Maternal Exposure to Secondhand Cigarette Smoke Primes the Lung for Induction of Phosphodiesterase-4D5 Isozyme and Exacerbated Th2 Responses: Rolipram Attenuates the Airway Hyperreactivity and Muscarinic Receptor Expression but Not Lung Inflammation and Atopy

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Maternal Exposure to Secondhand Cigarette Smoke Primes the Lung for Induction of Phosphodiesterase-4D5 Isozyme and Exacerbated Th2 Responses: Rolipram Attenuates the Airway Hyperreactivity and Muscarinic Receptor Expression but Not Lung Inflammation and Atopy

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Airway hyperreactivity (AHR), lung inflammation, and atopy are clinical signs of allergic asthma. Gestational exposure to cigarette smoke (CS) markedly increases the risk for childhood allergic asthma. Muscarinic receptors regulate airway smooth muscle tone, and asthmatics exhibit increased AHR to muscarinic agonists. We have previously reported that in a murine model of bronchopulmonary aspergillosis, maternal exposure to mainstream CS increases AHR after acute intratracheal administration of Aspergillus fumigatus extract. However, the mechanism by which gestational CS induces allergic asthma is unclear. We now show for the first time that, compared with controls, mice exposed prenatally to secondhand CS exhibit increased lung inflammation (predominant infiltration by eosinophils and polymorphs), atopy, and airway resistance, and produce proinflammatory cytokines (IL-4, IL-5, IL-6, and IL-13, but not IL-2 or IFN-γ). These changes, which occur only after an allergen (A. fumigatus extract) treatment, are correlated with marked up-regulated lung expression of M1, M2, and M3 muscarinic receptors and phosphodiesterase (PDE)4D5 isozyme. Interestingly, the PDE4-selective inhibitor rolipram attenuates the increase in AHR, muscarinic receptors, and PDE4D5, but fails to down-regulate lung inflammation, Th2 cytokines, or serum IgE levels. Thus, the fetus is extraordinarily sensitive to CS, inducing allergic asthma after postnatal exposure to allergens. Although the increased AHR might reflect increased PDE4D5 and muscarinic receptor expression, the mechanisms underlying atopy and lung inflammation are unrelated to the PDE4 activity. Thus, PDE4 inhibitors might ease AHR, but are unlikely to attenuate lung inflammation and atopy associated with childhood allergic asthma. The Journal of Immunology, 2009, 183: 2115–2121.

The adverse health effects of cigarette smoke (CS) are well recognized, and smoking is associated with increased risk for lung cancer and respiratory infections (1). Increasing evidence suggests that chronic exposure to environmental or secondhand CS (SS) also causes significant health effects (2–4). Moreover, strong epidemiological evidence indicates parental smoking, particularly maternal smoking during pregnancy, increases the risk of allergic asthma in children (4–10). Yet in the United States alone, nearly 12% of prospective mothers continue to smoke during pregnancy (11). Interestingly, prenatal and postnatal exposure to CS may affect immune and inflammatory responses differently (12, 13). For example, some allergic diseases and ulcerative colitis are less common in adult smokers than nonsmokers (1, 14–18), whereas ex-smokers are more likely to develop asthma than current smokers (19, 20). Moreover, in animal models, chronic exposure of adult animals to mainstream CS or nicotine suppresses innate and adaptive immune responses (1, 21–24), and even SS moderates some parameters of allergic asthma in mice (25). In Brown Norway rats, chronic exposure to nicotine attenuates the ragweed/house dust mite-induced lung inflammation and atopy (13). Thus, in adult humans and animals, chronic CS/nicotine exposure may attenuate some parameters of allergic inflammation in the lung. In contrast, in utero exposure to mainstream CS exacerbates allergic and inflammatory responses in the offspring (26), and it is likely that the mechanisms by which CS modulates the allergic responses in utero and during adult life do not totally overlap.

The mechanism(s) by which gestational exposure to CS affects the lung function in children is not clearly understood. In an established murine model of bronchopulmonary aspergillosis, in which Aspergillus fumigatus extract (Af) induces allergic asthma (26, 27), we have shown that exposure of mothers to mainstream CS throughout the gestational period increases airway hyperreactivity (AHR) after an acute exposure to the allergen (Af) and the increased AHR is related to elevated expression of phosphodiesterase (PDE)4 in the lungs of the progeny (26). However, unlike chronic Af sensitization (27), single exposure to the allergen did not induce significant lung inflammation and atopy (26). Therefore, in addition to the mechanism of allergen-induced increase in

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Abbreviations used in this paper: CS, cigarette smoke; Af, Aspergillus fumigatus extract; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; BALF, BAL fluid; EOS, eosinophil; FA, filtered air; i.t., intratracheal; mACHr, muscarinic acetylcholine receptor; MCh, methacholine; PDE, phosphodiesterase; qPCR, quantitative RT-PCR; Rl, airway resistance; RP, rolipram; SS, secondhand CS.
AHR, the effects of gestational CS exposure on lung inflammation and atopy are unknown. In this communication, we show that fetuses are highly sensitive to CS, and maternal exposure to even SS strongly exacerbates the allergen-induced AHR through up-regulated expression of M1, M2, and M3 muscarinic receptors and the PDE4 isozyme PDE4D5 in the lung. In addition, SS markedly intensifies lung inflammation, Th2 cytokine production, and atopy induced through allergic sensitization. Although the PDE4-selective inhibitor rolipram (RP) decreased muscarinic receptor expression and AHR, it essentially failed to affect the allergen-induced atopy and lung inflammation.

Materials and Methods

Animals

Pathogen-free BALB/c mice were obtained from the Frederick Cancer Research Facility. Animals were housed in shoe box-type plastic cages with hardwood chip bedding and conditioned to whole-body exposure in exposure chambers (H1000; Hazleton Systems) for 2 wk before exposure to SS. The chamber temperature was maintained at 26 ± 2°C, and lights were set to a 12-h on/off cycle. Food and water were provided ad libitum. All animal protocols used in this study were approved by Lovelace Respiratory Research Institute’s Institutional Animal Care and Use Committee.

Abs and reagents

Ab to PDE4 (ab14628) was obtained from Abcam. All the other reagents, unless otherwise stated, were bought from Sigma-Aldrich.

CS generation and exposure

Mice were exposed to whole-body SS (the smoke released from the burning end of a cigarette) or filtered air (FA) for 6 h/day, 7 days/wk, as described (26). Briefly, a smoking machine (type 1030; AMESA Electron- ics) generated two 64 cm³ puffs/min from a research cigarette (type 2Rl; Tobacco Health Research Institute), and the smoke was captured from the lit end of the cigarette with a plastic manifold placed above it. Mice were placed in whole-body chambers and exposed to either FA or SS (total particulate matter: 1.52 ± 0.41 mg/m³). This level of SS exposure simulates the conditions to which fetuses are likely to be exposed through maternal smoking, is slightly lower than the average SS concentration of 2 mg/m³ found in most smoking bars, and is ≈70-fold less than the amount of CS inhaled by an average two-pack/day smoker (26, 28). Adult (3- to 4-mo-old) male and female mice were separately acclimatized either to SS or FA for 2 wk and then paired for mating under the same exposure conditions. After ascertaining pregnancy by vaginal smear, pregnant mice were separated, housed singly in plastic cages, and continued to receive SS or FA until the pups were born. The mothers and pups continued to be exposed to FA or SS until the pups were weaned at 3 wk of age.

Allergen (Af) sensitization and challenge

A lyophilized culture filtrate preparation (29, 30) of Af was used as the allergenic sensitization, as described previously (27). Briefly, mice were sensitized intratracheally (i.t.) with Af (50 μg/0.1 ml sterile endotoxin-free saline or saline alone) and subsequently challenged i.t. with 100 μg/ml each of afrophitin, antipain, and leupeptin at 4°C. Protein content of the extracts was determined by the bicinchoninic acid protein assay kit (Pierce), according to the manufacturer’s directions. The samples were electrophoresed on 10% SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad). Membranes were blocked using 5% nonfat dry milk in TBST for 1 h at room temperature and probed with primary Ab to PDE4 (rabbit polyclonal ab14628; Abcam) overnight at 4°C. The blots were washed three times with TBST, incubated for 1 h at room temperature with HRP-conjugated anti-rabbit serum (Santa Cruz Biotechnology) at room temperature, and developed by ECL (Amersham Biosciences) on a x-ray film.

Bronchoalveolar lavage (BAL) and cell collection

Mice were anesthetized and euthanized by exsanguination 24 h after the last allergen (Af) challenge. Before excision of the lungs, the trachea was surgically exposed and cannulated. The left lung was tied off with a silk thread suture, and the right lung was lavaged with 1 ml of PBS (2×) and pooled for each animal. BAL cells were collected by centrifugation and resuspended in PBS. Approximately 5 × 10⁶ cells from each sample were cytospun on duplicate slides and stained with Diff Quik (Baxter Healthcare) to score eosinophils (EOS), macrophages, neutrophils, and lymphocytes using standard hemocytometric criteria. At least 200 cells from each slide were counted to obtain the differential cell count.

Analysis of cytokines/chemokines in the BAL fluid (BALF)

BALF was analyzed for cytokines and chemokines by the commercially available mouse cytokine Ten-plex ELISA kit (BioSource International; Invitrogen) and used according to the manufacturer’s directions. Cytokine concentrations were determined using Bioplex manager software with four-parameter analysis data. The sensitivity of the assay was <10 pg/ml.

Quantitative RT-PCR (qPCR) analysis

Total RNA was extracted from the lung samples using TRI reagent (Molecular Research Center) and quantified following the manufacturer’s instructions. The lung expression of cytokines (IL-4, IL-5, IL-6, and IL-13), chemokines receptors (SDF-1, M2, and M3), 18S RNA, and GAPDH was determined by qPCR using the One-Step RT-PCR Master Mix and specific labeled primer/probe set (Applied Biosystems). The relative expression of each mRNA was calculated, as previously described (13).

Total serum IgE

Blood from the mice was collected on the day of sacrifice, centrifuged in a serum separator, and stored at −80°C until analysis. Total serum IgE titers were determined by the ELISA method, as previously described (30).

Western blot analysis

Lung tissues were homogenized in radioimmunoprecipitation assay buffer (20 mM Tris, 150 mM NaCl, 0.1% SDS, 20 mM β-glycerol-phosphate, 1% Triton-X, 10 mM NaF, 5 mM EDTA, 1 mM Na3VO4, protease inhibitors, 1 mM PMSF, and 1 mM EGTA) at 4°C. Protein content of the extracts was determined by the bicinchoninic acid protein assay kit (Pierce), according to the manufacturer’s directions. The samples were electrophoresed on 10% SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad). Membranes were blocked using 5% nonfat dry milk in TBST for 1 h at room temperature and probed with primary Ab to PDE4 (rabbit polyclonal ab14628; Abcam) overnight at 4°C. The blots were washed three times with TBST, incubated for 1 h at room temperature with HRP-conjugated anti-rabbit serum (Santa Cruz Biotechnology) at room temperature, and developed by ECL (Amersham Biosciences) on an x-ray film.

Statistical analysis

All data were analyzed using GraphPad Prism software 3.0 using Student’s t test or by two-way ANOVA. Results are presented as the means ± SD of the combined experiments. The differences with p value of ≤0.05 were considered statistically significant.

Results

PDE4D5 expression is up-regulated in the lungs of mice exposed to maternal SS

We have previously shown that the activity of PDE4 is higher in animals exposed in utero to mainstream CS (26). However, the identity of the PDE4 isozyme(s) affected by gestational exposure to CS was not known. Also, it was not clear whether the increased enzymatic activity reflected increased PDE4 protein and whether the increase was independent of allergen sensitization. Although there are nine isoforms of PDE4, including alternative spliced products (31), under these conditions the Western blot analysis of the lung homogenates showed the presence of only three immuno-reactive forms of PDE4 isoforms in the mouse lung that correspond to 105 kDa (PDE4D5), 95 kDa (PDE4D3), and 79 kDa (PDE4A/B) (Fig. 1). Results indicate that prenatal exposure to SS or Af sensitization of control animals alone does not significantly

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alter their content in the lung. However, after Af sensitization, the lungs from SS-exposed animals (SS/Af) exhibit increased PDE4 proteins, particularly the PDE4D5 isoform of the enzyme. Treatment of SS/Af animals with the PDE4-selective inhibitor RP completely blocked the rise in PDE4 content, including that of the PDE4D5 isoform. These data suggest that prenatal exposure to CS per se does not elevate PDE4, but primes the lung to make more PDE4, particularly the PDE4D5 isoform, after an allergic challenge. Moreover, essentially all the increase in PDE4D5 is blocked by RP treatment.

Increased $R_L$ in Af-sensitized mice exposed to maternal CS

Children exposed to tobacco smoke either through maternal smoking or exposure of the mother to SS are at higher risk of developing asthma (32). We have demonstrated that prenatal exposure to mainstream CS increases AHR detected by Baxco plethysmography (26). However, the validity of the Baxco system to measure bronchoconstriction has been strongly questioned (33). Therefore, to determine whether maternal exposure to SS affected AHR, we used the Flexivent system to measure $R_L$ in response to MCh in animals exposed prenatally to SS. Results presented in Fig. 2A (inset) show that maternal exposure to SS slightly, but significantly, increased AHR at the baseline. Af sensitization of FA-exposed animals significantly increased the AHR response; however, compared with animals exposed to FA or FA plus Af, the increase in the $R_L$ in response to MCh of prenatally SS-exposed animals sensitized with Af was extremely dramatic (Fig. 2A). Moreover, as seen by changes in the ED$_{50}$ for MCh, treatment of animals with the PDE4-selective inhibitor RP significantly attenuated the MCh-induced $R_L$ in Af-sensitized SS-treated animals (Fig. 2B). Thus, gestational exposure to SS primes the lung for increased allergen-induced AHR, and the increase is at least partly related to the increased lung PDE4 expression after the allergic sensitization.

Maternal SS exposure increases lung leukocytic infiltration after allergic sensitization

Allergic asthma is associated with elevated numbers of EOS and neutrophils in the lung (34, 35). To determine whether maternal exposure to SS promoted leukocytic infiltration in the lung before and after an allergen challenge, prenatally FA- and SS-exposed animals were challenged through i.t. instillation with Af extracts. BAL cells were collected and stained for differential cell count. The total number of BAL cells in FA- and SS-exposed animals without Af sensitization was similar (6–8 $\times$ 10$^4$); however, after Af challenge, the number of cells in FA/Af rose to 44 $\pm$ 7 $\times$ 10$^4$, and the number was further increased to 96 $\pm$ 14 $\times$ 10$^4$ in SS/Af animals. Moreover, as shown in Fig. 3, before Af sensitization, macrophages represented the predominant cell population in FA- and SS-exposed animals; following Af sensitization, however, the composition of BAL cells changed. The percentage of EOS and polymorphs relative to macrophages increased significantly, and the increase was markedly higher in Af-sensitized animals exposed maternally to SS. Thus, in the 200 cells counted, neutrophils and EOS were over 3-fold higher in SS/Af mice than in the FA/Af mice (35 $\pm$ 12, and 31 $\pm$ 11 vs 9 $\pm$ 5 and 12 $\pm$ 3, respectively). Interestingly, RP did not significantly alter the total number or distribution of leukocyte subtypes in the SS/Af BAL. These results suggest that maternal exposure to SS primes the lung for increased inflammatory responses, and the increase is not significantly related to changes in the lung PDE4 activity.
Maternal exposure to SS increases the propensity of Th2 cytokine production in the lung

To ascertain whether the increased lung inflammation in maternally SS-exposed animals is associated with increased production of proinflammatory cytokines, we determined the expression of various proinflammatory cytokines by ELISA in the BALF and by qPCR of the lung mRNA in animals exposed prenatally to FA or SS before and after sensitization with Af. Compared with FA/Af, levels of the Th2 cytokines (IL-4, IL-5, IL-6, and IL-13) were significantly higher in the BALF from SS/Af animals (Fig. 4A). Similarly, the lung mRNA expression for IL-4, IL-5, IL-6, and IL-13 was significantly higher in the SS/Af than the FA/Af group (Fig. 4B). There was no detectable level of IFN-γ or IL-2 in any BALF sample, and the lung mRNA level of IFN-γ was low and unaltered by any treatment (data not shown). Moreover, RP had no significant effect on the expression of Th2 cytokines. Thus, it is likely that the maternal exposure to SS induces epigenetic changes that primarily increase the propensity of the lung to up-regulate the expression of Th2 cytokines after Af sensitization, and the SS-induced changes in Th2 cytokine expression are not significantly related to the increase in PDE4 activity.

Maternal exposure to SS increases serum IgE levels

Atopy is the hallmark of allergic asthma, and the Th2 cytokines IL-4/IL-13 are critical in the production of IgE (36). Moreover, Af sensitization stimulates IgE production in the murine model of aspergillosis (37). To determine the effect of maternal exposure to SS on IgE, total IgE was assayed in the serum from the FA- and maternal SS-exposed mice before and after Af sensitization. Sensitization with Af significantly increased the serum IgE concentration of FA and SS animals; however, compared with FA-exposed mice, the serum level of IgE was nearly 3-fold higher in the SS-exposed animals after Af sensitization. Moreover, RP treatment failed to significantly alter the serum concentration of IgE (Fig. 5). These results suggest that maternal SS exposure increases serum IgE in response to allergic sensitization, but the increased IgE levels are not linked to changes in the lung PDE4 activity.

Maternal exposure to SS up-regulates the muscarinic acetylcholine receptor (mAChR) expression in the lung

Lungs have an abundant expression of mAChRs. These receptors modulate airway smooth muscle contraction and affect AHR and asthma (38). To investigate whether increased AHR in animals maternally exposed to SS and sensitized with Af was related to the effects of SS exposure on the mAChR expression in the lung, we determined the expression of M1, M2, and M3 mAChRs in the lung by qPCR. These three mAChR subtypes have been detected in murine and human airways (39). It is clear from the results presented in Fig. 6 that compared with Af-sensitized FA animals, the mRNA expression of M1, M2, and M3 receptors is markedly up-regulated in Af-sensitized, SS-exposed animals by ~43-, 13-, and 6-fold, respectively. Moreover, RP treatment completely blocked the increase in expression of these mAChR subtypes, indicating a crucial role for PDE4 in the maternal smoke-induced elevated expression of mAChRs.

Discussion

In utero exposure to parental smoking is associated with an increased risk for development of childhood atopy and asthma (40), indicating that direct maternal smoking as well as exposure of mothers to SS may be significant factors in eliciting childhood asthma. To study the role of maternal SS exposure in childhood allergic asthma, we used the murine model of allergic bronchopulmonary aspergillosis, in which Aspergillus proteins act as allergens and increase the total and Aspergillus-reactive IgE in adult animals (27, 37). Allergic bronchopulmonary aspergillosis is also seen in humans, and IgE from these patients reacts with Aspergillus proteins (29). In the Af-sensitized animals, the allergic response is further aggravated after the administration of rIL-4, indicating a significant role of this cytokine in the allergic response.
In recent years, the pharmaceutical industry has actively followed the development of subtype-specific PDE4 inhibitors for the treatment of asthma, and several compounds such as roflumilast, filamastin (43), and cilomilast are in clinical trials (44). Results with novel PDE4 inhibitors BAY 19-8004 (45) and roflumilast (46) have shown only marginal clinical benefits in asthmatics and chronic obstructive pulmonary disease patients. Similarly, despite the initial optimism, the clinical trials of many other PDE4 inhibitors have been discontinued primarily due to the low therapeutic ratio that severely limits the dose that can be administered to humans (43). Our data clearly show that despite ameliorating AHR and blocking increased expression of PDE4D5, RP has no significant effect on lung inflammatory parameters, including leukocytic infiltration and Th2 cytokine/chemokine expression. These data are similar to those obtained in the PDE4D$^{-/-}$ mice, in which the disruption of the PDE4D gene attenuated AHR without affecting the inflammatory response in the lung (47). However, some PDE4 inhibitors have decreased lung inflammation in the murine OVA model (48). Although it is conceivable that some PDE4 inhibitors may have beneficial effects on lung inflammation (48, 49), at present it is not clear whether the anti-inflammatory properties of these inhibitors are related to PDE4 activity. Indeed, the anti-inflammatory property of theophylline is manifested at drug levels well below the $K_c$ for PDE inhibition and might relate to its histone-modulating activity (50, 51). In general, the problem is the complexity of PDE4, because it includes 4 gene families and 20 splice variants (52), and the expression of these isozymes in multiple cell types (44, 53). Given the critical role of cAMP as the second messenger in numerous biological functions, a global PDE4 inhibitor is likely to have undesirable side effects. The identification of PDE4D5 in these studies as the RP-sensitive PDE4D isoform suggests the possibility of targeting this isoform for therapeutic interventions to reduce AHR. With anti-inflammatory (particularly Th2-specific) drugs, it could be useful in controlling allergic asthma in children. An agent without PDE4D5 inhibition may lack sufficient therapeutic efficacy to control AHR in children exposed to maternal CS.

Although the precise mechanism by which PDE4D5 controls AHR is not clear, cAMP, the substrate for PDE4, is a bronchodilator (55), and decreased levels of cAMP in the lung (26) are likely to increase bronchoconstriction. However, the major effect of SS exposure on AHR is attained only after Af sensitization, which is associated with increased lung expression of muscarinic receptors M1, M2, and M3. Five muscarinic receptor subtypes (M1–M5) have been identified (56); however, the lung mainly expresses the M1, M2, and M3 subtypes (39, 56). Although M1, M2, and M3 receptors participate in bronchoconstriction, M3 accounts for most of the bronchoconstriction in the normal lung (39). Our data show that compared with controls, the expression of all three muscarinic receptors is strongly up-regulated in SS-exposed lungs. Therefore, it is likely that the increased muscarinic receptor expression accounts for the increased sensitivity of these animals to MCh-induced $R_A$, RP attenuates AHR and the increased muscarinic receptor expression in SS-exposed lungs, indicating a causal relationship between the PDE4D5 activity and muscarinic receptor expression in allergic asthma. However, the mechanistic basis of this relationship is not clear at present. Although RP essentially blocked the allergen-induced rise in PDE4D5 expression and muscarinic receptor expression, it did not completely eliminate the allergen-induced AHR. It is possible that other mediators, such as
mast cell-derived serotonin and histamine that cause bronchial hyperresponsiveness through receptors distinct from muscarinic receptors (57), are not affected by PDE inhibitors.

In addition to AHR and inflammation, the clinical signature of allergic asthma is atopy (25), and the serum IgE levels are significantly higher in Af-sensitized SS animals than in FA-exposed animals. However, as with lung inflammation, RP treatment did not decrease the allergen-induced IgE response, indicating that atopy in these animals is essentially independent of PDE4 activity. The inability of RP to control atopy is understandable, because the RP treatment failed to significantly suppress the production of IL-4/IL-13 and IL-6 in SS-exposed, Af-sensitized animals. These cytokines are critical in IgE isotype switching and B cell proliferation/differentiation, respectively (36, 58). Moreover, the development of marked lung eosinophilia persisted in RP-treated, SS-exposed animals, suggesting a lack of effect on IL-5 and eotaxin, the main EOS cytokine/chemokine production. This is likely through epigenetic mechanisms other than gene methylation such as covalent modifications of histone tails, nucleosome occupancy and turnover, and high-order chromatin folding (61) involved in the epigenetic modulation by gestational CS.

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