

Mechanism of glucocorticoid protection of airway smooth muscle from proasthmatic effects of long-acting β 2-adrenoceptor agonist exposure

Gustavo Nino, MD, Aihua Hu, MD, PhD, Judith S. Grunstein, MA, and Michael M. Grunstein, MD, PhD Philadelphia, Pa

Background: Chronic use of long-acting β 2-adrenergic receptor agonists (LABAs), resulting in β 2-adrenergic receptor desensitization, has been associated with increased asthma morbidity. When LABAs are used in combination with inhaled glucocorticoids, however, asthma control is improved, raising the following question: Do glucocorticoids inhibit the proasthmatic mechanism that mediates altered contractility in LABA-exposed airway smooth muscle (ASM)?

Objective: This study aimed to identify the potential protective role and mechanism of action of glucocorticoids in mitigating the effects of prolonged LABA exposure on ASM constrictor and relaxation responsiveness.

Methods: Cultured human ASM cells and isolated rabbit ASM tissues were examined for induced changes in agonist-mediated cyclic adenosine monophosphate accumulation, constrictor and relaxation responsiveness, and expression of specific glucocorticoid-regulated molecules after 24-hour exposure to the LABA salmeterol in the absence and presence of dexamethasone.

Results: Salmeterol-exposed ASM exhibited impaired cyclic adenosine monophosphate and relaxation responses to isoproterenol and increased acetylcholine-induced contractility. These proasthmatic effects of prolonged LABA exposure were attributed to upregulated phosphodiesterase 4 (PDE4) activity and were ablated by pretreatment with dexamethasone. Further studies demonstrated that (1) dexamethasone suppressed activation of the mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 (ERK1/2), which upregulate PDE4 expression in salmeterol-exposed ASM; and (2) the inhibitory actions of dexamethasone on salmeterol-induced ERK1/2 activation and resultant PDE4-mediated changes in ASM responsiveness were prevented by gene silencing or pharmacologic inhibition of dexamethasone-induced expression of mitogen-activated protein kinase phosphatase 1, an endogenous deactivator of ERK1/2 signaling.

Conclusion: Glucocorticoids prevent the adverse proasthmatic effects of prolonged LABA exposure on airway responsiveness

as a result of glucocorticoid-induced upregulation of mitogen-activated protein kinase phosphatase 1, which inhibits proasthmatic ERK1/2 signaling in the LABA-exposed ASM. (J Allergy Clin Immunol 2010;■■■■:■■■-■■■.)

Key words: Asthma, salmeterol, homologous β 2-adrenergic receptor desensitization, airway smooth muscle, phosphodiesterase, ERK1/2 phosphorylation, siRNA, MAPK phosphatase 1

Although inhaled β 2-adrenergic receptor (β 2AR) agonists are commonly used to treat asthma, their chronic use in long-acting preparations can result in airway tolerance to β 2AR stimulation. This loss of bronchodilator effect is a result of the development of homologous β 2AR desensitization and has been associated with worsening of bronchial hyperreactivity and an increased incidence of asthma morbidity.¹⁻⁴ Conversely, when used in combination with inhaled glucocorticoids, long-acting β 2AR agonists (LABAs) have been shown to improve asthma control and alleviate airway inflammation.⁵⁻⁹ Apart from reducing inflammation, the beneficial effects of this combinational therapy may reflect a complementary interaction between β 2AR and glucocorticoid signaling in regulating airway smooth muscle (ASM) function under proasthmatic conditions.¹⁰ Moreover, the therapeutic benefit of combined glucocorticoid and LABA therapy may also relate to attenuation of the adverse effects of β 2AR desensitization, because previous studies demonstrated that glucocorticoids can reverse β 2AR tolerance in isolated human bronchi and small airways exposed to a short-acting β 2AR agonist.^{11,12} To date, the role and mechanism by which glucocorticoids potentially mitigate the proasthmatic effects of homologous β 2AR desensitization on ASM constrictor and relaxation responsiveness remains unidentified.

We recently reported that prolonged exposure of cultured human ASM (HASM) cells and isolated rabbit ASM tissues to the LABA, salmeterol, leads to impaired acute β 2AR-induced cyclic adenosine monophosphate (cAMP) accumulation and proasthmatic changes in ASM tissue responsiveness that are mediated by upregulated phosphodiesterase 4 (PDE4) activity in the β 2AR-desensitized ASM.¹³ Moreover, the increase in PDE4 activity was attributed to β 2AR-induced activation of the inhibitory G protein $\beta\gamma$ subunit (Gi- $\beta\gamma$) via cAMP-dependent protein kinase A (PKA), which then activates the mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 (ERK1/2), which leads to transcriptional upregulation of PDE4 expression.¹³ Given this new information, the current study was undertaken to determine the potential role and mechanism of action of glucocorticoids in protecting ASM from the adverse effects of prolonged homologous β 2AR desensitization as a result of LABA exposure

From the Children's Hospital of Philadelphia Research Institute, University of Pennsylvania School of Medicine.

Supported by NIH grants HL-031467, HL-061038, and HL097739.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication October 29, 2009; revised February 3, 2010; accepted for publication February 4, 2010.

Reprint requests: Michael M. Grunstein, MD, PhD, Division of Pulmonary Medicine, Abramson Research Building, Children's Hospital of Philadelphia, 34th Street and Civic Center Blvd, Philadelphia, PA 19104. E-mail: grunstein@email.chop.edu.

0091-6749/\$36.00

© 2010 American Academy of Allergy, Asthma & Immunology

doi:10.1016/j.jaci.2010.02.007

Abbreviations used

ASM: Airway smooth muscle
 β 2AR: β 2-Adrenergic receptor
 cAMP: Cyclic adenosine monophosphate
 ERK1/2: Extracellular signal-regulated kinases 1 and 2
 HASM: Human airway smooth muscle
 LABA: Long-acting β 2-adrenergic receptor agonist
 MKP-1: Mitogen-activated protein kinase phosphatase 1
 PDE4: Phosphodiesterase 4
 scRNA: Scrambled RNA
 siRNA: Small interfering RNA
 Tmax: Maximal isometric contractile force

on ASM responsiveness. The results are the first to demonstrate that (1) glucocorticoid-treated ASM is protected from the proasthmatic effects of prolonged exposure to a LABA on ASM constrictor and relaxation responsiveness, and (2) this protective action is attributed to glucocorticoid-induced expression of mitogen-activated protein kinase phosphatase 1 (MKP-1), which suppresses ERK1/2 signaling and its consequent induction of PDE4 activity in the β 2AR-desensitized state. These new findings highlight a heretofore unidentified mechanism that may play a pivotal role in contributing to the known therapeutic efficacy of using glucocorticoids in combination with LABAs to treat asthma, and suggest that interventions targeted at this mechanism may yield new therapeutic approaches to improve asthma control.

METHODS**Materials**

Human ASM cells were obtained from Bio Whittaker, Inc (Walkersville, Md), and all chemicals were purchased from Sigma-Aldrich (St Louis, Mo) unless otherwise indicated.

Animals

Sixteen adult New Zealand White rabbits were used in this study, which was approved by the Biosafety and Animal Research Committee of the Research Institute at Children's Hospital of Philadelphia.

Culture and treatment of cells

Human ASM cells were cultured in growth medium supplemented with 10% FBS (Bio Whittaker, Inc, Walkersville, Md) and maintained in an incubator containing 5% CO₂ in air at 37 °C. After attaining ~95% confluence, the cells were starved in unsupplemented Ham's F12 media for 24 hours, subsequently treated with salmeterol or isoproterenol, and then examined for β 2AR agonist-induced cAMP accumulation, PDE activity, ERK1/2 phosphorylation, and PDE4D5 mRNA expression under different experimental conditions.

cAMP accumulation and PDE activity

Confluent HASM cells were pretreated for 24 hours with either vehicle alone or salmeterol (10 μ M/L) both in the absence and presence of a predetermined maximally effective concentration of dexamethasone (1 μ M/L). In the continued presence of these treatments, cells were subsequently exposed for 5 minutes to isoproterenol (10 μ M/L), and intracellular cAMP levels were detected by using a cAMP enzyme immunoassay kit (R&D Systems, Minneapolis, Minn).^{13,14} In separate studies, total cAMP PDE activity was measured in cell lysates by using a colorimetric enzymatic assay (Biomol, Plymouth Meeting, Pa)¹³ after exposure of cells for 24 hours to vehicle, salmeterol, or isoproterenol in the absence or presence of dexamethasone under different cotreatment conditions, as described. The levels of cAMP and PDE activity were standardized to the cellular protein content.

Detection of PDE4D5 mRNA

As previously described,^{13,14} PDE4D5 transcripts were detected after extraction of total RNA, and cDNAs were isolated by RT-PCR using the Superscript First Strand Synthesis System kit (Invitrogen, Carlsbad, Calif), with the following oligonucleotide primer sets (Integrated DNA Technologies, Coralville, Iowa): for PDE4D5, 5'-TGCCAGCTGTACAAAGTTGACC-3' (forward) and 5'TTCTCGGAGAGATCACTGGAGA-3' (reverse); and for β -actin, 5'-GAAGAGCTACGAGCTGCCTGAC-3' (forward) and 5'-CGGAGTACTT GCGCTCAGGAGGAG-3' (reverse). The reaction volume was 20 μ L, and cycling conditions used were 35 cycles of 30-second denaturation at 95 °C, followed by 30-second annealing at 60 °C and elongation at 72 °C for 30 seconds. Ex-Tag (Takara Biotechnology, Pittsburgh, Pa) was used as DNA polymerase.

Protein expression of ERK1/2 and MKP-1

Phosphorylated and total ERK1/2 and MKP-1 protein levels were detected by Western blotting in HASM cells before and after treatment with salmeterol, isoproterenol, or dexamethasone alone and in combination. After protein extraction, lysates were loaded on a 10% SDS-PAGE gel for immunoblotting and, after transfer to a polyvinylidene fluoride membrane, membranes were incubated overnight with monoclonal mouse antihuman primary antibodies directed against phospho-ERK1/2 and total ERK1/2, or a polyclonal goat antihuman primary antibody directed against MKP-1 (Imgenex Corp, San Diego, Calif). Extracellular signal-regulated kinase levels were detected by enhanced chemiluminescence after incubation with a 1:2000 dilution of horseradish peroxidase-conjugated rabbit antimouse secondary antibody, followed by exposure to autoradiography film. MKP-1 levels were detected with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Neb) by using a 1:10,000 dilution of donkey anti-goat secondary antibody. The protein band intensities were quantified by densitometry.

Small interfering RNA knockdown of MKP-1

Human ASM cells were seeded into 6-well plates and, at ~40% confluency, the medium was replaced with the reduced serum-containing medium Opti-MEM (Invitrogen, Carlsbad, Calif). As previously described,^{13,14} cells were then transfected twice during a 24-hour interval with a pool of 3 small interfering RNA (siRNA) duplexes targeted against human MKP-1 (Applied Biosystems, Foster City, Calif; siRNA identification nos. 5173,105846, and 146461), or with a nontargeted scrambled RNA (scRNA) duplex serving as a negative control, using Oligofectamine (Invitrogen, Carlsbad, Calif) as the transfection agent. Each siRNA preparation was administered in a final concentration of 100 nmol/L. In separate experiments, isolated rabbit ASM tissues were similarly transfected with the same siRNA preparations. On the basis of preliminary studies, this transfection approach was found to greatly enhance transfection efficiency, with dexamethasone-induced MKP-1 protein expression inhibited by 78% to 91% in the siRNA-treated cells, and MKP-1 mRNA expression inhibited by 64% to 79% in the ASM tissues.

Pharmacodynamic studies of constrictor and relaxation responsiveness in rabbit ASM tissues

After initial general anesthesia with intramuscular ketamine (50 mg/kg), rabbits were killed with an intravenous overdose of sodium pentobarbital (100 mg/kg). As previously described,^{13,14} the tracheae were excised, loose connective tissue and epithelium were removed, and the tracheae were divided into ring segments that were incubated for 24 hours with either vehicle (control) or salmeterol, each administered under different pretreatment conditions, as described. The tissues were then placed in organ baths containing the same concentrations of their respective pharmacological treatments in modified Krebs-Ringer solution aerated with 5% CO₂ in O₂ and attached to force transducers to monitor isometric tension. Cholinergic contractility to cumulatively administered acetylcholine (10⁻⁹ to 10⁻³ mol/L) was assessed and, after rinsing with fresh buffer to remove the administered acetylcholine, the pharmacologic agents were immediately reintroduced into the organ baths and, ~20 minutes thereafter, relaxation dose-response curves to isoproterenol (10⁻⁹ to 10⁻⁴ mol/L) were generated after the tissues were initially half-maximally contracted with acetylcholine. The constrictor and relaxation

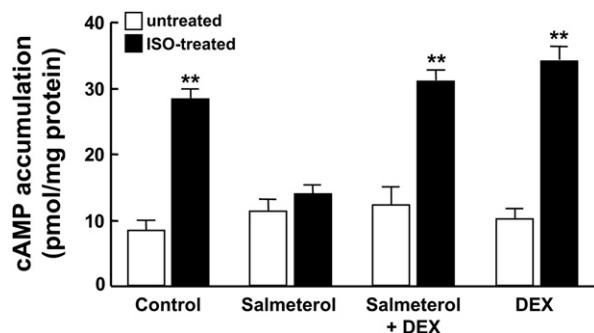


FIG 1. Dexamethasone (DEX) prevents impaired acute β 2AR-induced cAMP accumulation in salmeterol-exposed HASM cells. In contrast with control cells, acute isoproterenol (ISO)-induced cAMP accumulation is suppressed in HASM cells pre-exposed for 24 hours to salmeterol. By comparison, ISO-induced cAMP accumulation is preserved in salmeterol-exposed HASM cells that are cotreated with DEX. Data represent means $\pm$ SEs from 4 paired experiments. ** $P < .01$ compared with untreated cells by using the paired t test.

dose-response curves were analyzed with respect to each tissue's maximal isometric contractile force (Tmax) to acetylcholine and maximal relaxation response to isoproterenol.

Statistical analyses

Results are expressed as means $\pm$ SEs. Comparisons between groups were made by using the Student t test (2-tailed) or ANOVA with Tukey posttest analysis, where appropriate. A probability of $< .05$ was considered statistically significant. Statistical analyses were conducted by using the Prism computer program by Graph Pad Software Inc (San Diego, Calif).

RESULTS

Glucocorticoid prevention of homologous β 2AR desensitization in HASM cells

To evaluate the role of glucocorticoids in modulating ASM function in the β 2AR-desensitized state, we initially examined the effect of pretreatment with dexamethasone on isoproterenol-induced cAMP accumulation in HASM cells after their prolonged homologous β 2AR desensitization. Accordingly, acute changes in intracellular cAMP accumulation, detected at 5 minutes after administration of a near half-maximal effective concentration of isoproterenol ($1.0 \mu\text{mol/L}$),^{13,14} were compared in HASM cells that were pretreated for 24 hours with either vehicle alone (control) or a maximally effective concentration of the LABA salmeterol ($10 \mu\text{mol/L}$)¹³ both in the absence and presence of cotreatment with dexamethasone. As shown in Fig 1, without dexamethasone, salmeterol-exposed HASM cells exhibited homologous β 2AR desensitization, as evidenced by markedly suppressed cAMP responses to isoproterenol relative to those detected in control cells. Contrasting these observations, the cAMP responses to isoproterenol were preserved in salmeterol-exposed cells that were cotreated with a predetermined maximally effective concentration of dexamethasone ($1 \mu\text{mol/L}$), and similar to those detected in cells exposed to dexamethasone alone.

Glucocorticoids prevent altered ASM responsiveness induced by prolonged β 2AR desensitization

We recently demonstrated that prolonged exposure of isolated rabbit ASM tissues to salmeterol elicits proasthmatic-like changes in ASM constrictor and relaxation responsiveness that

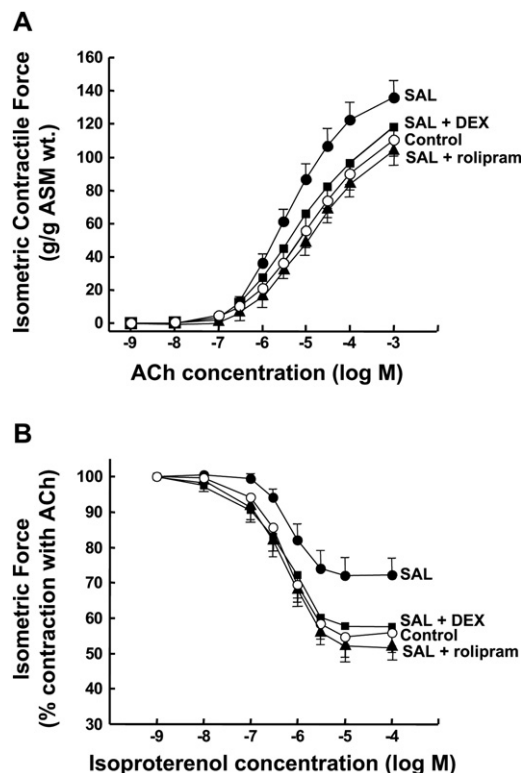


FIG 2. Dexamethasone (DEX) prevents salmeterol (SAL)-induced changes in agonist responsiveness in rabbit ASM tissues. Relative to controls, ASM tissues exposed to SAL exhibit significantly increased contractility to acetylcholine (ACh; A) and impaired relaxation to isoproterenol (B). Pretreatment with either rolipram or DEX prevents the SAL-induced changes in ASM responsiveness. Data represent means $\pm$ SEs from 6 to 7 experiments. ANOVA used for multiple comparisons of mean Tmax values.

are prevented by pretreating the tissues with the selective PDE4 inhibitor rolipram.¹³ To determine whether glucocorticoids exert a comparable protective action, agonist-induced constrictor and relaxation responses were compared in isolated rabbit ASM tissues that were exposed for 24 hours to either vehicle alone (control) or salmeterol ($10 \mu\text{mol/L}$), both in the absence and presence of pretreatment with either dexamethasone ($1 \mu\text{mol/L}$) or rolipram ($10 \mu\text{mol/L}$). Relative to their respective controls, the salmeterol-exposed ASM tissues exhibited significantly increased constrictor responsiveness to cumulative administration of acetylcholine (Fig 2, A), yielding a mean $\pm$ SE Tmax of 136.0 ± 10.3 versus 110.7 ± 7.2 g/g ASM weight in the control tissues ($P < .05$). Similar to the inhibitory effect of pretreatment with rolipram,¹³ this enhanced constrictor responsiveness was also completely abrogated in salmeterol-exposed tissues that were pretreated with dexamethasone.

Under the same treatment conditions, cumulative administration of isoproterenol produced dose-dependent acute relaxation of preconstricted ASM segments (Fig 2, B). Relative to controls, the relaxation responses were significantly attenuated in the salmeterol-exposed tissues, consistent with their development of homologous β 2AR desensitization. Accordingly, the mean $\pm$ SE maximal relaxation response in the salmeterol-exposed tissues amounted to $27.8 \pm 4.9\%$ versus $44.2 \pm 5.5\%$ in the controls ($P < .05$). This impaired relaxant responsiveness was also completely abrogated in salmeterol-exposed tissues that were pretreated with either rolipram or dexamethasone. Of note, in

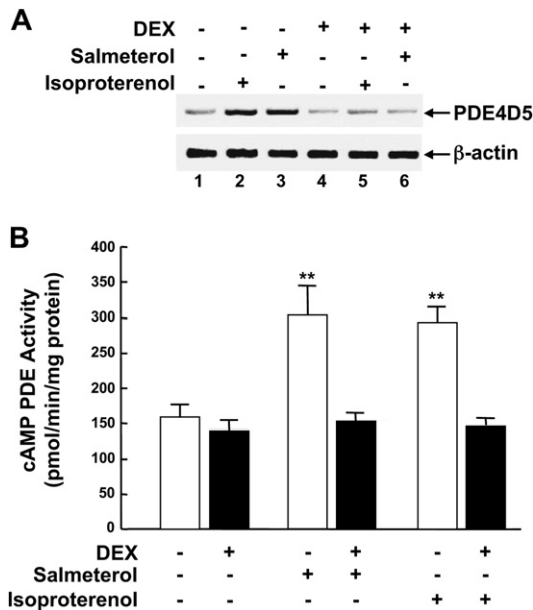


FIG 3. Dexamethasone (DEX) prevents β 2AR agonist-induced upregulation of PDE4 in HASM cells. Relative to untreated controls, PDE4D5 mRNA expression (**A**) and cAMP PDE activity (**B**) are upregulated in HASM cells exposed to either salmeterol or isoproterenol. β 2AR agonist-induced upregulation of PDE4D5 transcripts and PDE activity is abrogated in cells pretreated with DEX. Data are means $\pm$ SEs from 5 experiments. ** $P < .01$ compared with untreated cells by using the paired t test.

separate experiments, relative to controls, ASM tissues that were treated with either dexamethasone or rolipram alone showed no significant changes in responsiveness to either acetylcholine or isoproterenol (data not shown).

Glucocorticoid suppression of PDE4 expression in β 2AR-desensitized ASM

Because transcriptional upregulation of PDE4 was found to mediate the above rolipram-sensitive changes in ASM responsiveness after its prolonged homologous or heterologous β 2AR desensitization,^{13,14} we next examined whether dexamethasone modulates PDE4 expression in the β 2AR-desensitized state. HASM cells were initially exposed to vehicle, salmeterol (10 μ mol/L), or the shorter-acting β 2AR agonist isoproterenol (10 μ mol/L), all in the absence and presence of cotreatment with dexamethasone (1 μ mol/L), and then examined for induced changes in mRNA expression of PDE4D5, the functionally relevant PDE4 isoform in HASM.¹⁵ As previously described,¹³ relative to control cells (Fig 3, A, lane 1), HASM cells exposed to either isoproterenol or salmeterol for 6 hours (ie, time to maximal response) exhibited upregulated expression of PDE4D5 mRNA transcripts (lanes 2 and 3). Whereas constitutive transcript levels were essentially unaltered in cells exposed to dexamethasone alone (lane 4), induction of PDE4D5 mRNA transcripts by the β 2AR agonists was completely suppressed in cells that were cotreated with dexamethasone (lanes 5 and 6). As shown in Fig 3, B (open bars), comparable changes in total cAMP PDE activity were also detected under these experimental conditions. Accordingly, relative to control cells, HASM cells exposed for 24 hours to either isoproterenol or salmeterol exhibited similar significant increases in PDE activity that averaged approximately 2-fold above control ($P < .01$). Basal PDE activity was little affected in cells that were treated with

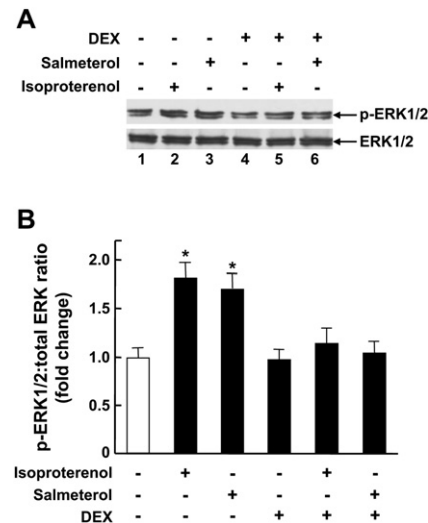


FIG 4. A, HASM cells exposed to either salmeterol or isoproterenol exhibit increased ERK1/2 phosphorylation that is prevented by pretreating the β 2AR agonist-exposed cells with dexamethasone (DEX). B, Corresponding densitometric analysis demonstrates similar increases in ERK1/2 phosphorylation (p-ERK1/2) in salmeterol-exposed and isoproterenol-exposed cells that are prevented by pretreatment with DEX. Data are means $\pm$ SEs from 4 determinations under each treatment condition. * $P < .05$ compared with untreated cells by using the paired t test.

dexamethasone alone, whereas induction of PDE activity by the β 2AR agonists was completely abrogated in ASM cells that were cotreated with dexamethasone (Fig 3, B, filled bars).

Glucocorticoid suppression of ERK1/2 activation in β 2AR-desensitized ASM

In relation to the above observations, it should be noted that we previously demonstrated that the salmeterol-induced upregulation of PDE4 activity and associated PDE4-dependent changes in ASM constrictor and relaxation responsiveness are completely prevented by pretreating the ASM tissues with an inhibitor of either PKA or ERK1/2, and that these effects are reflective of PKA-dependent ERK1/2 activation in the β 2AR-desensitized ASM.¹³ Given this evidence, we examined whether β 2AR agonist-induced activation of ERK1/2 is glucocorticoid-sensitive. As previously described,¹³ treatment of HASM cells with 10 μ mol/L of either isoproterenol or salmeterol acutely elicits increased phosphorylation of ERK1/2 proteins that peaks at 20 minutes and is sustained for at least 80 minutes. Accordingly, the effect of pretreatment with dexamethasone (1 μ mol/L $\times$ 24 hours) on ERK1/2 phosphorylation was assessed in HASM cells at 60 minutes after exposure to 10 μ mol/L salmeterol or isoproterenol. A representative immunoblot in Fig 4, A, demonstrates that, relative to control cells (lane 1), β 2AR agonist-exposed cells exhibit increased ERK1/2 phosphorylation (lanes 2 and 3) and, although pretreatment with dexamethasone alone has no effect (lane 4), the induction of ERK1/2 phosphorylation is suppressed in β 2AR agonist-exposed cells that are pretreated with dexamethasone (lanes 5 and 6). The results obtained in 4 experiments demonstrated that isoproterenol and salmeterol elicited similar increases in ERK1/2 phosphorylation, expressed as fold-changes in the densitometric ratios of phosphorylated ERK1/2 to total ERK1/2, that averaged $\sim$ 1.8-fold above that detected in control cells ($P < .01$), and that, in contrast with the lack of effect

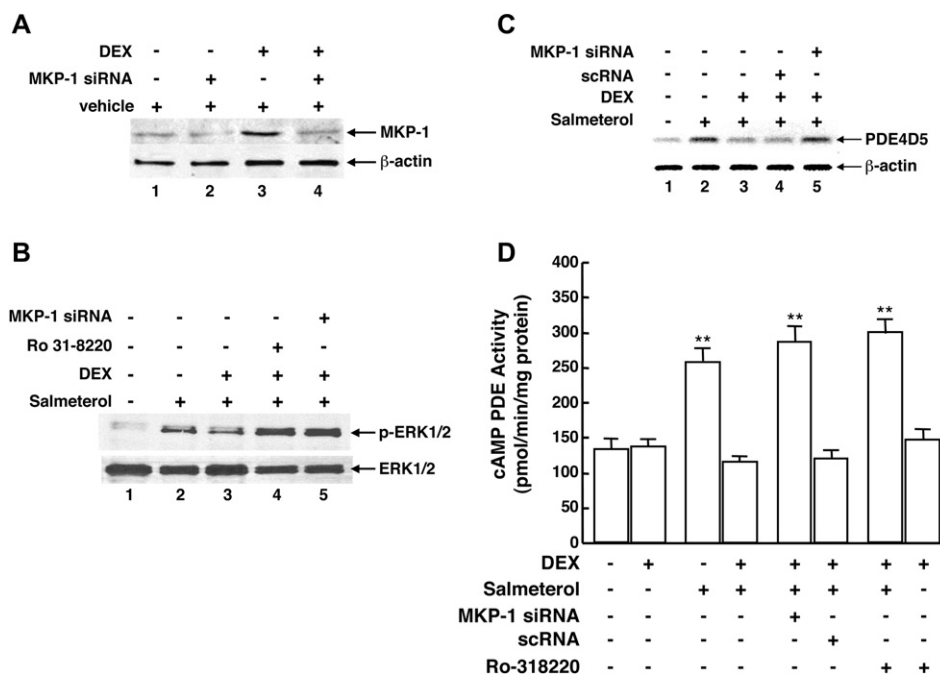


FIG 5. A, Dexamethasone (DEX)-induced upregulation of MKP-1 protein is prevented in HASM cells transfected with MKP-1 siRNA duplexes. DEX-induced suppression of upregulated ERK1/2 phosphorylation (B) and PDE4D5 mRNA (C) expression in salmeterol-exposed cells is abrogated by transfection with MKP-1 siRNAs, whereas transfection with scRNA (negative control) has no effect. D, DEX-induced suppression of upregulated PDE activity in salmeterol-exposed cells is prevented by transfection with the MKP-1 siRNAs or by pretreatment with Ro-31-8220. Data are means $\pm$ SEs from 5 experiments. $^{**}P < .01$ compared with untreated cells by using the paired *t* test.

of dexamethasone alone, β 2AR agonist-induced phosphorylation of ERK1/2 was completely suppressed in HASM cells that were pretreated with dexamethasone (Fig 4, B). Thus, these data demonstrate that β 2AR agonist-induced ERK1/2 activation in HASM cells is glucocorticoid-sensitive.

MKP-1 mediates glucocorticoid suppression of ERK1/2 signaling and PDE4 expression in β 2AR-desensitized ASM

Because glucocorticoids evoke synthesis of the dual-specificity phosphatase, MKP-1, which acts as a potent endogenous deactivator of ERK1/2 signaling by dephosphorylating its tyrosine and threonine residues,¹⁶ we next examined whether dexamethasone-induced suppression of ERK1/2-coupled upregulation of PDE4 in β 2AR-desensitized HASM cells is mediated by altered MKP-1 expression. As previously documented,^{17,18} our initial studies demonstrated that dexamethasone (1 μ M/L) acutely elicited increased MKP-1 protein expression in HASM cells that peaked at 2 hours and, as exemplified in Fig 5, A, this dexamethasone-induced MKP-1 expression was markedly suppressed (ie, between 78% and 91%) in cells that were transfected with a pool of 3 siRNA duplexes directed against human MKP-1. By comparison, transfection with a scrambled siRNA sequence (scRNA) serving as an inert negative control had no effect on dexamethasone-induced MKP-1 expression (data not shown). Accordingly, these siRNA preparations were then used to ascertain the role of MKP-1 in mediating dexamethasone-induced inhibition of ERK1/2 activation, PDE4D5 expression, and cAMP PDE activity in salmeterol-exposed HASM cells. Fig 5, B, demonstrates that

dexamethasone-induced inhibition of salmeterol-evoked ERK1/2 activation was prevented in HASM cells that were pretreated with the MKP-1 siRNAs, or with the nonselective MKP-1 inhibitor Ro-31-8220 (5 μ M/L).¹⁹ Comparably, suppression by dexamethasone of salmeterol-induced PDE4D5 mRNA expression was prevented in HASM cells transfected with the MKP-1 siRNAs, whereas transfection with scRNA had no effect (Fig 5, C). Finally, dexamethasone-induced suppression of upregulated PDE activity in salmeterol-exposed cells was also abrogated either by transfection with the MKP-1 siRNAs (whereas scRNA transfection had no effect) or by pretreatment with Ro-31-8220 (Fig 5, D).

MKP-1 mediates glucocorticoid-induced suppression of altered responsiveness in β 2AR-desensitized ASM

To ascertain the physiological relevance of these observations, we next examined whether suppression of MKP-1 impairs the ability of dexamethasone to prevent the PDE4-dependent changes in ASM responsiveness elicited in the β 2AR-desensitized state (as depicted in Fig 2). In these studies, agonist-induced ASM constrictor and relaxation responses were compared in rabbit ASM tissues that were exposed to salmeterol (10 μ M/L $\times$ 24 hours) after initial transfection with either scRNA (control) or the siRNA duplexes directed against MKP-1. Fig 6 demonstrates that, relative to controls, the significantly increased constrictor responses to acetylcholine (Fig 6, A) and impaired relaxation responses to isoproterenol (Fig 6, B) were abrogated in salmeterol-exposed ASM tissues that were pretreated with dexamethasone (1 μ M/L),

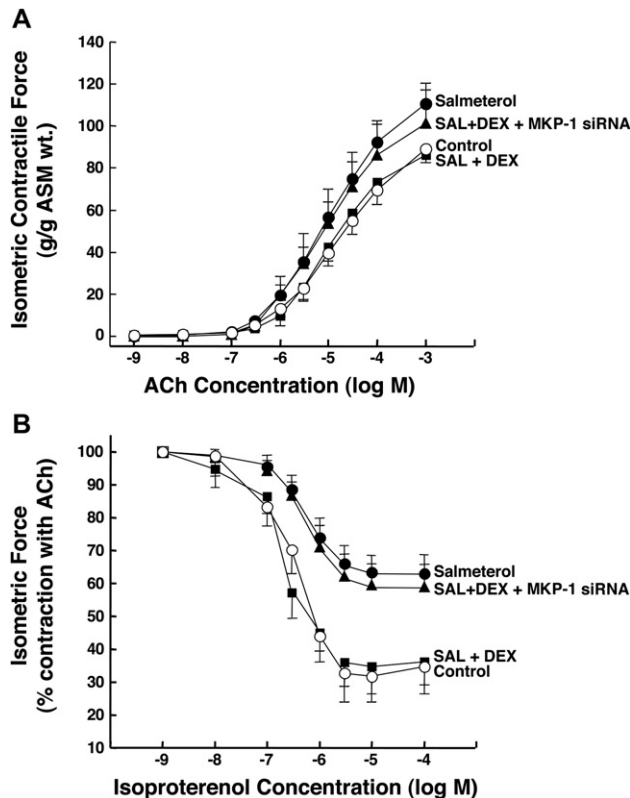


FIG 6. MKP-1 siRNA prevents dexamethasone (DEX)-induced suppression of the proasthmatic effects of salmeterol (SAL) on ASM responsiveness. Rabbit ASM tissues exposed to salmeterol exhibit significantly increased contractility to acetylcholine (ACh; **A**) and impaired relaxation responses to isoproterenol (**B**) that are prevented by pretreatment with DEX. The protective effects of DEX are abrogated in SAL-exposed tissues transfected with MKP-1 siRNAs. Data are means $\pm$ SEs from 5 to 7 paired experiments. ANOVA used for multiple comparisons of mean Tmax values.

and that the latter protective effects of dexamethasone were ablated in salmeterol-exposed tissues that were transfected with the MKP-1 siRNA preparations. In relation to these observations, it should be noted that in separate studies conducted on control and salmeterol-exposed ASM tissues that were not pretreated with dexamethasone, neither transfection with the MKP-1 siRNAs nor scRNA had a significant effect on the tissues' constrictor or relaxation responses (data not shown). Collectively, these data support the concept that prevention of the PDE4-mediated changes in ASM responsiveness elicited in the β 2AR-desensitized state by dexamethasone is attributed to its induced upregulation of MKP-1, which suppresses ERK1/2 activation and its associated induction of PDE4 in the β 2AR-desensitized ASM.

DISCUSSION

Chronic use of LABAs to treat asthma has been associated with impaired bronchodilation, heightened bronchoconstriction, and an increase in asthma morbidity,¹⁻³ presumably reflecting the adverse effects of prolonged homologous β 2AR desensitization of the airways.⁴ Although mechanisms implicated in mediating acute homologous desensitization of the β 2AR in ASM are well described, including uncoupling of the β 2AR from the stimulatory G protein, Gs, as a result of its phosphorylation by G protein-coupled receptor kinases and cAMP-dependent PKA,^{4,20,21}

these mechanisms do not readily explain the worsening of asthma symptoms caused by chronic use of LABAs. In this regard, we recently demonstrated that the induction of β 2AR desensitization in ASM after its prolonged exposure to a LABA, as well as other cAMP-elevating agents, is associated with proasthmatic-like changes in ASM responsiveness that are mediated by upregulated PDE4 activity evoked by cross-talk between the PKA and ERK1/2 signaling pathways, which leads to transcriptional upregulation of *PDE4D5*.^{13,14} We now provide new evidence that identifies the role and mechanism of action of glucocorticoids in preventing the proasthmatic effects of LABA exposure on ASM function. Specifically, the current results demonstrate that (1) dexamethasone prevents the induction of PDE4-mediated changes in ASM responsiveness evoked by prolonged exposure to the LABA salmeterol, and (2) this protective action of dexamethasone is attributed to its induced upregulation of MKP-1, which suppresses ERK1/2 activation and its consequent upregulation of PDE4 activity. These findings are the first to highlight a heretofore unidentified mechanism of action of glucocorticoids in ASM that may fundamentally contribute to the reported improved asthma control and reduced risk of severe asthma morbidity in patients using LABAs in combination with inhaled glucocorticoids.⁵⁻⁹

Although an earlier study suggested that reversal of β 2AR desensitization in human airways by glucocorticoid administration may be related to upregulation of β 2AR expression,¹¹ more recent studies have reported that the protective effects of glucocorticoids in β 2AR-desensitized rodent lung tissue²² and human small airways¹² are not readily attributed to glucocorticoid-induced changes in β 2AR expression, implying that glucocorticoids likely exert their protective action in ASM via an alternative mechanism. Our present observations demonstrated that (1) pretreatment with dexamethasone prevented the rolipram-sensitive changes in constrictor and relaxation responsiveness exhibited in homologous β 2AR-desensitized ASM (Fig 2), and (2) the protective action of dexamethasone was associated with its inhibition of upregulated PDE4D5 expression and cAMP PDE activity (Fig 3), coupled with inhibition of ERK1/2 activation (Fig 4), in the β 2AR-desensitized state. These observations raise certain relevant considerations. Accordingly, it should be noted that, although the assay used herein measured total PDE activity, we previously demonstrated that the upregulated PDE activity elicited in homologous β 2AR-desensitized ASM is specifically attributed to PDE4, because it is completely inhibited by pretreating salmeterol-exposed HASM cells with the PDE4-selective inhibitor rolipram.¹³ Moreover, the PDE4 activity most likely reflects that of PDE4D5 because it is the functionally relevant isoform in HASM cells.¹⁵ In relation to this evidence, it is noteworthy that recent studies have identified that PDE4 activity plays a critical role in regulating ASM contractility,²³ and that upregulated PDE4 activity mediates the constrictor hyperresponsiveness of the airways accompanying allergen challenge in subjects with asthma²⁴ and in animal models of allergic asthma.²⁵⁻²⁹ Moreover, in concert with our current findings, other studies have reported that glucocorticoids inhibit the upregulated PDE4 activity detected in the lungs of rodent models of allergic asthma.^{27,28} Given these findings, together with those of the current study, it is reasonable to speculate that (1) the induction of altered airway responsiveness after exposure to either a LABA or allergen in the sensitized asthmatic state is attributed to the same downstream mechanism that mediates upregulated PDE4 activity in ASM

under these proasthmatic conditions, and (2) the bronchoprotective effect of glucocorticoids on airway function is a result, at least in part, of the same mechanism that mediates glucocorticoid-induced suppression of PDE4 activity in ASM under these conditions. These possibilities remain to be systematically investigated.

The anti-inflammatory actions of glucocorticoids, including in ASM,^{10,30,31} are mediated by activation of the intracellular glucocorticoid receptor, which serves both to suppress transactivation of various key proinflammatory transcription factors and to promote transcription of genes that can acutely modulate the activities of various intracellular signaling cascades.^{32,33} Acute glucocorticoid induction of gene expression involves homodimerization of the activated glucocorticoid receptor complex, its nuclear translocation, and binding to palindromic glucocorticoid response elements in the promotor region of various glucocorticoid-responsive immediate-early response genes. The archetype of these glucocorticoid-induced immediate-early response genes is MKP-1, which was found to have at least 3 putative glucocorticoid response elements in its promotor region.³⁴ MKP-1 dephosphorylates activated mitogen-activated protein kinases and thereby serves a crucial anti-inflammatory function,¹⁶ including in ASM.^{17,18,35} Our current results support the concept that glucocorticoid-induced upregulation of MKP-1 and its consequent attenuation of ERK1/2 signaling play a pivotal role in preventing the PDE4-dependent changes in ASM constrictor and relaxation responsiveness that accompany prolonged homologous β 2AR desensitization. Specifically, the results demonstrated that (1) inhibition of MKP-1 activity by siRNA silencing of MKP-1 expression, or by the nonselective inhibitor Ro-31-8220, abrogated dexamethasone-induced suppression of ERK1/2 activation and its coupled upregulation of PDE4D5 expression and PDE4 activity in salmeterol-exposed HASM cells (Fig 5); and (2) dexamethasone-induced suppression of the PDE4-mediated changes in constrictor and relaxation responsiveness in salmeterol-exposed ASM tissues was also abrogated by siRNA silencing of MKP-1 (Fig 6). Of note, these observations are consistent with those in previous studies reporting that ERK1/2 activation plays a crucial role in regulating the synthesis and activity of PDE4 in ASM^{13,14,36,37} and in mediating airway hyperreactivity *in vivo* and altered ASM responsiveness *in vitro* in response to different proasthmatic stimuli,³⁸⁻⁴¹ and that glucocorticoids inhibit ERK1/2 activation by upregulating MKP-1 under various proinflammatory conditions in different cell systems,^{16,42} including ASM.¹⁸ Notwithstanding these considerations, it is important to indicate that the nature of LABA exposure and its effects examined under the *ex vivo* conditions herein may be significantly different from that related to LABA use in human beings with asthma wherein, moreover, the presence of allergic airway inflammation may further modulate β 2AR function.

In conclusion, this study is the first to report that (1) glucocorticoids prevent the PDE4-dependent proasthmatic changes in ASM responsiveness that accompany β 2AR tolerance induced by prolonged exposure to a LABA, and (2) this protective action of glucocorticoid is attributed to its induction of MKP-1 and, consequently, inhibition of ERK1/2-coupled upregulation of PDE4 activity in the β 2AR-desensitized ASM. These novel findings highlight a heretofore unidentified mechanism by which glucocorticoids suppress the proasthmatic effects of prolonged airway exposure to a LABA on airway responsiveness. Thus, future interventions targeted at this

mechanism may provide new therapeutic approaches to improve asthma control.

We thank David Kaltman for his technical assistance.

Key messages

- Glucocorticoids prevent the proasthmatic effects of prolonged homologous β 2AR desensitization as a result of exposure to a LABA on ASM function.
- This bronchoprotective action is attributed to glucocorticoid-induced upregulation of MKP-1 expression, which inhibits ERK1/2 activation and its consequent induction of PDE4-mediated changes in ASM responsiveness in the β 2AR-desensitized state.

Clinical implications: This newly identified mechanism of glucocorticoid action in preventing the proasthmatic effects of LABA exposure on ASM responsiveness may play a pivotal role in contributing to the therapeutic efficacy of using glucocorticoids in combination with LABAs for asthma control. Future interventions targeted at this mechanism may provide new approaches to treat asthma.

REFERENCES

1. Beasley R, Pearce N, Crane J, Burgess C. Beta-agonists: what is the evidence that their use increases the risk of asthma morbidity and mortality? *J Allergy Clin Immunol* 1999;104:S18-30.
2. Cheung D, Timmers MC, Zwinderman AH, Bel EH, Dijkman JH, Sterk PJ. Long-term effects of a long-acting beta 2-adrenoceptor agonist, salmeterol, on airway hyperresponsiveness in patients with mild asthma. *N Engl J Med* 1992;327:1198-203.
3. Nelson HS, Weiss ST, Bleecker ER, Yancey SW, Dorinsky PM. The Salmeterol Multicenter Asthma Research Trial: a comparison of usual pharmacotherapy for asthma or usual pharmacotherapy plus salmeterol. *Chest* 2006;129:15-26.
4. Giembycz MA, Newton R. Beyond the dogma: novel beta2-adrenoceptor signaling in the airways. *Eur Respir J* 2006;27:1286-306.
5. Woolcock A, Lundback B, Ringdahl N, Jaques LA. Comparison of addition of salmeterol to inhaled steroids with doubling of the dose of inhaled steroids. *Am J Respir Crit Care Med* 1996;153:1481-8.
6. Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, et al. Effect of inhaled formoterol and budesonide on exacerbations of asthma. *N Engl J Med* 1997;337:1405-11.
7. Kips J, O'Connor B, Inman M, Svensson K, Pauwels RA, O'Byrne PM. A long-term study of the anti-inflammatory effect of low-dose budesonide plus formoterol versus high-dose budesonide in asthma. *Am J Respir Crit Care Med* 2000;161:996-1001.
8. Bateman E, Nelson H, Bousquet J, Kral K, Sutton L, Ortega H, et al. Meta-analysis: effects of adding salmeterol to inhaled corticosteroids on serious asthma-related events. *Ann Int Med* 2008;149:33-42.
9. Ni Chroinin M, Lasserson TJ, Greenstone I, Ducharme FM. Addition of long-acting beta-agonists to inhaled corticosteroids for chronic asthma in children. *Cochrane Database Syst Rev* 2009; (3): CD007949.
10. Giembycz MA, Kaur M, Leigh R, Newton R. A Holy Grail of asthma management: toward understanding how long-acting beta(2)-adrenoceptor agonists enhance the clinical efficacy of inhaled corticosteroids. *Br J Pharmacol* 2008;153:1090-104.
11. Hauck RW, Harth M, Schulz C, Prauer H, Bohm M, Schomig A. Effects of β -adrenoceptor- and dexamethasone-treatment on relaxation and regulation of β -adrenoceptors in human bronchi and lung tissue. *Br J Pharmacol* 1997;121:1523-30.
12. Cooper PR, Panettieri RA Jr. Steroids completely reverse albuterol-induced β 2-adrenergic receptor tolerance in human small airways. *J Allergy Clin Immunol* 2008;122:734-40.
13. Nino G, Hu A, Grunstein JS, Grunstein MM. Mechanism regulating proasthmatic effects of prolonged homologous β 2-adrenergic receptor desensitization in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L746-57.

14. Hu A, Nino G, Grunstein JS, Fatma S, Grunstein MM. Prolonged heterologous β_2 -adrenoceptor desensitization promotes proasthmatic airway smooth muscle function via PKA/ERK1/2-mediated phosphodiesterase-4 induction. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L1055-67.
15. Billington CK, Le Jeune IR, Young KW, Hall IP. A major functional role for phosphodiesterase 4D5 in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 2008;38:1-7.
16. Abraham SM, Clark AR. Dual-specificity phosphatase 1: a critical regulator of innate immune responses. *Biochem Soc Trans* 2006;34:1018-23.
17. Issa R, Xie S, Khorasani N, Sukkar M, Adcock IM, Lee KY, et al. Corticosteroid inhibition of growth-related oncogene protein- α via mitogen-activated kinase phosphatase-1 in airway smooth muscle cells. *J Immunol* 2007;178:7366-75.
18. Kang BN, Jude JA, Panettieri RA Jr, Walseth TF, Kannan MS. Glucocorticoid regulation of CD38 expression in human airway smooth muscle cells: role of dual specificity phosphatase 1. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L186-93.
19. Beltman J, McCormick F, Cook SJ. The selective protein kinase C inhibitor, Ro-31-8220, inhibits mitogen-activated protein kinase phosphatase-1 (MKP-1) expression, induces c-Jun expression, and activates Jun N-terminal kinase. *J Biol Chem* 1996;271:27018-24.
20. Penn RB, Panettieri RA Jr, Benovic JL. Mechanisms of acute desensitization of the β_2 AR-adenylyl cyclase pathway in human airway smooth muscle. *Am J Respir Cell Mol Biol* 1998;19:338-48.
21. Shore SA, Moore PE. Regulation of β -adrenergic responses in airway smooth muscle. *Respir Physiol Neurobiol* 2003;137:179-95.
22. Mak JC, Hisada T, Salmon M, Barnes PJ, Chung KF. Glucocorticoids reverse IL-1 β -induced impairment of β -adrenoceptor-mediated relaxation and up-regulation of G-protein-coupled receptor kinases. *Br J Pharmacol* 2002;135:987-96.
23. Mehats C, Jin SL, Wahlstrom J, Law E, Umetsu DT, Conti M. PDE4D plays a critical role in the control of airway smooth muscle contraction. *FASEB J* 2003;17:1831-41.
24. van Schalkwyk E, Strydom K, Williams Z, Venter L, Leitch S, Schmid-Wirlitsch C, et al. Roflumilast, an oral, once-daily phosphodiesterase 4 inhibitor, attenuates allergen-induced asthmatic reactions. *J Allergy Clin Immunol* 2005;116:292-8.
25. Hansen G, Jin S, Umetsu DT, Conti M. Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D. *Proc Natl Acad Sci U S A* 2000;97:6751-6.
26. Kanehiro A, Ikemura T, Makela MJ, Lahn M, Joetham A, Dakhama A, et al. Inhibition of phosphodiesterase 4 attenuates airway hyperresponsiveness and airway inflammation in a model of secondary allergen challenge. *Am J Respir Crit Care Med* 2001;163:173-84.
27. Tang HF, Song YH, Chen JC, Chen JQ, Wang P. Upregulation of phosphodiesterase-4 in the lung of allergic rats. *Am J Respir Crit Care Med* 2005;171:823-8.
28. Sun JG, Deng YM, Wu X, Tang HF, Deng JF, Chen JQ, et al. Inhibition of phosphodiesterase activity, airway inflammation and hyperresponsiveness by PDE4 inhibitor and glucocorticoid in a murine model of allergic asthma. *Life Sci* 2006;79:2077-85.
29. Chapman RW, House A, Jones H, Richard J, Celly C, Prelusky D, et al. Effect of inhaled roflumilast on the prevention and resolution of allergen-induced late phase airflow obstruction in Brown Norway rats. *Eur J Pharmacol* 2007;571:215-21.
30. Hirst SJ, Lee TH. Airway smooth muscle as a target of glucocorticoid action in the treatment of asthma. *Am J Respir Crit Care Med* 1998;158:S201-6.
31. Panettieri RA Jr. Effects of corticosteroids on structural cells in asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2004;1:231-4.
32. Barnes PJ. Antiinflammatory action of glucocorticoids: molecular mechanisms. *Clin Sci* 1998;94:557-72.
33. Hakonarson H, Halapi E, Whelan R, Gulcher J, Stefansson K, Grunstein MM. Association between IL-1 β /TNF- α -induced glucocorticoid-sensitive changes in multiple gene expression and altered responsiveness in airway smooth muscle. *Am J Respir Cell Mol Biol* 2001;6:761-71.
34. Noguchi T, Metz R, Chen L, Mattéi M-G, Carrasco D, Bravo R. Structure, mapping and expression of erp, a growth factor-inducible gene encoding a nontransmembrane protein tyrosine phosphatase and effect of erp on cell growth. *Mol Cell Biol* 1993;13:5195-205.
35. Burgess JK, Lee JH, Ge Q, Ramsay EE, Poniris MH, Parmentier J, et al. Dual ERK and phosphatidylinositol 3-kinase pathways control airway smooth muscle proliferation: differences in asthma. *J Cell Physiol* 2008;216:673-9.
36. Liu H, Palmer D, Jimmo SL, Tilley DG, Dunkerley HA, Pang SC, et al. Expression of phosphodiesterase 4D (PDE4D) is regulated by both the cyclic AMP-dependent protein kinase and mitogen-activated protein kinase signaling pathways: a potential mechanism allowing for the coordinated regulation of PDE4D activity and expression in cells. *J Biol Chem* 2000;275:26615-24.
37. Houslay MD, Baillie GS. The role of ERK2 docking and phosphorylation of PDE4 cAMP phosphodiesterase isoforms in mediating cross-talk between the cAMP and ERK signalling pathways. *Biochem Soc Trans* 2003;31:1186-90.
38. Duan W, Chan JH, Wong CH, Leung BP, Wong WS. Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. *J Immunol* 2004;172:7053-9.
39. Ohnishi H, Takeda K, Domenico J, Lucas JJ, Miyahara N, Swasey CH, et al. Mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2-dependent pathways are essential for CD8 $^{+}$ T cell-mediated airway hyperresponsiveness and inflammation. *J Allergy Clin Immunol* 2009;123:249-57.
40. Grunstein MM, Veler H, Shan X, Larson J, Grunstein JS, Chuang S. Proasthmatic effects and mechanisms of action of the dust mite allergen, Der p 1, in airway smooth muscle. *J Allergy Clin Immunol* 2005;116:94-101.
41. Shan X, Hu A, Veler H, Fatma S, Grunstein JS, Chuang S, et al. Regulation of toll-like receptor 4-induced pro-asthmatic changes in airway smooth muscle function by opposing actions of ERK1/2 and p38 MAPK signaling. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L324-33.
42. Kassel O, Sancono A, Kratzschmar J, Kreft B, Stassen M, Cato AC. Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *EMBO J* 2001;20:7108-16.