

## Chapter 2

# Tiotropium Increases PPAR $\gamma$ and Decreases CREB in Cells Isolated from Induced Sputum of COPD Patients

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**Abstract** Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation and chronic inflammation of airways and lung parenchyma. Our aim was to assess two important elements of intracellular signaling involved in regulation of inflammation in COPD in patients subjected to long-acting beta2-agonist or long-acting beta2-agonist plus long-acting anti-muscarinic: peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) protein, which has antiinflammatory and immunomodulatory properties and cAMP response element binding protein (CREB) and activated (CREB-P) protein which has histone acetyltransferase activity and increases histone acetylation and transcriptional activation of chromatin. Twenty one stable COPD patients (18 males and 3 females, mean age 65 years) receiving 12  $\mu$ g B.I.D formoterol were assayed before and after 3 month add-on therapy, consisting of 18  $\mu$ g Q.D. tiotropium. In all patients, sputum induction, spirometry, lung volumes, and DLCO were performed before and after therapy. Sputum cells were isolated and processed to isolate cytosolic and nuclear fractions. PPAR $\gamma$ , CREB, or CREB-P proteins were quantified in subcellular fractions using Western blot. Tiotropium add-on therapy improved respiratory parameters: FEV1 and lung volumes. After therapy mean expression of PPAR $\gamma$  in cell nuclei was significantly increased by about 180%, while CREB and phosphorylated CREB levels in cytosol and nuclei were decreased by about 30%. Our data show that the mechanism whereby tiotropium reduces exacerbations may be associated not only with persistent increase in airway functions and reduced hyperinflation mediated by muscarinic receptors, but also with possible anti-inflammatory effects of the drug, involving increased PPAR $\gamma$  and decreased CREB signaling.

**Keywords** COPD • CREB • Histone acetylation • PPAR $\gamma$  • Tiotropium

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## 2.1 Introduction

Irreversible and progressive airflow limitation is a landmark of chronic obstructive pulmonary disease (COPD), the only major disease with an increasing death rate (Viegi et al. 2007). In COPD, airflow obstruction is caused by increased activity of parasympathetic system and chronic inflammation of the airways and lung parenchyma, very frequently associated with chronic tobacco smoking (Viegi et al. 2007). COPD is considered as a fatal disease, but it can be managed, controlled and slowed down, however a necessary step is smoking cessation. In pharmacotherapy of moderate to severe COPD long-acting bronchodilators are used (Global Strategy for the Diagnosis, Management and Prevention of COPD 2008). Currently approved drugs for the treatment of COPD are: long-acting beta2-agonists (LABA), i.e., formoterol, salmeterol, indacaterol, combined with long-acting antimuscarinic agents (LAMA) such as tiotropium bromide (Meyer et al. 2011). Formoterol, a selective LABA, increases adenylyl cyclase and cyclic adenosine monophosphate (cAMP) resulting in relaxation of bronchial smooth muscles (Kaur et al. 2008). Tiotropium acts as antagonist of M3 and M1 muscarinic receptors, modulating inositol 1,4,5-trisphosphate (IP3) and 1,2-diacyl-glycerol (DAG) signaling pathways (Casarosa et al. 2010). Drug binding produces prolonged improvement in clinical respiratory parameters and usually a single inhaled dose reverses compromised respiratory function (Kato et al. 2006). Our previous data indicate that tiotropium altered pharmacodynamic parameters of cholinergic M3 receptors and increased histone acetylation in chromatin of inflammatory cells migrating to the airways of COPD patients (Holownia et al. 2010). However, the possible anti-inflammatory mechanisms related to tiotropium are unknown. Our aim was to assess important elements of cytosolic and nuclear inflammatory signaling - expression and activation (Ser133 phosphorylation) of cAMP response element binding protein (CREB), and peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) in cells isolated from induced sputum of COPD patients before and after tiotropium therapy. CREB is an end-point and integration site of several signaling pathways, with histone acetyltransferase (HAT) activity (Lim et al. 2011), whether PPAR $\gamma$  acts as nuclear hormone receptor regulating glucose metabolism and the expression of inflammatory cytokines with possible histone deacetylase (HDAC) activity (Miard and Fajas 2005).

## 2.2 Methods

### 2.2.1 *Subjects and Treatment*

Twenty one (18 males and three females, mean age 65 years) COPD patients with stable disease, defined according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (Global Strategy for the Diagnosis, Management and Prevention of COPD 2008) were enrolled into the study. All patients with COPD had airflow limitation (FEV1 < 80% predicted, FEV1/FVC < 70%, GOLD stage 2–4) and received stable formoterol therapy for 4 weeks preceding inclusion. 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 weeks before inclusion, no inhaled or oral glucocorticosteroids in the 3 months preceding inclusion. All patients gave their informed consent after a full discussion of the nature of the study, which had been approved by a local Ethics Committee. No patient in the study had symptoms nor was treated for COPD exacerbation during at least 2 months preceding the day of inclusion.

The lung function and DLCO tests were performed with body box (Elite DL, Medgraphics, USA). The measurement was performed using standard protocols according to American Thoracic Society guidelines. Twenty one patients underwent 4 week stable therapy with 12  $\mu$ g B.I.D. formoterol and subsequently were subjected to sputum induction. Next patients were treated for 12 additional weeks with add-on 18  $\mu$ g Q.D. tiotropium and their sputum was collected.

## 2.2.2 Sputum Induction and Processing

Sputum was induced by the inhalation of a 4.5% hypertonic aerosol saline solution, generated by an ultrasonic nebulizer (Voyager, Secura Nova; Warsaw, Poland) (Loh et al. 2005). 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. Samples were processed within 15 min after the termination of the induction.

Induced sputum samples were solubilized in equal volumes of 0.1% dithiothreitol (Sigma Chemicals Co, Poznan, Poland) in Hanks solution and incubated for 15 min in an ice bath. Cell suspension was then rinsed twice with Hanks solution, filtered by a nylon membrane and centrifuged (1,000 rpm) on Histopaque 1077. Isolated cells were further processed to obtain cytosolic and nuclear fractions.

To isolate subcellular fractions, sputum cells were centrifuged, resuspended in cold hypotonic buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 50 mM dithiothreitol, 100 mM phenanthroline, 1 mg/ml pepstatin, 100 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane, 100 mM 3,4-dichloroisocoumarin, 10 mM NaF, 100 mM sodium orthovanadate, 25 mM b-glycerophosphate and centrifuged at 14,000  $\times$  g for 5 min at 4°C (Mroz et al. 2007). Cells were then lysed in a solution of the same buffer containing 0.2% (v/v) Nonidet P- 40 for 10 min on ice and centrifuged at 14,000  $\times$  g for 10 min at 4°C. The supernatant was collected as a cytosolic extract. The remaining pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% (v/v) glycerol, 100 mM 3,4-dichloroisocoumarin), incubated for 15 min at 4°C, and centrifuged at 14,000  $\times$  g for 10 min at 4°C. The supernatant including soluble nuclear protein was collected as a nuclear extract.

Cytosolic and nuclear fractions were evaluated for the expression of CREB and CREB-P proteins, while PPAR $\gamma$  was assessed only in nuclear fractions using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots. Sample proteins were separated in reducing conditions, transferred onto polyvinylidene difluoride (PVDF) membranes, and incubated with specific rabbit monoclonal antibodies against human CREB or PPAR $\gamma$  proteins (Abcam, Cambridge, USA). After washing, bound antibody was detected using appropriate secondary anti-rabbit antibody (Abcam, Cambridge, USA) linked to horseradish peroxidase. The bound complexes were detected using enhanced chemiluminescence (ECL, Amersham, GE Healthcare, Little Chalfont, UK) and quantified using Quantity One software (BioRad, Warsaw, Poland). The constitutively expressed protein –  $\beta$ -actin, served as a loading control and the data were quantified in respect to  $\beta$ -actin expression. For the negative control study, membranes were treated similarly but without the addition of primary antibody. Protein levels were measured using a BCA kit (Sigma-Aldrich, Poznan, Poland).

Statistical analysis was performed using statistical package – Statistica (Statsoft, Cracow, Poland) using nonparametric Wilcoxon test for paired data. The data were expressed as means  $\pm$  SD.  $P < 0.05$  was as considered statistically significant.

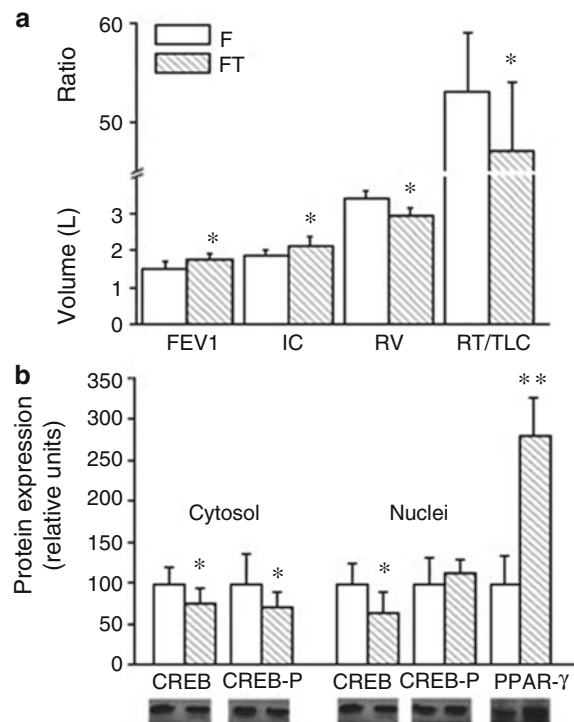
## 2.3 Results

Table 2.1 and Fig. 2.1 show respiratory parameters: forced expiratory volume in one second (FEV1), inspiratory capacity (IC), residual volume (RV) and residual volume divided by total lung capacity (RV/TLC) and biochemical data: cytosolic and nuclear CREB and phosphorylated CREB as well as nuclear PPAR $\gamma$  levels in cells isolated from induced sputum of COPD patients before and after tiotropium ad-on therapy. Representative Western blot pictures of CREB, CREB-P and PPAR $\gamma$  protein are shown (Fig. 2.1). Therapy improved lung function parameters. After therapy, mean FEV1 increased by 14% ( $P < 0.05$ ), IC increased by 14% ( $P < 0.05$ ), residual volume was reduced by 13% ( $P < 0.05$ ), and RV/TLC decreased by 11% ( $P < 0.05$ ) from baseline.

**Table 2.1** Respiratory parameters: forced expiratory volume in 1 s (FEV1), inspiratory capacity (IC), residual volume (RV) and residual volume divided by total lung capacity (RV/TLC) and cytosolic and nuclear CREB and phosphorylated CREB as well as nuclear PPAR $\gamma$  levels (relative units) in cells isolated from induced sputum of COPD patients before and after tiotropium add-on therapy

	Formoterol(F)		Formoterol+ Tiotropium (FT)	
FEV1 (L)	1.52 $\pm$ 0.18		1.73 $\pm$ 0.19*	
% predicted	52%		56%	
IC (L)	1.87 $\pm$ 0.13		2.14 $\pm$ 0.22*	
% predicted	62%		71%	
RV (L)	3.39 $\pm$ 0.21		2.92 $\pm$ 0.23*	
% predicted	152%		132%	
RV/TLC	53 $\pm$ 6%		47 $\pm$ 7%*	
	Cytosol	Nuclei	Cytosol	Nuclei
CREB	100.0 $\pm$ 21.3	100.0 $\pm$ 25.4	75.4 $\pm$ 18.4*	64.3 $\pm$ 26.2*
CREB-P	100.0 $\pm$ 37.2	100.0 $\pm$ 31.3	71.2 $\pm$ 19.3*	112.4 $\pm$ 7.5
PPAR $\gamma$	100.0 $\pm$ 33.9	–	281.0 $\pm$ 45.7**	–

\*P<0.05; \*\*P<0.01 – comparing to corresponding data from F-monotherapy



**Fig. 2.1** (a) **Respiratory parameters:** forced expiratory volume in one second (FEV1), inspiratory capacity (IC), residual volume (RV) and residual volume divided by total lung capacity (RV/TLC); (b) **Cytosolic and nuclear CREB, phosphorylated CREB, and nuclear PPAR $\gamma$**  (relative units) in cells isolated from induced sputum of COPD patients before and after tiotropium add-on therapy. Representative Western blot pictures of cytosolic and nuclear CREB, CREB-P, and PPAR $\gamma$  protein are also shown. F formoterol, FT formoterol + tiotropium; \*P<0.05 and \*\*P<0.01 compared with the corresponding data from F-monotherapy

Tiotropium decreased expressions of CREB and phosphorylated CREB in cytosol by about 25% ( $P < 0.05$ ) and 29% ( $P < 0.05$ ) for cytosolic CREB and CREB-P, respectively. In cell nuclei CREB was also decreased after tiotropium therapy by about 36% ( $P < 0.05$ ), but CREB-P levels were not altered. Comparing to values from patients on formoterol monotherapy, tiotropium significantly increased PPAR $\gamma$  in cell nuclei (increase by about 180%,  $P < 0.01$ ).

## 2.4 Discussion

We have previously shown that in cells isolated from induced sputum of COPD patients treated with tiotropium, acetylated H3 histone levels are significantly higher (Holownia et al. 2010). This observation may be important to inflammatory signaling, because acetylated histones represent a type of epigenetic tag which is responsible for gene transcription within chromatin. Histone acetylation mediated by HAT neutralizes the positive charge on the histone molecules (Khan and Khan 2010). As a consequence, chromatin is transformed into a more relaxed structure, associated with greater levels of gene transcription which is relevant to inflammatory signaling. In asthma, bronchial tissue and alveolar macrophages have increased HAT and decreased HDAC1 expression (Grabiec et al. 2008). In COPD formoterol and glucocorticosteroids were found to increase HAT-active CREB, especially in the cytosol of sputum cells (Mroz et al. 2007). In our patients cytosolic CREB (both inactive and phosphorylated) was slightly reduced after antimuscarinic therapy. In cell nuclei, unphosphorylated CREB was also lowered but phosphorylated, active CREB was not decreased after LAMA treatment. Given the important role of CREB in adrenergic signaling (Kaur et al. 2008), it seems that described changes may reflect adaptation of adrenergic receptors to chronic stimulation of  $\beta_2$  receptors by formoterol. It is interesting to note that the PPAR $\gamma$  agonist rosiglitazone can decrease adrenoceptor desensitization and increase salbutamol effects on airway smooth muscle (Fogli et al. 2011). On the other hand, it was shown, that downregulation of PPAR $\gamma$  increased airway inflammation (Belvisi and Mitchell 2009). We have found significant increase in PPAR $\gamma$  expression in sputum cells, not only after tiotropium therapy, but also after formoterol/inhaled corticosteroids treatment (Holownia et al. 2008). Although in the allergen challenge of asthmatic patients, rosiglitazone was associated with only modest reduction in the late asthmatic reaction (Richards et al. 2010), our data suggest that combined therapy of tiotropium and rosiglitazone in COPD may be more effective.

The role of tiotropium in regulation of histone signaling is not clear. However, interactions of anticholinergic drugs and immune system are well established. It is well known that chronic lung diseases are related to *in utero* nicotine exposure (Miller and Marty 2010). In COPD there is higher acetylcholine release, increased vagal tone, airway inflammation and increased mucus production, all efficiently blocked by tiotropium (Gosens et al. 2006). It has been shown that the bronchodilatory activity of tiotropium against acetylcholine-induced bronchoconstriction is in the same dose range as the anti-inflammatory activity (Wollin and Pieper 2010). In asthma, tiotropium bromide significantly reduced airway inflammation and the T helper cytokine production in bronchoalveolar lavage fluid (Ohta et al. 2010). Our earlier data showed that tiotropium therapy involved pharmacodynamic changes in cholinergic M3 receptors and alterations in histone acetylation (Holownia et al. 2010). The present data suggest that the latter effect is most probably not mediated by alterations in CREB signaling.

Our results show that the mechanism whereby tiotropium reduces COPD exacerbations and ameliorates respiratory parameters is not only a result of persistent increase in airway functions and reduced hyperinflation caused by the drug, but also of its anti-inflammatory effects, involving increased PPAR $\gamma$  and decreased CREB signaling.

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

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