

Salmeterol attenuates chemotactic responses in rhinovirus-induced exacerbation of allergic airways disease by modulating protein phosphatase 2A

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Background: β -Agonists are used for relief and control of asthma symptoms by reversing bronchoconstriction. They might also have anti-inflammatory properties, but the underpinning mechanisms remain poorly understood. Recently, a direct interaction between formoterol and protein phosphatase 2A (PP2A) has been described *in vitro*.

Objective: We sought to elucidate the molecular mechanisms by which β -agonists exert anti-inflammatory effects in allergen-driven and rhinovirus 1B-exacerbated allergic airways disease (AAD).

Methods: Mice were sensitized and then challenged with house dust mite to induce AAD while receiving treatment with salmeterol, formoterol, or salbutamol. Mice were also infected with rhinovirus 1B to exacerbate lung inflammation and therapeutically administered salmeterol, dexamethasone, or the

PP2A-activating drug (S)-2-amino-4-(4-[heptyloxy]phenyl)-2-methylbutan-1-ol (AAL[S]).

Results: Systemic or intranasal administration of salmeterol protected against the development of allergen- and rhinovirus-induced airway hyperreactivity and decreased eosinophil recruitment to the lungs as effectively as dexamethasone. Formoterol and salbutamol also showed anti-inflammatory properties. Salmeterol, but not dexamethasone, increased PP2A activity, which reduced CCL11, CCL20, and CXCL2 expression and reduced levels of phosphorylated extracellular signal-regulated kinase 1 and active nuclear factor κ B subunits in the lungs. The anti-inflammatory effect of salmeterol was blocked by targeting the catalytic subunit of PP2A with small RNA interference. Conversely, increasing PP2A activity with AAL(S) abolished rhinovirus-induced airway hyperreactivity, eosinophil influx, and CCL11, CCL20, and CXCL2 expression. Salmeterol also directly activated immunoprecipitated PP2A *in vitro* isolated from human airway epithelial cells.

Conclusions: Salmeterol exerts anti-inflammatory effects by increasing PP2A activity in AAD and rhinovirus-induced lung inflammation, which might potentially account for some of its clinical benefits. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Long-acting β_2 -agonist, salmeterol, formoterol, salbutamol, asthma, allergy, rhinovirus, exacerbation, chemokine, dexamethasone, (S)-2-amino-4-(4-[heptyloxy]phenyl)-2-methylbutan-1-ol, protein phosphatase 2A, nuclear factor κ B

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Asthma is characterized by airway hyperreactivity (AHR), mucus hypersecretion, and, commonly, airways inflammation.^{1,2} The degree of eosinophilic, neutrophilic, and lymphocytic accumulation in the airways correlates with disease severity, and clinical treatments aim to control and ameliorate episodic airways obstruction, the clinical hallmark of asthma.^{3,4} Airway inflammation in asthmatic patients is tightly regulated and involves the release of IL-4, IL-5, and IL-13 by T_H2 cells, as well chemokines, such as CCL11 (eotaxin-1), CCL20 (macrophage inflammatory protein 3 α), and CXCL2 (macrophage inflammatory protein 2 α), by residential lung and innate immune cells.⁵⁻⁹ A majority of all health care costs generated by the estimated 300 million asthmatic patients worldwide is attributed to exacerbations caused by respiratory viruses,¹⁰⁻¹² and rhinoviruses are the most commonly detected.^{13,14}

Current treatments for controlling asthma symptoms consist of— if indicated—combination therapy of inhaled corticosteroids and long-acting β_2 -agonists (LABAs).^{15,16} Inhaled corticosteroids have broad immunosuppressive effects through glucocorticoid receptor (GR) activation, whereas LABAs relax airway smooth muscle through activation of β_2 -adrenoreceptors to reopen obstructed

Abbreviations used

AAD:	Allergic airways disease
AAL(S):	(S)-2-amino-4-(4-[heptyloxy]phenyl)-2-methylbutan-1-ol
AHR:	Airway hyperreactivity
GR:	Glucocorticoid receptor
HDM:	House dust mite
LABA:	Long-acting β_2 -agonist
NF- κ B:	Nuclear factor κ B
OVA:	Ovalbumin
PAS:	Periodic acid–Schiff
p-ERK1:	Phosphorylated extracellular signal-regulated kinase 1
PP2A:	Protein phosphatase 2A
RV1B:	Rhinovirus 1B
siRNA:	Small interfering RNA

airways.^{17,18} The LABA salmeterol also possesses an anti-inflammatory mode of action in models of ovalbumin (OVA)–driven allergic airways disease (AAD),^{19,20} but the molecular mechanisms of these observations remain elusive. Interestingly, LABAs increase the activity of protein phosphatase 2A (PP2A) through direct interaction independent of β_2 -adrenoceptor signaling *in vitro*.²¹ PP2A is one of the most abundantly expressed cellular proteins and regulates kinase-driven signaling through dephosphorylation of numerous signaling molecules.^{22–24}

In this study we show that salmeterol attenuated AHR, eosinophilic inflammation, and local chemokine expression by increasing PP2A activity and suppression of downstream mitogen-activated protein kinase and nuclear factor κ B (NF- κ B) signaling pathways.

METHODS

For more detailed methods, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Mice and induction of AAD and rhinovirus exacerbation

Specific pathogen-free male BALB/c mice were intranasally sensitized and challenged with crude house dust mite (HDM) antigen (50 and 5 μ g, respectively; Greer Laboratories, Lenoir, NC) for 17 days, with β -agonists administered before and during the challenge phase (see [Fig E1, A–C](#), in this article's Online Repository at www.jacionline.org). Nonsensitized mice received sterile endotoxin-free saline (0.9%) only. In some experiments sensitized mice were intranasally infected with 1×10^7 virions of the minor group rhinovirus 1B (RV1B) or UV-inactivated RV1B on day 18 (see [Fig E1, D and E](#), in this article's Online Repository at www.jacionline.org).²⁵ All mice were killed 24 hours after the final challenge. The Animal Care and Ethics Committee of the University of Newcastle, Australia, approved all experiments, which were conducted and reported in accordance with the ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines.

AHR measurement

AHR to nebulized methacholine (increasing lung resistance) was measured, as previously described.⁸ Results were expressed as raw values of lung resistance and percentage change from control (baseline).

Enumeration of lung inflammation

Bronchoalveolar lavage fluid and blood smears were collected and stained with May–Grünwald–Giemsa. Lung slices were stained with Congo Red (CR),

periodic acid–Schiff (PAS), or toluidine blue to identify peribronchial eosinophils, goblet cells, and mast cells, respectively, as previously described.²⁶

Quantitative RT-PCR

Trachea and lungs were extracted from killed mice, and forceps were used to separate the airways from the parenchyma by means of blunt dissection.²⁷ Total mRNA was then extracted with TRIzol (Ambion, Carlsbad, Calif), and cDNA was generated with BioScript (Bioline, Alexandria, Australia). Quantitative PCR was performed with SYBR Green (Invitrogen, Mulgrave, Australia) by using primers detailed in [Table E1](#) in this article's Online Repository at www.jacionline.org.

Lymph node cell culture and ELISA

Single-cell suspensions of peribronchial lymph node cells were cultured for 6 days in the absence or presence of HDM (50 μ g/mL), as previously described.²⁶ ELISA (BD Biosciences PharMingen, San Jose, Calif) was used to determine concentrations of IL-5 and IL-13 in the supernatants of cell cultures.

Quantification of lung chemokines, phosphorylated extracellular signal-regulated kinase 1, and active NF- κ B subunits

Snap-frozen lung lobes were homogenized in buffers recommended by the manufacturer's instructions. Levels of CCL11, CCL20, CXCL2, and phosphorylated extracellular signal-regulated kinase 1 (p-ERK1) were determined in clarified lung lysate by means of ELISA (R&D Systems, Minneapolis, Minn), whereas quantitation of NF- κ B subunits was performed with the TransAM NF- κ B Transcription Factor Assay (Active Motif, Carlsbad, Calif). All concentrations were normalized to lung weight.

BEAS-2B cell culture and PP2A activity

BEAS-2B cells, an SV40-transformed airway bronchial epithelial cell line, were grown in Bronchial Epithelial Cell Growth Medium (BEGM; Clonetics)²⁸ until 80% confluence. After serum starvation, they either received dexamethasone, salmeterol, or both preceding HDM stimulation (50 μ g/mL) or were collected, and PP2Ac was immunoprecipitated. PP2A activity was assessed by using the Active PP2A DuoSet IC activity assay (R&D Systems), according to the manufacturer's instructions.

Statistical analysis

Statistical significance was analyzed with the Student *t* test or Mann–Whitney *U* test. All statistical analyses shown are comparisons with vehicle control groups, unless otherwise stated. Data are expressed as means $\pm$ SEMs.

RESULTS**Systemic salmeterol suppresses HDM-induced AHR and inflammation but not numbers of PAS-positive cells**

We administered salmeterol or vehicle control intraperitoneally 24 hours before and during the challenge phase in a mouse model of HDM-induced AAD (see [Fig E1, A](#)). Data collection was performed 24 hours after the last salmeterol administration to account for possible bronchodilator effects influencing airways resistance. This was based on the documented duration of action of salmeterol in human subjects,²⁹ as well as our own data from time course studies in naive mice showing protective effects on methacholine-induced AHR at 2 hours (see [Fig E2, A](#), in this article's Online Repository at www.jacionline.org), but not 24 hours (see [Fig E2, B](#)), after treatment. However, in allergic mice salmeterol significantly suppressed AHR compared with the vehicle

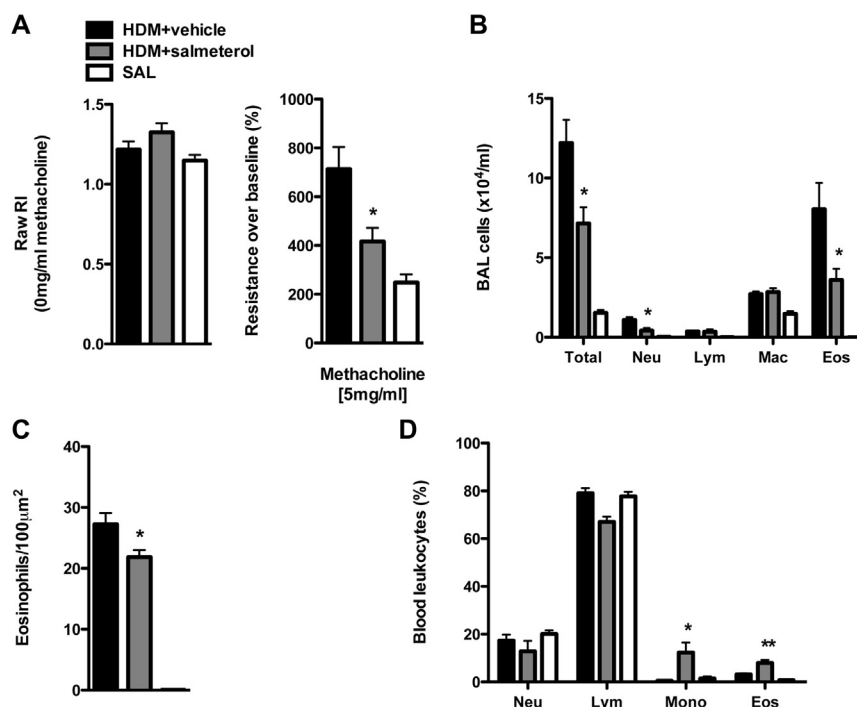


FIG 1. Salmeterol suppresses AHR and inflammation in AAD. **A**, Total lung resistance (*RI*) presented as raw baseline values and percentage change in response to methacholine ($n = 8$ mice per group). **B**, Cells in bronchoalveolar lavage (BAL) fluid. **C**, Tissue eosinophils per 100 μm^2 . **D**, Proportions of blood leukocytes. Results are presented as means $\pm$ SEMs ($n = 3$ –6 mice per group). * $P < .05$ and ** $P < .01$ compared with the vehicle control group. *Eos*, Eosinophils; *Lym*, lymphocytes; *Mac*, macrophages; *Neu*, neutrophils; *SAL*, sterile saline.

control 24 hours after treatment (Fig 1, A) concurrently with a reduction in the numbers of neutrophils and eosinophils in bronchoalveolar lavage fluid (Fig 1, B). This decrease in lung eosinophil numbers was also observed within the peribronchial tissue surrounding the airways (Fig 1, C) but associated with higher numbers of blood eosinophils (Fig 1, D). This suggests that salmeterol may exert its anti-inflammatory function by limiting eosinophil chemotaxis from the blood compartment to the lung tissue. Interestingly, salmeterol did not attenuate the number of PAS-positive cells (see Fig E3, A–D, in this article's Online Repository at www.jacionline.org) in the airways after HDM challenge.

Salmeterol suppresses chemokines but not T_H2 -associated cytokines

Salmeterol treatment had no effect on IL-4, IL-5, and IL-13 expression in the airways (Fig 2, A) nor did it attenuate T_H2 responses of peribronchial lymph node cells cultured *ex vivo* (see Fig E4 in this article's Online Repository at www.jacionline.org). In contrast, CCL11, CCL20, and CXCL2 levels were significantly reduced by salmeterol treatment (Fig 2, B) concurrently with a partial restoration of p-PP2A activity (Fig 2, C), as well as decreased levels of p-ERK1 and active NF- κ B subunits (Fig 2, D and E).

Local administration of other long- and short-acting β -agonists have comparable effects to salmeterol treatment

To investigate whether anti-inflammatory effects could be elicited by other β -agonists, we administered salmeterol, formoterol, and salbutamol via the airway route to mice during allergen

challenge (see Fig E1, B). An identical suppression of AHR and inflammation was observed for all 3 compounds (see Fig E5, A and B, in this article's Online Repository at www.jacionline.org), with numbers of PAS-positive cells remaining unaffected by treatment (see Fig E5, C). Consistent with our prior findings, local expression of chemokines was attenuated (see Fig E5, D), and there was a moderate level of PP2A activity restoration (see Fig E5, E).

Salmeterol-induced anti-inflammatory action is dependent on PP2A reactivation

To demonstrate a causal link between salmeterol and PP2A reactivation, we treated mice with salmeterol in combination with PP2Ac-targeting small interfering RNA (siRNA) or a nonsense control siRNA via the airway route during allergen challenge (see Fig E1, C). The efficacy of siRNA-mediated targeting of PP2Ac was confirmed by means of measurement of PP2A activity and PP2Ac protein levels (Fig 3, A). Inhibition of PP2Ac abolished salmeterol-induced suppression of chemokine expression (Fig 3, B) and eosinophil recruitment to the airways (Fig 3, C), whereas T_H2 -associated cytokines were unaffected (Fig 3, D). This confirms that salmeterol modulates PP2A activity to suppress airways inflammation.

Salmeterol suppresses AHR and inflammation in rhinovirus-exacerbated AAD

In a mouse model of RV1B-induced exacerbation of AAD (see Fig E1, D), we found salmeterol to be as effective as dexamethasone in suppressing rhinovirus-induced AHR (Fig 4, A) and eosinophil recruitment to the airways (Fig 4, B). However, salmeterol treatment did not attenuate T_H2 -associated cytokines, whereas

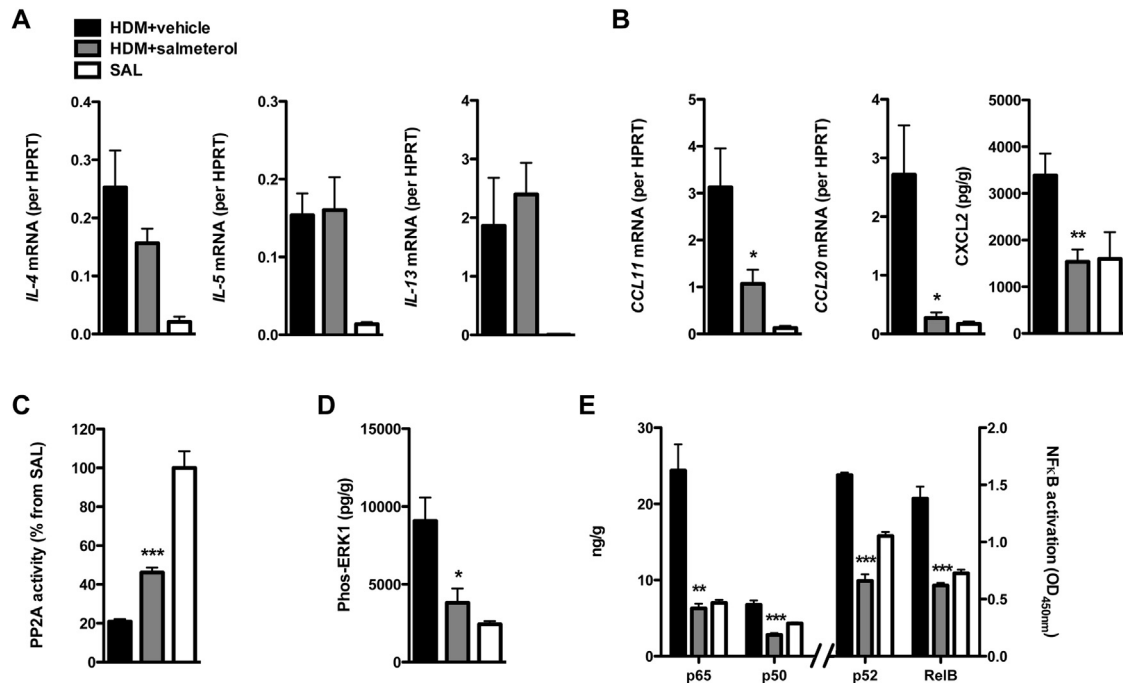


FIG 2. Salmeterol attenuates chemokine but not T_H2 cytokine expression. **A** and **B**, Cytokine/chemokine expression in lower airway tissue (Fig 2, **A** and **B**) and protein levels of CXCL2 (Fig 2, **B**). **C-E**, PP2A activity (Fig 2, **C**), p-ERK1 (Fig 2, **D**), and activated NF-κB subunits (Fig 2, **E**) quantified from lung lysates by using ELISA. Results are presented as means $\pm$ SEMs ($n = 3-6$ mice per group). * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with the vehicle control group. SAL, Sterile saline.

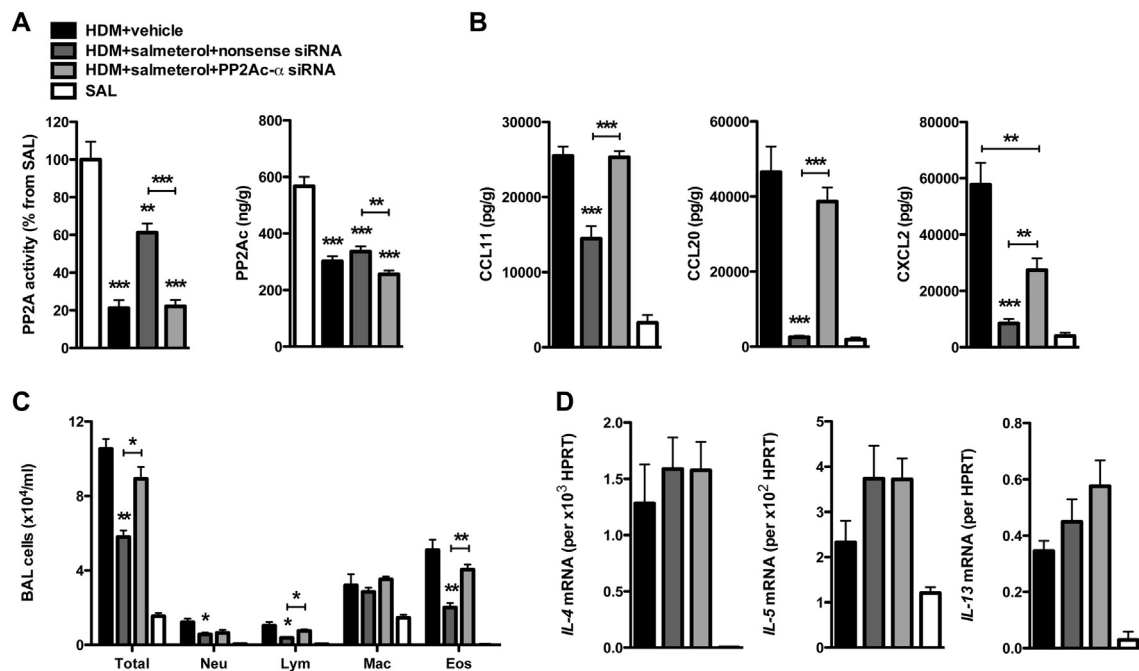


FIG 3. Inhibition of PP2A reverses the therapeutic effect of salmeterol. **A** and **B**, PP2A activity and PP2Ac protein (Fig 3, **A**) and chemokines (Fig 3, **B**) in lung lysates. **C**, Cells in bronchoalveolar lavage (BAL) fluid. **D**, Cytokine expression in lower airway tissue. Results are presented as means $\pm$ SEMs ($n = 3-6$ mice per group). * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with the vehicle control group or otherwise indicated. SAL, Sterile saline.

dexamethasone significantly suppressed IL-13 (Fig 4, **C**). Both treatments reduced CCL11 expression, but only salmeterol attenuated CCL20 and CXCL2 production (Fig 4, **D**). Salmeterol had

no effect on IFN-α and IFN-β expression (see Fig E6, **A**, in this article's Online Repository at www.jacionline.org), which correlated with rhinovirus replication (see Fig E6, **B**). In contrast,

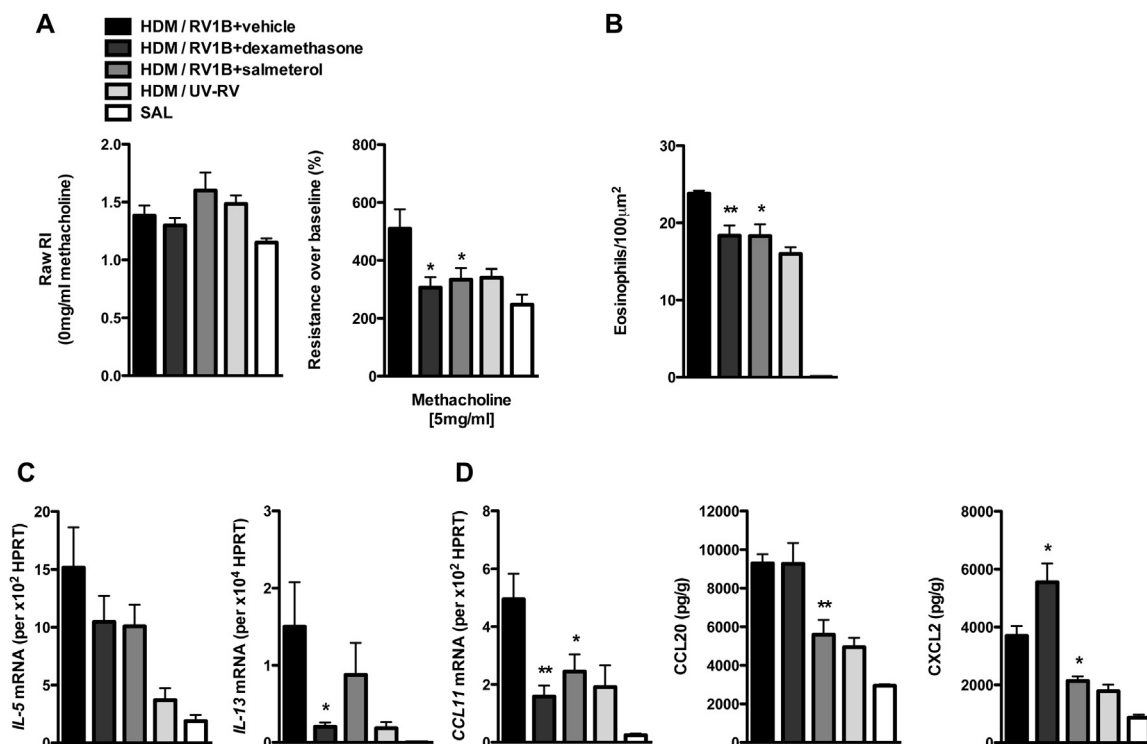


FIG 4. Salmeterol and dexamethasone suppress rhinovirus-induced exacerbation. **A**, Total lung resistance (*RI*) presented as raw baseline values and percentage change in response to methacholine ($n = 6-9$ mice per group). **B**, Tissue eosinophils per $100 \mu\text{m}^2$. **C** and **D**, Gene expression in lower airway tissue (Fig 4, **C** and **D**) and protein levels of CCL20 and CXCL2 (Fig 4, **D**). Results are presented as means $\pm$ SEMs ($n = 3-6$ mice per group). * $P < .05$ and ** $P < .01$ compared with the vehicle control group. SAL, Sterile saline.

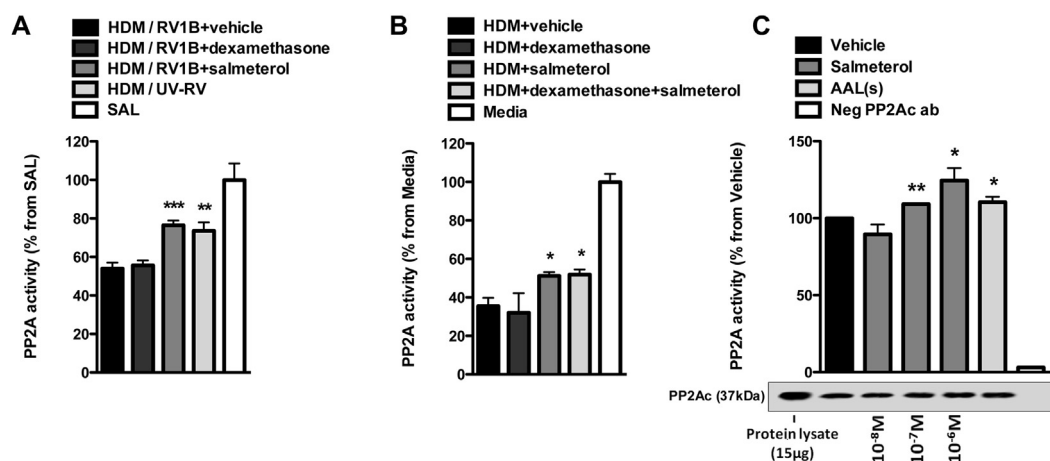


FIG 5. Salmeterol, but not dexamethasone, increases PP2A activity *in vivo* and *in vitro*. PP2A activity in lung lysates of rhinovirus-exacerbated mice (**A**), human BEAS-2B cells (**B**), or immunopurified PP2A complexes (**C**) after treatment with salmeterol, dexamethasone, or their combination is shown. Results are presented as means $\pm$ SEMs ($n = 4-6$ mice per group or 3 independent experiments). * $P < .05$, ** $P < .01$, and *** $P < .001$ compared with the vehicle control group. SAL, Sterile saline.

dexamethasone treatment led to increased rhinovirus replication, which resulted in an increase in IFN- α expression.

Salmeterol modulates phosphatase activity through direct interaction with the PP2A complex

As observed in models of HDM-induced AAD, salmeterol treatment correlated with a partial restoration of PP2A activity in

the lungs of rhinovirus-exacerbated mice, whereas dexamethasone had no effect (Fig 5, **A**). Next, we stimulated human epithelial cells (BEAS-2B) with HDM after pretreatment with salmeterol and dexamethasone (Fig 5, **B**). As observed *in vivo*, salmeterol, but not dexamethasone, partially restored PP2A activity. To confirm a direct molecular interaction between salmeterol and PP2A independent of, for example, β_2 -adrenoreceptors, we immunopurified PP2Ac from BEAS-2B cells, preserving the

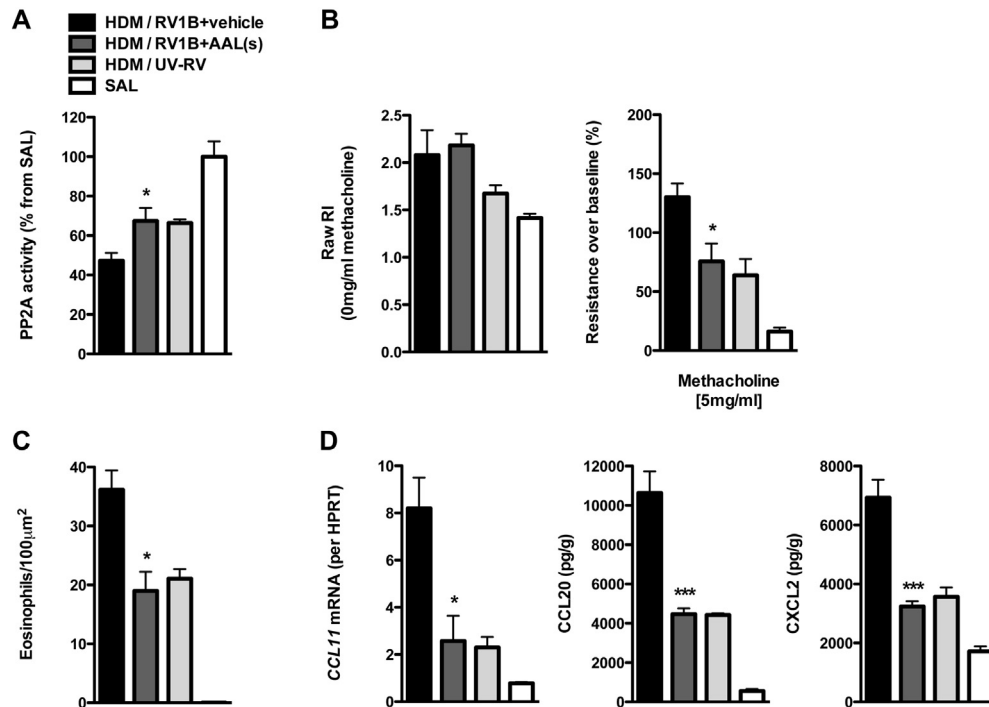


FIG 6. The PP2A agonist AAL(S) prevents rhinovirus-induced exacerbation. **A** and **B**, PP2A activity (Fig 6, **A**) and total lung resistance (*R*_T; Fig 6, **B**) are presented as raw baseline values and percentage change in response to methacholine (*n* = 4-6 mice per group). **C** and **D**, Tissue eosinophils per 100 μm^2 (Fig 6, **C**) and gene and protein expression of chemokines (Fig 6, **D**). Results are presented as means $\pm$ SEMs (*n* = 3-5 mice per group). **P* < .05 and ****P* < .001 compared with the vehicle control group.

bound protein complexes only, and treated them with salmeterol in a cell-free system (Fig 5, **C**). Importantly, we observed that salmeterol increased PP2A activity directly and in a dose-dependent manner comparable with the effect of the direct PP2A agonist (S)-2-amino-4-(4-[heptyloxy]phenyl)-2-methylbutan-1-ol (AAL[S]).

Reactivation of PP2A protects against AHR and suppresses eosinophilia in rhinovirus-exacerbated AAD

To provide evidence for the therapeutic potential of PP2A modulation by other drugs in rhinovirus-induced exacerbation of AAD, we treated mice with the nonphosphorylatable FTY720 analogue AAL(S) (see Fig E1, **E**). AAL(S) cannot be phosphorylated by sphingosine kinase 2 and therefore cannot bind to S1P receptors like phosphorylated FTY720.³⁰ We have previously shown that AAL(S) increases PP2A activity *in vivo* and is therapeutic in models of HDM-induced AAD.²⁵ Here we confirm these findings in rhinovirus-infected allergic mice (Fig 6, **A**). Notably, AAL(S) limited rhinovirus-induced AHR (Fig 6, **B**) and eosinophilic airways inflammation (Fig 6, **C**) in association with reduced CCL11, CCL20, and CXCL2 expression (Fig 6, **D**). There was no effect on type 1 interferon expression and viral replication of AAL(S) treatment (see Fig E7 in this article's Online Repository at www.jacionline.org).

Reactivation of PP2A prevented rhinovirus-induced neutrophilic inflammation

Naive mice received AAL(S) 24 hours before rhinovirus infection, which restored PP2A activity up to 24 hours after

infection (see Fig E8, **A**, in this article's Online Repository at www.jacionline.org) and conferred protection against rhinovirus-induced AHR 24 hours after infection (see Fig E8, **B**), neutrophilic inflammation (see Fig E8, **C**), and CXCL2 production (see Fig E8, **D**).

DISCUSSION

Using a well-characterized model of HDM-induced AAD,^{26,27} we observed suppression of AHR, as well as reduced eosinophilic airways inflammation, caused by systemic and local salmeterol treatment, whereas blood eosinophil levels mildly increased. This suggests that the anti-inflammatory effect of salmeterol might be associated with impaired eosinophilic chemotaxis rather than proliferation or maturation. This was supported by reduced levels of CCL11, which is crucial for eosinophil tissue homing,³¹⁻³³ whereas levels of T_H2 -associated cytokines, such as IL-5 and IL-13, were not suppressed by salmeterol treatments. The singular and cooperative role of IL-5 and CCL11 in the regulation of eosinophils has previously been dissected in an OVA-induced model of AAD,³⁴ and our data support a role of salmeterol in CCL11-mediated eosinophilic chemotaxis. Salmeterol also reduced CCL20 levels in this study, which is a critical signal for the expression of AAD through regulation of dendritic cell and T-cell recruitment.⁸ Interestingly, neutrophilic inflammation was also limited on salmeterol treatment, and this was associated with reduced levels of CXCL2, which is a potent chemoattractant for neutrophils.³⁵

IL-13 has been identified as a critical cytokine signal for the development of mucus hypersecretion and goblet cell hyperplasia

in patients with allergic asthma.^{34,36,37} In this study mucus, as assessed based on PAS staining concurrently with IL-13 expression, was not altered by salmeterol treatment. These data support previous findings showing that salmeterol treatment of OVA-sensitized and OVA-challenged mice did not attenuate mucus production in the airways.³⁸

PP2A activity was reduced in PBMCs from patients with severe asthma, and synthetic knockdown *in vitro* conferred resistance to corticosteroids.³⁹ This was associated with hyperphosphorylation of the GR, in addition to modulating GR nuclear translocation.⁴⁰ In this study salmeterol partially restored PP2A activity and suppressed *in vivo* levels of p-ERK1, which is known to play a key role in inflammatory gene expression in asthmatic patients⁴¹ and is dephosphorylated by PP2A.⁴⁰ Similarly, salmeterol treatment led to lower quantities of active NF- κ B, which is a key inflammatory mediator sequestered by I κ B α , another molecule dephosphorylated by PP2A.^{42,43} Notably, Hu et al²⁰ observed suppressed mitogen-activated protein kinase and NF- κ B activation in salmeterol-treated dendritic cells.

Rhinovirus-induced exacerbations of asthma are common and only partially responsive to corticosteroid treatment. In this study we used an established model of HDM-induced AAD with superimposed rhinovirus infection, leading to an exacerbation of AHR and inflammation greater than the level seen in allergic mice exposed to UV-inactivated rhinovirus.²⁵ Protection against the development of AHR after salmeterol treatment was equal to dexamethasone treatment in rhinovirus-exacerbated allergic mice. Although both treatments suppressed lung eosinophilia, the mechanisms were distinct. Namely, dexamethasone suppressed both IL-13 and CCL11, as well as mucus hypersecretion (data not shown) but did not inhibit other inflammatory chemokines or increase PP2A activity *in vivo* or *in vitro*. Additionally, only dexamethasone treatment was found to promote rhinovirus replication, leading to increased IFN- α production (see Fig E6). The effects of corticosteroids on interferons and rhinovirus replication remain largely unexplored,⁴⁴ but our results suggest that this treatment might adversely affect rhinovirus clearance, an observation reported previously.⁴⁵⁻⁴⁷

To confirm a causal link between PP2A activity and the anti-inflammatory effects exerted by salmeterol, we used siRNA directed against PP2Ac, which completely blocked the therapeutic effect of salmeterol. In support, the PP2A agonist AAL(S) yielded an identical response in rhinovirus-exacerbated mice as salmeterol treatment. Additionally, increasing PP2A activity suppressed neutrophilic inflammation and CXCL2 release induced by rhinovirus infection in naive mice. Finally, we demonstrated a direct modulation of PP2A activity by salmeterol in a cell-free system, suggesting that the anti-inflammatory effects of salmeterol-induced PP2A activation *in vivo* can occur, at least in part, independent of β_2 -adrenoreceptor activation. Further studies are now required to determine the precise interaction between PP2A subunits and salmeterol on a molecular level.

In summary, we provide a novel molecular mechanism for salmeterol-mediated suppression of AHR and airways inflammation that can be further exploited by optimized PP2A-activating drugs and β -agonists. We hypothesize that salmeterol and other β -agonists used as an add-on to steroid treatment have the potential to target distinct proinflammatory pathways unresponsive to corticosteroids in patients with asthma and virus-induced exacerbations.

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Key messages

- Salmeterol suppresses allergen- and rhinovirus-induced airways hyperreactivity, inflammation, and chemokine release by increasing PP2A activity.
- Increasing PP2A activity with AAL(S) also suppresses hallmark features of allergic and rhinovirus-induced airways disease.
- Salmeterol-induced modulation of PP2A activity might be of therapeutic benefit for the treatment of asthma and rhinovirus-induced exacerbation.

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