

Albuterol Isomers Modulate Platelet-Activating Factor Synthesis and Receptor Signaling in Human Bronchial Smooth Muscle Cells

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Key Words

Asthma · PAF synthesis · PAF acetylhydrolase · PAF receptor

Abstract

Background: Racemic albuterol is a 50:50 mixture of the (R)- and (S)-enantiomers of albuterol. Its clinical efficacy resides in the (R)-enantiomer (levalbuterol). Studies have shown that (S)-albuterol induces human bronchial smooth muscle cell (HBSMC) proliferation via a pathway linked to platelet-activating factor (PAF), but the underlying mechanism by which (S)-albuterol augments PAF effects is not clear. In this study, we compared effect of levalbuterol and (S)-albuterol on PAF receptor (PAFr)-mediated signaling and PAF metabolism by HBSMCs after incubation with the albuterol isomers. **Methods:** PAF binding and inositol phosphate (IP₃) release were studied on adherent cultured cells. PAFr protein expression was measured by Western blotting, PAF synthesis and catabolism were measured in membrane and cytosolic proteins of cells incubated with albuterol isomers. **Results:** Compared to control conditions, (S)-albuterol increased PAF binding by 70% after 30 min of preincubation and by 150% after 24 h of preincubation. Levalbuterol had no effect on PAF binding under both conditions. (S)-albuterol also augmented PAF stimulation of IP₃ release, while levalbuterol and the racemic mixture had no effect. WEB 2170, a PAFr antagonist, inhibited the ability of (S)-albuterol to in-

crease PAF binding or stimulate IP₃ release. (S)-albuterol stimulated PAFr protein expression. With PAF metabolism, (S)-albuterol treatment augmented PAF synthesis, but significantly inhibited PAF catabolism. **Conclusions:** Our data suggest that one mechanism by which (S)-albuterol stimulates HBSMC proliferation involves upregulation of PAFr-mediated effects including increased PAF synthesis and decreased PAF catabolism.

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Introduction

Asthma, a chronic lung disease with different etiologies, is characterized by reversible chronic airway obstruction, chronic airway inflammation, and airway hyperresponsiveness [1]. Airway inflammation and hyperresponsiveness in asthma result, in part, from release of pro-inflammatory molecules, cytokines and lipid mediators, by activated neutrophils, platelets and macrophages [2, 3]. Racemic albuterol (salbutamol), a 50:50 mixture of (R)-albuterol (levalbuterol) and (S)-albuterol, is a bronchodilator currently used in the management of asthma [4, 5]. Levalbuterol, the active component of the racemic mixture, elevates cyclic adenosine monophosphate (cAMP) level in airway smooth muscle, whereas (S)-albuterol, which was thought to be inert, may aggravate airway reactivity and lead to loss of asthma control after pro-

longed use [6–8]. Levalbuterol was reported to inhibit proliferation of human bronchial smooth muscle cells (HBSMCs) in vitro, by increasing cAMP production and augmenting activity of cAMP-dependent protein kinase A (PKA). On the other hand, (S)-albuterol induces cell proliferation by inhibiting levalbuterol-induced elevation of cAMP levels, by activating some intracellular mitogenic signaling pathways such as mitogen activated protein kinases (MAPK), nuclear factor- κ B (NF- κ B), p65 and retinoblastoma (Rb) proteins, among others [9, 10]. (S)-albuterol has also been reported to induce bronchial smooth muscle cell proliferation in culture via a pathway linked to platelet-activating factor (PAF) receptor activation.

PAF is a potent endogenous pro-inflammatory lipid synthesized by platelets and macrophages and it is implicated in the pathophysiology of asthma [1, 2]. Its release in asthma results in exacerbation asthmatic airway hyperresponsiveness and inflammation [11]. PAF level is high in serum and bronchoalveolar lavage of asthmatic patients [12, 13]. In experimental anaphylaxis, serum PAF level is high and is inversely related to the activity of PAF acetylhydrolase, the PAF catabolizing enzyme and the plasma PAF level decreased with a decrease in the severity of anaphylaxis [14]. Although clinical use of PAF receptor antagonists has not proved useful in therapeutic management of asthma [15, 16], studies have shown that use of recombinant PAF acetylhydrolase may offer some useful therapeutic potentials [17, 18]. Thus, the roles of PAF and PAF-mediated responses in asthma are not well understood and demand further investigation. Furthermore, the mechanism by which (S)-albuterol activates PAF receptor to promote HBSMC proliferation is not well understood.

The objective of this study was to employ HBSMCs to investigate how (S)-albuterol interacts with PAF and PAF receptor pathway to augment cell proliferation. Our hypothesis is that, in vivo, following prolonged use of racemic albuterol in management of asthma, the level of (S)-albuterol will increase in the lung, particularly in the airway smooth muscle cells, resulting in activation of PAF receptor-mediated signaling and aggravation of the inflammatory response in asthmatic patients.

Methods

Cell Culture

HBSMCs were purchased from Lonza and grown in 5% FBS in humidified incubator aerated with 5% CO₂ in air at 37°C. Cells were seeded at a density of 10⁴ cells/well in 6-well plates to study PAF binding to its receptors or a density of 10⁶ cells/dish in 150 mm Petri dishes to study PAF receptor protein expression, PAF

synthesis or PAF acetylhydrolase activity. All studies were done with cells at subconfluence.

Effect of (S)-Albuterol and PAF on HBSMC Proliferation

Cells were seeded at a density of 1×10^4 cells/well in 6-well plates and allowed to stabilize for 2 days. The cells were then starved by culturing in 0.1% FBS for 72 h. After 72 h in culture with 0.1% FBS cell starving media, the starving media were aspirated and the cells were treated with 2 μ M of racemic albuterol, 1 μ M each of (S)-albuterol and levalbuterol, 10 nM PAF or a co-culture of 1 μ M (S)-albuterol and 10 nM PAF in 5% FBS growth media together with 5 μ Ci/well of ³H-thymidine and incubated for 24 h more in 5% CO₂ in air. Cells in the 5% FBS culture media alone were used as the control in proliferation and protein expression studies.

Assay Procedure for ³H-Thymidine Incorporation

After 24 h in culture, unincorporated ³H-thymidine was aspirated and cells were washed with ice-cold, protein-free phosphate-buffered saline, followed with 5% trichloroacetic acid. Washed cells were treated with 0.5 N sodium hydroxide and aliquots of cell suspension were counted for ³H-thymidine radioactivity and quantified. Cell counting was done in some experiments to corroborate ³H-thymidine incorporation.

PAF Receptor Binding

PAF receptor binding to the HBSMCs was standardized as we previously reported [19]. Briefly, 0.1, 1, 10 and 100 nM of ³H-PAF was incubated for 30 min at 37°C or at 4°C for 24 h with and without 1,000 nM of authentic nonradiolabeled PAF standard. PAF bound to its receptor in the HBSMC were extracted on Whatman GF/C membranes filters as we previously reported [19]. PAF radioactivity was quantified and specific PAFr binding was calculated. The specific PAFr binding (fmol/10⁶ cells means $\pm$ SEM, n = 3 separate cell purchases) was as follows: 0.1 nM, 9.3 ± 1.43 ; 1 nM, 15.67 ± 3.1 ; 10 nM, 21.81 ± 2.53 ; 100 nM, 19.69 ± 2.43 . After this standardization, all PAFr binding studies were done with 1 nM ³H-PAF as PAF is active in lower concentrations and this concentration is in the linear range of PAF binding and consistently produced the most reproducible results. Also binding at 37°C for 30 min was higher than that at 4°C for 24 h for adherent cell studies. Effect of albuterol isomers on PAF receptor binding was studied on adherent cells to assess the interaction of the albuterol isomers on presynthesized receptors. The PAF receptor binding buffer was the smooth muscle cell media containing 0.1% BSA, pH 6.4. Subconfluent cells were preincubated for 30 min at 37°C with 2 μ M of racemic albuterol and 1 μ M each of levalbuterol and (S)-albuterol, or with the binding buffer and 1,000 nM nanradiolabeled PAF (Control) and then 1 nM of ³H-PAF was added to all flasks and incubated for 30 min more.

To study effect of albuterol isomers on PAF receptor synthesis and binding, sub-confluent cells were serum starved for 72 h and then fed 2 μ M of racemic albuterol, and 1 μ M each of levalbuterol, (S)-albuterol, or 10 μ M of the PAF receptor antagonist WEB 2170 (gift of Boehringer Ingelheim), or with 5% FBS growth media and incubated for 24 h more. Concentration of WEB 2170 used was based on a concentration-effect study of WEB 2170 (μ M): 0.001, 0.01, 0.1, 1.0, 10.0 and 100) against 1 nM of ³H-PAF. Inhibition of PAFr binding by the WEB concentrations were (%Control, PAF alone) means $\pm$ SEM, n = 3: 0.001 μ M, 95.6 ± 4.31 ; 0.01 μ M, 90.8

± 5.66 ; $0.1 \mu\text{M}$, 80.5 ± 3.6 ; $1.0 \mu\text{M}$, 73.8 ± 4.5 ; $10 \mu\text{M}$, 33.4 ± 2.3 ; $100 \mu\text{M}$, 8.8 ± 1.98 . Significant inhibition of PAFr binding by WEB 2170 did not occur until at $0.1 \mu\text{M}$ of WEB 2170. We then used $10 \mu\text{M}$ of WEB 2170 in all our studies because it produced the most significant and reproducible inhibition. Moreover, $100 \mu\text{M}$ WEB produced significant cell toxicity indicated by cell detachment. After 24 h of incubation of cells with the albuterol isomers or $10 \mu\text{M}$ WEB 2170, cells were washed with phosphate-buffered saline and then fed 1 nM ^3H -PAF and incubated for 30 min as in the assay standardization protocol. Receptor bound ^3H -PAF was extracted on GFC membranes and quantified as we previously reported [19].

Inositol Phosphate Production

Sub-confluent cells were pulsed with $10 \mu\text{Ci/ml}$ of ^3H -myo-inositol for 24 h in an inositol-free medium [19]. Unincorporated ^3H -myo-inositol was washed off and cells were stimulated separately with 10 nM PAF, $2 \mu\text{M}$ of racemic albuterol, $1 \mu\text{M}$ each of levalbuterol, (S)-albuterol, and $10 \mu\text{M}$ WEB 2170 and incubated for 20 min at 37°C . To determine involvement of PAF receptor in (S)-albuterol stimulation of IP_3 release, cells were pretreated with $10 \mu\text{M}$ WEB 2170 for 20 min, and then $1 \mu\text{M}$ (S)-albuterol was added and incubated for 20 min more. To determine if (S)-albuterol augments PAF stimulation of release of inositol phosphates, cells were pretreated with $1 \mu\text{M}$ (S)-albuterol for 20 min and then 10 nM PAF was added and incubated for 20 min more. Inositol phosphates produced in all treatments was extracted on AG 1-X8 ion exchange columns and quantified.

PAF Receptor Protein Expression

Subconfluent cells grown in 150-mm Petri dishes were serum starved with 0.1% FBS for 24 h. Cells were then incubated for 24 h more with $2 \mu\text{M}$ of racemic, $1 \mu\text{M}$ each of levalbuterol and (S)-albuterol in 5% FBS growth medium or 5% FBS medium alone (control). Cells were lysed with a lysis buffer containing a cocktail of protease inhibitors [19]. Cell lysates were spun for 1 h at $100,000 \text{ g}$ at 4°C to pellet membrane proteins. Membrane pellet was resuspended in the lysis buffer. Protein concentration was determined by the Bradford assay and then subjected to SDS PAGE to measure PAFr protein expression.

Culture of Cells with Albuterol Isomers for Study PAF Synthesis and Catabolism

Subconfluent HBSMCs were fed $2 \mu\text{M}$ of racemic, $1 \mu\text{M}$ each of levalbuterol, (S)-albuterol, $0.1 \mu\text{M}$ authentic PAF and a mixture of $0.1 \mu\text{M}$ PAF and $1 \mu\text{M}$ (S)-albuterol or 5% FBS alone, and cultured for 16 h in 5% carbon dioxide in air. Cells incubated in 5% FBS alone were used as control. After 16 h in culture, culture medium was aspirated and cells were lysed with a hypotonic lysis buffer containing a cocktail of protease inhibitors [19], and cell lysates from similarly treated cells were combined. Membrane protein ($100,000 \text{ g}$ pellet) was prepared and used to study PAF synthesis by PAF acetyltransferase. The soluble fraction was used to study PAF acetylhydrolase activity.

PAF Synthesis by Cell Membrane Proteins

PAF synthesis by the cell membrane proteins was studied by measuring the activity of lyso-PAF:acetyl-S-CoA acetyltransferase. For each membrane protein, $250\text{-}\mu\text{M}$ aliquots of lyso-PAF and $500 \mu\text{M}$ ^3H -acetyl-S-CoA (3.7 Ci/mmol) were placed in a poly-

propylene tube and warmed to 37°C in a shaker bath. Reaction was initiated by adding $100 \mu\text{g}$ of membrane protein of each treatment for a total volume of 1 ml of 30 mM Tris buffer, pH 7.4, containing $10 \mu\text{M}$ CaCl_2 , 1 mM dithiothreitol and 0.025% BSA, and incubating the mixture at 37°C for 15 min. After incubation, ^3H -acetyl-PAF synthesized was subjected to lipid extraction.

Study of PAF Catabolism by PAF-Acetylhydrolase

PAF acetylhydrolase activity was studied in soluble fraction of cell lysates prepared from cells cultured as described for PAF synthesis. PAF acetylhydrolase activity was studied by determining the rate of PAF catabolism as we previously reported [20]. Briefly, cell plasma proteins were incubated with $50 \mu\text{M}$ of ^3H -acetyl- C_{16} -PAF (27 Ci/mmol) in 30 mM Tris buffer containing 0.01% BSA and incubated for 15 min at 30°C . Enzyme activity was terminated by adding 1 ml of ice-cold methanol.

Extraction and Purification of PAF by Thin-Layer Chromatography and High-Performance Liquid Chromatography

Prior to lipid extraction, $25 \mu\text{g}$ each of authentic PAF and lyso-PAF were added to each tube to aid in establishing PAF and lyso-PAF bands on thin-layer chromatography (TLC) plate. Then, PAF and lyso-PAF were extracted from samples, dried with nitrogen, and resuspended in $300 \mu\text{l}$ of methylene chloride. Ten microliters of each sample was spotted on silica gel G plates and developed in a solvent mixture containing methylene chloride-methanol-water (65:35:5) against authentic radiolabeled and nonradiolabeled PAF and lyso-PAF standards. TLC spots were visualized under iodine vapor and spots corresponding in retention time (R_f values) with the standard PAF were scraped off pooled, re-extracted and further purified by high-performance liquid chromatography (HPLC) on an Ultramex C_{18} column as we previously reported [20]. The HPLC peaks corresponding in retention time to authentic PAF standard was collected and PAF radioactivity was measured by scintillation spectrometry (Beckman Coulter, Fullerton, Calif., USA) and quantified.

Measurement of Activity of the Purified PAF

Activity of the PAF synthesized by HBSMCs and purified by a combination of TLC and HPLC was determined on washed rabbit platelets by the serotonin release assay following a modification of the method reported by Pinckard et al. [21], with some modifications as we previously reported [20]. Authentic PAF standards were not added to the samples before lipid extraction or purification processes. Briefly, rabbit blood was collected in acid citrate dextrose and platelets were prepared with calcium-free Tyrode's buffer, pH 6.5, containing 0.2 mM EGTA and 0.25% (w/v) gelatin. The purified platelets were incubated for 45 min with $1 \mu\text{Ci}$ of ^3H -serotonin at 37°C . After incubation, unincorporated radioactivity was washed off the platelets with the calcium-free Tyrode's buffer without EGTA. The labeled platelets were transferred into tubes so as to give 10^7 platelets/ml (final volume) and used to study PAF stimulation of serotonin release. The authentic PAF standard as well as the purified PAF-sample extracts were dissolved in Tyrode's buffer with gelatin. The following treatment variables were done in replicates: Tyrode's buffer alone (used to determine spontaneous serotonin release), a calibration curve of 0.001 nM to $1.0 \mu\text{M}$ of authentic PAF in Tyrode's buffer was generated, and then the concentration of the purified PAF-sample ex-

tracts in Tyrode's buffer (0.1 ml) was determined from the standard curve. The tubes were incubated at room temperature for 90 s. Reaction was quenched with 9% glutaraldehyde after which the platelets were spun at 2,500 g for 15 min at 4°C to pellet the platelets. The supernatant was decanted and quantified radiometrically for serotonin release, then the radioactivity in the platelet pellet was also determined and used to calculate the total radioactivity used in each tube. The amount of serotonin released spontaneously was subtracted from the experimental values before quantification of PAF concentration from the standard curve and presented as pmol (nM) PAF/ μ g protein.

Data Analysis

In all instances, each data point represents an average of 2–4 repeat experiments done with different cell passages of the same Lot purchases. Data from different passages of the same lot purchase were averaged and denoted as $n = 1$. All numerical data were analyzed for statistical significance with a two-tailed Student t test at $p < 0.05$ level of significance.

Results

Effect of (S)-Albuterol and PAF on HBSMC Proliferation

Figure 1 shows the effect of racemic, levalbuterol, (S)-albuterol and PAF on HBSMC proliferation measured as DNA synthesis in ^3H -thymidine disintegrations per minute. Cell proliferation was compared to effect of 5% FBS which is the cell culture medium. Treatment with 2 μM of the racemic albuterol caused a 14% decrease in cell proliferation. Treatment with 1 μM levalbuterol also decreased cell proliferation by 47% compared to 5% FBS and by 38% compared to effect of the racemic mixture. On the other hand, 1 μM (S)-albuterol increased cell proliferation by 28% over 5% FBS alone. Treatment with 10 nM PAF alone increased cell proliferation by 58% compared to 5% FBS and by 28% compared to effect of (S)-albuterol alone. When cells were cultured with a combination treatment of 1 μM (S)-albuterol and 10 nM PAF, cell proliferation increased by 82% over 5% FBS control, 20% over PAF alone and 35% over (S)-albuterol treatments alone.

(S)-Albuterol Upregulates PAF Binding to Its Receptors

Figure 2a shows the effect of 2 μM racemic, 1 μM each of levalbuterol and (S)-albuterol, on PAF binding to its receptor. Compared to effect of 1 nM PAF, 30-min preincubation with 2 μM of racemic albuterol increased PAFr binding by 23%. On the other hand, pretreatment of cells with levalbuterol for 30 min produced no significant change in PAF binding to its receptor. The effect was similar to that of PAF alone. However, (S)-albuterol augmented PAF receptor binding by 70%.

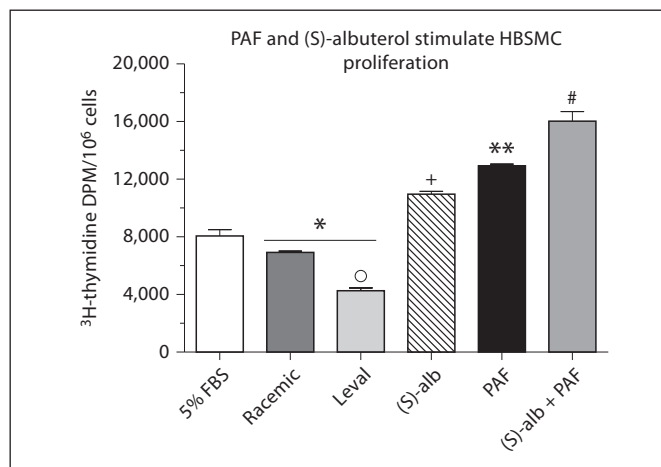


Fig. 1. Effect of (S)-albuterol on HBSMC proliferation. Data are means $\pm$ SEM, $n = 6$. Cells were studied as described in Methods. Levalbuterol (Leval) and the racemic mixture inhibited cell proliferation, while (S)-albuterol [(S)-alb] and PAF stimulated cell proliferation. Co-culture of (S)-albuterol and PAF stimulated cell proliferation more than (S)-albuterol or PAF alone. * $p < 0.05$, different from 5% FBS control; ° $p < 0.05$, different from racemic mixture; + $p < 0.05$, different from 5% FBS, racemic and levalbuterol; ** $p < 0.05$, different from (S)-albuterol and the preceding conditions; # $p < 0.05$, different from all other treatment conditions.

Effect of Albuterol Isomers and WEB 2170 on PAF Binding to Its Receptors

Figure 2b shows the effect of 24-hour preincubation of cells with 2 μM racemic albuterol, and 1 μM each of levalbuterol and (S)-albuterol on PAF binding to its receptor. Treatment of cell with racemic albuterol for 24 h increased PAF receptor binding by 50% compared to PAF alone. Pretreatment of cells with levalbuterol for 24 h did not change PAF binding compared to control (PAF alone), but less than effect of racemic mixture on PAF binding. On the other hand, pretreatment of cells with (S)-albuterol for 24 h increased PAF receptor binding by 150% compared to effect of PAF alone. (S)-albuterol's augmentation of PAF binding was attenuated by WEB 2170, a PAF receptor antagonist, but binding was still greater than effect of levalbuterol alone.

Effect of Albuterol Isomers on Release of Inositol Phosphates by HBSMCs

Figure 3 shows the profile of inositol phosphates release by cells treated with 2 μM of racemic albuterol and 1 μM each levalbuterol, (S)-albuterol and 10 nM of PAF for 20 min. Both the racemic mixture and levalbuterol each produced <12% increase in inositol phosphates re-

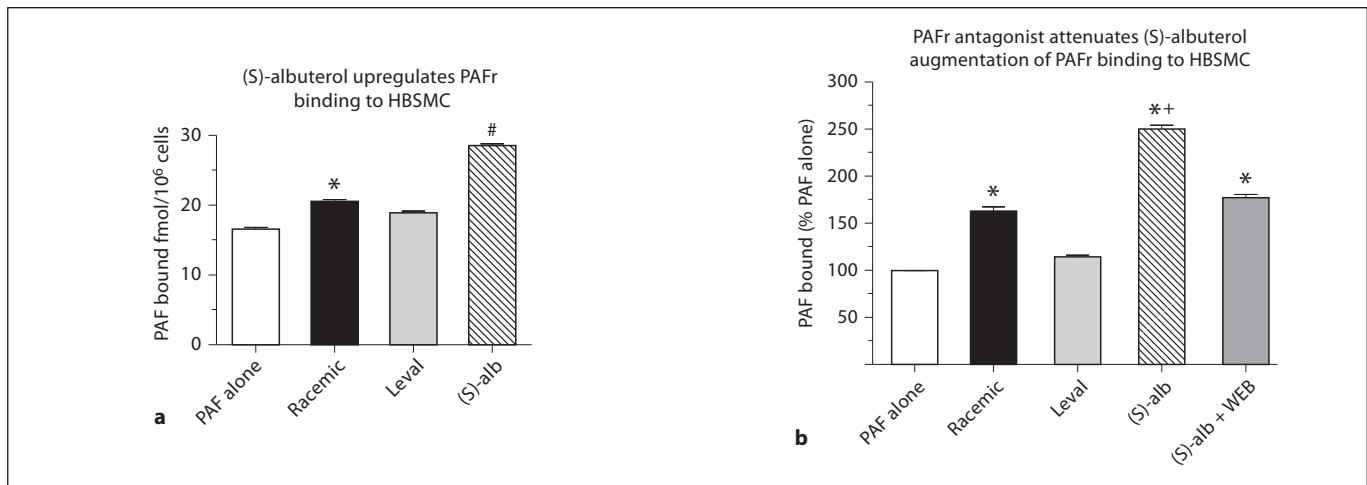
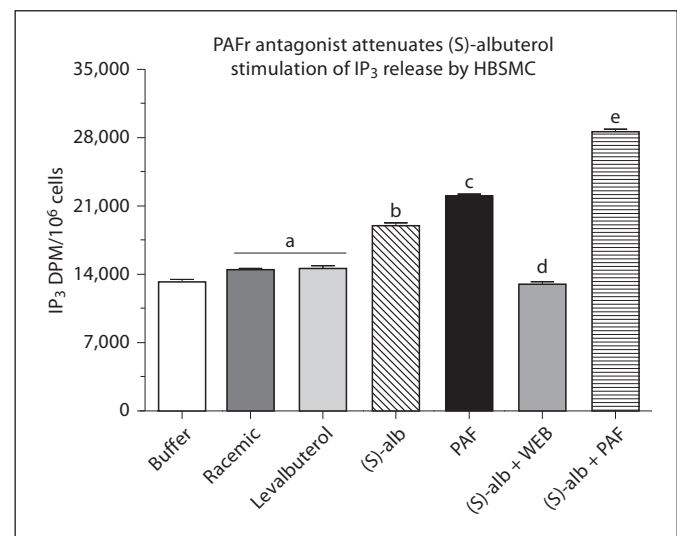


Fig. 2. a Effect of 30 min exposure to albuterols on PAF receptor binding. Data are means $\pm$ SEM, $n = 5$. Cells were preincubated for 30 min with 2 μ M of racemic mixture and 1 μ M each of levalbuterol or (S)-albuterol and PAF receptor binding was assayed as described in Methods. Racemic albuterol and (S)-albuterol treatment increased PAFr binding. Levalbuterol effect was not different from control. * $p < 0.05$, different from PAF alone (control); ⁺ $p < 0.05$, different from PAF alone, levalbuterol or racemic albuterol. **b** Effect of 24 h pretreatment of cells with albuterol

isomers on PAF binding to its receptors in HBSMCs. Data are means $\pm$ SEM, $n = 4$. Racemic albuterol and (S)-albuterol pretreatment for 24 h significantly increased PAF binding to its receptors and then binding in (S)-albuterol-treated cells was greater than binding in cells treated with racemic albuterol. WEB 2170 (WEB) attenuated stimulation of PAF receptor binding by (S)-albuterol. Effect of levalbuterol was not different from control. The statistics are: * $p < 0.05$, different from PAF alone (control); ⁺ $p < 0.05$, different from other treatments.

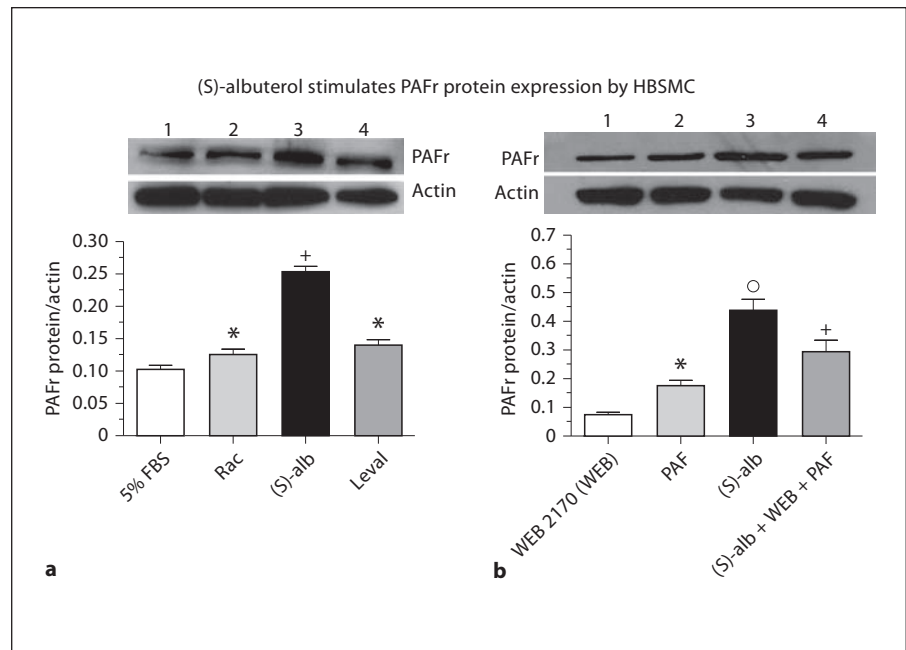
Fig. 3. Effect of albuterol isomers on stimulation of inositol phosphate (IP₃) release. Data are means $\pm$ SEM, $n = 6$. Cells were studied as described in Methods. Stimulation of IP₃ by levalbuterol and the racemic mixture was not different, but (S)-albuterol and PAF increased IP₃ release greater than either levalbuterol or racemic mixture. Combined treatment with (S)-albuterol and PAF increased IP₃ release over (S)-albuterol or PAF alone. WEB 2170 (WEB) attenuated (S)-albuterol's stimulation of IP₃ release suggesting a PAF receptor-mediated effect. ^a $p < 0.05$, different from buffer alone; ^b $p < 0.05$, different from buffer, racemic or levalbuterol alone; ^c $p < 0.05$, different from buffer, racemic mixture levalbuterol and (S)-albuterol; ^d $p < 0.05$, different from (S)-albuterol; ^e $p < 0.05$, different from all other treatments.



lease within the 20-min treatment compared to buffer alone. With respect to (S)-albuterol effect, 20-min treatment of cells with (S)-albuterol led to 43% increase in inositol phosphates release compared to buffer alone. Treatment with 10 nM PAF alone produced a 66% increase in inositol phosphates release compared to buffer

conditions and 17% increase compared to effect of (S)-albuterol alone. Pretreatment of cells with WEB 2170, a PAF receptor antagonist, attenuated (S)-albuterol-stimulated release of inositol phosphates by 68% compared to (S)-albuterol alone, but release was not different from effect of buffer alone. When cells were pulsed for 20 min

Fig. 4. Western analysis of PAFr protein expression by cells incubated for 24 h with the albuterol isomers (**a**) and with (S)-albuterol and PAF (**b**) normalized to total actin expression. Data are means $\pm$ SEM, $n = 3$. Western analyses were done as described in Methods. In each panel, the lanes are as identified on the plots. In **a**, (S)-albuterol significantly augmented PAFr protein expression compared to effect of levalbuterol or the racemic mixture. In **b**, both PAF and (S)-albuterol augmented PAFr protein expression above effect of WEB 2170 alone. Inclusion of WEB 2170 in co-culture of (S)-albuterol and PAF led to a significant attenuation of PAFr protein expression below effect of (S)-albuterol alone. **a** * $p < 0.05$, different from 5% FBS growth media, $^+ p < 0.05$, different from 5% FBS, racemic and levalbuterol; **b** * $p < 0.05$, different from WEB 2170; $^o p < 0.05$, different from PAF and WEB 2170; $^+ p < 0.05$, different from all other conditions.



with 10 nM PAF and then treated with (S)-albuterol, release of inositol phosphates increased by 52% compared to (S)-albuterol alone and 117% compared to buffer alone. Thus, WEB 2170 decreased (S)-albuterol stimulation of inositol phosphates release, while pretreatment with PAF augmented inositol phosphates release by (S)-albuterol.

Effect of Albuterol Isomers on PAF Receptor Protein Expression

Figure 4 shows the effect of albuterol isomers on PAF receptor protein expression (fig. 4a) and effect of WEB 2170 on (S)-albuterol stimulation of PAF receptor protein expression (fig. 4b). In figure 4a, treatment of cells with 2 μ M of the racemic mixture increases PAF receptor expression by 18% compared to 5% FBS control. There was no difference in PAF receptor expression between cells treated with 1 μ M levalbuterol and the racemic mixture. However, 1 μ M of (S)-albuterol increased PAF receptor protein expression by 60% compared to 5% FBS and more than effect of racemic mixture or levalbuterol. In figure 4b, PAF and (S)-albuterol induced more PAF receptor protein expression than WEB 2170 (WEB), a PAF receptor antagonist. Combined treatment of PAF and (S)-albuterol did not reverse the inhibitory effect of WEB compared to effect of PAF or (S)-albuterol alone.

Effect of Albuterol Isomers on the Activity of Lyso-PAF:Acetyl-S-CoA Acetyltransferase

Figure 5 shows the amount of PAF synthesized by membrane proteins of the treated cells. PAF synthesis (nM/ μ g protein, means $\pm$ SEM) by membrane proteins from cells incubated with 5% FBS growth media alone was 2.86 ± 0.93 . PAF synthesis by membrane proteins of cells treated with the 2 μ M of racemic albuterol was 6.56 ± 0.78 , which was over 3-fold greater than production by cell incubated in 5% FBS alone. With 1 μ M levalbuterol-treated cells, PAF synthesis was 30% less than synthesis by the racemic mixture, but was over 2-fold greater than production by 5% FBS-treated cells. Cells incubated with 1 μ M (S)-albuterol synthesized the greatest amount at 9.95 ± 1.19 nM of PAF, which was significantly greater than production by any of the other treatment conditions. Thus, (S)-albuterol induced the greatest activity of the lyso-PAF:acetyl-S-CoA acetyltransferase of the treated cells.

Effect of Albuterol Isomers on the Activity of PAF Acetylhydrolase

Figure 6 shows PAF acetylhydrolase activity in the soluble fraction of cells treated with the albuterol isomers or 5% FBS control. PAF acetylhydrolase activity (fmol lyso-PAF/ μ g protein) in protein of 5% FBS-treated cells was 18.0 ± 0.69 , which was not different from the activity in proteins of levalbuterol-treated cells. However, enzyme

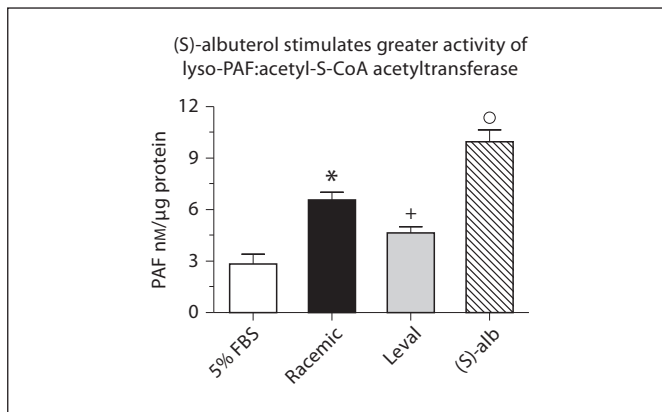


Fig. 5. Effect of albuterol isomer on stimulation of PAF synthesis. Data are means $\pm$ SEM, $n = 4$. PAF synthesis by the cells, and quantification of bioactivity were studied as described in Methods. Racemic, levalbuterol and (S)-albuterol all stimulated PAF synthesis by the cells. Levalbuterol had the least effect while (S)-albuterol had the greatest stimulatory effect. * $p < 0.05$, different from 5% FBS; ° $p < 0.05$, different from effect of 5% FBS, racemic mixture and levalbuterol; + $p < 0.05$, different from all other treatments.

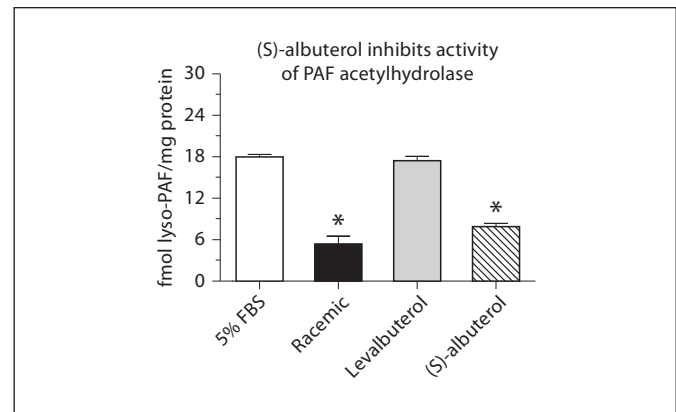


Fig. 6. Effect of albuterol isomers on PAF acetylhydrolase activity. Data are means $\pm$ SEM, $n = 4$. Effect of levalbuterol on PAF acetylhydrolase activity was not different from effect of 5% FBS. However, both the racemic mixture and (S)-albuterol inhibited PAF acetylhydrolase activity. * $p < 0.05$, different from 5% FBS control.

activity in protein of cells treated with the racemic mixture or (S)-albuterol-treated cells was significantly less than enzyme activity in 5% FBS or levalbuterol. Thus, racemic mixture and (S)-albuterol treatment inhibited PAF acetylhydrolase activity.

Discussion

Previous reports have shown that smooth muscle cells of systemic [23] and pulmonary vascular origin [20] synthesize PAF following an appropriate stimulus. In this study, we show that albuterol isomers stimulate PAF production by HBSMCs in culture. We also demonstrate that the racemic mixture and the albuterol isomers, levalbuterol and (S)-albuterol, produce different effects on HBSMCs in culture. In general, (S)-albuterol had stimulatory effects on cell proliferation and augmented PAF effects on the cells, while levalbuterol had inhibitory effects. In a previous study, Tomlinson et al. [24] showed that salbutamol, the racemic mixture of levalbuterol and (S)-albuterol, inhibited proliferation of human airway smooth muscle cells in culture. Later reports with HBSMC and racemic mixture of albuterol isomers [9, 10] corroborated this report by Tomlinson et al. [24] and further showed that treatment of the cells with levalbuterol alone resulted in inhibition of cell proliferation,

while (S)-albuterol treatment stimulated cell growth. The present report is also in agreement with the previous reports cited above and further lends some insight into the biochemical pathways of (S)-albuterol-induced HBSMC proliferation in culture. It is noteworthy that in this study, when (S)-albuterol was co-incubated with PAF, a known mitogen, cell proliferation was augmented beyond either (S)-albuterol or PAF alone. Thus, even if they were stimulating cell proliferation via two different pathways, they did not prevent each other's ability to stimulate cell growth. PAF is an endogenous pro-inflammatory lipid synthesized by smooth muscle cells, platelets, macrophages and endothelial cells. It is implicated in the pathophysiology of asthma [1, 2] and its production in asthma results in the exacerbation of asthmatic airway hyperresponsiveness and inflammation [11]. We found that (S)-albuterol stimulated greater PAF synthesis than cells treated with the racemic mixture or levalbuterol alone. However, levalbuterol-treated cells produced less PAF than the racemic mixture, suggesting that (S)-albuterol present in the racemic mixture may be interfering with the ability of levalbuterol to inhibit PAF synthesis. Previous reports [25–27] have shown that (S)-albuterol is preferentially metabolized and retained in the lung after prolonged use of the racemic albuterol in asthma therapy. This suggests that in asthma therapy with racemic albuterol, (S)-albuterol present in the race-

mic mixture may counteract the ability of levalbuterol to inhibit lyso-PAF:acetyl-S-CoA acetyltransferase. Thus, greater stimulation of PAF synthesis by (S)-albuterol will result in more PAF being present in the airway smooth muscle to induce greater PAF receptor protein expression, a condition that will in turn result in the upregulation of PAF receptor-mediated responses in the airway smooth muscle. Clinical use of PAF receptor antagonists has not proved useful in therapeutic management of asthma [15, 16], but studies have shown that use of recombinant PAF acetylhydrolase may offer some useful therapeutic potentials [17, 18]. Since PAF acetylhydrolase is the enzyme that catalyzes the degradation of PAF, the low activity of PAF acetylhydrolase in this cell culture model suggests that in vivo, pathological effect of PAF in asthma may be due to the inability of the cells in the asthmatic lung to effectively degrade PAF produced during asthma attack. Therefore, (S)-albuterol present in racemic albuterol used in therapeutic management of asthma may be inhibiting the ability of levalbuterol to induce the enzymatic degradation PAF produced in the smooth muscle cells. This condition, if it occurs in vivo, will increase PAF level in airway smooth muscle leading to airway smooth muscle hyperplasia and release of other pro-inflammatory cytokines. In this situation, the level of PAF in bronchial smooth muscle cells may increase to activate PAFr-mediated signaling and worsening of asthmatic conditions.

Racemic albuterol, the equimolar mixture of levalbuterol and (S)-albuterol, is a β_2 -adrenergic agonist used in the management of asthma [4]. However, recent pieces of evidence suggest that (S)-albuterol is not a true β_2 -adrenergic agonist [9, 10, 28–30]. Prolonged administration of racemic albuterol leads to increased airway responsiveness to spasmogens and may result in loss of asthma control [7, 8, 31]. In this study, WEB 2170, a PAF receptor antagonist, inhibited (S)-albuterol's effect in HBSMCs. This suggests that (S)-albuterol is not a true β_2 -adrenergic receptor agonist and also that (S)-albuterol may produce its effect on HBSMCs by interacting with other G protein-coupled receptors. Although this assertion is unclear at this time, (S)-albuterol may perhaps interact with a PAF receptor-related protein. Furthermore, the mechanism by which (S)-albuterol induces increased airway hyperresponsiveness is not well understood, but may involve activation of other inflammatory cell signaling pathways. We can speculate that one mechanism by which (S)-albuterol present in racemic albuterol induces the proliferation of HBSMCs in culture may be by inhibiting the ability of levalbuterol to stimulate cyclic adenosine monophosphate

(cAMP) production, by activation of PAF receptor-mediated pathway as well as by activation of some other intracellular mitogenic proteins [9, 10]. In the present study, we provide further evidence to show that (S)-albuterol induces the proliferation of HBSMCs in culture and that if it occurs in vivo, may exacerbate asthmatic conditions via some yet undefined mode of interaction with PAF receptor-linked signaling. Asthma and other chronic inflammatory diseases are characterized by release of inflammatory cytokines such as PAF [16, 32–34], which may also induce release of other inflammatory mediators, thereby sustaining asthmatic airway hyperresponsiveness.

In summary, this study shows that (S)-albuterol increases PAF receptor protein expression and PAF binding to its receptors which may be one mechanism whereby (S)-albuterol worsens the asthmatic airway hyperresponsiveness in repeated use of racemic albuterol in asthma therapy. The study also shows that levalbuterol inhibits while (S)-albuterol stimulates PAF production. And while levalbuterol had no effect on PAF catabolism, (S)-albuterol inhibited PAF catabolism by PAF acetylhydrolase. Taken together, these data suggest that, since (S)-albuterol has a longer half-life than levalbuterol, following prolonged use of racemic albuterol in asthma therapy, (S)-albuterol will increase PAF level in bronchial smooth muscle resulting in increased airway hyperresponsiveness and loss of asthma management.

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