BIOMARKERS, VOL. 9, NO. 6 (NOVEMBER–DECEMBER 2004), 461–469

Systemic markers of the redox balance in chronic obstructive pulmonary disease

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Received 15 May 2004, revised form accepted 10 November 2004

Chronic obstructive pulmonary disease (COPD) is highly prevalent and its pathogenesis is still not completely clarified. Clinically stable patients ($n=21$) and healthy subjects ($n=24$) were studied for blood markers of oxidative injury and antioxidant status. The plasma concentration of protein carbonyls was significantly increased in COPD patients, both ex-smokers (0.76 ± 0.28 nmol mg^{-1}) and smokers (0.99 ± 0.20 nmol mg^{-1}) versus controls (0.49 ± 0.14 nmol mg^{-1}). The concentration of total thiols was slightly enhanced in plasma of the COPD patients (ex-smokers 492 ± 23 μmol l^{-1} and smokers 505 ± 36 μmol l^{-1} versus controls 450 ± 67 μmol l^{-1}; $p<0.05$). The activity of the antioxidant enzyme superoxide dismutase was increased in erythrocytes (activity in U g^{-1} haemoglobin: ex-smokers 4460 ± 763 and smokers 4114 ± 1060 versus 3015 ± 851 in controls; $p<0.01$), while glutathione peroxidase activity was decreased in total blood (activity in U g^{-1} haemoglobin: ex-smokers 27 ± 9 and smokers 23 ± 9 versus 47 ± 25; $p<0.01$). Lower levels of selenium in plasma were also found for COPD patients (concentration in mg l^{-1}: ex-smokers 0.030 ± 0.019 and smokers 0.032 ± 0.024 versus 0.058 ± 0.023 in controls; $p<0.01$), being more evident in those with very low levels of arterial oxygen pressure. In addition, the levels of potassium and rubidium were increased in blood cells of the patient group. All these changes might reflect oxidant damage and an altered electrolytic homeostasis and can be interpreted as markers of COPD rather than as indicators of smoking habits.

Keywords: protein carbonyls, total thiols, superoxide dismutase (SOD), glutathione peroxidase (GPx), trace elements.

Introduction

Chronic obstructive pulmonary disease (COPD) is a highly prevalent and probably underestimated disease associated with airway and parenchyma inflammation leading to progressive impairment of pulmonary function. COPD is becoming one of the leading causes of morbidity all over the world (Shapiro 2000). In Portugal, it is estimated that it attains 5% of the population (and 6.8% at Lisbon region). Its impact in the quality of life and as an economic health care burden is recognized. In spite of the considerable progress in understanding the

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2004 Taylor & Francis Group Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500400024768

mechanisms of some other inflammatory diseases of the airway tract, only recently COPD has attracted attention and consideration. Cigarette smoking is the major environmental factor responsible for 80–90% of COPD cases, although some other inhaled irritants may also initiate the inflammatory response (Rahman and MacNee 1996, Croxton *et al*. 2002). However, it is not clear why only 10–20% of chronic heavy smokers develop COPD. Two main mechanisms are assumed in the pathogenesis of this disease: the oxidative stress resulting from oxidant/antioxidant equilibrium impairment (MacNee and Rahman 1999, 2001, Hageman *et al*. 2003) and the proteinase-antiproteinase imbalance (Sampson 2000), being the neutrophil the main cell that seems to orchestrate them. However, the cell recruitment and mutual influence of those mechanisms are far from being well known and much investigation is still needed.

Organic fluids, such as blood, may give significant systemic data about the factors involved in these mechanisms and thus on the individual physiological status. Relating clinical, functional and morphological changes with the biochemical markers in blood may provide an increased understanding of the importance of oxidative stress in the pathology.

The paper reports on data for blood biochemical parameters related to oxidant damage and antioxidant status in COPD patients. The contents of carbonyl groups in proteins, the activities of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx), and the concentrations of total thiols were the evaluated indicators. The levels of essential trace elements, playing a role in oxidant/antioxidant pathways — iron (Fe), copper (Cu), zinc (Zn) and selenium (Se) — were also determined. Several of these parameters were related to clinical and physiological data.

Materials and methods

Group characterization

The Pulmonary Diseases Centre of St Maria Academic Hospital, Lisbon, Portugal, approved the protocol of the study, and all subjects gave informed consent.

The COPD group involved 21 patients with an average age of 70 ± 7 years attending the Pneumology Department of the hospital. COPD diagnosis was based on clinical ground and respiratory functional criteria recommended by the Global Initiative for Chronic Obstructive Lung Disease (GOLD 2004), being 13 patients in stage II, six in stage III and two in stage IV. Emphysema was confirmed in all patients by high-resolution computer tomography. Patients were all clinically stable and no one was subjected to oxygen therapy or treated with inhaled or oral corticosteroids for at least the 6 previous weeks, nor were they supposed to have exacerbation episodes registered during the last year.

The determination of plasma levels of α1-antitrypsin (AAT) and albumin was carried out in all subjects. Functional data including parameters based on the post-bronchodilator forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC), arterial blood pressure of oxygen ($Pa\,O_2$) and of carbon dioxide ($Pa\,CO_2$), as well as AAT and albumin plasma levels are listed in table 1.

The reference group consisted of 24 healthy volunteer individuals, non-smokers, without respiratory complaints, nor under any anti-inflammatory therapy. The volunteers were also asked about their living habits and occupational exposure. This group had an average age of 74 ± 9 years.

Blood sample collection

Blood was collected by venopuncture and drawn into heparinized tubes. Portions of total blood were separated for enzyme activity evaluation, which was carried out as soon as possible after collection. The remaining blood was centrifuged at 1500*g* for 10 min at 4°C to obtain plasma, which was divided into

Table 1. COPD group characterization.

	Ex-smokers ($n=15$)	Smokers ($n=6$)
Age (years)	70 ± 7	69 ± 7
Smoking habits (packs × year)	45–95[a]	10–120[a]
FEV_1 (% of predicted)	54 ± 20	56 ± 9
FEV_1/FVC (% of predicted)	54 ± 13	58 ± 13
PaO_2 (mmHg)	71 ± 12	78 ± 9
$PaCO_2$ (mmHg)	42 ± 5	42 ± 2
Severity (GOLD 2004)	Stage II, $n=9$ Stage III, $n=4$ Stage IV, $n=2$	Stage II, $n=4$ Stage III, $n=2$ Stage IV, $n=0$
AAT[b] ($g\,l^{-1}$)	1.31 ± 0.34	1.31 ± 0.29
Albumin[c] ($g\,l^{-1}$)	49 ± 6	48 ± 4
Therapy	β-adrenergic agonists ($n=21$) theophylline ($n=9$) anticolinergics ($n=21$)	

[a]Minimums and maximums.
[b]Reference interval 1–2 $g\,l^{-1}$; control group, 1.34 ± 0.27 $g\,l^{-1}$.
[c]Reference interval 37–46 $g\,l^{-1}$; control group, 40 ± 3 $g\,l^{-1}$.

aliquots and stored at $-80^{\circ}C$, until analysis. For trace element determination, blood plasma and cells were stored at $-20^{\circ}C$.

Evaluation of protein carbonyl groups and total thiols

The carbonyl contents in plasma proteins were evaluated according to Levine *et al*. (1990). The method is based on the reaction of 2,4-dinitrophenylhydrazine with carbonyl groups and the protein-bound hydrazone was determined spectrophotometrically at 370 nm, using a molar absorption coefficient of 22 100 $M^{-1}\,cm^{-1}$. The carbonyl contents were expressed as nmol mg^{-1} protein.

Total plasma thiols were measured spectrophotometrically at 412 nm based on a method that uses 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) as the thiol reagent (Sedlak and Lindsay 1968). A molar absorption coefficient of 13 100 $M^{-1}\,cm^{-1}$ was used.

Determination of enzyme activities

Measurement of SOD activity in erythrocytes was carried out with the Ransod SD125 kit (Randox Laboratories, UK). The method uses xanthine and xanthine oxidase to generate superoxide radicals that react with a phenyltetrazolium derivative. Activity was measured by the degree of inhibition of this reaction. GPx activity was measured in total blood using the Ransel Randox RS506 kit. The method is based on that developed by Paglia and Valentine (1967) using cumene hydroperoxide and reduced glutathione as substrates. NADPH consumption by glutathione reductase was monitored as a measure of the formation of oxidized glutathione. For both enzyme activities, results were express per g haemoglobin.

Determination of elemental concentrations

Concentrations of Fe, Cu, Zn and Se in plasma and blood cells were determined by particle-induced X-ray emission. This technique also allowed one to evaluate the levels of potassium (K), rubidium (Rb) and calcium (Ca). The methodology applied is described by Barreiros *et al*. (2001). The analytical procedure was checked using Gent second-generation freeze-dried human serum reference material (Versieck *et al*. 1998), which was analysed together in each sample batch. Differences to the certified value were below 5% for Fe, Se and Rb, and below 10% for Cu, Zn, K and Ca. Results were expressed in $mg\,l^{-1}$ plasma or $mg\,kg^{-1}$ blood cells wet mass.

Statistical analysis

Data are expressed as mean ± SD. Significant differences between subject groups were determined using parametric, *t*-test, and non-parametric, Kruskal–Wallis, Mann–Whitney and Kolmogorov–Smirnov, approaches (Norusis 2001). Differences were considered significant when the two-tailed confidence interval of 95% was exceeded at a probability error (p level) <0.05 in all tests applied.

Results

All the COPD patients had airway obstruction and all had a smoking history, being ex-smokers or current smokers. Most patients showed a moderate to severe COPD and only two exhibited a very severe condition (table 1). When smoking habits were assessed relative to FEV_1 and FEV_1/FVC values, blood gas concentrations and haematological data, no significant differences between active smokers and ex-smokers were found.

Carbonyl contents in plasma proteins were determined as an indicator of oxidative damage (figure 1A). Protein carbonyls were higher in COPD patients than in reference subjects ($p < 0.01$). Total thiols were evaluated in plasma as an indicator of redox balance being slightly enhanced ($p < 0.05$) in the patient group (figure 1B).

The activities of two antioxidant enzymes SOD (Cu,Zn-SOD) and GPx were measured in blood in order to obtain information on the antioxidant enzymatic capacity associated with this pathology. SOD activity was increased in erythrocytes ($p < 0.01$) and GPx was decreased in total blood ($p < 0.01$) of patients with COPD (figure 2A, B, respectively). Smoking habits did not influence the variations observed, as no significant differences were determined between ex-smokers and current smokers within the COPD group.

The concentrations of several elements in plasma and in packed blood cells of the studied groups are shown in the tables 2 and 3, respectively. In COPD group plasma, Se was significantly decreased ($p < 0.01$) compared with the control group. Interestingly, significant higher values were observed for K ($p < 0.01$) and Rb ($p < 0.01$) in blood cells of COPD patients. For these three elements, no significant differences between ex- and current-smokers were observed within the COPD group (plasma Se in $mg\,l^{-1}$: ex-smokers 0.030 ± 0.019 and current smokers 0.032 ± 0.024; blood cells K in $mg\,kg^{-1}$ wet mass: ex-smokers 2304 ± 396 and current smokers 2277 ± 236; blood cells Rb in $mg\,kg^{-1}$ wet mass: ex-smokers 3.5 ± 0.8 and current smokers 4.6 ± 1.2).

A strong linear dependence was not observed between age, smoking habits, FEV_1, FEV_1/FVC ratio or blood arterial gas data and protein carbonyls, thiols, enzyme activities or trace element levels. Although, when patients with an FEV_1/FVC ratio below 50% were compared with those with higher ratios, the increase in total thiols contents (512 ± 21 $\mu mol\,l^{-1}$ for ratios below 50% versus

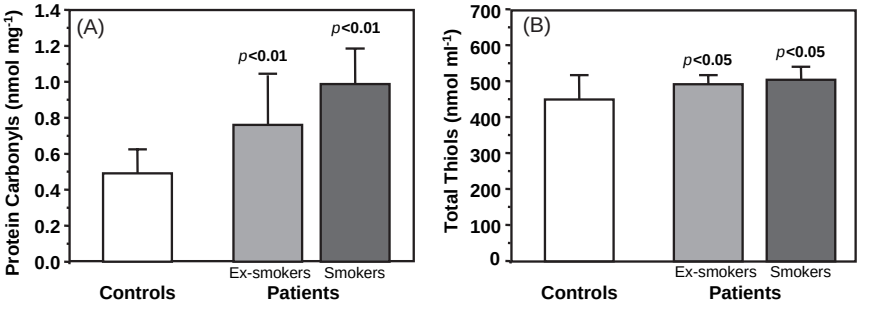

Figure 1. Protein carbonyl contents (A) and total thiols (B) in the plasma of control subjects and COPD patients. Significant differences to control values are indicated.

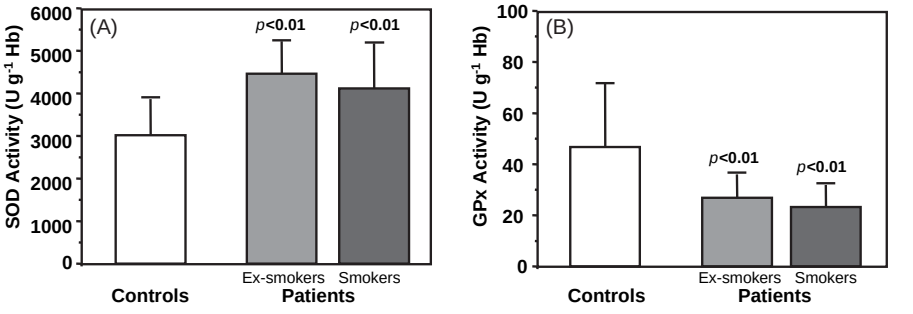

Figure 2. Erythrocytes SOD activity (A) and whole blood GPx activity (B) for control subjects and COPD patients. Significant differences to control values are indicated.

483 ± 26 μmol l^{-1}) and plasma Fe (1.2 ± 0.5 mg l^{-1} for ratios below 50% versus 0.8 ± 0.2 mg l^{-1}) were significant ($p < 0.05$). Also, if a cut-off in oxygen blood pressures is established at 70 mmHg, a significant decrease for carbonyl concentration in plasma proteins ($p < 0.05$) was found in COPD patients with lower $Pa\,O_2$ (figure 3A). Se levels in plasma also appeared to be lower in the same patients (figure 3B), although the difference was not significant ($p < 0.06$).

Discussion

There is increasing evidence that oxidant stress occurs in COPD and might represent an important event in the pathogenesis. It is known that powerful oxidants are generated during the inflammatory process due to the activation of neutrophils (Repine *et al*. 1997) and markers of oxidative stress can be found in systemic circulation. Numerous studies have reported evidence of oxidant-mediated injury in the air spaces and fluids of both smokers and patients with COPD (Croxton *et al*. 2002, Langen *et al*. 2003). Other studies have shown that lipid oxidation also occurs in the blood serum of subjects with the same pathology as well as alterations in the antioxidant capacity (Matés *et al*. 1999, Daga *et al*. 2003, Kinnula and Crapo 2003). The COPD group studied constituted a heterogeneous population concerning the disease severity, although the patients' conditions were stabilized. The number of subjects enrolled in the study limited the establishment of significant sub-groups concerning living habits (e.g. smoking), disease severity and/or significant classes within each sub-group for

Table 2. Plasma elemental concentrations (mg l^{-1}) evaluated in the studied subjects.

	Control group (mean ± SD)	COPD group (mean ± SD)	*p*
K	81 ± 15	98 ± 17	<0.1
Ca	51 ± 11	56 ± 7	>0.1
Fe	1.1 ± 0.4	1.0 ± 0.4	>0.1
Cu	1.3 ± 0.3	1.2 ± 0.2	>0.1
Zn	0.9 ± 0.1	0.9 ± 0.2	>0.1
Se	0.058 ± 0.023	0.032 ± 0.019	<0.01
Rb	0.20 ± 0.06	0.17 ± 0.08	>0.1

Values are given as mean ± SD and the significance of the differences is indicated by the *p* values.

Table 3. Elemental concentrations in blood cells (mg kg^{-1} wet mass) evaluated in the studied subjects.

	Control group (mean ± SD)	COPD group (mean ± SD)	*p*
K	1840 ± 196	2296 ± 355	<0.01
Ca	35 ± 17	47 ± 24	>0.1
Fe	785 ± 62	794 ± 36	>0.1
Cu	1.2 ± 0.2	1.2 ± 0.2	>0.1
Zn	11 ± 2	12 ± 1	>0.1
Se	0.19 ± 0.13	0.23 ± 0.14	>0.1
Rb	2.9 ± 0.6	3.8 ± 1.1	<0.01

Values are given as mean ± SD and the significance of the differences is indicated by the *p* values.

both physiological and clinical data. Despite this, alterations in the parameters measured, although suggesting oxidative stress, could not be associated with smoking history. In fact, the increase of carbonyl groups content in plasma proteins of the COPD patients studied might reflect oxidative damage at a systemic level. The level of oxidized proteins is dependent on the balance between pro-oxidant, antioxidant and proteolitic activities, and an imbalance between them might lead to the higher carbonyl contents observed. The protein injury can contribute to decreased proteinase inhibitor activity, which is thought to play a role in this disease pathogenesis (Rahman and MacNee 1996). When the protein carbonyls in plasma of patients were analysed according to PaO_2 classes, they were lower in those patients with decreased arterial oxygen pressure. The diminished oxygen level in blood might contribute for decreased protein oxidation relative to the patient class with highest oxygen pressure. Due to the few number of individuals studied so far, this relationship could not be confirmed. In contrast, Praticò *et al*. (1998) found a negative correlation between oxygen arterial pressure and the urinary excretion of isoprostanes, which indicates higher lipid peroxidation in COPD patients under worse conditions. In the present study, the level of total thiol groups in blood plasma of COPD subjects was slightly increased. Total thiols in plasma include protein sulphydryl groups and, in lower levels, non-protein ones such as those in the reduced form of gluthatione. Albumin contributes to most of the plasma thiols

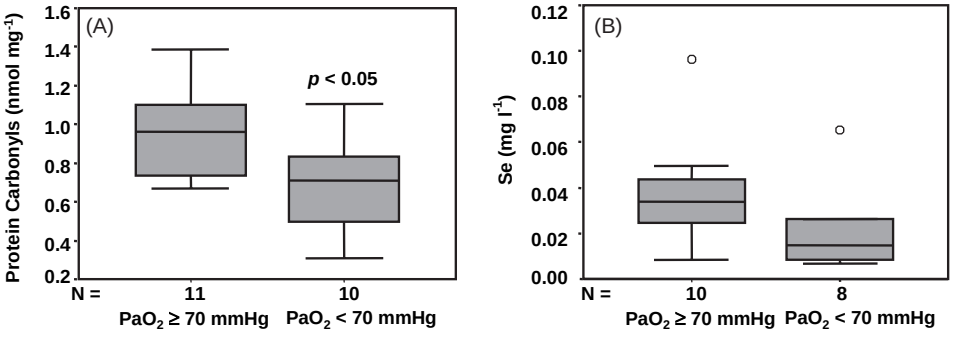

Figure 3. Box and whisker plots for plasma protein carbonyl contents (A) and for Se levels (B) according to two PaO_2 classes established for COPD patients. Graphs are based on medians (line across the box) and the interquartile range (boxes). Circles represent outlier values. The number of samples in each group is given as *N*; significant differences are indicated.

(Thomas and Evans 1975) and the COPD patients had significantly enhanced plasma albumin levels by report to the control group and to reference intervals established. Reduced glutathione could not contribute significantly to any increase in plasma total thiols, since its plasma levels are low compared with protein thiols, although it plays an important role in the inflammatory response modulation and its synthesis may be up-regulated at least in the lungs of smokers with and without COPD (Rahman and MacNee 2000). Hence, the increased albumin level might account for the higher plasma thiol concentration in the COPD group.

The alterations observed for both SOD and GPx activities emphasize the redox imbalance in clinically stable patients. SOD, which in erythrocytes is the Cu, Zn-dependent isoenzyme, catalyses the dismutation of superoxide anion radical leading to hydrogen peroxide generation. GPx, in turn, belongs to a family of enzymes that catalyse the reduction of hydrogen peroxide and organic peroxides using glutathione as the reductor. The increased SOD activity as observed in COPD patients could be a consequence of induction by an excess of oxidants. Similar alterations have been found for patients with asthma or rhinitis (Matés *et al*. 1999), but decreased SOD activity in erythrocytes has been reported in patients with COPD (Daga *et al*. 2003). However, the comparison of results from different studies should be taken cautiously and should account for several factors such as the tissues involved, the severity of disease and drug therapy. The higher SOD activity in erythrocytes occurred in parallel with lower GPx activity in the whole blood of COPD patients. Although the activity of catalase, an enzyme that eliminates hydrogen peroxide, has not been evaluated, an imbalance in the SOD/GPx ratio may lead to higher peroxide levels and, consequently, other reactive chemical species can be generated. The whole blood GPx activity is associated with two selenoproteins: cellular glutathione peroxidase (GPx-1) and plasma glutathione peroxidase (GPx-3) (Flohé *et al*. 1973, Rotruck *et al*. 1973, Allan *et al*. 1999). In the present study, plasma Se concentration was lower in the COPD group, mainly in those subjects with the lowest arterial oxygen pressure, and serum Se depletion has been also reported in chronic cigarette smokers (Rahman *et al*. 1996). The lower Se level might be related in part to the decreased GPx activity. Alterations in the elemental homeostasis probably occur in COPD as a result of impaired absorption and/or excretion or due to changes in cellular ion transport. In fact, significant changes in K and Rb concentrations were also observed in blood cells of COPD subjects. The alterations found for these elements might reflect an electrolytic imbalance, which indirectly could affect the Se concentration in plasma.

In summary, in stable COPD patients, an oxidant/antioxidant imbalance occurred leading to increased oxidative injury in plasma proteins. Trace element homeostasis was also affected. These observations and the relationship between the alterations observed in plasma for protein carbonyls and Se content, and the arterial blood oxygen pressure, show that systemic modifications can be linked to physiological parameters associated with the pathological condition rather than to smoking habits. These findings point out the relevance of blood markers in the characterization of COPD.

Acknowledgements

The work was supported by the Calouste Gulbenkian Foundation, Research Contract SDH.IC.I.01.22. The authors thank Ana Isabel Costa and Rute Pinheiro for technical assistance on sample collection and preparation.

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