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The Red Blood Cell as a Biosensor for Monitoring Oxidative Imbalance in Chronic Obstructive Pulmonary Disease: An *Ex Vivo* and *In Vitro* Study

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity in Western countries. The increased oxidative stress, caused by the release of reactive oxygen and nitrogen species (ROS/RNS) from inflammatory airways cells, contributes to the pathogenesis of the disease. The aim of the present study was to evaluate (a) whether the oxidative imbalance can lead to specific alterations of red blood cells (RBCs) from stable COPD patients; (b) whether treatment with *N*-acetyl-cysteine (NAC), in widespread use as mucolytic agent in clinical practice, can counteract these effects; and (c) whether an *in vitro* model represented by the exposure of RBC to ROS/RNS could mimic the *in vivo* situation. The results obtained clearly indicated that the RBC integrity and function are similarly altered in COPD patients and in ROS/RNS *in vitro*–treated samples and that NAC administration was capable of counteracting RBC oxidative modifications both *in vivo*, as detected by clinical and laboratory evaluations, and *in vitro*. Altogether these results point to RBC oxidative modifications as valuable bioindicators in the clinical management of COPD and indicate that *in vitro* RBC exposure to ROS/RNS as a useful tool in experimental studies aimed at the comprehension of the pathogenic mechanisms of the redox-associated diseases. *Antioxid. Redox Signal.* 8, 1171–1182.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is considered a leading cause of chronic morbidity and mortality throughout the world. It is a major and increasing global health problem, which is predicted to become the third commonest cause of death and the fifth cause of disability in the world by 2020 (19, 30). Tobacco smoking is considered the major known risk factor, accounting for >95% of cases in developed countries, but several other risk factors are known, like air pollution, occupational dust and chemicals, socioeconomic status, and infections (29, 30). According with the GOLD guidelines, COPD is defined as "a disease state characterized by airflow limitation that is not fully reversible but progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases." It is characterized by acceleration in the normal decline of lung function.

The pathogenetic mechanisms underlying COPD have only partially been elucidated (29). COPD has been described as a complex inflammatory disease that involves several types of inflammatory cells and a large number of inflammatory mediators in the distal airspace (5, 38). Moreover, oxidative stress plays an important role in the amplification of the inflammatory response (15). The plasmatic redox balance has been recently proposed as a specific marker of COPD (33).

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Thus, a current hypothesis in the pathogenesis of COPD includes a role for increased oxidant burden, caused directly by smoking or indirectly by the increase of ROS/RNS release from inflamed airspace leukocytes. Once not adequately counterbalanced by the lung antioxidant systems, this results in oxidative stress. In turn, this leads to activation of transcription factors, such as nuclear factor-κB (NF-κB) to inactivation of antiproteases, increased sequestration of neutrophils in the pulmonary microvasculature, and oxidative tissue injury (12, 20). These pathologic alterations in the lungs lead to pulmonary hypoxemia, thus limiting intramuscular oxidative metabolism and decreasing aerobic adenosine triphosphate (ATP) production (22). From a cellular point of view, it can be hypothesized that hypoxemia is related to compromised oxygen gas exchange between erythrocytes and lung cells. Furthermore, pilot studies (34) have shown alterations caused by oxidative stress in the red blood cells (RBCs) from patients with COPD. These altered RBCs have a reduced transport capacity and peripheral release of O_2, which, in association with reduced PaO_2 (hypoxemia) typical of COPD, could lead to muscle dysfunction (9). On the basis of these data and of recent works analyzing the prevalence of anemia in COPD (16), a study was thus undertaken on the peripheral blood with the aim of comparing oxidative changes occurring in RBCs from healthy subjects with those found in COPD patients. Enormous potential application exists of this cell type as a bioindicator for human health monitoring in clinical practice (7, 31). Two different approaches were followed: (a) an *ex vivo* study of blood samples from 40 patients with stable COPD that were compared with those of age-matched healthy donors, and (b) an *in vitro* study carried out on blood samples from healthy donors exposed to oxidative imbalance, possibly mimicking the redox imbalance occurring *in vivo*. For this purpose, RBCs were treated with peroxynitrite [$ONOO^-$; this term refers to both the anion oxoperoxynitrate (1^-), $ONOO^-$, and its conjugate acid hydrogen oxoperoxynitrate, ONOOH], the product of the fast radical-radical reaction between superoxide and nitric oxide. By virtue of its potent oxidant and nitrating ability, peroxynitrite has been proposed as an important mediator of inflammation-induced tissue injury and dysfunction as well as of degenerative disorders including COPD (6). Conversely, RBCs are considered the most important scavengers of peroxynitrite *in vivo*, because ~50% of peroxynitrite generated in the blood crosses the RBC membrane and is inactivated inside the cell almost exclusively by oxyhemoglobin (11, 32). We previously reported that, in the reaction with RBCs, peroxynitrite can induce several morphologic and functional modifications (23). All these modifications hijack RBCs toward senescence and apoptosis. Importantly, in these *in vitro* studies, *N*-acetylcysteine (NAC), acting as a reduced sulfhydryl donor, was able to provide a powerful protection against $ONOO^-$-induced oxidative changes in RBCs, at least in terms of cell-shape maintenance and "correct" expression of RBC senescence markers (23). With this in mind, we performed *ex vivo* (*i.e.*, in RBCs from COPD patients) and *in vitro* treatments with NAC to evaluate cellular and clinical effects of this drug. The results reported in this study clearly indicate that (a) erythrocytes from COPD underwent significant morphologic and functional alterations similar to those induced by peroxynitrite in healthy RBCs treated *in vitro*; (b) a correlation seems to exist between RBC alterations and clinical functional evaluations of COPD patients; and (c) NAC exerts a beneficial effect both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Patient selection

We studied 40 patients with clinically stable COPD with a mean age of 73 ± 3 years, 22 men and 18 women. All patients, recruited from one outpatient pulmonary clinic (Forlanini University Hospital, Rome), were first seen at a screening visit where inclusion/exclusion criteria, medical history, smoking history, and physical examination were performed. All patients underwent a pulmonary-function test performed with the patient seated. Forced vital capacity (FVC) and forced expiratory volume in the first second (FEV_1) were measured with a clinical spirometer (Quark PFT, COSMED Srl. Rome, Italy). We considered the predicted percentage value of air exhaled during the first second of forced expiration (pred FEV_1%) and the Tiffeneau index (FEV_1/FVC). The patients also underwent blood gas analysis to evaluate arterial blood gases (PaO_2 and PCO_2) and a 6-min walking test (6MWT) with an evaluation of dyspnea by the Borg Scale according to the American Thoracic Society Statement (2002) (1). The Borg scale is a 12-point ordinal scale ranging from 0 (no respiratory complaints) and 0.5 (very, very slight respiratory complaints) to 10 (maximal respiratory complaints) administered to the patients after the 6MWT (36). It is used to quantify dyspnea and discomfort and weakness occurring in patients during daily activities (simulated by the 6MWT); a lower score assigned by the patient is considered a clinical improvement.

A peripheral venous blood sample was taken for biochemical and cytologic analysis (Department of Drug Research and Evaluation, Section of Cell Aging and Degeneration, Istituto Superiore di Sanità, Rome).

These specific parameters were evaluated at the beginning (T_0) and at the end of the study (T_1) after 90 days.

To be eligible for the study, the patients had to meet the following criteria: a clinical diagnosis of stable COPD; current or former smoker; PaO_2 >50 mm Hg; 50% ≤FEV_1 ≤80% of predicted (stage 2 according with the GOLD guidelines); and use of short- or long-acting β_2-agonists, xanthine derivatives, and diuretics. Moreover, all individuals had never had hypertension or organ disease. The patients had no evidence of cor pulmonale or of metabolic, renal, hepatic, or neuromuscular disorders. They had not received pharmacologic treatments with antibiotics or systemic steroids in the last 2 weeks.

Patients enrolled in our study, after informed consent, were subdivided into two groups (A and B) and, with a 1:1 ratio randomization, received two different doses of NAC: 1,200 mg/day (group A, n = 20) and 1,800 mg/day (group B, n = 20). NAC was given in once at the same time every day. The study groups were compared with a control group of 13 age-matched healthy donors. Patients who had exacerbation of COPD that required a change in medication or hospital admission were excluded from the study.

Red blood cell preparation

Fresh human blood from healthy donors and COPD patients was drawn into heparin zed tubes and centrifuged for 10 min at 1,500 *g*. The plasma and buffy coat were removed and washed twice in Buffer G (50 m*M* Tris, 50 m*M* Hepes, 10 m*M* $MgCl_2$, 2 m*M* EDTA, 10 m*M* D-glucose, 10 m*M* $CaCl_2$, 50 m*M* NaCl, 5 m*M* ACL, and 0.1% bovine serum) and resuspended in the same buffer to the initial hematocrit concentration. No appreciable cell lysis was observed during the RBC preparation procedure.

Peroxynitrite preparation

Peroxynitrite was synthesized from sodium nitrite and hydrogen peroxide, stabilized by alkali as described (27), and processed as reported elsewhere (23).

In vitro treatments

Unless otherwise indicated, all chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.).

Oxidative stress. Washed RBCs were suspended in PBS at 0.25% hematocrit (50 μ*M* hemoglobin content) and treated with a bolus of 50 μ*M* $ONOO^-$ (final pH, 7.3 ± 0.1) under vigorous vortexing, centrifuged, and washed with PBS. No appreciable cell lysis was observed during the preparation procedure. To avoid metal-catalyzed reactions, the PBS was treated extensively with Chelex 100, and all samples contained 0.1 m*M* diethylenetriaminepentaacetic acid (DTPA). Control experiments were performed with decomposed $ONOO^-$ to exclude the participation of $ONOO^-$ contamination products. Decomposed $ONOO^-$ was obtained by adding $ONOO^-$ to the PBS/DTPA for 5 min at room temperature before the addition of biologic targets (reversed order of addition).

NAC treatment. Before the $ONOO^-$ treatment, cells were exposed to different concentrations of NAC (1, 3, 5, 10, and 15 m*M*) (Zambon, Milan, Italy) for 2 h.

Scanning electron microscopy (SEM)

RBCs from controls, from patients with COPD, and from those exposed to $ONOO^-$ (with and without NAC) were collected and plated on poly-L-lysine–coated slides, fixed with 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) at room temperature for 20 min. After postfixation in 1% OsO_4 for 30 min, these blood cells were dehydrated through graded ethanols, critical point-dried in CO_2, and gold-coated by sputtering with Balzers Union SCD 040 apparatus. The samples were examined with a Cambridge 360 scanning electron microscope.

Morphometric analyses

Altered erythrocyte shape was evaluated by counting ≥500 cells (50 erythrocytes for each different SEM field at a magnification of ×3,000) from healthy individuals, COPD patients, and samples exposed to $ONOO^-$ (with and without NAC) in triplicate. Statistical analysis was conducted by using Student's *t* test.

Analytic cytology

RBCs were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, and after washing in the same buffer, were permeabilized with 0.5% Triton X-100 in PBS for 5 min, also at room temperature. The erythrocytes were then stained with monoclonal antibodies (MAbs) to band 3 (Sigma), and after 30 min at 37°C, samples were washed and then incubated for 30 min at 37°C with fluorescein isothiocyanate (FITC)-labeled anti mouse (Sigma). Secondary antibody given alone was used as negative control. For glycophorin A (GA) detection, unfixed cells were stained with MAbs to GA directly conjugated to FITC (Dako, Glostrup, Denmark) for 30 min at 4°C. Appropriate FITC-conjugated immunoglobulin was used as negative control. Samples were analyzed by intensified charge-coupled device video microscopy (IVM) or by flow cytometry with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.) equipped with a 488-nm argon laser. At least 20,000 events have been acquired. The median values of fluorescence-intensity histograms were used to provide semiquantitative analysis. The values of the untreated RBCs were set to 100%, and the levels of the considered proteins in the different samples were reported relative to these.

Analysis of the redox balance

All RBC samples were incubated with 10 μ*M* dihydrorhodamine 123 (DHR 123; Molecular Probes), or 1 μ*M* dihydroethidium (DHE; Molecular Probes) to reveal hydrogen peroxide and superoxide anion production, respectively, or with 10 μ*M* 5-chloromethyl-2′,7′-dichlorodihydrofluoresceindiacetate (CM-H_2DCFDA, Molecular Probes) for the analysis of intracellular content of reduced thiols, as previously reported (23). The fluorescence signal of control cells was set to 100%, and the expression levels of the individual proteins in treated cells are reported relative to this.

Methemoglobin (MetHb) detection

Methemoglobin content of erythrocytes was evaluated spectrophotometrically (37) after cell lysis in cold hypotonic buffer (5 m*M* phosphate buffer, pH 8.0).

Determination of phosphotyrosine phosphatase (PTP-ase) activity

PTP-ase activity was detected in membranes (protein content, 1 mg/ml) prepared from $ONOO^-$-treated RBCs by using *p*-nitrophenyl phosphate (p-NPP) as substrate according to published procedures (39).

Statistical analyses

Statistical analyses were carried out by using Mann–Whitney nonparametric tests, and regression analyses, by using Statview 5.1 (software for Macintosh). Only p values of <0.01 were considered significant.

RESULTS

Structural analyses

Scanning electron microscopy analyses prompted us to a general view of cell-shape features and to visualize cell-surface alterations. Different forms of altered RBCs have been referred to as surface herniations (echinoid forms) or cup forms (stomatocytic forms). However, some other RBC morphologies have also been described, such as acanthocytic (RBCs with surface blebbing) or leptocytic (subtle and elongated RBCs) forms. This kind of qualitative analyses, once carried out in RBCs from COPD patients, clearly indicated the presence of several acanthocytic and few leptocytic forms (Fig. 1C). These altered forms were absent in control samples (Fig. 1A). Interestingly, 3 months after NAC treatment (1,200

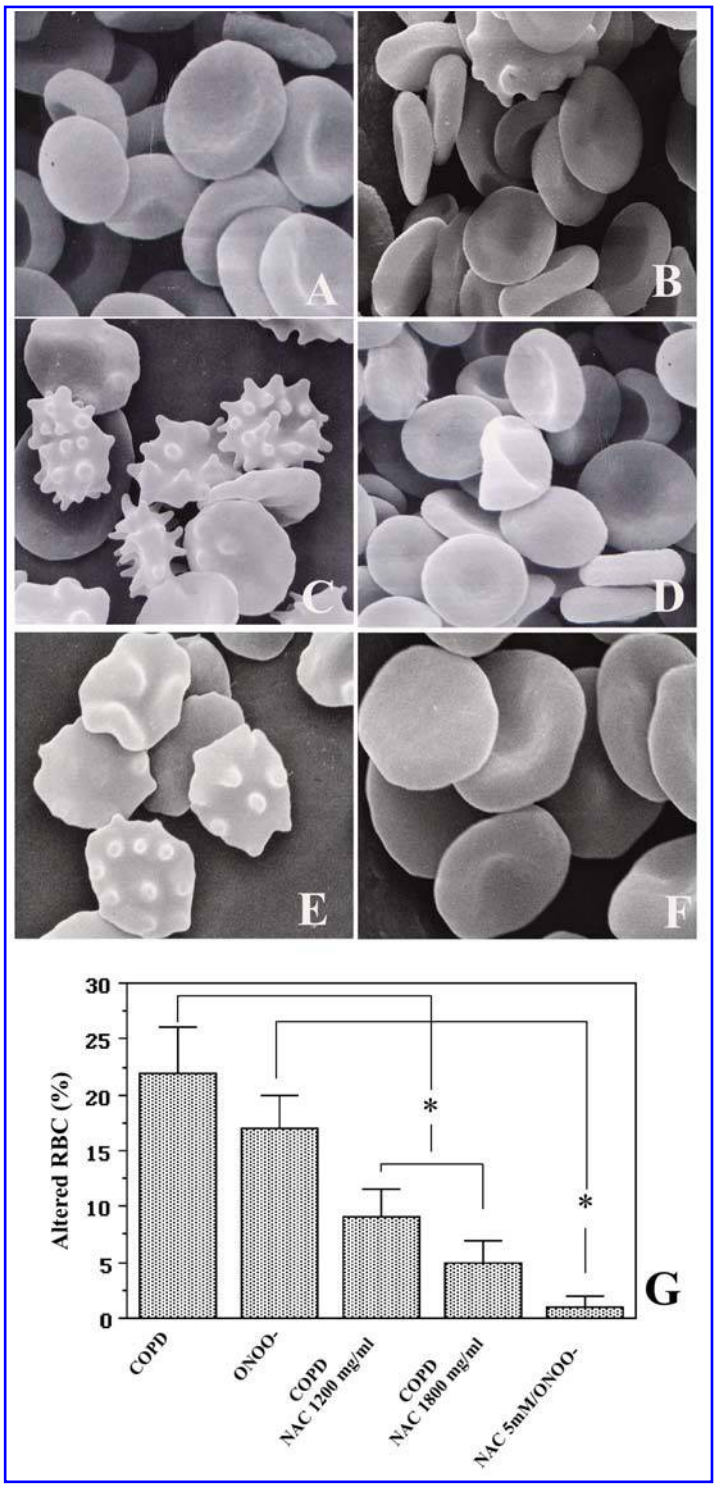

FIG. 1. Scanning electron microscopy analyses. Erythrocytes from a representative healthy donor and a chronic obstructive pulmonary disease (COPD) patient are shown in (**A**) and (**C**), respectively. Note that several red blood cells (RBCs) from the COPD patient appear subtle (leptocytes) and with numerous surface protrusions (acanthocytes). Three months of *in vivo N*-acetyl-cysteine (NAC) administration (1,200 mg/day or 1,800 mg/day) reestablished the normal shape in RBCs from a COPD patient (**B** and **D**, respectively). Similar changes were detectable in RBCs from a representative healthy donor exposed *in vitro* to $ONOO^-$ (**E**). Also in this case, 2-h pretreament with 5 m*M* NAC substantially prevented $ONOO^-$-induced RBC alterations (**F**). The morphometric analysis (**G**) clearly demonstrated that (a) in COPD subjects, >20% of RBCs were altered in comparison with those from healthy donors; (b) similar morphologic alterations were observed in RBCs exposed *in vitro* to $ONOO^-$; and (c) either *in vivo* or *in vitro* NAC administration reduced significantly the percentage of altered RBCs. The percentages of RBC alterations reported in (**G**) are expressed as a percentage with respect to control samples (RBC from healthy donors). $*p < 0.01$.

or 1,800 mg/day), the normal discoid shape of RBCs appeared reestablished (Fig. 1B and D, respectively). Moreover, morphologic alterations, similar to those observed in RBCs from COPD patients, were found in RBCs exposed *in vitro* to $ONOO^-$ (Fig. 1E). Also in these samples, several acanthocytes and leptocytes were observed. As expected, the normal discoid shape was generally maintained in RBC samples pretreated with 5 m*M* NAC before $ONOO^-$ administration (Fig. 1F). Lower concentrations of NAC (down to 1 m*M*) were able significantly to counteract the morphologic alterations induced by $ONOO^-$ (not shown).

To quantify the presence of altered erythrocytes, morphometric analyses were then performed. As shown in Fig. 1G, we found that (a) in COPD patients, the percentage of morphologically altered RBCs was significantly higher with respect to control samples (considered as 100%); and that (b) in RBCs from healthy donors, *in vitro* treatment with $ONOO^-$ induced similar morphologic alterations. Moreover, in accordance with these qualitative analyses, both *in vivo* and *in vitro* NAC exposure exerted a "protective" activity, significantly ($p < 0.01$) reducing the percentage of altered RBCs.

Increase of senescence markers in altered RBCs

Considering that structural and molecular modifications are associated with RBC senescence and degeneration (23), we evaluated whether the structural alterations shown earlier were accompanied by typical signs of RBC senescence. For this, two different "indicators" were selected: glycophorin A (GA) and band 3 molecules. The first is a glycoprotein widely expressed on the RBC surface and is downregulated during senescence; the second is a channel pumping unit that strongly contributes to erythrocyte homeostasis. The analysis of these molecules, carried out in parallel by both static and flow-cytometry analyses clearly demonstrated that (a) GA and band 3 expression significantly decreased either in RBCs from COPD patients or in those exposed *in vitro* to $ONOO^-$ (–48.6 ± 4.5% and –60.1 ± 5.7% for GA, and –30 ± 3.7% and –46.7 ± 7.3% for band 3) with respect to RBCs from control samples; and that (b) NAC treatment led to a substantial restoring of normal values (Figs. 2 and 3, left column, first panel). Immunofluorescence analyses, carried out by IVM, confirmed flow-cytometry results and, further, showed a dramatic rearrangement of these molecules either in RBCs from COPD patients (Figs. 2 and 3, left column, second panel; compare with right column, first panel) or in those exposed to $ONOO^-$ (Figs. 2 and 3, left column, third panel). This qualitative analysis clearly revealed the protective effect exerted by NAC both *in vivo* (Figs. 2 and 3, right column, second panel) and *in vitro* (Figs. 2 and 3, right column, third panel).

Redox balance

Next, a specific analysis of three important parameters that are known to influence the redox balance of the cell was performed. In particular, we analyzed production of superoxide anion and hydrogen peroxide as well as the intracellular content of reduced thiols in RBCs from COPD subjects and in RBCs treated *in vitro* with $ONOO^-$. No significant changes were detected in the ROS production in COPD patients with respect to healthy donors (not shown). In contrast, after treatment with $ONOO^-$, we found a significant increase in superoxide anion (+46 ± 3%) and hydrogen peroxide (+65 ± 7%). It was previously demonstrated that mild but persistent oxidative stress can lead to GSH depletion and to oxidation of thiol groups of skeletal proteins that oversee cell-shape maintenance (8). In consideration of the key role of these proteins in RBC shape changes, a measurement of thiol content, essentially referred to cytoskeletal thiol groups, was performed by semiquantitative flow-cytometry analysis both in RBCs from COPD patients and in those exposed to $ONOO^-$ *in vitro* (Fig. 4). We found a significant ($p < 0.01$) reduction of the intracellular content of reduced thiols either in RBCs from COPD patients or in RBCs treated *in vitro* with $ONOO^-$. Importantly, and in accord with data reported in Figs. 2 and 3, NAC was able to prevent the oxidation of thiol groups both *in vivo* and *in vitro*.

$ONOO^-$-dependent oxidation of hemoglobin and effects of NAC

RBCs are the most important scavengers of ROS/RNI *in vivo* mainly because of the presence of high concentrations (20 m*M*) of intracellular oxygenated hemoglobin (Hb). The ferrous heme of oxygenated Hb reacts with ROS/RNI-derived species with formation of ferric heme (methemoglobin; metHb), which is considered a good marker of RBC oxidative stress (11, 32). The physiologic amount of metHb in human blood is ≤1%. No metHb was detected in untreated RBCs (50 μ*M* as Hb) from healthy subjects or from COPD patients. In contrast, treatment of RBCs of healthy subjects with stoichiometric amounts of $ONOO^-$ induced the formation of 23.4 ± 2% metHb (Fig. 5A), confirming previous results reported by Matarrese *et al*. (23). More interestingly, metHb formation was significantly increased in $ONOO^-$-treated RBCs from COPD patients. This clearly indicates an increased susceptibility to oxidation of RBCs from COPD patients (Fig. 5A). The $ONOO^-$-dependent formation of metHb by RBCs was efficiently counteracted by the preincubation with NAC before the oxidative treatment (Fig. 5B). We observed a 75% inhibition at 0.5 m*M* and 100% inhibition at higher concentrations.

RBC PTP-ase activity

In RBCs, peroxynitrite stimulates a tyrosine-dependent signal-transduction pathway that leads to the enhancement of band 3 tyrosine phosphorylation, translocation of glycolytic enzymes to the cytoplasm, and a consequent increase in glucose metabolism (21). The increase in band 3 tyrosine phosphorylation appears to be mediated by a reversible inhibition of the activity of PTP-ase associated with the erythrocyte membrane. Since we reported a rearrangement of the band 3 in RBCs from COPD patients as well as in those of control samples exposed to $ONOO^-$ (Fig. 3), we hypothesized a modification in this signal-transduction pathway involving also the PTP-ase activity. We then compared the PTP-ase activity of RBCs from COPD patients with that of RBCs from healthy subjects taken as controls (Fig. 6; the PTP-ase activity is expressed as percentage of controls). Untreated RBCs from COPD patients showed basal levels of PTP-ase activity not significantly different from those found in untreated controls, despite a wide distribution of the values. As expected (21,

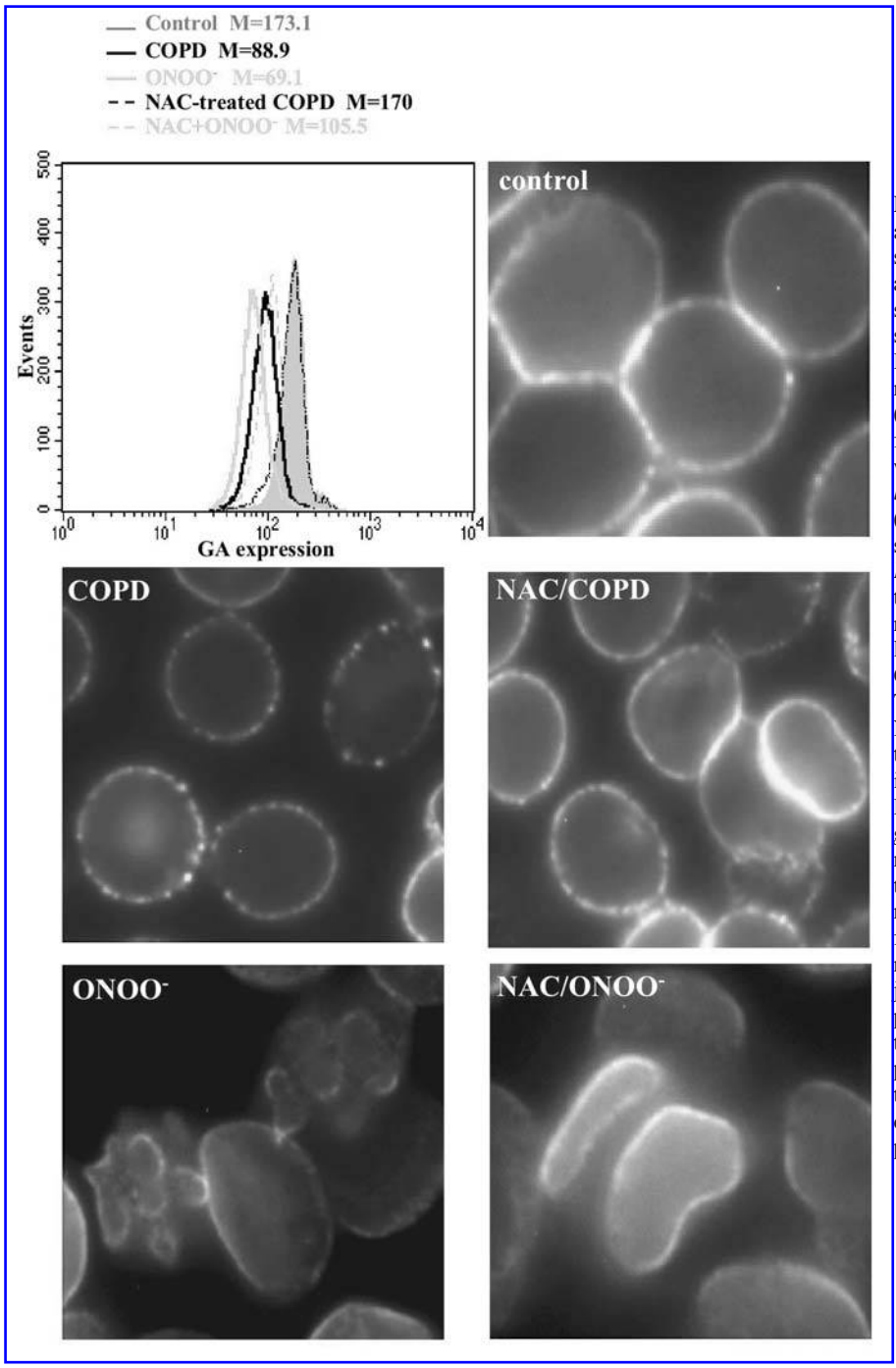

FIG. 2. Glycophorin A (GA) analysis. As demonstrated by a semiquantitative flow-cytometry analysis (left column, first panel), a significant ($p < 0.01$) decrease of surface GA was found either in RBCs from chronic obstructive pulmonary disease (COPD) patients (*continuous black line*) or in those from healthy donors treated *in vitro* with $ONOO^-$ (*continuous grey line*) with respect to untreated RBC (*grey histogram*). Statistical analysis also revealed that 3 months of *N*-acetyl-cysteine (NAC) administration *in vivo* (1,800 mg/day) significantly ($p < 0.01$) prevented the loss of surface GA in RBCs from COPD patients (*dashed black line*). Two-hour pretreatment with 5 m*M* NAC significantly ($p < 0.01$) counteracted the decrease of surface GA induced by $ONOO^-$ treatment in RBCs from healthy donors (*dashed grey line*). Numbers represent the median values of fluorescence intensity histograms. Qualitative immunofluorescence analysis confirmed **f**low-cytometry data and revealed, in RBCs from COPD subjects, the presence of a particular patching of GA that was not detectable in RBCs after $ONOO^-$ exposure *in vitro*. Only one representative experiment of six (for *in vitro* data) and a representative COPD patient (among 40) are shown.

23), we relieved the decrease of the PTP-ase activity of control RBCs after $ONOO^-$ treatment but, more interestingly, we also found a more severe inhibition when RBCs from COPD patients were submitted to oxidative treatment (Fig. 6). This confirms the increased susceptibility to oxidative stress detected in RBCs from COPD patients mentioned earlier.

Clinical evaluations and beneficial effects of NAC on COPD patients

Parallel studies were also conducted to verify whether NAC treatment could improve the clinical outcome of COPD patients. Specific functional parameters were taken into consideration. In particular, pulmonary function test (PFT), blood gas analysis, and 6-MWT with Borg Dyspnea Scale were evaluated in 38 COPD patients (two patients were excluded because of exacerbation) before and after 90 days of NAC treatment. Mean baseline predicted value of FEV_1 at the beginning of the study (T_0) was 72.6% ± 2. At the end of the study (T_1, after 90 days), a significant difference was found in FEV_1 into the two groups; we found an increase of 4.6% of FEV_1 predicted value (T_1, 77.2% ± 2) in group A patients (NAC, 1,200 mg/day) and an increase of 7.5% (T_1, 80.1% ± 2) in group B patients (NAC, 1,800 mg/day). No important

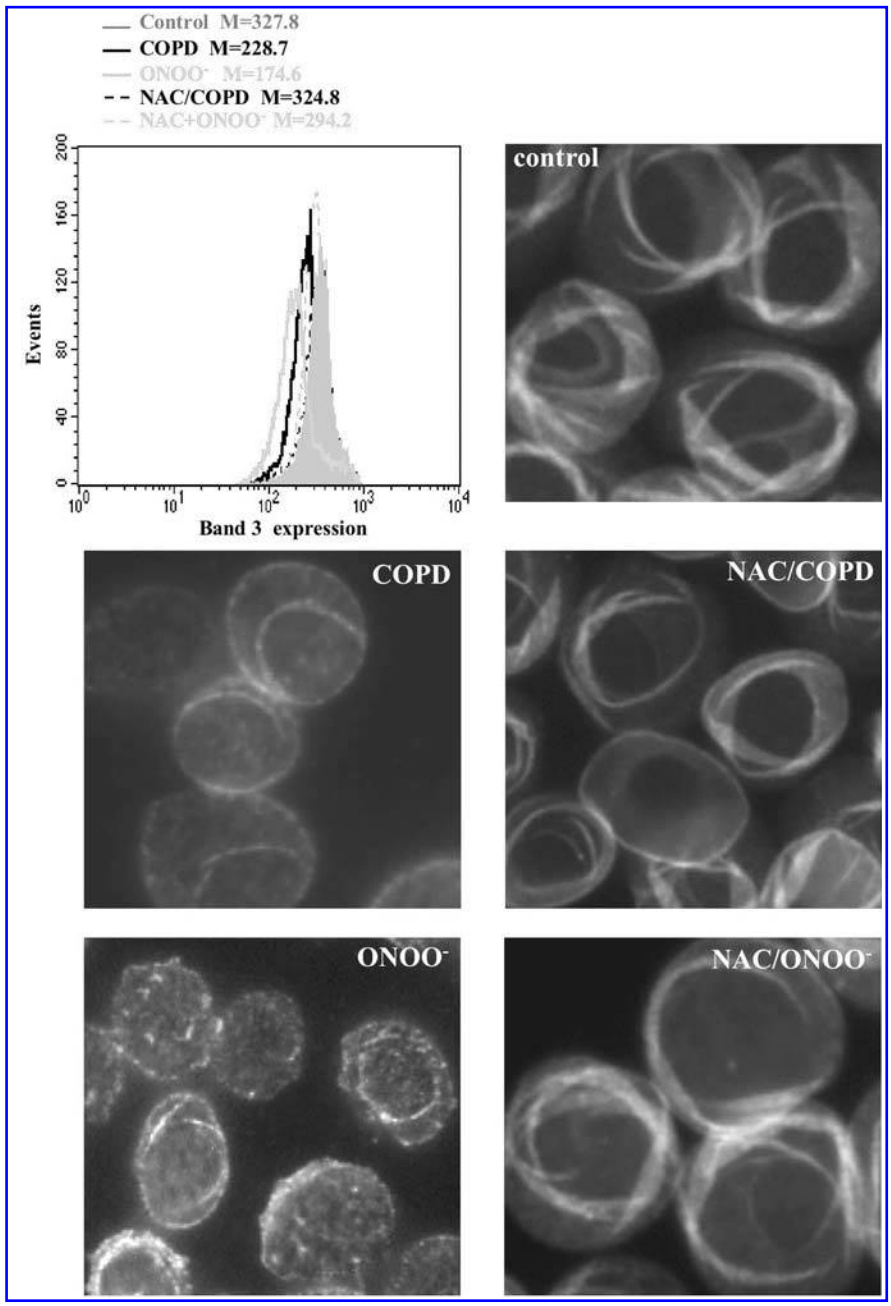

FIG. 3. Band 3 analyses. Band 3 expression levels as detected by a semiquantitative flow-cytometry analysis (left column, first panel). Both in RBCs from chronic obstructive pulmonary disease (COPD) patients (*continuous black line*) and in RBCs exposed to peroxynitrite *in vitro* (*continuous grey line*), a significant ($p < 0.01$) reduction of band 3 expression, with respect to RBCs from healthy donors (*grey histogram*), was detected. *N*-acetylcysteine (NAC) administration, either *in vivo* (*dashed black line*) or *in vitro* (*dashed grey line*) significantly ($p < 0.01$) impaired the downregulation of this protein. Numbers represent the median values of fluorescence intensity histograms. Qualitative immunofluorescence analysis revealed a redistribution of the band 3 in RBCs from COPD patients and in those from healthy donors treated *in vitro* with $ONOO^-$ (compare with control RBCs). This band 3 rearrangement was prevented by NAC treatment, either *in vivo* or *in vitro*. Only one representative experiment of six (for *in vitro* data) and a representative COPD patient (among 40) are shown.

modifications were detected in the Tiffeneau index (data not shown). In the two groups (A and B), we also found a significant ($p < 0.01$) correlation between the FEV_1 improvement and the reduction in percentage of altered RBCs, both *in vivo* and *in vitro*. In consideration of the muscle dysfunction, weakness and discomfort normally reported by patients with COPD, a specific clinical evaluation was carried out by using a dyspnea scale, which allowed us to measure the effects of NAC administration on this important parameter. We found a significant improvement of Borg Scale after 6MWT in both experimental groups, with a starting value of $4 \pm 1 (T_0)$ that was significantly reduced (2 ± 1 at T_1) for both study groups. Finally, a PaO_2 evaluation, of great importance in the present study regarding RBCs, also was carried out. Notably, the starting PaO_2 value was of 62.7 ± 2 mm Hg at T_0. This value was found considerably increased after NAC treatment for 90 days (T_1): pO_2, 68.4 ± 2 mm Hg for group A (+8.3% improvement) and 69.9 ± 2 mm Hg for group B (+10.3%). No clinical side effects were detected during the study. These results are summarized in Table 1.

DISCUSSION

COPD is a leading cause of death and disability and has only recently been studied from a biochemical perspective (5). It has been suggested that ROS/RNS may play a key role in the cellular and molecular mechanisms involved in COPD pathogenesis and evolution (5, 12). In addition, some recent studies point to nitric oxide defects in RBCs as key molecules

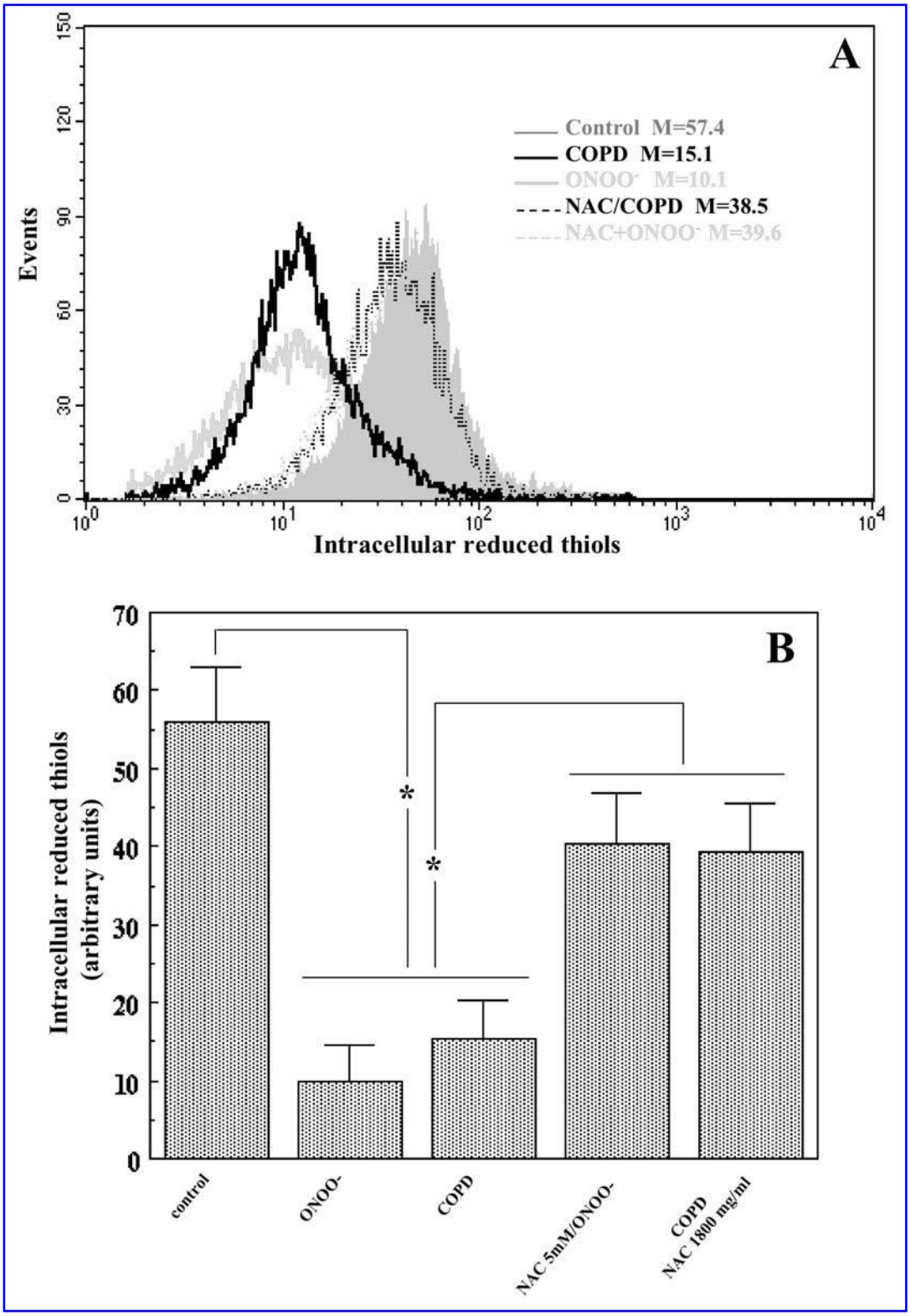

FIG. 4. Intracellular reduced thiols. Semiquantitative evaluation of the intracellular thiol groups performed by flow cytometry indicates that the –SH depletion was either found in red blood cells (RBCs) from chronic obstructive pulmonary disease (COPD) subjects (*continuous black line*) or induced by *in vitro* treatment of RBC from healthy donors with peroxynitrite (*continuous grey line*). This depletion was significantly ($p < 0.01$) counteracted by *N*-acetyl-cysteine (NAC) administration, both *in vivo* (*dashed black line*) and *in vitro* (*dashed grey line*). In (**A**), only one representative experiment of six (for *in vitro* data) and a representative COPD patient (among 40) are shown. Numbers represent the median values of fluorescence intensity histograms. In (**B**), the mean values ± SD obtained in six independent experiments (for *in vitro* data) or analyzing RBCs from 40 COPD patients are shown.

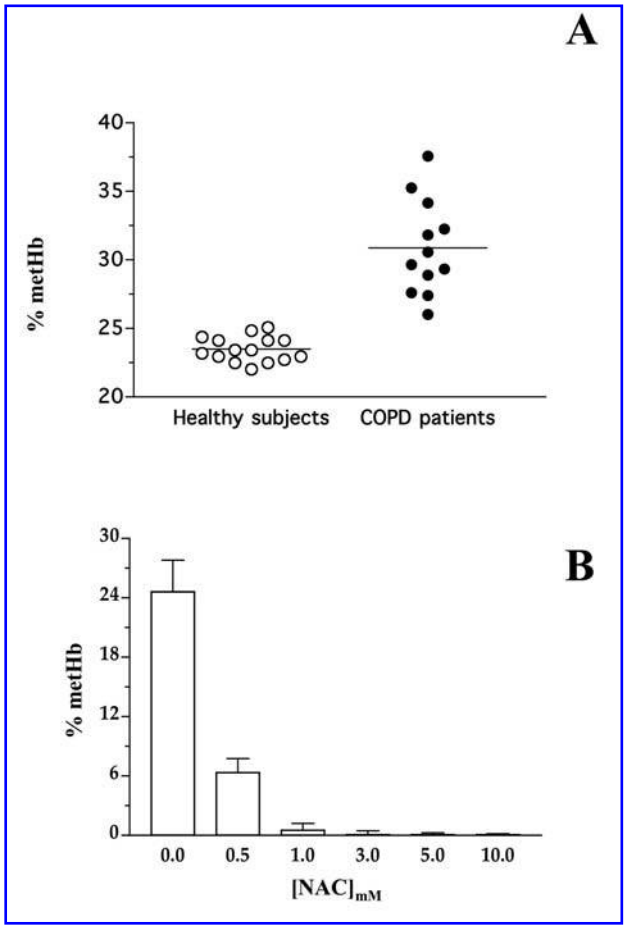

FIG. 5. ONOO⁻-dependent oxidation of RBC hemoglobin. **A:** Comparison of metHb formation induced in red blood cells (RBCs; 0.25% hematocrit, 50 μ*M* as Hb) of healthy subjects and chronic obstructive pulmonary disease (COPD) patients by the treatment with 50 μ*M* $ONOO^-$ (in PBS, pH 7.4, containing 0.1 m*M* DTPA). $ONOO^-$-dependent increase of metHb observed in RBCs from COPD patients was significantly ($p < 0.0001$) higher than that measured in RBCs from healthy donors. **B:** Dose-dependent effects of NAC on metHb formation induced by the treatment with 50 μ*M* $ONOO^-$ of RBCs (0.25% hematocrit, 50 μ*M* as Hb) from healthy subjects. The results represent the means ± SD of three different experiments.

in the assessment of new therapeutic strategies (17, 24). A current hypothesis is that the increased oxidant burden, caused by smoking or by the increase of the ROS/RNS release from airspace leukocytes in chronic inflammations, may not be adequately counterbalanced by the lung antioxidant systems, resulting in oxidative stress (12). An excess of oxidants may then lead to the oxidative tissue injury associated with COPD and, consequently, to a reduced function in alveolar gas exchange. Hence, results herein reported are in line with the idea that COPD could be considered a redox-associated disease and describe for the first time the possible implications of this imbalance for RBC integrity and function. Oxidative modifications result in biophysical and cytologic changes in a significant percentage of erythrocytes (18, 25) that undergo senescence and/or premature apoptosis ("erythroptosis") (23). In turn, this can lead to an impairment of oxygen transport to the periphery and to modifications of cell–cell interactions (*i.e.,* to alterations of delivery functions and to adhesive properties of RBCs, contributing to clot formation) (2). Rheologic changes, together with muscle weakness and clot formation, have been considered key events in the onset and/or in COPD (35).

Several lines of evidence have shown that structural and functional alterations of RBCs induced by ROS/RNS could damage their oxygen-transport capacity (34). The results reported in this work indicate that RBCs from COPD patients underwent modifications of morphologic and functional parameters such as cytoskeleton rearrangement, ultrastructural alterations, and decreased expression of glycophorin A. Altogether these alterations have generally been described as senescence-associated markers and have been hypothesized to modify RBC "plasticity" and deformability that represent key factors for erythrocyte function. Furthermore, RBCs from COPD patients also displayed a lower content of intracellular reduced thiols and an increased susceptibility to the exogenous oxidative stress (13) characterized by increased oxidation of oxyhemoglobin to methemoglobin, and decreased PTP-ase activity after $ONOO^-$ treatment. Importantly, NAC treatments were capable of significantly counteracting these alterations. Although the modification of cytoskeletal–membrane interaction seems to be involved in the morphologic changes found in RBCs from COPD, the possible involvement of membrane lipids is a plausible hypothesis. Further studies are in progress to investigate the contribution of membrane lipids to the observed alterations of RBCs from

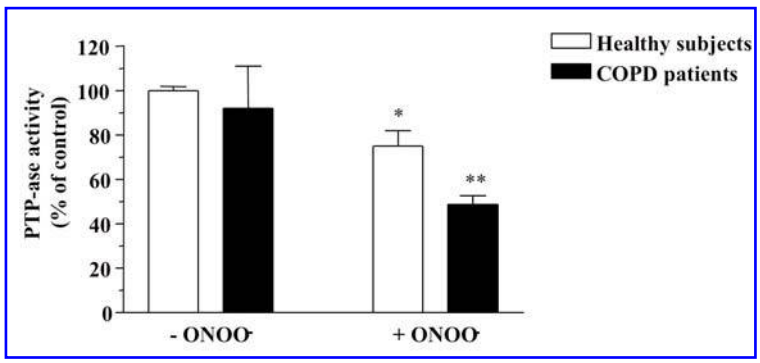

FIG. 6. Phosphotyrosine phosphatase (PTP-ase) activity in red blood cells (RBCs). PTP-ase activity was measured in membranes of RBCs (0.25% hematocrit, 50 μ*M* as Hb) of healthy subjects (*white column*) and chronic obstructive pulmonary disease (COPD) patients (*black columns*) untreated or treated *in vitro* with 50 μ*M* $ONOO^-$. PTP-ase activity was measured by using p-NPP as substrate. PTP-ase activity is expressed as a percentage of the activity measured in control samples. The results represent the mean ± SD of seven samples. *$p < 0.01$ *vs.* untreated control; **$p < 0.001$ *vs.* $ONOO^-$-treated RBCs of healthy patients.

Table 1. Main Clinical Data of COPD Patients

	FEV1		*PO2*		*Borg scale*	
Treatment	*T0*	*T1*	*T0*	*T1*	*T0*	*T1*
Group A (NAC 1,200 mg/day)	72.6±2%	77.2±2%	62.7±2	68.4±2	4±1	2±1
Group B (NAC 1,800 mg/day)		80.1±2%		69.9±2		2±2

Each number represents the mean ± standard deviation (SD) of the measurements obtained from 38 patients with stable chronic obstructive pulmonary disease (COPD). Pred FEV_1%, predicted percentage value of air exhaled during the first second of forced expiration; PO_2: partial O_2 pressure of arterial blood gases; Borg scale, dyspnea points assigned by patients; T_0, baseline values; T_1, values detected after 90 days of -acetyl-cysteine (NAC) administration.

COPD. Taken together, these changes are suggestive of an important role of redox alterations of RBCs in the pathogenetic mechanisms of COPD considered as a "systemic" disease.

In this work, we chose a $ONOO^-$ concentration producing (in control RBCs) a GA downregulation, and surprisingly, we found a similarity between COPD and ONOO-treated RBCs in other apparently unrelated parameters (*i.e.*, morphologic alterations, band 3 expression, and intracellular thiols). It is to be underlined, however, that RBCs from COPD patients did not show neither metHb formation or a significant production of superoxide or hydrogen peroxide (both indicators of Hb autoxidation). In contrast, *in vitro* treatment of RBCs with peroxynitrite induced superoxide, hydrogen peroxide, and a massive, but reversible, formation of metHb (23). At present, these results remain to be clarified. Furthermore, the fact that peroxynitrite is only one of the prooxidant species under COPD inflammatory conditions should be taken into account. We can only speculate that RBCs from stable COPD patients or from ROS/RNS *in vitro*–treated erythrocytes undergo similar oxidative alterations, suggesting that the metabolic machinery related to the maintenance of a correct redox status (*e.g.*, glycolysis and pentose shunt) can be greatly altered. Although erythrocyte hemoglobin is the major target of peroxynitrite, we cannot exclude that membrane lipids also could be modified. Indeed, nitrolipids with cell-signaling activity have been detected in both human plasma and erythrocyte membranes (4) and their involvement in human pathology, such COPD, remains to be established.

It is well known that oxidative stress can lead to the depletion of reduced glutathione (GSH) content and to oxidation of thiol groups of cytoskeletal proteins (that is, of the molecules that oversee cell-shape maintenance) (8). Reduced glutathione is the most important low-molecular-weight antioxidant in the lungs, and its content is reduced after short exposures to cigarette smoke and in severe exacerbation of COPD (12, 28). Fittingly, after peroxynitrite treatment, the intracellular GSH undergoes consistent depletion (3, 23). This can also explain the increased inhibition of PTP-ase activity measured in $ONOO^-$-treated RBCs from COPD patients (*i.e.*, in RBC susceptibility to oxidative stress). Furthermore, the fact that these enzymes contain critical cysteine residues in their active site, and that for full activity, these cysteine residues must be in the reduced form, should also be taken into consideration. Accordingly, we found that NAC can act directly on RBCs and indirectly on lung function–gas exchanges, two crucial causes of hypoxemia that can lead to the muscle dysfunction typical of COPD patients. NAC, acting as a reduced sulfhydryl donor, can possibly restore the RBC redox balance by reestablishing the GSH levels and by exerting a scavenging activity actually worsened in the pathophysiology of COPD (imbalance ox-redox).

In this scenario, NAC can be considered as crucial for the pharmacologic control of lung-function decline in the COPD evolution. It was observed that NAC not only reduces the risk of rehospitalization in COPD patients, thanks to its important mucolytic activity, but, thanks to an enhancement of airway clearance and to a specific effect on bacterial adhesive capacity (14), it can be considered an antiinflammatory drug with a significant antioxidant activity. Results obtained in mild COPD patients with a long-term treatment (3 months) with NAC, 1,200 or 1,800 mg/day, seem to suggest that the increased FEV_1 detected after treatment with NAC could have contributed to the improvement of the PO_2 of arterial blood and, consequently, led to a better oxygenation of peripheral tissues, as shown by a reduction of dyspnea during the walking tests (an indirect clinical evaluation of functional limitation typical of COPD patients) and to a reduction of COPD exacerbations (10, 26). Accordingly, at a subcellular level, NAC treatment induced a powerful protection against modifications induced by ROS/RNS in RBCs from both healthy subjects (*in vitro* studies) and COPD patients also in terms of oxidation of oxyhemoglobin to methemoglobin, of cytoskeleton rearrangement, and of structural alterations. This could indicate that NAC can exert a "protective" activity toward the increased susceptibility to oxidative stress that we found in RBCs from COPD patients.

In conclusion, the findings reported herein clearly indicate that oxidative modifications occurring in RBCs from COPD patients can be valuable in the peripheral blood samples and that these changes can partially be recapitulated by *in vitro* treatments with ROS/RNS. These oxidative changes can be referred to prematurely senescent erythrocytes and can be counteracted by NAC treatments both *in vivo* and *in vitro*. Altogether these considerations underline two possible clinical implications: (a) the possibility that erythrocyte oxidative modifications, playing a role in the pathogenetic mechanisms

of COPD (*e.g.*, in terms RBC rheologic properties), could represent a potential target in the management of the disease; and (b) that RBC alterations, as hypothesized previously by other authors (7, 31), could also represent real-time biosensors in monitoring the progression of the disease.

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ABBREVIATIONS

BSO, L-buthionine-[*S,R*]-sulfoximine; CM-H_2DCFDA, 5-chloromethyl-2',7'-dichloro-dihydrofluoresceindiacetate; COPD, chronic obstructive pulmonary disease; DHE, dihydroethidium; DHR, dihydrorhodamine; DTPA, diethylenetriaminepentaacetic acid; FEV_1, volume of air exhaled during the first second of forced expiration; FVC, forced vital capacity; GA, glycophorin A; GSH, reduced glutathione; Hb, hemoglobin; HBSS, Hanks' balanced salt solution; IVM, intensified charge-coupled device video microscopy; MetHb, methemoglobin; 6MWT, 6-min walking test; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; $ONOO^-$, peroxynitrite; ONOOH, acid hydrogen oxoperoxynitrate; $PaCO_2$, partial CO_2 pressure of arterial blood gases; PaO_2, partial O_2 pressure of arterial blood gases; PFT, pulmonary function test; p-NPP, *p*-nitrophenyl phosphate; PTP-ase, phosphotyrosine phosphatase; RBC, red blood cell; RNS, reactive nitrogen species; ROS, reactive oxygen species; SEM, scanning electron microscopy.

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