

**LONG-TERM EFFECTS OF  $\beta$ -2-ADRENERGIC RECEPTOR STIMULATION ON  
ALVEOLAR FLUID CLEARANCE IN MICE**

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**Running title: Downregulation of the  $\beta$ AR and alveolar fluid clearance**

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## ABSTRACT

Stimulation of active fluid transport with  $\beta$ -adrenergic receptor ( $\beta$ AR) agonists can accelerate the resolution of alveolar edema. However, chronic  $\beta$ AR-agonist administration may cause  $\beta$ AR-desensitization and -downregulation that may impair physiological responsiveness to  $\beta$ AR-agonist stimulation. Therefore, we measured baseline and terbutaline ( $10^{-3}$  M) stimulated alveolar fluid clearance in mice that received subcutaneously (mini-osmotic pumps) either saline or albuterol (2 mg/kg/day) for 1, 3, or 6 days. Continuous albuterol administration increased plasma albuterol levels ( $10^{-5}$  M), an effect that was associated with (1) a significant decrease in  $\beta$ AR density, and (2) attenuation, but not ablation, of maximal terbutaline-induced cAMP production. Forskolin-mediated cAMP-release was unaffected. Continuous albuterol-infusion stimulated alveolar fluid clearance on day 1, but did not increase alveolar fluid clearance on day 3 and 6. However, terbutaline-stimulated alveolar fluid clearance in albuterol-treated mice was not reduced compared to saline-treated mice. Despite significant reductions in  $\beta$ AR density and agonist mediated cAMP production by long-term  $\beta$ AR-agonist exposure, maximal  $\beta$ AR-agonist-mediated increase in alveolar fluid clearance is not diminished in mice.

## INTRODUCTION

Pulmonary edema is a life-threatening condition resulting from an imbalance between forces driving fluid into the air spaces and active transport mechanisms that remove edema fluid from the air spaces and interstitium of the lung. There is now strong evidence that vectorial sodium and chloride transport across the alveolar epithelium play important roles in creating the osmotic gradient that leads to water reabsorption in both the perinatal lung (13, 21, 34) and adult lung (26, 36, 39).

Several studies have demonstrated that endogenous and exogenous  $\beta$ AR agonists (in particular but not exclusively  $\beta_2$ AR-selective agonists) can markedly increase transepithelial sodium transport *in vitro* (26, 30), and upregulate alveolar fluid clearance both *ex vivo* in human lung (37), and *in vivo* when given acutely to animals (5, 16, 33). Consistent with these responses, both  $\beta_1$ ARs and  $\beta_2$ ARs have been detected on the alveolar epithelium (7). The stimulatory effect of  $\beta$ AR agonists on ion and water transport is, at least partly, mediated by cAMP-dependent mechanisms (18), is partly inhibited by amiloride (5, 13, 17, 25, 26, 33, 34), and is not related to changes in the pulmonary blood flow which is simultaneously induced by these  $\beta$ AR agonists (5).

Recent observations (2, 14, 38) have suggested that  $\beta$ AR stimulation of alveolar fluid clearance may be of potential use for the treatment and prevention of pulmonary edema. Subacute or chronic  $\beta$ AR agonist administration may however lead to downregulation of  $\beta$ ARs, as has earlier been shown in the airways (4). Little data is available evaluating long-term effects of  $\beta$ AR agonist administration on the capacity of the alveolar epithelium to respond to  $\beta$ AR agonist stimulation, nor assessing whether downregulation of  $\beta$ ARs would prevent or attenuate the  $\beta$ -

adrenergically mediated increases in alveolar fluid clearance (8, 31). Recent findings in rats demonstrated that desensitization of the alveolar fluid clearance response does not occur after 4 hours of continuous epinephrine exposure (8), whereas isoproterenol when given over 48 hours resulted in a down-regulation of the alveolar epithelial  $\beta$ ARs and an impaired response to additional air space  $\beta$ AR stimulation (31).

To determine whether a similar phenomenon was present after a more prolonged exposure to systemic  $\beta$ AR agonists and whether this functional impairment may be counterbalanced by high dose of acute intra-alveolar administration of  $\beta$ 2AR agonists, we measured total lung adrenergic-induced release of cAMP and  $\beta$ AR density, and compared both baseline and terbutaline-stimulated alveolar fluid clearance in *ex vivo* mice that received either saline or albuterol by continuous subcutaneous administration for 1, 3, or 6 days.

## **METHODS**

Male CD-1 mice weighing 25-35 g were used for all experiments. The mice were housed in air-filtered, temperature controlled units ( $20 \pm 2$  °C) and had food and water *ad libitum*. All procedures were approved by the UCSF committee on animal research.

### **Albuterol plasma concentration measurements**

Plasma albuterol levels were measured by high performance liquid chromatography. Albuterol levels reported in this study are the sum of the (R)- and (S)-enantiomer levels (15) and were measured by a technician blinded to the experimental condition at Sepracor, Inc. (Marlborough, MA).

### **Lung cAMP measurements**

As previously described (33), duplicate samples of distal lung tissue (25-30 mg) were washed in ice-cold 0.9% NaCl with 1 mM isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor, Sigma Chemical Co., St. Louis, MO), then incubated in 0.25 ml of 5 mM tris-hydroxymethyl-aminomethane (Merck, Darmstadt, Germany) in 0.9% NaCl (pH 7.4), 1 mM IBMX, 0.1 mM ascorbic acid (Merck), and 0.1 mM HCl (Merck). Baseline cAMP content was determined after incubation of the sample at 4 °C for 10 min, and baseline cAMP production was studied after 10 min incubation at 37 °C. Stimulation of cAMP generation was studied after the addition of either terbutaline ( $10^{-3}$  M, Sigma Chemical Co.) or forskolin ( $10^{-4}$  M; Sigma Chemical Co.), and incubation of the samples for 10 min at 37 °C. All reactions were stopped with 0.25 ml of

10% trichloroacetic acid (Sigma Chemical Co.). The samples were homogenized and centrifuged (4,000 x g, 15 min at 4 °C). The supernatants were extracted with ether (5:1) three consecutive times to remove the trichloroacetic acid. The remaining ether was evaporated in a 70 °C water bath for 30 min. The samples were stored at -70 °C until analysis. The cAMP content was determined with a radioimmunoassay (NEN-DuPont, Boston, MA). The cAMP content was normalized to mg lung tissue and the results were expressed as cAMP per mg of lung tissue as previously done (33).

### **b-adrenoceptor density**

Lung membranes were prepared from mice by homogenizing the left lung in 10 ml of hypotonic lysis buffer (5 mM Tris, pH 7.4, 2 mM EDTA), containing the protease inhibitors leupeptin, aprotinin, benzamide, and soybean trypsin inhibitor (10 µg/ml each). The homogenate was centrifuged at 40,000 x g for 10 minutes at 4 °C. The supernatant was removed and the pellets containing crude membrane particulates were resuspended in assay buffer (75 mM Tris, pH 7.4, 12.5 mM MgCl<sub>2</sub>, 2 mM EDTA). βAR expression was determined by radioligand binding with <sup>125</sup>I-iodocyanopindolol (ICYP, a non-selective βAR antagonist), as described previously (28, 29).

### **Alveolar Fluid Clearance Measurements**

#### *Preparation of Instillate*

The instillate consisted of 5 g/100 ml bovine serum albumin (Sigma Chemical Co.) in Ringer's lactate adjusted to 330 mOsm/kg H<sub>2</sub>O with NaCl to be isosmolar with mouse plasma, and 0.1 µCi of <sup>131</sup>I-labeled albumin (Merck-Frosst, Montreal, Canada) as the labeled alveolar

protein tracer (16). For measurements of stimulated alveolar fluid clearance, terbutaline ( $10^{-3}$  M, Sigma Chemical Co.) was added to the instillate.

### *Surgical Preparation*

The mice were euthanized by an overdose of pentobarbital sodium (200 mg/kg ip). The trachea was dissected and cannulated with a 20-gauge, trimmed Angiocathplastic needle (Becton Dickinson, Sandy, UT). The lungs were kept inflated with 5 cmH<sub>2</sub>O continuous positive airway pressure and oxygenated with 100% oxygen throughout the experiment. The body temperature was maintained at 37-38 °C, as done earlier (16).

### *General Protocol*

In all studies 13 ml/kg of the instillate was delivered over 30 seconds into both lungs through the tracheal cannula. After 30 minutes, an alveolar fluid sample (0.05-0.10 ml) was aspirated with a 1-ml syringe directly connected to the 20-gauge Angiocath. The aspirate was weighed and the radioactivity measured in a gamma-counter. Alveolar fluid clearance (% of instilled fluid volume) was calculated by measuring the increase in tracer-labeled albumin (<sup>131</sup>I-albumin) concentration in the instilled solution. Because the initial volume of the instilled solution and the initial and final radioactivity of the samples were known, alveolar fluid clearance (AFC) could be determined by using the following mass-balance equation:

$$\text{AFC} = (1 - \text{radioactivity in the instilled sample} / \text{radioactivity in the final sample}) \times 100$$

where AFC is expressed in percent of the initial volume of instillate that was cleared from the distal air spaces during the 30 minutes (8, 16).

### **Specific Protocols**

Baseline and terbutaline-stimulated alveolar fluid clearance were determined in mice receiving either continuous albuterol administration (2 mg/kg/day) or normal saline for 24 hours (*Group 1: Alveolar fluid clearance at day 1*) via a mini-osmotic pump (Alzet model 2001, Alzo Co., Palo Alto, CA) implanted subcutaneously after a rapid anesthesia with ketamine/xylazine (12). Similarly, baseline and terbutaline-stimulated alveolar fluid clearance were measured in mice that received either albuterol or normal saline for 72 h (*Group 2: Alveolar fluid clearance at day 3*) and 150 h (*Group 3: Alveolar fluid clearance at day 6*)

### **Statistical Analysis**

The data are summarized as mean  $\pm$  SD. Analysis of variance (ANOVA) and paired and unpaired *t*-test were used for comparisons as appropriate. We regarded as significant differences with a *P* value of <0.05.

## RESULTS

### *Albuterol plasma concentration*

Continuous subcutaneous albuterol administration (2 mg/kg/day) for 1, 3, and 6 days by a osmotic mini-pump provided a steady-state plasma albuterol concentration of approximately 11 ng/ml (or  $10^{-5}$  M) on day 1 that persisted through day 6 (**Figure 1**). Albuterol was not detected in control mice receiving continuous administration of normal saline.

### *Total lung cAMP measurements and $\beta$ AR density*

Basal cAMP production in lung slices from mice that received systemic albuterol for 1, 3, or 6 days were not significantly different from that of the saline-treated control mice. Addition of terbutaline to the saline-treated lungs caused a ~3-fold increase ( $P<0.05$ ) in cAMP release (**Figure 2**). Terbutaline also caused a significant increase in cAMP production in each of the albuterol treatment groups (days 1, 3, and 6). However, terbutaline-stimulated cAMP production in each of the albuterol-treated groups was ~40-50% lower than the agonist-stimulated levels achieved in the saline control groups (**Figure 2**). In contrast, cAMP levels stimulated by forskolin (a direct activator of the adenylyl cyclase) were not different in the saline- and albuterol-treated mice (**Figure 2**), indicating that systemic albuterol administration had no significant effect on  $\beta$ AR-independent cAMP production.

The attenuation of terbutaline-stimulated cAMP production in the albuterol-treatment groups suggested the possibility that chronic  $\beta$ AR agonist therapy was contributing to desensitization of the signal transduction pathway. Since downregulation (i.e., a decrease in

receptor number) is one mechanism that may underlie such desensitization, and has been observed for  $\beta$ ARs in the lung, we compared  $\beta$ AR density in whole lung homogenates from the saline- and albuterol-treated mice by radioligand binding with the nonselective  $\beta$ AR antagonist  $^{125}\text{ICYP}$ .  $\beta$ AR density in lungs from animals treated with albuterol for 1 and 3 days was decreased by ~25% ( $P < 0.05$ ) when compared to saline treated-control mice, and was decreased by ~50% in lungs from mice treated for 6 days (**Figure 3**).

### *Alveolar Fluid Clearance*

We and others have previously shown that endogenous and exogenous  $\beta$ AR-agonists significantly increase *in vivo* alveolar fluid clearance, suggesting that  $\beta$ AR activation may serve a protective function in the setting of pulmonary edema. Whether this effect on alveolar fluid clearance wanes in the presence of chronic  $\beta$ AR agonist exposure (i.e., tachyphylaxis), however, is unclear. We therefore measured alveolar fluid clearance in saline-treated mice and compared them to those in mice treated continuously with albuterol for up to 6 days. For each treatment group, alveolar fluid clearance was determined in the absence (basal alveolar fluid clearance) or presence of intra-alveolar terbutaline (stimulated alveolar fluid clearance). When compared to saline-treated controls, there was a small but significant increase in alveolar fluid clearance in mice treated with systemic albuterol for 1 day. However, basal alveolar fluid clearance rates in mice treated with albuterol for 3 and 6 days were not different from their respective controls (**Figure 4, panel A**).

To determine whether the maximal response of alveolar fluid clearance stimulation by  $\beta$ AR agonists was diminished, we added terbutaline directly to the alveolar instillate. When compared to the respective basal alveolar fluid clearance for each group, addition of terbutaline significantly increased alveolar fluid clearance in saline controls as well as the 1, 3, and 6 day

albuterol treatment groups ( $P < 0.05$  for all; **Figure 4, panel B vs. panel A**). Thus, despite the reductions in  $\beta$ AR number and agonist-stimulated cAMP production associated with chronic albuterol treatment, the magnitude of terbutaline-stimulated alveolar fluid clearance was not affected (**Figure 4, panel B**). Interestingly, there was even an apparent additive effect of albuterol and terbutaline in stimulating alveolar fluid clearance on days 1 and 3.

## DISCUSSION

Although continuous albuterol administration induced a significant downregulation of the  $\beta$ ARs in the lung, and attenuated the terbutaline-induced release of cAMP, the sustained albuterol-treatment over 6 days did not diminish the acute intra-alveolar  $\beta$ AR agonist-mediated stimulation of alveolar fluid clearance. These findings represent new information that may have clinical as well as physiological relevance.

Continuous release of albuterol subcutaneously with a mini-osmotic pump resulted in a sustained high plasma albuterol concentration throughout the entire experimental period. We thought it was important to directly measure albuterol plasma levels to ensure that the levels obtained were adequate to model the clinical setting where critically ill patients may have elevated levels of endogenous catecholamines or receive high levels of systemic  $\beta$ AR agonists.

Chronic albuterol administration resulted in downregulation of lung  $\beta$ ARs as demonstrated by a decrease in receptor number and attenuation of agonist-promoted cAMP release. To ensure that the effect of lung cAMP was due to  $\beta$ AR stimulation, we added forskolin, a direct activator of the adenylyl cyclase. Forskolin generated similar concentrations of cAMP in all experimental groups, indicating that the receptor-signaling defect occurred upstream of the adenylyl cyclase.

Both  $\beta_1$ AR and  $\beta_2$ AR subtypes are present in the lung, whereas, to the best of our knowledge, no data exists that demonstrates presence of the  $\beta_3$ AR subtype in the lung. Iodocyanopindolol is a non-selective  $\beta$ AR antagonist that recognizes both subtypes and thus measures total  $\beta$ AR density. However, the ratio of  $\beta_1$ ARs to  $\beta_2$ ARs in mouse lung parenchyma (i.e., peripheral lung) is 28:72 in favor of the  $\beta_2$ AR (20). Moreover, the majority of cells in

peripheral lung are those of the alveolar wall where the receptor ratio is 18:82 in favor of the  $\beta_2$ AR. Therefore, any in change in total  $\beta$ AR density in the peripheral mouse lung is most likely to represent changes in the  $\beta_2$ AR subtype.

The decline in  $\beta$ AR number that we observed with chronic  $\beta$ -agonist administration is consistent with previously published data in other experimental studies (24, 32). In particular, a similar exposure to albuterol in rats was associated with a reduction in lung  $\beta$ AR density comparable to the results in this study (12). Recent data in rats continuously infused with isoproterenol also demonstrated a similar downregulation of  $\beta$ AR in the lung (31). Thus, prolonged subcutaneous administration of  $\beta$ AR agonists provide a simple and reliable method to induce  $\beta$ AR tolerance in the lung, although some studies have indicated that these pulmonary receptors may be particularly resistant, when compared to other tissues, to desensitization/downregulation (4).

Despite the rapid attainment of high, steady-state plasma albuterol concentrations after subcutaneous implantation of the mini-osmotic pumps, albuterol therapy alone did not induce a sustained increase in cAMP release in the mouse lung. The lack of such an increase, which in another study was associated with a non-stimulated PKA activity (12), may have contributed to the relatively unaltered baseline alveolar fluid clearance in albuterol-treated mice compared to their controls. Consistent with recent data in rats (31), the failure of subcutaneous albuterol to stimulate alveolar fluid clearance at days 3 and 6 may be a manifestation of  $\beta$ AR desensitization.

However, as shown by similar terbutaline-stimulated alveolar fluid clearance measurements between control mice and those treated with albuterol for 3 and 6 days, a key finding in this study was that the albuterol-induced downregulation and desensitization of lung  $\beta$ ARs did *not* impair the

acute stimulatory effect on alveolar fluid clearance induced by high-dose intra-alveolar  $\beta$ AR agonists. These results are similar to the data demonstrating no influence of continuous systemic epinephrine administration for up to 4 hours on  $\beta$ AR stimulation of alveolar fluid clearance in rats (8), but contrast with recent data in the rat showing that delivery of systemic isoproterenol for 48 hours impaired the lung's ability to respond to air space  $\beta$ AR agonist stimulation with an increase in alveolar fluid clearance (31). These differences may have resulted from use of different species and techniques, but may also be due in part to the choice of agonist used for chronic administration. Whereas we used the  $\beta_2$ AR-selective partial agonist albuterol, Morgan and colleagues (31) used the non-selective and full agonist isoproterenol. Interestingly, recent data suggest that partial agonists may induce less  $\beta$ AR desensitization than full agonists (22). Thus, the extent of desensitization that we observed may have been less than that which occurred in the study by Morgan et al (31), thus accounting for the observed differences in physiological tolerance reported.

Desensitization/downregulation of  $\beta$ ARs may occur by two different mechanisms. One is short-term, within minutes or hours, involving phosphorylation of the  $\beta$ AR and uncoupling from stimulatory G-proteins (23, 35). The second is a long-term mechanism that may occur within several hours or over days that may involve internalization/degradation of the  $\beta$ ARs (3) and inhibition of  $\beta$ AR gene expression and transcription (19). Taken together, our results suggest that even if some receptor tolerance develops, acute intra-alveolar administration of relatively high doses of  $\beta_2$ AR agonists can counteract such effect and stimulate alveolar fluid clearance.

Another explanation may be that alveolar epithelial cells contain more  $\beta$ ARs than necessary to achieve a given response (spare receptors). In such a setting, the number of receptors that remain functional after downregulation may still be sufficient to obtain adequate cAMP levels inside the

epithelial cells to stimulate alveolar fluid clearance. Consistent with this hypothesis, alveolar type II cells have high levels of mRNA expression and high density of  $\beta$ ARs suggesting that part of the synthesized  $\beta$ ARs may constitute a functional pool-reserve (9, 40) that is rapidly available to counterbalance  $\beta$ AR down-regulation (4). While our data show that the maximal cAMP response to terbutaline in albuterol-treated lung tissue was less than that of saline-treated control mice, thereby suggesting an element of desensitization, it is important to note that the increase in cAMP over unstimulated levels in the albuterol-treated groups was nevertheless significant despite the decrease in receptor number. Given that the *in vivo* alveolar fluid clearance response to terbutaline was not diminished by chronic albuterol treatment, our findings suggest that a maximal cellular response with regards to cAMP may not be necessary for maximal physiological responsiveness (i.e., an increased alveolar fluid clearance). An alternative explanation is that a cAMP-dependent pathway is not the only mechanism responsible for  $\beta$ AR stimulation of alveolar fluid clearance (6, 17, 36).

These results may have potential clinical relevance. First, in humans, development of tolerance to  $\beta$ AR agonists has become an important issue, particularly with the introduction of long-acting inhaled  $\beta$ AR agonists for asthma treatment. Indeed, in normal healthy subjects, tolerance has been demonstrated after only one week of continuous therapy with inhaled  $\beta$ AR agonists (4). Of note, the systemic levels of  $\beta$ -agonist in these studies were similar to a concentration in edema fluid that enhanced alveolar fluid clearance in patients with ARDS (1). However, whether clinically significant tolerance develops with regards to alveolar fluid clearance has been unclear. Second and more importantly, an intact epithelial function with preserved respiratory transepithelial sodium, chloride, and fluid transport functions is necessary for clinical improvement in patients recovering from acute lung injury (27, 41, 42). Stimulation of vectorial

fluid transport by  $\beta$ AR agonists contributes both to prevention and/or acceleration of the resolution of pulmonary edema in experimental acute lung injury models (see reference 39 for review), as well as in a clinical study in subjects who are prone to high altitude pulmonary edema development (38).

If  $\beta$ AR downregulation acts to limit agonist-stimulated fluid clearance, then other strategies, such as stimulation by dopamine or even gene therapy with Na-K-ATPase (11) or  $\beta_2$ ARs (10) may be needed to counteract the lack of responsiveness. Our findings, however, suggest that while  $\beta$ AR down-regulation may occur with chronic systemic agonist administration the capacity of the alveolar epithelium to upregulate alveolar fluid clearance in response to intra-alveolar  $\beta$ -agonists can be maintained.

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## FIGURE LEGENDS

### Figure 1

Albuterol plasma concentration in mice receiving a continuous subcutaneous albuterol administration (2 mg/kg/day) for 1, 3, and 6 days or saline. There was no detectable albuterol in the plasma of mice receiving saline. n = 5 for each albuterol group, n = 3 for the saline group.

### Figure 2

Baseline (open columns), terbutaline- (filled columns), and forskolin-stimulated (dashed columns) cAMP release in total lung tissue of mice receiving either saline or albuterol for 1, 3, and 6 days. Represented values are mean + SEM. \*  $P < 0.05$  vs. saline. Values in controls at different time points were similar and therefore were pooled. n = 18 for controls, n = 6 for each albuterol group.

### Figure 3

$\beta$ AR number in total lung tissue of mice receiving either saline or albuterol for 1, 3, and 6 days. \*  $P < 0.05$  vs. saline. n = 3 for each group.

### Figure 4

Basal (**panel A**) and terbutaline-stimulated (**panel B**) alveolar fluid clearance in mice under continuous saline (baseline; open columns) or albuterol (filled columns) administration for 1, 3, and 6 days. \*  $P < 0.05$  vs. saline. At least 6 mice for each group.

# Intra-alveolar terbutaline administration significantly stimulated ( $P < 0.05$ ) alveolar fluid clearance in all groups of mice when compared to corresponding groups studied under baseline conditions. (**panel B** vs. **panel A**).

Figure 1

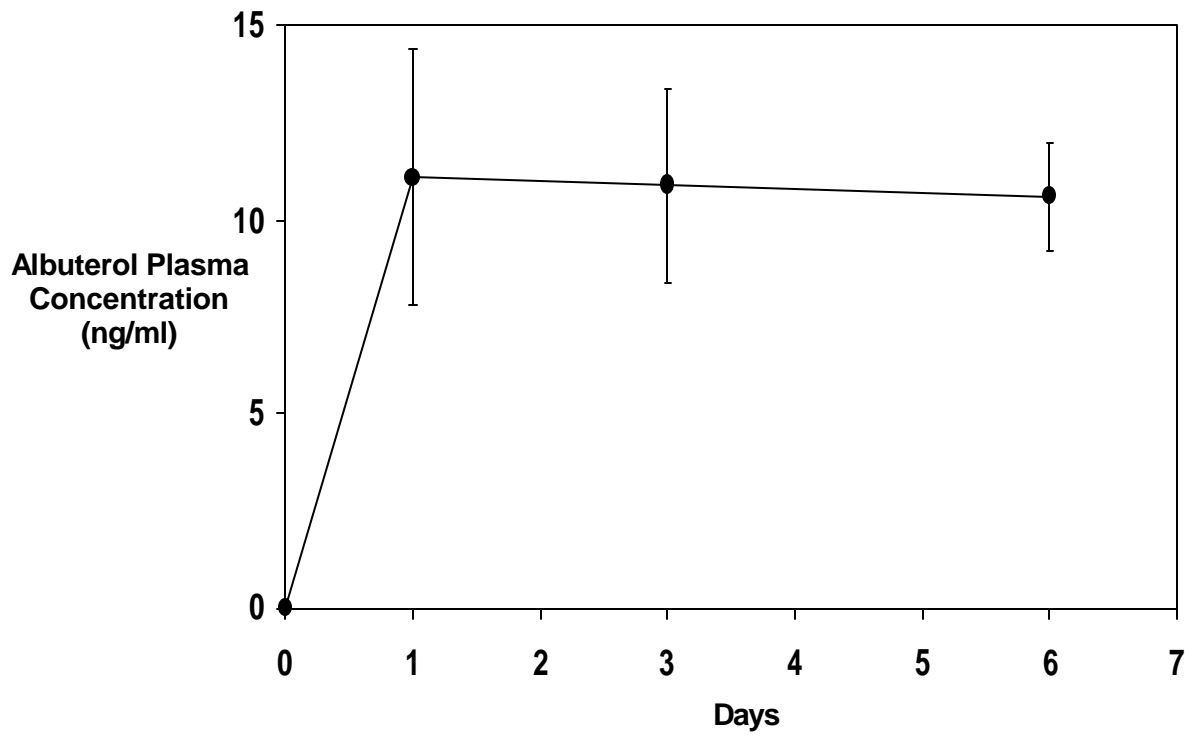


Figure 2

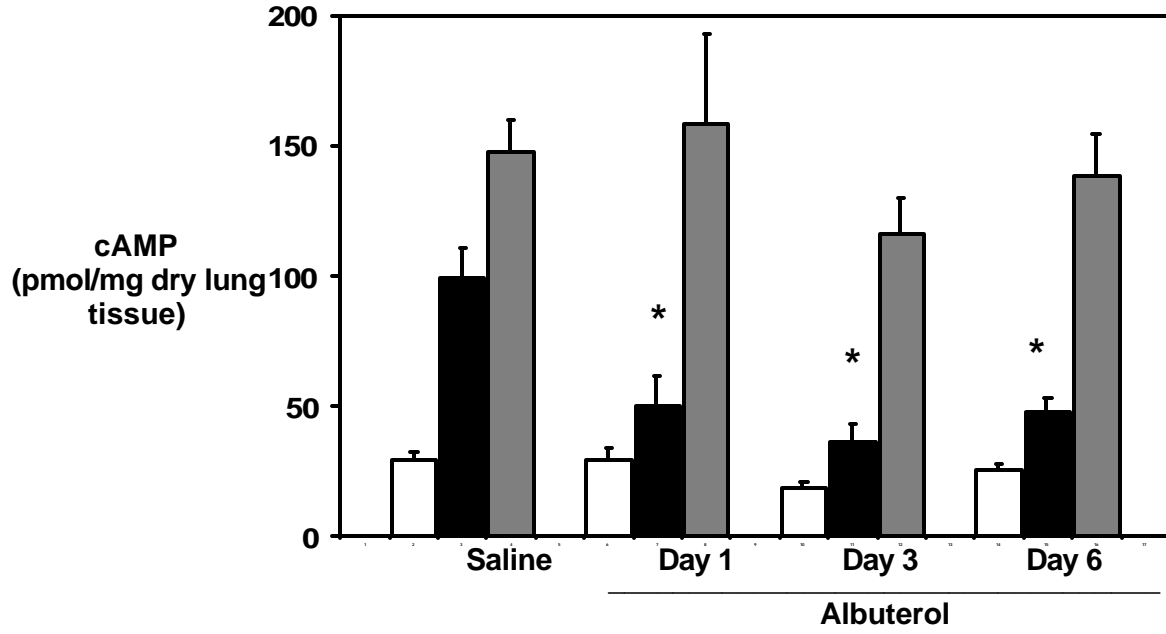


Figure 3

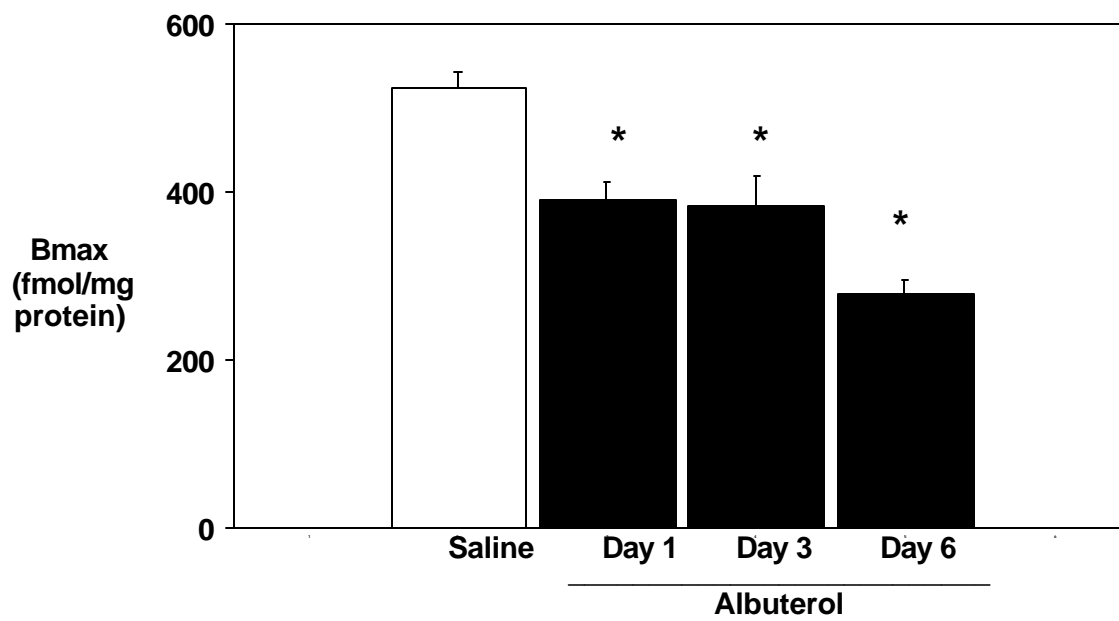


Figure 4

