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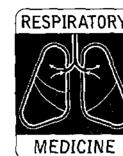
RESPIRATORY MEDICINE (1998) **92**, 1102–1109



Glucocorticoid receptor mRNA levels in bronchial epithelial cells of patients with COPD: influence of glucocorticoids

S. H. KORN, F. B. J. M. THUNNISSEN, G. J. WESSELING, J.-W. ARENDS AND E. F. M. WOUTERS

Departments of Pulmonology and Pathology, Maastricht University, Maastricht, The Netherlands

Glucocorticoids (gcs) are known to be effective in the treatment of asthma. In chronic obstructive pulmonary disease (COPD), however, no beneficial effects are demonstrated in most patients. Hypothetically, this may be explained by an overexpressed β-glucocorticoid receptor (GR) compared to the α-GR. The aim of this study was to investigate α- and β-GR mRNA levels and ratios in patients with COPD with or without glucocorticoid treatment.

GR and, as a control, metallothionein (MT) 2 mRNA levels were compared between patients with COPD receiving glucocorticoids (COPD + gcs), glucocorticoid naive COPD-patients (COPD − gcs) and non-COPD control patients not using gcs. Bronchoscopy was performed and bronchial epithelial cells were sampled with brushing.

Smoking did not influence α- and β-GR levels and ratios, nor the MT2 mRNA expression level. The α-GR mRNA expression was lower in the COPD − gcs group than in controls. Both GR forms were higher in the COPD + gcs patients than in the COPD − gcs patients, but not different from the levels measured in the controls. $\alpha 1/\beta$-GR mRNA ratios did not differ between the groups and averaged 1·7, suggesting no inhibitory effect of the β-GR on the $\alpha 1$ form. MT2 levels were upregulated in the COPD + gcs patients as compared to the COPD − gcs group, indicating a pharmacological glucocorticoid effect.

In the present study it is demonstrated that basal GR mRNA levels are lower in patients with COPD. Although this needs to be investigated further, this might explain, in part, the non-responsiveness of patients with COPD to gcs.

RESPIR. MED. (1998) **92**, 1102–1109

Introduction

Glucocorticoids (gcs) have proven to be effective in the long-term management of asthma. Symptoms and airway hyperresponsiveness are reduced, lung function improved and airway integrity restored (1). Only a small proportion of asthma patients do not respond to gcs. In contrast, effects of gcs in patients with chronic obstructive pulmonary disease (COPD) are limited (2,3). Callahan *et al.* demonstrated in only 10% of the patients a response to oral corticosteroid therapy, measured as a 20% increase in baseline FEV_1 (4). The beneficial effects of glucocorticoids in COPD are at present still under study (5), and the results of this investigation will be available soon. The reason for the limited response to glucocorticoids in patients with COPD is not yet elucidated.

Received 28 July 1997 and accepted in revised form 21 April 1998.
Correspondence should be addressed to: F. B. J. M. Thunnissen, Department of Pathology, Maastricht University, P.O. Box 5800, 6202-AZ Maastricht, The Netherlands.
This study was supported by a grant of ASTRA Pharmaceuticals, The Netherlands.

The molecular mechanisms involved in the anti-inflammatory actions of glucocorticoids are not completely understood. Glucocorticoids enter the cell by passive diffusion and bind to a cytoplasmic glucocorticoid receptor (GR). This receptor mediates the effect of glucocorticoids by translocating into the nucleus, binding to glucocorticoid responsive elements (GRE) in the DNA and modulating the transcription of genes (6). Another way of regulating the transcription of genes does not involve binding of the GR to DNA but the interaction of GR with other transcription factors. Cross-talk between the GR and proinflammatory transcription factors, like activator protein-1 (AP-1) (7,8) and nuclear factor κB (NFκB) (9,10), has been demonstrated within the cell. This interaction is believed to be important for the anti-inflammatory effect of glucocorticoids (11). Direct protein–protein interaction of the GR has not only been described with AP-1 and NFκB, but also with cAMP-responsive element binding protein (CREB) (12,13) and signal transducers and activators of transcription 5 (Stat5) (14). In asthma, steroid resistance might be due to a reduced binding of the activated glucocorticoid receptor to DNA, caused by high AP-1 concentrations (15). No information is available about the GR expression and interactions with other transcription factors in patients with COPD.

0954-6111/98/091102 + 08 $12.00/0
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As a result of alternative splicing, the glucocorticoid receptor exists in two forms, α and β (16). Both α and β-GR mRNAs are present in all tissues investigated (17,18), but because of its ligand binding capacity, the α form has been the primary target in research (16,18). The levels of GR expression vary between cell types and individuals, but in all cases higher α-GR mRNA expression levels have been observed compared to β-GR mRNAs (18). Recently, Oakley *et al.* (18), reported the existence of 2 α-GR isoforms, $\alpha 1$ and $\alpha 2$ with Northern blot analysis. The $\alpha 1$, $\alpha 2$, and β bands were demonstrated at 7 kb, 5·5 kb, and 4·3 kb, respectively. However, translation of both α forms results in the same GR protein. The fact that the β form is widely expressed in many cell types, indicates that it may play a role in the cellular response to glucocorticoids. It has been demonstrated that the β-GR acts as a dominant negative inhibitor on the transcriptional activity of the α-GR at an α/β ratio well below 1 (17). Thus, a predominant expression of the β-GR mRNA over the α-GR mRNA could explain a lack of response to steroids as observed in steroid resistant asthma and COPD. Therefore, it is important to study both GR forms in COPD.

The aim of the present study was (1) to investigate α and β-GR levels and ratios in bronchial epithelial cells of patients with COPD and (2) to assess the effect of glucocorticoids in these patients. Since the amount of cells obtained from human bronchial epithelial cells *in vivo* is limited and no antibodies are available to separately investigate both GR forms, we chose to study the α and β-GR at mRNA level. As a control gene, metallothionein mRNA levels were studied, since this gene is known to be upregulated by glucocorticoids.

Methods

PATIENTS

In this cross-sectional study, three patient groups were investigated. COPD patients receiving glucocorticoids (n=12), patients with COPD using no glucocorticoids (n=6), and controls, i.e. patients without pulmonary obstruction (n=14). COPD was defined according the standards of the American Thoracic Society (19). Pulmonary function tests and bronchoscopies were performed according to the guidelines of the European Respiratory Society (20) and American Thoracic Society (21), respectively. Smoking status was recorded and non-smoking was defined as 'stopped smoking for more than 5 years'. The control group existed of patients with lung cancer (n=10), patients with haemoptysis (n=3) and one patient with unexplained cough. From the patients with lung cancer, bronchial brushings were performed from second to fourth order bronchi of the non-malignant contralateral lung. β_2-agonists (at least $3 \times 400\,\mu g$ albuterol daily) were used by all patients with COPD using glucocorticoids (COPD+gcs) but by only two COPD patient in the glucocorticoid free group (COPD − gcs). The controls neither used glucocorticoids nor β_2-agonists. Patients in the COPD+gcs group were under maintenance gcs therapy in variable doses of inhaled glucocorticoids (ICS, at least $2 \times 400\,\mu g$ of either budesonide or beclamethasone). Two COPD patients were hospitalized during bronchoscopy and received additional i.v. prednisone. The interval between last dose of ICS and bronchoscopy was not standardized. Four patients underwent bronchoscopy 3 h after the last dose of ICS, three patients after a time interval of more than 10 h, three underwent bronchoscopy within 2 h of the last dose and of two patients the time between inhalation and bronchoscopy was unknown. The protocol has been approved by the local ethical committee.

RNA-ISOLATION, NORTHERN BLOTTING AND HYBRIDIZATION

RNA was isolated from the cells as described before by Korn *et al.* (22). In short: 10 brush samples were taken from the second to fourth order bronchi and cells were firmly shaken into 4 ml DMEM. Immediately after collection, an equal amount of 8 M GTC was added. Total RNA was isolated with the GTC/CsCl-method and 20 μg was run on gel for Northern blot analysis. The glucocorticoid receptor, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and metallothionein (MT2) probes were subsequently hybridized. Both α forms and the β form are detected with the GR-probe. The MT2 mRNA is known to be upregulated by glucocorticoids and served as a positive control. The gene has 2 GREs in its promotor sequence (DNA Stock Center, Japan). Sample signals were analyzed visually as well as semiquantitatively with a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA, U.S.A.). GR and MT2 mRNA levels were expressed relative to GAPDH levels of the same sample.

CYTOLOGY

To determine the cellular composition of the brushes, smears were made by spreading one additional brush on an object glass. Cells were Giemsa stained, and cellular composition analysed as described before (23,24).

STATISTICS

For all results mean ± standard deviation (SD) was calculated. To determine differences the Mann–Whitney U test or χ^2-square test were performed. Differences of P<0·05 were considered to be statistically significant.

Results

CLINICAL CHARACTERISTICS OF THE PATIENTS

Clinical characteristics and smoking status of the patients with COPD and the control group are shown in Table 1. Age, gender and smoking status did not differ between the three groups. As expected the FEV_1 was lower (P<0·05) in patients with COPD compared to the control group, 51%

TABLE 1. Clinical characteristics of the patients

Patient	Steroid +/−	Age year	Sex M/F	Disease	FEV_1 % pred.	FEV_1 % rev.	DLCO % pred.	Smoking y/n
1	−	64	M	COPD	72	10	124	y
2	−	52	F	COPD	66	2	46	y
3	−	72	M	COPD	47	3	47	y
4	−	74	M	COPD	71	6	104	y
5	−	74	M	COPD	48	4	74	n
6	−	73	M	COPD	62	0	59	y
7	+	67	M	COPD	33	0	43	y
8	+	73	F	COPD	41	n.d.	n.d.	y
9	+	62	M	COPD	65	5	68	y
10	+	67	F	COPD	32	0	59	y
11	+	68	M	COPD	32	5	62	n
12	+	62	M	COPD	45	2	70	y
13	+	73	F	COPD	69	0	74	n
14	+	67	F	COPD	38	2	93	n
15	+	71	M	COPD	70	n.d.	85	y
16	+	57	M	COPD	51	7	n.d.	y
17	+	67	F	COPD	41	5	51	y
18	+	72	M	COPD	34	4	72	n
19	−	48	M	Cancer	77	n.d.	124	y
20	−	58	M	Cancer	87	n.d.	133	y
21	−	77	M	Cancer	84	n.d.	54	y
22	−	61	F	Cancer	81	n.d.	n.d.	y
23	−	63	M	Cancer	83	n.d.	86	y
24	−	36	F	Cancer	103	n.d.	91	y
25	−	61	M	Haemoptoe	n.d.	n.d.	n.d.	y
26	−	72	M	Haemoptoe	111	n.d.	94	n
27	−	49	M	Haemoptoe	136	n.d.	123	n
28	−	61	M	Cough	96	n.d.	147	n
29	−	69	M	Cancer	96	n.d.	108	y
30	−	73	M	Cancer	86	n.d.	90	n
31	−	74	M	Cancer	93	n.d.	89	y
32	−	52	M	Cancer	100	n.d.	120	y

n.d., Not determined.

and 95%, respectively. Additionally, the FEV_1 in patients with COPD − gcs was higher ($P<0{\cdot}05$) compared to the COPD+gcs group. The percentage FEV_1 reversibility (gained to β_2-agonist) in the COPD+gcs patients was $3{\cdot}0 \pm 2{\cdot}4\%$ and from COPD − gcs patients was $4{\cdot}2 \pm 3{\cdot}2\%$. The diffusion capacity (DLCO) was lower ($P<0{\cdot}05$) in patients with COPD+gcs as compared to the controls: 68% and 105%, respectively. In the COPD − gcs group the DLCO (76%) tended to be lower than in the controls ($P=0{\cdot}08$).

CELL COUNTS

In Table 2 the cellular composition from the brush specimens is presented. The majority of the cell types were epithelial in origin (average >85%). Although the COPD patients on average seem to have more neutrophilic granulocytes, due to the large variation no significant difference was observed in cell differential counts obtained from brushes of patients with COPD and the non-obstructive control group. Neither was there a significant difference in cell types between brushes from smokers and non-smokers.

TABLE 2. The relative cellular distribution of brushes given in percentages

Cell types	COPD+gcs	COPD − gcs	Control brush
Epithelial cells	81 ± 20	78 ± 18	91 ± 8
Macrophages	2 ± 2	4 ± 7	1 ± 1
Neutrophils	12 ± 21	10 ± 13	4 ± 5
Eosinophils	0·1 ± 0·2	0·2 ± 0·4	0·2 ± 0·3
Lymphocytes	6 ± 4	7 ± 9	4 ± 4
Basophils	0	0·2 ± 0·3	0

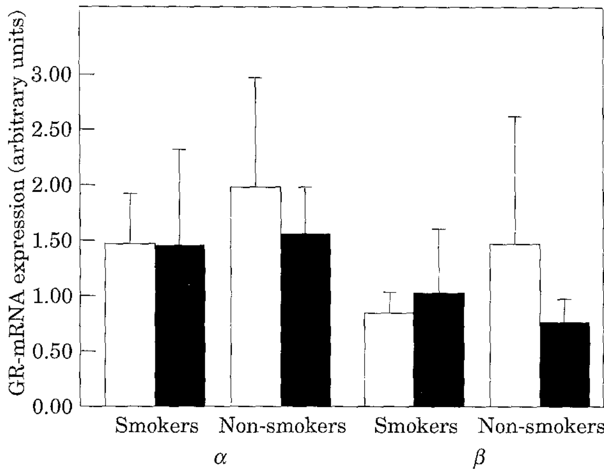

FIG. 1. The mean ± SD of α1- and β-GR mRNA expression in brushes of patients with COPD+gcs (□) and the age-matched controls (■) subdivided by smoking. Glucocorticoid therapy did not downregulate either α1- or β-GR mRNA in patients with COPD.

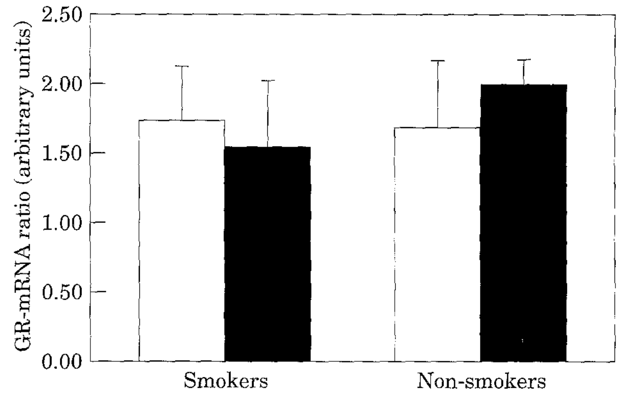

FIG. 2. $\alpha 1/\beta$-GR mRNA ratios in brush samples of patients with COPD+gcs (□) and controls (■). Shown are mean values ± SD of every group, subdivided by smoking.

GENE EXPRESSION

In five patients (two COPD+gcs, two COPD − gcs and one control) the GR mRNA signal was too weak for reliable quantitation. Because smoking is an important factor in COPD it is essential to check whether GR mRNA levels are affected by smoking. Therefore all groups were subdivided in current smokers and non-smokers, except for the COPD − gcs group which only has one smoker. This group is not included in the first three figures. Because the α2 band was not detected in these blots, only α1- and β-GR mRNA levels were determined.

The means and standard deviations of the α1- and β-GR mRNA levels in bronchial epithelial cells, subdivided by smokers and non-smokers from each group, are shown in Fig. 1. The $\alpha 1/\beta$ ratios in the same cells from the same groups are shown in Fig. 2. No difference in both α1- and β-GR mRNA expression levels or ratios was observed between smoking patients with COPD+gcs and smoking controls using no glucocorticoids. Similar results were obtained for the comparison between the non-smoking patients and for the comparison between smokers and non-smokers per patient group.

In Fig. 3 MT2 mRNA levels are given in human bronchial epithelial cells of COPD+gcs and controls, subdivided by smokers and non-smokers. MT2 mRNA levels were not influenced by smoking, not in the controls (P=0·3) nor in patients with COPD+gcs (P=0·6).

Because no effect of smoking on either α- and β-GR mRNA levels or ratios nor on MT2 mRNA levels was demonstrated, we concluded that smoking was not a confounding factor in this study. Therefore, for further analysis, the data within the COPD+gcs, COPD − gcs and control groups were combined.

In Fig. 4, α- and β-GR mRNA levels are shown and compared for all three groups. Lower GR mRNA levels

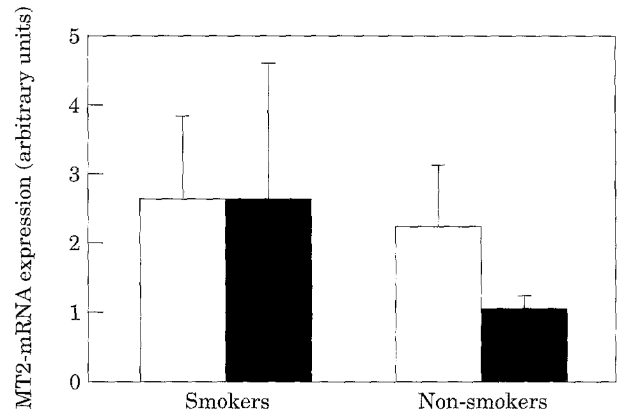

FIG. 3. MT2 mRNA expression in brush samples of patients with COPD+gcs (□) and an age-matched control group (■). No upregulation of MT2 mRNA was seen in patients with COPD+gcs compared to the controls, nor in the smokers compared to the non-smokers. Shown are mean values ± SD.

were demonstrated in the COPD − gcs patients than in the controls, which was significant for the α-GR (P<0·05). A clear difference in expression was observed between the COPD+gcs and COPD − gcs group for the α1-GR form, 1·7 ± 0·8 and 0·8 ± 0·2 (P=0·01) and for the β-GR 1·1 ± 0·8 and 0·5 ± 0·1 (P=0·03), respectively.

Comparing the α/β-GR mRNA ratios between the three groups no significant difference is observed (Fig. 5). The average ratio was 1·7 ± 0·4, clearly above 1, indicating no inhibitory effect of the β-GR on the α form.

In Fig. 6, MT2 mRNA levels are demonstrated. A higher expression level was seen in patients with COPD using glucocorticoids compared to the COPD − gcs group (P<0·05). There was no difference in expression between the controls and COPD − gcs group, nor between the controls and the COPD+gcs group.

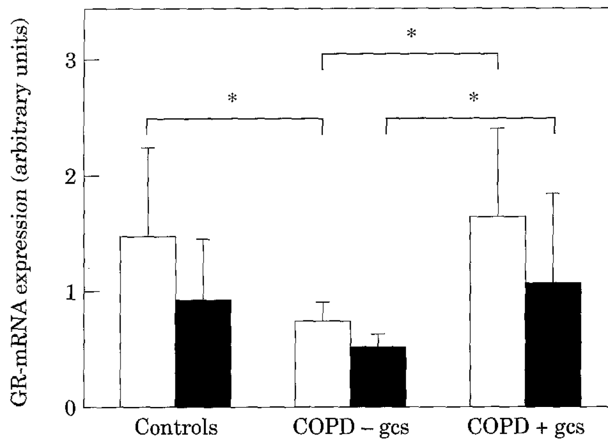

FIG. 4. Given are mean α1- (□) and β- (■) GR mRNA expressions in bronchial epithelial cells of patients with COPD − gcs, COPD+gcs and a control group. Basal GR mRNA levels are lower in patients with COPD − gcs compared to the controls. The COPD+gcs demonstrate an upregulated α- and β-GR mRNA expression compared to the COPD − gcs. $^*P<0.05$.

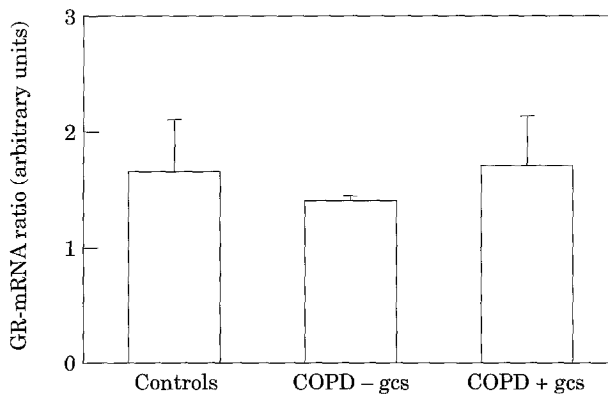

FIG. 5. The ratios between the α1- and β-GR mRNA are plotted. No difference is observed between the three groups.

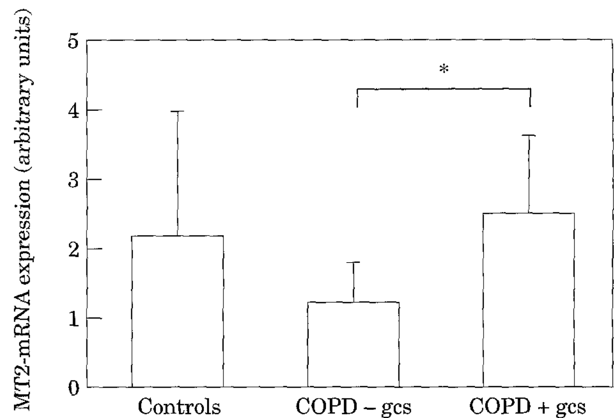

FIG. 6. Mean MT2 mRNA levels are shown for the COPD − gcs, COPD+gcs and controls. A clear higher expression of MT2 mRNA is observed in the patients using glucocorticoids (COPD+gcs) as compared to the COPD − gcs. $^*P<0.05$.

Discussion

The aim of this study was to investigate the GR mRNA expression in patients with COPD. Smoking did not appear to influence α1- and β-GR mRNA levels and ratios nor MT2 mRNA levels in the bronchial epithelial cells. The α1- and β-GR mRNA levels were lower in COPD − gcs than in controls. GR mRNA levels were similar between COPD+gcs and the control group, and significantly higher than in the COPD − gcs group. On average, a 1·7 times higher α-1 GR mRNA expression was seen compared to the β-GR mRNA levels. No difference in α/β-GR mRNA ratio was observed between the three groups, indicating no inhibitory function from the β-GR on the activity of the α-GR. MT2 levels were higher in the COPD+gcs group compared to the COPD − gcs, but not different from the levels in the controls, indicating a functional response to glucocorticoid.

Interestingly, basal expressions of the GR mRNA were lower in patients with COPD − gcs compared to the controls. This can be interpreted in two ways. First, it might be a pathophysiological phenomenon, resulting in a disturbed GR function in bronchial epithelial cells of patients with COPD. Since it has been demonstrated that the response of cells to glucocorticoids is dependent on GR protein levels (25–27), lower GR mRNA levels could explain the lack of response of patients with COPD to glucocorticoids. However, it is unknown if these lower mRNA amounts demonstrated in the COPD − gcs patients are also present at protein level and if the translational capacity of both receptor mRNA forms is equal. This needs to be investigated further. An alternative explanation for the lower α-1 and β-GR mRNA expression may be the number of patients investigated. Because glucocorticoids are commonly prescribed in COPD in The Netherlands, it was difficult to collect glucocorticoid naive COPD patients.

When comparing the patients with COPD − gcs to the COPD+gcs group, an upregulated α1- and β-GR mRNA expression is observed in the glucocorticoid treated group to similar levels as in the non-obstructive control patients. This 'normalization' of the GR mRNA expression is an unexpected finding, since in a previous study (22) we demonstrated a downregulation of the α1- and β-GR mRNA shortly after acute glucocorticoid therapy. However, after chronic (4 weeks daily) inhalation of 1600 μg budesonide, on average, no change in GR mRNA expression was observed. Only after correcting for the time between inhalation and bronchoscopy, an acute downregulation was observed. This downregulation occurred after 2 h and was reversed after 10 h. There are two explanations for the upregulated GR mRNA expression in patients with COPD after glucocorticoid use. Apparently, basal GR mRNA levels are decreased in patients with COPD. Therefore, in these patients, in the inflammatory process the balance of transcription factors is unfavourable for the GR. The mechanism behind this imbalance needs further investigation. Arguments for functioning GRs are

the upregulation of the MT2 levels as well as the 'normalization' of the GR mRNA after GCS use in patients with COPD. Alternatively, the use of β_2-agonists may provide an explanation. The patients of the COPD+gcs group all received β_2-agonists compared to only two of the patients from the COPD − gcs group. Since CREB is activated by β_2-agonists and is able to interact with the GR, this might play a role in the upregulated, 'normalized' GR mRNA expression.

In a recent study of Oakley *et al.* (18), the GR β isoform was studied. Similar findings as compared to the study of Bamberger *et al.* (17) were described regarding the negative inhibitory function of the β-GR on the activity of the α form. Striking in this article was the observation that with Northern blot analysis three GR mRNA forms were present. Instead of the previously frequently mentioned (16,28–30) α-GR mRNA (7 kb) and β-GR mRNA (5 kb), an α1 band of 7 kb, an α2-band of 5·5 kb and a β-band of 4·3 kb were demonstrated (18). In a previous study (22) we investigated the α1-GR mRNA form, which was abundantly expressed in both bronchial epithelial cells and alveolar macrophages and the β-band, which was also expressed in these cell types, although to a lesser extent. We determined the identity of these isoforms by the location of the 28S and 18S ribosomal bands. Occasionally in alveolar macrophages expressing higher amounts of GR, a faint third hybridization signal could be discerned, although in much lower levels as compared to the other two GR mRNA forms. This band was located just above the 28S band, whereas the β form was situated on the lower side of the 28S band, in agreement with the study of Oakley *et al.* (18). We were not able to detect the α3-GR form in the patients described in this study. The recent discovery of the β-GR as a negative inhibitor of the α-GR might be an important element in the poor response to glucocorticoids in steroid resistant asthma or COPD (17,18). Bamberger *et al.* (17) demonstrated in a model study an increasing inhibition of 50–85% on α/β ratios from 0·2 to 0·07, respectively. Importantly the $\alpha1/\beta$-mRNA ratio in the present study was 1·7, more than 10-fold higher than described in the article by Bamberger (17), rendering a possible inhibitory effect of the β-GR on GR-function unlikely in COPD.

Since the GR mRNA expression may vary among different cell types, it is important to determine the cellular composition in the brush specimens. However, in this study, no significant increase in neutrophils or other inflammatory cell types was found in patients with COPD and smokers, indicating that the GR gene expression studied mainly concerned the bronchial epithelial cell.

Smoking is known to induce neutrophilic inflammation in the airways (31), either by direct chemotactic effects of cigarette components, such as nicotine, or by the release of chemokines by alveolar macrophages (32). COPD is characterized by airway inflammation, represented by larger numbers of neutrophils in the bronchoalveolar lavage and induced sputum (33–35). A correlation exists between the amount of neutrophils present in induced sputum and the airway obstruction and decline in lung function (33,36). Inflammatory stimuli, like IL1β, IL2, IL4, IFNγ and LPS increase the number and decrease ligand binding affinity of GRs *in vitro* (37–40). If these effects could be extrapolated to the *in vivo* situation in COPD, higher GR mRNA levels might be expected rather than lower GR mRNA levels. However, we did not measure inflammatory mediators and therefore can not draw conclusions about the inflammatory status of these patients. MT2 mRNA levels were also investigated, since heavy metals, present in cigarettes, increase the transcription of the MT2 gene (41). No change in MT2 gene expression was observed in bronchial epithelial cells of smokers compared to non-smokers, despite the fact that heavy metals, like cadmium, are present in cigarette smoke (42).

The present study demonstrates that patients with COPD − gcs have lower α1- and β-GR mRNA levels compared to a control group not receiving any glucocorticoids. Although this needs to be investigated further, this might be a first explanation of the non-responsiveness of patients with COPD to glucocorticoids.

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