Mechanism of Inhibitory Effect of Prednisolone on RhoA Upregulation in Human Bronchial Smooth Muscle Cells

Kumiko GOTO, Yoshihiko CHIBA,* Hiroyasu SAKAI, and Miwa MISAWA

Department of Pharmacology, School of Pharmacy, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan. Received November 20, 2009; accepted January 31, 2010; published online February 3, 2010

RhoA plays an important role in Ca$^{2+}$ sensitization of bronchial smooth muscle in antigen-induced airway hyperresponsiveness (AHR). Glucocorticoids are now the most effective anti-inflammatory treatment for asthma, and inhaled corticosteroids are the most effective long-term control therapy for persistent asthma. To determine the mechanism of the inhibitory action of glucocorticoids on AHR in allergic bronchial asthma, that of prednisolone on RhoA upregulation was investigated using cultured human bronchial smooth muscle cells (hBSMCs). The upregulation of RhoA induced by interleukin (IL)-13 and tumor necrosis factor (TNF-α), major mediators for development of AHR, was observed in hBSMCs. Prednisolone partly inhibited the IL-13-induced RhoA upregulation and RhoA promoter activity, although prednisolone had no effects on the activations of signal transducers and activators of transcription (STAT6) and nuclear factor (NF)-κB. Increased expression and promoter activity of RhoA induced by TNF-α was completely inhibited by prednisolone, although the activation of NF-κB failed to be inhibited by prednisolone in hBSMCs. These findings suggest that prednisolone might inhibit NF-κB-induced transcription via interaction between glucocorticoid receptor (GR), resulting in an inhibition of RhoA upregulation induced by IL-13 and TNF-α.

Key words RhoA; prednisolone; nuclear factor-κB; signal transducer and activator of transcription 6; airway hyper responsiveness

Airway hyperresponsiveness (AHR) associated with heightened airway resistance and inflammation is an asthmatic characteristic feature. Although the importance of AHR in the pathogenesis of asthma has been suggested by its relevance to the severity of this disease, the pathophysiologic alterations leading to the hyperresponsiveness are still unclear. It has been demonstrated that smooth muscle responsiveness to contractile agonists was significantly increased in bronchial preparations from repeatedly antigen challenged rats.1)

Smooth muscle contraction is mainly regulated by an increase in cytosolic Ca$^{2+}$ concentration in myocytes. Recently, additional mechanism, termed Ca$^{2+}$ sensitization, has also been suggested to be involved in the agonist-induced smooth muscle contraction. It has been demonstrated that agonist stimulation increases myofilament Ca$^{2+}$ sensitivity in permeabilized smooth muscles of the rat coronary artery,2) guinea pig vas deferens,3) canine trachea,4) and rat bronchus.5) Although the detailed mechanism is not fully cleared, an involvement of RhoA, a monomeric GTP-binding protein, in agonist-induced Ca$^{2+}$ sensitization has been suggested by many investigators.6) Previously, we demonstrated that the Ca$^{2+}$ sensitization of the bronchial smooth muscle (BSM) contraction was markedly augmented concomitantly with an increased expression of RhoA protein in the AHR rats and mice.7,8) Moreover, an augmented RhoA-mediated Ca$^{2+}$ sensitization of smooth muscle contraction has been reported in experimental animal models of diseases.9,10) It is thus possible that RhoA-mediated signaling is a crucial key for understanding the abnormal contraction of diseased smooth muscles. Recent studies revealed that the RhoA upregulation is triggered by interleukin (IL)-13 and tumor necrosis factor (TNF)-α, both of which are known as major mediators for development of AHR, via activation of signal transducers and activators of transcription (STAT6) and nuclear factor (NF)-κB in human BSM cells (hBSMCs).7,11)

Glucocorticoids are by far the most effective anti-inflammatory treatment for bronchial asthma and have now become the first-line therapy in all patients with persistent asthma. The predominant effect of glucocorticoids such as prednisolone and beclomethasone is to switch off multiple inflammatory genes that have been activated during the inflammatory process. They have additional effects such as the synthesis of anti-inflammatory proteins and post-genomic effects. Recent studies have also demonstrated that glucocorticoids may affect diverse functions via glucocorticoid receptor (GR), such as an increase in β3 receptor expression in airway smooth muscle cells,12) decreases in chemokines and cytokines secretion in airway epithelial cells13,14) and so on. One mechanism by which GR influences gene expression is via direct physical association with other transcription factor, such as STAT6 and NF-κB to modulate their function.15,16) On the other hand, clinical studies also revealed that glucocorticoids could reduce, at least in part, the AHR.17–21) In addition, we have previously reported that glucocorticoids inhibited antigen-induced AHR22) and RhoA upregulation in BSMs.23) However, the mechanism of inhibitory effect of glucocorticoid on the upregulation of RhoA is unknown.

In the present study, to investigate the mechanisms of the inhibitory effect of prednisolone on the RhoA upregulation, the effects of prednisolone on activations of STAT6 and NF-κB induced by the IL-13 and TNF-α were examined in hBSMCs. Prednisolone partly inhibited the IL-13-induced RhoA upregulation and RhoA promoter activity, although prednisolone had no effects on the activations of STAT6 and NF-κB. Increased expression and promoter activity of RhoA induced by TNF-α were completely inhibited by prednisolone, although the activation of NF-κB failed to be inhibited by prednisolone in hBSMCs. These findings suggest that prednisolone might inhibit the NF-κB-induced transcription via interaction with GR, resulting in an inhibition of RhoA upregulation induced by IL-13 and TNF-α.

* To whom correspondence should be addressed. e-mail: chiba@hoshi.ac.jp © 2010 Pharmaceutical Society of Japan
MATERIALS AND METHODS

Cell Culture and Sample Collection Normal human BSM cells (hBSMCs; Cambrex Bio Science Walkersville, Inc., MD, U.S.A.) were maintained in SMIMedium (Cambrex) supplemented with 5% fetal bovine serum (FBS), 2 ng/ml human fibroblast growth factor-basic (hFGF-b), 0.5 ng/ml human epidermal growth factor (hEGF), 5 μg/ml insulin, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B as described previously.11,24) Cells were washed with phosphate buffered saline (PBS), immediately collected and disrupted with 1×SDS sample buffer (200 μl/well), and used for Western blot analyses.

Western Blot Analyses The samples (10 μg of total protein per lane) were subjected to 7.5% (for STAT6 and phosphorylated STAT6) and 10% (for p65) or 15% (for RhoA) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 3% gelatin or 3% skim milk, the PVDF membrane was incubated with polyconal rabbit anti-NF-κB p65 (1:1000 dilution; Biolegend, CA, U.S.A.), polyclonal rabbit anti-STAT6 (1:1000 dilution; Santa Cruz Biotechnology, Inc., CA, U.S.A.), polyclonal rabbit anti-phosphorylated STAT6 (1:1000 dilution; Santa Cruz Biotechnology, Inc.), or polyclonal rabbit anti-RhoA (1:2500 dilution; Santa Cruz Biotechnology, Inc.) overnight. Then the membrane was incubated with horseradish peroxidase-conjugated donkey antirabbit immunoglobulin (IgG) (1:2500 dilution; Amersham Biosciences, Co., NJ, U.S.A.), detected by an enhanced chemiluminescent system (Amersham Biosciences) and analyzed by a densitometry system. Detection of house-keeping gene was also performed on the same membrane by using monoclonal mouse anti-β-actin (1:5000 dilution; Santa Cruz Biotechnology, Inc.) for total protein samples to confirm the same amount of proteins loaded.

Construction of Rat RhoA-Luciferase Promoter Plasmid For the reporter assay, a rat RhoA genomic fragment from nt -1217 to +21 was obtained by polymerase chain reaction (PCR) amplification using the rat genomic DNA (Clontech, CA, U.S.A.) as a template. The reverse primer contained Nhe I restriction site. The PCR primer sets used were as follows: 5′-GGAACCCCATTTGGAATCCTG-GAGC-3′ (sense) and 5′-GAGGAGGAGCGCTAGCAAGC AG-3′ (antisense), which were designed from published sequence (GenBank Accession No. NW_047483). The PCR product was digested by Sac I (−1195) and Nhe I (+10), and inserted into the pGL4.10 vector (Promega, WI, U.S.A.).

Luciferase Assay The hBSMCs were seeded in 48-well tissue culture plates, grown to 80% confluence, and transfected with pGL4 reporter plasmids containing various lengths of the rat RhoA gene promoter using Lipofectamine 2000 (Invitrogen Life Technologies) according to manufacturer’s instructions. Cells were cultured without serum for 24 h before addition of recombinant human IL-13 or recombinant human TNF-α and prednisolone. Luciferase assay was performed using ONE-GloTM Luciferase assay system (Promega) 72 h after transfection in accordance with the manufacturer’s instructions. Luciferase activity was measured with a Wallac 1420 ARVOSx multilabel counter (Perkin Elmer, MA, U.S.A.).

Statistical Analyses All the data were expressed as the mean with S.E.M. Statistical significance of difference was determined by one-way analysis of variance (ANOVA) with post hoc Bonferroni/Dunn (StatView for Macintosh ver. 5.0, SAS Institute, Inc., NC, U.S.A.). A value of p<0.05 was considered as significant.

RESULTS

Effects of Prednisolone on the Activation of STAT6 and NF-κB Induced by IL-13 and TNF-α in hBSMCs To determine whether prednisolone directly inhibit the activation of STAT6 and NF-κB, we analyzed the effects of prednisolone on the IL-13- and TNF-α-induced phosphorylation of STAT6 and the translocation of NF-κB to nuclei in human bronchial smooth muscle cells (hBSMCs). As shown in Fig. 1A, a phosphorylation of STAT6 was observed when the hBSMCs were treated with IL-13 but not TNF-α for 1 h. This phenomenon failed to be inhibited by treatment with prednisolone for 1 h. The translocation of NF-κB to nuclei was observed when the hBSMCs were treated with both IL-13 and TNF-α for 0.5 h; treatment with prednisolone for 0.5 h had no effects on the IL-13- and TNF-α-induced translocation of NF-κB to nuclei (Fig. 1B).

Effects of Prednisolone on RhoA Upregulation Induced by IL-13 and TNF-α in hBSMCs To determine the effects of prednisolone on IL-13- or TNF-α-induced RhoA up-regulation, we analyzed the expression of RhoA protein and RhoA promoter activity. The expression of RhoA protein was significantly augmented by IL-13 and TNF-α for 24 h in hBSMCs (Fig. 2). Interestingly, the increased expression of RhoA protein induced by IL-13 was partly inhibited, although the levels of phosphorylated STAT6 and translocated NF-κB failed to be inhibited by treatment with prednisolone. Moreover, TNF-α-induced RhoA protein upregulation was abolished by prednisolone (Fig. 2). Next, the inhibitory effects of prednisolone on IL-13- and TNF-α-induced increases in RhoA promoter activities were assessed using luciferase assay (Fig. 3). As shown in Fig. 3, the promoter activity was markedly increased by IL-13 and TNF-α stimulations for 24 h in the hBSMCs transfected with RhoA promoter luciferase plasmid. The IL-13-induced increase in RhoA promoter activity was partly inhibited by prednisolone. Prednisolone also inhibited the increase in RhoA promoter activity induced by TNF-α (Fig. 3).

DISCUSSION

In the present study, although prednisolone inhibited the promoter activities and the upregulation of RhoA protein induced by IL-13 or TNF-α (Figs. 2, 3), it had no effect on the phosphorylation of STAT6 induced by IL-13, and on the translocation of NF-κB to nuclei induced by IL-13 or TNF-α in hBSMCs (Fig. 1). These findings suggest that prednisolone might inhibit the NF-κB-induced transcription via interaction with glucocorticoid receptor (GR), resulting in an inhibition of RhoA upregulation induced by IL-13 and TNF-α.

Although the exact RhoA promoter and/or enhancer re-
gions have not yet been identified, the transcriptional start site of rat RhoA was identified by 5'RACE (our personal communication). The DNA sequence analysis for the directly upstream region of the rat RhoA transcriptional start site (from −1 to −1195) using the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html) revealed 3 putative STAT6 binding sites—nt −640 to −632 (score 84.6), nt −323 to −316 (score 80.8), and nt −192 to −184 (score 85.6) and 2 putative p65 binding sites—nt −739 to −730 (score 81.0), and nt −197 to −188 (score 83.4). It is thus possible that antigen challenge causes the activation of STAT6 and NF-κB in bronchial smooth muscle, resulting in an increased RhoA transcription.

Glucocorticoids are the most commonly used agents to treat asthma due to their ability to attenuate both the pulmonary inflammatory response and airway hyperresponsiveness associated with this disorder. Glucocorticoids have bronchoprotective action to directly alter bronchial smooth muscle function by the following effects; 1) inhibition of bronchial smooth muscle contractility, 2) augmentation of bronchial smooth muscle relaxation, 3) suppression of bronchial smooth muscle proliferation, 4) prevention of the release of various cytokines and chemokines from stimulated bronchial smooth muscle, and other actions. We previously reported that glucocorticoids might inhibit the AHR by reducing the protein expression and activation of a monomeric GTP-binding protein, RhoA, which is involved in the agonist-induced Ca²⁺ sensitization of bronchial smooth muscle contraction. However, little is known about the mechanism of glucocorticoid action on RhoA expression.

Fig. 1. Effects of Prednisolone (10 μM) on Translocation of p65 and Phosphorylation of STAT6 Induced by IL-13 (100 ng/ml) or TNF-α (10 ng/ml) in Cultured Human Bronchial Smooth Muscle Cells (hBSMCs)

(A) Total proteins of hBSMCs were assayed for phosphorylation of STAT6 by immunoblotting. (Upper photos) Typical blots for phosphorylated STAT6 and total STAT6. The phosphorylation levels of STAT6 are summarized in lower panel. (B) Nuclear proteins of hBSMCs were assayed for p65 by immunoblotting. (Upper photos) Typical blots for p65 and histone H1. The expression levels of p65 in nucleus are summarized in lower panel. Values are the means with S.E.M. from 3—6 independent experiments. ***p<0.001 vs. Cont. by two-way ANOVA with post hoc Bonferroni/Dunn’s test.

Fig. 2. Effects of Prednisolone (10 μM) on the Upregulation of RhoA Induced by IL-13 (100 ng/ml) or TNF-α (10 ng/ml) in Cultured Human Bronchial Smooth Muscle Cells (hBSMCs)

Total proteins of hBSMCs were assayed for RhoA by immunoblotting. (Upper photos) Typical blots for RhoA and β-actin. The expression levels of RhoA are summarized in lower panel. Values are the means with S.E.M. from 3—6 independent experiments. ***p<0.001 vs. Cont., ***p<0.001 vs. IL-13 alone and +++p<0.001 vs. TNF-α alone by two-way ANOVA with post hoc Bonferroni/Dunn’s test.

Fig. 3. Effects of Prednisolone (10 μM) on the Rat Promoter Activity of RhoA Induced by IL-13 (100 ng/ml) or TNF-α (10 ng/ml)

Cultured hBSMCs were transfected with luciferase reporter plasmid of the rat RhoA gene promoter, and luciferase assays were performed in hBSMCs with no stimulation, IL-13 (100 ng/ml) or TNF-α (10 ng/ml) stimulation with vehicle or prednisolone for 24 h. Values are the means with S.E.M. from 5 independent experiments. ***p<0.001 vs. Cont., **p<0.01 vs. IL-13 alone and ***p<0.001 vs. NF-κB alone by two-way ANOVA with post hoc Bonferroni/Dunn’s test.
of inhibitory effect of glucocorticoid on the upregulation of RhoA induced by antigen-challenge.

Then, to determine whether prednisolone directly inhibit activation of transcription factors, STAT6 and NF-κB, the inhibitory effects of prednisolone on the phosphorylation of STAT6 induced by IL-13 and the translocation of NF-κB to nuclei induced by IL-13 and TNF-α in hBSMCs were investigated. As shown in Fig. 1A, prednisolone failed to inhibit the phosphorylation of STAT6 induced by IL-13 in hBSMCs. So et al.26) showed that methylprednisolone (1—100 μM) inhibited IL-4-induced STAT6 phosphorylation in mononuclear cells. Because our experimental system is quite different from their study, the effect of glucocorticoid on STAT6-induced transcription may depend on the cell type.

Several studies showed that glucocorticoids inhibited an activation of NF-κB in human airway smooth muscle cells.27) However, prednisolone had no effect on the translocation of NF-κB to nuclei induced by IL-13 or TNF-α in the present study (Fig. 1B). Glucocorticoids inhibit NF-κB-induced transcription via the interaction between glucocorticoid receptor and NF-κB.28,29) Thus, to examine whether prednisolone inhibits the STAT6- and NF-κB-induced transcription, we investigated the promoter activity of RhoA using luciferase vector containing three STAT6 binding sites and two p65 binding sites (Fig. 3). The increased promoter activity induced by IL-13 was partly inhibited by prednisolone, and that induced by TNF-α was completely inhibited by prednisolone. In addition, prednisolone also inhibited the IL-13- and TNF-α-induced RhoA upregulation in hBSMCs (Fig. 2). We demonstrated that the augmented promoter activity of rat RhoA by TNF-α was abolished by 1xκB kinase (IKK) inhibitor (data not shown). This observation suggests that the upregulation of RhoA is induced by TNF-α via only NF-κB, which was inhibited by prednisolone. On the other hand, the augmented promoter activity of rat RhoA by IL-13 was partly inhibited by STAT6 inhibitor or IKK inhibitor, and abolished by both STAT6 inhibitor and IKK inhibitor (data not shown). These findings suggest that the IL-13-induced RhoA upregulation is mediated via activation of both STAT6 and NF-κB. The inhibitory effects of prednisolone on the IL-13-induced RhoA promoter activity and the RhoA upregulation resulted from an inhibition of NF-κB, and prednisolone might not have ability of inhibition of STAT6-induced transcription.

In conclusion, the current study demonstrated that prednisolone inhibits the NF-κB-induced transcription, resulting in an inhibition of RhoA upregulation induced by IL-13 and TNF-α.

Acknowledgement We thank Mr. Taiki Kobayashi and Ms. Makiko Momata for their technical assistance.

REFERENCES