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Original Article

Overexpression of Rho/Rho kinase and M_3 cholinergic receptor is involved in formoterol-induced impairment of β_2-adrenoceptor bronchoprotection

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Abstract: Background: The mechanism of β_2-adrenoceptor hyposensitivity has not yet been thoroughly elucidated. The classic theory is that β_2-adrenoceptor desensitization is caused by a decline in the number of β_2-adrenoceptors. However, certain other experiments have shown that the number of β_2-adrenoceptors exhibits a positive relationship with airway responsiveness and that cross-talk between the G_s and G_q signaling pathways exists through PLC-β_1. Recently, the Rho/Rho kinase signaling pathway was suggested to be involved in β_2-adrenoceptor dysfunction. Objectives: We aimed to establish a mouse model that demonstrates formoterol-induced β_2-adrenoceptor hyposensitivity and to determine how β_2-agonists induce the loss of bronchoprotection. Methods: We combined chronic allergen exposure with the repeated administration of formoterol, formoterol plus budesonide, formoterol plus fasudil, or saline in allergen-treated Balb/c mice. The contribution of β_2-adrenoceptors to terbutaline-induced bronchoprotection was measured at 5 different time points. The associated changes in protein and gene expression (RhoA, Rho kinase1, M_3R, β_2R, and PLC-β_1) as well as in RhoA activation were also measured. Results: Chronic treatment with formoterol resulted in worsened airway inflammation, impaired bronchoprotection, and increased airway hyperresponsiveness. RhoA, Rho kinase 1 and M_3 receptor were overexpressed, whereas β_2-adrenoceptor and PLC-β_1 exhibited little change in the formoterol-treated groups. The addition of fasudil alone partially restored the bronchoprotection. Conclusions: This experiment reproduced the salient features of human asthma, including the loss of bronchoprotection, and mimicked the drug formulation and delivery route in humans. Ca^{2+} sensitization and M_3 receptor overexpression played key roles in formoterol-induced β_2-adrenoceptor hyposensitivity. Inhibiting Ca^{2+} sensitization partially reversed the impaired bronchoprotection.

Keywords: Asthma, bronchoprotection, Ca^{2+} sensitization, formoterol, Rho/Rho kinase

Introduction

β_2-Agonists are the first-line and most extensively prescribed therapeutic drugs for the treatment of asthma in clinical practice and are powerful bronchodilators; these drugs provide relief from smooth muscle (SM) bronchospasms and hence attenuate airway obstruction. In addition to causing bronchodilation, β_2-agonists mediate bronchoprotection, which is defined as the inhibition of induced bronchoconstriction [1]. Although β_2-agonists are effective at improving lung function, chronic use of these agonists diminishes their therapeutic efficiency and even induces serious adverse effects [2], including tolerance, impaired bronchoprotection, increased airway hyperresponsiveness (AHR) to allergens, increased severe asthma exacerbation events, and even death [3-7]. Although the mechanisms that underlie β_2-adrenoceptor dysfunction have not yet been thoroughly elucidated, they have been implicated in promoting asthma pathogenesis and worsening asthma control.

The classic theory is that β_2-adrenoceptor desensitization which was defined as declining numbers of β-adrenoceptors. In contrast, cer-

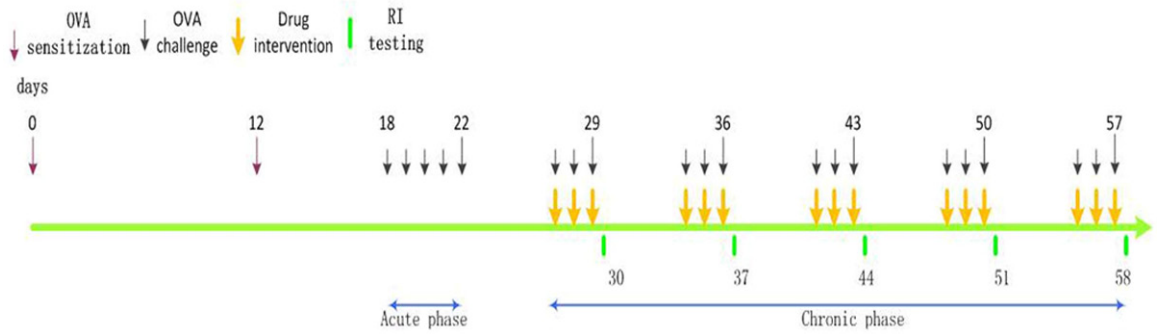

Figure 1. Schematic of the study protocol for the induction of a prolonged allergen challenge and for the administration of therapeutic drugs. Testing was performed on the days on which the animals in each group were sacrificed.

tain experiments have shown that the number of β-adrenoceptors exhibits a positive relationship with airway responsiveness and that cross-talk between the Gs and Gq signaling pathways may exist through phospholipase C-β_1 (PLC-β_1) [8]. Moreover, Nguyen et al. confirmed that β_2-adrenoceptor signaling is required for the development of an asthma phenotype, including the full development of AHR, mucous metaplasia, and airway inflammation, in a murine model [9].

Recently, the Rho/Rho kinase signaling pathway has been studied in more detail in terms of Ca^{2+} sensitization, which is defined as the lack of a change in the intracellular Ca^{2+} concentration when SM cell (SMC) contraction increases. This signaling pathway plays a significant role in almost all pathophysiological and pathological changes in asthma-AHR, airway remodeling, inflammatory cell migration, and mucus hypersecretion [10]. The obvious question is whether Rho/Rho kinase contributes to β_2-adrenoceptor dysfunction. In fact, certain ex vivo experiments suggest that Ca^{2+} sensitization may be involved in isoprenaline-induced impaired bronchoprotection [11].

Currently, most studies concerning the relationship between β_2-adrenoceptor and impaired bronchoprotection have focused on Gs-coupled β_2-receptor. However, the conclusions have been entirely different [12]. Most of our knowledge about GPCR regulation is derived from analyses employing overexpressed or knocked-out receptors in artificial systems, and few experiments have been performed in settings that mimic the natural disease process. Lin et al. showed that the β_2-adrenoceptor subtype is the major mediator of albuterol-induced bronchorelaxation in β_2-adrenoceptor-KO mice [13]. Their work demonstrated that these mice are a suitable model for studying β_2-adrenoceptor-mediated bronchoprotection.

We aimed to establish a mouse model that exhibits formoterol (FORM)-induced β_2-adrenoceptor dysfunction as well as to determine how β_2-agonists induce the loss of β_2-protection, especially whether Ca^{2+} sensitization was involved in.

Materials and methods

All the mouse care and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Sichuan University. In total, 100 specific-pathogen-free, 8-week-old female Balb/c mice weighing 20-25 g were purchased from the Experiment Animal Center at the Sichuan Academy of Medical Sciences. All the mice were randomly divided into five groups: control (PBS challenge), asthma (OVA challenge), FORM, FORM + fasudil (FAS), or FORM + budesonide (BUD). The mice were housed in a pathogen-free barrier facility.

Drug formulations Formoterol fumarate dihydrate (FORM) was purchased from Sigma-Aldrich, USA. The BUD/FORM compound formulation was prepared by adding BUD to the FORM suspension at concentrations of 200 mg/ml and 5.72 mg/ml, respectively. All the formulations were adjusted to pH 5.0 and were stored in the dark. The intratracheal instillation volumes were 1 ml/kg body weight for all the mice [14, 15].

Mice chronically treated with ovalbumin (OVA) and drugs

Mice were sensitized on days 0 and 12 via an i.p. injection of 20 mg of OVA (Sigma-Aldrich,

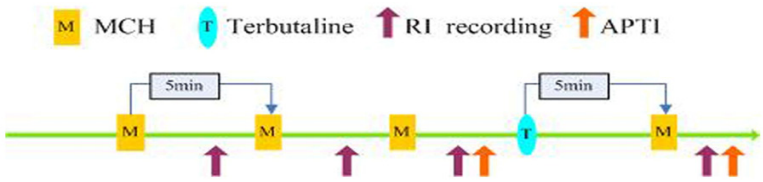

Figure 2. Schematic of the study protocol for detecting airway impedance and for administering drugs.

USA) and 2 mg of aluminum adjuvant diluted in saline as previously described [16]. Beginning on day 18 and continuing until day 56, the mice were challenged for 60 min per day for five days in the first week and three days a week thereafter, with four rest days in between the challenges with 1% OVA aerosol produced using an atomizer (PARI, Germany). During the chronic phase, 2 h after the OVA challenge, mice were modestly anaesthetized with isoflurane, and then 20 μl of FORM (0.29 μg/kg) or vehicle (0.9% saline) was dropped into the trachea after maximal oropharyngeal exposure using a mouse-specific laryngoscope. Animals were tested 24 h after the last challenge day of each week. FAS was administered via drinking water (100 mg/kg/day) as shown in **Figure 1** [17]. The control group was challenged with PBS and treated with vehicle, whereas the asthma group was challenged with OVA and treated with vehicle.

Evaluation of airway responsiveness: airway resistance and airway pressure time index (APTI)

Airway responsiveness was measured 24 h after the last challenge day of each week, and mice were anaesthetized and paralyzed with an i.p. injection of 80 mg/kg sodium pentobarbital and 0.25 mg/kg pancuronium bromide, respectively. Once the mice were anaesthetized, a tracheal cannula and a specially made caudal vein catheter were inserted and fixed with sutures. The mice were ventilated (Buxco, USA) at a constant volume of 7 ml/kg and a frequency of 160 breaths/min. We established that the administered dose of MCH was maximal (400 μg/kg) but not lethal for the mice and confirmed that the repeated MCH challenges resulted in consistent and reproducible increases in resistance. Mice were ventilated until the RI remained stable for 2 minutes before each drug injection, which occurred after a 5-minute washing interval. In brief, 400 μg/kg MCH in a 35 μl volume for each mouse was first injected, followed by injection of 30 μg/kg terbutaline in a 35 μl volume for each mouse and MCH (**Figure 2**). For APTI, peak tracheal pressure was continuously acquired from a tracheal cannula side port. APTI was calculated as the sum of the post MCH-induced changes in peak tracheal pressure (relative to pre-MCh peak tracheal pressure) integrated with respect to time (30 s) [13]. After finishing the experiment, the mice were executed by cervical dislocation.

β_2-adrenoceptor-mediated bronchoprotection protocol

The APTI response to combined i.v. MCh and terbutaline was then measured. Bronchoprotection was calculated as % change in MCh-induced response by the following equation: [[(APTI response to MCh)-(average APTI response to MCh + terbutaline)]/average APTI response to MCh × 100] [13, 18].

Hematoxylin and eosin (H&E) staining

Lung sections were stained with H&E to determine whether the asthmatic model was successfully established by assessing two points-perivascular and peribronchiolar inflammation. Airway inflammation was semi-quantitatively evaluated using a six-tiered scoring system of inflammation severity. Briefly, the inflammation score was determined based on the depth of peribronchiolar or perivascular inflammation and was further elevated if eosinophils were predominant [19].

Immunohistochemistry

Immunohistochemistry of RhoA were processed in the same manner as described before [20]. Image-Pro Plus software was utilized to semi-quantitatively evaluate RhoA. RhoA positive cells in bronchi were counted based on the ratio of the integrated optical density (IOD) to the detection area.

Protein samples of bronchial tissues

The airway tissues below the main bronchi to lungs were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution.

Table 1. Specific PCR primer sequences

Gene	Accession number	Primer sequence 5'-3'
RhoA-F	NM 016802	CTCGGAGTCCTCGCCTTGA
RhoA-R		CTCTGGGAACTGGTCCTTGCT
ROCK1-F	NM 009071.2	GCACCATCCTCAACTCTAC
ROCK1-R		TCACGGTCATCCATACTCT
Chrm3-F	NM 033269	GGTCATAGCACCATCCTCAACTC
Chrm3-R		GTCACGGTCATCCATACTCTTCTG
Adrb2-F	NM 007420	CAACGGCAGAACGGACTACAC
Adrb2-R		TTGGGAGTCAACGCTAAGGCT
Plcb1-F	NM 001145830	GAGCCACCGATCCACACAGA
Plcb1-R		GAGCCACCGATCCACACAGA
Actin-F	NM 007393	CCGTAAAGACCTCTATGCCAACAC
Actin-R		GAGCCACCGATCCACACAGA
GAPDH-F	NM 008084	CATGGCCTTCCGTGTTCCTA
GAPDH-R		TGCTTCACCACCTTCTTGATG

The bronchial tissue (containing the main and intrapulmonary bronchi) segments were quickly frozen with liquid nitrogen, and the tissue was crushed to powder with a mortar. The followed procedure was as described before [21, 22].

Real-time quantitative PCR

Total RNA extraction, cDNA reverse transcription and real-time PCR were performed according to standard protocols [23]. Specific primers for murine RhoA, Rho kinase1, β_2, M_3, PLC-β_1, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Premier 5 and synthesized by DNA Technology (Shengong, China). The primer sequences are shown in **Table 1**. Two housekeeping genes, β-actin and GAPDH, were simultaneously evaluated. The relative amount of mRNA was presented as the CT value of the target gene mRNA normalized to that of the housekeeping gene in the same sample.

Rho activation assay and western blot analysis

We used the Rho-binding domain (RBD), which binds specifically to the active GTP-bound form of Rho, to measure RhoA-GTP according to the manufacturer's manual (Cytoskeleton, USA). The RhoA-GTP bands were visualized by chemiluminescence [13, 20]. Quantity One software was utilized to semi-quantitatively evaluate the relative density of each lane.

β_2, M_3, Rho kinase 1, and PLC-β_1 expression (western blot)

For the western blot analyses, samples were lysed in RIPA supplemented with PMSF and separated by 10% SDS-PAGE. Western blotting was performed with rabbit anti-mouse primary antibody (1:1000; Abcam, USA) and horseradish peroxidase-conjugated horse anti-rabbit secondary antibody (1:6000; CST, USA) using chemiluminescence detection.

Statistics

The data are presented as the mean ± SEM. The statistical analyses were performed using SPSS 10. Significant differences among groups were identified by ANOVA. Differences with *P*-values less than 0.05 were considered statistically significant.

Results

Airway responsiveness

As shown in **Figure 3**, all groups exhibited a wave-like trend in the APTI values: declined in the first two weeks and then increased. Chronic FORM treatment significantly increased the APTI in response to MCH compared to the other groups in the last week ($P < 0.05$), while significant differences were detected between the asthma group and control group ($P < 0.05$) in the last week. The APTI was moderately lower in the FORM + FAS group than in the FORM group ($P > 0.05$) but was not significantly different compared with the other groups ($P > 0.05$). The APTI in the asthma group was significantly higher than the control group.

Bronchoprotection

The percent change in bronchoprotection followed a wavy pattern: it increased in the first three weeks and then declined in the last two weeks (**Figure 4**). In the last two weeks, the FORM group had the lowest percent change in bronchoprotection, whereas the control group had the greatest level of bronchoprotection ($P < 0.05$). These data implied that FORM exerted maximal bronchoprotection in the sixth week; thereafter, the effect on bronchoprotection

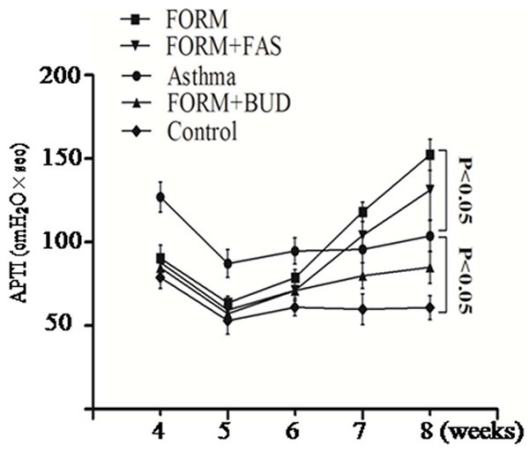

Figure 3. APTI induced by MCH as measured over five consecutive weeks. FORM-treated animals showed a wave-like trend in APTI: it declined in the first two weeks and increased thereafter relative to the control group. The other groups, with the exception of the control group, also experienced a modest increasing trend over five consecutive weeks. In the last weeks, a significant difference in the APTI was observed in the FORM group compared to the other groups ($P < 0.05$), and the APTI in the asthma group was higher than that in the control group ($P < 0.05$). The data from 4 mice per group are presented as the mean ± SE.

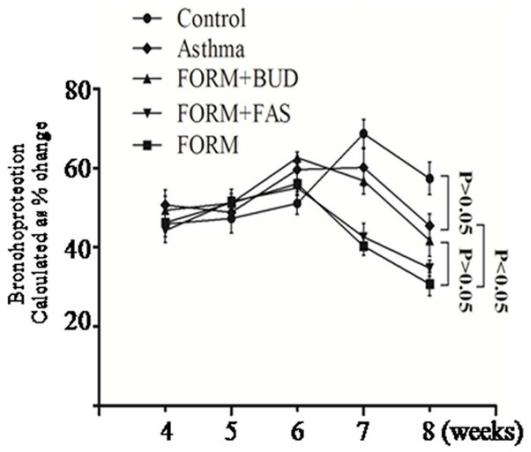

Figure 4. Bronchoprotection was calculated as the percent change in the MCH-induced response. In the first three testing weeks, no statistically significant differences were detected among the groups. In the last two weeks, the control group had the highest bronchoprotection, while the FORM-treated group had the lowest. The addition of FAS or BUD could not significantly reverse the impaired bronchoprotection compared to the FORM group ($P > 0.05$).

gradually decreased. The addition of FAS partially reversed the impaired bronchoprotection, which is weaker than adding BUD ($P > 0.05$). Taken together, these results demonstrate that our mouse model successfully reproduced the association between chronic long acting beta agonist (LABA) use and β-adrenoceptor dysfunction or tolerance that has been observed in certain asthmatics.

H&E staining

The asthma group demonstrated peribronchiolar and perivascular inflammation accompanied by epithelial shedding and epithelial layer damage, whereas no such changes were observed in the control group. Although treating the chronic OVA-challenged mice with FORM increased the peribronchiolar and perivascular inflammation, no significant difference was observed compared to the asthma group ($P > 0.05$). Chronic treatment with FORM/BUD significantly inhibited the inflammatory response ($P < 0.05$), whereas the addition of FAS inhibited this response to some extent ($P > 0.05$) compared to the FORM group (**Figure 5**).

Immunohistochemistry

The positive immunohistochemical staining of the lungs with the RhoA antibody was intense in the FORM group and moderate in the FORM + BUD group; the addition of FAS only partially decreased the RhoA staining. The asthma and control groups showed no significant differences in RhoA staining (**Figure 6**).

Real-time quantitative PCR

The concentration of RhoA mRNA relative to β-actin mRNA exhibited a wave-like trend in consecutive weeks in control and asthma groups, while it exhibited gradually increasing trends in the other three containing FORM groups in the last three weeks. RhoA mRNA had the highest level in the FORM group, while it was lower in the FORM + FAS group in the last week ($P > 0.05$) (**Figure 7A**). Rho kinse 1 mRNA was significantly inhibited by FAS compared to the other groups ($P < 0.05$). M_3R mRNA was overexpressed in FORM-group compared to the other groups, except for the FORM + FAS group ($P < 0.05$) (**Figure 7B**), whereas β_2R and PLC-β_1 mRNA expression showed little change in the FORM-treated groups compared to the other groups (data not shown).

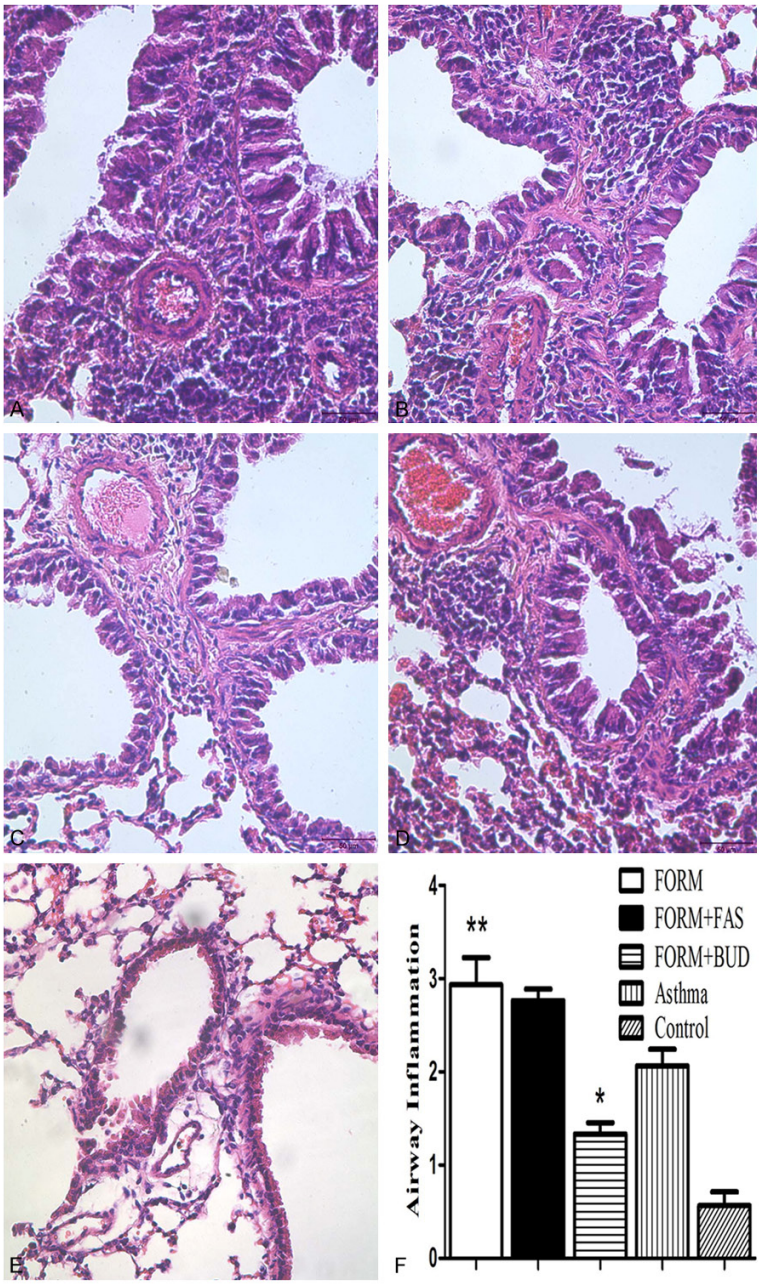

Figure 5. H&E staining. A-F: FORM, FORM + FAS, FORM + BUD, asthma, and control groups, respectively. Semiquantitative H&E staining in each group; magnification, × 400. ※※P>0.05 compared with the asthma group. ※P<0.05 compared with the control group.

RhoA activation assay

Consistent with RhoA mRNA expression, the concentration of activated RhoA (GTP-RhoA) in the FORM group gradually increased over consecutive weeks and was the highest during the last week compared with the other weeks ($P < 0.05$) (**Figure 8A**). Compared to the other groups, GTP-RhoA levels were highest in the FORM group and lowest in the control group (**Figure 8B**). The addition of BUD significantly inhibited RhoA activation; Adding FAS inhibited RhoA activation to some extent, but the inhibition was much weaker than that observed with BUD ($P > 0.05$).

Rho kinase 1, β_2R, M_3R, and PLC-β_1 expression

Rho kinase 1 was gradually increased first and then declined in consecutive weeks in FAS-group. Compared to the other groups, Rho kinase 1 level was the lowest in FAS-group, which was the highest in FORM-group ($P < 0.05$) (**Figure 9**). M_3R was overexpressed, whereas β_2R and PLC-β_1 exhibited little difference in expression in the FORM group compared to the other groups (**Figure 10**). The addition of BUD decreased M_3R expression, whereas the addition of FAS had no effect on this parameter.

Discussion

LABAs are traditionally prescribed as add-on therapies to inhaled corticosteroids to control moderate and severe asthma, such as Symbicort Turbuhaler. However, when used regularly, the effectiveness of LABAs declines, and these therapies may gradually enhance disease progression. This study is the first to provide detailed descriptions of the effects of chronic FORM administration on chronic allergen-challenged asthmatic mice and of the dynamic changes in bronchoprotection and AHR over 8 consecutive weeks. These findings are crucial because they confirm that chronic administration of FORM can exacerbate airway inflammation, responsiveness and impair bron-

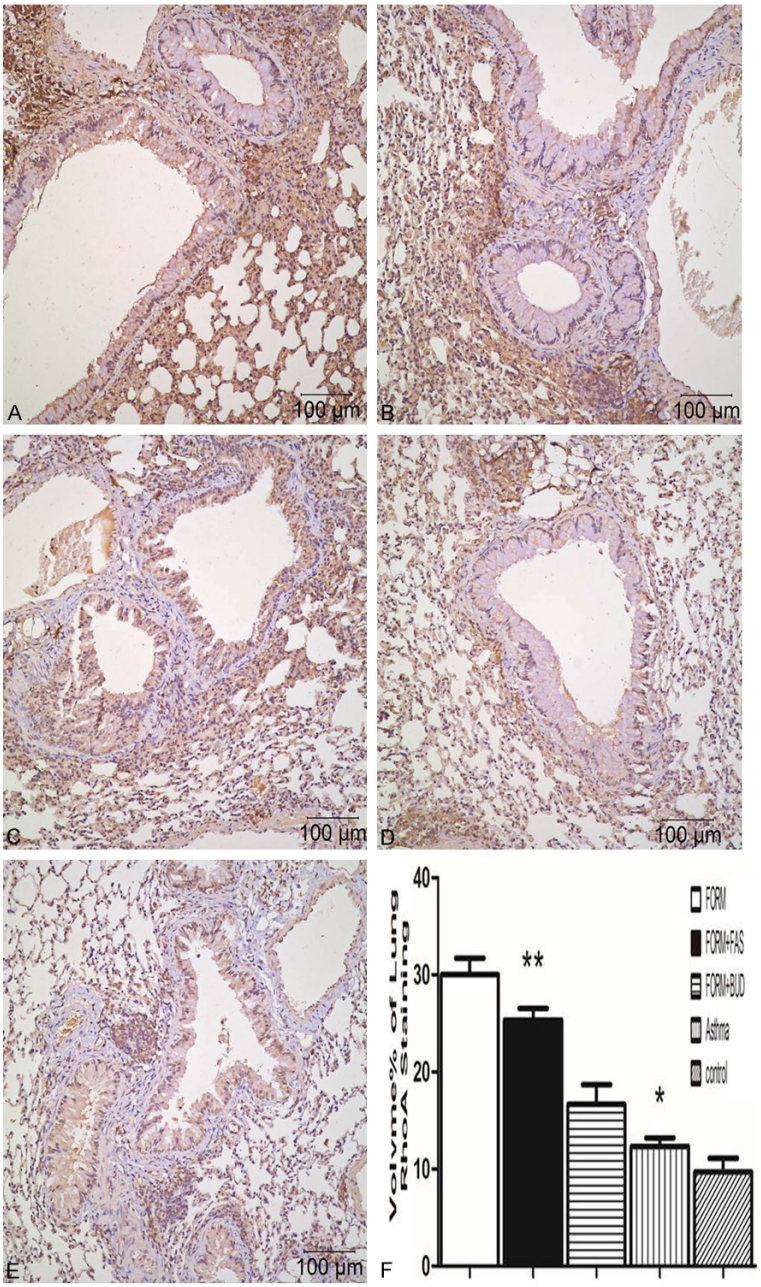

Figure 6. Immunohistochemical staining of murine lungs using anti-RhoA antibody. A-E: FORM, FORM + FAS, FORM + BUD, asthma, and control groups, respectively. Magnification, × 200. ※※P < 0.05 compared with the control group. ※P > 0.05 compared with the control group.

choprotection. The airway inflammatory score did not significantly differ between the FORM group and asthma group, but the bronchoprotection dramatically differed, which suggested that the impaired bronchoprotection had no direct relationship with airway inflammation. Thus far, only a few animal studies have evaluated the effects of chronic β-agonist treatment on bronchoprotection; however, only indirect effects on bronchoprotection or albuterol were evaluated in these studies [13, 24].

Rho/Rho kinase is widely expressed in many types of cells, e.g., eosinophils, T-lymphocytes, macrophages, Schwan cells, epithelial cells, endothelial cells and SMCs as shown in **Figure 6** [25-29]. Rho/Rho kinase causes inflammatory cell migration and sustained SMC contraction by inhibiting myosin phosphatase target subunit 1 (MYPT1) [30]. After a thorough literature review, we found that Rho/Rho kinase exerts positive feedback only with other kinase/signal transducers and activators of transcription, receptor tyrosine kinase signaling pathways and reactive oxygen species, which seem to comprise certain signaling loops. Through these signaling loops, inflammatory mediators (leukotriene) and other cytokines (interleukin) are maintained at high levels, thereby inducing pathological and pathophysiological changes that contribute to the pathogenesis of asthma [10]. Rho has isoforms of A-E and G, however, most of the functions of Rho are described, based on the studies of RhoA. Rho kinase has two isoforms Rho kinase 1 and Rho kinase 2, while Rho kinase 1 is involved in destabilizing the actin cytoskeleton through regulating MLC phosphorylation and peripheral actomyosin contraction [10]. Therefore we choose RhoA and ROCK1 to represent this signal pathway.

In the current study, GTP-RhoA levels did not significantly differ in the asthma and control groups, indicating that Ca^{2+} sensitization worked under physiological conditions to ma-

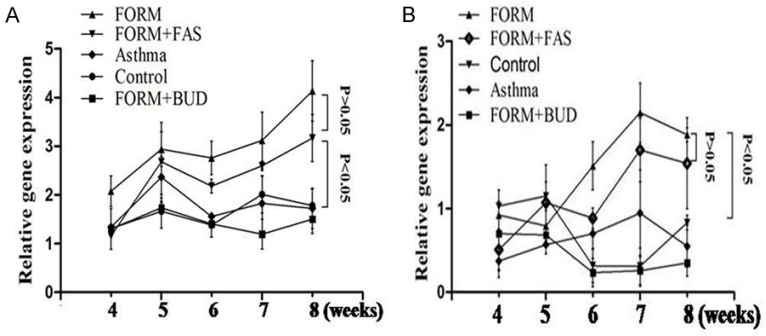

Figure 7. RhoA and M3 mRNA expression over five consecutive weeks in each group. RhoA and M3 mRNA expression levels in the FORM group were highest in the last week.

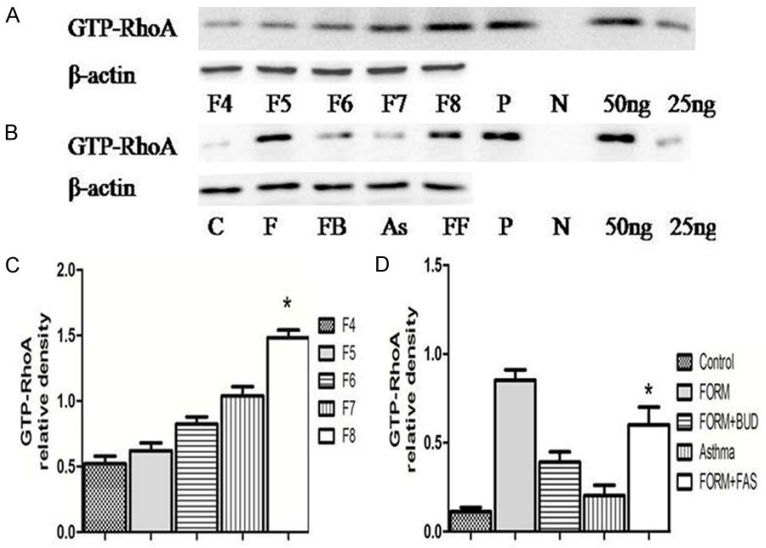

Figure 8. Detection of activated (GTP-bound) RhoA (24 kDa) by western blot analysis. A: Representative data for the FORM group over five consecutive weeks. F4-F8 represent weeks 4 to 8, respectively. B: Representative data for each group at week 8. P, positive control: total cell lysates loaded with GTPγS for the pulldown assay. N, negative control: total cell lysates loaded with GDP. C: Relative density of A; ※P < 0.05 compared with week 4. D: Relative density of B; ※P < 0.05 compared with the asthma group.

intain some basic cell functions and was not activated by modest inflammatory cell infiltration. In fact, Forstermann and his colleagues discovered that endothelium-dependent vasodilation in healthy subjects tends to worsen with FAS therapy compared with placebo treatment [31]. The key observation is that the effects of FAS treatment on the FORM + allergen group did not significantly differ in terms of bronchoprotection or RhoA gene and protein expression compared to the FORM + allergen group without FAS treatment. Does this finding imply that the RhoA/Rho-kinase signal pathway is not involved in the response? The answer is definitely negative. Three points need to be highlighted here. First, our study confirmed that Ca^{2+} sensitization was over-activated after chronic administration of FORM, as proven by GTP-RhoA and Rho mRNA expression. Compared with the asthma group, the possibility of allergen-induced effects on Ca^{2+} sensitization can be ruled out in this case. However, why was Ca^{2+} sensitization over-activated, when the sole addition of FAS only somewhat reversed β_2-adrenoceptor sensitivity, which was even weaker than in BUD-treated animals? The second point is that the current study confirmed that the chronic administration of FORM induced the overexpression of M_3R, suggesting that both Ca^{2+} sensitization and Ca^{2+} mobilization could be over-activated in response to the same stimulus. Therefore, only inhibiting Ca^{2+} sensitization (FAS) could not completely reverse the impaired β_2-protection, while adding BUD could inhibit both Ca^{2+} sensitization and Ca^{2+} mobilization. This observation also suggests that chronic FORM use by asthmatics causes not only beta-adrenoceptor dysfunction but also pro-contractile GPCR signaling pathway overactivation. In fact, Mak et al. demonstrated that exposing ASM cells ex vivo to beta-agonists upregulates histamine$_1$ (H_1) and neurokinin$_2$ (NK_2) receptors [32, 33]. Third, as an inhibitor of Ca^{2+} sensitization, FAS has only 3-fold more selectivity for Rho kinase than does PKA, and FAS has a weaker affinity for Rho kinase than does Y-27632, as demonstrated by its inhibition constant for Rho kinase [34]. Rho kinase is downstream of RhoA, and theoretically, FAS could inhibit Rho kinase and has little effect on RhoA. Moreover, we administered FAS orally, which may further inhibit its effects.

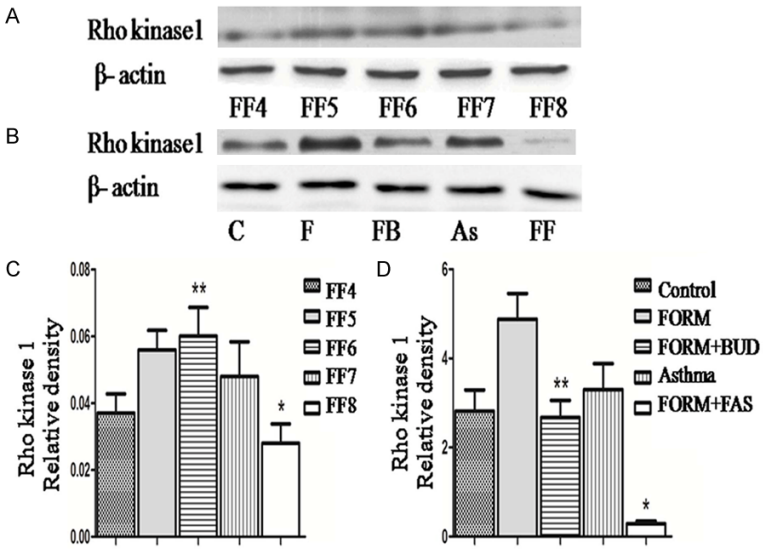

Figure 9. Detection of Rho kinase 1 expression by western blot. A: Representative data for the FORM + FAS group over five consecutive weeks. FF4-FF8 represent weeks 4 to 8, respectively. B: Representative data for each group at week 8. C: Relative density of A; ※P < 0.05 compared with week 6. ※※P < 0.05 compared with week 4. D: Relative density of B; ※P < 0.05 compared with the FORM + BUD group. ※※P > 0.05 compared with asthma group.

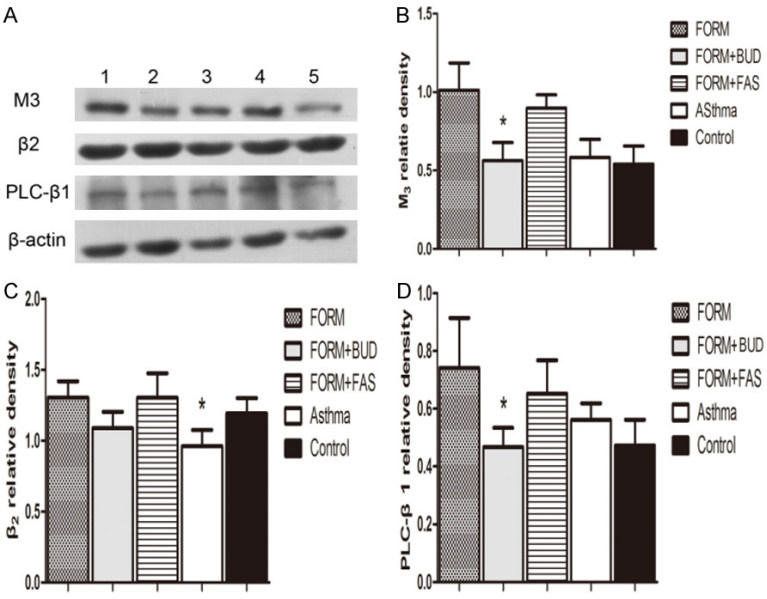

Figure 10. A: Detection of M_3R, β_2R, PLC-β_1 and β-actin expression by western blot. Lanes 1-5: FORM, FORM + BUD, FORM + FAS, asthma, and control groups, respectively. B: Relative density of M_3R; ※P < 0.05 compared with the FORM + FAS group. C: Relative density of β_2R; ※P > 0.05 compared with the FORM + FAS group. D: Relative density of PLC-β_1; ※P > 0.05 compared with the FORM + FAS group.

Our study has some limitations. Further research may focus on how Ca^{2+}-sensitization may be activated by G_s coupled β_2-receptor which is traditionally recognized as inhibitor of Ca^{2+}-sensitization as well as the distribution of β_2-receptor (on or under the surface of SMCs) and the effect of adding anticholinergic drugs based on this model.

Conclusion

First, this animal model provided a useful research tool that we utilized to study the chronic effects of β-adrenoceptor agonists and to determine the underlying mechanism of β-adrenoceptor dysfunction. Second, we demonstrated that chronic FORM treatment induced the overactivation of Ca^{2+} sensitization as well as of Ca^{2+} mobilization, whereas β_2-adrenoceptor and PLC-β_1 exhibited little change during these natural pathologic processes. Third, inhibiting Ca^{2+} sensitization signaling could partially inhibit airway responsiveness and reverse the loss of bronchoprotection, which may provide a novel strategy for the treatment of asthma.

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Disclosure of conflict of interest

None.

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