Budesonide Prevents Cytokine-Induced Decrease of the Relaxant Responses to Formoterol and Terbutaline, but Not to Salmeterol, in Mouse Trachea

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ABSTRACT

During asthma exacerbations, increased airway inflammation may impair the effects of β2-adrenoceptor (β2AR) agonists. It is unclear whether this impairment is prevented by inhaled glucocorticoids (GCs). We have investigated the relaxation of carbachol-contracted mouse tracheal segments to the β2AR agonists formoterol, terbutaline, and salmeterol. The segments were pre-exposed for 4 days to the proinflammatory cytokines tumor necrosis factor α (100 ng/ml) and interleukin-1β (10 ng/ml) with or without the GC, budesonide (1 μM). Formoterol and terbutaline induced greater maximal relaxation (Rmax) than salmeterol. The cytokines decreased Rmax of all β2AR agonists, whereas budesonide had no effect. However, after concomitant treatment with cytokines and budesonide, the Rmax values of formoterol and terbutaline were not impaired, whereas budesonide did not prevent the decrease in the Rmax of salmeterol. A similar pattern was observed for cAMP production by the agonists. In tracheal smooth muscle, β2AR mRNA was not affected by the cytokines but increased with budesonide. However, the cytokines markedly increased cyclooxygenase (COX)-2 mRNA expression, which may lead to heterologous desensitization of β2AR. It is noteworthy that the cytokine-induced increase of COX-2 was blocked by concomitant budesonide suggesting that heterologous desensitization of β2AR by the cytokines may be prevented by budesonide treatment. Budesonide prevented cytokine-induced impairment of the tracheal relaxation and β2AR/cAMP signaling for formoterol but not for salmeterol. This suggests that differences exist between formoterol and salmeterol in β2AR coupling/activation and/or signal transduction upstream of cAMP. These results imply that maximal bronchodilator effects of formoterol, but not of salmeterol, are maintained by budesonide treatment during periods with increased inflammation, such as asthma exacerbations.

Asthma, a chronic inflammatory disease in the airways, is most successfully treated with inhalation of a glucocorticoid (GC) in combination with a long-acting β2-adrenoceptor (β2AR) agonist (Pauwels et al., 1997). During periods of increased disease activity, this therapy has further potential to intensify both the activation of GC and the β2AR pathways to prevent exacerbations (O’Byrne et al., 2005; Rabe et al., 2006; Kuna et al., 2007). The exact mechanisms responsible for the superior effect of this drug combination are not completely understood, but may involve beneficial effects of GCs on β2AR function, which is known to be decreased in asthmatic airways (Barnes and Pride, 1983; Bai, 1990).

Several proinflammatory cytokines are increased in the airways of asthmatic subjects (Broide et al., 1992; Tonnel et al., 2001). Among them, interleukin-1β (IL-1β) has been shown to attenuate the responses to β2AR agonists in human smooth muscle cells in vitro (Shore et al., 1997) and in vivo in rats (Koto et al., 1996). This was also observed in animal whole tissue preparations (Wills-Karp et al., 1993; Hakonarson et al., 1996) when IL-1β was applied together with tumor necrosis factor α (TNFα). Thus, increased levels of IL-1β and TNFα in the airways of asthmatic subjects, especially during exacerbation periods, may impair bronchodilating effects of β2AR agonists. It is conceivable that this process may be counteracted by treatment with GCs (O’Byrne et al., 2001), which are known to switch off activated genes of multiple proinflammatory cytokines.

ABBREVIATIONS: GC, glucocorticoid; β2AR, β2-adrenoceptor; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; COX, cyclooxygenase; PCR, polymerase chain reaction; Ct, cycle threshold; EIA, enzyme immunoassay; Gs, stimulatory G-protein; Gi, inhibitory G-protein; ANOVA, analysis of variance; CYP2D6, CYP3A4.

[2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulfonate; ICI 118551, (-)-1-[2,3-dihydro-7-methyl-1H-inden-4-yl]oxy]-3-[1-methylethy]lamino]-2-butanol, BUD, budesonide.
The aim of this study was to investigate 1) the extent to which relaxation responses of long-acting β2AR agonists, formoterol and salmeterol, and short-acting terbutaline, were impaired in smooth muscle exposed to the proinflammatory cytokines IL-1β and TNFα, and 2) the effect of concomitant budesonide treatment on the impairment of these responses by proinflammatory cytokines. To determine the β2AR responses in inflamed airways, organ culture of mouse tracheal segments were used because of their ability to maintain the smooth muscle function after long-term treatment with inflammatory agents (Adner et al., 2002). To further investigate the mechanisms behind the effects of cytokine exposure and drug treatments, the expression of the β2AR, the induction of cyclooxygenase (COX)-2 by the cytokines (which may lead to heterologous desensitization of β2AR), and the cAMP production induced by formoterol and salmeterol were also studied.

Materials and Methods

Materials. Dulbecco’s modified Eagle’s medium, carbachol, indomethacin, (±)-propranolol, CGP 20712A, and IC 118551 were obtained from Sigma-Aldrich (St. Louis, MO), penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA), and recombinant murine tumor necrosis factor α and interleukin 1β were from R&D Systems Europe Ltd (Abingdon, Oxfordshire, UK). 3-isobutyl-1-methylxanthine (dissolved in methanol) was obtained from Fluka (Buchs, Switzerland). Formoterol (dissolved in methanol), terbutaline (dissolved in saline), salmeterol, and budesonide (both dissolved in ethanol) were supplied from AstraZeneca R&D Lund (Lund, Sweden).

Tissue Preparation and Organ Culture. Nine- to 10-week-old male BALB/c mice were killed by cervical dislocation. All animal experiments were approved by the regional committee of animal experimentation ethics (permission N152/06). Whole lungs with tracheas were rapidly removed and placed into Dulbecco’s modified Eagle’s medium (4500 mg/l Na-glucose, 110 mg/l sodium pyruvate, 584 mg/l 1-glutamine), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Individual tracheas were thereafter dissected free from adherent tissue under sterile conditions. For in vitro pharmacology experiments, each trachea was divided into four segments with two or three cartilage rings, whereas for mRNA extraction the whole tracheas were used. Parenchymal tissues were cut into longitudinal strips not exceeding 10 × 1 × 1 mm. The tracheal tissues and parenchymal strips were incubated in Dulbecco’s modified Eagle’s medium at 37°C in humidified 5% CO2 air for 4 days (Adner et al., 2002) under control conditions or with TNFα (100 ng/ml) + IL-1β (10 ng/ml) and with/without concomitant budesonide (1 μM). The tissues were moved into a new well containing fresh media and substances every day. For mRNA expression analysis, the tracheal segments were, after the culture period, placed in RNAlater (QIAGEN GmbH, Hilden, Germany), and the smooth muscle layer was dissected free. The parenchymal strips for the radioligand-binding analysis were frozen at −20°C.

Isometric Force Measurement. Tracheal smooth muscle reactivity was analyzed in temperature-controlled (37°C) myographies (Organ Bath model 700MO; J. P. Trading, Aarhus, Denmark) containing Krebs-Henseleit buffer solution composed of 143 mM Na+, 5.9 mM K+, 1.5 mM Ca2+, 2.5 mM Mg2+, 128 mM Cl−, 1.2 mM H2PO4−, 1.2 mM SO42−, 25 mM HCO3−, and 10 mM d-glucose. The solution was continuously equilibrated with 5% CO2 and 95% O2 resulting in a pH of 7.4. The tracheal segments were mounted on two L-shaped metal prongs. One prong was connected to a force-displacement transducer for continuous recording of isometric tension by the Chart software (ADInstruments Ltd., Hastings, UK). The other prong was connected to a displacement device, allowing adjustment of the distance between the two parallel prongs. To avoid any effect of prostanoid secretion in the preparations (Bachar et al., 2005), indomethacin was always present 30 min before and during agonist administration.

In Vitro Pharmacology. After 1 h equilibration at the optimal tension of 0.8 mN (Adner et al., 2002), a concentration-effect curve of carbachol was obtained. After a 30-min resting period, the segments were precontracted to generate a submaximal carbachol contraction approximately 50 to 60% of the maximal contraction (EMax) for each segment. A post hoc analysis of the contractile level before β2AR antagonism showed 61.2 ± 2.0, 66.8 ± 2.1, 58.8 ± 1.9, and 59.5 ± 2.7% of Emax for the control, the budesonide, the TNFα + IL-1β, and the budesonide together with TNFα + IL-1β groups, respectively (there were no statistically significant differences between the groups). Subsequently, at a stable contractile level, concentration-effect curves to formoterol, terbutaline, and salmeterol were obtained by cumulative dosing.

mRNA Expression in Mouse Tracheal Smooth Muscle. Strips of tracheal smooth muscle layer from two mice were pooled into each sample, and mRNA extraction was performed by use of the RNeasy Mini kit (QIAGEN). The purity of total RNA was ensured spectrophotometrically by a wavelength absorption ratio (260/280 nm) between 1.6 and 2.1 in all preparations. Reverse transcription of total RNA to cDNA was performed by use of Omniscript reverse transcriptase kit (QIAGEN) in a 20-μl volume at 37°C for 1 h by using a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, St. Leon-Rot, Germany). Real-time PCR was performed in 25-μl volumes and with/without budesonide treatment on the impairment of these responses in inflamed airways, organ culture of mouse tracheal segments for this method, the ligand binding to β2ARs was determined by subtracting the Ct values for these genes from the Ct value for the housekeeping gene β-actin (ΔCt). Specific primers for the mouse β2AR and β1AR, and the housekeeping gene β-actin were designed by use of Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and synthesized by DNA Technology AS (Aarhus, Denmark). Sequences as follows: β2AR: forward, 5′-TGCGATTTGGCTCACCAACA-3′; reverse, 5′-CGGAACCCCGCGAGTAC-3′; β1AR: forward, 5′-CTATCGATTGTTACACCGAGGAGA-3′; reverse, 5′-AATAGACAAAGAGCCATCACCACA-3′; COX-2: forward, 5′-ATACCACTGTGTTACACCGAGGAGA-3′; reverse, 5′-CTGCTGCTGCTGCTGCTG-3′; β-actin: forward, 5′-TGG GTC AGA AGG ACT CCT AGT TG-5′; reverse, 5′-CTT CCC ACT TGG TAA CAA TGC-3′. The amount of mRNA of β2AR, β1AR, and COX-2 was expressed as the number of moles per 100,000 mol of β-actin.

Radioligand Binding in Lung Tissue. Radioligand-binding studies were performed to explore how the receptor protein was affected by exposure to the cytokines and treatment with budesonide. Because of the insufficient amount of smooth muscle in tracheal segments for this method, the ligand binding to β2ARs was studied in membrane prepared from lung parenchyma. The frozen lung tissue was thawed, minced, and homogenized for 2 × 10 s in ice-cold buffer (50 mM Tris-HCl; pH 7.5, at room temperature). The homogenate was centrifuged at 1000g for 10 min at 4°C, and the pellet was discarded. Thereafter, the supernatant was centrifuged at 46,000g for 30 min at 4°C, the resulting pellet was resuspended, and the obtained membranes were used in the binding assay. The membranes were incubated at 25°C with [3H]-labeled dihydroalprerol (PerkinElmer Life and Analytical Sciences, Waltham, MA; dissolved in Tris-HCl at 4 μM and used as an estimate of Emax) in the absence (total binding) and the presence (nonspecific binding) of 10 μM (±)-propranolol. The difference between total and nonspecific bind-
ing was defined as the specific binding. In the displacement studies, the ability of the βAR antagonists CGP 20712A (β2AR selective; 100 nM) and/or ICI 118551 (β2AR selective; 70 nM) to compete with 3H-labeled dihydralprenolol for the binding sites was determined. The incubation was terminated after 30 min, and the samples were diluted with 1 ml of ice-cold buffer followed by filtration through glass fiber filters (Whatman GF/C; Whatman, Maidstone, UK) under reduced pressure. Thereafter, the membranes were washed with 3 × 5 ml ice-cold buffer. The radioactivity retained on the filters was assessed by liquid scintillation counting. The density of β2AR was given as femtomoles per milligram protein.

**cAMP Measurement.** After culture, the tracheae were placed in a tissue bath containing Krebs-Henseleit buffer solution and continuously equilibrated with 5% CO2 and 95% O2 at 37°C as during the isometric force measurement. The tissue was equilibrated for 45 min; thereafter, 200 μM 3-isobutyl-1-methylxanthine, a nonspecific inhibitor of phosphodiesterases, was administered, and after a further 25 min the tracheae were precontracted with carbachol as described above. After a further 15 min, the segments were exposed to a single concentration of formoterol or salmeterol for 5 min (a time period that was found to give a stable relaxant effect for both agonists) and snap-frozen in liquid nitrogen until preparation for the enzyme immunoassay (EIA; 181001, Cayman Chemical, Ann Arbor, MI). The purification of the tissue samples, three tracheae in each pool, was performed in accordance with the manufacturer’s instruction for the EIA. In brief, the frozen tissues were placed in 5% trichloroacetic acid and homogenized on ice. After centrifugation at 1500g for 10 min, the trichloroacetic acid was extracted from the supernatant by water-saturated ether. Before analysis by EIA, the ether was removed by heating to 70°C. The detection limit of the assay was 0.1 pmol/ml.

**Data Analysis.** All data are expressed as mean values ± S.E.M. To provide estimates of maximal relaxation (Rmax) and midpoint location (pEC50), agonist concentration-effect curve data from individual tissues were fitted to the Hill equation by use of an iterative, least-square method (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Statistical analysis was performed by use of one-way analysis of variance (ANOVA), and if the null hypothesis was rejected (p < 0.05), predetermined, pairwise comparisons with Bonferroni correction were performed between the groups.

### Results

**In Vitro Pharmacology.** All tracheal segments precontracted with carbachol relaxed to formoterol, terbutaline, and salmeterol in a concentration-dependent manner (Fig. 1). The average maximal relaxation (Rmax) achieved was similar for formoterol and terbutaline, whereas the relaxation to salmeterol was significantly smaller (Table 1). Formoterol was the most potent agonist in the induction of relaxation with a significant 76- and 7-fold higher potency than terbutaline and salmeterol, respectively (Table 1).

To evaluate the effect of proinflammatory cytokines on smooth muscle relaxation by different βAR agonists, tracheal segments were cultured for 4 days with 100 ng/ml TNFα and 10 ng/ml IL-1β before contraction with carbachol and addition of β2AR agonists. Treatment with TNFα and IL-1β significantly decreased the maximal relaxation to all three agonists, compared with the control segments, without affecting their potency (Table 1; Fig. 2). The cytokines decreased the maximal relaxation to formoterol by 15 ± 5%, to terbutaline by 27 ± 3%, and to salmeterol by 40 ± 6% (p < 0.01 for formoterol versus salmeterol; Fig. 3).

The effect of GCs on the relaxant responses to the β2AR-agonists was investigated by the addition of 1 μM budesonide, alone or together with TNFα and IL-1β, to the tracheal segment culture for 4 days before segments were contracted

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**TABLE 1**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Treatment</th>
<th>n</th>
<th>Rmax</th>
<th>pEC50</th>
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</thead>
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<tr>
<td>Formoterol</td>
<td>Control</td>
<td>14</td>
<td>94.8 ± 1.3</td>
<td>8.29 ± 0.13</td>
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<td></td>
<td>BUD</td>
<td>9</td>
<td>97.6 ± 1.0</td>
<td>8.15 ± 0.28</td>
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<td></td>
<td>TNFα + IL-1β</td>
<td>13</td>
<td>79.4 ± 5.2</td>
<td>7.95 ± 0.15</td>
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<td>TNFα + IL-1β + BUD</td>
<td>8</td>
<td>89.2 ± 1.9</td>
<td>6.41 ± 0.15</td>
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<tr>
<td>Terbutaline</td>
<td>Control</td>
<td>12</td>
<td>81.5 ± 4.6</td>
<td>6.27 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>BUD</td>
<td>9</td>
<td>81.5 ± 4.6</td>
<td>6.27 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>TNFα + IL-1β</td>
<td>12</td>
<td>65.1 ± 3.1</td>
<td>6.19 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>TNFα + IL-1β + BUD</td>
<td>8</td>
<td>86.5 ± 3.6</td>
<td>6.41 ± 0.21</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>Control</td>
<td>12</td>
<td>80.9 ± 3.4</td>
<td>7.44 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>BUD</td>
<td>11</td>
<td>75.0 ± 6.6</td>
<td>7.91 ± 0.23</td>
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<tr>
<td></td>
<td>TNFα + IL-1β</td>
<td>12</td>
<td>65.8 ± 5.2</td>
<td>7.81 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>TNFα + IL-1β + BUD</td>
<td>11</td>
<td>54.7 ± 4.4</td>
<td>7.18 ± 0.19</td>
</tr>
</tbody>
</table>

BUD, budesonide.

Rmax and pEC50 denote maximal relaxation and midpoint location, respectively (n = number of animals), p < 0.05 for formoterol vs terbutaline controls (a), formoterol vs salmeterol controls (b), terbutaline vs salmeterol controls (c), control vs TNFα + IL-1β (d), control vs TNFα + IL-1β + BUD (e), BUD vs TNFα + IL-1β (f), BUD vs TNFα + IL-1β + BUD (g); and TNFα + IL-1β vs TNFα + IL-1β + BUD (h).
with carbachol and relaxant responses to the β2AR-agonists were studied. Compared with the control segments, treatment with budesonide alone had no effect on either the maximal relaxation or potency for any of the β2AR agonists investigated. However, when the segments were treated with budesonide in the presence of the cytokines, the cytokine-induced impairment of relaxation to formoterol and terbutaline was completely prevented by budesonide (Figs. 2, A and B, and 3). In contrast, budesonide did not significantly prevent the cytokine-induced impairment of the relaxation to salmeterol (Figs. 2C and 3).

mRNA Expression of β1- and β2ARs in Mouse Tracheal Smooth Muscle. To examine whether the exposure to cytokines and treatment with budesonide affected the receptor gene expression, the mRNA expression of β1- and β2ARs were investigated. The mRNA of β1AR was not significantly affected by the different treatments (p = 0.92 by ANOVA; Fig. 4). In contrast, the mRNA of β2AR was significantly altered (p < 0.0001 by ANOVA; Fig. 4). Compared with the control treatment (5.4 ± 0.6 mol/100,000 mol β-actin), exposure to the cytokines had no effect on β2AR mRNA expression (9.2 ± 2.3 mol/100,000 mol β-actin), whereas treatment with budesonide, both alone and in the presence of cytokines (20.6 ± 2.2 and 12.9 ± 2.3 mol/100,000 mol β-actin, respectively), significantly increased the expression (p < 0.001 and p < 0.01, respectively).

mRNA Expression of COX-2 in Mouse Tracheal Smooth Muscle. To investigate whether the exposure to cytokines increased expression of COX-2, which may result...
in heterologous desensitization of $\beta_2$AR, and whether this response was affected by budesonide treatment, the mRNA expression of COX-2 was investigated. The cytokines increased COX-2 mRNA expression nearly 12-fold compared with the control conditions (7797 ± 2025 versus 677 ± 231 mol/100,000 mol $\beta$-actin; $p < 0.01$). Budesonide alone had no significant effect (1073 ± 372 mol/100,000 mol $\beta$-actin), whereas concomitant exposure to budesonide and the cytokines significantly counteracted ($p < 0.01$) the cytokine-induced increase of COX-2 mRNA so that the level obtained (2062 ± 484 mol/100,000 mol $\beta$-actin) was not significantly different from the control level (Fig. 5).

Radioligand Binding in Lung Tissue. The analysis of the radioligand binding to $\beta_2$ARs in membranes prepared from mouse lung parenchyma showed that exposure to the cytokines did not decrease receptor binding. Although a slight increase of binding was seen in tissues treated with budesonide alone (327 ± 54 fmol/mg protein), or with budesonide together with the cytokines (341 ± 25 fmol/mg protein), compared with control tissue (289 ± 24 fmol/mg protein) and tissue exposed to the cytokines alone (309 ± 26 fmol/mg protein), no statistically significant differences between the groups were obtained ($p = 0.47$ by ANOVA; Fig. 6). The ligand binding to $\beta_1$ARs was less than 10% of the total binding for all treatment groups.

**cAMP Measurement.** To investigate a possible relation between relaxant effects of $\beta_2$AR agonists and their production of cAMP, concentration-response curves for cAMP production were investigated at experimental conditions similar to those of studies on relaxant effects. The basal cAMP production was not significantly altered by the exposure to the cytokine and/or budesonide. Both formoterol and salmeterol caused a concentration-dependent increase of cAMP (Fig. 7). At the control conditions (no exposure to cytokines or budesonide), maximal cAMP production by formoterol was somewhat higher than that by salmeterol (525 ± 80 versus 306 ± 14 pmol/ml, but without reaching statistical significance; $p = 0.14$). It is noteworthy that both formoterol- and salmeterol-stimulated cAMP production was attenuated by exposure to the cytokines. For formoterol, this decrease was prevented by budesonide present during cytokine exposure. In contrast, budesonide did not prevent cytokine-induced impairment of cAMP production by salmeterol (Fig. 7).

**Discussion**

The present study showed that the proinflammatory cytokines TNF$\alpha$ and IL-1$\beta$ decreased relaxation to the $\beta_2$AR agonists in carbachol-contracted mouse tracheal segments, and that this effect was significantly more pronounced for salmeterol than for formoterol and terbutaline. Furthermore, this study demonstrated that, although budesonide alone did not affect the relaxation induced by the $\beta_2$AR agonists, when...
applied together with the cytokines, it completely prevented the cytokine impairment of relaxation to formoterol and terbutaline but not to salmeterol. The similar pattern was observed for cAMP production by the agonists. The cytokines did not affect β2AR mRNA in tracheal smooth muscle, but budesonide increased it irrespective of the presence of the cytokines. On the other hand, the cytokines markedly increased COX-2 mRNA expression, which may lead to heterologous desensitization of β2AR. It is noteworthy that the cytokine-induced increase of COX-2 was counteracted by concomitant budesonide, suggesting that heterologous desensitization of β2AR by the cytokines may be prevented by budesonide treatment. These results together suggest that the differences between the β2AR agonists, regarding their relaxant responses in the presence of the proinflammatory cytokines and budesonide, depend on differences in β2AR signaling upstream of cAMP generation.

The differences between the β2AR agonists in the present study, where formoterol and terbutaline induced greater maximal relaxation of the mouse tracheal segments than salmeterol did, and where formoterol was the most potent agonist followed by salmeterol, are similar to the results obtained in human bronchus (Källström et al., 1994; Mollmand et al., 1998). In this study, the exposure to TNFα and IL-1β reduced the maximal relaxation achieved by any of the three β2AR agonists, but their potency was not significantly affected. Similar impairment of relaxation by these cytokines, with or without changes in potency, have been shown for isoproterenol in guinea pig, rabbit, and rat tracheal segments (Wills-Karp et al., 1993; Hakonarson et al., 1996; Koto et al., 1996) and in several studies in dispersed human airway smooth muscle cells (Shore and Moore, 2003). This is the first time that this phenomenon has been shown in the mouse and after long-term culture with the cytokines; i.e., 4 days, whereas not more than 1 day of culture was used in the studies described above. Thus, the present study better mimics chronic inflammation and demonstrates that the cytokine-induced reduction of the β2AR relaxation is not species-specific and is not an acute, transient reaction. The impairment of salmeterol relaxation by the cytokines, in the present study, was greater than those of formoterol and terbutaline (40, 16, and 27%, respectively). As a consequence, although maximal relaxation to salmeterol at control conditions was only 10 to 15% lower than that with formoterol and terbutaline, it was nearly 40% lower in the presence of the cytokines (Table 1). This confirms the notion that agonists with lower intrinsic efficacy, such as salmeterol, are more susceptible to decreases in tissue response capability than agonists with higher intrinsic efficacy, such as formoterol and terbutaline (Kenakin, 1997; Anderson, 2000). Furthermore, the cytokine-induced decrease of the relaxation to formoterol and terbutaline was prevented by concomitant exposure to budesonide, whereas the impairment of salmeterol relaxation was not significantly affected. These findings suggest both quantitative and qualitative differences in the action of salmeterol compared with formoterol and terbutaline.

Tissue response capability depends on both receptor density and efficiency of stimulus-response mechanism. Therefore, in an attempt to identify mechanisms responsible for the effects of the cytokines and budesonide on responses of β2AR agonists, we have investigated β2AR expression and formoterol- and salmeterol-induced cAMP production in tracheal segments. In lung tissue membranes, the binding of β2AR represented more than 90% of the total binding. After treatment with the cytokines there was no reduction of the β2AR protein in lung tissue membranes or β2AR mRNA in tracheal smooth muscle, and in the literature either no effect (Wills-Karp et al., 1993), a decrease (Koto et al., 1996; Mak et al., 2002), or an increase (Stern and Kunos, 1988) has been reported. In the present study, the β2AR mRNA in tracheal smooth muscle was significantly increased by budesonide, both in the absence and presence of the cytokines, and a similar pattern was seen on β2AR protein expression in lung tissue membranes. This agrees with the findings of others showing that GCs increase the β2AR expression in the airways and lung (Mak et al., 1995; Baraniuk et al., 1997; Kalavantavanich and Schramm, 2000), indicating that GC treatment is beneficial for maintaining the β2AR effect. However, the increased β2AR expression by budesonide cannot explain the full preservation of the relaxation to formoterol and terbutaline in the current study because budesonide alone did not affect the relaxant responses of the agonists, and there was no significant difference between the β2AR expression after exposure to the cytokines alone and after concomitant treatment with budesonide. Furthermore, budesonide did not preserve relaxation to salmeterol in the presence of the cytokines. This further indicates that the findings of this study cannot be explained on the level of β2AR expression, but may involve differential effects of budesonide on receptor activation and/or downstream signaling for agonists with high versus low intrinsic activity.

To study the β2AR signaling pathway, the production of the intracellular second messenger molecule cAMP was studied. The pattern of the agonist cAMP responses to formoterol and salmeterol was similar to the relaxant responses in the present study; that is, formoterol induced a higher level of cAMP than salmeterol did, and for both agonists the concentration-effect curves were lower in the cytokine-exposed segments than in control tracheal segments. Furthermore, as for the relaxant responses, although budesonide alone had no effect on cAMP levels induced by formoterol or salmeterol, the cytokine-induced impairment of the cAMP response to formoterol was restored to control level by concomitant treatment with budesonide, whereas the salmeterol response was not restored. These results suggest that cytokine-induced impairment of β2AR agonist relaxant responses was due to an impaired signal transduction upstream of cAMP generation.

Several mechanisms may be involved in the impairment of β2AR signaling by TNFα and IL-1β. One is IL-1β-induced increase of expression and activity of G-protein-coupled receptor kinase 2 leading to desensitization of β2AR, as demonstrated in vivo in rat lung after intratracheal instillation of IL-1β, where dexamethasone completely prevented the increased expression of G-protein-coupled receptor kinase 2 and G-protein-coupled receptor kinase activity by IL-1β (Mak et al., 2002). Another mechanism is heterologous desensitization of β2AR through the induction of COX-2 by the cytokines, subsequent release of prostaglandin E2, and activation of protein kinase A via prostaglandin EP2 receptors, leading to uncoupling of β2AR from stimulatory G-protein (Gs), as shown in mouse trachea (Bachar et al., 2005) and in human airway smooth muscle cells (Shore et al., 1997). GCs are known inhibitors of the COX-2 induction, and thus have ability to prevent this process. Indeed, in the present study,
the cytokines increased COX-2 mRNA expression nearly 12-fold, and this increase was blocked by concomitant budesonide, suggesting that GCs may prevent heterologous desensitization of β2AR via this mechanism. This finding may therefore explain why budesonide prevented the cytokine-induced impairment of β2AR responses to formoterol and terbutaline in the present study.

The question remains to be explained in the present study is why salmeterol responses impaired by the cytokines were not prevented by concomitant treatment with budesonide in contrast to the complete restoration of responses to formoterol and terbutaline. The explanation may relate to the fact that β2AR stimulation by structurally different agonists leads to stabilization of different active states of the receptor and this may result in activation of several different signaling pathways (McGraw and Liggett, 2005). In addition to the activation of GSs, which is considered to be the main pathway for stimulation of adenyl cyclase via the β2AR, it has been shown that this receptor can also activate inhibitory G-protein (Gi) (Daaka et al., 1997). The extent of Gi involvement may determine the degree of β2AR activation, because Gi signaling opposes GS stimulation of adenyl cyclase (Pönicke et al., 2006). It is noteworthy that IL-1β leads to a marked increase of the expression of Gia subunit (Gio; or specifically Gio2) in guinea pig trachea (Wills-Karp et al., 1993), in tracheal smooth muscle in guinea pig (Hirata et al., 1994) and in rabbit (Wills-Karp et al., 1993; Hakonarson et al., 1996), in rat trachea and lungs (Koto et al., 1996), and in cultured human endothelial cells (Lee et al., 1989). The increase of Gio2 was also shown in several animal models with relevance to asthma (reviewed in McGraw et al., 2007). The Gi signaling leads to decreased cAMP accumulation, and the increase of Gi impairs β2AR-agonist-mediated bronchodilatation (Wills-Karp et al., 1993; McGraw et al., 2007). Thus, it seems possible that β2AR stimulation by salmeterol involves Gi activation to a greater extent than by formoterol or terbutaline and that IL-1β, through the up-regulation of the Gi signaling, attenuates salmeterol responses to a greater extent than responses of formoterol and terbutaline. Furthermore, Gi activation by salmeterol may explain why budesonide did not counteract the cytokine-induced impairment of salmeterol responses in the present study, because Gia protein was shown to be insensitive to GC treatment (Kalavan-tanvich and Schramm, 2000).

In a clinical setting both formoterol and salmeterol, when added to inhaled GC treatment, reduce asthma exacerbations (information available from Global Initiative for Asthma, at www.ginasthma.org). However, when added to GC treatment compared with the same dose of GC plus placebo, only formoterol reduces asthma-related hospital admissions, as shown in a recent systematic review (Jaeschke et al., 2008). This finding is also in agreement with the largest double-blind study comparing fixed-dose combination therapy with budesonide-formoterol versus salmeterol-fluticasone at equivalent GC doses (Kuna et al., 2007), which found a lower rate of asthma-related hospitalizations/emergency room visits in formoterol-treated patients.

In summary, these results confirm that the inflammatory cytokines TNFα and IL-1β reduce the ability of β2AR agonists to relax airway smooth muscle, and show that this effect is significantly more pronounced for salmeterol, an agonist with low intrinsic efficacy, than for formoterol and terbutaline, which have high intrinsic efficacy. Furthermore, this study demonstrates that concomitant treatment with budesonide completely counteracted the effect of the cytokines on responses to formoterol and terbutaline, but not to salmeterol. This discrepancy may result from the inherent differences in agonist molecular structure with partly divergent activation of GS and Gi proteins by higher- and lower-efficacy agonists. These results raise the possibility that the maximal bronchodilator effects of a higher-efficacy agonist, such as formoterol, can be maintained by combined treatment with budesonide during periods with increased inflammation, such as during asthma exacerbations, whereas salmeterol activity may be impaired despite combined therapy with inhaled GCs during these periods.

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