Transgenic Overexpression of $\beta_2$-Adrenergic Receptors in Airway Smooth Muscle Alters Myocyte Function and Ablates Bronchial Hyperreactivity

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$\beta_2$-Adrenergic receptors ($\beta_2$AR) act to relax airway smooth muscle and can serve to counteract hyperresponsiveness, although the effect may not be ablative even in the presence of exogenous agonist. Within this signaling cascade that ultimately transduces smooth muscle relaxation, a significant “spare receptor” pool has been hypothesized to be present in the airway. In order to modify the relationship between $\beta_2$AR and downstream effectors, transgenic mice (TG) were created overexpressing $\beta_2$AR ~75-fold in airway smooth muscle using a mouse smooth muscle $\alpha$-actin promoter. While >90% of these receptors were expressed on the smooth muscle cell surface, the percentage of receptors able to form the agonist-promoted high affinity complex was less than that found with nontransgenic (NTG) cells ($R_0 = 18$ versus 36%). Nevertheless, $\beta_2$AR signaling was found to be enhanced. Intact airway smooth muscle cells from TG had basal cAMP levels that were greater than NTG cells. A marked increase in agonist-stimulated cAMP levels was found in the TG (~200% stimulation over basal) compared with NTG (~50% over basal) cells. Adenylyl cyclase studies gave similar results and also showed a 10-fold lower EC$_{50}$ for TG cells. Tracheal rings from TG mice that were precontracted with acetylcholine had an enhanced responsiveness (relaxation) to $\beta_2$-agonist, with a 60-fold decrease in the ED$_{50}$ indicating that the enhanced signaling imposed by overexpression results in an increase in the coordinated function of the intact airway cells. In vivo studies showed a significantly blunted airway resistance response to the inhaled bronchoconstrictor methacholine in the TG mice. Indeed, with $\beta_2$-agonist pretreatment, the TG mice displayed no response whatsoever to methacholine. These results are consistent with $\beta_2$AR being the limiting factor in the transduction system. Increases in the initial component of this transduction system (the $\beta_2$AR) are sufficient to markedly alter signaling and airway smooth muscle function to the extent that bronchial hyperresponsiveness is ablated, consistent with an anti-asthma phenotype.

Asthma is a chronic inflammatory disorder of the airways in which airflow obstruction occurs due to an active constriction of airway smooth muscle of the bronchi and airway mucous accumulation. Bronchial smooth muscle cells express numerous G protein-coupled receptors that modulate contractility including $\beta_2$-adrenergic receptors ($\beta_2$AR),1 which act to relax, and muscarinic receptors, which act to contract, the muscle (1). Agonists to the former and antagonists to the latter receptors are utilized clinically for reversal of bronchoconstriction. The propensity for airway smooth muscle to constrict in asthma has been termed airway hyperresponsiveness (2). Thus, a hallmark physiological finding in patients with asthma is hyperresponsiveness of the bronchi to inhalation of constractive agents such as the muscarinic agonist methacholine. This constrictive response in asthmatics is thought to be ultimately due to sensitization of pathways, such as the cholinergic system, that culminate in airway smooth muscle contraction. Typically, nonasthmatics have no detectable airway response to inhalation of methacholine, and thus airway hyperreactivity has become a defining physiologic parameter.

$\beta_2$AR are cell surface receptors that couple to the stimulatory guanine nucleotide-binding protein (G$_s$), activating adenylyl cyclase. Increased intracellular cAMP mediates relaxation of airway smooth muscle by activation of protein kinase A. Protein kinase A acts to phosphorylate myosin light chain kinase, cell surface K$^+$ channels, a Na$^+$/K$^+$ ATPase, phospholamban, and one or more pumps that lead to sarcoplasmic reticulum uptake of Ca$^{2+}$ and acts to inhibit the production of inositol phosphates (3, 4). The net effect is a decrease in intracellular Ca$^{2+}$ and phosphorylation of contractile proteins leading to relaxation. The $\beta_2$AR subtype is the predominant $\beta_2$AR expressed on bronchial smooth muscle in humans (5), although there are some conflicting data regarding the role of the $\beta_2$AR subtype in bronchodilation in various other species (6–9). Within the airway, luminal epithelial cells also express $\beta_2$AR, and some evidence suggests that activation of these receptors contributes to smooth muscle relaxation via an unknown mediator (10).

The hierarchy of signaling pathways that establishes physiologic bronchomotor tone is not well established. The principle endogenous agonist for airway $\beta_2$AR is epinephrine. Thus, in the absence of significant elevations in circulating epinephrine, adrenergic control of smooth muscle tone may be primarily due to “basal” coupling of receptor to its effector. Chronic exposure to exogenous agonists in the treatment of bronchospasm can result in desensitization of the $\beta_2$AR response (tachyphylaxis)

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1 The abbreviations used are: $\beta_2$AR, $\beta_2$-adrenergic receptor(s); G$_s$, stimulatory guanine nucleotide-binding protein; Penh, enhanced pause; NTG, nontransgenic; kb, kilobase; ORF, open reading frame; PBS, phosphate-buffered saline; CYP, cyanopindolol.

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(11). Furthermore, the asthmatic inflammatory milieu itself appears to promote a desensitized β2AR (12). These issues have promoted the concept that maintenance or augmentation of nonagonist (basal), and agonist-promoted, β2AR function in the airway could favorably alter myocyte signaling and airway physiology to effectively block hyperresponsiveness. Due to the dynamic nature of β2AR regulation, such attempts by pharmacologic or genetic means may lead to feedback regulation of the receptor, Gs, or other downstream components of the transduction pathway, thus minimizing the impact at the cellular and physiologic level. Such overexpression has also been shown to cause “promiscuous” coupling of receptors to G proteins that are not natively activated in cells with physiologic levels of receptor (13). Extensive functional coupling of airway smooth muscle β2AR to Gq could serve to inhibit adenylyl cyclase and possibly promote mitogen-activated protein kinase activation (14), adversely affecting cell growth and possibly airway contractility itself (3). Promiscuous Gq coupling would mimic M3 possibly promote mitogen-activated protein kinase activation that increasing the number or function of these receptors would have no discernible effect on signaling or physiologic function. 

The current study was undertaken to further understand the relationship between airway smooth muscle β2AR expression and signaling, smooth muscle function, and airway responses within the context of bronchial hyperreactivity and to specifically address the multiple issues regarding augmentation of β2AR function as stated above. We thus created transgenic mice overexpressing the β2AR on smooth muscle using a smooth muscle α-actin promoter. This was carried out in FVB/N mice, a strain that displays a moderate degree of bronchial hyperresponsiveness in the absence of antigenic challenge. Such overexpression had profound effects on cellular signaling, smooth muscle function, and bronchial hyperresponsiveness.

**EXPERIMENTAL PROCEDURES**

*Transgenic Mice*—Smooth muscle-specific expression of the human β2AR in transgenic mice was achieved by using the mouse smooth muscle α-actin promoter (18) (a gift from Dr. A. Strauch). The construct was prepared by cloning the 1.5-kb HindIII/PsaA1 fragment encoding the human β2AR (1.2 kb of ORF and 0.3 kb of 3′-untranslated region) upstream of the SV40 polyadenylation sequence in the plasmid pNN03. The 3.6-kb upstream smooth muscle α-actin promoter fragment, termed SMP8 (18), was then subcloned into a BamHI site 5′ to the β2AR ORF. Orientation of each fragment was confirmed by sequence analysis and restriction enzyme digestion. The transgenic construct (~5.9 kb) was excised from the plasmid by NotI digestion and microinjected into male pronuclei of fertilized zygotes from superovulated FVB/N mice. Surviving zygotes were implanted into pseudopregnant foster mothers who gave birth to founders. Transgene-positive founder mice were identified by Southern blot analysis of genomic DNA derived from tail clips. Genomic DNA was extracted from the offspring and analyzed by PCR amplification with primers specific for the transgene. BamHI restriction digestion of the plasmid containing the transgene-expressing construct was used to screen founder mice for the presence of the transgene. A transgene-positive founder was identified and designated Tyr1 (19). The Tyr1 founder was bred to FVB/N mice to generate the Tyr1/FVB line. Subsequent screening for the hemizygous transgene-positive founder mice was performed by Southern blot analysis of genomic DNA derived from tail clips. Genomic DNA from offspring of Tyr1/FVB mice was isolated using a standard DNA isolation protocol. Genomic DNA from Tyr1/FVB mice was digested with BamHI, and the DNA blot was hybridized with a radiolabeled fragment specific for the transgene. Genomic DNA from offspring of Tyr1/FVB mice was isolated using a standard DNA isolation protocol. Genomic DNA from Tyr1/FVB mice was digested with BamHI, and the DNA blot was hybridized with a radiolabeled fragment specific for the transgene. Genomic DNA from offspring of Tyr1/FVB mice was isolated using a standard DNA isolation protocol. Genomic DNA from Tyr1/FVB mice was digested with BamHI, and the DNA blot was hybridized with a radiolabeled fragment specific for the transgene.

**Radioiodinated Binding and cAMP Studies**—To prepare membranes from lung tissue, lungs from an individual mouse were homogenized with a Polytron (Brinkman) in 10 ml of hypotonic lysis buffer (5 mM Tris, pH 7.4, 2 mM EDTA) containing the protease inhibitors leupeptin, antipain, and soybean trypsin inhibitor (10 μg/ml each). After homogenization, the mixture was centrifuged at 40,000 × g for 10 min at 4 °C. The pellets were washed and centrifuged two additional times, after which the pellets were suspended in assay buffer (50 mM Tris, pH 7.4, 12.5 mM MgCl2, 2 mM EDTA). For determination of receptor expression, radioiodinated binding was carried out with [125I]-CYP as described. The fraction of receptors in the high affinity binding site was determined by competition experiments performed in the absence of GTP using 40 pm [125I]-CYP and concentrations of isoproterenol ranging from 10 to 10-10 μM as described previously (23). Competition data were fit to a two-site model when this fit was statistically (p < 0.05) better than a one-site fit by F-test, using Prizm software (GraphPad, San Diego, CA). Receptor density on the cell surface was assessed using methods previously described (24). Briefly, cells grown in monolayers were detached with 0.25% trypsin for 5 min at 37 °C. After trypsin activity was neutralized by the addition of fetal calf serum, the cells were washed with PBS and resuspended in Dulbecco’s modified Eagle’s medium. The resuspended cells were incubated in a volume of 500 μl with 400 pm [125I]-CYP at 37 °C for 60 min in the absence or presence of the hydrophobic β2AR antagonist propranolol (1 μM) or the hydrophobic α1A-adrenergic antagonist CGP 12177 (10 μM). Binding was measured throughout the cell was defined as that displaced by propranolol, while cell surface binding was defined as that displaced by CGP12177 (24). Bound radioactivity was separated by filtration and washing over GF/C glass fiber filters. Cyclic AMP content of attached mouse smooth muscle cells in culture exposed to 10 μM isoproterenol or carrier for 10 min was measured by an acetylated radium immunomassay method as described.
Adenylyl cyclase activity was measured in membranes prepared as above using column chromatography as described previously (25).

Ex Vivo Smooth Muscle Studies—Studies of mouse tracheal contractility have been reported in detail elsewhere (26). Briefly, tracheas were excised and dissected free of surrounding tissues and cut into rings of approximately 5 mm in length. The tracheal rings were mounted on stainless steel wires connected to isometric force transducers. The rings were then immersed in a physiologic saline solution (118 mM NaCl, 4.73 mM KCl, 1.2 mM MgCl₂, 0.026 mM EDTA, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaH₂CO₃, and 11 mM glucose) maintained at 37 °C and bubbled with 95% O₂, 5% CO₂ to maintain a pH of 7.4. Each tracheal ring was attached to a tension of 5 millinewtons, an optimal passive tension for maximizing active force (27). After a 20-min equilibration period, contraction/relaxation cycles with 10 μM acetylcholine were performed until consistent forces were observed. Cumulative concentration-isometric force curves were then generated to acetylcholine (1 nM to 30 μM). After rinsing, tracheas were contracted with 10 μM acetylcholine (approximately the ED₅₀), and cumulative concentration-relaxation curves were generated to isoproterenol (30 pM to 10 μM). Concentration-response relations were fitted to a logistic equation (26).

In Vivo Airway Physiology—Airway responsiveness to methacholine was measured noninvasively in conscious, unrestrained mice using a whole body plethysmograph (Buxco Electronics, Troy, NY) (28). Using this system, the volume changes that occur during a normal respiratory cycle are recorded as the pressure difference between the 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 is a unitless value that is a function of the peak inspiratory (PIP) and peak expiratory (PEP) pressures and the timing of expiration and is calculated as follows:

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\text{Penh} = \frac{\text{Pause} \times \text{PEP}}{\text{PIP}} \quad \text{(Eq. 1)}
\]

where Pause is the ratio of time spent in the last third of expiration relative to early expiration. Penh has been shown to closely correlate with invasive measurements of airway resistance (28) and was used as the measure of airway responsiveness in this study. Mice were placed in the chamber and allowed to adjust to their surroundings for 10 min. They were then exposed to aerosolized PBS (to establish baseline), followed by increasing concentrations of methacholine (2.5–80 mg/ml). Aerosolization was for 3 min, and respiratory measurements were recorded and averaged for the subsequent 5 min after each dose. The degree of bronchoconstriction was expressed as the percentage change in Penh relative to the PBS base line. On a separate day, the mice were submitted to the same protocol except that they were first treated with aerosolized albuterol (1.0 mg/ml) for 20 min. The concentration-response data for each individual mouse were fit to a sigmoid curve by an interactive least squares technique, and the dose of methacholine required to double baseline Penh (ED₂₀₀) was derived.

RESULTS AND DISCUSSION

From a total of 40 mice screened, three SMP8-β₂AR founder mice were identified. Subsequent matings with nontransgenic mice showed that one of the three founders was mosaic. In lines established from the other two founder mice (denoted as lines 79 and 95), the transgene was inherited in approximately 50% of the offspring with equal distribution between male and female mice. Hemizygous mice from these two lines were further characterized with respect to transgene copy number, mRNA expression, receptor density, and histologic analysis. Southern blot analysis of genomic DNA prepared from tail clips showed no difference in smooth muscle of stomach, colon, and uterus (data not shown). Hybridization or radioligand binding showed increased expression in smooth muscle of stomach, colon, and uterus (data not shown).

β₂AR expression was further quantitated by radioligand binding assays with [³²P]CYP. Initial studies with membrane preparations from whole lung homogenates showed no differences in receptor density between transgenic and nontransgenic mice. However, previous studies have shown that >90% of β₂ARs in the lung are localized to cells (type I and type II pneumocytes and capillary endothelium) that line the alveoli (5, 7). Indeed, based on the densities of β₂AR as assessed by autoradiography (5, 7) and the extensive surface area of alveoli (29), the ratio of total airway smooth muscle versus alveolar β₂AR is >1:1000 or more. Thus, we felt it was unlikely that using a whole lung preparation would detect enhanced smooth muscle expression in the transgenic mice. Since the β₂AR transcript was confined to airway smooth muscle, and to a lesser extent in pulmonary vascular smooth muscle, with no signal observed in the bronchial epithelium or alveolar lining cells (Fig. 2, A and B). Additional studies with in situ hybridization or radioligand binding showed increased expression in smooth muscle of stomach, colon, and uterus (data not shown).

β₂AR expression has been shown to be expressed in the lung of the transgenic mice but absent in nontransgenic mice. We therefore used the same species-specific cRNA probe described above for the ribonuclease protection assay analysis so that we could limit detection to that of the transgene only. As shown in Fig. 2, specific hybridization (appearing as white dots) was observed in the SMP8-β₂AR mice (A and B) but was absent in nontransgenic mice (C and D). These studies clearly showed that expression of the SMP8-β₂AR transcript was confined to airway smooth muscle, and to a lesser extent in pulmonary vascular smooth muscle, with no signal observed in the bronchial epithelium or alveolar lining cells (Fig. 2, A and B). Additional studies with in situ hybridization or radioligand binding showed increased expression in smooth muscle of stomach, colon, and uterus (data not shown).
the βAR was significantly overexpressed in tracheal smooth muscle cells from transgenic mice when compared with the level of expression in smooth muscle cells derived from nontransgenic mice (Fig. 4). βAR density in airway smooth muscle cells from transgenic lines 79 and 95 were both ~75 times greater than that of cells from nontransgenic mice (2510 ± 229 and 2218 ± 167 versus 33 ± 6 fmol/mg protein, respectively, n = 4, p < 0.001). Given that the levels of transgene mRNA and receptor protein were equivalent in the two transgenic lines, the majority of the remaining pharmacological and physiological studies were carried out with mice of line 95.

Radioligand binding with 125I-CYP was also carried out in whole cells, using the competitors propranolol (hydrophobic) and CGP12177 (hydrophilic) to identify total cellular versus cell surface βAR, respectively. These studies showed that the vast majority of βAR (95 ± 1.5%, n = 4) of transgenic smooth muscle cells were expressed on the cell surface. This distribution was similar to what was found in cells from nontransgenic mice, although quantitation was difficult due to the low expression of receptors in those cells. Having determined that the transgenic βAR have a normal cellular distribution, we next assessed whether they had the capacity to form the high affinity agonist/receptor/Gs complex. In cells derived from nontransgenic and transgenic mice, agonist (isoproterenol) competition data in studies carried out in the absence of guanine nucleotide were best fit to a two-site model, while in the presence of 100 μM GTP the data were best fit to a single site model. However, in the absence of GTP, the proportion of receptors in the high affinity state (%R1) was lower in membranes from transgenic cells compared with those of nontransgenic cells (%R1 = 18 ± 4.2 versus 36 ± 6.7). Taken together, the radioligand binding studies indicate that expression of βAR is significantly greater in transgenic smooth muscle cells compared with nontransgenic and that such expression is localized primarily to the cell surface but that a smaller fraction of these receptors is capable of physically coupling to Gs.

To assess whether receptor-mediated adenylyl cyclase stimulation was enhanced in SMP8-βAR mice, we measured both cAMP content (Fig. 5A) in intact tracheal smooth muscle cells and adenylyl cyclase activities in smooth muscle cell membranes (Fig. 5B). We found that basal cAMP contents in airway smooth muscle cells from transgenic mice were ~2.8-fold greater than those of cells from nontransgenic mice (3.70 ± 0.41 versus 1.34 ± 0.05 pmol/mL, respectively, n = 5, p < 0.001). Similarly, isoproterenol-stimulated cAMP content in transgenic airway smooth muscle cells was also significantly greater than that of cells from the nontransgenic controls (11.78 ± 1.62 versus 2.05 ± 0.10 pmol/mL, respectively, n = 5, p < 0.001). When assessed as percentage of stimulation over basal levels,
the isoproterenol-stimulated cAMP levels of cells from the transgenic mice (~200%) was markedly greater than what was observed in nontransgenic cells, which amounted to only ~50% over basal. Studies of adenylyl cyclase activity in cell membranes gave similar results. Basal activities were greater in transgenic as compared with nontransgenic membranes (273 ± 39.3 versus 172 ± 7.8, pmol/min/mg, n = 4, p < 0.05), as were maximal isoproterenol-stimulated activities (609 ± 74.7 versus 251 ± 7.3, pmol/min/mg, n = 4, p < 0.005). The percentage of isoproterenol stimulation over basal level was ~125 versus ~46%, respectively. The dose-response curve for isoproterenol stimulation of adenylyl cyclase from SMP8-β2AR membranes was left shifted compared with nontransgenic, with an EC50 of 21 ± 1.4 versus 213 ± 107 nM (n = 4, p < 0.05). Of note, a less than doubling of basal adenylyl cyclase or cAMP levels by isoproterenol in cultivated airway smooth muscle cells from other species has been previously reported (30), although higher levels have also been noted (21). This variability is probably due to assay conditions or species variation. However, in our work, smooth muscle cells from the two lines were studied under identical conditions in a paired manner, so it is the difference observed between transgenic and nontransgenic cells that is the critical finding.

These studies thus clearly demonstrate that overexpression of β2AR in airway smooth muscle results in enhanced adenylyl cyclase activity at baseline and in response to agonist. The data are consistent with the multistate model of G protein-coupled receptors, where in the non-agonist-bound state a small proportion of receptors spontaneously achieve the active conformation (R°). With overexpression, the number of receptors in this state at any one time is increased sufficiently to alter “basal” coupling, as shown by the increased cAMP levels and adenylyl cyclase activities of airway smooth muscle cells in the absence of agonist. Maximal constitutive activation of Gαs-adenyl cyclase was not observed, however, since isoproterenol resulted in yet further stimulation of cAMP in the transgenic derived cells. The percentage stimulation by agonist over base line was significantly greater as compared with nontransgenic littermates. Taken together, the data are consistent with the β2AR being a limiting factor in the receptor-Gαs-adenyl cyclase cascade in native airway smooth muscle cells. This is in direct contrast to other studies (15–17), which have concluded that there is a substantial receptor reserve on airway smooth muscle. Although β2AR expression was increased ~75-fold in the current study, we did not observe commensurate increases in basal or agonist-stimulated cAMP responses. A very similar finding has been reported with transgenic β2AR overexpression in the heart. In one such study, we obtained a ~45-fold overexpression of wild-type human β2AR in the hearts of transgenic mice, yet basal and isoproterenol-stimulated adenylyl cyclase activities were increased only ~3–4-fold over nontransgenic activities (31). Milano et al. (32) had a 200-fold increase in cardiac β2AR expression, with 2-fold increases in basal and isoproterenol-stimulated activities. These findings have been interpreted as being consistent with other elements of the transduction cascade (Gαs, adenylyl cyclase) becoming limiting factors when β2AR expression is markedly increased, such that proportional increases in signaling in relation to receptor expression are not observed. Indeed, in the current study a smaller percentage of β2AR in transgenic smooth muscle cells can form the high affinity receptor-Gαs complex as compared with nontransgenic cells, probably indicating insufficient Gαs to accommodate all of the overexpressed receptors.

To determine whether this enhanced signaling observed in cells results in regulation of coordinated smooth muscle function of the airway, we measured responses to agonist ex vivo using tracheal ring preparations. For these studies, tracheal rings dissected from SMP8-β2AR and nontransgenic mice were mounted in the same organ bath, and relaxation in response to isoproterenol was measured. Initial studies showed that the constriction response to acetylcholine was equivalent between rings derived from transgenic and nontransgenic mice (Fig. 6A). For isoproterenol dose-response experiments, rings were preconstricted by incubation with 10 μM acetylcholine. The sensitivity to isoproterenol was found to be markedly enhanced in the tracheal rings from SMP8-β2AR mice (Fig. 6B). As shown, response curves for isoproterenol for these mice were shifted to the left (60-fold decrease in ED50) compared with nontransgenic tracheal rings (ED50 = 0.64 ± 0.07 versus 40.0 ± 7.1 nM, respectively, n = 4, p < 0.001). However, the maximal extent of relaxation was equivalent. This enhancement of isoproterenol-induced relaxation in SMP8-β2AR mice was blocked by pretreatment with the selective β2AR antagonist ICI 118,551 (0.1 μM) (Fig. 6C), indicating that the response observed in these mice was directly the result of β2AR activation from transgenic overexpression of the receptor rather than a change in some other factor caused by insertion of the transgene. While these differences in smooth muscle relaxation ex vivo are quite significant, the fact that the maximal degree of agonist-mediated relaxation in the transgenic rings was not greater than that of the nontransgenics suggests that at the level of this physiologic response there may be other factors that limit further relaxation regardless of the number of β2AR expressed. Since in intact smooth muscle cells we do observe a

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**Fig. 5. cAMP production and adenylyl cyclase activity in isolated tracheal smooth muscle cells.** A, cAMP levels were measured in intact smooth muscle cells that were grown in 24-well plates and treated with either vehicle or various concentrations of isoproterenol for 10 min at 37°C. Reactions were stopped by the addition of HCl, and cAMP was measured by radioimmunoassay. Results are from four independent experiments. B, adenylyl cyclase activity was measured in membranes prepared from tracheal smooth muscle cells of transgenic and nontransgenic mice. Reactions were carried out in the presence of the indicated concentrations of isoproterenol for 10 min at 37°C. Detection of [32P]cAMP produced was determined by column chromatography. Shown is a representative dose-response curve. Data for basal and maximal (10 μM isoproterenol) agonist-stimulated activity in pmol/min/mg are summarized in the bar graph (n = 4 experiments).
leftward shift in the response curve as well as an increase in the maximal response, the limiting factor(s) may be in elements that are necessary after the early signal transduction events.

The above results indicated that transgenic mice overexpressing the β2AR in airway smooth muscle have enhanced signaling in isolated cells and tracheal rings. To determine whether this resulted in altered physiologic function of the airways in vivo, studies were carried out in intact mice using a rodent whole body plethysmography system. We hypothesized that this persistent β2AR signaling would result in a state of relative hyporesponsiveness to bronchoconstriction by methacholine. Thus, the responses to inhaled methacholine alone and methacholine after inhalation of the β-agonist albuterol were measured (Fig. 7). These results show that the maximal level of bronchoconstriction induced by methacholine in the SMP-β2AR mice was significantly less than that of nontransgenic mice (Penh = 250 ± 26 versus 558 ± 42% of base line, respectively, p < 0.001). In addition, the sensitivity to methacholine was altered in the SMP-β2AR mice, with the ED_{200} = 35.7 ± 10.6 mg/ml as compared with 12.1 ± 1.90 mg/ml in the nontransgenic species (p < 0.02). Even more striking were the responses to methacholine after exposure to the β-agonist albuterol. As shown, when SMP8-β2AR mice were pretreated with albuterol, methacholine caused no increase in Penh, even at the highest dose used in this study (80 mg/ml) (Fig. 7). In contrast, in nontransgenic mice methacholine responsiveness after albuterol was present, with the maximal bronchoconstriction being 373 ± 33% of base line. It is interesting to note that the extent of bronchoconstriction in nontransgenic mice treated with albuterol was greater than that observed in the transgenic mice in the absence of agonist (Penh = 373 ± 33 versus 250 ± 26% of base line, p = 0.01). These results are entirely consistent with the intact smooth muscle cell studies, where maximal cAMP content after agonist exposure in the nontransgenic cells was similar to non-agonist-exposed levels in the transgenic cells. The in vivo studies are also consistent with the ex vivo organ bath results. Here, trachea overexpressing β2AR maximally relaxed in the presence of low concentrations of agonist, while nontransgenic rings showed no demonstrable response at these concentrations (Fig. 6B). It should be noted, however, that while cellular cAMP/adenyl cyclase, tracheal ring, and whole body plethysmography studies are complementary, each has constraints that limit interpretation in isolation. In cells, our studies are confined to measurement of effector (adenyl cyclase) activity or its product (cAMP). While these experiments and radioligand binding studies can assess coupling of the receptor to this pathway, they do not provide information regarding post-cAMP events relevant to smooth muscle function (i.e., relaxation). The tracheal ring studies require preconstriction with acetylcholine in order to derive a signal and may not directly relate relaxation to airflow in vivo, the latter being influenced by additional physiologic variables. The whole animal plethysmography studies are limited by the doses of the drugs that are tolerated, could additionally be influenced by endogenous catecholamines, and are not easily amenable to measurements in response to multiple doses of agonist. Given the whole cell cAMP and the in vivo plethysmography results, it would not be unexpected for the tracheal ring studies to show a decreased basal force (tension) and a leftward shift in the acetylcholine dose response for the transgenic rings. However, tracheal rings must be stretched to some extent in order to obtain a transducer signal. Thus, basal as well as acetylcholine-induced bronchoconstriction are under conditions in such preparations that are not analogous to our whole cell or in vivo studies.

Our studies constitute the first transgenic overexpression of β2AR in a tissue where the receptor acts to inhibit a physiologic...
response. Previous reports with transgenic overexpression of the receptor in the heart, where the receptor stimulates contraction, have shown increased cardiac inotropy and chronotropy (31, 32). These studies suggested that β2AR overexpression might be useful therapeutically to overcome the depressed chronotropic state in congestive heart failure. Subsequent studies with very high β2AR overexpression in genetic models of cardiac hypertrophy/heart failure have revealed an increased mortality (33, 34). In our current work, we have overexpressed the receptor in a cell type where the physiologic response of β2AR is a decrease, rather than an increase, in contraction. In mice up to 14 months old, we have not found structural remodeling of the airway, alterations in smooth muscle morphology, or pathologic consequences in the lung of β2AR overexpression in airway smooth muscle. Our results clearly indicate that the β2AR-mediated relaxation response in airway smooth muscle can accommodate increased receptor levels with an increase in baseline and agonist-promoted function. The notion that there are sufficient spare receptors on native airway smooth muscle to limit the effectiveness of enhanced expression or function is thus not supported, at least in mouse lung.

The potential for transgenic overexpression of β2AR in smooth muscle to decrease cellular levels of Gs has been suggested in studies of transfection of β2AR into NG108 cells (35). Such a decrement might have consequences for β2AR and other Gs-coupled receptors endogenously expressed on the airway and could limit the effectiveness of the transgene. Using the isolated airway smooth muscle cells, Gs content as assessed by Western blots was not different in cells derived from transgenic mice compared with nontransgenic littermates assessed by Western blots was not different in cells derived from transgenic mice compared with nontransgenic littermates (data not shown); nor were the levels of the Gq isoform Gq12 different. We also examined the potential for agonist-promoted desensitization of physiologic β2AR responses in this setting of transgenic overexpression of the receptor. Transgenic mice were implanted with osmotic minipumps administering isoproterenol for 3 continuous days and then studied by plethysmography. The methacholine concentration-response curve in transgenic mice pretreated with isoproterenol remained essentially flat after acute albuterol, with a maximal Penh of 128 ± 1% of baseline (compared with 146 ± 12% in the absence of isoproterenol pretreatment, p > 0.05). So agonist-promoted desensitization of β2AR function at the physiologic level is not observed in the SMP8-β2AR transgenic mice, probably due to the extensive overexpression to a point such that some fraction of spare receptors are in fact present.

In conclusion, we have shown that the β2AR of airway smooth muscle represent a limiting element of the signal transduction pathway. This constraint is alleviated by increased expression, which enhances basal and isoproterenol-stimulated levels of intracellular cAMP. Such an increase has a significant impact on airway smooth muscle function, ultimately decreasing bronchial hyperresponsiveness. Given the central importance of bronchial hyperresponsiveness to the asthmatic phenotype, these mice can be considered to be in an anti-asthmatic state. As such, overexpression of β2AR in airway smooth muscle may be a potential genetic therapy for asthma.

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