Reduced Intracellular Oxygen Radical Production in Whole Blood Leukocytes From COPD Patients and Asymptomatic Smokers*

Lena Wehlin, PhD; Magnus Löfdahl, MD; Joachim Lundahl, MD, PhD; and Magnus Skölld, MD, PhD

Rationale and objectives: COPD is characterized by irreversible airflow obstruction. It has, however, become clear that COPD also is a systemic disease. In the present study, we sought to investigate its impact on different peripheral leukocyte subpopulations that are recognized as important effector cells in the lung tissue.

Methods: We enrolled 20 patients with stable, moderate COPD (FEV₁, 33 to 69%). Ten asymptomatic smokers and 10 nonsmokers served as control groups. Flow cytometry and whole blood analysis were used to minimize unwanted ex vivo modulation. Oxidative burst and adhesion molecule mobilization were analyzed on freshly drawn cells and after in vitro activation.

Measurements and main results: We found reduced oxidative burst in neutrophils, monocytes, and eosinophils after in vitro stimulation with tumor necrosis factor (TNF) and the bacterial peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) in both COPD patients and asymptomatic smokers as compared to nonsmoking control subjects. Vascular involvement was determined as increased soluble intercellular adhesion molecule-1 (sICAM-1) in the COPD group. There were no differences in adhesion molecule expression among the three groups. However, in COPD patients who had smoked the same morning prior to blood sampling, we found a reduced ability to mobilize adhesion molecule CD11b after TNF plus fMLP activation in all investigated cell types. “Acute” smoking did not significantly alter respiratory burst measurements.

Conclusions: Both COPD patients and asymptomatic smokers have increased levels of sICAM-1 and a reduced intracellular oxidative burst in vitro, indicating a vascular endothelial activation and a possible state of refractoriness in circulating phagocytes in COPD. Although expression and mobilization of adhesion molecules were similar between groups, the acute smoke effect on CD11b points out the value of information on smoking behavior when analyzing function of peripheral inflammatory cells in a smoking population.

(CHEST 2005; 128:2051–2058)

Key words: adhesion molecules; COPD; eosinophil; flow cytometry; monocyte; neutrophil; oxidative burst

Abbreviations: fMLP = N-formyl-methionyl-leucyl-phenylalanine; oxLDL = oxidized low-density lipoprotein; sICAM-1 = soluble intercellular adhesion molecule-1; TNF = tumor necrosis factor; TNF-RI = tumor necrosis factor receptor I; TNF-RII = tumor necrosis factor receptor II

COPD is a heterogeneous disease that is characterized by airflow obstruction that is not fully reversible. The major risk factor for COPD is long-term tobacco smoking, leading to airway inflammation and remodeling.¹ At present, cessation of smoking is the only therapeutic option in order to stop the accelerating decline in lung function. While tobacco smoke primarily targets the lung, it has been recognized that COPD is a disease that exerts effects remote from the lung. Thus, COPD is considered to be a systemic inflammatory disorder that influences the peripheral leukocytes and plasma proteins, causes weight loss, and affects the cardiovascular system.² In a recent meta-analysis,³ it was shown that...

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This work was supported by grants from The Swedish Heart-Lung Foundation and Karolinska Institutet.

Manuscript received January 31, 2005; revision accepted April 7, 2005.

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patients with stable COPD have increased levels of several systemic inflammatory markers as compared to healthy control subjects, including total leukocytes, C-reactive protein, fibrinogen, and tumor necrosis factor (TNF)-α. The increased oxidant load in the lungs due to an antioxidant/oxidant imbalance and reactive oxygen and nitrogen species in tobacco smoke is proposed to be a major cause for the systemic effects of COPD.4

Long-term cigarette smoking has several effects on immune cells. It causes neutrophilia with increased numbers of band cells indicating early bone marrow release.5 Neutrophils from smokers have an increased concentration and activity of myeloperoxidase,6,7 and they express more formyl peptide receptors8 than neutrophils from nonsmokers. Activation of peripheral monocytes has been reported in addition to increased concentrations of soluble intercellular adhesion molecule-1 (sICAM-1).9 In vitro cigarette smoke or nicotine exposure has inhibitory effects on neutrophil expression of adhesion molecules,10,11 although in vivo no such clear relationship seems to exist in the literature. Regarded as a systemic disorder, one must consider the effect the disease may have on the function of the circulating inflammatory cells as well as adjacent inflammatory mediators.

Increased cellular infiltration into the lungs of COPD patients has been demonstrated for neutrophils, macrophages, dendritic cells, and T-lymphocytes,12 and during exacerbations also increased number of eosinophils.13 The inflammatory response includes multiple actions, of which the recruitment of leukocytes from the circulation constitutes a key event. Given the pivotal role for these cells at the inflammatory site, their activation state in the circulation is of vital importance; and studies14–16 have demonstrated altered adhesion molecule expression, oxidative burst, and apoptosis on isolated peripheral neutrophils from COPD patients.

In view of these findings of activated peripheral neutrophils in COPD, we hypothesized that COPD may have an impact on a variety of circulating inflammatory cells claimed to play a pathophysiological role in disease development. To test this hypothesis, we enrolled patients with stable COPD, and asymptomatic smokers and nonsmokers as control subjects. We investigated the expression of adhesion molecules and oxidative burst before and after in vitro stimulation in peripheral neutrophils, monocytes, and eosinophils.

Considering the potential influence of increased levels of circulating inflammatory mediators on peripheral cells, we used whole blood analysis and flow cytometry to minimize ex vivo modulation. Soluble inflammatory markers including C-reactive protein, sICAM-1, and TNF receptor II (TNF-RII) were run in parallel with cell analysis.

**Materials and Methods**

**Patient Characteristics**

The study was approved by the local ethics committee, and informed consent was obtained from all participants. Twenty patients with COPD recruited via the Division of Respiratory Medicine, Karolinska University Hospital, Stockholm, Sweden were enrolled in the study. Ten smokers with normal lung function and 10 nonsmokers were recruited by advertisement at the local blood bank. The subjects were age matched, and the COPD patients and smokers who smoked 1 h prior to blood sampling, No. 6 10 0.

### Table 1—Subject Characteristics*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>COPD (n = 20)</th>
<th>Smokers (n = 10)</th>
<th>Nonsmokers (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>57 (30–69)</td>
<td>58 (44–66)</td>
<td>54 (47–64)</td>
</tr>
<tr>
<td>Male/female gender, No.</td>
<td>10/10</td>
<td>3/7</td>
<td>6/4</td>
</tr>
<tr>
<td>Smoking history, pack-yr</td>
<td>35 (15–54)</td>
<td>30 (10–100)</td>
<td>0</td>
</tr>
<tr>
<td>Current smokers, No.</td>
<td>18</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Present cigarette consumption (current smokers), per day</td>
<td>20 (5–20)</td>
<td>18 (8–40)</td>
<td></td>
</tr>
<tr>
<td>COPD patients and smokers who smoked &lt; 1 h prior to blood sampling, No.</td>
<td>6</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.5 (21.6–29.7)</td>
<td>26.5 (19.2–30)</td>
<td>23.9 (15.1–30)</td>
</tr>
<tr>
<td>FEV₁, % predicted†</td>
<td>50 (33–69)†</td>
<td>95 (83–135)</td>
<td>108 (98–114)</td>
</tr>
<tr>
<td>Reversibility, % improvement</td>
<td>13 (0–46)†</td>
<td>3 (0–8)</td>
<td>2 (0–7)</td>
</tr>
<tr>
<td>Total leukocytes, 10⁶/mL</td>
<td>8.0 (6.1–14.4)</td>
<td>7.6 (5.2–10.5)</td>
<td>6.0 (5.2–10.0)</td>
</tr>
<tr>
<td>Platelets, 10⁹/mL</td>
<td>278 (206–396)</td>
<td>276 (236–419)</td>
<td>290 (191–345)</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>142 (123–160)</td>
<td>144 (131–160)</td>
<td>139 (116–158)</td>
</tr>
<tr>
<td>High-sensitivity C-reactive protein, mg/L</td>
<td>1.7 (0.2–9.4)</td>
<td>1.0 (0.3–3.4)</td>
<td>1.7 (0.4–6.5)</td>
</tr>
</tbody>
</table>

*Data are presented as median (range) unless otherwise indicated.
†Measured after bronchodilation.
‡Significant difference (p < 0.05), COPD patients vs nonsmokers.
§Significant difference (p < 0.05), COPD patients vs asymptomatic smokers.
leukocytes were incubated in 0.2 mL of 2 mol/L Na-citrate (Vacutainer; Becton Dickinson; Plymouth, UK). Blood was hemolyzed in NH4Cl and washed, and leukocytes were thereafter kept on ice until further analysis.

For in vitro stimulation, leukocytes were incubated for 30 min at +37°C in TNF-α (final concentration, 10 ng/mL) [Genzyme Diagnostics; Cambridge, MA] or in buffer alone. The prestimulated cells were then stimulated for 15 min at +37°C in N-formyl-methionyl-leucyl-phenylalanine (fMLP; final concentration, 10^6 mol/L) [Sigma; St. Louis, MO] or in buffer alone. The oxidative burst in eosinophils followed a similar pattern as neutrophils but at a lower level, and there was a reduced oxidative burst in smokers and COPD patients. Stimulation with TNF-α alone did not affect production. There were no differences in oxidative burst in neutrophils between smokers and COPD patients (Fig 1, top left, A). The induced oxidative burst in monocytes was not as potent as in neutrophils (Fig 1, top right, B). The oxidative burst in eosinophils followed a similar pattern as neutrophils but at a lower level, and there was a reduced oxidative burst in smokers and COPD patients after fMLP stimulation, as compared to healthy control subjects (Fig 1, bottom, C). We found no correlations with measured lung function parameters (as determined by nonparametric Spearman rank-order correlation test).

Adhesion Molecules

Mobilization of adhesion molecules is a fundamental mechanism in leukocyte activation, endothelial adhesion, and transmigration. To evaluate possible in vivo activation and responsiveness, we measured expression of CD11b and CD35 on freshly drawn and in vitro activated neutrophils, monocytes, and eosinophils.

Whole blood analysis of complement receptor CD11b did not reveal any differences among the three study groups on any of the cell populations. This was also true for cellular responsiveness, measured as mobilization of CD11b in response to in vitro stimulation with TNF-α and fMLP (Fig 2, top left, A). All study groups mobilized the intracellular pool of CD35 equally in response to stimulation in all cell types with no synergistic effect of TNF-α and fMLP (data not shown).

Effect of Smoke Exposure In Vivo on Mobilization of Adhesion Molecules and Oxidative Burst

We examined the acute effect of smoking in currently smoking COPD patients (n = 18) by dividing the group into those who had smoked in the morning on the day of examination (n = 6) from...
those who had refrained from smoking \( (n = 12) \). We found that monocytes, neutrophils, and eosinophils from patients who had smoked prior to blood sampling had a reduced ability to mobilize CD11b in response to a combined TNF-\( \alpha \) and fMLP exposure. This was also observed in neutrophils exposed to TNF-\( \alpha \) only (Fig 2, top right, B). There were no differences in oxidative burst in either cell population.

**Serum Factors**

sICAM-1 levels were significantly increased in serum from COPD patients and asymptomatic smokers compared to nonsmoking control subjects as determined by nonparametric Mann-Whitney \( U \) test (Fig 3, left, A). Serum level of TNF-RII was similar in all study groups (Fig 3, right, B). oxLDL was measured as a marker for lipid peroxidation in plasma. We found no differences between the study groups in the total content of oxLDL or in the proportion of oxLDL in total low-density lipoprotein or cholesterol (data not shown). There were no correlations with measured lung function parameters (as determined by nonparametric Spearman rank-order correlation test).

**Discussion**

In this comparative study, we demonstrate reduced stimulated oxidative burst in peripheral, un-separated, neutrophils, monocytes, and eosinophils, accompanied by increased levels of sICAM-1, in COPD patients and asymptomatic smokers. We found no differences in the capacity to mobilize adhesion molecules among COPD patients, smokers, and nonsmokers, but we report an “acute” smoking
Figure 2. Cell surface expression of CD11b in COPD patients, asymptomatic smokers, and nonsmokers at basal conditions and after in vitro activation on neutrophils (top left, A), monocytes (center left, C), and eosinophils (bottom left, E). Shown is also the effect of acute smoking on CD11b at baseline and after in vitro stimulation in currently smoking COPD patients in neutrophils (top right, B), monocytes (center right, D), and eosinophils (bottom right, F). COPD m-sm = smoked the same morning; COPD n-s = did not smoke the same morning. Data are presented as median, interquartile range, and percentile range. See Figure 1 legend for expansion of abbreviation.
An antioxidant/oxidant imbalance is proposed to be one of the major attributes of the systemic effects of COPD. This could be due to both increased production and release of oxygen free radicals from neutrophils and macrophages in the lungs, but also from oxidative particles in cigarette smoke per se. In the current study, we sought to investigate whether the imbalance in oxidative burst in COPD patients was present also in peripheral blood leukocytes.

We used TNF and fMLP as in vitro stimuli, separately and in combination. TNF is a potent proinflammatory cytokine, and fMLP is a chemotractant for neutrophils but can also activate eosinophils and monocytes. The rationale for using a combination of TNF and fMLP is given by the notion that TNF priming augments the fMLP-induced neutrophil response, with enhanced CD11b mobilization, chemotaxis, and cellular stiffness as a consequence. In line with this, neutrophils as well as eosinophils from the nonsmoking control group responded with increased oxidative burst after fMLP activation, a response that was further pronounced after the additional TNF priming. However, both COPD patients and smokers had a reduced response as compared to healthy control subjects. This was not restricted to neutrophils but was also present in monocytes and eosinophils. A reduced radical production is not in line with the general concept of increased oxidant load in COPD patients. Noguera et al also reported a different oxygen radical production in isolated neutrophils from COPD patients compared to control subjects. However, they reported an increased oxidative burst after stimulation with the receptor independent phorbolester phorbol-myristate-acetate in COPD patients but not in smoking control subjects. The reason for this discrepancy is not fully understood but might be explained by the different methodologic approaches. Isolation of specific granulocyte population by gradient centrifugation will cause activation and degranulation, and the use of different stimuli will, due to different intracellular signaling pathways, generate divergent responses. The choice of fluorescent probe also has impact on the result. In addition, 18 of 20 patients included in the present study were current smokers, while the 10 patients included in the study by Noguera et al were not. In this context, it is interesting to note that neutrophils exposed in vitro to extracts of cigarette smoke have been shown to decrease and delay the intracellular production and increase and accelerate the extracellular production of oxygen species in response to stimuli such as zymosan and phorbol-myristate-acetate. This indicates a time-dependent shift toward an extracellular production of oxygen radicals after tobacco smoke exposure. Also, alveolar macrophages from smokers have been shown to have a decreased intracellular radical production as compared to nonsmokers.

Matheson et al found that smokers have increased numbers of fMLP receptors on isolated peripheral neutrophils. This might suggest an augmented responsiveness to formyl-peptides with subsequent release of oxidative products, but in the present study we did not gain data in support for this assumption. Also, the expression of complement receptors was not different between groups after fMLP activation. One might speculate that increased fMLP receptor expression on neutrophils from smokers is a consequence of decreased receptor turnover rate, which would affect the cellular response to fMLP stimulation. fMLP receptor desensitization in neutrophils has been demonstrated previously, and we cannot rule out the possibility that an unknown mediator affects the responsiveness to fMLP in COPD patients and smokers. However, our data on the ex vivo effects of TNF priming followed by fMLP stimulation might suggest an existing refractory mechanism in leukocytes from smokers and COPD patients. The physiologic response to TNF is regulated by availability of its two receptors. The TNF receptor I (TNF-RI) is constitutively expressed...
on virtually all cells and is internalized on ligand binding, while TNF-RII is mostly expressed on inflammatory cells and is shed from activated cells. This given, it is possible that an ongoing inflammation would reduce the cellular TNF response. We did not find differences in serum TNF-RII levels between our study groups. Vernooij et al previously reported increased levels of TNF-RII in patients with COPD and nonsmokers, or COPD patients in CD11b and CD35 expression at baseline or after nonsmokers, or COPD patients in CD11b and CD35 that can be mobilized on activation. In our study, we could not, in either cell population, find any dissimilarities among asymptomatic smokers, nonsmokers, or COPD patients in CD11b and CD35 expression as compared to control subjects. However, Dentener et al reported no differences in TNF-RII but increased levels of TNF-RI. Both these studies used ethylenediamine tetra-acetic acid plasma for analysis.

We found that both asymptomatic smokers and COPD patients have vascular involvement as measured by increased concentration of sICAM-1. This has been described earlier in serum and bronchial lavage fluid from COPD patients and in asymptomatic smokers. However, opposite results exist showing decreased sICAM-1 in both stable and exacerbated COPD.

Neutrophil granulocytes encompass several distinct granules that are mobilized to the cell surface on appropriate stimulation. Also, monocytes and eosinophils have intracellular pools of CD11b and CD35 that can be mobilized on activation. In our study, we could not, in either cell population, find any dissimilarities among asymptomatic smokers, nonsmokers, or COPD patients in CD11b and CD35 expression at baseline or after in vitro activation. However, we found that TNF/fMLP-stimulated neutrophils, monocytes, and eosinophils from COPD patients who had smoked prior to blood sampling had a reduced CD11b mobilization capacity compared to patients who refrained from smoking. It has previously been shown that smoking generates an acute peripheral response irrespective of lung disease. For example, Selby et al showed that neutrophils exposed to cigarette smoke, in vivo and ex vivo, are less adherent than control cells without changes in adhesion molecule expression. The possibility that cigarette smoke affects the cytoskeleton of circulating leukocytes has also been proposed. Speer et al demonstrated partially inhibited CD11b and totally inhibited CD11a expression in neutrophils after in vitro nicotine exposure. The few studies on acute effects of cigarette smoking in vivo on peripheral leukocyte adhesion molecule expression have shown unaltered expression of CD18 and unaltered or increased expression of L-selectin. On isolated neutrophils, increased expression of CD11b has been reported previously in COPD patients both under basal and TNF stimulated conditions in smoking patients who did not smoke 8 h prior to blood sampling, or in patients who were nonsmokers.

These discrepancies in cellular receptor expression and oxidative burst between different studies raise questions. First of all, current smoking habits of COPD patients and the timing of blood sampling in relation to smoking must evidently be taken into consideration when comparing studies. Secondly, when isolated by gradient centrifugation, neutrophils and monocytes degranulate with considerable up-regulation of several epitopes including CD11b on cell surfaces. One ought therefore to consider that the possible subtle in vivo influence on peripheral cells in COPD patients will be enhanced or distorted after the isolation procedures. In this study, we used whole blood analysis by flow cytometry and separated different cell populations by their light-scattering properties only to minimize the impact of the cell-handling procedure on the cellular phenotype.

To summarize, COPD patients and smokers with normal lung function have a reduced intracellular oxidative burst. We found no differences in expression and mobilization of adhesion molecules, although a short-term effect of smoking was identified in cells from COPD patients after in vitro activation. Furthermore, COPD patients and smokers had increased sICAM-1. Taken together, this indicates a vascular endothelial involvement and a potential state of refractoriness in circulating phagocytes in COPD patients. And finally, these results suggest that smoking behavior must be taken into consideration while examining systemic effects of COPD.

ACKNOWLEDGMENT: The authors thank Ms. Margitha Dahl, Mrs. Gunnel de Forest, and Mrs. Helene Blomqvist for assistance with blood sampling.

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