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Does oxidative stress modulate limb muscle atrophy in severe COPD patients?

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ABSTRACT: Oxidative stress may differentially regulate protein loss within peripheral muscles of severe chronic obstructive pulmonary disease (COPD) patients exhibiting different body composition.

Oxidation levels of proteins, myosin heavy chain (MyHC) and myonuclei, superoxide anion, antioxidants, actin, creatine kinase, carbonic anhydrase-3, ubiquitin–proteasome system, redox-signalling pathways, inflammation and muscle structure, and damage were quantified in limb muscles of severe COPD patients with and without muscle wasting, and in sedentary controls.

Compared with controls, in the quadriceps of muscle-wasted COPD patients, levels of protein carbonylation, oxidation of MyHC and myonuclei, superoxide anion production, superoxide dismutase, total protein ubiquinitation, $E2_{14k}$, atrogin-1, FoxO1 and p65 were higher, while content of MyHC, creatine kinase, carbonic anhydrase-3, myogenin, and fast-twitch fibre size were decreased. Importantly, in nonwasted COPD patients, where MyHC was more oxidised than in controls, its content was preserved. Muscle inflammation and glutathione levels did not differ between patients and controls. In all patients, muscle structure abnormalities were increased, while muscle force and exercise capacity were reduced.

In severe COPD, while muscle oxidative stress increases regardless of their body composition, protein ubiquitination and loss of MyHC were enhanced only in patients exhibiting muscle atrophy. Oxidative stress does not seem to directly modulate muscle protein loss in these patients.

KEYWORDS: Muscle protein loss, oxidative stress, quadriceps muscle dysfunction, severe chronic obstructive pulmonary disease, signalling pathways, ubiquitin–proteasome system

Highly prevalent conditions, such as chronic obstructive pulmonary disease (COPD) are frequently associated with muscle loss and skeletal muscle dysfunction. These systemic manifestations have a considerable impact on the exercise tolerance and quality of life of the patients, and are also associated with increased mortality [1, 2]. The ubiquitin–proteasome has been identified as the major proteolytic system involved in the degradation of muscle proteins of several catabolic states, including cancer-induced cachexia [3, 4] and COPD, in both limb [5–7] and respiratory [8] muscles of patients with relatively well-preserved body composition. Despite this progress, in severe COPD, the degree of total protein ubiquitination or the upstream signal regulation of muscle protein degradation has not yet been fully explained. In line with this, the family of forkhead box O (FoxO) transcription factors has been shown to regulate atrogin-1 in skeletal muscles of COPD patients [6], in cancer cachectic muscles [9] and in the atrophying diaphragm of patients exposed to mechanical ventilation [10]. However, it remains to be elucidated whether other cellular signalling pathways, such as mitogen activated protein kinase (MAPK), nuclear factor (NF)-κB, myostatin and myogenin, may also contribute to muscle atrophy in patients with severe COPD.

Among several aetiological factors [11, 12], redox imbalance, which has consistently been shown to be involved in the muscle dysfunction of severe COPD patients [12–21], may also contribute to signalling pathways that modulate muscle loss and atrophy [22]. In this regard, increased oxidant levels within cells may act as second messengers that regulate pathological signalling leading to enhanced proteolysis and muscle atrophy. Nevertheless, whether increased oxidative stress may trigger muscle proteolysis within the peripheral

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muscles of severe COPD patients remains an open question. On this basis, it was hypothesised that oxidative stress may differentially regulate protein loss within the muscles of patients with severe COPD exhibiting a wide range of body composition. Therefore, in the present study, our objectives were to assess the following potentially interrelated molecular events within the limb muscles of a population of severe COPD patients exhibiting the following different degrees of body composition: 1) redox balance (protein oxidation, superoxide anion content in myonuclei and muscle fibre compartments, antioxidant enzymes and reduced glutathione (GSH)); 2) levels of redox-sensitive signalling pathways, total protein ubiquitination and markers of the ubiquitin–proteasome system; 3) inflammation; 4) the content of key contractile and functional proteins, previously shown to be consistently oxidised and prone to degradation in COPD muscles [12, 15, 23], such as myosin heavy chain (MyHC), actin, creatine kinase and carbonic anhydrase-3; and 5) muscle structural abnormalities. A group of healthy sedentary control subjects was also recruited in the investigation.

METHODS

See the online supplementary material for additional information.

Human study subjects

29 stable Caucasian male severe COPD patients [24] were recruited from the COPD clinics at Hospital del Mar and Hospital Clinic (Barcelona, Spain). Patients were further subdivided into those exhibiting low weight and reduced muscle mass (body mass index (BMI) $\leqslant$21 kg·m^{-2} and fat-free mass index (FFMI) $\leqslant$18 kg·m^{-2}, muscle-wasted patients; n=18) and normal weight and muscle mass (BMI >21 kg·m^{-2} and FFMI >18 kg·m^{-2}, nonwasted patients; n=11) following previously published studies [25, 26]. Additionally, 10 healthy male sedentary control subjects were also recruited from the general population. Approval was obtained from the institutional Ethics Committees on Human Investigation (Hospital del Mar and Hospital Clinic, Barcelona). Informed written consent was obtained from all individuals.

Anthropometric and functional assessment

Anthropometric evaluation included BMI and FFMI [17, 27]. Lung function and quadriceps muscle strength were evaluated in both patients and controls as previously described [28–31].

Muscle biopsies

Muscle samples were obtained from the vastus lateralis of severe COPD patients and controls using the open muscle biopsy technique [12, 13, 15–17].

Muscle biology analyses

All muscle biology analyses were conducted blind in the same laboratory by the same investigators, at Hospital del Mar-IMIM (Barcelona).

Immunoblotting of one-dimensional electrophoresis

Markers of proteolysis, signalling pathways, redox balance, muscle proteins and carbonylated MyHC were determined using immunoblotting [12–17, 23, 32].

Detection of superoxide anion within myonuclei

The presence of superoxide anion was detected using the fluorescent probe dihydroethidium (DHE) on 3-μm muscle paraffin-embedded sections from all study subjects following previously published methodologies [33].

Detection of superoxide anion radicals in muscle compartments

Superoxide anion levels were detected in cytosolic, membrane and mitochondrial compartments following previous published methodologies [23].

Reduced GSH in muscles

GSH content was measured using the Glutathione Assay (Northwest Life Sciences Specialties, Vancouver, WA, USA) following the specific manufacturer's instructions and published methodologies [34].

Cytokine ELISA

Protein levels of the cytokines tumour necrosis factor (TNF)-α, interferon-γ and vascular endothelial growth factor (VEGF) were quantified in the muscles of all study subjects using specific sandwich ELISA kits (RayBiotech, Norcross, GA, USA) following previously published methodologies [16].

Muscle structure

On 3-μm muscle paraffin-embedded sections from both patients and controls, MyHC-I and -II isoforms were identified using specific antibodies as published elsewhere [12–14, 16, 23, 32]. The area fraction of normal and abnormal muscle was also evaluated following previously published methodologies [35].

Statistical analysis

Data are presented as mean±SD. Comparisons of physiological and biological variables among healthy controls and either wasted or nonwasted severe COPD patients were analysed using one-way ANOVA and Tukey's *post hoc* test. Correlations between physiological and biological variables were explored using the Pearson's correlation coefficient.

RESULTS

Clinical and functional characteristics

As shown in table 1, both BMI and FFMI were significantly decreased in the muscle-wasted severe COPD patients compared with both nonwasted patients and control subjects. Lung function parameters were significantly impaired in all COPD patients compared with the controls, and muscle-wasted patients exhibited a more severe lung disease than patients with preserved body composition. Compared with control subjects, both groups of severe COPD patients exhibited reduced quadriceps muscle force and exercise capacity, the latter being dramatically impaired in the muscle-wasted patients. Importantly, airway obstruction (forced expiratory volume in 1 s; FEV_1) and muscle mass (FFMI) were positively correlated among COPD patients (r=0.380, p=0.014).

Muscle redox balance

Nuclear superoxide anion detection

Compared with healthy controls (fig. 1a and d), the vastus lateralis of both nonwasted and wasted COPD patients exhibited greater levels of nuclear DHE fluorescence (fig. 1b, c, e and f).

TABLE 1 Anthropometric characteristics and functional status of severe chronic obstructive pulmonary disease (COPD) patients and controls

	Control subjects	Nonwasted severe COPD patients	Muscle-wasted severe COPD patients
Subjects n	10	11	18
Age yrs	65±9	67±5	63±11
BMI kg·m^{-2}	28.2±4.4	28.5±4.6	18.9±2.6***,###
FFMI kg·m^{-2}	22.5±2.5	22.7±2.4	15.9±1.7***,###
FEV_1 % pred	95±22	45±19***	32±14***,#
FVC % pred	95±14	78±18**	55±12***,#
FEV_1/FVC	75±11	44±11***	42±11***
RV %	110±3	169±46**	208±62***
TLC % pred	113±18	109±12	116±17**
RV/TLC	43±2	54±10**	67±10***,##
$D_{L,CO}$ % pred	106±18	58±20***	34±12***,##
K_{CO} % pred	97±21	39±26***	38±21***
P_{a,O_2} kPa	12.6±1.7	9.6±1.8*	9.7±0.9*
P_{a,CO_2} kPa	4.9±0.5	5.8±0.7*	5.8±1.1*
$V'_{O_2,max}$ % pred	98±13	77±18*	29±7***,###
W_{peak} % pred	95±15	66±18**	23±11***,###
QMVC kg	39.3±3.7	30.7±3.2***	28.4±2.4***

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; FFMI: fat-free mass index; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; $D_{L,CO}$: diffusing capacity of the lung for carbon monoxide; K_{CO}: transfer coefficient of the lung for carbon monoxide; P_{a,O_2}: arterial oxygen tension; P_{a,CO_2}: arterial carbon dioxide tension; $V'_{O_2,max}$: maximal exercise oxygen uptake; W_{peak}: peak work rate; QMVC: quadriceps isometric maximum voluntary contraction. *: $p \leqslant 0.05$; **: $p \leqslant 0.01$; ***: $p \leqslant 0.001$, between any of the groups of severe COPD patients and controls. #: $p \leqslant 0.05$; ##: $p \leqslant 0.01$; ###: $p \leqslant 0.001$, between nonwasted and wasted severe COPD patients.

Superoxide anion in muscle compartments

Compared with healthy subjects, levels of superoxide anion were significantly increased in the membrane and cytosolic fractions of both nonwasted and wasted COPD patients (fig. 2a and b), while superoxide anion from the mitochondrial compartment was significantly increased only in the vastus lateralis of the wasted patients (fig. 2c).

Protein carbonylation

Total protein carbonylation levels were significantly greater in limb muscles of both muscle-wasted and nonwasted COPD patients than in the controls (fig. 3a). Among patients, protein carbonylation levels correlated with superoxide anion levels within the membrane and cytosolic muscle fractions ($r=0.691$, $p=0.039$ and $r=0.0621$, $p=0.074$, respectively).

Antioxidants

Mn-superoxide dismutase (SOD) protein content was higher in limb muscles of muscle-wasted patients compared with both healthy controls and nonwasted patients (fig. 3b). CuZn-SOD protein levels were also significantly increased in limb muscles of muscle-wasted patients compared with control subjects (fig. 3c). Muscle protein content of the antioxidants catalase, glutathione peroxidase-I, peroxiredoxin-II, peroxiredoxin-III and GSH did not differ between patients and controls (table 2).

Contractile and functional muscle proteins

Protein levels of MyHC were significantly reduced in limb muscles of muscle-wasted COPD patients compared with both control subjects and nonwasted patients (fig. 3d). No differences were observed in MyHC content between nonwasted patients and healthy controls (fig. 3d). Nevertheless, levels of carbonylated MyHC were significantly increased in limb muscles of both groups of patients compared with healthy controls (fig. 3e). Levels of muscle actin were not different between severe COPD patients and control subjects (fig. 4a). Compared with controls, protein content of creatine kinase was significantly reduced in limb muscles of muscle-wasted COPD, whereas muscles of the nonwasted patients showed a tendency to be decreased ($p=0.09$, fig. 4b). Limb muscles of both muscle-wasted and nonwasted patients exhibited a significant decline in carbonic anhydrase-3 content compared with control subjects (fig. 4c). Among the COPD patients, no significant correlations were found between the content of any of these proteins and either physiological or molecular variables.

Ubiquitin–proteasome system

Total protein ubiquitination levels were greater in the vastus lateralis of the muscle-wasted patients compared with control subjects (fig. 5a). Muscle content of the 20S proteasome subunit C8 did not differ between any of the patients and control subjects (fig. 5b). However, levels of the ubiquitin-conjugating enzyme $E2_{14k}$ and of the E3 ligase atrogin-1 (muscle atrophy F-box) were greater in the vastus lateralis of muscle-wasted COPD patients than in control muscles (figs 5c and d, respectively). Limb muscles of the nonwasted patients exhibited an almost significant increase ($p=0.08$) in atrogin-1 protein levels compared with the controls (fig. 5d). Muscle content of the E3 ligase MURF-1 did not differ between any of the study groups (fig. 5e).

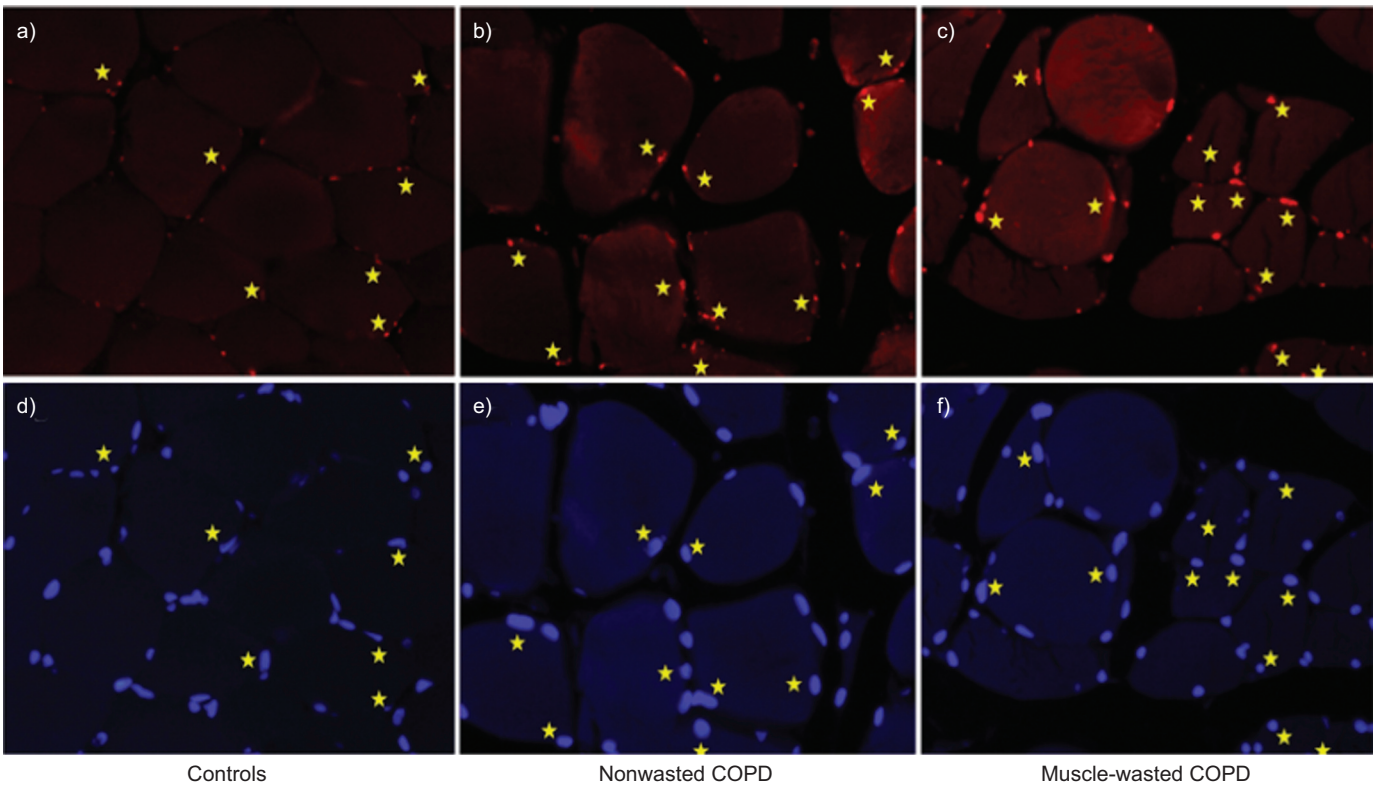

FIGURE 1. Superoxide anion detection with the fluorescent probes a–c) dihydroethidium (DHE; red staining) and e–f) 4′,6-diamidino-2-phenylindole (DAPI; blue staining) in the vastus lateralis of a, d) a healthy control subject (magnification ×200), b, e) a nonwasted severe chronic obstructive pulmonary disease (COPD) patient (magnification ×200), and c, f) a wasted COPD patient (magnification ×200). Yellow stars indicate nuclei that were positively stained for both DHE and DAPI. g) The percentage of positively stained nuclei (mean±SD) for both DHE and DAPI was significantly higher in the vastus lateralis of nonwasted and wasted severe COPD patients than in the control muscles. ***: $p<0.001$.

Signalling pathways of muscle proteolysis

FoxO pathway

Muscle content of FoxO1 transcription factor was significantly higher in the vastus lateralis of the muscle-wasted patients than in control subjects (fig. 6a). Among COPD patients, muscle FoxO1 levels correlated with total muscle protein ubiquitination ($r=0.570$, $p=0.003$).

MAPK pathway

Muscle levels of MAPK subfamilies c-Jun terminal (JNK), extracellular regulated kinases (ERK)1/2, and p38 did not differ between COPD patients and healthy controls (table 2).

NF-κB pathway

In the limb muscles, no differences were observed between patients and healthy controls regarding protein levels of the transcription factor p50 (fig. 6b). Protein content of the p65 factor, however, exhibited a significant rise in the vastus lateralis of the muscle-wasted patients compared with control subjects (fig. 6c). Levels of p65 also showed a tendency to be increased in limb muscles of the nonwasted patients compared with control muscles (fig. 6c). Among COPD patients, p65 protein content positively correlated with total muscle protein ubiquitination ($r=0.526$, $p=0.008$).

Muscle growth and differentiation

Muscle levels of myostatin protein did not differ between patients and healthy controls (table 2). Nevertheless, protein content of myogenin was significantly reduced within limb muscles of both muscle-wasted and nonwasted severe COPD patients compared with healthy controls (fig. 6d). Among all

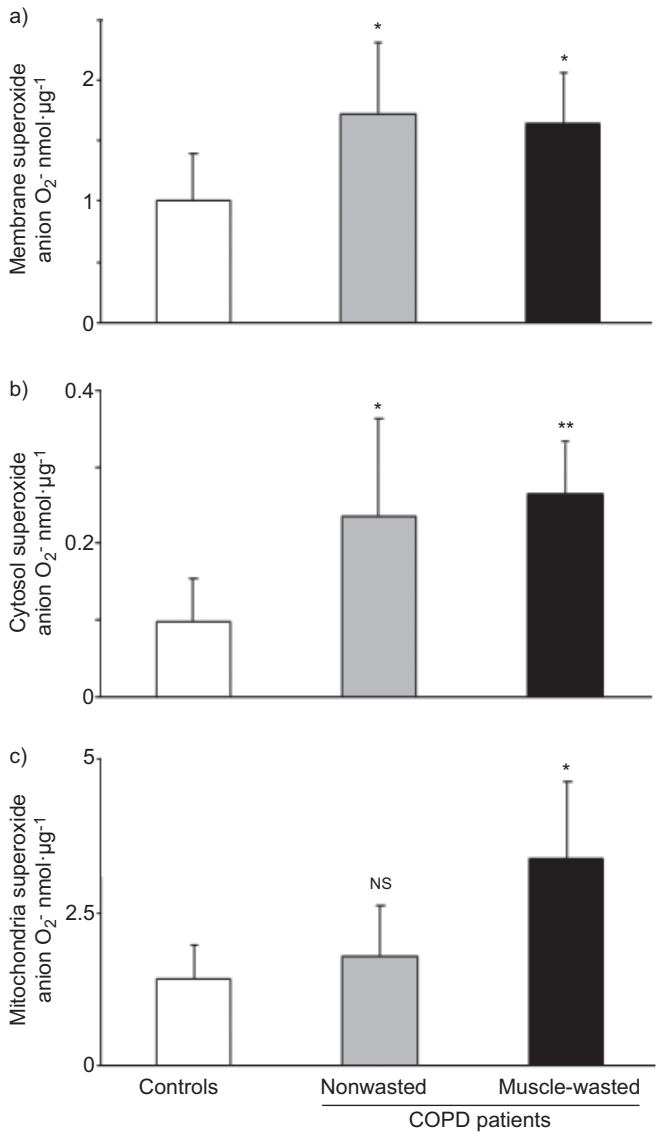

FIGURE 2. a) Mean±SD of superoxide anion production (nmol·µg^{-1}) in the membrane fraction of the vastus lateralis in both nonwasted and wasted severe chronic obstructive pulmonary disease (COPD) patients were significantly greater than in control subjects (*: $p<0.05$). b) Mean±SD of superoxide anion production (nmol·µg^{-1}) in the cytosol compartment of the vastus lateralis in both nonwasted and wasted severe COPD patients were significantly greater than in control subjects (*: $p<0.05$ and **: $p<0.01$). c) Mean±SD of superoxide anion production (nmol·µg^{-1}) in the mitochondrial fraction of the vastus lateralis in wasted severe COPD patients were significantly greater than in control subjects (*: $p<0.05$). Muscle levels of mitochondrial superoxide anion, however, did not significantly differ between healthy controls and nonwasted severe COPD patients. NS: nonsignificant.

COPD patients, myogenin levels were significantly associated with FFMI ($r=0.402$, $p=0.046$).

Inflammatory cytokines

ELISA levels of interferon-γ, TNF-α, and VEGF did not significantly differ in the limb muscles between patients and controls (table 2).

Muscle structure

Fibre type composition

Proportions of type I fibres were significantly reduced within limb muscles of muscle-wasted patients compared with healthy controls (table 3). Additionally, the size of type II fibres was decreased (21%) in vastus lateralis of muscle-wasted patients compared with both healthy controls and nonwasted patients (table 3 and fig. S4).

Muscle abnormalities

Proportions of abnormal muscle were greater in the vastus lateralis of both muscle-wasted and nonwasted patients than in healthy controls (table 3). No significant differences were observed between the two groups of COPD patients regarding muscle structural abnormalities (table 3).

DISCUSSION

A major novel finding in the present investigation is that levels of oxidative stress markers, such as superoxide anion, within the different muscle compartments and myonuclei, total protein carbonylation and MyHC oxidation were increased within the limb muscles of all severe COPD patients regardless of their body composition, while a decline in MyHC content and atrophy of type II fibres was seen only in limb muscles of the muscle-wasted patients. Moreover, compared with the controls, the vastus lateralis of the latter patients also showed a significant rise in the levels of several markers of the proteolytic ubiquitin–proteasome system, such as total protein ubiquitination and atrogin-1 and $E2_{14k}$ protein content, and of the redox-sensitive signalling pathways FoxO1 and NF-κB (p65). Levels of muscle inflammatory parameters did not differ between COPD patients and healthy controls. Additionally, levels of functional muscle proteins such as creatine kinase and carbonic anhydrase-3 were decreased among the patients. On the basis of the current findings, it would be possible to conclude that oxidative stress does not seem to directly modulate loss of contractile proteins, within the atrophied muscles of patients with severe COPD. Such a conclusion is partly counter to our initial hypothesis.

Exercise capacity and peripheral muscle function and structure

As previously demonstrated [12, 16–21], in the current investigation, COPD patients with muscle wasting exhibited severe impairment of aerobic capacity together with moderate reduction in quadriceps muscle force. The latter parameter was similar within the two groups of patients regardless of their muscle mass. The greater proportions of type II fibres observed within the limb muscles of the muscle-wasted patients, albeit of smaller size, could be a contributing factor. Furthermore, muscle mass was shown to be directly associated with disease severity as measured by FEV_1.

A novel finding in the study refers to the degree of muscle structure abnormalities, which was increased and similar in both groups of patients. These findings suggest that factors related to COPD, rather than body composition, are more likely to account for the increase in muscle structure alterations among the patients. Furthermore, the vastus lateralis muscle of the muscle-wasted patients exhibited a less resistant phenotype (decreased proportion of slow-twitch fibres) together with signs of atrophy of the fast-twitch fibres. These findings are in agreement with earlier investigations in which limb muscles of

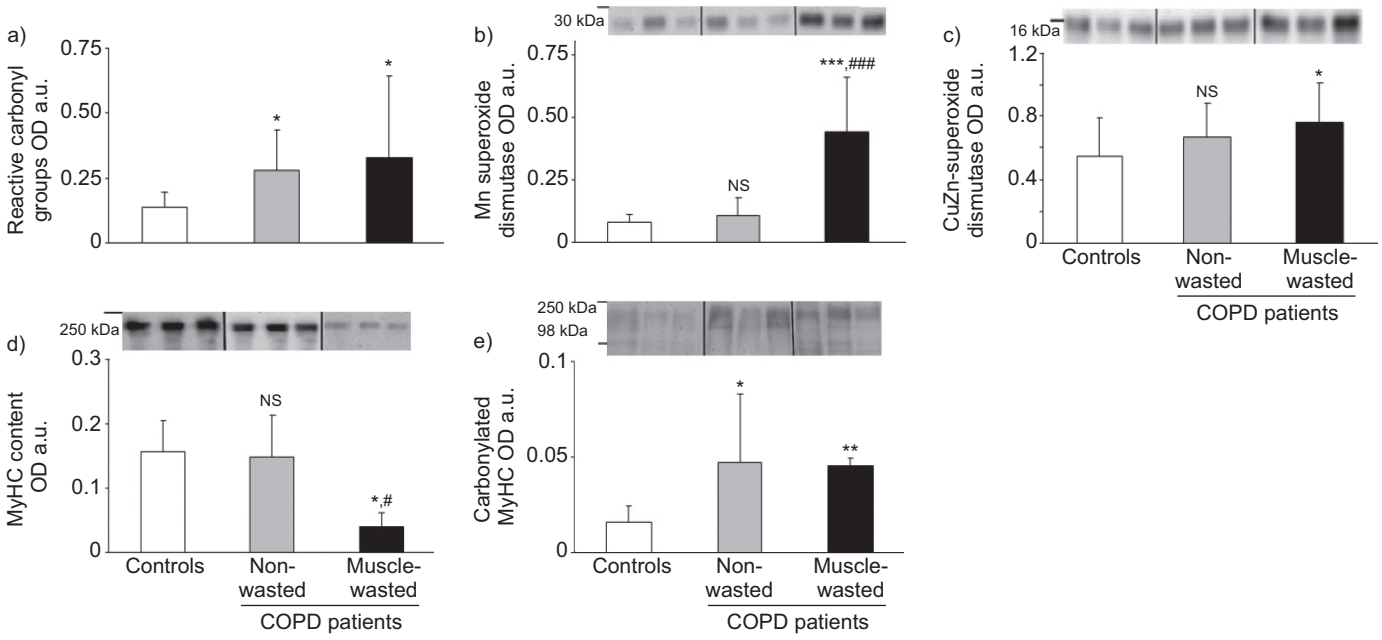

FIGURE 3. a) Mean±SD of total carbonylated protein levels were significantly greater (*: $p<0.05$) in the limb muscles of both nonwasted and muscle-wasted COPD patients than in the healthy sedentary controls. b) Representative immunoblots of Mn-superoxide dismutase protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of protein content of Mn-superoxide dismutase were significantly higher in the vastus lateralis of the muscle-wasted COPD patients than in both healthy sedentary controls and nonwasted patients (***: $p<0.001$ and $^{###}$: $p<0.001$, respectively). c) Representative immunoblots of CuZn-superoxide dismutase protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of protein content of CuZn-superoxide dismutase were significantly higher in the vastus lateralis of the muscle-wasted COPD patients than in the healthy sedentary controls (*: $p<0.05$). Protein levels of CuZn-superoxide dismutase did not significantly (NS: nonsignificant) differ between nonwasted COPD patients and healthy controls. d) Representative immunoblots of myosin heavy chain (MyHC) protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of protein content of contractile MyHC were significantly lower in the vastus lateralis of the muscle-wasted COPD patients than in both healthy sedentary controls (*: $p<0.05$) and nonwasted patients ($^{#}$: $p<0.05$). e) Representative immunoblots of carbonlyated MyHC protein in healthy controls, nonwasted (*: $p<0.05$) and wasted (**: $p<0.01$) severe COPD patients, respectively. Mean±SD of carbonylated MyHC were significantly greater in the limb muscles of both nonwasted and muscle-wasted COPD patients than in the healthy sedentary controls. Note that noncontiguous gel lanes are demarcated by black lines. Optical densities (OD) are expressed in arbitrary units (a.u.).

TABLE 2 Biological markers in the vastus lateralis of severe chronic obstructive pulmonary disease (COPD) patients and healthy controls

	Control subjects	Nonwasted severe COPD patients	Muscle-wasted severe COPD patients
Antioxidants			
Catalase OD a.u.	0.148±0.063	0.177±0.032	0.165±0.018
GPx-1 OD a.u.	0.635±0.376	0.741±0.280	0.609±0.344
Prx-II OD a.u.	0.304±0.149	0.294±0.118	0.272±0.063
Prx-III OD a.u.	0.107±0.039	0.129±0.040	0.117±0.031
GSH μM	17.631±7.288	21.090±8.691	23.137±11.701
Signalling pathways			
JNK OD a.u.	0.110±0.062	0.120±0.070	0.109±0.054
ERK-1/2 OD a.u.	0.508±0.240	0.483±0.126	0.473±0.187
p38 OD a.u.	0.228±0.103	0.189±0.092	0.219±0.119
Myostatin OD a.u.	0.330±0.063	0.327±0.064	0.326±0.064
Inflammation			
IFN-γ pg·mL^{-1}	50.190±15.140	40.480±22.630	55.850±25.004
TNF-α pg·mL^{-1}	28.25±4.830	26.990±5.600	26.320±6.550
VEGF pg·mL^{-1}	10.660±4.740	8.280±3.580	8.820±5.062

Data are presented as mean±SD. OD: optical density; a.u.: arbitrary units; GPx: glutathione peroxidase; Prx: peroxiredoxin; GSH: reduced glutathione; JNK: c-Jun N-terminal kinase; ERK: extracellular-signal-regulated kinase; p38: p38 mitogen-activated protein kinase; IFN: interferon; TNF: tumour necrosis factor; VEGF: vascular endothelial growth factor.

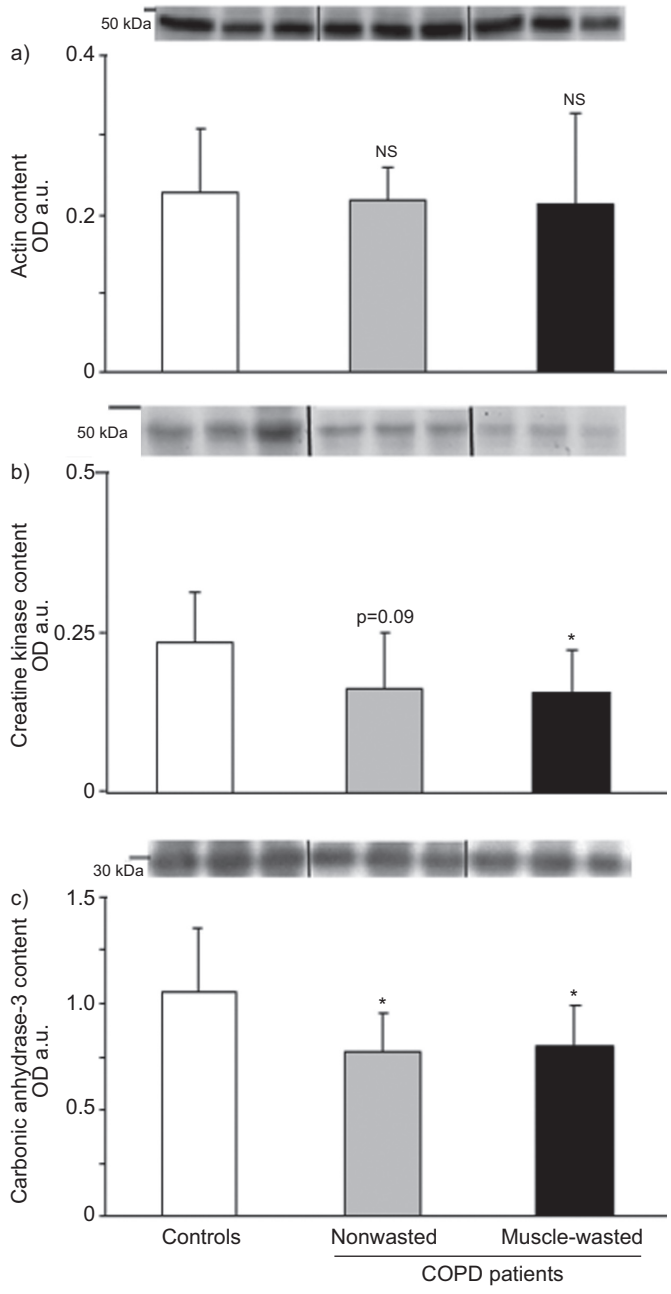

FIGURE 4. a) Representative immunoblots of actin protein content in healthy controls, nonwasted and wasted severe chronic obstructive pulmonary disease (COPD) patients, respectively. Mean±SD of contractile actin did not significantly (NS: nonsignificant) differ among healthy controls and any of the groups of COPD patients. b) Representative immunoblots of creatine kinase protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of creatine kinase content were significantly lower in the limb muscles of the muscle-wasted COPD patients (*: $p<0.05$) than in the healthy sedentary controls. Protein levels of creatine kinase showed a strong tendency to be decreased ($p=0.09$) in the vastus lateralis of the nonwasted patients compared with the controls. c) Representative immunoblots of carbonic anhydrase-3 protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of carbonic anhydrase-3 content were significantly lower in the limb muscles of both muscle-wasted and nonwasted COPD patients (*: $p<0.05$) than in the healthy sedentary controls. Optical densities (OD) are expressed in arbitrary units (a.u.). Note that noncontiguous gel lanes are demarcated by black lines.

severe COPD patients with abnormal body composition exhibited a shift towards a lower resistant phenotype [12, 16, 17, 36]. Besides, they are also in line with another investigation from some of us [32], in which the size of type II fibres was of smaller size in the gastrocnemius of rats with cancer-induced cachexia. Altogether, these findings suggest that fast-twitch rather than slow-twitch fibres are likely to be a major target for enhanced muscle proteolysis, at least in the models in question.

Redox balance and content of key muscle proteins

We and other investigators [12–20, 23, 34, 37] have already shown, on several occasions, that protein oxidation is increased in the muscles of patients with COPD. Indeed, oxidative stress has widely been proposed as one of the most important mechanisms involved in the aetiology of peripheral muscle dysfunction in COPD. In the current study, we confirm once more that lower limb muscle proteins undergo severe oxidation. Moreover, we also report that both mitochondrial Mn-SOD and cytosolic CuZn-SOD protein content was greater in the vastus lateralis of the muscle-wasted COPD patients, whereas levels of catalase, glutathione peroxidase-I, peroxiredoxin-II and -III, and GSH did not differ between patients and controls. This is in agreement with previous reports [13, 17], leading to the concept that superoxide anion is likely to be a major player in the oxidative cascade of limb muscles in COPD.

Importantly, compared with both sedentary controls and non-wasted patients, limb muscles of the muscle-wasted patients exhibited a four-fold decrease in MyHC content (26% of the control levels). A second relevant finding in the study is related to the three-fold increase in carbonylation levels of MyHC within peripheral muscles of both muscle-wasted and nonwasted patients. These results are in line with a previous report from some of us [23], in which MyHC content was dramatically decreased in the diaphragms of severe COPD patients, while also exhibiting a substantial rise in carbonylation levels. Considering all these findings taken together, it could be argued that while oxidative stress is clearly involved in the pathophysiology of COPD muscle dysfunction [14, 16, 17], enhanced contractile protein breakdown may rely on other mechanisms not directly linked to increased oxidative stress within the myofibres, at least in peripheral muscles. Other factors, such as the degree of the airway obstruction, diffusion capacity, hypoxia and deconditioning, may have also influenced enhanced MyHC protein loss in COPD patients. However, these aspects were not addressed in the current investigation and will be the focus of future research.

In the current investigation, an attempt to assess the content of key muscle proteins previously shown to be targeted by oxidants [12, 15, 23], as well as by the proteolytic systems [8, 38], has been made. We found that levels of the enzymes creatine kinase and carbonic anhydrase-3, but not contractile actin, were reduced in the limb muscles of muscle-wasted and nonwasted patients. In previous studies [12, 15, 23], when compared with healthy controls, the respiratory and limb muscles of the severe patients exhibited a significant decrease in creatine kinase content and activity, while carbonic anhydrase-3 levels did not differ between groups in any of the muscles [12, 15, 23]. Considering differences in disease severity, patients were more severely diseased in the current study than in the previous reports [12, 15, 23], and in body composition may account for the discrepancies observed among the three studies. Future investigations are

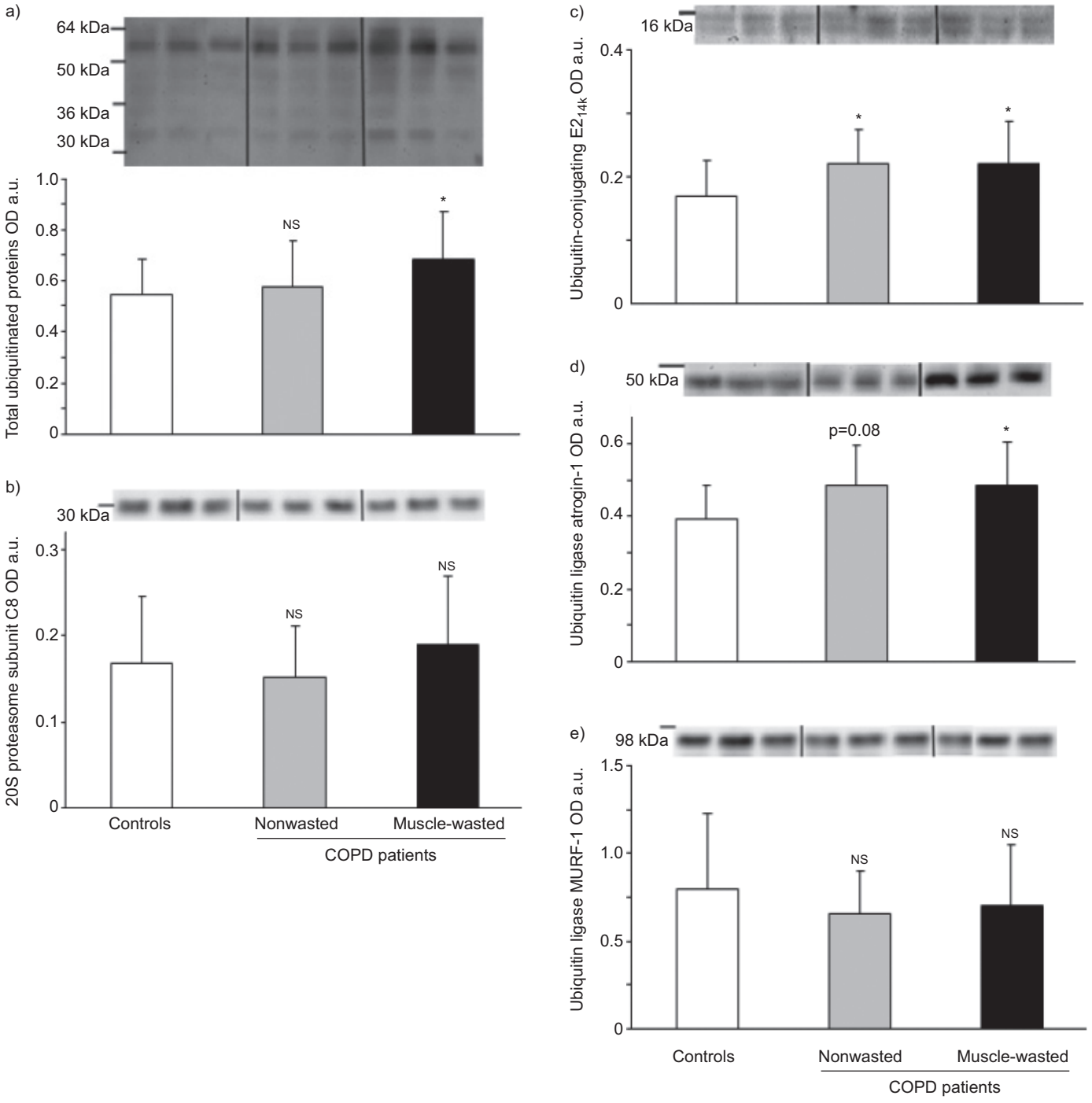

FIGURE 5. a) Representative immunoblots of ubiquitinated proteins in healthy controls, nonwasted and wasted severe chronic obstructive pulmonary disease (COPD) patients, respectively. Mean±SD of total ubiquitinated protein levels were significantly higher in the quadriceps of the wasted COPD patients (*: $p<0.05$) than in the healthy sedentary control subjects. Levels of total ubiquitinated proteins did not significantly (NS: nonsignificant) differ between nonwasted COPD patients and healthy controls. b) Representative immunoblots of 20S proteasome C8 subunit content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of the 20S proteasome C8 subunit did not significantly differ among healthy controls and any of the groups of COPD patients. c) Representative immunoblots of $E2_{14k}$ protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of the ubiquitin-conjugating enzyme $E2_{14k}$ were significantly greater in the quadriceps of both nonwasted and wasted COPD patients (*: $p<0.05$) than in the healthy sedentary control subjects. d) Representative immunoblots of atrogin-1 protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of the ubiquitin-ligase enzyme atrogin-1 were significantly greater in the limb muscles of the muscle-wasted COPD patients (*: $p<0.05$) than in the healthy sedentary control subjects. Protein levels of atrogin-1 showed a strong tendency to be increased ($p=0.08$) in the vastus lateralis of the nonwasted patients compared with the controls. e) Representative immunoblots of MURF-1 protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of the ubiquitin-ligase enzyme MURF-1 did not significantly differ among healthy controls and any of the groups of COPD patients. Optical densities (OD) are expressed in arbitrary units (a.u.). Note that noncontiguous gel lanes are demarcated by black lines.

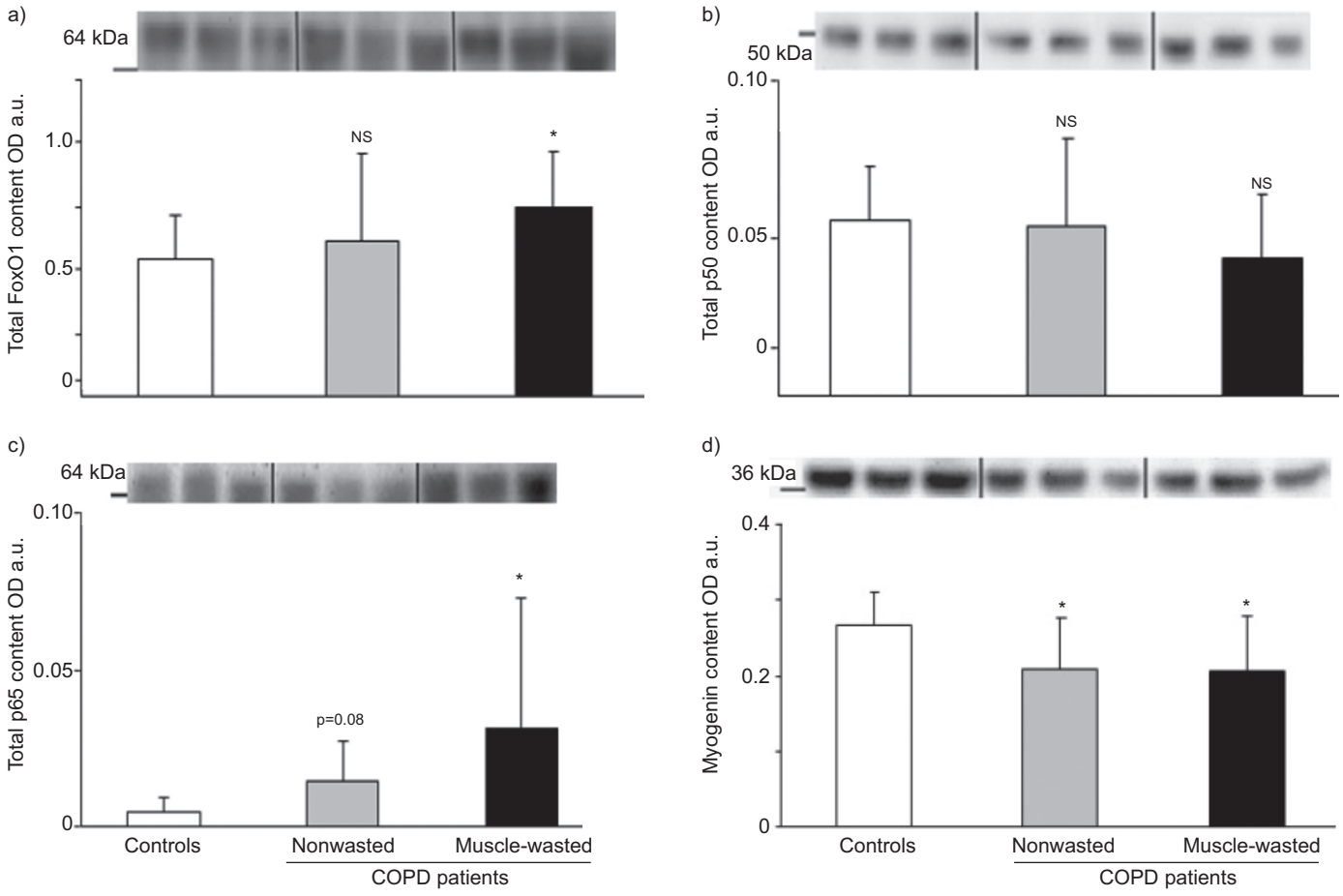

FIGURE 6. a) Representative immunoblots of FoxO1 protein content in healthy controls, nonwasted and wasted severe chronic obstructive pulmonary disease (COPD) patients, respectively. Mean±SD of transcription factor FoxO1 were significantly greater in the limb muscles of the wasted COPD patients (*: $p<0.05$) than in the healthy sedentary controls. Protein levels of FoxO1 did not significantly (NS: nonsignificant) differ between nonwasted COPD patients and healthy controls. b) Representative immunoblots of p50 protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of the NF-κB pathway, as measured by the transcription factor p50, did not significantly differ among healthy controls and any of the groups of COPD patients. c) Representative immunoblots of p65 protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of the transcription factor p65 were significantly greater in the limb muscles of the muscle-wasted COPD patients (*: $p<0.05$) than in the healthy sedentary controls. Protein levels of p65 showed a strong tendency to be increased ($p=0.08$) in the vastus lateralis of the nonwasted patients compared with the controls. d) Representative immunoblots of myogenin protein content in healthy controls, nonwasted and wasted severe COPD patients, respectively. Mean±SD of the myogenic transcription factor myogenin were significantly lower in the vastus lateralis of both nonwasted and muscle-wasted COPD patients (*: $p<0.05$) than in the healthy sedentary controls. Optical densities (OD) are expressed in arbitrary units (a.u.). Note that noncontiguous gel lanes are demarcated by black lines.

TABLE 3 Structural characteristics of the vastus lateralis muscle in severe chronic obstructive pulmonary disease (COPD) patients and healthy controls

	Control subjects	Nonwasted severe COPD patients	Muscle-wasted severe COPD patients
Type I fibres %	30±5	29±7	23±6*,#
Type II fibres %	70±5	71±7	77±6*,#
Cross-sectional area μm²			
Type I fibres	2581±278	2452±425	2243±514
Type II fibres	2909±540	2880±361	2306±533*,#
Normal muscle %	98.5±0.4	97.5±0.7**	97.5±0.9*
Abnormal muscle %	1.5±0.4	2.5±0.7**	2.5±0.9*

Data are presented as mean±SD. *: $p\leqslant0.05$; **: $p\leqslant0.01$, between any of the groups of severe COPD patients and controls; #: $p\leqslant0.05$, between muscle-wasted and nonwasted COPD patients.

required to specifically disentangle the factors that may contribute to alterations in the content of functional muscle proteins in severe COPD.

Ubiquitin–proteasome system and signalling pathways

The ubiquitin–proteasome system degrades both cytosolic and nuclear proteins, and myofibrillar proteins, which are the predominant proteins in skeletal muscles. In the current investigation, levels of the ubiquitin-conjugating enzyme $E2_{14k}$ and of the E3 ligase atrogin-1, but not MURF-1, were significantly greater in the vastus lateralis of the muscle-wasted COPD patients than in the healthy sedentary controls. This is partly in agreement with a previous report [6] in which mRNA levels of atrogin-1 and MURF-1 were shown to be up-regulated in the limb muscles of stable, relatively well-nourished, severe COPD patients. In that study [6], however, the increase in atrogin-1 protein content observed in the severe COPD patients did not reach the statistical significance. More recently, limb muscle mRNA levels of atrogin-1, but not MURF-1, were also shown to be upregulated in stable nonwasted severe COPD patients [7]. Additionally, MURF-1 and atrogin-1 mRNA levels were shown to be upregulated in the vastus lateralis of hospitalised nonwasted patients during an acute exacerbation [5]. Differences in the number of patients, their body composition, study conditions and in the methodologies employed in each case (mRNA expression *versus* protein content herein) might account for the minor discrepancies between studies. Another novel finding in the investigation is that muscle-wasted severe COPD patients exhibited greater protein ubiquitination levels in their atrophying muscles. In view of the present findings and of those previously published in limb [5–7] and respiratory muscles [8, 39] of severe COPD patients, it could be assumed that the ubiquitin–proteasome system seems to play a relevant role in the process of muscle atrophy in severe COPD patients.

In severe COPD, the upstream signalling regulation of muscle protein degradation, especially of the ubiquitin–proteasome system, has not yet been fully elucidated. The line has been put forward that the transcription factors FoxO play crucial roles in the regulation of muscle wasting [4, 40]. For instance, FoxO1 has been shown to regulate atrogin-1 in skeletal muscles of COPD patients [6], in cancer cachectic muscles [9] and in the atrophying diaphragm of patients exposed to mechanical ventilation for several days [10]. In the present investigation, protein levels of FoxO1, in addition to being increased within the vastus lateralis of the muscle-wasted patients, also exhibited a positive correlation with levels of total muscle protein ubiquitination among all patients. The latter finding suggests that enhanced muscle protein ubiquitination may be signalled, at least to some extent, by FoxO1 in severe COPD.

The MAPK cascade leads to the activation of protein kinases and transcription factors through phosphorylation, resulting in signal transduction, hence playing a key role in cell signalling within tissues. In the current study, protein content of different phosphorylated MAPKs was not explored. Total protein levels, however, of the best characterised MAPK subfamilies JNK, ERK1/2 and p38 were not different between COPD patients and control subjects. These results suggest that despite the relevance of MAPK signalling pathway in a variety of physiological and pathophysiological processes, it may not play a significant role in the COPD-associated muscle atrophy.

NF-κB is one of the most relevant signalling pathways leading to skeletal muscle loss. It is composed of a family of five members (p65, Rel-B, c-Rel, p50 and p52), which are all expressed within skeletal muscles. The NF-κB pathway was recently shown to be upregulated in the diaphragm of severe COPD patients [39]. In the muscle-wasted patients of the current study, protein expression of p65, but not p50, was greater in their vastus lateralis, and also showed a positive correlation with levels of total muscle protein ubiquitination. Altogether, the present findings suggest that FoxO1 and NF-κB, but probably not MAPK, are likely to play a major role in the regulation of muscle contractile protein loss through enhanced ubiquitination in severe COPD.

Myostatin, which is a member of the transforming growth factor-β superfamily, is almost exclusively expressed in skeletal muscles and is a potent negative regulator of muscle mass. It has recently been shown that myostatin expression was increased in the vastus lateralis [7, 41] and diaphragm [39] of severe COPD patients. It has also been suggested that resistance training reduces myostatin levels in the limb muscles of nonwasted COPD patients [42, 43], eventually contributing to enhanced muscle mass in these patients. In the present investigation, protein levels of myostatin did not differ between any of the study groups. Differences in the study design, in the degree of muscle wasting and in the number of patients, together with the lack of a control group of healthy subjects in some of the studies [42, 43], could account for discrepancies among investigations.

Another important myogenic transcriptor factor required for muscle development during embryonic and fetal life is myogenin. Myogenin also plays a key role in skeletal muscle differentiation, maintenance and repair, regulating muscle metabolism and energy utilisation. In the present study, myogenin protein levels were decreased in both groups of severe COPD patients. These findings lead to the conclusion that the repair mechanisms are probably defective within the limb muscles of severe COPD patients, irrespective of their nutritional status. In reality, muscle structural abnormalities were also encountered in both groups of patients.

Study limitations

A first limitation in this study was that patients exhibiting impaired muscle mass were also those showing worse lung function. Importantly, a significant positive association was observed between airway obstruction as measured by FEV_1 and the FFMI. In fact, it has been established that weight loss and increasing COPD severity are not entirely separate phenomena and that their associations imply a poorer prognosis of the disease.

A second limitation in the present investigation is related to the use of sedentary individuals as the healthy controls. This explains the relatively low proportions of slow-twitch fibres observed within the limb muscles of the control subjects. Nevertheless, we reasoned that physical activity might influence to a great extent redox balance and the expression of the different markers and signalling pathways investigated herein. As the severe COPD patients were all sedentary, this

was, indeed, our major argument to specifically recruit healthy sedentary individuals as the control group.

Conclusions

In contrast to our initial hypothesis, in severe COPD patients, while muscle protein oxidation is increased regardless of their body composition, loss of contractile MyHC and total protein ubiquitination were enhanced only in patients exhibiting muscle atrophy. This process appears to be signalled by FoxO and NF-κB pathways. Oxidative stress, however, does not seem to modulate muscle protein loss directly in these patients. Other factors, such as disease and emphysema severity, hypoxia, and deconditioning, may also influence muscle atrophy in severe COPD.

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STATEMENT OF INTEREST

None declared.

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