

## RESEARCH PAPER

# Chronic treatment *in vivo* with $\beta$ -adrenoceptor agonists induces dysfunction of airway $\beta_2$ -adrenoceptors and exacerbates lung inflammation in mice

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## BACKGROUND AND PURPOSE

Inhalation of a  $\beta$ -adrenoceptor agonist ( $\beta$ -agonist) is first-line asthma therapy, used for both prophylaxis against, and acute relief of, bronchoconstriction. However, repeated clinical use of  $\beta$ -agonists leads to impaired bronchoprotection and, in some cases, adverse patient outcomes. Mechanisms underlying this  $\beta_2$ -adrenoceptor dysfunction are not well understood, due largely to the lack of a comprehensive animal model and the uncertainty as to whether or not bronchorelaxation in mice is mediated by  $\beta_2$ -adrenoceptors. Thus, we aimed to develop a mouse model that demonstrated functional  $\beta$ -agonist-induced  $\beta_2$ -adrenoceptor desensitization in the context of allergic inflammatory airway disease.

## EXPERIMENTAL APPROACH

We combined chronic allergen exposure with repeated  $\beta$ -agonist inhalation in allergen-treated BALB/C mice and examined the contribution of  $\beta_2$ -adrenoceptors to albuterol-induced bronchoprotection using FVB/NJ mice with genetic deletion of  $\beta_2$ -adrenoceptors (KO). Associated inflammatory changes – cytokines (ELISA), cells in bronchoalveolar lavage and airway remodelling (histology) and  $\beta_2$ -adrenoceptor density (radioligand binding) – were also measured.

## KEY RESULTS

$\beta_2$ -Adrenoceptors mediated albuterol-induced bronchoprotection in mice. Chronic treatment with albuterol induced loss of bronchoprotection, associated with exacerbation of the inflammatory components of the asthma phenotype.

## CONCLUSIONS AND IMPLICATIONS

This animal model reproduced salient features of human asthma and linked loss of bronchoprotection with airway pathobiology. Accordingly, the model offers an advanced tool for understanding the mechanisms of the effects of chronic  $\beta$ -agonist treatment on  $\beta$ -adrenoceptor function in asthma. Such information may guide the clinical use of  $\beta$ -agonists and provide insight into development of novel  $\beta$ -adrenoceptor ligands for the treatment of asthma.

## Abbreviations

$\alpha$ -SMA,  $\alpha$ -smooth muscle actin; APTI, airway pressure time index; BAL, bronchoalveolar lavage; bHABP, biotinylated hyaluronan-binding protein;  $B_{max}$ , maximal binding; H&E, haematoxylin and eosin; ICYP, iodocyanopindolol; LABA, long-acting  $\beta$ -agonist; MCh, methacholine; OVA, ovalbumin; PAS, periodic acid Schiff; PEEP, positive end expiratory pressure; SABA, short-acting  $\beta$ -agonist

## Introduction

Asthma is a chronic disease characterized by airway inflammation, hyperresponsiveness and remodelling. Arguably, the most debilitating symptoms associated with this disease are wheezing and shortness of breath, both of which ultimately result from increased bronchomotor tone. Agonists of the  $\beta$ -adrenoceptor ( $\beta$ -agonists) are the oldest and most commonly prescribed therapeutic agents for the management of asthma and are potent bronchodilators that relieve asthmatic bronchospasm and thereby resolve airway obstruction. In addition to mediating bronchodilation,  $\beta$ -agonists also confer bronchoprotection; that is, inhibition of induced bronchoconstriction (Abisheganaden and Boushey, 1998). Although  $\beta$ -agonists are very effective at improving lung function acutely, chronic use limits their therapeutic efficacy and, in some cases, leads to deleterious effects (Taylor, 2009).

Numerous clinical studies demonstrate that chronic use of  $\beta$ -agonists (both short- and long-acting) results in loss of bronchoprotection (Cheung, 1992; Bhagat *et al.*, 1995; Drotar *et al.*, 1998; Lipworth *et al.*, 1998; Jokic *et al.*, 2001). The clinical significance of this experimental observation is its association with a reduced ability to oppose allergen-mediated bronchoconstriction and cross-tolerance to rescue  $\beta$ -agonists. Additionally, the mechanisms of desensitization of  $\beta_2$ -adrenoceptors that underlie loss of bronchoprotection have been implicated in promoting asthma pathogenesis and worsening of asthma control (see Deshpande and Penn, 2006).

Although many clinical studies report that  $\beta$ -agonist treatment is efficacious and safe (Drazen *et al.*, 1996; Dennis *et al.*, 2000; Bateman *et al.*, 2008), reports linking chronic  $\beta$ -agonism to worsening asthma control and asthma-related death are sufficient in number to raise legitimate questions about how best to clinically utilize this class of drugs to treat asthma (Stolley and Schinnar, 1978; Spitzer *et al.*, 1992; Pearce *et al.*, 1995; Sears, 2002; Salpeter *et al.*, 2006). An important step towards addressing these concerns is to gain a mechanistic understanding of how, in a disease setting,  $\beta$ -adrenoceptor function and regulation are altered by chronic  $\beta$ -agonist activation and how this differs from what occurs during acute activation. It is difficult to imagine how a drug that is so beneficial acutely could lead to detrimental effects when administered chronically. However, this  $\beta$ -adrenoceptor paradox is not unique to asthma (Bond, 2001).

A classic example of the  $\beta$ -adrenoceptor paradox comes from the study of heart failure where chronic use of  $\beta$ -agonists causes not only desensitization to their inotropic effect but also leads to increased cardiac morbidity and mortality (Packer, 1989; Petrofski and Koch, 2003). An understanding of how excessive  $\beta$ -adrenoceptor activation in asthma can transform the beneficial effects of  $\beta$ -agonists into

deleterious ones is currently lacking due to methodological and ethical constraints on human research and the lack of a comprehensive animal model.

Although animal models exist for studying the physiological effects of either chronic allergen exposure (Kamachi *et al.*, 2001; Sugiura *et al.*, 2007) or chronic  $\beta$ -agonist administration (Finney *et al.*, 2000; 2001; Tamaoki *et al.*, 2004), few combine these two treatments. Moreover, of the few that do, the treatment duration is insufficient to demonstrate enhanced disease (Kamachi *et al.*, 2001; Callaerts-Vegh *et al.*, 2004; Riesenfeld *et al.*, 2010; Lundblad *et al.*, 2011), and only one has directly assessed whether or not  $\beta_2$ -adrenoceptors become functionally desensitized (Callaerts-Vegh *et al.*, 2004). Perhaps one reason why murine models of  $\beta_2$ -adrenoceptor desensitization in the context of allergic airway inflammation are not widely reported is the speculation that murine airways have the ability to bronchorelax via  $\beta_1$ -adrenoceptors.

$\beta_1$ -Adrenoceptors are expressed in greater abundance than are  $\beta_2$ -adrenoceptors in murine tracheal smooth muscle and are functionally predominant in mediating tracheal smooth muscle relaxation (Henry and Goldie, 1990; Henry *et al.*, 1990). However, the diameter of airways distal to the trachea is the main determinant of airway resistance during bronchoconstriction, and yet localization of  $\beta_2$ -adrenoceptor subtypes in mouse bronchioles has not been examined. The density of  $\beta_2$ -adrenoceptors increases with increasing airway generation in humans (see Johnson, 1998), and murine airways are not devoid of functional  $\beta_2$ -adrenoceptors. Taken together, it is plausible that  $\beta_2$ -adrenoceptors are the functionally predominant  $\beta$ -adrenoceptor in murine bronchioles.

The goal of our study was to demonstrate that  $\beta_2$ -adrenoceptors mediated bronchorelaxation in mice and to develop an animal model that mimicked both the loss of bronchoprotection (an indicator of  $\beta_2$ -adrenoceptor desensitization) and worsening of asthma (defined by enhanced inflammatory phenotype), observed in some human asthmatics. To this end, we tested the magnitude of  $\beta$ -agonist-mediated bronchoprotection in  $\beta_2$ -adrenoceptor-KO mice and combined chronic allergen exposure with repeated  $\beta$ -agonist inhalation in allergen-sensitized BALB/C mice.

## Methods

All animal care and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center and were carried out in accordance with the standards established by the US Animal Welfare Acts. Male BALB/C mice aged 6–8 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME).  $\beta_2$ -Adrenoceptor-KO male mice (Rohrer *et al.*, 1999) aged

6–10 weeks and FVB/NJ WT mice aged 6–8 weeks (purchased from The Jackson Laboratory, stock # 1800) were kept in a pathogen-free barrier facility.

### *Mouse chronic ovalbumin (OVA) and albuterol treatment*

Mice were sensitized on days 0 and 14 by i.p. injection of 10  $\mu$ g ovalbumin (OVA) (Sigma, St. Louis, MO, USA) adsorbed to 2 mg of alum adjuvant (Pierce Biotechnology Inc., Rockford, IL, USA) diluted in saline, as previously described (Walker *et al.*, 2003). Beginning on day 21 and continuing until day 62, mice were exposed for 60 min three times a week, with a rest day in between, to a 1% OVA-in-saline aerosol generated using a six-jet atomizer (TSI Inc., St. Paul, MN, USA). This OVA sensitization and challenge protocol is henceforth referred to as *OVA treatment*. Several hours after OVA aerosol exposure, mice were lightly anaesthetized with isoflurane and subjected to 50  $\mu$ L oropharyngeal instillation of 1 mg·mL<sup>-1</sup> albuterol sulphate (Sigma) or vehicle (0.9% saline). Animals were phenotyped on day 63, 24 h after the last albuterol or vehicle instillation. We administered the  $\beta$ -agonist by oropharyngeal instillation to mimic the route of delivery in humans and thereby negate potentially confounding effects of systemic  $\beta$ -agonist administration and control for the potential long-term effects of  $\beta$ -agonists on airway epithelial cells (McGraw *et al.*, 2000; Nishimura *et al.*, 2002).

### *Assessment of airway responsiveness: airway pressure time index (APTI) and impedance*

Airway responsiveness was measured in a terminal experiment where mice were anaesthetized, paralysed and ventilated through a tracheal cannula (Walker *et al.*, 1999). In brief, once mice were anaesthetized with i.p. 65 mg·kg<sup>-1</sup> sodium pentobarbital, a jugular vein catheter and tracheal cannula were inserted and secured with sutures (Lundbeck, Inc., Deerfield, IL, USA). The animal was paralysed with 0.25 mg·kg<sup>-1</sup> pancuronium bromide and ventilated with 100% oxygen (Sigma, St. Louis, MO, USA) at a constant volume of 7 mL·kg<sup>-1</sup> and a frequency of 180 breaths·min<sup>-1</sup>. Mice were hyperinflated to 25 cmH<sub>2</sub>O 2 min before each injection of methacholine (MCh) (Sigma) to establish a constant volume history and respiratory mechanics. Drugs were injected at 5 min intervals. For APTI, peak tracheal pressure was continuously acquired from a tracheal cannula side port. APTI was calculated as the sum of the post MCh-induced changes in peak tracheal pressure (relative to pre-MCh peak tracheal pressure) integrated with respect to time (30 s). APTI is a measure that others have validated for its ability to provide a reasonable index of airway responsiveness (Levitt and Mitzner, 1988) as assessed by the more specific mechanical variables of resistance and compliance. For impedance measurements, mice were ventilated at 150 breaths·min<sup>-1</sup> by a computer-controlled small animal ventilator (flexiVent, SCIREQ, Montreal, PQ, Canada) which generated sine wave volume perturbations (Schuessler and Bates, 1995). Following approximately 5 min of regular ventilation at a positive end-expiratory pressure (PEEP) of 3 cmH<sub>2</sub>O, a standard lung volume history was established followed by the acquisition of three baseline respiratory input impedance measurements. Bronchospasm was induced by aerosolizing 5 and 40 mg·mL<sup>-1</sup> MCh (in saline) solutions using an ultrasonic nebulizer (flex-

iVent, SCIREQ). The aerosol was delivered to the airway opening by diverting the inspiratory ventilator flow through the aerosol chamber of the nebulizer for a total of 12 breaths (8 s aerosol) at a tidal volume of 10 mL·kg<sup>-1</sup> at a rate of 150 breaths·min<sup>-1</sup>. Once the MCh had been delivered, the impedance measurements were made every 5–6 s, alternating between quickprime and snapshot measurements for a period of approximately 2 min. A 5 min washout period that included two deep sighs occurred before the next MCh challenge. The resultant total lung impedance signal contains information about the resistance and elastance properties of the lung from which Newtonian resistance can be calculated (Schuessler and Bates, 1995). In an idealized lung model, Newtonian resistance is a good indicator of the luminal diameter of the conducting airways (Proskocil and Fryer, 2005). APTI measurements were made on chronic OVA-treated BALB/C mice, whereas impedance measurements were made on  $\beta_2$ -adrenoceptor-KO and FVB/NJ control mice.

### *$\beta$ -Agonist-mediated bronchoprotection protocol*

To assess  $\beta$ -agonist-mediated bronchoprotection, we first established (data not shown) that the dose of MCh administered was submaximal, and that after priming the bronchoconstrictor response with one low dose of MCh (50  $\mu$ g·kg<sup>-1</sup>), repeated administration of MCh over 30 min resulted in similar magnitude bronchoconstrictor responses. In other words, the bronchoconstrictor response to MCh did not desensitize, a finding consistent with that of Finney *et al.* (2001). We then defined the bronchoconstrictor response to MCh by averaging the APTI response to two sequential doses of i.v. 100  $\mu$ g·kg<sup>-1</sup> MCh [a physiological dose (Walker *et al.*, 1999)]. The APTI response to combined i.v. 100  $\mu$ g·kg<sup>-1</sup> MCh and 30  $\mu$ g·kg<sup>-1</sup> albuterol was then measured. Bronchoprotection was calculated as % change in MCh-induced response by the following equation: [(APTI response to MCh + albuterol) – (average APTI response to MCh)]/average APTI response to MCh  $\times$  100]. Whereas bronchoprotection in humans is calculated by comparing the protective effect of  $\beta$ -agonist against bronchoconstrictor stimuli measured at screening with that measured after several weeks of treatment (Cheung *et al.*, 1992), repeat measures of lung mechanics in mice are rarely undertaken. Since inbred mice are nearly genetically identical, we compared the bronchoprotective effect of  $\beta$ -agonist in vehicle-treated mice with that measured in mice chronically treated with albuterol.

### *$\beta$ -Adrenoceptor subtype mediating bronchoprotection*

To assess the physiological importance of  $\beta$ -adrenoceptor subtypes in mediating airway bronchoprotection, we measured lung impedance and calculated airway resistance in wild-type and  $\beta_2$ -adrenoceptor-KO mice using the flexiVent (SCIREQ). Baseline measurements were collected prior to each aerosol treatment. Mice were first exposed to a 20 s delivery of 15 mg·mL<sup>-1</sup> albuterol aerosol or vehicle (0.9% saline) followed, sequentially, by 5 and 40 mg·mL<sup>-1</sup> MCh aerosol exposures interspersed with another 15 mg·mL<sup>-1</sup> albuterol or vehicle aerosol. Delivery of MCh and albuterol/control aerosols lasted 8 and 20 s respectively. The percent change in resistance was calculated as follows: [(pre-aerosol baseline

resistance – MCh-induced resistance)/pre-aerosol baseline resistance  $\times 100$ ].

### Whole lung lavage

At the termination of APTI measurements, mice were killed, and lungs were lavaged with saline. Cytokine concentrations and differential cell counts in lung lavage were determined as previously described (Walker *et al.*, 2003). In brief, the percent of lavage cell types was calculated by differentiating, according to standard morphological criteria, 200 cells on slides stained using a Hema-3 staining kit (Fisher Scientific, Springfield, NJ, USA). Cytokine detection limits can be found at [http://www.bio-rad.com/webroot/web/pdf/lst/literature/Bulletin\\_3156.pdf](http://www.bio-rad.com/webroot/web/pdf/lst/literature/Bulletin_3156.pdf).

### Radioligand binding

Total  $\beta$ -adrenoceptor density expressed on plasma membranes of mouse lungs was measured by radioligand binding as previously described (Deshpande *et al.*, 2008). In brief, whole lung plasma membranes were prepared in ice-cold homogenization buffer (75 mM Tris-HCl, 12.5 mM MgCl<sub>2</sub>, 2 mM EDTA, pH 7.4). Samples of lung plasma membranes (5  $\mu$ g protein) were used for the radioligand binding assay where  $\beta$ -adrenoceptor total binding was determined by incubation with a saturating concentration (500 pM) of [<sup>125</sup>I]-iodocyanopindolol (ICYP; Perkin Elmer, Waltham, MA, USA) at 37°C for 40 min, a duration that achieves a steady state as verified by experiments in which longer incubations did not significantly alter specific binding. Inclusion of propranolol (20  $\mu$ M; Sigma) defined non-specific binding and, although propranolol is a lipophilic antagonist, use of hydrophilic ligands such as isoprenaline (100  $\mu$ M) and CGP 12 177 (1  $\mu$ M) produced similar non-specific binding results (data not shown). Specific binding was calculated by subtracting non-specific binding from the total binding and maximal binding ( $B_{\max}$ ) was estimated as specific binding per mg protein. As some treatment groups were chronically and/or acutely (just prior to lung collection) treated with albuterol, we first produced saturation binding curves using 5 to 750 pM ICYP to demonstrate that 500 pM is a saturating concentration for all treatment groups and represented approximately seven times the  $K_D$ .

### Hyaluronan staining and BAL content

Slide-mounted lung sections were stained with biotinylated hyaluronan-binding protein (bHABP) (2  $\mu$ g·mL<sup>-1</sup>) (Associates of Cape Cod Incorporated, East Falmouth, MA, USA) for 2 h and then developed using a Vectastain-Elite-ABC kit (Vector Laboratories, Burlingame, CA, USA). Hyaluronan concentrations in bronchoalveolar lavage (BAL) fluid of mice were measured with a competitive ELISA-like assay using bHABP as described previously (Teder *et al.*, 2002).

### Histological staining and quantitative assessment

Inflated lungs were fixed in 4% paraformaldehyde and stored overnight at 4°C prior to cleaning. Lungs were subsequently embedded in paraffin and sectioned in 4  $\mu$ m thick slices. After individual staining of slides, 10 images of randomly chosen variable size airways (but not terminal bronchioles) were pho-

tographed at 20 $\times$  magnification. The method of slide analysis and semi-quantitation depended upon the structure being assessed and the stain used, as described below.

### Haematoxylin and eosin (H&E) staining

Lung tissue inflammation was semi-quantitatively determined, without knowledge of the treatments, from H&E-stained sections using a six-tiered scoring system of inflammation severity (Hollingsworth *et al.*, 2010). In brief, the inflammation score increased with increasing depth of perivascular or peribronchiolar inflammation and was further elevated when eosinophils were predominant.

### Periodic acid Schiff (PAS) staining

To assess airway mucin production, lung sections were analysed using PAS staining (Hollingsworth *et al.*, 2010). The scale ranged from 0 to 4, where 0 indicated no staining within the airway epithelium, 4 indicated greater than 75% airway epithelium staining, and scores 1, 2 and 3 represented up to 25%, 50% and 75% staining respectively.

### Morphometric analysis of structural changes (collagen, $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), elastin)

Hart's method and Masson-trichrome stain were used to stain lung sections for elastin (Veness-Meehan, 2002) and collagen, respectively. To identify  $\alpha$ -SMA, antibody clone 1A4 was used with a biotinylated horse-anti-mouse secondary antibody.  $\alpha$ -SMA was quantitated by using the thresholding method. To semi-quantitate the thickness of airway wall collagen, the area between the outer extent of the total collagen layer in the submucosal region and the basement membrane was digitally traced. Using the colour segmentation function of the Image Pro  $\oplus$  software (Media Cybernetics, Inc., Bethesda, MD, USA), the area inside the tracing that was positively Masson trichrome-stained for collagen was quantitated. The basement membrane length was digitally calculated to normalize the 'collagen area'. ImageJ software was used to semi-quantitatively assess  $\alpha$ -SMA and elastin.  $\alpha$ -SMA-positive cells in only lung bronchioles were counted. The surrounding tissue of the airways was erased in Adobe Photoshop, and a colour deconvolution module was employed to automatically threshold only the tissue expressing  $\alpha$ -SMA. The volume percent of  $\alpha$ -SMA-positive cells relative to total tissue volume was calculated and reported. An identical approach was taken to calculate the percentage of lung elastin except that the colour deconvolution profile was customized to account for the black colour of the Hart stain.

### Statistics

Data are expressed as mean  $\pm$  SEM. Statistical calculations were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Significant differences among groups were identified by ANOVA and a Tukey honest significant difference *post hoc* test was used for individual comparisons. When variances were unequal, Welch's correction was applied. When only two groups were compared, a one- or two-tailed Student's *t*-test was applied as appropriate. A *P*-value of less than 0.05 was considered statistically significant.



## Results

### Airway responsiveness and bronchoprotection

As shown in Figure 1A, chronic OVA treatment resulted in a significant increase in airway response to MCh, relative to alum-treated mice. Although animals treated with OVA-albuterol showed a trend for increased responsiveness to MCh relative to OVA-treated animals, no statistically significant difference was measured. Thus, as is characteristic of human asthmatics, mice chronically treated with OVA demonstrate increased airway responsiveness.

As shown in Figure 1B, i.v. albuterol substantially abated airway responsiveness to MCh in mice treated with OVA alone. However, mice chronically treated with OVA-albuterol were significantly refractory to the bronchoprotective effect of intravenous albuterol. Thus, our mouse model reproduced the association between chronic  $\beta$ -agonist use and  $\beta$ -adrenoceptor tolerance, or dysfunction, observed in human asthmatics.

### Functional $\beta$ -adrenoceptor subtype

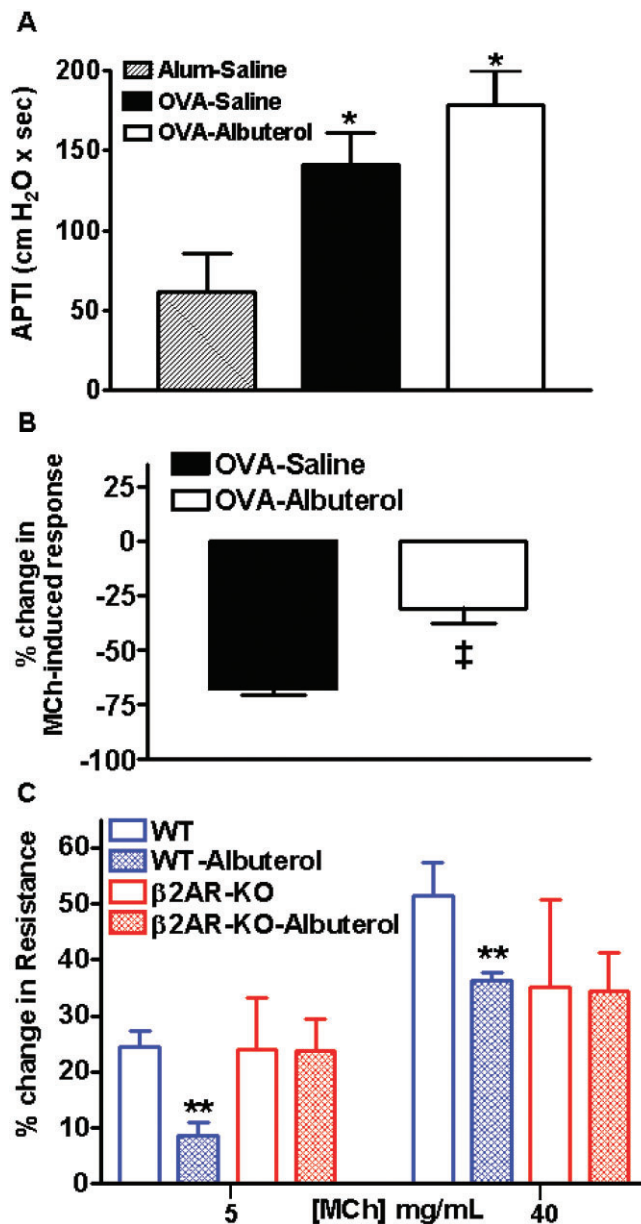
Although studies show that the  $\beta_1$ -adrenoceptor is the predominant adrenoceptor expressed in mouse trachea, localization of the  $\beta$ -adrenoceptor subtypes in more distal mouse airways has not been described. Since albuterol is only moderately selective for  $\beta_2$ - over  $\beta_1$ -adrenoceptors (Baker, 2005), we determined which  $\beta$ -adrenoceptor subtype mediated airway smooth muscle relaxation in our model. Thus, we compared the bronchoprotective effect of albuterol in  $\beta_2$ -adrenoceptor-KO mice versus wild-type mice. As shown in Figure 1C, the percent change in airway resistance was significantly diminished by albuterol, relative to vehicle at 5 and 40 mg·mL<sup>-1</sup> bronchoconstricting doses of MCh in naïve WT, but not naïve  $\beta_2$ -adrenoceptor-KO mice. These data indicate that, as in humans, bronchoprotection was primarily mediated by  $\beta_2$ -adrenoceptors.

### Lung inflammation

Our mouse model of chronic allergic airway inflammation demonstrates many other features of human chronic asthma. For example, chronically OVA-treated mice show increased numbers of eosinophils, lymphocytes and neutrophils in BAL (Figure 2A), increased airway mucin staining (Figure 3A–D) and enhanced peribronchiolar and perivascular inflammation (Figure 3E–H). The addition of albuterol to the chronic OVA treatment caused a significant increase in peribronchiolar and perivascular lung inflammation (Figure 3E) and mucin production (Figure 3A) but did not influence the differential cell counts in BAL (Figure 2A). In contrast to human asthma, the levels of Th2-type cytokines (IL-4 and IL-13) as well as IL-1 $\beta$  and the chemokine CCL5 (RANTES) were not elevated in OVA-treated mice, relative to alum-treated mice (Figure 2B) (Barnes, 2008). However, consistent with human asthma, IL-5 was significantly elevated after 6 weeks of OVA treatment. Albuterol treatment did not alter the BAL concentration of any of the measured cytokines, except for that of IL-13 which was reduced (Figure 2B).

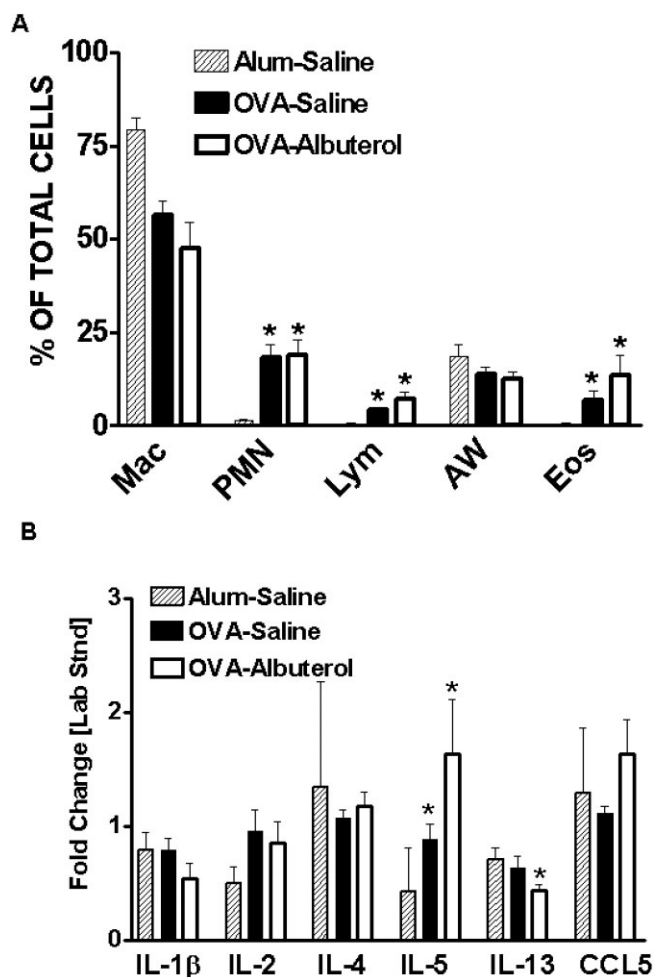
### Airway and lung remodelling

Subepithelial collagen deposition is a classic characteristic of airway remodelling in human asthma, as is thickening of



**Figure 1**

Assessment of airway responsiveness and bronchoprotection. (A) Relative to alum-treated mice, chronic OVA-treatment increased airway responsiveness to 100  $\mu$ g·kg<sup>-1</sup> MCh that is not significantly altered by additional chronic administration of albuterol (open bar). (B) The airway response to MCh in OVA-treated mice that are naïve to chronic oropharyngeal albuterol treatment is significantly abated by i.v. administration of albuterol during MCh challenge. However, the effectiveness of i.v. 30  $\mu$ g·kg<sup>-1</sup> albuterol to functionally neutralize the airway response to MCh is significantly diminished in mice chronically treated with OVA and oropharyngeal albuterol. Values are mean  $\pm$  SE from three to nine mice per group. (C) The percent change in airway resistance was significantly diminished by albuterol relative to vehicle at 5 and 40 mg·mL<sup>-1</sup> aerosolized MCh in naïve wild type (WT), but not naïve  $\beta_2$ -adrenoceptor KO ( $\beta_2$ AR-KO) mice. Values are mean  $\pm$  SE from three to four mice per group. \* $P$  < 0.05 compared with alum-saline; ‡ $P$  < 0.05 compared with OVA-saline; \*\* $P$  < 0.05 compared with WT using a one-tailed Student's  $t$ -test.



**Figure 2**

Assessment of lung inflammation. Relative to alum-treated mice, chronic OVA treatment increased (A) lung lavage neutrophils (PMNs), lymphocytes (Lym) and eosinophils (Eos), but not macrophages (Mac) or airway epithelial cells (AW). (B) Cytokine IL-5 was elevated by OVA treatment. Chronic administration of albuterol to OVA-treated mice had no significant effect on any of these measurements except that IL-13 was reduced. Values are mean  $\pm$  SE from 3 to 13 mice per group. \* $P < 0.05$  compared to alum-saline.

airway smooth muscle (Vignola *et al.*, 2003). To assess airway remodelling in chronically allergen-challenged mice, we stained lung sections with Masson-trichrome and morphometrically semi-quantitated the intensity of collagen staining per length of basement membrane in the airway subepithelial layer (Figure 4A–D). Chronic OVA treatment led to increased subepithelial collagen deposition, and this was not appreciably altered by chronic albuterol treatment. To assess morphological changes in airway smooth muscle, slides were semi-quantitated for volume % of  $\alpha$ -SMA staining. We found no effect of chronic OVA, or OVA-albuterol treatment on the volume of smooth muscle around the airway (data not shown). However, we observed that lungs from mice chronically treated with OVA-albuterol displayed a significant increase in lung parenchymal  $\alpha$ -SMA expression relative to alum-saline-treated mice (Figure 4E–H). As hyaluronan, a

pro-fibrotic glycosaminoglycan, is associated with collagen regulation, we measured its levels in BAL. OVA treatment significantly increased BAL-hyaluronan relative to alum-treated control mice, and this was further significantly increased by the addition of albuterol treatment (Figure 5A–D). Some asthmatics display obstruction resulting from loss of elastic recoil and we therefore assessed elastin deposition in lung parenchyma (Figure 5E). Lung elastin deposition was not significantly altered by OVA or OVA-albuterol treatment. This mouse model displayed many of the airway remodelling features characteristic of human asthma.

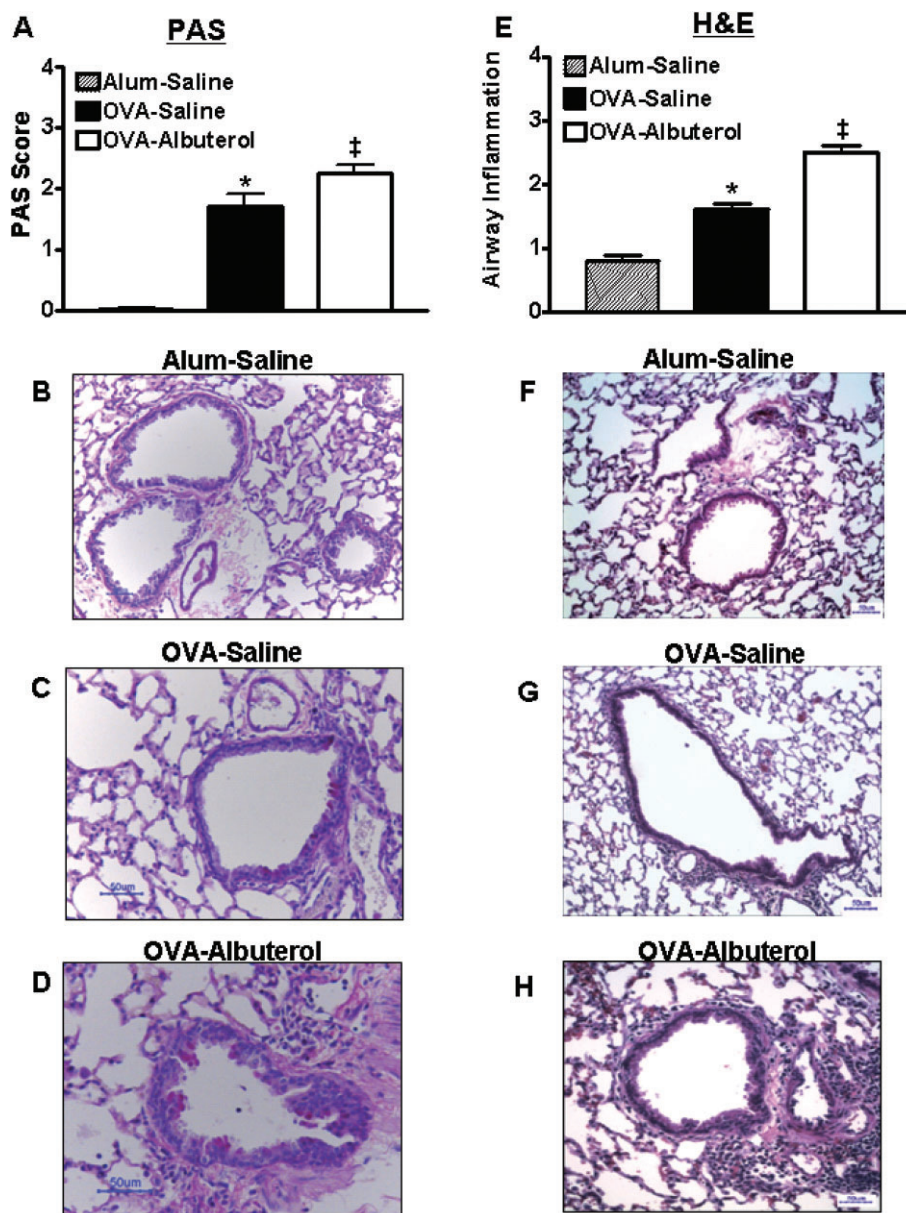
### Lung $\beta$ -adrenoceptor expression

Since  $\beta$ -agonist effectiveness depends, in part, on  $\beta_2$ -adrenoceptor expression, we measured lung  $\beta$ -adrenoceptor density by radioligand binding. As shown in Figure 6, lung expression of  $\beta$ -adrenoceptors was significantly diminished in association with chronic OVA treatment but was not further altered by the addition of chronic albuterol treatment. Importantly, the  $K_D$  values remained unchanged between treatment groups (alum-saline,  $75.7 \pm 7.0$  pM; OVA-saline,  $84.4 \pm 8.9$  pM; OVA-albuterol,  $63.7 \pm 3.3$  pM), showing that neither OVA nor albuterol treatment influenced  $\beta$ -adrenoceptor density.

### Discussion

$\beta$ -Agonists are very important therapeutic agents for the treatment of asthma. However, when used chronically, their effectiveness wanes, and they may actually promote disease progression. This report provides two important advances to the study of chronic  $\beta$ -agonist effects on  $\beta_2$ -adrenoceptor function and regulation in asthma. Initially, this study provides the first unequivocal evidence that, as in humans, murine bronchorelaxation is mediated by the  $\beta_2$ -adrenoceptors. This finding establishes the mouse as a suitable species with which to model loss of bronchoprotection and  $\beta_2$ -adrenoceptor desensitization. Secondly, this is the first report that provides a detailed description of the pro-inflammatory effects of chronic  $\beta$ -agonist treatment in the context of allergic inflammatory airway disease and associates these changes with functional loss of bronchoprotection. This finding is important because it provides evidence that chronic exposure to  $\beta$ -agonists can exacerbate allergic inflammation, implicates  $\beta_2$ -adrenoceptor dysfunction as an underlying mechanism of inflammation and provides an animal model with which to further explore this association.

Mice have been widely used as a model to study allergic inflammatory airway disease; however, their suitability as a model for the study of  $\beta$ -adrenoceptor-mediated bronchodilation and desensitization has been questioned. Prior to this report, the distribution, density and function of  $\beta$ -adrenoceptor subtypes in murine airways distal to the trachea were unknown. Moreover, based on work from Henry and colleagues, it appeared that, in contrast to the functional dominance of  $\beta_2$ -adrenoceptors clearly demonstrated in humans, mice might mediate bronchorelaxation via  $\beta_1$ -adrenoceptors. They showed that the density and function of  $\beta_1$ -adrenoceptors predominated in murine tracheal smooth



**Figure 3**

Assessment of airway mucous metaplasia and inflammation. Relative to alum–saline-treated mice, chronic OVA treatment increased (A–D) airway mucous metaplasia and (E–H) peribronchiolar and perivascular inflammation. The additional chronic administration of albuterol exacerbated both of these phenotypes. Values are mean  $\pm$  SE from 8 to 10 mice per group. \* $P < 0.05$  compared with alum–saline; ‡ $P < 0.05$  compared with alum– and OVA–saline.

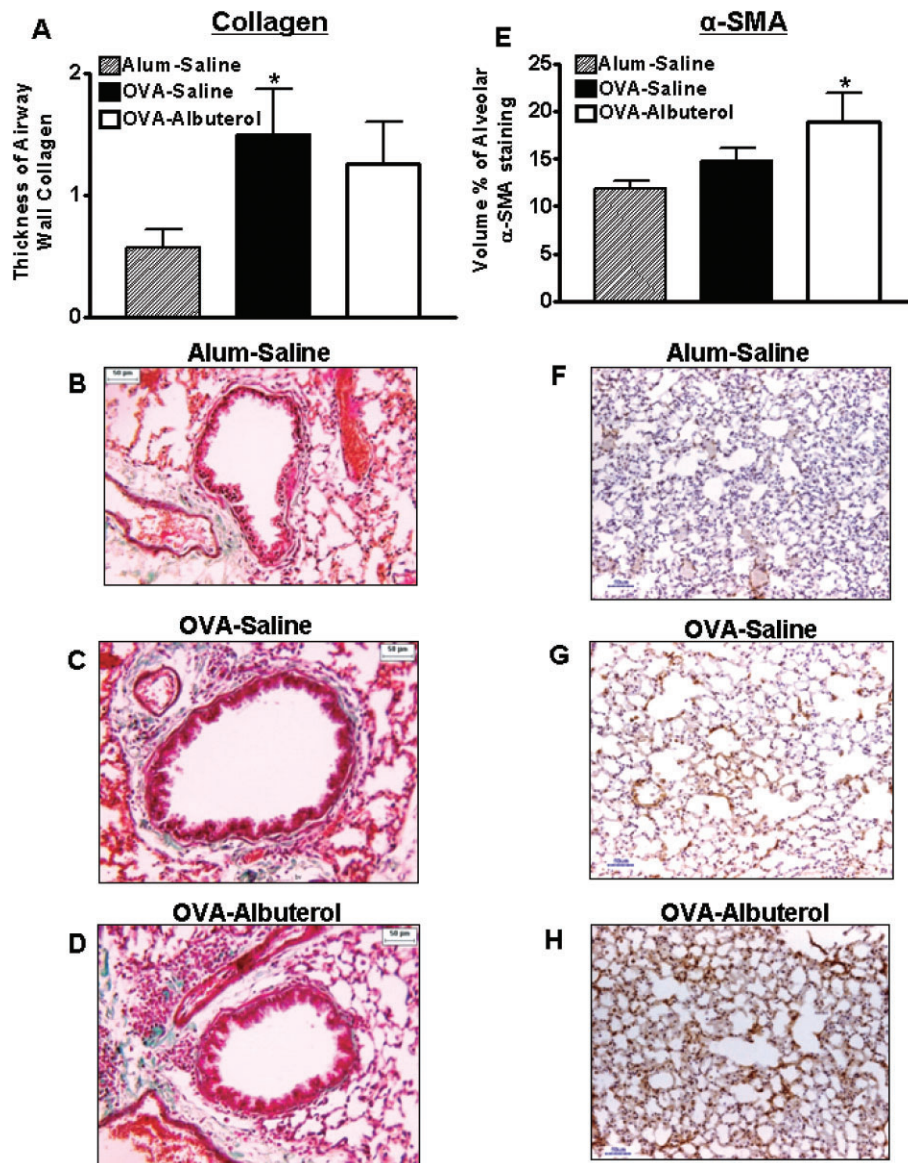
muscle; however, no one had mapped the smooth muscle distribution or density of  $\beta$ -adrenoceptor subtypes in murine bronchioles, where the greatest resistance to airflow occurs in healthy and asthmatic humans. Although we did not directly localize  $\beta$ -adrenoceptor subtypes in mouse airways, the observation that albuterol provides no significant bronchoprotection in  $\beta_2$ -adrenoceptor-KO mice demonstrated that the  $\beta_2$ -adrenoceptor subtype was the predominant mediator of albuterol-induced bronchorelaxation in mice.

It is likely, but not certain, that the functional dominance of murine bronchiolar  $\beta_2$ -adrenoceptors is coincident with

elevated receptor density. The idea that the density of  $\beta_2$ -adrenoceptors may increase along the tracheobronchial tree in mice is supported by a similar pattern demonstrated in human airways (Johnson, 1998). Additionally, other species, such as guinea pig and dog demonstrate a predominant  $\beta_2$ -adrenoceptor density and function (Tamaoki *et al.*, 1993; Kompa *et al.*, 1995) respectively, in bronchial smooth muscle.

Our study cannot completely rule out a role for  $\beta_1$ -adrenoceptors in mediating bronchorelaxation; however, our finding adds credibility to those murine studies where only the relatively weak selectivity of albuterol for  $\beta_2$ , over





**Figure 4**

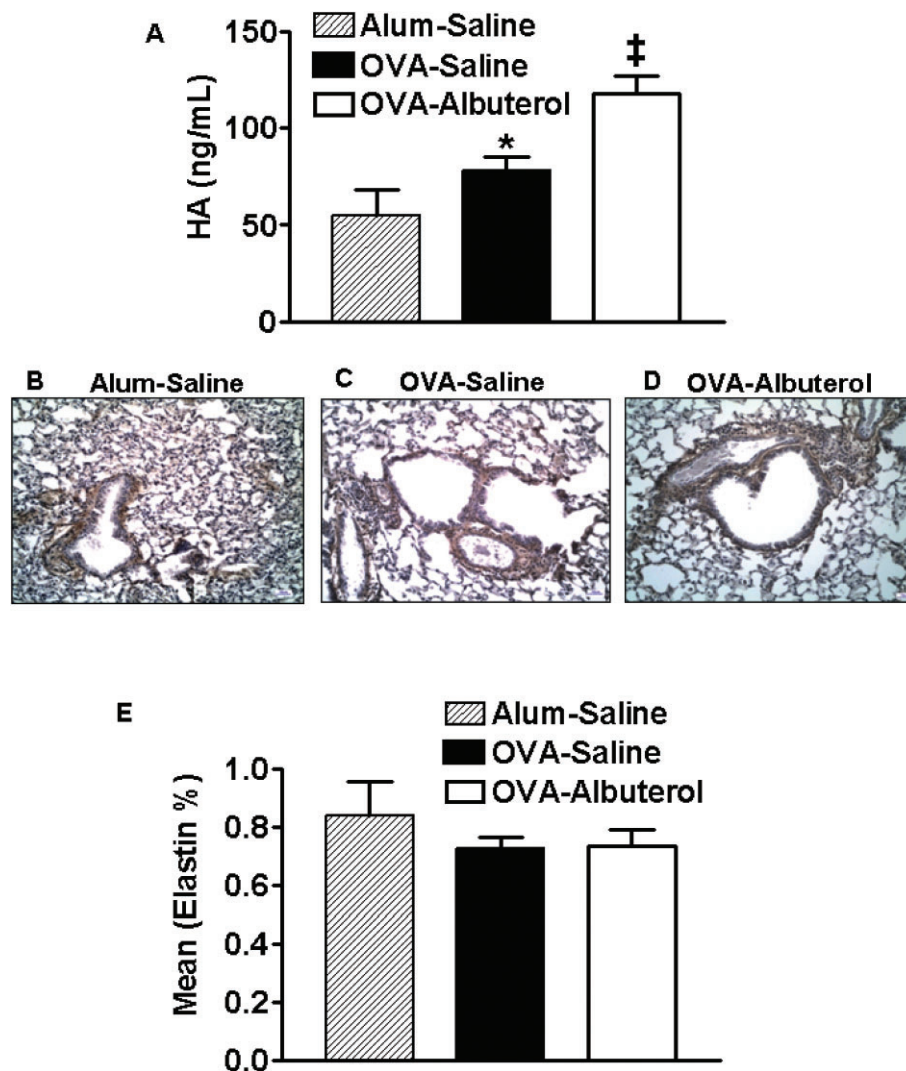
Assessment of airway remodelling. (A–D) Airway wall collagen thickness was significantly elevated in chronically OVA-treated mice, but not those that additionally received albuterol. (E–H) Only mice that chronically received combined OVA–albuterol treatment demonstrated a significant increase in alveolar  $\alpha$ -SMA. Values are mean  $\pm$  SE from 6 to 14 mice per group. \* $P < 0.05$  compared with alum–saline.

$\beta_1$ -adrenoceptors was used as evidence of  $\beta_2$ -adrenoceptor-mediated bronchorelaxation (Finney *et al.*, 2000; Callaerts-Vegh *et al.*, 2004).

Tolerance or subsensitivity to the bronchoprotective effect of  $\beta$ -agonists is a reproducible event in humans (Jackson and Lipworth, 2004) and is demonstrated in our mouse model. Chronic  $\beta$ -agonist-induced loss of bronchoprotection is clinically relevant for at least three reasons. First, although there are reports to the contrary (Wilding *et al.*, 1997; Korosec *et al.*, 1999), studies have shown that loss of bronchoprotection associated with repeated use of long-acting  $\beta$ -agonists (LABAs) can result in cross-tolerance to short-acting  $\beta$ -agonists (SABAs) (Newnham *et al.*, 1994; 1995;

Grove and Lipworth, 1995), thereby reducing the effectiveness of rescue bronchodilators. Secondly, the inability of  $\beta$ -agonists to inhibit bronchoconstriction mediated by naturally clinically important stimuli (such as allergens) may have significant deleterious consequences during an asthma exacerbation (Cockcroft *et al.*, 1993; Jokic *et al.*, 2001). Finally, the  $\beta_2$ -adrenoceptor desensitization mechanism underlying loss of bronchoprotection has been implicated in exacerbating asthma pathogenesis and worsening of asthma control (see Deshpande and Penn, 2006) presumably via pro-inflammatory effects [reviewed in (Cazzola *et al.*, 2011)]. It is theorized that, in the setting of asthma, chronic  $\beta$ -agonist treatment drives  $\beta_2$ -adrenoceptor signalling through a non-





**Figure 5**

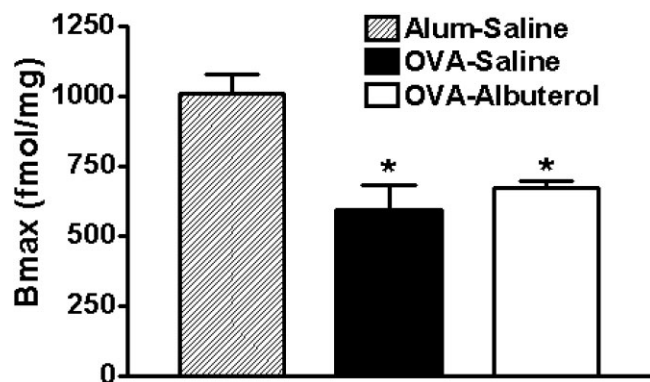
Assessment of hyaluronan and airway remodelling. (A) Hyaluronan (HA) levels in BAL were significantly elevated in chronically OVA-treated mice and this was significantly enhanced in OVA-albuterol-treated mice. (B–D) Representative micrographs of hyaluronan-stained lung slices are shown for all three treatment groups. (E) Neither OVA nor combined OVA-albuterol treatment was associated with significant changes in alveolar elastin. Values are mean  $\pm$  SE from 6 to 10 mice per group. \* $P$  < 0.05 compared with alum-saline; ‡ $P$  < 0.05 compared with alum- and OVA-saline.

canonical,  $\beta$ -arrestin-dependent, adverse signalling pathway that exacerbates airway inflammation and mucin phenotypes (Dickey *et al.*, 2010; Walker *et al.*, 2011).

It comes as no surprise that studies describing the effect of  $\beta$ -agonist therapy on asthmatic airway inflammation have provided mixed results. Whereas some clinical studies report anti-inflammatory effects of  $\beta$ -agonists (see Remington and Digiovine, 2005), others suggest inflammation is unaffected or increased by inhaled  $\beta$ -agonist therapy (reviewed in Loza *et al.*, 2008). More recent reviews conclude that the body of evidence from human-based studies is weighted towards  $\beta$ -agonists being pro-inflammatory (see Taylor, 2009; Cazzola *et al.*, 2011). This conclusion is compellingly supported by work from the Bond laboratory that has shown, through pharmacological and genetic methods, that  $\beta_2$ -adrenoceptor signalling is required for full development of the asthma

phenotype in mice (Callaerts-Vegh *et al.*, 2004; Nguyen *et al.*, 2008; 2009). Although Bond's work focused on loss of  $\beta_2$ -adrenoceptor function, and our present work focuses on enhanced  $\beta_2$ -adrenoceptor function, taken together, these studies support the notion that chronic therapeutic use of  $\beta$ -agonists could accelerate asthma severity.

Efforts to reveal the mechanisms underlying  $\beta_2$ -adrenoceptor desensitization and associated deleterious events have been hampered by clinical research limitations including the relative dearth of  $\beta$ -agonist naïve patients, the supposition that only a subpopulation of asthmatics will experience  $\beta$ -agonist induced adverse events (Taylor, 2009) and restrictions on the amount and type of tissue that can be collected from humans. Advances in our understanding have also been slowed by animal models that fail to combine the optimal dose, route and duration of  $\beta$ -agonist treatment with



**Figure 6**

Assessment of  $\beta$ -adrenoceptor expression. Radioligand binding experiments showed that relative to alum-saline-treated mice, chronically OVA-treated mice demonstrate a decrease in lung  $\beta$ -adrenoceptor expression, that is not significantly altered by additional chronic oropharyngeal administration of albuterol. Values are mean  $\pm$  SE from four mice per group. \* $P < 0.05$  compared with alum-saline.

sufficient duration of allergen exposure, and which lack an in-depth assessment of the asthma phenotype. In particular, airway hyperresponsiveness to the bronchoconstrictor MCh is typically reported for allergen-treated mice (Lundblad *et al.*, 2011) rather than an assessment of bronchoprotection. Despite being influenced by  $\beta$ -adrenoceptor expression (McGraw *et al.*, 2003), MCh (or other bronchoconstrictor) responsiveness is not a direct reflection of  $\beta$ -adrenoceptor desensitization. Only one animal study to date has assessed the effect of chronic  $\beta$ -agonist treatment on bronchoprotection in the context of allergen sensitization and challenge; however, only a cursory description of the inflammatory phenotype was included in that study (Callaerts-Vegh *et al.*, 2004). Consistent with our results, they showed that chronic albuterol treatment led to loss of bronchoprotective effect, and because they administered albuterol via osmotic pump, we further conclude that the route of  $\beta$ -agonist delivery may not affect the process of  $\beta_2$ -adrenoceptor desensitization.

The observation in humans and animals that repeated  $\beta_2$ -adrenoceptor activation leads to functional receptor desensitization is not surprising since receptor desensitization is a normal homeostatic process that presumably serves to protect cells from excessive stimulation (Penn and Benovic, 2008). One obvious mechanism by which chronic  $\beta$ -agonist treatment might lead to  $\beta_2$ -adrenoceptor tolerance is through receptor down-regulation. However, our results, which are consistent with those of others (Callaerts-Vegh *et al.*, 2004), showed that OVA-sensitization and -challenge significantly down-regulated lung  $\beta$ -adrenoceptor expression, but that repeated albuterol treatment did not further reduce receptor expression. Thus,  $\beta_2$ -adrenoceptor subsensitivity in the present study was likely to have derived from receptor modification or altered signalling independent of whole cell receptor loss, and this conclusion is in keeping with *in vitro* studies on human cells that point to changes in  $\beta$ -adrenoceptor signalling elements and regulatory events as the explanation for agonist-induced loss of  $\beta_2$ -adrenoceptors function (see Desh-

pande and Penn, 2006). One caveat to our interpretation of the binding data is that our measurements were made using whole lung membranes and a non-receptor subtype specific radioligand (ICYP), so we cannot rule out the possibility that a change in receptor expression in airway smooth muscle is being masked by an equal and opposite change in another lung tissue or that a decline in  $\beta_2$ -adrenoceptor expression is hidden by a concomitant increase in  $\beta_1$ -adrenoceptors expression.

The chronic OVA treatment that we employed in this study successfully reproduced in mice many of the remodelling features characteristic of human asthma including mucous metaplasia (see Sugiura *et al.*, 2007), increased basement membrane collagen deposition (Vignola *et al.*, 2003) and the appearance of myofibroblasts in the lung parenchyma (Bergeron *et al.*, 2005). However, the loss of bronchoprotection observed in our model cannot be explained by changes in airway remodelling.

Despite the fact that subepithelial collagen deposition (Kuhn *et al.*, 2000; McParland *et al.*, 2003) and lung parenchymal elastin content can affect airway mechanics, neither was significantly changed by  $\beta$ -agonist treatment in this study. Similarly, although myofibroblasts can develop mechanical tension in small airways (Sugiura *et al.*, 2007),  $\beta$ -agonist treatment did not significantly alter  $\alpha$ -SMA staining. Thus, myofibroblast activity does not contribute to  $\beta_2$ -adrenoceptor tolerance in our model. Interestingly, myofibroblasts can release the tissue repair and remodelling regulatory molecule, hyaluronan, which is an extracellular matrix glycosaminoglycan. Hyaluronan becomes fragmented in the context of lung inflammation and, in so doing, becomes pro-inflammatory and pro-fibrotic (Jiang *et al.*, 2007). The mechanism by which chronic  $\beta$ -agonist treatment significantly elevated BAL-hyaluronan accumulation in OVA-treated mice is unknown but is consistent with the idea that such treatment is pro-inflammatory. In contrast to models of lung fibrosis, where hyaluronan is positively associated with collagen accumulation, our results demonstrate no enhanced increase in airway wall collagen in OVA-albuterol-treated mice. However, in pulmonary fibrosis, the pro-fibrotic effect of hyaluronan is manifest outside airways (Jiang *et al.*, 2007), a location not assessed in our study.

Consistent with other chronic allergen models, we demonstrated airway epithelial mucous metaplasia in chronically OVA-treated mice. Interestingly, the addition of chronic albuterol treatment resulted in a modest, but significant, increase in this remodelling phenotype, which is consistent with previous reports (Kamachi *et al.*, 2001; Nguyen *et al.*, 2009). It appears that combined  $\beta$ -agonist and allergen treatment needs to be of sufficient duration to elicit the enhanced airway mucous phenotype, as others who used acute models showed no effect of albuterol treatment on mucin content in the airway epithelium (Nguyen *et al.*, 2008) and no effect of albuterol on airway epithelial density of PAS-positive cells (Riesenfeld *et al.*, 2010). However, based on their associated lung mechanics and microCT-imaging results, Riesenfeld *et al.*, concluded that a physiologically significant change in epithelial mucous cell density was indeed associated with prolonged  $\beta$ -agonist administration. If  $\beta$ -agonist administration can promote epithelial cell mucous metaplasia in humans, then this might provide one explanation for the

$\beta$ -agonist associated worsening of asthma control observed in some human asthmatics.

## Conclusion

The animal model described here provides an advanced tool that can be used to facilitate our mechanistic understanding of the temporal effects of  $\beta$ -agonism on  $\beta$ -adrenoceptor function in asthma. Additionally, the chronic effects of other  $\beta$ -adrenoceptor ligands (i.e. ultra-long acting  $\beta$ -agonists) or  $\beta$ -adrenoceptor modulating drugs (i.e. corticosteroids) on  $\beta_2$ -adrenoceptor desensitization and severity of asthma phenotype could be assessed using this model. Finally, the outcomes measured in this model, which extend beyond standard pulmonary mechanics and conventional reductionist endpoints, may aid the discovery of new  $\beta$ -adrenoceptor ligands for the treatment of asthma.

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## Conflicts of interest

None.

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