Increased Exhalation of Hydrogen Peroxide in Patients with Stable and Unstable Chronic Obstructive Pulmonary Disease

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An imbalance between oxidative stress and antioxidative capacity is thought to play an important role in the development and progression of chronic obstructive pulmonary disease (COPD). To assess the lung oxidative status in patients with COPD, we studied whether exhaled hydrogen peroxide (H_2O_2) is increased in breath condensate of patients with stable COPD (n = 12, mean FEV_1 51% pred) and in patients with exacerbated COPD (n = 19, actual FEV_1 36% pred) compared with a healthy control group (n = 10, FEV_1 108% pred). Expired breath condensate during 15 min of tidal breathing was collected by cooling. The concentration of H_2O_2 was measured spectrophotometrically by means of horse radish peroxidase–catalyzed oxidation of tetramethylbenzidine. Concentrations of H_2O_2 (mean ± SEM) were significantly elevated at 0.205 ± 0.054 μM in patients with stable COPD compared with 0.029 ± 0.012 μM in the control group ($p < 0.05$) and were further increased to 0.600 ± 0.075 μM in patients with acutely exacerbated COPD ($p < 0.001$ compared with patients with stable COPD). Patients with pulmonary infiltrates on chest radiograph showed similar values compared with patients without obvious infiltrates. These findings demonstrate that patients with stable COPD exhibit increased oxidant production in the airways and that oxidant production increases further during exacerbations.

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Reactive oxygen-derived species (ROS) have been implicated in the pathogenesis and pathophysiology of tobacco smoke–induced chronic obstructive pulmonary disease (COPD) (1). This contention is supported by several findings. Cigarette smoke is a rich source of oxidants. Smokers have increased numbers of macrophages and neutrophils in their alveoli (2), and these cells are activated and produce increased amounts of ROS (3, 4). Oxidative inactivation of methionine residues of α_1-proteinase inhibitor may contribute to the onset and progression of emphysema (5).

Production of ROS is likely to increase further during an acute exacerbation because of the accompanying increased numbers of inflammatory cells in the lower airways (6). In addition, alveolar macrophages (AM) in smokers with a recent lower respiratory tract infection were found to release increased numbers of ROS (7).

Direct *in vivo* evidence of increased concentrations of ROS in patients with COPD has not been provided, however. In this respect, exhalation of hydrogen peroxide (H_2O_2) is of potential interest. Hydrogen peroxide is a harmful ROS because it is relatively stable, it can cross membranes due to its small size and its lack of charge, and it can generate the highly reactive hydroxyl radical in the presence of superoxide anions and iron (8). Clinically, increased levels of exhaled H_2O_2 have been demonstrated in children with asthma (9) and in patients with adult respiratory distress syndrome and acute hypoxemic respiratory failure (10, 11).

We hypothesized that an increased oxidative burden in the lungs of patients with COPD would be reflected in increased levels of exhaled H_2O_2. The present study was undertaken to answer two questions: 1) Is the concentration of exhaled H_2O_2 increased in patients with stable COPD compared with normal subjects, and 2) Are levels of exhaled H_2O_2 increased even further during exacerbations of COPD?

METHODS

Subjects. Three groups of subjects were studied (Tables 1 and 2): normal control subjects, patients with stable COPD, and patients with acutely exacerbated COPD. Normal control subjects (n = 10; mean [± SEM] age, 53 ± 4 yr) were never-smokers with no pulmonary disorders and no signs of upper or lower respiratory tract infection in the previous

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TABLE 1

CHARACTERISTICS OF PATIENTS WITH STABLE COPD

Patient	Age, sex	Smoking*	Pa_{O_2} (*kPa*)	Pa_{CO_2} (*kPa*)	FEV_1 (% *pred*)†	H_2O_2 (μM)
1	63, M	C	9.2	5.8	63	0.000
2	72, M	E	8.9	5.6	36	0.196
3	55, M	C	8.3	4.8	57	0.157
4	73, M	E	6.6	5.8	38	0.052
5	86, M	E	7.2	5.2	55	0.210
6	74, M	E	9.7	6.3	18	0.052
7	58, M	C	9.4	4.8	61	0.000
8	64, F	C	8.6	4.6	63	0.151
9	82, F	E	6.2	5.8	62	0.571
10	60, M	E	10.7	4.0	70	0.212
11	74, M	E	9.3	5.7	49	0.512
12	80, F	E	8.2	5.3	45	0.345
Mean	70		8.5	5.3	51	0.205
SEM	3		0.4	0.2	4	0.054

* Smoking: C = current, E = ex.
† FEV_1: actual value at the morning of collection of breath condensate.

3 mo (FEV_1 108 ± 6% pred [12]). Patients with COPD were included if postbronchodilator FEV_1 was below 60% pred and/or FEV_1/vital capacity ratio was below 60%. All patients were classified as smokers or exsmokers (i.e., discontinuation of smoking for at least 3 mo) based on medical history. Exclusion criteria were pulmonary disorders at present or in the past possibly contributing to chronic airflow obstruction (e.g., tuberculosis, sarcoidosis, bronchiectasis, and asthma), clinical signs of bronchial hyperreactivity and/or acute response to an inhaled bronchodilator of more than 15% of predicted value, chronic airflow obstruction without smoking in the past, regular intake of vitamin C and E, and treatment with oral or inhaled *N*-acetylcysteine. Patients with stable COPD (n = 12; mean [± SEM] age, 70 ± 3 yr; mean FEV_1, 51 ± 4% pred) were defined as having no increase in symptoms and no exacerbations in the previous 3 mo. They were on maintenance therapy with inhaled bronchodilators; one used inhaled corticosteroids, and none received oral steroids. An exacerbation was defined by an acute deterioration of breathlessness, mostly with increased coughing and production of purulent sputum, for which additional medication was indicated (inhaled bronchodilators, oral or intravenous corticosteroids, or antibiotics). In this group, 19 patients were studied (mean [± SEM] age, 69 ± 2 yr; FEV_1 measured within 6 mo before the exacerbation, 49 ± 4% pred). Patients with signs of upper respiratory tract infection were excluded. Measurements in the acute patients were performed on the first or second day after consultation at the outpatient clinic or at hospitalization. Only patients who sought medical attention within 1 wk after the start of exacerbation symptoms were included in the study. All received supplemental oxygen, which was discontinued for at least 30 min before the collection of exhaled breath condensate. The study was approved by the hospital ethics committee; informed consent was obtained from all subjects.

Collection of expired breath and measurement of H_2O_2. The samples were collected in the morning, approximately 1 h after inhalation of the patient's own bronchodilator. Current smokers were requested to refrain from smoking after midnight. First, FEV_1 was measured. Subsequently, the participants were breathing through a face mask with a two-way valve. The expired air was conducted through a tube with a col-

TABLE 2

CHARACTERISTICS OF PATIENTS WITH EXACERBATED COPD

Patient	Age, sex	Systemic steroids	Smoking*	Pa_{O_2}	Pa_{CO_2}	FEV_1 (% *pred*)†	H_2O_2 (μM)‡
1	65, M	x	E	6.7	5.6	25	0.148
2	75, M	x	E	8.5	5.7	26	0.276
3	73, M	x	E	8.2	4.9	55	0.591
4	62, M	x	E	8.7	4.5	32	0.307
5	72, M		E	8.5	5.3	20	1.111
6	59, F		C	8.4	4.6	28	0.719
7	83, M	x	E	8.4	4.9	29	0.559
8	51, F	x	E	8.8	4.9	46	0.837
9	60, M	x	E	7.3	6.8	21	0.636
10	82, F	x	E	8.3	5.7	78	0.343
11	76, M	x	E	8.9	5.1	23	1.023
12	79, F	x	E	6.2	5.8	n.m.	0.396
13	70, F	x	E	6.3	8.4	20	0.761
14	57, M	x	E	7.1	5.2	22	0.558
15	60, M	x	E	9.6	5.6	31	0.326
16	76, F	x	E	7.6	5.3	63	0.329
17	63, M	x	C	9.7	5.7	29	0.204
18	64, M	x	E	8.5	5.3	70	1.063
19	74, M	x	E	7.0	5.6	35	1.208
Mean	69			8.0	5.5	36	0.600
SEM	2			0.2	0.2	4	0.075

* Smoking: C = current, E = ex.
† FEV_1: actual value at the morning of the collection of breath condensate.
‡ Arterial blood gas obtained after at least 30 minutes of breathing ambient air.
n.m. = not measured.

lecting system which was placed in ice. The collecting system was connected to a 2 ml sterile plastic tube. In this way, approximately 1 ml of breath condensate was collected within 15 min of tidal breathing. The samples were immediately placed in liquid nitrogen. Measurements of H_2O_2 were performed within 6 h after sample collection, since preliminary data showed that H_2O_2 concentrations did not change during this period. The method described by Gallati and Pracht was applied (13). Briefly, 100 μl of 420 μM 3′,3,5,5′-tetramethylbenzidine (dissolved in 0.42 M citrate buffer, pH 3.8) and 10 μl of 52.5 U/ml of horseradish peroxidase (HRP; Sigma Chemicals) were added to 100 μl of the condensate. The reaction proceeded for 20 min at room temperature. Subsequently, the mixture was acidified to a pH of 1 with 10 μl of 18 N sulfuric acid. The reaction product was measured spectrophotometrically at 450 nm using an automated microplate reader (model EL312; Bio-Tek Instruments Inc.). The absorbance is directly proportional to the concentration of H_2O_2. The detection limit was approximately 0.1 μM H_2O_2. All samples were measured in duplicate; mean values were used for subsequent analysis.

Statistical analysis. Data between the three groups were compared using one-way analysis of variance followed by Duncan's multiple range test. A Wilcoxon test was performed to test differences between patients with or without pulmonary infiltrates. Spearman correlation tests were performed to detect a correlation between the concentration of exhaled H_2O_2 and clinical markers of severity of the disease such as FEV_1 and Pa_{O_2}. All values are presented as mean ± SEM; statistical significance was assumed at $p < 0.05$.

RESULTS

Normal subjects had the lowest concentration of H_2O_2 in breath condensate (0.029 ± 0.012 μM) (Figure 1). No H_2O_2 was detected in 6 of 10 healthy subjects. In contrast, patients with stable COPD exhibited an increased concentration of exhaled H_2O_2 (0.205 ± 0.054 μM, $p < 0.05$ compared with normal subjects). Hydrogen peroxide was not detected in 2 of these 12 subjects. Finally, H_2O_2 was detected in all samples of the patients with exacerbated COPD and was elevated in comparison with patients with stable COPD (0.600 ± 0.075 μM, $p < 0.001$).

Patients with an exacerbation with pulmonary infiltrates on chest radiograph (n = 5) showed similar values compared with patients without obvious infiltrates (n = 14) (0.601 ± 0.166 μM and 0.599 ± 0.087 μM, respectively). In the group of patients with stable COPD, those who still smoked (n = 4, FEV_1 61 ± 1% pred) exhaled 0.077 ± 0.044 μM, whereas the exsmokers (n = 8, FEV_1 47 ± 6% pred) exhaled 0.269 ± 0.068 μM (current versus exsmokers, $p = 0.094$). No significant correlations were found between the levels of H_2O_2 and FEV_1 ($r = 0.13$) or Pa_{O_2} ($r = 0.16$) in patients with stable and unstable COPD.

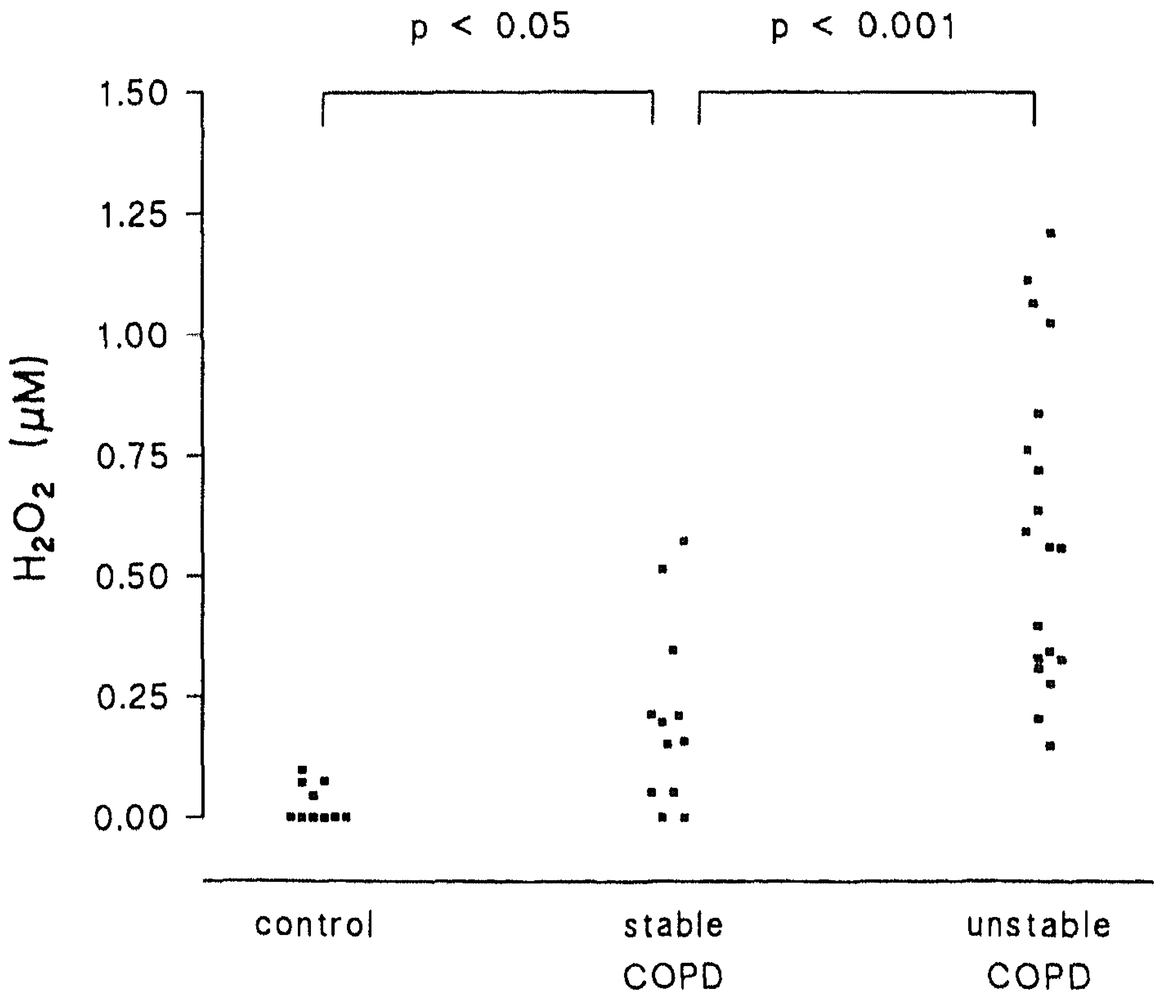

Figure 1. Individual data for H_2O_2 concentrations in breath condensate.

DISCUSSION

This study shows increased H_2O_2 concentrations in exhaled breath condensate in patients with stable COPD, providing direct evidence of increased production of ROS in the airways of these patients. A further increase was observed in patients with an exacerbation.

Concentrations of H_2O_2 measured in exhaled breath condensate may be considered as the net result of production versus scavenging of H_2O_2. An increased production of H_2O_2 may be caused by an increased number of lung inflammatory cells and/or increased production of H_2O_2 by these cells (7). An increased number of these cells have been found in the lungs of patients with stable COPD. Both bronchial lavage and bronchoalveolar lavage fluid obtained from smokers with COPD contained more neutrophils than found in fluid obtained from healthy nonsmokers (14, 15). Increased numbers of macrophages and T lymphocytes were present in the bronchial mucosa of patients with chronic bronchitis and airflow obstruction compared with healthy nonsmokers (16). Exacerbations of chronic bronchitis in patients with mild COPD (mean FEV_1 approximately 66% pred) were associated with increased numbers of eosinophils, neutrophils, and T lymphocytes in the bronchial wall (6).

Indications of increased activation and production of ROS by these cells have been observed in smokers (3, 4, 7, 17). Bronchoalveolar lavage fluid from smokers contained increased numbers of AM with higher densities, which produced more superoxide anion radicals (17). In addition, AM from smokers with a recent lower respiratory tract infection released increased quantities of H_2O_2 (7).

The production of ROS may be reduced, at least *in vitro*, by medications such as β_2 sympathomimetic drugs and theophylline (18). Whether this effect also occurs *in vivo* is unknown. Natural protection against an increased concentration of H_2O_2 is provided by catalase and—of particular importance in the lungs—glutathione. Only a few studies have addressed the antioxidative capacity of the lungs of smokers (4, 15, 19). Reduced activities of superoxide dismutase, glutathione S-transferase, and glutathione peroxidase were found in the AM of elderly smokers (4). In contrast, enhanced activities of superoxide dismutase and catalase, but not of glutathione peroxidase, were shown in the AM of younger smokers (19). Linden and colleagues (15) demonstrated an increased concentration of total glutathione (free and disulfide bound) in bronchoalveolar lavage fluid obtained from patients with COPD who were current smokers. It is unknown whether this increase in total glutathione is caused by continued smoking or by COPD itself (i.e., also occurring in exsmokers).

In contrast to our findings, Sznajder and colleagues (10) found significantly higher concentrations of exhaled H_2O_2 in patients with acute respiratory failure and pulmonary infiltrates compared with those without. These authors also noted that central nervous system involvement in these patients increased exhaled H_2O_2 levels compared with those patients without involvement; moreover, patients with infiltrates and sepsis exhaled higher levels of H_2O_2 than those without sepsis. Thus, the differences observed in the study by Sznajder and colleagues may not be attributed solely to the presence or absence of infiltrates.

The current smokers in the group with stable COPD tended to exhale lower concentrations of H_2O_2 compared with the exsmokers. The number of patients studied, however, was too small to make any conclusions about the effects of smoking in this respect. In addition, bias may have occurred in our separation

of smokers and exsmokers, which was based solely on medical history.

Hydrogen peroxide is produced not only in the lower but also in the upper airways. Therefore, patients with clinically suspected upper airway infection were excluded from the study in order to avoid a major contribution of these airways to total H_2O_2 exhalation. Differences in minute ventilation and breathing pattern may have occurred among the different groups. This, however, was not likely to account for the differences observed in exhaled H_2O_2 levels, as changes in minute ventilation and breathing pattern did not alter H_2O_2 exhalation in animal experiments (10).

In conclusion, our data show that increased H_2O_2 occurs in subjects with stable COPD and even more so in patients with exacerbated COPD. These data are consistent with the concept that ongoing airway inflammation with elevated production of ROS increases lung oxidative stress in patients with stable COPD.

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