

Modulation of GM-CSF release by enantiomers of β -agonists in human airway smooth muscle

Bill T. Ameredes, PhD, and William J. Calhoun, MD Pittsburgh, Pa

Background: β_2 -Adrenergic receptor agonists can reduce the release of GM-CSF by human airway smooth muscle cells (HASMCs). These effects are considered anti-inflammatory and are ascribed to the activity of the (R)-enantiomer within the racemate of the agonist. However, the effect of the (S)-enantiomer on GM-CSF release, once thought to be inert, has not been extensively explored.

Objective: We hypothesized that the (S)-enantiomer may counter the effects of the (R)-enantiomer, potentially increasing GM-CSF release. Therefore, the effects of administration of individual and combined enantiomers on GM-CSF release were examined.

Methods: Cultured HASMCs were stimulated with IL-1 β , TNF- α , and IFN- γ and treated with (R)-enantiomers and (S)-enantiomers of albuterol and formoterol, with and without propranolol and ICI-118,551, and in combination with dexamethasone. GM-CSF in the resulting conditioned media was assessed by ELISA.

Results: (R)-enantiomers significantly reduced GM-CSF release by as much as 41% ($P < .05$), which was reversible with propranolol. In contrast, (S)-enantiomers significantly increased GM-CSF release by as much as 34% ($P < .05$) over release with no drug, and by 25% to 40% ($P < .05$) when added with (R)-enantiomers. The decremental effect of dexamethasone was amplified by (R)-enantiomers but inhibited by (S)-enantiomers. Both propranolol and ICI-118,551 alone increased GM-CSF release in a concentration-dependent fashion, similar to (S)-enantiomers.

Conclusion: We conclude that GM-CSF release by HASMC is downregulated by (R)-enantiomers and enhanced by (S)-enantiomers. The reversal of (R)-enantiomer and dexamethasone effects by the (S)-enantiomer suggests

suppression of their anti-inflammatory effects, perhaps through an antagonistic mechanism similar to propranolol. (J Allergy Clin Immunol 2005;116:65-72.)

Key words: Albuterol, β -receptors, cAMP, formoterol, inverse agonist, levalbuterol, propranolol, racemic

Airway smooth muscle cells are a major constitutive and contractile cell type within human airways. Their contractile characteristics regulate airway caliber; however, they also have been shown to possess paracrine-like activity with regard to cytokine release. For example, it is known that human airway smooth muscle cells (HASMCs) produce cytokines considered to be proinflammatory, such as GM-CSF,¹ which promotes eosinophil activation and survival.^{2,3} Given that eosinophils have been linked to airway inflammation in diseases such as asthma,⁴ and that measurable levels of GM-CSF have been found in bronchoalveolar lavage fluid from symptomatic patients with asthma,⁵ production of GM-CSF by HASMC may be important in promoting the process of inflammation and the development of asthma. Furthermore, although HASMCs are known to possess large numbers of β_2 -adrenergic receptors that can modulate contractile function, it has also been shown that application of β_2 -adrenergic receptor agonists can modulate GM-CSF release by HASMC.⁶ Therefore, a mechanism likely exists for anti-inflammatory modulation of GM-CSF release through β_2 -adrenergic receptor agonism.⁷ However, there is evidence that β_2 -adrenergic agonists can result in paradoxical worsening of asthma symptoms through mechanisms that exacerbate airway inflammation and airway hyperresponsiveness.⁸ The sources of these mechanisms remain poorly understood but may be related to application of racemic mixtures of β_2 -adrenergic agonists in the treatment of asthma.⁹⁻¹¹

Racemic mixtures refer to a 50:50 mix of (R)-enantiomer and (S)-enantiomer of β_2 -adrenergic agonists typically present in commercially available preparations such as albuterol. Because of the preferential β_2 -adrenergic receptor binding of the (R)-enantiomer and the poor affinity of the (S)-enantiomer for β_2 adrenergic receptors, the (S)-enantiomers typically have been considered inactive.^{12,13} Indeed, stereoselectivity for the (R)-enantiomers has been shown to play a role in the determination of anti-inflammatory effects of β_2 -adrenergic receptor agonists, such as elevation in intracellular cyclic adenosine monophosphate (cAMP) and IL-10 production, and

From the Asthma, Allergy, and Airway Research Center, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh Medical Center.

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Reprint requests: Bill T. Ameredes, PhD, Research Assistant Professor of Medicine, Asthma, Allergy, and Airway Research Center, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, 628NW Montefiore University Hospital, 3459 Fifth Ave, Pittsburgh, PA 15213. E-mail: ameredesbt@upmc.edu.

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Abbreviations used

cAMP: Cyclic adenosine monophosphate
 HASMC: Human airway smooth muscle cell

decrements in TNF- α production.¹⁴ However, there has been some evidence that (S)-enantiomers may not be biologically inactive and may be responsible for some paradoxical or deleterious responses,^{11,15} including promotion of increased intracellular calcium levels.¹⁶ Moreover, it has been shown that metabolic clearance of (S)-albuterol occurs much less rapidly than (R)-albuterol,¹⁷ and thus the paradoxical responses may persist after the beneficial effects of (R)-albuterol have occurred.¹⁰

Therefore, we hypothesized that (S)-enantiomers of β_2 -adrenergic agonists applied to HASMC would produce effects counter to those produced by (R)-enantiomers. On the basis of this hypothesis, we predicted that (R)-albuterol and (R,R)-formoterol would be effective in reduction of GM-CSF release by HASMC, and that this effect would be diminished by propranolol, a classic β_2 -adrenergic receptor antagonist. Conversely, we predicted that (S)-albuterol and (S,S)-formoterol applied alone might increase GM-CSF release by HASMC and perhaps negate the reduction of GM-CSF by their (R)-enantiomer counterparts when applied in combination with either (R)-enantiomers or dexamethasone. Because agonist binding to the β_2 -adrenergic receptor typically produces an intracellular response resulting in elevations in cAMP, we measured intracellular cAMP, and we also tested whether addition of an intracellular cAMP analogue might result in GM-CSF reduction in our model, suggesting one potential pathway through which the (R)-enantiomers may produce their effects.

METHODS

Cell culture and stimulation

Human airway smooth muscle cells collected postmortem from normal donors were purchased (BioWhittaker Inc, Clonetics Prod., San Diego, Calif) and cultured (37°C; 5% CO₂) to passage 6 over a period of 7 days in modified smooth muscle cell basal media (SmBM; BioWhittaker Inc, Clonetics Prod.). The media contained 4.4 mg/L L-glutamine, 10 μ g/mL human epidermal growth factor, 1 μ g/mL human fibroblastic growth factor, 5 mg/mL insulin, 50 mg/mL gentamicin, and 50 μ g/mL amphotericin B, and was free of epinephrine and other β -adrenergic receptor agonists. Cells from a total of 3 different donors were studied in the experiments described. The cells were plated at 1 million cells per well and serum-starved for 24 hours to synchronize growth at the G₀ phase.¹⁸ After the 24-hour interval, they were serum-replenished and simultaneously incubated with cytomix (IL-1 β , 100 U/mL; TNF- α , 500 U/mL; IFN- γ , 100 U/mL) + LPS (0.1 μ g/mL) for an additional 24 hours. In all experiments, at the end of the 24-hour incubation period, the conditioned media was harvested and frozen at -80°C for later assay.

The stimulus mixture and growth model was chosen to provide a strong, proinflammatory cytokine stimulus signal that could be considered synergistic¹ and might be present in the asthmatic airway⁵ in the case of a potent bacterially related allergic response in cells

undergoing proliferation as part of an airway remodeling process,^{19,20} with subsequent treatment by β_2 -adrenergic receptor agonists. All cytokines were purchased from R&D Systems, Inc (Minneapolis, Minn), and all other nonenantiomer chemicals were purchased from Sigma (St Louis, Mo).

Single and combined enantiomers

In one set of experiments, one of each β_2 -adrenergic receptor agonist enantiomers was added alone—(R)-albuterol, (S)-albuterol, (R,R)-formoterol, or (S,S)-formoterol—at 2 concentrations (10 nmol/L and 10 μ mol/L), at the same time of serum replenishment and cytomix was added, after the 24-hour serum starvation period. A previous study in HASMC indicated suppression of GM-CSF release with 10 μ mol/L fenoterol²¹ and falls within expected concentrations that would be obtained in the large airways (1000 μ mol/L to generation 8) and small airways (100 μ mol/L for generations 9-16) on the basis of calculations with inhalation of 2.5 mg β_2 -adrenergic receptor agonist.²²⁻²⁴ Furthermore, the clinical relevance of 10 μ mol/L was demonstrated in measurements of attained distal airway concentrations, in which levels of 1 μ mol/L to 10 μ mol/L were measured with conventional dosing of albuterol.²⁵ A low concentration of 10 nmol/L was chosen as representative of that attained in plasma with administration of inhaled albuterol,²⁶ representing a lower-end theoretical equilibration point between the blood and airway compartments. All β_2 -adrenergic receptor agonist enantiomers were provided by Sepracor, Inc (Marlborough, Mass).

In one series of experiments, β_2 -adrenergic receptor antagonism was achieved with a single effective concentration of propranolol (10 μ mol/L^{27,28}), added simultaneously with each of the enantiomers. In another series of experiments, increasing concentrations of propranolol were tested against 1 effective concentration of either (R)-albuterol or (R,R)-formoterol. Finally, the effects of β_2 -adrenergic receptor antagonism with sole administration of either propranolol or ICI-118,551, a potent β_2 -adrenergic receptor antagonist, were assessed.

In another series of experiments, the potential that (S)-enantiomers might act through a muscarinic receptor-associated mechanism was evaluated by adding atropine (100 nmol/L) in combination with (S)-enantiomers 24 hours after serum starvation. Also, atropine was added in combination with (R)-enantiomers as another control for its effects.

Dexamethasone experiments

A concentration-response relationship was obtained with application of dexamethasone with no enantiomers or propranolol, to determine a dexamethasone concentration that was effective in significantly reducing GM-CSF release, such that an additional change in GM-CSF release with addition of (R)-enantiomers or (S)-enantiomers might be observed. All administration of dexamethasone was performed at 24 hours after the initiation of serum starvation, as described.

cAMP experiments

Exogenous dibutyryl cAMP (N⁶,2'-O-dibutyryladenosine-3', 5'-cyclic monophosphate), a cell-permeant cAMP analogue that activates cAMP-dependent protein kinase A,²⁹ was added as described, with conditioned media collected 24 hours later. A computer program (SigmaPlot v8.0; SPSS Inc, Chicago, Ill) with a regression fitting a 4-parameter sigmoidal curve to the data was used, with the equation

$$y = y_0 + a / (1 + e^{-(x-x_0/b)})$$

allowing stipulation of the initial GM-CSF release as the starting point of each curve.

GM-CSF measurement

The release of GM-CSF was measured by ELISA for human GM-CSF (Quantikine; R&D Systems, Inc). The sensitivity of the assay was <3 pg/mL, and the assay was highly specific, with no significant cross-reactivity with TNF- α , IFN- γ , or IL-1 β . Resultant GM-CSF values (pg/mL) were typically expressed as a function of the stimulated control GM-CSF value, in which no drugs were added.

cAMP measurement

Measurement of intracellular cAMP was performed by using a kit (Biomol, Plymouth Meeting, Pa) on HASMC lysates prepared according to the kit manufacturer's directions. Values were determined in picomol/mL and normalized to initial control levels present with no drugs added.

Statistics

GM-CSF levels were compared between conditions of no cell stimulus and with stimulus but no drugs by using an independent sample *t* test. Trials of either (R)-enantiomers and (S)-enantiomers or mixtures, corresponding inhibition with propranolol, sole administration of propranolol or ICI-118,551, and drugs in combination with dexamethasone were analyzed by using repeated measures ANOVA (SigmaStat v3.0, SPSS Inc). When parametric criteria were unmet, a Friedman repeat-measures ANOVA on ranks was performed. Post hoc testing of discrete data on acquisition of a significant *F* statistic or χ^2 was performed by using Student-Newman-Keuls analysis. All tests were performed on GM-CSF or cAMP raw data expressed in concentration units. Results of all tests were considered significant at levels of $P < .05$.

RESULTS

Elicitation of GM-CSF release

Baseline (nonstimulated) GM-CSF production was very low or not detectable (3 pg/mL $\pm$ 2 pg/mL [SE]). With cytomix stimulation, GM-CSF in the conditioned media increased significantly, averaging 1055 pg/mL $\pm$ 102 pg/mL ($P < .05$), but sometimes as much as 3000 pg/mL. Because of this variation in levels of stimulated GM-CSF release between experiments, results were expressed relative to the stimulated control within a given experiment to allow comparisons across experiment repetitions and treatments.

Effects of enantiomers

Addition of (R)-albuterol at 10 μ mol/L or (R,R)-formoterol at 10 nmol/L or 10 μ mol/L to stimulated cells typically decreased GM-CSF release from ~25% to 50% ($P < .05$), with (R,R)-formoterol more effectively decreasing GM-CSF compared with (R)-albuterol ($P < .05$). Addition of (S)-albuterol at 10 nmol/L and 10 μ mol/L and (S,S)-formoterol at 10 nmol/L typically produced variable but significant increases of GM-CSF 10% to 50% greater than the control with no enantiomers added ($P < .05$), which were significantly greater (30% to 80%) than the levels produced by treatment with the (R)-enantiomers alone ($P < .05$). Addition of (S)-enantiomers of albuterol and formoterol in combination with respective (R)-enantiomers resulted in elevated GM-CSF levels compared with the (R)-enantiomers alone (Fig 1).

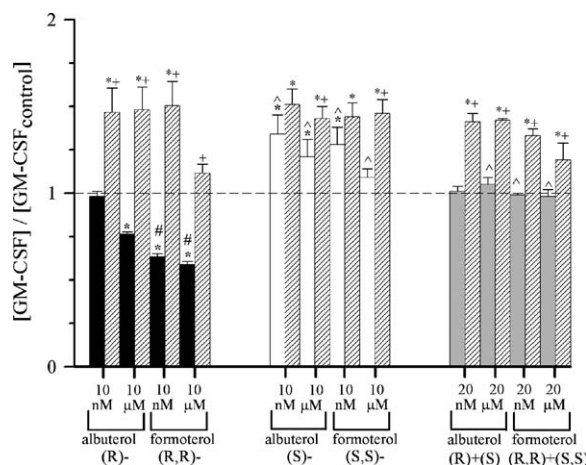


FIG 1. GM-CSF release with enantiomers and propranolol (10 μ mol/L; hatched bars) relative to control (dashed line; [enantiomer] = 0). (R)-albuterol (n = 6), (S)-albuterol (n = 4), (R)- + (S)-albuterol (n = 4), (R,R)-formoterol (n = 6), (S,S)-formoterol (n = 4), or (R,R)- + (S,S)-formoterol (n = 4). Symbols indicate $P < .05$ for comparison versus *respective control, +respective enantiomer at same concentration, ^respective (R)-enantiomer at same concentration, and # (R)-albuterol at same concentration.

Effects of propranolol and ICI-118,551

Application of propranolol alone increased GM-CSF release in stimulated cells in a concentration-dependent fashion (Fig 2, A), with a concentration of 10 μ mol/L reliably resulting in an approximate 50% increase in GM-CSF release. Although this suggested that there may have been a basal β_2 -adrenergic receptor-associated responsiveness in our model, we also inferred that it may have been indicative of propranolol acting as an antagonist or inverse agonist with respect to GM-CSF release. The maximal antagonist ICI-118,551 strongly potentiated GM-CSF release to an even greater degree than propranolol, resulting in GM-CSF levels more than 3 times greater than 0 drug at the highest concentration tested (Fig 2, A). Intracellular cAMP levels were significantly decreased with increasing ICI-118,551 concentrations (Fig 2, B) and were numerically decreased in a similar fashion with increasing propranolol concentrations.

In all cases of β_2 -adrenergic receptor agonism by enantiomeric compounds, application of the competitive antagonist propranolol increased GM-CSF release by 25% to 50% over control ($P < .05$), by 25% to 100% over (R)-enantiomers ($P < .05$), and by 15% to 40% over (S)-enantiomers (Fig 1). A significant decrease of this effect of propranolol was observed when tested against (R,R)-formoterol at the higher concentration.

In competitive dose concentration-response experiments, propranolol at increasing dosages versus a single concentration of (R)-enantiomers resulted in reversal of GM-CSF reductions, with significant amplification at the higher concentrations (Fig 3). As expected on the basis of the greater intrinsic activity of (R,R)-formoterol, the propranolol concentration-dependent increases in GM-CSF were similar in pattern between (R)-enantiomers

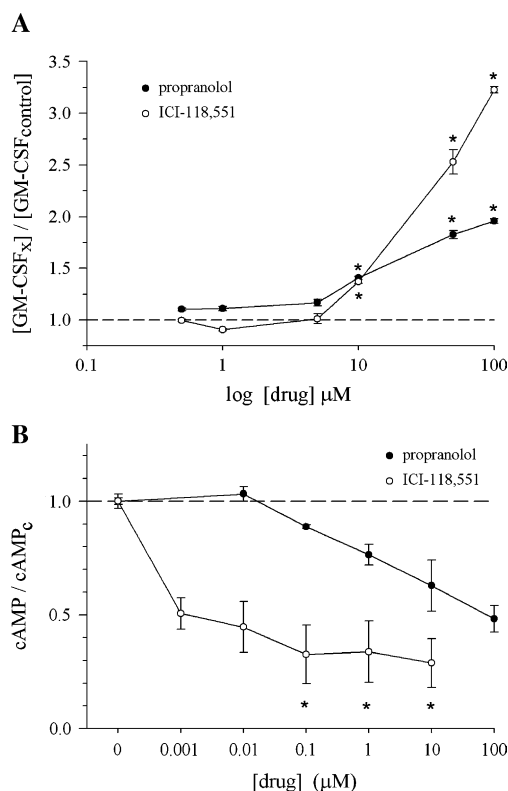


FIG 2. A, GM-CSF release by HASMC with propranolol (○) and ICI-118,551 (●). * $P < .05$ compared with control (0 $\mu\text{mol/L}$ propranolol = 467 pg/mL $\pm$ 4 pg/mL; 0 $\mu\text{mol/L}$ ICI-118,551 = 1079 pg/mL $\pm$ 28 pg/mL; $n = 5$ experiments per concentration. **B**, Intracellular cAMP levels in HASMC with propranolol ($n = 2$) and ICI-118,551 ($n = 4$); significance as in A; mean baseline cAMP = 1.9 pmol/mL $\pm$ 0.1 pmol/mL.

but reduced in magnitude with (R,R)-formoterol compared with (R)-albuterol. Administration of increasing concentrations of (R)-albuterol increased intracellular cAMP levels in HASMC (Fig 4), which was right-shifted with application of propranolol (10 $\mu\text{mol/L}$).

Effects of atropine

Application of atropine did not alter the release of GM-CSF from HASMC treated with either (S)-albuterol or (S,S)-formoterol (data not shown). GM-CSF release also was not modified by atropine added in combination with (R)-albuterol or (R,R)-formoterol (data not shown). These data suggested that the effects of (S)-enantiomers were not a result of a muscarinic receptor-associated mechanism.

cAMP-induced GM-CSF modulation

Application of dibutyryl cAMP resulted in a concentration-dependent reduction in GM-CSF release (Fig 5). The magnitude of reductions in GM-CSF obtained with 10 $\mu\text{mol/L}$ to 100 $\mu\text{mol/L}$ dibutyryl cAMP (20% to 50%) approximated reductions in GM-CSF observed with the (R)-enantiomers. Administration of increasing concentrations of dibutyryl cAMP during treatment with a maximal

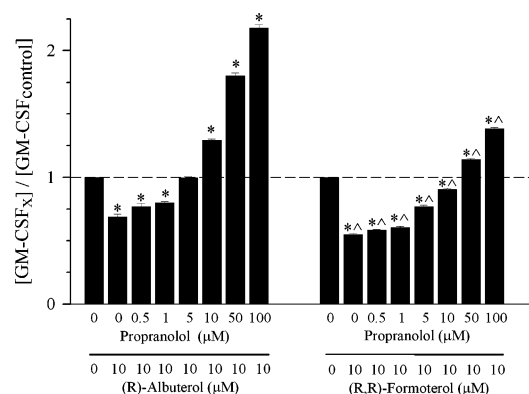


FIG 3. Propranolol concentration and GM-CSF release response relationship versus single concentration (10 $\mu\text{mol/L}$) of either (R)-albuterol (left) or (R,R)-formoterol (right) in HASMC. Symbols indicate $P < .05$ for comparison versus* respective control (0 $\mu\text{mol/L}$ propranolol), ^ (R)-albuterol at same concentration; $n = 5$ experiments/bar.

effect concentration of propranolol (100 $\mu\text{mol/L}$) significantly reduced GM-CSF release, suggesting a mechanism for the reversal of the antagonistic effects of propranolol.

Effects of dexamethasone

Concentrations of 0.1 nmol/L, 1 nmol/L, 10 nmol/L, and 100 nmol/L dexamethasone resulted in significant decrements (all $P < .05$) of GM-CSF release that were 29%, 59%, 74%, and 75% of control (0 nmol/L dexamethasone; 2202 pg/mL $\pm$ 17 pg/mL). As previously observed (Fig 1), (R)-albuterol alone (10 $\mu\text{mol/L}$) significantly reduced GM-CSF release by 16% ($P < .05$) (Fig 6). Addition of dexamethasone (0.1 nmol/L) in combination with 10 $\mu\text{mol/L}$ (R)-albuterol further reduced GM-CSF release by another 22%, suggesting an additive effect of corticosteroid + (R)-enantiomer. (R,R)-formoterol alone at a low dose concentration (10 nmol/L) was effective at significantly reducing GM-CSF release by 26%, and further reduced GM-CSF release at the higher concentration (10 $\mu\text{mol/L}$) by 34% (data not shown). In combination with dexamethasone, 10 nmol/L and 10 $\mu\text{mol/L}$ (R,R)-formoterol further reduced GM-CSF release by an additional 14% to 20% (data not shown).

In contrast, (S)-albuterol at 10 $\mu\text{mol/L}$ increased GM-CSF release (14%) and reversed the reduction induced by dexamethasone to only 12% less than control. Most importantly, the (S)-albuterol-induced change in GM-CSF release in combination with dexamethasone represented a significant increase (26%) over that of 10 $\mu\text{mol/L}$ (R)-albuterol in combination with dexamethasone. (S,S)-formoterol in combination with dexamethasone resulted in smaller additional decrements of GM-CSF release (5% to 13%) (data not shown). However, similar to albuterol enantiomers, (S,S)-formoterol + dexamethasone increased GM-CSF release by 10% to 16% (NS) compared with dexamethasone + (R,R)-formoterol at the same concentrations (data not shown).

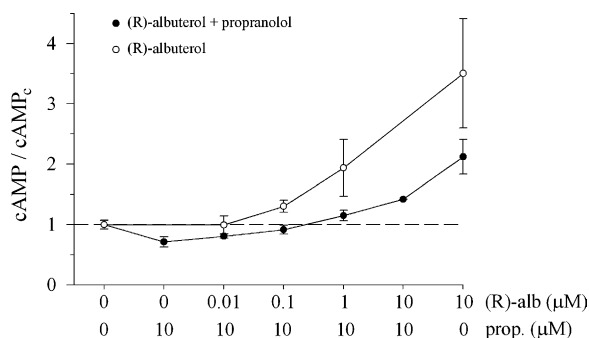


FIG 4. Intracellular cAMP with increasing (R)-albuterol alone (□; n = 2) and with increasing (R)-albuterol plus propranolol (●; 0 μmol/L or 10 μmol/L; n = 2), expressed as a function of baseline intracellular cAMP in HASMC with no drugs added (horizontal dashed line; 1.2 pmol/mL ± 0.4 pmol/mL).

DISCUSSION

The main findings of this study indicate a difference of effects between (R)- and (S)-enantiomers—that is, potentially a beneficial effect of (R)-enantiomers, and a possibly detrimental effect of (S)-enantiomers—with regard to GM-CSF release by HASMC. Our finding of β₂-adrenergic receptor–modulated GM-CSF reduction is consistent with previous reports in which racemic agonists isoprenaline (isoproterenol), salbutamol (albuterol), and fenoterol were studied.^{6,21} However, this study is the first report of the relative singular and combined effects of the enantiomeric constituents on proinflammatory cytokine release in HASMC.

Critique of methods

The mixture of cytokines and LPS that modeled a strong proinflammatory stimulus^{30,31} was effective in raising HASMC release of GM-CSF. Combinations of T_H1 cytokines have been shown previously to have a synergistic effect in eliciting a strong T_H2 cytokine release response in cultured HASMCs.^{1,32} IL-1β has been reported to be the most important of the cytokine constituents in stimulation of GM-CSF release by HASMCs,¹ and others have suggested synergism between IL-1β and TNF-α, whereas IFN-γ may be countermodulatory.³³ However, we cannot currently say which of the agents within the stimulation mixture was the principal overdeterminant of the responses we observed, or whether they truly synergized to produce these responses.

Application of cytokines to cultured HASMC has been shown to result in the phenomenon of heterologous desensitization of the β₂-adrenergic receptors,³⁴ such that they become less responsive to direct β₂-adrenergic receptor stimulation. It is possible that this may explain why the lowest concentration of (R)-albuterol was ineffective at altering GM-CSF release. Although propranolol was used to verify a β₂-receptor–mediated process, it is possible that the cytokine stimulus may have been too strong to be altered by the lowest concentration of (R)-albuterol that we used.

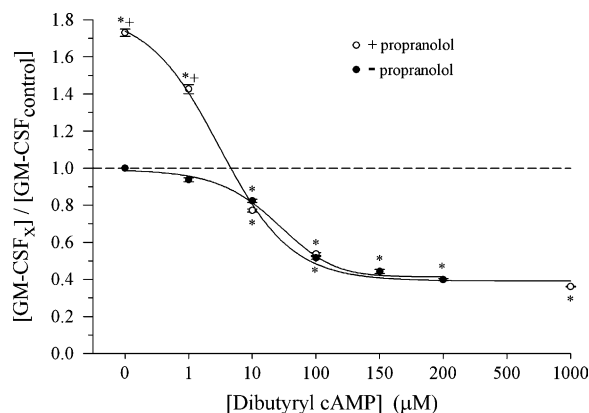


FIG 5. GM-CSF release with dibutyl cAMP alone (●) and with dibutyl cAMP plus propranolol (○; 100 μmol/L), **P* < .05; versus 0 μmol/L dibutyl cAMP, †*P* < .05; versus dibutyl cAMP alone at same concentration; *r*² > .99 for regression of both data sets; horizontal dashed line indicates control GM-CSF release with 0 μmol/L dibutyl cAMP (1672 pg/mL ± 22 pg/mL); n = 5 experiments per set.

Finally, the serum-supported growth cycle we used in the current experiments differs from some others. After the serum starvation phase, in which the cells were quiescent, were synchronized to the G₀ phase, and tended toward a contractile or constitutive form,¹⁸ they were exposed to a serum replenishment phase. This step was taken to provoke induction of a synthetic form to model the proliferative phase of airway remodeling, in which HASMC numbers are known to increase in diseases such as asthma.¹⁹ Thus, the β₂-adrenergic receptor responses we observed were likely a function of this approach, perhaps differing somewhat from previous studies focusing on contractile forms of HASMC. This also may be another contributing factor explaining the concentrations of agonists and antagonist necessary to modulate GM-CSF release in HASMC under these conditions. However, we chose the enantiomer concentrations, in part on the basis of their potential achievement *in vivo* when administered to patients.²⁵ Thus, this combination of proliferative activity, strong multifactorial proinflammatory stimulation, and clinical concentration relevance was important in our experimental design.

(R)-enantiomer reduction of GM-CSF release

The reduction in GM-CSF release by (R)-enantiomers, the significant reversal by propranolol, and the blunting of the reduction by the addition of (S)-enantiomers suggest that GM-CSF release in our model is linked to enantiomer-specific β₂-receptor–mediated mechanisms. One important finding was the attenuated amplitude of the pattern of reversal and amplification of GM-CSF release produced with propranolol versus (R,R)-formoterol (Fig 3). These findings, coupled with the greater effects of (R,R)-formoterol alone (Fig 1), further indicated that (R,R)-formoterol was the most potent and efficacious β₂-adrenergic receptor agonist we tested, in agreement with the known greater potency of formoterol.³⁵

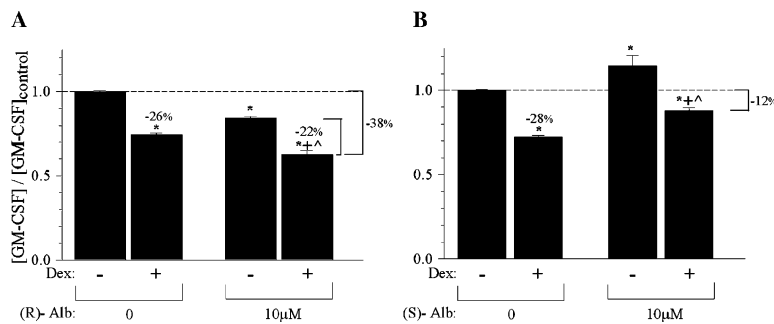


FIG 6. Effects of dexamethasone (Dex) in combination with enantiomers. GM-CSF expressed as ratios relative to -Dex, 0 enantiomer control (range: 1800-2700 pg/mL); percentage change values as shown. * $P < .05$ vs -Dex, 0 agonist (no drugs) control; + $P < .05$ vs -Dex, 10 μ M/L agonist; ^ $P < .05$ vs +Dex, 0 agonist; n = 4-10 experiments/bar.

The association between (R)-enantiomer reductions in GM-CSF release, increases in intracellular cAMP levels, and the apparently similar reduction in GM-CSF produced by addition of intracellular cAMP suggest a potential mechanism of action for the (R)-enantiomers. A reduction in HASMC GM-CSF release previously has been linked to increased intracellular cAMP in experiments using racemic β -agonists (isoprenaline and salbutamol) and dibutyl cAMP.^{6,36} The current study suggested that the (R)-enantiomers of albuterol and formoterol likely work through a similar cAMP-dependent mechanism. This mechanism may in turn be linked to regulation of nuclear factor- κ B activation in exerting what are considered anti-inflammatory effects.^{7,37} Further study is necessary to identify this as a potential mechanism with regard to (R)-enantiomers and (S)-enantiomers.

The addition of (R)-albuterol with dexamethasone resulted in amplification of the inhibitory effects of dexamethasone. (R,R)-formoterol resulted in the greatest reduction of GM-CSF release in combination with dexamethasone, consistent with its known stronger effects. Overall, these effects might be expected on the basis of previous work showing amplification of anti-inflammatory effects with combinations of corticosteroids and racemic β_2 -adrenergic receptor agonists²⁷; however, these studies are the first to show the additive nature of these effects by active enantiomers of β_2 -adrenergic receptor agonists in HASMC. The mechanism is currently unknown but is possibly similar to that shown for the interaction between formoterol and budesonide, in which their combination resulted in synchronized activation of transcription factors.²⁷

(S)-enantiomer modulation of GM-CSF release

In contrast with (R)-enantiomers alone, (S)-albuterol and (S,S)-formoterol alone typically increased GM-CSF release. These findings indicate that those (S)-enantiomers are not biologically inactive, as conventionally held, but seem to be paradoxical or counterproductive, where GM-CSF release is concerned. This (S)-enantiomer-associated increase of a proinflammatory agent and the blunting or masking of the (R)-enantiomer-associated reduction in GM-CSF release are similar to actions reported previously

in mast cells³⁸ and T cells³⁹ and are consistent with the notion that (S)-enantiomers can antagonize the actions of (R)-enantiomers.⁴⁰ This notion is supported by previous reports indicating that (S)-albuterol reduces HASMC intracellular cAMP levels⁴¹ and that β_2 -agonist-dependent elevations in cAMP are associated with reductions in GM-CSF release in HASMC.^{6,36} In all, these data are consistent with the idea that (R)-enantiomers and (S)-enantiomers can have opposing actions.

Several previous studies have suggested that (S)-enantiomers might act through a mechanism associated with the muscarinic receptor,^{16,42} which would provide an attractive counterbalance to the β_2 -adrenergic receptor effects produced by the (R)-enantiomers. However, our findings with atropine suggest that this mechanism was not significant in our model; therefore, our focus remains on the potential of β_2 -adrenergic receptor effects of the (S)-enantiomers.

Propranolol: A potential model for action of (S)-enantiomers

We initially set out to use propranolol as a classic β -adrenergic receptor antagonist in competitive studies with enantiomers of β_2 -adrenergic receptor agonists. The direction of increased GM-CSF release with propranolol in competition with the (R)-enantiomers was similar to that observed when (S)-enantiomers were added. This led us to theorize that the (S)-enantiomers were possibly acting as antagonists, or perhaps inverse agonists, countering the effects of the (R)-enantiomers.

The classical concept of inverse agonism holds that certain ligands can stabilize G-protein-coupled receptors in an inactive conformation that inhibits the typical mechanism initiated with binding of an agonist.⁴³ This mechanism might explain the blunting of (R)-enantiomer effects that we observed when (S)-enantiomers were added, similar to the mechanism suggested in T cells.³⁹ Furthermore, we were surprised to find that propranolol had a potent amplifying effect on GM-CSF release in HASMC by itself, similar to that observed with (S)-enantiomers alone. This effect might be explained by the concept that a fraction of the β_2 -adrenergic receptor pool may exist in an activated conformation because of

stimulation by cytokines in the absence of a β_2 -adrenergic receptor agonist.⁴⁴ This effect also might be explained by β_2 -adrenergic receptor–linked upregulation of inhibitory G-proteins that could suppress the action of protein kinase A and reduce the production of intracellular cAMP,⁴⁵ ultimately leading to increased GM-CSF release. A recent study has shown that (S)-albuterol increases $\text{Gi}\alpha_1$ in HASMC,⁴¹ consistent with this possibility. However, we note that many of the concentrations of propranolol that we investigated were at the high end or above the range of this drug typically used for studies of inverse agonism of the β -adrenergic receptor (1–10 $\mu\text{mol/L}$),^{28,46} and therefore, the possibility exists that the effects we observed were caused by its action through a non- β -adrenergic receptor pathway, perhaps through stimulation of the p42/44-mitogen-activated protein kinase pathway.²⁸ Further studies are necessary to determine this possibility.

Potential clinical relevance

With administration of racemic albuterol, (S)-albuterol has been shown to persist in the plasma as much as 10-fold longer than (R)-albuterol,¹⁷ and it likely persists in the lung as well.²⁶ Furthermore, the concentrations of enantiomers used in the current experiments are within the range that could be expected within the airways with administration of nebulized drug.^{22,25} On the basis of those facts and our data, it is conceivable that persisting levels of (S)-enantiomers with racemic albuterol use may elevate GM-CSF release *in vivo*. Elevated levels of GM-CSF could promote eosinophil survival,³ early eosinophil-mediated alterations HASMC hyperresponsiveness, and perhaps airway remodeling over time, with frequent (S)-enantiomer exposure. This mechanism would be consistent with data showing that (S)-albuterol elevates eosinophil superoxide production,⁴⁷ which can have a potent effect on airway hyperresponsiveness. It may also be relevant to the clinically observed paradoxical worsening of asthma with chronic β_2 -adrenergic receptor agonist use,^{9,11} such that persistence of the (S)-enantiomer could lead to asthma exacerbations through persistent production of proinflammatory mediators in the lung.

One of the most important and perhaps clinically relevant findings of the current study was the reversal of the effects of dexamethasone by (S)-enantiomers. Combinations of corticosteroids and β_2 -adrenergic receptor agonists have been used with effectiveness in treating asthma in recent years, and indeed, have become standard of therapy in many instances.⁴⁸ It is thought that the success of this combination approach may be a result of a β_2 -adrenergic receptor agonist–mediated complementary effect on the action of the corticosteroid.⁴⁹ Similar to other studies of racemic β_2 -adrenergic receptor agonists, our data demonstrate a potentially beneficial amplification of anti-inflammatory effects of the combination of steroid and the (R)-enantiomers. However, the suppression of this beneficial steroid effect by (S)-albuterol, for example, suggests that some combination therapies may be sub-optimal because of opposing actions of the (S)-enantiomers obtained through administration of the racemate.

This notion is consistent with findings demonstrating that racemic β_2 -adrenergic receptor agonists (albuterol, salmeterol, and isoproterenol) can block both the dexamethasone-mediated inhibition of eosinophil respiratory burst and dexamethasone-mediated increases in eosinophil apoptosis,⁵⁰ further suggesting a proinflammatory role for the (S)-enantiomer that counteracts the beneficial anti-inflammatory effects of corticosteroids. Thus, additional clinical trials evaluating the effects of combinations of corticosteroids, enantiomers, and racemates are necessary to establish whether further optimization of combination therapy might be attainable with omission of the (S)-enantiomers.

In summary, the current study suggests that the anti-inflammatory properties of β_2 -adrenergic receptor agonists in HASMC are a result of the (R)-enantiomer, and that the (S)-enantiomer can have opposing effects leading to proinflammatory responses, such as increased GM-CSF release. Thus our data and those of others indicate that the (S)-enantiomers of β_2 -adrenergic receptor agonists cannot be considered inactive in several relevant airway and immune cell models, including HASMC. Further research is necessary to determine the kinetics and nature of these HASMC mechanisms.

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