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Increased FKBP51 in Induced Sputum Cells of Chronic Obstructive Pulmonary Disease Patients after Therapy

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Abstract
Objective: Immunophilin FKBP51 assists polypeptide folding, participates in glucocorticoid actions and may play a role in glucocorticoid resistance. FKBP51 is altered in patients with asthma, but its role in chronic obstructive pulmonary disease (COPD) characterized by dysregulation of several pro/antiinflammatory genes is less clear.
Methods: We assessed changes in nuclear/cytosolic FKBP51 protein using SDS-PAGE/WB and FKBP51 mRNA by qRT-PCR in cells isolated from induced sputum of stable COPD patients treated with formoterol/budesonide or formoterol/budesonide/theophylline for 4 wk.
Results: Expression of FKBP51 was higher in formoterol/ budesonide/theophylline-treated patients, compared with formoterol/budesonide group in both cytosolic and nuclear fractions by about 57% and 31%, respectively ($P<0.001$, $P<0.01$). FKBP51 mRNA was only slightly, but not significantly, higher in patients on formoterol/ budesonide/theophylline.
Conclusions: Increased FKBP51 in COPD patients treated with formoterol/ budesonide/theophylline may be important in altering signaling from corticosteroid receptors.

Key words: budesonide, FKBP51, formoterol, glucocorticoids, theophylline

Introduction

Glucocorticoids effectively switch off pro-inflammatory genes in asthma, but are ineffective in chronic obstructive pulmonary disease (COPD) [1]. It is possible that glucocorticoid resistance in COPD is related to altered glucocorticoid signaling. FK506-binding protein (FKBP), the peptidyl prolyl cis-trans isomerase and the member of a large immunophilin family assists proper folding of polypeptides, responds to bronchodilator drugs and alters glucocorticoid effects [2, 3]. There are two functional FKBP proteins interacting with glucocorticoid receptor (GR)-Hsp90 complex: FKBP51 coded by FKBP5 gene, which binds unliganded GR and FKBP52 coded by FKBP4 gene, which interacts with liganded GR and activate GR complex [4]. This complex called transportosome is transported to the nucleus and transactivates or transrepresses specific genes or transcription factors [4]. It has been shown that increased levels of FKBP51 caused glucocorticoid resistance in New World primates [5]. The role of FKBP51 in COPD, characterized by disregulation of several pro/anti-inflammatory genes and signaling proteins remain largely unknown, but expression of FKBP51 is altered in asthma [6] and affected by glucocorticoids [4, 7], theophylline (Th) [8], or inhaled β2-receptor agonists [9].

Theophylline may restore steroid responsiveness in COPD patients via normalization of reduced histone deacetylase [1, 10], the drug has both antiinflammatory and antioxidant properties and affects glucocorticoid response [11, 12]. The possible Th-dependent pathway involves increased cyclic AMP, activation of cyclic AMP response element binding protein (CREB) and chaperone system [13], since the levels of phosphorylated CREB correlated with increased expression of human hsp90β gene [14]. The goal of the present study was to assess FKBP51 protein expression and nuclear/cytosolic protein distribution and possible changes in FKBP51 mRNA in cells isolated from induced sputum of stable COPD patients treated with formoterol/budesonide or formoterol/ budesonide/theophylline.

Subjects and Methods

All patients included in the study gave their consent after a full discussion of the nature of the study and the study was approved by a local Ethics Committee.

Sputum was induced in 36 COPD patients with stable disease, defined according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (15). All patients with COPD had airflow limitation (FEV1 <80% predicted, FEV1/FVC <70%, GOLD stage 2-2) and received no COPD therapy for 4 wk. Lung function and DLCO tests were performed with a body box (Elite DL, Medgraphics, USA). The measurement was performed using standard protocols according to American Thoracic Society guidelines. All subjects were characterized with respect to sex, age, smoking history, COPD symptoms, comorbidity, and current medical treatment. 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 mo before inclusion. No patient in the study had symptoms

nor was treated for COPD exacerbation during at least 2 mo preceding the day of inclusion.

Treatment

All patients underwent a 4 wk washout salbutamol only on demand therapy. At the beginning of the treatment patients were stratified to following treatments: formoterol/budesonide (F/ICS, n=18) and formoterol/budesonide/theophylline (F/ICS/Th); n = 18) b.i.d. for 4 wk.

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) [16]. Samples were processed within 15 min after the termination of 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 FEV1 was recorded. Induction of sputum was stopped if the FEV1 value fell by at least 20% from baseline or if troublesome symptoms occurred.

Induced sputum samples were processed to isolate mRNA (qPCR-grade RNA isolation kit, SABiosciences, Frederick, USA) or were homogenized for 1 min in the 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 -glycerophosphate, a protease inhibitor tablet, 0.2 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotenin, and 10% Nonidet P-40. The samples were then incubated on ice for 15 min and 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 aprotenin, 50 mM -glycerophosphate, and a protease inhibitor tablet (Complete Mini; Roche Diagnostics, Mannheim, Germany). The samples were then incubated on a rotating platform for 30 min at 4°C followed by centrifugation at 13000 x g for 5 min. The resulting nuclear and cytosolic fractions were evaluated for the expression of FKBP51.

Protein levels were measured using a BCA kit (Sigma-Aldrich, Poznan, Poland).

FKBP51 Protein Levels

FKBP51 protein levels were assessed in nuclear and cytosolic fractions using SDS/PAGE/ Western blots. Nuclear or cytosolic proteins were separated by SDS/PAGE in reducing conditions, transferred onto polyvinylidene difluoride membranes, and incubated with specific rabbit antibodies against human FKBP51 (Abcam, Cambridge, USA). After washing, bound antibody was detected using appropriate secondary anti-rabbit antibody (Abcam) linked to horseradish peroxidase. The bound complexes were detected using enhanced chemiluminescence (ECL, Amersham, GE Healthcare, Little Chalfont, UK) 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.

FKBP51 mRNA

Expression of FKBP5 gene coding for immunophilin FKBP51 was assessed using SYBR Green based qRT-PCR after total RNA extraction from induced sputum cells, RNA purification and template cDNA synthesis was performed with commercial PCR master mixes containing appropriate controls and reference dyes (SABiosciences, Frederic, USA) as described earlier [17]. Samples were run on ABI 7900HT instrument and data were analyzed and quantified using SABiosciences software based on the ΔΔCt method with normalization of the raw data to either housekeeping genes or an external RNA control.

Statistical Analysis

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

Results

Table 1 demonstrates the cytosolic and nuclear FKBP51 proteins and the FKBP51 mRNA in cells of

Table 1. Cytosolic and nuclear FKBP51 protein and FKBP51 mRNA in cells of patients treated with F/ICS and F/ICS/Th for 4 weeks.

			F/ICS	F/ICS/Th
FKBP51	Protein	Cytosol (C)	100.0 ± 18.1	157.6 ± 21.4**
		Nuclei (N)	61.0 ± 11.9	79.5 ± 14.7*
		Ratio (C/N)	1.64 ± 0.36	1.98 ± 0.38**
	mRNA	100.0 ±46.8	132.7 ± 51.1	

Relative content of cytosolic and nuclear FKBP51 protein and FKBP51 mRNA in cells isolated from induced sputum of stable COPD patients treated with formoterol +budesonide (F/ICS; n=18) and formoterol + budesonide + theophylline (F/ICS/Th; n=18) b.i.d. for 4 wk. Expressions of FKBP51 were equalized in each sample for loading and numerized with density software. The mean FKBP51 expression in cytosolic fraction of cells isolated from F/ICS-treated patients was set as 100 relative units. *P<0.05 compared with the F/ICS-treated group; **P<0.01 compared with the F/ICS-treated group.

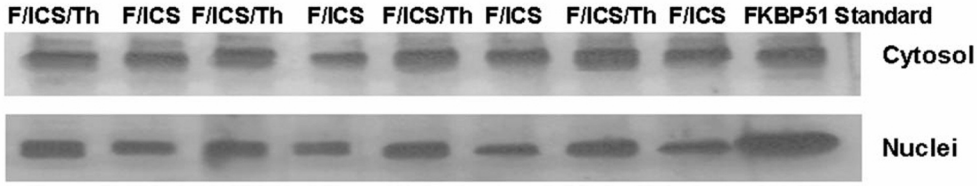

Fig. 1. Relative cytosolic and nuclear FKBP51 protein expression in cells isolated from induced sputum of stable COPD patients treated with Formoterol + Budesonide (F/ICS) or Formoterol + Budesonide + Theophylline (F/ICS/Th) b.i.d. for 4 weeks.

patients treated with F/ICS and F/ICS/Th for 4 wk. Corresponding representative Western Blot pictures are shown in Fig. 1. The expression of FKBP51 was higher in the F/ICS/Th-treated patients, compared with the F/ICS treated patients in both cytosolic and nuclear fractions by about 58% ($P<0.01$) and 31% ($P<0.05$), respectively. The cytosolic/nuclear FKBP51 ratio was significantly ($P<0.01$) increased in the F/ICS/Th patients compared with the F/ICS-treated patients indicating relative accumulation of FKBP51 in the cytosol. FKBP51 mRNA in cells isolated from the F/ICS/Th patients was highly variable between patients, and although the mean expression in this group was slightly higher than in F/ICS patients, the difference was not statistically significant.

Discussion

Theophylline, a non-specific phosphodiesterase inhibitor, is a narrow therapeutic index drug and must be monitored due to significant side effects. In COPD, theophylline is used as one of main bronchodilators, aside from inhaled anticholinergics and long acting β2 agonists. It has been shown that thophylline withdrawal in severe COPD patients may worsen their clinical status [18, 19]. Thephylline signaling pathways are not clearly identified, but experimental and clinical data indicate that it has a broad spectrum of actions including antioxidant, immunomodulatory, and antiinflammatory effects [11, 12]. It inhibits activation of nuclear transcription factor NF-κB in human pulmonary epithelial cell [20] and may even reverse steroid resistance in COPD [10]. It has been shown, that theophylline alters the expression of several genes, downregulates IL-13 mRNA, and upregulates mRNA of FKBP38-cochaperone protein involved in apoptosis [20]. In the present study, the expression of cochaperone immunophilin FKBP51 was higher in F/ICS/Th-treated patients, compared with the F/ICS group in both cytosolic and nuclear fractions, but increased cytosolic/nuclear FKBP51 ratio indicates that FKBP51 is mostly accumulated in the cytosol. It is possible that this increase may be transcriptionally regulated, which, however, should be verified on higher numbers of patients. GR-GC-heat shock protein 90kD (Hsp90) complex-associated immunophilins compete with each other and it is possible that the relative concentrations of the different immunophilins bound to the complex might control GR activation. Thus, the possible functional consequence of increased FKBP51 protein may be an altered nuclear transport of GR [21]. FKBP51 is a major immunophilin in inactive GR–Hsp90 complexes, and in the presence of glucocorticoids it is replaced by another cochaperone - FKBP52 - with subsequent nuclear translocation [4, 21]. Mouse knockout experiments support a major role of FKBP52 in GR activation [22, 23], while other studies point to the contribution of elevated FKBP-51 protein to glucocorticoid resistance [5]. The present results indicate that high doses of theophylline in combined therapy with b2agonists and glucocorticoids may possibly decrease the glucocorticoid response via discriminative FKBP51 binding, but this is not consistent with the published data indicating that theophylline can improve clinical status of some COPD patients or restore the antiinflammatory action of glucocorticoids in cells subjected to oxidative stress [1, 10]. Interestingly, steroid hormones themselves regulate immunophilin levels and may increase systemic FKBP51 expression, producing partial functional feedback inhibition of GR [24, 25]. Thus, the present study indicates that theophylline and glucorticoids in polytherapy may act synergistically in this aspect, but functional consequences of increased FKBP51 in COPD patients remain to be established.

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