

# Altered antioxidant status in peripheral skeletal muscle of patients with COPD

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## Altered antioxidant status in peripheral skeletal muscle of patients with COPD<sup>☆</sup>

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**Summary** Despite the growing field of interest in the role of pulmonary oxidative stress in chronic obstructive pulmonary disease (COPD), barely any data are available with respect to antioxidant capacity in the peripheral musculature of these patients. The main objective of this study was to assess in detail the antioxidant status in skeletal muscle of patients with COPD. Biopsies from the vastus lateralis of 21 patients with COPD and 12 healthy age-matched controls were analysed. Total antioxidant capacity, vitamin E, glutathione, and uric acid levels were determined and the enzyme activities of superoxide dismutase, glutathione reductase, glutathione peroxidase, and glutathione-S-transferase were measured. Malondialdehyde was measured as an index of lipid peroxidation. The total antioxidant capacity and the uric acid levels were markedly higher in COPD patients than in healthy controls (25%,  $P = 0.006$  and 24%,  $P = 0.029$ , respectively). Glutathione-S-transferase activity was also increased (35%;  $P = 0.044$ ) in patients compared to healthy subjects. Vitamin E level was lower in patients than in controls ( $P < 0.05$ ). The malondialdehyde level was not different between the two groups. It can be concluded that the muscle total antioxidant capacity is increased in patients with COPD. Together with the reduced vitamin E levels, the increased glutathione-S-transferase activity and normal levels of lipid peroxidation products, these findings suggest that the antioxidant system may be exposed to and subsequently triggered by elevated levels of reactive oxygen species.

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## Introduction

Evidence is accumulating that oxidative stress plays an important role in chronic obstructive pulmonary disease (COPD). Considerable attention has especially been paid to oxidative stress in the pulmonary compartment during the last decade.<sup>1</sup> Free radical formation is associated with local inflammation and cigarette smoking and may result in the inactivation of antiproteases, airspace epithelial damage, mucus hypersecretion, increased influx of neutrophils into lung tissue, and the expression of pro-inflammatory mediators.<sup>1,2</sup> In addition to the pulmonary impairment, skeletal muscle wasting and muscle dysfunction are hallmarks of COPD.<sup>3,4</sup> Likewise, oxidative stress may also be involved in the systemic consequences of COPD. Oxidative stress occurs when the balance between oxidants, for instance reactive oxygen species (ROS), and antioxidants shifts in favour of the ROS. A few studies indicate that the antioxidant status is impaired at the systemic level: increased levels of lipid peroxidation products have been demonstrated in serum of patients with stable COPD.<sup>5</sup> In patients with acute exacerbations, but not in clinical stable condition, plasma antioxidant capacity was reduced.<sup>5</sup> After exercise, serum levels of lipid peroxidation products and oxidized glutathione were increased in patients compared to controls.<sup>6-8</sup> The latter findings indicate that exercise-induced oxidative stress occurred and it is therefore most likely that the source of oxidative stress is the exercising muscle. A direct indication of oxidative stress in skeletal muscle comes from a study performed by Allaire et al. who found increased accumulation of lipofuscin, a marker of oxidative damage, in vastus lateralis biopsies from COPD patients.<sup>9</sup> Many diseases are associated with oxidative stress and the use of antioxidant supplements became very popular the last decades. However, the adverse side effects and toxicity of these supplements are not always recognized.<sup>10</sup>

Moreover, a detailed study is required to establish if and at what level the muscular antioxidant defence system is impaired in COPD in order to create a platform from which targeted intervention strategies can be developed.

Therefore, the aim of the present study was to assess in detail the antioxidant status in skeletal muscle of patients with COPD compared to healthy controls in rest. For this purpose the total antioxidant capacity was measured. In addition, the activities of several key antioxidant enzymes were measured, including superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione-S-transferase

(GST). Also, the contents of total glutathione, uric acid, and vitamin E were measured. Finally, malondialdehyde (MDA) was determined as an index of lipid peroxidation.

## Methods

A group of 21 patients with moderate to severe airflow obstruction and 12 healthy age-matched volunteers was studied. All patients had COPD according to ATS guidelines<sup>11</sup> chronic airflow limitation, defined as measured forced expiratory volume in 1 s (FEV<sub>1</sub>) less than 70% of reference FEV<sub>1</sub>. Furthermore, patients had irreversible obstructive airway disease (less than 10% improvement of FEV<sub>1</sub> predicted baseline after  $\beta_2$ -agonist inhalation). They were in clinically stable condition and not suffering from a respiratory tract infection or an exacerbation of their disease at least 4 weeks prior to the study. Exclusion criteria were malignancy, cardiac failure, distal arteriopathy, recent surgery, severe endocrine, hepatic or renal disorders and use of anticoagulant medication. The healthy age-matched control subjects were volunteers recruited through advertisement in a local newspaper. Written informed consent was obtained from all subjects and the study was approved by the medical ethical committee of the University Hospital Maastricht (Maastricht, The Netherlands).

## Pulmonary function tests

All patients and control subjects underwent spirometry to determine FEV<sub>1</sub>. Forced vital capacity (FVC) was assessed by whole-body plethysmography and diffusion capacity for carbon monoxide (DL<sub>CO</sub>) was measured by using the single-breath method (Masterlab, Jaeger, Wurzburg, Germany). All values obtained were related to a reference value and expressed as percentage of the predicted value.<sup>12</sup> Arterial oxygen tension (PaO<sub>2</sub>) was determined (ABL 330; Radiometer, Copenhagen, Denmark) while breathing room air.

## Assessment of body composition

Body height and weight were assessed. Whole-body fat-free mass (FFM) was determined by bioelectrical impedance (Xitron 4000b, Xitron technologies, San Diego, California, USA) as described previously.<sup>13</sup> Weight parameters were adjusted for body surface, resulting in the body mass index (BMI) and FFM index (FFMI).<sup>14</sup>

## Muscle strength

Isokinetic muscle strength of the dominant knee extensor (quadriceps) was measured using a Biodex dynamometer (Biodex Corporation, Shirley, NY, USA).<sup>15</sup> The highest torque value (expressed as Nm) of three sequential voluntary maximal contractions at an angular velocity of 60°/s was used.

## Exercise capacity

Exercise capacity was assessed by means of cycle ergometry. Subjects performed an incremental (10 W/min for patients and 15–25 W/min for controls) cycle ergometry test as described previously.<sup>16</sup> Expired gases were investigated using breath by breath analysis through a breathing mask (Oxycon Beta<sup>®</sup>, Jaeger, Würzburg, Germany). Peak VO<sub>2</sub> and peak load were measured at the moment of cessation of the exercise.

## Collection and processing of muscle tissue

Postabsorptive muscle biopsies of the lateral part of the quadriceps femoris were obtained under local anaesthesia by the needle biopsy technique<sup>17</sup> and the specimens were immediately frozen in liquid nitrogen. The frozen biopsies were weighed and subsequently grinded with a mortar cooled in liquid nitrogen. The frozen powder was resuspended in buffer (100 mM sodium phosphate, pH 7.4) to a final concentration of 250 mg tissue/ml and directly re-frozen in liquid nitrogen and stored at –80°C until use. For the determination of vitamin E, an aliquot of the total muscle biopsy homogenate was used. For analysis of the other parameters the homogenate was centrifuged (5 min at 14,000 × g and 4°C) and the muscle biopsy supernatant was used. Total protein content in the homogenates was assayed according to Smith et al.<sup>18</sup> using BSA as standard.

## Measurements of antioxidants and lipid peroxidation

GST activity was determined by the GST catalysed reaction of chlorodinitrobenzene (Sigma Chemical Co., St. Louis, USA) with reduced glutathione (GSH), resulting in a yellow coloured product, glutathione-dinitrobenzene, of which the increase in absorbance was measured (2 min at 340 nm).<sup>19</sup> After correction for the non-enzymatic reaction, the activity of GST in the sample was calculated using the molar absorptivity coefficient of glutathione-dinitrobenzene. The GR activity was de-

termined by measuring the decrease in absorbance (2 min at 340 nm) by the consumption of NADPH (Sigma) in the enzymatic reduction of oxidized glutathione (GSSG) to GSH by GR.<sup>20</sup> The activity was calculated using the molar absorptivity coefficient of NADPH. The selenium dependent GPX activity, with H<sub>2</sub>O<sub>2</sub> as substrate, was also determined by measuring the decrease in absorbance (3 min at 340 nm) due to the consumption of NADPH.<sup>21</sup> GPX activity was calculated using the molar absorptivity coefficient of NADPH. Total glutathione level was determined by measuring the increase in absorbance (2 min at 405 nm), caused by the GSH driven reduction of 5'5'-dithiobis 2-nitrobenzoic acid (ICN Biomedicals Inc., Costa Mesa, USA), using the recycling method described by Vandeputte et al.<sup>22</sup> The activity of the SOD was measured using the xanthine-xanthine oxidase system (Sigma) for the generation of superoxide anions and nitro blue tetrazolium (NBT) (Sigma): NBT is reduced by superoxide anions to the blue formazan which can be measured at 560 nm. SOD scavenges superoxide anions resulting in less reduction of NBT. The increase in absorbance was measured (2 min at 560 nm).<sup>25</sup> After adding an internal standard, vitamin E nicotinate (Sigma), vitamin E was extracted by hexane extraction. Vitamin E concentration was determined with the HPLC method as previously described by van Haften et al.<sup>23</sup> using an Agilent HPLC system (Agilent, Palo Alto, CA, USA) and a Nucleosil C18 column (125 × 4 mm<sup>2</sup>, particle size 5 μm, Agilent). Uric acid was determined in ultra filtrates with the HPLC method described by Lux et al.<sup>24</sup> using a Hypersil BDS C18 end-capped column (125 × 4 mm<sup>2</sup>, particle size 5 μm, Agilent). The total antioxidant capacity was measured by means of the Trolox equivalent antioxidant capacity (TEAC) assay, as described by van den Berg et al.<sup>25</sup> MBS samples were deproteinated with trichloro-acetic acid. The absorbance (at 734 nm) of the radical solution was 0.7 ± 0.02. Then the decrease in absorbance, caused by antioxidant capacity in the sample, was measured and related to that of trolox standards. The determination of MDA was based on the formation of a coloured adduct of MDA with 2-thiobarbituric acid, which was measured according to the HPLC method described by Lepage et al.<sup>26</sup> using a Nucleosil C18 column (150 × 3.2 mm<sup>2</sup> particle size 5 μm, Supelco Inc., Bellefonte, PA, USA).

## Statistical analysis

Data were analysed with the unpaired Student's *t*-test (corrected for unequal variances if appropriate) or the Pearson correlation test, as

appropriate.<sup>27</sup> Data are represented as the means  $\pm$  SD. Differences in gender between the control and the patient groups were examined using the  $\chi^2$ -test. A two-tailed probability value of less than 0.05 was considered statistically significant.

## Results

Subject characteristics are presented in Table 1. There were no statistically significant differences in age and gender between the control and patient groups. Lung function was clearly impaired in patients with COPD. Patients had a lower body mass index, a reduced peripheral muscle strength, and a decreased exercise capacity compared to control subjects.

The results of muscle biopsy analysis are shown in Table 2. Total antioxidant capacity, as measured by the TEAC, was significantly increased in COPD, being 25% higher than in healthy controls. Muscle tissue uric acid levels were also significantly higher (24%) in patients compared to control subjects. The antioxidant capacities of the TEAC and uric acid can be directly compared on a one-to-one basis. From this it can be calculated that the difference in uric acid level (0.25 nmol/mg protein) accounts only for 2% of the difference in TEAC (12 nmol/mg protein). Glutathione levels were normal in patients. The activities of all four antioxidant enzymes tended to be somewhat higher in COPD, but only GST activity was statistically significant higher (35%). The level of vitamin E, a lipid soluble scavenger, was reduced in patients, being 66% of

control values. MDA, the marker of lipid peroxidation, was not different between patients and controls.

Muscle antioxidant status was not related to disease severity as measured by the FEV<sub>1</sub>. In addition, the increased antioxidant capacity can not be explained by differences smoking history. There were also no relations between antioxidant status and muscle strength or exercise capacity.

## Discussion

The most striking results of this study are that the overall antioxidant capacity of peripheral skeletal muscle in rest was higher in patients with COPD compared to healthy age-matched control subjects, whereas the vitamin E level was reduced. These are very important findings, because they indicate that the muscular antioxidant status is chronically altered in COPD patients characterized by peripheral muscle weakness and wasting. Probably the most important way by which the antioxidant defence system can be triggered is exposure to ROS.<sup>28-30</sup> It is therefore likely that the observed increased antioxidant capacity is a compensatory adaptation to elevated ROS formation in skeletal muscle of patients with COPD.

Oxidative stress occurs when the balance between oxidants, i.e. ROS, and antioxidants shifts in favour of the ROS. The reactive superoxide anions may subsequently give rise to hydrogen peroxide, hydroxyl radicals, and other redox-reactive molecules. An inadequate defence system may be

**Table 1** Subject characteristics.

	Controls	COPD
N (Female/Male)	12 (4/8)	21 (3/18)
Age (yr)	65 $\pm$ 7	65 $\pm$ 8
FEV <sub>1</sub> (% pred)	113 $\pm$ 25	40 $\pm$ 23***
DL <sub>CO</sub> (% pred)	114 $\pm$ 16	64 $\pm$ 23***
FVC (% pred)	118 $\pm$ 18	86 $\pm$ 19***
PaO <sub>2</sub> (kPa)	10.5 $\pm$ 1.4	9.7 $\pm$ 1.1
SaO <sub>2</sub> (%)	95 $\pm$ 2.0	94 $\pm$ 1.9
<i>Systemic characteristics:</i>		
BMI (kg/m <sup>2</sup> )	27.3 $\pm$ 2	24.1 $\pm$ 4**
FFMI (kg/m <sup>2</sup> )	19.8 $\pm$ 1.8	17.3 $\pm$ 2.9**
Exercise capacity as peak VO <sub>2</sub> (ml/min)	2175 $\pm$ 889	894 $\pm$ 343***
Exercise capacity as peak load (W)	193 $\pm$ 78	68 $\pm$ 29***
Leg muscle strength as peak torque (Nm)	154 $\pm$ 43	84 $\pm$ 47**
Smoking history (pack years)	15 $\pm$ 5	51 $\pm$ 7***

Values are mean  $\pm$  SD; Significance of difference compared to controls: \*\* $P < 0.01$ ; \*\*\* $P \leq 0.001$ . BMI: body mass index; FFMI: fat-free mass index; DL<sub>CO</sub>: diffusion capacity for carbon monoxide; FEV<sub>1</sub>: forced expiratory volume in one second; FVC: forced vital capacity; peak VO<sub>2</sub>: peak oxygen consumption.

**Table 2** Muscle biopsy data.

	Controls	COPD
SOD (U/mg protein)	6.9 ± 5.0	9.5 ± 4.0
GR (U/mg protein)	12.8 ± 2.5	13.3 ± 3.6
GST (U/mg protein)	0.17 ± 0.07	0.23 ± 0.08*
GPX (nmol NADPH/min/mg protein)	20.7 ± 6.7	23.1 ± 6.4
Glutathione (nmol/mg protein)	18.5 ± 4.9	19.6 ± 5.2
TEAC (nmol Trolox Eq/mg protein)	46 ± 8.8	58 ± 12.7**
Uric acid (nmol/mg protein)	1.04 ± 0.21	1.29 ± 0.31*
Vitamin E (µg/mg tissue)	21.6 ± 0.5	17.3 ± 0.5*
MDA (nmol/mg protein)	0.69 ± 0.17	0.77 ± 0.37

Values are mean ± SD; Significance of difference compared to controls: \* $P < 0.05$ ; \*\* $P < 0.01$ . GPX: glutathione peroxidase; GR: glutathione reductase; GST: glutathione S-transferase; SOD: superoxide dismutase; TEAC: Trolox equivalent antioxidant capacity; MDA: malondialdehyde.

overwhelmed by the ROS, leading to damage of proteins, lipids, and DNA, which in turn may result in extensive cell and tissue damage. SOD catalyses the dismutation of superoxide anions to hydrogen peroxide, which in turn is converted to harmless water and oxygen by the glutathione system. This system includes the enzymes GPX, GST, and GR. GPX and GST activities require reduced glutathione (GSH) as a co-factor, GPX activity results in oxidized glutathione (GSSG) and the accumulating GSSG can subsequently be recycled to GSH via the enzyme GR.<sup>31</sup> Uric acid has been suggested as a ROS scavenger, although its action in muscle has not been studied systematically.<sup>31</sup> ROS can also react with lipids, forming lipid radicals and lipid peroxyl radicals. The lipid peroxyl radicals can be scavenged by vitamin E, a lipid-soluble antioxidant that plays an important role in the protection of phospholipid membranes against oxidative damage.<sup>31</sup> Vitamin E can be recycled by, for example GSH. Lipid peroxidation products can be detoxified by GST. But if the scavenging capacity is insufficient, lipid peroxidation products may accumulate, as reflected by increased MDA levels.

In the present study, the total antioxidant capacity, as measured by the TEAC, was markedly higher in COPD than in controls and, as mentioned earlier, oxidative stress may very well be the trigger. The only direct indication for muscular oxidative stress in COPD has recently been provided by Allaire et al., who reported accumulation of intramuscular lipofuscin, a marker of oxidative stress.<sup>9</sup> An interesting side-note is that lipofuscin contains iron, which may in turn catalyse further oxidative reactions.<sup>32</sup> The fact that vitamin E levels were decreased in patients also supports this notion, because this may reflect its utilization during oxidative stress. Interestingly, reduced vitamin E was reported for COPD recently in blood

as well.<sup>8</sup> However, in the current study no significantly increased lipid peroxidation products (MDA) were found in the muscle biopsies from COPD patients. There are two possible explanations for this. The first option is that, in rest, there is no muscular oxidative stress; thus there is a balance between the increased antioxidant capacity and the, supposedly elevated, free radical formation. In this respect, it is also possible that the observed increase in GST activity provides extra potential to detoxify lipid peroxidation products, thereby preventing a rise in MDA. Skeletal muscle fibres generate ROS at a relative low rate continuously, because ~3% of total oxygen consumed leaks away from the mitochondrial electron transport in the form of superoxide anions. ROS formation is dramatically increased during exercise.<sup>31</sup> It is therefore still possible that exercise induced oxidative stress is responsible for reduced vitamin E levels and the stimulated TEAC. Unlike lipofuscin, MDA does not accumulate in the muscle cells over time, but diffuses away from the muscle and can be metabolized. Therefore, a second option is that, in rest, oxidative stress and subsequent lipid peroxidation do occur, but at such a low rate that MDA may not be a sensible marker.

Although the present study was not designed to determine the mechanism behind and the sources of muscular oxidative stress in COPD, some possibilities will be briefly discussed below. First of all, mitochondria in the muscle cells are a major source of ROS, especially during exercise. Normally, the antioxidant status in the oxidative type I fibres is higher compared to the less oxidative type II fibres.<sup>31,33</sup> However, despite the currently observed increased total antioxidant capacity, a I→II fibre type shift and a reduction of oxidative capacity have consistently been observed in the vastus lateralis of patients with COPD.<sup>34–37</sup> This would

suggest that ROS formation in the remaining type I fibres may be higher than normal, which is supported by the recent study in which increased lipofuscin accumulation was indeed greater in type I fibres than in type II fibres.<sup>9</sup> Because COPD patients are certainly not more physically active than healthy subjects, this suggests that relatively more free radicals are produced in exercising muscles of these patients. Baarends et al. recently reported that leg muscle mechanical efficiency is less in COPD compared to controls.<sup>38</sup> This and the reduced capacity of oxidative energy metabolism mentioned earlier may lead to the incomplete oxygen reduction and subsequent ROS formation.

A second source of ROS is inflammation. Systemic inflammation is indeed common in COPD and has been associated with muscle wasting in these patients.<sup>39-41</sup> There is also evidence that immune cells that are activated during inflammation are a source of ROS in the skeletal muscles.<sup>42,43</sup> In addition, there are indications that the muscle cells themselves can be triggered by circulating inflammatory mediators to produce ROS.<sup>44-47</sup>

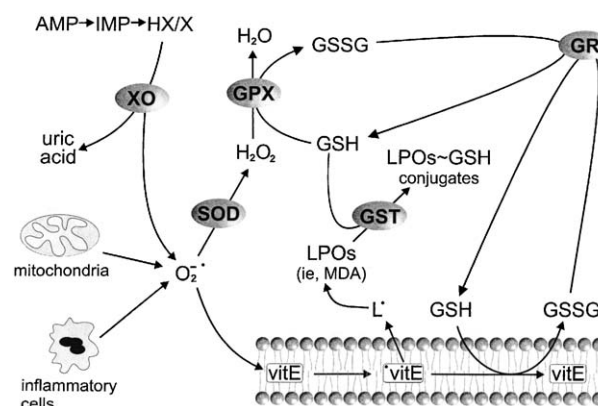
A third alternative source of ROS is the xanthine oxidase, which is an enzyme that catalyses the conversion of hypoxanthine into uric acid, hypoxanthine accumulates in situations of metabolic stress (i.e. exercise and hypoxia) as a result from AMP degradation.<sup>33</sup> Heunks et al. demonstrated, for COPD, that the oxidation of glutathione in blood during exercise can be inhibited by treatment with allopurinol, a xanthine oxidase inhibitor.<sup>6</sup> In this study we found elevated levels of uric acid in the muscle biopsies from patients with COPD. Although it can be considered as an antioxidant itself, the observed increase of uric acid is probably of minor importance compared to the increased total antioxidant capacity in patients, as measured by TEAC. However, an increased uric acid level may also indicate increased xanthine oxidase activity, since uric acid is a degradation product of AMP degradation. This is in line with a previous study in which elevated IMP levels were observed in skeletal muscle biopsies from COPD patients, since IMP is an intermediate in AMP degradation.<sup>48</sup>

The present data indicate that the total muscular glutathione level was normal in this COPD group. Rabinovich et al. also demonstrated that muscle glutathione was normal in COPD compared to controls.<sup>49</sup> Previously, Engelen et al. reported reduced glutathione levels in vastus lateralis biopsies from emphysema patients, which was associated with reduced levels of its precursor, glutamate.<sup>50</sup> This suggests that glutathione homeostasis may differ between COPD sub-groups. Alternatively, the discrepancy may reflect differ-

ences between the methodologies that were used: Engelen et al. expressed glutathione levels per wet weight of muscle tissue, whereas glutathione levels were corrected for total protein level in the other two studies.

The present data provide some new starting points for future research. For example, the elevated TEAC remains largely unexplained. Because the TEAC is measured in a deproteinated sample, the contribution of enzymes can be ruled out. Identification of the antioxidant(s) that is (are) responsible for this increase may provide perspectives for new therapies. The findings also suggest that vitamin E supplementation might be beneficial for COPD patients. Although evidence for a protective effect of vitamin E intake on respiratory symptoms and lung function are inconsistent,<sup>51</sup> there are some indications that vitamin E may prevent oxidative stress induced muscle damage, for example in immobilized rats<sup>52</sup> or in humans during surgical ischemia/reperfusion.<sup>53</sup> More research is thus required to establish whether antioxidant supplementation in COPD patients is beneficial from a "muscular" point of view.

In summary, to our knowledge, this is the first detailed study in which several key antioxidants in peripheral skeletal muscle were examined in COPD. The collected data strongly suggest that the production and handling of oxygen free radicals is altered in muscles of COPD patients. It remains to be explored whether this alteration is instrumental in muscle weakness in COPD patients (Fig. 1).



**Figure 1** A schematic representation of antioxidants and ROS formation. AMP: adenosine monophosphate; IMP: inosine monophosphate; XO: xanthine oxidase; HX: hypoxanthine; X: xanthine; SOD: superoxide dismutase; GPX: glutathione peroxidase; GSH: reduced glutathione; GSSG: oxidized glutathione; GR: glutathione reductase; GST: glutathione S-transferase; L: lipid radical; LPOs: lipid peroxidation products; MDA: malondialdehyde; vitE: vitamin E.

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