Objective: Heat shock proteins assist cellular protein folding and are required for the normal activity of steroid receptors. In this study we assessed nuclear HSP90 and HSP70 proteins and mRNA levels in cells isolated from induced sputum of chronic obstructive pulmonary disease patients treated for 4 weeks with formoterol (F) or formoterol+budesonide (F/ICS).

Methods: Nuclear heat shock protein levels were assessed by Western blot and specific mRNAs were quantified in cell lysates using qRT-PCR.

Results: Both HSP90 and HSP70 protein levels were higher in the F/ICS-treated patients in comparison with the F-treated group (by 31%, P<0.05 and 28%, P<0.05, respectively), while specific mRNAs were lowered. HSP86/HSP89 and D6S182/HSP90-BETA were repressed by about 40% (P<0.05) while HSP70-1/HSP70-1A, HSP70-1B/HSP70-2, and HSP70-HSC54/HSC70 were repressed by 47% (P<0.01), 57% (P<0.01) and 65% (P<0.01), respectively.

Conclusions: It is possible that increased nuclear heat shock proteins may play a role in the attenuation of the response to glucocorticoids in COPD patients.

Key words: COPD, formoterol, glucocorticoids, HSP70, HSP90

INTRODUCTION

Heat shock proteins (HSP) play important role in the physiology and pathology [1]. They act as intracellular chaperones for other proteins, assist their proper folding, prevent aggregation, stabilize partially unfolded proteins, and participate in intracellular protein distribution [1, 2]. HSP are induced by several kinds of stress, including high and low temperatures, toxins, metals, and also by hypoxia, infection, inflammation, physical exercise, and others [3]. The chaperone system is highly coordinated and several chaperones are involved not only in protein folding, but also in their functional, conformational regulations. There are several data indicating that steroid hormone receptors are under a tight control of HSP [4, 5]. A critical role in glucocorticoid receptors (GR) signaling is played by HSP90 and HSP70 which bind unliganded cytosolic receptor and dissociate when the hormone level increases [6]. HSP90 facilitates GC binding to the GR and forms a GR-HSP90 heterocomplex in concert with HSP70 and several nonessential cochaperones [7]. GR and HSP90 are co-transported into the cell nuclei and regulate ligand-dependent transcriptional activity of the GR [8]. It has been shown that an altered HSP90/GR ratio may be responsible for steroid resistance in asthma [9, 10]. Both asthma and chronic obstructive pulmonary disease (COPD) are characterized by chronic airway inflammation and major structural changes of lung tissue. However, inhaled glucocorticoids and long-acting β2-receptor agonists targeting both inflammation and bronchoconstriction, which are usually efficient in the treatment of asthma, are not effective in COPD. The role of HSP in COPD is unknown, but recent data show that HSP are induced in COPD patients [11, 12]. Since molecular chaperones strongly affect GR, the aim of our study was to assess the nuclear levels of HSP90 and HSP70 in the cells isolated from induced sputum of stable COPD patients treated with formoterol (F) or formoterol+glucocorticoids (F/ICS).

MATERIAL AND METHODS

SUBJECTS

All patients included in the study gave their consent after a full discussion of the nature of the study, which had been approved by a local Ethics Committee.

Induced sputum samples were obtained from 34 stable COPD patients. COPD was defined according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [13]. All patients with COPD had airflow limitation (FEV1<80% predicted, FEV1/FVC<70%, GOLD stage 2-3) and received no COPD therapy for four weeks. 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 months before inclusion. No patient in the study had symptoms nor was treated for COPD exacerbation during at least two 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.

**TREATMENT**

All patients included in the study underwent 4-week of washout therapy with Salbutamol only on demand therapy. At the beginning of the treatment, patients were stratified to the following treatments: formoterol alone (F; n = 16), formoterol+budesonide (F/ICS, n = 18) b.i.d. for 4 weeks.

**SPUTUM INDUCTION AND PROCESSING**

Sputum was induced by the inhalation of a 4.5% hypertonc aerosol saline solution, which was generated by an ultrasonic nebulizer (Voyager, Secura Nova; Warsaw, Poland) [14]. Samples were processed within 15 min after 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-hydroxypiperazin-N'-ethane sulfonic acid, 10 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid, 0.2 mM NaF, 50 mM glycrophosphate, 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. Thereafter, the samples were incubated on ice for 15 min and then centrifuged at 13000 x g for 30 min. The cell pellets containing nuclei were retained and resuspended in extracting buffer (50 mM N-2-hydroxypiperazin-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 α-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 fraction was evaluated for the expression of HSP90 and HSP70.

**WESTERN BLOTTING**

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 HSP90 or HSP70 (Abcam, Cambridge, USA; rabbit antibodies against human HSP). 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 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.

**QUANTITATIVE RT-PCR**

Expression of specific HSP genes was assessed using SYBR Green based quantitative RT-PCR after total RNA extraction from induced sputum cells, RNA purification and template cDNA synthesis with commercial PCR master mixes containing appropriate controls and reference dyes (SABiosciences, Frederick, USA) [15]. The expression of HSP86/HSP89A and D6S182/HSP90-BETA coding for cytosolic proteins HSP90AA1 and HSP90AB1, respectively was quantified. In HSP70 family, HSP70-1/HSP70-1A, HSP70-1B/HSP70-2 and HSC54/HSC70 gene products were assessed for coding for HSPA1A, HSPA1B and HSPA8 proteins, respectively. The first two HSP70 proteins are the most abundant heat inducible members of HSP70 family, while HSPA8 is the cognate HSPA, and an essential house-keeping HSPA member. All 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. Protein levels were measured using BCA kit (Sigma-Aldrich, Poznan, Poland).

**STATISTICAL ANALYSIS**

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

**RESULTS**

Table 1 shows a relative content of nuclear HSP90 and HSP70 proteins and mRNA expression coding for essential HSP90 or HSP70 proteins in cells isolated from induced sputum of stable COPD patients treated with formoterol (F; n = 16) and formoterol + budesonide (F/ICS, n = 18) b.i.d. for 4 weeks. The expressions of HSP were equalized in each sample for loading and numerated with density software. The mean specific protein expression in the nuclear fraction of cells isolated from F-treated patients was set as 100 relative units.

Representative Western blot pictures are shown in Fig. 1. In F/ICS treated patients, higher levels of HSP90 and HSP70 (31% increase; P<0.05 and 28% increase, P<0.05 for HSP90 and HSP70, respectively) were observed compared with the corresponding data in the F-treated patients. HSP90 and HSP70 mRNAs were lower in patients treated with F/ICS compared with the F-treated group. Both HSP86/HSP89 and D6S182/HSP90-BETA genes coding for cytosolic HSP90 were repressed by about 40% (P<0.05), while the HSP70 family genes, i.e., HSP70-1/HSP70-1A, HSP70-1B/HSP70-2 and the gene coding for constit-
tutive HSC70-HSC54/HSC70 were all repressed by 47% (P<0.01), 57% (P<0.01), and 65% (P<0.01), respectively.

**DISCUSSION**

COPD is accompanied by increased cellular stress and inflammation which is steroid resistant but its pathomechanisms remain poorly understood [16]. Since, there is no safe and efficient treatment replacing glucocorticosteroids in asthma control, their precise role in COPD treatment is still under discussion. Moreover, several new molecular aspects of GR activation and repression have been identified. The activity of GR is clearly affected by cytosolic molecular chaperones, mostly by HSP90 and HSP70 [6, 7, 8]; however, several cochaperones can further modulate transcriptional activity of GR [17, 18]. There is also accumulating evidence on important role of nuclear HSP, especially in regulating GR-DNA binding [19, 20]. Both HSP90 and HSP70 participate in GR nuclear recycling and retention [4]. Our objective was to assess the levels of HSP90 and HSP70 in nuclear fractions of cells isolated from induced sputum of COPD patients subjected to formoterol (F) and formoterol + budesonide (F/ICS) b.i.d. for 4 weeks. We found that in F/ICS treated patients, nuclear HSP90 and HSP70 protein levels were higher than in F-treated patients, while mRNAs coding for major HSP90 and HSP70 proteins were repressed. Although recent study has shown increased HSP in blood cells of COPD patients and proposed specific serum HSP as a diagnostic marker of COPD, the present study is the first to demonstrate increased HSP90 and HSP70 in the cell nuclei of COPD patients [11]. The functional aspect of this observation may be more relevant, as it is clear that alterations in chaperone machinery may be related to altered GR activity. In asthma, HSP90 expression increases with worsening of inflammation and with duration of the disease [10]. Accordingly, the more HSP90 gene expression, the less T lymphocytes were inhibited in asthma by dexamethasone [21]. Higher HSP expression was also found in glucocorticoid resistant acute lymphoblastic leukemia [22]. Until now, there were no data on downregulation of HSP90 and HSP70 mRNA in F/ICS-treated COPD patients. However, about 2-fold decrease in HSP90 mRNA levels was observed in an in vitro system after 48 h treatment with dexamethasone [23]. Downregulation of HSP70 in asthma was also observed after Fluticasone propionate therapy [24]. Our results indicate that nuclear HSP90 and HSP70 expression in COPD patient treated with ICS may be regulated at the post–mRNA levels. It is also possible that overexpressed nuclear HSP90 and HSP70 in COPD patients subjected to F/ICS therapy may participate in the inhibition of transcriptional activation of GR-dependent target genes and glucocorticoid resistance. We believe that further studies determining the precise role of HSP in COPD pathophysiology may provide the basis for the new treatment options.

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

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**Table 1.** Nuclear HSP90 and HSP70 proteins and mRNAs in cells isolated from induced sputum of COPD patients treated with formoterol (F, n = 16) and formoterol + budesonide (F/ICS, n = 18) b.i.d. for 4 weeks.

|          | **HSP90** | **HSP70** |
|----------|-----------|-----------|
| **F**    | Protein   | 100 ± 28  | 100 ± 26  |
|          | mRNA      | HSP90AA1  | HSP90AB1  |
|          |           | 3.5 ± 2.1 | 1.6 ± 1.1 |
| **F/ICS**| Protein   | 131 ± 43* | 128 ± 42* |
|          | mRNA      | HSP90AA1  | HSP90AB1  |
|          |           | 2.1 ± 1.5*| 0.9 ± 0.8*| 0.7 ± 0.5 | 6.0 ± 3.7 |

*P<0.05 compared with F-treated group; **P<0.01 compared with F-treated group.

**Fig. 1.** Western blot analysis of HSP90 and HSP70 proteins in cells isolated from induced sputum of stable COPD patients treated with Formoterol (F) and Formoterol + Budesonide (F/ICS) b.i.d. for 4 weeks. Standard HSP90 and HSP70 proteins and a loading control (β-actin) are labeled on the right-hand side.
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