

(S)-Albuterol activates pro-constrictory and pro-inflammatory pathways in human bronchial smooth muscle cells

Devendra K. Agrawal, PhD,^a Krishan Ariyaratna, BS,^a and Peter W. Kelbe, BS^b

Omaha, Neb, and Marlborough, Mass

Background: Pro-constrictory and proinflammatory properties of (S)-albuterol have been widely reported both under in vivo and in vitro conditions. However, underlying mechanisms are unclear.

Objective: We examined and compared the cellular effects of albuterol enantiomers on key intracellular molecules involved in constrictory and inflammatory pathways in human bronchial smooth muscle cells (hBSMCs).

Methods: Primary hBSMCs were grown in culture and treated with various concentrations of albuterol enantiomers for various periods. Methacholine was used to stimulate cells. The expression and activity of G_s and G_i proteins, the intracellular free calcium concentration ($[Ca^{2+}]_i$), the activity of phosphatidylinositol 3'-OH-kinase (PI3) kinase, and the transcriptional nuclear factor κB (NF- κB) level were examined.

Results: There was a significant increase in the expression and activity of $G_{i\alpha-1}$ protein and a decrease in the expression of G_s protein in hBSMCs after 8 hours of treatment with (S)-albuterol. These effects of (S)-albuterol were observed in a dose-dependent manner. Nonreceptor-mediated activation of adenylate cyclase by forskolin was attenuated with (S)-albuterol. Treatment of the cells for 24 hours with (S)-albuterol significantly increased $[Ca^{2+}]_i$ on stimulation with methacholine. Interestingly, the effect of (R)-albuterol was opposite to that of (S)-albuterol. The effect of the racemic albuterol in some cases was similar to that of (S)-albuterol. (S)-Albuterol significantly activated both PI3 kinase and NF- κB in hBSMCs. **Conclusion:** These studies demonstrated an (S)-albuterol-induced increase in the expression and activity of pro-constrictory pathways involving $G_{i\alpha-1}$ protein and $[Ca^{2+}]_i$ and a decrease in the activity of the bronchodilatory pathway involving G_s proteins in hBSMCs. Additionally, (S)-albuterol activated proinflammatory pathways involving PI3 kinase and NF- κB . Because (S)-albuterol metabolizes at least 10-fold slower than (R)-albuterol and has a longer elimination half-life, these cellular effects of (S)-albuterol might explain the detrimental effect seen with chronic administration of racemic albuterol in the treatment of airway diseases, such as bronchial asthma. (J Allergy Clin Immunol 2004;113:503-10.)

Key words: Albuterol, asthma, bronchial smooth muscle cells, enantiomers, G-proteins, inflammation, levalbuterol, PI3 kinase, pro-constriction, (S)-albuterol

Airway obstruction in bronchial asthma occurs in 2 phases: an immediate asthmatic reaction begins 15 to 30 minutes after exposure to an antigen, followed by the late-phase response, which might begin hours after exposure. The late phase can last as long as several days and is characterized by symptoms seen in chronic asthma. β_2 -Adrenergic receptor (β_2 -AR) agonists are effective in treating bronchoconstriction during the immediate asthmatic reaction but have less of an effect on the late-phase response.^{1,2} In addition, short-acting β -agonists cannot protect against antigen challenge several hours after dosing, leading to increased use with no benefit. Patients might in fact use 3 to 4 times the recommended number of inhalations per month to reduce symptoms,³ possibly to their detriment: excessive β -agonist use can lead to worsened symptom control and an increased risk of mortality.^{4,5}

Because β -agonists contain a chiral carbon, chemical synthesis of these drugs typically produces a racemic (equal) mixture of stereoisomers. Thus racemic albuterol is a 1:1 mixture of the active (R)-isomer and its distomer, (S)-albuterol.⁶ Only (R)-albuterol has bronchorelaxant activity, whereas the pharmacologic effect of (S)-albuterol is unknown: it has been generally accepted to be inactive.⁷ The ability to produce pure preparations of stereoisomers has led to the hypothesis that (S)-albuterol might cause paradoxical effects. Studies with (RS)-albuterol initially ascribed paradoxical effects to tachyphylaxis or receptor desensitization,⁸ but in vitro studies of the racemic mixture and purified isomers support a role for the (S)-enantiomer in these negative effects. Hoshiko and Morley⁹ found that sustained treatment (6 days) with ($\pm$)-salbutamol lead to heightened airway sensitivity in guinea pigs. This response was not a consequence of β_2 -AR desensitization.¹⁰ During shorter treatment periods, racemic albuterol treatment always reduced the airway response to histamine, but (S)-albuterol caused large increases in airway resistance.^{10,11} However, it remains controversial as to whether (S)-albuterol does in fact have any clinically relevant adverse effects in patients with asthma.^{12,13}

Nevertheless, the experimental animal studies, together with clinical studies in the emergency department,¹⁴ link (S)-albuterol to airway hyperresponsiveness (AHR), and studies in cells raise the possibility that (S)-albuterol also has proinflammatory effects that could negatively

From ^athe Center for Allergy, Asthma and Immunology, Creighton University School of Medicine, Omaha, and ^bSepracor, Inc, Marlborough. Supported by a research grant from Sepracor Inc, Marlborough, Mass. Received for publication July 24, 2003; revised November 24, 2003; accepted for publication December 4, 2003.

Reprint requests: Devendra K. Agrawal, PhD, FAAAAI, Professor of Medicine, Medical Microbiology, and Immunology and Biomedical Sciences, Creighton University School of Medicine, CRISS I Room 131, 2500 California Plaza, Omaha, NE 68178.

0091-6749/\$30.00

© 2004 American Academy of Allergy, Asthma and Immunology
doi:10.1016/j.jaci.2003.12.039

Abbreviations used

AHR:	Airway hyperresponsiveness
AR:	Adrenergic receptor
ASM:	Airway smooth muscle
[Ca ²⁺] _i :	Intracellular free calcium concentration
cAMP:	Cyclic adenine monophosphate
GTP:	Guanosine triphosphate
hBSMC:	Human bronchial smooth muscle cell
NF-κB:	Nuclear factor κB
PI3 kinase:	Phosphatidylinositol 3'-OH-kinase
PKB:	Protein kinase B

affect long-term asthma control.¹⁵⁻¹⁹ In contrast, these studies have shown that (R)-albuterol either has no effect on inflammation or induces an anti-inflammatory response. The mechanisms for the potential effects of racemic albuterol, as observed under in vitro conditions or in experimental animals, on the inflammatory response are not well known, but several experiments have examined responses downstream of the β₂-AR and other G protein-coupled receptors in airway smooth muscle (ASM) that control muscle contractility. Signaling through G protein-coupled receptors in ASM controls the intracellular free calcium concentration ([Ca²⁺]_i), which in turn regulates ASM contraction. Relaxant responses through the β₂-AR are effected through activation of G_{sα} and adenylate cyclase, leading to an increase in cyclic adenine monophosphate (cAMP) and a decrease in [Ca²⁺]_i.²⁰ An antagonistic pathway also exists: activation of G_{iα-1} and G_{iα-3} can reduce adenylate cyclase activity and allow the release of intracellular calcium stores, promoting ASM cell contraction.²¹ The bronchorelaxant activity of (R)-albuterol has been correlated with increases in intracellular cAMP levels and a decrease in [Ca²⁺]_i,²² but treatment with (S)-albuterol or racemic albuterol had the opposite effect, resulting in cell shortening and an increase in [Ca²⁺]_i.

Signaling through either the β₂-AR/G_{sα} or the G_{iα} pathway activates feedback mechanisms that regulate the sensitivity of these pathways. Continual stimulation of the β₂-AR leads to desensitization through several rapid and sustained mechanisms, including uncoupling of G_{sα} from β₂-AR, receptor degradation, and downregulation of G_{sα} expression.²³ Downregulation of the β₂-AR/G_{sα} pathway effectively sensitizes ASM to contractile responses mediated by the G_{iα} pathway.²⁴ Given the paradoxical calcium responses observed on treatment of ASM with (S)-albuterol, we examined the effects of albuterol enantiomers in human bronchial smooth muscle cells (hBSMCs) to determine whether the enantiomers could be differentiated on the basis of their effect on G-protein signal transduction and activation of nuclear factor κB (NF-κB).

METHODS**Cells and culture conditions**

Primary hBSMCs (Clonetics, Inc, San Diego, Calif) in passage 2 were grown in growth factor-supplemented Smooth Muscle Cell

Basal Medium (Clonetics, Inc). In all experiments cells were grown to confluence in T-25 flasks at 37°C with 5% CO₂ before being harvested. Cells were used in passages 4 to 6. Smooth muscle phenotype was regularly confirmed by means of positive staining with smooth muscle α-actin. Unless otherwise specified, harvested cells were treated with (R)-albuterol, (S)-albuterol, or racemic albuterol for 1, 8, or 24 hours and then activated with methacholine (30 nmol/L or 1 μmol/L) for the period specified in each experiment.

Western blots

Cells after treatment with enantiomers of albuterol for either 1, 8, or 24 hours were collected and lysed in lysis buffer (60 mmol/L Tris HCl [pH 7.4], 1% NP-40, 0.15% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L sodium orthovanadate, 5 mmol/L NaF, 20 μg/mL leupeptin, and 5 μg/mL aprotinin), and the protein concentration of the lysate was determined by using the BioRad protein assay (BioRad, Hercules, Calif). Cleared lysates were subjected to SDS-PAGE on 10% to 20% tricine gels (NOVEX, San Diego, Calif) to resolve cellular proteins (10-20 μg per experiment). After electrophoresis at 30 or 35 mA of constant current for 3 to 3.5 hours in electrophoresis buffer (25 mmol/L Tris-base [pH 8.3], 192 mmol/L glycine, and 0.1% SDS), proteins were transferred to nitrocellulose. Blots were blocked by means of incubation in 5% milk powder/Tris-buffered saline, pH 7.8/0.1% Tween 20 and probed with (rabbit) antibodies to (rat) G_{iα-1} (I-20 clone), G_{iα-3} (C-10 clone), G_{sα} (K-20 clone), p65, or p110 for activated phosphatidylinositol 3'-OH-kinase (PI3) kinase (Santa Cruz Biotechnology, Santa Cruz, Calif), or to phospho-IκB (New England Biolabs, Beverly, Mass). According to the manufacturer's description, the rabbit anti-rat antibodies to G proteins cross-react with human products. Immunoreactive proteins were detected with secondary antibodies. After extensive washing in PBS, the protein bands were visualized by using enhanced chemiluminescence (Amersham Corp, Piscataway, NJ), and membranes were exposed to Kodak film. Images of the bands were corrected for the intensity against the background, and actin was routinely run in parallel as an internal control for quantitative analysis. The density of the immunoblots was quantified as mean optical density after correction for background and internal control by means of computer-enhanced video densitometry by using the National Institutes of Health image analysis program.

[³⁵S]-Guanosine triphosphate γS binding assay

Confluent hBSMCs were initially treated with 10 to 1000 nmol/L (S)-albuterol for 8 hours and harvested in Tris buffer with 0.03% digitonin, and aliquots containing equal amounts of cellular protein were prepared. Cells were further incubated for 1 hour at 23°C in buffer containing 1 μmol/L methacholine, 30 μmol/L guanosine diphosphate, and 190 pmol/L [³⁵S]-guanosine triphosphate (GTP) γS. Nonspecific binding was determined by treating cells under the same conditions with 30 μmol/L unlabeled GTPγS. At the conclusion of treatment, cells were lysed, and G_{iα-1} was immunoprecipitated; bound [³⁵S]-GTPγS was quantitated with a liquid scintillation counter. Assays were repeated 3 times for each (S)-albuterol concentration.

Intracellular cAMP measurement

Confluent cells were harvested and pretreated for 8 hours with (S)-albuterol (10-5000 nmol/L) before the addition of forskolin (5 μmol/L). After an additional 15 minutes of incubation, intracellular cAMP was measured by using Direct Biotrak EIA (Amersham Biosciences, Arlington Heights, Ill), according to the manufacturer's instructions. Each data point represents 4 to 5 separate experiments.

Measurement of cytosolic calcium concentrations

hBSMCs at passage 5 were grown for 24 hours with (R)-albuterol (10^{-6} mol/L), (S)-albuterol (10^{-6} to 10^{-8} mol/L), or racemic albuterol (10^{-6} mol/L). Harvested cells were resuspended in Krebs-Ringer solution and loaded with Fura-2AM ($4 \mu\text{mol/L}$) for 20 minutes at room temperature. Methacholine (30 nmol/L) was added to activate the cells, which were then maintained in a stirring cuvette-based fluorescence spectrophotometer (Photon Technology International, Princeton, NJ). Fura-2AM excitation at 340 nm and 380 nm was monitored at 510 nm over 30 minutes to determine the F340/F380 ratio; calcium concentrations were calculated from the F340/F380 ratio values by using the Grynkiewicz equation.²⁵ Calibrated values were obtained by means of treatment with $10 \mu\text{mol/L}$ ionomycin in Krebs-Ringer solution plus 10 nmol/L calcium or calcium-free Krebs-Ringer solution/ 20 mmol/L ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid.

Statistical analysis

Data are reported as the arithmetic means $\pm$ SDs or SEMs (as indicated in the legends to the figures) calculated from 3 to 6 independent experiments. Analysis of repeated measures was performed by using ANOVA, and comparison between groups was performed by using the Student *t* test.

RESULTS

Expression of G_{α} proteins

The bronchorelaxant pathway is represented in ASM by $G_{s\alpha}$, whereas 2 members of the $G_{i\alpha}$ family ($G_{i\alpha-1}$ and $G_{i\alpha-3}$) contribute to constrictive responses in ASM. There was no observed effect after a 1-hour treatment with albuterol enantiomers on the expression of G proteins. However, treatment with either (RS)-albuterol or (S)-albuterol for 8 hours strongly increased the expression of $G_{i\alpha-1}$ of 42 kd of protein (Fig 1). The effect of (S)-albuterol on increased expression of $G_{i\alpha-1}$ was significantly greater than that of (RS)-albuterol. There was no significant effect of (R)-albuterol on $G_{i\alpha-1}$ protein expression in hBSMCs (Fig 1). There was no significant effect of albuterol stereoisomers on $G_{i\alpha-3}$ protein of 43 kd (data not shown).

In contrast to the effect on $G_{i\alpha-1}$ proteins, 8 hours of treatment with (S)-albuterol significantly reduced the expression of $G_{s\alpha}$ protein (46-kd band) to approximately 50% of the basal level (Fig 2). There was no significant effect of either (RS)-albuterol or (R)-albuterol at up to 8 hours of treatment on $G_{s\alpha}$ protein expression in hBSMCs. Because the 1-hour treatment with enantiomers had no significant effect on $G_{i\alpha-1}$ protein induction, all future experiments were performed after either 8 or 24 hours of treatment, as specified.

Activity of G_{i-1} protein

On activation, GTP binds to the $G_{i\alpha-1}$ subunit and dissociates from the $G_{i\beta\gamma-1}$ subunit to inhibit adenylate cyclase activity. Therefore to examine whether the treatment of hBSMCs with (S)-albuterol affects the activity of G_{i-1} protein, we examined the binding of nonhydrolysable analog of GTP to $G_{i\alpha-1}$ protein after treatment of hBSMCs with various doses to (S)-albuterol for 8

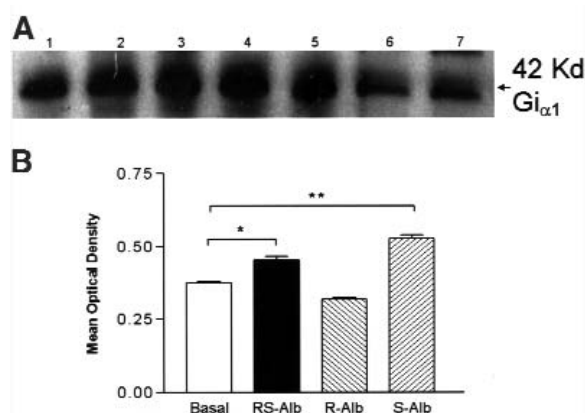


FIG 1. A, Effect of albuterol stereoisomers on the expression of $G_{i\alpha-1}$ in hBSMCs. Cells were incubated for 8 hours with medium (1), $0.1 \mu\text{mol/L}$ (RS)-albuterol (2), $1 \mu\text{mol/L}$ (RS)-albuterol (3), $0.1 \mu\text{mol/L}$ (S)-albuterol (4), $1 \mu\text{mol/L}$ (S)-albuterol (5), $0.1 \mu\text{mol/L}$ (R)-albuterol (6), and $1 \mu\text{mol/L}$ (R)-albuterol (7). B, Densitometric analysis (mean $\pm$ SEM, $n = 4-5$) of $G_{i\alpha-1}$ protein bands in hBSMCs. Basal, Media; RS-Alb, $1 \mu\text{mol/L}$ (RS)-albuterol; R-Alb, $1 \mu\text{mol/L}$ (R)-albuterol; S-Alb, $1 \mu\text{mol/L}$ (S)-albuterol. * $P < .05$, ** $P < .01$.

hours, followed by a 1-hour exposure to 1 mmol/L methacholine. We observed a dose-dependent effect of (S)-albuterol in the increased activation of G_{i-1} protein in hBSMCs (Fig 3).

Forskolin-induced adenylate cyclase activity

To examine whether (S)-albuterol affects nonreceptor-mediated activation of adenylate cyclase, we used forskolin, which bypasses cell-surface receptors and directly activates adenylate cyclase. Treatment of hBSMCs for 8 hours with (S)-albuterol decreased the forskolin-induced increase in intracellular cAMP levels in a dose-dependent manner, with a statistically significant effect at a concentration of 1 and $5 \mu\text{mol/L}$ (Fig 4).

$[Ca^{2+}]_i$

One of the key events that modulate hBSMC shortening and contraction is an increase in $[Ca^{2+}]_i$. Therefore we examined $[Ca^{2+}]_i$ levels after pretreatment of hBSMCs with various doses of (S)-albuterol. (S)-Albuterol increased $[Ca^{2+}]_i$ in a dose-dependent manner, with a statistically significant effect at concentrations of 0.1 and $1 \mu\text{mol/L}$ (Fig 5). Both (RS)-albuterol and (R)-albuterol decreased $[Ca^{2+}]_i$ levels (Fig 5).

Activity of PI3 kinase

Although the exact function of PI3 kinase in hBSMCs has not been completely characterized, PI3 kinases have been implicated in several inflammatory pathways. Furthermore, ligand-activated heterotrimeric G-protein activation in response to inflammatory stimuli activates PI3 kinase, followed by activation of downstream targets, including PDK1, Akt/protein kinase B (PKB), p70 ribosomal S6 kinase, and the transcription factor FKHR (forkhead in human rhabdomyosarcoma).²⁶ Therefore we examined the expression of p110 (and thus of PI3 kinase activity)²⁷ in hBSMCs after treatment with enan-

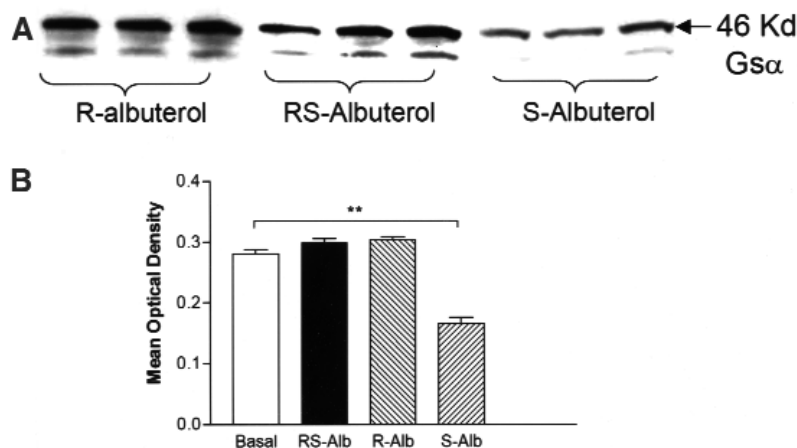


FIG 2. A, Effect of albuterol stereoisomers on $G_{s\alpha}$ protein in hBSMCs. The same amount of protein was loaded in each lane, and bands are shown from 3 separate samples for each experimental group. B, Densitometric analysis of $G_{s\alpha}$ protein in hBSMCs was performed in 4 to 5 separate experiments. Data are shown as means $\pm$ SEMs. ** $P < .01$.

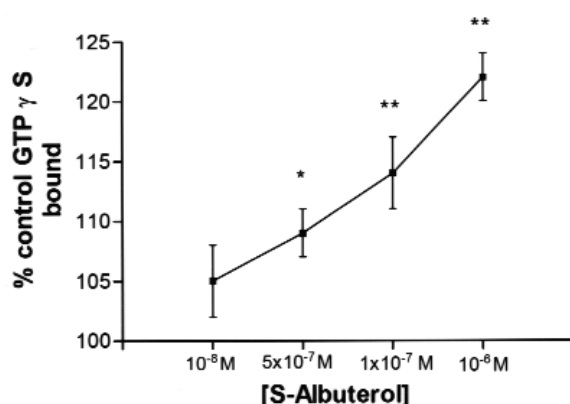


FIG 3. Effect of (S)-albuterol on agonist-stimulated $GTP\gamma S$ binding in hBSMCs. Data represent means $\pm$ SDs of 4 to 5 individual experiments. * $P < .05$, ** $P < .01$.

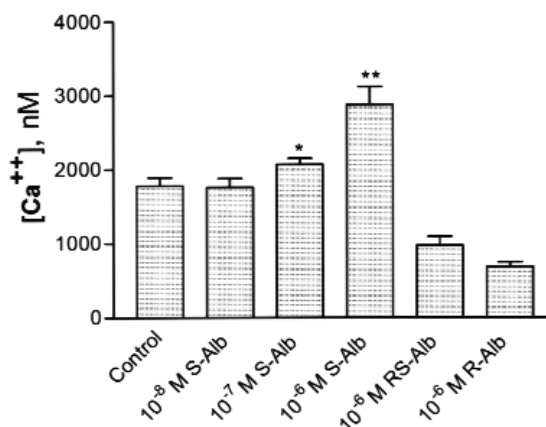


FIG 5. Effect of albuterol stereoisomers on $[Ca^{2+}]_i$. Cells were treated with enantiomers for 24 hours, followed by activation with methacholine. Data represent means $\pm$ SEMs from 4 independent experiments. * $P < .05$ and ** $P < .01$ compared with the control group.

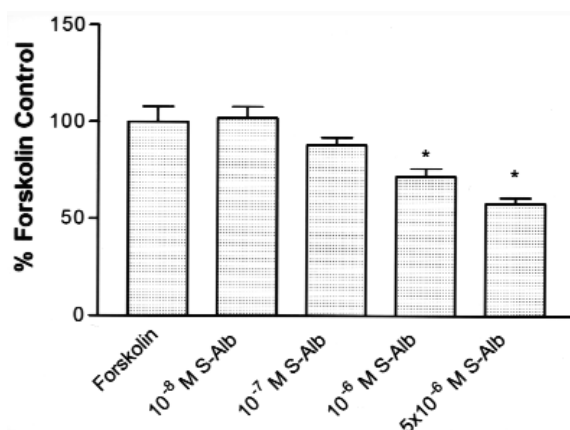


FIG 4. Effect of albuterol stereoisomers on intracellular cAMP. Confluent hBSMCs were treated with (S)-albuterol (10 nmol/L, 100 nmol/L, 1 μ mol/L, and 5 μ mol/L) for 10 minutes before and 15 minutes during exposure to 5 mmol/L forskolin. Results are shown as means $\pm$ SDs ($n = 4-5$). * $P < .05$.

tiomers of albuterol for 24 hours, followed by 15 minutes of treatment with 1 μ mol/L methacholine. We observed a significant increase in PI3 kinase activity in the cells treated with either 1 μ mol/L (S)-albuterol or 1 μ mol/L (RS)-albuterol (Fig 6). Interestingly, (R)-albuterol at a concentration of 1 μ mol/L significantly reduced the PI3 kinase activity in hBSMCs (Fig 6).

Activation of NF- κ B

There was a significant increase in the expression of I κ B-P protein in hBSMCs treated with either 1 μ mol/L (RS)-albuterol or (S)-albuterol (Fig 7). However, the increase in NF- κ B activity was much more pronounced (approximately 1.5-fold greater) with (S)-albuterol compared with that seen after (RS)-albuterol treatment (Fig 7). There was no significant difference in the expression of p65 protein between any of the experimental groups (data not shown).

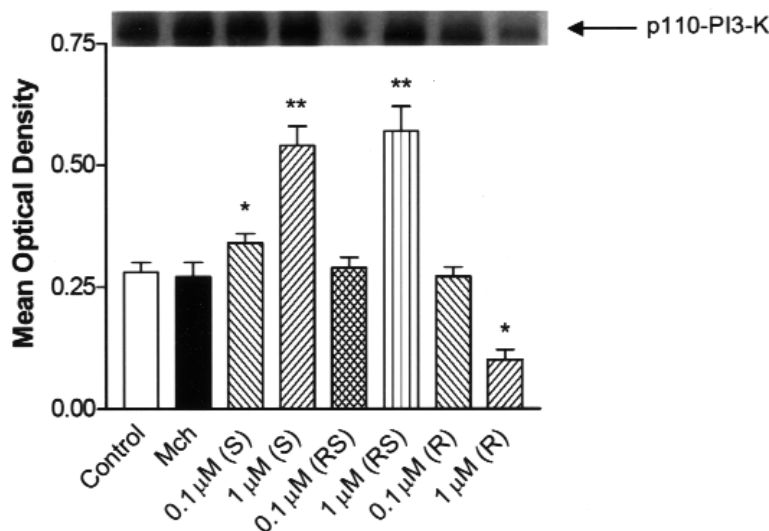


FIG 6. A representative blot of the effect of albuterol stereoisomers on the expression of p110 protein in hBSMCs. Densitometric analysis of the individual treatment groups is shown as mean $\pm$ SD ($n = 3$). *Mch*, Methacholine; (S), (S)-albuterol; (RS), (RS)-albuterol; (R), (R)-albuterol. * $P < .05$, ** $P < .01$.

DISCUSSION

β_2 -Agonists remain the first-line treatment in alleviating the symptoms of immediate allergic response in bronchial asthma. However, recent reports have raised some concerns as to the potential detrimental effects of racemic β_2 -agonists on long-term treatment. (S)-Enantiomers of β_2 -agonists are considered to be inactive molecules that stabilize the (R)-isomer. However, our initial in vivo study in guinea pigs with racemic and individual isomers of albuterol revealed increased inflammatory response on treatment with (S)-albuterol.²⁸ Several in vitro studies in isolated ASM cells also demonstrated the proinflammatory and pro-constrictory effects of (S)-albuterol.^{15-19,29-32} The underlying mechanisms of such effects were not clear. In this study we examined the effect of the enantiomers of albuterol on intracellular transmembrane signaling pathways involved in the activation of β -agonists and interaction with kinases and transcription factor in hBSMCs.

In this study the (S)-albuterol-induced increase in total protein and activity of G_{i-1} proteins and decrease in response to G_s protein suggest that (S)-albuterol is capable of desensitizing the intracellular pathway involved in bronchodilation and causing resultant hypersensitization of the pathways involved in bronchoconstriction. The premise was further supported by an increase in $[Ca^{2+}]_i$ observed on exposure of the hBSMCs to (S)-albuterol. Whether this increase in $[Ca^{2+}]_i$ is due to depletion of intracellular stores, an increase in the entry of extracellular calcium, or both is not clear. Interestingly, the effect of (R)-albuterol was opposite to that of (S)-albuterol. These data might provide a mechanistic explanation for the findings of several investigators that long-term treatment with racemic albuterol was detrimental in several patients with bronchial asthma.³⁻⁵ Whether such a detri-

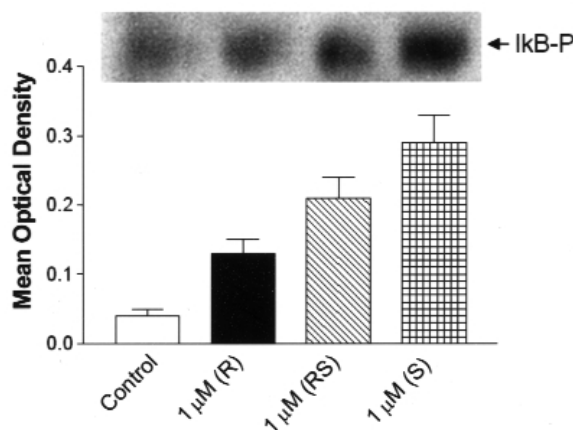


FIG 7. Effect of albuterol stereoisomers on NF- κ B activation in hBSMCs. (R), (R)-Albuterol; (RS), (RS)-albuterol; (S), (S)-albuterol. Western blot is representative of 3 separate experiments, and densitometric analysis from 3 separate experiments is shown as the mean $\pm$ SD. * $P < .05$, ** $P < .01$.

mental effect of racemic and (S)-albuterol is dependent on the genotype or phenotype of β_2 -adrenoceptors in hBSMCs is yet to be determined.

The PI3 kinase family is a family of enzymes subdivided into 3 classes on the basis of their structural motifs and activators. Class I PI3 kinases can be subdivided into IA and IB. Class IB p110 subunits of PI3 kinase are activated by heterotrimeric G proteins and are of the γ (p110 γ) subtype.³³ The ability of the catalytic p110 subunit to bind to phospholipids in the cell membrane might be a critical point at which PI3 kinases, in conjunction with Akt/PKB, are further regulated in various inflammatory conditions. In this study we observed a significant increase in p110 (and thus of PI3 kinase activity) after 24 hours of treatment with (S)-albuterol. It has been

reported in the literature that most physiologic stimuli that increase intracellular cAMP levels also activate both PI3 kinase and PKB activity in several cell systems.^{34,35} Whether it is true with hBSMCs is not known. Indeed, our data do not support this theory because (S)-albuterol attenuated cAMP levels in response to a nonreceptor-mediated stimulus, forskolin. This can be substantiated by the findings that forskolin can activate PKB in a PI3 kinase-independent manner.³⁶ This could be due to the fact that cAMP modulates the same phosphorylation events that are stimulated by PI3 kinase-dependent PKB-activating stimuli. We have not examined PKB activation in this study. However, it is likely that (S)-albuterol activated PI3 kinase through some alternative mechanisms involved in the inflammatory effects of hBSMCs. Recently, Naga-Prasad et al³⁷ demonstrated that β -AR kinase 1 and PI3 kinase form a cytosolic complex that leads to β -AR kinase 1-mediated translocation of PI3 kinase to the membrane, which resulted in rapid interaction with the receptor, which is of functional importance because inhibition of PI3 kinase activity attenuates β -AR sequestration. Therefore it is possible that (S)-albuterol might be inducing, at least in part, desensitization of β -ARs through activation of PI3 kinase.

We have previously reported the exaggeration of late allergic response, AHR, and airway inflammation by (S)-albuterol in ovalbumin-sensitized and challenged guinea pigs.²⁸ Proinflammatory pathways involve the activation of several transcription factors, including NF- κ B.^{38,39} Indeed, the primary mechanism underlying the anti-inflammatory effect of corticosteroids includes the inhibition of NF- κ B activation.⁴⁰ In the inactive state NF- κ B consists of 3 subunits, p50, p65, and I κ B, and on phosphorylation of the I κ B subunit, p50-p65 subunits dissociate from the I κ B subunit and dimerize. It then migrates to the nucleus, and on binding to the NF- κ B-binding elements on DNA in the upstream of the promoter region, it modulates the transcription of several inflammatory mediators. In this study we observed that (S)-albuterol increased the activity of NF- κ B transcription factor in hBSMCs. This might be responsible for (S)-albuterol-induced increased airway inflammation. This could be further supported by data from a phase III clinical study by Nelson et al,⁴¹ who observed that steroids inhibited (or masked) the negative effects of (S)-albuterol. In fact, racemic albuterol decreased the FEV₁ at 28 days in patients not receiving concomitant steroids. This effect of racemic albuterol on a decrease in FEV₁ was not present in placebo or levalbuterol recipients or in any groups receiving steroids. Although in this study we did not examine the effect of steroids on (S)-albuterol-induced activation of NF- κ B, the study by Nelson et al⁴¹ further supports our findings that (S)-albuterol has a direct effect on the proinflammatory transmembrane-signaling pathway, which is sensitive to steroids. Also, (S)-albuterol in high doses over a short period of time might antagonize the beneficial effects of steroids in the treatment of acute episodes of allergic response in the airways.

The effects of (S)-albuterol on proinflammatory pathways were observed only when the cells were treated for

at least 8 hours. The half-life of levalbuterol is approximately 3 hours. However, a recent study suggested that (S)-albuterol is differentially metabolized and excreted at one tenth the rate of levalbuterol, and that pathway has finite and comparatively limited capacity.^{42,43} This raises the potential for accumulation of a high amount of (S)-albuterol in tissues to induce deleterious effects. Indeed, Dhand et al⁴⁴ reported a significantly increased accumulation of (S)-albuterol in the lungs of patients who died of asthma exacerbations.

There are several reports in the literature supporting our data on the pro-constrictive and proinflammatory effects of (S)-albuterol in hBSMCs. In bovine tracheal smooth muscle cells, carbachol also causes an increase in [Ca²⁺]_i, and treatment with (S)-albuterol before carbachol exposure increased the magnitude of this response; in contrast, (R)-albuterol reduced the carbachol-induced calcium response.⁴⁵ The increase in [Ca²⁺]_i after (S)-albuterol treatment was linked to activation of the inositol-1,4,5-triphosphate pathway in bovine smooth muscle cells.²² (S)-albuterol has also been reported to enhance AHR to spasmogens in experimental animals.^{9,10} Cho et al¹⁵ reported that (S)-albuterol increased the secretion of histamine and IL-4 in IgE-stimulated murine mast cells. Proallergic effects of (S)-isomers of β_2 -agonists have also been observed in eosinophils, epithelial cells, and lymphocytes.¹⁶⁻¹⁹

In an elegant study Baramki et al¹⁶ demonstrated that addition of (S)-albuterol to (R)-albuterol mediated a dose-dependent increase in T-cell proliferation. Furthermore, the combination of (R)-albuterol and (S)-albuterol stimulated production of IL-2 and IL-13. These data clearly demonstrated the proinflammatory effects of excess (S)-albuterol. In this study the authors did not examine the underlying cellular and molecular mechanisms involved. Because we observed a significant increase in the activity of the transcription factor NF- κ B, and this transcription factor is known to be an inflammatory mediator during the allergic response that leads to the production of proinflammatory cytokines, it is likely that (S)-albuterol might be activating NF- κ B in T lymphocytes as well.

Whether such effects of (S)-albuterol are mediated through β_2 -ARs or some independent mechanism is not clear. Racemic albuterol possesses a high affinity for β_2 -adrenoceptors on the surface of hBSMCs. Although the affinity of (R)-albuterol for β_2 -ARs is greater than that of racemic albuterol, the binding affinity of (S)-albuterol is 500 to 1000 times less than that of racemic albuterol for β_2 -ARs in a variety of cell systems and human cloned receptors.⁴⁶ Indeed, there was no significant binding of (S)-albuterol in clinically achievable levels. However, in this study an equimolar concentration of (S)-albuterol, as compared with (R)-albuterol, had an opposite and negative effect on bronchodilatory pathways in bronchial smooth muscle cells. This suggests that the detrimental effect of (S)-albuterol is due to its binding on a site on β_2 -ARs, which is different from the (R)-albuterol-binding site. Alternatively, chronic exposure of the cell to (S)-

albuterol switches the coupling of the β_2 -ARs to pro-constrictory G proteins, such as G_{i-1} .⁴⁷ Indeed, this could be supported by our findings that (S)-albuterol significantly increased the expression and activity of $G_{i\alpha-1}$ protein in hBSCs. There is also a possibility that (S)-albuterol binds to a site other than the β_2 -ARs, possibly intracellularly. This can be further supported by our observation that (S)-albuterol also attenuated the response to forskolin, which directly activates adenylate cyclase and increases cAMP in a nonreceptor-mediated fashion. These data do not support the potential inverse agonist theory for the activity of (S)-albuterol.⁴⁸ Nevertheless, the exact binding site for (S)-albuterol on hBSCs is still unclear. However, very recently, we observed strong specific binding of tritiated (S)-albuterol in intracellular membrane fractions of hBSCs, which is not displaceable by (R)-albuterol (our unpublished data). Further studies are currently under investigation.

In summary, these data suggest that (S)-albuterol, which elicited dose-dependent and time-dependent effects, increased G_{i-1} protein and its activity and decreased G_s protein in hBSCs. (S)-Albuterol also enhanced $[Ca^{2+}]_i$ and decreased cAMP on agonist stimulation. In addition, (S)-albuterol activated both PI3 kinase and NF- κ B. These findings further enlighten us as to the potential mechanisms underlying the detrimental effect of racemic albuterol or (S)-albuterol observed in experimental animals and studies in isolated cells. Our study does not directly provide supporting evidence for its clinical implication. Perhaps clinical relevance to these in vitro findings will become apparent at high doses, such as those used in an intensive care unit for severe acute asthma.^{49,50} However, this remains to be demonstrated.

REFERENCES

- Nelson HS. β -Adrenergic bronchodilators. *N Engl J Med* 1994;333:499-506.
- Dutta EJ, Li JT. Beta-agonists. *Med Clin North Am* 2002;86:991-1008.
- Sears M, Taylor D, Print C, et al. Regular inhaled beta-agonist treatment in bronchial asthma. *Lancet* 1990;336:1391-6.
- Spitzer WO, Suissa S, Ernst P, et al. The use of β -agonists and the risk of death and near death from asthma. *N Engl J Med* 1992;326:501-6.
- Cockcroft DW, O'Byrne PM, Swystun VA, Bhagat R. Regular use of inhaled albuterol and the allergen-induced late asthmatic response. *J Allergy Clin Immunol* 1995;96:44-9.
- Handley DA, Anderson AJ, Koester J, Snider ME. New millennium bronchodilators for asthma: single isomer beta agonists. *Curr Opin Pulm Med* 2000;6:43-9.
- Ramsay CM, Cowan J, Flannery E, McLachlan C, Taylor DR. Bronchoprotective and bronchodilator effects of single doses of (S)-salbutamol, (R)-salbutamol and racemic salbutamol in patients with bronchial asthma. *Eur J Clin Pharmacol* 1999;55:353-9.
- Cockcroft DW, Swystun VA. Functional antagonism: tolerance produced by inhaled β_2 agonists. *Thorax* 1996;51:1051-6.
- Hoshiko K, Morley J. Exacerbation of airway hyperreactivity by ($\pm$) salbutamol in sensitized guinea pigs. *Jpn J Pharmacol* 1993;63:159-63.
- Mazzoni L, Naef R, Chapman ID, Morley J. Hyperresponsiveness of the airways following exposure of guinea pigs to racemic mixtures and distomers of beta2-selective sympathomimetics. *Pulm Pharmacol* 1994;7:367-76.
- Templeton AGB, Chapman ID, Chilvers ER, Morley J, Handley DA. Effects of S-albuterol on human isolated bronchus. *Pulm Pharmacol Ther* 1998;11:1-6.
- Lotvall J, Palmqvist M, Arvidsson P, Maloney A, Ventresca P, Ward J. The therapeutic ratio of R-albuterol is comparable with that of RS-albuterol in asthmatic patients. *J Allergy Clin Immunol* 2001;108:726-31.
- Ahrens R, Weinberger M. Levalbuterol and racemic albuterol: are there therapeutic differences? *J Allergy Clin Immunol* 2001;108:681-4.
- Emerman C, Nowak R. Inverse correlations between (S)-albuterol levels and FEV1 in the emergency department [abstract]. *Acad Emerg Med* 2002;9:404.
- Cho SH, Hartleroad JY, Oh CK. (S)-albuterol increases the production of histamine and IL-4 in mast cells. *Int Arch Allergy Immunol* 2001;124:478-84.
- Baramki D, Koester J, Anderson AJ, Borish L. Modulation of T-cell function by (R)- and (S)-isomers of albuterol: Anti-inflammatory influences of (R)-isomers are negated in the presence of the (S)-isomer. *J Allergy Clin Immunol* 2002;109:449-54.
- Volchek GW, Gleich GJ, Kita H. Pro- and anti-inflammatory effects of β -adrenergic agonists on eosinophil response to IL-5 [abstract]. *J Allergy Clin Immunol* 1998;101(suppl):S35.
- Leff AR, Herreiter A, Naclerio RM, Baroody FM, Handley DA, Munoz NM. Effect of enantiomeric forms of albuterol on stimulated secretion of granular protein from human eosinophils. *Pulm Pharmacol Ther* 1997;10:97-104.
- Frieri M, Pergolizzi R, Millan C, et al. Cytosine, chemokine, and nitric oxide (NO) release in stimulated small airway epithelial cells (SAEC) treated with beta2-agonist enantiomers of albuterol [abstract]. *J Allergy Clin Immunol* 2000;105(suppl):S292.
- Johnson M. The beta-adrenoceptor. *Am J Respir Crit Care Med* 1998;158(suppl):S146-53.
- Billington CK, Hall IP, Mundell SJ, et al. Inflammatory and contractile agents sensitize specific adenylyl cyclase isoforms in human airway smooth muscle. *Am J Respir Cell Mol Biol* 1999;21:597-606.
- Mitra S, Ugur M, Ugur O, Goodman H, McCullough J, Yamaguchi H. (S)-albuterol increases intracellular free calcium by muscarinic receptor activation and a phospholipase C-dependent mechanism in airway smooth muscle. *Mol Pharmacol* 1998;53:347-54.
- McGraw DW, Liggett SB. Heterogeneity in beta-adrenergic receptor kinase expression in the lung accounts for cell-specific desensitization of the beta2-adrenergic receptor. *J Biol Chem* 1997;272:7338-44.
- Lefkowitz RJ, Pitcher J, Krueger K, Daaka Y. Mechanisms of beta-adrenergic receptor desensitization and resensitization. *Adv Pharmacol* 1998;42:416-20.
- Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440-50.
- Missy K, Van Poucke V, Raynal P, et al. Lipid products of phosphoinositide 3-kinase interact with Rac1 GTPase and stimulate GDP dissociation. *J Biol Chem* 1998;273:30279-86.
- Yu J, Wjasow C, Backer JM. Regulation of the p85/p110 α phosphatidylinositol 3'-kinase: distinct roles for the N-terminal and C-terminal SH2 domains. *J Biol Chem* 1998;273:30199-203.
- Agrawal DK, Clark KC, Townley RG. Differential effects of albuterol enantiomers on pulmonary functions and bronchoalveolar lavage (BAL) eosinophilia [abstract]. *Am J Respir Crit Care Med* 2000;161:191A.
- Zhang X, Zhu F, Olszewski MA, Robinson NE. Effects of enantiomers of β_2 -agonists on Ach release and smooth muscle contraction in the trachea. *Am J Physiol* 1998;74:L32-8.
- Johansson F, Rydberg I, Aberg G, Andersson RGG. Effects of albuterol enantiomers on in vitro bronchial reactivity. *Clin Rev Allergy Immunol* 1996;14:57-64.
- Perrin-Fayolle M, Blum P, Morley J, Grosclaude M, Chambe MT. Differential responses of asthmatic airways to enantiomers of albuterol. *Clin Rev Allergy Immunol* 1996;14:139-47.
- Handley DA. The asthma-like pharmacology and toxicology of (S)-isomers of β -agonists. *J Allergy Clin Immunol* 1999;104(suppl):S69-76.
- Arcaro A, Volinia S, Zvelebil MJ, et al. Human phosphoinositide 3-kinase C2 β , the role of calcium and the C2 domain in enzyme activity. *J Biol Chem* 1998;273:33082-90.
- Tilton B, Andjelic M, Didichenko SA, Hemmings BA, Thelen M. G-protein-coupled receptors and Fcgamma-receptors mediate activation of Akt/protein kinase B in human phagocytes. *J Biol Chem* 1997;272:28096-101.
- Coffer PJ, Schweizer RC, Dubois GR, Maikoe T, Lammers J-WJ, Koen-

- derman L. Analysis of signal transduction pathways in human eosinophils activated by chemoattractants and the T-helper-2-derived cytokines interleukin-4 and interleukin-5. *Blood* 1998;91:2547-57.
36. Sable CL, Filippa N, Hemmings B, Van Obberghen E. cAMP stimulates protein kinase B in a Wortmannin-insensitive manner. *FEBS Lett* 1997;409:253-7.
 37. Naga-Prasad SV, Barak LS, Rapacciuolo A, Caron MG, Rockman HA. Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by beta-adrenergic receptor kinase 1. A role in receptor sequestration. *J Biol Chem* 2001;276:18953-9.
 38. Barnes PJ. Nuclear factor-kappa B. *Int J Biochem Cell Biol* 1997;29:867-70.
 39. Blackwell TS, Christman JW. The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol* 1997;17:3-9.
 40. Das J, Chen CH, Yang L, Cohn L, Ray P, Ray A. A critical role for NF-kappa B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat Immunol* 2001;2:45-50.
 41. Nelson HS, Bensch G, Pleskow WW, et al. Improved bronchodilation with levalbuterol versus racemic albuterol in patients with asthma. *J Allergy Clin Immunol* 1998;102:943-52.
 42. Schmekel B, Rydberg I, Norlander B, Sjosward KN, Ahlner J, Andersson RGG. Stereoselective pharmacokinetics of S-salbutamol after administration of the racemate in healthy volunteers. *Eur Respir J* 1999;13:1230-5.
 43. Gumbhir-Shah K, Kellerman D, DeGraw S, Koch P, Jusko W. Pharmacokinetic and pharmacodynamic characteristics and safety of inhaled albuterol enantiomers in healthy volunteers. *J Clin Pharmacol* 1998;38:1096-106.
 44. Dhand R, Goode M, Reid R, Fink J, Fahey P, Tobin M. Preferential pulmonary retention of (S)-albuterol after inhalation of racemic albuterol. *Am J Respir Crit Care Med* 1999;160:1136-41.
 45. Yamaguchi H, McCullough JR. (S)-Albuterol exacerbates calcium responses to carbachol in airway smooth muscle cells. *Clin Rev Allergy Immunol* 1996;14:47-55.
 46. Penn R, Frielle T, McCullough JR, Aberg G, Benovic J. Comparison of R-, S-, and RS-albuterol interaction with human β_1 - and β_2 -adrenergic receptors. *Clin Rev Allergy Immunol* 1996;14:37-45.
 47. Daaka Y, Luttrell LM, Lefkowitz RJ. Switching of the coupling of the β_2 -adrenergic receptor to different G proteins by protein kinase A. *Nature* 1997;390:88-91.
 48. Milligan G, Bond R. Inverse agonism and the regulation of receptor number. *Trends Pharmacol Sci* 1997;18:468-74.
 49. Milgrom H, Skoner DP, Bensch G, Kim KT, Claus R, Baumgartner RA. Low-dose levalbuterol in children with asthma: Safety and efficacy in comparison with placebo and racemic albuterol. *J Allergy Clin Immunol* 2001;108:938-45.
 50. Truitt T, Witko J, Halpern M. Levalbuterol compared to racemic albuterol: efficacy and outcomes in patients hospitalized with COPD or asthma. *Chest* 2003;123:128-35.