The exercise-induced oxidative stress paradox in COPD

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The exercise-induced oxidative stress paradox in COPD

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The exercise-induced oxidative stress paradox in COPD

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. mr. G.P.M.F. Mols,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 13 februari 2009 om 14.00 uur

door

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Als sterren konden spreken,
Als sterren konden zien,
Zou je dan heel eventjes willen zwaaien,
En twinkelen als een groet,
Zodat ik hier beneden weet,
Dat het goed met je gaat.

Opgedragen aan mijn oma

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CHAPTER 1

General introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive and irreversible airflow obstruction. COPD is a major public health problem and its prevalence keeps increasing yearly. No effective therapeutic strategy currently exists. COPD is associated not only with an abnormal pulmonary inflammatory process but also by extra-pulmonary manifestations including systemic inflammation and skeletal muscle weakness (for review see (1)). Peripheral muscle dysfunction is a prominent and disabling systemic feature of patients with COPD and is characterized by specific anatomic changes (e.g. shift in fiber type composition and muscle fiber atrophy) and functional changes (e.g. decreased strength, decreased endurance and altered metabolic enzyme activities) (2). Peripheral muscle dysfunction significantly contributes to exercise intolerance and impaired quality of life in these patients (2). Importantly, reduced muscle mass is also a strong predictor of mortality in COPD, independent of the degree of lung function impairment (3). Currently, pulmonary rehabilitation and anabolic hormones have been investigated as therapeutic strategies of peripheral muscle dysfunction. The scientific rationale for undertaking pulmonary rehabilitation for patients with COPD is well established since it has been consistently shown in Cochrane reviews that it relieves dyspnea and improves exercise capacity and health-related quality of life (HRQL) in spite of irreversible abnormalities in lung function (4, 5). Exercise training has been shown to be the cornerstone of a pulmonary rehabilitation program in patients with moderate to severe COPD (6-8). To optimize the training stimulus during pulmonary rehabilitation, it is important to tailor exercise training programs to individual patients based on appropriate phenotyping and function tests. Several interventions (i.e. endurance training, interval training and resistance training) or a combination thereof can be applied (9, 10). It is generally acknowledged that endurance types of exercise (i.e. treadmill walking and ergometry cycling) are indicated for patients with moderate COPD who have a cardio-circulatory limitation during a symptom-limited incremental exercise test, while interval types of exercise are indicated for patients with severe COPD who are ventilatory limited at the end of a symptom-limited incremental exercise test (11). Whole body endurance training at a high intensity resulted in significant improvements in HRQL, exercise capacity and peripheral muscle force in COPD patients (10, 12, 13). In practice, however, patients are not always able to execute high intensity endurance exercises because of obvious symptoms of dyspnea and leg fatigue (14). For these patients, who generally suffer from severe muscle weakness or profound ventilatory limitation to exercise, resis-

tance training is advised. Resistance training, an exercise modality in which small muscle groups are trained by repetitively lifting heavy weights has been reported to induce only moderate cardiac and ventilatory responses (15). Combination of both training interventions was shown to have complementary effects (i.e. effect on strength and endurance) (10, 16). Therefore, the combination of resistance and endurance training is advised in pulmonary rehabilitation in advanced COPD.

Figure 1. Model of peripheral muscle dysfunction in COPD.

Several mechanisms for peripheral muscle dysfunction have been proposed but the precise contribution of each is yet unknown. Chronic inflammation and oxidative stress are prominent features of COPD and may be implicated in the etiology of peripheral muscle dysfunction (17, 18) (see Figure 1). Increased levels of circulating cytokines (interleukin (IL)-6,-8, and tumor necrosis factor- α (TNF- α)) and acute-phase proteins (C-reactive protein (CRP)) have been reported in stable COPD patients at rest, reflecting a low-grade systemic inflammation in these patients (19-23). Moreover, elevated CRP levels and cytokines are associated with impaired exercise capacity in patients with COPD (24, 25). It is expected that physical exercise will further increase this inflammatory response. However, contradictory results have been reported for the exercise-induced inflammatory response in COPD (26-

28). At present, limited information is available regarding exercise as modulator of the systemic inflammatory response and also locally in skeletal muscle of patients with COPD.

Several investigations have shown that markers of oxidative stress are increased in patients with COPD and even more pronounced during exacerbations (29, 30). Oxidative stress refers to an imbalance in the pro- and antioxidant status in favour of the former. Increased production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) can cause oxidative modifications of proteins, lipids and DNA. Exercise training may be beneficial for patients with COPD, in part by improving exercise intolerance (endurance and strength), but paradoxically, exercise is known, if sufficiently intense, to elicit an oxidative stress response in healthy subjects (for review see (31)). The paradox arises if under these circumstances exercise is advisable to COPD patients. Recent evidence indicates that exercise results in increased systemic and muscle oxidative stress in patients with moderate-to-severe COPD (32-35), strongly suggesting that these patients are frequently exposed to oxidative damage as they perform their daily living activities. In addition, an impairment of the adaptive response to reduce oxidative stress has also been described in COPD patients (36, 37), thus amplifying the deleterious effects of ROS. These data are indicative of increased oxidative stress in COPD, especially during exercise, being the result of enhanced oxidant formation and/or impaired antioxidant capacity.

Besides the detrimental effects of ROS, there is increasing evidence that ROS have a critical role in muscle adaptation to exercise-induced oxidative stress by modulating gene expression via redox-sensitive transcription pathways (38-40). One of the most important signaling pathways that could be modulated by ROS involves the transcription factor nuclear protein κ B (NF- κ B) (for review see (41)). NF- κ B is involved in a wide variety of physiological processes such as immunity and inflammatory responses, antioxidant defense, as well as cell growth and survival. *Chronic* activation of this signaling pathway by oxidative stress has been suggested to play a role in the etiology of the systemic features of COPD including muscle wasting and altered insulin sensitivity (42-44). Paradoxically, evidence exists that an *acute* bout of exercise potently activates NF- κ B signaling in skeletal muscle of healthy subjects. The functions of exercise-stimulated increases in NF- κ B activity are currently unknown, but it is suggested that NF- κ B acts as a beneficial mediator of exercise-induced adaptations to cellular stress. Thus far, the physiological role of NF- κ B pathway during exercise is still elusive in COPD.

In addition, there is substantial evidence to support the notion that impaired muscle oxidative capacity (as shown by reduced activity of the enzymes citrate synthase and hydroxyacetyl-CoA dehydrogenase (45, 46)) may contribute to peripheral

muscle dysfunction in patients with COPD. Besides a disturbed oxidant/antioxidant balance also an impaired oxidative capacity can result in increased muscle lipid peroxidation. Indeed, increased levels of intramuscular lipid peroxidation products have been reported for COPD at baseline and in response to exercise (35, 47). Emerging literature strongly supports the notion that uncoupling protein-3 (UCP3) plays a role in the protection of mitochondria against lipotoxicity, since it is capable of reducing ROS production by lowering the mitochondrial membrane potential and/or the removal of fatty acid anions from the mitochondrial matrix (48-50). Accordingly, Gosker et al. (51) previously showed that muscle UCP3 levels are increased by exercise training in COPD patients with a blunted response of the β -oxidation, suggesting that UCP3 was upregulated in response to an insufficient capacity to oxidize free fatty acids. Until now, the effect of acute exercise on muscular UCP3 in COPD is still lacking.

The metabolic consequences of changes in oxidative capacity in COPD are reflected in alterations in muscle energy status. Pouw et al. (52) found higher inosine monophosphate levels, which were negatively related with ATP/ADP ratios, indicative for an imbalance between the utilization and resynthesis of ATP in resting muscle of patients with COPD. Moreover, a significant muscle adenine nucleotide loss during exercise has been demonstrated in patients with COPD suggesting that ATP resynthesis is unable to meet the energy demands of exercise even at the low absolute exercise intensities COPD patients can achieve (53). Recently, it has been shown in COPD patients that by modulating skeletal muscle oxidative energy metabolism using dichloroacetate (DCA) infusion before a maximal exercise challenge, blood lactate and ammonia accumulation decreased and exercise performance improved (54). However, this study did not address possible effects on exercise-induced oxidative stress. It can be hypothesized that by increasing muscle energy status in COPD, DCA could additionally decrease oxidative stress. This modulation of oxidative stress could also play a role in the therapeutic effect of DCA.

AIM AND OUTLINE OF THE THESIS

The overall aim of this thesis was to evaluate the effects of acute and prolonged exercise on pulmonary, systemic and muscle oxidative stress, and inflammatory responses in patients with COPD.

The primary aims of this thesis are:

- To evaluate the 'exercise paradox' by exploring the effect of pulmonary rehabilitation on exercise-induced oxidative stress in COPD.
- To establish test protocols to examine exercise-induced oxidative stress and systemic inflammatory responses in COPD.
- To explore potential differential effects on NF- κ B mediated gene expression in response to acute exercise in COPD versus healthy controls.
- To examine the effect of acute exercise on muscular UCP3 gene expression in patients with COPD compared to healthy controls.
- To assess the effect of a pharmacological agent that specifically target skeletal muscle oxidative energy metabolism on systemic exercise-induced oxidative stress and inflammation in COPD patients.

Despite the clear health benefits of exercise training in COPD, exercise-induced oxidative stress can adversely affect outcome in COPD. 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, **Chapter 2** presents the effects of maximal and submaximal exercise and the effect of rehabilitation on systemic and pulmonary oxidative stress. Besides commonly used oxidative stress markers such as malondialdehyde, the comet assay in peripheral blood mononuclear cells was introduced as a new method. The comet assay is a sensitive technique for analyzing ROS-induced DNA damage. Whether peripheral skeletal muscle is a potential source of exercise-induced oxidative stress and inflammation was investigated in two ways. **Chapter 3** describes a study aimed at investigating the effect of a constant work rate single leg ergometer test on oxidative stress and inflammation in patients with COPD. The advantage of this exercise modality is that during a localized quadriceps exercise, work is performed almost exclusively by one muscle group, which minimizes cardiac and ventilatory stress responses (55). Additionally, this exercise test reflects the metabolic stress during resistance type of exercise training that is recommended for COPD patients with severe ventilatory impairment. In **Chapter 4**, NF- κ B mediated gene expression, inflammation, survival and muscle differentiation as well as the antioxidant status was examined in skeletal muscle of patients with COPD versus healthy controls in response to an acute bout of exercise. Muscle biopsies of the vastus lateralis were obtained before, immedi-

ately after and 2h after a submaximal cycle ergometer test of standardized duration and exercise intensity. Besides a disturbed muscle oxidant/antioxidant balance, an impaired oxidative capacity (46) could result in increased muscle lipid peroxidation and this could be exaggerated in response to an acute exercise bout. In **Chapter 5**, we tested the hypothesis that in COPD patients muscle gene expression of UCP3 is upregulated during acute exercise as an adaptation to a blunted response of the antioxidant defense system and β -oxidation. The effect of dichloroacetate (DCA), a pharmacological agent known to activate muscle pyruvate dehydrogenase complex, on systemic exercise-induced oxidative stress and inflammation was examined in COPD patients in **Chapter 6**. Finally, in **Chapter 7**, a general discussion of this thesis, together with some further prospects is presented.

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CHAPTER 2

Rehabilitation decreases exercise-induced oxidative stress in chronic obstructive pulmonary disease

ABSTRACT

The effect of exercise at different intensities as well as the effect of intensive supervised pulmonary rehabilitation on oxidative stress was studied for chronic obstructive pulmonary disease (COPD). Eleven COPD patients and eleven healthy age-matched controls performed a maximal and submaximal exercise cycle ergometry test at 60% of peak workload. COPD patients performed these tests before and after 8 weeks of pulmonary rehabilitation. Measurements were done before, immediately after and 4 hours after both exercise tests. At rest, increased oxidative stress was observed in patients compared to controls, as measured by urinary malondialdehyde (MDA) ($p < 0.05$) and hydrogen peroxide (H_2O_2) in breath condensate ($p < 0.05$). In healthy controls, a significant increase in urinary MDA was observed 4 hours after both exercise tests ($p = 0.05$), whereas H_2O_2 significantly increased immediately after maximal exercise ($p < 0.05$). In COPD patients, before rehabilitation, reactive oxygen species (ROS)-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 ROS-induced DNA damage was detected after exercise at submaximal intensity despite increased exercise duration of 73%. In summary, COPD patients 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.

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INTRODUCTION

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 (TBARs), and increased protein oxidation (1-3). Additional evidence supporting increased exercise-induced systemic oxidative stress in COPD patients 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 et al. (4) reported increased hydrogen peroxide (H_2O_2) in exhaled breath condensate (EBC) in COPD patients compared to 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 COPD patients and in healthy age-matched controls. As an additional marker of systemic oxidative stress, the comet assay in peripheral blood mononuclear cells (PBMCs) was introduced as a new method and yet unexplored in COPD. It is a sensitive technique for analysing reactive oxygen species (ROS)-induced DNA damage in peripheral blood mononuclear cells (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 COPD patients was assessed by analysing 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 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 in-patient pulmonary rehabilitation program on weekdays over 8 weeks (10, 11) ($FEV_1 < 60\%$ predicted and $FEV_1/FVC < 70\%$ and $< 10\%$ predicted improvement in FEV_1 after β_2 -agonist inhalation) 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 weeks prior to 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 non-smoking 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 β_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 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, COPD patients 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, anthropometric measurements and they had to wear a physical activity monitor during 9 consecutive days. First, they completed an incremental cycle ergometry exercise test until exhaustion and after four days 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_2O_2 were obtained at baseline, immediately after and 4 hours 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 COPD patients.

Pulmonary Function Tests

All participants underwent flow volumes including measurements of FEV_1 and FVC with the highest value from at least three properly performed measurements being used for analysis. Also lung volumes and diffusion capacity were obtained in COPD patients. Residual volume (RV) and total lung capacity (TLC) were assessed by whole-body plethysmography and diffusion capacity for carbon monoxide (Dlco)

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 Watt/minute) cycle ergometry test as described previously (14). Expired gases were analysed using breath-by-breath analysis through a breathing mask (Oxygen-β, Jaeger, Würzburg, Germany). Simultaneously, heart rate was registered every minute (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 minutes. For the control group the endurance time was standardized at 12 minutes, which was the mean endurance time of the COPD patients at baseline. Also 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 analyse 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 (EDTA)-containing tubes (Venoject). All blood samples were immediately put on ice, and kept on ice during sample preparation. Plasma was obtained by centrifugation (800 x g for 10 minutes 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 x g for 10 minutes 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 (Greiner) of peripheral blood mononuclear cells (PBMCs). After centrifugation at 800 x *g* for 30 minutes at 4°C, gradient-separated PBMCs were recovered, resuspended in 10 ml cold PBS and centrifuged again at 250 x *g* for 10 minutes 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 (LMPA) of 37°C and layered onto pre-chilled agarose-coated (1.5% agarose in PBS) microscope slides. The slides were covered with a coverslip and kept at 4°C for 5 minutes to allow the LMPA 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 Na₂EDTA, 10 mM Tris (pH 10), 1% Triton X-100, 10% DMSO). For reasons of stability of the DNA during lysis, the PBMCs were kept at 4°C for a period of maximally 6 weeks (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 Na₂EDTA pH 12.5-13.5) for 20 minutes to allow for DNA unwinding and expression of alkali-labile sites. The electrophoresis was subsequently conducted at 4°C, for 20 minutes, 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 microscope analysis, 50 µl ethidium bromide (20 µg/ml) was added to each slide and fifty randomly selected cells were analysed from each sample. Comet measurements were made by image analysis using a fluorescence microscope and the Comet Assay III software (Perceptive Instruments). 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 standard error of the mean of tail moment were calculated from each sample. Median of each sample was taken since data had a non-normal distribution.

Online Figure E1. Representative comet image and comet measurements Tail Moment = Tail Length (μm) * Tail DNA (%) (Tail DNA = Tail fluorescence / Total fluorescence)

MDA in Plasma and Urine

The determination of MDA was assayed by measuring TBARs according to the high-performance liquid chromatography (HPCL) method described by Lepage et al. (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% (w/v) trichloroacetic acid (TCA), centrifuged and uric acid was assayed by a HPLC method described by Lux et al. (19) using a Hypersil BDS C18 end-capped column (125 x 4 mm, particle size μm , Agilent). Additional details on this method are available in the online supplement.

Collection of EBC and Measurement of H_2O_2

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 a period of 10 minutes. The EBC samples were immediately divided into aliquots, stored at -80°C and thawed at room temperature just before analysis.

EBC H_2O_2 was measured by means of horseradish peroxidase-catalysed oxidation of tetramethylbenzidine according to the method previously described by Gallati and

Pracht and modified for microtiter plate-based analysis (21). All samples were measured in duplicate and a separate standard curve for H_2O_2 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 analysed 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 COPD patients. Non-parametric 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, USA).

RESULTS

Anthropometric and Spirometric Data

As shown in table 1, anthropometric data were not significantly different between COPD patients and control participants. The COPD group showed a moderate to severe airflow obstruction with an FEV_1 of $39.4 \pm 4.1\%$ predicted. In patients, FVC, TLC and RV were significantly ($p < 0.05$) improved after rehabilitation, while BMI and FFMI were not significantly different and within the normal range (12). The Pam Score for healthy controls classified them moderately active, whereas the COPD patients had a low activity level (Table E1 in the online supplement). Physical activity level of COPD patients was significantly ($+19\%$, $p < 0.05$) increased after rehabilitation.

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, years	59.7 ± 1.5	56.7 ± 2.0		
Pack-years of smoking	1.9 ± 3.6	33.2 ± 3.7*		
Pam Score	34.2 ± 6.8		14.5 ± 2.5 [†]	17.2 ± 2.8 [‡]
BMI, kg/m ²	26.3 ± 1.1		25.6 ± 1.6	25.6 ± 1.3
FFMI, kg/m ²	18.7 ± 0.9		17.5 ± 0.7	17.4 ± 0.5
FEV ₁ , % predicted	111.1 ± 4.6		39.4 ± 4.1*	43.8 ± 4.6
FVC, % predicted	117.2 ± 5.6		76.6 ± 3.2*	93.1 ± 4.9 [§]
TLC, % predicted			136.5 ± 5.4	131.4 ± 6.3 [‡]
RV, % predicted			230.1 ± 19.7	198.7 ± 14.8 [§]
DLco, % predicted			61.1 ± 5.9	74.0 ± 9.5

Definition of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary disease; DLco = diffusion capacity; FFMI = fat-free mass index; Pam = Physical Activity Monitor; RV = residual volume; TLC = total lung capacity. Data are presented as mean ± SEM. *Significant difference compared with the healthy control subjects, $p < 0.001$ (Mann-Whitney). [†]Significant difference compared with the healthy control subjects, $p < 0.01$ (Mann-Whitney). [‡]Significant difference compared with values before rehabilitation, $p < 0.05$ (Wilcoxon signed ranks). [§]Significant difference compared with values before rehabilitation, $p < 0.01$ (Wilcoxon signed ranks).

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 COPD patients when compared with healthy controls ($p < 0.001$). Also peak lactate was significantly lower in COPD patients than in healthy controls ($p < 0.01$). Nevertheless, the COPD patients exceeded their maximum voluntary ventilation ($\dot{V}_E/MVV > 100\%$), as a consequence of their ventilatory limitation, maximum exercise intensity was reached, whereas the heart rate reserve was preserved. The healthy controls were within the normal range for these two parameters. Additional details on these measurements are provided in the online supplement. The COPD patients 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%, $p < 0.05$).

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	COPD Before	COPD After
	(n=11)	(n=11)	(n=11)
Incremental exercise			
Peak workload, watt	201.7 ± 16.6	68.3 ± 7.4*	84.8 ± 8.8 [‡]
Peak $\dot{V}_E$ O ₂ , mL/min	2120 ± 182	1060 ± 74*	1265 ± 83 [§]
Peak lactate, mmol/L	5.9 ± 0.6	3.1 ± 0.4 [†]	3.2 ± 0.5
Peak HR, beat/min	159 ± 4	126 ± 9 [†]	129 ± 7
HRreserve, %	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
Constant work-rate exercise			
Duration time, minutes	11.5 ± 0.5	12.2 ± 3.3	21.2 ± 3.6 [§]
$\dot{V}_E$ O ₂ , mL/min	1700 ± 160	986 ± 88 [†]	1082 ± 78
Δ 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). [†]Significant difference compared with the healthy control subjects, $p < 0.01$ (Mann-Whitney). [‡]Significant difference compared with before rehabilitation, $p < 0.02$ (Wilcoxon signed ranks). [§]Significant difference compared with before rehabilitation, $p < 0.02$ (Wilcoxon signed ranks).

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 COPD patients compared with the healthy controls ($p < 0.05$ respectively; Table 3). No significant difference in the baseline values of plasma uric acid was found between both groups (Table 3). H₂O₂ in breath condensate, considered to be a pulmonary oxidative stress marker, was also significantly increased in the COPD patients compared to the healthy controls at baseline ($p < 0.05$; Table 3).

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)	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	$0.38 \pm 0.02^*$
Plasma Uric acid, μM	238 ± 17	279 ± 22
Exhaled H_2O_2 , $\mu\text{mol/min}$	0.005 ± 0.001	$0.020 \pm 0.041^*$

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; MDA = malondialdehyde. Data are presented as mean $\pm$ SEM of the two baseline values measured in both groups. *Significant difference compared with the healthy control subjects, $p < 0.05$ (Mann-Whitney).

Exercise-induced systemic and pulmonary oxidative stress

The comet assay was applied to measure ROS-induced DNA damage (tail moment) in PBMCs. In COPD patients, a significant increase in ROS-induced DNA damage was observed immediately after the maximal exercise test, which was not returned to baseline values after 4 hours ($p < 0.05$; 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).

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 $\pm$ SEM. * $p < 0.05$; *** $p < 0.01$ significantly different from baseline values (Wilcoxon signed ranks); ** $p < 0.02$ significantly different from values before PR (Wilcoxon signed ranks). COPD = chronic obstructive pulmonary disease.

In addition to measurements of MDA in plasma, we also determined MDA in urine since 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 after and 4 hours after the maximal exercise test, the concentration of MDA in urine was significantly elevated in the COPD patients ($p < 0.05$, $p < 0.02$ 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 controls, a significant increase in urinary MDA was found only 4 hours after both exercise tests ($p = 0.05$; Figure 2).

Maximal exercise

Submaximal exercise

Figure 2. Concentration of urinary malondialdehyde (MDA) before and after pulmonary rehabilitation (PR) at different time points after the maximal and submaximal exercise test. Values are expressed as mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.02$; $^{\S}p = 0.05$ significantly different from baseline values (Wilcoxon signed ranks); $^{##}p < 0.02$ 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.

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

Maximal exercise **Submaximal exercise**

Figure 3. Concentration of plasma uric acid before and after pulmonary rehabilitation (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 ranks); # $p < 0.05$ 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.

Exhaled H_2O_2 was determined as an index of exercise-induced pulmonary oxidative stress. In COPD patients, mean production of H_2O_2 was significantly elevated 4 hours after the maximal exercise test relative to baseline, whereas for healthy controls a significant increase was observed immediately after the maximal exercise test ($p < 0.02$; $p < 0.05$ 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 controls 4 hours after both exercise tests ($p < 0.01$; $p < 0.05$ respectively; Figure 4).

Maximal exercise

Submaximal exercise

Figure 4. Production of hydrogen peroxide (H_2O_2) measured in exhaled breath condensate before and after pulmonary rehabilitation (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 ranks); # $p < 0.05$; ### $p < 0.01$ 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.

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 to 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 hours after exercise, whereas immediately after the submaximal exercise test a significant effect was found ($p < 0.05$; $p < 0.02$ 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 COPD patients compared to healthy age-matched controls, 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-TBA and other TBARs 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 COPD patients (4, 24, 25). This implies that COPD patients have a persistent increased pulmonary oxidative burden. Although statistical significance was not reached, plasma uric acid concentration was slightly increased in the COPD patients.

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 COPD patients after exercise. In contrast, in the healthy controls, 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 participants at various intensities and durations of exercise. Several studies have demonstrated exercise-induced DNA damage at 24-72 hours following exhaustive treadmill running or a marathon race (26-28). It was also reported that no enhanced DNA damage was found in healthy controls 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 COPD patients 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). In the present study, we 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 COPD patients. Exercise had no significant effects either on plasma MDA in the healthy controls. 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 aldehydes, like MDA, are formed and enter the circulation they are rapidly excreted.

In contrast to plasma levels, in COPD patients, the MDA excretion in urine, as a marker of 'whole body' oxidative stress, was significantly elevated immediately after both exercise tests and also 4 hours 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 controls, a significant increase was observed only 4 hours 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 COPD patients. In the present study, the absence of an immediate exercise-induced systemic oxidative stress response in healthy controls 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 minutes, since otherwise the difference in duration could have been too large compared to the COPD patients. It is also likely that in healthy controls the antioxidant defence 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 defence system can be overwhelmed by the ROS leading to an increased systemic oxidative stress response after exercise.

In our study, plasma uric acid, as a marker of xanthine oxidase activity, was significantly increased 4 hours after the maximal and submaximal exercise test, whereas in healthy controls, a significant increase was observed only 4 hours after the maximal exercise test. This finding is consistent with previous studies reporting increased plasma uric acid in response to strenuous exercise in healthy controls (38, 39). It is expected that the activation of xanthine oxidase is more prominent for the maximal exercise test, since 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. Further, in the muscle cells, AMP is continuously degraded to hypoxanthine that 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). Whereas under aerobic conditions, this reaction may be catalysed by xanthine dehydrogenase, which uses NAD 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 controls. This was reported earlier by Hellsten et al. (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 COPD patients was significantly increased 4 hours after the maximal exercise test, whereas in healthy controls 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. As ventilatory demands increase more during the maximal compared to the submaximal exercise test, patients with COPD tend to breath more shallow and with higher frequency, resulting in dynamic lung hyperinflation. This results in increased physiologic dead space ventilation, which is attendant with a 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 COPD patients 4 hours after the maximal exercise test. Earlier, Schleiss et al. (45) reported that the hydrogen peroxide concentration in exhaled air is dependent on expiratory flow rates, as 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.

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 COPD patients, since 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, as trained participants had less DNA damage compared to untrained participants after exercise.

Based on 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 found no longer 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 et al. (47) reported that patients with COPD had a reduced ability to adapt to endurance training, as reflected by a lower capacity to synthesise reduced glutathione. The reduced exercise-induced oxidative stress response may be attributable to an improved oxidative metabolism rather than to an upregulation of antioxidant defences, 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_2O_2 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_2O_2 production at the submaximal exercise test was significantly higher compared to 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_2O_2 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 life style factors, especially passive smoking behaviour may have been different from those in the pulmonary rehabilitation center.

Methodological 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 oxidative stress at isotime would probably have led to even more pronounced differences between measurements before and after rehabilita-

tion, 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 to age-matched healthy controls, are characterised by increased systemic and pulmonary oxidative stress markers both at rest as well as induced by exercise. This suggests that healthy controls are able to tolerate exercise more effectively than COPD patients. 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 COPD patients 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 pharmacological modulation on exercise-induced oxidative stress, will be subject of further investigations

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Online Data Supplement for:

**Rehabilitation decreases Exercise-induced Oxidative Stress in
Chronic Obstructive Pulmonary Disease**

Evi M. Mercken, Geja J. Hageman, Annemie M.W.J. Schols, Marco A. Akkermans,
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METHODS

Study population

Pulmonary rehabilitation program

All patients participated in a standardized inpatient pulmonary rehabilitation program, consisting of exercise training, education and when appropriate psychosocial and behavioural intervention. The physical exercise training consisted of a combination of endurance and strength training. All patients performed two activity sessions made up of 20 minutes of submaximal cycle ergometry, 20 minutes of treadmill exercise, 30 minutes of gymnastics and 1 session of unsupported arm exercise training daily. Also dynamic strength training exercises of the upper and lower extremities were implemented in the program. Because the exercise program was offered in an inpatient setting there was a thorough control on the compliance of the patients to the different aspects of the program. Furthermore, since the patients in general trained together, they exerted an additional control on the compliance of each other. The diet that was offered to the patients was standardized as follows from the inpatient setting and none of the studied COPD patients received nutritional support. Furthermore, when the patients started their rehabilitation program, they were not allowed to smoke in or outside the rehabilitation centre. During the rehabilitation period, the patients HbCO was measured several times to see if the patients had smoked. None of patients included in the study had an elevated HbCO.

Level of physical activity

Participants had to wear the Personal Activity Monitor (Pam) accelerometer during 9 consecutive days. The Pam could easily be clipped to the belt or clothes and was attached to the right hip. It measures the acceleration in both horizontal and vertical direction. Furthermore, Pam has the ability to store data continuously for 112 days and has computer-uploading capabilities through a special website (www.pam.com). In this way, Pam users can observe the physical activity pattern of recent days/weeks.

Malondialdehyde in plasma and urine

The determination of malondialdehyde (MDA) was assayed by measuring the thiobarbituric acid reactive material according to the high-performance liquid chromatography (HPCL) method described by Lepage et al. (E1). A 200 µl plasma or urine sample was added to 1 ml of a reagent [containing 0.12 M TBA, 0.32 M *o*-phosphoric acid, and 0.01% (mass/vol) EDTA, 0.68 mM butylated hydroxytoluene (BHT)]. After this, the mixture was incubated for 1h at 100°C in a water bath. After cooling, the MDA-products were extracted with 500 µl butanol. Malondialdehyde

was then determined using HPLC (Agilent, Palo Alto, CA) and 30 µl of the butanol layer were injected and separated on a Nucleosil C18 column (150 X 3.2 mm) particle size 5 µm (Supelco, PA). Samples were eluted with 65% (vol/vol), 0.05% trifluoroacetic acid (TFA) in MilliQ and 35% (vol/vol) methanol. The MDA-TBA complex was detected with a fluorescence detector with excitation and emission wavelengths of 532 and 553 nm, respectively. A calibration curve was constructed using malonaldehyde bis(diethylacetal) as standard. The urinary MDA concentrations were corrected for creatinine content. Creatinine in urine samples was measured in triplicate using the Jaffé reaction as described by Falco et al. (E2). Briefly, creatinine reacts with the alkaline picrate to form a coloured complex, which is measured at 492 nm. The values of MDA in urine are expressed per mmol creatinine.

Plasma uric acid

An aliquot of 150 µl of plasma was deproteinized by mixing with an equal volume of 10% (w/v) trichloroacetic acid (TCA), centrifuged (800 x g for 5 minutes at 4°C) and uric acid was assayed by a HPLC method described by Lux et al. (E3) with minor modification. Twenty-five µl of the deproteinized plasma was injected on the HPLC. The system (Agilent, Palo Alto, CA, USA) consisted of an Hypersil BDS C-18 end-capped column, 125 x 4 mm, particle size 5 µm (Agilent, Palo Alto, CA, USA) with a mobile phase of 5 mM sodium phosphate buffer pH = 3.3. Detection was performed using a variable wavelength detector set on a wavelength of 292 nm. The uric acid concentration was calculated using a calibration curve of uric acid.

Collection of exhaled breath and measurement of H₂O₂

Exhaled breath condensate (EBC) hydrogen peroxide (H₂O₂) was measured by means of horseradish peroxidase-catalysed oxidation of tetramethylbenzidine according to the method previously described by Gallati and Pracht and modified for microtiter plate-based analysis (E4). Briefly, 100 µl 3,3',5,5'-tetramethylbenzidine (dissolved in 0.2 M citrate buffer, pH 3.95) and 10 µl of 50 U/ml horseradish peroxidase (HRP; Sigma Chemicals) were added to 50 µl EBC. The reaction mixture was incubated for 20 min at room temperature. Subsequently, the reaction mixture was acidified to pH 1 with 20 µl of 2 M sulphuric acid. The absorbance of the reaction product was measured spectrophotometrically at 450 nm.

RESULTS

Anthropometric and Spirometric Data

Pam utilizes a unique and patented universal index, known as the “Pam Score[®]” (Table E1). It is the ratio between the amount of energy you use while active and the amount of energy you use while at rest, multiplied by 100%.

Table E1. The suggested range of daily Pam Scores for each activity level, segmented by age category.

Age	< 20 yr	20-40 yr	40-60 yr	> 65 yr
Activity level	Pam Score			
Low	< 30	< 20	<15	< 10
Light	30-40	20-30	15-25	10-20
Medium	40-60	30-40	25-35	20-30
Active	60-80	40-60	35-50	30-45
Very Active	80-100	60-80	50-70	45-60
Extremely Active	> 100	> 80	> 70	> 60

Exercise capacity

COPD patients exceeded their maximum voluntary ventilation ($\dot{V}_E/MVV > 100\%$), as a consequence of their ventilatory limitation, maximum exercise intensity was reached, whereas the heart rate reserve was preserved. The healthy controls were within the normal range for these two parameters. Peak ventilatory (VE) reserve was calculated as $100\% - (100 \times \text{peak VE}) / (FEV_1 \times 37.5)$ (E5, E6) and peak heart rate (HR) reserve was calculated as $100\% - (100 \times \text{peak HR}) / (220 - \text{age})$ (E5).

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CHAPTER 3

Systemic and pulmonary oxidative stress after single leg exercise in COPD

ABSTRACT

Background: The aim of this study was to disentangle the contribution of muscular versus pulmonary oxidative stress during endurance exercise in patients with COPD. *Methods:* Fifteen COPD patients and 10 healthy age-matched controls performed a continuously submaximal single leg ergometer test (40% of peak workload) for 20 minutes or until they stopped (Tlim). Venous blood, urine samples and exhaled breath condensate were sampled before, immediately and 2h after exercise. *Results:* Tlim was lower in COPD than controls ($p<0.01$). No exercise-induced systemic inflammation (IL-6, TNF- α) was found in the groups. Urinary malondialdehyde (MDA) and uric acid ($p<0.05$) as well as erythrocyte GSSG/GSH ($p=0.08$) were increased in COPD compared to controls after exercise. Despite the relatively low cardioventilatory response to this localized muscle exercise, hydrogen peroxide (H_2O_2) in breath condensate significantly increased in COPD ($p<0.01$). NF- κ B DNA binding activity of p50 in peripheral blood monocytes was elevated after exercise in both COPD ($p<0.01$) and controls ($p<0.05$), whereas p65 protein was not altered. *Conclusion:* COPD patients showed increased pulmonary and systemic oxidative stress after localized leg muscle exercise compared to healthy controls without evidence of increased levels of systemic inflammation.

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INTRODUCTION

Peripheral muscle dysfunction is common in chronic obstructive pulmonary disease (COPD). Previous studies showed that peripheral muscle strength and endurance¹⁻³ are reduced and susceptible to fatigue⁴ in patients with COPD compared to healthy subjects. Understanding the underlying mechanisms of peripheral muscle impairment in COPD is clinically relevant since skeletal muscle dysfunction is linked to disability and impaired quality of life.⁵ Chronic inflammation and oxidative stress may be implicated in the etiology of peripheral muscle dysfunction.⁶ Increased systemic and pulmonary oxidative stress has consistently been reported in patients with severe COPD after constant work rate cycle exercise.^{7, 8} This type of exercise however stresses the whole body and limitations of the ventilatory system in COPD patients in particular.⁹ Moreover, under these circumstances, it is difficult to disentangle the relative contribution of lungs versus muscles to the exercise-induced oxidative stress in COPD.

We assumed that a localized limb exercise may obviate exercise-induced ventilatory and cardiac stress and thus be an ideal exercise modality to study the contribution of skeletal muscle to exercise-induced oxidative stress in COPD. Recently, Couillard and colleagues indeed provided evidence for localized quadriceps exercise-induced systemic and muscle oxidative stress in patients with COPD^{2, 10}, but no data were reported regarding the pulmonary oxidative stress response. Furthermore, in these studies endurance time was tested on an exercise bench according to the technique of Serres et al.³ In this model, the movement is based on knee extension and flexion in regular pace with periods of rest in between. A more suitable model to study exercise endurance time is the model of Andersen et al.¹¹ in which knee extension is performed continuously. The aim of the present study was therefore to investigate if and to what extent systemic inflammation and oxidative stress as well as pulmonary oxidative stress are differentially triggered by contracting peripheral muscle of COPD patients versus healthy controls and whether this stress response is associated with peripheral reduced muscle endurance in COPD.

MATERIALS AND METHODS

Study population

Fifteen stable patients who met GOLD criteria for COPD were recruited on admission to the Centre for Integrated Rehabilitation Organ failure (CIRO), Horn, The Netherlands.¹² All patients were ex-smokers and had not experienced a respiratory tract infection or exacerbation of their disease for at least 4 weeks prior to the study. Exclusion criteria were rheumatoid arthritis, chronic colitis, diabetes, cardiovascular

diseases, renal diseases, liver diseases or mental diseases. None of the subjects were taking antioxidants or vitamin supplements. All patients received standardized medical treatment according to the GOLD guidelines including anticholinergic, β_2 -agonists and inhaled corticosteroids. Two patients also received theophylline and oral corticosteroids as maintenance therapy. Ten healthy sedentary age-matched non-smoking control subjects were recruited through newspaper advertisements. Written informed consent was obtained from all participants, and the study was approved by the medical ethics committee of the University Hospital Maastricht.

Subject characteristics

Body composition¹³, pulmonary function¹⁴, and Physical Activity Scale for the Elderly questionnaire¹⁵ were determined as described previously.

Exercise capacity

Participants performed an incremental and a day later a submaximal cycling exercise test (80% of peak workload) as described previously.¹⁶ A week later, they performed a maximal and 3 days later a submaximal single leg ergometer test (40% of peak workload) for which subjects were instructed to cycle for 20 min. The exercise equipment and methodology were designed based on the model of Andersen et al.¹¹ The duration time of the exercise test was called 'limit time' (Tlim). Venous blood, urine samples and exhaled breath condensate samples were obtained at baseline, immediately and 2h after the exercise test. Blood lactate was analyzed (Cobas Mira; Roche, Basel, Switzerland). Dyspnea and muscle fatigue were evaluated using the Borg scale (0 to 10).

Markers of systemic inflammation and oxidative stress

Venous blood samples were drawn into EDTA-containing tubes (Venoject). Plasma was obtained by centrifugation (800 x g for 10 minutes at 4°C) and stored at -80°C until analysis. The volume of the remaining blood was brought to 15 ml with cold phosphate-buffered saline and layered on an equal volume of Lymphoprep medium in a 50-ml tube with a filter (Greiner) of peripheral blood mononuclear cells (PBMCs). After centrifugation at 800 x g for 30 min at 4°C, gradient-separated PBMCs were recovered, resuspended in 10-ml cold PBS, and centrifuged again at 250 x g for 10 min at 4°C. The cells were resuspended in 1 ml cold PBS and centrifuged at 800 x g for 5 min and the pellet was used to prepare nuclear extracts for NF- κ B determination. Nuclear extracts were prepared as described by Hofmann et al.¹⁷ Urine samples were stored at -20°C until further analysis. Plasma IL-6 and TNF- α concentrations were determined using a quantitative high-sensitivity enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). Urinary MDA and uric acid were assessed by HPCL.^{18, 19} Erythrocyte glu-

tathione was measured in its reduced (GSH) and oxidized (GSSG) form.²⁰ The GSSG-to-GSH ratio, another marker of oxidative stress was calculated. NF- κ B concentrations were determined in nuclear extracts of peripheral blood mononuclear cells (TransAM NF- κ B p50 and p65 transcription Factor Assay Kit; Active Motif Europe, Rixensart, Belgium).

Markers of pulmonary oxidative stress

Exhaled breath condensate (EBC) was collected as described previously.²¹ Hydrogen peroxide (H₂O₂) was measured by means of horseradish peroxidase-catalyzed oxidation of tetramethylbenzidine.²²

Statistical analyses

All data are expressed as mean $\pm$ SEM. Differences between patients and controls were tested using Student's *t*-test for independent samples. When normality was not obtained, a Mann-Whitney U-test was used. The Wilcoxon signed rank test was used to evaluate the effect of single leg exercise on biological markers within subgroups. Differences between the exercise tests (single leg ergometer, maximal and submaximal cycle exercise) were determined by ANOVA with post hoc LSD test. A *p*-value of ≤ 0.05 was considered statistically significant. Statistical analyses were analyzed with SPSS for Windows (version 13.0; SPSS, Inc., Chicago, IL).

RESULTS

Subject characteristics

As shown in Table 1, the COPD group was characterized by moderate to severe airflow obstruction with a FEV₁ of $46.6 \pm 5.2\%$ predicted. Physical activity assessed by PASE score was significantly lower in COPD patients compared to control subjects (*p*<0.001).

Table 1. Characteristics of the study population

	Controls (n=10)	COPD patients (n=15)
Sex, M/F	4/6	10/5
Age, yr	55.5 ± 1.8	56.5 ± 2.3
Pack-yr	11.9 ± 5.0	38.0 ± 6.2
BMI, kg/m ²	27.6 ± 1.4	24.6 ± 1.1
FFMI, kg/m ²	17.3 ± 0.7	15.9 ± 0.4
FEV ₁ , % pred.	115.9 ± 7.0	46.6 ± 5.2*
FVC, % pred.	126.1 ± 10.0	112.8 ± 8.6
FEV ₁ /FVC, % pred.	78.3 ± 1.5	35.2 ± 2.5*
PASE	235.3 ± 16.0	85.1 ± 9.4*

Definition of abbreviations: BMI: body mass index; FFMI: fat-free mass index; PASE: Physical Activity Scale for the Elderly. Data are presented as mean ± SEM. *p<0.001 compared with healthy control subjects.

Single leg ergometer test

The results of the submaximal single leg ergometer test are presented in Table 2. All healthy controls except for one reached the 20-min time limit whereas only 5 COPD patients were able to complete the entire duration of the test. Workload and quadriceps endurance (Tlim) were significantly lower in COPD patients compared to controls (p<0.001 and p<0.01 respectively). Ventilation ($\dot{V}E/MVV$) was significantly higher in COPD compared to controls (p<0.001), whereas the heart rate reserve was similar between both groups. Subjective assessment of muscle fatigue showed greater leg fatigue in COPD compared to controls (p<0.05), whereas perceived breathlessness was not significantly different between the groups.

Table 2. Physiological response to submaximal single leg ergometer test

	Controls (n=10)	COPD patients (n=15)
Workload, W	15.6 ± 1.6	6.4 ± 1.0 ^{***}
Tlim, min	19.0 ± 1.0	11.0 ± 1.8 ^{**}
$\dot{V}O_2$, mL/min		
Rest	493.7 ± 49.6	366.8 ± 44.1
End-exercise	867.0 ± 67.2	610.6 ± 62.2
$\dot{V}CO_2$, mL/min		
Rest	390.0 ± 54.6	304.4 ± 43.0
End-exercise	770.7 ± 64.5	560.9 ± 64.4
HR reserve, %		
End-exercise	42.4 ± 6.7	41.5 ± 2.9
$\dot{V}E/MVV$		
End-exercise	0.22 ± 0.01	0.57 ± 0.09 ^{***}
SaO ₂ , %		
Rest	96.3 ± 0.9	96.5 ± 0.6
End-exercise	97.0 ± 0.8	96.8 ± 0.5
RER		
End-exercise	0.89 ± 0.02	0.91 ± 0.02
Δ Lactate, mmol/L	0.80 ± 0.28	0.98 ± 0.22
Δ Dyspnea Borg Score	0.5 ± 0.8	1.8 ± 0.4
Δ Leg fatigue Borg Score	0.9 ± 0.8	1.6 ± 0.3 [*]

Definition of abbreviations: Tlim: limit time; HR: heart rate; MVV: maximum voluntary ventilation; SaO₂: oxygen saturation; RER: respiratory exchange ratio. Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 compared with healthy control subjects.

Comparison between whole body and local exercise

Physiological responses to whole body exercise tests (maximal and submaximal cycle exercise) and submaximal localized quadriceps exercise (single leg ergometer test) are compared in Table 3. In COPD, the single leg ergometer test resulted in significantly lower peak $\dot{V}O_2$, peak $\dot{V}CO_2$ (both; p<0.001) and higher oxygen saturation (p<0.01 and p<0.05 respectively) compared to whole body maximal and submaximal cycle exercise tests. Moreover, voluntary ventilation ($\dot{V}E/MVV$) was significantly lower after single leg ergometer test compared to maximal cycle exercise, whereas the heart rate reserve was more preserved after the single leg ergometer test (both; p<0.01) in COPD. For COPD, dyspnea perception (p<0.01) and to a lesser extent also leg fatigue perception (p<0.05) during single leg exercise were less when compared to whole body exercise.

Table 3. Comparison between local *versus* whole body exercise tests in COPD

COPD (n=15)	Single leg exercise	Maximal cycle exercise	Submaximal cycle exercise
Tlim, minutes	11.0 ± 1.8	-	8.3 ± 1.6
$\dot{V}O_2$, mL/min	610.6 ± 62.6	1231.1 ± 110.6 ^{***}	1228.9 ± 89.1 ^{***}
$\dot{V}CO_2$, mL/min	560.9 ± 64.4	1184.4 ± 111.0 ^{***}	1200.7 ± 83.9 ^{***}
SaO ₂ , %	96.6 ± 0.6	92.1 ± 1.0 ^{**}	93.2 ± 0.7 [*]
HR, beat/min			
Rest	82.6 ± 5.2	67.9 ± 3.2	-
End-exercise	96.4 ± 4.9	95.1 ± 11.6	-
HR reserve, %			
End-exercise	41.5 ± 2.9	21.4 ± 2.7 ^{**}	-
$\dot{V}E$, L/min			
Rest	15.0 ± 1.9	13.9 ± 1.0	-
End-exercise	29.4 ± 2.7	46.4 ± 3.8	-
$\dot{V}E/MVV$			
End-exercise	0.57 ± 0.09	0.95 ± 0.31 ^{**}	-
Δ Dyspnea Borg Score	1.8 ± 0.4	5.0 ± 0.7 ^{**}	4.9 ± 0.5 ^{**}
Δ Leg fatigue Borg Score	1.6 ± 0.3	3.3 ± 0.7	3.9 ± 0.5 [*]

Definition of abbreviations: HR: heart rate; MVV: maximum voluntary ventilation. Data are presented as mean ± SEM. ^{*}p<0.05, ^{**}p<0.01, ^{***}p<0.001 compared with single leg exercise.

Markers of systemic inflammation and oxidative stress

At baseline, only IL-6 was significantly higher in COPD compared to controls (0.7 ± 0.2 controls vs. 2.0 ± 0.4 COPD; $p<0.01$). Systemic as well as pulmonary oxidative stress markers at baseline did not differ between both groups. No exercise-induced inflammatory changes were observed in both groups (data not shown). As shown in Figure 1, urinary MDA was significantly elevated immediately after exercise in COPD patients whereas in controls, a significant decrease was found 2h after exercise (both $p<0.05$). Additionally, a significant difference in the response to exercise in urinary MDA between both groups was observed ($p<0.02$). Urinary uric acid was significantly increased immediately after exercise in COPD ($p<0.05$; Figure 2). Moreover, the exercise-induced uric acid response was significantly higher in COPD compared to controls ($p<0.05$).

Figure 1. Concentration of urinary malondialdehyde (MDA) in healthy control subjects and COPD patients immediately after and 2h after submaximal single leg exercise. Data represent differences with baseline values. Values are expressed as mean $\pm$ SEM. * $p < 0.05$ significantly different from baseline values; # $p < 0.02$ significantly different between the groups.

Figure 2. Concentration of urinary uric acid in healthy control subjects and COPD patients immediately after and 2h after submaximal single leg exercise. Data represent differences with baseline values. Values are expressed as mean $\pm$ SEM. * $p < 0.05$ significantly different from baseline values; # $p < 0.05$ significantly different between the groups.

Furthermore, in control subjects erythrocyte GSH levels decreased 2h after exercise ($p < 0.05$; Figure 3A), whereas in COPD GSSG levels and the GSSG-to-GSH ratio tended to be increased immediately after exercise (both $p = 0.08$; Figure 3B+3C). In COPD, H_2O_2 concentration in exhaled breath condensate (EBC) was significantly elevated immediately and 2h after exercise (both $p < 0.01$; Figure 4), whereas for controls no significant effect of exhaled H_2O_2 concentration was observed.

Figure 3. Erythrocyte GSH levels (A), GSSG levels (B) and GSSG/GSH ratio (C) in healthy control subjects and COPD patients immediately after and 2h after submaximal single leg exercise. Data represent differences with baseline values. Values are expressed as mean $\pm$ SEM. * $p < 0.05$, § $p = 0.08$ significantly different from baseline values.

Figure 4. Production of hydrogen peroxide (H₂O₂) measured in exhaled breath condensate in healthy control subjects and COPD patients immediately after and 2h after submaximal single leg exercise. Data represent differences with baseline values. Values are expressed as mean $\pm$ SEM. *p<0.01 significantly different from baseline values.

NF- κ B nuclear translocation

As shown in Figure 5, basal NF- κ B DNA binding activity of p50 and p65 in PBMCs did not differ between the groups. 2h after exercise, NF- κ B DNA binding activity of p50 in PBMCs significantly increased to a similar extent in controls and COPD (p<0.05 and p<0.01 respectively), whereas exercise did not alter p65 protein in both groups.

Figure 5. NF- κ B DNA binding activity of p50 (A) and p65 (B) was determined in peripheral blood mononuclear cells in healthy control subjects and COPD patients 2h after submaximal single leg exercise. Values are expressed as mean $\pm$ SEM. *p<0.05, **p<0.01 significantly different from baseline values.

DISCUSSION

The results of this study indicate that quadriceps endurance is impaired in clinically stable COPD patients compared to healthy controls. In addition, patients with COPD showed a greater susceptibility to local exercise-induced systemic and pulmonary oxidative stress without evidence of increased levels of systemic inflammation during exercise.

Validity of the local endurance test

To study muscle endurance (Tlim), we used a local continuous work quadriceps exercise test based on the model of Andersen et al.¹¹ In COPD, the increase in dyspnea, ventilation and heart rate after this single leg exercise was of small amplitude, whereas muscle fatigue was the main factor limiting exercise. This response pattern indicates that continuously localized exercise minimizes cardiorespiratory responses and nicely reflects muscle response to exercise. Conversely, during whole body cycling exercise, impairment of ventilatory function represented the main limiting factor for exercise capacity. This paralleled the differences in observed subjective perception of leg fatigue and dyspnea during the different exercise modalities in patients and controls.

Local exercise-induced systemic inflammatory and oxidative stress response

In accordance with a previous study²³, plasma inflammatory cytokine levels were unchanged by the exercise test in both groups, suggesting that local muscle exercise does not induce systemic inflammation. However, COPD patients showed an increased systemic oxidative stress response after exercise compared to controls as measured by elevated urinary MDA and urinary uric acid levels. In healthy controls, the ubiquitous antioxidant GSH decreased 2h after exercise. Previously, it has been shown that limb skeletal muscle mass of COPD patients is reduced compared to controls.^{1, 24} Therefore, it is feasible that GSH demand during exercise was lower in COPD compared to controls. Although statistical significance was not reached, GSSG and GSSG-to-GSH ratio slightly increased in COPD following exercise which indicates an increased oxidative stress response after localized exercise in COPD. This finding is in agreement with previous studies that reported increased GSSG-to-GSH ratio after exhaustive whole body exercise.^{7, 25} A reduced endurance of the quadriceps occurs in a substantial number of patients with COPD. In the present study, different patterns were observed for exercise-induced NF- κ B DNA binding activity of p50 and p65 in PBMCs but no differences between the two groups. Only DNA binding activity of the p50 subunit was significantly elevated 2h after exercise in both COPD and controls. This indicates that after a local exercise the induced NF- κ B DNA binding consist mostly of p50 homodimers. It is well appre-

ciated that p50 homodimers can act as suppressors of NF- κ B dependent inflammatory cytokine transcription.²⁶ This is in agreement with the present finding that plasma inflammatory cytokine levels were unchanged by the exercise test in both groups.

Remarkably, even despite the low cardioventilatory responses, we observed that H₂O₂ concentration in the EBC of patients with COPD was significantly elevated immediately and 2h after local exercise, whereas in controls no significant increase was observed, indicating an enhanced production of ROS in the airways of these patients. Previously, we showed that H₂O₂ concentration was increased in COPD patients after maximal whole body cycling exercise⁸, which could be explained by the increased ventilatory responses during this exercise modality. However, in the present study, the immediate increase of H₂O₂ concentration in EBC of COPD patients after local exercise is likely to be derived from a different mechanism since a local exercise is attended by less pronounced increased ventilatory responses. Until now, no other studies have investigated the effect of exercise on H₂O₂ concentration in EBC. However, basal differences have been attributed to an enhanced activity of xanthine oxidase (XO) in the lungs COPD.^{27, 28} Additionally, an increased number of inflammatory cells have been found in the bronchoalveolar lavage fluid of stable COPD patients compared to healthy non-smokers.²⁹ Still, it could be speculated that XO activity in epithelial cells of COPD patients is increased after exercise compared to controls leading to the immediate increase in H₂O₂ concentration in COPD observed in this study. Moreover, it could be hypothesized that the pulmonary inflammatory response is intensified in COPD after exercise. The immediate increased H₂O₂ concentration may also originate from enhanced and/or inappropriate NADPH oxidase activation (for review see³⁰). Further research will have to elucidate whether these or other mechanisms also play a role in the exercise-induced H₂O₂ concentration.

It has been suggested that oxidative stress plays a role in peripheral muscle dysfunction in patients with COPD. However, no correlations were observed between markers of oxidative stress and quadriceps endurance in COPD. This is in contrast with a previous study showing an inverse relationship between muscle lipid peroxidation and oxidized proteins with quadriceps endurance in COPD.²

Systemic inflammation and oxidative stress at rest

In the present study, resting values of plasma IL-6 were increased in clinically stable COPD patients compared to controls³¹, reflecting a low-grade systemic inflammation in these patients. However, no differences in basal plasma TNF- α between both groups were observed. Conflicting results have been reported regarding the pro-inflammatory cytokine TNF- α in plasma of COPD patients.^{23, 32} Contrary to previous studies, no differences in oxidative stress markers (urinary MDA, urinary uric acid

and H_2O_2 in breath condensate) were noted in COPD patients compared to controls.^{8, 22} These discrepancies may be attributable to a difference in disease severity in terms of an altered oxidant/antioxidant balance, with the patients of the present study less severely affected than those of our previous study.⁹ Additionally, no differences between reduced GSH and oxidized GSSG levels were observed between both groups, which are in agreement with the results of previous studies.^{7, 33} Moreover, no differences in NF- κ B DNA binding activity of p50 and p65 were found between the two groups. Data regarding DNA binding activity of both subunits in PBMCs of COPD were not previously available.

In summary, the results of this study strongly suggest that COPD patients have a more pronounced exercise-induced systemic oxidative stress response to localized leg muscle exercise without evidence of increased levels of systemic inflammation compared to healthy controls when tested at a similar relative intensity. Moreover, despite the low cardioventilatory responses after a localized exercise, COPD showed a greater susceptibility to local pulmonary oxidative stress response than controls. Future studies are indicated to study the effects of antioxidant modulation on the acute exercise-induced oxidative stress response as well as the long term effects of nutritional or pharmacological modulation as adjunct to exercise training on skeletal muscle endurance capacity.

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ABBREVIATION LIST

COPD: chronic obstructive pulmonary disease
 CIRO: Centre for Integrated Rehabilitation Organ failure
 EBC: exhaled breath condensate
 ELISA: enzyme-linked immunosorbent assay
 GSH: reduced glutathione
 GSSG: oxidized glutathione
 H_2O_2 : hydrogen peroxide
 MDA: malondialdehyde
 PBMCs: peripheral blood mononuclear cells
 Tlim: muscle endurance
 XO: xanthine oxidase

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CHAPTER 4

Reduced NF- κ B mediated gene expression in skeletal muscle of patients with COPD in response to acute exercise

ABSTRACT

Background: NF- κ B activation and oxidative stress are physiological responses of skeletal muscle to exercise but may be impaired in patients with COPD and decreased exercise capacity. To test this hypothesis we investigated NF- κ B activity and expression of NF- κ B regulated genes in quadriceps muscle of COPD patients and healthy controls at rest and after an acute exercise bout. Methods: Quadriceps specimens were obtained before, immediately and 2h after a standardized cycle ergometer test (10 min at 70% of peak workload) from 7 COPD patients and 7 controls. NF- κ B DNA binding activity in skeletal muscle and peripheral blood mononuclear cells (PBMCs) was determined using electrophoretic mobility shift assay and ELISA respectively. mRNA expression and protein carbonylation were measured by real-time PCR and Western blot, respectively. Results: In COPD, only IL-6 mRNA was increased in skeletal muscle after exercise, whereas in controls IL-6, I κ B α , TNF- α and IL-1 β mRNA were increased. Moreover, only in controls mRNA transcripts encoding superoxide dismutase, thioredoxin, heme oxygenase 1 and heat shock protein70 were upregulated after exercise. Exercise-induced mRNA levels of anti-apoptotic Bcl2 were lower in COPD compared to controls. Basal muscle protein oxidation was higher in COPD than in controls, but attenuated in response to exercise. No exercise-induced changes in NF- κ B DNA binding activity in skeletal muscle and PBMCs of both groups were detected. However, basal NF- κ B activity in PBMCs was lower in COPD compared to controls. Conclusion: Skeletal muscle of COPD patients is characterized by an impaired response to acute exercise of a set of NF- κ B regulated genes encoding inflammatory cytokines, antioxidants, stress proteins and survival factors.

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INTRODUCTION

Chronic inflammation and oxidative stress are prominent features of chronic obstructive pulmonary disease (COPD) and may be implicated in the etiology of peripheral muscle dysfunction and muscle wasting.[1] Whereas the transient inflammatory response in the circulation and muscle following exercise is well-established in healthy controls, limited information is available regarding exercise as modulator of the inflammatory response in skeletal muscle of patients with COPD. In contrast exercise-induced oxidative stress has been well documented in the pulmonary, blood and muscle compartments of patients with COPD.[2, 3]

Different mechanisms could contribute to augmented formation of reactive oxygen species (ROS) in response to exercise including mitochondrial respiration, NADPH oxidase and xanthine oxidase. The presence of ROS not only results in tissue damage, but recent research indicates a critical role in muscle adaptation by modulating gene expression. A major redox-sensitive transcriptional regulator is nuclear factor protein κ B (NF- κ B).[4] Sustained activation of NF- κ B by oxidative stress has been suggested to play a central role in the etiology of the systemic features of COPD.[5] In particular, a growing body of evidence from experimental models supports the involvement of NF- κ B in the pathogenesis of muscle wasting.[6, 7] In contrast, transient activation of NF- κ B is also involved in a wide variety of physiological processes such as antioxidant defense, cell growth, survival and differentiation as well as in immunity and inflammatory responses. In particular, NF- κ B mediates gene expression of superoxide dismutase (SOD) and thioredoxin (Trx) which provide cellular protection to oxidative stress.[8, 9] Additionally, expression of the stress proteins heme oxygenase 1 (HO-1) and heat shock protein-70 (Hsp70) involves regulation by NF- κ B and yields cellular protection against oxidative damage.[10, 11] NF- κ B also mediates the expression of Bcl2 protein which is involved in cell survival as well as the expression of the myogenic differentiation factor (MyoD).[12, 13] Furthermore, NF- κ B is an important regulator of cytokines (IL-6, TNF- α and IL-1 β).

Thus far, the physiological role of NF- κ B pathway activation during exercise is still elusive in COPD. Therefore, the purpose of this study was to investigate if NF- κ B is activated in peripheral blood mononuclear cells and if NF- κ B activity is induced and NF- κ B mediated gene expression is upregulated in skeletal muscle of patients with COPD in response to an acute exercise bout. To evaluate this, quadriceps muscle biopsies were obtained from patients with COPD and healthy age-matched controls before and after standardized cycle ergometry.

MATERIALS AND METHODS

Study population

Seven clinically stable male patients with moderate COPD as defined by GOLD guidelines participated in this study.[14] All patients were ex-smokers and had not experienced respiratory tract infection or exacerbation of their disease for at least 4 weeks prior to the study. Exclusion criteria were rheumatoid arthritis, chronic colitis, diabetes, cardiovascular diseases, renal diseases, liver diseases or mental diseases. Seven healthy sedentary age-matched non-smoking participants were recruited as control group. All patients received standardized medical treatment according to the GOLD guidelines (see online supplement). 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

Participants performed an incremental cycle ergometer test and four days later a submaximal constant work rate exercise test. Venous blood and muscle biopsies were obtained at baseline, immediately after and 2 hours after the submaximal exercise test. The test was performed and samples were collected at the same time point of the day.

Anthropometry and spirometry

Body composition and flow volumes were determined as described previously (see online supplement).[15, 16]

Exercise capacity

Based on the outcome of the incremental (10 Watt/minute) cycle ergometer test, patients performed a submaximal (70% of peak workload) cycle ergometer test as described previously for a standardized duration of 10 minutes (see online supplement).[17]

Muscle biopsies

Percutaneous biopsy specimens of vastus lateralis muscle of the dominant leg at baseline and immediately after exercise and of the non-dominant leg 2 hours after exercise were obtained as described by Bergström.[18] This time point was chosen based on the literature to determine the early gene response after an acute bout of exercise.[19]

Markers of systemic inflammation and oxidative stress

Details on sample preparation are provided in the online supplement. Plasma IL-6, TNF- α and IL-1 β concentrations were determined using a quantitative high-sensitivity enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). Levels of systemic protein carbonyls were measured by means of an ELISA.[20]

NF- κ B DNA-binding

NF- κ B concentrations were determined in nuclear extracts of peripheral blood mononuclear cells (PBMCs) or muscle biopsies (see online supplement), and protein concentrations were determined using the method of Bradford (Biorad).

NF- κ B p50 DNA-binding was determined in equal amounts of protein from nuclear extracts of PBMCs (TransAM NF- κ B p50 transcription Factor Assay Kit; Active Motif Europe, Rixensart, Belgium). Alternatively, for nuclear extracts from biopsies electrophoretic mobility shift assay (EMSA) was used to analyze RelA DNA binding to an oligonucleotide containing a κ B consensus sequence (Santa Cruz, Santa Cruz, CA, USA). 50 μ g of nuclear protein was used per binding reaction and protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.25X Tris-borate-EDTA buffer at 120V for 2 hours. Gels were dried and exposed to film (X-Omat Blue XB-1, Kodak, Rochester, NY, USA). Shifted complexes were quantified by phosphor-imager analysis (Biorad, Hercules, CA, USA). Supershift reactions were performed by pre-incubation of the nuclear extracts with an antibody specific to the RelA subunit of NF- κ B (Santa cruz, Santa cruz, CA, USA).

Skeletal muscle analysis

mRNA levels of FOXO1, I κ B α , IL-6, TNF- α , IL-1 β , MnSOD, Trx, HO-1, Hsp70, Bcl2 and MyoD were determined by quantitative real-time PCR, and muscle protein carbonylation was determined by Western blot. Details on the methods and primer sequences (Table E1) are provided in the online supplement.

Statistical analyses

Data are presented as mean $\pm$ SEM. Between-group comparisons were analyzed using the Mann-Whitney U test and the Wilcoxon signed rank test was used to evaluate the effect of submaximal exercise within the groups. For analysis of baseline gene expression the Δ Ct was used and for the effect of exercise on gene expression the $\Delta\Delta$ Ct. Nonparametric tests were used because the normality assumption was not obtained. A difference with $p \leq 0.05$ was considered statistically significant. Statistical analyses were analyzed with SPSS for Windows (version 13.0; SPSS, Inc., Chicago, IL).

RESULTS

Baseline and exercise characteristics of the study population

The subject characteristics are presented in Table 1. Mean FEV₁ of the COPD patients was $50.6 \pm 5.7\%$ predicted. The anthropometric data did not show any significant differences between patients and healthy controls.

Table 1. Characteristics of the study population

	Healthy control subjects (n = 7)	Patients with COPD (n = 7)
Age, yr	60.7 ± 1.0	61.9 ± 1.4
Pack-yr of smoking	11.6 ± 4.5	31.4 ± 8.7
BMI, kg/m ²	26.8 ± 1.3	25.0 ± 1.3
FFMI, kg/m ²	18.5 ± 0.5	17.2 ± 0.6
FEV ₁ , % predicted	108.5 ± 9.7	$50.6 \pm 5.7^{**}$
FVC, % predicted	113.9 ± 9.5	96.1 ± 7.6
Inflammation		
IL-6 (pg/mL)	1.93 ± 0.25	2.43 ± 0.28
TNF-α (pg/mL)	1.97 ± 0.15	1.95 ± 0.13
IL-1β (pg/mL)	0.31 ± 0.02	0.35 ± 0.03
Oxidative stress		
Carbonyls (nM)	0.03 ± 0.01	$0.10 \pm 0.03^*$

Definitions of abbreviations: COPD = chronic obstructive pulmonary disease; BMI = body mass index; FFMI = fat-free mass index. Data are presented as mean $\pm$ SEM.

Significant difference compared with healthy control subjects, * $p < 0.05$; ** $p < 0.01$.

The results of the exercise tests are presented in Table 2. As expected, peak exercise capacity was decreased in COPD patients when compared with healthy control subjects (111 ± 16 vs. $238 \pm 16\%$; $p < 0.01$). During incremental cycle ergometry patients reached their ventilatory threshold whereas heart rate reserve was significantly higher compared to the healthy controls ($p < 0.01$). Baseline forkhead transcription factor O1 (FOXO1) mRNA expression was not different between both groups and as expected FOXO1 mRNA was significantly and to the same extent upregulated during the submaximal exercise test ($p < 0.05$). This indicates that the exercise intensity of the chosen exercise test was sufficient to induce transcriptional changes in both patients and controls.

Table 2. Exercise characteristics of healthy subjects and patients with chronic obstructive pulmonary disease

	Healthy control subjects (n = 7)	Patients with COPD (n = 7)
Incremental exercise		
Peak workload, W	238.3 ± 16.3	111.4 ± 15.7**
Peak $\dot{V}O_2$, ml/min	2440 ± 136	1353 ± 107**
Peak HR, beat/min	166 ± 6	133 ± 2**
HR reserve, %	-0.4 ± 3.5	16.1 ± 1.3**
Peak $\dot{V}E$, L/min	93.5 ± 5.3	57.1 ± 5.9**
$\dot{V}E$ reserve, %	20.8 ± 10.8	-1.3 ± 8.7
Constant work-rate exercise		
Workload, W	166.9 ± 11.4	78.4 ± 10.9**
$\dot{V}O_2$, ml/min	1939 ± 270	1177 ± 150
$\dot{V}E$, L/min	72.3 ± 7.1	43.6 ± 4.0*
ΔCt FOXO1	8.8 ± 0.2	8.6 ± 0.2
$2^{-\Delta\Delta Ct}$ FOXO1	2.1 ± 0.44 [‡]	1.6 ± 0.25 [‡]

Definitions of abbreviations: COPD = chronic obstructive pulmonary disease; HR = heart rate; MVV = maximum voluntary ventilation; ΔCt FOXO1 = baseline mRNA levels forkhead transcription factor O1; $2^{-\Delta\Delta Ct}$ = fold changes. Data are presented as mean ± SEM.

*Significant difference compared with healthy control subjects, $p < 0.02$.

**Significant difference compared with healthy control subjects, $p < 0.01$.

[‡]Significant difference compared with baseline, $p < 0.05$.

Markers of systemic inflammation and oxidative stress

As expected (according to disease severity and body composition of the patients) at baseline, no significant differences of plasma inflammatory markers were observed between COPD patients and healthy controls (Table 1). Also in line with previous studies, COPD patients showed increased levels of protein oxidation compared to healthy controls ($p < 0.05$; Table 1). In the healthy control group but not in the patients the exercise bout resulted in elevated systemic IL-6 levels 2h after exercise (1.93 ± 0.25 before vs. 2.22 ± 0.15 2h after exercise; $p < 0.05$).

NF- κ B DNA binding activity

As shown in Figure 1A, the basal NF- κ B DNA binding activity in peripheral blood mononuclear cells was significantly lower in COPD patients compared to healthy controls ($p < 0.05$). No effect of exercise on NF- κ B in peripheral blood mononuclear cells was observed in both groups. Furthermore, no exercise-induced changes in NF- κ B DNA binding activity in skeletal muscle of both groups could be detected (Figure 1B).

Figure 1. NF- κ B DNA-binding activity was determined in (A) peripheral blood mononuclear cells and (B) skeletal muscle in healthy control subjects and patients with COPD before, immediately after, and 2h after exercise. * $p < 0.05$ significantly different between healthy control subjects and COPD patients. Values are expressed as mean $\pm$ SEM; healthy controls ($\square$) and COPD (hatched).

NF- κ B related gene expression

In this study, all genes were normalized to GAPDH, although similar results were obtained using Cyclophilin A as housekeeping gene (data not shown). Between group comparison revealed that at baseline, only MyoD mRNA content was significantly decreased in COPD compared to controls ($9.6 \pm 0.2 \Delta\text{Ct}$ controls vs. $10.5 \pm 0.3 \Delta\text{Ct}$ COPD; $p < 0.05$).

Exercise-induced cytokine response. Healthy controls: As shown in Figure 2, muscle I κ B α mRNA levels increased 1.5-fold immediately after exercise. IL-6, TNF- α and IL-1 β mRNA levels increased 27-, 2.5- and 5.5-fold respectively. Also 2h after exercise a 2-fold increase of IL-6 mRNA levels was observed. Patients: Only IL-6 mRNA levels were increased (20-fold) immediately after exercise. Moreover, the response of IL-1 β to exercise was significantly lower in COPD than in healthy controls ($p < 0.05$; Figure 2). In both groups, there was no significant correlation between the changes in plasma cytokines and the increases in cytokine muscle mRNA levels.

Figure 2. Relative gene expression of muscle IκBα and cytokines IL-6, TNF-α and IL-1β in COPD patients and healthy controls at different time points after the exercise test. A $-\Delta\Delta C_t$ value above 0 indicates increased gene expression. Fold changes are presented in boxes (f): * $p < 0.05$; # $p < 0.02$ significantly different from baseline values; ‡ $p < 0.05$ significantly different between healthy control subjects and subjects with COPD. Values are expressed as mean $\pm$ SEM; outliers (o); healthy controls (□) and COPD (■).

Exercise-induced antioxidants response. Two hours after exercise, MnSOD mRNA levels increased 2-fold in healthy controls (Figure 3). No changes in MnSOD were observed in skeletal muscle of COPD. In contrast, Trx mRNA levels decreased 0.7-fold in COPD patients immediately after exercise (Figure 3). This decrease tended to be significant ($p=0.091$). Additionally, Trx response significantly differed between COPD and healthy controls after exercise ($p<0.05$).

Figure 3. Relative gene expression of muscle antioxidant genes Mn-superoxide dismutase (MnSOD) and thioredoxin (Trx) in COPD patients and healthy controls at different time points after the exercise test. A $-\Delta\Delta Ct$ value above 0 indicates increased gene expression. Fold changes are presented in boxes (f): $^*p<0.05$; $^+p=0.091$ significantly different from baseline values; $^{\ddagger}p<0.05$ significantly different between healthy control subjects and subjects with COPD. Values are expressed as mean $\pm$ SEM; outliers ($\circ$); healthy controls ($\square$) and COPD ($\blacksquare$).

Exercise-induced stress proteins. HO-1 mRNA increased 3.5-fold only in healthy controls 2h after exercise ($p<0.05$; Figure 4). Moreover, immediately after exercise Hsp70 mRNA levels increased in healthy controls ($p<0.05$; Figure 4) but no changes were observed in skeletal muscle of COPD.

Figure 4. Relative gene expression of muscle stress proteins, heme oxygenase-1 (HO-1) and heat shock protein 70 (Hsp70) in COPD patients and healthy controls at different time points after the exercise test. A $-\Delta\Delta Ct$ value above 0 indicates increased gene expression. Fold changes are presented in boxes (f); * $p < 0.05$ significantly different from baseline values. Values are expressed as mean $\pm$ SEM; outliers ($\circ$); healthy controls ($\square$) and COPD ($\blacksquare$).

Exercise-induced apoptosis and myogenic differentiation related genes. No significant exercise-induced change in Bcl2 mRNA expression was observed in any groups (Figure 5). However, Bcl2 response significantly differed between COPD and healthy controls after exercise ($p = 0.05$; Figure 5). For MyoD mRNA expression, no exercise-induced changes were observed in both groups (data not shown).

Figure 5. Relative gene expression of muscle apoptosis related Bcl2 in COPD patients and healthy controls at different time points after the exercise test. A $-\Delta\Delta C_t$ value above 0 indicates increased gene expression. § $p < 0.05$ significantly different between healthy control subjects and subjects with COPD. Values are expressed as mean $\pm$ SEM; outliers ($\circ$); healthy controls ($\square$) and COPD ($\blacksquare$).

Marker of muscle oxidative stress

Representative immunoblots of protein oxidation (detected as total carbonyls) in skeletal muscle of control subjects and patients with COPD are shown in Figure 6A. Four to five bands with molecular weight ranging from 20 to 68 kD were detected in both groups. The migration pattern of these bands was similar between the two groups. Mean values of cumulative carbonyl band intensity in patients with COPD were significantly higher at baseline compared to healthy controls ($p < 0.05$; Figure 6B). Moreover, 2h after exercise, total protein carbonylation levels were significantly lower in COPD patients compared to healthy controls ($p < 0.05$; Figure 6B).

Figure 6. (A) Representative immunoblots of muscle protein carbonyl groups in healthy controls and patients with COPD before (t0) and 2h after exercise (t1). Four to five bands with molecular weight varying from 20 to 68 kD were detected in both groups. Negative control (underivatized protein) is also shown. (B) Mean values of total carbonyl optical densities in skeletal muscles of healthy controls (□) and patients with COPD (■). * $p < 0.05$ significantly different from baseline values; # $p < 0.05$ significantly different between healthy control subjects and subjects with COPD.

DISCUSSION

The data in this study reveal that in patients with moderate COPD and in absence of muscle wasting or an enhanced systemic inflammatory response, NF- κ B activity in PBMCs is diminished. Reduced basal NF- κ B activity in PBMCs coincided with a consistently blunted exercise-induced response of NF- κ B mediated gene expression in skeletal muscle.

NF- κ B is a critical intracellular mediator of inflammatory cytokines, antioxidants and survival factors. Limited information is available regarding the exercise-induced inflammatory response in patients with COPD. In contrast to earlier studies in patients with severe COPD and muscle atrophy, we did not find elevated basal plasma inflammatory cytokine levels (IL-6, TNF- α and IL-1 β) in this moderate COPD study group.[21, 22] Furthermore, plasma inflammatory cytokine levels were unchanged by the exercise test in COPD patients, suggesting that exercise did not induce systemic inflammation. Contradictory results have been reported for the exercise-induced inflammatory response in COPD.[21, 23] These discrepancies could be due to differences in patient population, study design and methodology. A

striking feature of our study was the markedly reduced basal levels of NF- κ B activity in peripheral blood mononuclear cells in COPD patients compared to healthy controls. Reduced NF- κ B DNA binding activity might be explained by the therapeutic use of glucocorticoids which have been shown to be potent inhibitors of activation of NF- κ B through multiple mechanisms.[24] Alternatively, higher levels of endogenous steroids such as cortisol might be present in COPD.

Intriguingly, muscle I κ B α gene expression was only significantly upregulated after exercise in healthy controls, indicative of an increased NF- κ B activation in healthy controls after exercise.[25] In line with the increased I κ B α expression in healthy controls, muscle IL-6, TNF- α and IL-1 β mRNA gene expression increased after exercise indicating an inflammatory response in exercised muscle of healthy controls. Exercise-induced increases in muscle inflammatory cytokines, particularly IL-6 mRNA gene expression have been reported previously in healthy subjects.[26] In the present study, however, no significant changes were observed in muscle TNF- α and IL-1 β mRNA expression after exercise in patients with COPD in agreement with a previous study.[23] In contrast to TNF- α and IL-1 β , muscle IL-6 was increased immediately after exercise in COPD, although this increase was attenuated in comparison to healthy controls. Furthermore, the upregulated transcription of cytokines in skeletal muscle was not reflected in increased plasma cytokine levels. This can be explained by a different time-frame required for protein synthesis, but may also indicate that cytokines produced by skeletal myocytes act in a paracrine/autocrine fashion.[27] Although several studies have associated pro-inflammatory cytokines to skeletal muscle injury that occurs with strenuous exercise, there is evidence that under normal physiological conditions these cytokines contribute to repair processes. Opposing evidence from experimental studies regarding the role of TNF- α and inflammatory signalling in regeneration has been documented. It appears that, depending on timing, duration, magnitude and composition of the inflammatory response, muscle regeneration may beneficially or detrimentally be affected by TNF- α in a nuclear factor NF- κ B-dependent fashion.[28, 29] Expression of IL-1 β mRNA has also been linked to muscle tissue repair and regeneration.[30] Therefore, it is tempting to hypothesize that the observed higher expression of these inflammatory cytokines mediated by NF- κ B in healthy controls may be a requirement for the remodelling and regeneration processes following exercise. Thus, the attenuated response observed in COPD may reflect the impaired regenerative capacity of skeletal muscles and may ultimately contribute to muscle wasting. This attenuated regenerative capacity may also be reflected by the decreased muscle mRNA levels of MyoD in COPD patients at baseline, since MyoD has a key role in regulating muscle differentiation and regeneration.[31] No exercise-induced significant differences were observed for this gene in both groups. This however

could be due to the timing of the biopsies, since MyoD mRNA expression has been reported to peak between 4-12 hours after exercise and not as early as 2 hours.[32] Another class of NF- κ B regulated genes that rapidly responds to ROS/inflammation includes antioxidant enzymes, such as MnSOD and Trx. The inability of muscle of COPD patients to respond to acute exercise was also apparent from the expression of these antioxidant genes. To date, no studies are available that have evaluated the effect of exercise on muscle MnSOD and Trx mRNA expression in COPD. MnSOD mRNA expression was increased in exercised muscle of healthy controls only. In addition, Trx showed a tendency to be lower in skeletal muscle of COPD than healthy controls. Despite the increased expression of antioxidants, healthy controls showed enhanced levels of muscle protein carbonylation 2h after exercise. A likely explanation for the absence of an increase in protein carbonylation in COPD patients after exercise might be attributed to the already elevated levels at rest. With respect to stress proteins with antioxidant properties, the present investigation indicates a reduced exercise-induced expression of HO-1 and Hsp70 at mRNA level in skeletal muscle of patients with COPD when compared to healthy controls. No comparison with other studies is possible since data regarding the effects of an acute exercise on HO-1 or Hsp70 expression in skeletal muscle of COPD are not available in literature. Both stress proteins appear to be highly inducible by exercise and represent a major factor acting against exercise-induced oxidative stress.[19, 33] The protective effects of HO-1 activity have been attributed to the antioxidant properties of its byproducts (biliverdin, carbon monoxide and ferritin). It is still controversial as to how exercise-generated ROS induce upregulation of Hsp70. Taken together, these data show that COPD patients have a reduced muscle antioxidant defense system in response to exercise when compared to healthy controls.

It has also been suggested that NF- κ B plays an important role in programmed cell death or apoptosis.[34] Conflicting results have been reported regarding the incidence of apoptosis in skeletal muscles from patients with COPD.[35, 36] In the present study, the anti-apoptotic protein Bcl2 response was significantly lower in COPD than in healthy controls after exercise. This might imply that skeletal muscle from COPD patients is less well protected from apoptosis, although more markers of survival and apoptosis should be measured to properly address this hypothesis.

Strikingly, despite the activation of the NF- κ B regulated genes in response to exercise, the present study showed no exercise-induced changes in NF- κ B DNA binding activity in skeletal muscle of both groups. Results from earlier studies investigating NF- κ B activity have been contradictory, demonstrating either increased activity after an acute exercise [37, 38] or a reduction in NF- κ B nuclear binding activity after exercise.[39] Differences in duration of the exercise protocols likely explain differences between studies. Nevertheless, alterations in the expression of NF- κ B regulated genes are not solely governed by translocation, but also by post-translational

modification of RelA. For instance, phosphorylation [40] and acetylation [41] strongly enhance transcriptional activity of RelA, which may explain the increased expression of NF- κ B target genes observed in healthy controls in absence of alterations in DNA binding activity.

Limitations of the study

A limitation of the present study is the relatively small number of subjects studied. Because this was a complex study design that involved multiple muscle biopsies, recruitment typically was difficult. Moreover, we studied clinically and weight stable COPD patients with moderate disease and normal fat-free mass to exclude potential changes in muscle NF- κ B activity resulting from disease exacerbations or pulmonary cachexia. Yet, despite the relatively small number of subjects studied, we found consistent results in terms of a blunted exercise-induced response of NF- κ B mediated gene expression in skeletal muscle. We were also able to detect between-group comparisons of the exercise response.

Conclusions

These data clearly reveal differential NF- κ B mediated gene expression of skeletal muscle in response to exercise in patients with moderate COPD compared to healthy age-matched controls. The impaired response to exercise in COPD patients is reflected by a blunted expression of inflammatory cytokines, antioxidants, heat shock proteins and survival factors. Further research is needed to delineate the specific role of redox-regulated pathways in exercise-induced adaptations in COPD as well as the modulating effect of rehabilitation.

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Chapter 4

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Online Data Supplement:

Reduced NF- κ B mediated gene expression in skeletal muscle of patients with COPD in response to acute exercise

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MATERIALS AND METHODS

Study population

All patients were ex-smokers and had not experienced respiratory tract infection or exacerbation of their disease for at least 4 weeks prior to the study. Clinically and weight stable COPD patients with moderate disease and normal fat-free mass were included to exclude potential changes in muscle NF- κ B activity resulting from disease exacerbations or pulmonary cachexia. This group also reflects the COPD population for which exercise training is indicated as integrated part of disease management. In addition, all participants were questioned on their dietary habits to ensure that none were taking antioxidants or vitamin supplements. All patients received anticholinergic and β 2-agonists as bronchodilator therapy, five patients received inhaled corticosteroids and one patient theophylline. None were treated with oral corticosteroids.

Anthropometry and spirometry

Body composition was determined by bioelectrical impedance analysis (BIA; Xitron technologies, San Diego, CA, USA) as described previously (E1). All participants underwent flow volumes including measurements of FEV₁ and FVC with the highest value from at least three properly performed measurements being used for analysis. The values obtained were expressed as a percentage of the reference value (E2).

Exercise capacity

Participants performed an incremental (10 Watt/minute) cycle ergometer test as described previously (E3). Expired gases were analyzed using breath-by-breath analysis through a breathing mask (Oxygen- β , Jaeger, Würzburg, Germany). Peak ventilatory ($\dot{V}E$) reserve was calculated as $100\% - (100 \times \text{peak } \dot{V}E) / (\text{FEV}_1 \times 37.5)$ and peak heart rate (HR) reserve was calculated as $100\% - (100 \times \text{peak HR}) / (220 - \text{age})$ (E4, E5). The submaximal exercise test was performed at 70% of peak workload achieved during the incremental exercise test. This exercise protocol was chosen since it is comparable with regular exercise training as part of pulmonary rehabilitation. The endurance time was standardized at 10 minutes.

Markers of systemic inflammation and oxidative stress

Venous blood samples (10 ml) were drawn into ethylenediaminetetraacetic acid (EDTA)-containing tubes (Venoject). All blood samples were immediately put on ice, and kept on ice during sample preparation. Plasma was obtained by centrifugation ($800 \times g$ for 10 minutes at 4°C) and stored at -80°C until analysis.

Preparation of nuclear extracts

PBMCs After removal of the plasma, the 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 (Greiner) of peripheral blood mononuclear cells (PBMCs). After centrifugation at 800 x *g* for 30 min at 4°C, gradient-separated PBMCs were recovered, resuspended in 10-ml cold PBS, and centrifuged again at 250 x *g* for 10 min at 4°C. The cells were resuspended in 1 ml cold PBS and centrifuged at 800 x *g* for 5 min and the pellet was used to prepare nuclear extracts for NF- κ B determination. Nuclear extracts were prepared as described by Hofmann et al. (E6).

Skeletal muscle. Frozen muscle was homogenized in ice-cold buffer A (20 mM HEPES pH 7.8, 20 mM KCl, 4mM MgCl₂, 0.2 mM EDTA pH 8.0, 1 mM DTT, 0.2 mM NaVanadate, 0.4 mM PMSF, 0.3 mg/ml Leupeptin, 0.2 mM NaFl). After homogenizing samples were kept on ice for 15 minutes and incubated on a rotating platform at 4°C for 45 minutes. Buffer B (Nonidet P40) was added, samples were mixed vigorously and centrifuged for 5 minutes at 14000 rpm. Supernatant was removed (=cytoplasmatic fraction), pellets were washed in buffer A+B, mixed and centrifuged. Supernatant was removed and nuclei were lysed in buffer C (100 mM HEPES pH 7.8, 100 mM KCl, 600 mM NaCl, 0.2 mM EDTA pH 8.0, 20% glycerol, 1 mM DTT, 0.2 mM NaVanadate, 0.4 mM PMSF, 0.2 mM NaFl) by a 20 minute incubation at a rotating platform at 4°C. Samples were centrifuged (5 min at 14000 rpm), supernatant was transferred to a new eppendorf tube and stored at -80°C.

Skeletal muscle analysis

Gene expression analyses by quantitative real-time PCR. Biopsies were analyzed for FOXO1, I κ B α , IL-6, TNF- α , IL-1 β , MnSOD, Trx, HO-1, Hsp70, Bcl2 and MyoD expression. For this, total RNA was isolated from ~ 60 mg of muscle tissue which was homogenized (POLYTRON® PT 2100, Kinemaitca AG) in presence of TRIzol® reagent (Gibco BRL). Total RNA was extracted according to the manufacturer's instructions. The RNeasy® Mini Kit (Qiagen) was used to purify total RNA from salts and residual DNA. Total RNA concentration was determined spectrophotometrically. cDNA was generated from 0.4 μ g of total RNA according to the manufacturer's instructions using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Aliquots were used for real-time PCR on the BioRad MyiQ iCycler Single Color real-time PCR detection system using iQ™ SYBR® Green Supermix, containing all ingredients; iTaq Polymerase, dNTPs, SYBR® Green I and buffers. GAPDH, Cyclophilin A and β -actin were used as housekeeping genes and all reactions were performed in duplicate. The expression of β -actin appeared to be influenced by exercise, as described previously, but GAPDH and Cyclophilin A were stable in response to exercise (E7). Therefore, β -actin was left out in the further

analyses. To avoid inter-assay variability, all samples were analyzed together in one single real-time PCR run. The data were analyzed using ΔCt and $\Delta\Delta\text{Ct}$ (E8-E10). To compare the relative gene expression between COPD patients and healthy controls at baseline ΔCt was used. The $\Delta\Delta\text{Ct}$ was used to calculate relative changes in gene expression within each group as a result of the exercise test. Fold changes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. PCR primers are shown in Table E1.

Table E1. Primer sequences.

Target mRNA	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
FOXO1	CGCCCTCGAACTAGCTCAAA	GCGGGTACACCATAGAATGCA
I κ B α	CTACACCTTGCCTGTGAGCA	TCCTGAGCATTGACATCAGC
IL-6	CCAGGAGCCCAGCTATGAAC	CCCAGGGAGAAGGCAACTG
TNF- α	TCAATCGGCCCGACTATCTC	CAGGGCAATGATCCCAAAGT
IL-1 β	CTGAGCTCGCCAGTGAAATG	TTTAGGGCCATCAGCTTCAAA
MnSOD	GTGGTCCATGAAAAAGCAGATGA	CACAAGCCAAACGACTTCCA
Trx	TGTGGATGACTGTCAGGATGTTG	TTGTCCCTTCTTAAAAAACTGGAAT
HO-1	CTTCTTCACCTTCCCAACA	GCTCTGGTCCTTGGTGTCAAT
Hsp70	TCGAGGTGGCCGTTAGTTG	AGGCGAGCGACGTTAGGA
Bcl2	TCGCCCTGTGGATGACTGA	CAGAGACAGCCAGGAGAAATCA
MyoD	CACAGCGCGGTTTTTTCC	TGAACCTAGCCCCTCAAGGTT

Definition of abbreviations: FOXO1: forkhead transcription factor O1; MnSOD: Mn-superoxide dismutase; Trx: thioredoxin; HO-1: heme oxygenase 1; Hsp70: heat shock protein-70; MyoD: myogenic differentiation factor.

Western blotting. Frozen muscle samples were homogenized in an aqueous buffer containing Tris-HCl (20mM), NaCl (150mM), Nonidet P-40 (1% v/v), Dithiothreitol (1mM), Sodium orthovanadate (1mM), Phenylmethylsulphonyl fluoride (1mM), Leupeptin (10 $\mu\text{g}/\text{ml}$) and Aprotinin (1%) (pH=7.4). Samples were then centrifuged at 10.000 x g at 4°C for 30 min. The pellet was discarded and the supernatant was designated as a crude homogenate. Total muscle protein level in each sample was determined with the Bradford (Biorad Inc., Hercules, CA) technique. Changes in protein carbonylation in crude homogenates were detected using a commercial kit (Oxyblot kit; Chemicon International Inc.). To evaluate the selectivity of carbonyl measurements, muscle protein samples also underwent protein carbonyl detection procedure without the derivatization step (negative control). The blots were scanned with an imaging densitometer and optical densities (OD) of protein bands were quantified using Image Master VDS (Amersham Pharmacia Biotech, Uppsala, Sweden) software. Total carbonyls in each muscle sample were calculated by adding OD of individual carbonylated protein bands.

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CHAPTER 5

Acute exercise triggers muscle UCP3 in COPD: adaptive mechanism against lipotoxicity?

ABSTRACT

Peripheral muscle dysfunction is a hallmark of chronic obstructive pulmonary disease (COPD) in which an important role for oxidative stress is evolving. We hypothesized that in COPD muscle gene expression of uncoupling protein-3 (UCP3), which protects mitochondria against lipotoxicity, is upregulated during acute exercise as an adaptation to a blunted response of the antioxidant defense system and β -oxidation. Vastus lateralis biopsies were obtained before and 2h after a 10 min submaximal (70% of peak workload) cycle ergometry exercise from 7 COPD patients and 6 healthy age-matched controls. Gene expression of UCP3, PPAR γ co-activator-1 α (PGC-1 α ; regulator of β -oxidation) and antioxidant Mn-superoxide dismutase (MnSOD) were measured using RT-PCR. Lipid peroxidation (4-hydroxy-2-nonenal) and over-oxidized peroxiredoxin III were determined by Western blot. UCP3 was upregulated after exercise in COPD (2-fold; $p < 0.05$), but not in controls. Exercise induced a marked increase in PGC-1 α in controls (9-fold; $p < 0.05$), but to a lower extent in COPD (6-fold; $p < 0.05$). MnSOD was only upregulated in controls after exercise (2-fold; $p < 0.05$). COPD patients showed enhanced muscle lipid peroxidation after exercise ($p < 0.05$). These data are supportive of an inadequate response of the antioxidant and β -oxidation capacities to acute exercise leading to adaptive upregulation of UCP3 in peripheral muscle of patients with COPD.

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INTRODUCTION

Oxidative stress is a prominent feature of chronic obstructive pulmonary disease (COPD) and has been proposed as a potential mechanism of peripheral muscle dysfunction and impaired exercise tolerance (1). Increased levels of intramuscular lipid peroxidation products have been reported for COPD at baseline and in response to exercise (2, 9). Moreover, on the opposite side of the balance, reduced muscle antioxidant levels were observed in COPD (9, 25). These data are indicative of increased muscular oxidative stress in COPD, especially during exercise, being the result of enhanced oxidant formation and/or impaired antioxidant capacity. Exercise in general has long been known to elicit an increase in the production of reactive oxygen species (ROS) as a result of increases in mitochondrial oxygen consumption and electron transport flux (7). With exercise the supply of free fatty acids (FFAs) to the muscles is also increased, which may lead to the accumulation of fatty acid anions in the mitochondrial matrix (29). This combination of higher ROS production and increased fatty acids anion levels enhances the risk of oxidation of these fatty acid anions (i.e. lipid peroxidation). Several defense mechanisms exist: The β -oxidation of FFAs prevents them from being oxidized in the first place. In mitochondria, superoxide anion is converted to hydrogen peroxide by manganese superoxide dismutase (MnSOD), and subsequently converted to water by the enzyme glutathione peroxidase and the thioredoxin-dependent enzyme peroxiredoxin III (Prx III) (8). The mammalian peroxiredoxin is a novel family of peroxidases and has six members expressed in several subcellular compartments. Prx III is specifically expressed in mitochondria (33). Finally, uncoupling protein-3 (UCP3) is considered to be a key player in the protection of mitochondria against lipotoxicity, because it is capable of reducing ROS production by lowering the mitochondrial membrane potential and/or the removal of fatty acid anions from the mitochondrial matrix (4, 18, 28). Accordingly, we previously showed that muscle UCP3 levels increased by exercise training in COPD patients with a blunted response of the β -oxidation, suggesting that UCP3 was upregulated in response to an insufficient capacity to oxidize FFAs (14). It is likely that the peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) is involved in this impaired response of the β -oxidation, as it is a master regulator of mitochondrial oxidative capacity (31). Considering the evidence for increased muscular oxidative stress in COPD in combination with the incapability of the muscle to sufficiently improve antioxidant defense and β -oxidation in response to exercise training, we hypothesized that the exercise-induced upregulation of antioxidants enzymes such as MnSOD and the β -oxidation is impaired resulting in a compensatory upregulation of UCP3. To test this hypothesis, quadriceps muscle biopsies were obtained before and after acute exercise from seven patients with COPD and six healthy age-matched controls.

MATERIALS AND METHOD

Study population

Seven clinically stable male patients with moderate COPD as defined by GOLD guidelines participated in the study (12). All patients were ex-smokers and had not experienced respiratory tract infection or exacerbation of their disease for at least 4 weeks prior to the study. Exclusion criteria were rheumatoid arthritis, chronic colitis, diabetes, cardiovascular diseases, renal diseases, liver diseases or mental diseases. None of the patients went through a rehabilitation program before inclusion. All patients received anticholinergic and β 2-agonists as bronchodilator therapy, five patients received inhaled corticosteroids and one patient theophylline. None were treated with oral corticosteroids. Six healthy, sedentary age-matched non-smoking males were recruited as controls. None of the subjects were taking antioxidants or vitamin supplements. Written informed consent was obtained from all participants, and the study was approved by the medical ethics committee of the University Hospital Maastricht (Maastricht, The Netherlands).

Study Design

The participants were instructed to abstain from strenuous physical activity and to consume a light breakfast on the test day and the exercise tests were performed at the same time point of the day. First, they completed an exhaustive incremental cycle ergometry test and four days later a submaximal constant work rate cycle ergometry test. This exercise protocol was chosen since it is comparable with regular exercise training as part of pulmonary rehabilitation. Biopsies of vastus lateralis were obtained at baseline and 2 hours after the submaximal exercise test. This time point was chosen to determine the early gene response after an acute bout of exercise (22).

Assessment of body composition

Body composition was determined by bioelectrical impedance analysis (BIA; Xitron technologies, San Diego, CA, USA) as described previously (27).

Pulmonary Function Tests

All participants underwent flow volumes including measurements of FEV₁ and FVC with the highest value from at least three properly performed measurements being used for analysis. The values obtained were expressed as a percentage of the reference value (24).

Exercise tests

Participants performed an incremental (10 Watt/minute) cycle ergometry test as described previously (13). Expired gases were analyzed using breath-by-breath analysis through a breathing mask (Oxygen-β, Jaeger, Würzburg, Germany). Peak ventilatory ($\dot{V}E$) reserve was calculated as $100\% - (100 \times \text{peak } \dot{V}E) / (\text{FEV}_1 \times 37.5)$ and peak heart rate (HR) reserve was calculated as $100\% - (100 \times \text{peak HR}) / (220 - \text{age})$ (6, 32). The submaximal exercise test was performed at 70% of the peak workload achieved during the incremental exercise test. The endurance time was standardized at 10 minutes. Also oxygen consumption was monitored as described in the incremental exercise test.

Muscle biopsies

Percutaneous biopsy specimens of vastus lateralis muscle of the dominant leg at baseline and the non-dominant leg post-exercise were obtained as described by Bergström (3). Muscle samples were immediately frozen in liquid nitrogen and then stored at -80°C until further analysis.

Skeletal muscle analyses

Gene expression analyses by real-time PCR. Biopsies were analyzed for UCP3, PGC-1 α and MnSOD mRNA expression. For this, total RNA (~ 60 mg) was isolated by means of a TRIzol[®] reagent (Gibco BRL), using a Polytron homogenizer (POLYTRON[®] PT 2100, Kinemaitca AG). Total RNA was extracted according to the manufacturer's instructions. The RNeasy[®] Mini Kit (Qiagen) was used to purify total RNA from salts and residual DNA. cDNA was generated from 0.4 μg of total RNA according to the manufacturer's instructions using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Aliquots were used for real-time PCR on the BioRad MyiQ iCycler Single Color real-time PCR detection system using iQ[™] SYBR[®] Green Supermix. GAPDH, Cyclophilin A and β -actin were used as house-keeping genes and all reactions were performed in duplicate. The expression of β -actin was influenced by exercise, but GAPDH and Cyclophilin A were equally stable in response to exercise. Therefore, β -actin was left out in the further analyses. We have chosen to show the results for gene expression corrected by both GAPDH and Cyclophilin A because in the literature a substantial variability of housekeeping genes following an acute exercise has been reported (16, 19). To avoid inter-assay variability, COPD patients as healthy controls and the two time points for each gene were run together in one single real-time PCR run. The data were analyzed using the expression ΔCt and $\Delta\Delta\text{Ct}$. To compare the relative gene expression between COPD patients and healthy controls at baseline the expression ΔCt was used. The expression $\Delta\Delta\text{Ct}$ was used to calculate relative changes in gene expression within

each group as a result of the exercise test. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. PCR primers are shown in Table 1.

Table 1. Primer sequences used for PCR analysis

	Forward	Reverse
UCP3	CGTGGTGATGTTTCGTAACCTATG	CGGTGATTCCCGTAACATCTG
PGC-1 α	GACCAGTGCTACCTGAGAGAGACTT	GCTCGGCTCGGATTTCCT
MnSOD	GTGGTCCATGAAAAAGCAGATGA	CACAAGCCAAACGACTTCCA

Definitions of abbreviations: Uncoupling protein-3 (UCP3), PPAR γ co-activator-1 α (PGC-1 α); Mn-superoxide dismutase (MnSOD)

Western blotting. Frozen muscle samples were homogenized in ice-cold Tris-EDTA buffer at pH=7.4. Samples were then centrifuged at 10,000 x *g* at 4°C for 30 min. The pellet was discarded and the supernatant was designated as a crude homogenate. Equal amounts of protein were loaded on to a 10-15% SDS PAGE gel, and electrophoresis was performed using a Mini-PROTEAN 3 Electrophoresis Cell system (Bio-Rad, USA). We used polyclonal anti-HNE antibody diluted 1:10000 (Calbiochem, San Diego, CA) and polyclonal anti-peroxiredoxin-SO₃ diluted 1:500 (LabFrontier, Seoul, South Korea) antibodies. The secondary antibody was horse anti-rabbit peroxidase. Detection was performed using SuperSignal West Dura Extended Duration Substrate (Pierce Boston Technology Centre, Woburn, MA, USA) on a CL-Xposure Film (Pierce Boston Technology Centre). The blots were scanned with an imaging densitometer and optical densities (OD) of protein bands were quantified using Image Master VDS (Amersham Pharmacia Biotech, Uppsala, Sweden) software. Total 4HNE in each muscle sample were calculated by adding OD of individual protein bands.

Venous blood analysis

Determination of plasma free fatty acids (FFAs). Blood samples were drawn into EDTA-containing tubes (Venoject; Terumo Corporation, Leuven, Belgium), and plasma was obtained by centrifugation (800 x *g* for 10 min at 4°C) and stored at -80°C until analysis. Plasma FFAs were determined using the Wako NEFA C test kit (Wako Chemicals, Neuss, Germany).

Statistical Analyses

Data are presented as mean $\pm$ SEM. Between-group comparisons were analyzed using the Mann-Whitney U test and the Wilcoxon signed rank test was used to evaluate the effect of submaximal exercise within the groups. For analysis of baseline gene expression ΔCt was used and for the effect of exercise on gene expression $\Delta\Delta Ct$. 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 13.0; SPSS, Inc., Chicago, IL).

RESULTS

Anthropometric and Spirometric Data

The subject characteristics are presented in Table 2. The anthropometric data did not show any significant differences between COPD patients and healthy controls. The COPD group was characterized by moderate airflow obstruction with a FEV₁ of $50.6 \pm 5.7\%$ predicted.

Table 2. Characteristics of the study population

	Healthy subjects (n = 6)	Patients with COPD (n = 7)
Age, yr	60.7 ± 1.0	61.9 ± 1.4
Pack-yr of smoking	11.6 ± 4.5	31.4 ± 8.7
BMI, kg/m ²	26.8 ± 1.3	25.0 ± 1.3
FFMI, kg/m ²	18.5 ± 0.5	17.2 ± 0.6
FEV ₁ , % predicted	108.5 ± 9.7	$50.6 \pm 5.7^*$
FVC, % predicted	113.9 ± 9.5	96.1 ± 7.6

Definitions of abbreviations: BMI = body mass index; COPD = chronic obstructive pulmonary disease; FFMI = fat-free mass index; FEV₁ = forced expiratory volume in 1 s; FVC = forced vital capacity. Data are presented as mean $\pm$ SEM.

*Significant difference compared with the healthy control subjects, $p < 0.01$.

Exercise tests

The results of the exercise tests are presented in Table 3. As expected, exercise capacity was limited in COPD patients when compared with healthy control subjects ($p < 0.01$). Although COPD patients reached their maximal voluntary ventilation ($\dot{V}_{E\text{reserve}} \approx 0$), this parameter was not significantly different between both groups, whereas the heart rate reserve was preserved in COPD ($p < 0.01$). Baseline plasma free fatty acids were similar between both groups, and as expected were significantly increased immediately after submaximal exercise in the healthy controls, which indicates that the exercise intensity/duration of the chosen exercise test was sufficient ($p < 0.05$).

Table 3. Exercise characteristics of healthy subjects and COPD patients

	Healthy subjects (n = 6)	Patients with COPD (n = 7)
Incremental exercise		
Peak workload, W	238.3 ± 16.3	111.4 ± 15.7*
Peak $\dot{V}O_2$, ml/min	2440 ± 136	1353 ± 107*
Peak HR, beat/min	166 ± 6	133 ± 2*
HR reserve, %	-0.4 ± 3.5	16.1 ± 1.3*
Peak $\dot{V}E$, L/min	93.5 ± 5.3	57.1 ± 5.9*
$\dot{V}E$ reserve, %	20.8 ± 10.8	-1.3 ± 8.7
Constant workload exercise		
Workload, W	166.9 ± 11.4	78.4 ± 10.9*
$\dot{V}O_2$, ml/min	1939 ± 270	1177 ± 150
$\dot{V}E$, L/min	72.3 ± 7.1	43.6 ± 4.0 [§]
Δ free fatty acids, μmol/L	70.5 ± 20.1 [‡]	86.5 ± 53.2

Definitions of abbreviations: COPD = chronic obstructive pulmonary disease; HR = heart rate. Data are presented as mean ± SEM.

*Significant difference compared with the healthy control subjects, $p < 0.01$.

[§]Significant difference compared with the healthy control subjects, $p < 0.02$.

[‡]Significant difference compared with baseline, $p < 0.05$.

Gene expression

Basal gene expression. In COPD, significantly decreased baseline mRNA content of UCP3 (-0.6 ± 0.2 ΔCt controls vs. 0.5 ± 0.4 ΔCt COPD; $p < 0.05$), normalized to Cyclophilin A, were observed. PGC-1α and MnSOD mRNA levels were similar in patients with COPD and healthy controls. The same pattern was found when glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeper.

Exercise-induced gene expression. Relative gene expression of UCP3, MnSOD and PGC-1α are shown in Figure 1. In COPD patients, we observed a 2-fold increase of UCP3 mRNA levels ($p < 0.05$), normalized to Cyclophilin A, in response to exercise, whereas no difference of UCP3 gene expression was found in the controls. Moreover, UCP3 response tended to be higher in COPD than in healthy controls after exercise ($p = 0.073$). The same tendency was observed with GAPDH. Exercise induced a 2-fold increase in MnSOD mRNA levels in the healthy controls ($p < 0.05$), but no changes were observed in the COPD patients. In addition, the MnSOD response tended to be lower in COPD than controls ($p = 0.073$). These results were consistent for both housekeepers. With Cyclophilin A, PGC-1α mRNA levels increased 6- and 9-fold after exercise in COPD patients and healthy controls, respectively (both $p < 0.05$). Moreover, with GAPDH the PGC-1α response in the healthy controls was significantly higher than in COPD patients 2h after exercise ($p < 0.05$).

Figure 1. Relative gene expression of uncoupling protein-3 (UCP3), Mn-superoxide dismutase (MnSOD) and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α) in COPD patients and healthy controls (HC) before and 2 h after exercise. * $p < 0.05$ significantly different from baseline values; # $p < 0.05$, ‡ $p = 0.073$ significantly different between healthy control subjects and subjects with COPD.

Markers of muscle oxidative stress

As illustrated in Figure 2A, the anti-HNE antibody detected several protein bands, with apparent molecular weights ranging from 30 to 98 kD, in the muscles of both patients and control subjects. The skeletal muscles of patients with COPD showed higher levels of HNE-protein adducts 2h after exercise ($p < 0.05$; Figure 2B). However, there were no correlations between UCP3 mRNA expression and HNE levels. No differences could be detected in the expression of over-oxidized Prx III between COPD patients and control subjects. Additionally, exercise did not induce any significant change in over-oxidized Prx III in either patients or controls (data not shown).

Figure 2. (A) Representative examples of lipid peroxidation (detected with anti-HNE antibody) in vastus lateralis biopsies of healthy controls and patients with COPD before and after exercise. Several HNE-protein adducts were detected. (B) Individual values of total HNE optical densities in skeletal muscles of healthy controls and patients with COPD. * $p < 0.05$ significantly different compared with values before exercise.

DISCUSSION

The main finding of the present study is that an acute bout of exercise upregulate UCP3 gene transcription in skeletal muscle of COPD, but not in healthy controls, despite the higher absolute workload in healthy control subjects. This response is associated with a blunted upregulation of MnSOD and PGC-1 α gene transcription in COPD in combination with enhanced intramuscular lipid peroxidation, as assessed by 4-HNE. These findings are supportive of a role for UCP3 in protecting the muscle cell against ROS-induced lipotoxicity.

It has been proposed that UCP3 plays a role in lowering the ROS production (4) (see also Figure 3). The mitochondrial electron transport chain is a major non-enzymatic source for the formation of ROS (7). One to two percent of total oxygen consumed leaks away in the form of superoxide anions. ROS production, especially of superoxide, is increased during exercise as a result of the build-up of a proton gradient across the inner mitochondrial membrane through increased mitochondrial oxygen consumption and electron transport flux. Consequently, the enhanced formation of superoxide may in turn result in lipid peroxidation (21). It has been suggested that UCP3, through mild uncoupling, decreases the mitochondrial membrane potential and hence attenuates the rise in ROS production (4, 11, 18). This negative feedback loop would protect the cell against ROS-induced cellular damage. In the present study, baseline UCP3 mRNA levels in the limb muscle of patients with COPD were reduced compared to healthy controls, which is in agreement with previous studies (15, 26). However, in contrast to healthy subjects, only COPD patients responded to an acute exercise by increasing skeletal muscle UCP3 gene expression which was accompanied by a blunted induction of MnSOD. The absence of an immediate increased UCP3 expression after exercise in healthy controls could be explained by the fact that their antioxidant defense system, in particular MnSOD, is able to cope with the increased production of ROS generated during the exercise test. The mitochondrial isoform MnSOD has the ability to eliminate superoxide that arises from the electron transport chain to generate hydrogen peroxide. Consequently, hydrogen peroxide is degraded by Prx III and glutathione peroxidase. In the present study, COPD patients showed increased intramuscular lipid peroxidation after exercise compared to healthy controls, as assessed by 4-HNE adduct proteins. This finding is in agreement with a previous study who observed increased muscle thiobarbituric acid reactive substances in COPD patients after local exercise (9). However, in the current study, an acute bout of exercise did not induce any change in the over-oxidized Prx III levels in the groups. Hence, the increased muscle lipid peroxidation observed after exercise in patients with COPD could be related to the reduced MnSOD mRNA expression. It has been suggested that both superoxide and lipid peroxidation breakdown products, such as 4-HNE, activate UCP3 thereby lowering the mitochondrial membrane potential and attenuate the rise in ROS production (21). Apart from a role in lowering ROS production, UCP3 has also been postulated to play a role in fatty acid metabolism, serving as a fatty acid anion exporter from the mitochondrial matrix (10) (see also Figure 3). Fatty acids entering the mitochondrial matrix are normally oxidized through fatty acid β -oxidation. Interestingly, 3-hydroxyacyl-CoA dehydrogenase (HAD), a key enzyme of the β -oxidation of fatty acids, has consistently been shown to be reduced in COPD patients compared to healthy controls, which implies a reduced fatty acid oxidation in COPD (20). PGC-1 α is considered a master regulator of mitochondrial biogenesis and metabolism, including the components of the fatty acid β -oxidation,

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especially in response to exercise (23, 31). In this study, PGC-1 α mRNA expression was indeed significantly increased 2h after exercise in COPD patients as well in the healthy control subjects, but interestingly, this response was significantly lower in COPD. Exercise is known to increase plasma FFAs and due to the blunted PGC-1 α response observed in this study and as a result of an impaired oxidative capacity in COPD, it is likely that the fatty acid supply to the muscle exceeds its oxidizing capacity (20). As consequence, cytosolic fatty acids will accumulate and *flip-flop* into the mitochondrial matrix as fatty acid anions where they can neither be metabolized nor cross the mitochondrial membrane (30). These fatty acid anions are prone to be attacked by superoxide inevitably leading to muscle lipid peroxidation, which we indeed observed in COPD. Upregulation of UCP3 could prevent the accumulation of fatty acid anions in the mitochondrial matrix (17) and/or lower the proton gradient across the inner mitochondrial membrane, thereby reducing ROS production (5) and the risk of lipid peroxidation.

Figure 3. Schematic model of mitochondrial lipid peroxidation. 1) *ROS production.* Superoxide ($O_2^{\bullet-}$) is generated primarily by the electron transport chain (ETC). Superoxide is dismutated to hydrogen peroxide (H_2O_2) by Mn-superoxide dismutase (MnSOD). UCP3 attenuates superoxide production through mild uncoupling, thereby decreasing mitochondrial membrane potential. The enzyme peroxiredoxin III (Prx III) and glutathione peroxidase (Gpx) degrades H_2O_2 to water (H_2O) and thereby decreases hydroxyl radical formation and subsequent muscular lipid peroxidation products. 2) *Accumulation of fatty acid anions.* Free fatty acids (FFAs) entering the mitochondrial matrix will be oxidized through fatty acid β -oxidation, which is regulated by peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α). Exercise increases plasma FFAs and if the supply of FFAs to the muscle exceeds the capacity to oxidize them, they will accumulate in the mitochondrial matrix as fatty acid anions. These fatty acid anions will be deprotonated by superoxide anions leading to lipid peroxidation. UCP3 acts as a fatty acid anion exporter, thereby preventing them from being peroxidized.

Limitations of the study

We found significant differences of exercise within in the groups. A main limitation of this study however is related to a relatively small sample size which may not have had enough statistical power for the between-group comparison of the exercise response. However, we still found that the exercise response of PGC-1 α was

significantly different between COPD and healthy controls. Also, the UCP3 and MnSOD response tended to be different between both groups. Another limitation is that we did not assess protein levels in the present study. Although large responses on the protein level are not expected immediately after exercise we can only assume that the results found for the gene transcripts also reflect a protein response on the longer term. This study was primarily designed to investigate the acute exercise-induced effects on gene regulation.

Conclusion

Since exercise-induced muscle oxidative stress is a consistent finding for COPD, this disease provides a unique model to study the physiologic response of UCP3 to oxidative stress *in vivo*. Taken together, these data suggest that the impaired oxidative capacity, reflected in a blunted PGC-1 α response and a previously reported reduced HAD-activity, in combination with an increased production of superoxide as a result of a blunted MnSOD response, increase exercise-induced muscle lipid peroxidation. The upregulation of UCP3 mRNA in patients with COPD could very well be a compensatory mechanism against this lipotoxicity. The differences found between COPD patients and healthy controls emphasize the importance to further unravel the underlying mechanisms by which UCP3 affects the ROS-induced lipotoxicity. Eventually, this may result in novel targets for therapies for peripheral muscle dysfunction in this disease.

LIST OF ABBREVIATIONS

BIA: bioelectrical impedance analysis
 COPD: chronic obstructive pulmonary disease
 FFAs: free fatty acids
 GAPDH: glyceraldehyde 3-phosphate dehydrogenase
 HAD: 3-hydroxyacyl-CoA dehydrogenase
 4-HNE: 4-hydroxy-2-nonenal
 HR: heart rate
 MnSOD: Mn-superoxide dismutase
 OD: optical density
 PGC-1 α : peroxisome proliferator-activated receptor γ coactivator-1 α
 PrxIII: peroxiredoxin III
 ROS: reactive oxygen species
 UCP3: uncoupling protein-3
 VE: ventilation per unit time

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CHAPTER 6

Dichloroacetate reduces exercise-induced systemic stress response in COPD

ABSTRACT

Background: Impaired skeletal muscle function contributes to exercise intolerance in patients with chronic obstructive pulmonary disease (COPD). Exercise-induced oxidative stress may initiate or accelerate impaired muscle function. Dichloroacetate (DCA) activates muscle pyruvate dehydrogenase complex (PDC) at rest, reducing inertia in mitochondrial energy delivery at the onset of exercise and thereby diminishing anaerobic energy production. This study aimed to determine whether DCA infusion may also reduce exercise-induced systemic oxidative stress in COPD patients. *Methods:* A randomized, double-blind crossover design was used in which thirteen COPD patients performed maximal cycle exercise after an intravenous infusion of DCA (50mg/kg body mass) or saline (placebo). Venous blood was sampled before, immediately after, 30 min and 2h after exercise. Urine samples were obtained before and 2h after exercise. *Results:* Peak workload improved significantly after DCA compared to placebo (10%; $p<0.01$). Urinary uric acid after exercise was significantly lower in the DCA condition when compared to placebo, whereas no significant difference was observed for urinary malondialdehyde. Oxidized glutathione (GSSG) was significantly increased 2h after exercise in the placebo condition ($p<0.02$), but not after treatment. No changes in reduced glutathione (GSH), GSSG/GSH ratio and superoxide dismutase activity were observed. Plasma IL-6 significantly increased 2h after exercise only in the DCA condition ($p<0.01$). *Conclusions:* This study shows that the improved performance after a pharmacological intervention known to activate PDC was accompanied by an enhanced IL-6 response and a reduced exercise-induced systemic oxidative stress.

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INTRODUCTION

Peripheral skeletal muscle dysfunction is a common feature in patients with chronic obstructive pulmonary disease (COPD) and contributes to exercise intolerance, disability and poor quality of life and survival [1]. Chronic inflammation and oxidative stress have been implicated in the etiology of peripheral muscle dysfunction [2, 3]. Increased systemic oxidative stress has been demonstrated in COPD patients at rest which is further intensified after exercise [4, 5]. Furthermore, a reduction in skeletal muscle mitochondrial oxidative energy capacity has been observed in COPD, which may also contribute to exercise intolerance [1, 6-8]. Additionally, it has been reported that muscle adenine nucleotide loss occurs at low exercise absolute workload in COPD [9]. This suggests that ATP resynthesis is unable to meet the energy demands of exercise even at the low work rates patients with COPD can achieve [9].

There is a delay in skeletal muscle mitochondrial ATP production at the onset of exercise, which results in the reliance on non-oxidative sources of energy production to meet this shortfall in ATP supply in the early stages of exercise. Recent evidence suggests that this metabolic inertia resides at the level of the pyruvate dehydrogenase complex (PDC), a mitochondrial multi-enzyme complex which regulates carbohydrate entry into the tricarboxylic acid cycle [10] and catalyses the irreversible oxidation of pyruvate to mitochondrial acetyl CoA.

Dichloroacetate (DCA), a pharmacological agent, increases mitochondrial aerobic energy production through the activation of PDC. The mechanism of action is by inhibition of PDC kinase [11]. DCA has been used in humans for the treatment of mitochondrial diseases and lactic acidosis [12]. Moreover, several studies have investigated the effects of DCA on skeletal muscle energy metabolism in healthy controls after exercise [13, 14]. Recently, Calvert et al. [15] demonstrated that by activating PDC blood lactate and ammonia accumulation decreased during exercise and improved maximal exercise performance in COPD. However, the effect of DCA on exercise-induced oxidative stress and inflammatory markers has not been explored in humans yet. Therefore, the purpose of this study was to assess the effect of DCA infusion on exercise-induced systemic oxidative stress and inflammation in COPD patients. We assume that metabolic stress, as indicated by significant adenine nucleotide loss, leads to oxidative stress in part through the activity of xanthine oxidase which is a known source of free radicals. Given that DCA increases mitochondrial energy production, thereby improving the supply of ATP and attenuating adenine nucleotide loss, as measured by a reduction in ammonia accumulation [16], we hypothesized that DCA would reduce exercise-induced oxidative stress. We were also interested in exploring whether modifying the meta-

bolic response by DCA might alter the inflammatory response to exercise. We evaluated these questions by investigating the effect of DCA treatment on systemic variables of oxidative stress and inflammation during a maximal cycle exercise in COPD patients who were participating in the original trial [15].

MATERIALS AND METHODS

Study population

The decision to undertake the current investigation was made after the start of the original trial and therefore thirteen of the 18 stable COPD patients examined in the study of Calvert et al. [15] were included. Patients met clinical and spirometric GOLD criteria for COPD [17, 18]. Patients were excluded if taking maintenance oral corticosteroids, unable to perform exercise tests, exercise desaturation ($\text{SaO}_2 < 80\%$), cardiac dysfunction, exacerbation of COPD within the previous 6 weeks, or pulmonary rehabilitation within the last year. Full approval was obtained from the Leicestershire Research Ethics Committee and all participants provided informed written consent.

Assessment of body composition

Body mass index was calculated from height (measured by wall mounted stadiometer to the nearest 0.1 cm) and weight (measured in light clothing to the nearest 0.1 kg (SECA, UK)). Fat free mass (FFM) was estimated (Bodystat 1500, Bodystat Ltd, Douglas, UK) and calculated using disease-specific regression equations [19].

Pulmonary function tests

Spirometry was performed to ERS standards (Vitalograph Model R, Buckingham, UK). Predicted values were calculated from ERS regression equations [18].

Study design

Details on the study design have been reported previously [15]. Briefly, subjects performed a maximal incremental exercise test on an electrically braked cycle ergometer. After a two-week washout period, subjects repeated the exercise challenge. Prior to exercise participants received either 50mg/kg body mass of Dichloroacetate (DCA; 25mg/ml, sodium salt) or an equivalent volume of normal saline as an intravenous infusion into a forearm vein over 45 minutes. Peripheral venous blood samples were taken at rest, immediately after, 30 min and 2h after the maximal incremental exercise test. Urine samples were obtained at rest and 2h after the exercise test. Moreover, arterialized-venous blood samples for lactate and

ammonia analysis were taken at different time points during and after exercise as described previously [15].

Sample preparation

Venous blood samples were drawn into ethylenediaminetetraacetic acid-containing tubes. Plasma was obtained by centrifugation (800 x *g* for 10 minutes at 4°C) and stored at -80°C until analysis. Urine samples were collected in sterile containers and stored at -20°C until further analysis.

Markers of systemic oxidative stress

Urinary malondialdehyde (MDA) and urinary were assessed by HPCL described by Lepage et al. [20] and by Lux and colleagues [21] respectively. Both urinary MDA and uric acid concentration were corrected for creatinine contents [22].

Erythrocyte superoxide dismutase (SOD) activity was determined according to the method of Sun et al. [23]. Hemoglobin (Hb) concentration was determined [24].

Erythrocyte glutathione was measured in its reduced [25] and oxidized [26] form.

Markers of systemic inflammation

Plasma concentrations of IL-6 and TNF- α were determined using a quantitative high-sensitivity enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Data are expressed as mean $\pm$ SD or as mean $\pm$ SEM. A paired *t*-test was used to test the effect of treatment on exercise-induced biological markers. Putative correlations between variables were evaluated using Pearson correlation coefficients. A difference with $p < 0.05$ was considered statistically significant. Statistical analyses were analyzed with SPSS for Windows (version 13.0; SPSS, Inc., Chicago, IL).

RESULTS

Patient characteristics

Anthropometric and spirometric data are shown in Table 1. The COPD group was characterized by moderate airflow obstruction. Subjects were either ex-smokers ($n=8$) or current smokers ($n=5$).

Table 1. Characteristics of the study population

	COPD (n = 13)
Age (years)	65.8 ± 6.5
Sex, M/F	11/2
FEV ₁ (% pred)	48.0 ± 15.1
FEV ₁ (L)	1.24 ± 0.50
BMI (kg/m ²)	30.3 ± 6.2
FFMI (kg/m ²)	19.8 ± 2.6
Oxygen saturation at rest (%)	95.2 ± 1.7

Data are expressed as mean ± SD. FEV₁= forced expiratory volume in 1 second; BMI= body mass index; FFMI= fat free mass index.

Exercise capacity

The results of the incremental exercise test performed after DCA and normal saline infusion are presented in Table 2. Peak workload increased significantly after DCA treatment (10%; $p < 0.01$). Moreover, both peak oxygen consumption and peak ventilation were significantly increased after DCA infusion compared with placebo (9%; $p < 0.01$ and 12%; $p < 0.02$ respectively). RER at peak exercise was significantly greater following DCA ($p < 0.001$). DCA infusion also significantly reduced blood lactate and ammonia accumulation compared to placebo ($p < 0.01$ and $p < 0.001$ respectively)[15].

Table 2. Exercise data from maximal cycle exercise in COPD patients after DCA and saline infusion

COPD patients (n=13)		
	Saline	DCA
Peak workload (watts)	70.0 ± 27.6	78.1 ± 29.8 ^{**}
$\dot{V}O_{2\text{ peak}}$ (ml/min)	1314.9 ± 470.0	1448.6 ± 530.9 ^{**}
Peak $\dot{V}_E$ (L/min)	36.9 ± 14.0	41.8 ± 15.8 [*]
Peak $\dot{V}_E$ (%MVV)	89 ± 28	98 ± 28 [*]
Peak RER	0.92 ± 0.06	0.95 ± 0.06 ^{***}
Peak HR (%pred)	75 ± 9	74 ± 9
Δ Lactate	1.46 ± 0.74	1.06 ± 0.58 ^{**}
Δ Ammonia	32.4 ± 13.6	19.5 ± 8.6 ^{***}

Data are expressed as mean ± SD. MVV: maximum voluntary ventilation (calculated as FEV₁ x 35); RER: respiratory exchange ratio; HR: heart rate (calculated as age-220).

^{*} $p < 0.02$; ^{**} $p < 0.01$; ^{***} $p < 0.001$ significantly different compared to saline.

Markers of systemic oxidative stress and inflammation

The results of the systemic oxidative stress and inflammatory markers at rest and 2h after exercise following placebo and DCA infusion are shown in Table 3.

Table 3. Markers of systemic oxidative stress and inflammation in COPD patients at rest and 2h after exercise following DCA and saline infusion

COPD patients (n=13)				
	Saline		DCA	
	before	2h after	before	2h after
Erythrocyte GSH ($\mu\text{mol/gHb}$)	6.3 ± 0.7	6.1 ± 0.4	6.4 ± 0.9	7.1 ± 1.1
Erythrocyte GSSG ($\mu\text{mol/gHb}$)	0.64 ± 0.07	$0.74 \pm 0.08^*$	0.71 ± 0.06	0.75 ± 0.08
Erythrocyte GSSG/GSH	0.15 ± 0.05	0.13 ± 0.02	0.15 ± 0.03	0.12 ± 0.02
Erythrocyte SOD (U/mgHb)	1208 ± 146	1420 ± 186	1236 ± 131	1293 ± 122
Plasma IL-6 (pg/ml)	2.5 ± 0.3	3.2 ± 0.5	2.4 ± 0.2	$4.5 \pm 0.7^{**}$
Plasma TNF- α (pg/ml)	2.6 ± 0.5	2.6 ± 0.5	2.5 ± 0.5	2.6 ± 0.5

Data are expressed as mean $\pm$ SEM. GSH: reduced glutathione; GSSG: oxidized glutathione; SOD: superoxide dismutase.

* $p < 0.02$; ** $p < 0.01$ significantly different from baseline values.

Baseline values of systemic oxidative stress and inflammation were not significantly different between both conditions (data not shown). Urinary uric acid was 2h after exercise significantly lower in the DCA condition when compared to placebo ($p < 0.05$; Figure 1A). For urinary MDA, no significant differences between both groups were observed 2h after exercise (Figure 1B).

DCA reduces exercise-induced systemic stress response

Figure 1. Concentration of urinary uric acid (A) and urinary malondialdehyde (MDA) (B) 2h after exercise in placebo and DCA condition. *p<0.05 significantly different 2h after exercise between placebo versus DCA.

No significant effect of exercise was observed for erythrocyte GSH levels after both conditions, whereas erythrocyte GSSG levels were significantly elevated 2h after exercise in the placebo condition (p<0.02; Figure 2). However, levels of GSSG-to-GSH ratio were unchanged after both conditions. Furthermore, no significant exercise-induced change in erythrocyte SOD activity was observed after both conditions.

Figure 2. Effect of exercise on erythrocyte GSSG levels in placebo and DCA condition. *p<0.02 significantly different from baseline values.

Remarkably, as shown in Figure 3, the exercise-induced IL-6 response was significantly higher in the DCA condition compared to placebo ($p < 0.001$). Moreover, IL-6 levels were significantly increased 2h after exercise following DCA treatment ($p < 0.01$). No significant exercise-induced effects were observed for TNF- α after both conditions. However, a negative correlation was observed between exercise-induced changes in IL-6 and TNF- α levels ($r = -0.80$, $p < 0.01$; Figure 4) after DCA treatment, indicating that a rise in IL-6 might be a physiological response to attenuate exercise induced TNF- α .

Furthermore, no significant correlations were found between changes in blood markers of the metabolic response and markers of oxidative stress and inflammation.

Figure 3. Effect of exercise on plasma IL-6 levels in placebo and DCA condition. * $p < 0.01$ significantly different from baseline values; # $p < 0.001$ significantly different between placebo versus DCA.

Figure 4. There was an inverse and significant correlation between exercise-induced changes in IL-6 and TNF- α after DCA condition ($r=-0.80$, $p<0.01$).

DISCUSSION

This study shows that a pharmacological intervention with DCA, which has been previously reported to improve exercise performance, also resulted in a reduced exercise-induced systemic oxidative stress and enhanced IL-6 response in patients with COPD. Another important finding of the present study was the inverse relationship between exercise-induced changes in IL-6 and TNF- α levels after DCA treatment, suggesting that DCA treatment may have an anti-inflammatory effect in COPD. It is known that DCA improves the supply of ATP and thereby attenuates adenine nucleotide loss, as measured indirectly by a reduction in ammonia accumulation [15], and consequently this enhanced mitochondrial (oxidative or aerobic) energy production could play a role in decreasing the exercise-induced systemic oxidative stress and pro-inflammatory response in COPD.

During strenuous exercise, ATP is consumed at a greater rate than it can be replenished, leading to adenine nucleotide degradation, followed by increases in the plasma concentrations of hypoxanthine, xanthine, and uric acid. Uric acid is an indicator of xanthine oxidase activity, which is seen as an important source of reactive oxygen species (ROS). In the present study, urinary uric acid after exercise was significantly lower in the DCA condition when compared to placebo which could point to an increased production of free radicals due to xanthine oxidase activity in

the placebo group. In addition, the higher exercise-induced urinary uric acid levels after placebo condition might also reflect greater muscle adenine nucleotide loss during exercise, since the formation of uric acid is irreversible. This finding is supported by previously published data of Calvert et al. [15] who observed increased blood ammonia accumulation after placebo condition compared to DCA. This is also in keeping with previous data showing that adenine nucleotide loss occurs at low absolute exercise work rates in COPD which is associated with a rise in blood ammonia [9, 16]. These observations indicate that ATP resynthesis is unable to meet the energy demands of exercise after placebo treatment suggesting that metabolic stress occurs in COPD patients during maximal exercise. This aberrant energy production might be countered by DCA that has been reported to activate PDC at the onset of exercise leading to an increase in the provision of mitochondrial acetyl CoA which was paralleled by an exponential rise in mitochondrial ATP production [25, 27, 28]. In the present study, urinary MDA levels were reduced after DCA infusion providing additional evidence for reduced exercise-induced oxidative stress in these COPD patients. Furthermore, we observed increased blood GSSG levels after placebo treatment, also indicating increased oxidative stress. However, no differences were observed for neither GSH nor for the GSSG-to-GSH ratio between the two conditions, nor for the activity of the antioxidant enzyme SOD. Therefore, the reduced exercise-induced systemic oxidative stress following DCA is likely to be attributable to a more efficient oxidative metabolism, as DCA is known to increase the overall contribution of oxidative pathways at the onset of exercise. DCA infusion activates muscle PDC at rest, reducing inertia in mitochondrial energy delivery at the onset of exercise and thereby diminishing non-oxidative (anaerobic) energy production. PDC is a key enzyme in controlling the selection of different respiratory substrate fuels. To meet the demands of an increasing workload as occurs during strenuous exercise, PDC has been reported to stimulate the relative contribution of glucose at the expense of fatty acids as substrate. Therefore, it is reasonable to assume that shifting the balance from fatty acids to carbohydrate oxidation results in greater efficiency of ATP production since oxidation of glucose requires less oxygen per unit ATP produced than oxidation of fatty acids. This is supported by higher RER after DCA observed in this study, which might indicate a preferential utilization of carbohydrate over fatty acids during exercise. It has also been hypothesized that decreasing fatty acid oxidation and increasing glucose oxidation improves the “coupling” between glycolysis and glucose oxidation, thereby preventing proton accumulation and hence less intracellular acidosis [29]. This is in agreement with lower blood lactate accumulation after DCA observed in this study. In the placebo condition, on the other hand, a reduced aerobic ATP production lead to a greater reliance on non-oxidative sources through anaerobic pathways and increased blood lactate levels. These conditions may induce an increased leakage of electrons from a disrupted respiratory chain and the generation of more ROS

[30]. Taken together, these results indicate that in COPD, a more efficient oxidative metabolism during exercise could reduce the exercise-induced systemic oxidative stress response, although we were unable to find significant correlations between changes in markers of oxidative and metabolic stress. This may be due to differences in kinetics of formation and disposal of ammonia, lactate and oxidation products. However, further research is necessary to elucidate the underlying mechanisms responsible for the reduced exercise-induced oxidative stress response following DCA treatment.

A marked increase of plasma IL-6 levels after exercise has been a consistent finding in healthy controls [31]. It has therefore been suggested that contraction-induced IL-6 production in skeletal muscle may mediate several diverse metabolic and physiological effects. IL-6 has been proposed to mediate glucose homeostasis during exercise by stimulation of hepatic glucose production. In the present study, plasma IL-6 levels were significantly increased after exercise in the DCA condition only. Moreover, a negative correlation between changes in IL-6 and TNF- α levels was observed after DCA treatment. This is in line with previous studies showing that increased IL-6 levels inhibit the production of the pro-inflammatory cytokine TNF- α [32-34]. Furthermore, data have been provided by Petersen et al. [35] that support the hypothesis that IL-6 has important anti-inflammatory effects in relation to exercise. Altogether, the above proposed functions suggest that the exercise-induced increased IL-6 levels after DCA are a normal physiological response.

Clinical implications

Our data suggest that exercise in the absence of DCA dampened the IL-6 response, and given the inverse relationship of IL-6 with TNF- α after DCA treatment it could be speculated that DCA may have an anti-inflammatory effect in COPD. Moreover, DCA attenuated the systemic oxidative stress response during exercise in COPD. It is suggested that daily life activities can cause regularly bursts of systemic inflammation and oxidative stress in COPD that may be involved in muscle wasting. In this respect, DCA might have important clinical implications especially in the subgroup of COPD patients with muscle wasting. In this point of view it would be interesting to study the role of DCA in muscle wasted COPD patients.

Limitations of the study

We found significant differences of exercise within both conditions. A main limitation of this study however is related to a relatively small number of patients included which may not have enough statistical power for the between-group comparison of the exercise response. However, an exercise-induced change of urinary uric acid and plasma IL-6 was found between both conditions. Another limitation of the

current study is that we did not directly measure the activity of PDC, but the data on activation by DCA in this dose is fairly robust [13, 36, 37].

In summary, we have demonstrated that DCA infusion administered before a maximal exercise test reduces exercise-induced systemic oxidative stress which was accompanied by an improved exercise performance. In light of the current observation, it would be of interest to study PDC as a target to modulate the exercise-induced oxidative stress response.

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ABBREVIATION LIST

COPD: chronic obstructive pulmonary disease

DCA: dichloroacetate

FFM: fat free mass

GSH: reduced glutathione

GSSG: oxidized glutathione

Hb: hemoglobin

MDA: malondialdehyde

PDC: pyruvate dehydrogenase complex

RER: respiratory exchange ratio

ROS: reactive oxygen species

SOD: superoxide dismutase

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CHAPTER 7

General discussion and future directions

The data presented in this thesis show that COPD patients are characterized by an increased pulmonary and systemic oxidative stress response at rest as well as induced by exercise compared to healthy controls, which was not paralleled by enhanced pro-inflammatory cytokines. Intriguingly, in contrast to healthy controls, our data suggest a blunted exercise-induced expression of cytokines, antioxidants, heat shock proteins and survival factors in skeletal muscle of COPD patients. Moreover, our data are supportive of an inadequate antioxidant and β -oxidation capacity leading to an adaptive upregulation of uncoupling protein-3 (UCP3) during exercise in skeletal muscle of patients with COPD. Interestingly, in COPD patients not suffering from muscle wasting we showed that increased exercise capacity after pulmonary rehabilitation was associated with reduced exercise-induced oxidative stress. These intriguing observations will be placed in a broader perspective in the following section.

THE EXERCISE-INDUCED OXIDATIVE STRESS PARADOX

Exercise-induced oxidative stress and inflammation have gained a lot of attention in the last decade. Previous studies showed an increased systemic oxidative stress response after strenuous whole body exercise in patients with COPD (1-3). However, the effect of exercise intensity on systemic oxidative stress and potential differential effects on pulmonary oxidative stress have not been clearly defined and are described in **Chapter 2**. This study showed that clinically and weight stable patients with moderate COPD are chronically exposed to low-grade pulmonary as well as systemic oxidative stress, which further increased after both maximal (high-intensity) and submaximal (moderate intensity) exercise. Therefore, these patients might also frequently be exposed to repeated episodes of oxidative stress during activities of daily living. These studies focused on whole body exercise during which the ventilatory system is in most patients the dominant limiting factor of exercise intolerance (4, 5). Moreover, under these circumstances, it is difficult to identify the specific source of exercise-induced oxidative stress in COPD: lungs, peripheral skeletal muscles, respiratory muscles or maybe even other sites. Therefore, in **Chapter 3**, the effect of a constant work rate single leg ergometer test on systemic inflammation and oxidative stress as well as on pulmonary oxidative stress was evaluated in patients with COPD. In line with the increased systemic oxidative

stress response observed after whole body exercise, COPD patients also showed a greater susceptibility to local exercise-induced systemic oxidative stress, without evidence of increased levels of systemic inflammation during exercise. The increased pulmonary oxidative stress observed after maximal whole body exercise in COPD, as described in **Chapter 2**, could be contributed to increased ventilatory demands. Remarkably, even despite the low cardioventilatory responses, an increased pulmonary oxidative stress response was also observed after a localized quadriceps exercise in patients with COPD, but not in healthy controls indicating an enhanced production of reactive oxygen species (ROS) in the airways of these patients. Possible mechanisms that might play a role in the exercise-induced pulmonary stress response include increased xanthine oxidase activity in epithelial cells, an inappropriate NADPH oxidase activation and/or an intensified pulmonary inflammatory response. Further research will have to elucidate whether these or other mechanisms may indeed be involved in the exercise-induced pulmonary oxidative stress response. Interestingly, our data demonstrated that the susceptibility of exercise intolerance was not homogenous among patients with COPD as was reflected in a subgroup that had to quit the exercise prematurely. These so called quitters were characterized by a reduced metabolic capacity, indicating a greater reliance on anaerobic metabolism, which may limit local quadriceps endurance capacity. Moreover, they showed a less pronounced oxidative stress response compared to non-quitters which could be explained by the fact that the oxidative stress threshold was not reached due to early muscle fatigue in the quitters. Based on these findings, it could be speculated that nutritional or pharmacological intervention will be more beneficial in specific subgroups of patients and that more extensive phenotyping of COPD patients is indicated to achieve optimal benefits of pulmonary rehabilitation.

Besides increased basal systemic oxidative stress, we observed enhanced oxidative stress in skeletal muscle of patients with COPD, as documented in **Chapter 4**. Intriguingly, the higher oxidative stress within the skeletal muscle of COPD patients was not paralleled by elevated pro-inflammatory cytokines. This suggests that inflammation and oxidative stress do not coexist in COPD patients at rest. Increased oxidative stress in skeletal muscle of patients with COPD is a relative consistent finding in the literature (6-8). However, a controversy exists regarding inflammatory markers at rest in skeletal muscle of COPD patients. Some studies support the presence of local inflammation in skeletal muscle of patients with COPD (9, 10) whereas others do not support this finding (11-13). To date, it is still unclear whether a local muscle inflammatory response is beneficial or detrimental in patients with COPD. Although several reports have clearly linked cytokine-mediated effects, particularly tumor necrosis factor alpha (TNF- α), to muscle dysfunction, muscle atrophy and inhibition of muscle regeneration (14-16), there is also evidence that

these cytokines are essential to repair processes (17-20). It appears that, depending on timing, duration, magnitude, and composition of inflammatory response, muscle regeneration may be beneficially or detrimentally affected by TNF- α . Consequently, further studies should be designed to address this apparent controversy. Whether peripheral skeletal muscle is a potential source of exercise-induced oxidative stress and inflammation was further confirmed with needle biopsies of the vastus lateralis in **Chapter 3 and Chapter 4**. Herein, it was demonstrated that a submaximal cycle ergometer test can indeed produce oxidative stress within the skeletal muscle. Conversely, Rabinovich and colleagues (21) were unable to document increased muscle oxidative stress after whole body exercise at a lower intensity. However, Couillard et al. (22) showed that a local quadriceps exercise performed until exhaustion produced oxidative stress within the contracting muscle. Therefore, it could be argued that both whole body and local exercise, if sufficiently intense, are able to produce oxidative stress within the skeletal muscle of patients with COPD.

Although exercise training is highly recommended in the pulmonary rehabilitation setting for the purpose of improving physical performance and health related quality of life, exercise-induced increases in oxidative stress may adversely affect outcome in COPD (3, 22). This 'exercise paradox' was evaluated in **Chapter 3** by exploring the effects of pulmonary rehabilitation on markers of exercise-induced oxidative stress in COPD patients. Interestingly, in COPD patients, pulmonary rehabilitation resulted in significant improvements in exercise capacity which was associated with reduced exercise-induced systemic oxidative stress, especially after a submaximal exercise. Therefore, this study indicates that exercise training is a preventive treatment of exercise-induced systemic oxidative stress in COPD and hence it is reasonable that physical exercise training can reduce potential adverse effects associated with daily activities. The reduced exercise-induced systemic oxidative stress response may be attributed to adaptive responses involving a more efficient oxidative metabolism or an increased capacity of endogenous antioxidative systems. Knowledge about adaptation of the skeletal muscle antioxidant defense system to exercise training in COPD is limited. However, the former may likely occur since Rabinovich and colleagues (21) reported that patients with COPD had a reduced ability to adapt to endurance training, as reflected by a lower capacity to synthesize reduced glutathione.

In view of both an increased resting and exercise-induced oxidative stress in COPD and the positive effect of exercise training on oxidative stress, it is of interest to investigate whether nutritional or pharmacological supplementation with antioxidants as adjunct to exercise training further modifies basal or exercise-induced systemic oxidative stress and thereby improves outcome of rehabilitation. Only limited data are available on the effects of specific nutritional intervention on oxida-

tive stress in COPD. Firstly, it has been shown that exercise-induced oxidative stress can be (partially) prevented by the antioxidant N-acetylcysteine (NAC) in COPD (1). Secondly, it has also been reported that oral supplementation with NAC reduced basal disturbance in the pro-oxidant system and prevented the exercise-induced oxidative stress in patients with COPD (3). Moreover, short-term high dose NAC treatment improved quadriceps endurance in COPD (3). However, long-term improvement of patients is obviously the ultimate goal. At present, as part of the ENIGMA (European Network for investigating the global mechanisms of muscle abnormalities in COPD) project, the effects of prolonged NAC supplementation (3 months) on muscle function and oxidative stress at rest is investigated in patients with COPD. Moreover, we are currently performing a double-blind randomized study in which the effect of nutritional supplementation enriched with antioxidants as adjunct to exercise training on exercise-induced systemic oxidative stress and exercise capacity is assessed in COPD.

More recent studies have underlined the importance of an altered energy metabolism as mechanism for development of peripheral muscle dysfunction. Several reports have shown a reduced oxidative energy metabolism in skeletal muscle from COPD patients, which is even more compromised after exercise, indicating an imbalance between the utilization and resynthesis of ATP in COPD (23-26). Recently, it has been shown in COPD patients that by modulating skeletal muscle oxidative energy metabolism using dichloroacetate (DCA) infusion before a maximal exercise challenge, blood lactate and ammonia accumulation decreased and exercise performance improved (27). Whether an increased muscle energy status could additionally decrease exercise-induced oxidative stress was explored in **Chapter 6**. It was shown that by triggering skeletal muscle oxidative energy metabolism in COPD with DCA, a reduced exercise-induced systemic oxidative stress response was observed compared to placebo. A likely explanation for the reduced systemic exercise-induced oxidative stress following DCA is attributable to a more efficient oxidative (aerobic) metabolism, as DCA has the ability to reduce the inertia in aerobic mitochondrial ATP generation and the reliance on anaerobic ATP resynthesis at the onset of exercise. Therefore, based on the results described in **Chapter 3**, it could be hypothesized that triggering skeletal muscle energy metabolism would probably benefit exercise performance more in quitting COPD patients since they already have a more compromised metabolic oxidative capacity than non-quitters.

UCP3: AN ADAPTIVE MECHANISM AGAINST LIPOTOXICITY?

At present, the functional role of mitochondrial uncoupling protein-3 (UCP3) in skeletal muscle metabolism is still controversial and incomplete. The existing evidence points towards a function for UCP3 in the prevention of ROS formation (28) and/or the export of fatty acid anions from the mitochondrial matrix (29). However, regardless of the exact mechanism of UCP3, the physiological implication of these two functions is that UCP3 is related to the protection of mitochondria against lipotoxicity. In this context, it is noteworthy to mention that COPD patients, in addition to a blunted muscle antioxidant capacity as documented in this thesis, are characterized by an impaired fatty acid β -oxidation (25), which both may result in oxidative stress-induced muscle lipotoxicity. Interestingly, 3-hydroxyacyl-CoA dehydrogenase (HAD), a key enzyme of the β -oxidation of fatty acids, has consistently been shown to be reduced in COPD patients compared to healthy controls, which implies a reduced fatty acid oxidation in COPD (25). Accordingly, Gosker and colleagues (30) previously showed that muscle UCP3 levels increased by exercise training in COPD patients with a blunted response of the β -oxidation, suggesting that UCP3 was upregulated in response to an insufficient capacity to oxidize free fatty acids (FFAs). Because an acute bout of exercise not only results in increased oxidative stress but also increases FFAs levels and fat oxidation, we tested the hypothesis whether muscle UCP3 gene expression in COPD is upregulated during an acute bout of exercise as an adaptation to a blunted response of the antioxidant defense system and β -oxidation (**Chapter 5**). This work indicated that COPD patients, but not healthy controls responded to an acute bout of exercise by increasing skeletal muscle UCP3. Concomitantly, this response was associated with a blunted antioxidant system and impaired oxidative capacity which led to enhanced exercise-induced muscle lipid peroxidation, as assessed by 4-hydroxy-2-nonenal (4-HNE). These findings are supportive of an adaptive role for UCP3 in protecting the muscle cell against ROS-induced lipotoxicity. Moreover, these results provide a plausible link between the two proposed physiological functions of UCP3. It is likely that due to an impaired β -oxidation observed in COPD, fatty acid delivery to the muscle exceeds its oxidizing capacity, and will accumulate and *flip-flop* into the mitochondrial matrix as fatty acid anions (31). Moreover, as a result of the blunted antioxidant system, increased ROS production in the mitochondria might attack these fatty acid anions inevitably leading to the formation of lipid peroxidation products, such as 4-HNE. Interestingly, 4-HNE has recently been identified as a strong activator of UCP3 and subsequently reduced mitochondrial ROS formation (32). Consequently, therapeutic strategies to decrease ROS production should be focused on two approaches, selective uncoupling of mitochondria, and increasing the antioxidant defense. Therefore, in light of the current observation it would be of interest to study

the effect of antioxidant supplementation combined with exercise training on UCP3 mRNA and protein levels. It is important to keep in mind that in peripheral muscle of COPD there is a shift in muscle fiber type from type I (slow twitch, oxidative fibers) to type II (fast twitch glycolytic) fibers (24). Nevertheless, the marked increase in the proportion of type II is accompanied by selective atrophy of type II fibers, as reported recently (24). Additional studies need to be undertaken to explore whether the exercise-induced UCP3 upregulation in COPD is related to muscle fiber composition, since UCP3 is predominantly expressed in glycolytic type II fibers (33). Moreover, further research is required in order to unravel the underlying mechanism behind this adaptation that is likely to be shared by other chronic conditions, such as chronic heart failure. Eventually, this may result in novel targets for therapies for peripheral muscle dysfunction in this disease.

DISTINCTIVE ROLE OF NF- κ B BETWEEN PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL CONDITION (EPISODIC / CHRONIC)

Existing data indicate that an acute bout of exercise activates the expression of a number of genes, including genes associated with stress, inflammation, survival and tissue repair in skeletal muscle of healthy controls. It is conceivable that this response to exercise is necessary for adequate muscle contractile function, adequate remodeling of injured skeletal muscle and to provide considerable protection against oxidative damage. Whether the adaptive response of skeletal muscle of patients with COPD to exercise is different compared with healthy controls was examined in **Chapter 4**. Intriguingly, in contrast to healthy controls, our findings suggest that skeletal muscle of patients with COPD had an impaired response to acute exercise which was reflected in a blunted expression of inflammatory cytokines, antioxidants, heat shock proteins and survival factors. The blunted cellular protective response strongly indicates that COPD patients are more susceptible to oxidative stress/damage which can negatively affect muscle function and contribute to the development of fatigue, a prominent feature of peripheral muscle dysfunction (34). There is evidence that under normal physiological conditions inflammatory cytokines may be necessary for an adequate remodeling of injured skeletal muscle. In this regard, the attenuated response observed in COPD may impair the regenerative capacity of skeletal muscles and ultimately contribute to muscle wasting. This attenuated response is reflected in decreased levels of myogenic differentiation factor (MyoD) in COPD patients, since MyoD has a key role in regulating muscle differentiation and regeneration. A key question that remains to be determined is whether this attenuated ability of skeletal muscle to adapt to the oxidative exercise-induced challenge may be due to altered redox signaling cascades. There is now

considerable evidence that the ROS generated by skeletal muscle play an important role in influencing redox-regulated processes that control, at least some of, the adaptive responses to exercise. Thus, ROS generation during exercise may have a 'two faced' character: the infliction of oxidative stress and damage, and the stimulation of adaptive responses favoring long-term protection. One of the most important signaling pathways that could be modulated by ROS involves the transcription factor nuclear protein κ B (NF- κ B) (for review see (35)). NF- κ B has been studied most extensively for its pivotal role in the regulation of innate immunity, inflammation and apoptosis. However, its physiological and pathological roles in tissues outside of the immune system are less well understood. Sustained activation of NF- κ B by oxidative stress has been suggested to play a central role in the etiology of systemic features of COPD (36). In particular, recent findings have highlighted an important role for chronic unopposed activation of NF- κ B in cachexia and other muscle wasting syndromes (16, 37). This raises intriguing questions about what effect episodic activation of NF- κ B, as occurs during exercise, might have on skeletal muscle of COPD. Interestingly, consistent with the blunted expression of inflammatory cytokines, antioxidants and heat shock proteins in response to exercise, a reduced I κ B α expression in skeletal muscle of COPD patients compared to healthy controls was demonstrated, indicating an attenuated NF- κ B activation in COPD after exercise (**Chapter 4**). One may argue that the reduced NF- κ B mediated gene expression observed in COPD may be due to an altered intracellular redox state by the changes in the rates of ROS production. This might emphasize a fine-tuned balance between the production of oxidants which is necessary for the protective adaptive response of skeletal muscle or excessive production of oxidants, as likely occurs in COPD, which will lead to cell damage. In this context, it is plausible that administration of antioxidants during physical exercise may be a requirement for augmented tolerance of skeletal muscle to exercise-induced oxidative stress. Collectively, exercise-mediated NF- κ B activation has probably many different roles, but at least three potential roles conferred at the transcriptional level of regulation are postulated; antioxidant defense, tissue regeneration and metabolism. Therefore, it emerges that NF- κ B has diverse roles, particularly in the context of episodic versus chronic cellular stress. Additionally, it seems that differential NF- κ B regulated genes may be selectively activated in skeletal muscle of patients with COPD compared to healthy age-matched controls in response to exercise (Figure 1). Obviously, further research is necessary to delineate the specific role of redox-regulated pathways in exercise-induced adaptations in patients with COPD. More insight into redox mechanisms and oxidative stress in skeletal muscle will provide a clearer picture of muscle function and will broaden our understanding of the process of muscle fatigue and muscle damage. A major challenge that lies ahead in this field is to understand how exercise exerts its effects on gene and protein expression in skeletal

muscle under physiological and pathophysiological conditions. Also the link between redox-regulated gene expression and adaptation to exercise training in COPD is at an exciting stage and merits further clarification.

Figure 1. NF-κB paradox

FUTURE DIRECTIONS

The research conducted in this thesis focused on well-designed clinical studies and clearly indicates that COPD patients are characterized by increased oxidative stress, both at rest and induced by different types of exercise. However, as indicated above, further research is necessary to delineate these findings. An intriguing observation that is not addressed in this thesis is that oxidative stress seemed to be associated with apoptosis which may eventually lead to muscle atrophy. Skeletal muscle atrophy is a prominent and disabling feature of COPD, and even an independent predictor of mortality. Because muscle atrophy is associated with reduced exercise capacity and quality of life in patients with COPD, the elucidation of its mechanisms appears to be relevant with respect to therapeutic interventions.

Recently, apoptosis has emerged as an important contributor to muscle disuse-induced atrophy. However, to date only few studies have been undertaken to identify the molecular mechanisms underlying this cell loss in patients with COPD. Thus the challenge is to unravel the underlying mechanisms responsible for linking oxidative stress to apoptosis. One molecular mechanism that might induce apoptosis is poly-(ADP-ribose) polymerase-1 (PARP)-mediated myocyte cell death. Preliminary evidence showed that the nuclear enzyme PARP-1 is activated after exercise in skeletal muscle of patients with COPD. PARP-1 is a multifunctional protein important for maintenance of genomic integrity and RNA transcription in cells (38, 39). During conditions of oxidative stress, as observed in COPD, PARP-1 is overactivated and may contribute to myocyte cell death (38). A possible molecular mechanism behind PARP-mediated myocyte cell death is that PARP-1 overactivation depletes cellular NAD^+ content, thereby suppressing the activity of other NAD^+ -dependent cellular processes. Another crucial family of proteins that is greatly affected by changes in cellular NAD^+ levels is the class III histone deacetylases, also called sirtuins. They have been implicated in chromatin silencing, cell survival and aging (for review see (40)). Therefore, in future research we will investigate the possible role of repression of SIRT1 by PARP-1 in myocyte cell death *ex vivo* and subsequently in translational studies. This could ultimately lead to new therapeutic strategies aimed at repressing apoptosis as opposed to currently used anabolic stimuli of the skeletal muscle.

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CHAPTER 8

Summary

Peripheral muscle dysfunction is a common and disabling systemic feature of patients with chronic obstructive pulmonary disease (COPD) which contributes to exercise intolerance, impaired quality of life and survival. There are several mechanisms proposed that could explain the peripheral muscle dysfunction but the precise contribution of each is yet unknown. Among them, chronic inflammation and oxidative stress are prominent features of COPD that may be implicated in the etiology of peripheral muscle dysfunction. Exercise training is beneficial for patients with COPD, in part by improving exercise intolerance, but paradoxically, exercise is known, if sufficiently intense, to elicit an oxidative stress response in healthy controls. This thesis describes the effects of acute and prolonged exercise on pulmonary, systemic and muscle oxidative stress, and inflammatory responses in patients with COPD.

In **Chapter 2**, we examined the effects of different exercise intensities and the effect of intensive supervised pulmonary rehabilitation on systemic and pulmonary oxidative stress. Clinically and weight stable patients with moderate COPD were chronically exposed to low-grade pulmonary and systemic oxidative stress compared to healthy age-matched controls, which was observed to further increase in COPD patients after both maximal and submaximal exercise. This suggests that healthy control subjects are able to tolerate exercise more effectively than patients with COPD. Importantly, intensive pulmonary rehabilitation was associated with reduced systemic exercise-induced oxidative stress. This decrease was accompanied by a significant improvement in exercise capacity after rehabilitation in patients with COPD. As COPD patients are characterized by an increased oxidative stress response at rest as induced by exercise, it will be interesting to study whether specific nutritional or pharmacological modulation as adjunct to exercise training can reduce this oxidative stress even further.

Most studies to date have focused on whole body exercise in which the ventilatory system is, in most patients, the dominant limiting factor of exercise intolerance. Under these circumstances, it is difficult to disentangle the relative contributions of lung versus muscle to exercise-induced oxidative stress in COPD. We examined in **Chapter 3** whether and to what extent systemic inflammation and oxidative stress as well as pulmonary oxidative stress are differentially triggered by contracting peripheral muscle of COPD patients versus healthy controls. In line with the in-

creased systemic oxidative stress response observed after whole body exercise (Chapter 2), COPD patients also showed a greater susceptibility to local exercise-induced systemic oxidative stress, without evidence of increased levels of systemic inflammation. An important finding was that, even despite the low cardioventilatory responses, an increased pulmonary oxidative stress response was observed after a localized quadriceps exercise in patients with COPD.

Chronic activation of the transcription factor NF- κ B by oxidative stress has been suggested to play a role in the etiology of the systemic features of COPD including muscle wasting. Paradoxically, evidence exists that an acute bout of exercise potently activates NF- κ B signaling in skeletal muscle of healthy controls. Therefore, in **Chapter 4** the question we addressed was whether NF- κ B is activated and if NF- κ B activity and downstream effectors were induced in skeletal muscle of patients with COPD in response to an acute exercise bout. Our data reveal differential NF- κ B mediated gene expression in skeletal muscle of patients with COPD compared to healthy age-matched controls in response to acute exercise. Skeletal muscle of COPD patients was characterized by an impaired response to acute exercise as reflected by a blunted expression of inflammatory cytokines, antioxidants, heat shock proteins and survival factors. This suggests that muscle NF- κ B activation is a physiological response to exercise-induced adaptations to cellular stress that may be impaired in COPD and it may contribute to peripheral muscle dysfunction in COPD. Therefore, NF- κ B emerges as having diverse roles, particularly in the context of episodic versus chronic cellular stress.

Besides a disturbed muscle oxidant/antioxidant balance, COPD patients are characterized by an impaired oxidative capacity. Both may result in oxidative stress-induced muscle lipotoxicity which could be exaggerated after exercise. Uncoupling protein-3 (UCP3) is considered to be a key player in the protection of mitochondria against lipotoxicity. Therefore, **Chapter 5** focused on the role of muscular UCP3 gene expression after an acute exercise in patients with COPD compared to healthy controls. The main finding of the present study was that an acute bout of exercise upregulates UCP3 gene transcription in skeletal muscle of COPD, but not in healthy controls. This response is associated with a blunted antioxidant defense system and β -oxidation in COPD in combination with enhanced intramuscular lipid peroxidation. We hypothesize that the upregulation of UCP3 in peripheral muscle of patients with COPD could very well be a compensatory mechanism against this lipotoxicity. The differences found between COPD patients and healthy controls emphasize the importance to further unravel the underlying mechanisms by which UCP3 affects the ROS-induced lipotoxicity.

A reduction in skeletal muscle mitochondrial oxidative energy capacity has been observed in patients with COPD, which may also contribute to exercise intolerance. Therefore, the effect of a pharmacological agent, dichloroacetate (DCA), on systemic exercise-induced oxidative stress and inflammation in COPD patients was explored in **Chapter 6**. DCA specifically targets skeletal muscle oxidative energy metabolism and improves exercise performance in COPD. We demonstrated that DCA infusion administered before a maximal exercise test reduces exercise-induced systemic oxidative stress and enhanced the anti-inflammatory cytokine interleukin (IL)-6 response in patients with COPD. Another important finding of the present study was the inverse relationship between exercise-induced changes in IL-6 and the pro-inflammatory cytokine tumour necrosis factor- α (TNF- α) levels after DCA treatment, suggesting that DCA treatment may have an anti-inflammatory effect in patients with COPD. It is known that DCA improves the supply of ATP and thereby attenuates adenine nucleotide loss, as measured indirectly by a reduction in ammonia accumulation. Consequently this enhanced mitochondrial energy production could play a role in decreasing the exercise-induced systemic oxidative stress and pro-inflammatory response in COPD patients.

In conclusion, this thesis has demonstrated that COPD patients are characterized by an increased pulmonary and systemic oxidative stress response at rest as well as induced by exercise when compared to healthy controls. We hope that our findings contribute to tailored therapies, from physical to nutritional or pharmacological interventions to improve the quality and prospective of life for this complex and heterogenic chronic disorder.

CHAPTER 9

Samenvatting

Verlies van spiermassa is een veel voorkomend probleem bij patiënten met chronisch obstructief longlijden (COPD) en draagt bij tot een verminderd inspanningsvermogen, een verminderde kwaliteit van leven en een verhoging van het sterfterisico. Ondanks het feit dat de preciese oorzaak van het verlies van spiermassa nog niet is opgehelderd, wordt verondersteld dat een sluimerende chronische ontstekingsactiviteit en oxidatieve stress een belangrijke rol spelen in de etiologie van spierfunctieverlies. Oxidatieve stress is een toestand waarbij de balans verstoord is tussen de vorming (pro-oxidatieve werking) en de eliminatie (anti-oxidatieve werking) van toxische zuurstofverbindingen. Lichamelijke inspanning is gunstig om het inspanningsvermogen van COPD patiënten te verbeteren maar aan de andere kant is bij gezonde mensen aangetoond dat zeer intensieve inspanning een oxidatieve stress respons teweeg kan brengen. Dit proefschrift beschrijft de acute en lange termijn effecten van lichamelijke inspanning op de ontstekingsrespons en op de oxidatieve stress respons die systemisch (hele lichaam), pulmonaal (long) en in de spier optreedt in patiënten met COPD.

In **Hoofdstuk 2**, hebben we zowel het effect van verschillende inspanningsintensiteiten als het effect van een intensieve longrevalidatie op systemische en pulmonale oxidatieve stress onderzocht. In vergelijking met gezonde controles, die met de patiëntengroep overeenkwamen wat betreft leeftijd en geslacht, zijn patiënten met matig COPD continu blootgesteld aan pulmonale en systemische oxidatieve stress. Deze oxidatieve stress respons was zelfs meer uitgesproken na zowel een maximale als een submaximale inspanningstest in COPD patiënten. Dit suggereert dat gezonde controles beter in staat zijn om oxidatieve stress te tolereren dan patiënten met COPD. Een opmerkelijke bevinding was dat regelmatig bewegen (longrevalidatie) een gunstig effect had op de inspannings-geïnduceerde oxidatieve stress respons in COPD. Bovendien hadden COPD patiënten een verbeterde inspanningscapaciteit na revalidatie. Omwille van de verhoogde oxidatieve stress respons zowel in rust als gedurende inspanning, en vanwege het gunstige effect van regelmatig bewegen is het interessant om nader te onderzoeken of een specifieke voedings- of farmacologische modulatie in combinatie met inspanningstraining deze oxidatieve stress respons verder kan doen afnemen.

Tot op heden lag de focus in de meeste studies op een inspanning waarbij het hele lichaam actief betrokken is. Bij deze inspanningstest is het ventilatoire systeem in

het merendeel van de COPD patiënten de grootste beperkende factor voor het verminderde inspanningsvermogen. Bovendien is het onder deze omstandigheden moeilijk om de relatieve bijdrage van de longen ten op zichte van de skeletspieren aan de inspannings-geïnduceerde oxidatieve stress respons in COPD te onderscheiden. Daarom werd in **Hoofdstuk 3** onderzocht in welke mate een lokale beenspiertest oxidatieve stress induceert in COPD patiënten in vergelijking met gezonde controles. In lijn met de toegenomen systemische oxidatieve stress na een lichaamsinspanning (hoofdstuk 2), vertoonden COPD patiënten ook een grotere gevoeligheid voor een door lokale inspannings-geïnduceerde systemische oxidatieve stress respons. Echter, dit ging gepaard zonder een verhoogde systemische ontstekingsrespons. Een belangrijke bevinding was dat ondanks de minimale cardiale (hart)- en ventilatoire belasting van de lokale beenspiertest, een toegenomen oxidatieve stress respons in de longen werd aangetoond in patiënten met COPD.

Chronische activatie van de transcriptiefactor NF- κ B vervult een belangrijke rol in spiervezelverschuiving en verlies van spiermassa bij COPD. NF- κ B wordt echter acuut geactiveerd in de skeletspier van gezonde controles na een lichamelijke inspanning. Naar aanleiding hiervan hebben we in **Hoofdstuk 4** de effecten van acute inspanning op NF- κ B activiteit en NF- κ B gemedieerde genexpressie onderzocht bij patiënten met COPD. Onze data toonden aan dat NF- κ B gemedieerde gen expressie in de skeletspier van COPD patiënten na een acute inspanning verschillend was in vergelijking met leeftijdsgematchte gezonde controles. De skeletspier van COPD patiënten werd na een acute inspanning gekenmerkt door een verstoorde respons die zich weerpspiegelde in een verlaagde expressie van ontstekings-eiwitten, antioxidanten, stress eiwitten, en overlevingfactoren. Dit suggereert dat NF- κ B activiteit in de spier een normale reactie is op inspannings-geïnduceerde stress. Deze inspannings-geïnduceerde adaptatie lijkt verstoord te zijn in COPD en dit zou vervolgens kunnen bijdragen aan spierfunctieverlies in COPD. Hieruit blijkt dat NF- κ B diverse functies heeft, in het bijzonder in de context van acute versus chronische cellulaire stress.

Naast een verstoorde oxidant/antioxidant balans in de spier worden COPD patiënten ook gekenmerkt door een verminderde oxidatieve capaciteit en beiden kunnen resulteren in oxidatieve stress geïnduceerde lipotoxiciteit (stapeling van vetten) in de spieren. Dit proces zou versneld kunnen worden door lichamelijke inspanning. Uncoupling protein-3 (UCP3) wordt verondersteld een belangrijke sleutelrol te vervullen in de bescherming van mitochondria (energiefabriekjes van de cel) tegen stapeling van vetten in de spier. In **Hoofdstuk 5** werd de rol van UCP3 in de spier na een acute inspanning in gezonde controles en patiënten met COPD nader

onderzocht. Opvallend was dat UCP3 genexpressie in de skeletspier van patiënten met COPD wel verhoogd was na een acute inspanning, maar niet in gezonde controles. Deze respons ging gepaard met een verlaagd antioxidant afweersysteem en β -oxidatie (vetafbraak) in COPD wat resulteerde in een verhoogde vetperoxidatie (vorming van toxische oxidatieproducten van vetten) in de spier. Deze resultaten geven aan dat de verhoging van UCP3 in de skeletspier van patiënten met COPD een compensatoir mechanisme zou kunnen zijn tegen deze stapeling van toxische vetoxidatieproducten. De verschillen tussen COPD patiënten en gezonde controles in deze studie benadrukken het belang van de verdere opheldering van de onderliggende regulatiemechanismen van UCP3.

Er zijn aanwijzingen gevonden voor een afname van het oxidatieve energiemetabolisme in mitochondriën in de skeletspier van patiënten met COPD. Dit zou kunnen bijdragen aan de beperkte inspanningscapaciteit in deze patiënten. Het effect van een farmacologische interventie met dichloroacetate (DCA), op de inspannings-geïnduceerde systemische oxidatieve stress en ontstekingsrespons wordt beschreven in **Hoofdstuk 6**. DCA prikkelt specifiek het oxidatief energiemetabolisme in de skeletspier en verbetert acuut de inspanningscapaciteit bij COPD. In deze studie toonden we aan dat DCA, toegediend kort vóór een maximale inspanningstest, de inspannings-geïnduceerde systemische oxidatieve stress respons verlaagd. Daarnaast was het ontstekingsremmende cytokine interleukin (IL)-6 in patiënten met COPD verhoogd na inspanning. De inspannings-geïnduceerde veranderingen in IL-6 na DCA behandeling waren omgekeerd evenredig gerelateerd aan de niveau's van het ontstekingsbevorderende cytokine tumor necrose factor-alfa (TNF- α). Dit suggereert dat DCA behandeling een ontstekingsremmend effect zou kunnen hebben in patiënten met COPD. Het is algemeen bekend dat DCA de voorraad van ATP aanvult en daardoor adenine nucleotide verlies afzwakt, zoals indirect gemeten was door een verlaging van ammonia ophoping. Geconcludeerd kan worden dat de verhoogde mitochondriële energieproductie een rol kan spelen in de verlaagde inspannings-geïnduceerde systemische oxidatieve stress- en ontstekingsremmende respons die werd gevonden in COPD patiënten na DCA toediening.

We concluderen in dit proefschrift dat COPD patiënten in vergelijking met gezonde controles gekarakteriseerd worden door een verhoogde pulmonale en systemische oxidatieve stress respons zowel in rust als gedurende inspanning. We hopen dat onze bevindingen leiden tot de ontwikkeling van verschillende soorten therapieën, van fysieke tot voedings- of farmacologische interventies, die resulteren in een verbeterde kwaliteit van leven en een verhoogde levensverwachting in patiënten met COPD.

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CURRICULUM VITAE

Evi Mercken was born on the first of March 1978 in Bilzen, Belgium. After obtaining her secondary degree, she started to study Nutrition at the Rega School in Leuven, where she graduated in 2000. Thereafter, she started studying 'Biological Health Science' at the Faculty of Health Sciences at the University of Maastricht, The Netherlands. The topic of her graduate research project was "Specific inhibition of CMV-transcripts by RNA interference mediated by 21-nucleotide dsRNAs (small interfering and short hairpin RNAs)". From June 2003 until June 2004, she worked as a research assistant at the Department of Respiratory Medicine at the University of Maastricht on a research project named poly(ADP-ribose) polymerase (PARP) activation during exercise-induced oxidative stress in patients with chronic obstructive pulmonary disease (COPD): its effects on NAD⁺ status, cellular energy metabolism and skeletal muscle weakness. In June 2004, she continued working at the Department of Respiratory Medicine as a PhD student. The title of her thesis is "The exercise-induced oxidative stress paradox in COPD". At the ESPEN conference in 2008 she was awarded a Travel Fellowship and she received a Travel Grant for Best Posters from the European Respiratory Society. In December 2007, she received a Scientific Travel award from the Dutch Asthma Foundation and she was able to perform a 6 months fellowship at the Laboratory of Experimental Gerontology (headed by Dr. de Cabo) at the National Institute on Aging (NIA), NIH, U.S.A. Currently, she has a position as a visiting post-doctoral fellow at the Laboratory of Experimental Gerontology at the NIA. Her research will focus on the role of skeletal muscle atrophy in aging and its impact on metabolism, health-span and longevity.