

# Rehabilitation Decreases Exercise-induced Oxidative Stress in Chronic Obstructive Pulmonary Disease

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The effect of exercise at different intensities as well as the effect of intensive supervised pulmonary rehabilitation on oxidative stress were studied for chronic obstructive pulmonary disease (COPD). Eleven patients with COPD and 11 healthy age-matched control subjects performed a maximal and submaximal exercise cycle ergometry test at 60% of peak workload. Patients with COPD performed these tests before and after 8 wk of pulmonary rehabilitation. Measurements were done before, immediately after, and 4 h after both exercise tests. At rest, increased oxidative stress was observed in patients compared with control subjects, as measured by urinary malondialdehyde (MDA;  $p < 0.05$ ) and hydrogen peroxide ( $H_2O_2$ ) in breath condensate ( $p < 0.05$ ). In healthy control subjects, a significant increase in urinary MDA was observed 4 h after both exercise tests ( $p = 0.05$ ), whereas  $H_2O_2$  significantly increased immediately after maximal exercise ( $p < 0.05$ ). In patients with COPD, before rehabilitation, reactive oxygen species-induced DNA damage in peripheral blood mononuclear cells, urinary MDA, and plasma uric acid were significantly increased after both exercise tests ( $p < 0.05$ ), whereas no significant increase was observed in plasma MDA. In contrast, exhaled  $H_2O_2$  was only significantly increased after maximal exercise ( $p < 0.02$ ). Although after rehabilitation peak workload was increased by 24%, a similar oxidative stress response was found. Remarkably, a decrease in reactive oxygen species-induced DNA damage was detected after exercise at submaximal intensity despite increased exercise duration of 73%. In summary, patients with COPD had increased pulmonary and systemic oxidative stress both at rest and induced by exercise. In addition, pulmonary rehabilitation increased exercise capacity and was associated with reduced exercise-induced oxidative stress.

**Keywords:** chronic obstructive pulmonary disease; exercise capacity; oxidative stress; rehabilitation

Several studies have shown an increased systemic oxidative stress response after strenuous exercise in patients with chronic obstructive pulmonary disease (COPD) (1–3). This was derived from increased lipid peroxidation, measured as thiobarbituric acid–reactive substances, and increased protein oxidation (1–3). Additional evidence supporting increased exercise-induced systemic oxidative stress in patients with COPD is still lacking, especially possible differential effects of exercise at different intensities. Moreover, no studies are presently available that have evaluated the effect of exercise on pulmonary oxidative stress. Dekhuijzen and colleagues (4) reported increased hydro-

gen peroxide ( $H_2O_2$ ) in exhaled breath condensate (EBC) in patients with COPD compared with healthy participants, which was further increased during acute exacerbations. Therefore, as a first step, the present study was designed to examine the changes in exercise-induced systemic and pulmonary oxidative stress response at maximal and submaximal exercise levels in patients with COPD and in healthy age-matched control subjects. As an additional marker of systemic oxidative stress, the comet assay in peripheral blood mononuclear cells (PBMCs) was introduced as a new method and is as yet unexplored in COPD. It is a sensitive technique for analyzing reactive oxygen species (ROS-)–induced DNA damage in PBMCs. The comet assay detects double- and single-strand breaks, incomplete excision repair sites, cross-links, and alkali-labile sites (5, 6). In addition to the comet assay, plasma and urine malondialdehyde (MDA) were determined as markers of systemic oxidative stress. Furthermore, uric acid in plasma was measured as an indicator of xanthine-oxidase activity. Pulmonary oxidative stress in patients with COPD was assessed by analyzing  $H_2O_2$  concentration in EBC (4).

A major component of pulmonary rehabilitation to improve physical performance and health-related quality of life is exercise training (7, 8). However, exercise-induced increases in oxidative stress may adversely affect outcome in COPD (3, 9). For that reason, it is important to understand whether or not exercise training during a pulmonary rehabilitation program can reduce exercise-induced oxidative stress. Therefore, the second aim of this study was to evaluate the effects of pulmonary rehabilitation on markers of exercise-induced oxidative stress in patients with COPD.

## METHODS

### Study Population

Eleven clinically stable patients with stage II–IV disease according to the Global Initiative for Chronic Obstructive Lung Disease guidelines were consecutively recruited on admission to a pulmonary rehabilitation center (Asthma Center Hornerheide, Horn, The Netherlands) and participated in an inpatient pulmonary rehabilitation program on weekdays over 8 wk ( $FEV_1 < 60\%$  predicted and  $FEV_1/FVC < 70\%$  and  $< 10\%$  predicted improvement in  $FEV_1$  after  $\beta_2$ -agonist inhalation) (10, 11). Additional information on the rehabilitation program is available in the online supplement. All patients were ex-smokers, were not depleted (12), did not use oxygen supplementation, and had not experienced respiratory tract infection or exacerbation of their disease for at least 4 wk before the study. Exclusion criteria were no other chronic diseases, such as rheumatoid arthritis and chronic colitis. Also, patients with diabetes, cardiovascular diseases, renal diseases, liver diseases, or mental diseases were excluded from the study. Eleven healthy, age-matched, nonsmoking participants were recruited as the control group. In addition, all participants were questioned on their dietary habits to ensure that none were taking antioxidants or vitamin supplements. Furthermore, all patients received anticholinergic and  $\beta_2$ -agonists as bronchodilator therapy as well as inhaled corticosteroids. Three patients also received theophylline and one patient was on oral corticosteroid therapy. This maintenance medication remained unchanged during the

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study. Written, informed consent was obtained from all participants, and the study was approved by the medical ethics committee of the University Hospital Maastricht.

### Study Design

The participants were instructed to abstain from strenuous physical activity on the test day. Moreover, patients with COPD were not allowed to take antioxidants or vitamin supplements during the rehabilitation program. At the beginning of the study, all participants underwent pulmonary function testing and anthropometric measurements, and they had to wear a physical activity monitor during 9 consecutive d. First, they completed an incremental cycle ergometry exercise test until exhaustion and, after 4 d, a submaximal constant work-rate exercise test was performed at 60% of the maximal power output achieved on the incremental exercise test. Peripheral venous blood, urine samples, and samples for measurements of breath condensate H<sub>2</sub>O<sub>2</sub> were obtained at baseline, immediately and 4 h after both exercise tests. All tests were performed and samples were collected at the same time point of the day. The same procedure was strictly followed at the end of the pulmonary rehabilitation program for patients with COPD.

### Pulmonary Function Tests

All participants underwent flow volume tests, including measurements of FEV<sub>1</sub> and FVC, with the highest value from at least three properly performed measurements being used for analysis. In addition, lung volumes and diffusion capacity were obtained in patients with COPD. Residual volume and total lung capacity (TLC) were assessed by whole-body plethysmography, and diffusion capacity for carbon monoxide (DL<sub>CO</sub>) was measured by using the single-breath method (Masterlab; Jaeger, Würzburg, Germany). The values obtained were expressed as a percentage of the reference value (13).

### Level of Physical Activity

The daily physical activity level was assessed using the Physical Activity Monitor (Pam) accelerometer. Pam (type AM 100; Pam B.V., The Netherlands) is a small-sized, lightweight, unidirectional accelerometer. This accelerometer provides a valid and reliable method to estimate the energy expenditure of the whole body during walking patterns (*see* the online supplement for additional information).

### Exercise Capacity

Participants performed an incremental (10 W/min) cycle ergometry test as described previously (14). Expired gases were analyzed using breath-by-breath analysis through a breathing mask (Oxygen-β; Jaeger, Würzburg, Germany). Simultaneously, heart rate was registered every min (Polar Electro Cy, Kempele, Finland).

The submaximal exercise test was performed at 60% of the peak workload achieved during the incremental exercise test. The patients were instructed to cycle as long as possible but for a maximum of 30 min. For the control group, the endurance time was standardized at 12 min, which was the mean endurance time of the patients with COPD at baseline. In addition, oxygen consumption and heart rate were monitored as described in the incremental exercise test. Antecubital venous blood samples were taken before and at the end of both exercise tests to analyze lactate. Plasma lactate was determined enzymatically using an automated system (Cobas Mira; Roche, Basel, Switzerland).

### Sample Preparation

Venous blood samples (10 ml) were drawn into ethylenediaminetetraacetic acid-containing tubes (Venoject; Terumo Corporation, Leuven, Belgium). All blood samples were immediately put on ice, and kept on ice during sample preparation. Plasma was obtained by centrifugation (800 × *g* for 10 min at 4°C) and stored at -80°C until analysis. Urine samples were collected in sterile containers and two 10-ml aliquots of urine were stored at -20°C until further analysis. Plasma, urine, and breath samples of one patient, gathered before and after rehabilitation, were processed in the same run for (bio)chemical analyses.

### Comet Assay

The comet assay (single-cell gel electrophoresis) was adapted from the method of Singh and colleagues (15) with minor modifications. After

removal of the plasma (800 × *g* for 10 min at 4°C), the volume of the remaining blood was brought to 15 ml with cold phosphate-buffered saline (PBS) and layered on an equal volume of Lymphoprep medium in a 50-ml tube with a filter (Bio-One GmbH; Greiner, Frickenhausen, Germany) of PBMCs. After centrifugation at 800 × *g* for 30 min at 4°C, gradient-separated PBMCs were recovered, resuspended in 10-ml cold PBS, and centrifuged again at 250 × *g* for 10 min at 4°C. The cells were resuspended in 1 ml cold PBS. For the analysis of DNA damage, 10 μl of the cell suspension was mixed with 90 μl of 1% low-melting-point agarose of 37°C and layered onto prechilled agarose-coated (1.5% agarose in PBS) microscope slides. The slides were covered with a coverslip and kept at 4°C for 5 min to allow the low-melting-point agarose to solidify. Then, the coverslips were removed and the slides were immersed in a jar containing cold lysing solution (2.5 M NaCl, 100 mM Na<sub>2</sub> ethylenediaminetetraacetic acid, 10 mM Tris [pH 10], 1% Triton X-100, 10% dimethyl sulfoxide). For reasons of stability of the DNA during lysis, the PBMCs were kept at 4°C for a maximum of 6 wk (16). All samples of one patient taken before rehabilitation were processed in one run, and samples taken after rehabilitation in a separate run. After lysis, the slides were drained and placed in a horizontal ice-cold electrophoresis box filled with fresh alkaline electrophoresis buffer of 4°C (10 N NaOH, 200 mM Na<sub>2</sub> ethylenediaminetetraacetic acid [pH 12.5–13.5]) for 20 min to allow for DNA unwinding and expression of alkali-labile sites. The electrophoresis was subsequently conducted at 4°C, for 20 min, at 25 V and 300 mA. After electrophoresis, the slides were washed three times with cold neutralization buffer (0.4 M Tris, pH 7.5) and stored at 4°C until further analysis. All of these steps were conducted under dim light to prevent the occurrence of additional DNA damage. For microscopic analysis, 50 μl ethidium bromide (20 μg/ml) was added to each slide and 50 randomly selected cells were analyzed from each sample. Comet measurements were made by image analysis using a fluorescence microscope and the Comet Assay III software (Perceptive Instruments, Suffolk, UK). ROS-induced DNA damage was measured as tail moment (based on the product of the percentage of DNA in the tail and tail length). A representative comet image is shown in Figure E1 in the online supplement. Median, mean, and SE of the mean of tail moment were calculated from each sample. The median of each sample was taken because data had a nonnormal distribution.

### MDA in Plasma and Urine

The determination of MDA was assayed by measuring thiobarbituric acid-reactive substances according to the HPCL method described by Lepage and coworkers (17). The urinary MDA concentration was corrected for creatinine content. Creatinine in urine samples was measured in triplicate using the Jaffé reaction as previously described (18). The values of MDA in urine are expressed per mmol creatinine. Additional details for these measurements are provided in the online supplement.

### Plasma Uric Acid

An aliquot of 150 μl of plasma was deproteinized with 10% (wt/vol) trichloroacetic acid and centrifuged, and uric acid was assayed by an HPLC method described by Lux and colleagues (19) using a Hypersil BDS C18 end-capped column (125 × 4 mm, particle size μm; Agilent, Palo Alto, CA). Additional details on this method are available in the online supplement.

### Collection of EBC and Measurement of H<sub>2</sub>O<sub>2</sub>

The collection of EBC was performed using a commercially available condenser as previously described (20). Participants were asked to breathe at a normal frequency and tidal volume for 10 min. The EBC samples were immediately divided into aliquots, stored at -80°C, and thawed at room temperature just before analysis.

EBC H<sub>2</sub>O<sub>2</sub> was measured by means of horseradish peroxidase-catalyzed oxidation of tetramethylbenzidine according to the method previously described by Gallati and Pracht (21) and modified for microtiter plate-based analysis. All samples were measured in duplicate, and a separate standard curve for H<sub>2</sub>O<sub>2</sub> was constructed for each assay. Mean values were used for subsequent statistical analysis. Additional detail on the method for making these measurements is provided in the online supplement.

## Statistical Analyses

All data are expressed as mean  $\pm$  SEM. Between-group comparisons were analyzed using the Mann-Whitney U test. The Wilcoxon signed rank test was used to evaluate the effect of the maximal and submaximal exercise-induced oxidative stress response within the groups and the effect of pulmonary rehabilitation on oxidative stress in patients with COPD. Nonparametric tests were used because the normality assumption was not obtained. A difference with  $p < 0.05$  was considered statistically significant. Statistical analyses were analyzed with SPSS for Windows (version 11.0; SPSS, Inc., Chicago, IL).

## RESULTS

### Anthropometric and Spirometric Data

As shown in Table 1, anthropometric data were not significantly different between patients with COPD and control participants. The COPD group showed a moderate to severe airflow obstruction, with an FEV<sub>1</sub> of 39.4  $\pm$  4.1% predicted. In patients, FVC, TLC, and residual volume were significantly ( $p < 0.05$ ) improved after rehabilitation, whereas body mass index and fat-free mass index were not significantly different and within the normal range (12). The Pam score for healthy control subjects classified them as moderately active, whereas the patients with COPD had a low activity level (Table E1). The physical activity level of patients with COPD was significantly increased (+19%,  $p < 0.05$ ) after rehabilitation.

### Exercise Capacity

The results of both exercise tests are presented in Table 2. One patient was excluded from the submaximal exercise test because of respiratory infection, which occurred a few days after the maximal exercise test. As expected, exercise capacity was severely impaired in the patients with COPD when compared with healthy control subjects ( $p < 0.001$ ). In addition, peak lactate was significantly lower in patients with COPD than in healthy control subjects ( $p < 0.01$ ). Nevertheless, the patients with COPD exceeded their maximal voluntary ventilation ( $\dot{V}_E/\dot{V}_{MV} > 100\%$ ). Because of their ventilatory limitation, maximum exercise intensity was reached, whereas the heart rate reserve was preserved. The healthy control subjects were within the normal range for these two parameters. Additional details on these measurements are provided in the online supplement. The patients with COPD

responded to the rehabilitation program by a significant increase in peak workload (+24%,  $p < 0.01$ ), maximal  $\dot{V}O_2$  (+19%,  $p < 0.05$ ) and exercise duration time (+73%,  $p < 0.05$ ).

### Oxidative Stress Markers Compared between Patients with COPD and Healthy Control Subjects

**Systemic and pulmonary oxidative stress markers at rest.** Because of variation between electrophoresis runs, it is not possible to apply data for detection of differences in baseline values between patients and control subjects for ROS-induced DNA damage. Baseline values of plasma MDA were not significantly different between both groups. In contrast, urinary MDA excretion was significantly higher in the patients with COPD compared with the healthy control subjects ( $p < 0.05$ ; Table 3). No significant difference in the baseline values of plasma uric acid was found between both groups (Table 3). H<sub>2</sub>O<sub>2</sub> in breath condensate, considered to be a pulmonary oxidative stress marker, was also significantly increased in the patients with COPD compared with the healthy control subjects at baseline ( $p < 0.05$ ; Table 3).

**Exercise-induced systemic and pulmonary oxidative stress.** The comet assay was applied to measure ROS-induced DNA damage (tail moment) in PBMCs. In patients with COPD, a significant increase in ROS-induced DNA damage was observed immediately after the maximal exercise test, which did not return to baseline values after 4 h ( $p < 0.05$ ; Figure 1). After the submaximal exercise test, the same pattern of DNA damage was seen ( $p < 0.01$ ; Figure 1). In contrast, no significant effect for ROS-induced DNA damage was found after both exercise tests for the control group (Figure 1).

In addition to measurements of MDA in plasma, we also determined MDA in urine because urinary MDA is considered to be a marker of "whole body" oxidative stress. We found no significant increase in plasma MDA after the maximal or submaximal exercise test for both groups (data not shown). Conversely, immediately and 4 h after the maximal exercise test, the concentration of MDA in urine was significantly elevated in the patients with COPD ( $p < 0.05$  and  $p < 0.02$ , respectively; Figure 2). Also, immediately after the submaximal exercise test, we observed a significant increase in urinary MDA ( $p < 0.05$ ; Figure 2). In healthy control subjects, a significant increase in urinary MDA was found only 4 h after both exercise tests ( $p = 0.05$ ; Figure 2).

**TABLE 1. CHARACTERISTICS OF HEALTHY CONTROL SUBJECTS AND PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE BEFORE AND AFTER THE PULMONARY REHABILITATION PROGRAM**

	Healthy Control Subjects	Patients with COPD	COPD Before	COPD After
Sex, M/F	5/6	6/5		
Age, yr	59.7 $\pm$ 1.5	56.7 $\pm$ 2.0		
Pack-yr of smoking	1.9 $\pm$ 3.6	33.2 $\pm$ 3.7*		
Pam score	34.2 $\pm$ 6.8		14.5 $\pm$ 2.5 <sup>†</sup>	17.2 $\pm$ 2.8 <sup>‡</sup>
BMI, kg/m <sup>2</sup>	26.3 $\pm$ 1.1		25.6 $\pm$ 1.6	25.6 $\pm$ 1.3
FFMI, kg/m <sup>2</sup>	18.7 $\pm$ 0.9		17.5 $\pm$ 0.7	17.4 $\pm$ 0.5
FEV <sub>1</sub> , % predicted	111.1 $\pm$ 4.6		39.4 $\pm$ 4.1*	43.8 $\pm$ 4.6
FVC, % predicted	117.2 $\pm$ 5.6		76.6 $\pm$ 3.2*	93.1 $\pm$ 4.9 <sup>§</sup>
TLC, % predicted			136.5 $\pm$ 5.4	131.4 $\pm$ 6.3 <sup>§</sup>
RV, % predicted			230.1 $\pm$ 19.7	198.7 $\pm$ 14.8 <sup>§</sup>
D <sub>LCO</sub> , % predicted			61.1 $\pm$ 5.9	74.0 $\pm$ 9.5

*Definition of abbreviations:* BMI = body mass index; COPD = chronic obstructive pulmonary disease; D<sub>LCO</sub> = diffusion capacity; FFMI = fat-free mass index; Pam = Physical Activity Monitor; RV = residual volume; TLC = total lung capacity.

Data are presented as mean  $\pm$  SEM.

\* Significant difference compared with the healthy control subjects,  $p < 0.001$  (Mann-Whitney).

<sup>†</sup> Significant difference compared with the healthy control subjects,  $p < 0.01$  (Mann-Whitney).

<sup>‡</sup> Significant difference compared with values before rehabilitation,  $p < 0.05$  (Wilcoxon signed rank).

<sup>§</sup> Significant difference compared with values before rehabilitation,  $p < 0.01$  (Wilcoxon signed rank).

**TABLE 2. EXERCISE CHARACTERISTICS OF HEALTHY CONTROL SUBJECTS AND PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE BEFORE AND AFTER THE PULMONARY REHABILITATION PROGRAM**

	Healthy Control Subjects (n = 11)	COPD Before (n = 11)	COPD After (n = 11)
<b>Incremental exercise</b>			
Peak workload, W	201.7 ± 16.6	68.3 ± 7.4*	84.8 ± 8.8 <sup>‡</sup>
Peak $\dot{V}O_2$ , ml/min	2120 ± 182	1060 ± 74*	1265 ± 83 <sup>‡</sup>
Peak lactate, mmol/L	5.9 ± 0.6	3.1 ± 0.4 <sup>†</sup>	3.2 ± 0.5
Peak HR, beat/min	159 ± 4	126 ± 9 <sup>†</sup>	129 ± 7
HR reserve, %	0.3 ± 2.5	22.7 ± 5.0*	20.3 ± 3.6
Peak $\dot{V}E$ , L/min	81.0 ± 7.2	45.4 ± 3.8*	53.6 ± 4.7
Peak $\dot{V}E/MVV$	0.66 ± 0.04	1.21 ± 0.15*	1.26 ± 0.13
<b>Constant work-rate exercise</b>			
Duration time, min	11.5 ± 0.5	12.2 ± 3.3	21.2 ± 3.6 <sup>§</sup>
$\dot{V}O_2$ , ml/min	1700 ± 160	986 ± 88 <sup>†</sup>	1082 ± 78
$\Delta$ Lactate, mmol/L	2.4 ± 0.4	2.1 ± 0.8	1.2 ± 0.4
HR, beat/min	140.1 ± 4.0	126.4 ± 10.9	125.0 ± 12.2

*Definition of abbreviations:* COPD = chronic obstructive pulmonary disease; HR = heart rate; MVV = maximum voluntary ventilation.

Data are presented as mean ± SEM.

\* Significant difference compared with the healthy control subjects,  $p < 0.001$  (Mann-Whitney).

<sup>†</sup> Significant difference compared with the healthy control subjects,  $p < 0.01$  (Mann-Whitney).

<sup>‡</sup> Significant difference compared with before rehabilitation,  $p < 0.01$  (Wilcoxon signed rank).

<sup>§</sup> Significant difference compared with before rehabilitation,  $p < 0.02$  (Wilcoxon signed rank).

Plasma uric acid was assessed as a marker of xanthine-oxidase activity. Plasma uric acid in patients with COPD was significantly increased 4 h after the maximal and submaximal exercise tests ( $p < 0.01$  and  $p < 0.05$ , respectively; Figure 3). In healthy control subjects, a significant increase was observed only 4 h after the maximal exercise test, whereas immediately after the submaximal exercise test, a significant decrease in plasma uric acid was found ( $p < 0.02$  and  $p < 0.01$ , respectively; Figure 3). Moreover, the exercise-induced increase in plasma uric acid was significantly different between patients with COPD and healthy control subjects immediately and 4 h after the submaximal exercise test ( $p < 0.05$ ; Figure 3).

Exhaled  $H_2O_2$  was determined as an index of exercise-induced pulmonary oxidative stress. In patients with COPD, mean production of  $H_2O_2$  was significantly elevated 4 h after the maximal

exercise test relative to baseline, whereas for healthy control subjects, a significant increase was observed immediately after the maximal exercise test ( $p < 0.02$  and  $p < 0.05$ , respectively; Figure 4). In contrast, we found no significant effect on exhaled  $H_2O_2$  production after the submaximal exercise test for both groups (Figure 4). Exhaled  $H_2O_2$  production was significantly different between patients and control subjects 4 h after both exercise tests ( $p < 0.01$  and  $p < 0.05$ , respectively; Figure 4).

### Exercise-induced Oxidative Stress after Rehabilitation in Patients with COPD

A significant increase in ROS-induced DNA damage was found immediately after the maximal exercise test ( $p < 0.05$ ; Figure 1), which was similar to values observed before rehabilitation. In contrast to the data obtained before rehabilitation, we found no significant increase of ROS-induced DNA damage after the submaximal exercise test (Figure 1). Moreover, the differences with baseline values immediately after the submaximal exercise were significantly lower compared with values obtained before rehabilitation ( $p < 0.02$ ; Figure 1).

After rehabilitation, we found no significant increase in plasma MDA after the maximal and submaximal exercise test (data not shown). For the maximal exercise test, we only observed a significant effect of urinary MDA 4 h after exercise, whereas immediately after the submaximal exercise test, a significant effect was found ( $p < 0.05$  and  $p < 0.02$ , respectively; Figure 2). After rehabilitation, changes in plasma uric acid found after the maximal exercise test were similar to those observed before rehabilitation. In contrast to data obtained before rehabilitation, we detected no significant increase in plasma uric acid after the submaximal exercise test (Figure 3).

In addition, we observed no significant increase in pulmonary  $H_2O_2$  production after the maximal and submaximal exercise test. Remarkably, baseline values of the submaximal exercise test were significantly higher when compared with baseline values of the maximal exercise test ( $p < 0.01$ ; Figure 4).

## DISCUSSION

### Oxidative Stress Markers Compared between Patients with COPD and Healthy Control Subjects

*Systemic and pulmonary oxidative stress markers at rest.* In the present study, the resting values of plasma MDA were not significantly different between both groups. This result is in agreement with previous studies (2, 22). In contrast, increased oxidative stress at rest was observed in patients with COPD compared with healthy age-matched control subjects, as measured by urinary MDA. Plasma and urinary MDA, biomarkers of lipid peroxidation induced by oxidative stress, were determined using HPLC, which allows a good separation between MDA–thio-barbituric acid and other thiobarbituric acid–reactive substances, and minimizes spectrophotometric interference (23). In accordance with other studies, the pulmonary oxidative stress marker  $H_2O_2$  in breath condensate was significantly higher in the patients with COPD (4, 24, 25). This implies that patients with COPD have a persistent increased pulmonary oxidative burden. Although statistical significance was not reached, plasma uric acid concentration was slightly increased in the patients with COPD.

*Exercise-induced systemic and pulmonary oxidative stress.* This study is the first to demonstrate a significant increase in ROS-induced DNA strand breaks in PBMCs of patients with COPD after exercise. In contrast, in the healthy control subjects, no significant increase in ROS-induced DNA damage was observed after both exercise tests. The comet assay has already been applied to evaluate systemic oxidative stress in healthy partici-

**TABLE 3. OXIDATIVE STRESS MARKERS IN HEALTHY AGE-MATCHED CONTROL SUBJECTS AND PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE AT REST**

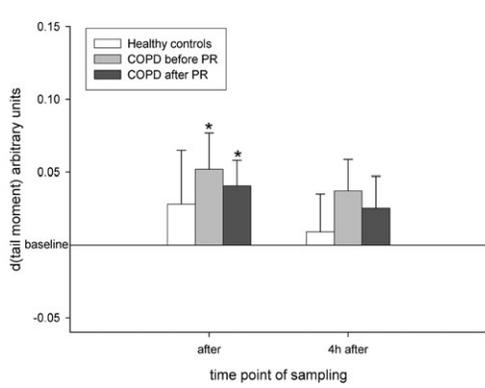
	Healthy Control Subjects (n = 11)	Patients with COPD (n = 11)
Plasma MDA, $\mu\text{mol/L}$	0.47 ± 0.03	0.56 ± 0.07
Urinary MDA, $\mu\text{mol/mmol creatinine}$	0.28 ± 0.04	0.38 ± 0.02*
Plasma uric acid, $\mu\text{M}$	238 ± 17	279 ± 22
Exhaled $H_2O_2$ , $\mu\text{mol/min}$	0.005 ± 0.001	0.020 ± 0.041*

*Definition of abbreviations:* COPD = chronic obstructive pulmonary disease; MDA = malondialdehyde.

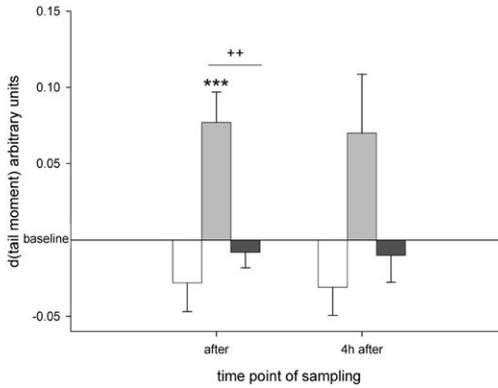
Data are presented as mean ± SEM of the two baseline values measured in both groups.

\* Significant difference compared with the healthy control subjects,  $p < 0.05$  (Mann-Whitney).

**Maximal exercise**



**Submaximal exercise**



**Figure 1.** Reactive oxygen species–induced DNA damage (tail moment) in peripheral blood mononuclear cells before and after pulmonary rehabilitation (PR) at different time points after the maximal and submaximal exercise test. Data represent differences with baseline values. Values are expressed as mean ± SEM. \**p* < 0.05; \*\*\**p* < 0.01 significantly different from baseline values (Wilcoxon signed rank); \*\**p* < 0.02 significantly different from values before PR (Wilcoxon signed rank). COPD = chronic obstructive pulmonary disease.

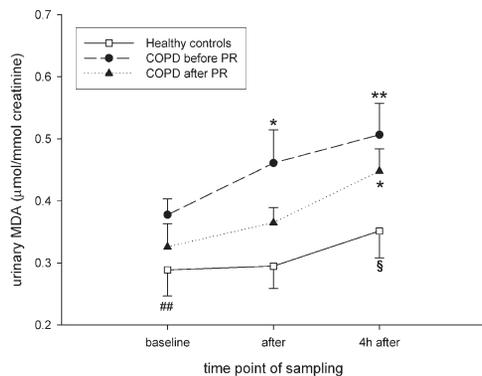
pants at various intensities and durations of exercise. Several studies have demonstrated exercise-induced DNA damage at 24 to 72 h after exhaustive treadmill running or a marathon race (26–28). It was also reported that no enhanced DNA damage was found in healthy control subjects after moderate exercise that did not exceed the anaerobic threshold (29). The fact that DNA damage in this study was found immediately after exercise in patients with COPD indicates that it is likely to be a consequence of exercise-induced ROS (30). This is remarkable because in previous studies with healthy participants, oxidative DNA damage was detected at later time points after exercise, suggesting that it might be a consequence of exercise-induced tissue injury and inflammation (26, 27, 29, 31).

The present study found no significant changes in plasma MDA from baseline values after the maximal and submaximal exercise test, in contrast to reports (1–3) that indicate increased plasma MDA after exercise in patients with COPD. Exercise also had no significant effects on plasma MDA in the healthy control subjects. Results from earlier studies investigating the changes in plasma MDA after exercise have also been inconclusive: some studies showed an effect of exercise on plasma MDA (32, 33), whereas other studies found no effect (34, 35). Possible explanations for the discrepancy may be due to the methodologies used in the different studies, the time points examined, level of training of the participants, or differences in exercise conditions. Our results indicate that the ROS production during both exercise tests was not extensive enough to detect a significant increase in plasma MDA after exercise because once alde-

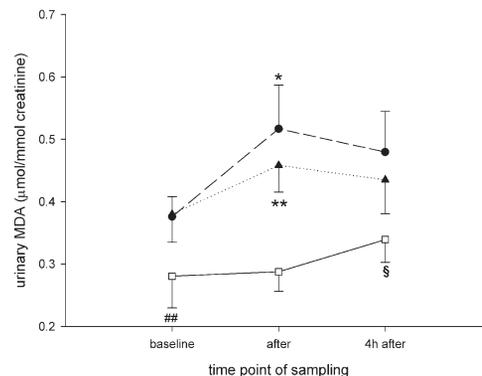
hydes, like MDA, are formed and enter the circulation, they are rapidly excreted.

In contrast to plasma levels, in patients with COPD, the MDA excretion in urine, as a marker of “whole body” oxidative stress, was significantly elevated immediately after both exercise tests and also 4 h after the maximal exercise test, providing evidence for exercise-induced oxidative damage. Potential sources for increased ROS generation during exercise include leakage from the mitochondrial electron transport system (36), the xanthine-oxidase/dehydrogenase system (1, 37), and the inflammatory response. In healthy control subjects, a significant increase was observed only 4 h after both exercise tests. This delayed effect on urinary MDA excretion after exercise might be related to exercise-induced tissue injury. Until now, no other studies have examined the effect of exercise on urinary MDA in patients with COPD. In the present study, the absence of an immediate exercise-induced systemic oxidative stress response in healthy control subjects is probably due to the fact that the duration of the exercise tests was too short to cause immediate exercise-induced oxidative damage. The duration of the submaximal exercise test in the control group was standardized at 12 min, because otherwise the difference in duration could have been too large compared with the patients with COPD. It is also likely that, in healthy control subjects, the antioxidant defense system is able to cope with an increased production of ROS generated by those two exercise tests. However, in patients with COPD, apparently the antioxidant defense system can be overwhelmed by the ROS, leading to an increased systemic oxidative stress response after exercise.

**Maximal exercise**

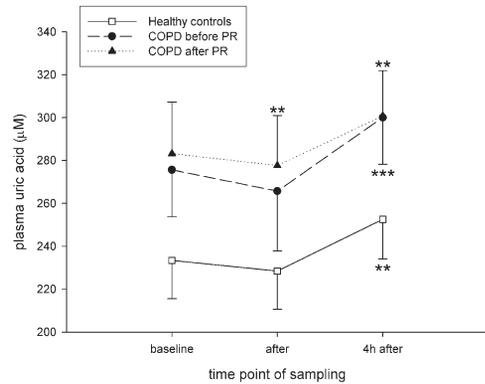


**Submaximal exercise**

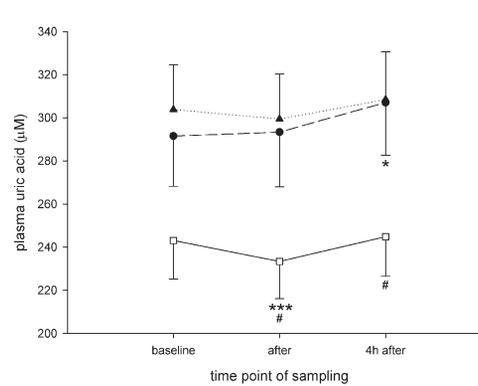


**Figure 2.** Concentration of urinary malondialdehyde (MDA) before and after PR at different time points after the maximal and submaximal exercise test. Values are expressed as mean ± SEM. \**p* < 0.05; \*\**p* < 0.02; §*p* = 0.05 significantly different from baseline values (Wilcoxon signed rank); ##*p* < 0.02 significantly different between healthy control subjects and subjects with COPD before PR (Mann-Whitney). There were no statistically significant differences between values before and after PR.

## Maximal exercise



## Submaximal exercise



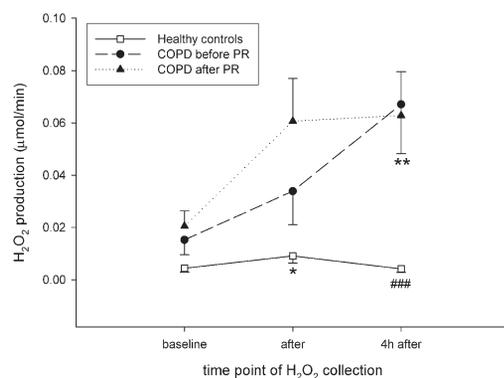
**Figure 3.** Concentration of plasma uric acid before and after PR at different time points after the maximal and submaximal exercise test. Values are expressed as mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.02$ ; \*\*\* $p < 0.01$  significantly different from baseline values (Wilcoxon signed rank); # $p < 0.05$  significantly different between healthy control subjects and subjects with COPD before PR (Mann-Whitney). There were no statistically significant differences between values before and after PR.

In our study, plasma uric acid, as a marker of xanthine-oxidase activity, was significantly increased 4 h after the maximal and submaximal exercise test, whereas in healthy control subjects, a significant increase was observed only 4 h after the maximal exercise test. This finding is consistent with previous studies reporting increased plasma uric acid in response to strenuous exercise in healthy control subjects (38, 39). It is expected that the activation of xanthine oxidase is more prominent for the maximal exercise test, because during strenuous exercise, in combination with insufficient oxygen supply, ATP is consumed faster than it can be regenerated, resulting in a build-up of AMP. Furthermore, in the muscle cells, AMP is continuously degraded to hypoxanthine, which may be converted to xanthine and subsequently to uric acid by xanthine oxidase. Xanthine oxidase uses molecular oxygen as an electron acceptor, resulting in the formation of superoxide radicals (40). Although under aerobic conditions this reaction may be catalyzed by xanthine dehydrogenase, which uses nicotinamide adenine dinucleotide as electron acceptor, rather than xanthine oxidase (41). In addition, immediately after the submaximal exercise test, a significant decrease in plasma uric acid was observed in healthy control subjects. This was reported earlier by Hellsten and colleagues (38), who found that uric acid was extracted by the muscle immediately after termination of strenuous exercise, in part via uptake from plasma.

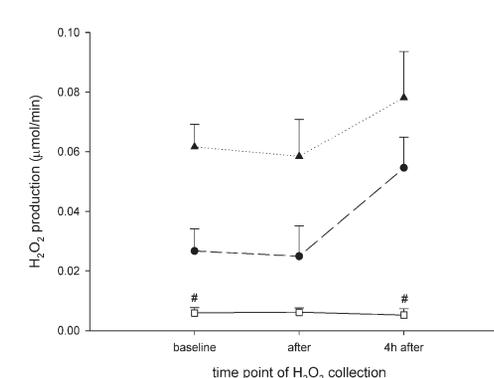
In the present study, we observed that  $H_2O_2$  concentration in the EBC of patients with COPD was significantly increased 4 h after the maximal exercise test, whereas in healthy control

subjects, a slight, but significant increase immediately after maximal exercise was found, indicating an enhanced production of ROS in the airways of these participants. In contrast, we found no significant increase after the submaximal exercise test for both groups. This suggests that exercise-induced pulmonary oxidative stress measured by  $H_2O_2$  seems to be more related to exercise intensity than to duration. A likely explanation for this effect can be attributed to the differences in pulmonary ventilatory recruitment between the maximal and submaximal exercise test. Because ventilatory demands increase more during the maximal compared with the submaximal exercise test, patients with COPD tend to take more shallow breaths and to breathe with higher frequency, resulting in dynamic lung hyperinflation. This results in increased physiologic dead space ventilation, with an attendant drop in alveolar oxygen partial pressure, causing hypoxia. Consequently, decreased alveolar oxygen could induce lung inflammation (42–44), which may lead to the increased  $H_2O_2$  production that was observed in patients with COPD 4 h after the maximal exercise test. Earlier, Schleiss and coworkers (45) reported that the  $H_2O_2$  concentration in exhaled air is dependent on expiratory flow rates, because  $H_2O_2$  levels increased with decreasing flow rates, although in animal experiments, changes in minute ventilation and breathing pattern did not alter  $H_2O_2$  exhalation (46). In our study, we did not control expiratory flow rates. However, assuming that the expiratory flow rate increases after exercise and in particular after the maximal exercise test, our data are likely to be an underestimation of the  $H_2O_2$  production and are therefore still valid.

## Maximal exercise



## Submaximal exercise



**Figure 4.** Production of hydrogen peroxide ( $H_2O_2$ ) measured in exhaled breath condensate before and after PR at different time points after the maximal and submaximal exercise test. Values are expressed as mean  $\pm$  SEM. \* $p < 0.05$ ; \*\* $p < 0.02$  significantly different from baseline values (Wilcoxon signed rank); # $p < 0.05$ ; ### $p < 0.01$  significantly different between healthy control subjects and patients with COPD before PR (Mann-Whitney). There were no statistically significant differences between values before and after PR.

### Exercise-induced Oxidative Stress after Rehabilitation in Patients with COPD

After rehabilitation, we observed a similar increase in DNA damage and plasma uric acid levels after the maximal exercise test as we did before rehabilitation. An additional finding was that the concentration of plasma uric acid slightly, but significantly decreased immediately after the maximal exercise test. The most striking finding in the present study was that, after rehabilitation, we did not detect a significant increase in ROS-induced DNA damage after the submaximal exercise test. This decrease in ROS-induced DNA damage may be attributable to an improved training status of the patients with COPD, because the patients had an increased exercise capacity and physical activity level, as indicated by the Pam score, after rehabilitation. This is in line with a previous study (31), which reported that the extent of DNA damage was dependent on the training status of the healthy participants, because trained participants had less DNA damage compared with untrained participants after exercise.

On the basis of these results, we can conclude that intensive supervised pulmonary rehabilitation reduces exercise-induced DNA damage, when performed at moderate intensity. Moreover, the submaximal exercise test seems to be more discriminative to evaluate the effect of pulmonary rehabilitation.

After rehabilitation, there were no statistically significant changes in plasma MDA after both exercise tests. For urinary MDA, results were similar to those observed before rehabilitation, with the exception of the maximal exercise test, for which we no longer found a significant increase immediately after the exercise test.

In general, we observed a tendency for a decrease in systemic exercise-induced oxidative stress after rehabilitation, when also taking into account that the intensity and duration were higher after rehabilitation. This could be a consequence of an improved oxidative metabolism or an increased capacity of endogenous antioxidative systems. Previously, Rabinovich and colleagues (47) reported that patients with COPD had a reduced ability to adapt to endurance training, as reflected by a lower capacity to synthesize reduced glutathione. The reduced exercise-induced oxidative stress response may be attributable to an improved oxidative metabolism rather than to an upregulation of antioxidant defenses, although we cannot rule out the possibility that the effects were caused by the induction of antioxidant adaptations. Further studies are needed to clarify the mechanisms involved in the exercise-induced oxidative stress response and possible adaptations after pulmonary rehabilitation.

After rehabilitation, we observed no significant increase in exhaled H<sub>2</sub>O<sub>2</sub> after both exercise tests, despite a significant increase in intensity and duration. This effect would probably be more pronounced if we had measured at similar intensity and duration before and after rehabilitation. Another striking effect deriving from the data presented is that baseline pulmonary H<sub>2</sub>O<sub>2</sub> production at the submaximal exercise test was significantly higher compared with data obtained from the maximal exercise test. This effect was also found before rehabilitation, but was not significant. It can be speculated that the increased baseline H<sub>2</sub>O<sub>2</sub> production might be due to the fact that the patients performed the submaximal exercise test after the weekend. The patients spent the weekend at home, and lifestyle factors, especially passive-smoking behavior, may have been different from those in the pulmonary rehabilitation center.

### Methodologic Considerations/Limitations of the Study

In this study, the maximal and submaximal exercise tests were not performed at similar workload and duration time (isotime) before and after rehabilitation. However, measurements of ox-

idative stress at isotime would probably have led to even more pronounced differences between measurements before and after rehabilitation, because it has been suggested that oxidative stress increases with the intensity and duration of the exercise.

### Conclusions

In conclusion, the present study clearly indicates that patients with COPD, when compared with age-matched healthy control subjects, are characterized by increased systemic and pulmonary oxidative stress markers both at rest as well as induced by exercise. This suggests that healthy control subjects are able to tolerate exercise more effectively than patients with COPD. Moreover, this study showed for the first time that intensive supervised pulmonary rehabilitation was associated with decreased systemic exercise-induced oxidative stress, especially after submaximal exercise. This decrease was accompanied by a significantly improved exercise capacity in patients with COPD after rehabilitation. The observed improvement may be attributable to adaptive responses involving a more efficient oxidative metabolism or an increased capacity of endogenous antioxidative systems. The possible mechanisms underlying improved exercise capacity and decreased exercise-induced oxidative stress after intensive supervised pulmonary rehabilitation, and the outcome of specific nutritional or pharmacologic modulation on exercise-induced oxidative stress, will be the subject of further investigations.

**Conflict of Interest Statement:** E.M.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.J.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.M.W.J.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.A.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.F.M.W. serves as a consultant to GlaxoSmithKline (GSK) and is a member of scientific advisory boards for GSK, Boehringer Ingelheim, AstraZeneca, Centocor, and Numico. He has received lecture fees from GSK, AstraZeneca, Boehringer Ingelheim, Pfizer, and Numico, and also received research grants between 2001 and 2004 from GSK, AstraZeneca, Boehringer Ingelheim, Centocor, and Numico.

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