EXPRESSION OF CREB-BINDING PROTEIN AND PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA DURING FORMOTEROL OR FORMOTEROL AND CORTICOSTEROID THERAPY OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

1Department of Clinical Pharmacology and 2Pneumology Department, Bialystok Medical University, Bialystok, Poland

We assessed the effect of therapy on nuclear signaling related to inflammatory processes in sputum cells of patients with chronic obstructive pulmonary disease (COPD). Patients were treated with formoterol (F) or formoterol plus budesonide (F/ICS) b.i.d. for 4 weeks, their sputum cells were isolated and subjected to RNA extraction or lysis, followed by differential centrifugation. Signaling protein levels were assessed by Western blots, their specific mRNAs were quantified using qRT-PCR, while 8-isoprostane levels were examined using enzyme immunoassay kit. Cytosolic 8-isoprostane levels and nuclear glucocorticoid receptor expression (protein and mRNA) were not significantly different in both groups, while nuclear cAMP response element binding protein (CREB; protein and mRNA) and peroxisome proliferator-activated receptor gamma (PPARγ; protein and mRNA) were significantly higher in cells from F/ICS-treated patients. CREB-binding protein (CBP; protein and mRNA) levels were significantly lower in F/ICS patients. These changes indicate increased anti-inflammatory signaling in F/ICS-treated patients and seem to be beneficial.

Key words: CBP, COPD therapy, CREB, glucocorticoids, PPARγ

INTRODUCTION

Glucocorticoids are commonly used anti-inflammatory drugs, which efficiently switch off pro-inflammatory genes in asthma but are not effective in chronic obstructive pulmonary disease (COPD) (1). We have previously
described increased expression and activation of nuclear cyclic AMP-response element binding protein (CREB) in COPD patients treated with inhaled corticosteroids (ICS) (2). It is possible that CREB activation can shift pro/antiinflammatory balance toward inflammation and account for poor responses of COPD patients to glucocorticoid therapy.

Experimental data show that anti-inflammatory effects of glucocorticoids may be independent of their DNA binding (3), pointing to the transrepressive mechanisms of steroid drugs. Nuclear CREB and its co-activator CREB-binding protein (CBP) are interacting with fast transcriptional on-off switches and are important in inflammatory diseases (4). Particularly, CBP is a co-activator molecule, integrating several signaling pathways, which are important in inflammatory diseases, since it anchors NF-κB, AP-1, and GR to the RNA polymerase (5), mediates activation of peroxisome proliferators activated receptor γ (PPARγ) (6), and alters chromatin remodeling and transcription. In inflammatory diseases, an interaction between ligand-activated glucocorticoid receptor (GR) and other transcription factors has been described; particularly an antagonism of NF-κB-mediated transcription by GR has been reported in many cell types involved in the immune/inflammatory response (7). In the present study, we investigated how formoterol or formoterol plus budesonide therapy of COPD patients affects nuclear signaling proteins and their mRNAs, particularly CREB, CBP, and PPARγ in relation to the level of oxidative stress and GR expression.

MATERIAL AND METHODS

The study was approved by a local Ethics Committee and all patients gave their informed consent after a full discussion of the nature of the study. COPD was defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines. Spirometry and lung volumes were performed with body box (Elite DL, Medgraphics, USA). The measurement was performed using standard protocols according to the American Thoracic Society guidelines. All patients had airflow limitation (FEV₁ <80% predicted, FEV₁/FVC <70%, GOLD stage II-IV). All subjects were characterized with respect to sex, age, smoking history, COPD symptoms, co-morbidity, and current medical treatment. All COPD patients had a smoking history of 10 pack-years or more. Exclusion criteria included other systemic diseases, other lung diseases apart from COPD and lung tumors, pulmonary infection, and antibiotic treatment 6 wk before inclusion and inhaled or oral glucocorticoids in the 3 months before inclusion. No patient in the study had symptoms or was treated for COPD exacerbation during at least two months before the day of inclusion. At the beginning all patients underwent a four-week wash-out with salbutamol rescue medication only. Then, the patients were stratified to the following treatment: formoterol alone (F; n=13), formoterol/budesonide (F/ICS; n=12) b.i.d. for 4 weeks.

Sputum was induced by the inhalation of a 4.5% hypertonic aerosol saline solution, which was generated by an ultrasonic nebulizer (Voyager, Secura Nova; Warsaw, Poland) (8). Samples were processed within 15 min after the termination of the induction. Throughout the procedure, the subjects were encouraged to cough and to expectorate into a plastic container. Three flow volume curves were performed before and after each inhalation, and the best FEV₁ was recorded. Induction
of sputum was stopped if the FEV\textsubscript{1} value fell by at least 20% from the baseline level or if troublesome symptoms occurred.

Induced sputum samples were directly used for RNA extraction and, in part, homogenized for 1 min in lysis buffer containing 10 mM N-2-hydroxyethylpiperazine-N’-ethane sulfonic acid, 10 mM KCl, 2 mM MgCl\textsubscript{2}, 1 mM dithiothreitol, 0.1 mM ethylenediamine-tetraacetic acid, 0.2 mM NaF, 50 mM β-glycerophosphate, a protease inhibitor tablet, 0.2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 10% Nonidet P-40. Thereafter, the samples were incubated on ice for 15 min and then centrifuged at 13000 x g for 30 s. The cell pellets containing nuclei were retained and resuspended in extracting buffer (50 mM N-2-hydroxyethylpiperazine-N’-ethane sulfonic acid, 50 mM KCl, 300 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid, 0.2 mM NaF, 0.2 mM Na-orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 50 mM β-glycerophosphate, and a protease inhibitor tablet (Complete Mini; Roche Diagnostics, Mannheim, Germany). The samples were incubated on a rotating platform for 30 min at 4°C followed by centrifugation at 13,000 x g for 5 min. The resulting nuclear extract and cytosol were evaluated for the expression of signaling molecules or 8-isoprostane levels.

For Western blots, 10 µg of nuclear proteins were separated by SDS/PAGE in reducing conditions, transferred onto polyvinylidene difluoride membranes, and incubated with specific antibodies against CREB, CBP, PPAR\textsubscript{γ}, or GR (Abcam or Santa Cruz rabbit specific antibodies against human proteins). After washing, bound antibody was detected using appropriate anti-rabbit antibody (Abcam) linked to horseradish peroxidase, and the bound complexes were detected using enhanced chemiluminescence (ECL, Amersham) and quantified using Image Quant software. The data were quantified with respect to the loading controls.

Changes in the expression of individual genes were quantified using SYBR Green based quantitative RT-PCR (SuperArray, Frederick, USA) after total RNA extraction from induced sputum cells, RNA purification, and template cDNA synthesis with customized PCR master mixes containing appropriate controls and reference dyes (SuperArray, Frederick, USA). PCR was performed on ABI 7900HT instrument and data were analyzed based on the ∆∆Ct method with normalization software. 8-isoprostanes were quantified in the cytosolic fraction using 8-isoprostane EIA Cayman kit (Ann Arbor, Michigan, USA). Protein levels were measured using BCA kit (Sigma-Aldrich, Poznan Poland).

The data were expressed as means ±SD. Statistical significance was calculated using one-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test for selected pairs of data.

RESULTS

Fig. 1 shows a relative expression of nuclear GR protein and GR mRNA levels and the quantity of cytosolic 8-isoprostanes in cells isolated from induced sputum of the stable COPD patients treated for 4 weeks with F or F/ICS. 8-isoprostane levels were not significantly different in the F and F/ICS patients, although slightly increased values were observed in the F/ICS group. In the F/ICS-treated patients, both GR protein and GR mRNA levels were not significantly different from the corresponding data of the F-treated patients. Fig. 2 shows the CREB, CBP, and PPAR\textsubscript{γ} protein expression and their specific mRNAs in the COPD patients treated for 4 weeks with F or F/ICS. Significantly higher (by ~ 20%; P<0.05) levels of the nuclear CREB and CREB mRNA were observed. Both CBP
mRNA and CBP protein were significantly lower (by 77 and 45%, respectively; P<0.01), while the PPARγ expression was significantly higher in the F/ICS-treated patients than those in the F-treated patients - PPARγ mRNA was increased by 181%; (P<0.01) and PPARγ protein was increased by 211%; P<0.01).

Fig. 1. Relative nuclear levels of GR mRNA, GR protein, and cytosolic 8-isoprostan e in cells isolated from induced sputum of stable COPD patients treated with formoterol (F; n = 13) and formoterol + budesonide (F/ICS; n = 12) b.i.d. for 4 weeks. The mean levels in the F-treated patients were set as 100 relative units ±SD.

Fig. 2. CREB, CBP, and PPARγ mRNAs, and proteins in cells isolated from induced sputum of stable COPD patients treated with formoterol (F; n = 13) and formoterol + budesonide (F/ICS; n = 12) b.i.d. for 4 weeks. The mean levels in the F-treated patients were set as 100 relative units ±SD. *P<0.05, **P<0.01 compared with the F-treated group.
DISCUSSION

To date, there is no effective treatment of a long-term decline in lung function, which is the hallmark of COPD. Current experimental COPD therapies target mostly intracellular signaling molecules or cytoplasmic receptors (9), but also focus on glucocorticoid resistance (10). Glucocorticoids, which are effective in many inflammatory diseases including asthma, are by far less beneficial in COPD. A recently proposed mechanism of steroid resistance in COPD involves a chronic generation of oxidative stress which impairs the activity of histone desacetylase 2 (HDAC2), resulting in altered chromatin remodeling, modification of pro/anti-inflammatory signaling balance, and increased inflammation (11). In the present study, we did not detect altered 8-isoprostane levels in COPD patients during F or F/ICS therapy, but a reduction of oxidative stress as a result of addition of theophylline to the F/ICS therapy was reported by the others (12).

It is now evident that chromatin remodeling and activation of histone acetyltransferase (HAT) is closely associated with proinflammatory changes (13). Several signaling molecules, including CREB, have HAT activity and may play a role in proinflammatory changes, but the clinical data are limited. We have previously shown increased nuclear and cytosolic CREB, and Ser 133 phosphorylated (activated) CREB expression increased in the cytosol and nuclei of cells isolated from sputum of COPD patients subjected to ICS therapy (2). It is, therefore, possible that therapy may increase CREB-mediated transcriptional activity. Similar activation was described in rat C6 glioma cells, where the mRNA of several CREB isoforms was significantly increased by dexamethasone (14). To further characterize alterations in nuclear signaling in COPD patients undergoing glucocorticoid therapy, we examined the transcriptional co-integrator CBP which binds CREB and mediates anchoring of the proinflammatory NF-κB and AP-1 molecules (15). The main signaling pathway of glucocorticoids is related to GR activation and transcriptional repression, thus interactions between activated GR and inflammatory signaling molecules, like NF-κB, CREB, and AP-1, are very important. Our present data indicate that in the F/ICS treated patients, GR protein expression and GR mRNA levels are not significantly different from the corresponding data in the F-treated patients, while the nuclear CREB and its mRNA are elevated. However, CBP mRNA and protein are significantly lower in the F/ICS-treated patients compared with the F-treated patients, which may result in decreased CREB-mediated signaling.

It is known that NF-κB and GR agonists have opposing effects in affecting inflammatory responses (16). Antagonism of the NF-κB-mediated transcription by GR has been reported in many cell types involved in the immune/inflammatory response (17). GR can directly sequester the activated form of NF-κB and increase the expression of the natural inhibitor CBP, which as co-integrator molecule has been reported to bind, and possibly co-activate both GR and NF-κB (18). It is
possible that CBP may play a key role as a restrictive molecule required for both NF-kB and GR in COPD patients during therapy, although it has been shown that CBP does not physically interact with the antagonist-bound GR (19), but overexpression of CBP partially represses the GR transactivation (20).

Combined β2-agonist and glucocorticoid therapy can strongly activate peroxisome proliferator-activated receptor gamma PPARγ which represses the AP-1 and NF-kB transcriptional activity (21). This negative cross-talk occupies an important role not only in cancer (22), but also in inflammatory diseases (23). In the present study, PPARγ mRNA and protein were significantly higher in the F/ICS-treated than in F-treated patients. PPARγ is negatively correlated with proinflammatory signaling, while altered CBP expression may result in its reduced recruitment to the transcriptional initiation complex on the promoter region of various genes, and both changes may decrease proinflammatory signaling at least in sputum cells.

To conclude, combined F/ICS therapy seems to have positive effects on basal nuclear signaling related to anti-inflammatory reactions. However, it remains to be established weather similar alterations take place in lung tissue.

*Conflicts of interest:* The authors had no conflicts of interest to declare in relation to this article.

**REFERENCES**


Received: August 8, 2008
Accepted: August 22, 2008

Author’s address: A. Holownia, Department of Clinical Pharmacology, Medical University of Białystok, Zurawia 14 St., 15-540 Białystok, Poland; phone: +48 85 7450649; e-mail: holow_sinai@hotmail.com