Nitrosative Stress, Heme Oxygenase-1 Expression and Airway Inflammation During Severe Exacerbations of COPD*

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Study objectives: The aim of this study was to examine the relationship between airway inflammation, nitrosative stress, heme-oxygenase expression, and acute severe exacerbations of COPD.

Design: We measured heme oxygenase (HO)-1, inducible nitric oxide (NO) synthase expression and nitrotyrosine formation, as well as eosinophilic cationic protein, myeloperoxidase (MPO), interleukin (IL)-8, and granulocyte macrophage-colony stimulating factor levels in induced sputum samples from 12 COPD patients (mean ± SD; FEV1 40 ± 14% predicted) at the onset of an acute severe exacerbation of COPD requiring hospital admission and 16 weeks after remission.

Results: We demonstrated increased percentages (p = 0.001) and absolute numbers (p = 0.028) of total nitrotyrosine positive (+ve) inflammatory cells (i.e., polymorphonuclear cells and macrophages), increased percentages (p = 0.04) and absolute numbers (p = 0.05) of total HO-1 +ve inflammatory cells, and increased MPO (p = 0.005) and IL-8 levels (p = 0.028) during severe exacerbation compared with the stable state.

Conclusions: Our results support the hypothesis of an involvement of inflammatory and nitrosative stress in severe COPD exacerbations. Future therapeutic strategies may aim at regulating inflammation and NO synthesis during COPD exacerbations.

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Key words: chronic bronchitis; emphysema; exacerbation; heme oxygenase; inducible nitric oxide synthase; inflammation; nitrotyrosine; oxidative stress; sputum

Abbreviations: CV = coefficient of variation; DTT = dithiothreitol; ECP = eosinophilic cationic protein; GM-CSF = granulocyte macrophage-colony stimulating factor; HO = heme oxygenase; IL = interleukin; iNOS = inducible nitric oxide synthase; MPO = myeloperoxidase; NO = nitric oxide; +ve = positive

COPD is characterized by periodic exacerbations and remissions.1,2 As lung function worsens, exacerbations become more frequent and severe. In patients with stage III to IV COPD (the Global Initiative for Chronic Obstructive Lung Disease classification), exacerbations become an important feature of the disease with a considerable negative impact on the quality of life.3,4 In addition, severe exacerbations often require hospital admission and are associated with high morbidity and mortality rates.3

Inflammatory and oxidant stimuli induce the cellular expression of inducible nitric oxide synthase (iNOS) and heme oxygenase (HO)-1.4,5 HO-1 confers protection against oxidative stress conditions, through antioxidant, antiapoptotic, and antiinflammatory actions.6 On the contrary, iNOS generates nitric oxide (NO), which shifts the cellular redox potential to a more oxidized state. NO under aerobic conditions reacts with oxygen and superoxide anion radicals to yield nitrite and peroxynitrite.7 Peroxynitrite or peroxidase-dependent nitrite oxidation leads to tyrosine nitration. Nitrotyrosine is an indicator of the involvement of NO in irreversible oxidative reactions and has been associated with altered protein function.8,9 Although a number of studies have related COPD exacerbations to increased airway inflammation and oxidative stress, there are relatively limited data regarding severe COPD exacerbations requiring hospital admissions.5 This is mainly due to technical difficulties in studying lung tissue.

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samples obtained from COPD patients during exacerbation. On the contrary, sputum induction is a relatively noninvasive and safe technique, which can be performed on COPD patients even at the time of an exacerbation.

The aim of our study was to test the hypothesis that severe COPD exacerbations are related to increased airway inflammation and oxidative stress. Changes in sputum HO-1, iNOS expression and nitrotyrosine formation in patients with severe COPD exacerbation compared to stable condition were assessed by immunostaining of sputum inflammatory cells. Changes in airway inflammation were assessed by measurement of eosiโนphlic cationic protein (ie, the eosinophil activation marker), myeloperoxidase (MPO) [ie, neutrophil activation marker], interleukin (IL)-8 (ie, neutrophil chemotactic factor), and granulocyte-macrophage colony stimulating factor (GM-CSF) levels in sputum supernatant. Our results showed significant increases in inflammatory mediators, nitrotyrosine, and HO-1 immunopositivity, which are associated with severe COPD exacerbation.

**Study Subjects**

Twelve patients with COPD were studied at the onset of an acute severe exacerbation and while in stable condition. A diagnosis of COPD was made according to the Global Initiative for Chronic Obstructive Lung Disease criteria. In order to enter the study, the patient had to have satisfied the following inclusion criteria: (1) be an ex-smoker with a history of at least 20 pack-years of smoking; (2) have fixed airflow limitation with salbutamol reversibility of <12% and <200 mL. Exclusion criteria included the following: (1) a history of allergy; (2) current smoking or a history of having smoked within 2 years prior to the study; (3) the use of other medications than inhaled salbutamol, short-acting anticholinergic agents, and inhaled steroids before the study, the patient had to have satisfied the following inclusion criteria: (4) have fixed airflow limitation with a history of at least 20 pack-years of smoking; (5) long-term oxygen therapy.

**Study Design**

The recruitment of patients was made from those who were admitted for a severe COPD exacerbation to the Department of Thoracic Medicine at the University Hospital of Heraklion. An exacerbation was defined as being severe if a patient was experiencing acute respiratory failure while breathing room air that required hospital admission. COPD patients requiring treatment in the ICU were excluded from the study. Between January and June 2002, a total of 156 hospital admissions were recorded. Fourteen patients satisfied the inclusion criteria and were enrolled into the study.

All patients were treated with long-acting β₂-agonists, short-acting anticholinergic agents, and inhaled steroids before the exacerbation. All patients received systemic steroids (mean ± SD dose, 50 ± 12 mg of methyl prednisolone), short-acting β₂-agonists, short-acting anticholinergic agents, and oxygen therapy until remission of the exacerbation. No change in any of the patients' regular medication was introduced after recovery from the exacerbation to ensure that all patients were receiving the same medical treatment at the onset of the exacerbation and while in a stable state.

Induced sputum samples were obtained during the exacerbation before any therapeutic intervention. A chest radiograph and blood gas analysis were performed. Twelve of the 14 patients were reassessed on a scheduled visit after remission of the exacerbation. Two patients were excluded from the study because they had experienced another exacerbation and/or an acute respiratory tract infection within the 16-week period after the first exacerbation. Induced sputum samples were collected again while the patient was in stable condition; spirometry, skin-prick reaction tests, and blood gas analyses were also performed.

Induced sputum samples obtained from the same patients during an exacerbation and while in a stable state were processed for the analysis of inflammatory mediators and cellular iNOS, nitrotyrosine, and HO-1 expression, and they were tested for the presence of common microorganisms (ie, Gram stain and quantitative bacterial cultures for detecting common microorganisms). An acute bacterial infection during an exacerbation was defined when a new pathogenic organism was cultured from sputum on the hospital admission day of the exacerbation, but was not cultured while the patient was in a stable state. The Hospital Ethics Committee approved the protocol and all subjects gave their consent.

**Materials and Methods**

**Sputum Induction and Analysis**

Sputum was induced and processed as previously described. Total and differential cell counts were performed using standard methods. The supernatant to be analyzed for released amounts of ECP and MPO was diluted 1:1 with 0.4% cetyltrimethylammonium bromide (H5882; Sigma Chemical; St. Louis, MO) before it was stored at −80°C to prevent unspecified absorption.

**Immunocytochemical Analysis**

Sputum cytospin samples were fixed in acetone for 5 min and rehydrated in Tris-maleate buffer (Trizma, T3128; Sigma) containing saponin (S4521; Sigma) at pH 7.6. Immunostaining procedures were performed using the goat antirabbit, antirabbit immunostaining system (Envision System; DAKO; Carpinteria, CA) with alkaline phosphatase (K1396; DAKO). Nonspecific labeling was blocked by coating with normal goat serum (X0907 [diluted 1:20]; DAKO) for 20 min at room temperature. The following primary antibodies were used: for HO-1 staining, the anti-HO-1 rabbit polyclonal antihuman antibody (sc10789 [diluted 1:50]; Santa Cruz Biotechnology, Inc) overnight at 4°C; for iNOS staining, the anti-iNOS rabbit polyclonal antihuman antibody (sc510 [diluted 1:50]; Santa Cruz Biotechnology, Inc) overnight at 4°C; and for nitrotyrosine staining, the anti-nitrotyrosine rabbit antihuman antibody (diluted 1:100) [Upstate; Lake Placid, NY] for 1 h at room temperature. After being incubated with the primary antibody, the slides were washed, followed by staining with the secondary antibody–alkaline phosphatase anti-alkaline phosphatase complex for 30 min at room temperature. After washing, the slides were stained with Fast Red for 15 min at room temperature. Dilutions and washing steps were performed with the preincubation solution. Slides were counterstained with hematoxylin for 5 min and mounted (Glycergel, G0563; DAKO). Negative control experiments for
nonspecific binding were performed in a similar manner but in the absence of the primary antibody. The primary antibody was replaced by nonspecific Ig of the same species as the primary antibody or the preincubation solution. On immuno-stained slides, polymorphonuclear cells and macrophages were identified by morphologic analysis, and positively stained cells within 300 cells of each population were counted using a light microscope. Results were expressed as the percentage of positive cells within each leukocyte population. Within the polymorphonuclear cell population, there were mostly neutrophils and very few eosinophils, as was shown on slides stained with May-Grunwald-Giemsa stain (Table 2). However, to be precise we use the term *polymorphonuclear cells* for the entire analysis of the study. All analyses were performed in a blind fashion by two investigators, and the results were averaged. Three replicate measurements were performed by each observer in 10 randomly selected slides. Both intraobserver and interobserver coefficient of variation (CV) were < 10%.

**Measurement of Soluble Mediators**

Commercial enzyme-linked immunosorbent assays were used to measure IL-8 (No. IM.2237; Immunotech; Marseille, France) and GM-CSF (No.1989; Immunotech). For IL-8, the sensitivity of the assay was 8 pg/mL, with an intraassay CV of 2 to 5% and an interassay CV of 7 to 10%. For GM-CSF, the sensitivity of the assay was 5 pg/mL, with an intraassay CV of 3% and an interassay CV of 4 to 13%. ECP was measured using a fluoroenzyme-immunoassay (UniCAP, No. 10–9261-01; Pharmacia; Uppsala, Sweden), with a sensitivity of 0.5 μg/L, and intraassay CV of 2 to 3%, and an interassay CV of 4 to 5%. MPO was measured using a competitive radioimmunoassay (No. 52–55115–06/01; Pharmacia) with a sensitivity of 8 μg/L, an intraassay CV of 5 to 6%, and an interassay CV of 7 to 12%. For ECP and MPO measurements, sample dilutions were made with 0.2% cetrimidethyllammonium bromide (CTAB). All measurements were performed twice for the same sample, and the results were averaged. The mean intrasample CV was < 4%.

**Statistical Analysis**

Differences between stable and exacerbation states were tested using the Wilcoxon signed rank test for nonnormally distributed variables and the paired t test for normally distributed variables. Normality was tested by the Shapiro-Wilk test. Correlations were made using the Spearman correlation coefficient (p). A statistical software package (StatsDirect; Camcode; Cambridge, UK) was used for the entire analysis. A p value of < 0.05 was considered to be significant.

All variables compared in the study were evaluated against the criterion α. According to the data and the results of this study, the power of this study was always > 80% for the number of patients (ie, the sample size), the differences obtained for all variables tested (ie, the effect size), and the criterion α = 0.05.

**RESULTS**

**Subjects’ Characteristics**

We studied 1 female and 11 male COPD patients (mean ± SD; FEV₁ 40 ± 14% predicted; mean age, 69 ± 7 years) who were ex-smokers with a mean smoking history of 68 ± 23 pack-years (Table 1). All patients had negative results of skin-prick reaction tests and radioallergosorbent tests for a battery of common allergens. Patients’ exacerbation and stable-state characteristics are given in Table 1. A clear deterioration in cough, sputum, and blood gas analysis was observed in all subjects during an exacerbation. In addition, patients were unable to perform acceptable spirometry during an exacerbation. All patients showed acute respiratory failure at the time of the exacerbation (Pao₂ < 60 mm Hg) [Table 1]. Five patients had positive sputum culture results during exacerbations (for *Streptococcus pneumoniae*, three patients; for *Pseudomonas aeruginosa*, two patients). Chest radiographs at the time of exacerbation did not reveal pneumonia or pneumothorax in any patient.

**Sputum Cytology**

The median viability of cells recovered by sputum processing was 89% (range, 59 to 99%) during exacerbations (for *Streptococcus pneumoniae*, three patients; for *Pseudomonas aeruginosa*, two patients). Chest radiographs at the time of exacerbation did not reveal pneumonia or pneumothorax in any patient.

### Table 1—Characteristics of the Subjects During Exacerbation and in the Stable State*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ES</th>
<th>SS</th>
</tr>
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<tbody>
<tr>
<td>FEV₁, % predicted</td>
<td>40 ± 14</td>
<td></td>
</tr>
<tr>
<td>FEV₁/FVC, % predicted</td>
<td>53 ± 11</td>
<td></td>
</tr>
<tr>
<td>ΔFEV₁, %</td>
<td>4 (0–4.9)</td>
<td></td>
</tr>
<tr>
<td>Pao₂, mm Hg</td>
<td>51 ± 7</td>
<td>72 ± 11</td>
</tr>
<tr>
<td>PaCo₂, mm Hg</td>
<td>53 ± 8</td>
<td>30 ± 51</td>
</tr>
<tr>
<td>Purulent sputum, No.</td>
<td>6/12</td>
<td>0/12</td>
</tr>
<tr>
<td>Cough, No.</td>
<td>9/12</td>
<td>4/12</td>
</tr>
<tr>
<td>Dyspnea at rest, No.</td>
<td>11/12</td>
<td>3/12</td>
</tr>
</tbody>
</table>

*Values given as mean ± SD, unless otherwise indicated. ΔFEV₁ = changes in FEV₁ after the inhalation of 200 μg of salbutamol; ES = exacerbation state; SS = stable state. 
†Value in parentheses is percentage change from baseline. 
‡p < 0.05.

### Table 2—Total Nonsquamous Cell Count, Neutrophil, Macrophage, Eosinophil, and Lymphocyte Count*

<table>
<thead>
<tr>
<th>Variables</th>
<th>ES</th>
<th>SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell count, ×10⁶ cells/g</td>
<td>38 (6, 100)</td>
<td>23.5 (6, 77)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac ××10⁶ cells/g</td>
<td>35.9 (14.9, 95)</td>
<td>18 (5.9, 68.5)</td>
</tr>
<tr>
<td>%</td>
<td>98 (90, 100)</td>
<td>83.5 (56, 99)</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac ××10⁶ cells/g</td>
<td>0.5 (0.5)</td>
<td>2.9 (0.18, 0.9)</td>
</tr>
<tr>
<td>%</td>
<td>1 (0.8)</td>
<td>13.5 (0, 42)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac ××10⁶ cells/g</td>
<td>0 (0, 0.5)</td>
<td>0.03 (0, 2.5)</td>
</tr>
<tr>
<td>%</td>
<td>0 (0, 2)</td>
<td>0.25 (0, 4)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ac ××10⁶ cells/g</td>
<td>0.2 (0, 0.9)</td>
<td>0.15 (0, 1.3)</td>
</tr>
<tr>
<td>%</td>
<td>0.75 (0, 2)</td>
<td>1 (0, 2)</td>
</tr>
</tbody>
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*Values given as median (minimum, maximum). ac = absolute count. See Table 1 for abbreviations not used in the text. 
|p < 0.05.

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exacerbation and 89% (range, 63 to 98%) in the stable state. The percentage of squamous epithelial cells among total cells was 0.2% (range, 0.04 to 1%) during exacerbation and 1.3% (range, 0.1 to 4.7%) in the stable state. No statistically significant difference was observed in cell viability or squamous cell contamination between samples obtained from patients during an exacerbation and while in the stable state. The volume of sputum obtained from COPD patients during an exacerbation was greater than that taken while in the stable state. However, this does not affect our analysis, because the results were expressed as the number of cells per gram of sputum, and levels of mediators were expressed per gram of sputum. Table 2 shows sputum cellularity in both states. The percentages of neutrophils were significantly higher during exacerbations (p = 0.002), and the percentages of macrophages were significantly higher in the stable state (p = 0.003). No statistically significant difference was observed in total nonsquamous cell count, eosinophil count, or lymphocyte count.

Fluid-Phase Inflammatory Mediators, and Cellular iNOS, Nitrotyrosine, and HO-1 Expressions

Levels of soluble mediators during exacerbation and in the stable state are shown in Table 3. MPO and IL-8 levels were increased during exacerbation (p = 0.005) when compared to the stable state (p = 0.028) [Table 3, Fig 1]. The mean values of GM-CSF (p > 0.09) and ECP (p > 0.16) were also increased, although not significantly (Table 3).

Percentages and absolute counts of iNOS, nitrotyrosine, and HO-1 positive (+ve) polymorphonuclear cells and macrophages are shown in Table 4. The percentages of nitrotyrosine +ve (p = 0.001) and HO-1 +ve (p = 0.004) inflammatory cells were significantly increased during exacerbations (Fig 2). When absolute counts of immunoreactive cells were compared, increased numbers of nitrotyrosine +ve inflammatory cells (p = 0.028) and marginally increased numbers of HO-1 +ve inflammatory cells (p = 0.05) were found during exacerbations compared to those found in the stable state (Fig 2). Total

<table>
<thead>
<tr>
<th>Table 3—Sputum ECP, MPO, IL-8, and ECP Levels*</th>
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<tbody>
<tr>
<td>Variables</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>ECP, μg/g</td>
</tr>
<tr>
<td>MPO, μg/g</td>
</tr>
<tr>
<td>IL-8, ng/g</td>
</tr>
<tr>
<td>GM-CSF, pg/g</td>
</tr>
</tbody>
</table>

*Values given as median (minimum, maximum). See Table 1 for abbreviations not used in the text.

†p < 0.05.

percentages and absolute counts of sputum iNOS +ve inflammatory cells were not significantly increased on exacerbation.

Changes in the level of soluble markers and in immunopositive cell counts did not differ between patients with documented bacterial infection during exacerbation (n = 5) or not (n = 7). No significant correlation was found between changes in absolute cell counts and changes in levels of cell chemoattractants (ie, IL-8 and GM-CSF) during exacerbation.

Discussion

We have used induced sputum samples and counted cells expressing HO-1, iNOS, and nitrotyrosine, and levels of soluble inflammatory mediators in patients with COPD who were hospitalized for severe exacerbation compared to stable state. The results showed significantly increased MPO, IL-8, nitrotyrosine, and HO-1 expression in patients during exacerbation relative to the stable state. These findings provide further insight into the pathogenesis of COPD exacerbations.

There are several limitations regarding our study. First, we did not examine them for evidence of acute viral infection, or for infection with chlamydia or mycoplasma, which are often detected during COPD exacerbations. Second, it was difficult to accurately differentiate between chronic bacterial colonization of the respiratory tract and acute infection. We tried to overcome this difficulty by comparing sputum samples obtained from patients during exacerbations with those obtained from the same patients in the stable state. Third, due to significant comorbidities in the majority of COPD patients who were admitted for severe exacerbations, we were only able to recruit a relatively limited number of patients. However, the power of our results was tested, and it was found to be > 80%. Therefore, a type 1 statistical error is unlikely. Fourth, the present study used sputum analysis to assess airway inflammation. It is known that dithiothreitol (DTT), a reducing agent that is regularly used to homogenize sputum, may affect the detection of inflammatory mediators in the sputum sol phase. However, other investigators have shown good recovery of sputum ECP, MPO, IL-8, and GM-CSF using commercial immunoassays, as well as no effect of DTT on their standard curves. Even if DTT affects any of these measurements, comparability between samples obtained from patients during exacerbations or while in the stable state was preserved.

Previously published studies have associated inflammation with COPD exacerbations. Increased numbers of inflammatory cells and elevated levels of
various mediators have been reported in biopsy, BAL, and induced sputum samples obtained from COPD patients during exacerbations. Differences in airway inflammation have been reported between patients with mild or severe exacerbations. Specifically, airway eosinophilia has been related to mild exacerbation, while airway neutrophilia has been related to severe exacerbation.\textsuperscript{19–22} Neutrophil chemokines IL-8 and epithelial-derived neutrophil attractant-78 (CXCL5) and chemokine receptors CXCR1 and CXCR2 appear to play an important role in airway neutrophilia, which is characteristic of severe exacerbations.\textsuperscript{22}

The present study demonstrated increased sputum neutrophil levels, and increased sputum IL-8 and MPO levels at the onset of a severe COPD exacerbation requiring hospital admission (Fig 1). MPO is a granule proteolytic protein that is released from neutrophils during their activation, and proteolysis could be a primary mechanism of neutrophil-induced injury during COPD exacerbation. IL-8 is primarily known for its chemotactic action on neutrophils. Qiu et al\textsuperscript{22} have reported a positive association between the numbers of neutrophils and cells with messenger RNA that is positive for IL-8 in endobronchial biopsy specimens from COPD patients during severe exacerbation. However, we failed to find any correlation between sputum IL-8 levels and sputum neutrophils. This discrepancy could be attributed to the fact that we have studied protein while Qiu et al\textsuperscript{22} studied messenger RNA, or that we have studied sputum samples while Qiu et al\textsuperscript{22} studies endobronchial biopsy specimens. Moreover, the recruitment of neutrophils in the airways of COPD patients is induced by a combination of neutrophil chemokines, which often makes it difficult to correlate a specific chemokine (eg, IL-8) with neutrophil numbers. Increased levels of leukotriene B4 have also been found during COPD exacerbations, and it may be a more powerful neutrophil chemoattractant than IL-8.\textsuperscript{23,25–28}

Sputum neutrophilia and increased MPO and IL-8 levels were observed independently of the presence of bacterial infection during exacerbation. Similarly, Aaron et al\textsuperscript{24} found no relationship between airway inflammation and acute respiratory tract infection during exacerbation. Such results are not entirely unexpected. Factors other than infections (eg, oxidative stress) may also induce airway neutrophilia during COPD exacerbations. Oxidative stress induces the transcription of genes of various inflammatory mediators, including the IL-8 gene.\textsuperscript{29}

In the study by Aaron et al\textsuperscript{24} only 1 of 14 COPD patients had bacterial positive exacerbation. In this study, 5 of 12 patients had bacterial positive exacerbation. The increased number of patients with bacterial airway infection may be due to the fact that we have studied patients with severe COPD who were hospitalized for severe exacerbation.

<table>
<thead>
<tr>
<th>Variables</th>
<th>ES</th>
<th>SS</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS +ve MACR + PMN</td>
<td>80 (50, 98)</td>
<td>69 (20, 90)</td>
<td>NS</td>
</tr>
<tr>
<td>%</td>
<td>31 (3, 69)</td>
<td>13 (3, 70)</td>
<td>NS</td>
</tr>
<tr>
<td>ac ×10\textsuperscript{6} cells/g</td>
<td>77 (39, 99)</td>
<td>38 (13, 76)</td>
<td>0.001</td>
</tr>
<tr>
<td>Nit +ve MACR + PMN</td>
<td>29 (3, 100)</td>
<td>7 (2, 27)</td>
<td>0.028</td>
</tr>
<tr>
<td>%</td>
<td>19 (0, 50)</td>
<td>12 (0, 25)</td>
<td>0.043</td>
</tr>
<tr>
<td>ac ×10\textsuperscript{6} cells/g</td>
<td>6 (0, 30)</td>
<td>2 (0, 10)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Values given as median (minimum, maximum), unless otherwise indicated. MACR = macrophages; PMN = polymorphonuclear cells; Nit = nitrotyrosine; NS = not significant. See Tables 1 and 2 for abbreviations not used in the text.
It should be noted that, unlike the dramatic increases in sputum neutrophils, IL-8, and MPO levels during exacerbation, a similar increase in eosinophils, ECP, or GM-CSF level was not observed. Considering that ECP is a cytotoxic protein that is released from eosinophils on activation and that GM-CSF is a cytokine with multiple functions, among which are the recruitment, survival, and degranulation of eosinophils, the involvement of eosinophils in severe COPD exacerbations seems to be unlikely, which further supports the hypothesis of a different inflammatory profile for exacerbations of different severity, such as neutrophilia during severe exacerbations and eosinophilia during mild exacerbations.30,31

For the purposes of the present study, we also measured immunoreactivity for iNOS and nitrotyrosine. iNOS expression is induced under inflammatory and oxidant conditions, and nitrotyrosine formation is increased in the presence of reactive oxygen species, as in the airways of stable COPD patients.32,33 Although previous investigators have suggested28,34–37 that oxidative stress is further increased during COPD exacerbations, we did not find a significant increase in total sputum iNOS +ve inflammatory cells (percentages and absolute counts) during severe COPD exacerbations. An explanation of this discrepancy could be that inhaled steroid treatment suppressed increased iNOS expression.38 It may be that further studies, including those with steroid-naive patients, are needed to identify the changes in iNOS expression during COPD exacerbations. On the other hand, we found significantly increased percentages and absolute numbers of nitrotyrosine +ve sputum inflammatory cells. These observations indicate that NO consumption through oxidant mechanisms is increased during a severe COPD exacerbation. NO elicits a diverse array of physiologic and pathophysiologic effects.7 Nitrosative stress and nitration of proteins may inhibit physiologic protein function and induce oxidative DNA damage during COPD exacerbations.5,9 There might be a clinical benefit from inhibiting iNOS expression and nitrotyrosine formation during exacerbation, which supports the potential role of NO modulators in the treatment of exacerbations. Preliminary results have suggested39 that NO synthase inhibitors and NO donors that are administered regularly may have clinical benefits for COPD patients, but there are no data regarding COPD exacerbations.

Under physiologic conditions, inflammation and
oxidative stress induce HO-1, which protects against inflammatory and oxidant-mediated cellular injury. The mechanisms by which HO-1 mediates these cytoprotective functions are not clear. However, the three major catalytic byproducts, carbon monoxide, ferritin, and bilirubin represent potential targets. Increased HO-1 expression has been reported in smokers, and decreased HO-1 expression has been reported in patients with severe COPD. Having demonstrated increased airway inflammation and nitrosative stress in COPD patients during severe exacerbations, we expected and found an increase in HO-1 expression (Fig 2). However, it is possible that this increase was not sufficient to protect the patient from lung injury. This is an intriguing hypothesis, which further highlights the possibility for a different therapeutic approach to COPD exacerbations in the future. For example, the exogenous administration of HO-1 via transgene delivery in rats provides cytoprotection against hyperoxia.

In conclusion, the present study of sputum samples that were obtained at the onset of a severe COPD exacerbation demonstrated increased airway inflammation and nitrosative stress during exacerbation, accompanied by an increase in HO-1 expression. These observations may improve our understanding of the pathogenetic pathways of severe COPD exacerbations and might help in the discovery of novel therapeutic modalities to treat COPD exacerbations. However, further studies are needed to verify this hypothesis.

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