

Enantiomer-Specific Effects of Albuterol on Airway Inflammation in Healthy and Asthmatic Cats

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Key Words

Cat • R-Albuterol • S-Albuterol • Inhalation

Abstract

Background: Racemic (R,S)-albuterol is a 1:1 mixture of an R-enantiomer which has bronchodilatory and anti-inflammatory effects, and an S-enantiomer which is associated with increased airway hyperreactivity and proinflammatory effects. Proinflammatory effects of regularly inhaled and S-albuterol have not been studied in a whole-animal model. We hypothesized that regular administration of R,S-albuterol or S-albuterol, but not R-albuterol, would induce airway inflammation in healthy and asthmatic cats. **Methods:** Six healthy and 5 experimentally asthmatic cats were randomized to receive inhaled R,S-albuterol, S-albuterol, R-albuterol, or placebo (saline) twice daily for 2 weeks, followed by a 6-week washout before crossover to the next treatment. Bronchoalveolar lavage fluid was collected for cell counts and cytokine analysis prior to and at the end of each 2-week treatment. **Results:** Healthy and asthmatic cats receiving R,S- and S-albuterol had higher total lavage cell numbers ($p = 0.04$ and $p = 0.02$, respectively) than those receiving R-albuterol and placebo. The number of lavage eosinophils and the TNF- α bioactivity was higher in asthmatic cats receiving R,S- and S-albuterol compared with those receiving the other

treatments ($p = 0.03$ and $p = 0.004$, respectively). In healthy cats, the number of lavage neutrophils was higher when they received R,S- and S-albuterol compared with other treatments ($p = 0.04$). **Conclusion:** Airway inflammation is induced in both healthy and asthmatic cats with regular inhalation of racemic and S-albuterol, but not with R-albuterol.

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Introduction

Racemic albuterol (R,S-albuterol), a 1:1 mixture of an R-enantiomer and an S-enantiomer, is the most commonly prescribed short-acting β_2 -agonist in the USA [1]. R-Albuterol (levalbuterol) is responsible for the clinical bronchodilator activity of racemic albuterol, and when given at proportionally equivalent nebulized doses as racemic albuterol, it possesses superior bronchodilatory properties [2]. Several investigators have shown that S-albuterol is not inert, but likely works in opposition to R-albuterol; in a number of in vitro and in vivo studies, administration of S-albuterol was associated with increased airway hyperreactivity and proinflammatory effects [3–8]. After regular use of racemic albuterol, the S-enantiomer is thought to accumulate in the lung partly due to a stereoselective metabolism of the two isomers and due to

preferential retention of the *s*-isomer [9, 10]. However, the effects of albuterol enantiomers on airway hyperreactivity and inflammation are controversial, and previous *in vitro* and *in vivo* studies using murine cells or allergen challenge models of asthma have been criticized for not translating into studies relevant to humans with asthma [3, 11, 12]. Additionally, in human asthmatics, some investigators failed to find a benefit to administering R-albuterol over the racemate [13–15].

A variety of animal models have been used to study the major immunologic and pathologic mechanisms of human asthma, but only the cat naturally and commonly develops spontaneous asthma that replicates all of the major features of the disease in humans [16–18]. These features include clinical signs of cough, wheeze, and episodic respiratory distress; bronchoconstriction reversible with β -agonists, eosinophilic inflammation of the airways, and remodeling changes of the airways. Repeated airway challenge using a clinically relevant allergen (Bermuda grass allergen, BGA) led to development of a model of allergic asthma in the cat with many features of human asthma, including elevated BGA-specific IgE, airway hyperreactivity, airway eosinophilia, an acute T helper 2 cytokine profile, and histologic evidence of smooth muscle hyperplasia, epithelial erosion, and goblet cell/submucosal mucous gland hyperplasia [19]. This feline model is therefore an excellent preclinical model for the study of allergen-induced human asthma. In a preliminary study we were surprised to find that regular inhalation of racemic albuterol for 1 week produced airway inflammation in healthy cats [20]. In the present study, we conducted a prospective, placebo-controlled investigation to determine the effects of regular inhalation of racemic and single isomer preparations of albuterol on airway inflammation in healthy and experimentally asthmatic cats. We hypothesized that regularly inhaled R,S-albuterol and *s*-albuterol would be associated with increased inflammatory cells in bronchoalveolar lavage fluid (BALF) compared with inhalation of R-albuterol or placebo.

Material and Methods

Animals

Six mixed-breed male intact cats aged 6 months were obtained from a commercial vendor (Liberty Research, Waverly, N.Y., USA) for the healthy cat group, and 5 cats aged 3 months (4 male, 1 female, all intact) were obtained from high-responder asthmatic cat breeding pairs (School of Veterinary Medicine, University of Missouri, Columbia, Mo., USA). Animals were cared for according to the principles outlined in the NIH Guide for the Care and Use of



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Fig. 1. Delivery of inhaled medication was accomplished by placing a tight-fitting face mask attached to an Omron Micro Air vibrating mesh nebulizer over the muzzle of the cat while awake and spontaneously breathing. Cats received each arm of this treatment protocol twice daily for 2 weeks.

Laboratory Animals. Unlike the asthmatic cats, healthy cats were not subject to injections of allergen or adjuvants, and did not receive aerosol challenges (see 'Induction of Asthma' below).

Induction of Asthma

Allergen sensitization and challenge using BGA (Greer Laboratories, Lenoir, N.C., USA) was performed in 5 cats as previously described [19]. After confirmation of an asthmatic phenotype, allergen aerosol challenges were performed at weekly intervals for the duration of the study. All samples of BALF were collected in a blind fashion 48 h after allergen aerosol challenges.

Protocol

The study was conducted in a blinded, randomized, placebo-controlled crossover design. Cats in each group were assigned using a table of random numbers to receive one of the following inhaled medications (5 mg/ml) twice daily for 15 treatments: racemic albuterol (2 mg/cat/treatment in 0.4 ml PBS), *s*-albuterol (1 mg/cat/treatment in 0.2 ml PBS), R-albuterol (1 mg/cat/treatment in 0.2 ml PBS), and PBS (0.2 ml; placebo control). Drugs were nebulized using an Omron Micro Air mesh nebulizer (Model NE-U22 SN 4401322A; Omron Health Care, Bannockburn, Ill., USA) via a tight fitting anesthetic face mask while awake and spontaneously breathing as illustrated in figure 1. This nebulizer produces a heterodisperse aerosol of albuterol with a mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of 4.5 ± 0.2 and 2.0 ± 0.1 μ m, respectively [21]. A clinical scoring system was used to grade the severity of symptoms the cats displayed immediately after delivery of allergen via aerosol in their chamber (table 1).

At baseline and after the 2-week treatment period, BALF was collected and processed as described previously so that each cat had BALF collected a total of 8 times over the course of the study [22]. Total nucleated cell counts were obtained from the BALF us-

Table 1. As a clinical correlate for severity of allergen-induced airway hyperreactivity/bronchoconstriction, a clinical scoring system was employed to grade the symptoms the cats displayed immediately after delivery of allergen via aerosol in their chamber

Clinical signs	
0 points	no signs
5 points	mild to moderate increase in respiratory effort but not enough to affect activity or mentation; spontaneous cough
10 points	moderate signs of respiratory difficulty (expiratory push or rapid, shallow breathing)
15 points	moderate to severe signs of respiratory difficulty (nostrils flaring, impaired activity, obvious distress)
20 points	severe signs (open mouth breathing, lateral collapse, obtunded)

Cats were randomized to receive each of the following treatments in a crossover design: inhaled PBS, R,S-albuterol, S-albuterol and R-albuterol. The clinical scoring system was performed by the same investigator after administration of 11 treatments of each compound (i.e., 48 h prior to collection of BALF).

ing a Coulter counter (Z1 single threshold analyzer; Beckman Coulter, Fullerton, Calif., USA). Remaining BALF was centrifuged at 300 g for 10 min, and the supernatant harvested in aliquots for storage at -80°C . Cats were then allowed a washout period of 6 weeks. This was followed by crossover to the next randomly assigned treatment with baseline and posttreatment BALF collections. The study continued until each of the treatments had been administered.

Cytokine Analysis

Banked aliquots of BALF supernatant stored at -80°C for up to 24 months were thawed and used for determination of concentrations of IL-4 (Th2 cytokine) and IFN- γ (Th1 cytokine) in asthmatic cats, and IL-8 (neutrophil chemokine) in healthy cats. All samples were run in duplicate using commercially available ELISA kits (R&D Systems, Minneapolis, Minn., USA) according to manufacturer's instructions. The ranges of detection of these assays are 62.5–4,000 pg/ml. Samples were also normalized to total protein concentrations which were run in duplicate using a commercially available Bradford-type protein assay following the manufacturer's instructions (BioRad Laboratories, Hercules, Calif., USA). The range of detection of this assay is 0.0625–0.5 mg/ml. An IL-4:IFN- γ ratio was also calculated in asthmatic cats.

Tumor necrosis factor- α (TNF- α) activity was evaluated in BALF from both study groups using a cell-killing bioassay. Briefly, 3.0×10^5 cells from mouse fibroblast cell (L929) were cultured on 96-well plates for 12 h followed by the addition of 50 μl of BALF samples to the wells. Cells were incubated in MEM (Gibco, Invitrogen Corporation, Auckland, New Zealand) plus horse serum

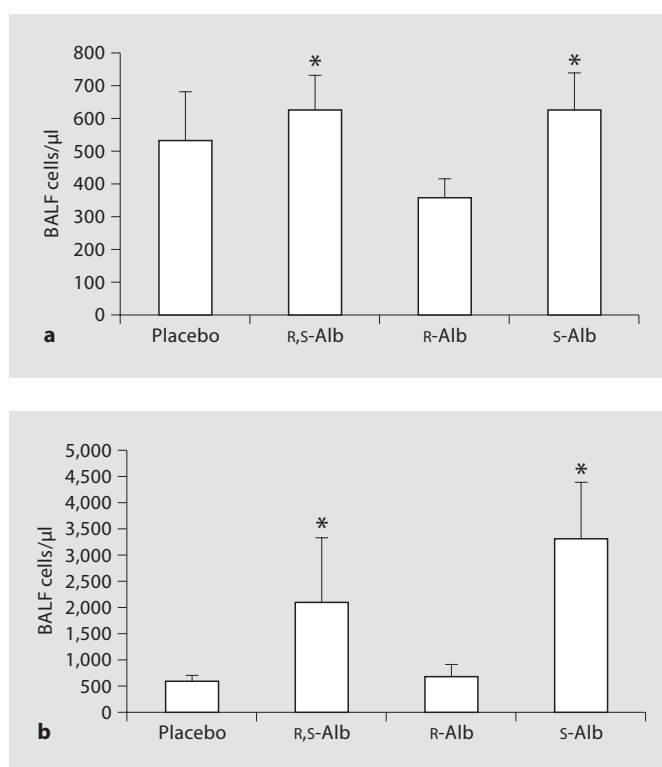


Fig. 2. Group mean total numbers of BALF cells in 6 healthy (a) or 5 experimentally asthmatic cats (b) administered a 2-week course of inhaled saline (placebo), R,S-albuterol (R,S-Alb), R-albuterol (R-Alb), or S-albuterol (S-Alb). Cats were randomized to receive each of the aforementioned treatments, followed by a 6-week washout period between treatments, before crossover to the next treatment. Cats with experimentally induced asthma received weekly aerosol challenges of allergen, and collection of BALF at the end of the 2 week treatment was performed 48 h after allergen challenge. Cats in both groups receiving R,S-albuterol and S-albuterol had significantly (*) higher group mean total numbers of BALF cells/ μl than when receiving R-albuterol or placebo (healthy cats, $p = 0.04$; asthmatic cats, $p = 0.02$). Note that the scale of the y-axes differs between the two graphs. Bars represent SEM.

(1%) (Gibco) and actinomycin D (3 $\mu\text{g}/\text{ml}$; Sigma-Aldrich, St. Louis, Mo., USA), for 20 h, followed by addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl tetrazolium bromide (MTT; Sigma-Aldrich). Cells were incubated for an additional 2.5 h. Formazan crystals were solubilized in dimethylformamide (50%) and SDS (20%) (both from Fisher Scientific International, Pittsburgh, Pa., USA) and color development was measured at 630 nm. Feline recombinant TNF- α (Fisher) was used to construct a standard curve (from 0 to 10 ng/ml) to quantify the TNF- α activity in the BALF samples. The range of detection of this assay is 0.25–10 ng/ml.

Data Analysis

All analyses were performed using PROC MIXED in SAS (SAS Institute, Cary, N.C., USA). Data were analyzed by 2-way uni-

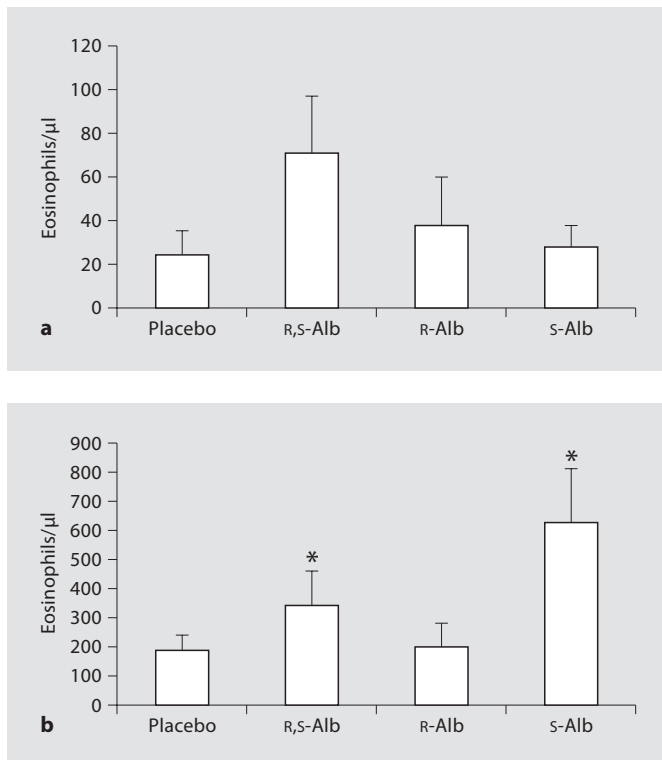


Fig. 3. Group mean absolute numbers of BALF eosinophils in (a) 6 healthy or (b) 5 experimentally asthmatic cats administered a 2-week course of inhaled saline (placebo), R,S-albuterol (R,S-Alb), R-albuterol (R-Alb), or S-albuterol (S-Alb). There were no significant differences between the treatments for the healthy cats. However, asthmatic cats receiving R,S-albuterol and S-albuterol had significantly (*) higher group mean absolute numbers of BALF eosinophils/μl than when receiving R-albuterol or placebo ($p = 0.03$). Note that the scale of the y-axes differs between the two graphs. Bars represent SEM.

variate repeated-measures ANOVA. Responses were modeled as a function of both the treatment (therapy vs. placebo) and time. Each model additionally included a random effect for the animal to account for the correlation between measurements made upon the same animal. All models were assessed to ensure that necessary assumptions were met, and effects were considered to be statistically significant when the p values were less than 0.05.

Results

Inhalation of any of the formulations of albuterol in either healthy and asthmatic cats did not produce any clinically observed respiratory problems such as cough, wheeze, elevated respiratory rate, or respiratory distress.

In the asthmatic cat group after aerosol challenge with allergen, the mean $\pm$ SEM of the clinical sign score for each treatment was: placebo 12.5 ± 1.3 ; R,S-albuterol 13.8 ± 2.8 ; R-albuterol 11.3 ± 2.2 ; S-albuterol 18.8 ± 2.2 . There was a trend towards an increase in severity of clinical signs in cats administered R,S-albuterol and S-albuterol compared with R-albuterol and placebo ($p = 0.0975$).

No significant differences were noted in the baseline (pretreatment) values for the total number of BALF cells, the percentage or absolute number of eosinophils, and the percentage or absolute number of neutrophils for either the healthy or asthmatic cat groups.

Significantly higher group mean total BALF cell numbers were noted in healthy ($p = 0.04$) and asthmatic ($p = 0.02$) cats when administered R,S-albuterol and S-albuterol, compared with R-albuterol and placebo (fig. 2). While there were no significant differences in the group mean absolute number of BALF eosinophils between treatments in the healthy cat group, the group mean absolute number of BALF eosinophils was significantly higher in asthmatic cats receiving R,S- and S-albuterol compared with R-albuterol and placebo ($p = 0.03$; fig. 3). The group mean absolute number of BALF neutrophils was significantly higher in healthy cats ($p = 0.04$) and there was a trend toward statistical significance in asthmatic cats ($p = 0.09$) receiving R,S- and S-albuterol compared with R-albuterol and placebo (fig. 4).

In asthmatic cats, there were no significant differences in the BALF IL-4 or IFN- γ concentrations or in the concentration of either of these BALF cytokines normalized to total protein, or in the BALF IL-4:BALF IFN- γ ratio (data not shown). In the asthmatic cat group, there was a significantly higher concentration of TNF- α in cats treated with R,S-albuterol and S-albuterol compared with placebo and R-albuterol (fig. 5; $p = 0.04$). In healthy cats, BALF IL-8 was below the limits of detection of the assay in over half of the samples, preventing meaningful analysis. There were no significant differences in the TNF- α concentrations between treatments in the healthy cat group (data not shown).

Discussion

Induction of airways inflammation occurred in both healthy and experimentally asthmatic cats after regular inhalation of R,S-albuterol and S-albuterol; these inflammatory changes were not noted after regular inhalation

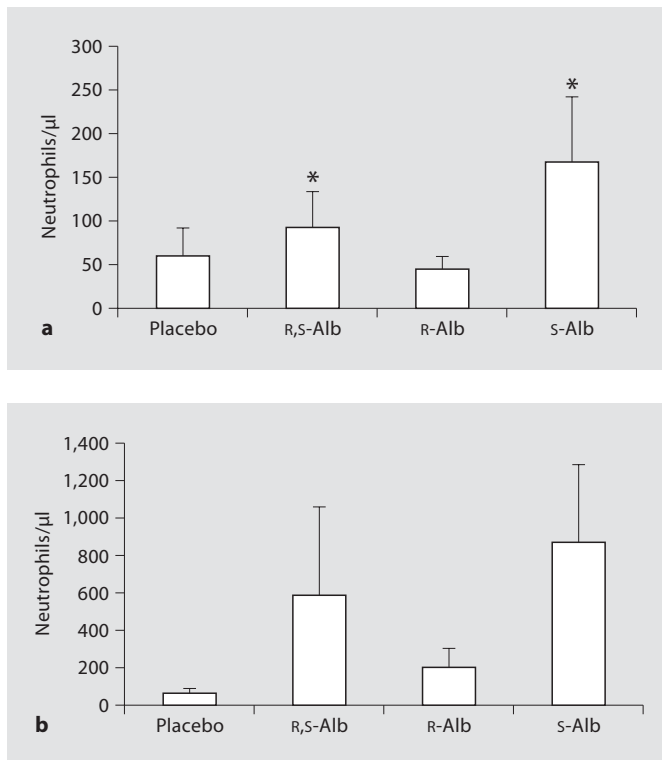


Fig. 4. Group mean absolute numbers of BALF neutrophils in 6 healthy (a) or 5 experimentally asthmatic cats (b) administered a 2-week course of inhaled saline (placebo), R,S-albuterol (R,S-Alb), R-albuterol (R-Alb), or S-albuterol (S-Alb). Healthy cats receiving R,S-albuterol and S-albuterol had significantly (*) higher group mean absolute numbers of BALF neutrophils/ μ l than when receiving R-albuterol or placebo ($p = 0.04$). Asthmatic cats receiving R,S-albuterol and S-albuterol had group mean absolute numbers of BALF neutrophils/ μ l that tended towards statistical significance compared to R-albuterol or placebo ($p = 0.09$). Note the scale of the y-axes differs between the two graphs. Bars represent SEM.

of R-albuterol. This is compelling evidence that intermediate-term delivery of the single isomer S-albuterol has proinflammatory effects in healthy research cats without evidence of lung disease as well as in a preclinical feline model of asthma. After regular inhalation of R,S-albuterol and S-albuterol (but not R-albuterol), significant increases in total BALF cell numbers and neutrophils were observed in healthy research cats. In asthmatic cats, significant increases in total BALF cell numbers, eosinophils, and TNF- α bioactivity were found and there was a trend towards higher symptom scores after allergen challenge with treatment containing the S-albuterol isomer as observed in a number of previous studies [3, 5, 6].

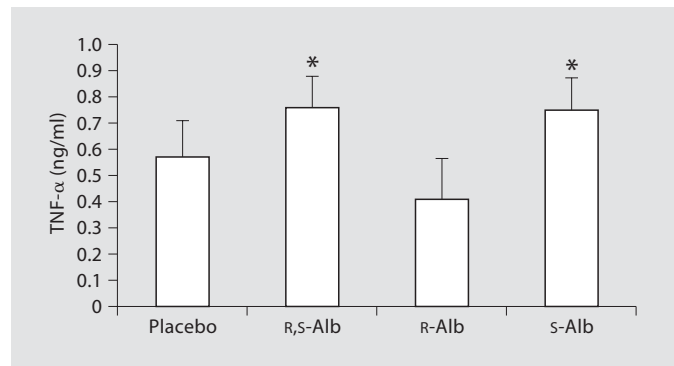


Fig. 5. Group mean BALF TNF- α bioactivity detected using a cell killing bioassay in 5 experimentally asthmatic cats administered a 2-week course of inhaled saline (placebo), R,S-albuterol (R,S-Alb), R-albuterol (R-Alb), or S-albuterol (S-Alb). There was a significantly (*) higher concentration of TNF- α bioactivity (ng/ml) in cats treated with R,S-albuterol and S-albuterol compared with placebo and R-albuterol ($p = 0.04$). Bars represent SEM.

While a clear significant treatment effect with use of albuterol containing the S-isomer in both healthy and asthmatic cats was documented, the clinical significance of a relatively small increase in BALF cell numbers induced by treatment in healthy cats without a parallel increase in cytokines may be questioned. However, in healthy cats, only IL-8 and TNF- α were evaluated; it is possible that other cytokines might have been altered with treatment.

In asthmatic cats, not only did treatment with the S-isomer increase BALF cellularity in comparison to treatment with R-albuterol, but parallel increases in TNF- α and a trend toward increased clinical signs after allergen challenge were noted. While IL-4 was used as a measure of Th2 cytokine bias in asthmatic cats, IL-5 is the cytokine directly involved in eosinophil maturation, differentiation and survival, and would have been an ideal choice to correlate with increased numbers of eosinophils with S-albuterol. However, at this time, a feline-specific IL-5 ELISA is not available.

Differential effects of the single isomer forms of albuterol in animal models of asthma were evaluated previously by only two groups of investigators [8, 23]. The first study, using a murine model of asthma, concluded that both isomers reduced airway eosinophil trafficking, but that only the S-enantiomer increased airway hyperresponsiveness [8]. However, three major issues limit the ability to extrapolate the results of that study to humans with asthma: (1) the murine model used ovalbumin

(OVA) to sensitize and challenge mice, and OVA is not a clinically relevant allergen in humans; (2) R- and S-enantiomers were administered systemically by insertion of a subcutaneous pump, and not by inhalation, and (3) the investigators failed to use racemic albuterol as a control, which is important since other *in vitro* studies have shown proinflammatory effects or alterations in mucociliary clearance only when the two isomers are administered simultaneously [4, 24]. In the second study, guinea pigs were sensitized to OVA and administered 3 doses of racemic or single isomers of albuterol by inhalation [23]. Animals showed no differences in the numbers or types of inflammatory cells in the BALF between treatment and placebo groups [23]. The major limitations to that study were the use of OVA as an allergen, the method of aerosol delivery not accurately modeling the route of delivery in humans (i.e. aerosols were delivered in a 9-liter cage in which animals were allowed to move freely), and the short time course in which evaluation of airway inflammation was performed (i.e. 3 doses were given over a 24-hour period). Our model of feline chronic allergic asthma has the advantages of using a clinically relevant allergen to humans (BGA), of being able to mimic a more typical route of administration of albuterol in humans (inhalation in awake and spontaneously breathing cats by use of a tight-fitting face mask; fig. 1), of administration for an intermediate-term study (2 weeks of treatment) and of being able to perform multiple BALF collections whereby cats serve as their own control.

In vitro studies are useful in dissecting the mechanisms by which the single isomer forms of albuterol exert their beneficial or negative effects. For example, S-albuterol (but not R-albuterol) activates murine mast cells to produce the proinflammatory mediators histamine and IL-4 [3]. Incubation of IL-5-activated eosinophils with R-albuterol inhibited superoxide production for up to 60 min, whereas S-albuterol enhanced superoxide production after 60 min [7]. With tetanus-specific cell lines, R-albuterol was shown to inhibit T cell proliferation and IL-2 and IFN- γ production, racemic albuterol had minimal effects on T cell proliferation or cytokine production, and addition of the S-isomer produced a dose-dependent increase in T cell proliferation and cytokine production [4]. Likewise, S-albuterol activated proinflammatory pathways involving both PI3 kinase and NF- κ B in human bronchial smooth muscle cells [5]. While these studies detail effects of the isomers of albuterol on immune cells, cytokines, and airway hyperactivity, and others show detrimental effects on airway smooth muscle proliferation [5, 6, 25] and function of the

mucociliary apparatus [24], ultimately preclinical models must be employed to evaluate the overall effects of the racemic and single-isomer forms of albuterol in an intact host.

Interpretation of studies evaluating adverse effects of racemic albuterol must take into consideration the duration of exposure and differences in metabolism of its two isomers. Additionally, the kinetics of the response between the two isomers may be influenced by differences in the pathways of cellular activation [6, 7]. Equimolar concentrations of R- and S-albuterol may fail to produce adverse effects [4, 26], whereas altering the ratio in favor of the distomer may result in proinflammatory influences [4]. When R,S-albuterol is given by inhalation, preferential retention of S-albuterol in the lung could lead to increasing concentrations of S-albuterol compared with R-albuterol over time [10]. Moreover, slower metabolism of S-albuterol compared to R-albuterol leads to maximum plasma concentrations and area under the curve of S-albuterol being 2–4 times higher than that of R-albuterol after inhalation of R,S-albuterol [27, 28]. Furthermore, after inhalation of R,S-albuterol, the elimination half-life of S-albuterol is approximately 50% longer than that of R-albuterol [29]. Thus, repeated inhalations of R,S-albuterol lead to a successive increase in the S:R ratio [28, 30]. After several days of regular R,S-albuterol inhalation, the airways are probably exposed to levels of S-albuterol that are several times higher than those of R-albuterol [31]. In this context, the observation by Baramki et al. [4] that incubation of human T cells with equivalent concentrations of isomers had no effect on proliferation compared with control, but a 10-fold higher concentration of S-albuterol compared to R-albuterol led to enhanced T cell proliferation is intriguing. The results of the present study are in agreement with previous observations [32–34] that regular exposure to racemic albuterol for 1 week or more promotes inflammatory responses in patients with asthma, and they extend previous observations by showing that airway inflammation may be due to the detrimental effects of the S-isomer.

A limitation of the study was the use of a crossover design; this design was employed to decrease the number of animals evaluated. Crossover studies have been previously advocated to remove inter-subject variability [35]. To overcome this, we performed BALF collection at the end of each washout period prior to crossover drug treatment and we found no differences in any of the pretreatment values. Another limitation of the study was the high variability in responses between cats. This is to be expected in outbred animal species and is parallel to testing

human subjects. Despite the relatively small numbers of cats studied and their variable responses, we found significant increases in measures of airway inflammation after regular inhalation of R,S- and S-albuterol.

In summary, we have shown for the first time that regular inhalation of either R,S-albuterol or S-albuterol (but not R-albuterol) induces airway inflammation in vivo in both healthy cats and in experimentally asthmatic cats. Further research is required to determine if differences in airway inflammation with long-term use of racemic albuterol and levalbuterol are also observed in patients with asthma.

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