Rapid publication

Steroids completely reverse albuterol-induced β_2-adrenergic receptor tolerance in human small airways

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Background: Evidence suggests that chronic stimulation of β_2-adrenergic receptors (β_2-ARs) induces receptor tolerance that limits the efficacy of β-agonists in the treatment of asthma. The precise mechanisms that induce β_2-AR tolerance remain unclear.

Objective: We sought to determine whether steroids modulate albuterol-induced β_2-AR tolerance in human small airways.

Methods: β_2-AR responsiveness to isoproterenol was characterized in human precision-cut lung slices (PCLSs) precontracted to carbachol after pretreatment with albuterol.

Results: Incubation of PCLSs with albuterol for 3, 6, or 12 hours attenuated subsequent isoproterenol-induced relaxation in a dose- and time-dependent manner. A 40% decrease ($P < .0001$) in maximum relaxation and a 45% decrease ($P = .0011$) in airway sensitivity from control values occurred after the maximum time and concentration of albuterol incubation. Desensitization was not evident when airways were relaxed to forskolin. Dexamethasone pretreatment of PCLSs (1 hour) prevented albuterol-induced β_2-AR desensitization by increasing the maximum drug effect ($P = .0023$) and decreasing the log half-maximum effective concentration values ($P < .0001$) from that of albuterol alone. Albuterol (12-hour incubation) decreased the β_2-AR cell-surface number ($P = .013$), which was not significantly reversed by 1 hour of preincubation with dexamethasone.

Conclusion: These data suggest that β_2-AR desensitization occurs with prolonged treatment of human small airways with albuterol through mechanisms upstream of protein kinase A and that steroids prevent or reverse this desensitization. Clarifying the precise molecular mechanisms by which β_2-AR tolerance occurs might offer new therapeutic approaches to improve the efficacy of bronchodilators in asthma and chronic obstructive pulmonary disease. (J Allergy Clin Immunol 2008;122:734-40.)

Key words: *Airway smooth muscle, airway remodeling, asthma, chronic obstructive pulmonary disease*

Abbreviations used
β_2-AR: β_2-Adrenergic receptor
COPD: Chronic obstructive pulmonary disease
EC_{50}: Half-maximum effective concentration
E_{max}: Maximum drug effect
PCLS: Precision-cut lung slices
SABA: Short-acting β_2-adrenergic receptor agonist

Inhaled β_2-adrenergic receptor (β_2-AR) agonists are among the most common therapies for asthma and chronic obstructive pulmonary disease (COPD). Prolonged use of short-acting β_2-AR agonists (SABAs) in patients, however, might induce receptor tolerance that limits the efficacy of these drugs and potentially increases exacerbations and hospitalization.[1,2] In combination with steroids, these drugs can reduce the number of exacerbations in asthmatic subjects and therefore improve asthma control.[3-5]

Potentially, 3 mechanisms of receptor tolerance exist that can limit SABA efficiency, and these include uncoupling of the receptors from adenylate cyclase, internalization of uncoupled receptors, and phosphorylation of internalized receptors. Despite considerable research efforts with animal models or human cells, the precise mechanisms of inducing β_2-AR tolerance in patients with asthma and COPD remain undetermined. Unfortunately, interspecies variations and cell-culture model systems have limited the translation of these findings to that observed in human small airways, the primary site of airway obstruction in asthma and COPD.

In this study using precision-cut lung slices (PCLSs) of human lung, we demonstrate that chronic albuterol exposure profoundly decreases the efficacy of β-agonists to abrogate carbachol-induced luminal narrowing. Importantly, we now show that preincubation of slices with steroids completely restores β-agonist–induced sensitivity and prevents β_2-AR desensitization through a mechanism in part independent of receptor surface upregulation. Identifying the precise molecular mechanisms by which β_2-AR tolerance occurs might offer new therapeutic approaches to improve the efficacy of bronchodilators in patients with asthma and COPD.

METHODS

Reagents

Reagents used were carbachol, isoproterenol, low-melting-point agarose (IX-A), dexamethasone, and Ham's F-12 medium (supplemented with 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL Fungizone, 50 μg/mL gentamycin, and 1 mol/L HEPES [pH 7.6]). All reagents were obtained from Sigma (St Louis, Mo), unless otherwise stated.

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PCLS preparation and airway function

Healthy whole lungs were received from the National Disease Research Interchange. The smallest lobe was cut free, exposing its main bronchiole, and inflated with 2% (wt/vol) low-melting-point agarose solution. Once the agarose had solidified, the lobe was sectioned. Cores of 8 mm in diameter were made in which a small airway was visible. The cores were placed in a Krumdieck tissue slicer (Alabama Research & Development Model no. MD4000), and the speed was set to produce slices at approximately 1 per 30 seconds. PCLSs (thickness, 250 μm) were transferred in sequence to wells containing Ham's F-12 medium to identify contiguous airway segments. Suitable airways on slices were selected on the basis of the following criteria: presence of a full smooth muscle wall (ie, cut perpendicular to direction of airway), presence of beating cilia and internal folding of epithelium to eliminate blood vessels, and presence of unshared muscle walls at airway branch points to eliminate possible counteracting contractile forces. Slices were then incubated at 37°C on a rotating platform in a humidified air/CO_2 (95%/5%) incubator. Trauma caused by tissue slicing contracts the airway, presumably through the release of mediators. Media were therefore changed every 2 to 3 hours during the remainder of day 1 and all of day 2 to minimize trauma and reduce airway tone, as well as to remove any remaining agarose in the tissue. On day 3, lung slices were placed in a 12-well plate in 1.0 mL of buffer and were held in place by using a platinum weight with nylon attachments. The airway was located with a microscope (Nikon ECLIPSE, model no. TE2000-U; magnification ×40) connected to a live video feed (Evolution QEi, model no. 32-0074A-130 video recorder). A baseline image was taken (0% contraction) followed by the addition of the lowest concentration of carbachol to begin the concentration response (10^{-8} to 10^{-4} mol/L). Images were collected 10 minutes after each dose. Once the airway had reached about 80% to 90% full contraction or would not contract further, the β-adrenoceptor activity was examined. A concentration response to isoproterenol (10^{-9} to 10^{-4} mol/L) was added in the presence of the final concentration of carbachol, with images taken 5 minutes after each dose until no further relaxation occurred. Previous in-house experiments have determined that these time points are sufficient to allow the maximal effect at each concentration. Airway lumen area was measured with a macro written within Image Pro-Plus (version 6.0) software (Media Cybernetics, Inc, Bethesda, Md) and presented in square micrometers. After functional studies, the area of each airway at baseline and at the end of each dose of agonist was calculated by using the same macro written within Image Pro-Plus software. A log half-maximum effective concentration (EC_{50}) and maximum drug effect (E_{max}) value for each airway was derived from a concentration-response curve. The minimum lumen area after contraction was normalized to 0% relaxation, and effects of the β-agonist were measured with respect to this measurement and with respect to the original baseline value.

PCLSs were incubated with albuterol for 3, 6, and 12 hours at concentrations of 1.0, 0.1, and 0.01 μmol/L, and the airways were then contracted to carbachol followed by a full concentration response to isoproterenol, as described above. A concentration response to forskolin, which directly activates adenylyl cyclase, was also performed after incubation with albuterol for 12 hours at a concentration of 1.0 μmol/L. PCLSs were pretreated with dexamethasone (1.0 μmol/L) for 1 hour before albuterol (0.1 μmol/L) addition (ie, 13 hours in total) to determine the kinetics of steroidal effects on β_2-AR tolerance. The albuterol and dexamethasone were thoroughly washed out before the addition of isoproterenol. Two airways from each donor were used per condition; n values indicate the number of donors. Data are expressed as means ± SEM. Statistical difference was shown by using a nonpaired *t* test.

β_2-AR binding assay

In 5 experiments PCLSs were treated for 12 hours in media in either albuterol (1.0 μmol/L) or dexamethasone (1.0 μmol/L) in combination or absence. At the end of the incubation, the slices were snap-frozen and subsequently used for radioligand binding studies to quantitate β_2-AR expression. Sections were homogenized in 5 mmol/L Tris and 2 mmol/L EDTA (pH 7.4) buffer at 4°C and centrifuged at 100,000*g*, and the pellet was resuspended in 75 mmol/L Tris, 12 mmol/L $MgCl_2$, and 2 mmol/L EDTA (pH 7.4) buffer. Radioligand binding was carried out as previously described by Schwinn et al[6] with the β-AR antagonist iodine 125–labeled cyanopindolol using 1.0 μmol/L ICI118551 (a β_2-AR–specific antagonist) to define nonspecific binding. Membranes were incubated in triplicate at 37°C for 2 hours, and the reaction was stopped by means of dilution with cold buffer and bound versus free radioligand separated with vacuum filtration over glass-fiber filters. The filters were counted in a gamma counter at 65% efficacy. Specific binding was calculated as the total binding minus the nonspecific binding and normalized to the added protein and is presented as femtomoles per milligram. Protein concentrations were determined by using the copper bicinchoninic acid method.[7]

RESULTS

Carbachol induces airway luminal diameter narrowing in a dose-dependent manner

Slices were incubated with cumulative doses of carbachol and luminal narrowing was determined as shown in Fig 1, *A*, to determine whether agonists induce small airway narrowing in PCLSs. Carbachol abrogated airway luminal diameter with a log EC_{50} value of −0.39 ± 0.06 μmol/L. Additionally, the E_{max} (86.0% ± 3.1%) of this effect was observed at a concentration of 30 μmol/L. Carbachol dose responses inducing luminal diameter narrowing were performed at 24, 48, 72, and 96 hours *ex vivo* to assess the viability of the PCLS model over time. Over the 4-day study interval, the carbachol-induced luminal narrowing log EC_{50} and E_{max} values were relatively unaffected. Furthermore, the epithelial integrity and viability, as demonstrated by cilia beat frequency and mucus secretion, were comparable over the same interval (data not shown). Slices were precontracted to 80% to 90% with 30 μmol/L carbachol and then treated with increasing doses of isoproterenol to address whether β-agonists reverse carbachol-induced closure. Isoproterenol nearly completely reversed carbachol-induced bronchoconstriction, as shown in Fig 1, *B*. The log EC_{50} and E_{max} values of isoproterenol-induced bronchodilation were −1.41 ± 0.07 μmol/L and 84.4% ± 2.8%, respectively. Collectively, these data suggest that the PCLS model serves as a unique *ex vivo* human model to study agonist-induced airway narrowing and β-agonist–induced relaxation.

Chronic exposure to albuterol induces β_2-AR desensitization in a dose- and time-dependent manner

Incubation with albuterol decreased the sensitivity (increase in log EC_{50} value) and maximum effect (decrease in E_{max} value) to the β_2-AR agonist isoproterenol at 3, 6, and 12 hours of incubation in a concentration- and time-dependent manner. An incubation of 18 hours with the 1 μmol/L albuterol concentration produced results identical to those seen at the corresponding 12-hour time point, suggesting no further effect beyond this time point (data not shown). Importantly, treatment with albuterol had no effect on the maximum contractile effect to carbachol (data not shown). A 40% decrease in maximum relaxation was seen after the maximum time and concentration of albuterol exposure compared with the time-matched control value ($P < .0001$), as well as a 45% decrease in airway sensitivity, as demonstrated by an increase in log EC_{50} values from −1.28 ± 0.12 to −0.71 ± 0.20 μmol/L ($P = .0011$), as shown in Fig 2.

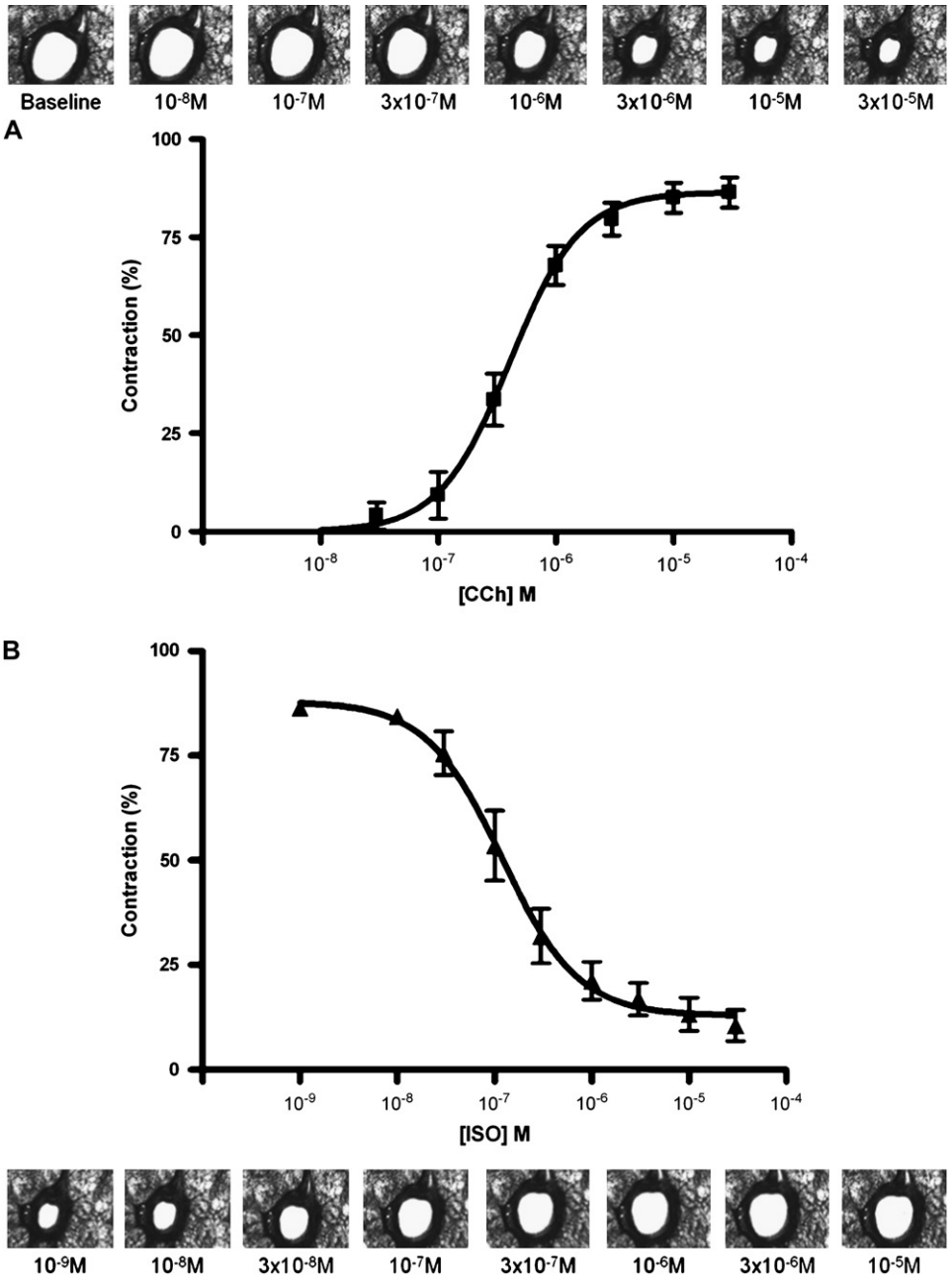


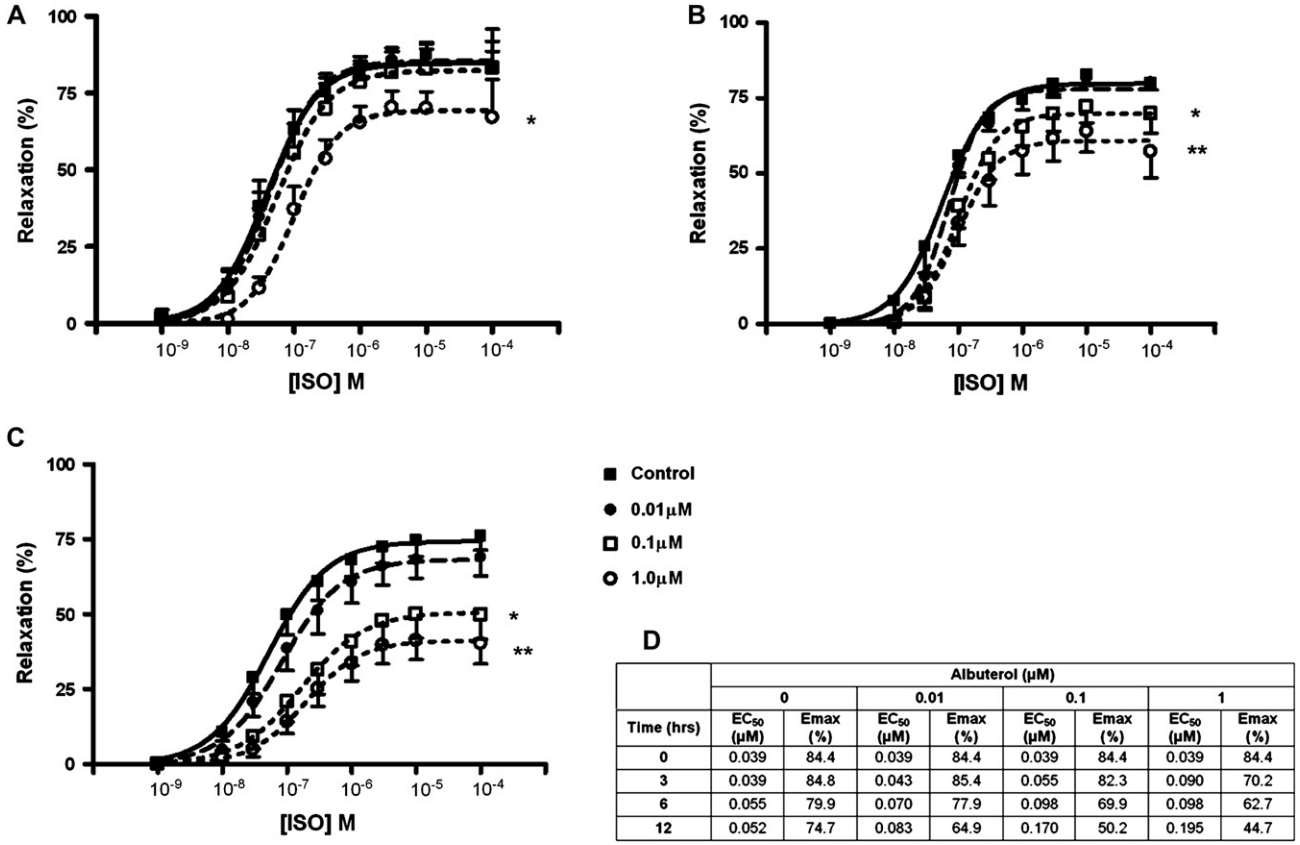
FIG 1. Concentration responses to carbachol *(CCh)* and isoproterenol *(ISO)* inducing airway luminal area narrowing and increasing airway luminal area. **A**, A typical contraction of a human small airway to carbachol and a mean concentration-response curve. **B**, The same airway as shown in panel A, relaxing to increasing concentrations of isoproterenol. The mean concentration-response curve is also shown. Data are expressed as means ± SEMs. Each group contains 2 airways from each of the 5 donors (10 total airways).

Activation of adenylyl cyclase that induces small airway relaxation remains unaffected by β_2-AR desensitization

A concentration response was performed to forskolin on human small airways incubated with albuterol for 12 hours at a concentration of 1.0 μmol/L to determine whether the mechanism of β_2-AR desensitization occurs upstream of adenylyl cyclase activation. There was little change in the maximum relaxation or sensitivity to forskolin after the albuterol incubation compared with the control value, as shown in Fig 3, which is unlike that previously seen to the β-agonist isoproterenol.

Steroids prevent β-agonist–induced β_2-AR desensitization

PCLSs were treated 1 hour before the chronic SABA exposure to address whether steroids modulate albuterol-induced β_2-AR tolerance. A 1-hour preincubation with dexamethasone prevented



D

	Albuterol (μM)							
	0		0.01		0.1		1	
Time (hrs)	EC_{50} (μM)	Emax (%)	EC_{50} (μM)	Emax (%)	EC_{50} (μM)	Emax (%)	EC_{50} (μM)	Emax (%)
0	0.039	84.4	0.039	84.4	0.039	84.4	0.039	84.4
3	0.039	84.8	0.043	85.4	0.055	82.3	0.090	70.2
6	0.055	79.9	0.070	77.9	0.098	69.9	0.098	62.7
12	0.052	74.7	0.083	64.9	0.170	50.2	0.195	44.7

FIG 2. Chronic exposure to albuterol induces β_2-AR desensitization in a dose- and time-dependent manner. Concentration-response curves to isoproterenol (*ISO*; 10^{-9} to 10^{-4} mol/L) after the incubation of albuterol (1.0, 0.1, or 0.01 μmol/L) after 3 **(A)**, 6 **(B)**, or 12 **(C)** hours with lung slices containing human small airways are shown. *Solid squares*, Control; *solid circles*, 0.01 μmol/L albuterol; *open squares*, 0.1 μmol/L albuterol; *open circles*, 1.0 μmol/L albuterol. **D**, Table of mean EC_{50} and E_{max} values of isoproterenol were calculated from airways. Each group contains 2 airways from each of the 4 donors (8 total airways).

the (0.1 μmol/L) albuterol-induced decrease in airway sensitivity and maximum relaxation to isoproterenol. The log EC_{50} values were returned to 152% of the baseline value, suggesting an additive beneficial effect of steroids over SABAs in decreasing small airway responsiveness. In a similar occurrence the E_{max} value was returned to 93% of the baseline value. Dexamethasone alone had little effect on maximum relaxation or on carbachol-induced PCLS contraction (data not shown) but did increase the sensitivity to isoproterenol, as shown in Fig 4. In separate experiments dexamethasone treatment for 6 hours after albuterol was also capable of reversing β_2-AR desensitization as effectively as the 1-hour pretreatment with dexamethasone; however, a 30-minute incubation to dexamethasone was ineffective at altering albuterol-induced β_2-AR desensitization (data not shown).

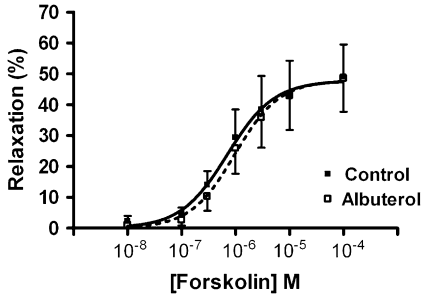


FIG 3. β_2-AR desensitization occurs upstream of adenylyl cyclase activation. Concentration responses to forskolin (10^{-8} to 10^{-4} mol/L) on human small airways after incubation with albuterol for 12 hours at a concentration of 1.0 μmol/L versus control airway values is shown. *Solid squares*, Control; *open squares*, albuterol (1.0 μmol/L; 12 hours). Each group contains 4 airways from each of the 2 donors (8 total airways).

Albuterol decreases β_2-AR cell-surface numbers in human PCLSs, whereas dexamethasone modestly restores albuterol-induced loss of β_2-AR numbers

In separate experiments lung slices were treated for 12 hours with vehicle, albuterol, dexamethasone, or their combination; membranes were then prepared to ascertain β_2-AR cell-surface numbers (Fig 5). β_2-AR expression decreased approximately 3-fold in the albuterol-treated group ($P = .013$ versus the untreated group). Dexamethasone alone had little effect on expression, although the variation was greater than in the other conditions. Expression increased approximately 2-fold between the albuterol treatment group and the albuterol plus dexamethasone group, which was not significant ($P = .14$). These data are surprising because dexamethasone completely reversed the albuterol-induced β_2-AR desensitization to control levels, as measured based on luminal diameter narrowing.

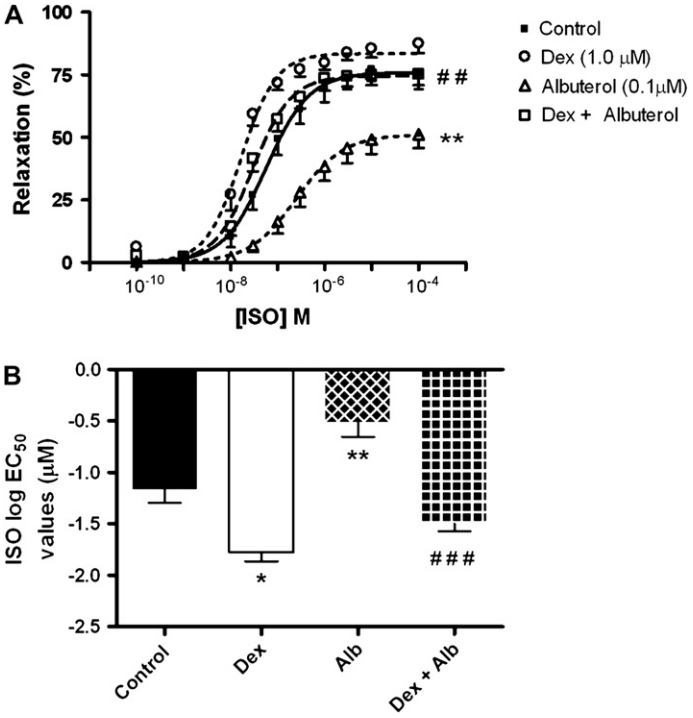


FIG 4. Steroids prevent β-agonist–induced β_2-AR desensitization. **A**, Concentration-response curves to isoproterenol (*ISO*; 10^{-9} to 10^{-4} mol/L). **B**, Log EC_{50} values of isoproterenol were calculated from airways. $*P < .05$ and $** P < .01$ versus the control group or $###P < .001$ versus the albuterol group. *Solid squares*, Control; *open circles*, 1.0 μmol/L dexamethasone *(Dex)*; *open triangles*, 0.1 μmol/L albuterol *(Alb)*; *open squares*, 1.0 μmol/L dexamethasone plus 0.1 μmol/L albuterol. Each group contains 2 airways from each of the 4 donors (8 total airways).

DISCUSSION

This study is the first to demonstrate a model of β_2-AR tolerance in human small airways. The short-acting bronchodilator albuterol induced a concentration- and time-dependent decrease in β_2-AR activity at 3, 6, and 12 hours and at concentrations of 0.01, 0.1, and 1.0 μmol/L, with no difference in relaxation to forskolin shown after incubation with albuterol versus control values. This study has also shown that a 1-hour incubation with dexamethasone prevented the β_2-AR desensitization from occurring without increasing an albuterol-induced downregulation of β_2-ARs.

The use of human PCLSs in the current study allows a direct measurement of human small airway smooth muscle function. The preparation of the slices is comparable with that done by Wohlsen et al[8] in that an entire lobe is inflated from the main bronchus. Indeed, the muscarinic agonist EC_{50} value obtained in the present study of 0.41 μmol/L and the E_{max} value were identical to those obtained by Wohlsen et al. Concentration responses to the β-agonist isoproterenol have not been published with human small airways, but studies involving isolated human bronchi have yielded an EC_{50} value of 0.06 μmol/L,[9] which is comparable with the 0.04 μmol/L value obtained in the current study.

Incubation with albuterol did have a slight effect on small airway sensitivity to carbachol but no effect on E_{max} values. Although interesting, there appears to be little correlation between contractile EC_{50} values and relaxation E_{max} values in isolated human bronchi,[9] and therefore changes in the maximum relaxation of the human small airways are likely due to properties of the β_2-AR and not to the β_2-agonists' interaction with the muscarinic receptor. Increased concentrations of albuterol decreased the

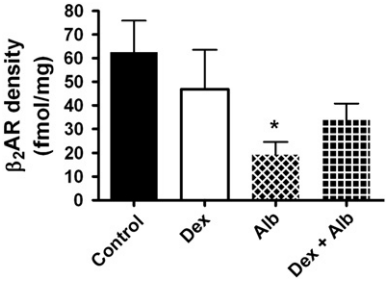


FIG 5. Effects of albuterol *(Alb)* and dexamethasone *(Dex)* on β_2-AR expression. Lung slices were homogenized, and quantitative radioligand binding with iodine 125–labeled cyanopindolol was carried out, as described in the Methods section. $*P = .013$ versus the untreated control group. *Solid squares*, Control; *open circles*, 1.0 μmol/L dexamethasone; *open triangles*, 0.1 μmol/L albuterol; *open squares*, 1.0 μmol/L dexamethasone plus 0.1 μmol/L albuterol. Each group contains 5 airways from each of the 5 donors (25 total airways).

sensitivity and maximum relaxation to human small airways in a time-dependent manner, a finding that is consistent with the development of airway β_2-AR desensitization to agonists in human tissue. Previous studies have also shown a tolerance to short-acting β_2-agonists in human[10] and animal[11] models. Investigators using animal models have used β_2-agonists[11] or IL-1β[12,13] *in vivo* to study β_2-AR tolerance. Although exposure of airway smooth muscle to IL-1β evokes β_2-AR desensitization, the exact mechanism by which this occurs might not be identical to that which occurs in asthmatic patients. Tolerance to SABAs has been

demonstrated *in vivo* by means of subcutaneous infusion of rats with albuterol for 7 days. There was no decrease in acetylcholine-induced bronchoconstriction seen in albuterol-infused rats after a secondary intravenous administration of albuterol compared with that after saline infusion, suggesting loss of bronchoprotection, whereas no difference was seen after an intravenous administration of forskolin, suggesting that β_2-AR occurred at the level of the receptor expression or coupling to the G protein.[11]

A concentration response to forskolin was performed on airways incubated with albuterol for 12 hours at a concentration of 1.0 μmol/L to determine whether β_2-AR desensitization occurs at the level of the receptor or effective downstream pathways. Forskolin increases levels of cyclic AMP and in part promotes relaxation by direct activation of adenylyl cyclase. Incubation with albuterol failed to induce desensitization to forskolin, suggesting β-agonist–induced tolerance likely occurs at the level of the β_2-AR or at the coupling between the β_2-AR and α s subunit of G proteins. In human models of β_2-AR desensitization that use cultured human airway smooth muscle cells,[10] the accumulation of cyclic AMP levels in airway smooth muscle are used as a surrogate signaling molecule to demonstrate β_2-AR tolerance. Penn et al[10] demonstrated that incubation of human airway smooth muscle with 1 μmol/L isoproterenol for 30 minutes decreased the production of cyclic AMP after an isoproterenol stimulus, whereas forskolin failed to attenuate the cyclic AMP decrease. Although the use of human airway smooth muscle cells lacks a functional output, their results, along with the results of the current study and the *in vivo* animal study by Finney et al,[11] suggest that the mechanism of β_2-AR tolerance occurs upstream of PKA.

In the study by Penn et al,[10] the number of receptors on the cell surface of airway smooth muscle decreased by 50% after just 30 minutes of incubation with isoproterenol at a concentration of 1 μmol/L without altering the total number of receptors in the airways. It could be suggested that if 50% of the cell-surface receptors are remaining, then a concurrent decrease in the efficacy to β_2-agonists would also be evident. The current study, however, shows a β_2-AR efficacy decrease of 19.7% after 3 hours of incubation with 1 μmol/L albuterol, suggesting that other mechanisms might be involved in β_2-AR tolerance.

Because β_2-agonists and glucocorticosteroids are a mainstay of asthma therapy, we questioned whether steroids modulate β_2-AR desensitization. Evidence also suggests that steroids increase β_2-AR transcription in human lung,[14] and therefore the identical aforementioned protocol in the present study was performed with the exception that slices were incubated with dexamethasone 1 hour before albuterol treatment and remained with the tissue for the entire incubation. The concentration of dexamethasone chosen in this study approximates that used clinically in patients with asthma and COPD and is the maximum concentration that induces glucocorticoid response element promoter activation in human airway smooth muscle cells.[15] One hour of preincubation with dexamethasone prevented β_2-AR tolerance, as shown by a lack of attenuation of the β_2-AR activity when human PCLSs were incubated with dexamethasone and albuterol. Dexamethasone was also able to fully prevent desensitization at the higher albuterol concentration of 1.0 μmol/L (data not shown).

Our data reveal a substantial desensitization of human airway β_2-AR function caused by agonist exposure and a virtual elimination of this effect with glucocorticoid coexposure. To begin to address the mechanism of both phenomena, β_2-AR expression was determined by using quantitative radioligand binding. In these experiments β_2-AR expression was clearly decreased by the 12-hour albuterol exposure. Interestingly, dexamethasone alone did not increase expression, which has been reported in other species[16] and in some immortalized non–smooth muscle human cell lines or circulating cells.[17,18] Whether this is a function of human airway smooth muscle cells or factors that are related to our measuring airway smooth muscle receptors in their intact environment within the airway remains to be investigated. Functional β_2-AR desensitization was abrogated by coexposure to dexamethasone; however, β_2-AR expression increased only by approximately 33% compared with untreated baseline values. The albuterol-induced downregulation of β_2-AR expression was similar to that seen in the albuterol plus dexamethasone group. These data suggest a degree of "spare receptors" in human airway smooth muscle or an effect of dexamethasone on other components of β_2-AR signal transduction, such as expression of G proteins, regulators of G protein signaling proteins, other effectors, G protein–coupled receptor kinase–mediated phosphorylation of the receptors, or β-arrestin recruitment. Additional studies will be required to assess these possibilities.

This is the first study to report β_2-AR functional tolerance in human small airways and to observe the prevention of β_2-AR tolerance by a steroid. The model of human PCLSs used in the current study allows a direct measurement of small airway luminal change, therefore permitting direct observation of functional changes of airway smooth muscle induced by incubations with β_2-agonists. This study provides a platform to further define the exact mechanisms of β_2-AR desensitization in human small airways and to help determine mechanisms to prevent β_2-AR tolerance in human airway disease. Furthermore, these data might support the use of combined therapy that includes β_2-AR agonists and steroids to enhance the efficacy of bronchodilators.

Clinical implications: Combination therapy in asthma that includes a steroid and a β-agonist might offer therapeutic benefit over either therapy alone.

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