Phosphodiesterase 4 Inhibitors Attenuate the Asthma Phenotype Produced by β_2 -Adrenoceptor Agonists in Phenylethanolamine N-Methyltransferase–Knockout Mice

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Abstract

Mice lacking the endogenous β_2 -adrenoceptor (β_2 AR) agonist epinephrine (phenylethanolamine N-methyltransferase [PNMT]-knockout mice) are resistant to developing an “asthma-like” phenotype in an ovalbumin sensitization and challenge (Ova S/C) model, and chronic administration of β_2 AR agonists to PNMT-KO mice restores the phenotype. Based on these and other studies showing differential effects of various β_2 AR ligands on the asthma phenotype, we have speculated that the permissive effect of endogenous epinephrine and exogenous β_2 AR agonists on allergic lung inflammation can be explained by qualitative β_2 AR signaling. The β_2 AR can signal through at least two pathways: the canonical $G_{\alpha s}$ -cAMP pathway and a β -arrestin-dependent pathway. Previous studies suggest that β -arrestin-2 is required for allergic lung inflammation. On the other hand, cell-based assays suggest antiinflammatory effects of $G_{\alpha s}$ -cAMP signaling. This study was

designed to test whether the *in vitro* antiinflammatory effects of phosphodiesterase 4 inhibitors, known to increase intracellular cAMP in multiple airway cell types, attenuate the asthma-like phenotype produced by the β_2 AR agonists formoterol and salmeterol *in vivo* in PNMT-KO mice, based on the hypothesis that skewing β_2 AR signaling toward $G_{\alpha s}$ -cAMP pathway is beneficial. Airway inflammatory cells, epithelial mucus production, and airway hyperresponsiveness were quantified. In Ova S/C PNMT-KO mice, formoterol and salmeterol restored the asthma-like phenotype comparable to Ova S/C wild-type mice. However, coadministration of either roflumilast or rolapram attenuated this formoterol- or salmeterol-driven phenotype in Ova S/C PNMT-KO. These findings suggest that amplification of β_2 AR-mediated cAMP by phosphodiesterase 4 inhibitors attenuates the asthma-like phenotype promoted by β -agonists.

Keywords: asthma model; β_2 -adrenoceptor; biased signaling

Asthma is a chronic inflammatory disease of the airways, characterized by inflammatory cell infiltration into the lungs, excess mucus production, and airway hyperresponsiveness (AHR). The mainstays of asthma therapy include inhaled

corticosteroids and β_2 -adrenoceptor (β_2 AR) agonists (1). The β_2 AR agonists used for managing asthma have been modified over the years to enhance their selectivity for the β_2 AR, to reduce off-target effects, and to increase their duration of action to

improve patient adherence. These changes led to the development of preferential and long-acting β_2 AR agonists (2). Despite these changes, the chronic use of β_2 AR agonists is associated with a loss of asthma control, and β_2 AR agonists with long

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Clinical Relevance

Our previous work suggests that β_2 -adrenoceptor (β_2 AR) ligands may contribute to the asthma phenotype via a β -arrestin-dependent mechanism. The current study demonstrates that cAMP-augmenting agents, such as phosphodiesterase 4 inhibitors, help reverse this effect. Thus, our data now implicate opposing effects for two pathways activated by β_2 AR signaling.

duration of action, such as salmeterol, have shown a small, but significant, increase in asthma-related mortality (3).

We have previously shown, using an ovalbumin (Ova) sensitization and challenge (Ova S/C) murine asthma model, that β_2 AR-knockout (KO) mice (β_2 AR-KO mice) and phenylethanolamine N-methyltransferase (PNMT)-KO mice (which lack the enzyme, phenylethanolamine N-methyltransferase, required for the synthesis of epinephrine, the endogenous ligand for the β_2 AR) have an attenuated “asthma-like” response (4, 5). Those reports showed that ligand activation of the β_2 AR was required for developing the asthma phenotype. PNMT-KO mice do not exhibit any overt phenotypes. They breed normally, have no developmental defects, and have normal resting blood pressure and body temperature (6–8).

The β_2 AR can signal through at least two pathways: the canonical $G_{\alpha s}$ -cAMP pathway and the β -arrestin signaling pathway that can activate multiple pathways, including those of the mitogen-activated protein kinase family, such as extracellular signal-regulated kinase (ERK) 1/2 (9–12). Numerous studies have shown that ligands can preferentially activate signaling pathways relative to the activation profile of a reference ligand at that receptor (10, 13). This preferential activation of a signaling pathway of the receptor by a ligand has become known as “biased signaling” (9, 10, 11).

Studies suggest that the β -arrestin-2 (also termed arrestin 3) signaling pathway is an important mediator of the asthma phenotype in murine models. For example, β -arrestin-2 null mice show an attenuated asthma-like phenotype (14), and, as shown by a recent study, where β -arrestin-2 was deleted after the asthma-like phenotype had been established, β -arrestin-2 is essential

for perpetuation of AHR (15). Furthermore, we have recently identified that certain “ β -blockers” (such as carvedilol) that lack agonism toward the $G_{\alpha s}$ signaling pathway, yet *activate* ERK1/2 in cell-based assays, restore the asthma-like phenotype in PNMT-KO mice, and are ineffective at attenuating the phenotype in wild-type (WT) mice (16).

On the other hand, there are few *in vivo* data about the role of preferentially activating, the canonical β_2 AR- $G_{\alpha s}$ -cAMP pathway, in the development or maintenance of the asthma-like phenotype. This gap in knowledge is due, at least in part, to the fact that no orthosteric $G_{\alpha s}$ -biased drugs for the β_2 AR have been identified to date. For the β_2 AR- $G_{\alpha s}$ -cAMP pathway, there are no ligands with the opposite signaling profile of carvedilol, a ligand that preferentially activates ERK1/2 via β -arrestin-2 (12). However, numerous cell-based studies using normal and asthmatic human bronchial epithelial and airway smooth muscle cells have indirectly examined the role of the β_2 AR- $G_{\alpha s}$ -cAMP signaling pathway using phosphodiesterase (PDE) 4 inhibitors to increase β_2 AR-cAMP accumulation. PDE4 is the isozyme responsible for the breakdown of cAMP in numerous inflammatory cells, and in airway epithelial cells (17). The results of these studies suggest that enhancing cAMP accumulation produces an antiinflammatory effect through various mechanisms, including suppression of inflammatory gene expression (18, 19). Data also suggest that asthmatic airway smooth muscle cells have a decreased cAMP response, and this impaired cAMP accumulation is due to up-regulation of PDE4D-mediated cAMP breakdown (20). Indeed, enhancing antiinflammatory responses by increasing β_2 AR- $G_{\alpha s}$ -cAMP accumulation led to the development of GS-5759, a novel bifunctional molecule, where potent β_2 AR agonists have been covalently linked with a PDE4 inhibitor (GSK256066). This dual ligand (GS-5759) has also shown potent antiinflammatory properties in *in vitro* assays (21). Although combining a β_2 AR-agonist with a PDE4 inhibitor is not a classical example of a β_2 AR- $G_{\alpha s}$ -cAMP-biased ligand, it represents a convenient means of skewing signaling qualitatively, similar to what would happen with a β_2 AR-cAMP-biased ligand.

Thus, there are numerous mechanistic studies in human cells suggesting that amplification of the β_2 AR- $G_{\alpha s}$ -cAMP pathway would be protective in asthma. There are also reports using murine models showing that administration of roflumilast resulted in attenuation of asthma-like phenotypes (22, 23). However, there is little *in vivo* evidence to suggest that the attenuation of the asthma phenotype results from biasing signaling toward the β_2 AR- $G_{\alpha s}$ -cAMP pathway. The present studies were performed to assess the *in vivo* role of qualitatively skewing β_2 AR- $G_{\alpha s}$ -cAMP signaling by administering PDE4 inhibitors in conjunction with long-acting β_2 AR agonists compared with administration of the β_2 AR agonist alone. To assure, as much as possible, that the results were due to PDE4 inhibition increasing β_2 AR agonist-induced increases in cAMP, two different PDE4 inhibitors were used (roflumilast and rolipram), as well as two clinically relevant β_2 AR agonists (salmeterol and formoterol). These studies have the added novelty of being the first to examine the interaction of long-acting β_2 AR agonists and PDE4 inhibition *in vivo* in mice lacking the endogenous β_2 AR hormone, epinephrine. This not only eliminated the confounding influence of varying levels of circulating endogenous β_2 AR agonist, but also allowed a more direct comparison with the conditions used in the *in vitro* cell-based studies.

Materials and Methods

Experimental Animals

WT SvJ/129 mice (6- to 8-wk old; Jackson Laboratory, Bar Harbor ME) and mating pairs of PNMT-KO mice, obtained as a gift from Stephen Ebert of University of Central Florida (Orlando, FL), were bred in house (6) and used for the experiments. All animal experiments were conducted in conformity to strict rules and regulations of the Institutional Animal Care and Use Committee of the University of Houston (Houston, TX). Mice were randomly assigned to the various treatment or control groups, and the parameters were measured in blinded fashion.

Antigen S/C Protocol

Briefly, mice were sensitized to 2 mg/kg/day Ova with 2 mg alum intraperitoneally on

Figure 1. Treatment protocol. Phenylethanolamine N-methyltransferase (PNMT)-knockout (KO) and wild-type (WT) mice received ovalbumin (Ova; 2 mg/kg/day in 2 mg of alum) intraperitoneally (i.p.), as indicated, on Days 0, 7, and 14, followed by intranasal (i.n.) challenge with Ova (1 mg/kg/day) or saline on Days 41–45 (arrows). (A) Groups of Ova sensitization and challenge (Ova S/C) PNMT-KO and WT mice received twice-daily formoterol (FOR; 5 μ g/kg, intraperitoneally) or salmeterol (SAL; 1.5 μ g/kg, intraperitoneally). (B) Groups of Ova S/C PNMT-KO and WT mice were administered roflumilast (ROF; 5 mg/kg) or rolipram (ROL; 5 mg/kg) alone via oral gavage, or groups were coadministered ROF (5 mg/kg) or ROL (5 mg/kg) twice daily with either FOR (5 μ g/kg) or SAL (1.5 μ g/kg). Duration of all drug administration was 12 days.

Days 0, 7, and 14, followed by once-daily intranasal challenge with 1 mg/kg/day Ova or saline for 5 consecutive days, as shown in Figures 1A and 1B and as previously described (5). For intranasal challenge, a noninvasive inoculation technique was performed on mice anesthetized with isoflurane.

Administration of β_2 AR Agonists and PDE4 Inhibitors

Ova S/C WT and PNMT-KO mice received twice-daily intraperitoneal injections of either 5 μ g/kg formoterol (saline: DMSO, 99.95/0.05, vol/vol; Sigma-Aldrich, St. Louis, MO) (24–27) or 1.5 μ g/kg salmeterol (saline: methanol, 99.95/0.05, vol/vol; Sigma-Aldrich) (28–30); or an equal volume of the appropriate vehicle via intraperitoneal injection. Ova S/C WT and PNMT-KO mice were coadministered 5 mg/kg roflumilast in 4% methylcellulose solution (24, 31–33) or 5 mg/kg rolipram in 0.1% methylcellulose (34, 35) (Figure 1B), or an equal volume of the appropriate vehicle, via oral gavage. The doses chosen for all drugs were taken on the low end of what has been used in other murine studies. Our rationale for this was that, for the β_2 AR agonists, we

were attempting to “restore” β_2 AR agonist signaling in the PNMT-KO mice, not necessarily produce a drug effect (24–29, 32, 36). Similarly, for the PDE4 inhibitors we did not want the dose to be highly antiinflammatory, but simply a concentration that would skew β_2 AR signaling toward the G α s-cAMP pathway (22, 31–35).

All drugs were administered for 12 days, as shown in Figures 1A and 1B. Control mice received an equal volume of vehicle solution.

Bronchoalveolar Lavage

Mice were killed with 45 mg/kg pentobarbital intraperitoneal (Sigma-Aldrich), and then 400 μ l of cold sterile saline was perfused into the right lung lobe to obtain bronchoalveolar lavage (BAL) fluid. Total and differential cell counts were obtained from the BAL fluid using a hemacytometer (Hausser Scientific, Horsham, PA) and Wright-Giemsa staining (Sigma-Aldrich), as previously described (37).

Mucin Analysis

Formalin-fixed, paraffin-embedded lungs were cut into 5- μ m sections transversely and stained with periodic acid fluorescent Schiff's stain (Sigma-Aldrich).

Morphometric quantification of mucin was based upon a previous report (38). Different investigators, who were blinded to the treatment groups, performed image acquisition and morphometric quantification.

AHR

AHR was measured using the forced oscillation method with the FlexiVent (SCIREQ, Montreal, PQ, Canada). Briefly, mice were anesthetized with ketamine (240 mg/kg) and xylazine (48 mg/kg), and then methacholine was nebulized at doses of 3.125, 6.25, 12.5, 25, and 50 mg/ml. Three peak airway resistance values were averaged at each dose to determine the airway resistance (5).

Statistical Analysis

Data are expressed as mean ($\pm$ SEM) and one-way ANOVA with Tukey's *post hoc* test or two-way ANOVA with Holm-Sidak's *post hoc* test were performed. Statistical significance was set at *P* less than 0.05. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA).

Results

Effect of Drug Administration on Total Inflammatory Cells in the Airways

To determine the inflammatory response of mice to Ova S/C with different drug administration, we examined the infiltration of inflammatory cells into the airways (Figures 2A and 2B). Consistent with previously published results, the total cells in the airway markedly increased in vehicle-treated Ova S/C WT mice, but not in vehicle-treated Ova S/C PNMT-KO mice (Figures 2A and 2B). Compared with vehicle treatment, formoterol administration to Ova S/C PNMT-KO mice caused an increase in total cells to levels comparable to that in Ova S/C WT mice (*P* < 0.05) (Figures 2A and 2B). Salmeterol administration to Ova S/C PNMT-KO mice also increased the total cells in airways relative to control PNMT-KO mice, and this increase was comparable to that after formoterol administration (*P* < 0.05; Figures 2A and 2B). Ova S/C WT mice administered either formoterol or salmeterol did not show a change in total cells in the airways compared with vehicle-treated Ova S/C WT mice (Figures 2A and 2B).

Figure 2. Effect of ROF or ROL administration, with or without FOR or SAL coadministration, on total cell count. Total cells in bronchoalveolar lavage (BAL) fluid of saline challenged (control [CTL]) and vehicle (VEH)-treated Ova S/C PNMT-KO (solid bars) and WT mice (open bars). Groups of Ova S/C PNMT-KO and WT mice were administered different drug combinations. In these groups of mice, either (A) ROF (5 mg/kg) or (B) ROL (5 mg/kg) were administered once daily via oral gavage, or coadministered twice-daily dosages of either FOR (5 μ g/kg, intraperitoneally) or SAL (1.5 μ g/kg, intraperitoneally) for a duration of 12 days. The lungs were lavaged with 400 μ l of sterile saline through a sterile cannula, and BAL fluid was collected 24 hours after the last challenge. Cells were counted using a hemacytometer. Data represent the mean ($\pm$ SEM) from $n = 5-8$ mice in each group. * $P < 0.05$ significance compared with respective PNMT-KO or WT CTL; # $P < 0.05$ significance compared with respective PNMT-KO or WT treatment groups in the absence of ROF or ROL.

Total cell number in BAL fluid of Ova S/C PNMT-KO mice or Ova S/C WT mice administered roflumilast or rolipram was not different from vehicle-treated Ova S/C PNMT-KO mice or vehicle-treated Ova S/C WT mice, respectively (Figures 2A and 2B). The increase in total cell infiltration produced by administration of formoterol or salmeterol to Ova S/C PNMT-KO mice was attenuated when either of the two long-acting β_2 AR agonists (formoterol or salmeterol) was coadministered with either roflumilast or rolipram ($P < 0.05$; Figures 2A and 2B).

Effect of Drug Administration on Eosinophilic Infiltration in the Airways

The Ova murine model of asthma is mainly an eosinophil-driven inflammatory model. Therefore, we quantified the number of eosinophils in BAL fluid using the Wright-Giemsa staining. As shown in previous studies, vehicle-treated Ova S/C PNMT-KO mice, unlike Ova S/C WT mice, did not demonstrate significant eosinophilia in the BAL fluid ($P < 0.05$; Figures 3A and 3B). However, administration of formoterol restored the number of eosinophils in BAL fluid from Ova S/C PNMT-KO mice ($P < 0.05$; Figures 3A and 3B), comparable to those seen in vehicle-treated Ova S/C WT mice. Similar to

formoterol, salmeterol also significantly restored the number of eosinophils in Ova S/C PNMT-KO mice ($P < 0.05$; Figures 3A and 3B). Ova S/C WT mice administered formoterol or salmeterol exhibited no change in eosinophils compared with vehicle-treated Ova S/C WT mice (Figures 3A and 3B).

There was no difference in eosinophil cell counts between Ova S/C PNMT-KO mice administered with roflumilast or rolipram compared with their respective control mice (Figures 3A and 3B). In Ova S/C WT mice, rolipram, but not roflumilast, lowered eosinophil counts in the airways (Figures 3A and 3B). The increased eosinophil cell count in the airways observed in Ova S/C PNMT-KO and Ova S/C WT mice treated with formoterol or salmeterol was attenuated by the coadministration of roflumilast or rolipram ($P < 0.05$; Figures 3A and 3B).

Effect of Drug Administration on Mucus Production in the Airways

As shown in Figures 4A and 4B, vehicle-treated Ova S/C WT mice ($P < 0.05$), but not vehicle-treated Ova S/C PNMT-KO mice, exhibited an increase in mucus production (assessed by morphometric quantification of periodic acid fluorescent Schiff's staining; Figure 4C). In Ova S/C

PNMT-KO mice, either formoterol or salmeterol administration restored mucus production in the airways ($P < 0.05$; Figures 4A and 4B). There was no difference in mucus production between Ova S/C PNMT-KO mice administered roflumilast (Figure 4A) or rolipram (Figure 4B) compared with their respective control mice. The mucus production in Ova S/C WT mice administered either roflumilast (Figure 4A) or rolipram (Figure 4B) was not different from the mucus production in vehicle-treated Ova S/C WT mice (Figure 4D).

The restored mucus production observed with formoterol or salmeterol administration in Ova S/C PNMT-KO mice was attenuated when coadministered with either roflumilast (Figure 4A) ($P < 0.05$) or rolipram ($P < 0.05$) (Figure 4B). Ova S/C WT mice administered formoterol and roflumilast, as well as salmeterol with either roflumilast or rolipram, also displayed similar reduction in mucus production to that observed in similarly treated PNMT-KO mice ($P < 0.05$) (Figures 4A–4D).

Effect of Roflumilast Administration, with or without Formoterol Coadministration, on AHR

As shown in Figures 5A and 5B, 25 and 50 mg/ml of methacholine increased airway

Figure 3. Effect of ROF or ROL administration, with or without FOR or SAL coadministration, on airway eosinophil count. Eosinophils in BAL fluid of saline-challenged (CTL) and VEH-treated Ova S/C PNMT-KO (solid bars) and WT mice (open bars). Groups of Ova S/C PNMT-KO (solid bars) and WT mice (open bars) were administered with different drug combinations as indicated. In these groups of mice, either (A) ROF (5 mg/kg) or (B) ROL (5 mg/kg) were administered once daily via oral gavage or coadministered with twice-daily FOR (5 μ g/kg, intraperitoneally) or SAL (1.5 μ g/kg, intraperitoneally) for 12 days. Differential counting of cells was done with Wright-Geimsa staining. Data represent the mean ($\pm$ SEM) from $n = 5-7$ mice in each group. * $P < 0.05$ significance compared with respective PNMT-KO or WT CTL; # $P < 0.05$ significance compared with respective PNMT-KO or WT treatment groups in the absence of ROF or ROL.

resistance in vehicle-treated Ova S/C WT, but not in vehicle-treated Ova S/C PNMT-KO mice ($P < 0.05$). In Ova S/C PNMT-KO mice, formoterol administration restored airway resistance ($P < 0.05$; Figure 5A). There was no difference in airway resistance between Ova S/C PNMT-KO mice administered roflumilast compared with control mice (Figure 5A). Airway resistance in Ova S/C WT mice administered roflumilast was not different from vehicle-treated Ova S/C WT mice (Figure 5B). The increase in airway resistance observed with formoterol administration in Ova S/C PNMT-KO mice was attenuated when coadministered with roflumilast ($P < 0.05$; Figure 5A).

Discussion

We have previously shown that β_2 AR signaling is required for development of the asthma-like phenotype in murine models, because either (1) chronic treatment with certain β_2 AR inverse agonists (such as nadolol and ICI 118,551), or (2) genetic deletion of the β_2 AR, attenuated the asthma phenotype (4, 37). We also demonstrated, through both pharmacologic and genetic approaches, that ligand activation of the β_2 AR was required for development of the asthma phenotype (5). Furthermore, we have used ligands that modify the

asthma-phenotype and shown that their effect is not observed in β_2 AR-KO mice. Finally, we have also shown that nadolol blocks the effect of alprenolol in maintaining the asthma-like phenotype, suggesting that the ligands are competing for the β_2 AR (4). Thus, numerous lines of evidence indicate that ligand binding to the β_2 AR is responsible for promoting the development of the asthma phenotype.

However, the β_2 AR can signal through multiple effector pathways downstream of the receptor after agonist binding (10). There are two signaling pathways that have been well characterized for the β_2 AR. One is the canonical $G_{\alpha s}$ -cAMP pathway, which, by increasing cAMP, leads to PKA activation. The other is activation of mitogen-activated protein kinases (usually ERK1/2), which are most often activated in a β arrestin-dependent manner (9, 10, 12, 39).

There are numerous *in vitro* and *in vivo* studies implicating the β arrestin-ERK1/2 pathway as proinflammatory (reviewed in Ref. 40). *In vivo* studies have reported that β arrestin-2 null mice have shown an attenuated asthma phenotype, and we have recently shown that administration of β_2 AR ligands capable of activating ERK1/2 *in vitro* (10, 12) restore the asthma-like phenotype in PNMT-KO mice, and do not attenuate the asthma-like phenotype in

WT mice (16). There are even small clinical studies in subjects with asthma showing administration of nadolol, a ligand that inhibits β_2 AR-mediated activation of the ERK1/2 pathway (12), causes a dose-dependent reduction in AHR (reflected as an increase in the provocative concentration of methacholine causing a 20% drop in FEV₁) in subjects with mild asthma (41, 42). Conversely, administration of propranolol, a ligand that activates the ERK1/2 pathway via the β_2 AR (12), had no effect in a different subset of subjects with asthma concurrently using inhaled corticosteroids (43).

Whereas the existence of well established β arrestin-biased β_2 AR ligands has enabled a clear picture of the proinflammatory effect of skewing β_2 AR signaling toward β arrestin signaling, similar studies assessing the effects of skewing β_2 AR signaling toward the β_2 AR- $G_{\alpha s}$ -cAMP signaling pathway are lacking. An ideal strategy for such studies would be to use a β_2 AR ligand that preferentially activated the $G_{\alpha s}$ -cAMP pathway, while inhibiting the β arrestin pathway (i.e., a β_2 AR- $G_{\alpha s}$ -cAMP-biased ligand). However, no such compound has yet been identified. In addition, although it would seem tempting to have studied the $G_{\alpha s}$ signaling pathway in the β arrestin-2 null mice, thereby isolating the pathway, it

Figure 4. Effect of ROF or ROL administration, with or without FOR or SAL coadministration, on mucus production. (A and B) Morphometric quantification of the mucin volume density, and (C and D) representative microscopic images of mucin (red) content in the airway epithelia (green) assessed from saline-challenged (CTL) and VEH-treated Ova S/C PNMT-KO and WT mice. Groups of Ova S/C PNMT-KO (solid bars) and WT mice (open bars) were administered different drug combinations, as indicated. In these groups of mice, either (A) ROF (5 mg/kg) or (B) ROL (5 mg/kg) were administered alone via oral gavage or coadministered with twice-daily dosages of either FOR (5 μ g/kg, intraperitoneally) or SAL (1.5 μ g/kg, intraperitoneally) for 12 days. Data represent the mean ($\pm$ SEM) from $n = 5-8$ in each group (PNMT-KO) and from $n = 5-13$ in each group (WT). * $P < 0.05$ significance as compared with respective CTL; # $P < 0.05$ significance as compared with respective treatment groups in the absence of ROF or ROL. (C) Representative images of mucin content by either FOR or SAL administration. (D) Representative images of mucin content effect after phosphodiesterase 4 inhibition by either ROF or ROL administration. Scale bars: 50 μ m.

Figure 5. Effect of ROF administration, with or without FOR coadministration, on airway resistance. (A and B) Airway resistance responses to increasing doses of nebulized methacholine (0–50 mg/ml) were measured using forced oscillation technique in Ova S/C PNMT-KO (A) and WT (B) mice administered different drug combinations. Airway resistance was determined by averaging the three highest resistance responses recorded for each mouse at each methacholine dose. In these groups of mice, ROF (5 mg/kg) was administered once daily via oral gavage or coadministered twice daily with FOR (5 μ g/kg, intraperitoneally) for 12 days. Data represent the mean ($\pm$ SEM) from $n = 4$ –9 mice in each group. * $P < 0.05$ significance compared with respective PNMT-KO or WT CTL; # $P < 0.05$ significance compared with respective PNMT-KO or WT treatment groups in the absence of ROF. AHR, airway hyperresponsiveness.

must be recalled that such mice already have a greatly attenuated asthma phenotype, and thus allow little room to measure any further improvement or attenuation of the asthma phenotype by an alternate pathway (14).

In the absence of a G α s-biased β_2 AR ligand, we chose the strategy used in cell-based assays to increase cAMP levels downstream of the β_2 AR using PDE4 inhibitors. PDEs, including PDE4, break down cAMP and cGMP and play a key role in inflammatory and immune responses (44). PDE4s are the major isozymes highly expressed in immune cells, airway epithelial cells, and smooth muscle cells (17). The antiinflammatory effects of combining a β_2 AR with a PDE4 inhibitor were promising enough that a new chemical entity (GS-5759) for possible use in obstructive airway diseases was created by Gilead Sciences by covalently binding the β_2 AR agonists, indacaterol and carmoterol, to a potent PDE4 inhibitor, GSK256066 (21, 45). In our studies, to more rigorously test whether the results that we observed were due to the “amplified” β_2 AR–cAMP resulting from coadministration of a PDE4 inhibitor, we tested four different combinations of a PDE4 inhibitor and a β_2 AR agonist (rolipram or roflumilast in combination

with either formoterol or salmeterol), and compared the results to administration of the β_2 AR agonist alone (Figures 1A and 1B).

We observed that, similar to our previous results assessing formoterol (5), another long-acting β_2 AR agonist, salmeterol, also restored inflammatory cell infiltration (Figures 2 and 3) and mucus production (Figure 4) in the airways of Ova S/C PNMT-KO mice. However, all concurrent administrations of either PDE4 inhibitor, roflumilast or rolipram, with either β_2 AR agonist, formoterol or salmeterol, to Ova S/C PNMT-KO mice attenuated the increases in inflammatory cell infiltration (Figures 2 and 3) and mucus production (Figure 4) that occurred with treatment with the β_2 AR agonists alone.

Ova S/C WT mice treated with roflumilast or rolipram alone did not show any significant reduction in inflammatory cell infiltration (Figures 2 and 3) or mucus production (Figure 4) in the airways. In addition, cotreatment with either formoterol and roflumilast or salmeterol and rolipram did not significantly reduce total inflammatory cells in the airways of Ova S/C WT mice (Figures 2A and 2B). The reason for this discrepancy of a lack of significant effect of roflumilast and rolipram when administered in the

presence of endogenous epinephrine rather than formoterol or salmeterol is unclear. However, the β_2 AR agonists, salmeterol and formoterol, have been shown to be β arrestin–ERK1/2 activation biased relative to epinephrine (13). It is possible that the “balancing” of signaling produced by amplification of the G α s–cAMP pathway of β_2 AR signaling may have a more protective effect in the presence of a β arrestin–ERK1/2–biased ligand than in the presence of epinephrine, which, by definition, is the reference-neutral (unbiased) ligand. Furthermore, one of the major advantages of our studies is the ability to study the β_2 AR agonist/PDE4 inhibition in the absence of the confounding influence of the circulating β_2 AR agonist, epinephrine. Given the natural variability and circadian fluctuations of the endogenous β_2 AR hormone, it is perhaps not surprising that differences between PNMT-KO and WT mice treated with PDE4 inhibitors would occur. Especially noteworthy is that we only observed quantitative, not qualitative, differences (Figures 2–4).

In all of our previous studies, the effect of drug therapies on inflammatory cell infiltration and mucus production has paralleled the effect on AHR and lung function parameters. In the present

study, we also observed that concurrent administration of roflumilast and formoterol to Ova S/C PNMT-KO mice significantly reduced airway resistance compared with administration of formoterol alone (Figure 5A).

In summary, we have demonstrated that amplification of specific β_2 AR-G α s-cAMP

signaling by two distinct PDE4 inhibitors attenuates cardinal features of the asthma-like phenotype when PNMT-KO mice were chronically treated with formoterol or salmeterol. Although further studies are needed to fully comprehend the role of the various β_2 AR signaling pathways, taken together with the results implicating

the β arrestin-2 pathway as proinflammatory (14, 16, 46), our results suggest that the ideal β_2 AR drug for asthma management may be a G α s-biased ligand. ■

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

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