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Skeletal muscle inflammation and nitric oxide in patients with COPD

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ABSTRACT: In chronic obstructive pulmonary disease (COPD) the presence of systemic inflammation has been associated with peripheral muscle abnormalities and weight loss.

To study whether inflammatory factors are important in these processes, the present study compared the skeletal muscle levels of nitrite, nitrate, nitrotyrosine, neuronal, endothelial and inducible nitric oxide synthases (nNOS, eNOS, and iNOS, respectively), and inflammatory markers (tumour necrosis factor (TNF)-α, CD154 and CD163) in 15 patients (forced expiratory volume in one second 43±11%) and 14 controls. All these markers were also compared between patients with normal and low body weight.

Nitrite (12.5±2.6 *versus* 17.0±3.4 μmol·mg^{-1} protein), nitrate (20.7±2.4 *versus* 24.4±4.5 μmol·mg^{-1} protein) and eNOS (31.9±4.6 *versus* 43.6±7.5 ng·mg^{-1} protein) were lower in COPD patients than in controls. Nitrotyrosine (25.6±5.4 *versus* 6.6±3.3 ng·mg^{-1} protein), iNOS expression (32±9.5 *versus* 7.16±2.7 ng·mg^{-1} protein), TNF-α (257±160 *versus* 48.3±4.4 pg·mg^{-1} protein) and CD163 (6.4±2.1 *versus* 0.8±0.4 ng·mg^{-1} protein) were higher in COPD patients than in controls. CD154 levels were 15.7±7.0 ng·mg^{-1} protein in COPD patients and undetectable in controls. Similar levels of all these markers were observed in COPD patients with normal and low body weight.

In conclusion, these findings suggest the presence of an inflammatory process in the muscle tissue of chronic obstructive pulmonary disease patients, and argue in favour of its participation in the pathogenesis of skeletal muscle abnormalities.

KEYWORDS: Chronic obstructive pulmonary disease, emphysema, skeletal muscle

Chronic obstructive pulmonary disease (COPD) is a pulmonary disorder associated with significant extra-pulmonary manifestations, including peripheral muscle dysfunction and unexplained weight loss [1–4]. The inflammation of COPD primarily affects the airways and lung parenchyma, but it may also play a role in the systemic consequences of the disease [4–8].

Despite the fact that peripheral muscle dysfunction and weight loss are probably the most extensively studied systemic effects of COPD, the mechanisms responsible for their occurrence are still poorly understood. They are probably multi-factorial in origin, but there is strong evidence suggesting a link with systemic inflammation [4–8].

In peripheral tissues, nitric oxide (NO) is generated enzymatically by at least three different isoforms of nitric oxide synthases (NOS), which oxidises L-arginine. NO formed by endothelial constitutive NOS (eNOS) is responsible for maintaining low vascular tone and preventing leukocytes and platelets from adhering to the vascular wall, while NO formed by neuronal constitutive NOS (nNOS) acts as a neuromodulator or neuromediator. The activation of the inducible isoform (iNOS) through inflammatory stimulants produces NO in 1,000-fold larger quantities, which mediates defense and pathological processes.

The simultaneous release of superoxide anion (O_2^-) and the over production of NO can form peroxynitrite (ONOO-), which in turn is responsible for the cytotoxic effects of NO. As ONOO- is a transient species with a biological half-life even shorter than that of free radical NO, it cannot be directly measured, and its presence must be inferred by indirect methods. One of these is the presence and level of nitrotyrosine, a product formed by nitration of the tyrosine residues of proteins. Nitrotyrosine formation has attracted considerable interest because: 1) it can alter protein function; 2) it has been associated to acute and chronic disease status; and 3) it is

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considered an important indicator of disorders caused by $ONOO^-$-dependent tissue damage [9, 10].

Two recent studies have assessed the iNOS expression in the skeletal muscle of patients with COPD [6, 7]. BARREIRO *et al*. [6] could not detect iNOS in the skeletal muscle of patients with moderate COPD. In contrast, AGUSTÍ *et al*. [7] have shown nuclear factor (NF)-κB activation and iNOS induction occurring in the skeletal muscle of severe COPD patients, in particular in those with low body weight. Although abnormal NO regulation may contribute to muscle alteration in COPD, the generation of NO and the role of inflammation in the peripheral muscle of these patients remains unclear.

The aim of the current study was to investigate cross-sectionally the presence of inflammatory markers (tumour necrosis factor (TNF)-α, CD154 and CD163), and the concentration of NOS and NO end products (nitrites (NO_2^-), nitrates (NO_3^-) and nitrotyrosine) in the skeletal muscles of patients with COPD. Inmunohistochemical techniques (CD68) and electron microscopy were used to evaluate the possible presence of inflammatory cells in muscle sections of some patients. The enzymes, NO end products and inflammatory mediators were also compared between normal and low body weight COPD patients to determine the potential association between inflammation and the unexplained weight loss.

METHODS

Study subjects

The study group consisted of 15 (eight males and seven females) clinically stable ex-smoker patients with COPD (64±8 yrs), recruited from the pulmonary clinic of the Hospital Universitario de Caracas (Caracas, Venezuela). In total, 14 (six males and eight females) healthy sedentary nonsmoking subjects (59±8 yrs) served as controls. The committee on human research (Hospital Universitario de Caracas, Venezuela) approved the study and all subjects signed the informed consent.

COPD was defined as a post-bronchodilator forced expiratory volume in one second/forced vital capacity (FEV1/FVC) <0.7 [11], and a history of smoking >20 pack-yrs. Patients were in a clinically stable condition, defined as no acute exacerbation of COPD, for 6 weeks prior to entry into the study, and were receiving optimal medical therapy without regular use of systemic corticosteroids. Patients were excluded if they had reversibility of FEV1 after bronchodilators >12% and 200 mL. Patients with congestive heart failure, neoplastic and metabolic diseases were also excluded, as well as those involved in regular exercise training. The subjects in the control group led a sedentary life style and did not perform regular exercise.

To determine whether NO end products and inflammatory markers occur in the skeletal muscle of patients with COPD and low body weight, the patients were divided into two groups according to body mass index (BMI). Low body weight was defined as a BMI of $\leqslant$21 kg·m^{-2}.

Pulmonary function tests and incremental exercise test

The FEV1, FVC and FEV1/FVC were calculated according to the recommendations of the American Thoracic Society [12]. Normal values for pulmonary measurements were taken from a standard reference source [13].

An exercise test was performed on a cycle ergometer (CardiO2 System; MedGraphics Corporation, St Paul, MN, USA), using a standard 1 min incremental cycle exercise protocol [14]. Patients were started with a 2-min period of unloaded pedalling at 60 cycles·min^{-1}, followed by a 15 watt increment·min^{-1}. The patients were strongly encouraged to cycle until discomfort or exhaustion was reported (symptom limited exercise test). Minute ventilation and its components were measured using a pneumotachograph. The concentration of expired O_2 and CO_2 were analysed breath-by-breath with a zirconium dioxide cell O_2 analyser and an infrared CO_2 analyser, respectively. Heart rate reserve was determined from the maximal heart rate obtained and maximal predicted heart rate (HRmax=220–age). Predicted maximal oxygen consumption ($V'O_2$ max) was calculated according to JONES and CAMPBELL [15]. Maximal voluntary ventilation was directly determined over a 12-s period.

Muscle biopsy

A muscle biopsy was taken from the vastus lateralis of the quadriceps muscle using a Bergström needle, under local anaesthetic and antiseptic conditions. The muscle sample was divided into three sections. One was processed for ultrastructural analysis, one frozen in isopentane then cooled in liquid nitrogen for immunohistochemistry, and the third frozen directly in liquid nitrogen for cytokine, NO products and enzymes determination.

Assessment of NO_2^- and NO_3^-

Frozen muscle samples were weighed and the tissue was homogenised with a hand-made glass pestle (Krogh Institute, Copenhagen, Denmark) with 1 mL buffer (Tris-HCl 10 mmol·L^{-1}, pH 7.5), NaCl (150 mmol·L^{-1}), EDTA (5 mmol·L^{-1}), triton x-100 (1% (v/v)), leupeptin (10 μg·Ml^{-1}), aprotinin (10 μg·mL^{-1}) and pepstatin (2.5 g·mL^{-1}). The homogenised tissue was centrifuged at 90×*g* for 5 min. The supernatant was used for the rest of the assays. Protein concentration was assessed using the BCA protein assay kit (Pierce Biochemicals, Rockfort, IL, USA).

NO levels were determined indirectly by quantification of their oxidised products of degradation (NO_2^- and NO_3^-), using nitrate reductase and Griess reagent [16] according to the method of MOSHAGE *et al*. [17]. Briefly, the supernatants of homogenised tissue were diluted four-fold with distilled water and incubated with nitrate reductase from *Aspergillus* spp. in order to quantify the total amount of nitric oxide products (NO_2^- plus NO_3^-). In the absence of the enzyme, only NO_2^- concentrations were determined. After 30 min incubation at 37℃ in the presence of the enzyme and its cofactors, NADPH and $FADH_2$, further incubation occurred for 10 min with sodium pyruvate and lactic dehydrogenase to degrade excess NADPH. The samples were deproteinised with zinc sulphate, and 100 μL of the supernatant was mixed with 100 μL of Griess reagent. A standard curve was obtained with sodium nitrate dissolved in water or in a pool of normal sera. The NO_2^- concentration was determined at 540 nm using an ELISA plate reader (MCC/340; Labsystems Multiscan, Helsinki, Finland).

Determination of NOS, nitrotyrosine, CD163 and CD154 by ELISA

Sandwich ELISA assays were used to assess eNOS, nNOS and iNOS. First, eNOS (NOS3) was detected using a commercial kit purchased from R & D systems (Minneapolis, MN, USA), using the manufacturer's instructions. The sensitivity was 25 pg·mL^{-1}. Secondly, nNOS (NOS1) and iNOS (NOS2) were assessed using standard ELISA sandwich techniques, as described previously [18]. For the detection of nNOS, the monoclonal and polyclonal antibodies were purchased from BD Biosciences (San Diego, CA, USA) and the recombinant enzyme for the standard curve was purchased from Calbiochem (San Diego, CA, USA). iNOS was assessed using a pair of antibodies purchased from Serotec (Kidlington, Oxford, UK). The sensitivity of both assays was 25 pg·mL^{-1}

The total amount of nitrotyrosine was determined by ELISA as explained previously [18]. The mouse immunoglobulin (Ig)-G monoclonal antibody, the polyclonal antibody against nitrotyrosine and the polyclonal goat anti-rabbit IgG-peroxidase antibody were purchased from Upstate Biotechnology (Lake Placid, NY, USA). The quantification of nitrotyrosine was performed using a standard curve with known concentrations of nitrotyrosine from chemically modified bovine serum albumin. The sensitivity of the assay was 50 pg·mL^{-1}.

A sandwich ELISA assay was performed to determine CD163 as described by SULAHIAN *et al*. [19], with minor modifications. Briefly, a monoclonal anti-CD163 (clone ED-Hu1; Serotec) was used as to capture CD163 and the biotinylated clone RM3/1 was used to detect the molecule. The homogenised muscle samples were diluted 1:10 in distilled water prior to being added to the plates and incubated overnight. The rest of the procedure was performed as described by SULAHIAN *et al*. [19]. However, the CD163 was purified from the supernatant of phormol myryistate acetate-activated monocytes by affinity chromatography using the monoclonal clone ED-Hu1, and the purified protein was used to perform the standard curve. The detection limit was 100–10,000 pg of purified CD163. The specificity was confirmed by immunoprecipitation of CD163 in homogenised muscle tissue prior to the assay.

CD154 levels were detected by a commercial sandwich inmunoenzymatic assay purchased from Chemicon Corporation (Temécula, CA, USA). The only modification was that the samples were incubated for 18 h at 4℃. The quantitative analysis was performed with a standard curve. The detection limit was 1 ng CD154 per mg of protein.

Assessment of TNF-α

TNF-α was assessed by a third generation TNF-α quantikine assay (R & D systems) following the manufacturer's instructions, except that samples were diluted as suggested for serum samples and incubated for 18 h instead of 3 h. The sensitivity of the assay is 0.5–3 pg·mL^{-1}.

Immunohistochemical analysis

In order to demonstrate the presence of leukocytes in muscle sections, immunohistochemical analysis was performed in samples of four patients and four control subjects. Anti-CD68, clone MB11 (Dako, Carpintería, CA, USA) was used as described by the manufacturer. The staining was performed with the Vecstain ABC Elite chemical for rapid staining (Vector Laboratories, Burlingame, CA, USA) using manufacturer's instructions.

Ultrastructural analysis

Part of the muscle sample was fixed in 3% glutaraldehyde in phosphate buffer, at pH 7.4 and 320 mOsmol, post-fixed in 1% OsO_4 and embedded in epon. Sections were cut with a diamond knife in a Porter-Blum MT2 ultramicrotome and stained with uranyl acetate and lead citrate. They were observed in a Hitachi H-500 transmission electron microscope at an acceleration voltage of 100 kV.

Statistical analysis

Data are presented as mean±SD. A nonparametric Mann-Whitney U-test was used to compare the levels of NO end products, enzymes, leukocytes and inflammatory markers between COPD patients and control subjects. All of these markers were also compared between COPD patients with normal and low body weight. An acceptable level of statistical significance for the test was a probability value $\leqslant 0.05$.

RESULTS

The mean values for the clinical characteristics and spirometric data of the patients and controls are detailed in table 1. There were no differences in age, weight or height between patients and controls. However, BMI was significantly lower in COPD patients than in controls. As expected, resting pulmonary function values were lower in COPD patients compared with controls and the patients showed severe airflow obstruction.

The peak values for the physiological variables measured during the exercise test are shown in table 2. The patients had reduced exercise capacity and functional capacity. Accordingly, the mean V'_{O_2}·kg·min^{-1} of all the patients places them in a functional impairment class with moderate-to-severe exercise limitation (V'_{O_2} 10–16 mL·kg^{-1}·min^{-1}). The patients also had reduced breathing reserve and abnormal O_2 delivery indices (O_2 pulse).

The results of skeletal muscle NO end products (NO_2^- NO_3^- and nitrotyrosine), iNOS, eNOS, TNF-α and CD163 of the patients and the healthy subjects are shown in figures 1–4. The

TABLE 1 Anthropometric and spirometric data in chronic obstructive pulmonary disease (COPD) patients and control group

Variables	COPD	Control	p-value
Age yrs	64±8	59±8	NS
Height cm	160±11	163±11	NS
Weight kg	56±17	66±12	NS
BMI	21.5±4.6	25.6±3.3	<0.01
FVC % pred	74±14	97±7	<0.001
FEV_1 % pred	43±11	95±7	<0.001
FEV_1/FVC %	46±6	80±3	<0.001

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; % pred: % predicted; NS: nonsignificant.

TABLE 2 Exercise testing data

Variables	Mean±SD
$V'O_2$ % pred	55.1±13.2
$V'O_2$ mL·kg^{-1}·min^{-1}	14.9±4.3
AT % pred	43.3±12.2
$V'E$max L·min^{-1}	38.2±14.4
$V'E$/MVV %	91.1±25.2
HRmax beats·min^{-1}	128±12
HRR % pred	82.6±7.7
O_2 pulse % pred	68.1±15.8

$V'O_2$: peak oxygen uptake; AT: anaerobic threshold; $V'E$max: peak minute ventilation; $V'E$: minute ventilation; MVV: maximal voluntary ventilation; HRmax: peak heart rate; HRR: heart rate reserve; O_2 pulse: peak O_2 pulse.

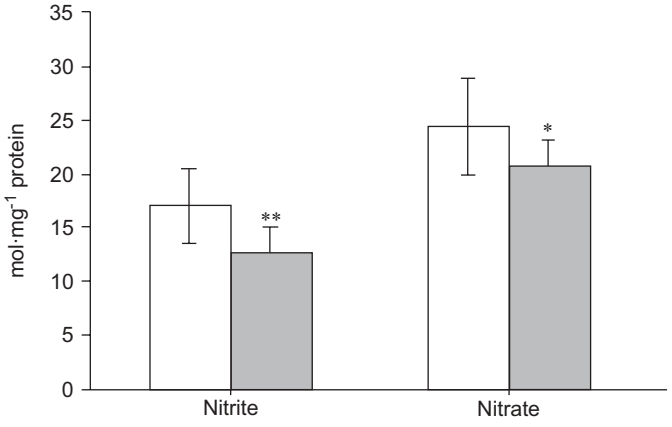

FIGURE 1. Nitrite and nitrate levels in patients with chronic obstructive pulmonary disease (■) and control subjects (□). *: p<0.05; **: p<0.01.

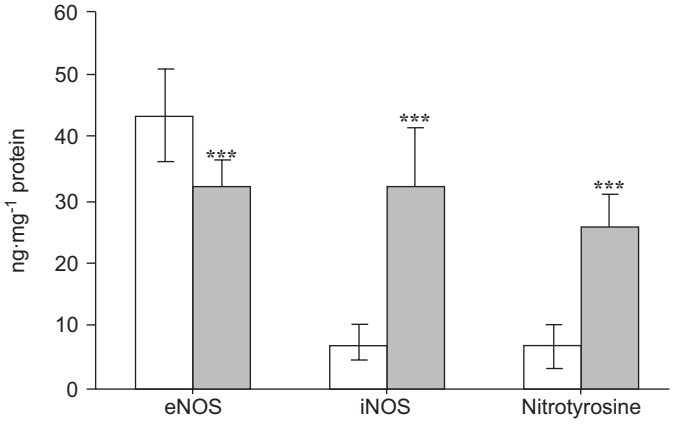

FIGURE 2. Endothelial constitutive nitric oxide synthases (eNOS), inducible isoform nitric oxide synthases (iNOS) and nitrotyrosine levels in patients with chronic obstructive pulmonary disease (■) and control subjects (□). ***: p<0.001.

NO_2^-, NO_3^- and eNOS expression levels were significantly lower in COPD patients compared with controls (figs. 1 and 2). nNOS was also lower in COPD patients compared with

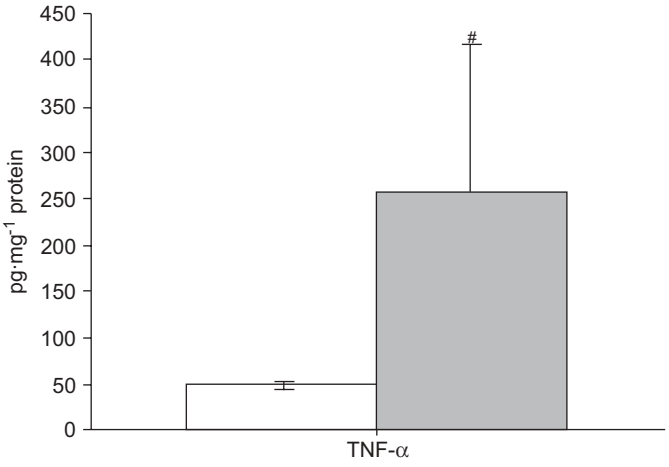

FIGURE 3. Tumour necrosis factor (TNF)-α levels in patients with chronic obstructive pulmonary disease (■) and control subjects (□). #: p<0.0001.

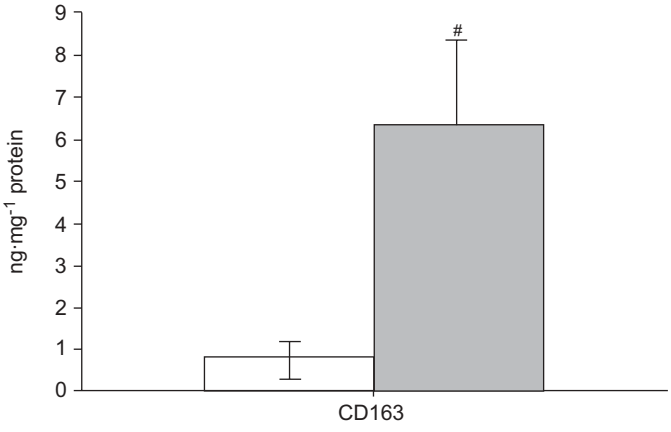

FIGURE 4. CD163 levels in patients with chronic obstructive pulmonary disease (■) and control subjects (□). #: p<0.0001.

controls (92.3±21.4 and 134.6±35.6 ng·mg^{-1} protein, respectively; p<0.01). In contrast, the skeletal muscle nitrotyrosine, iNOS expression, TNF-α and CD163 levels were markedly higher in COPD patients compared with healthy subjects (figs. 2–4). CD154 was only observed in homogenates of COPD patients (15.7±7.0 ng·mg^{-1} protein), but not in the control subjects, which was below the sensitivity of the method (<1 ng·mg^{-1} protein).

The characteristics of the COPD patients with normal and low BMI are shown in table 3. As expected, weight and BMI were lower in the low BMI group. They also had lower height compared with normal BMI COPD patients. Both groups of patients had a similar degree of airflow obstruction.

As reported in table 4, similar skeletal muscle NO_2^-, NO_3^-, nitrotyrosine, iNOS, eNOS, nNOS, TNF-α, CD154 and CD163 levels were observed in both groups of COPD patients.

The immunohistochemical analysis of the muscle tissue showed that the number of infiltrating leukocytes was markedly higher in the patients compared with healthy

TABLE 3 Anthropometric and spirometric data in low and normal weight chronic obstructive pulmonary disease (COPD)

Variables	Low weight COPD	Normal weight COPD	p-value
n	7	8	
Age yrs	68±7	66±9	NS
Height cm	154±10	166±8	<0.05
Weight kg	42±5	67±14	<0.001
BMI	17.9±1.2	24.2±3.7	<0.001
FVC % pred	75±13	71±14	NS
FEV_1 % pred	45±12	42±7	NS
FEV_1/FVC %	47±7	47±8	NS

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; % pred: % predicted; NS: nonsignificant.

TABLE 4 Skeletal muscle levels of inflammatory markers in low and normal weight chronic obstructive pulmonary disease (COPD)

Variables	Low weight COPD	Normal weight COPD	p-value
Nitrites μmol·mg^{-1} protein	11.4±2.0	13.6±3.1	NS
Nitrates μmol·mg^{-1} protein	19.5±2.2	24.5±2.9	NS
Total μmol·mg^{-1} protein	30.9±3,5	34.3±2.8	NS
Nitrotyrosine ng·mg^{-1} protein	24.5±6.9	25.2±0.1	NS
iNOS ng·mg^{-1} protein	27.1±7.6	36.6±9.4	NS
eNOS ng·mg^{-1} protein	32.2±6.2	31.9±3.1	NS
nNOS ng·mg^{-1} protein	85.3±18.4	101.7±23.1	NS
TNF-α pg·mg^{-1} protein	201±93	267±207	NS
CD163 ng·mg^{-1} protein	6.4±0.7	6.8±2.7	NS
CD154 ng·mg^{-1} protein	14.3±5.9	17.6±8.5	NS

Data are presented as mean±SD, unless otherwise stated. iNOS: inducible isoform nitric oxide synthase; eNOS: endothelial constitutive nitric oxide synthase; nNOS: neuronal constitutive nitric oxide synthase; TNF-α: tumour necrosis factor-α; CD163: macrophages marker; CD154: T-cell marker; NS: nonsignificant.

subjects (296±98 cells·mm^{-3} *versus* 6±3 cells·mm^{-3}, respectively; $p<0.05$). A representative picture illustrating the presence of infiltrating leukocytes in COPD muscle is shown in figure 5.

The ultrastructural analysis of the muscle in all the COPD patients shows the presence of macrophages in the intercellular space. The macrophages frequently presented cytoplasmic prolongations surrounding the capillaries (fig 6).

DISCUSSION

Several studies have reported increased inflammatory markers in the skeletal muscle of patients with different conditions including COPD [6, 7, 20–23]. The results of the present study expand this observation by evaluating several inflammatory

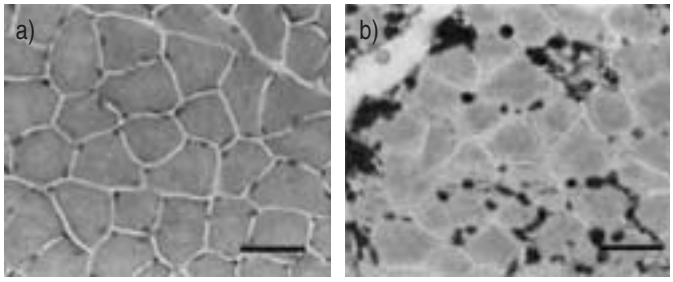

FIGURE 5. Transversal section of the vastus lateralis part of quadriceps muscle. Immunohistochemical reaction with anti-CD68, clone MB11. a) Control subject, male aged 68 yrs. The black dots are muscle fibres and endothelial cell nuclei. b) Chronic obstructive pulmonary disease patient, male aged 68 yrs. The black stain represents macrophages infiltrate. Scale bar=50 μm.

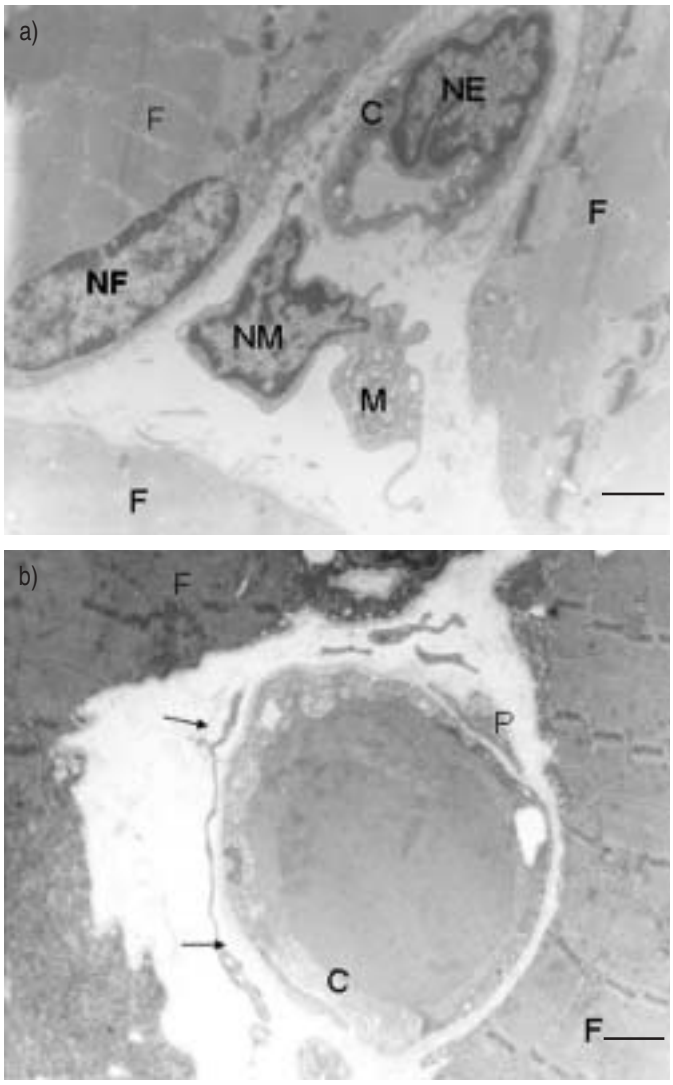

FIGURE 6. a) Oblique section of vastus lateralis part of quadriceps muscle in a 70-yr old female chronic obstructive pulmonary disease (COPD) patient. b) Longitudinal section of vastus lateralis part of quadriceps muscle in a 69-yr-old female COPD patient. Arrows show prolongations of macrophage surrounding capillary. M: macrophage; NM: nucleus of macrophage; F: muscle fibres; NF: nucleus of muscle fibre; C: capillary; NE: nucleus of capillary endothelial cell; P: pericyte. Scale bars=1 μm.

and nitrosative stress markers and the presence of inflammatory cells in the biopsy of the skeletal muscle of patients with COPD.

The current authors' observed that skeletal muscle nitrotyrosine and iNOS expression levels were markedly higher, whereas NO_2^- and NO_3^- levels were reduced in patients with COPD compared with healthy subjects. The increment of nitrotyrosine level indicates that most of the NO produced probably reacts with O_2^- to generate $ONOO^-$ rather than NO_2^- and NO_3^-. NO production kinetics by iNOS differs greatly from the production by eNOS or nNOS. Inducible NOS produces very large, toxic amounts of NO in a sustained manner, whereas constitutive NOS isoforms produce NO within seconds and its activities are direct and short acting. The pathway followed by NO depends partly on the local availability of O_2^-, O_2 and water [24]. During the course of an inflammatory response, large amounts of NO formed by iNOS surpass the physiological amounts of NO, which are usually made by nNOS or eNOS. Large amounts of inflammatory NO from myeloid cells are usually generated side by side with large amounts of O_2^-. These two can form $ONOO^-$ which mediates the cytotoxic effects of NO, and produces tyrosine nitration. There are other mechanisms in addition to iNOS expression that could eventually explain the increased level of nitrotyrosine and the decreased NO_2^- and NO_3^- levels in the skeletal muscle of these patients. In addition to the $ONOO^-$ pathway, activated leukocytes may generate nitrotyrosine by hypoclorous acid plus NO_2^-, and a chloride-independent mechanism involving myeloperoxidase, NO_2^-, and hydrogen peroxide. NO_2^- utilisation as a substrate to catalyze tyrosine nitration in proteins could eventually explain the reduced levels of NO_2^- [9, 25, 26]. The present results stress the need to better determine why the increased NO present in the skeletal muscle of COPD patients causes protein nitrotyrosination rather than generation of NO_2^- and NO_3^-. A better understanding of this phenomenon could open up avenues to intervene and modulate this potentially damaging pathway.

In the skeletal muscle biopsy sample there were muscle fibres, connective tissue, microvessels and nerve fibres. The presences of eNOS and iNOS has been described in the endothelial cells and muscle fibres, while nNOS has been found in nerve and muscle, therefore, the origin of the measured enzymes cannot be differentiated. It is possible that the eNOS detected is partly from capillary endothelium, and the lower level of eNOS found in the current study could be related to the reduced number of capillaries reported in the skeletal muscle of these patients [1]. The nNOS is originated in the muscle fibres and its reduction in the skeletal muscle of patients with COPD could be related to the abnormal fibre type distribution shown in these patients [1]. Conversely, the current authors hypothesise that iNOS could be mainly induced in the macrophages as is supported by the elevated levels of CD163 and the increased number of these cells that were detected in the muscle samples of these patients.

The present results contrast, in part, with those reported by BARREIRO *et al*. [6]. They neither detected iNOS expression in the skeletal muscle of COPD patients and controls, nor observed difference in the eNOS and nNOS levels between patients and controls. However, in agreement with those authors, elevated levels of nitrotyrosine were found in the skeletal muscle of the patients. The discrepancies observed between the current findings and those of BARREIRO *et al*. [6] could probably be explained by the different techniques used for the determination of NOS isoforms in the two studies. A quantitative method (ELISA assays) was used in the current study, whereas they used a semi-quantitative determination. Another factor that may explain the differences in the results is the severity of the airflow obstruction of the patients in both groups. They assessed patients with mild-to-moderate COPD (FEV_1 54±14% and FEV_1/FVC 61±7%), whereas the current authors included patients with more severe airflow obstruction (FEV_1 43±11% and FEV_1/FVC 46±6%). The inverse correlation that they reported between nitrotyrosine level and FEV_1 ($r=-0.65$; $p<0.05$) support the latter possibility.

AGUSTÍ *et al*. [7], also using semi-quantitative methods, reported very low iNOS expression in six healthy subjects. However, if a relation is made with the mean values of iNOS, in normal weight COPD patients and control subjects with data from that study, it is similar with the one observed in the present study subjects (patients iNOS/control iNOS; 3.4 *versus* 3.9, respectively).

Enhanced levels of TNF-α were found in the skeletal muscle of the COPD patients. This is in agreement with the findings reported by RABINOVICH *et al*. [27], who studied nine patients and six healthy controls and found that at rest, TNF-α mRNA expression was significantly higher in the COPD patients.

GOSKER *et al*. [28] used immunohistochemical techniques (CD68) for the detection of macrophages and leukocytes in the skeletal muscle of patients with COPD. Those authors did not find abnormal numbers of these inflammatory cells in the muscle of patients compared with controls subjects. In the current study, using the same antibody, it was found that the number of macrophages in the muscle tissue were markedly higher in the COPD patients compared with healthy subjects. Macrophages were also observed in all the COPD ultrastructural studied samples examined, their presence contrasted with their absence in normal subjects in which they were rarely observed. The differences observed between the present findings and those of GOSKER *et al*. [28] could be due to the increased numbers of macrophages that they found in their control subjects. The normal subjects in the present study showed a low number of macrophages, similar to those reported by MALM *et al*. [29].

To the current authors' knowledge, no previous study has evaluated the levels of CD163 (a macrophage marker) in the skeletal muscle of patients with COPD. Although the total value of CD163 was measured, there is a probability that it also reflects the soluble part of the moiety. Still, the increase in CD68 immunoreactivity supports the notion that the increase in CD163 reflects an increase in macrophages rather that soluble CD163. In addition CD154 was measured, which is a T-cell marker. Chronic T-cell activation depends upon over-expression of CD154. NO is generated by macrophages during the process of antigen presentation to T-cells and the activated T helper (Th) cell expresses co-stimulatory molecules that, together with several cytokines, induce NO production in the macrophage. One of the T cell co-stimulatory molecules is CD154 (CD40L) [30]. The combined results of the increased

CD163 and CD154 levels with the detection of increased number of macrophages in the muscle tissue are consistent with the hypothesis that an inflammatory process is probably occuring in the skeletal muscle of COPD patients, with activation of Th cells and NO macrophage production.

Recently, AGUSTÍ *et al*. [7] reported that NF-κB activation and iNOS expression occur in the skeletal muscle of COPD patients with low body weight. The authors speculated that these changes might contribute to the molecular pathogenesis of weight loss in these patients. The results of the present study contrast with those reported by these authors, since similar levels of NO_2^-, NO_3^-, nitrotyrosine, iNOS, nNOS, eNOS, TNF-α, CD154 and CD163 were observed in the skeletal muscle of normal and low body weight patients. The differences observed between the current findings and those of AGUSTÍ *et al*. [7] could partly be explained by the difference in severity of airflow obstruction of the patients in both studies. Both groups of patients in the current study had similar values of FEV1. This was 45±12% in patients with low BMI compared with 42±7% in patients with normal BMI. In contrast the FEV1 for the low BMI group in the study by AGUSTÍ *et al*. [7] was significantly lower (28±2%) compared with that of the patients with normal BMI (39±2%).

Finally, a limitation of the present study deserves discussion. Due to the invasive nature of procedure used to obtain the skeletal muscle samples, a relatively small amount of subjects was included. This probably limits the direct extrapolation of the present findings to the entire population of COPD patients. However, 15 patients with COPD and 14 normal subjects comprise the largest number ever reported using peripheral muscle biopsy for the assessment of inflammatory markers.

In summary, the present study supports the presence of local inflammation in the skeletal muscle of patients with chronic obstructive pulmonary disease. There are increased levels of the cytokines tumour necrosis factor-α, CD163 and CD154 all suggesting the presence of macrophages and activated T-cells, respectively. The concentrations of the enzymes endothelial constitutive nitric oxide synthase and neuronal constitutive nitric oxide synthase, as well as the nitric oxide end products (nitrite and nitrate) were reduced. Increased expression of inducible isoform nitric oxide synthase and nitrotyrosine was also observed, indicating that most of the nitric oxide produced results in nitrotyrosine, probably with the contribution of macrophage inducible isoform nitric oxide synthase expression. Finally, no differences in all of these markers was found between normal and low body weight chronic obstructive pulmonary disease patients that could eventually aid in the explanation of weight loss and its possible relation with skeletal muscle inflammation.

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