Transgenic overexpression of \( \beta_2 \)-adrenergic receptors in airway epithelial cells decreases bronchoconstriction

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McGraw, Dennis W., Susan L. Forbes, Judith C. W. Mak, David P. Witte, Patricia E. Carrigan, George D. Leikau, and Stephen B. Liggett. Transgenic overexpression of \( \beta_2 \)-adrenergic receptors in airway epithelial cells decreases bronchoconstriction. Am J Physiol Lung Cell Mol Physiol 279: L379–L389, 2000.—Airway epithelial cells express \( \beta_2 \)-adrenergic receptors (\( \beta_2 \)-ARs), but their role in regulating airway responsiveness is unclear. With the Clara cell secretory protein (CCSP) promoter, we targeted expression of \( \beta_2 \)-ARs to airway epithelium of transgenic (CCSP-\( \beta_2 \)-AR) mice, thereby mimicking agonist activation of receptors only in these cells. In situ hybridization confirmed that transgene expression was confined to airway epithelium, and autoradiography showed that \( \beta_2 \)-AR density in CCSP-\( \beta_2 \)-AR mice was approximately twofold that of nontransgenic (NTG) mice. Airway responsiveness measured by whole body plethysmography showed that the methacholine dose required to increase enhanced pause to 200% of baseline (ED\(_{200}\)) was greater for CCSP-\( \beta_2 \)-AR than for NTG mice (345 ± 34 vs. 157 ± 14 mg/ml; P < 0.01). CCSP-\( \beta_2 \)-AR mice were also less responsive to ozone (0.75 ppm for 4 h) because enhanced pause in NTG mice acutely increased to 77% over baseline (P < 0.05) but remained unchanged in the CCSP-\( \beta_2 \)-AR mice. Although both groups were hyperreactive to methacholine 6 h after ozone exposure, the ED\(_{200}\) for ozone-exposed CCSP-\( \beta_2 \)-AR mice was equivalent to that for unexposed NTG mice. These findings show that epithelial cell \( \beta_2 \)-ARs regulate airway responsiveness in vivo and that the bronchodilating effect of \( \beta \)-agonists results from activation of receptors on both epithelial and smooth muscle cells.

G protein-coupled receptor; adenosine 3’,5’-cyclic monophosphate; adenylyl cyclase; ozone

The airway epithelium represents a critical interface between the bronchial smooth muscle and the external environment. As such, the epithelium can be a significant regulator of airway responsiveness (reviewed in Ref. 58). First, the epithelium forms a mechanical barrier that protects the underlying bronchial structures from exposure to environmental spasmogens (19, 42). Second, the epithelium has enzymatic activity that can degrade a variety of bronchospastic substances (12, 47, 53). Third, mucociliary clearance is regulated by the ciliary activity of the epithelium (10), as is the production and content of the epithelial lining fluid (4). These functions of the epithelium affect airway responsiveness indirectly by limiting the exposure of airway smooth muscle to luminal spasmogens.

In addition to these indirect effects, airway epithelial cells may regulate bronchial tone directly through the release of substances that either relax or contract airway smooth muscle. Known epithelium-derived substances that relax airway smooth muscle include PGE\(_2\) (22, 59) and nitric oxide (NO) (26). Some studies (3, 14) have also suggested the presence of a nonprostanoid epithelium-derived factor that inhibits contraction. However, the presence of such a factor has not been a consistent finding, and its significance remains speculative (24). Contractile factors produced by the bronchial epithelium include leukotrienes (23), PGF\(_{2\alpha}\) (22), and endothelin-1 (29). Airway epithelial cells produce a number of different cytokines and adhesion molecules that could affect smooth muscle responsiveness as well (8).

This notion that the epithelium is an important regulator of airway tone is further supported by clinical observations in asthma. Asthma is a chronic inflammatory disorder of the airways characterized by bronchial hyperresponsiveness to a variety of nonspecific stimuli. The pathogenesis of bronchial hyperresponsiveness in asthma is unclear, but there is evidence that disruption of the bronchial epithelium may be a contributing factor. Indeed, alteration of the epithelium may be present even in mild asthma (27, 28). Furthermore, the amount of epithelial damage can be correlated to the degree of airway hyperreactivity (5).

Results of several studies suggest that these inhibitory functions of the bronchial epithelium are modulated by \( \beta_2 \)-adrenergic receptors (\( \beta_2 \)-ARs). The \( \beta_2 \)-AR is a G protein-coupled membrane receptor that acts to raise intracellular cAMP levels via stimulation of ad-
Enkyl cyclase (18). The receptor is expressed on airway smooth muscle cells where it acts to relax the muscle and promote bronchodilation (reviewed in Ref. 2). However, the airway epithelium contains a high density of β2-ARs as well (6, 9, 21, 49). Although neither receptor autoradiography nor in situ hybridization have localized the β2-ARs to specific epithelial cell types, these receptors have been shown to regulate ion transport (7, 38), mucus secretion (33), ciliary beat frequency (10), and Clara cell secretory activity (36, 37). The regulatory role of the epithelial cell β2-AR is further supported by the observation that mechanical removal of the epithelium from tracheal rings decreases the bronchodilator effect of isoproterenol in vitro (14, 16, 51). This finding has been interpreted to indicate that β-agonists stimulate the epithelial release of relaxant factors that act directly on smooth muscle. However, this effect has not been consistently observed (1, 3), leaving the concept of a physiologically relevant β2-AR-regulated, epithelium-derived relaxant factor in some doubt. The physiological and pathophysiological significance of epithelial cell β2-ARs therefore remains unclear, primarily because the effects of epithelial cell β2-ARs in vivo cannot be easily differentiated from the direct bronchodilator effects of β2-ARs on airway smooth muscle.

In the current study, we selectively overexpressed the β2-AR in the airway epithelium of transgenic (TG) mice and examined the role that these receptors play in the regulation of airway tone. The use of such a targeted strategy permitted the epithelial β2-AR signaling pathway to be selectively enhanced without directly affecting the density or activity of smooth muscle β2-ARs. Because the receptor is not a secreted product, we reasoned that the effects of epithelial cell β2-AR activation could be distinguished in vivo from those of other cells by comparing airway responsiveness of nontransgenic (NTG) mice to that of TG Clara cell secretory protein (CCSP)-β2-AR mice. Our results showed that overexpression of the β2-AR in the airway epithelium of TG mice reduced airway responsiveness, thereby supporting a role for this population of receptors in the regulation of airway tone.

**METHODS**

**TG mice.** Targeted expression of the human β2-AR to the airway epithelium was achieved with the use of the rat CCSP promoter (50). The CCSP-β2-AR transgene was composed of a 2.4-kb Hind III-Hind III fragment from the rat CCSP promoter, a 1.5-kb Hind III-PshI fragment from the human β2-AR [1.2 kb of open reading frame (ORF) and 0.3 kb of 3′-untranslated region (UTR)], and a 0.85-kb Xho I-BamHI fragment encoding the SV40 small T intron and polyadenylation [poly(A)] signal. The fragments were ligated together in the vector pUC18, with the orientation of each fragment confirmed by sequence analysis and restriction enzyme digestion. A 4.75-kb NotI fragment (Fig. 1) was then isolated for injection. The DNA was gel purified and dialyzed against 5 mM Tris·HCl (pH 7.4) and 0.1 mM EDTA. Transgene DNA was injected into fertilized eggs of FVB/N mice and implanted into pseudopregnant females as previously described (54). Three founder mice were identified by Southern blot analysis of genomic DNA prepared from tail clips. Independent lines of heterozygous CCSP-β2-AR mice were maintained by mating TG mice with NTG FVB/N mice. Subsequent screening for the heterozygous progeny was by PCR analysis of the genomic DNA with a forward primer from the β2-AR ORF (5′-GGAGCAGAGTGGATATCACG-3′) and a reverse primer from the SV40 poly(A) region (5′-GTCACACCCA-GAAATGAAGG-3′). Heterozygous mice from generations 2 to 4 between the ages of 10 and 14 wk were used for all studies.

**Transgene expression and localization.** Screening for CCSP-β2-AR transgene expression in the lung was initially done by RT-PCR. Total RNA was extracted from freshly isolated lungs with Tri-Reagent (Molecular Research Center, Cincinnati, OH). An aliquot of RNA was reverse transcribed with random hexamers with murine leukemia virus reverse transcriptase (PerkinElmer) as previously described (13). Transgene cDNA was amplified with 150 nM each of a sense primer from the CCSP promoter (5′-CATCAGCGACACAT-TAGACAG-3′) and an antisense primer from the β2-AR ORF (5′-GACCAGCACATTGCACAACAC-3′). The

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*Fig. 1. A: Clara cell secretory protein (CCSP)-β2-adrenergic receptor (AR; CCSP-β2-AR) transgene was composed of the rat CCSP promoter, the human β2-AR open reading frame (ORF), and the SV40 small T intron and polyadenylation [poly(A)] site. UTR, untranslated region. B: Southern blot analysis of genomic DNA from tail clips showed that the 2 founder mice (lines 714 and 717) used to establish transgenic (TG) lines each had >40 copies of the transgene DNA. NTG, nontransgenic.*
PCR was started at 95°C for 120 s, followed by amplification for 35 cycles at 95°C for 60 s and 60°C for 60 s, followed by a final extension at 72°C for 7 min. The PCR products were detected in agarose gels stained with ethidium bromide. To quantitatively assess transgene expression among the independent TG lines, ribonuclease protection assays were performed as previously reported (40) with a 32P-labeled antisense riboprobe corresponding to the distal 500 bp of the human β2-AR ORF (40). Lung RNA (20 μg) and β2-AR riboprobe were hybridized overnight, digested with RNase, and subjected to denatured PAGE analysis. The gels were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed with the ImageQuant software package (Molecular Dynamics).

The distribution of transgene expression within the lung was assessed by in situ hybridization as previously reported (57). Tracheae and lungs were rapidly dissected, fixed in 4% paraformaldehyde, cryoprotected with 30% sucrose in PBS, and frozen in optimum cutting temperature compound. Cryostat sections (7 μm) were then mounted on silane-coated slides. An antisense cRNA probe for the human β2-AR was prepared as described above for the ribonuclease protection assays except that the probe was labeled with 35S-UTP. A sense cRNA probe was prepared with SP6 polymerase for use as a negative control. Hybridization was performed with 0.5–1.0 × 106 counts/min of labeled probe in a final volume of 30 μl/slide. After overnight incubation at 42°C, the sections were treated with 50 μg/ml of RNase A and 100 U/ml of RNase T1 for 30 min at 37°C and washed to a final stringency with 0.1× saline-sodium citrate at 50°C. Slides were dipped in NTB2 emulsion (Eastman Kodak) diluted 1:1 with 0.6 M ammonium acetate and exposed for 2 wk, after which they were developed with D19 developer (Eastman Kodak) and counterstained with hematoxylin and eosin.

Receptor density and function. Lung membranes were prepared from individual mice by homogenizing the entire lung or trachea in 10 ml of hypotonic lysis buffer (5 mM Tris, pH 7.4, and 2 mM EDTA) containing the protease inhibitors leupeptin, aprotinin, benzamidine, and soybean trypsin inhibitor (10 μg/ml each). Homogenates were centrifuged at 40,000 g for 10 min at 4°C. The supernatant was removed, and the pellets containing the crude membrane particulates were suspended in assay buffer (75 mM Tris, pH 7.4, 12.5 mM MgCl2, and 2 mM EDTA). Receptor expression was determined by radioligand binding with [125I]iodocyanopindolol (ICYP) as described (40). Adenyl cyclase activity in these membrane preparations was assessed by a column method as previously reported (40). Adenylyl cyclase activity in the lungs was inflated with tissue embedding fluid diluted 1:4 with PBS. The tracheae and lungs were removed as a unit and homogenized in 25 mM Tris-HCl (pH 7.4), 1 mM ascorbic acid, and 0.25% polyethyleneglycol 8000. The tracheae were subsequently sectioned (10 μm) on a cryostat at −30°C and thaw mounted onto slides coated with gelatin. Slides were stored at −80°C and showed no loss of binding capacity under these conditions. For autoradiography, the slides were allowed to warm to room temperature, after which they were washed for 15 min in incubation buffer (25 mM Tris-HCl, pH 7.4, 154 mM NaCl, 1.1 mM ascorbic acid, and 0.25% polyethylene glycol). Binding reactions were then carried out at 37°C for 120 min in incubation buffer containing 25 pM 125ICYP. Specific binding of β2-ARs was determined in the presence of 100 μM CGP-20712A, a selective β2-AR antagonist. Non-specific binding was determined by incubation of adjacent tissue sections with 125ICYP in the presence of 200 μM (-)-isoproterenol. After the incubation period, the slides were washed twice for 15 min in ice-cold 25 mM Tris-HCl (pH 7.4), rinsed in cold distilled water to remove buffer salts, and dried rapidly under a stream of cold air. The slides were postfixed in paraformaldehyde vapor at 60°C for 30 min and then dipped directly in Ilford K-5 emulsion. After 3 days at 4°C, the emulsion was developed and fixed. Sections were stained with hematoxylin and examined under a Zeiss microscope equipped with dark- and bright-field illumination. Grain counts, assessed as optical density, were measured with the Image Analysis System (Seescan, Cambridge, UK) with a constant magnification (×200) and corrected for background and nonspecific binding. The results represent the means of nine separate fields (300 μm2/field) taken from three different mice, each within the NTG and TG groups (27 fields total/group).

Pulmonary function. Airway responsiveness (i.e., bronchoconstriction) was assessed noninvasively in conscious, unrestrained mice with a whole body plethysmograph (Buxco Electronics, Troy, NY). Previous studies with this system, the volume changes that occur during a normal respiratory cycle are recorded as the pressure difference between an animal-containing chamber and a reference chamber. The resulting signal is used to calculate respiratory frequency, minute volume, tidal volume, and enhanced pause (Penh). Penh was used as the measure of bronchoconstriction and was calculated from the formula: Penh = peak expiratory pressure/peak inspiratory pressure, where pause is the ratio of time required to exhale the last 30% of tidal volume relative to the total time of expiration (20). Mice were placed in the plethysmograph and the chamber was equilibrated for 10 min. They were exposed to aerosolized PBS (to establish baseline) followed by incremental doses of methacholine (1–640 mg/ml). Each dose of methacholine was aerosolized for 2 min, and respiratory measurements were recorded for 2 min afterward. During the recording period, an average of each variable was derived from every 30 breaths (or 30 s, whichever occurred first). The maximum Penh value after each dose was used to measure the extent of bronchoconstriction. On a separate day, the mice were submitted to the same protocol except that they were first treated with aerosolized albuterol (1 mg/ml) for 20 min. The concentration-response data for each mouse were fit to a curve by an iterative least squares technique, and the dose of methacholine required to increase Penh to 200% of baseline (ED200) was derived.

Ozone exposure. Mice were exposed for 4 h to filtered room air containing 0.75 ppm ozone in a 0.32-m3 stainless steel inhalation chamber that was capable of complete air exchange every 2 min. Zhao et al. (60) have previously shown that this level of exposure does not result in inflammation or structural lesions. Ozone was generated from 100% extra-dry oxygen (Matheson, Columbus, OH) with a model V1-0 ultraviolet ozonator (OREC, Phoenix, AZ), and its concentration was measured continuously with an ultraviolet photometric ozone analyzer (model 1008-PC, Dasibi Environmental, Glendale, CA). This instrument has an internal calibration system and was routinely checked and calibrated against a US Environmental Protection Agency transfer standard. The status of the mice and the concentration of ozone were checked hourly throughout the exposure period. Penh was measured by whole body plethysmography for 2-min intervals at 5, 15, 30, 45, 60, 90, and 120 min after ozone exposure. Methacholine challenge testing was performed 6 and 24 h postozone exposure with the protocol described in Pulmonary function. In studies carried out to characterize the increase in
that we observed in NTG mice after ozone exposure, mice were pretreated with either aerosolized albuterol or atropine. Albuterol (1 mg/ml) markedly reduced the increase in P\textsubscript{eh} induced by ozone (2.47 ± 0.41 without albuterol, 1.26 ± 0.07 with albuterol; n = 4 animals; P < 0.03). These results confirmed that bronchospasm accounted for most, although not all, of the increase in P\textsubscript{eh} that occurred acutely after exposure to ozone. In contrast, atropine (0.6 mg/ml) had little or no effect (<20% reduction, which was not significant) on the ozone response of NTG mice, consistent with a minimal role for cholinergic mechanisms in mediating the acute ozone-induced bronchospasm.

Bronchoalveolar lavage studies. To perform bronchoalveolar lavage (BAL), mice were killed by a lethal injection of pentobarbital sodium and exsanguinated via transection of the abdominal aorta. The trachea were then exposed and cannulated with a polyethylene catheter. BAL was performed by instilling 1 ml of PBS warmed to 37°C. The lavage fluid was allowed to dwell for 5 min, after which it was withdrawn by gentle aspiration. The typical fluid return was ~0.8 ml, with no difference observed between the TG and NTG mice. The BAL fluid was centrifuged at 1,000 g for 10 min at 4°C to remove cells, and the resulting supernatant was frozen and stored at −80°C until further use. Levels of PGE\textsubscript{2} in BAL fluid were measured by a colorimetric enzyme immunoassay from a commercial kit (Amersham Pharmacia Biotech, Piscataway, NJ). Duplicate samples (50 μl) from each BAL specimen were assayed directly in a 96-well plate. Optical density was read at 450 nm. The PGE\textsubscript{2} content of each sample, measured in triplicate, was determined from a reference curve of known PGE\textsubscript{2} standards. NO content in BAL fluid was determined by a spectrophotometric method based on the Griess reaction to measure nitrite (55). To enzymatically reduce BAL fluid nitrate to nitrite, samples were first incubated with 80 U/l of nitrate reductase, 1 μM NADPH, 0.5 mM glucose 6-phosphate, and 160 U/l of glucose-6-phosphate dehydrogenase for 3 h at 25°C. The sample (50 μl) was mixed with 50 μl of 1% sulfanilamide in 5% H\textsubscript{3}PO\textsubscript{4} and 50 μl of 0.1% N-(1-naphthyl)ethylenediamine. After incubation for 10 min, absorbance was read at 550 nm. The nitrite content of BAL samples was subsequently calculated from a reference curve that was generated from the absorbance values of sodium nitrite standards. The nitrite concentration for each sample was determined from the mean of duplicate measurements. To standardize BAL recovery, the urea content of BAL fluid was determined with a colorimetric assay (Sigma, St. Louis, MO). The manufacturer’s protocol was followed except that sample size was increased to 50 μl, and the total reaction volume was reduced to 1 ml. These conditions provided for a linear calibration curve. Aliquots from each BAL sample were measured in duplicate.

Statistical analysis. Data are reported as means ± SE. Statistical comparisons between NTG and TG groups were performed with a two-tailed Student’s t-test. For the whole body plethysmography studies, the effect of a given treatment within the NTG or TG group was assessed by paired analysis when appropriate. Differences were considered significant at the P < 0.05 probability level.

RESULTS

CCSP-β\textsubscript{2}-AR founder mice were identified by Southern blot analysis of genomic DNA with a cDNA probe corresponding to the SV40 poly(A) fragment of the transgene. The transgene was detected in 3 of 36 mice initially screened. These founder mice, designated lines 714, 717, and 721, were mated with NTG FVB/N mice to generate heterozygous offspring. Genotypic analysis of the progeny showed that the transgene was inherited in ~50% of the progeny, with equal distribution between males and females. Quantitative Southern blotting showed that heterozygous mice from lines 714 and 717 contained ~40 copies of the transgene (Fig. 1), whereas line 721 contained <5 copies (data not shown).

Expression of the CCSP-β\textsubscript{2}-AR transgene was initially confirmed by RT-PCR. Total RNA prepared from whole lung homogenates was reverse transcribed and subjected to PCR with a forward primer from the CCSP promoter and a reverse primer from the β\textsubscript{2}-AR ORF. As shown in Fig. 2A, only mice with positive genomic screens were found to express the transgene mRNA. No PCR products were detected in the absence of reverse transcriptase. CCSP-β\textsubscript{2}-AR transgene expression was quantitated by ribonuclease protection assays (Fig. 2B). For these studies, total cellular RNA pre-
pared from whole lung homogenates was hybridized with an antisense riboprobe corresponding to the distal 500 bp of the human β2-AR ORF. Because this region has only ~70% homology with the mouse gene, the riboprobe does not protect the endogenous mouse transcript from RNase digestion. Therefore, the full-length protected fragment (Fig. 2B, arrow) was only detected in mice expressing the CCSP-β2-AR transgene. With this technique, we found that transgene expression in the lungs of mice from lines 714 and 717 was equivalent and much greater than that of line 721. The two higher expressing lines were subsequently propagated for further studies.

After confirming that the β2-AR transgene could be detected in whole lung RNA, we next used in situ hybridization to assess its distribution within the lung. With the human-specific β2-AR riboprobe, we found that transgene expression in the CCSP-β2-AR mice was limited to the airway epithelium (Fig. 3A). No specific binding was observed in lung sections from NTG mice (Fig. 3B). Similarly, no specific binding occurred in either CCSP-β2-AR (Fig. 3C) or NTG (data not shown) mice when probed with the sense riboprobe.

A previous study (6) has demonstrated that >90% of all β2-ARs in the lung are localized to the alveolar wall. Because the human TG receptor cannot be differentiated from the endogenous mouse receptor with β-AR radioligands, we anticipated that receptor overexpression in one population of cells (i.e., those of the bronchial epithelium) might go undetected in receptor binding studies with membranes prepared from whole lung homogenates. Indeed, our initial radioligand assays with [125I]ICYP showed no difference in β2-AR density of membranes prepared from CCSP-β2-AR lungs or trachea versus those prepared from NTG mice. Analysis of adenyl cyclase activities in these membranes also showed no difference. We therefore used receptor autoradiography with [125I]ICYP to establish that the receptor protein was overexpressed in the airway epithelium. To specifically assess β2-AR density, CGP-20712A was included to inhibit [125I]ICYP binding to the β1-AR.

Compared with NTG mice, CCSP-β2-AR mice had increased grain density over the bronchial epithelium (Fig. 4, A and D). Grain counts over the airway epithelium of the NTG mice were approximately twofold greater than those of the NTG mice (1.07 ± 0.07 vs. 0.59 ± 0.04 optical density, respectively, P < 0.05). No difference in grain density was observed for other lung structures (bronchial smooth muscle, alveoli, and vessels), indicating that receptor overexpression was confined to the airway epithelium. (−)-Isoproterenol inhibited [125I]ICYP binding in both NTG and CCSP-β2-AR mice (Fig. 4, C and D, respectively), confirming that binding was due to the β2-AR.

Long-term observation of CCSP-β2-AR TG mice (up to 20 mo) showed that epithelial cell overexpression of the β2-AR was not associated with increased morbidity or mortality. CCSP-β2-AR mice develop and grow normally compared with their NTG littermates. The gross structural anatomy of the lungs of CCSP-β2-AR mice was unremarkable. Examination of hematoxylin and eosin-stained lung sections showed no histological abnormalities (Fig. 5, A and D). Additional staining with Masson’s trichrome showed no evidence of increased collagen deposition (Fig. 5, B and E), and staining with Alcian blue showed no differences in the number of mucus-secreting cells (Fig. 5, C and F). Remodeling of the lung, at least as detected by these techniques, did not occur in CCSP-β2-AR mice.
To examine the potential effects of targeted epithelial cell β2-AR overexpression on airway responsiveness, we measured methacholine-induced bronchoconstriction in vivo so that the integrity and function of the epithelium could be maintained. For these experiments, we used whole body plethysmography to measure Penh, a derived parameter that has been shown to correlate highly with airway resistance as measured by invasive methods (20). Conscious, unrestrained NTG and CCSP-β2-AR mice were exposed to incremental doses of aerosolized methacholine for 2 min followed by recording of respiratory parameters for 2 min. As shown in Fig. 6, the CCSP-β2-AR mice required higher doses of methacholine to induce a change in Penh compared with the NTG mice. The ED200 for methacholine in CCSP-β2-AR mice was approximately twice that of NTG mice (345 ± 634 vs. 157 ± 614 mg/ml, respectively; P < 0.01; n = 10). These results therefore indicated that overexpression of the β2-AR in bronchial epithelial cells afforded protection against cholinergic-mediated bronchoconstriction in vivo. To confirm that this effect was the result of β2-AR activation, NTG and CCSP-β2-AR mice were treated with the β-antagonist propranolol (0.5 g/l in the drinking water) for 6 days. Afterward, the mice were exposed to 160 mg/ml (the ED200 for NTG mice) of aerosolized methacholine. The Penh value for the 160 mg/ml concentration before propranolol treatment was then compared with that after treatment. For NTG mice, the Penh value before propranolol treatment (1.55 ± 0.12; n = 10) was not different from that after treatment (1.49 ± 0.35; n = 9). In contrast, the Penh of untreated CCSP-β2-AR mice increased significantly after treatment with propranolol (0.99 ± 0.10 vs. 1.72 ± 0.33; P < 0.03; n = 10 and 7, respectively).

The results of the aforementioned experiments indicated that the CCSP-β2-AR TG mice were hyporesponsive to methacholine and that this effect was specifically mediated rather than an artifact of transgene insertion in the genome. Our next goal was to assess whether the in vivo response of CCSP-β2-AR mice to β-agonists was also different from that of NTG mice. For these experiments, NTG and CCSP-β2-AR mice were pretreated with aerosolized albuterol (1 mg/ml) for 20 min before methacholine challenge. Figure 7 shows that albuterol caused a rightward shift in the methacholine dose response of NTG mice, increasing the ED200 from 157 ± 14 to 388 ± 68 mg/ml (P < 0.01; n = 10). Of note, the ED200 value for the albuterol-treated NTG mice was not statistically different from that of the untreated CCSP-β2-AR mice. In CCSP-β2-AR mice, pretreatment with albuterol also raised the ED200 for methacholine (345 ± 34 to 455 ± 62 mg/ml; n = 10), but this difference was small and did not reach significance. Thus in these mice, epithelial cell overexpression of the β2-AR was as effective as inhaled albuterol that acts on both epithelial cell and smooth muscle cell receptors.
We next examined whether the acute response to ozone was altered in CCSP-β2-AR mice. For these experiments, NTG and CCSP-β2-AR mice were exposed to 0.75 ppm ozone for 4 h. Penh was measured at frequent intervals during the initial 2-h postexposure period. As shown in Fig. 8, Penh immediately increased to 77% over baseline in NTG mice and then remained significantly elevated for 1 h after exposure (P < 0.05).

In contrast, CCSP-β2-AR mice showed no increase in Penh during the same time period.

To assess whether the extent of ozone-induced hyperresponsiveness was also affected in CCSP-β2-AR mice, methacholine challenge testing was performed 6 and 24 h after ozone exposure. Ozone-induced airway hyperresponsiveness was observed for both NTG and CCSP-β2-AR mice (Fig. 9). Compared with the pre-ozone values, the ED200 for methacholine 6 h after ozone exposure decreased from 157 ± 14 to 56 ± 12 mg/ml (P < 0.01) in NTG mice and from 345 ± 34 to 143 ± 35 mg/ml (P < 0.05) in CCSP-β2-AR mice. The postexposure ED200 for methacholine for the CCSP-β2-AR mice remained significantly greater than that of the exposed NTG mice (P < 0.05) and, in fact, was not
after ozone decreased from 157 ± 6 to 56 ± 12 mg/ml (P < 0.01) in NTG mice and from 345 ± 34 to 143 ± 35 (P < 0.05) in CCSP-β2-AR mice. The ED200 for ozone-treated CCSP-β2-AR was not significantly different from that of nonexposed NTG mice. At 24 h postexposure, hyperresponsiveness to methacholine had resolved in both groups.

The results of the whole body plethysmography studies showed that CCSP-β2-AR mice were less responsive to methacholine and ozone than were NTG mice. To address the possibility that these effects were due to the release of bronchodilator substances by the bronchial epithelial cells, we measured the concentration of two candidates, PGE2 and NO, in BAL fluid. For these measurements, mice were lavaged with 1 ml of PBS that was allowed to dwell for 5 min. In another group of mice, 10−6 M isoproterenol was added to the lavage fluid so that the response to β-agonists could be assessed. PGE2 levels in the BAL fluid from NTG (0.56 ± 0.15 ng/ml) and CCSP-β2-AR (0.66 ± 0.07 ng/ml) mice were not different (Fig. 10A). Addition of 1 μM isoproterenol to the lavage fluid had no effect on PGE2 levels in BAL fluid from either group even though cAMP content in the BAL fluid was approximately four times greater than that in the untreated samples (data not shown). Likewise, basal levels of nitrite in BAL fluid, as measured by the Griess reaction, in NTG (1.05 ± 0.16 μM) and CCSP-β2-AR (1.35 ± 0.11 μM) mice were not different (Fig. 10B). Exposure to isoproterenol had no effect on nitrite content in the BAL fluid from either CCSP-β2-AR or NTG mice. Normalization of PGE2 and NO measurements to either urea or protein had no impact on the results.

**DISCUSSION**

The β2-AR is present on many different cell types within the lung, including airway epithelial cells. In vitro studies (14, 16, 51) have demonstrated that the airway epithelium can modulate bronchial tone and that this interaction may be at least partly regulated by the β2-AR signal transduction pathway. However, the physiological and potential pharmacological significance of epithelial cell β2-ARs have not been addressed in a manner identical to that evoked by agonists. In transfected Chinese hamster fibroblasts, Green et al. (17) observed a direct correlation between receptor density and basal adenyl cyclase activity. Furthermore, a mutant β2-AR that exhibited decreased agonist-stimulated adenyl cyclase activation had proportionately decreased basal levels of activation as well (17). Similarly, Turki et al. (54) and others (41) have shown that TG mice overexpressing the β2-AR in the heart have increased basal adenyl cyclase activities relative to their NTG litter-
mates. Various modeling techniques have shown that the increased basal signaling that occurs with β2-AR overexpression is due to spontaneous conversion of receptors to the active (R*) conformation (46). Thus by selectively targeting expression to a specific cell type, one has the capability to distinguish the effect(s) of β2-AR activation in cell types that are anatomically in close proximity and otherwise not amenable to selective activation by agonists.

With the use of this strategy, our results revealed that β2-AR activation in Clara cells had a striking effect on bronchomotor tone as assessed in vivo by whole body plethysmography. In response to the inhaled bronchoconstrictor methacholine, CCSP-β2-AR mice were significantly less reactive than their NTG littermates. Remarkably, the degree of protection afforded to the CCSP-β2-AR mice was the same as that achieved by inhalation of the β-agonist albuterol in NTG mice. Although there was a trend toward increased protection in CCSP-β2-AR mice treated with albuterol, suggesting that epithelial β2-ARs act in an additive fashion with smooth muscle β2-ARs to bronchodilate, the difference did not reach significance. These data suggest, then, that epithelial cell β2-ARs can have a substantial impact on airway smooth muscle responsiveness and are thus indicative of a response not previously quantifiable.

The protective effect of β2-AR overexpression was not limited to methacholine because the acute response to ozone was also reduced. Unlike methacholine, ozone does not pass beyond the epithelial layer (45) and therefore does not act directly on airway smooth muscle. Instead, the effects of ozone are due to ozonation and oxidation of proteins and lipids in the epithelium and epithelial lining fluid that either act directly as bronchoconstrictors or lead to the production of other bronchospastic substances (reviewed in Ref. 32). Ozone caused an acute increase in $P_{\text{enh}}$ of NTG mice that persisted up to 1 h after exposure. In contrast, $P_{\text{enh}}$ was unchanged in the CCSP-β2-AR mice over the same time period. Because $P_{\text{enh}}$ parallels measures of airway resistance, the acute response to ozone-exposed NTG mice could conceivably be due to edema from inflammation, mucosal damage, or frank mucus plugging. However, several lines of evidence indicate that bronchospasm was the primary component. First, spontaneous resolution was rapid (<2 h). Second, the response was markedly attenuated by the β-agonist bronchodilator albuterol. Third, mice exposed to these concentrations of ozone showed no evidence of cellular inflammation, edema, or plugging for up to 4 h after exposure (60). Furthermore, it would not be expected that edema and mucus plugging would be readily reversed by a smooth muscle relaxant. Taken together, these findings indicate that smooth muscle contraction is the major contributing factor that accounts for the postozone increase in $P_{\text{enh}}$. The protection afforded to CCSP-β2-AR mice was therefore effective against two different bronchoconstrictors (i.e., methacholine and ozone). These bronchospastic agents appear to act through different mechanisms because the anticholinergic agent atropine had little effect on the $P_{\text{enh}}$ response after ozone.

Epithelial cell β2-AR overexpression did not, however, prevent the development of ozone-induced airway hyperresponsiveness. The $E_{200}$ for methacholine measured 6 h after ozone was 36% of basal level in NTG mice and 41% of basal level in CCSP-β2-AR mice. The relative protective effect of β2-AR overexpression was maintained, though, because the $E_{200}$ for the ozone-exposed TG mice was still significantly less than that for the treated NTG mice. In fact, the $E_{200}$ of ozone-exposed CCSP-β2-AR mice was not different from that for unexposed NTG mice. CCSP-β2-AR mice were therefore protected in the sense that their response to methacholine after ozone was submaximal.

As mentioned earlier, airway epithelial cells could regulate airway responsiveness through one or more of several different mechanisms. Overexpression of the β2-AR in airway epithelial cells could have caused chronic remodeling of the airways. However, histological analysis showed no structural alterations in the airways of the CCSP-β2-AR mice, and the protection afforded to the TG mice was inhibited by short-term treatment with propranolol. Clara cells also have enzymatic activity (e.g., acetylcholinesterase) that could potentially be upregulated by β2-AR activation and thus limit the response to an agent such as methacholine. However, the observation that CCSP-β2-AR mice were also protected against ozone-induced bronchospasm (which was noncholinergic) suggests the presence of a generalized pathway rather than one specific for a given agent.

The bronchial epithelium is the source of a number of substances that may directly affect airway smooth muscle tone. We therefore considered the possibility that activation of epithelial cell β2-ARs stimulates the release of a relaxant factor. The two candidates we chose to consider were PGE$_2$ and NO because both are known to relax airway smooth muscle (11, 52) and both are produced by Clara cells (30, 48). To investigate whether either of these substances could account for the inhibition of bronchoconstriction in the CCSP-β2-AR mice, we measured the content of PGE$_2$ and NO in BAL fluid from NTG and CCSP-β2-AR mice. Evaluation of this fluid showed no differences in the levels of PGE$_2$ or NO, suggesting that neither agent was contributing to the protective effect of β2-AR overexpression. However, epithelial release of these mediators may be primarily directed toward the serosal surface (25, 34) and therefore underestimated in BAL fluid. Thus we cannot conclusively exclude PGE$_2$, NO, or other factors as mediators of the epithelial cell β2-AR-mediated relaxation at this time. It is also possible that overexpression altered the production and release of Clara cell secretory products that in some way modify airway compliance or reactivity. β-Agonists have previously been shown to increase the secretory activity of Clara cells (37). An increase in such a component of the epithelial lining fluid could thus potentially act to limit the effect of methacholine and ozone by stabilization of the airways, making them more resistant to closure.

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Baseline lung function and histology, however, were not different in the CCSP-β2-AR TG mice, suggesting that this scenario is unlikely.

Regardless of the mechanism, our findings clearly show that overexpression of the β2-AR in epithelial cells modulates airway responsiveness to contractile agents. Previous ex vivo studies that used airway ring preparations have been inconsistent in demonstrating a modulatory role for the epithelial cell β2-ARs. This may be due to the fact that the bronchoactive substances have direct access to airway smooth muscle via the exposed serosal surface, thus potentially bypassing protective effects of the epithelium. Furthermore, mechanical removal of the epithelium may introduce artificial confounding variables caused by mast cell degranulation (15). Because our goal was to assess the physiological relevance of this pathway on airway responsiveness in vivo, we assessed bronchoconstriction with whole body plethysmography to measure Penh. Penh is a derived measure of bronchoconstriction that has been shown to strongly correlate with airway resistance as measured by traditional invasive methods (20). Whole body plethysmography also permits assessment of airways distal to the trachea (20). This is particularly relevant with regard to the CCSP-β2-AR mouse because Clara cells comprise up to 50% of the epithelial cells lining the terminal bronchioles of murine airways (43, 44). Furthermore, at least two studies (51, 56) have demonstrated that the effect of epithelial cell β2-ARs varied among different airway generations, with their effect being most pronounced in the smaller airways.

In summary, we have found that TG mice overexpressing β2-ARs in airway epithelial cells exhibit decreased responsiveness to methacholine and ozone. Although the mechanism by which this protection is afforded is unclear, these findings show that the bronchial epithelium is capable of modulating airway tone and that this interaction is at least partly regulated by β2-ARs present on these cells. Furthermore, the effect of epithelial cell β2-AR activation is distinct from the effects of β2-AR activation in other lung cell types. The inhibition of bronchoconstriction in TG mice overexpressing the β2-AR in the airway epithelium suggests the intriguing possibility that delivery of this gene in vivo could be used in the management of bronchospastic lung disease.

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REFERENCES


