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## Reduction in Peripheral Blood Leukocyte Heat Shock Proteins 27 and 70 Expression in Chronic Obstructive Pulmonary Disease

Lada Rumora,<sup>a,\*</sup> Lara Milevoj,<sup>b</sup> Sanja Popović-Grle,<sup>c</sup> Karmela Barišić,<sup>a</sup>  
Tihana Žanić Grubišić,<sup>a</sup> and Ivana Čepelak<sup>a</sup>

<sup>a</sup>*Department of Medical Biochemistry and Haematology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Domagojeva 2, HR-10000 Zagreb, Croatia*

<sup>b</sup>*Department for Laboratory Diagnostics, University Hospital of Traumatology, Draškovićeveva 19, HR-10000 Zagreb, Croatia*

<sup>c</sup>*University Hospital for Lung Diseases Jordanovac, School of Medicine, University of Zagreb, Jordanovac 104, HR-10000 Zagreb, Croatia*

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Chronic obstructive pulmonary disease (COPD) is characterized by chronic local and systemic inflammation, and increased oxidative stress. Changes in peripheral blood leukocyte counts or in the fractions of leukocyte subsets might be implicated in the development of this disease. In this study, increased monocyte fraction was observed in COPD non-smokers. In light COPD smokers, the fraction of neutrophils was significantly increased along with significantly decreased lymphocyte fraction and lung function parameters. Heat shock proteins (Hsps) could stimulate antioxidant defence of cells by decreasing intracellular levels of reactive oxygen particles (ROS) and by neutralizing toxic effects of oxidized proteins. This study showed that the expression of Hsps in leukocytes was influenced by health and smoking status. Hsp70 and Hsp27 were significantly decreased in COPD ex-smokers and healthy smokers, but the most striking suppression in Hsps expression was detected in COPD smokers. It is suggested that this decline in Hsps intracellular levels could be implicated in the pathogenesis of COPD.

### INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by slowly progressive and poorly reversible development of airflow limitation and by abnormal inflammatory response in the airways. COPD is now recognized as a major and increasing global health problem, and is predicted to be the third most common cause of death and the fifth most common cause of disability in the world by the year 2020.<sup>1</sup> Pathological hallmarks of COPD in-

clude chronic obstructive bronchitis with fibrosis and obstruction of small airways, emphysema with destruction of lung parenchyma, loss of lung elasticity and obstruction of peripheral airways.<sup>2,3</sup> The critical role of the local inflammatory process in the pathogenesis of COPD is recognized and generally accepted, and it is associated with the influx of neutrophils into the airway lumen and increased macrophage and T lymphocyte numbers in the airway wall. However, less is known about the so called

\* Author to whom correspondence should be addressed. (E-mail: lrumora@pharma.hr)

low-grade systemic inflammation present in COPD. Many studies have reported changes in oxidative status, and levels of inflammatory cells and mediators in the circulation of COPD patients.<sup>3,4</sup> Cigarette smoking is the major etiological factor responsible for COPD and more than 90 % of COPD patients are smokers. However, only 15–20 % of chronic smokers develop COPD. These observations indicate that additional risk factors, possibly genetic, contribute to the development and the severity of COPD.<sup>4–6</sup> There is now considerable evidence of increased oxidative stress in smokers and in COPD patients.<sup>7–9</sup> Cigarette smoke is a complex mixture of more than 4700 chemical compounds, including high concentrations of free radicals and other oxidants. In addition, activated circulating neutrophils, monocytes and eosinophils release reactive oxygen species (ROS). Oxidative burden is also propagated by increased ROS production from alveolar macrophages and epithelial cells.<sup>8,10</sup> ROS may affect the target cells directly or indirectly, via activation of signal transduction pathways and via formation of oxidized mediators.<sup>3,7,8</sup> Inappropriate activation of signalling pathways could occur during acute and chronic oxidative stress as a result of protein misfolding, protein aggregation, or disruption of regulatory complexes.

The network of heat shock proteins (Hsps) represents an emerging paradigm for the coordinated, multistep regulation of apoptotic signalling events to provide protection from damaging stimuli and to facilitate cellular recovery. These proteins act as molecular chaperones by assisting the correct folding of nascent and stress-accumulated misfolded proteins, and by preventing their aggregation. Hsp synthesis is tightly regulated at the transcriptional level by heat shock transcription factors (HSFs), especially by HSF-1. In resting cells, HSF-1 is a monomer; however, active HSF-1 exists as a trimer and binds to the heat shock elements (HSEs) present in the promoters of the heat shock-inducible genes.<sup>11,12</sup>

It was suggested that circulating neutrophils might be implicated in the pathogenesis of COPD, but the role of other peripheral blood cell types in COPD is still unclear. In this study, we investigated the changes in total peripheral blood leukocyte counts, fractions of leukocyte subsets (neutrophils, eosinophils, basophils, monocytes and lymphocytes) and lung function parameters (FEV<sub>1</sub>, FEV<sub>1</sub>/FVC) in patients with COPD and in healthy volunteers. They were all men with different smoking histories. Chronic inflammation and increased oxidative stress play a major role in the pathogenesis of COPD. It has been shown that Hsps can modulate the cell's immune response and inflammatory response. In particular, it has been demonstrated that both Hsp27 and Hsp70 control inflammation by regulating the expression of the pro- and anti-inflammatory genes. In addition, these proteins, especially Hsp27, could protect cells from oxidative damage by decreasing ROS production, neutralizing the toxic effects

of oxidized proteins, and by affecting cellular glutathione levels. Thus, we assessed the level of Hsp27 and Hsp70 expression in the leukocytes of COPD patients and healthy volunteers, and we hypothesized that their expression would be affected by the person's health status and smoking history.

## EXPERIMENTAL

### *Subjects*

The study included 28 male COPD subjects (11 smokers, 9 ex-smokers, 8 non-smokers) and 42 healthy male subjects (15 smokers, 13 ex-smokers, 14 non-smokers) of similar age (50–70 years old). Participants had no history of bronchial asthma, respiratory tract infectious diseases 1 month prior to the study, no cardiac, renal, hepatic, gastrointestinal or endocrine diseases. Control subjects were taking no medications. COPD patients were receiving bronchodilators only, and no inhaled or oral corticosteroids. Their medication therapy consisted of a combination of long-acting (salbutamol) and short-acting (salmeterol)  $\beta_2$ -agonists as needed, an anticholinergic (ipratropium bromide) and a xanthine preparation (theophylline). All patients were considered to be clinically stable because none of them had required medical attention and/or any change in their regular therapy during the previous 3 months. COPD patients had moderate ( $n = 11$ ) or severe to very severe COPD ( $n = 17$ ) according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The study protocol was approved by the Ethics Committee of the University Hospital for Lung Diseases Jordanovac. All participants gave their written consent after being fully informed of the nature, characteristics, risks, and potential benefits of the study.

### *Spirometry Measurements*

Before starting the measurements, the patient's height and weight were measured. Testing was performed in the sitting or standing position. After applying nose clips, the subject was instructed to take a full inspiration, and then exhale without hesitation through a mouthpiece into the spirometer (MasterLab Pro Body version 3.1, E. Jaeger, Wurzburg, Germany) as forcefully and completely as possible, but at least for six seconds. The test was repeated a minimum of three times, and a maximum of eight times, until two reproducible efforts were obtained. The two largest forced vital capacities (FVC) and forced expiratory volumes in one second (FEV<sub>1</sub>) had to show <5 % variability. After spirometry measurements were taken according to the standard procedure, the patient inhaled 400  $\mu\text{g}$  of salbutamol from a metered dose inhaler. After 15–30 minutes, spirometry was repeated with three measurements, and the highest FEV<sub>1</sub> value was recorded. Before testing, regularly prescribed bronchodilators were withheld for 6 hours for inhaled short-acting  $\beta_2$ -agonists, 12 hours for long-acting  $\beta_2$ -agonists, 6 hours for inhaled anticholinergics, 12 hours for short-acting theophylline preparations, and 24 hours for long-acting theophylline preparations. The bronchodilatory test was considered negative

if the FEV<sub>1</sub> value after salbutamol was less than 200 mL and/or 12 % of that before testing. FEV<sub>1</sub> was expressed in percents with respect to referent values which were used according to the lung parameter standard of the European Community for Coal and Steel (ECCS).<sup>13</sup>

### Blood Sampling

Peripheral blood was collected from all subjects after an overnight fast into tubes containing lithium heparin as anticoagulant. Total leukocyte blood counts and leukocyte differential blood counts were determined using an automated haematology analyzer (Sysmex KX-21N). The fractions of leukocyte subsets (neutrophils, eosinophils, basophils, monocytes and lymphocytes) were expressed in percents.

### Leukocyte Isolation

Leukocytes were isolated according to the method of Percy and Brady.<sup>14</sup> Whole blood and dextran (50 g of dextran in 1 dm<sup>-3</sup> of 120 mmol dm<sup>-3</sup> NaCl solution) were mixed in a 5:1 ratio and left to sediment for one hour at room temperature. The supernatant, containing leukocytes, was separated from the sediment containing erythrocytes. The supernatant was centrifuged at 500 g for 10 minutes at 4 °C. The sediment was suspended in 150 mmol dm<sup>-3</sup> NaCl solution, erythrocyte impurities were removed by hypotonic lysis, and the sample was centrifuged for 10 minutes at 4 °C. This purifying procedure was repeated three times. Purified leukocytes obtained from 5 mL of whole blood were suspended in 150 µL of ice-cold whole-cell lysis buffer (50 mmol dm<sup>-3</sup> Tris-HCl pH = 8.0, 137 mmol dm<sup>-3</sup> NaCl, 1 % Non-ident P-40, 10 % glycerol, and a Complete Protease Inhibitor Cocktail Tablet).

### Leukocyte Lysis

Leukocyte lysates were prepared by repeated freezing/thawing procedures in addition to sonication, and finally cell lysates were centrifuged at 25000 g for 20 minutes at 4 °C. Protein concentration was determined by the method of Lowry *et al.*<sup>15</sup> Samples were denatured by boiling for 3 minutes with 6x Laemmli sample buffer (0.375 mol dm<sup>-3</sup> Tris-HCl pH = 6.8, 12 g/100 mL SDS, 3 % glycerol, 0.2 g/100 mL bromophenol blue, 12 % β-mercaptoethanol, in distilled water) at 4:1 dilution.

### Western Blotting

50 µg of total protein was loaded for each sample onto a 12 % polyacrylamide gel, usually run at 100 V. Transfer onto the nitrocellulose membrane was conducted at 250 mA for 90 minutes. Membranes were blocked for one hour with blocking buffer containing 1 g/100 mL BSA and 1 g/100 mL chicken egg albumin in TBS+T (25 mmol dm<sup>-3</sup> Tris pH = 7.6, 150 mmol dm<sup>-3</sup> NaCl, 0.05 % Tween 20). Membranes were probed overnight at 4 °C with anti-Hsp27 (Stressgen, Biotechnologies) diluted 1:5000 in blocking buffer, anti-Hsp70 (Stressgen, Biotechnologies) diluted 1:1000 in blocking buffer or anti-actin (Sigma) diluted 1:1000 in blocking

buffer. A horseradish peroxidase-conjugated secondary antibody diluted 1:4000 in 5 % low fat milk in TBS+T was utilized to allow detection of the appropriate bands using enhanced chemiluminescence reagent (ECL<sup>TM</sup> Western Blotting Detection Reagents, Amersham Biosciences) and film (Lumi-Film Chemiluminescent Detection film, Roche). Membranes were stripped by incubating at 65 °C for half an hour in stripping buffer (100 mmol dm<sup>-3</sup> β-mercaptoethanol, 2 g/100 mL SDS and 62.5 mmol dm<sup>-3</sup> Tris-HCl pH = 6.7). All experiments were conducted at least three times. Films were developed and scanned, and densitometry measurements were performed using the ScionImage software for Windows (Scion Corporation).

### Statistical Analysis

Analyses were performed using the Sigmapstat software for Windows, version 3.5 (Systat Software Inc.). Variables were all expressed as median (interquartile range) and tested for normality using the Kolmogorov-Smirnov test. The *t*-test and Mann-Whitney rank sum test were used for between-group difference testing of parametric and nonparametric data, respectively. Differences between multiple groups were tested using the one-way ANOVA. Relationships between pairs of variables were tested using Spearman's rank correlation. For Western blot analysis, statistical significance was determined by Student's *t*-test, and data were expressed as mean ± SD. Values of *P* < 0.05 were considered statistically significant.

## RESULTS

### White Blood Cells and Lung Function Parameters in COPD Patients and in Healthy Subjects

Cigarette smoking is known to increase neutrophil blood counts and is negatively associated with postbronchodilator FEV<sub>1</sub>.<sup>7,16</sup> We therefore wanted to explore changes in total peripheral blood leukocyte counts, fractions of leukocyte subsets and lung function parameters in COPD patients and in healthy control subjects subdivided according to their smoking history (Table I). In COPD patients, a statistically significant difference in monocyte fraction was observed between ex-smokers and non-smokers. On the other hand, healthy smokers had significantly higher total leukocyte counts than ex-smokers and non-smokers.

To further study the influence of cigarette smoking on peripheral white blood cells and lung function, we have subdivided COPD and healthy smokers and ex-smokers in two groups according to the median value for daily cigarette consumption: light smokers (daily cigarette consumption < 22, Table II) and heavy smokers (daily cigarette consumption ≥ 22, Table III). Light COPD smokers had a significantly higher neutrophil fraction and a significantly lower lymphocyte fraction, FEV<sub>1</sub> (expressed

TABLE I. Total leukocyte counts, fractions of leukocyte subtypes, and lung function parameters in COPD patients and healthy individuals subdivided according to their smoking histories<sup>(a)</sup>

	COPD patients ( <i>n</i> = 28)			<i>P</i>	Healthy subjects ( <i>n</i> = 42)			<i>P</i>
	Smokers ( <i>n</i> = 11)	Ex-smokers ( <i>n</i> = 9)	Non-smokers ( <i>n</i> = 8)		Smokers ( <i>n</i> = 15)	Ex-smokers ( <i>n</i> = 13)	Non-smokers ( <i>n</i> = 14)	
Total leukocyte counts / 10 <sup>9</sup> dm <sup>-3</sup>	7.3 (6.5–8.7)	7.0 (5.1–8.1)	5.5 (5.1–8.7)	0.338	7.1 (6.7–7.8)	5.3 (4.9–5.9)	5.6 (4.9–6.9)	≤0.001 <sup>(b),(c)</sup>
Neutrophils / %	69.0 (60.8–72.3)	63.0 (62.0–68.5)	59.5 (56.0–70.5)	0.611	58.0 (52.8–61.8)	58.0 (54.3–61.3)	56.0 (54.0–60.0)	0.979
Eosinophils / %	1.0 (1.0–3.0)	2.0 (1.8–2.3)	0.0 (0.0–2.5)	0.270	3.0 (1.0–3.0)	3.0 (1.0–3.0)	0.5 (0.0–2.0)	0.156
Lymphocytes / %	24.0 (19.8–29.8)	27.0 (24.3–30.3)	31.5 (17.5–35.5)	0.926	36.0 (31.8–38.8)	33.0 (29.8–36.3)	38.5 (31.0–41.0)	0.813
Monocytes / %	6.0 (4.0–7.8)	5.0 (5.0–6.0)	8.0 (6.5–8.5)	0.069 <sup>(d)</sup>	4.0 (2.0–6.5)	5.0 (4.0–7.3)	5.0 (3.0–7.0)	0.495
FEV <sub>1</sub> / % <sup>(e)</sup>	52.6 (34.9–55.5)	45.0 (43.0–47.0)	47.1 (40.6–58.9)	0.630	99.0 (87.8–115)	112 (96.5–115)	103.0 (94.0–109)	0.515
(FEV <sub>1</sub> /FVC) / %	56.4 (48.4–59.8)	64.3 (53.2–66.4)	61.1 (56.9–66.7)	0.116	82.0 (75.0–84.8)	82.0 (77.3–89.0)	81.0 (79.0–84.0)	0.643

<sup>(a)</sup>Fraction of basophiles was unchanged and close to zero for all conditions tested. FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity. Data are presented as median and interquartile range in parentheses. Differences between multiple groups were tested using one-way ANOVA, and between two groups by the *t*-test and Mann-Whitney rank sum test for parametric and nonparametric data, respectively.

<sup>(b)</sup>Significant difference between the multiple groups (COPD smokers, ex-smokers and non-smokers; healthy smokers, ex-smokers and non-smokers), *P*<0.05.

<sup>(c)</sup>Significant difference between healthy smokers and healthy ex-smokers, and between healthy smokers and healthy non-smokers, *P*<0.05.

<sup>(d)</sup>Significant difference between COPD ex-smokers and COPD non-smokers, *P*<0.05.

<sup>(e)</sup>With respect to referent, predicted value.

TABLE II. Total leukocyte counts, fractions of leukocyte subtypes, and lung function parameters in light COPD and healthy smokers<sup>(a)</sup>

	Daily cigarette consumption < 22					
	Total COPD ( <i>n</i> = 12)	Total healthy ( <i>n</i> = 13)	COPD smokers ( <i>n</i> = 6)	Healthy smokers ( <i>n</i> = 10)	COPD ex-smokers ( <i>n</i> = 6)	Healthy ex-smokers ( <i>n</i> = 3)
Total leukocyte counts / 10 <sup>9</sup> dm <sup>-3</sup>	6.9 (5.3–8.2)	6.9 (6.5–7.7)	6.9 (6.5–7.3)	7.0 (6.6–7.7)	6.7 (5.2–8.2)	5.4 (4.7–8.4)
Neutrophils / %	69.5 <sup>(b)</sup> (63.0–76.5)	58.0 (55.8–61.3)	71.5 <sup>(b)</sup> (69.0–77.0)	59.0 (56.0–62.0)	64.5 <sup>(c)</sup> (63.0–76.0)	57.0 (55.5–59.3)
Eosinophils / %	1.5 (1.0–2.0)	3.0 (1.0–3.0)	1.0 (1.0–2.0)	2.0 (1.0–3.0)	2.0 (1.0–2.0)	3.0 (1.5–3.0)
Lymphocytes / %	26.0 <sup>(b)</sup> (17.0–29.5)	35.0 (33.0–37.3)	24.5 <sup>(b)</sup> (17.0–29.0)	35.5 (34.0–38.0)	27.0 (16.0–30.0)	34.0 (30.0–35.5)
Monocytes / %	5.0 (4.0–5.5)	4.0 (2.0–5.8)	4.0 (3.0–5.0)	3.0 (2.0–5.0)	5.0 (5.0–6.0)	8.0 (5.0–9.5)
FEV <sub>1</sub> / % <sup>(d)</sup>	45.9 <sup>(b)</sup> (42.2–53.3)	99.0 (89.8–111.0)	53.3 <sup>(b)</sup> (40.7–61.0)	100.5 (90.0–114.0)	44.5 <sup>(b)</sup> (43.7–46.7)	98.0 (91.3–107.0)
(FEV <sub>1</sub> /FVC) / %	59.9 <sup>(b)</sup> (51.8–65.0)	82.0 (74.5–85.0)	57.4 <sup>(b)</sup> (45.7–65.1)	82.5 (73.0–85.0)	62.9 <sup>(b)</sup> (53.6–64.9)	82.0 (79.8–87.3)

<sup>(a)</sup>Fraction of basophiles was unchanged and close to zero for all conditions tested. Total COPD = total COPD smokers and ex-smokers, Total healthy = total healthy smokers and ex-smokers; FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity. Data are presented as median and interquartile range in parentheses. Differences between total COPD subjects and total healthy subjects, COPD and healthy smokers, and COPD and healthy ex-smokers were tested by the *t*-test and Mann-Whitney rank sum test for parametric and nonparametric data, respectively.

<sup>(b)</sup>Significant difference between the groups (total COPD and total healthy; COPD smokers and healthy smokers; COPD ex-smokers and healthy ex-smokers), *P*<0.05.

<sup>(c)</sup>Trend towards significance, 0.05<*P*<0.1.

<sup>(d)</sup>With respect to referent, predicted value.

TABLE III. Total leukocyte counts, fractions of leukocyte subtypes, and lung function parameters in heavy COPD and healthy smokers<sup>(a)</sup>

	Daily cigarette consumption $\geq 22$					
	Total COPD (n = 8)	Total healthy (n = 15)	COPD smokers (n = 5)	Healthy smokers (n = 5)	COPD ex-smokers (n = 3)	Healthy ex-smokers (n = 10)
Total leukocyte counts / $10^9 \text{ dm}^{-3}$	7.6 <sup>(b)</sup> (6.3–8.6)	5.8 (5.0–6.8)	8.5 (7.3–9.6)	7.7 (6.7–8.1)	7.0 <sup>(c)</sup> (5.4–7.5)	5.2 (5.0–5.8)
Neutrophils / %	63.0 (59.5–66.0)	58.0 (50.5–61.8)	63.0 (56.8–67.5)	55.0 (47.0–62.5)	63.0 (60.0–64.5)	58.5 (52.0–62.0)
Eosinophils / %	2.5 (1.5–4.0)	3.0 (1.3–3.8)	3.0 (0.8–3.5)	3.0 (1.8–5.8)	2.0 (2.0–4.3)	3.0 (1.0–3.0)
Lymphocytes / %	28.5 <sup>(c)</sup> (23.0–30.5)	33.0 (30.3–38.5)	24.0 (21.3–33.8)	36.0 (28.0–42.5)	30.0 (27.8–30.8)	33.0 (30.0–37.0)
Monocytes / %	7.0 <sup>(c)</sup> (5.5–8.5)	5.0 (4.0–7.0)	8.0 (7.0–9.3)	7.0 (3.5–8.3)	5.0 (5.0–5.8)	5.0 (4.0–7.0)
FEV <sub>1</sub> / % <sup>(d)</sup>	43.0 <sup>(b)</sup> (33.1–53.8)	112.0 (92.5–118.5)	33.2 <sup>(b)</sup> (32.5–53.8)	94.0 (85.3–126.0)	45.0 <sup>(b)</sup> (42.0–52.2)	112.5 (112.0–117.0)
(FEV <sub>1</sub> /FVC) / %	55.3 <sup>(b)</sup> (48.9–63.2)	82.0 (75.8–87.8)	43.9 <sup>(b)</sup> (48.5–59.1)	82.0 (78.0–85.0)	66.2 (55.4–78.3)	82.0 (75.0–89.0)

<sup>(a)</sup>Fraction of basophiles was unchanged and close to zero for all conditions tested. Total COPD = total COPD smokers and ex-smokers, Total healthy = total healthy smokers and ex-smokers; FEV<sub>1</sub>, forced expiratory volume in 1 second; FVC, forced vital capacity. Data are presented as median and interquartile range in parentheses. Differences between total COPD subjects and total healthy subjects, COPD and healthy smokers, and COPD and healthy ex-smokers were tested by the *t*-test and Mann-Whitney rank sum test for parametric and nonparametric data, respectively.

<sup>(b)</sup>Significant difference between the groups (total COPD and total healthy; COPD smokers and healthy smokers; COPD ex-smokers and healthy ex-smokers),  $P < 0.05$ .

<sup>(c)</sup>Trend towards significance,  $0.05 < P < 0.1$ .

<sup>(d)</sup>With respect to referent, predicted value.

in percents with respect to referent values) and FEV<sub>1</sub>/FVC ratio (expressed in percents) compared to light healthy smokers. On the other hand, in light COPD ex-smokers only a significant decrease in FEV<sub>1</sub> and FEV<sub>1</sub>/FVC was observed compared to light healthy ex-smokers. Heavy COPD smokers had a significantly lower FEV<sub>1</sub> and FEV<sub>1</sub>/FVC than heavy healthy smokers, while only FEV<sub>1</sub> was significantly reduced in heavy COPD ex-smokers as compared to healthy ex-smokers.

#### Expression of Hsp70 and Hsp27 in Leukocytes of COPD Patients and in Healthy Subjects

It has been shown that an increase in Hsps expression protects cells from various kinds of stressors.<sup>17,18</sup> We assessed the level of expression of Hsp70 and Hsp27 in the peripheral blood leukocytes of COPD patients and healthy individuals (Figures 1 and 2). Expression of both Hsp70 and Hsp27 was dependent on health status (subjects with or without COPD) and smoking status (smokers, ex-smokers and non-smokers). Expression of Hsp70 and Hsp27 was significantly decreased in COPD ex-smokers and healthy smokers, but the most striking suppression of Hsps expression was detected in COPD smokers as compared to healthy non-smokers.

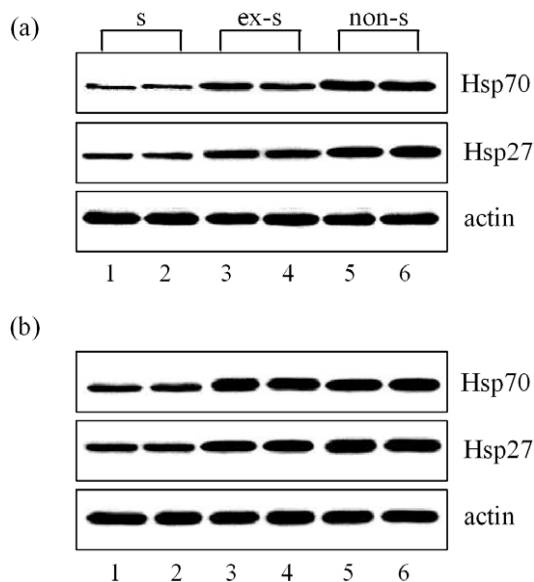


Figure 1. Expression of Hsp70 and Hsp27 in the peripheral blood leukocytes of COPD patients (a) and healthy individuals (b). Participants were subdivided according to their smoking history in three groups: smokers (s; lines 1 and 2), ex-smokers (ex-s; lines 3 and 4) and non-smokers (non-s; lines 5 and 6). Western blot analysis was performed as described in Experimental. Representative blots for Hsp70 and Hsp27 expression in the leukocytes of COPD patients (a) and healthy individuals (b) from three independent experiments are shown.  $\beta$ -actin was detected to ensure that equal amounts of proteins were loaded for each sample.

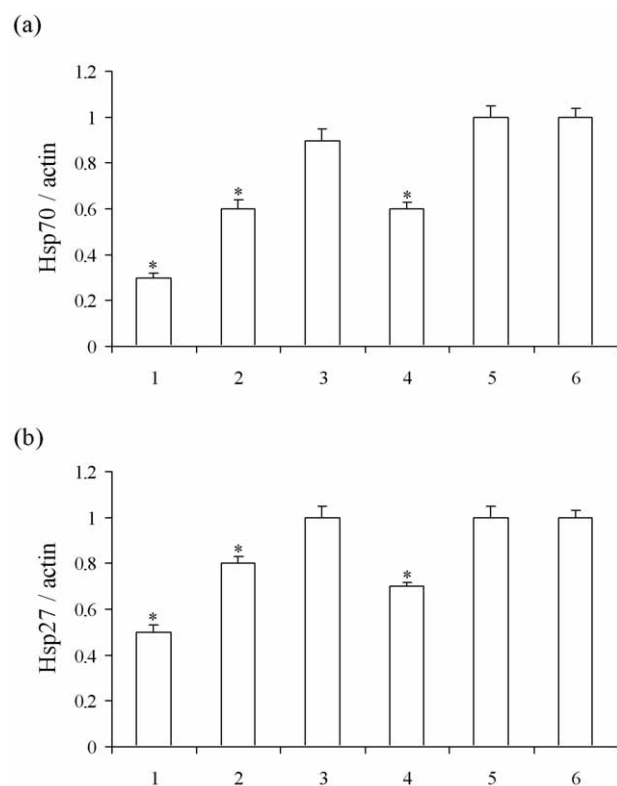


Figure 2. Graphic presentation of the levels of Hsp70 (a) and Hsp27 (b) expression in the peripheral blood leukocytes of COPD patients (1, 2, 3) and healthy individuals (4, 5, 6). Participants were subdivided according to their smoking history in three groups: smokers (1, 4), ex-smokers (2, 5) and non-smokers (3, 6). Data were expressed as mean  $\pm$  SD. Densitometric values of Hsps levels were normalized to the housekeeping protein  $\beta$ -actin. The normalized expression of Hsps in the leukocytes of healthy non-smokers was taken as 1. Asterisk denotes significant change compared to healthy non-smokers. Differences were considered significant at  $P < 0.05$ .

## DISCUSSION

COPD is characterized by chronic local and systemic inflammation, and increased oxidative stress. Cigarette smoking is the major etiological factor responsible for COPD and, so far, smoking cessation is the only intervention able to reduce the progression of COPD.<sup>19</sup> However, several studies have shown that airway inflammation persists even after smoking cessation.<sup>20,21</sup> Neutrophils play an important role in the pathogenesis of airway inflammation in COPD due to their ability to release a number of mediators such as elastases, metalloproteases and oxygen-derived radicals which promote tissue inflammation and damage.<sup>22</sup> Cigarette smoking is known to increase neutrophil blood counts and to cause sequestration of the neutrophils in the lung capillaries by decreasing their deformability.<sup>23</sup> In addition, epidemiological studies have shown a relationship between the number of circulating neutrophil and FEV<sub>1</sub> and between

smoking and FEV<sub>1</sub>.<sup>7,16</sup> In contrast to neutrophils, the possible association between other peripheral blood cell types and COPD is less clearly understood. In our study, increased monocyte fraction was observed in COPD non-smokers. In »light« COPD smokers (daily cigarette consumption  $< 22$ ), the fraction of neutrophils was significantly increased along with a significantly decreased lymphocyte fraction and lung function parameters. On the other hand, we could not detect any significant change in leukocyte subtypes in »heavy« COPD smokers (daily cigarette consumption  $\geq 22$ ), and only FEV<sub>1</sub> and FEV<sub>1</sub>/FVC were significantly decreased in those subjects. Unfortunately, due to the small number of COPD and healthy participants, it would be inappropriate to infer from these observations that any peripheral blood leukocyte subtype is necessarily actively involved in the pathogenesis of COPD.

In determining prophylactic and/or therapeutic strategies against COPD and other human diseases, it is reasonable to regard the intracellular molecules that might regulate their mechanisms, such as heat shock proteins (Hsps), as appropriate targets. There is increasing evidence that Hsps may function at multiple checkpoints in the apoptotic signalling pathway, and these proteins could increase the antioxidant defence of cells by decreasing the intracellular levels of ROS and by neutralizing the toxic effects of oxidized proteins.<sup>17,24,25</sup> Imbalance between oxidants and antioxidants is considered to play a role in the pathogenesis of COPD.

Our data show that the expression of both Hsp70 and Hsp27 in the peripheral blood leukocytes is down-regulated in COPD ex-smokers, healthy smokers and especially in COPD smokers, which could further promote oxidative burden within the cell. This is in agreement with the results presented by Zhao and colleagues, who reported a decrease in Hsp70 mRNA and Hsp70 protein levels in the lymphocytes of COPD patients.<sup>26</sup> Some studies refer to the reduced transcriptional activity and binding of HSF to the HSE as being the cause of the decrease in Hsp production.<sup>27,28</sup> It was demonstrated that mitogen-activated protein kinases ERK and JNK could bind to and phosphorylate HSF-1 at distinct sites, leading to suppression of its transcriptional activity.<sup>18,25,29</sup> On the other hand, Hsp70 negatively regulates stress kinases JNK and p38 MAPK.<sup>30</sup> However, various stressful conditions cause depletion of free cellular Hsp70 by accumulated abnormal proteins, which induce activation of stress kinases.<sup>30</sup> Indeed, we have observed a strong activation of JNK and p38 MAPKs in the contexts where Hsps expression was decreased, *e.g.*, in COPD smokers, COPD ex-smokers and in healthy smokers (unpublished data).

Decreased intracellular Hsps concentration could also reflect their increased release from the cell. Extracellular Hsps were present in the peripheral circulation of healthy individuals; however, elevated levels can be

found in various disease conditions, including inflammation, bacterial and viral infection, atherosclerosis, hypertension and renal disease.<sup>17,31,32</sup> Although the exact mechanism by which intracellular Hsps leave cells is still incompletely understood, it has been suggested that Hsps are released by both passive (necrotic) and active (physiological) mechanisms.<sup>17,31–36</sup>

Extracellular Hsp27 and Hsp72 possess potent chaperone and cytokine activity, a term now referred to as the chaperokine ability of Hsps.<sup>31, 37</sup> Exogenously added Hsps activate signal transduction cascades, leading to the stimulation of an immune response by inducing the production and release of various cytokines,<sup>37–39</sup> which could lead to continuous propagation of inflammation observed in COPD. Therefore, Hsps might become recognized as attractive pharmacological targets for the treatment of chronic inflammatory diseases, including COPD.

In conclusion, in this study we have observed down-regulation of the expression of Hsp70 and Hsp27 in the peripheral blood leukocytes from patients with COPD (smokers and ex-smokers) and from healthy smokers. We suggest that this decline in the Hsps intracellular levels could be implicated in the pathogenesis of COPD. A challenge to our future research is to determine the levels of Hsp27 and Hsp70 in sera of large groups of COPD patients carefully selected according to disease severity, smoking history and receiving therapy, and to test the hypothesis that extracellular Hsps may act as an effective danger signal to the immune system.

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## SAŽETAK

### **Promjene u udjelu pojedinih tipova leukocita u krvi i u ekspresiji proteina toplinskoga šoka u kroničnoj opstruktivskoj plućnoj bolesti**

**Lada Rumora, Lara Milevoj, Sanja Popović-Grle, Karmela Barišić, Ivana Čepelak  
i Tihana Žanić Grubišić**

Temeljne su karakteristike kronične opstruktivske plućne bolesti (KOPB; engl. *Chronic Obstructive Pulmonary Disease*, COPD) kronična upala i oksidacijski stres. Moguće je da promjene u broju ukupnih leukocita u krvi ili u pojedinim tipovima leukocita utječu na razvoj ove bolesti. U istraživanju su utvrđeni povišeni monociti kod nepušača s KOPB-om. S druge strane, kod pušača s KOPB-om koji su dnevno pušili do 22 cigarete opaženi su povišeni neutrofil i sniženi limfociti te sniženi parametri koji upućuju na funkciju pluća. Proteini toplinskoga šoka (engl. *Heat shock proteins*, Hsp) djeluju na oksidirane proteine tako da neutraliziraju njihovo toksično djelovanje, a smanjuju i koncentraciju reaktivnih kisikovih spojeva u stanici. U našem se ispitivanju pokazalo da ekspresija Hsp-a ovisi i o zdravstvenom stanju ispitanika i o tome puši li. Razine Hsp70 i Hsp27 značajno su potisnute kod bivših pušača s KOPB-om i kod zdravih pušača, a osobito kod pušača s KOPB-om. Rezultati ovih istraživanja upućuju na mogućnost da su Hsp70 i Hsp27 upleteni u patogenezu KOPB-a.