Overexpression of Rho/Rho kinase and M₃ cholinergic receptor is involved in formoterol-induced impairment of β₂-adrenoceptor bronchoprotection

Wei Luo¹, Ya-Lun Li²*, Qi Yu³, Tao Wang⁴, Chun-Tao Liu²

¹Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, PR China; ²Department of Respiratory and Critical Care Medicine, West China Hospital, Sichuan University, Chengdu, Sichuan, PR China; ³Department of Pediatrics, Leshan People’s Hospital, Leshan, Sichuan, PR China; ⁴Laboratory of Pulmonary Disease, West China Hospital, Sichuan University, Chengdu, Sichuan, PR China. *Equal contributors.

Received January 12, 2016; Accepted March 24, 2016; Epub April 1, 2016; Published April 15, 2016

Abstract: Background: The mechanism of β₂-adrenoceptor hyposensitivity has not yet been thoroughly elucidated. The classic theory is that β₂-adrenoceptor desensitization is caused by a decline in the number of β₂-adrenoceptors. However, certain other experiments have shown that the number of β₂-adrenoceptors exhibits a positive relationship with airway responsiveness and that cross-talk between the Gₛ and Gₚ signaling pathways exists through PLC-β₁. Recently, the Rho/Rho kinase signaling pathway was suggested to be involved in β₂-adrenoceptor dysfunction. Objectives: We aimed to establish a mouse model that demonstrates formoterol-induced β₂-adrenoceptor hypo-sensitivity and to determine how β₂-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 β₂-adrenoceptors to terbutaline-induced bronchoprotection was measured at 5 different time points. The associated changes in protein and gene expression (RhoA, Rho kinase 1, M₃R, β₂R, and PLC-β₁) 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 M₃ receptor were overexpressed, whereas β₂-adrenoceptor and PLC-β₁ 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. Ca²⁺ sensitization and M₃ receptor overexpression played key roles in formoterol-induced β₂-adrenoceptor hyposensitivity. Inhibiting Ca²⁺ sensitization partially reversed the impaired bronchoprotection.

Keywords: Asthma, bronchoprotection, Ca²⁺ sensitization, formoterol, Rho/Rho kinase

Introduction

β₂-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, β₂-agonists mediate bronchoprotection, which is defined as the inhibition of induced bronchoconstriction [1]. Although β₂-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 β₂-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 β₂-adrenoceptor desensitization which was defined as declining numbers of β₂-adrenoceptors. In contrast, cer-
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

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-β1 (PLC-β1) [8]. Moreover, Nguyen et al. confirmed that β2-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 Ca2+ sensitization, which is defined as the lack of a change in the intracellular Ca2+ 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 β2-adrenoceptor dysfunction. In fact, certain ex vivo experiments suggest that Ca2+ sensitization may be involved in isoprenaline-induced impaired bronchoprotection [11].

Currently, most studies concerning the relationship between β2-adrenoceptor and impaired bronchoprotection have focused on Gs-coupled β2-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 β2-adrenoceptor subtype is the major mediator of albuterol-induced bronchorelaxation in β2-adrenoceptor-KO mice [13]. Their work demonstrated that these mice are a suitable model for studying β2-adrenoceptor-mediated bronchoprotection.

We aimed to establish a mouse model that exhibits formoterol (FORM)-induced β2-adrenoceptor dysfunction as well as to determine how β2-agonists induce the loss of β2-protection, especially whether Ca2+ 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,
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

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.

**β₂-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: 

\[
\text{Bronchoprotection} = \left( \frac{\text{APTI response to MCh} - \text{average APTI response to MCh + terbutaline}}{\text{average APTI response to MCh} \times 100} \right)
\]

**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.
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

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, β₂, M₃, PLC-β₁, 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.

Table 1. Specific PCR primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Primer sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhoA-F</td>
<td>NM 016802</td>
<td>CTCGGAGTCTCTGGCCTTGA</td>
</tr>
<tr>
<td>RhoA-R</td>
<td></td>
<td>CTCGGAGAATCGTGCTTGGCT</td>
</tr>
<tr>
<td>ROCK1-F</td>
<td>NM 009071.2</td>
<td>GCACACTTCAAACCTTACT</td>
</tr>
<tr>
<td>ROCK1-R</td>
<td></td>
<td>TCACGGTCATCCATCCTCT</td>
</tr>
<tr>
<td>Chrm3-F</td>
<td>NM 033269</td>
<td>GGTATGACCATCCCATCTCACTC</td>
</tr>
<tr>
<td>Chrm3-R</td>
<td></td>
<td>GTACGCTTCATCATCTCTCTGG</td>
</tr>
<tr>
<td>Adrb2-F</td>
<td>NM 007420</td>
<td>CAACGCCAGAACGGACTAC</td>
</tr>
<tr>
<td>Adrb2-R</td>
<td></td>
<td>TTGGGAGTCAAAGCTAAGGCT</td>
</tr>
<tr>
<td>Plcb1-F</td>
<td>NM 001145830</td>
<td>GAGCCACCAGTCCACACAGA</td>
</tr>
<tr>
<td>Plcb1-R</td>
<td></td>
<td>GAGCCACCAGTCCACACAGA</td>
</tr>
<tr>
<td>Actin-F</td>
<td>NM 007393</td>
<td>CCGTAAGACCTCTATGCCAACAC</td>
</tr>
<tr>
<td>Actin-R</td>
<td></td>
<td>GAGCCACCAGTCCACACAGA</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>NM 008084</td>
<td>CATGGCTTCTCGGTCTCTTA</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td></td>
<td>TGCTTCACACCCTTCTTTGATG</td>
</tr>
</tbody>
</table>

β₂, M₃, Rho kinase 1, and PLC-β₁ 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
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

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 kinase 1 mRNA was significantly inhibited by FAS compared to the other groups (P < 0.05). M3R mRNA was overexpressed in FORM-group compared to the other groups, except for the FORM + FAS group (P < 0.05) (Figure 7B), whereas β2R and PLC-β1 mRNA expression showed little change in the FORM-treated groups compared to the other groups (data not shown).
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

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).

\[ \text{Rho kinase 1, } \beta_R, M_3R, \text{ and } PLC-\beta_1 \text{ expression} \]

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_R 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-
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

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, Schwann 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-
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

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.

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 β2-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 M3, 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 β2-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 histamine1 (H1) and neurokinin1 (NK1) 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.
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

Our study has some limitations. Further research may focus on how Ca²⁺-sensitization may be activated by Gₛ-coupled β₂-receptor which is traditionally recognized as inhibitor of Ca²⁺-sensitization as well as the distribution of β₂-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 over-activation of Ca²⁺ sensitization as well as of Ca²⁺ mobilization, whereas β₂-adrenoceptor and PLC-β₁ exhibited little change during these natural pathologic processes. Third, inhibiting Ca²⁺ sensitization signaling could partially inhibit airway responsiveness and reverse the loss of bronchoprotection, which may provide a novel strategy for the treatment of asthma.

Acknowledgements

The authors are grateful for the support from the Natural Science Foundation of China (grant 81170031).

Disclosure of conflict of interest

None.

Address correspondence to:

Chun-Tao Liu, Department of Respiratory and Critical Care Medicine, West China Hospital, Sichuan University, Chengdu 610-041, Sichuan, PR China. Tel: +86 28 85423872; Fax: +86 28 85423872; E-mail: LuoBao97@163.com
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection

References


[24] Callaerts-Vegh Z, Evans KLJ, Dudekula N, Cuba D, Knoll BJ, Callaerts PFK, Giles H, Shardonofsky FR, Bond RA. Effects of acute and chronic administration of beta-adrenoceptor ligands on airway function in a murine mod-
Overexpression of Rho/Rho kinase contribute to impaired bronchoprotection


