Inflammation in chronic obstructive pulmonary disease: its assessment and the effects of corticosteroids
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Detection of glucocorticosteroid receptor α and β mRNA in airway epithelial cells and alveolar macrophages from COPD patients; a pilot study

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Abstract

The limited anti-inflammatory efficacy of glucocorticosteroids in chronic obstructive pulmonary disease (COPD) may be related to a reduced local expression of the glucocorticosteroid receptor α (GRα) which mediates the anti-inflammatory action of glucocorticosteroids, and/or an enhanced local expression of the glucocorticosteroid receptor β (GRβ), which competes with GR activity.

In a pilot study we aimed to assess the presence of GRα mRNA and GRβ mRNA by a semi-quantitative PCR relative to transcripts of a house-keeping gene, β2-microglobulin mRNA, in epithelial cells from a single brush and in bronchoalveolar lavage cells or purified alveolar macrophages from 14 patients with mild to moderate COPD (mean age 66 y, mean FEV₁ 66% of predicted).

GRα mRNA was detectable in both epithelial BAL cells and alveolar macrophages, provided β2-microglobulin mRNA could be detected in 23 PCR cycles. GRβ mRNA was not detected in these samples, which was not due to a technical error, as GRβ mRNA in CemC7 cells was detected. GRβ mRNA expression was at least 18-fold lower, compared to that of GRα.

In conclusion, quantitative assessment of GRα but not of GRβ mRNA in airway epithelial cells and alveolar macrophages is feasible on material obtained respectively by a single brush and by a lavage. These preliminary data indicate that GRβ mRNA expression in the airways of patients with COPD is much lower compared to that of GRα and therefore enhanced expression of GRβ is unlikely to underlie the limited effects of glucocorticosteroids in COPD.
Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory airways disease characterized by progressive and irreversible airway obstruction, potentially affecting 10% of the world population.¹;² The anti-inflammatory effects of glucocorticosteroids in COPD are limited, in marked contrast to that for asthma, another major inflammatory airways disease.³ Glucocorticosteroids act by binding to the intracellular glucocorticosteroid receptor (GR) of which two forms, GRα and GRβ, exist.⁴ Binding of the glucocorticosteroid to GRα exerts an anti-inflammatory effect. GRβ lacks the binding site for glucocorticosteroids, but inhibits the action of GRα. Therefore, the efficacy of glucocorticosteroids may be attenuated by a reduced GRα expression,⁵;⁶ and/or by a relative abundance of the inactive GRβ.⁷ In a previous study,⁸ with steroid-naïve COPD patients, using Northern blot analyses on material obtained by 10 bronchial brushes per patient, reduced GRα mRNA levels in airway epithelial cells were found, and the GRα/GRβ ratio was high (approximately 1.7), though similar to that of controls. Both airway epithelial cells and alveolar macrophages are considered to drive airway inflammation in COPD, and thus are potential targets for glucocorticosteroids. As part of a larger study investigating the clinical and anti-inflammatory effects of systemic and inhaled corticosteroids in patients with COPD,⁹ we performed a pilot study to determine whether GRα and GRβ mRNA could be determined in airway epithelial cells, obtained by a single brush, and in alveolar macrophages, obtained by bronchoalveolar lavage fluid.

Methods

Fourteen out of 19 patients with clinically stable, smoking-related COPD, who participated in a glucocorticosteroid intervention study,⁹ agreed to two additional visits with a bronchoscopy, with bronchoalveolar lavage and subsequent epithelial brush. These bronchoscopies were performed as described earlier,¹⁰ before and after three-weeks, open, oral prednisolone treatment period (30 mg once daily), which was performed after an initial run-in period of eight weeks in which all corticosteroids were withheld. Written informed consent was obtained from all patients and the study was conducted according to the declaration of Helsinki and after approval by the local medical ethics committee. Portions 4 to 7 out of eight successive 20 ml lavages with prewarmed 0.9% saline were pooled and centrifuged at 500 g at 4°C. The cell pellet was resuspended in 1-2 ml phosphate-buffered saline (PBS; pH7.2), after which a cell count was performed using a Bürker-Turk chamber, and 4 cytospins (20,000 cells per slide) were made for cell differentiation. The remainder of the cells, when sufficient in number (>2x10⁶), were subjected to FACS sorting of alveolar macrophages by auto-fluorescence in a gate.
containing CD15 and HLA-DR expressing cells. Otherwise, BAL cells were used without sorting. Cells obtained by brush were resuspended in 5 ml of cold 0.5 % (w/v) bovine serum albumin and 0.02% (w/v) K-EDTA-containing PBS by vortexing, counted and collected by centrifugation. Subsequently, cells were treated with Trizol (Invitrogen) and RNA was purified according to the recommended procedure. Single stranded cDNA was synthesized from total RNA using 250 ng of oligo(dT)15 and 50 Units of Superscript II (Gibco/BRL) in a final 25 μl. Expression of GRα, GRβ and β2-microglobulin (β2M) mRNA (to normalize for variable RNA input) were analyzed by quantitative real-time PCR (Lightcycler: Roche), in which conditions were optimized so that the amplification for each mRNA (GRα, GRβ and β2M) was comparable, allowing semi-quantitative comparison. Two μl of cDNA was used in the PCR reaction (2 μl SYBR green; 4, 8, 2.8 or 3.2 μl 25 mM MgCl2 (for GRα, GRβ and β2M, respectively) and 1 μl of each primer (0.1 μg/μl)) in a 20 μl final volume. PCR conditions were: for GRα: 35 cycles of 45 sec. 95°C, 2 sec. 95 °C, 10 sec. 52 °C and 30 sec. 72 °C; for GRβ: 39 cycles of 45 sec. 95 °C, 2 sec. 95 °C, 10 sec. 56 °C, 15 sec. 72 °C, and for β2M: 32 cycles of 30 sec. 95 °C, 2 sec. 95 °C, 10 sec. 57 °C and 10 sec. 72 °C. The following primers were used: 5'-TGG-AGA-TCA-TAT-AGA-CAA-TCA-3' (GRα forward); 5'-TCA-CTT-TTG-ATG-AAA-CAG-AAG-3' (GRα reverse); 5'-GCT-GTA-TGT-TTC-CTC-TGA-GTT-A-3' (GRβ forward); 5'-TTT-TTG-AGC-GCC-AAG-ATT-GTT-G-3' (GRβ reverse), 5'-CCA-GCA-GAG-AAT-GGA-AAG-TC-3' (β2M forward), 5'-GAT-GCT-GCT-TAC-ATG-TCT-CG-3' (β2M reverse). Whether the resulting products were single products was verified by melting curves. The quantity of GR and β2M mRNA is expressed in threshold cycle values (Ct-values), which is the number of amplification cycles to reach a detectable mRNA amount (fluorescence set at 1). The GR Ct value was subsequently corrected for variable RNA input by deducing the corresponding β2M Ct value. A Ct-value of one unit lower corresponds with a two-fold higher gene expression. RNA from CemC7 (GRβ-expressing cell line) was used to generate cDNA for the GRβ standard curves and RNA from NCI-H292 airway epithelial-like cells was used to generate cDNA for GRα and β2M standard curves. In separate experiments the 507 bp, 324 bp and 268 bp rt-PCR products for GRα, GRβ and β2M, respectively, were purified on 2% agarose gel, cut-out, purified and served as standard curve material.

Results

Patients

Characteristics of patients are summarized in Table 1. One patient refused the second bronchoscopy procedure, but, in general, the bronchoscopy, the lavage and brush were well tolerated. Two patients experienced an airway infection a few days after the bronchoscopy, both after prednisolone treatment.
Table 1. Demographic and baseline data of the patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male / female</td>
<td>10 / 4</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>63.0 ± 6.7 (51 – 74)</td>
</tr>
<tr>
<td>Current-/ex-smoker</td>
<td>11 / 3</td>
</tr>
<tr>
<td>Pack-years</td>
<td>37.1 ± 20.0 (17 – 90)</td>
</tr>
<tr>
<td>ICS-user / non-user</td>
<td>9 / 5</td>
</tr>
<tr>
<td>FEV₁ (L)</td>
<td>2.07 ± 0.99 (0.91 – 3.94)</td>
</tr>
<tr>
<td>FEV₁ (% pred.)</td>
<td>66.4 ± 22.0 (34 – 98)</td>
</tr>
<tr>
<td>FEV₁–reversibility (% pred.)</td>
<td>4.8 ± 4.4 (.03 – 11.3)</td>
</tr>
<tr>
<td>FEV₁ / VC</td>
<td>0.46 ± 0.15 (0.25 – 0.69)</td>
</tr>
<tr>
<td>GOLD-stage 1 / 2 / 3</td>
<td>5 / 6 / 3</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD (range) or in actual numbers. ICS: inhaled corticosteroid; lung function data are post-bronchodilator values, measured prior to the ICS-free run-in period; FEV₁: forced expiratory volume in one second; pred: predicted; GOLD: global initiative for chronic obstructive lung disease; VC: vital capacity.

**GRα and GRβ in airway epithelial cells**

Brush material from 11 out of 14 patients obtained before, and from 7 out of 13 patients obtained after prednisolone treatment was sufficient to allow quantification of β2M (Ct <32). In 8 of these 11 patients and in 2 of the 7 patients, respectively, also GRα mRNA could be determined (Ct <35; see Figure 1). GRα mRNA could not be quantified in samples with a β2M Ct value >23. In the two samples obtained after prednisolone treatment the amount of GRα mRNA was somewhat higher (i.e. lower Ct values) than in the 8 samples obtained prior to prednisolone treatment, but no paired data was available of the same patient before and after treatment.

GRβ mRNA could not be detected in any of the samples (Ct <39), whereas GRβ mRNA was detectable in RNA from CemC7 cells. The smallest difference between the quantity

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**Figure 1.** Expression of Glucocorticosteroid Receptor α-mRNA in bronchial brush material before and after three weeks prednisolone treatment, and corrected for variable RNA input.
of GRβ mRNA and GRα mRNA was 4.21 Ct, indicating that the quantity of GRβ mRNA was at least 18-fold lower than the quantity of GRα mRNA in these samples.

**GRα and GRβ in alveolar macrophages**

BAL cells were predominantly macrophages (median 91% before and 90% after prednisolone treatment). In unpurified BAL cells, β2M mRNA was detectable in 8 out of 9 samples, and GRα mRNA was detectable in 4 of these 8 samples (Figure 2). A β2M Ct value of <24 was generally required to detect GRα mRNA, although one sample with a Ct value of 17.37 failed to reveal GRα mRNA. For purified alveolar macrophages, in 6 out of 20 samples β2M mRNA was detectable and in 5 of these 6 samples also GRα mRNA (in samples with β2M Ct values of <23; Figure 2). We had one paired sample, before and after glucocorticosteroid treatment, showing similar quantities of GRα mRNA. GRβ was not detectable in any of the samples; in samples with detectable GRα mRNA, the smallest difference between GRα mRNA and GRβ mRNA was 6.06 Ct units for the BAL cells and 7.38 Ct units for alveolar macrophages, indicating that the quantity of GRβ mRNA was at least 67-fold respectively 167-fold lower than that of GRα mRNA.

![Figure 2. Expression of Glucocorticosteroid Receptor α-mRNA in BAL cells (open symbols) and sorted alveolar macrophages (filled symbols) before and after three weeks prednisolone treatment, and corrected for variable RNA input. For one patient we had paired data (dashed line).](image)

**Discussion**

In the present study we found that GRα mRNA was detectable in epithelial cells obtained by a single brush, in BAL cells as well as in purified alveolar macrophages, provided that enough RNA was obtained to yield a β2M Ct value of <23. GRβ mRNA was not detectable in any of these samples. We exclude a technical error in the detection of GRβ mRNA, since the positive controls yielded a GRβ signal. The PCR assays for GRα, GRβ and β2M cDNA have been optimized, so that the amplification for each of these cDNA was comparable, which facilitates a direct but relative quantitative comparison. This indicates that GRβ mRNA compared to GRα mRNA is at least 18-fold lower in epithelial cells and 67-fold lower in BAL cells. These relative quantities in airway epithelial cells are
markedly lower than the values reported previously. The most likely explanation is that this difference is due to the large methodological differences. Assuming that mRNA levels for the GRs corresponded with GR protein levels and despite the limited number of samples with detectable GRα and GRβ, we conclude that the lack of effect of glucocorticosteroids in COPD is unlikely to be due to the competitive presence of GRβ in the airway epithelium. This contrasts with previously published data, where the GRβ could be detected in airway tissue of patients with COPD. Additionally, though we had paired data before and after prednisolone treatment for one patient only, the present data does not support a decreased presence of GRα after systemic corticosteroid treatment or a change in the relative presence of GRα and GRβ. However, more recent data, published after completing the present study, supports the present observation: in inflammatory bowel disease, glucocorticosteroid response was not linked to increased expression of GRβ, and in healthy subjects the ratio of the two GR isoforms was not related to sensitivity to dexamethasone induced adrenal suppression. In the future, the assessment of GRα and GRβ mRNA may be carried out preferably on slightly more material than analyzed here. In addition, it will be essential to also detect protein levels for both GRα and GRβ, as the proteins are directly responsible for the anti-inflammatory effects of glucocorticosteroids.

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References


