ABSTRACT

In vitro studies suggest that glucocorticoids may counteract \( \beta \)-agonist-induced desensitization of \( \beta \)-adrenoceptors by actions at the transcriptional level, but the clinical relevance of such findings is not clear. Oral terbutaline treatment decreases \( \beta \)-adrenoceptor sensitivity in alveolar macrophages in vivo. This effect is not counteracted by inhaled or orally taken steroids. We therefore examined whether inhaled terbutaline elicited a similar effect on \( \beta_2 \)-adrenoceptor sensitivity in alveolar macrophages, and if co-treatment with an inhaled steroid, budesonide, would prevent such down-regulation. Bronchoalveolar lavage (BAL) and lung function tests, including bronchodilator responses to inhaled terbutaline, were performed before and after 2 weeks of regular inhalation of terbutaline, 0.5 mg three times daily, and budesonide, 400 \( \mu \)g twice daily, or placebo, in 24 healthy volunteers. Four untreated subjects served as controls. A marked, approx. 90\%, decrease in isoprenaline-induced cAMP accumulation in alveolar macrophages was found in both treatment groups after 2 weeks, with no difference between placebo and budesonide (\( P = 0.45 \)). In the untreated control group, cAMP responses to both isoprenaline and prostaglandin E\(_1\) tended to be lower on the second occasion. A limited, non-specific desensitization of adenylate cyclase activity thus contributed to the marked desensitization elicited by terbutaline inhalations. The bronchodilator response to inhaled terbutaline did not change after treatment in any of the three groups (\( F = 0.9, P = 0.50 \)). In conclusion, inhalation of a \( \beta \)-agonist induced marked down-regulation of \( \beta_2 \)-adrenoceptor sensitivity in alveolar macrophages in vivo without influencing the bronchodilator response to a \( \beta_2 \)-agonist in healthy subjects. Co-treatment with an inhaled steroid failed to counteract the desensitization of alveolar macrophage \( \beta_2 \)-adrenoceptors.

INTRODUCTION

Early studies claimed that tolerance to \( \beta \)-mediated bronchodilatation developed in healthy subjects after treatment with \( \beta \)-agonists [1,2]. Later studies, in asthmatic subjects, failed to confirm those findings, and there is no evidence that anti-asthmatic therapy with \( \beta \)-agonists induces tolerance to the bronchodilator effect [3–5]. The question of pulmonary \( \beta \)-adrenoceptor desensitization during \( \beta_2 \)-agonist treatment is, however, complex, since receptor regulation in inflammatory cells may also be involved. Thus it has been shown that continuous treatment with a \( \beta \)-agonist leads to partial tolerance to the protective effects against bronchoconstrictor stimuli.
even though bronchodilator responses to \(\beta\)-adrenoceptor stimulation were intact [6–8]. The mechanism behind this tolerance has, however, not been established.

Short-lasting, intense \(\beta\)-adrenoceptor stimulation in vitro leads to rapid desensitization due to uncoupling of the receptor and the \(G\) protein, whereas more prolonged stimulation leads to internalization of the receptor [9,10], and eventually to permanent loss and degradation of the receptors. Restoration of responsiveness in this situation requires synthesis of new receptors [11].

\(\beta\)-Agonist-induced down-regulation of \(\beta\)-adrenoceptors can be reversed by corticosteroids. For example, desensitization of \(\beta\)-adrenoceptors on blood lymphocytes may be counteracted after a single dose of methylprednisolone [12], and glucocorticoids increase the transcription of \(\beta\)-adrenoceptors in cell lines and in human lung tissue [13,14]. However, glucocorticoid effects on receptor regulation have not been unambiguous. Thus Hall et al. [15] found no influence of dexamethasone on isoprenaline-induced desensitization in human airway smooth muscle. We have previously found marked \(\beta\)-adrenoceptor desensitization in alveolar macrophages during oral treatment with the \(\beta\)-agonist terbutaline [16]. This effect was not attenuated by inhaled or orally administered glucocorticoids [16].

The exact role of alveolar macrophages in asthma is not clear, but they may participate in the asthmatic reaction through several mechanisms. T-cells, which seem to have a central role in the complex cell–cell interactions in asthma [17], are activated upon antigen presentation by different cell types, including alveolar macrophages [18]. Alveolar macrophages are also capable of synthesizing and secreting a number of mediators, which may influence chemotaxis (leukotriene \(B\)\(_{\text{4}}\), interleukin-8 and platelet activating factor), cell activation (interleukin-1 and tumour necrosis factor–\(\alpha\)), maturation of eosinophilic granulocytes (granulocyte/macrophage colony-stimulating factor), and cause airway smooth-muscle contraction (leukotriene \(C\)\(_{\text{4}}\)) (for reviews see [19–22]).

The aims of the present study were to find out whether inhaled terbutaline desensitizes \(\beta\)-adrenoceptors in alveolar macrophages, and bronchodilator responses to terbutaline, and whether co-treatment with an inhaled steroid, budesonide, prevents such possible effects. Isoprenaline-induced cAMP accumulation was used as a marker for macrophage \(\beta\)-adrenoceptor sensitivity [23]. For comparison, responses to prostaglandin \(E\)\(_{\text{2}}\) (PGE\(_{\text{2}}\)) were examined to determine if possible changes were homologous or heterologous.

**MATERIAL AND METHODS**

**Subjects**

A total of 28 healthy smoking volunteers participated in the study (Table 1). Since smoking does not confound conclusions regarding \(\beta\)-adrenoceptor function [3], smokers (>10 cigarettes/day for more than 5 years) were selected to ensure a sufficient yield of alveolar macrophages. Prior to the study, an anamnesis was taken, and each subject underwent physical examination, routine laboratory tests, chest X-ray and spirometry. The subjects were included only if they had no history of allergy or airways disease and neglected airways symptoms during the last 2 weeks prior to the study. The subjects took no other drugs than the study medications, and had not been regularly treated with any drug prior to the study. Informed consent was obtained from all subjects, and the study was performed in accordance with the Declaration of Helsinki (1989), and approved by the Ethics Committee of the Karolinska Institute.

**Study design**

Before treatment, all subjects underwent bronchoalveolar lavage (BAL), and 2 days later spirometry, including a cumulative dose–response curve to inhaled terbutaline. Directly after the lung function tests, 24 of the subjects were randomly allocated to either of two treatment groups. One group (\(n = 12\)) started treatment with inhalation of terbutaline 0.5 mg three times daily and budesonide 400 \(\mu\)g twice daily. The other group (\(n = 12\)) inhaled the same dose of terbutaline and placebo (for budesonide). All drugs were inhaled by Turbuhaler* (Astra Draco, Lund, Sweden). Four subjects served as a control group receiving no treatment. After 2 weeks, a second BAL was performed 12 h after the last dosing. All subjects then continued the treatment for another 2 days, after which a second spirometry and dose–response curve for inhaled terbutaline were performed, 24 h after the last dosing.

**BAL**

Bronchoscopy was performed through the nose with a flexible fibre-optic bronchoscope under local anaesthesia by 2% lignocaine (Xylocain; Astra, Södertälje, Sweden), after premedication with morphine and scopolamine. The bronchoscope was wedged in a middle lobe bronchus

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**Table 1 Details of the participating subjects**

<table>
<thead>
<tr>
<th></th>
<th>Budesonide</th>
<th>Placebo</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n)</td>
<td>12</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Female/male</td>
<td>9/3</td>
<td>7/5</td>
<td>2/2</td>
</tr>
<tr>
<td>Mean age (range)</td>
<td>30 (19–45)</td>
<td>30 (21–45)</td>
<td>27 (23–30)</td>
</tr>
<tr>
<td>FEV(_1) (litres)</td>
<td>3.3 ± 0.2</td>
<td>3.9 ± 0.3</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>FEV(_1) (% predicted)</td>
<td>94.7 ± 3.9</td>
<td>101.9 ± 4.2</td>
<td>98.8 ± 6.0</td>
</tr>
<tr>
<td>VC (litres)</td>
<td>4.4 ± 0.2</td>
<td>5.0 ± 0.4</td>
<td>5.5 ± 0.9</td>
</tr>
<tr>
<td>VC (% predicted)</td>
<td>107.5 ± 3.7</td>
<td>107.7 ± 4.3</td>
<td>115.2 ± 10.4</td>
</tr>
<tr>
<td>FEV/VC</td>
<td>0.76 ± 0.02</td>
<td>0.80 ± 0.02</td>
<td>0.74 ± 0.04</td>
</tr>
</tbody>
</table>
and five 50 ml aliquots of sterile saline at 37 °C were instilled. Each aliquot was gently aspirated, collected in a siliconized plastic bottle, and kept on ice. There were no complications after the BAL procedures.

Cell isolation procedure
The BAL fluid was passed through a Millipore AP 32 dacron filter (Millipore Co., Bedford, MA, U.S.A.), after which the cells were pelleted by centrifugation at 400 g for 5 min at 4 °C. The pellet was resuspended in equal parts of Hanks balanced salt solution and 0.17 M Tris (pH 7.4), containing 1% BSA (RIA grade; Sigma). Alveolar macrophages were then isolated by elutriation, as previously described [24], using a Beckman J6B centrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.) equipped with a JE 5 rotor for elutriation. After elutriation, the macrophage fraction was centrifuged (400 g for 5 min) and the pellet was resuspended in incubation buffer. All glassware and plastic materials used were siliconized to reduce cell loss. RIA grade albumin was added to the elutriation buffer to counteract aggregation of the macrophages and thus increase the cell yield. The recovery of macrophages is approx. 80% with this technique, and the final cell preparation consists of 99% macrophages (viability 97%, as assessed by Trypan Blue exclusion) and 0.2% lymphocytes [23].

cAMP accumulation
The procedure described previously [23] was followed. Macrophages were counted in a Bürker chamber and diluted in PBS containing 10⁻⁴ mol/l of the phosphodiesterase inhibitor isobutylmethylxanthine at 37 °C. The final cell concentration was 2.5×10⁶ cells/ml, and the incubation volume was 200 μl. Isoprenaline (10⁻⁶–10⁻⁴ mol/l; kindly given by Riker Laboratories) and PGE₁ (10⁻⁶ and 10⁻⁴ mol/l; Sigma) were used separately to enhance cAMP accumulation. Ascorbic acid (20 μg/ml) was added as antioxidant to protect isoprenaline during incubation. Incubations were performed for 2 min, and terminated by heating to 95 °C for 3 min. cAMP contents of the incubates were determined by a competitive protein-binding assay, as described previously [23,25].

Lung function tests
Forced expiratory volume in 1 s (FEV₁), vital capacity (VC) and flow volume curves were performed using a low-resistance rolling seal spirometer (OHIO 840; OHIO Medical products, Madison, WI, U.S.A.). FEV₁ was stated as the highest value of three forced expirations with a difference of < 10%. Three slow VCs were measured and VC was stated as the highest value of three forced and three slow expirations. Residual volume and specific airway conductance were measured at a breathing frequency of 15 breaths/min in a volume-constant body plethysmograph (Eric Jaeger, GmbH, Würzburg, Germany). FEV₁ and VC were measured 10 min after inhalation of increasing doses of terbutaline (1.0, 2.0 and 4.0 mg of Bricanyl®, Astra Draco) through an inhalation device (Nebulator®, Astra Draco). Local reference values were used [26,27].

Statistical methods
Results are presented as mean values ± S.E.M. Comparisons were made by two-factor repeated-measures analyses of variance with Fischer’s PLSD test (SuperAnova version 1.11; Abacus Concepts, Inc., on a Macintosh computer). P values < 0.05 were considered statistically significant.

RESULTS
Isoprenaline sensitivity
Basal cAMP accumulation was similar before and after treatment in all three groups (Figure 1). Before treatment, alveolar macrophage cAMP accumulation increased concentration-dependently in response to isoprenaline in all three groups, with no significant difference between the groups (F = 0.75, P = 0.67). After 2 weeks of treatment there was a significant difference between the three groups with regard to the isoprenaline-induced cAMP accumulation (F = 43.5, P < 0.0001). Significant difference was found between the control group and the other two groups (P < 0.001), whereas no difference was found between the budesonide and placebo groups (P = 0.45).

After 2 weeks of terbutaline treatment the cAMP accumulation at 10⁻⁴ mol/l isoprenaline decreased by 86 ± 5% in the placebo group, 88 ± 3% in the budesonide group and 40 ± 9% in the control group. There was no difference between the budesonide and placebo group in this respect (P = 0.73; Figure 1), whereas both terbutaline-treated groups had more markedly depressed responses than the controls (P < 0.001).

PGE₁
Basal cAMP accumulation was also similar before and after treatment in all three groups (Figure 2). Alveolar macrophage cAMP accumulation increased concentration-dependently in response to PGE₁ before treatment in all three groups, with no significant difference between the groups (F = 0.09, P = 0.99). After 2 weeks treatment, responses were slightly decreased in all groups, but there was no significant difference between the three groups with regard to cAMP accumulation after PGE₁ stimulation (F = 0.59; P = 0.67). In the placebo group there was no significant difference between pre- and post-treatment PGE₁-induced cAMP-response (F = 0.39, P = 0.68), whereas the difference approached
Figure 1  Isoprenaline-induced cAMP accumulation in alveolar macrophages before and after 2 weeks of treatment (a, b) and in untreated controls (c)
Mean values are shown ± S.E.M. No significant difference between the groups with regard to pre-treatment response (F = 0.75, P = 0.67). Post-treatment responses differed significantly between controls and the two treated groups (P < 0.001), whereas no significant difference was found between the placebo and budesonide groups (P = 0.45).

Lung function tests
Baseline lung function data are shown in Table 1. Terbutaline induced significant bronchodilatation in all three groups (F = 14.0, P < 0.001), with no difference between the groups (F = 0.89, P = 0.51). After 2 weeks treatment, the response to inhaled terbutaline was not altered in any group compared with pre-treatment responses (F = 0.002, P = 0.96; Figure 3). Flow volume curves, specific airway conductance and VC did not add any further information than FEV₁ (results not shown).

Figure 2  PGE₁-induced cAMP accumulation in alveolar macrophages before and after 2 weeks of treatment (a, b) and in untreated controls (c)
Mean values are shown ± S.E.M. No significant difference between the groups with regard to pre-treatment response (F = 0.89, P = 0.99). No significant post-treatment differences between the groups was found (F = 0.59, P = 0.67).

DISCUSSION
In the present study, regular treatment with terbutaline induced a 90% loss of alveolar macrophage β₂-adrenoceptor function, measured by cAMP accumulation upon isoprenaline stimulation. This desensitization was not counteracted by inhaled budesonide. The major component of the desensitization phenomenon observed was homologous, since PGE₁-induced cAMP accumulation was only affected by terbutaline treatment to a small extent. In addition, similar attenuation of the cAMP responses to both isoprenaline and PGE₁ was found in the alveolar macrophages obtained from non-treated controls on the second occasion. Repeated BAL procedures may thus have some non-specific influence on the alveolar macrophage adenylate cyclase activity, whereas β₂-agonist treatment had a powerful effect over and above this. It should be mentioned that no significant
β-adrenoceptor desensitization in human macrophages

Figure 3 Cumulative bronchodilator dose–response curves to terbutaline before and after 2 weeks treatment (a, b) and in untreated controls (c)

Significant bronchodilatation was found in all three groups (P < 0.001), with no difference between the groups (F = 0.89, P = 0.51). No difference between pre- and post-treatment bronchodilatation was found (F = 0.002, P = 0.96).

β-selective difference in the alveolar macrophage response to stimulation was found in the time-control experiments; however, the control group consisted of only four subjects, which makes interpretation difficult. The present results are in accordance with our previous findings [16], which showed that oral terbutaline treatment induced marked homologous alveolar macrophage β-adrenoceptor desensitization. Also in that study [16], steroids, whether given orally or by inhalation, failed to counteract the development of tolerance. The depression of the β-adrenoceptor response was similar in magnitude whether the β-agonist was taken orally or by inhalation. For example, the depression of cAMP accumulation at 10^{-6} mol/l isoprenaline was 86% after inhaled terbutaline (the present study) compared with 82% after oral treatment [16].

Several studies in vitro, both in cell lines and human peripheral lung tissue, have demonstrated that steroids may increase the number of β-adrenoceptors, and that this occurs due to events at the transcriptional level [11,14]. Glucocorticoids may increase the levels of β-adrenoceptor mRNA within 2–4 h, both in the presence and absence of a β-agonist in vitro. With more long-lasting exposure to β-agonists, the cells remain capable of down-regulating the receptor expression in vitro, even in the presence of a glucocorticoid [13]. This is in accordance with our findings in vivo, where treatment with therapeutic doses of terbutaline during 2 weeks resulted in marked desensitization that was not prevented by inhaled budesonide. Thus glucocorticoid treatment does not seem to counteract the β-adrenoceptor desensitization occurring in inflammatory cells during continuous treatment with β-agonists, either orally or by inhalation. The reason for this finding is unclear. It is not known at all whether glucocorticoids counteract desensitization of β-adrenoceptors in alveolar macrophages. This needs to be established in future in vitro experiments. Even if such a counteraction of β-adrenoceptor down-regulation occurs, the glucocorticoid concentration obtained in the alveolar space during inhalation of doses commonly used in clinical practice (and in the present study) may be too low to affect β-adrenoceptor synthesis.

The regulation of β-adrenoceptors in lung tissue seems to differ between cell types. Many studies have demonstrated the relative resistance of airway smooth muscle to tolerance development with regard to bronchodilator responses to β-stimulation [6,7]. The present results in healthy volunteers are in accordance with these findings. On the other hand, β-adrenoceptors on inflammatory cells are readily down-regulated, as shown in the present and a previous study [16]. The tissue differences may be explained by the fact that airway smooth muscle cells have very high levels of β-adrenoceptor mRNA, whereas their β-adrenoceptor density is relatively low [28]. This facilitates compensation by β-adrenoceptor synthesis in smooth muscle, and resistance to down-regulation. In contrast, alveolar cells have a high density of β-adrenoceptors and low levels of mRNA for the receptor, and are therefore more sensitive to down-regulation [28,29].

It is unlikely that the lack of steroid effect is related to poor accessibility of inhaled budesonide to the alveolar macrophages, since inhaled terbutaline and budesonide reach the same level in the airway tree when administered via the Turbuhaler® device. From a theoretical point of view, a drug that is inhaled from a powder inhalator should reach the alveolar space [30]. However, when Nassari et al. [31] administered a β-agonist in the upper airways of chronic obstructive pulmonary disease patients with specially adapted endotracheal tubes, the bronchodilating effect was the same as after inhalation. The link between deposition and clinical effect is thus not entirely clear, as β-agonist deposition in the trachea may induce generalized bronchodilation, perhaps via distri-
bution through the bronchial circulation. Our findings indicate that β-adrenergceptors on alveolar macrophages are similarly desensitized after inhaled and orally administered terbutaline. It is thus unlikely that desensitization of the alveolar cells took place before the macrophages entered the alveolar space.

Alveolar macrophages are the most abundant cells in the peripheral airways and may therefore serve as a model for studies of β-adrenergceptors regulation on inflammatory cells, such as mast cells, eosinophils and neutrophils, all of which are endowed with β2-adrenergceptors [32–34]. In the present study we have demonstrated that an inhaled β2-agonist reaches the alveolar macrophages, and probably other cells in the alveolar space, in sufficient amounts to cause β2-adrenergceptor desensitization. Previously, it has been clearly shown that the protection against a bronchoconstrictor stimulus, but not bronchodilatation, is reduced during regular treatment with β2-agonists, indicating a tolerance to the β2-agonist-induced protective (but not bronchodilator) effect [6,7]. The impairment of bronchoprotection is partial, and a residual protection remains during long-term treatment with the β2-agonist. It could be assumed that the initial loss of protection by a β2-agonist in asthmatic subjects is related to the development of β2-adrenergceptor desensitization on cell types other than smooth muscle cells (e.g. inflammatory cells). The present data clearly show that such a desensitization of β2-adrenergceptors occurs. However, the role of alveolar macrophages in general, and the β2-adrenergceptors on those cells in particular, remains to be established in asthmatic. Thus we are not able to draw firm conclusions regarding the importance of our findings in relation to the clinical situation when treating asthma patients with β2-agonists.

In conclusion, long-term treatment with inhalation of the β-agonist terbutaline in healthy subjects induced a marked homologous depression of the β-adrenergceptor response on alveolar macrophages, which was not counteracted by concomitant treatment with inhaled budesonide. The present findings are in accordance with our previous findings with oral terbutaline. Asthma is known to be an inflammatory disease with a complex interaction between cells in the airways, and the alveolar macrophage is one of the inflammatory cells participating in the asthmatic reaction. It is hypothesized that β-adrenergceptor stimulation may influence the function of the alveolar macrophage, as well as of other inflammatory cells, and thereby influence the inflammatory component of the disease.

ACKNOWLEDGMENTS

We wish to thank Maj-Christina Johansson, Maud Daleksog and Britt-Marie Sundblad for technical assistance and Astra Draco for gifts of terbutaline, budesonide and placebo. We gratefully acknowledge the support by the Swedish Heart-Lung Foundation and King Oscar II’s Foundation.

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Received 21 July 2000/23 October 2000; accepted 8 January 2001

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