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# Altered Histone Deacetylase Activity and iNOS Expression in Cells Isolated from Induced Sputum of COPD Patients Treated with Tiotropium

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## Abstract

Chronic obstructive pulmonary disease (COPD) is the only major disease with increasing death rate. In COPD, progressive reduction in quality of life is closely related to the increasing limitation of airflow due to chronic bronchitis, cell hyperplasia, fibrosis, and irreversible lung damage. Signaling pathways involved in inflammatory processes in COPD and inflammatory response to therapy are unknown. Our aim was to isolate cells from induced sputum of COPD patients treated with formoterol or formoterol + tiotropium and assess enzymatic activity of histone deacetylases (HDACs) acetylated histone 4 (AcH4) and expression of inducible nitric oxide synthase (iNOS). HDACs are important in signal transduction and inflammation. iNOS is generating nitric oxide (NO) relevant to blood pressure regulation, inflammation and infections. Thirty stable COPD patients (21 males and 9 females, mean age 67 years) receiving 12 µg b.i.d. formoterol were assayed before and after 3 months add-on therapy consisting of 18 µg q.i.d. tiotropium. In all patients, spirometry, lung volumes, and DLCO were performed before and after tiotropium therapy and all patients were subjected to sputum induction. Sputum cells were isolated and processed to obtain cytosolic and nuclear fractions. HDAC activity was measured in nuclear fraction using colorimetric assay. Expression AcH4 and iNOS was quantified using Western blot. In patients receiving both drugs, FEV1 and lung volumes significantly improved compared with formoterol-only treated patients. Mean HDAC activity was slightly decreased ( $P < 0.05$ ), while AcH4 levels and

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iNOS expression were significantly elevated in tiotropium-treated patients (increase by about 65 %;  $P < 0.01$  and 77 %;  $P < 0.01$  respectively). Our data show that beneficial effects of tiotropium in add-on therapy to formoterol may be related to altered histone signaling and increased iNOS expression.

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**Keywords**

COPD • HDAC • iNOS • Induced sputum • Tiotropium

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## 1 Introduction

Chronic obstructive pulmonary disease (COPD) is the only major disease with increasing death rate and an important health problem. In COPD, progressive reduction in quality of life is closely related to the increasing limitation of airflow due to chronic bronchitis, cell hyperplasia, fibrosis, narrowing of the airways, and irreversible lung damage. Apart from inflammation, additional airflow obstruction in COPD is caused by increased activity of parasympathetic system (Viegi et al. 2007). In pharmacotherapy of the disease several drugs are used, particularly long acting anticholinergics, like tiotropium or beta-2 mimetics like formoterol (GOLD 2008; Kaur et al. 2008). At early stages of the disease, a single inhaled tiotropium dose usually reverses compromised respiratory function due to relatively high affinity and low internal activity of the drug to muscarinic M3 and M1 receptors regulating both mucus secretion and vagally-induced contraction of airway smooth muscles (Kaur et al. 2008; Kato et al. 2006). Inflammation in COPD may also be related to increased acetylcholine levels. Published data from clinical and experimental studies (Santus et al. 2012) and our earlier results (Holownia et al. 2010, 2013) indicate that tiotropium increases antiinflammatory signaling and possibly also decreases airway inflammation and remodeling. In the current approach, our aim was to assess in cells isolated from induced sputum of COPD patients, before and after add-on tiotropium therapy, two important elements of nuclear and cytosolic inflammatory signaling – enzymatic activity of histone deacetylases (HDAC) and the

level of acetylated histone H4 as well as cytosolic expression of inducible nitric oxide synthase (iNOS). HDACs are important in COPD because altered activity of HDAC enzymes affects chromatin remodeling and alters inflammatory gene transcription regulated by histone acetylation/deacetylation (Yao and Rahman 2012). iNOS produces nitric oxide (NO) involved in blood pressure regulation and in functional regulation of respiratory system (Malerba and Montuschi 2012). NO is usually increased in inflammation (Malerba and Montuschi 2012). iNOS is expressed both in lung epithelial cells, inflammatory cells, and also in skeletal muscle and experimental data suggest that it may serve as a therapeutic target in COPD (Marques et al. 2012).

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## 2 Methods

### 2.1 Subjects and Treatment

Thirty patients included into the study gave their consent after a full discussion of the nature of the study, which had been approved by a Local Ethics Committee. All patients were stable COPD patients (21 males and 9 females, mean age 67 years) and were characterized with respect to sex, age, smoking history, COPD symptoms, comorbidity, and current medical treatment. Patients had stable disease, defined according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines including airflow limitation ( $FEV_1 < 80$  % predicted,  $FEV_1/FVC < 70$  %, GOLD stage 2–3). No patient in the study had symptoms nor

was treated for COPD exacerbation during at least 2 months preceding the day of inclusion. Exclusion criteria included other systemic diseases, other lung diseases apart from COPD, and lung tumors, pulmonary infection and antibiotic treatment 4 week before inclusion or inhaled/oral glucocorticosteroids in the 3 months before inclusion. 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 underwent 4 weeks' washout therapy with Salbutamol. After that, they were treated for 4 weeks with 12 µg b.i.d. formoterol and then subjected to the sputum induction. Subsequently all patients were treated for 3 months with add-on 18 µg q.i.d. Tiotropium and their sputum was collected again.

## 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). 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 about 15 min after termination of the induction. Samples were solubilized in equal volumes of 0.1 % dithiothreitol (Sigma Chemicals, 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 1,077. Isolated cells were homogenized in a lysis buffer containing 10 mM N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 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 13,000 × g for 30 sec. The cell pellets containing nuclei were retained and resuspended in extracting buffer (50 mM N-2-hydroxyethyl piperazine-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 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 then incubated on a rotating platform for 30 min at 4 °C followed by centrifugation at 13,000 × g for 5 min. The resulting nuclear extract and cytosol were evaluated for the activity of HDAC and iNOS expression, respectively. To assess Ach4 histone extraction was performed in cells treated for 30 min in ice with lysis buffer 10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, and 1.5 mM phenylmethylsulfonyl fluoride and hydrochloric acid at a final concentration of 0.2 M and subsequently, lysed cells were centrifuged at 11,000 × g for 10 min at 4 °C. Supernatant containing acid-soluble proteins was dialyzed for 1 h, against 0.1 M acetic acid and then overnight against H<sub>2</sub>O and frozen until assayed (Chadee et al. 1999).

iNOS and Ach4 were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblotting with specific monoclonal antibodies recognizing human iNOS protein (Abcam, UK) or acetylated histone H4 (Upstate Biotech., UK). iNOS was run on 10 % SDS gels while Ach4 proteins were separated in 20 % polyacrylamide gels along with molecular weight markers (Bio-Rad, Hercules, CA) and loading controls. Gels were transferred onto 0.45 µm PVDF membranes (BioRad, Warsaw, Poland). Species-specific alkaline phosphatase secondary antibodies were purchased from Sigma (Sigma, Poznan, Poland). Protein bands were quantified using Quantity One software (BioRad, Warsaw, Poland).

HDAC activity was measured in nuclear fraction using colorimetric assay (Enzo Life Sci.

HDAC kit, Switzerland) adapted to cell homogenate (Yang et al. 2006). Protein levels were measured using a BCA kit (Sigma-Aldrich, Poznan, Poland).

Statistical analysis was performed using a 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 considered as statistically significant.

### 3 Results

Table 1.1 and Fig. 1.1 show respiratory parameters: forced expiratory volume in 1 s (FEV1) and inspiratory capacity (IC) in COPD patients before and after 3 months of tiotropium add-on therapy. Therapy improved clinical status of COPD patients and ameliorated their individual respiratory parameters. After 3 months of tiotropium, both FEV1 and CV increased by about 11 % ( $P < 0.05$ ) compared with the corresponding values before tiotropium therapy.

Table 1.1 and Fig. 1.2 show biochemical data: specific nuclear HDAC activity and AcH4 levels as well as cytosolic iNOS expression in cells isolated from induced sputum of COPD patients before and after add-on tiotropium therapy. After tiotropium specific activity of HDAC was slightly lower ( $P < 0.05$ ), while AcH4 levels were significantly elevated (by about 65 %;  $P < 0.01$ ). Expression of iNOS was also increased (by about 77 %;  $P < 0.01$ ).

**Table 1.1** Respiratory parameters: forced expiratory volume in 1 s (FEV1), inspiratory capacity (IC), and nuclear specific HDAC activity and acetylated histone 4

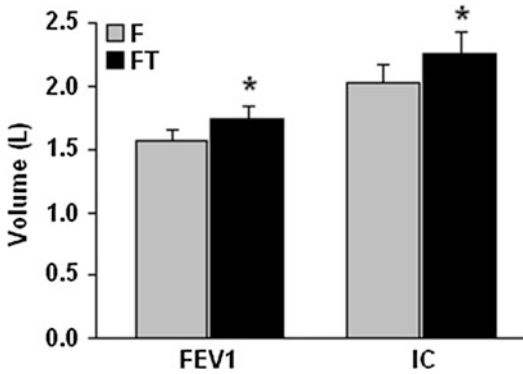
	Formoterol (F)	Formoterol + Tiotropium (FT)
FEV1 (L)	1.57 $\pm$ 0.09 53 %	1.74 $\pm$ 0.11* 57 %
IC (L)	2.03 $\pm$ 0.14 67 %	2.26 $\pm$ 0.17* 74 %
HDAC	100.0 $\pm$ 32.4	78.0 $\pm$ 25.4*
AcH4	100.0 $\pm$ 27.5	165.0 $\pm$ 33.3**
iNOS	100.0 $\pm$ 34.1	177 $\pm$ 51.3**

\* $P < 0.05$ ; \*\* $P < 0.01$  – compared with corresponding data from F-monotherapy

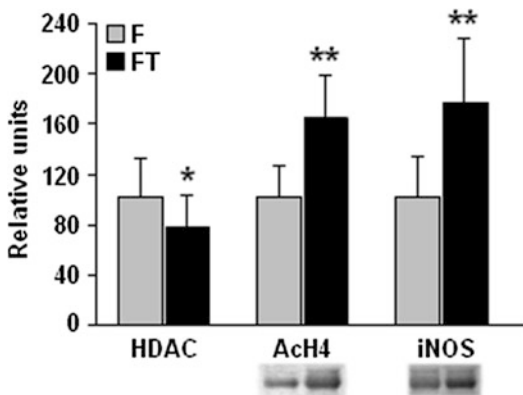
### 4 Discussion

In COPD, an irreversible and progressive disease, reduced lung function is associated with local and systemic inflammation (Viegi et al. 2007; GOLD 2008). COPD patients are usually treated with bronchodilators such as long-acting beta-2 agonists frequently combined with long-acting antimuscarinic agents like tiotropium bromide or/and steroids producing respiratory benefits and better patient survival but signaling pathways involved in inflammatory processes in COPD and inflammatory response to the therapy are not known. We have previously shown that that in cells isolated from induced sputum of COPD patients subjecting to add-on tiotropium therapy acetylated H3 histone levels are significantly higher (Holownia et al. 2010). A similar increase was observed in our patients in AcH4. It appears that histone acetylation/deacetylation balance in tiotropium-treated patients is shifted toward hyperacetylation. Histones are important in inflammatory signaling since they are 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. Several signaling molecules including CREB (cyclic AMP response element binding protein) have histone acetyltransferase (HAT) activity (Bedford and

(AcH4) levels as well as cytosolic iNOS expression in cells isolated from induced sputum of COPD patients before and after add-on tiotropium therapy



**Fig. 1.1** Respiratory parameters: forced expiratory volume in 1 s (FEV1) and inspiratory capacity (IC) in COPD patients before and after 3 months of tiotropium add-on therapy. Tiotropium ameliorated ( $P < 0.05$ ) respiratory parameters of COPD patients and improved their clinical status. *F* formoterol, *FT* formoterol + tiotropium. \* $P < 0.05$  – compared with corresponding data from F-monotherapy



**Fig. 1.2** Specific HDAC activity and acetylated histone 4 (AcH4) levels in cell nuclei, and cytosolic iNOS expression in cells isolated from induced sputum of COPD patients before and after 3 months of tiotropium add-on therapy. Representative Western blot pictures of AcH4 and iNOS are also shown. *F* formoterol, *FT* formoterol + tiotropium. \* $P < 0.05$ ; \*\* $P < 0.01$  – compared with corresponding data from F-monotherapy

Brindle 2012). HDACs are responsible for removing acetyl groups from histone  $\epsilon$ -N-acetyl lysines allowing the histones to tightly wrap the DNA and to limit or even block DNA

transcription (Khan and Khan 2010). Several recent reports stress important role of HDAC in COPD and HDAC activity is clearly decreased in oxidative and/or nitrosative stress (Winkler et al. 2012; To et al. 2012). In asthma bronchial tissue and alveolar macrophages have increased HAT and decreased HDAC (Bouchecareilh and Balch 2012). Our previous reports indicate that HDAC2 expression is not altered after tiotropium therapy (Holownia et al. 2010). Present data indicate however that enzymatic activity of HDAC in cell nuclei is lower. Unfortunately, there is no information on isotype specificity of the substrate which was used in this reaction, but it appears that lower HDAC activity may be responsible, at least in part, for histone hyperacetylation and altered expression of different inflammatory molecules.

In the present study we found increased expression of iNOS after tiotropium therapy. Expression of iNOS may be increased by several factors including inflammatory cytokines, but considering a better clinical status of our patients after tiotropium it seems that this increase is not relevant to pathology. iNOS inhibitors have been shown to block inflammation in a mouse model of COPD (Hesslinger et al. 2009), but were not efficient in asthma (Prado et al. 2011). High nitrosative stress may clearly cause airway inflammation, hyperresponsiveness, and remodeling (Sugiera and Ichinose 2011), while lower NO levels are relevant to airways regulation, especially to smooth muscle relaxation and bronchodilation (Ghosh and Erzurum 2011). We have no data on our IS cellular profiles and we did not assess NO levels which appear to be necessary to explain the reasons and possible roles of increased iNOS. Moreover, there is no published data on the role of histone hyperacetylation in iNOS regulation. However, considering an improvement in clinical status of our patients after tiotropium therapy it seems that increased iNOS expression and altered histone acetylation may have positive consequences.

**Conflict of Interest Statement** The authors had no conflicts of interest to declare in relation to this article.

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