p38 Mitogen-Activated Protein Kinase-γ Inhibition by Long-Acting β₂ Adrenergic Agonists Reversed Steroid Insensitivity in Severe Asthma[S]

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ABSTRACT

Corticosteroid insensitivity (CI) is a major barrier to treating severe asthma. Despite intensive research, the molecular mechanism of CI remains uncertain. The aim of this study was to determine abnormality in corticosteroid action in severe asthma and to identify the molecular mechanism of the long-acting β₂-adrenergic agonists (LABAs) formoterol and salmeterol on restoration of corticosteroid sensitivity in severe asthma in vitro. Peripheral blood mononuclear cells (PBMCs) were obtained from 16 subjects with severe corticosteroid-insensitive asthma, 6 subjects with mild corticosteroid-sensitive asthma, and 11 healthy volunteers. Corticosteroid (dexamethasone) sensitivity was determined on tumor necrosis factor-α (TNF-α)-induced interleukin (IL)-8 production. Glucocorticoid receptor (GR) phosphorylation and kinase phosphorylation were evaluated by immunoprecipitation-Western blotting analysis and kinase phosphorylation array in IL-2/IL-4-treated corticosteroid insensitive model in PBMCs. In vitro corticosteroid sensitivity on TNF-α-induced IL-8 production was significantly lower in patients with severe asthma than in healthy volunteers and patients with mild asthma. This CI seen in severe asthma was associated with reduced GR nuclear translocation and with hyperphosphorylation of GR, which were reversed by LABAs. In IL-2/IL-4-treated PBMCs, LABAs inhibited phosphorylation of Jun-NH₂-terminal kinase and p38 mitogen-activated protein kinase-γ (p38MAPK-γ) as well as GR. In addition, cells with p38MAPK-γ knockdown by RNA interference did not develop CI in the presence of IL-2/IL-4. Furthermore, p38MAPK-γ protein expression was up-regulated in PBMCs from some patients with severe asthma. In conclusion, p38 MAPK-γ activation impairs corticosteroid action and p38 MAPK-γ inhibition by LABAs has potential for the treatment of severe asthma.

Introduction

Most patients with asthma, symptoms are now effectively controlled with inhaled corticosteroids. However, approximately 5% of patients with asthma do not respond well to corticosteroids or require high-dose inhaled or oral corticosteroids to control asthma symptoms, although side effects are still a problem. Thus, corticosteroid insensitivity (CI) presents considerable management problems, accounting for a disproportionate amount of healthcare spending in asthma (Leung and Szefler, 1998; Adcock and Ito, 2004).

The biological actions of corticosteroids are mediated by glucocorticoid receptors (GRs), which are normally located in cell cytoplasm. Corticosteroids cross the cell membrane and bind to GR, which then translocates into the nucleus, and its homodimers bind to DNA at glucocorticoid response elements in the promoter region of corticosteroid-responsive anti-inflammatory genes, such as secretory leukoprotease inhibitor (*SLPI*), mitogen-activated kinase phosphatase-1 (*MKP-1*), and glucocorticoid inducible leucine zipper (*GILZ*), increasing gene transcription. As well as this GR-glucocorticoid response element binding, GR may directly influence proinflammatory signaling by forming inhibitory interactions with proinflammatory DNA-binding transcription factors such as

ABBREVIATIONS: CI, corticosteroid insensitivity; GR, glucocorticoid receptor; NF-κB, nuclear factor-κB; JNK, c-Jun-NH₂-terminal kinase; MAPK, mitogen-activate protein kinase; IL, interleukin; LABA, long-acting β₂-agonist; SB203580, 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1*H*-imidazol-5-yl]pyridine; FEV₁, forced expiratory volume in 1 s; PBMC, peripheral blood mononuclear cell; FITC, fluorescein isothiocyanate; Dex, dexamethasone; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; HSP, heat shock protein; KD, knockdown; FOR, formoterol; SAL, salmeterol; HV, healthy volunteer(s); SA, severe asthma; MA, mild glucocorticoid-sensitive asthma; PP, protein phosphatase.

activator protein-1 and nuclear factor-κB (NF-κB), or by recruitment of corepressors such as histone deacetylase 2 (Ito et al., 2006a,b). GR nuclear translocation, therefore, is an essential and critical step for corticosteroid action. However, as we reported previously (Matthews et al., 2004), some patients with severe asthma showed defect of GR nuclear translocation.

Numerous studies demonstrated possible mechanisms of corticosteroid insensitivity, such as overexpression of transcription factors (Adcock et al., 1995), histone deacetylase reduction (Cosío et al., 2004; Hew et al., 2006) and increased decoy receptor (Leung et al., 1998). Post-translational modifications of GR, such as phosphorylation, acetylation, and ubiquitinition, are also important components for the mechanism of corticosteroid resistance (Ito et al., 2006a,b). For example, Rogatsky et al. (1998) demonstrated that the ability of GR of transcriptional activation was reduced once Ser467 of rat GR (equivalent to Ser226 of human GR) was phosphorylated by c-Jun N-terminal kinases (JNK). Irusen et al. (2002) showed that an inhibitor of p38 mitogen-activate protein kinase (MAPK)-α and -β isoforms inhibited interleukin (IL)-2/IL-4-induced GR phosphorylation in whole-cell extracts though Rogatsky et al. (1998) showed that GR at Ser-246 was not phosphorylated by p38 MAPK. Thus, GR phosphorylation is reported to be associated with CI, but GR phosphorylation has not been detected in clinical samples.

p38 MAPK-γ is one of four isoforms of p38MAPKs (Mertens et al., 1996; Cuenda et al., 1997). This kinase is also called stress-activated protein kinase-3, extracellular signal-regulated kinase 6, or MAPK12 and is able to phosphorylate postsynaptic density 95/disc-large/zona occludens motif-containing proteins, such as stress-activated protein (SAP) 90 and SAP97. p38MAPK-γ is activated by environmental stress, such as oxidative stress and osmotic stress, or proinflammatory cytokines, and it phosphorylates several downstream targets. p38 MAPK-γ is expressed in T lymphocytes, macrophages, and skeletal muscle cells, but its function is not certain.

The combinations of a long-acting β₂-agonist (LABA) with a low dose of inhaled corticosteroid have been reported to achieve better asthma control than either drug alone or a higher dose of inhaled corticosteroid alone (Reynolds et al., 2005; Miller-Larsson and Selroos, 2006). LABAs alone have been shown to induce GR nuclear translocation in smooth muscle cells and fibroblasts (Eickelberg et al., 1999) and enhance corticosteroid actions in vitro and in vivo (Pang and Knox, 2000; Roth et al., 2002; Usmani et al., 2005). In this way, LABAs may enhance the anti-inflammatory action of corticosteroids, but the molecular mechanism has not been fully elucidated. Here we show that p38 MAPK-γ causes corticosteroid insensitivity in severe asthma through hyperphosphorylation of GR. In addition, we found that the steroid sensitivity and the defective mechanism are reversed by LABAs.

Materials and Methods

Materials. Formoterol [*rac*-(*R,R*)-*N*-[2-hydroxy-5-[1-hydroxy-2-[1-(4-methoxyphenyl)propan-2-ylamino]ethyl] phenyl]formamide] and salmeterol [(*R,S*)-2-(hydroxymethyl)-4-{1-hydroxy-2-[6-(4-phenylbutoxy) hexylamino]ethyl}phenol] were provided by AstraZeneca (Lund, Sweden) and GlaxoSmithKline (Greenford, UK), respectively. Dexamethasone

[(8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16- trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one] and SB203580 [4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1*H*-imidazol-5-yl]pyridine] were purchased from Sigma-Aldrich (Poole, UK).

Subjects. Eleven healthy nonsmoking subjects (mean age ± S.E.M., 35.8 ± 1.6 years; three women; mean forced expiratory volume in 1 s (FEV₁ ± S.E.M., 98.2 ± 2.2% of predicted), six patients with mild asthma (41.5 ± 3.3 years; three women; FEV₁, 84.5 ± 4.6% of predicted), and 16 patients with severe asthma (age, 35.1 ± 2.6 years; 11 women; FEV₁, 55.0 ± 3.4% predicted) were recruited (Table 1). This study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals National Health Service Trust, and all subjects gave written informed consent. Fifty milliliters of blood were taken, and PBMCs were separated by Ficoll-Paque (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) gradients.

Corticosteroid-Insensitive Model. PBMCs from healthy volunteers were incubated with human recombinant IL-2 (2 ng/ml) and IL-4 (10 ng/ml) for 48 h.

FITC-Dexamethasone Incorporation. PBMCs were incubated with FITC-conjugated dexamethasone (FITC-Dex; 10^{-6} M) for 30 min at 37°C. Nonspecific FITC diffusion was determined in the presence of 10^{-5} M nonconjugated Dex and subtracted from the total FITC fluorescence value. The nuclear fraction was prepared by hypotonic buffer (Active Motif, Rixensart, Belgium) for 10-min incubation, followed by pulse vortexing with 0.1% NP-40 containing PBS. FITC-dexamethasone in nuclei was extracted with 0.5% NP-40 containing PBS on ice for 20 min. The concentration of FITC-Dex was determined using a standard curve to different concentrations of FITC-Dex. FITC was detected at 488 nm in a fluorescent plate reader.

Detection of GR and Phosphorylated GR. Whole-cell extracts were prepared with modified radioimmunoprecipitation assay buffer (Ito et al., 2000). For the detection of GR, whole-cell extracts were separated by Tris-glycine SDS/polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The GR level was normalized to β-actin expression. For the detection of phosphorylated GR, GR was immunopurified using anti-GR antibody conjugated agarose A/G (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and separated by SDS-polyacrylamide gel electrophoresis/Western blotting. Phosphorylation level was determined with anti-pan-phosphoserine antibody (Santa Cruz Biotechnology Inc.) and was normalized to GR expression. In addition, GR phosphorylated at Ser226 was determined using anti-phosphorylated (S226) GR antibody (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK). The band density was calculated by densitometry (UVP Bioimaging Systems, Cambridge, UK) using Labworks software (Ultra-Violet Products, Cambridge, UK).

Reverse Transcription-PCR. Cells were harvested for total RNA isolation. Commercially available kits were used to extract total cellular RNA (RNeasy; QIAGEN, Crawley, UK) and to perform reverse transcription (Omniscript RT; QIAGEN). Gene transcript

TABLE 1

Characteristics of the subjects recruited

	Healthy Volunteers	Mild asthma	Severe asthma
N	11	6	16
Sex, no. male/female	8/3	3/3	5/11
Age, years	35.8 ± 1.6	41.5 ± 3.3	35.1 ± 2.6
Asthma duration, years	N.A.	N.D.	16.3 ± 2.6
Atopy	2/11	2/6	6/15
FEV₁, % pred.	98.2 ± 2.2	84.5 ± 4.6	55.0 ± 3.4
FEV₁/FVC, %	96.8 ± 3.1	79.9 ± 3.2	46.6 ± 12.6
Oral steroid, mg	0	0	16.8 ± 4.0
Inhaled steroid, μg	0	0	1860 ± 206
Others	N.A.	Albuterol on demand	Albuterol on demand

N.A., not applicable, N.D.: not determined; FVC, forced vital capacity.

level of p38 MAPK-α, -γ, and -δ and the housekeeping gene *GNB2L1* or *GAPDH* were quantified by real-time PCR using a TaqMan PCR kit (Applied Biosystems, Warrington, UK) on a Rotor-Gene 3000 PCR apparatus (Corbett Research, Mortlake, NSW, Australia).

ELISA. Cells were treated with dexamethasone (10^{-12}–10^{-6} M) for 30 min in the presence or absence of LABA and then stimulated overnight with either TNF-α (1 ng/ml) or a combination of anti-human CD3 (10 μg/ml) and CD28 antibodies (8 μg/ml) (BD Biosciences, Oxford, UK). IL-8 and IL-2 levels in supernatant were determined by sandwich ELISA (Duoset ELISA for human IL-8; R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions.

Kinase Profiling. The phosphorylation of 19 different kinases was evaluated using the Human Phospho-MAPK Array Kit Proteome Profiler (R&D Systems Europe) according to the manufacturer's instructions. HSP27 (phosphorylated and total) and p38MAPK-γ (phosphorylated p38MAPK/stress-activated protein kinase and total) were detected by Western blotting. All antibodies were purchased from R&D Systems Europe.

Measurement of Phosphorylated and Total p38MAPK-γ in Cells. Phosphorylated p38MAPK-γ and total p38MAPK-γ were detected in PBMCs obtained from healthy subjects using p38MAPK-γ (Thr183/Tyr185) phosphorylation and total cell-based ELISA (Duoset intracellular ELISA). In brief, cells were stimulated with human recombinant IL-2 (2 ng/ml) and IL-4 (10 ng/ml) for 48 h and then treated with formoterol, salmeterol, or salbutamol for 20 min. Cells were collected and lysed using lysis buffer according to the manufacturer's instructions.

RNA Interference. Short interference RNA (siRNA) of the p38 MAPK-δ (MAPK13) and p38 MAPK-γ (MAPK 12) were purchased from Dharmacon Inc. (Colorado Springs, CO, USA) and transfected by nucleofection using AMAXANucreofector (Lonza GmbH, Cologne, Germany) according to the manufacturer's instructions (100 nM each). Cells were incubated for 24 h and then stimulated with IL-2/IL-4 for further 48 h. Nonspecific control duplex (scrambled oligonucleotide, 47% GC content) were also purchased from Dharmacon RNA Technologies (Lafayette, CO).

Statistical Analysis. Results are expressed as means ± S.E.M. Analysis of variance was done by Kruskal-Wallis analysis; when significant, comparisons were made by Mann Whitney *U* test using the PC analysis package SPSS 10.0 (SPSS Inc., Chicago, IL) or Prism 4 (GraphPad Software, San Diego, CA). The differences between treatment groups in the in vitro data were analyzed by Welch's *t* test. The correlation between two parameters was determined by Spearman methods. A *p* value < 0.05 was considered statistically significant.

Results

PBMCs From Severe Asthma Were Corticosteroid-Insensitive Because of Defects of GR Nuclear Translocation. As shown in Fig. 1A, PBMCs produced IL-8 when stimulated with TNF-α in patients with severe asthma (SA; 1430 ± 286 pg/ml), to a level similar to that seen in healthy volunteers (HV; 1650 ± 304 pg/ml), although the IL-8 pro-

duction was significantly higher in patients with mild asthma (MA; 2160 ± 94.9 pg/ml) than that in HV. In contrast, when 50% inhibitory activity of dexamethasone (Dex-IC$_{50}$) on TNF-α-induced IL-8 release was calculated as an index of corticosteroid sensitivity, the Dex-IC$_{50}$ values in PBMCs from patients with SA (181 ± 28.7 nM) were significantly higher than those from HV (15.5 ± 4.2 nM; $p < 0.01$) and patients with MA (20.0 ± 3.8 nM; $p < 0.01$) (Fig. 1B).

In PBMCs, there were no significant differences in GRα mRNA expression (GRα/GNB2L1: SA, 70.4 ± 17.8; HV, 102.4 ± 34.4; MA, 89.3 ± 24.3), GRβ mRNA expression (GRβ/GNB2L1: SA, 0.00042 ± 0.00015; HV, 0.000002 ± 0.0000005; MA, 0.00043 ± 0.00029) or in GRα protein expression (GRα/β-actin: SA, 1681 ± 205; HV, 4914 ± 763; MA, 3050 ± 670).

As a marker of GR nuclear translocation after ligand binding, we determined the amount of FITC-Dex in nuclei. Because the anti-inflammatory efficacy of FITC-Dex was 10 times lower than that of unlabeled Dex (IC$_{50}$ values, FITC-Dex, 5.6×10^{-8} M versus unlabeled Dex, 4.3×10^{-9} M on IL-1β-induced IL-8 production in A549 cells), we used a relatively high concentration (10^{-6} M) of FITC-Dex for the assay. As shown in Fig. 1C, GR nuclear translocation was significantly impaired in SA cells [FITC-Dex in nuclei, 4.8 ± 0.9 nM; $p < 0.01$ versus HV (11.0 ± 1.5 nM); $p < 0.05$ versus MA (9.7 ± 1.4 nM)]. In addition, there was a significant, negative correlation between the IC$_{50}$-Dex value on TNF-α-induced IL-8 release and the amount of FITC-Dex in nuclei (Spearman $r = -0.55$; $p = 0.0035$), suggesting that less Dex efficacy was associated with defects of GR nuclear translocation.

Formoterol Reversed Corticosteroid Insensitivity in PBMCs from Patients with SA by Enhancing GR Nuclear Translocation. Treatment with formoterol (FOR, 1 nM) decreased the Dex-IC$_{50}$ value for TNF-α induced IL-8 release in PBMCs from patients with SA (Dex-IC$_{50}$ with versus without FOR, 42.8 ± 21.0 versus 182.5 ± 28.2 nM, respectively; $n = 6$, $p < 0.05$; Fig. 2A) and a similar change but with a lower effect was found with salmeterol (SAL, 100 nM) (Dex-IC$_{50}$ with versus without SAL, 83.7 ± 16.7 versus 180.7 ± 28.7 nM, respectively; $n = 15$, $p < 0.05$) (Table 2). In patients with SA, treatment with FOR 1 nM showed better improvement index than SAL (for SA: 4.3 for FOR versus 2.2 for SAL; Table 2), but the efficacy was not significantly different in FOR and SAL. Neither FOR nor SAL significantly changed corticosteroid sensitivity in HV (Dex-IC$_{50}$, with FOR, 21.5 ± 4.8 nM; with SAL, 31.0 ± 8.3 nM; no treatment, 15.5 ± 4.2 nM; Table 2) or in patients with MA (Dex-IC$_{50}$ with SAL, 14.7 ± 3.5 nM versus no treatment, 20.0 ± 3.8 nM; FOR was not tested in patients with MA). As well as reduced
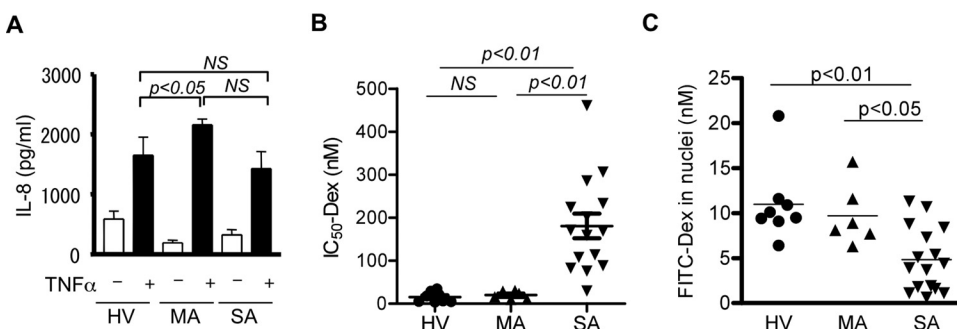

Fig. 1. TNF-α-induced IL-8 production (A), dexamethasone sensitivity to inhibit this response (IC$_{50}$-Dex; B), and GR nuclear translocation by FITC-Dex incorporation assay (C) were determined in PBMCs obtained from HV and patients with MA or SA. NS, not significant.
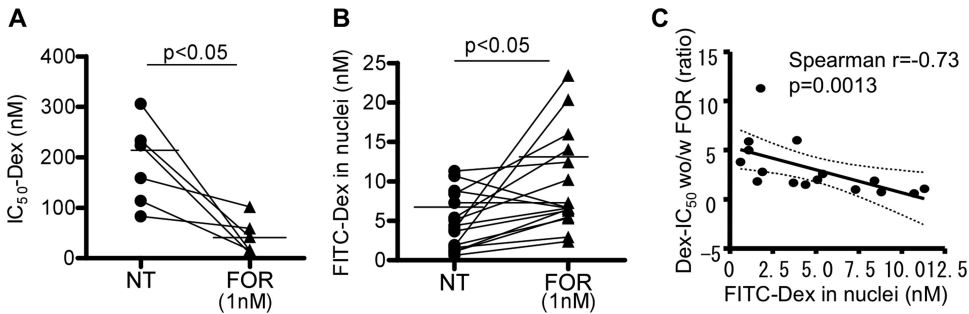
Fig. 2. Effects of FOR (1 nM) on dexamethasone sensitivity to inhibit TNF-α-induced IL-8 production (IC$_{50}$-Dex; A), and GR nuclear translocation determined by FITC-Dex incorporation assay (B) were evaluated in PBMCs obtained from patients with SA. C, correlation between FITC-Dex in nuclei and ratio of Dex-IC$_{50}$ without versus with FOR, as an index of the improvement of corticosteroid sensitivity with FOR.

TABLE 2
Effect of treatment with formoterol and salmeterol on corticosteroid sensitivity

The IC$_{50}$ values of dexamethasone are shown. Values in square brackets are Dex-IC$_{50}$ without treatment/Dex-IC$_{50}$ with LABAs.

	No. Patients Studied	Nontreated	Formoterol (1 nM)	Salmeterol (100 nM)
		nM	*nM*	*nM*
Healthy	7	15.5 ± 4.2	21.5 ± 4.8 [0.72]	31.0 ± 8.3 [0.5]
Mild asthma	6	20.0 ± 3.8	N.D.	14.7 ± 3.5 [1.4]
Severe asthma	6	182.5 ± 28.2	42.8 ± 21.0 [4.3]	N.D.
	15	180.7 ± 28.27	N.D.	83.7 ± 16.7 [2.2]

N.D., not determined.

corticosteroid sensitivity to TNFα-induced IL-8 release, corticosteroid sensitivity to CD3/CD28-induced IL-2 release from PBMC was also decreased in SA (Dex-IC$_{50}$, 77.6 ± 25.4 nM in SA, $n = 8$, versus 11.5 ± 3.3 nM in HV, $n = 7$, and 40.1 ± 16.4 nM in MA, $n = 6$) and reversed by SAL (100 nM) (Dex-IC$_{50}$ with SAL, 30.4 ± 14.4 nM; $p = 0.0065$; FOR was not tested), suggesting that SAL increased Dex sensitivity. In addition, in SA, FOR (1 nM) enhanced GR nuclear translocation as evaluated by FITC-Dex (nuclear FITC-Dex with versus without FOR, 9.5 ± 1.5 versus 4.8 ± 0.9 nM; $p < 0.05$; Fig. 2B). Similar results were found with SAL (100 nM) (nuclear FITC-Dex, with versus without SAL, 14.2 ± 2.7 versus 4.8 ± 0.9 nM; $p < 0.05$; $n = 16$). The improvement in Dex-IC$_{50}$ by FOR (increased ratio of Dex-IC$_{50}$ without FOR versus Dex-IC$_{50}$ with FOR) correlated well with the improvement of FITC-Dex accumulation in nuclei by FOR (decreased ratio of FITC-Dex in nuclei without FOR versus FITC-Dex in nuclei with FOR; Spearman $r = -0.77$, $p = 0.042$). In addition, the improvement in Dex-IC$_{50}$ by FOR was also negatively correlated with FITC-Dex values in nuclei (Spearman $r = -0.73$, $p = 0.0013$; Fig. 2C), suggesting that PBMCs with defect of GR nuclear translocation are more sensitive to FOR treatment.

GRs Were Highly Phosphorylated in PBMCs of Severe Asthma and Dephosphorylated by Formoterol. As shown in Fig. 3, A and B, GR in cytoplasm of PBMCs was highly phosphorylated at serine residues in SA (ratio of phospho-GR/GR, 0.48 ± 0.065; Fig. 3B) compared with those of HV (0.22 ± 0.083; Fig. 3B) and MA (0.25 ± 0.050; not shown). FOR significantly inhibited phosphorylation of GR after 20-min incubation (ratio of phospho-GR/GR, 0.58 ± 0.093 in SA, 0.22 ± 0.037 with 10 nM FOR, 0.25 ± 0.050 with 1 M FOR; $p < 0.05$ versus nontreated SA; Fig. 3C).

IL-2/IL-4 treatment of PBMCs from HV induced corticosteroid insensitivity with impaired GR nuclear translocation in

PBMCs (FITC-Dex in nuclei, with versus without IL-2/IL-4, 2.3 ± 1.1 versus 10.7 ± 1.3 nM; $p < 0.05$). IL-2/IL-4 treatment of PBMCs also induced phosphorylation of GR (ratio of phospho-GR/GR, with versus without IL-2/IL-4, 0.42 ± 0.047 versus 0.11 ± 0.024; $p < 0.05$; Fig. 3, D and E), and FOR significantly inhibited this effect (ratio of phospho-GR/GR, 0.17 ± 0.044 with 10 nM FOR; 0.17 ± 0.048 with 1 nM FOR; for both, $p < 0.05$ versus 0.42 ± 0.047 without FOR; Fig. 3, D and E). In addition, the GR phosphorylated at Ser 226 was determined in same samples. As shown in Fig. 3F, GR phosphorylation at Ser 226 was also significantly ($p < 0.05$) elevated in PBMCs from patients with SA.

p38 MAPK-γ Activation Caused Corticosteroid Insensitivity and Was Inhibited by Formoterol. To determine the kinase activated by 48-h cell incubation with IL-2 and IL-4, which may be involved in the GR phosphorylation (directly or indirectly), kinase phosphorylation array analysis was performed in PBMCs from healthy volunteers. The 48-h exposure to IL-2/IL-4 up-regulated phosphorylation of all four isoforms of p38 MAPK (α, β, γ, and δ), RSK1 and -2, Akt2 (and pan-Akt), JNK2, JNK3 (and pan-JNK), MSK1, HSP27, glycogen synthase kinase-3α and -β, and p70S6 (Fig. 4, A and B). In this experiment, higher concentration of FOR (10 nM) was used to maximize the effect. FOR significantly inhibited only phosphorylation of pan-JNK and p38 MAPK-γ (Fig. 4, A and B). Phosphorylation of p38 MAPK-α and -β was up-regulated by IL-2/IL-4 but was not inhibited by FOR. Western blot analysis also showed that FM (1 and 10 nM) decreased p38MAPK-γ phosphorylation but not p38α phosphorylation (Fig. 4C). In contrast, although p38MAPK α/β inhibitor SB203580 inhibited phosphorylation of HSP27, a p38MAPK-α downstream molecule, it did not inhibit p38MAPK-γ phosphorylation (Fig. 4C). These results were also quantified and shown in Supplemental Fig. 1. Furthermore, inhibitory effects of β-adrenoceptor agonists on phosphorylation of p38MAPK-γ were evaluated by cell-based ELISA. As shown in Fig. 4D, FOR and SAL concentration-dependently inhibited phosphorylation of p38MAPK-γ, and the IC$_{50}$ values were 0.97 and 26 nM, respectively, although salbutamol showed partial inhibition at 100 nM.

siRNAs against p38 MAPK-γ and -δ were transfected to PBMCs from healthy volunteers to obtain knockdowns (KD). After 24 h, mRNA levels of p38MAPK-γ and -δ were reduced by more than 75%. KD cells (and normal cells) were treated with IL-2 and IL-4 for 48 h and stimulated with CD3/CD28 in the presence or absence of Dex to determine Dex-IC$_{50}$ on IL-2 production. The expected IL2/IL-4-induced corticosteroid resistance to CD3/CD28-induced IL-2 release was not seen in cells with p38 MAPK-γ KD by RNA interference (Fig. 5A). By contrast, p38 MAPK-δ KD did not prevent corticosteroid in-

A

p-GR

GR

SA   SA   SA   HV   HV

B

p<0.05

HV   SA

C

p<0.05

FOR   –   –   10nM 1nM

HV   SA

D

pGR

GR

FOR   –   –   –   10nM   1nM

IL-2/IL-4

E

p<0.05

FOR   –   –   10nM 1nM

IL-2/IL-4

F
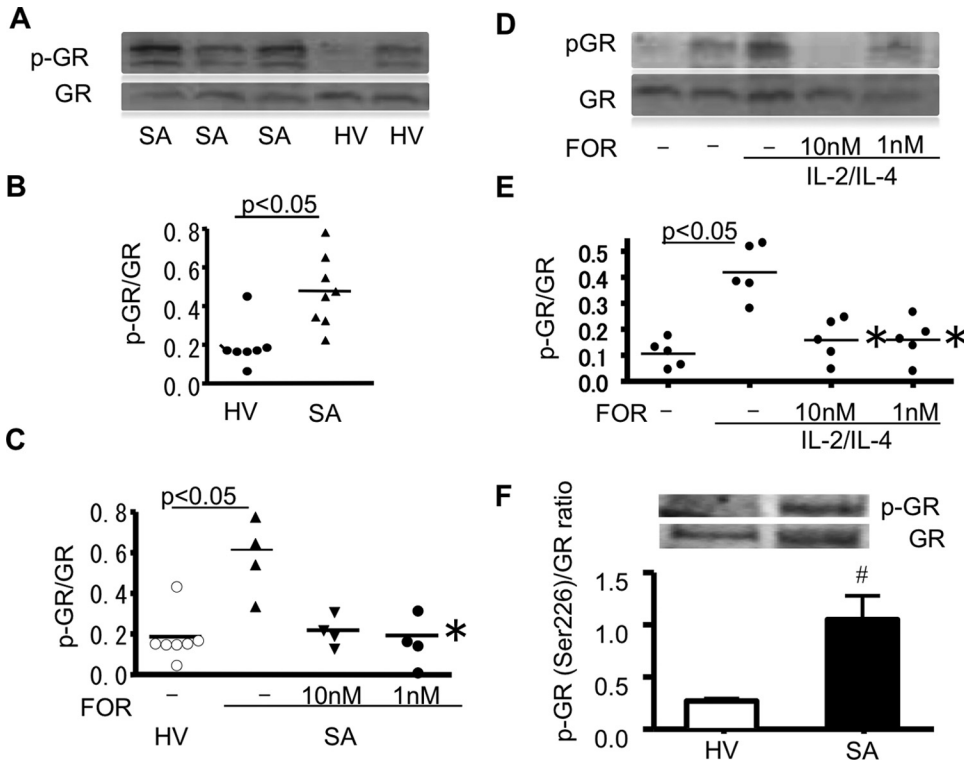

p-GR

GR

p-GR (Ser226)/GR ratio

#

HV   SA

Fig. 3. GR phosphorylation in severe asthma and restoration by formoterol. Serine phosphorylation of immunoprecipitated GR in PBMCs of patients with SA and HV (A and B). Effects of FOR on GR phosphorylation in PBMCs obtained from patients with SA (C) and in IL-2/IL-4-treated PBMCs from HV (D and E). pGR, phosphorylated GR. $*$, $p < 0.05$ versus severe asthma PBMCs (C) or versus IL-2/IL-4-treated PBMCs without FOR (E) by Wilcoxon ranked-pair test. F, GR phosphorylation at Ser226 was also detected in PBMCs from HV ($n = 4$) and patients with SA ($n = 6$); #, $p < 0.05$ for SA versus HV.
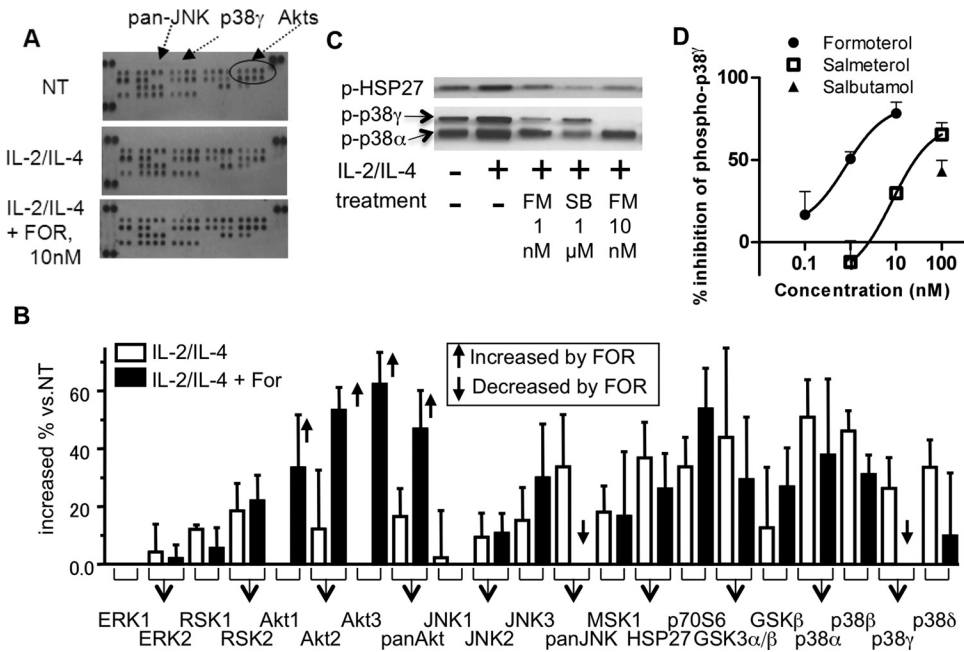
A

pan-JNK   p38γ   Akts

NT

IL-2/IL-4

IL-2/IL-4
+ FOR,
10nM

C

p-HSP27

p-p38γ
p-p38α

IL-2/IL-4   –   +   +   +   +
treatment   –   –   FM   SB   FM
                    1   1   10
                    nM μM nM

D

% inhibition of phospho-p38γ

● Formoterol
□ Salmeterol
▲ Salbutamol

Concentration (nM)

B

☐ IL-2/IL-4
■ IL-2/IL-4 + For

↑ Increased by FOR
↓ Decreased by FOR

increased % vs.NT

ERK1 RSK1 Akt1 Akt3 JNK1 JNK3 MSK1 p70S6 GSKβ p38β p38δ
ERK2 RSK2 Akt2 panAkt JNK2 panJNK HSP27 GSK3α/β p38α p38γ

Fig. 4. Kinase profiling in IL-2/IL-4-treated PBMCs from healthy volunteers. Representative image (A) and densitometric analysis (B) of kinase phosphorylation array. PBMCs were incubated with IL-2/IL-4 for 48 h and then incubated with FOR (10 nM) for 20 min. The density of each dot was calculated, and the percentage increase over nontreatment was determined. Open bars indicate treatment with IL-2/IL-4 alone, and closed bars indicate IL-2/IL-4 treatment with FOR. Significant induction or reduction by FOR are indicated by the bold arrows. Data were plotted as means ± S.E.M. of $n = 3$ independent experiments. C, Western blot of phosphorylation of p38MAPK-α and -γ and HSP27. Cells were treated as shown in A. SB, SB203580. D, cell-based ELISA of p38MAPK-γ phosphorylation. Formoterol, salmeterol, and salbutamol were treated 20 min after 48-h treatment of IL-2/IL-4, and inhibitory effects versus IL-2/IL-4 control were calculated and plotted.

sensitivity (Fig. 5A). Although FOR (1 nM) reversed IL-2/IL-4-dependent corticosteroid insensitivity, SB203580, a selective p38 MAPK-α and -β inhibitor, did not restore corticosteroid sensitivity in IL-2/IL-4-treated PBMCs (Fig. 5A). Improvement of Dex-IC$_{50}$ on CD3/CD28-induced IL-2 release by FOR correlated well with the improvement by p38 MAPK-γ KD ($r = 0.53$, $p = 0.0079$; Fig. 5B) in the same subjects.

U937 cells (a monocytic cell line) were transfected with siRNAs against p38 MAPK-γ or scrambled oligonucleotides as control for 24 h. As shown in Fig. 5C, p38MAPK-γ was clearly knocked down in this condition. Cells were then

treated with IL-2/IL-4 for 48 h and stimulated with TNF-α (1 ng/ml) in the presence or absence of Dex to determine Dex-IC$_{50}$ values. Dex inhibited TNFα-induced IL-8 production with an IC$_{50}$ of 4.6 nM, but Dex potency was decreased in the presence of IL-2/IL-4 (Dex-IC$_{50}$, 36 nM). However, p38 MAPK-γ KD shifted the dose-response curve of Dex leftward (Dex-IC$_{50}$, 9.6 versus 36 nM with scrambled oligonucleotide treatment as a control; all cells were treated with IL-2/IL-4; $p < 0.05$), suggesting that Dex counteracts the development of IL-2/IL-4-induced corticosteroid insensitivity (Fig. 5D). In contrast, KD of p38 MAPK-δ did not affect IL-2/IL-4-induced corticosteroid insensitivity (Dex-IC$_{50}$, 21 versus 36 nM with
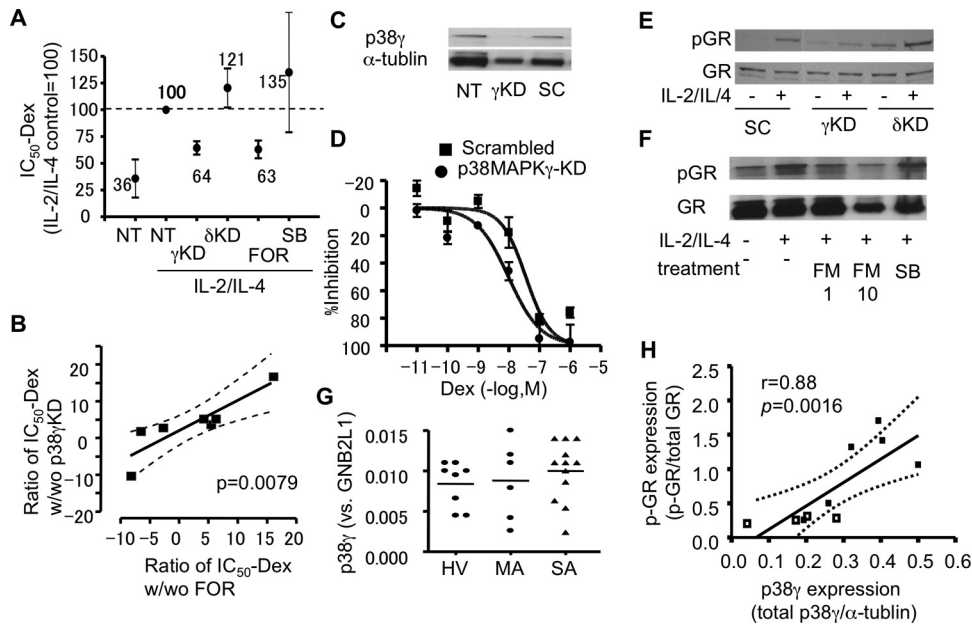
Fig. 5. p38 MAPK-γ causes corticosteroid insensitivity. A, effects of p38 MAPK-γ or -δ KD by RNA interference on IL2/IL-4-induced dexamethasone resistance to CD3/CD28-induced IL-2 release sensitivity in PBMCs from healthy volunteers. FOR (1 nM) or SB-203580 (SB, 1 μM) were also preincubated for 20 min before CD3/CD28 treatment. B, Spearman correlation analysis between formoterol efficacy and p38 MAPK-γ KD efficacy in each sample from healthy volunteer. C, representative image of p38MAPK-γ knockdown in U937 cells. SC, scrambled oligonucleotides. E, effect of p38 MAPK-γ on anti-inflammatory action of dexamethasone in U937 cells on TNF-α-induced IL-8 (D) and GR phosphorylation by IL-2/IL-4. F, effects of FOR (1 or 10 nM) or SB-203580 (SB; 1 μM) on GR phosphorylation in U937 cells. G, p38 MAP-γ mRNA expression in PBMCs from patients with SA, HV, and patients with MA. H. The relationship between phosphorylated GR (p-GR) corrected to total GR expression and p38MAPK-γ protein expression corrected to α-tublin, evaluated by Western blot analysis in PBMCs from three healthy volunteers and six patients with SA (Supplemental Fig. 2).

scrambled oligonucleotide treatment; not significant; data not shown). Furthermore, p38 MAPK-γ KD, but not p38 MAPK-δ KD, also inhibited GR phosphorylation by IL-2/IL-4 (Fig. 5E) where FOR, but not SB203580, inhibited GR phosphorylation in this U937 system (Fig. 5F). When mRNAs of p38MAPKs were determined in PBMCs, there was no significant difference between HV and patients with SA in mRNA encoding p38 MAPK-α, -β, and -δ (data not shown) or p38MAPK-γ (Fig. 5G). However, there was a good correlation between mRNA expression of p38 MAPK-γ and GR nuclear translocation (nuclear FITC-Dex) ($p < 0.05$, date not shown) in patients with SA, suggesting that higher p38MAPK-γ causes defect of GR function. More importantly, when p38MAPK-γ protein expression was analyzed and the level was normalized to α-tubulin expression (a housekeeping gene) in PBMCs from three HV and six patients with SA (Supplemental Fig. 2, there was significant correlation between GR phosphorylation and p38MAPK-γ protein expression (Fig. 5H). For further confirmation of role of p38MAPK-γ in corticosteroid insensitivity, p38MAPK-γ was also overexpressed in U937 cells, and Dex-IC_{50} was determined. The U937 cells with p38MAPK-γ overexpression showed significantly higher Dex-IC_{50} value on TNFα-induced IL-8 release after IL-2/IL-4 treatment (Supplemental Fig. 3).

Discussion

Severe asthma is characterized by corticosteroid insensitive inflammation. We showed here that IC_{50} value of Dex on TNF-α stimulated IL-8 release in PBMCs of patients with SA was higher by approximately 10-fold than those of HV or patients with MA, suggesting that PBMCs from patients with SA were also steroid-insensitive in vitro.

At a molecular level, the reduction in corticosteroid responsiveness observed in cells from patients with SA has been ascribed to a reduced number of GR, altered affinity of the ligand for GR, reduced ability of the GR to bind to DNA, increased expression of inflammatory transcription factors (such as activator protein-1) that compete for DNA binding, or reduction of histone deacetylase-2 (Adcock et al., 2006). In this study, there were no significant differences in GRα mRNA and protein expression. GRβ mRNA expression likely increased in patients with SA but the difference was not significant, possibly because of lack of power; however, several reports have shown that GRβ overexpression was not critical to corticosteroid insensitivity in patients with SA (Irusen et al., 2002; Torrego et al., 2004). Irusen et al. (2002) also demonstrated that GR affinity in nuclei was decreased in patients with SA, although we did not analyze the GR function in nuclei in present study (Irusen et al., 2002). In contrast, we found the defect of GR in cytoplasm.

An increase of activated GR in nuclei is critical for GR action. For the detection of ligand-bound GR in nuclei, we determined the amount of FITC-conjugated Dex in nuclei instead of using classic immunocytochemistry or Western blotting methods, which are not quantitative, but are time-consuming and require a large number of cells. Because the anti-inflammatory efficacy of FITC-conjugated Dex is 10-fold weaker than that of unlabeled Dex on IL-1β-induced IL-8 production in A549 cells, we used a relatively high concentration (10^{-6} M) of FITC-Dex for the assay. There was a good correlation between percentage of GR positive nuclei in immunocytochemistry assay and the absolute value of FITC-Dex in nuclei in PBMC of healthy volunteers (data not shown, $r = 0.65$, $p < 0.05$), suggesting that this FITC-Dex

method is useful for quantification of GR nuclear translocation in a small number of cells. We demonstrated that GR nuclear translocation was significantly impaired in SA PBMCs (Fig. 1C), which is supported by previous report (Matthews et al., 2004). In addition, there was a significant, negative correlation between the IC_{50}-Dex value on TNF-α-induced IL-8 release and the amount of FITC-Dex in nuclei, indicating that patients with less GR nuclear translocation are more corticosteroid insensitive (Spearman $r = -0.55$ $p = 0.0035$). LABAs are reported to be able to enhance corticosteroid sensitivity in several in vitro systems and clinical trials, even in our PBMC systems; we also confirmed that add-on-treatment with FOR (1 nM) and SAL (100 nM) decreased the Dex-IC_{50} value for TNF-α-induced IL-8 release in PBMCs from patients with SA (Fig. 2A), although the efficacy of SAL on restoration of corticosteroid sensitivity was weaker than that of FOR. Neither FOR nor SAL changed corticosteroid sensitivity in HV and patients with MA (Table 2). In addition, FOR (1 nM) enhanced GR nuclear translocation evaluated with FITC-Dex in patients with SA (Fig. 2B). The levels of corticosteroid sensitivity and restoration by FOR or SAL were not affected by current medication (oral steroid or inhaled steroid therapy; data not shown). The improvement in Dex-IC_{50} with FOR (ratio of Dex-IC_{50} with versus without FOR) correlated well with the improvement of FITC-Dex accumulation in nuclei by FOR (Spearman $r = -0.77$, $p = 0.042$), suggesting that FOR reversed corticosteroid insensitivity by enhancement of GR nuclear translocation. The improvement in Dex-IC_{50} with FOR was negatively correlated with basal FITC-Dex values in nuclei (Spearman $r = -0.73$; $p = 0.0013$; Fig. 2C), suggesting that patients with less GR nuclear translocation are more sensitive to FOR-dependent reversal of corticosteroid resistance.

GR are reported to be phosphoproteins, and phosphorylation of inactive GR may block subsequent hormone binding and affect GR subcellular localization and GR nuclear cytoplasmic trafficking through the nuclear pore complex (Ismaili and Garabedian, 2004). We demonstrated that GR in cytoplasm was highly phosphorylated at serine residues in patients with SA compared with those of HV and patients with MA (Fig. 3, A and B), and there was a good correlation between GR phosphorylation and FITC-Dex nuclear translocation (as an index of capability of GR on nuclear translocation). Phosphorylation of Ser226 is reported to cause defect of GR-mediated transcriptional activation (Rogatsky et al., 1998) or enhancement of nuclear export (Itoh et al., 2002). Very interestingly, the level of GR phosphorylation at Ser226 was higher in patients with SA compared with HV (Fig. 3F). Even more importantly, FOR significantly inhibited phosphorylation of GR (Fig. 3C). IL-2/IL-4 treatment, which is known to mimic corticosteroid insensitivity seen in SA (Kam et al., 1993; Larsson et al., 1997; Irusen et al., 2002), also induced phosphorylation of GR (Fig. 3, D and E) and significantly inhibited by FOR (Fig. 3, D and E).

Several kinases, such as MAPK, cyclin-dependent kinase, glycogen synthase kinase-3, and JNK are reported to phosphorylate GR, each of them having distinct specificities for potential phosphorylation sites (Ito et al., 2006). Kinase phosphorylation array analysis demonstrated that IL-2/IL-4 treatment up-regulated phosphorylation of several stress kinases, including p38 MAPK (α, β, γ, δ) and JNK (Fig. 4, A and B). Very interestingly FOR (10^{-8} M) significantly inhibited

only phosphorylation of pan-JNK and p38 MAPK-γ (Fig. 4, A and B). GR Ser226 phosphorylation is reported to be catalyzed by JNK and to inactivate GR (Rogatsky et al., 1998). The leucine-rich sequences flanking Ser^{246} in rat GR (Ser226 in human GR) are also reported to be involved in nuclear export (Itoh et al., 2002), and phosphorylation of this site may increase GR nuclear export as the means of inactivating GR transcriptional enhancement, leading eventually to accumulation of phosphorylated GR in the cytoplasm. Thus, JNK will be a key kinase on GR phosphorylation in this finding, but the roles of p38 MAPK-γ on GR phosphorylation and corticosteroid effects have not previously been reported.

In U937 cells and PBMCs, where p38 MAPK-γ was knocked down by RNA interference, IL2/IL-4 exposure did not induce corticosteroid insensitivity (Fig. 5, A and D). Furthermore, p38 MAPK-γ KD, but not p38 MAPK-δ KD, inhibited GR phosphorylation by IL-2/IL-4 (Fig. 5E). Thus, p38 MAPK-γ seems to be a key kinase regulating corticosteroid sensitivity, probably by phosphorylation of cytoplasmic GR. In fact, PBMCs with higher levels of GR phosphorylation at Ser226 showed higher levels of mRNA expression of p38 MAPK-γ (Fig. 5H). The p38MAPK-γ protein expression was also higher in patients with SA than in healthy subjects (Supplemental Fig. 2). Further analysis with p38MAPK-γ overexpression in U937 cells (Supplemental Fig. 3) supported the finding that p38MAPK-γ overexpression in SA is likely to be one of molecular mechanisms of steroid insensitivity.

In our system, FOR converted phosphorylated GR and p38 MAPK-γ to nonphosphorylated forms within 20 min after IL-2/IL-4 treatment for 48 h, suggesting that FOR might dephosphorylate GR via dephosphorylation of p38 MAPK-γ rather than by inhibiting p38 MAPK-γ directly. In fact, FOR (or SAL) (100, 1000 nM) did not directly inhibit p38 MAPK-γ kinase activity [kinase profiler assay (Millipore, Billerica, MA; data not shown]. That is, FOR might enhance a specific phosphatase to dephosphorylate p38 MAPK-γ. The phosphatase that specifically dephosphorylates p38 MAPK-γ has not yet been identified. However, cAMP-PKA signals are reported to enhance activity of protein phosphatase (PP)2A (Feschenko et al., 2002). PP2C is also known as cAMP-coupled phosphatase (Yokoyama et al., 1995), and PP1 and PP5
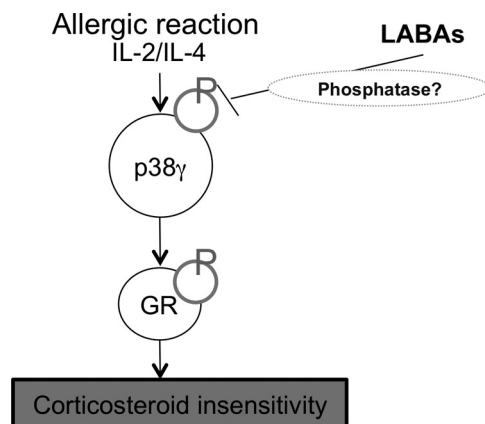

Fig. 6. Precise mechanism of formoterol on restoration of corticosteroid sensitivity in severe asthma. Severe allergic inflammation associated with production of IL-2 and IL-4 induced corticosteroid insensitivity via GR phosphorylation by p38MAPK-γ activation. Formoterol inhibits p38MAPK-γ phosphorylation, possibly via phosphatase activation, and inhibits GR phosphorylation.

are reported to be involved in GR localization (DeFranco et al., 1991; Dean et al., 2001; Hinds and Sánchez, 2008). Further studies will be required to clarify the molecular mechanism of LABA on dephosphorylation of p38MAPK-γ (and GR).

Thus, LABAs restored corticosteroid sensitivity defected by phosphorylation (Fig. 6). Corticosteroid itself is reported to increase β-adrenoceptor expression (Aksoy et al., 2002) and also LABAs are reported to enhance GR nuclear translocation(Eickelberg et al., 1999; Usmani et al., 2005). This should be a self-enforcing loop induced by the combination therapy of LABA and inhaled corticosteroid. Corticosteroid-insensitive severe diseases are heterogeneous, but our study demonstrated that at least a subpopulation of patients with SA is characterized by defective GR nuclear translocation and GR hyperphosphorylation, which were reversed by LABA via a p38 MAP kinase-γ-dependent mechanism (Fig. 6). Our studies provide new insights into the regulation of inflammation and raise the prospects of new classes of compounds to treat SA and other inflammatory diseases.

Authorship Contributions

Participated in research design: Mercado, To, Adcock, and Ito.
Conducted experiments: Mercado, To, Kobayashi, and Ito.
Performed data analysis: Mercado, To, Kobayashi, and Ito.
Wrote or contributed to the writing of the manuscript: Barnes and Ito.

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