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Changes in β_2-adrenoceptor and other signaling proteins produced by chronic administration of 'β-blockers' in a murine asthma model

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Abstract

Background—We have previously reported that chronic treatment with certain 'β-blockers' reduces airway hyperresponsiveness (AHR) to methacholine in a murine model of asthma.

Methods—Airway resistance was measured using the forced oscillation technique in ovalbulmin-sensitized and ovalbulmin-challenged mice treated with several β-adrenoceptor (β-AR) ligands. We used the selective β_2-AR ligand ICI 118,551 and the preferential β_1-AR ligand metoprolol to investigate the receptor subtype mediating the beneficial effect. Expression of β-ARs was evaluated using immunofluorescence. We evaluated several signaling proteins by western blot using lung homogenates, and measured the relaxation of the isolated trachea produced by EP_2 and IP receptor agonists.

Results—Four findings were associated with the decreased AHR after chronic β-blocker treatment: (1) the highly selective β_2-AR antagonist/inverse agonist, ICI 118,551 produced the bronchoprotective effect; (2) β_2-AR up-regulation resulted from chronic 'β-blocker' treatment; (3) reduced expression of certain proteins involved in regulating bronchial tone, namely, G_i, phosphodiesterase 4D and phospholipase C-β1; and (4) an enhanced bronchodilatory response to prostanoid agonists for the IP and EP_2 receptors.

Conclusions—These data suggest that in the murine model of asthma, several compensatory changes associated with either increased bronchodilator signaling or decreased bronchoconstrictive signaling, result from the chronic administration of certain 'β-blockers'.

Keywords

β-blockers; Asthma; Mouse trachea; Forced oscillation technique; Bronchial relaxation; Airway hyperresponsiveness; Prostaglandin receptors; β-Adrenoceptors; Inverse agonists

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1. Introduction

β_2-Adrenoceptors (β_2-ARs) are typical G protein-coupled receptors (GPCRs), that when activated play an important role in reversing the airway hyperresponsiveness (AHR) of asthmatic patients [1]. Typically, β_2-ARs couple to G_s proteins, activating signaling pathways that lead to bronchial smooth muscle relaxation. Thus, agonists for these receptors have been shown to be more efficacious than any other clinically used bronchodilators [2–5]. Consistent with this, short acting β_2-AR agonists are recommended as first line therapy for asthma exacerbations, while long-acting β_2-AR agonists (LABAs) are currently prescribed only with corticosteroids for maintenance therapy in patients refractory to other asthma controller medications [6]. However, chronic use of β_2-AR agonists has been associated with worsening of bronchial hyperresponsiveness to spasmogens [7], loss of vasthmacontrol [8], longer asthma exacerbations [9], and increased mortality [7,10,11]. These outcomes with β-AR agonist drugs are analogous to the results observed in congestive heart failure (CHF). This disease is characterized by low cardiac output. As a consequence, the body increases the release of adrenaline and noradrenaline to activate cardiac β-ARs in an attempt to improve cardiac function. Thus, the disease was managed for decades by using β-AR agonists such as dobutamine. However, clinical trials with these drugs in CHF showed that, as in asthma, chronic β-AR agonist therapy resulted in increased mortality [12,13].

On the other hand, β-AR antagonists/inverse agonists ('β-blockers') are contraindicated in asthma, because of reports that single doses or short-term administration of these drugs may cause severe worsening of asthma and even fatalities [14–16]. Again similar to asthma, 'β-blockers' were contraindicated in CHF because initially they make patients feel worse and decreases cardiac contractility [17]. However, large clinical trials using escalating doses of certain 'β-blockers' to decrease the initial adverse effects, have shown that chronic administration of certain 'β-blockers' increase cardiac inotropy and decrease mortality [18, 19].

Thus, we hypothesized that duration of treatment is a major determinant of the observed clinical response to β-AR drugs. Experiments with chronic use of 'β-blockers' in asthma were the last analogy to examine between CHF and asthma. That is, that certain 'β-blockers' are acutely detrimental, but chronically could be beneficial. In fact, in a murine model of asthma we have shown that nadolol and carvedilol acutely increased AHR to methacholine, but chronically reduced it, while alprenolol, a 'β-blocker' with weak agonist properties, did not reduce AHR with chronic administration [20].

Using an antigen-driven murine model of asthma, we have examined biochemical changes following chronic 'β-blocker' treatment in the levels of some of the proteins in the adenylate cyclase and phospholipase C (PLC) signaling pathways that regulate bronchial tone. We have also analyzed if chronic 'β-blocker' treatment can affect other G_s-coupled pathways in the airways. The results suggest that chronic treatment with certain 'β-blockers' induces several compensatory changes in the signaling pathways regulating bronchial tone, and potentiates the effects of other bronchodilating receptors in the lung. These findings are consistent with the paradoxical effect on airway resistance (R_{aw}) observed with chronic administration of certain β-blockers [20].

2. Materials and methods

2.1. Animals

Male, Balb/cJ mice aged 6 weeks (The Jackson Animal Laboratory, Bar Harbor, Maine) were used throughout this study. Mice were housed under specific pathogen-free conditions and given free access to water and chicken ovalbumin-free chow. The mice were treated as

humanely as possible and the Institutional Animal Care and Use Committee of the University of Houston approved all experiments reported in this investigation.

2.2. Antigen sensitization and challenge protocols

Mice were systemically sensitized with ovalbumin adsorbed to aluminum hydroxide by three intraperitoneal injections administered one week apart (protocol days 1, 8 and 15) and challenged by five daily nasal inhalation of the same antigen on protocol days 24–28 [21]. A group of non-ovalbumin-sensitized non-challenged mice served as controls. Ovalbumin-sensitized and ovalbumin-challenged mice, and non ovalbumin-sensitized non-challenged mice will be abbreviated as S/C and NS/NC mice, respectively.

2.3. Drug administration

For the determination of the β-AR subtype mediating the effect of chronic 'β-blocker' treatment, during the last 7 days of the protocol, S/C mice were chronically treated with either ICI 118,551 (a β_2-AR selective antagonist, dose: 10 mg/kg/d), or metoprolol (a preferential β_1-AR antagonist, at a dose of 10 or 20 mg/kg/d) by subcutaneous mini-osmotic pump® implantation at day 21. Doses were chosen from previous reports of effectiveness in mice [22,23]. One group of S/C mice was treated with dimethyl sulfoxide (DMSO) 50%, because it was the vehicle for ICI 118,551. All drugs were purchased from Sigma® (St. Louis, MO). Mini-osmotic pumps® (models 2001, Alzet, Durect Corporation, CA, USA) were implanted under anesthesia obtained after an intraperitoneal injection of 4–5 μl/g of a solution containing ketamine (40 mg/ml) and xylazine (6 mg/ml). Absences of corneal reflex and motor response to nociceptive stimuli were verified before the surgical procedures.

For the remaining experiments, we used 28 days of treatment with nadolol. For nadolol administration, S/C mice were fed (ad libitum) from protocol days 1 to 28 with mouse chow containing the drug at a concentration of 250 ppm. Previous experiments administering nadolol for 28 days after the sensitization process was complete (protocol days 18–45), produced identical results to administering nadolol from protocol days 1 to 28, meaning that β-AR ligand treatment does not interfere with the sensitization process. Non-treated NS/NC or S/C mice were fed with normal mouse chow.

2.4. Animal preparation and measurement of pulmonary function

On protocol day 28, mice were anesthetized with an intraperitoneal injection of 3–5 μl/g of a mixture containing ketamine (42.8 mg/ml), xylazine (8.6 mg/ml) and acepromazine (1.4 mg/ml). This slightly modified solution compared to the one described above for implantation of the mini-osmotic pumps® produced a longer lasting anesthesia. Once the animals lost corneal reflex, the absence of motor response to nociceptive stimuli was verified. Subsequently the tail vein was cannulated with a 27 × 3/8, 8 in. butterfly needle (Abbott, North Chicago, IL, USA). The animals were ventilated (10 ml/kg; 180 breaths/min) through a tracheal cannula connected to a computer-controlled small animal ventilator (Flexivent, Scientific Respiratory Equipment, Montreal). Airway resistance was measured by using the forced oscillation technique [24,25]. The complex input impedance of the respiratory system was computed as previously described [21], and the value of the real part of respiratory system impedance at 19.75 Hz was taken to reflect the magnitude of R_{aw}. To induce airway constriction, a solution containing 150 μg/ml of acetyl-*a*-methylcholine chloride (methacholine) (Sigma®, St. Louis, MO) was infused intravenously, using a syringe infusion pump (Raze Scientific Instruments, Stanford, CT, USA). The methacholine infusion was started at 0.0085 ml/min, and its rate was doubled stepwise up to a maximum of 0.136 ml/min. Each methacholine dose was administered for 5 min, during which data were sampled at 1 min intervals and then averaged. To examine the degree of airway responsiveness of each animal, the values for R_{aw} as a function of

methacholine doses were plotted. Peak R_{aw} is the maximal increase in R_{aw} measured during the methacholine dose–response curve.

2.5. Immunofluorescence

After deep anesthesia (as described above for the measurement of pulmonary function), lungs were exsanguinated by perfusing 10 ml of phosphate-buffered saline (PBS) via the right cardiac ventricle. Fixative (10 ml 4% paraformaldehyde in PBS, pH 7.0) was infused via the right cardiac ventricle and intratracheally *in situ* for 10 min at room temperature. Subsequently, the lungs and heart were removed from the thoracic cavity, washed three times with PBS, and placed into increasing sucrose concentrations (10%, 20%, and 30% sucrose in PBS) until tissues settled to the bottom of the tubes. Tissues were embedded in frozen tissue matrix (OCT®), cut into 12 μm sections longitudinally, and allowed to air dry before being fixed with 4% paraformaldehyde for 15 min. Three slides were obtained from each mouse, with 4–5 sections per slide. After three washes in PBS (10 min each), all sections were incubated in 0.1% Triton X-100 for 10 min, and then 10% normal goat serum in PBS for 1 h. Two of the three slides were then incubated overnight at 4 °C with either β_2-AR or β_1-AR C-terminal antibody (1:200, Santa Cruz Biotechnology). After incubation with or without primary antibodies, all slides were washed in PBS for 1 h and incubated with secondary antibody (1:200; Cy3-goat anti-rabbit IgG, Molecular Probes) overnight at 4 °C. Tissues incubated only with secondary antibodies were used as negative controls. Images were acquired using a Quantix EEV57 charge-coupled device (CCD) camera driven by ISee Imaging software (Raleigh, NC). Exposure times were the same for all samples, and the contrast was adjusted to give the same gray levels for background (usually <125). Morphometric analysis of the images was accomplished using the ISee Imaging software. For images of the lungs, a grid (2 × 7 μm) was projected onto the airway epithelium, and the gray levels were measured. The extent of immunostaining on the airway epithelium is expressed as the sum total of gray levels from each mouse lung divided by the number of bronchi examined (approximately 30 bronchi examined per mouse; n = 3–6 mice).

2.6. Quantitative immunoblotting

To measure the expression of $G\alpha_i$, PDE4D, $G\alpha_s$, GRK2, and GRK3 in lung homogenates, frozen lung tissues were chopped and homogenized in ice-cold homogenization buffer (25 mM Tris–HCl, 0.32 M sucrose, pH7.4) using a polytron homogenizer with three 30 s bursts at a setting of three. One Complete™ protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IL) was used per every 25 ml homogenization buffer. Homogenates were centrifuged at 1000 g for 10 min at 4 °C. The supernatant was filtered through cotton gauze and further centrifuged at 40,000 *g* for 20 min at 4 °C. The supernatant from this second centrifugation was stored at –80 °C as cytosolic fraction. The pellet was suspended in an ice-cold 25 mM Tris–HCl buffer (pH7.4) and centrifuged at 40,000 *g* for 20 min at 4 °C. The final pellet was suspended in 200 μl of the same buffer and stored at –80 °C as membranous fraction. To measure the expression of PLC-β1, the trachea and large bronchi were carefully dissected out and homogenized. This is because changes were not observed using whole lung homogenates and a previous study showed the change to occur in the airway smooth muscle cells [26]. Therefore, we used the trachea and large bronchia as a more concentrated source of airway smooth muscle. The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). The membranous or cytosolic fractions were re-suspended in SDS (0.7 μg/ml) sample buffer and heated at 95 °C for 5 min. This was followed by SDS–PAGE electrophoresis through 10% Tris–HCl gels (Bio-rad, ready gel). Proteins were electroblotted to immobilon filters (Millipore) and blocked by 5% non-fat milk for 1 h. The blot was then incubated with anti-$G\alpha_{i3}$ (Santa Cruz, 1:1000), anti-PDE4D (Febgennix, 1:500), anti-$G\alpha_s$ (Santa Cruz, 1:1000), anti-GRK2 (Santa Cruz, 1:500), anti-GRK3 (Santa Cruz, 1:100) or anti- PLC-β1 (Santa Cruz, 1:500) in 5% non-fat milk overnight. Again, the blots were washed

and blocked by non-fat milk, and incubated with secondary antibody (Jackson immunoresearch, 1:10,000). The bands were visualized by chemiluminescence (Pierce, IL), and the digital images were collected by CCD camera (Flurochem™). The blot was then stripped and reprobed by anti-β-actin (Santa Cruz, 1:1000), which was used as a loading control. The density of $G\alpha_{i3}$, $G\alpha_s$, GRK2, GRK3, and PDE4D bands were normalized to actin bands and quantified using AlphaEase software (Alpha Innotech, San Leandro, CA). After normalizing the density of PLC-β1 to β-actin, the ratio of PLC-β1/β-actin in different treatment groups was compared to the control group.

2.7. Isometric tension studies on tracheas

After 28 days of nadolol in food or control chow, mice were killed with a lethal dose of pentobarbital (0.1 ml of 65 mg/ml). Tracheas were carefully isolated, then dissected free of connective tissue and placed in cold and gassed (5% CO_2 in O_2) Krebs–Henseleit solution (K–H) (mmol/l): NaCl 120, KCl 4.7, $MgCl_2 \cdot 6H_2O$ 1.2, $NaHCO_3$ 25, $NaH_2PO_4 \cdot H_2O$ 1.2, D-Glucose 11, $CaCl_2$ 2.5. Approximately equal size tracheal rings were suspended in organ baths (15 ml) with one end attached to an isometric force transducer (Harvard Apparatus, Inc., Holliston, MA) and the other to a tissue holder. After setting resting tension at 0.5 g, the tissues were equilibrated in K–H at 37 °C and bubbled with 5% CO_2 in O_2 for 1 h. The bath solution was changed every 15 min. After equilibration, the tracheas were contracted with methacholine with 3 or 10 μM (approximately the EC_{80} methacholine concentration) and changes in tone were recorded using a polygraph (model 7754A, Hewlett Packard, USA). After a stable level of contraction to methacholine was achieved, the IP receptor agonist cicaprost (Schering Akiengesellschaft) or the EP_2 receptor agonist CAY 10399 (Cayman Chemical) were cumulatively added to the baths to test their relaxant effects. For tracheas from chronically treated mice, the K–H solution was supplemented with nadolol (10^{-5} M) to maintain exposure of the receptor to the drug.

2.8. Data analysis

Results were expressed as mean ± S.E.M. Comparisons of R_{aw} dose–response curves were performed by one-way ANOVA followed by Bonferonic post-test. Western blots densities, and the tracheal relaxation in the organ bath experiments between β-AR ligand-treated and non-treated mice were performed using one-way ANOVA followed by Dunnett's multicomparison test. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of treatment with the preferential β-AR subtype ligands on airway responses to methacholine

The intravenous administration of methacholine increased R_{aw} in a dose-dependent manner (Fig. 1A). There was no significant difference between S/C mice without treatment receiving regular mouse chow or treated with vehicle (DMSO), but both groups exhibited a significantly greater bronchoconstrictor response to methacholine compared to NS/NC mice (Fig. 1A). In S/C mice treated with ICI 118,551, there was a significant reduction in the peak R_{aw} response to methacholine compared with S/C vehicle-treated mice (Fig. 1B). When the S/C mice were treated with metoprolol 10 mg/kg/d for 7 days, their average methacholine dose–response relationship was similar to that obtained in non-treated S/C mice (Fig. 1C). Increasing the dose of metoprolol to 20 mg/kg/d did result in a significant reduction in peak R_{aw} (Fig. 1D).

3.2. Effect of chronic nadolol treatment on airway responses to methacholine

Treatment for 28 days with the non-selective β-AR antagonist/inverse agonist, nadolol, was again [20] able to significantly decrease the maximal airway constriction induced by

methacholine (Fig. 1E). Since nadolol produced similar reductions in AHR to ICI 118,551, we used nadolol for further experiments because of the tremendous cost differential (ICI 118,551 is over 500 times more expensive than nadolol) and ease of administration (nadolol is administered in the chow while ICI 118,551 requires implantation of osmotic minipumps due to its short half-life).

3.3. Immunofluorescence staining for β_1-ARs and β_2-ARs

Airways immunostained for β_1-ARs and β_2-ARs displayed changes in fluorescence intensity amongst the different treatment groups. Results show the β_1-AR (weak fluorescence intensity) and the β_2-AR (strong fluorescence intensity) expression is localized predominantly to the airway epithelium (Fig. 2, column A and B). In S/C mice, there was a decreased expression for β_2-ARs compared to NS/NC mice (Fig. 2, column B). However, expression for β_2-ARs was restored in S/C mice treated with ICI 181,551, and to a lesser extent with metoprolol 10 mg/kg/d (Fig. 2, column B). Treatment with metoprolol 20 mg/kg/d showed a further increase in β_2-ARs compared to the 10 mg dose (Fig. 2, column B). No appreciable difference in expression for β_1-ARs was seen amongst the different treatment groups (Fig. 2, column A). Though we had previously reported an increase in total β-AR density using radioligand binding assays, these experiments show that the upregulation is mainly of the β_2-AR subtype and occurs primarily in the airway epithelial cells. The bar graph shows quantified β_1-AR and β_2-AR fluorescence intensity in the airway epithelium (Fig. 3A). The β_1-AR and β_2-AR expression in the left cardiac ventricle of the heart from NS/NC mice were used to further assess the specificity of the antibodies (Fig. 3B).

3.4. Expression of proteins involved in receptor pathways modulating bronchial tone

Immunoblotting studies were conducted to investigate the expression of PDE4D, $G\alpha_{i3}$, $G\alpha_s$, GRK2, and GRK3 in S/C mouse lungs, with or without nadolol treatment and in NS/NC mouse lungs. In the cytosolic fraction of lung homogenates, chronic treatment with nadolol decreased the expression of PDE4D; whereas, no significant difference between NS/NC and S/C mice was identified (Fig. 4A). $G\alpha_{i3}$ expression was higher in the membrane fraction of lung homogenate from S/C mice than NS/NC mice, and $G\alpha_{i3}$ expression was decreased to NS/NC levels by chronic nadolol treatment (Fig. 4B). Compared to NS/NC mice, S/C mice had higher levels of PLC-β1 in trachea and large bronchial homogenates. Again chronic nadolol treatment significantly reduced the levels of PLC-β1 to the level in NS/NC (Fig. 4C). No changes were observed in the levels of $G\alpha_s$, GRK2, and GRK3, in any of the treatment groups (data not shown).

3.5. Tracheal responses to cicaprost and CAY 10399

The effect of chronic nadolol treatment on the tracheal responses to IP and EP_2 receptor agonists was measured by contracting the tracheas with methacholine and then performing a dose–response curve with cicaprost, and CAY 10399, respectively. Cicaprost and CAY 10399-induced concentration-dependent relaxations of the methacholine contracted mouse tracheal preparations. Chronic nadolol treatment produced a significant enhancement in the tracheal relaxant responses to cicaprost and CAY 10399 at concentrations $\geq 0.3\ \mu M$ ($P < 0.05$). Starting methacholine-induced tension was similar among the groups and the absolute decrease in tension produced by either cicaprost or CAY 10399 was also larger after chronic nadolol treatment (Fig. 5 and legend).

4. Discussion

We have previously shown that the non-selective 'β-blockers' nadolol and carvedilol, reduced AHR to methacholine when administered chronically, although they increased it with acute administration [20]. Mice airways express both β_1 and β_2-AR subtypes [27], and the previous

study used nadolol and carvedilol, which are non-selective β-blockers. Therefore, we investigated which β-AR subtype mediated the chronic bronchoprotective effects by using the selective β_2-AR antagonist ICI 118,551 (β_2:β_1≈550:1) or the preferential antagonist for the β_1-AR metoprolol (β_1:β_2≈2.3:1) [28]. We also examined the effects of chronic nadolol treatment on proteins in some of the signaling pathways involved in regulating bronchial smooth muscle tone. Though ICI 118,551, nadolol and metoprolol can function as β-AR inverse agonists in systems with sufficient constitutive receptor activity, we will refer to them as antagonists in this study since we know that they do not possess any agonist properties, but have no evidence that they function as inverse agonists in our murine model.

The drugs most commonly prescribed for acute relief of asthma symptoms are targeted to activate the β_2-AR subtype, which mediates airway relaxation. Besides this effect, activation of β_2-ARs in lungs are thought to mediate a number of beneficial effects in asthma, including enhanced mucociliary function of epithelial cells, decreased permeability of the vascular endothelium, decreased histamine release from mast cells, and a modification of neurotransmitter release from the autonomic nervous system [2,29]. However, persistent β_2-AR stimulation results in receptor desensitization, including receptor uncoupling from G_s, internalization and downregulation [30]. Chronic β_2-AR stimulation has also been associated with tolerance [31], bronchial hyperresponsiveness [7], loss in bronchoprotection [7], and asthma mortality [10,32]. We hypothesized that if chronic treatment with agonists can induce these detrimental effects, chronic β_2-AR antagonist treatment may produce the opposite effects, that is: receptor sensitization, upregulation, bronchoprotection, and perhaps a decrease in asthma mortality.

In this study, we show that chronic treatment with the selective β_2-AR antagonist ICI 118,551 decreases AHR in a murine model of asthma (Fig. 1B), while the preferential β_1-AR antagonist metoprolol, at a dose of 10 mg/kg/d, did not change the airway response compared to sensitized and challenged (S/C) animals (Fig. 1C). These results suggest the β_2-AR is mediating the beneficial effect of chronic treatment with antagonist. Metoprolol at a higher dose of 20 mg/kg/d significantly reduced AHR (Fig. 1D). The decrease in AHR with the higher dose could be explained because of metoprolol's low selectivity for β_1-ARs. Thus, higher doses of metoprolol would bind to β_2-ARs and affect that pathway. Indeed immunofluorescence of the β_2-AR showed an upregulation in S/C mice chronically treated with metoprolol 20 mg/kg/d, though this increase was only slightly larger than the upregulation produced by metoprolol 10 mg/kg/d (Figs. 2, column B and 3A). However, the results with the highly selective β_2-AR antagonist ICI 118,551 strongly implicate the β_2-AR as responsible for the beneficial effect on AHR.

Besides demonstrating that it is the β_2-AR subtype that is upregulated, the immunofluorescence for β_2-ARs showed that the fluorescence intensity in NS/NC mice is located predominantly on the airway epithelium. The fluorescence intensity from β_2-ARs was decreased in S/C mice (Figs. 2, column B and 3A), while there was no appreciable difference in the staining for β_1-ARs amongst the different treatment groups (Figs. 2, column A and 3A). These results could imply a pathophysiological impairment of β_2-AR signaling in this murine model, and the AHR to bronchoconstrictors may be explained by the reduced signaling through this bronchodilating pathway. Although an alteration of the β-AR relaxant pathway in smooth muscle cells has been theorized in human asthma [33,34], changes in the β_2-AR densities have not been found in lungs of severe asthmatics [35]. However, these measurements were not made during, or just after, an asthma attack, and reductions in β-ARs have been shown in lymphocytes after bronchial provocation with antigen [36], and in acute asthma [37]. These data show that chronic 'β-blocker' administration producing a reduction in AHR is associated with increased β_2-AR densities. These data suggest that the beneficial effect of certain 'β-blockers' is mediated by the β_2-AR. A theory confirmed by the fact that the highly selective β_2-AR antagonist, ICI

118,551 is fully capable of attenuating the observed AHR in S/C mice, and upregulating the β_2-ARs (Figs. 1B and 2, column B).

Since the upregulation of receptor density occurs in the presence of a drug that is presumably inactivating at least a portion of the receptor population, this may not fully explain the beneficial effects of chronic 'β-blocker' treatment. Also, we have used the catecholamine-depleting agent, guanadrel, and the corticosteroid, dexamethasone, to produce a comparable receptor upregulation to nadolol and have not seen an improvement in AHR (data not shown). Therefore, since receptor upregulation alone may not be sufficient to produce the bronchoprotective effect, we further investigated the influence of chronic β-blocker treatment on lung β_2-AR signaling. The majority of functions of the β_2-AR are mediated through G_s protein in a cAMP-dependent manner. PDE4D is the major PDE species degrading cAMP in airway epithelia, and smooth muscle [38–41]. PDE4D knockout mice, with or without antigen challenge, were resistant to cholinergic airway contraction [42,43]. In our animal model, chronic nadolol treatment decreased PDE4D expression in lung homogenates, mimicking the phenotype of PDE4D knockout mice [42,43].

Though we saw no changes in G_s, an increasing body of evidence has demonstrated that the β_2-AR also couples to G_i in rodent, canine and human hearts [42–47]. Also, in a rabbit antigen-driven model of asthma, the tracheal relaxant response to the β-AR agonist isoproterenol was impaired compared to control rabbits. This impairment of the β-AR mediated relaxation was corrected by inactivation of G_i proteins by incubating the trachea with pertussis toxin, suggesting the presence of β_2-AR-G_i coupling [48]. The G_i coupling pathway decreases the activity of AC and also contributes to the desensitization of β_2-ARs. Although we saw no changes in the levels of GRK2 or GRK3, we did observe an increase expression of $G_{\alpha i3}$ protein in our murine model of asthma as has been reported in the bronchial smooth muscle of rats with an antigen-induced AHR [49]. In our study, nadolol treatment decreased the elevated expression of $G_{\alpha i3}$, perhaps attenuating G_i signaling.

Studies on transgenic mice with overexpressed β_2-AR on airway smooth muscle show an increase in the expression of PLC-β1, while the β_1/β_2-AR double knockout down-regulates the enzyme [26]. In our studies with the murine asthma model, PLC-β1 was higher in S/C as compared to NS/NC mice, suggesting that G_q signaling was enhanced, perhaps resulting in hypersensitivity to spasmogens that stimulate G_q-coupled receptors such as the methacholine-mediated AHR. Chronic treatment with nadolol resulted in a reduction of PLC-β1 in the S/C mice suggesting that inactivation of the β_2-AR by nadolol may produce a down-regulation of PLC-β1. This data is consistent with the proposed cross-talk between β_2-AR and G_q-coupled receptors, suggested by Liggett and colleages [26].

In tracheas from S/C mice chronic nadolol treatment enhances the relaxant responses of the EP_2 receptor agonist CAY 10399, as well as the IP receptor agonist cicaprost. Studies showing that chronic 'β-blocker' treatment can produce enhanced responses via other receptor systems have previously been reported. The use of preferential β_1-AR blockers has been shown to produce enhanced signaling via β_2-AR [50], 5-HT_4 receptors [51], and H_1/H_2 receptors [52]. Also, cellular studies using inverse agonists of the serotonin (5-HT_{2C}) receptors have shown inverse agonism of these receptors results in sensitization of purinergic receptors [53]. There are also reports of very close interactions and cross-talk between prostanoid receptors and the β_2-AR system [26,54], and even reports of chronic 'β-blocker' treatment producing enhanced cAMP production in response to prostacyclin in lymphocytes from hypertensive patients [55].

In conclusion, the present study has provided evidence for four findings potentially related to the reduction of AHR produced by chronic 'β-blocker' treatment in a murine model of asthma:

(1) β_2-ARs are the main subtype responsible for decreasing the hyperresponsiveness to methacholine; (2) β_2-ARs are upregulated; (3) treatment with nadolol downregulates the expression of some molecules implicated in airway contraction such as G_i, PDE4D, PLC-β1; and (4) chronic nadolol treatment enhances the bronchodilatory responses of IP and EP_2 receptor agonists. Further studies are needed to conclusively explain the paradoxical effect of chronic treatment with certain 'β-blockers' on AHR in this murine model. After all, in the CHF field, where many laboratories have been searching for (a) definitive mechanism(s) for over a decade, there is still no conclusive answer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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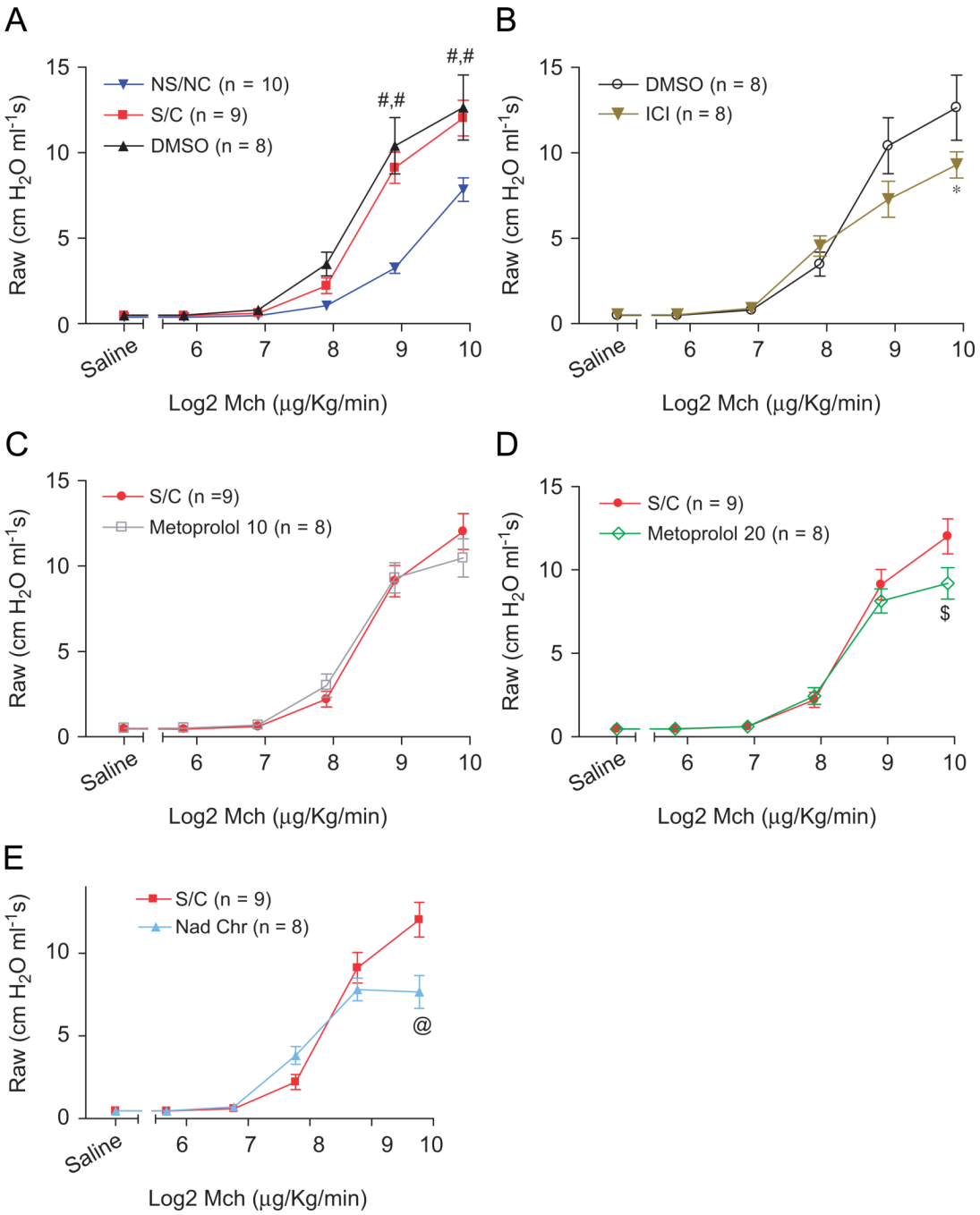

Fig. 1.

Effects of treatment with subtype specific *β*-AR ligands and nadolol on airway hyperresponsiveness to methacholine in murine model of asthma. Cumulative concentration–response curves to methacholine-induced increases in airway resisitance (R_{aw}) in NS/NC and S/C mice treated with or without 50% DMSO (A), S/C mice treated with or without ICI118,551 (ICI) in 50% DMSO (B), and S/C mice treated with or without metoprolol 10 mg/kg (C), metoprolol 20 mg/kg (D) for 7 days, and nadolol 250 ppm (E) for 28 days. For the *β*-AR subtype experiments, treatment started from day 21 to day 28. For nadolol, treatment was from days 1 to 28. Values represent mean and vertical lines show S.E.M. (n = 7–10). #, $P < 0.001$ compared

with NS/NC; *, $P < 0.05$ compared with DMSO; $, $P < 0.01$, @, $P < 0.001$ compared with S/C.

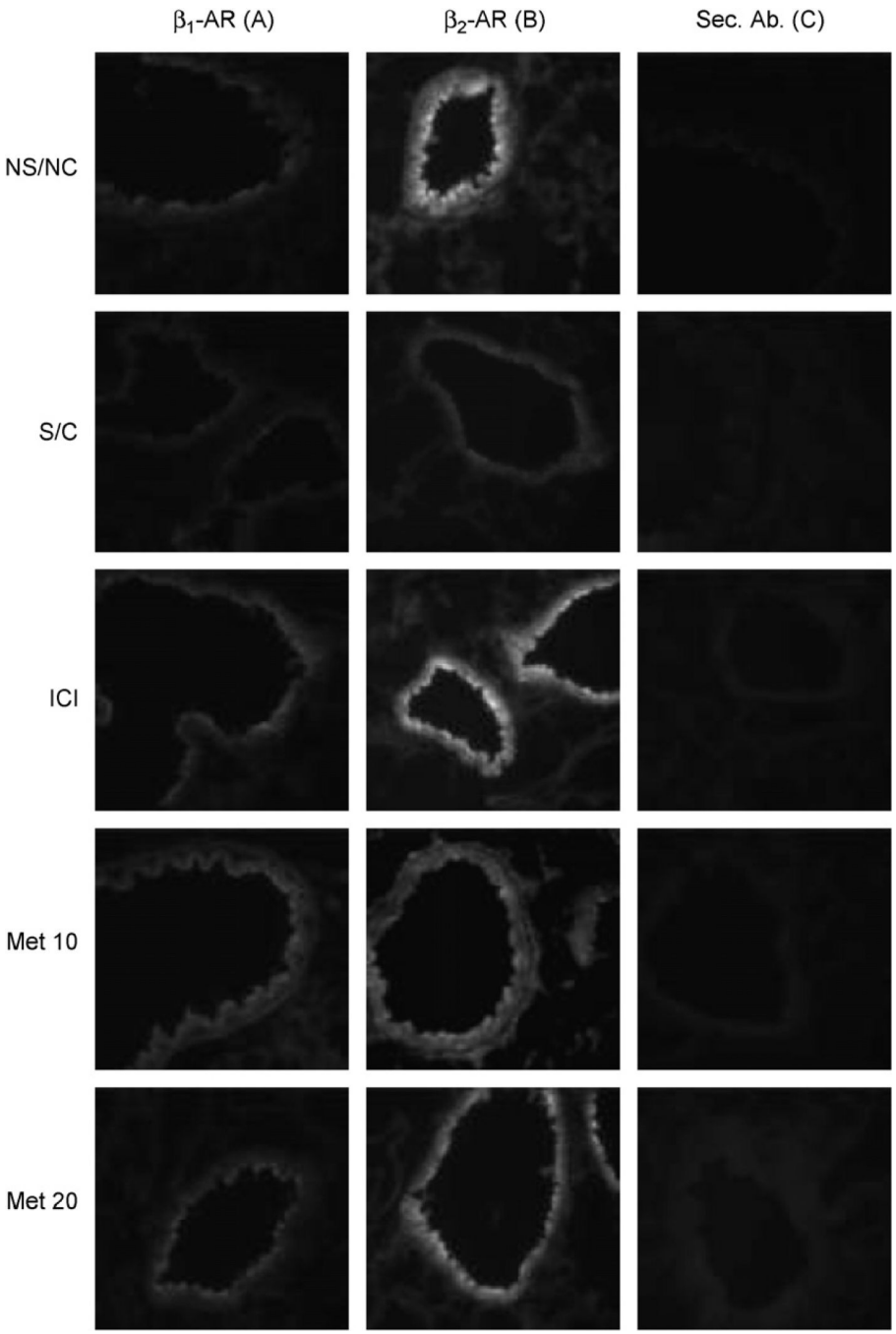

Fig. 2.

Immunofluoroscence staining for specific *β*-AR subtypes. Tissue sections of the lung obtained from NS/NC (*n* = 6), S/C (*n* = 5) ICI 118,551 (ICI; *n* = 6), metoprolol 10 mg/kg/d (Met 10; *n* = 3), and metoprolol 20 mg/kg/d (Met 20; *n* = 3), were immunostained with β_1-AR (A) and β_2-AR (B) specific antibodies. The fluorescence intensity of the images was localized predominately to the airway epithelium. Tissues incubated only with secondary antibodies (C) were used as negative controls. Original magnification is 20 ×.

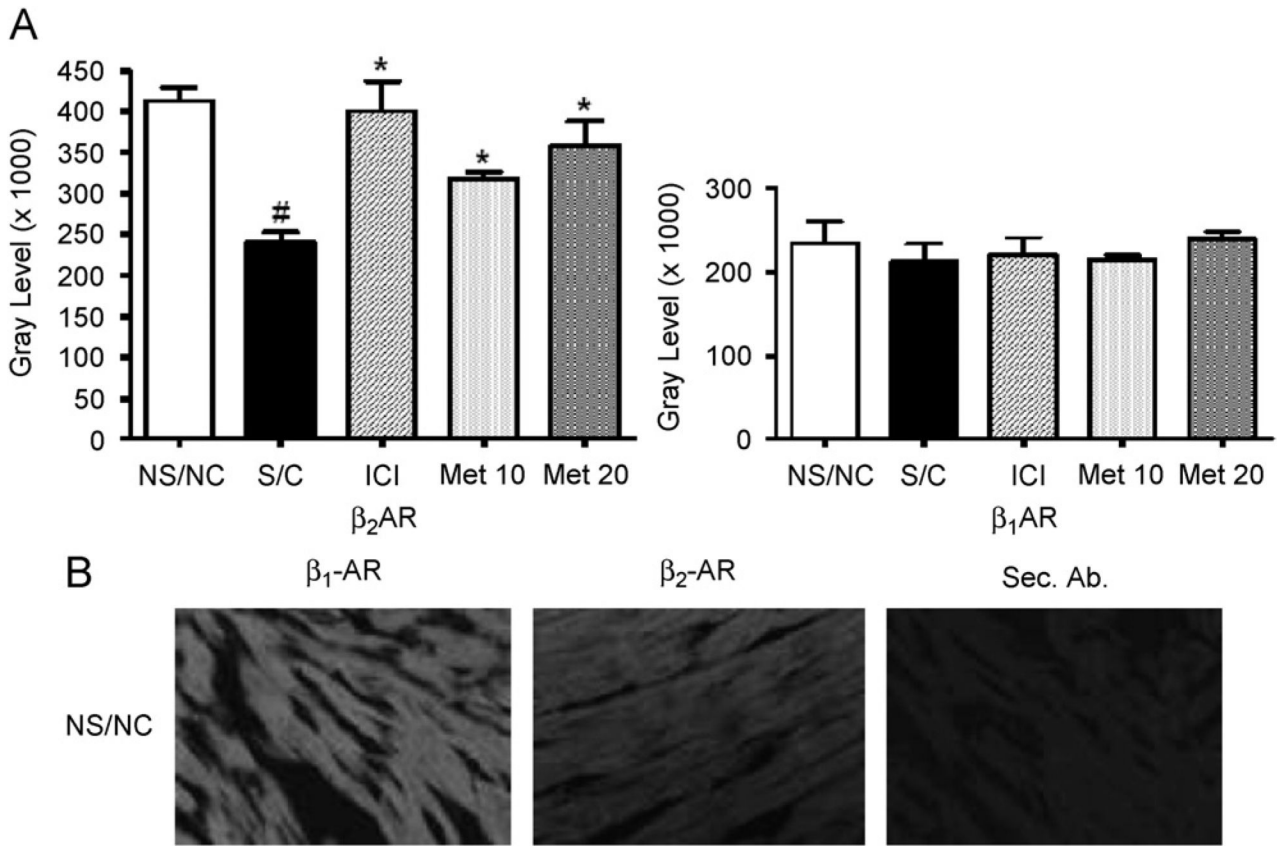

Fig. 3.

Morphometric analysis to quantify the expression of β-AR subtypes. Measured fluorescence intensity from the airway epithelium of NS/NC and S/C mice, and S/C mice treated with ICI 118,551 (ICI), metoprolol 10 mg/kg/d (Met 10), and metoprolol 20 mg/kg/d (Met 20) (A); tissue sections immunostained with β_1-AR and β_2-AR specific antibodies from the left cardiac ventricle of NS/NC (n = 6) mice (B). Tissues incubated only with secondary antibodies were used as negative controls. Original magnification is 20 ×. Data represents the mean ± S.E.M. (n = 3–6) mice. *, $P < 0.05$ compared with S/C; #, $P < 0.05$ compared with NS/NC.

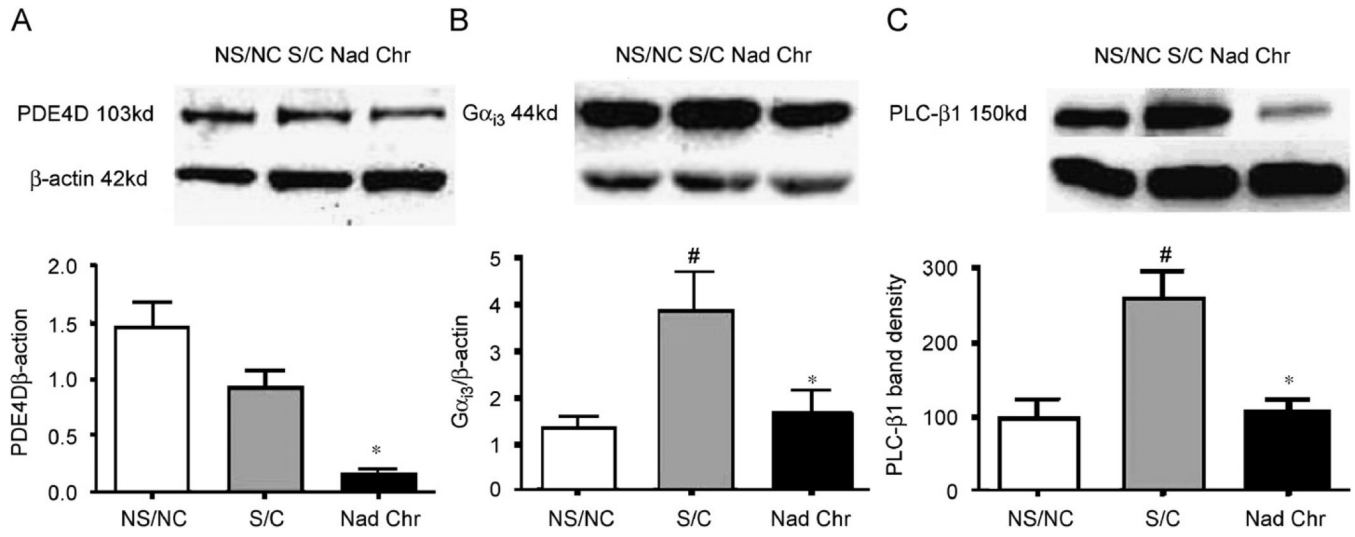

Fig. 4.

Immunoblotting of PDE4D, $G\alpha_{i3}$ and PLC-β1. Western blotting of PDE4D in cytosolic fraction (A), $G\alpha_{i3}$ in membranous fraction of lung homogenates (B) and PLC-β1 in trachea and large bronchi homogenate (C). Expression of β-actin was also measured as a loading control. Representative band images are shown in the upper panels. Densitometric analysis of protein of interest/β-actin ratios are shown in the lower panels as mean ± S.E.M. (n = 6–13). Nad Chr (nadolol chronic). *, $P < 0.05$ compared with S/C; #, $P < 0.05$ compared with NS/NC.

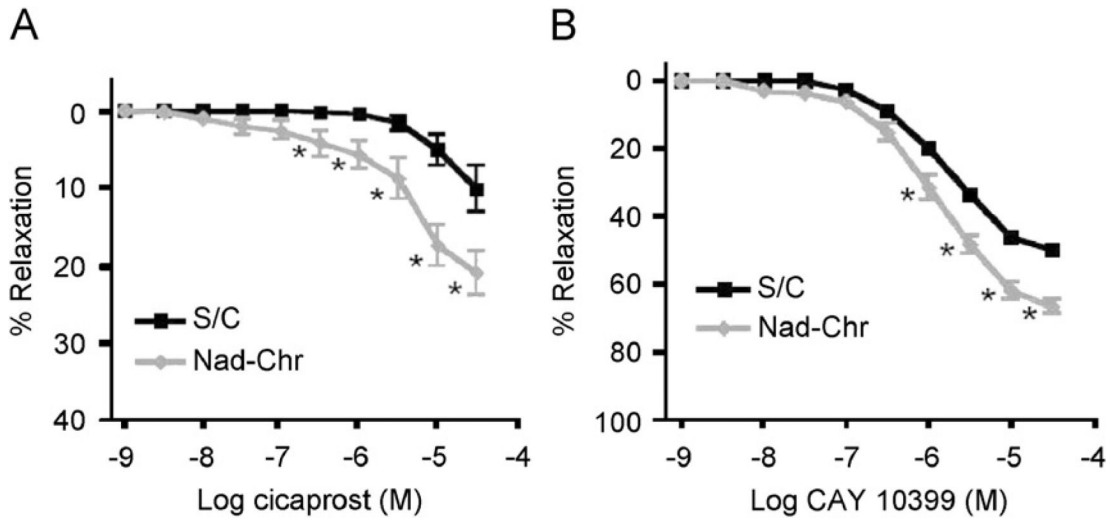

Fig. 5.

Effects of nadolol chronic treatment on isolated hyperresponsive mouse trachea. Cumulative concentration–response curves to cicaprost (A) and CAY 10399 (B) in the mouse isolated trachea after methacholine precontraction. Each point represents mean and vertical lines show S.E.M. (n = 5–6). The methacholine contraction was obtained by the addition of the approximate EC_{80} methacholine, 3 μM for (A) and 10 μM for (B) methacholine and produced a contraction of 2.436 ± 0.362 g and 2.646 ± 0.451 g, respectively. Nad Chr (nadolol chronic). *, $P < 0.05$ compared with S/C.