cAMP responsive element binding protein (CREB) plays an important role in transcriptional machinery. CREB signaling is altered in patients with asthma. However, the role of CREB in chronic obstructive pulmonary disease (COPD) is less clear. In the present study we assessed changes in subcellular CREB distribution and activation (CREB-P) in 35 stable COPD patients treated with formoterol (F), formoterol + budesonide (F/ICS), and formoterol + budesonide + theophylline (F/ICS/Th) b.i.d. for 4 weeks, using SDS-PAGE/WB in cytosol and nuclear extracts of induced sputum cells. The expression of CREB was increased after F/ICS in both cytosolic and nuclear fractions by about 40% and 24%, respectively (P<0.001, P<0.01), while CREB-P increased after F/ICS by about 50% (P<0.01) in both compartments. These changes were not affected by theophylline. In F/ICS-treated patients, relative accumulation of CREB in cytosol was observed. These findings indicate, that poor response to ICS therapy may be related to increased CREB-associated signaling.

Key words: COPD, cytosolic CREB, histone signaling, nuclear CREB, Ser133-phosphorylated CREB

INTRODUCTION

Glucocorticoids and long-acting β2-receptor agonists targeting both inflammation and bronchoconstriction are efficient in the treatment of asthma, but not in chronic obstructive pulmonary disease (COPD) (1). In recent years,
several new approaches have been developed to improve COPD therapy, including antagonists of tumour necrosis factor, phosphodiesterase, nuclear factor-kappaB and phosphoinositide-3 kinase-gamma inhibitors, as well as antioxidants and antagonists of inducible nitric oxide synthase or leukotriene B4 receptors and others (2). However, it is now becoming clear that a single target approach cannot be effective.

Currently approved COPD therapy includes combination of long-acting β2-receptor agonists, theophylline, anticholinergic drugs, and glucocorticoids. All these drugs alter distinct signaling pathways (3, 4). The cellular levels of cyclic adenosine monophosphate (cAMP), a common signaling molecule, are usually increased by both β2-adrenergic agonists (3) and theophylline (5). However, glucocorticoids may either increase and decrease cAMP levels and the glucocorticoid receptor (GR) function depends strongly on interactions with β-agonists (6). cAMP regulates histone acetylation through cAMP-responsive element binding protein (CREB)-binding protein (CBP) (7). Acetylation of core histones through the CBP (histone acetyltransferase) and desacetylation by histone desacetylase enzymes (HDACs) is very important in inflammatory airway diseases (8). cAMP-dependent transcription factor, CREB, due to its HAT activity, can alter chromatin signaling (9). Hyperacetylated histones were shown to correlate with activated transcriptional status and were associated with enhanced inflammatory gene expression (10). Glucocorticoids recruit histone deacetylase enzymes to the site of active gene expression and reduce inflammation. Increased HAT activity and decreased HDAC activity were observed in patients with asthma and those changes were reversed or partially reversed by corticosteroids (11). In COPD, however, HDAC activity is low (12) and it appears that this reduction maintains increased levels of chemotactic factors, chemokines inflammation and preserves relative glucocorticoid resistance.

The aim of our study was to assess changes in CREB expression, its subcellular distribution, and cytoplasmic and nuclear CREB activation via phosphorylation of this transcription factor at Ser133, in cells isolated from induced sputum of stable COPD patients during therapy.

MATERIAL AND METHODS

Subjects

Induced sputum samples were obtained from 35 stable COPD patients. COPD was defined according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines. All patients with COPD had airflow limitation (FEV₁<80% predicted, FEV₁/FVC< 0%, GOLD stage II-IV). All patients received no COPD therapy for 4 wk of washout treatment, with salbutamol rescue medication, if needed. All subjects were characterized with respect to sex, age, smoking history, COPD symptoms, comorbidity, and current medical treatment. All COPD patients had a smoking history of 10 pack-years or more. Exclusion criteria included the following: other systemic
diseases, other lung diseases apart from COPD and lung tumors, pulmonary infection and antibiotic
treatment 4 wk before inclusion or inhaled or oral glucocorticoids in the 3 months before inclusion
in the study. All patients of this study gave their informed consent after a full discussion of the
nature of the study. The study was approved by a local Ethics Committee. No patient in the study
had symptoms nor was treated for COPD exacerbation during at least two months preceding the day
of inclusion.

Treatment

All patients included in the study underwent 4 wk of washout therapy with Salbutamol only as
a rescue medication. At the beginning of the treatment, patients were stratified to the following
treatment modes: formoterol alone (F), formoterol + budesonide (F/ICS), and formoterol + ICS +
theophylline (F/ICS/Th) b.i.d. for 4 wk.

Spirometry

Spirometry was performed with a computerized system (Lungtest 1000; Krakow, Poland). The
lung function and DLCO tests were performed with a body box (Elite DL, Medgraphics, USA). The
measurement was performed using standard protocols according to the American Thoracic Society
guidelines.

Sputum induction and processing

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) (13). Samples were
processed within 15 min after the termination of the induction. Throughout the procedure, 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 FEV$_1$ was recorded. Induction of
sputum was stopped if the FEV$_1$ value fell by at least 20% from baseline or if troublesome
symptoms occurred.

Induced sputum samples were homogenized for 1 min in lysis buffer containing 10 mM N-2-
hydroxyethylpiperazine-N’-ethane sulfonic acid, 10 mM KCl, 2 mM MgCl$_2$, 1 mM dithiothreitol,
0.1 mM ethylenediaminetetraacetic acid, 0.2 mM NaF, 50 mM β-glycerophosphate, a protease
inhibitor tablet, 0.2 mM sodium 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 centrifuged at 13000 × 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 13000 × g for 5 min. The resulting nuclear
extract and cytosol were evaluated for the expression of CREB and Ser 133 phosphorylated CREB
(CREB-P).

Western blotting

For Western blots, 15 µg of cytosolic or nuclear proteins were separated by SDS/PAGE in
reducing conditions, transferred onto polyvinylidene difluoride membranes, and incubated with
antibodies against CREB or CREB-P (Abcam rabbit antibodies against human CREB or CREB-P).
After washing, bound antibody was detected using an 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 constitutively expressed protein, β-actin, served as a loading control, and the data were quantified in respect to β-actin expression. Protein levels were measured using a BCA kit (Sigma-Aldrich, Poznan Poland).

Statistical analysis

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

RESULTS

Table 1 shows relative expression of CREB and Ser133 phosphorylated CREB (CREB-P) levels in nuclear and cytosolic fractions of cells isolated from induced sputum of stable COPD patients treated with formoterol (F), formoterol + budesonide (F/ICS), and formoterol + budesonide + theophylline (F/ICS/Th). Representative Western blot pictures are shown in Fig. 1. In F/ICS treated patients, increased levels of CREB (40% increase; P<0.01) and CREB-P (50% increase; P<0.01) were observed in the cytosol compared with the corresponding data from F-treated patients, while nuclear CREB and CREB-P levels increased by 23% (P<0.05) and by 51% (P<0.01) after F/ICS and by 26% (P<0.05) and 57% (P<0.01) after F/ICS/Th respectively, compared with F-treated patients.

Relative content of CREB and CREB-P in nuclear and cytosolic fractions of cells isolated from induced sputum of stable COPD patients treated with formoterol (F, n=12), formoterol + budesonide (F/ICS, n=12), and formoterol + ICS + theophylline (F/ICS/Th, n=11) b.i.d. for 4 weeks. Expression of CREB was equalized in each sample for loading and numerized with density software. The mean CREB expression in cytosol from F-treated patients was set as 100 relative units. *P<0.05 compared with F-treated group; **P<0.01 compared with F-treated group.

Table 1. Relative content of CREB and CREB-P in nuclear and cytosolic fractions.

<table>
<thead>
<tr>
<th></th>
<th>F</th>
<th>F/ICS</th>
<th>F/ICS/Th</th>
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<tr>
<td><strong>Nuclei</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CREB</td>
<td>360 ±31</td>
<td>443 ±34**</td>
<td>453 ±41**</td>
</tr>
<tr>
<td>CREB-P</td>
<td>125 ±21</td>
<td>189 ±22**</td>
<td>193 ±24**</td>
</tr>
<tr>
<td>CREB-P/CERB</td>
<td>0.35 ±0.08</td>
<td>0.42 ±0.08</td>
<td>0.43 ±0.07*</td>
</tr>
<tr>
<td><strong>Cytosol</strong></td>
<td></td>
<td></td>
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<tr>
<td>CREB</td>
<td>100 ±16</td>
<td>140 ±18**</td>
<td>139 ±21**</td>
</tr>
<tr>
<td>CREB-P</td>
<td>51 ±9</td>
<td>77 ±11**</td>
<td>76 ±11**</td>
</tr>
<tr>
<td>CREB-P/CERB</td>
<td>0.51 ±0.09</td>
<td>0.55 ±0.10</td>
<td>0.55 ±0.09</td>
</tr>
<tr>
<td>Nuclear/cytosolic CREB</td>
<td>3.6 ±0.3</td>
<td>3.18 ±0.5*</td>
<td>3.24 ±0.4</td>
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</table>
About 50% of cytosolic CREB in ICS-treated patients was phosphorylated, while in nuclear fractions the corresponding value was 35%. Cytosolic and nuclear CREB expression and activation was not significantly different in patients treated with F/ICS or F/ICS/Th.

In patients treated with F/ICS/TH, the CREB-P/CREB ratio in nuclear, but not cytosolic fractions, was higher than in subjects treated with F only. A relative increase in CREB-P content was statistically significant (P<0.05) only in F/ICS/Th–treated patients, although a slightly elevated ratio could also be observed in F/ICS–treated patients. F/ICS treatment significantly (P<0.05) decreased the nuclear/cytosolic CREB ratio, compared with F-treated patients, which most probably reflects the relative accumulation of CREB in the cytosol.

**DISCUSSION**

COPD is a progressive disease of increasing frequency, and until now it has no specific treatment. Therefore, COPD is usually treated as other inflammatory airway diseases, most frequently with β2-receptor agonist, anticholinergics, theophylline and glucocorticoids. However, most patients are glucocorticoid resistant and some alleviation is usually observed only in initial stages of the disease (14). Experimental COPD therapies target mostly intracellular signaling molecules or cytoplasmic receptors. A promising and valuable therapeutic target or even biochemical marker of COPD is still missing. Recent protein microarray-based assays indicate disease specific panels of biomarkers (15), but they are not yet validated. The pathomechanism of glucocorticoid resistance in COPD also is
unclear. However, clinical and experimental data show that COPD is related to alteration in several signaling pathways.

β2-agonists, theophylline, and glucocorticoids are widely used in COPD treatment. They alter intracellular cAMP resulting in CREB activation and changes in chromatin signaling (6, 8). In this study we showed that ICS therapy not only increases CREB expression in cytosol and nuclei but also increases CREB activation via its Ser133 phosphorylation in the two cellular compartments. Thus, prolonged ICS therapy seems to increase CREB-mediated transcriptional activity, and this observation is surprising, because glucocorticoids usually attenuate intracellular signaling. There are conflicting data on interactions of β2-adrenergic agonists and glucocorticoids. It has been shown that β2-agonists interfere with the GR in cultured human bronchial epithelial cells when given simultaneously, but this interaction was absent when the cells were pretreated first with glucocorticoids and then treated with β2-agonists (16). In another study, chronic activation of neuronal GR inhibited transcriptional activity of CREB, most probably due to decreased CREB phosphorylation (17). In rat C6 glioma cells, the intracellular levels of mRNA of several CREB isoforms were significantly increased by dexamethasone (18). Moreover, in steroid-resistant asthma, high concentrations of β2-agonists may induce a secondary resistance via interaction between CREB and GR (19). It seems that CREB and GR may function additively or antagonistically to each other depending on their relative concentrations and type of target tissue, resulting in sensitivity or insensitivity of tissues to glucocorticoids.

We have used induced sputum, which can be easily taken from COPD patients, and was previously found to be a valuable material for estimation of changes in NF-kappaB, chemokines, lipid mediators, antioxidants and other molecules (20). It should be stressed, that in some assays, sputum processing should skip preliminary dithiothreitol solubilization, due to a possible redox-mediated changes (13). Our results indicate that CREB-mediated signaling in COPD patients treated with F/ICS is increased. It is possible that this increase affects nuclear transcription, since changes in cytosolic and nuclear signaling molecules clearly alter gene transcription. It was shown, that smoking cessation in patients with COPD was found to be associated with increased histone H3 acetylation (21).

In the present study we observed a significantly higher CREB accumulation in the cytosol than in nuclei. This may be due to alterations of organelle-specific CREB turnover and/or due to impaired nuclear CREB transport. Published data show that CBP protein, which is essential in mediating nuclear CREB effects, depends on nuclear transport proteins (22). Another transcription factor with clearly opposing to GR functions in the modulation of immune/inflammatory responses - nuclear factor-kappaB (NF-kappaB) is increased in human lung tissue of COPD patients due to nuclear translocation (23). It seems that CBP may play a role in this interaction, since it enhances physical interaction between NF-
kappaB and GR. However, due to the absence of experimental data this assumption is only speculative.

Surprisingly, theophylline, which inhibits phosphodiesterase and prevents the intracellular breakdown of cAMP, did not significantly affect any CREB alterations induced by ICS. It is possible that the already induced CREB may be relatively resistant to additional stimulation, but molecular mechanisms for the anti-inflammatory and immunomodulatory action of theophylline are still not clear.

Our data show that combined β2-agonist and glucocorticoid therapy can possibly activate CREB signaling, assuming that the accumulated and activated CREB is functional. This activation should result in altered histone signaling. This mechanism merits further study, particularly because a significant decrease in different histone desacetylase isoenzymes (HDACs) has been described in alveolar macrophages of COPD patients (24). According to the generally accepted model, activated CREB recruits the CBP (histone acetyltransferase) to activate transcription. In contrast, HDACs induce termination of CREB-dependent transcription. Our results support the hypothesis that increased corticosteroid sensitivity in COPD patients might be achieved not only by bringing back HDAC activity, but probably also by slowing down CREB-mediated signaling.

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Author address: Robert M. Mroz, Department of Pneumology, Bialystok Medical University, Bialystok, Zurawia 14 St., 15-540 Bialystok, Poland; phone: +48 85 7409530, fax +48 85 6545419, e-mail: robmroz@wp.pl