

## Effects of (*R*)- and (*S*)-isomers of $\beta$ -adrenergic agonists on eosinophil response to interleukin-5

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### Summary

**Background** Racemic  $\beta_2$ -adrenergic receptor agonists ( $\beta_2$ -agonists) are used frequently to treat patients with asthma. Potential differences in the biological activities and clinical efficacies among racemic  $\beta_2$ -agonists and their isomers are controversial, and research into these possible differences is limited.

**Objective** We hypothesized that the (*S*)- and the (*R*)-isomers of  $\beta_2$ -agonists have opposing effects on the activation of inflammatory cells.

**Methods** Isolated human eosinophils were pretreated with 1 : 1 racemic (*R,S*)-, (*R*)- or (*S*)-albuterol, isobutyl methylxanthine (IBMX), and stimulated with IL-5. The kinetics of superoxide production were examined by reduction of cytochrome *c*, and the effects of pharmacological agents on superoxide production were monitored for 180 min.

**Results** (*R,S*)-albuterol inhibited IL-5-induced superoxide production. This inhibition was enhanced by a cyclic adenosine monophosphate (cAMP) phosphodiesterase inhibitor, IBMX, and was reversed by the selective  $\beta_2$ -adrenergic receptor antagonist, ICI 118,551, verifying the involvement of both cAMP and the  $\beta_2$ -adrenergic receptor. In addition, (*R*)-albuterol alone, similarly to (*R,S*)-albuterol, significantly inhibited IL-5-induced superoxide production up to 60 min ( $P < 0.05$ ,  $n = 4$ ), but the inhibition was lost with longer incubation. In contrast, (*S*)-albuterol with IBMX did not inhibit IL-5-induced superoxide production before 60 min, but it significantly enhanced IL-5-mediated superoxide production after 60 min ( $P < 0.05$ ,  $n = 4$ ). When both were present as racemic (*R,S*)-albuterol, the inhibitory effect of (*R*)-albuterol was not affected by (*S*)-albuterol.

**Conclusion** When incubated with IL-5-activated eosinophils, (*R*)-albuterol shows anti-inflammatory effects and (*S*)-albuterol shows pro-inflammatory effects in the presence of IBMX. The kinetics of these effects are different, and when used simultaneously, (*R*)-albuterol predominates. When marked usage of the (*S*)-isomer is anticipated, racemic (*R,S*)-albuterol should be used clinically with caution.

**Keywords** albuterol, asthma,  $\beta_2$ -agonist, eosinophils, inflammation, IL-5, isomer, superoxide

Submitted 18 August 2004; revised 19 June 2005; accepted 8 August 2005

### Introduction

The eosinophil is a pro-inflammatory granulocyte that is implicated in the pathophysiology of bronchial asthma. Cationic proteins, secreted by eosinophils, including major basic protein, eosinophil cationic protein and eosinophil peroxidase, are toxic to bronchial epithelium [1, 2]. While a wide variety of molecules, including cytokines, chemokines, complement fragments, lipid mediators and immunoglobulins, can affect the dynamics and pro-inflammatory functions

of eosinophils, IL-5 is likely one of the critical cytokines involved in eosinophil-associated inflammation. IL-5 enhances various functions of eosinophils, such as adhesion, chemotaxis, survival and mediator release [3]. Increased levels of IL-5 protein and mRNA were observed in the airways of patients with asthma [4, 5]. Recent clinical studies showed that blockade of IL-5 abolished blood and airway eosinophilia [6] and inhibited tissue eosinophilia by 50% [7].

$\beta_2$ -adrenergic receptor agonists ( $\beta_2$ -agonists) have been used widely in the treatment of asthma since the 1940s. In the early 1990s, controversies emerged regarding the daily use of  $\beta_2$ -agonists and potential associations with increased risk of death [8], poor control [9] and accelerated loss of lung function in patients with asthma [10]. Furthermore, it appears that significant tolerance develops to the non-bronchodilating effects of  $\beta_2$ -agonists; namely, the protection against the bronchoconstriction caused by allergens, methacholine or

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exercise [11–13]. Therefore, a hypothesis has been suggested that the (*R*)- and (*S*)-enantiomers of the racemic  $\beta_2$ -agonists may have differential effects on the  $\beta$ -adrenergic receptors expressed in inflammatory cells and airway smooth muscle cells [14]. Indeed, while (*R*)-albuterol inhibited cell proliferation and cytokine production in tetanus-specific T cell lines, the addition of (*S*)-albuterol to (*R*)-albuterol paradoxically stimulated cellular responses [15]. In contrast, (*S*)-albuterol showed no significant effect on eosinophil degranulation stimulated with formyl-met-leu-phe plus cytochalasin B [16].

To address this controversy and to further clarify the effects of  $\beta_2$ -agonists on human eosinophils, we studied the effects of a 1 : 1 racemic mixture of albuterol [(*R,S*) albuterol], and the single isomers, (*R*)- and (*S*)-albuterol, with and without isobutyl methylxanthine (IBMX), on superoxide production stimulated with IL-5. This experimental system was selected to use a physiologic agonist and cell type relevant to asthma and allergic diseases and to employ a robust assay, which is capable of quantitative kinetic measurements. We tested the hypothesis that the (*S*)- and the (*R*)-isomer of  $\beta_2$ -agonists have opposite effects on the activation of eosinophils.

## Materials and methods

### Reagents

The pharmaceuticals, albuterol [a 1 : 1 racemic mixture of (*R*)- and (*S*)-albuterol, (*R,S*)-albuterol], IBMX, and a highly selective  $\beta_2$ -adrenergic receptor antagonist, ICI 118,551 HCl, were purchased from Sigma Chemical Co. (St Louis, MO, USA). The single isomers, (*R*)- and (*S*)-albuterol, were supplied by Sepracor Inc. (Marlborough, MA, USA). Human IL-5 was a generous gift from Schering-Plough Research Institute (Kenilworth, NJ, USA). IBMX was dissolved in absolute ethanol at  $8 \times 10^{-2}$  M and diluted in reaction medium before use. At the concentration employed in this study, ethanol (0.0375%) had no deleterious effects on eosinophil viability and functions. All the other reagents were directly dissolved and diluted in reaction medium.

### Isolation of eosinophils

Eosinophil isolation was performed, with minor modifications of the method described by Hansel et al. [17], using a magnetic cell separation system (Becton Dickinson, San Jose, CA, USA). Briefly, venous blood, anti-coagulated with 50 U/mL heparin, was obtained from healthy volunteers and diluted with phosphate-buffered saline at a 1 : 1 ratio. Diluted blood was overlaid on isotonic Percoll solution (density 1.085 g/mL, Sigma Chemical Co.) and centrifuged at 1000 *g* for 30 min at 4 °C. The supernatant and mononuclear cells at the interface were removed, and the sedimented erythrocytes were lysed by two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with piperazine-*N,N'*-bis-2-ethanesulphonic acid (Pipes) buffer (25 mM Pipes, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose, pH 7.4) with 1% defined calf serum (DCS) (Hyclone Laboratories, Logan, UT, USA), and an approximately equal volume of anti-CD 16 antibody conjugated with magnetic particles (Miltenyi Biotec Inc., Auburn, CA, USA) was added to the

cell pellet. After 60 min on ice, cells were loaded onto the separation column, and eosinophils were eluted with Pipes buffer with 1% DCS. The purity of eosinophils counted by Randolph's stain was greater than 95%.

### Measurement of superoxide anion production by purified eosinophils

Generation of superoxide by eosinophils was measured by superoxide dismutase inhibitable reduction of cytochrome *c*, as described previously [18] with slight modifications. We used 96-well flat bottom tissue culture plates (Becton Dickinson) blocked with 2.5% human serum albumin to inhibit non-specific activation of eosinophils as a result of adhesion to bare plastic surface. Freshly isolated eosinophils were washed and suspended in Hanks balanced salt solution (HBSS) with 10 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulphonic acid (HEPES) and with 100  $\mu$ M cytochrome *c* (Sigma) at  $1 \times 10^6$  cells/mL. One hundred microlitres of cell suspension was dispensed onto plates and mixed with 25  $\mu$ L of a phosphodiesterase inhibitor, IBMX ( $3 \times 10^{-6}$  M final concentration), or control medium for 15 min at room temperature. Cells were then incubated with 25  $\mu$ L of serial dilutions of albuterol and its isomers for another 15 min. In some experiments, to confirm the involvement of  $\beta_2$ -adrenergic receptor, eosinophils were pre-incubated with a selective antagonist, ICI 118,551 HCl ( $10^{-5}$  M final concentration), for 15 min prior to the addition of albuterol. Eosinophil superoxide production was initiated by adding 50  $\mu$ L of IL-5 (10 ng/mL final concentration) diluted in HBSS with HEPES or control medium. Immediately after addition of stimuli, the reaction wells were measured for absorbance at 550 nm in a microplate autoreader (Thermomax, Molecular Devices, Menlo Park, CA, USA), followed by repeated readings. Between absorbance measurements, the plate was incubated at 37 °C. Each reaction was carried out in duplicate. Superoxide anion generation was calculated with an extinction coefficient of  $21.1 \times 10^3$  L/mol cm for reduced cytochrome *c* at 550 nm and was expressed as nanomoles of cytochrome *c* reduced per  $1 \times 10^5$  cells. The rate of superoxide production was calculated by subtracting the amounts of superoxide production between the two time-points (nanomoles of cytochrome *c* reduced per  $1 \times 10^5$  cells) and by dividing the value by time (minutes) needed for the reaction.

### Statistical analysis

Statistical significance of the differences among various treatment groups was assessed with paired or unpaired Student's *t*-test. *P*-values less than 0.05 were considered significant. All data are expressed as means  $\pm$  SEM.

## Results

### Effects of (*R,S*)-albuterol on interleukin-5-induced superoxide production by eosinophils

$\beta_2$ -agonists and other agents, which elevate intracellular cyclic adenosine monophosphate (cAMP), inhibit eosinophil degranulation *in vitro* when eosinophils are stimulated with IgG or IgA immobilized to Sepharose 4B beads (Amersham

Biosciences, Piscataway, NJ, USA) [19]. To address the effects of albuterol on eosinophils stimulated with IL-5, we first examined the kinetics of superoxide production by eosinophils incubated with 10 ng/mL of IL-5. As shown in Fig. 1a, eosinophils stimulated with IL-5 produce superoxide in a time-dependent manner. Superoxide production was detectable as early as 20 min after stimulation and reached a plateau after approximately 180 min. Eosinophils incubated without IL-5 produced negligible superoxide.

When eosinophils were pre-incubated with a 1:1 racemic mixture of albuterol, namely (*R,S*)-albuterol, for 15 min and then stimulated with IL-5 for various times, (*R,S*)-albuterol inhibited superoxide production at 30 or 60 min (Fig. 1b, left panel). However, these inhibitory effects were lost when cells were incubated longer than 180 min. As reported earlier, the blocking of cAMP phosphodiesterase inhibits the degradation of cAMP and enhances accumulation of cAMP [20]. As shown in Fig. 1b, right panel, when eosinophils were pretreated with a cAMP phosphodiesterase inhibitor, IBMX, the inhibitory effects of albuterol were clearly enhanced. In the presence of IBMX,  $10^{-7}$  M (*R,S*)-albuterol suppressed superoxide production significantly by 30% and 18% at 30 and 60 min, respectively;  $10^{-6}$  M (*R,S*)-albuterol had a more pronounced suppression of superoxide production by 40% and 24% at 30 and 60 min, respectively. On the other hand, at 180 min, (*R,S*)-albuterol did not show any significant effects even in the presence of IBMX. Finally,  $3 \times 10^{-6}$  M IBMX (without albuterol) did not show any significant effects on IL-5-induced superoxide production (data not shown).

To verify the specificity of the effects of albuterol, eosinophils were first pre-incubated with the selective  $\beta_2$ -adrenergic receptor antagonist,  $10^{-5}$  M ICI 118,551 followed by  $10^{-6}$  M (*R,S*)-albuterol and then stimulated with IL-5. The inhibitory effects of  $10^{-6}$  M (*R,S*)-albuterol were completely reversed when cells were pretreated with ICI 118,551 ( $29 \pm 5\%$  inhibition vs.  $4 \pm 5\%$  inhibition at 60 min, without or with ICI 118,551, respectively). Altogether, these findings suggest that racemic (*R,S*)-albuterol inhibits activation of eosinophils stimulated with IL-5, and that this effect is mediated through the  $\beta_2$ -adrenergic receptor and intracellular cAMP.

#### Effect of (R)- and (S)-albuterol on interleukin-5-induced superoxide production

After establishing the specificity and kinetics of the inhibitory effects of racemic (*R,S*)-albuterol, we examined the effects of each isomer. Eosinophils were pre-incubated with IBMX and treated with equal concentrations ( $10^{-6}$  M) of (*R,S*)-albuterol, (*R*)-albuterol or (*S*)-albuterol for 15 min, and then stimulated with IL-5. Representative kinetic data are shown in Fig. 2. From 0 to 60 min, both (*R*)- and (*R,S*)-albuterol inhibited IL-5-induced superoxide production, similarly to the results in Fig. 1. However, after 60 min, there was no further inhibition of superoxide production by (*R,S*)- or (*R*)-albuterol. In contrast, (*S*)-albuterol did not inhibit superoxide production by 60 min and even enhanced superoxide production after 60 min compared with the control without albuterol. (*S*)-albuterol without IL-5, or without IBMX, did not induce superoxide production (data not shown).

Figure 3 summarizes the results of four experiments that compare (*R*)-, (*S*)- and (*R,S*)-albuterol. At 30 min, (*R*)- and

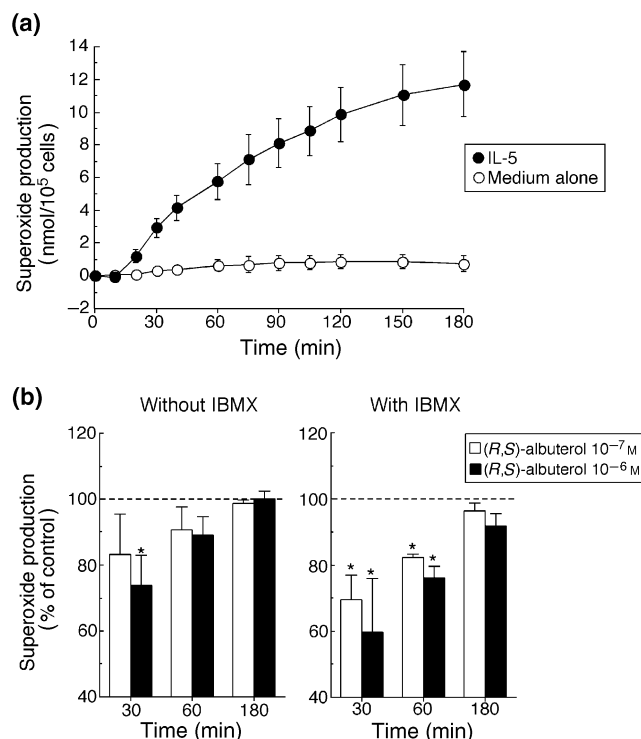


**Fig. 1.** Superoxide production was measured from eosinophils that were stimulated with IL-5 (10 ng/mL). (a) The average superoxide production was  $2.9 \pm 1.2$ ,  $5.7 \pm 2.2$  and  $11.7 \pm 3.9$  nmol/ $10^5$  eosinophils at 30, 60 and 180 min, respectively (mean  $\pm$  SEM,  $n = 6$ ); eosinophils incubated with media only did not produce detectable superoxide. (b) (Left panel) Superoxide production when eosinophils were incubated with (*R,S*)-albuterol ( $10^{-7}$ ,  $10^{-6}$  M) and stimulated with IL-5 (10 ng/mL) at the times indicated. The superoxide production is expressed as mean percentage of control  $\pm$  SEM,  $n = 4$ . The control values for eosinophils and IL-5 were  $2.6 \pm 0.5$  at 30 min,  $6.2 \pm 1.1$  at 60 min and  $13.1 \pm 2.2$  nmol/ $10^5$  eosinophils at 180 min. \*Significant difference ( $P < 0.05$ ) from control. (b) (Right panel) Superoxide production when eosinophils were incubated with (*R,S*)-albuterol ( $10^{-7}$ ,  $10^{-6}$  M) and isobutyl methylxanthine (IBMX) ( $3 \times 10^{-6}$  M) and stimulated with IL-5 (10 ng/mL) at the times indicated. The superoxide production is expressed as in (b), left panel,  $n = 4$ . The control values for eosinophils, IBMX and IL-5 were  $2.0 \pm 0.2$  at 30 min,  $5.0 \pm 0.4$  at 60 min and  $11.5 \pm 0.5$  nmol/ $10^5$  eosinophils at 180 min. \*Significant difference ( $P < 0.05$ ) from control.

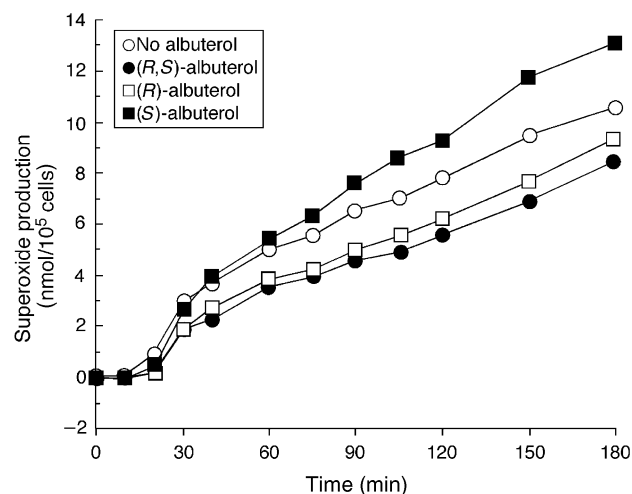


**Fig. 2.** Eosinophils were pre-incubated with  $10^{-6}$  M of the various  $\beta$ -agonists and with isobutyl methylxanthine ( $3 \times 10^{-6}$  M) and were stimulated with IL-5 (10 ng/mL). Superoxide production was measured (nmol/ $10^5$  eosinophils) at various times; results of a representative experiment are shown.

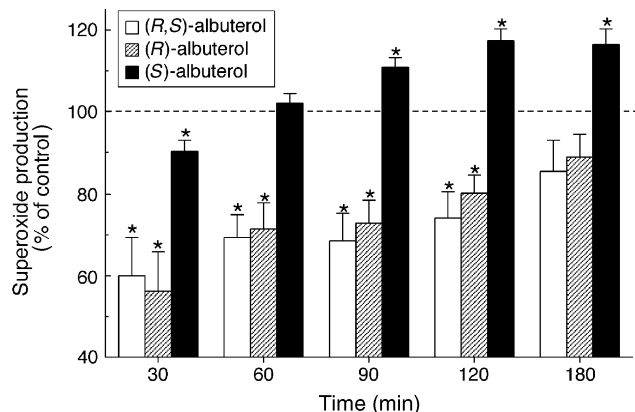


**Fig. 3.** Eosinophils were pre-incubated with the various  $\beta$ -agonists ( $10^{-6}$  M) and isobutyl methylxanthine (IBMX) ( $3 \times 10^{-6}$  M) and were stimulated with IL-5 (10 ng/mL). Superoxide production was measured at the times indicated. Superoxide production is expressed as mean percentage of control  $\pm$  SEM,  $n = 4$ . The control values for eosinophils, IBMX and IL-5 were  $2.6 \pm 0.8$  at 30 min,  $4.6 \pm 1.2$  at 60 min and  $10.3 \pm 2.1$  nmol/ $10^5$  eosinophils at 180 min. \*Significant difference ( $P < 0.05$ ) from control.

(*R,S*)-albuterol significantly inhibited superoxide production by 44% and 40%, respectively ( $P < 0.05$ ). The inhibition by (*R,S*)- and (*R*)-albuterol continued up to 120 min, but the inhibition was attenuated at longer times. No significant differences were observed between the inhibitory effects of (*R*)- and (*R,S*)-albuterol. In contrast, (*S*)-albuterol showed minimal (10%) inhibitory effects at 30 min and showed no inhibition at 60 min. Interestingly, at later times from 90 to 180 min, (*S*)-albuterol significantly increased superoxide production by 10–17% compared with the control ( $P < 0.05$ ). Thus, (*R*)-albuterol, similarly to (*R,S*)-albuterol, inhibits superoxide production especially at early times. In contrast, (*S*)-albuterol minimally inhibits superoxide production, but enhances IL-5-induced superoxide production at later times. Furthermore, no differences were observed in the inhibitory effects of (*R,S*)- and (*R*)-albuterol.

#### Superoxide production rate

To validate the results in Fig. 3, the rate of superoxide production by IL-5-stimulated eosinophils was calculated and compared over two time periods, between 0 and 60 min and between 60 and 120 min. The superoxide production rate was normalized to the value without albuterol as 100%. As shown in Fig. 4, left, between 0 and 60 min, the rate of IL-5-mediated superoxide production was significantly decreased when eosinophils were incubated with (*R*)- or (*R,S*)-albuterol ( $P < 0.01$  and  $P < 0.05$ , respectively). (*S*)-albuterol showed no inhibition or stimulation. As shown in Fig. 4, right, between 60 and 120 min, (*R*)- and (*R,S*)-albuterol no longer inhibited IL-5-induced superoxide production. (*S*)-albuterol, however, significantly enhanced the rate of superoxide production by 40% in eosinophils stimulated with IL-5 ( $P < 0.05$ ).

#### Discussion

Herein, we studied the effects of racemic (*R,S*)-, (*R*)- and (*S*)-albuterol on superoxide production by IL-5-activated eosi-

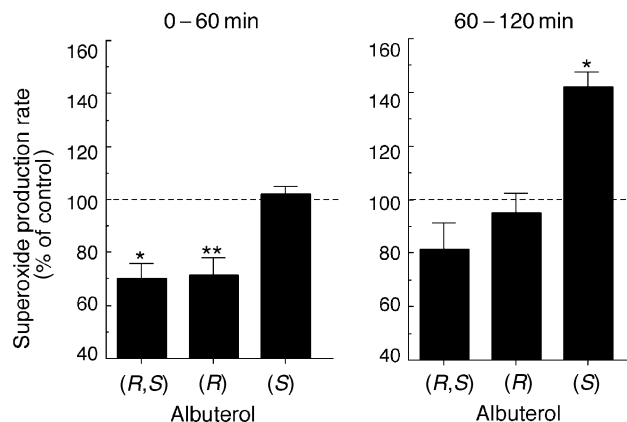


**Fig. 4.** Eosinophils were incubated with the various  $\beta$ -agonists ( $10^{-6}$  M) and isobutyl methylxanthine ( $3 \times 10^{-6}$  M) and stimulated with IL-5 (10 ng/mL). Rates of superoxide production (nmol/ $10^5$  eosinophils/min) were calculated from 0 to 60 min and 60 to 120 min and presented as a percentage of the control. For the controls from 0 to 60 minutes, the rate of superoxide production was  $0.077 \pm 0.021$  nmol/ $10^5$  eosinophils/min (mean  $\pm$  SEM,  $n = 4$ ); from 60 to 120 min, the rate of superoxide production was  $0.053 \pm 0.007$  nmol/ $10^5$  eosinophils/min (mean  $\pm$  SEM,  $n = 4$ ). \*Significant difference ( $P < 0.05$ ) from control; \*\*significant difference ( $P < 0.01$ ) from control.

nophils. In the past, the effects of  $\beta_2$ -agonists, mainly racemic (*R,S*)-forms, have been studied in a number of tissues and cell types. In addition to decreasing eosinophil mediator release [19],  $\beta_2$ -agonists inhibited mast cell degranulation and cutaneous reactions to allergens [21], decreased superoxide production by human neutrophils [22], and inhibited plasma leakage in response to inflammatory stimuli [23]. We now report that conventional racemic (*R,S*)-albuterol inhibits IL-5-induced superoxide production, suggesting that  $\beta_2$ -agonists fundamentally have an anti-inflammatory property. However, interestingly, our study also suggests that by itself (*S*)-albuterol up-regulates eosinophil superoxide production induced by IL-5, in the presence of IBMX. This stimulatory effect of (*S*)-albuterol was not observed before 60 min of incubation; it was pronounced from 60 to 120 min of incubation. In contrast, the (*R*)-isomer of albuterol inhibited IL-5-induced eosinophil activation, and the inhibitory effect of this (*R*)-albuterol was most apparent at times up to 60 min. Thus, (*S*)- and (*R*)-albuterol showed opposite effects on eosinophil functions with different kinetics. Importantly, the effects of (*R*)-albuterol seem to dominate (*S*)-albuterol when these isomers co-exist; the effects of the 1:1 racemic (*R,S*)-albuterol were almost identical to those of (*R*)-isomer alone, in both their inhibitory potencies and kinetics.

The remaining questions are why does (*S*)-albuterol lack inhibitory effects and how does (*S*)-albuterol stimulate IL-5-induced superoxide production with such delayed kinetics. Although we do not have definite answers for these questions, a review of the literature provides some insights. (*S*)-albuterol does not bind or binds with 100-fold less affinity to the  $\beta_2$ -adrenergic receptor [24]. Therefore, the cytoplasmic penetration of (*S*)-albuterol and its direct effects on signal transduction molecules could be considered. Alternatively, one study suggests that  $\beta_2$ -adrenergic receptors can be coupled to Gs or Gi proteins, depending on the phosphorylation status of the receptors [25]. Therefore, (*R*)-albuterol may show a strong affinity for the  $\beta_2$ -adrenergic receptor coupled to Gs proteins,

and (S)-albuterol may show weak affinity for the  $\beta_2$ -adrenergic receptor coupled to Gi proteins. If so, the (R)-isomer alone would activate the Gs proteins and adenylyl cyclase and show inhibitory effects on eosinophil functions through the increase in intracellular cAMP levels, and the (S)-isomer alone would enhance eosinophil functions through cell activation pathways mediated by Gi proteins, such as mitogen-activated protein (MAP) kinases. Consistent with this speculation, in tracheal smooth muscle cells, (S)-albuterol alone increased intracellular calcium concentrations [26], whereas (R)-albuterol alone decreased intracellular calcium concentrations. Calcium signals are often required, although they may not be sufficient, for effector functions of eosinophils [27]. Furthermore, recent studies suggest that IL-5 provokes various intracellular signals in eosinophils, including activation of the JAK-STAT pathways, the Ras-MAPK pathway, and PI-3K [28]. Our preliminary studies showed that the Ras-MAPK pathway was not activated by (R)- or (S)-albuterol alone (data not shown). Therefore, the calcium signal generated by the (S)-albuterol may synergize with signals generated by the IL-5 receptor, resulting in up-regulation of superoxide production by eosinophils.

The results of our studies are different from a previous study by other investigators, who examined the effects of (R)- and (S)-albuterol on eosinophil degranulation [16]. Although several factors, including differences in the stimuli and readouts, can be considered, the length of incubation may be the most critical. The previous study incubated eosinophils for a maximum of 30 min [16]; in our study, the stimulatory effects of (S)-albuterol were observed only after 60 min of incubation.

Clinically, (R)-albuterol appears to be more efficacious than racemic (R,S)- or (S)-albuterol in improving forced expiratory volume in 1 s [29]. In this large study comparing the  $\beta_2$ -agonists, daily nebulized doses of 1.25 mg (R)-albuterol showed superior efficacy to 2.5 mg (R,S)-albuterol. Because equal amounts of (R)-albuterol were contained in 1.25 mg (R)-albuterol and 1:1 racemic 2.5 mg (R,S)-albuterol, (S)-albuterol may decrease the clinical efficacy of the racemic albuterol. The concept that (S)-albuterol may have detrimental effects is also supported by the studies comparing racemic (R,S)- and (R)-albuterol in the hospital [30] and emergency room [31] settings. Because (S)-albuterol seems to have a pro-inflammatory effect by itself in eosinophils (this study) or in combination with (R)-albuterol in lymphocytes [15], perhaps a combination of such processes contributes to the clinical outcomes. Furthermore, frequent dosing of (R,S)-albuterol or  $\beta_2$ -agonists may exaggerate the potentially harmful effects of (S)-isomer because this isomer has a longer pharmacological half-life than the (R)-isomer [32] and therefore, it may accumulate in the airways.

In summary, this study shows that (R)-albuterol decreases superoxide production in IL-5-stimulated eosinophils; conversely, (S)-albuterol increases superoxide production. The anti-inflammatory effects of the (R)-albuterol occur early whereas the pro-inflammatory effects of (S)-albuterol occur later. The anti-inflammatory effects of (R)-albuterol were not reversed by (S)-albuterol when they were used as the 1:1 racemic (R,S) albuterol in this *in vitro* system. Further *in vitro* and *in vivo* studies are warranted to elucidate the different

biological activities between (R)- and (S)-enantiomers of the albuterol molecule and their molecular mechanisms.

## Acknowledgements

H. K. is supported by grants from the National Institutes of Health, AI 34486 and from the Mayo Foundation.

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