Beclomethasone rapidly ablates allergen-induced β2-adrenoeceptor pathway dysfunction in human isolated bronchi

LORENZO BRICHETTO, MANLIO MILANESE, PINGFANG SONG, MAURO PATRONE, EMANUELE CRIMI, KAI REHDER, AND VITO BRUSASCO

Dipartimenti di Scienze Motorie e Riabilitative, di Medicina Interna, e di Medicina Sperimentale, Università di Genova, 16132 Genoa, Italy

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Brichtetto, Lorenzo, Manlio Milanesi, Pingfang Song, Mauro Patrone, Emanuele Crimi, Kai Rehder, and Vito Brusasco. Beclomethasone rapidly ablates allergen-induced β2-adrenoeceptor pathway dysfunction in human isolated bronchi. Am J Physiol Lung Cell Mol Physiol 284: L133–L139, 2003. First published August 16, 2002; 10.1152/ajplung.00217.2002.—Bronchial rings from nonatopic humans were passively sensitized with serum from allergic subjects. Allergen challenge significantly reduced the relaxant effect of salbutamol on carbachol-induced contractions, suggesting β2-adrenoeceptor (β2-AR) pathway dysfunction. Incubation of challenged rings for 3 h with 3 × 10−6 M beclomethasone dipropionate (BDP) restored the relaxant effect, suggesting reversal of β2-AR pathway dysfunction. Incubation with the Gα protein-stimulating cholera toxin attenuated contractile responses to carbachol significantly less in challenged than in unchallenged rings. Treatment of challenged rings with BDP resulted in an inhibitory effect of cholera toxin that was similar to the effect in unchallenged rings. Gα protein expression was not significantly altered by BDP, suggesting that the activity of Gα protein was increased. Relaxation of challenged rings by forskolin was not significantly affected by BDP, suggesting that β2-AR pathway dysfunction was proximal to the adenylyl cyclase. In conclusion, short-term (3-h) treatment with BDP after allergen challenge ablated β2-AR pathway dysfunction by increasing the activity of the Gα protein in human isolated bronchi. Studies from this laboratory have shown that allergen challenge of passively sensitized human isolated bronchi caused β-AR pathway dysfunction (29). This dysfunction could be prevented by a leukotriene receptor antagonist (27) and was associated with a reduced activity, but not expression, of the receptor-coupled Gα protein (stimulatory guanine nucleotide-binding protein) (28), suggesting that mechanisms other than downregulation were involved.

The aim of the present study was to investigate whether beclomethasone dipropionate (BDP), a steroid widely used in the treatment of asthma, can rapidly (within 3 h) restore β2-adrenoeceptor (β2-AR) pathway function in passively sensitized and allergen-challenged human isolated bronchi. The activity and expression of Gα protein and the activity of adenylyl cyclase were determined to investigate possible effects of BDP at these levels of the β2-AR pathway.

METHODS

Tissue Preparation

Bronchi were obtained from 18 nonasthmatic male patients (50–70 yr) undergoing thoracotomy for lung cancer. None of the patients had received β2-agonists, theophylline, or anticholinergic drugs before or during surgery. After removal of tissue for pathological examination, bronchi were obtained from a site as far as possible from the malignancy. They were immersed in cold (4°C) and aerated (95% O2-5% CO2) physiological salt solution (PSS) of the following composition (in mM): 110.5 NaCl, 3.4 KCl, 2.4 CaCl2, 0.8 MgSO4, 1.2 KH2PO4, 25.7 NaHCO3, and 5.6 dextrose. Immediately after arrival in the laboratory, bronchial rings (3–5 mm ID, 4–5 mm long) were prepared, with care taken to avoid epithelial damage.

Passive Sensitization

An atopic serum was obtained by pooling sera from asthmatic subjects with high concentrations of specific IgEs (>17.5 radio-allergo-sorbent test units/ml; Pharmacia, Uppsala, Sweden) against Dermatophagoides pteronyssinus and Dermatophagoides farinae and low total IgE concentrations (215 ± 34 IU/ml by paper radioimmunosorbent test). Bronchial rings were passively sensitized with the atopic serum for 48 h before challenge with Dermatophagoides farinae and Dermatophagoides pteronyssinus (34 IU/ml). Results were compared with rings that had been sensitized with normal sera from nonatopic donors. Bronchial rings were then challenged with extracts of Dermatophagoides farinae and Dermatophagoides pteronyssinus (34 IU/ml).

Corticosteroids are highly effective in the control of asthma, because they inhibit expression of proinflammatory cytokines, thus blocking influx and activation of inflammatory cells (2). In addition, corticosteroids upregulate β-adrenoeceptor (β-AR) function by promoting gene transcription and receptor expression in lung tissues and cells (5, 11, 12). In vitro, within 12–24 h, β-AR downregulation induced by β-agonists is reversed by corticoids (18). Administration of corticosteroids parenterally (16) or by inhalation (23) improves lung function in patients with acute asthma and restores β-AR sensitivity (6, 18, 31). Importantly, this occurs faster than the time required for the gene transcriptional process.

Address for reprint requests and other correspondence: V. Brusasco, Dipartimento di Medicina Interna, Università di Genova, Viale Benedetto XV, 6, 16132 Genoa, Italy (E-mail: brusasco@dism.unige.it).

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chial rings were incubated overnight for 18 h with 1 ml of the atopic serum diluted in 9 ml of PSS, while they were continuously aerated at room temperature with 95% O₂-5% CO₂.

**Allergen Challenge**

After repeated washes with PSS, sensitized rings were incubated for 60 min at 37°C with 5 ml of aerated PSS containing 200 arbitrary units of *Dermatophagoides* mix (challenged rings) or with PSS only (unchallenged rings).

**Isometric Force Measurements**

Bronchial rings were placed in water-jacketed 25-ml tissue baths containing aerated PSS at 37°C. They were connected via a stirrup to a stationary hook at the bottom and via a silk string at the top of a force transducer (model FT03D, Grass Medical Instruments, Quincy, MA). The signal from the force transducer was continuously recorded (model TA 4000, Gould, Valley View, OH). The rings were allowed to equilibrate for 2 h, while they were washed every 20 min and progressively stretched to a resting force of 1 g (29). This length (i.e., the optimal length) was maintained throughout the studies.

**Verification of Passive Sensitization**

At the end of experiments, one sensitized ring from each of 14 patients was incubated for 60 min with 25 ml of aerated PSS containing 1,000 arbitrary units of *Dermatophagoides* mix. Passive sensitization was considered successful if >0.5 g of contractile force was recorded.

**Response to Salbutamol**

One unchallenged and one challenged ring from each of six patients was treated for 3 h with 3 × 10⁻⁶ M BDP. Two other paired rings from the same six patients were incubated with PSS containing only the solvent for BDP, dimethyl sulfoxide (DMSO). In one patient, an unchallenged ring was not available. After 3 h of incubation, all rings were contracted with 10⁻⁶ M carbachol, and, after steady contractions had been achieved, salbutamol was added cumulatively (10⁻⁹–10⁻⁴ M, in half-log increments).

**Gₛ Protein Function and Expression**

Two unchallenged and two challenged rings from each of six patients were incubated with 10⁻⁶ M carbachol. After a steady state had been achieved, the carbachol concentration was cumulatively increased to 10⁻⁴ M (in half-log increments). Thereafter, all rings were washed with PSS until resting force was reestablished. One challenged ring from each patient was incubated with 3 × 10⁻⁶ M BDP and 10 μg/ml cholera toxin (CTX) for 3 h. Two rings from each patient, one unchallenged and one challenged, were incubated for 3 h with 10 μg/ml CTX and no BDP. Second complete sets of carbachol concentration-response curves were then obtained for all rings. To correct for the effect of time, one unchallenged ring was incubated with PSS (30).

The expression of Gₛ subunit was assayed by Western blot analysis in eight challenged bronchial fragments from four separate patients. Paired muscles were incubated only with DMSO or with BDP for 3 h at 37°C in aerated (95% O₂-5% CO₂) PSS. Aliquots of tissue derived from the bronchial fragments were suspended in 20 mM Tris·HCl-buffered solution (pH 7.4) of the following composition: 140 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml leupeptin. Tissues were homogenized in an ice bath, and cells were lysed by sonication (6 bursts of 10-s duration each) at 0°C. After centrifugation at 12,000 g for 5 min, supernatants of samples were collected and suspended in SDS-PAGE loading buffer. Samples were immediately heated to 95°C for 4 min and submitted to SDS-PAGE on a 10% (wt/vol) slab gel (21) and then electroblotted onto a pure nitrocellulose membrane (Amersham Pharmacia Biotech, Milan, Italy). Gₛ-olf protein was identified by using the specific anti-Gₛ-olf antibody (C18) rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then probed with a peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) (14) and developed with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Blots were quantified by scanning analysis with a densitometer (model CS-9000, Shimadzu, Kyoto, Japan). The relative amount of each immunoreactive band was calculated by determining the areas of the densitometric peaks in square millimeters.

**Adenylyl Cyclase Activity**

One challenged ring from each of four patients was incubated with 3 × 10⁻⁶ M BDP for 3 h, and another challenged ring was incubated with the solvent DMSO only. All rings were then contracted with 10⁻⁶ M carbachol. After the contractile responses stabilized, forskolin was added cumulatively (10⁻⁹–10⁻⁴ M, in half-log increments).

**Data Analysis**

Isometric forces developed by bronchial rings used for salbutamol and forskolin relaxation studies are expressed as percentage of contractile responses to 10⁻⁶ M carbachol. The time-corrected force developed from rings used for the CTX studies is expressed as percentage of contractile responses to 10⁻⁴ M carbachol. The concentrations of salbutamol inhibiting 50% of carbachol-induced contraction (IC₅₀) were calculated by linear interpolation between the two adjacent points of the salbutamol relaxation curves.

Concentration-response curves were analyzed by two- or three-factor repeated-measures ANOVA with Newman-Keuls post hoc test. Bronchial ring characteristics were compared by a between-within-groups mixed ANOVA. Differences were considered to be statistically significantly different at P < 0.05. Values are means ± SD.

**Drugs**

Salbutamol free base, carbachol (carbamylcholine chloride), CTX, forskolin, and PTX were purchased from Sigma-Aldrich (Milan, Italy). *D. pteronyssinus* and *D. farinae* were purchased from Laboratorio Farmaceutici Lofarma (Milan, Italy). BDP was generously provided by Chiesi Farmaceutici (Parma, Italy). BDP was dissolved in DMSO and forskolin in absolute ethanol. All other drugs were dissolved in distilled water.
RESULTS

Successful sensitization was demonstrated in bronchial rings from 14 patients; in 2 patients used for the salbutamol study, verification was impossible because of insufficient amounts of tissue.

The mean weight of 55 bronchial rings from 18 patients (rings used for verification of sensitization not included) was 101 ± 37 mg, and the mean resting force was 0.9 ± 0.4 g. Mean weights and resting forces were not significantly different between experimental groups (P = 0.4 and P = 0.4, respectively). There were no significant differences (P = 0.6) in contractile forces induced by 10^{-6} M carbachol between the experimental groups.

Response to Salbutamol

Salbutamol relaxed all rings in a concentration-dependent manner (P < 0.001; Fig. 1). At 10^{-6}–10^{-5} M salbutamol, the relaxation was significantly greater (P < 0.05) in BDP-treated challenged rings than in non-BDP-treated challenged rings. There was no significant difference in relaxation between non-BDP-treated unchallenged rings and BDP-treated challenged rings (P = 0.4).

The mean IC_{50} values were significantly (P < 0.05) greater for non-BDP-treated challenged rings than for non-BDP-treated unchallenged, BDP-treated unchallenged, and BDP-treated challenged rings (Table 1).

G_{s}α Protein Function and Expression

Before incubation with CTX, there were no significant differences in the maximal contractile forces (10^{-4} M carbachol) between the experimental groups. Incubation with CTX at 10 μg/ml displaced the mean carbachol concentration-response curves significantly downward (P < 0.01; Fig. 2). The displacement was significantly less in non-BDP-treated challenged rings than in the other rings (P < 0.001). In BDP-treated challenged rings, the mean concentration-response curve was displaced significantly more downward than in non-BDP-treated challenged rings, so there was no significant difference between mean concentration-response curves of BDP-treated challenged and unchallenged rings (P = 0.44).

Expression of G_{s}α protein was not significantly different between non-BDP-treated challenged rings and BDP-treated challenged rings (Table 2).

Adenylyl Cyclase Activity

Forskolin relaxed non-BDP-treated and BDP-treated challenged rings in a concentration-related manner (P < 0.001). There was no significant difference (P = 0.3) between the two groups (Fig. 3).

G_i Protein Function

In nonsensitized unchallenged rings, incubation with PTX at 1 μg/ml enhanced the relaxant effect of salbutamol, with no difference between BDP-treated and non-BDP-treated rings (Fig. 4).

DISCUSSION

This study confirms the attenuated responses to β_{2}-AR and G_{s}α protein stimulation in passively sensitized and allergen-challenged human isolated bronchi (28). The new major finding is that 3 h of incubation with BDP increased the responses of challenged rings to β_{2}-AR or G_{s}α protein stimulation. This finding suggests that BDP rapidly restored the β_{2}-AR pathway function, and this effect involved G_{s}α protein.

Comments on Methodology

Isolated bronchi were passively sensitized by incubation with human serum containing low levels of total IgEs but high levels of specific IgEs (27–29). The method of sensitization used in this study does not alter contractile responses of human isolated airway smooth muscles, indicating that differences in contractions are not due to the force-generating capacity of the muscles. For this reason, unchallenged rings were used as control rings for the CTX studies.

Table 1. Salbutamol concentrations inhibiting 50% of active force

<table>
<thead>
<tr>
<th>Allergen Challenge</th>
<th>n</th>
<th>BDP</th>
<th>IC_{50}, -log M</th>
</tr>
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<tbody>
<tr>
<td>No</td>
<td>6</td>
<td>No</td>
<td>6.05 ± 0.67</td>
</tr>
<tr>
<td>No</td>
<td>5</td>
<td>Yes</td>
<td>6.00 ± 0.73</td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>No</td>
<td>5.69 ± 0.46*</td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
<td>Yes</td>
<td>6.09 ± 0.48</td>
</tr>
</tbody>
</table>

Values are means ± SD. BDP, beclomethasone dipropionate; IC_{50}, salbutamol concentration resulting in 50% inhibition of active force. *P < 0.05 vs. all other conditions.

Adenylyl Cyclase Activity

Forskolin relaxed non-BDP-treated and BDP-treated challenged rings in a concentration-related manner (P < 0.001). There was no significant difference (P = 0.3) between the two groups (Fig. 3).
The relaxant effects of salbutamol and forskolin were studied by precontracting bronchial rings with 10^{-6} M carbachol (Figs. 1 and 3). Normalization to 10^{-6} M carbachol was justified, because BDP, sensitization, and challenge with allergen did not alter the force-generating capacity of the muscles.

The selective β2-AR agonist salbutamol and CTX activate the β2-AR pathway in any cell type, including those in prejunctional parasympathetic nerve endings. It is unlikely, however, that the results of this study reflect prejunctional effects, because it has been suggested that functional prejunctional β-ARs are not present on parasympathetic nerve endings of human airways (4).

A 3-h incubation with BDP was chosen, because β2-AR function after homologous desensitization is restored in vivo within this time frame (6, 17, 31). The long-term effect of BDP was not the subject of interest of this study. Although the restored effects of salbutamol and CTX suggest an effect of BDP on Gs protein function, definite proof of Gs protein dysfunction in challenged rings and the effect of BDP on Gs protein function requires direct measure(s) of Gs protein activation. Finally, the results of this study should be carefully extrapolated to in vivo conditions, inasmuch as only cells in the bronchial wall are involved and the BDP concentrations achievable by inhalation in the airways are uncertain. These limitations do not invalidate the conclusions of the study.

Comments on Results

Hyporesponsiveness of β2-ARs can present a major problem in the treatment of acute asthma (1) and may...
intracellular signal transmission between receptor and effector.

Treatment of challenged rings with BDP for only 3 h restored the inhibitory effect of β2-AR stimulation in carbachol-precontracted rings, which suggests a restoration of the β2-AR pathway function. This conclusion seems justified, because BDP alone had no effect on relaxation by salbutamol in unchallenged rings (Fig. 1).

$G_{i\alpha}$ protein function and expression. CTX catalyzes ADP-ribosylation of the α-subunit of CTX-sensitive G proteins, which irreversibly activates the α-subunit. This activation results in increased intracellular cAMP concentration, reduced intracellular calcium concentration, and reduced contractile response. These effects are the underlying mechanisms for the attenuated contractile responses to carbachol in the presence of CTX (Fig. 2). The greater contractile response to carbachol of non-BDP-treated CTX-incubated allergen-challenged rings (Fig. 2B) is consistent with a dysfunction of $G_{i\alpha}$ protein.

The attenuation of contractile response to carbachol by CTX was greater in BDP-treated than in non-BDP-treated challenged rings. Importantly, the contractile response to carbachol in these rings was not significantly different from that in unchallenged rings, which suggests that BDP treatment for only 3 h restored the inhibitory effect of CTX on airway smooth muscle contraction. Western blot analysis suggests that this was not due to an increased expression of $G_{i\alpha}$ protein. There are many β-ARs on the epithelium that do not contribute to the relaxation response. To exclude the effects of these β-ARs on the results of the Western blot analysis, the same analysis was carried out in two epithelium-denuded rings from an additional patient. The densitometric peaks were 621 mm$^2$ for the non-BDP-treated ring and 520 mm$^2$ for the BDP-treated ring, which supports the previous conclusion.

Adenylyl cyclase activity. Forskolin stimulates the activity of adenylyl cyclase, thus increasing cAMP concentration and relaxing smooth muscle (25). BDP did not alter the relaxant effect of forskolin in carbachol-precontracted muscles (Fig. 3), suggesting that the effect of BDP on the restoration of the β2-AR signal transmission pathway was proximal to adenylyl cyclase. Consistent with this conclusion is the report that the response to forskolin is not reduced in allergen-challenged human isolated passively sensitized bronchi (28). Also the results of studies using other models of β2-AR hyporesponsiveness (19) are consistent with this conclusion.

Possible underlying mechanisms. Corticosteroids may interfere with β2-AR pathway function by promoting gene transcription, which regulates expression of β2-AR and $G_{i\alpha}$ protein. However, gene transcription is unlikely the underlying mechanism for the results of this study, because it has not been observed within 3 h (13) and BDP did not increase $G_{i\alpha}$ protein expression. Second, interleukin-1β (IL-1β) may interfere with coupling of $G_{i\alpha}$ protein to β2-ARs and, thus, activation of adenylyl cyclase (8, 26). Corticosteroids may ablate this effect by decreasing the stability of mRNA for
IL-1β (15). The effect of corticosteroids on IL-1β seems to be mediated by inhibition of prostanoid formation (19). Blockade of prostanoid formation by inhibition of cyclooxygenase does not prevent β2-AR dysfunction (27), suggesting that this mechanism cannot explain our results. In addition, no detectable levels of IL-1β were found in the majority of supernatants after 2 h of allergen challenge of sensitized human airway tissues (10). Furthermore, corticosteroids were administered before and not after β2-AR dysfunction (13, 19). Finally, corticosteroids can modulate Na+/-K+ pump-mediated relaxation (24). Again, these mechanisms cannot explain the present data, because relaxation of unchallenged rings was not affected by incubation with BDP.

In this study the nonselective muscarinic agonist carbachol was used to contract airway smooth muscle. Stimulation of postjunctional M2 receptors inhibits adenyl cyclase through the activity of the inhibitory Gi protein. Any change in Gi expression or function induced by challenge or BDP would have, therefore, affected the responses to β2-AR pathway stimulation. It seems unlikely that the results of the present study were determined by changes in Gi protein for several reasons: 1) allergen-induced dysfunction of the β2-AR pathway in this model was not associated with an increase in expression or function of Gi protein (28); 2) the relaxant effect of salbutamol was similar in unchallenged BDP-treated and non-BDP-treated rings (Fig. 1); 3) 24 h of incubation with dexamethasone did not increase Gi protein expression in bovine trachealis (13); and 4) the Gi protein-specific blocker PTX enhanced the relaxant effect of salbutamol similarly in BDP-treated and non-BDP-treated rings from two additional patients (Fig. 4).

Allergen-induced β2-AR dysfunction in human isolated sensitized bronchi can be prevented by leukotriene receptor blockade (27) and seems to be due to a dysfunction of the Gsα protein (28). Presumably, isoforms of protein kinase C (7, 22) are activated along the diacylglycerol pathway and cause phosphorylation of β2-AR and/or the coupled Gi protein. It is tempting to speculate that BDP may ablate this process.

Conclusions. The results of the present study in human isolated sensitized bronchi demonstrate that BDP rapidly reversed the dysfunction of the intracellular β2-adrenergic signal transmission pathway induced by allergen challenge. This effect does not appear to be dependent on gene transcription. Restoration of the intracellular β2-AR effector pathway may contribute to the efficacy of corticosteroids in the acute treatment of asthma. Elucidation of the underlying mechanisms for the effect of BDP on Gsα protein awaits further studies.

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