-muenchen.de).

CHRONIC AIRWAY DISEASES INCLUDING recurrent wheezing, chronic bronchitis and asthma constitute a major cause of morbidity in young children and evidence from epidemiologic studies identified tobacco smoke exposure as an important risk factor for the development of chronic airway disease in early childhood (9, 29, 37). However, current knowledge on the in vivo pathogenesis of airway disease induced by environmental tobacco smoke in young children, and how tobacco smoke exposure interacts with endogenous risk factors and underlying chronic lung disease remains poorly understood.

In adult smokers, chronic tobacco smoke exposure is a key risk factor of chronic bronchitis associated with influx of macrophages and neutrophils into the airways, goblet cell metaplasia and mucus hypersecretion, and structural lung damage (14, 16, 20, 31, 32), and these pathologies are at least in part recapitulated by chronic cigarette smoke (CS) exposure in adult mice (3, 5, 13, 19, 36). In comparison, studies on the effect of CS in children or short-term CS exposure in neonatal mice more closely reflecting environmental exposure in young children remain limited. In children who died of sudden infant death syndrome, it was found that maternal smoking is associated with airway wall thickening (8), and a study of newborn mice exposed to 2 wk of CS straight after birth reported an increase in the presence of alveolar macrophages, albeit to a lesser extent than in adult mice (27). However, the consequences of short-term CS exposure with regard to airway inflammation, mucus hypersecretion, and tissue remodeling in neonates in vivo remains to be fully elucidated.

The aim of this study was, therefore, to determine the in vivo effects of acute CS exposure on airway inflammation and lung morphology in neonatal wild-type mice as a model of parental CS exposure of heathy infants. Second, we used the β-subunit of the epithelial sodium ion channel (β-ENaC)-transgenic mouse as an established model of cystic fibrosis (CF) lung disease to assess the impact of CS exposure in the context of chronic underlying lung disease. These mice overexpress β-ENaC, a key protein in regulating airway surface liquid (26),
along with chloride ion channels (33), under the control of the club cell secretory protein promoter, causing enhanced airway Na⁺ absorption, which results in airway surface dehydration and reduced mucus clearance. The mice develop spontaneous early onset lung disease that shares key features with CF in children, including airway mucus plugging, chronic airway inflammation, and structural lung damage (24, 25). Specifically, we hypothesized 1) that neonatal wild-type mice are susceptible to develop airway disease even after short-term CS exposure; and 2) that adverse effects of CS are enhanced in β-ENaC-transgenic mice with obstructive lung disease. To test these hypotheses, we exposed neonatal wild-type and β-ENaC-transgenic mice to CS for 4 days and compared indexes of airway inflammation, epithelial, vascular, and alveolar remodeling, and airway mucus obstruction after this short-term CS exposure. Using this in vivo model of postnatal CS exposure, we demonstrate that tobacco smoke produces acute airway disease in neonatal mice, and that adverse effects are enhanced in concurrent chronic obstructive lung disease. These results provide novel insights into the link between tobacco smoke exposure and increased respiratory morbidity early in life and are consistent with its adverse effects on lung health in young children.

MATERIALS AND METHODS

Experimental animals. β-ENaC-transgenic mice on a C57BL/6 background (17, 24) were bred in-house with C57BL/6NCrI mice (Charles River Laboratories, Sulzfeld, Germany). Mice were housed under specific pathogen-free conditions at a constant temperature and humidity with a 12:12-h light cycle and allowed food and water ad libitum. All animal experiments were performed according to strict governmental and international guidelines and were approved by the local government for the administrative region of Upper Bavaria.

CS exposure. Ten-day-old β-ENaC-transgenic mice and their wild-type littermate controls (males and females) were whole body exposed to 100% mainstream CS at 500 mg/m³ total particulate matter generated from 3R4F research cigarettes (filter removed; Tobacco Research Institute, University of Kentucky, Lexington, KY), for 50 min twice per day for 4 days. Pups were separated from their mothers during the smoking period. In brief, to mimic natural human smoking habits, CS was generated with 2 s of puff and 4 s of break by a membrane pump and drawn into the exposure chamber (18). Filtered air (FA)-exposed mice were generated with 2 s puff and 4 s break by a membrane pump and drawn into the exposure chamber (18). Filtered air (FA)-exposed mice were generated with 2 s puff and 4 s break by a membrane pump and drawn into the exposure chamber (18).

Quantitative intrapulmonary real-time RT-PCR. Mouse cDNA was synthesized using random hexamers and reverse transcriptase (Applied Biosystems, Darmstadt, Germany) from right lung isolated total RNA. Mouse Mac3 (forward 5'-ATCGAGAGGAGCGTGTCGAC-3', reverse 5'-ATCGACCGCTTGGTAGGAC-3'), Mac5b (forward 5'-AGAAACTGGAGCTGGCTC-3', reverse 5'-TGACTGTCTCGGTAGGTTT'-3'), Cxcl1 (forward 5'-CCGAAGTCATTAGCCACAC-3', reverse 5'-TGCCATAGAGGAGCCTT3'), and Mmp12 (forward 5'-TGTTACCCCCAATACTAGTTTCA-3', reverse 5'-CCATAGAGGAGGCTAATGGTAGTCT-3') gene expression levels were analyzed using Platinum SYBR Green qPCR SuperMix (Applied Biosystems) on a StepOnePlus 96 well real-time-PCR system (Applied Biosystems, Carlsbad, CA), and calculated relative to the housekeeping gene Hprt1 (forward 5'-AGCTACTGTAAAGTACGTCGCAA-3', reverse 5'-AGGGTCCTTTTCCAGGCA-3'). Relative expression is defined as 2⁻ΔCt, where change in cycle threshold (ΔCt) = Ct_target - Ct_housekeeping.

Immunohistochemistry. Deparaffinized and rehydrated lung sections were analyzed using an Olympus BX51 light microscope equipped with the new Computer Assisted Stereological Toolbox (newCAST, Visiopharm, Hoersholm, Denmark), as described previously (19, 28). Lung air space enlargement was analyzed by quantifying the mean linear intercept of (MLI) of the H&E-stained lung sections. Briefly, 30 random fields of view per lung were superimposed with points and a line grid. The points hitting air space (P_a) and intercepts of lines with alveolar septa (I_septa) were counted to calculate the MLI, using MLI = P_a / L(p) / I_septa / 0.5, where L(p) is the line length per point.

For quantitative assessment of airway mucus accumulation, the PAS-stained lung sections were analyzed using a volume counting toolbox across 100 random fields of view per lung. The volume of airway mucus (V_mucus) was calculated as the number of points hitting on positive stained mucousubstances (P_mucus) normalized to the number of line intercepts with airway basement membrane (I_airway), using the formula V_mucus = P_mucus / L(p) / I_airway.

To calculate the percentage of goblet cells, a frame grid was superimposed on lung section images across 100 fields of view per lung. The percentage of points hitting on PAS positively stained airway epithelial cells compared with the points hitting on all airway epithelial cells were calculated.
The thickness of the airway epithelium was calculated as the number of points hitting epithelial cells (P_{epithelium}) normalized to the number of I_{airway}, using the formula V_{epithelium} = \sum P_{epithelium} \times 0.5/10^3 I_{airway}.

Muscularization of pulmonary arterial vessels. The muscularization of pulmonary arterial vessels was determined as previously described (34) from lung paraffin sections. The sections were stained with a 1:900 diluted α-smooth muscle actin antibody (clone 1A4, Sigma-Aldrich, Munich, Germany) to visualize the muscle layer and a 1:900 dilution of the anti-human von Willebrand-factor antibody to allow identification of vessels (Dako, Hamburg, Germany). The degree of muscularization of small vessels (20–70 µm) was microscopically determined by vascular morphometry using the Qwin software (Leica, Wetzlar, Germany) and expressed as averaged percentage of the vessel circumference being α-smooth muscle actin positive. Eighty-five vessels were analyzed from each lung lobe in a randomized and blinded fashion.

Statistical analysis. Data were analyzed with GraphPad Prism 6 software (GraphPad Software, La Jolla, CA) and presented as mean values ± SE. Groups of n = 12–16 mice were exposed to CS or FA, with the specific numbers used for each analysis given in the appropriate figure legend. Statistical analyses were performed using a two-tailed unpaired t-test or one-way ANOVA following Bonferroni posttesting, as indicated. P < 0.05 was taken to indicate statistical significance.

RESULTS

CS exposure causes acute airway inflammation in neonatal wild-type mice and exacerbates inflammation in β-ENaC-transgenic mice. To determine whether acute CS exposure causes airway inflammation, we measured the number of inflammatory cells in the bronchoalveolar lavage fluid (BALF) of neonatal (15-day-old) wild-type and β-ENaC-transgenic mice after 4 days of exposure to CS or FA. There was a clear increase in the total cell count in BALF of wild-type mice following acute CS exposure (Fig. 1A), which was predominantly due to an increase in macrophage and neutrophil numbers (Fig. 1, B and C). β-ENaC-transgenic mice had a higher inflammatory total cell count in the BALF compared with wild-type controls after exposure to FA (Fig. 1A). This was
largely composed of macrophages (Fig. 1B), with a trend toward increased neutrophils compared with FA wild-type controls (Fig. 1C), but this did not reach statistical significance. Following acute exposure to CS, there was no change in the number of macrophages present in the BALF of β-ENaC-transgenic mice, and this was similar to the number observed in CS-exposed wild-type mice (Fig. 1B). There was, however, a strong increase in the number of neutrophils detected in the BALF of β-ENaC-transgenic mice following CS exposure, which was significantly increased compared with CS-exposed wild-type mice (Fig. 1C).

For further characterization of the inflammation triggered by acute CS exposure, we undertook immunohistochemistry using anti-galectin-3 antibody for macrophages and anti-Gr1 antibody for neutrophils on lung sections from mice that had not undergone the BALF procedure. Figure 1D clearly shows that there is an increase in the number of septal tissue macrophages present in the lungs of wild-type mice following exposure to CS. Furthermore, more macrophages are detectable in the lungs of β-ENaC-transgenic mice exposed to FA than their wild-type counterparts, and these do not significantly increase in number following exposure to CS (Fig. 1D). Figure 1E demonstrates that no neutrophils are detectable in the lungs of wild-type mice exposed to FA, but a very small number can be seen associated with the airway following acute CS exposure. FA-exposed β-ENaC-transgenic mice showed Gr1-positive cells mainly in the airway lumen that increased in number following acute CS exposure (Fig. 1E).

Next, we determined transcript levels of the neutrophil chemoattractant Cxcl1 (KC) and Mmp12 as a marker of activated macrophages. Wild-type mice exposed to acute CS exhibited higher mRNA levels for Cxcl1 (KC) and Mmp12 in total lung homogenate compared with FA controls (Fig. 2, A and B). Concomitant with the increased airway neutrophil number observed in β-ENaC-transgenic mice (Fig. 1D), FA-exposed β-ENaC-transgenic mice demonstrated higher mRNA levels for Cxcl1 than their wild-type counterparts (Fig. 2A), which increased further following CS exposure. Mmp12 mRNA levels were also greater in the lungs of FA-exposed β-ENaC-transgenic mice compared with wild-type FA controls (Fig. 2B), which increased further following CS exposure and was significantly greater than that exhibited in wild-type CS-exposed mice (Fig. 2B). The changes in Mmp12 gene expression were also confirmed at the protein level by immunohistochemistry on lung tissue sections. Figure 2C demonstrates that airway epithelial cells were positively stained for Mmp12 in wild-type animals only following acute CS, whereas positively stained airway epithelial cells could also be detected in the FA-exposed β-ENaC-transgenic mice, which was further enhanced upon CS exposure. Mmp12-positive macrophages, surprisingly, could not be detected in the lungs of wild-type mice, even after CS exposure, whereas a large number of Mmp12-positively stained macrophages could be observed in the lungs of the β-ENaC-transgenic mice following acute CS exposure, with a large number accumulating in the airways (Fig. 2C). Interestingly, albeit to a lesser extent, Mmp12-positively stained macrophages could also be detected in the airways of FA-exposed β-ENaC-transgenic mice (Fig. 2C). Taken together, these data suggest that short-term CS induces a robust inflammatory response in neonatal mice that is exacerbated in predisposed β-ENaC-transgenic mice.

**Acute CS exposure causes remodeling of the airway epithelium and pulmonary vessels.** We next investigated whether acute CS exposure had effects on the morphology of the airway epithelium and pulmonary vasculature. Exposure of neonatal (10-day-old) wild-type mice to 4 days of CS increased the thickness of the airway epithelium compared with FA controls (Fig. 3A). This was confirmed by analysis of morphological quantification using the newCAST system (Fig. 3B). The airway epithelium of neonatal β-ENaC-transgenic mice after exposure to FA did not differ from that of wild-type FA controls (Fig. 3A and B). After exposure to CS, the β-ENaC-transgenic mice also appeared to have thicker airway epithelium compared with FA-exposed controls (Fig. 3A), but this did not reach statistical significance (Fig. 3B).

In addition to acute effects on the airway epithelium, we found that short-term CS exposure of neonatal wild-type mice had a significant effect on pulmonary vessels. Specifically, we observed a pronounced increase in the muscularization of small pulmonary vessels (20–70 μm in diameter) compared with FA controls (Fig. 4). Similar to their wild-type counterparts, β-ENaC-transgenic mice exposed only to FA demonstrated a low level of small-vessel muscularization, which increased significantly following exposure to acute CS (Fig. 4).
was no difference in the level of small vessel muscularization between the wild-type and β-ENaC-transgenic mice following CS exposure (Fig. 4B). These results demonstrate that even a short CS exposure in neonatal mice is sufficient to trigger remodeling of the airway epithelium and pulmonary vessels.

Air space size and lung function were not altered by acute CS exposure. We next investigated effects of short-term CS exposure on lung function and distal air space morphology. As shown in Fig. 5, acute exposure to CS had no effect on alveolar morphology (Fig. 5A), MLI (Fig. 5B), or lung function parameters, including dynamic compliance, elasance, or resistance of the lung (Fig. 5, C–E). As expected from previous studies (25, 42) FA-exposed β-ENaC-transgenic mice showed emphysema-like distal air space enlargement (Fig. 5A), which was confirmed by quantitative morphometry (MLI of 24.60 ± 1.92 μm in β-ENaC-transgenic vs. 15.55 ± 0.94 μm in wild type, *P < 0.01, Fig. 5B). There was, however, no further enlargement of the air spaces following acute CS exposure in the β-ENaC-transgenic mice (Fig. 5, A and B). In line with this, we did not detect changes in the level of cell death; however, a slight reduction in cell proliferation was observed following exposure of either wild-type or β-ENaC-transgenic mice to CS in lung sections stained by immunohistochemistry for cleaved (active)-caspase 3 and Ki67, respectively (data not show). Consistent with the spontaneous emphysema observed in the FA-exposed β-ENaC-transgenic mice, these animals also displayed impaired lung function with increased dynamic compliance and reduced total lung resistance compared with the wild-type controls (Fig. 5, C–E). Exposure of the β-ENaC-transgenic mice to acute CS for 4 days did not alter the lung function further (Fig. 5, C–E), indicating that, despite remodeling of airway and pulmonary vessels, the spontaneous emphysema of the β-ENaC-transgenic mice is not aggravated by acute CS exposure.

Acute CS exposure induces goblet cell metaplasia and increased mucin expression in β-ENaC-transgenic mice. Finally, we studied effects of acute CS exposure on goblet cell numbers, mucin expression, and intraluminal mucus content in neonatal wild-type and β-ENaC-transgenic mice. Consistent with previous studies (25), PAS staining of lung sections revealed mucus accumulation in the airways of 15-day-old β-ENaC-transgenic mice that was not detectable in wild-type controls (Fig. 6A). Acute CS exposure did not induce mucus accumulation in the airways of wild-type mice, but significantly increased the density and volume of the accumulated mucus in β-ENaC-transgenic mice compared with CS-exposed wild-type animals, as determined by morphometric analysis using the newCAST system (Fig. 6B). In addition, we observed metaplasia of airway goblet cells in the β-ENaC-transgenic but not in wild-type mice post-CS exposure (Fig. 6, C and D). This was quantified (Fig. 6D) as the percentage of PAS-positive airway epithelial cells, with the CS-exposed β-ENaC-transgenic mouse demonstrating 43.12 ± 2.98% goblet cells, compared with 6.74 ± 2.51% (P < 0.001) in wild-type mice exposed to CS and 18.77 ± 6.32% (P < 0.01) goblet cells in β-ENaC-transgenic mice exposed to FA. Similarly, Muc5ac and Muc5b gene expression in total lung homogenate were only increased in the β-ENaC-transgenic mice exposed to CS (Fig. 6E). Taken together, these data suggest that acute CS exposure triggers increased mucus production in β-ENaC-transgenic mice.
Mmp12 macrophage numbers, which was accompanied by increased wild-type mice also demonstrate a strong increase in BALF confirmed (Figs. 1 and 2) that acute CS-exposed neonatal increase in the presence of alveolar macrophages (27). Here we exposed to 2 wk of CS straight after birth also reported an increase in the occurrence of alveolar macrophages and neutrophils (6, 11, 18). A study of newborn mice CS results in a strong inflammatory response driven by macrophages and neutrophils (1, 2), but these are tumor-prone mice exposed chronically. Meanwhile, exposure to CS for 10 days across a 2-wk period in adult Balb/c mice induced airway epithelial hyperplasia (1, 2), but these are tumor-prone mice exposed chronically. This response is then enhanced following short-term exposure to CS, suggesting that young children with CF or other underlying chronic obstructive lung disease may require little CS exposure to exacerbate their condition.

A significant finding from our study is that, in both wild-type and β-ENaC-transgenic mice, we observe that an acute exposure to CS at neonatal ages results in thickening of the airway epithelium (Fig. 3). It has been previously reported that exposure of Swiss albino mice or H mice from birth for 120 days to CS resulted in pronounced hyperplasia of the bronchial epithelium (1, 2), but these are tumor-prone mice exposed chronically. Meanwhile, exposure to CS for 10 days across a 2-wk period in adult Balb/c mice induced airway epithelial hyperplasia and mucous cell metaplasia that was inhibited by blockade of DP2, a prostaglandin D2 receptor (38). Of note, CS-induced airway epithelial thickening occurred to a similar extent in both wild-type and β-ENaC-transgenic mice, suggesting that direct effects of CS rather than chronic inflammation is the driving force behind airway epithelial cell hyperplasia. Given the relatively small diameter of airways in infants, this mucosal thickening may contribute to airflow obstruction and recurrent wheezing in young children exposed to CS.

We also demonstrate that exposure to acute CS in newborn mice results in pronounced increases in muscularization of the small pulmonary vessels (20–70 μm in diameter), in both wild-type and β-ENaC-transgenic mice (Fig. 4). It has been previously shown in adult rats that short-term exposure to CS induced pulmonary vascular remodeling (45), but most studies have focused on the effect of chronic CS exposure (34, 43, 44).

DISCUSSION
In this study, we have established a new mouse model of postnatal CS exposure and CS-induced exacerbation of CF-like chronic obstructive lung disease in neonatal mice. We demonstrate that exposure of neonatal (10-day-old) wild-type mice to 4 days of acute CS resulted in a macrophage and neutrophil predominant inflammation of the airways. Furthermore, we show for the first time in young mice that the inflammation triggered by such a short exposure period to CS is accompanied by airway epithelial thickening and pulmonary vessel remodeling. Then, using neonatal β-ENaC-transgenic mice as an established model of CF-like lung disease featuring spontaneous mucus hypersecretion, airway mucus plugging, and inflammation in the first week of life, we demonstrate that short-term CS exacerbated the chronic bronchitis phenotype in this model. These results are consistent with the clinical observation and epidemiological data, suggesting that environmental tobacco smoke exposure is a major risk factor for the development of airway disease in children (9, 29, 37).

It is widely appreciated that exposure of adult mice to acute CS results in a strong inflammatory response driven by macrophages and neutrophils (6, 11, 18). A study of newborn mice exposed to 2 wk of CS straight after birth also reported an increase in the presence of alveolar macrophages (27). Here we confirmed (Figs. 1 and 2) that acute CS-exposed neonatal wild-type mice also demonstrate a strong increase in BALF macrophage numbers, which was accompanied by increased Mmp12 mRNA expression in total lung homogenates and positively stained airway epithelial cells in immunohistochemically stained sections. Aside from Mmp12 production by alveolar macrophages following short-term exposure to CS in mice (7), CS extract has been shown to upregulate Mmp12 in human airway-like epithelial cells (21). Interestingly, the β-ENaC-transgenic mice exposed only to FA have increased macrophage numbers in the BALF compared with wild-type controls, accompanied by higher levels of total lung Mmp12 gene expression and Mmp12 positive airway macrophages and epithelial cells (Figs. 1 and 2). These data confirm that impaired mucociliary clearance and mucus stasis triggered by airway surface dehydration in β-ENaC-transgenic mice already triggers a strong inflammatory response at neonatal ages (40). This response is then enhanced following short-term exposure to CS, suggesting that young children with CF or other underlying chronic obstructive lung disease may require little CS exposure to exacerbate their condition.
Pulmonary vascular remodeling is prevalent amongst patients with CS-induced COPD (30) and has been shown to contribute to emphysema development in chronic CS-exposed mice (34). Acute CS exposure does not lead to emphysema development; however, β-ENaC-transgenic mice spontaneously develop emphysema-like changes, but we show that this is independent of pulmonary vascular remodeling. Nevertheless, that short-term CS exposure can lead to pulmonary vascular remodeling in the newborn should be carefully considered as a potential contributing factor to respiratory disease and pulmonary hypertension later in life.

A strength with our model is the accumulation of mucus in the airways of β-ENaC-transgenic mice (Fig. 6). The pathophysiology of chronic bronchitis in patients and the associated increase in airway infections is closely related to intraluminal obstruction of the airways caused by mucus adhesion, mucus plaques, and, in severe cases, mucus plugging (4, 23). A weakness with existing models of CS exposure in mice is that wild-type mice exposed to CS, even chronically out to 6 mo, do not demonstrate evidence of goblet cell metaplasia or changes to Muc5ac or Muc5b gene expression, major secreted mucins (39). Interestingly, in the neonatal β-ENaC-transgenic mice with underlying chronic obstructive airway disease, acute CS smoke exposure resulted in goblet cell metaplasia and increased expression of Muc5ac and Muc5b. We speculate that reduced mucociliary clearance in β-ENaC-transgenic mice may cause impaired clearance of CS, which may lead to the more severe neutrophilia, producing higher levels of neutrophil elastase, acting as a potent stimulus of goblet cell metaplasia in the airways (10, 41). Interestingly, in a recent study, Seys et al. (35) exposed adult β-ENaC-transgenic mice to CS for up to 8 wk. They confirmed that adult β-ENaC-transgenic mice have increased goblet cells and mucus production compared with wild-type controls, but, unlike our observation with newborn mice, adult β-ENaC-transgenic mice did not exhibit goblet cell metaplasia after CS exposure. The likely reason for this is the inherent difference in

Fig. 6. Acute CS exposure exacerbates mucus hypersecretion in neonatal β-ENaC-transgenic mice. The 10-day-old β-ENaC-transgenic mice and WT littermate controls were exposed to CS or FA for 4 days. A–D: lung sections were obtained from 3 mice per group, which had not undergone BALF collection, and stained with PAS. A: representative photomicrographs highlighting enhanced volume and density of accumulated mucus in the airways of β-ENaC-transgenic mice exposed to CS. Scale bar: 100 μm. B: mucus volume was quantified by design-based stereology using the newCAST system. C: representative photomicrographs depicting goblet cell hyperplasia in β-ENaC-transgenic mice following exposure to CS. Scale bar: 30 μm. D: the percentage of airway goblet cells (PAS positive) relative to total airway epithelial cell number was quantified using the newCAST system. E: the level of Muc5ac and Muc5b gene expression in total lung homogenate relative to Hprt1 was determined by qPCR (n = 5 and 8 mice for FA and CS groups, respectively). Values are means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001 following one-way ANOVA with Bonferroni posttesting. #P < 0.05 following two-tailed unpaired t-test.
the number of goblet cells in the airways between young and adult mice (22). Taken together, these data suggest that young children with CF and other chronic airway diseases may be more susceptible to CS exposure.

β-ENaC-transgenic mice also demonstrate emphysema-like structural lung damage (10, 25, 40), which we confirmed by lung function analysis and quantification of MLI on H&E-stained lung sections to be present in our 15-day-old transgenic mice (Fig. 5). Interestingly, crossing the β-ENaC-transgenic mice onto a neutrophil elastase or Mmp12-deficient background reduced inflammation and emphysema in these mice (10, 40). Despite an increase in inflammation and Mmp12 upregulation in the lungs of β-ENaC-transgenic mice following exposure to acute CS, we do not see any deterioration in lung function or air space enlargement. This is not surprising, as our laboratory has previously shown that, in wild-type mice, we require 4 mo of CS exposure to induce emphysematous changes to the lungs (19). However, the CS-exposed β-ENaC-transgenic mice demonstrated both increased mucus production and epithelial thickening of the airways expected to cause increased airway resistance and airflow limitation due to airway obstruction. We speculate that increased airway resistance was not detected in our lung function measurements, because the FlexiVent uses a one-compartment model integrating the resistances of the conducting airways and distal air spaces in the lung parenchyma, where the reduction in resistance caused by the emphysema present in the β-ENaC-transgenic mice most likely overrides any changes due to airway obstruction. One would hypothesize that emphysema progression would be accelerated in the β-ENaC-transgenic mice following a longer exposure to CS, and that we only assessed acute CS exposure is a limitation of this current study. Although we saw no changes to air space enlargement following acute exposure to CS in the neonatal mice, it is important to again highlight that we did observe airway remodeling occurring in these young animals after CS exposure (Fig. 3). Hogg et al. (15) proposes that emphysema development stems from an initial lesion in the small airways; we should, therefore, consider that these early pathological changes may sow the seed for chronic obstructive lung disease later in life. Interestingly, in the study by McGrath-Morrow et al. (27), they following up mice at 8 wk of age that received 2 wk of CS from birth and reported reduced alveolar number and an increase in MLI size compared with 8-wk-old mice that did not receive the CS exposure at birth.

In summary, we established a novel neonatal murine model to assess in vivo effects of postnatal CS exposure in wild-type and β-ENaC-transgenic mice with CF-like obstructive lung disease. In this model, we demonstrate for the first time that short-term CS exposure is sufficient to induce acute airway disease characterized by neutrophilic airway inflammation, mucosal thickening, and vascular remodeling in neonatal wild-type mice. Furthermore, we show that CS exacerbates airway inflammation and mucus hypersecretion in neonatal β-ENaC-transgenic mice with chronic CF-like lung disease. These results provide novel insights into the link between CS exposure and increased respiratory morbidity during early life and demonstrate that even short-term CS exposure has substantial adverse effects on lung health and exacerbates underlying chronic lung disease such as CF in vivo. We expect that this novel model will be useful for studies of therapeutic intervention, as well as further elucidation of the factors that determine resolution vs. chronicity of CS-induced respiratory pathology and thus define the development of disease phenotypes, such as recurrent wheezing, chronic bronchitis, and asthma that are more common in infants and young children who are exposed to parental tobacco smoke.

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DISCLOSURES

M. A. Mall is listed on a patent application filed by the University of North Carolina, describing the β-ENaC-overexpressing mouse (patent no.: 7514593; filing date: May 2003). Of note, the β-ENaC-overexpressing mouse has been deposited at the Jackson Laboratory.

AUTHOR CONTRIBUTIONS


REFERENCES


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