Neutrophil Activation in Severe, Early-Onset COPD Patients versus Healthy Non-Smoker Subjects in vitro: Effects of Antioxidant Therapy

Javier Milara^{a, c} Gustavo Juan^{b, d} Teresa Peiró^a Adela Serrano^a
Julio Cortijo^{a, c, e}

^aResearch Unit and ^bRespiratory Unit, University General Hospital Consortium, ^cCIBERES, Health Institute Carlos III, and Departments of ^dMedicine and ^ePharmacology, Faculty of Medicine, University of Valencia, Valencia, Spain

For editorial comment see p. 103

Key Words

Neutrophil · Early-onset COPD · N-Acetyl-L-cysteine · Antioxidant · Inflammation

Abstract

Background: Neutrophils and oxidative stress have been implicated in the pathogenesis of COPD. Severe, early-onset COPD is characterized by a rapid decline in the lung function at an early age; however, nothing is known about neutrophil activation in COPD patients. **Objectives:** The aim of this study was to evaluate peripheral blood neutrophil activation in severe, early-onset COPD patients versus healthy non-smokers and the effect of N-acetyl-L-cysteine (NAC) on neutrophil activation in vitro. **Methods:** Neutrophils were isolated from 15 severe, early-onset COPD patients and 15 age-matched healthy subjects and stimulated with N-formyl-Met-Leu-Phe (fMLP) in the presence or absence of NAC (10 μ M to 10 mM). Neutrophil chemotaxis, elastase release, reactive oxygen species (ROS), intracellular thiols and apoptosis were measured by Boyden chamber, spectrofluorometry, CMFDA and H₂DCF-DA dyes and by annexin V-FITC, respectively. **Results:** Chemotaxis of peripheral blood neutrophils from COPD patients in response to fMLP was 30% more increased than that observed in healthy subjects. Elastase release in response to fMLP was 2-fold higher in neutrophils

from COPD patients versus healthy subjects. Intracellular thiol levels were 30% lower in COPD and ROS was approximately 30% higher in COPD versus healthy neutrophils. Spontaneous apoptosis showed no differences in both groups of patients and fMLP-induced apoptosis was higher in COPD. Pre-treatment with the antioxidant NAC effectively attenuated neutrophil chemotaxis, elastase release and ROS as well as effectively increased thiol levels in COPD. **Conclusions:** Neutrophils in severe, early-onset COPD patients are highly activated and this is alleviated by NAC in vitro.

Copyright © 2011 S. Karger AG, Basel

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by airway and systemic inflammation, which develops in an airway obstruction not completely reversible.

Neutrophils play a crucial role in COPD pathogenesis and are considered the primary effector cells involved in this inflammatory process [1].

In COPD, peripheral blood neutrophils are activated by different inflammatory mediators, and this essential step precedes their extravasation to the lung tissue [2]. Thus, activated neutrophils migrate to lung tissue where

they release a number of proteases such as elastase and matrix metalloproteases contributing to emphysema formation, and pro-inflammatory mediators such as IL-1 β , IL-8 and TNF- α contributing to lung inflammation and inflammatory cell recruitment to the lungs [3]. It is also considered an important feature of neutrophil activation, the neutrophil apoptotic resistance, since this phenomenon prolongs the inflammatory action of neutrophils perpetuating lung injury [4]. Oxidative stress burden is a common feature of COPD that contributes to the progression of this disease. This oxidative stress promotes activation of circulating neutrophils, which is translated in the secretion of several pro-inflammatory mediators [5]. Nowadays, there is a clear evidence of the existence of different COPD phenotypes. However, genetic and cellular characteristics of each type of COPD are poorly understood. One of the most severe phenotypes is described as severe, early-onset COPD phenotype which is characterized by a rapid decline in lung function at an early age and is typically represented by genetic α_1 -antitrypsin deficiency [6]. However, severe, early-onset COPD has also been observed in patients without genetic α_1 -antitrypsin deficiency which is in agreement with the polygenetic nature of this disease [6–10]. According to the COPD pathogenic theory of the oxidant-antioxidant imbalance, rapid FEV₁ decline in severe, early-onset COPD patients was recently related with genetic alterations of the glutathione S-transferase (GST) supergene family [8] which appears to be critical in protection against oxidative stress generated by cigarette smoke. Thus, it seems that early-onset COPD patients are more sensitive to oxidative stress than other phenotypes. However, to our knowledge, cellular characteristics of this type of COPD patients have not been described.

Increasing evidence closely relates inflammatory processes in COPD with oxidative stress [11]. As oxidative stress is highly related with the progression and consequently with the exacerbations of this disease, the use of antioxidants is likely to have a beneficial outcome [12]. The antioxidant agent N-acetyl-L-cysteine (NAC) inhibits oxidative stress changing the cellular redox status and acting as a direct reactive oxygen species (ROS) scavenger. In fact, NAC can prevent the excessive expression of inflammatory cytokines and chemokines by mitigating the stimulation of NF- κ B by ROS and cytokines [13, 14]. NAC has a substantial antioxidant activity and thus can maintain thiol groups in the reduced state, as demonstrated in previous *in vitro* experiments [15]. Furthermore, NAC has been shown to reduce the frequency of exacerbations and could have a role in the treatment of

patients with recurrent exacerbations [16]. However, the efficacy of the antioxidant treatment may vary depending on the COPD phenotype [5], so it would be important to study the effect of NAC in severe, early-onset COPD patients.

In this study, we have addressed neutrophil activation status in peripheral blood neutrophils from early-onset COPD patients versus non-smoker patients as this might contribute to the worsening of the disease. Since oxidative stress is up-regulated in COPD, we also studied how the antioxidant treatment could modulate neutrophil activation.

Subjects and Methods

Subjects

Severe, early-onset COPD patients and age-matched, healthy, non-smoker volunteers were enrolled. The inclusion criteria for COPD patients were: age <56 years, past smokers with a smoking history of >30 pack-years, FEV₁ <30% predicted, FEV₁/FVC <70%, rapid decliners (defined as change in FEV₁ <-160 $\pm$ 5 ml/year over a period of 5 years) and no evidence of α_1 -antitrypsin deficiency. Study was started after 5 years of follow-up of FEV₁ (<-160 $\pm$ 5 ml/year). Patients were all not current smokers (no smokers 2 years before the start of the study), exhibited serious COPD at an early age and did not show exacerbations at the moment of sample collection. None of the patients exhibited clinical evidence of viral or parasite infection, bronchial asthma, allergic rhinitis, pneumonia or lung cancer as well as other co-morbidity such as diabetes or cardiovascular diseases. All were clinically stable, as defined by the absence of any change in their regular treatment and/or need to seek medical attention during the previous three months. All patients were treated with inhaled β_2 -agonists, anticholinergic bronchodilators and inhaled steroids. None of them were current smokers or received antibiotics or oral steroids in the last three months. All healthy subjects had a normal lung function and were never smokers and did not show any comorbidity. The protocol of the study conformed to the Declaration of Helsinki and was approved by the local research and independent ethics committee of the University General Hospital of Valencia, Spain, with the approval No. 2692. Informed written consent was obtained from each participant.

The clinical patients' characteristics are summarized in table 1. Pulmonary function tests were performed prior to sample collection and FEV₁ and FVC values correspond to serious COPD of degree 4, according to GOLD criteria [17].

Human Neutrophil Isolation

Neutrophils were isolated from peripheral venous blood by standard laboratory procedures [18]. In brief, peripheral venous blood was mixed with dextran 500 at 3% (in 0.9% saline) in a proportion of 2:1. This mixture was incubated at room temperature for 30 min until erythrocytes were sedimented. The upper phase was carefully collected and added on Ficoll-Paque Histopaque 1077 (Amershan Pharmacia Biotech) density gradient in a proportion of 3:1. The two phases generated were centrifuged at 150 g,

Table 1. Subject clinical features

	COPD patients	Non-smokers	p value
Number	15	15	
Sex, female/male	1/14	3/12	
Age, years	50 ± 5	53 ± 5	0.1115
Tobacco consumption, pack-years	48.4 ± 8.3	0	
Current smokers	0	0	
Ex-smokers	15	0	
FEV ₁ , % predicted	26.8 ± 5.2	98.6 ± 4.3	<0.0001*
FVC, % predicted	65.3 ± 7.8	99.3 ± 5.4	0.0442*
FEV ₁ /FVC	43.3 ± 4.5	86.4 ± 4.5	<0.0001*
Total peripheral leukocytes	16.6 ± 1.3 × 10 ⁹ /l	6.4 ± 0.4 × 10 ⁹ /l	<0.0001*
Total peripheral neutrophils	12.2 ± 0.5 × 10 ⁹ /l	3.7 ± 0.6 × 10 ⁹ /l	<0.0001*
Total peripheral lymphocytes	1.9 ± 0.4 × 10 ⁹ /l	2.2 ± 0.5 × 10 ⁹ /l	0.0494*
Total peripheral blood monocytes	1.8 ± 0.1 × 10 ⁹ /l	0.4 ± 0.4 × 10 ⁹ /l	0.0008*
Total peripheral blood eosinophils	0.6 ± 1.3 × 10 ⁹ /l	0.1 ± 0.4 × 10 ⁹ /l	0.1656

* Significant values of COPD patients versus non-smokers.

4°C for 30 min. Then, supernatant was eliminated and the pellet obtained (which consisted a mixture of neutrophils and low proportion of residual erythrocytes and traces of eosinophils and basophils) was resuspended in an erythrocyte lysis buffer (Biolegend) for 5 min in ice. Cell suspension was washed two times with phosphate-buffered saline (PBS). The preparations were >97% pure in neutrophils, as assessed by Giemsa staining, and had a viability of >99%, measured by Trypan blue exclusion. Neither purity nor viability were affected in the study's different experimental conditions.

Neutrophil Chemotaxis

Neutrophil migration was measured with the Boyden chamber technique as previously outlined [19]. In brief, neutrophils (0.5 × 10⁶ cells/ml) were placed in the upper compartment of the chamber, previously incubated for 15 min in the absence or presence of NAC (Sigma) 0.5 mM. The chemotactic factor N-formyl-Met-Leu-Phe (fMLP; Sigma) 10 nM was placed in the lower compartment, followed by 30 min of incubation at 37°C. After migration, the filters were fixed and stained with Diff Quick (Baxter Diagnostics AG) and the distance (μm) travelled into the filter was determined according to the leading front technique by microscopy examination. In each chemotactic assay, the migration distance was determined at five different filter sites.

Elastase Release

Elastase release was measured with a spectrofluorometric method, as previously described [18]. In brief, neutrophils (2 × 10⁶ neutrophils/ml) were suspended in a buffer (composition in mM: NaCl 147, KCl 5, CaCl₂ 1.5, MgSO₄ 0.3, MgCl₂ 1, KH₂PO₄ 1.9, Na₂HPO₄ 1.4, glucose 5.5; pH 7.4) containing MeO-Suc-Ala-Ala-Pro-Val-MCA (substrate, 20 μmol/l) and cytochalasin B (5 μg/ml; Sigma). The cell suspension was incubated in either vehicle, NAC (0.5 mM, 1 mM) or dextran sulphate (DS, 60 μg/ml; Sigma) as a positive control for 15 min at 37°C before addition of stimulus. Then, either vehicle or fMLP (30 nM; approx. EC₅₀ according to

previous experiments [20]) was added. The initial rate of signal increase was determined and the enzyme rate was calculated using methylcoumarin amide as a standard. The enzyme rate is a reflection of the amount of elastase released from neutrophils.

Intracellular Thiol and ROS Determination

Intracellular thiol levels were measured by a flow cytometry assay using chloromethyl fluorescein diacetate (CMFDA; Molecular Probes) as previously described [21]. Intracellular ROS were measured by a flow cytometry assay using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA; Molecular Probes) as previously outlined [22]. In brief, neutrophils (1 × 10⁶ cells/ml) were suspended in PBS, 1% fetal calf serum (FCS) with HEPES (10 mM) and were incubated with NAC (10 μM to 10 mM) or with vehicle (PBS/FCS/HEPES) during 15 min at 37°C. CMFDA was added for 15 min before stimulus to a final concentration of 5 μM to measure intracellular thiols. In other experiments, H₂DCF-DA was added for 15 min before stimulus to a final concentration of 5 μM to measure intracellular ROS. The thiol alkylating agent N-ethylmaleimide (NEM; Sigma) 10 μM was added as stimulus after pharmacological treatments to reduce intracellular thiol levels. The bacterial peptide fMLP was added as stimulus at 1 μM for 30 min to generate intracellular ROS [23]. Cells were washed (PBS/FCS/HEPES), centrifuged (300 g) and fixed with paraformaldehyde 1%. Flow-cytometric analysis was performed using an Epics Profile II flow cytometer (Coulter Electronics).

Since neutrophil isolation procedure may alter its properties, we measured same thiol levels and intracellular ROS using fresh whole blood from healthy and COPD patients. Blood neutrophils were selected using CD16-Alexa Fluor 647 antibody (Molecular Probes) as neutrophil marker in an Epics Profile II flow cytometer. A total of 1 ml of whole blood was pre-incubated at 37°C for 30 min with NAC (10 μM to 10 mM) and CMFDA 5 μM (in case of thiol measurement) or H₂DCF-DA (in case of ROS measurement), followed by NEM (in case of thiols) or fMLP (in case of ROS) stimulation for another 30 min. Then, erythrocytes were

Fig. 1. fMLP-induced neutrophil chemotaxis is increased in severe, early-onset COPD patients and prevented by NAC. Peripheral blood neutrophils were isolated from severe, early-onset COPD and healthy subjects. **a** The chemoattractant fMLP (10 nM) significantly increased neutrophil chemotaxis in COPD versus healthy control. Pre-incubation with the antioxidant NAC attenuated the fMLP-induced neutrophil chemotaxis only at 10 mM in

neutrophils from healthy subjects (**b**), while in neutrophils from early-onset COPD patients NAC significantly reduced neutrophil chemotaxis from 100 μM to 10 mM (**c**). Data are means ± SEM of neutrophils from 7 different patients. * $p < 0.05$ vs. control; + $p < 0.05$ vs. fMLP.

lysed using a commercial erythrocyte lysis buffer (Biolegend) for 10 min. Fluorescent intensity was measured in the polymorphonuclear region of whole blood in an Epics Profile II flow cytometer.

Spontaneous and Induced Neutrophil-Apoptosis

Assessment of apoptosis was performed by flow cytometry using annexin V-FITC. The protocol indicated by the manufacturer (annexin-V-FITC; Roche Applied Science) was used as previously outlined [19]. Neutrophils (0.01×10^6) cultured in a 96-well plate in the absence or presence of NAC (10 μM to 10 mM) for 24 h at 37°C and 5% CO₂. Spontaneous apoptosis was evaluated by addition of 5 μM annexin V-FITC and analyzed in a Beckman Coulter Epics XL. Results were expressed as percent apoptotic cells versus non-incubated cells. In other experiments isolated neutrophils were also incubated with NAC (10 μM to 10 mM) for 30 min and stimulated with fMLP 100 nM for 12 h as previously outlined [24]. Then, annexin-V-FITC fluorescence intensity was measured in a Beckman Coulter Epics XL. Results were expressed as percent apoptotic cells versus non-stimulated cells.

Since neutrophil isolation procedure may alter its properties, we measured the same annexin-V-FITC using fresh whole blood from healthy and COPD patients. Blood neutrophils were selected using CD16-Alexa Fluor 647 antibody (Molecular Probes) by flow cytometry. A total of 1 ml of whole blood was incubated for 24 h at 37°C and 5% CO₂, with NAC (10 μM to 10 mM). Then, erythrocytes were lysed as describe above, and annexin-V-FITC 5 μM was added for 10 min. In other experiments whole blood was pre-incubated with NAC for 30 min followed by the stimulation with fMLP for 12 h at 37°C and 5% CO₂. Then, erythrocytes were lysed,

annexin-V-FITC 5 μM was added for 10 min and green fluorescent intensity was monitored in neutrophil region of the whole blood using a Beckman Coulter Epics XL.

IL-8 Measurement

Neutrophils (2×10^6 ml⁻¹) were cultured in the absence or presence of fMLP (100 nM) for 8 h at 37°C with 5% CO₂ in the absence or presence of NAC (10 μM to 10 mM) added from 1 h before the stimulus with fMLP until the end of the experiment. IL-8 was selected as important in the recruitment of neutrophils. The release of IL-8 into cell supernatant was measured by enzyme-linked immunosorbent assay following the indications of the manufacturer (Dialclone).

Statistical Analysis

Data are expressed as means ± SEM of n experiments. Statistical analysis of results was carried out by non parametric Mann-Whitney U test or Kruskal-Wallis test as appropriate, followed by appropriate post hoc analysis (Graph-Pad Software Inc.). Significance was accepted when $p < 0.05$.

Results

Increased fMLP-Induced Neutrophil Chemotaxis Is Reduced by NAC in Early-Onset COPD Patients

In experiments examining chemotaxis, migration distance amounted to 23.6 ± 5.4 μm in healthy subjects and

Fig. 2. fMLP-induced neutrophil elastase release is increased in severe, early-onset COPD patients versus healthy, non-smoker subjects. **b, c** Pre-incubation with the antioxidant NAC only reduced the fMLP-induced elastase release at 10 mM concentration in healthy subjects (**b**) and COPD patients (**c**), whilst the inhibitor-positive control DS effectively suppressed elastase release in both groups. Data are means $\pm$ SEM of neutrophils from 4–8 different patients. * $p < 0.05$ vs. healthy subjects; $^+ p < 0.05$ vs. control.

COPD in comparison to neutrophils from healthy, non-smoker subjects. **b, c** Pre-incubation with the antioxidant NAC only reduced the fMLP-induced elastase release at 10 mM concentration in healthy subjects (**b**) and COPD patients (**c**), whilst the inhibitor-positive control DS effectively suppressed elastase release in both groups. Data are means $\pm$ SEM of neutrophils from 4–8 different patients. * $p < 0.05$ vs. healthy subjects; $^+ p < 0.05$ vs. control.

$30.6 \pm 6.3 \mu\text{m}$ in early-onset COPD patients (fig. 1a). After stimulation with fMLP (10 nM), the distance was $69.6 \pm 8.3 \mu\text{m}$ in healthy subjects and $100.4 \pm 17.3 \mu\text{m}$ in COPD patients (fig. 1a; $p < 0.05$). NAC significantly reduced the fMLP-induced neutrophil chemotaxis only at 10 mM (fig. 1b; $p < 0.05$). However, in neutrophils from early-onset COPD patients, NAC significantly reduced neutrophil chemotaxis at 100 μM to 10 mM (fig. 1c; $p < 0.05$).

Neutrophils from Early-Onset COPD Patients Show a Significant Increase in fMLP-Induced Elastase Release Compared to Neutrophils from Healthy Subjects

Elastase release after stimulation with fMLP (30 nM) was 177 ± 33 ng elastase/10⁶ cells in healthy subjects and 361.8 ± 110 ng elastase/10⁶ cells in COPD patients reflecting a significant increase in elastase release from COPD patients (fig. 2a; $p < 0.05$). However, no difference was observed in elastase basal release between healthy and COPD patients after 15 min of incubation (fig. 2a). Pretreatment with NAC only attenuates elastase release at 10 mM concentration in both healthy subjects (127.7 ± 25.7 ng elastase/10⁶ cells; fig. 2b; $p < 0.05$ vs. fMLP) and COPD patients (276.9 ± 31.2 ng elastase/10⁶ cells; fig. 2b;

$p < 0.05$ vs. fMLP). The positive control DS effectively suppressed elastase release in both groups.

Neutrophils from Early-Onset COPD Patients Show Decreased Thiol Levels and Increased ROS That Are Partially Reversed by NAC

Basal thiol levels in isolated neutrophils from COPD patients were 68.7 ± 16.4 significantly lower than those observed for healthy subjects (98.1 ± 14.2 ; fig. 3a; $p < 0.05$). Decreased basal thiol levels, which imply higher oxidative stress, were not influenced by 500 μM and 1 mM NAC incubation (data not shown). In contrast, the negative thiol regulator NEM (10 μM) decreased thiol levels that were partially prevented by NAC (100 μM to 10 mM) in COPD patients and totally suppressed in healthy subjects (fig. 3a). Basal levels of intracellular ROS in isolated neutrophils from healthy subjects were 103 ± 17.3 , significantly lower than in COPD patients (168 ± 17.3 ; fig. 3b; $p < 0.05$ vs. basal healthy levels). fMLP increased intracellular ROS in neutrophils from healthy subjects to 201 ± 12 (fig. 3b) and in COPD patients (298 ± 31 ; fig. 3b; $p < 0.05$ vs. fMLP condition in healthy subjects). NAC exposure dose-dependently decreased fMLP-induced ROS which was significant at

Fig. 3. Oxidative stress burden is increase in peripheral neutrophils from severe, early-onset COPD patients. Peripheral blood neutrophils were isolated from severe, early-onset COPD and healthy subjects, and intracellular thiol and ROS levels were measured by means of fluorescence CMFDA dye and H_2DCF -DA dye, respectively, using flow cytometry. **a** Intracellular thiol levels were found decreased in neutrophils from COPD patients versus healthy subjects. Pre-incubation with the antioxidant NAC partially suppressed the NEM-induced thiol down-regulation in COPD patients and totally suppressed in healthy donors. **b** Intra-

cellular ROS were found increased in neutrophils from COPD patients versus healthy subjects. Pre-incubation with NAC dose-dependently decreased the fMLP-induced ROS generation in both COPD and healthy subjects. **c, d** Experiments were reproduced in whole peripheral blood using CD-16 Alexa 645 to identify the neutrophil population in whole blood by flow cytometry. Data are the mean $\pm$ SEM of neutrophils from 4–6 different patients. $^{\circ}$ $p < 0.05$ vs. healthy subjects; * $p < 0.05$ vs. control; $^+$ $p < 0.05$ vs. stimulus NEM or fMLP.

1 mM in healthy neutrophils and at 500 μ M in COPD patients (fig. 3b).

Since the isolation process of neutrophils may change some of the properties of neutrophils, we performed parallel experiments on whole blood, selecting neutrophil population by means of CD16 antibody in a flow cytometer. Results observed for intracellular thiols and ROS were similar to that found in isolated neutrophils (fig. 3c, d).

Neutrophil Apoptosis from Early-Onset COPD Patients and Healthy Subjects

The spontaneous survival of neutrophils in culture suffered a time-dependent decay during the 24-hour observation period. Differences were not observed in isolated neutrophil apoptosis from healthy subjects and COPD patients (fig. 4a–c) as well as in neutrophils from whole blood (fig. 4d–f). Additionally, NAC had no influence on neutrophil survival in the two study populations,

Fig. 4. Neutrophil spontaneous apoptosis is not modified in severe, early-onset COPD patients. Peripheral blood neutrophils were isolated from severe, early-onset COPD and healthy subjects and plated in a 96-well plate in presence or absence of NAC. Neutrophil apoptosis was measured by the annexin V-FITC fluorescence by means of a flux cytometer. **a** Neutrophil spontaneous apoptosis

did not vary between COPD and healthy donors. NAC pre-incubation was unable to reduce spontaneous apoptosis in both healthy subjects (**b**) and COPD (**c**). **d-f** The experiments were reproduced in whole peripheral blood using CD-16 Alexa 645 to identify the neutrophil population in whole blood by flow cytometry. Data are means $\pm$ SEM of neutrophils from 4–7 different patients.

in both isolated neutrophils and neutrophils from whole blood (fig. 4b, c, e, f).

Isolated neutrophils stimulated with fMLP (100 nM) for 12 h increased apoptosis 1.8-fold over control in healthy subjects and 1.3-fold over control in COPD patients, suggesting an apoptotic resistance in neutrophils from COPD patients (fig. 5a; $p < 0.05$ vs. healthy subjects). NAC pre-incubation only significantly decreases apoptosis at 1 and 10 mM in healthy subjects but not in COPD patients (fig. 5a). The same experiments were conducted in whole blood, where we observed similar results (fig. 5b).

Neutrophils from Early-Onset COPD Patients Show a Higher Increase in IL-8 Release than Those from Healthy Subjects

Basal IL-8 release in neutrophils from healthy subjects was 613.7 ± 126.2 pg/ml, significantly lower than in neu-

trophils from COPD patients ($1,141.7 \pm 271.7$ pg/ml; fig. 6; $p < 0.05$ vs. basal condition in healthy subjects). fMLP 100 nM stimulation for 8 h significantly increased IL-8 release, although that increase was significantly lower in healthy subjects ($1,604.3 \pm 213.7$ pg/ml) than in COPD patients ($4,091.8 \pm 572.9$ pg/ml; fig. 6; $p < 0.05$ vs. fMLP condition in healthy subjects). NAC dose-dependently decreased the IL-8 secretion induced by fMLP, reaching statistical significance at 1 mM in COPD patients and 10 mM in healthy subjects (fig. 6).

Discussion

This is the first study which evaluates peripheral blood neutrophil activation status in severe, early-onset COPD patients and how an antioxidant treatment may

Fig. 5. Neutrophils from early-onset patients show an increased resistance to fMLP-induced apoptosis. Peripheral blood neutrophils were isolated from severe, early-onset COPD and healthy subjects and plated in a 96-well plate in presence or absence of NAC. Neutrophil apoptosis was measured by the annexin V-FITC fluorescence by means of a flux cytometer. **a** fMLP increased percent of apoptotic neutrophils during 12 h of incubation being significantly higher in neutrophils from healthy

subjects than from COPD patients. NAC incubation dose-dependently reduced the fMLP-induced neutrophil apoptosis in healthy subjects but not in COPD patients. **b** Experiments were reproduced in whole peripheral blood using CD-16 Alexa 645 to identify the neutrophil population in whole blood by flow cytometry. Data are means $\pm$ SEM of neutrophils from 4 different patients. ° $p < 0.05$ vs. healthy subjects; * $p < 0.05$ vs. control; + $p < 0.05$ vs. stimulus fMLP.

Fig. 6. Neutrophils from early-onset COPD patients show an enhanced fMLP response to IL-8 release. Peripheral blood neutrophils were isolated from severe, early-onset COPD and healthy subjects and plated in a 96-well plate in presence or absence of NAC. In COPD patients, the fMLP-induced IL-8 release was significantly higher than in healthy subjects. NAC dose-dependently attenuated IL-8 release in COPD patients and in a lesser extent in healthy subjects. Data are means $\pm$ SEM of neutrophils from 4 different patients. ° $p < 0.05$ vs. healthy subjects; * $p < 0.05$ vs. control; + $p < 0.05$ vs. stimulus fMLP.

reduce those markers related with neutrophil activation in vitro.

In this study, we found an increased neutrophil chemotaxis secondary to fMLP in severe, early-onset COPD patients versus healthy subjects, which is in agreement with previous observations [25]. However, recently, another study has found a decreased chemotactic response in isolated peripheral blood neutrophils from patients with moderate to severe COPD, with this decrease being related to disease severity [26]. These authors concluded that chemotaxis is decreased in isolated neutrophils from COPD patients versus healthy subjects and that the increased number of neutrophils in the airways of COPD patients may be related to the increase in neutrophil chemotactic factors in the lungs such as IL-8 or by the increase in adhesion molecules expression on pulmonary endothelial cells which is present in COPD patients [26–28]. In this work, we selected the Boyden chamber technique to measure neutrophil chemotaxis, since this is a well-experimented technique known for long by our group and others [18, 19, 29], and do not need to introduce nor manipulate cells with fluorescent dye incubations such as fluorescence calcein [26] which could modify neutrophil properties. In this line, there are few works

that have studied peripheral blood chemotaxis in COPD patients versus healthy subjects, so discrepancy between previous observations and results shown in this study may be explained by different methods used to quantify chemotaxis and by differences in COPD patients, as the severe, early-onset patients in our study present a more advanced disease and therefore a possibly different neutrophil activation status.

Neutrophil accumulation in the airways could also be a result of a prolonged survival. Apoptosis is the major mechanism controlling the longevity of neutrophils at inflamed sites [30]. Furthermore, neutrophilic inflammation is resolved by neutrophil apoptosis followed by their removal by macrophages, helping to minimize and prevent damage. However, increased levels of inflammatory chemokines and cytokines delay neutrophil apoptosis [31] and, in addition, the chronic inflammatory environment in the airway might inhibit the normal recognition mechanism whereby macrophages engulf apoptotic neutrophils [32]. Recent studies have shown that apoptosis is decreased in peripheral venous neutrophils from COPD patients during their exacerbations [33], while neutrophil apoptosis from COPD patients during its stable phase does not show differences versus neutrophils from healthy subjects [34]. In this work, early-onset COPD patients studied were in their stable phase, and we did not observe statistical differences in spontaneous apoptosis of isolated neutrophil versus healthy subjects, which is in line with previous studies on COPD [34]. However, a decreased rate (although not significant) of spontaneous apoptosis in neutrophils of whole blood from early-onset COPD was observed (fig. 4d), which may suggest that in these patients, circulating neutrophils may show some apoptotic resistance. In contrast, neutrophil apoptosis induced by fMLP was significantly higher in healthy patients than in early-onset COPD patients, suggesting that neutrophils from early-onset COPD patients are resistant to neutrophil apoptosis under inflammatory conditions relevant to exacerbations, which is in accordance with previous studies [33].

There is accumulating evidence of systemic oxidative stress in patients with COPD. In this respect, an increased superoxide anion production in peripheral blood neutrophils obtained from patients with acute exacerbations of COPD has been shown previously [35]. There is also good evidence of oxidative stress reaching the circulation, since plasma antioxidant capacity is impaired during COPD exacerbations [36]. Epidemiological studies have shown a correlation between circulating neutrophil number and FEV₁ as well as with the oxidative stress mea-

sured as thiobarbituric acid-reactive substance in plasma [37]. In this work, severe, early-onset COPD patients showed an increase of peripheral neutrophil number in their clinical stable situation which may be a specific characteristic of the severity of these types of patients. In this sense, our results show a reduced intracellular thiol level and an increase in intracellular ROS in peripheral blood neutrophils from patients with severe, early-onset COPD which demonstrate a systemic increase of the oxidative stress, even in a clinically stable situation.

The role of neutrophil elastase in emphysema has been a cornerstone of the protease-antiprotease hypothesis. Neutrophil elastase has also a dramatic effect on both neutrophil and macrophage accumulation in the lungs in response to cigarette smoke [38]. In this work, we have found that neutrophils from severe, early-onset COPD patients release higher amounts of elastase in response to fMLP stimulus than those neutrophils from healthy subjects. These results agree with previous observations, where neutrophils from patients with emphysema generated a significantly larger amount of ROS and neutrophil elastase compared to healthy donors [39]. However, we did not observe differences on the basal elastase release in both healthy and severe early-onset COPD neutrophils, which suggests that differences of the elastase release are only expected under inflammatory conditions.

Since severe, early-onset COPD patients seem to have an oxidant/antioxidant imbalance, an optimal antioxidant therapy could attenuate the rapid FEV₁ decline observed in this type of patients. In this regard, the antioxidant compound most assayed in *in vitro* and *in vivo* studies is NAC. The cysteine-donating compound NAC acts as a cellular precursor of glutathione and becomes deacetylated in the gut to cysteine after oral administration. Although the *in vitro* effects of NAC on cellular outcomes relative to COPD have been attracted a broad attention [40], human *in vivo* studies are limited. Thus, the utility of NAC in the COPD treatment seems to influence exacerbation rate (20–30% of reduction) and limit the number of hospitalization days; however, little or no influence has been observed on the lung function parameters [41–44]. The reason for the variability of the current clinical studies using NAC may be due to the low oral bioavailability (6–10%) and the low dose assayed in different clinical trials, which supports the idea that higher doses or modifications of the molecule could improve NAC bioavailability and efficacy [5]. In this respect, it seems that oral NAC at 1,200 mg/day corresponding to approximately 9 μ M serum levels has antioxidant and anti-inflammatory properties in COPD patients [45].

In this study, we chose in vitro effective doses of NAC (10 μ M to 10 mM), trying to reproduce the oral effect of NAC in severe, early-onset COPD patients. We showed that NAC (100 μ M to 10 mM) significantly prevents the increase in neutrophil chemotaxis secondary to fMLP in severe, early-onset COPD patients whilst only showing weak inhibitory effect (at 10 mM) in healthy subjects. Since neutrophils from severe, early-onset COPD patients show an increase in intracellular ROS (fig. 3b, c), the superior effect of NAC inhibiting neutrophil chemotaxis from COPD patients suggests that oxidative stress burden in neutrophils is an important factor governing neutrophil chemotaxis [46], and that the reduction in intracellular ROS could be a good strategy to attenuate neutrophil lung extravasations in vivo. In this regard, NAC fully suppresses the NEM-induced thiol down-regulation and fMLP-induced ROS generation in healthy subjects as well as in severe, early-onset COPD patients. The NAC dose needed to inhibit ROS generation is controversial. Some authors found an increase in glutathione content in neutrophils only after 10 mM NAC [47], and others reported that NAC at 100–200 μ M in vitro inhibits ROS generation [48]. Our findings relative to oxidative stress and chemotaxis are in accordance with other studies and might have pharmacological significance, since a 100- μ M concentration of NAC is enough to inhibit both processes in vitro. In contrast, NAC was less effective in preventing elastase release. In this sense, we only appreciated a significant reduction at 10 mM, the highest concentration assayed, which was also in accordance with previous studies [47]. Recent studies have shown that NAC may ameliorate lung emphysema induced by elastase or cigarette smoke in animal models [49, 50], which indicates that an active antioxidant therapy could be useful to ameliorate the emphysema progression in humans. Since neutrophil apoptosis resistance is a marker of neutrophil activation and oxidative stress and chronic inflammation

increase neutrophil survival, it would be expected that NAC prevents apoptosis resistance. As we have commented above, spontaneous apoptosis was similar in neutrophils from healthy subjects and severe, early-onset COPD patients. Furthermore, NAC did not modify spontaneous apoptosis. However, apoptosis induced by fMLP was inhibited by NAC in healthy subjects as previously mentioned [51], but in early-onset COPD patients NAC did not significantly reduce neutrophil apoptosis. This is a novel finding which suggests that NAC does not promote neutrophils survival in this type of patients. However, the limitations of the in vitro characteristics of this study require in vivo studies to confirm these observations. In addition to its antioxidant properties, anti-inflammatory effects of NAC have also been observed in human neutrophils. In this regard, NAC was able to reduce neutrophil IL-1 β , IL-8 and TNF- α secretion induced by several inflammatory stimuli [45]. In this work, NAC effectively inhibited IL-8 secondary to fMLP exposure at 1–10 mM in severe, early-onset COPD neutrophils and at 10 mM in healthy neutrophils.

Therefore, findings observed in this in vitro work indicate a broad inhibitory role of NAC in isolated neutrophils as well as in neutrophils from whole peripheral blood of severe, early-onset patients which may be of potential value to support future in vivo studies with NAC in this type of patients.

Acknowledgments

This work was supported by grants SAF2008-03113 and SAF2009-08913, CIBERES (CB06/06/0027) from the Ministry of Science and Innovation and Health Institute 'Carlos III' of the Spanish Government and research grants (Prometeo/2008/045 and Emerging Groups GE-029/10) from Regional Government ('Generalitat Valenciana'). This study received no support from the drug industry.

References

- 1 Turato G, Zuin R, Saetta M: Pathogenesis and pathology of COPD. *Respiration* 2001; 68:117–128.
- 2 Koenderman L, Kanters D, Maesen B, Raaijmakers J, Lammers JW, de Kruif J, Logtenberg T: Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. *J Leukoc Biol* 2000;68:58–64.
- 3 Kim V, Rogers TJ, Criner GJ: New concepts in the pathobiology of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2008;5:478–485.
- 4 Plataki M, Tzortzaki E, Ryttilä P, Demosthenes M, Koutsopoulos A, Siafakas NM: Apoptotic mechanisms in the pathogenesis of COPD. *Int J Chron Obstruct Pulmon Dis* 2006;1:161–171.
- 5 Rahman I: Antioxidant therapeutic advances in COPD. *Ther Adv Respir Dis* 2008;2: 351–374.
- 6 Silverman EK, Chapman HA, Drazen JM, Weiss ST, Rosner B, Campbell EJ, O'Donnell WJ, Reilly JJ, Ginns L, Mentzer S, Wain J, Speizer FE: Genetic epidemiology of severe, early-onset chronic obstructive pulmonary disease. Risk to relatives for airflow obstruction and chronic bronchitis. *Am J Respir Crit Care Med* 1998;157:1770–1778.
- 7 Foreman MG, DeMeo DL, Hersh CP, Reilly JJ, Silverman EK: Clinical determinants of exacerbations in severe, early-onset COPD. *Eur Respir J* 2007;30:1124–1130.

- 8 He JQ, Ruan J, Connett JE, Anthonisen NR, Pare PD, Sandford AJ: Antioxidant gene polymorphisms and susceptibility to a rapid decline in lung function in smokers. *Am J Respir Crit Care Med* 2002;166:323–328.
- 9 Hersh CP, DeMeo DL, Al-Ansari E, Carey VJ, Reilly JJ, Ginns LC, Silverman EK: Predictors of survival in severe, early onset COPD. *Chest* 2004;126:1443–1451.
- 10 Silverman EK, Palmer LJ, Mosley JD, Barth M, Senter JM, Brown A, Drazen JM, Kwiatkowski DJ, Chapman HA, Campbell EJ, Province MA, Rao DC, Reilly JJ, Ginns LC, Speizer FE, Weiss ST: Genomewide linkage analysis of quantitative spirometric phenotypes in severe early-onset chronic obstructive pulmonary disease. *Am J Hum Genet* 2002;70:1229–1239.
- 11 Sadowska AM, van Overveld FJ, Gorecka D, Zdrzal A, Filewska M, Demkow UA, Luyten C, Saenen E, Zielinski J, De Backer WA: The interrelationship between markers of inflammation and oxidative stress in chronic obstructive pulmonary disease: modulation by inhaled steroids and antioxidant. *Respir Med* 2005;99:241–249.
- 12 Isajevs S, Taivans I, Svirina D, Strazda G, Ko-peika U: Patterns of inflammatory responses in large and small airways in smokers with and without chronic obstructive pulmonary disease. *Respiration* 2011;81:362–371.
- 13 Flohe L, Brigelius-Flohe R, Saliou C, Traber MG, Packer L: Redox regulation of NF- κ B activation. *Free Radic Biol Med* 1997;22:1115–1126.
- 14 Vlahopoulos S, Boldogh I, Casola A, Brasier AR: Nuclear factor- κ B-dependent induction of interleukin-8 gene expression by tumor necrosis factor α : evidence for an antioxidant sensitive activating pathway distinct from nuclear translocation. *Blood* 1999;94:1878–1889.
- 15 Rajmakers MT, Schilders GW, Roes EM, van Tits LJ, Hak-Lemmers HL, Steegers EA, Peters WH: N-Acetylcysteine improves the disturbed thiol redox balance after methionine loading. *Clin Sci (Lond)* 2003;105:173–180.
- 16 Pela R, Calcagni AM, Subiaco S, Isidori P, Tubaldi A, Sanguinetti CM: N-acetylcysteine reduces the exacerbation rate in patients with moderate to severe COPD. *Respiration* 1999;66:495–500.
- 17 Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS: Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO global initiative for chronic obstructive lung disease (GOLD) workshop summary. *Am J Respir Crit Care Med* 2001;163:1256–1276.
- 18 Dalli E, Milara J, Cortijo J, Morcillo EJ, Cosin-Sales J, Sotillo JF: Hawthorn extract inhibits human isolated neutrophil functions. *Pharmacol Res* 2008;57:445–450.
- 19 Buenestado A, Cortijo J, Sanz MJ, Naim-Abu-Nabah Y, Martinez-Losa M, Mata M, Issekutz AC, Marti-Bonmati E, Morcillo EJ: Olive oil-based lipid emulsion's neutral effects on neutrophil functions and leukocyte-endothelial cell interactions. *J Parenter Enteral Nutr* 2006;30:286–296.
- 20 Cortijo J, Villagrasa V, Navarrete C, Sanz C, Berto L, Michel A, Bonnet PA, Morcillo EJ: Effects of SCA40 on human isolated bronchus and human polymorphonuclear leukocytes: Comparison with rolipram, SKF94120 and levcromakalim. *Br J Pharmacol* 1996;119:99–106.
- 21 Hedley DW, Chow S: Evaluation of methods for measuring cellular glutathione content using flow cytometry. *Cytometry* 1994;15:349–358.
- 22 Milara J, Ortiz JL, Juan G, Guizarro R, Almudever P, Martorell M, Morcillo EJ, Cortijo J: Cigarette smoke exposure up-regulates endothelin receptor B in human pulmonary artery endothelial cells: molecular and functional consequences. *Br J Pharmacol* 2010;161:1599–1615.
- 23 Elbim C, Pillet S, Prevost MH, Preira A, Girard PM, Rogine N, Hakim J, Israel N, Gougerot-Pocidal MA: The role of phagocytes in HIV-related oxidative stress. *J Clin Virol* 2001;20:99–109.
- 24 Kettritz R, Falk RJ, Jennette JC, Gaido ML: Neutrophil superoxide release is required for spontaneous and fMLP-mediated but not for TNF α -mediated apoptosis. *J Am Soc Nephrol* 1997;8:1091–1100.
- 25 Burnett D, Chamba A, Hill SL, Stockley RA: Neutrophils from subjects with chronic obstructive lung disease show enhanced chemotaxis and extracellular proteolysis. *Lancet* 1987;2:1043–1046.
- 26 Yoshikawa T, Dent G, Ward J, Angco G, Nong G, Nomura N, Hirata K, Djukanovic R: Impaired neutrophil chemotaxis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;175:473–479.
- 27 Beeh KM, Kornmann O, Buhl R, Culpitt SV, Giembycz MA, Barnes PJ: Neutrophil chemotactic activity of sputum from patients with COPD: role of interleukin 8 and leukotriene B₄. *Chest* 2003;123:1240–1247.
- 28 Woolhouse IS, Bayley DL, Stockley RA: Sputum chemotactic activity in chronic obstructive pulmonary disease: Effect of α_1 -antitrypsin deficiency and the role of leukotriene B₄ and interleukin 8. *Thorax* 2002;57:709–714.
- 29 Milara J, Mata M, Mauricio MD, Donet E, Morcillo EJ, Cortijo J: Sphingosine-1-phosphate increases human alveolar epithelial IL-8 secretion, proliferation and neutrophil chemotaxis. *Eur J Pharmacol* 2009;609:132–139.
- 30 Haslett C: Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 1999;160:S5–S11.
- 31 Barnes PJ, Shapiro SD, Pauwels RA: Chronic obstructive pulmonary disease: Molecular and cellular mechanisms. *Eur Respir J* 2003;22:672–688.
- 32 Whyte MK, Hardwick SJ, Meagher LC, Savill JS, Haslett C: Transient elevations of cytosolic free calcium retard subsequent apoptosis in neutrophils in vitro. *J Clin Invest* 1993;92:446–455.
- 33 Pletz MW, Ioanas M, de Roux A, Burkhardt O, Lode H: Reduced spontaneous apoptosis in peripheral blood neutrophils during exacerbation of COPD. *Eur Respir J* 2004;23:532–537.
- 34 Noguera A, Sala E, Pons AR, Iglesias J, MacNee W, Agusti AG: Expression of adhesion molecules during apoptosis of circulating neutrophils in COPD. *Chest* 2004;125:1837–1842.
- 35 Rahman I, Skwarska E, MacNee W: Attenuation of oxidant/antioxidant imbalance during treatment of exacerbations of chronic obstructive pulmonary disease. *Thorax* 1997;52:565–568.
- 36 Rahman I, Morrison D, Donaldson K, MacNee W: Systemic oxidative stress in asthma, COPD, and smokers. *Am J Respir Crit Care Med* 1996;154:1055–1060.
- 37 Yeung MC, Buncio AD: Leukocyte count, smoking, and lung function. *Am J Med* 1984;76:31–37.
- 38 Shapiro SD, Goldstein NM, Houghton AM, Kobayashi DK, Kelley D, Belaaouaj A: Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. *Am J Pathol* 2003;163:2329–2335.
- 39 Kanazawa H, Kurihara N, Hirata K, Fujimoto S, Takeda T: The role of free radicals and neutrophil elastase in development of pulmonary emphysema. *Intern Med* 1992;31:857–860.
- 40 MacNee W, Bridgeman MM, Marsden M, Drost E, Lannan S, Selby C, Donaldson K: The effects of n-acetylcysteine and glutathione on smoke-induced changes in lung phagocytes and epithelial cells. *Am J Med* 1991;91:60S–66S.
- 41 Boman G, Backer U, Larsson S, Melander B, Wahlander L: Oral acetylcysteine reduces exacerbation rate in chronic bronchitis: report of a trial organized by the Swedish society for pulmonary diseases. *Eur J Respir Dis* 1983;64:405–415.
- 42 Poole PJ, Black PN: Oral mucolytic drugs for exacerbations of chronic obstructive pulmonary disease: systematic review. *BMJ* 2001;322:1271–1274.
- 43 Poole PJ, Black PN: Preventing exacerbations of chronic bronchitis and COPD: therapeutic potential of mucolytic agents. *Am J Respir Med* 2003;2:367–370.
- 44 Stey C, Steurer J, Bachmann S, Medici TC, Tramer MR: The effect of oral n-acetylcysteine in chronic bronchitis: a quantitative systematic review. *Eur Respir J* 2000;16:253–262.

- 45 Sadowska AM, Manuel YKB, De Backer WA: Antioxidant and anti-inflammatory efficacy of NAC in the treatment of COPD: discordant in vitro and in vivo dose-effects: a review. *Pulm Pharmacol Ther* 2007;20:9–22.
- 46 Yanagisawa S, Koarai A, Sugiura H, Ichikawa T, Kanda M, Tanaka R, Akamatsu K, Hirano T, Matsunaga K, Minakata Y, Ichinose M: Oxidative stress augments Toll-like receptor 8 mediated neutrophilic responses in healthy subjects. *Respir Res* 2009;10:50.
- 47 Sadowska AM, Manuel-y-Keenoy B, Vertongen T, Schippers G, Radomska-Lesniewska D, Heytens E, De Backer WA: Effect of N-acetylcysteine on neutrophil activation markers in healthy volunteers: in vivo and in vitro study. *Pharmacol Res* 2006;53:216–225.
- 48 Stolarek R, Bialasiewicz P, Nowak D: N-acetylcysteine effect on the luminol-dependent chemiluminescence pattern of reactive oxygen species generation by human polymorphonuclear leukocytes. *Pulm Pharmacol Ther* 2002;15:385–392.
- 49 Cai S, Chen P, Zhang C, Chen JB, Wu J: Oral N-acetylcysteine attenuates pulmonary emphysema and alveolar septal cell apoptosis in smoking-induced COPD in rats. *Respirology* 2009;14:354–359.
- 50 Rubio ML, Martin-Mosquero MC, Ortega M, Peces-Barba G, Gonzalez-Mangado N: Oral N-acetylcysteine attenuates elastase-induced pulmonary emphysema in rats. *Chest* 2004;125:1500–1506.
- 51 Watson RW, Rotstein OD, Jimenez M, Parodo J, Marshall JC: Augmented intracellular glutathione inhibits FAS-triggered apoptosis of activated human neutrophils. *Blood* 1997;89:4175–4181.