

**OXIDIZED PROTEINS AND SUPEROXIDE ANION PRODUCTION IN THE
DIAPHRAGM OF SEVERE COPD PATIENTS**

Corresponding author: Dr. Esther Barreiro, Muscle and Respiratory System Research Unit, IMIM-Hospital del Mar, PRBB, Dr. Aiguader, 88, E-08003 Barcelona, Spain, Telephone: +34 93 316 0385, Fax: +34 93 316 0410, e-mail: ebarreiro@imim.es

Requests for reprints: Dr. Esther Barreiro

Judith Marin-Corral¹ MD, Joan Minguella¹ MD, PhD, Alba Lucía Ramírez-Sarmiento¹ PhD, Sabah N.A. Hussain² MD, PhD, Joaquim Gea¹ MD, PhD, and Esther Barreiro¹, MD, PhD

¹ Muscle Research and Respiratory System Unit, Respiratory Medicine and Thoracic Surgery Departments, IMIM-Hospital del Mar, *Centro de Investigación en Red de Enfermedades Respiratorias (CibeRes)*, Health and Experimental Sciences Department (CEXS), *Universitat Pompeu Fabra*, and *Universitat Autònoma de Barcelona*, PRBB, Dr. Aiguader, 88, Barcelona, Catalonia, E-08003 Spain.

² Critical Care and Respiratory Divisions, Royal Victoria Hospital and Meakins-Christie Laboratories, McGill University, 687, Pine Avenue west, Montreal, Quebec, H3A 1A1 Canada.

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ABSTRACT

In the diaphragms of COPD patients, the nature of the oxidatively modified proteins and superoxide anion production were explored.

Methods: Diaphragm specimens were obtained through thoracotomy because of localized lung lesions in COPD patients (sixteen severe and eight moderate) and ten control subjects. Lung and respiratory muscle functions were evaluated. Oxidized proteins were identified using immunoblotting and mass spectrometry. Protein and activity levels of the identified proteins were determined using immunoblotting and activity assays. Lucigenin-derived chemiluminescence signals in a luminometer were used to determine superoxide anion levels in muscle compartments (mitochondria, membrane, and cytosol) using selective inhibitors.

Results: In severe COPD patients compared to controls: respiratory muscle function was impaired; creatine kinase, carbonic anhydrase III, actin and myosin were oxidized; myosin carbonylation levels were increased five-fold; creatine kinase content and activity and myosin protein were reduced; superoxide anion levels were increased in both mitochondria and membrane compartments; and the percentage of superoxide anion inhibition achieved by rotenone was significantly greater.

Conclusions: In severe COPD patients, oxidation of diaphragm proteins involved in energy production and contractile performance is likely to partially contribute to the documented respiratory muscle dysfunction. Furthermore, generation of superoxide anion was increased in the diaphragms of these patients.

Word count: 200

KEY WORDS: COPD, muscle compartments, oxidized proteins, respiratory muscles, superoxide anion.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a highly prevalent condition which imposes a substantial burden in terms of disability and impairment of quality of life [1]. COPD is also a leading cause of death worldwide, projected to increase within the next decade, since the prevalence of cigarette smoking continues to rise and the population ages [2, 3]. Poor exercise tolerance and reduced quality of life partially owing to muscle dysfunction are major complaints in patients with COPD.

Several reports have shown that in severe COPD, the main inspiratory muscle, the diaphragm, undergoes adaptive modifications that probably render the overloaded muscle more resistant to fatigue [4-8]. However, there is growing evidence that *in vitro* fiber contractile function is also impaired in the diaphragms of patients with COPD [9], even at early stages of their disease, as was shown by reduced myosin heavy chain (MyHC) content and increased protein degradation via the ubiquitin-proteasome pathway [10, 11]. Oxidative stress, among other factors, was also suggested as a contributor to this process of muscle dysfunction and wasting [12-14], although its specific role is yet to be fully elucidated. The oxidative posttranslational modifications of proteins may cause loss of protein function as a result of oxidation of residues in the protein active site. In other cases, oxidation of critical residues may lead to increased proteolytic degradation of the modified proteins [15]. Indeed, it has also been suggested that reactive oxygen species (ROS) cascade regulates pathophysiological signaling, leading to proteolysis and apoptosis when synthesized at high levels within the myofibers [16].

In a previous study [14], we showed that diaphragm oxidative stress could be a contributor to the intrinsic respiratory muscle dysfunction of severe COPD patients. In that study, however, neither the nature of the oxidatively modified proteins nor the generation of ROS within the diaphragm fibers of COPD patients were explored. On this basis, it is now

hypothesized that excessive ROS production in the diaphragm muscles of severe COPD patients, chronically exposed to inspiratory overloads, would target specific proteins involved in muscle contractile function, thereby contributing to the recognized *in vivo* ventilatory muscle dysfunction. Accordingly, our objectives were first to identify the nature of the oxidatively modified proteins (protein carbonylation) in the diaphragms of patients with both severe and moderate COPD and in control subjects, and second to explore the generation of superoxide anion within several muscle compartments in the diaphragms of the same patients and control individuals.

METHODS

Subjects

(See the online supplement for additional information).

Twenty-four Caucasian male patients with stable COPD (eight moderate and sixteen severe patients) [17] and ten healthy male age-matched sedentary controls were recruited on an out-patient basis. Moderate and severe COPD were defined in accordance with the Global Initiative for Obstructive Lung Disease (GOLD) guidelines [17]. All subjects underwent thoracotomy for a localized lung neoplasm. All patients were clinically stable at the time of the study. Eight COPD patients (three moderate and five severe) also participated in a former study aimed at assessing the associations between oxidative stress and respiratory muscle dysfunction in COPD [14]. The current investigation was designed in accordance with both the ethical standards on human experimentation in our institution and the World Medical Association guidelines for research on human beings. The Ethics Committee on Human Investigation at *IMIM-Hospital del Mar* approved all experiments. Informed written consent was obtained from all individuals.

Nutritional and Functional Assessment

Nutritional evaluation included body mass index and analytical parameters. Both pulmonary and respiratory muscle functions were evaluated.

Biopsies

During thoracotomy, because of localized lung lesions, diaphragm biopsy specimens were obtained from the anterior costal diaphragm lateral to the insertion of the phrenic nerve [5, 14].

Biological Muscle Studies

Immunoblotting of 1-D electrophoresis. The levels of oxidative and nitrosative stress and those of muscle creatine kinase, carbonic anhydrase III, alpha-actin, and MyHC were assessed as described elsewhere [13, 14, 18].

Identification of carbonylated proteins: 1-D and 2-D electrophoresis and mass spectrometry. Oxidized proteins (protein carbonylation) were separated and identified in the diaphragms of COPD patients and control subjects as previously described [11, 13, 18].

Creatine kinase activity assay. Total muscle creatine kinase activity was measured with a commercial kit (Diagnostic Chemicals Ltd., PEI, Canada) as described elsewhere [13, 18].

Muscle fiber counts and morphometry. Proportions of type I, type II, and hybrid fibers were determined as previously described [14]. The size of type I and type II fibers was also measured.

Measurements of superoxide anion production by lucigenin-derived chemiluminescence. Lucigenin-derived chemiluminescence signals were determined in all muscle samples using a luminometer as formerly described [19, 20].

Statistical Analysis

Data are presented as mean (SD). One-way analysis of variance (ANOVA) together with Tukey's test to adjust for multiple comparisons was employed in order to compare variable results among the three groups. Pearson's correlation coefficient was employed to assess relationships among different variables within COPD patients. A *P* value ≤ 0.05 was considered significant.

RESULTS

Characteristics of the Study Subjects

Table 1 indicates the main characteristics of the study subjects. No significant differences in age or nutritional status, as assessed by body mass index and analytical

parameters, were observed between control subjects and either severe or moderate COPD patients. However, forced expiratory volume in one second (FEV₁), forced vital capacity, the ratio of FEV₁ to forced vital capacity, diffusion capacity, and arterial oxygen partial pressure were significantly lower, while residual volume and the ratio of residual volume to total lung capacity were significantly higher in the severe COPD patients. Global respiratory muscle strength, as measured by maximal inspiratory pressure (MIP), and maximal transdiaphragmatic pressure (Pdi_{max}) were moderately reduced in severe COPD patients.

Table 1. Anthropometric characteristics and functional status of the study groups

| | Control subjects n=10 Mean (SD) | Patients with moderate COPD, n=8 Mean (SD) | Patients with severe COPD, n=16 Mean (SD) |
|--|--|---|--|
| Age, yrs, | 64 (8) | 66 (10) | 67 (4) |
| BMI, Kg /m ² | 25.8 (3.2) | 23.2 (3.4) | 26.5 (4.6) |
| FEV ₁ , % pred | 88 (8) | 62 (4) *** | 42 (6) ***, ††† |
| FVC, % pred | 91 (12) | 71 (7) *** | 59 (9) ***, ††† |
| FEV ₁ /FVC | 74 (5) | 63 (5)** | 53 (8) ***, ††† |
| RV, % | 101 (26) | 119 (25) | 163 (51) **, † |
| TLC, % pred | 93 (9) | 93 (6) | 101 (17) |
| RV/TLC | 40 (6) | 47 (6)* | 60 (11) ***, †† |
| FRC, % pred | 97 (8) | 95 (13) | 125 (36)*, † |
| DLco, % pred | 84 (12) | 69 (9)* | 65 (8)* |
| KCO, % pred | 89 (13) | 77 (12) | 68 (11)* |
| PaO ₂ , kPa | 12.1 (0.9) | 10.6 (1.0) ** | 9 (0.8) ***, †† |
| PaCO ₂ , kPa | 5.2 (0.5) | 5.2 (0.5) | 5.7 (0.5)*, † |
| MIP, % pred | 87 (7) | 83 (9) | 62 (13) *, †† |
| PeS _{max} , cmH ₂ O | N.A. | -80 (34) | -69 (26) |
| Pdi _{max} , cmH ₂ O | N.A. | 108 (21) | 90 (19) † |
| Type I fibers, % | 50.5 (7.0) | 52.0 (7.1) | 62.5 (7.8) **, †† |
| Type II fibers, % | 47.8 (6.8) | 46.6 (7.0) | 32.4 (7.1) ***, ††† |
| Hybrid fibers, % | 1.7 (0.6) | 1.4 (0.4) | 5.1 (2.3) ***, ††† |
| Cross sectional area, type I fibers, μm ² | 2155 (594) | 2242 (509) | 2105 (685) |
| Cross sectional area, type II fibers, μm ² | 2166 (1021) | 2112 (325) | 2384 (850) |

Data are presented as mean (SD).

Definition of abbreviations: SD, standard deviation; BMI, body mass index; FEV₁, forced expiratory volume in one second; pred, predicted; FVC, forced vital capacity; RV, residual volume; TLC, total lung capacity; DLco, carbon monoxide transfer; KCO,

Krogh transfer factor; PaO₂, arterial oxygen partial pressure; PaCO₂, arterial carbon dioxide partial pressure; MIP, maximal inspiratory pressure, Pes_{max}, maximal esophageal pressure; Pdi_{max}, maximal transdiaphragmatic pressure; NA, not available.

Statistical significance of the results is expressed as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, between either severe or moderate COPD patients and controls; † $p \leq 0.05$, †† $p \leq 0.01$, ††† $p \leq 0.001$, between severe and moderate COPD patients.

Muscle Structure

In the diaphragms of severe COPD patients, proportions of both type I and hybrid fibers were higher, while those of type II fibers were lower than in the muscles of either moderate COPD or control subjects (Table 1). When considering all COPD patients as a group, proportions of type I fibers negatively correlated with FEV₁ ($r=-0.499$, $p=0.042$).

Identification of carbonylated proteins

Total carbonyl group formation. As illustrated in Figure 1A, anti-DNP antibody detected different positive protein bands, with apparent masses ranging from 67 to 29 kDa, in the muscles of both patients and controls. The diaphragms of severe COPD patients showed higher levels of total carbonyl content than those of the controls or moderate patients (Figure 1B).

Carbonylated proteins. The identification of the different carbonylated proteins revealed that muscle creatine kinase, muscle carbonic anhydrase III, alpha-1 sarcomeric actin, and MyHC were specifically oxidized in the diaphragms of both groups of patients and control subjects (Table 2, Figure 1C, Figure 1D). Carbonyl group formation in MyHC protein was significantly greater in the diaphragms of the severe COPD patients compared to either moderate COPD or control subjects (Figure 1D). Indeed, there was a five-fold increase in MyHC carbonylation in the diaphragms of the severe patients compared to the controls (Figure 1D). Albumin (\cong 58-67 kDa) was also consistently oxidized in all the diaphragm specimens (arrows in Figure 1C).

Table 2. Identified carbonylated proteins in the diaphragms of COPD patients (moderate and severe) and control subjects

| Study Subjects | Identified Carbonylated Proteins | Accession No. | Mass | MASCOT score | Peptide Matched |
|-----------------------|---|----------------------|-------------|---------------------|------------------------|
| Ctl and COPD | Muscle creatine kinase | gi/21536288 | 43,302 | 207 | 38 |
| Ctl and COPD | Carbonic anhydrase III | gi/224979 | 29,707 | 182 | 50 |
| Ctl and COPD | Alpha-1 actin precursor | gi/4501881 | 42,366 | 117 | 29 |
| Ctl and COPD | Myosin, heavy polypeptide 7 | gi/148342499 | 223,684 | 195 | 34 |

Definition of abbreviations: Ctl, control; COPD, chronic obstructive pulmonary disease.

Expression and activity of identified carbonylated proteins

Muscle creatine kinase content was significantly lower in the diaphragms of severe COPD patients compared to either moderate patients or control subjects (Figures 2A and 2B). Protein levels of carbonic anhydrase-III and actin were not significantly different between COPD patients and controls (Figures 2A and 2B). Moreover, creatine kinase activity was significantly reduced in the diaphragms of both severe and moderate COPD patients compared to controls (Figure 2C). Importantly, MyHC content was significantly reduced in the diaphragms of the severe COPD patients compared to either moderate COPD or control subjects (Figure 2D). In fact, in the severe patients, diaphragm MyHC levels were 32% of those detected in the controls.

Protein tyrosine nitration

Several nitrated proteins were identified with apparent masses ranging from 64 to 30 kDa (Figure 3A). Levels of diaphragm protein nitration did not differ among the study groups (Figure 3B).

Diaphragm superoxide anion production

Diaphragm levels of superoxide anion were significantly higher in the mitochondrial fraction of severe COPD patients compared to those in the controls (Figure 4A, top panel). Likewise, muscle superoxide anion levels were greater in the membrane fraction of severe COPD patients compared to either moderate COPD or controls (Figure 4A, middle panel).

However, diaphragm superoxide anion production from the cytosolic fraction did not significantly differ between COPD patients and control subjects (Figure 4A, bottom panel).

Interestingly, the percentage of superoxide anion inhibition elicited by rotenone (respiratory chain inhibitor) was significantly greater in the diaphragms of both severe and moderate COPD patients compared to controls (Figure 4B, top panel). Conversely, the percentage of superoxide anion inhibition elicited by the inhibitors apocynin (specific nicotinamide adenine dinucleotide phosphate hydrogen [NADPH] oxidase inhibitor) and oxypurinol (inhibitor of xanthine oxidase), respectively, did not differ between COPD patients and controls (Figure 4B, middle and bottom panels, respectively).

DISCUSSION

The novel findings in this study are that in the diaphragms of severe COPD patients compared to control subjects: 1) total muscle protein carbonylation rather than protein nitration was increased, 2) muscle creatine kinase, carbonic anhydrase III, actin, and myosin were oxidized, 3) carbonylation levels in MyHC were five-fold greater, 4) creatine kinase content and activity were reduced and MyHC levels were 32% of those in the controls, 5) superoxide anion levels were increased in the mitochondria and membrane compartments, and 6) the percentage of superoxide anion inhibition achieved by the mitochondrial inhibitor rotenone was significantly greater.

Respiratory muscle dysfunction and structure

In the current investigation, the severe COPD patients exhibited a moderate reduction in respiratory muscle strength, as measured by Pdi_{max} and MIP. Although this level of respiratory muscle dysfunction may not be clinically relevant at rest, it could have significant clinical implications under certain circumstances such as during an exacerbation or physical exercise. Importantly, our observations were consistent with previous reports, where *in vivo*

respiratory muscle and diaphragm strength were also shown to be compromised [14, 21, 22]. In keeping with, it has recently been proposed [23] that in severe COPD, several molecular mechanisms contribute to the etiology of this respiratory muscle dysfunction such as myosin loss [10, 11], sarcomeric injury [24], oxidative stress [14], and cross-bridge cycling kinetics alterations [8], resulting in reduced diaphragm isometric force. This raises serious questions concerning whether the adaptive mechanisms encountered in the diaphragms of the severe COPD patients [4-8] are sufficient to make this muscle more efficient, since impaired contractile properties of the diaphragm muscle fibers have been clearly demonstrated [9-11].

In the present study, the diaphragm fiber type shift towards a fatigue-resistant phenotype exhibited by the severe COPD patients is in agreement with previous investigations [4, 14]. Moreover, we provide evidence of the proportions of hybrid fibers in the human diaphragm. The significant increase in hybrid fiber proportions observed in the diaphragms of the severe patients is likely to be another adaptive mechanism to their respiratory overloads. Interestingly, the size of the diaphragm fibers did not differ between patients and controls. In fact, discrepancies exist in the literature regarding diaphragm fiber cross sectional areas in COPD patients. While in several studies [4, 6, 25], the size of the diaphragm fibers was reduced in these patients, no significant differences were found between patients and controls in other investigations [5, 24, 26]. The number of patients examined, which was greater in the latter studies and in the current one, the methodologies employed in each study, such as the use of frozen or paraffin-embedded tissues, or the identification of the muscle fiber types using histochemical or immunohistochemical procedures, and the absence in our patients of nutritional abnormalities, which have been shown to influence diaphragm fiber sizes [25, 27], could account for such discrepancies.

Diaphragm carbonylated proteins

Over the last decade a growing body of evidence has shown that oxidative stress is one of the mechanisms clearly involved in the skeletal muscle dysfunction of COPD patients [12-14]. Carbonyl formation is an important detectable marker of protein oxidation in tissues. The present study is the first to report that carbonylation of diaphragm proteins in severe COPD and in control subjects includes proteins involved in key muscle cellular processes such as ATP metabolism (creatine kinase), hydration of carbon dioxide (carbonic anhydrase-III), and contractile function (actin and MyHC).

Diaphragm carbonic anhydrase-III content was not different in COPD patients from that in the controls. This is in keeping with a previous study [13], in which quadriceps carbonic anhydrase levels did not differ between COPD patients and controls. Creatine kinase activity, however, was reduced in the diaphragms of both moderate and severe COPD patients. Indeed, this is consistent with previous reports, where creatine kinase was shown to be a major target for the *in vitro* and *in vivo* exposure of ROS, resulting in enzyme inactivation [28, 29]. In our study, although patients with moderate COPD did not exhibit a significant reduction in diaphragm creatine kinase content, it could be hypothesized that moderate levels of ROS in their muscles may have already influenced enzyme activity, since their levels were indeed similar to those of the severe patients. Moreover, it has been well established that slow-twitch oxidative fibers exhibit the lowest creatine kinase activity in skeletal muscles [30]. On this basis, it is possible to conclude that the decreased creatine kinase activity in the diaphragms of our severe COPD patients could be partly due to their fiber-type shift towards a more fatigue-resistant phenotype. Eventually, although in the current investigation, it is not possible to define the precise contribution of reduced creatine kinase activity to respiratory muscle dysfunction in severe COPD patients, it is likely to play a

role, since absence of creatine kinase activity in mice led to profound reductions in exercise performance [31] and to myocardial dysfunction [32].

In contrast to the current findings, in a previous study [7], total creatine kinase activity levels in the diaphragms of severe COPD patients were shown not to differ from those in the control muscles. Discrepancies between the two studies could be explained by the different methodologies employed to determine creatine kinase activity and by the fact that measurements were only conducted in saponin-skinned fibers in the previous study [7]. In line with the current findings, the quadriceps of severe COPD patients also exhibited a significant reduction in creatine kinase content and activity both at rest [13] and after exercise training [33]. However, creatine kinase activity in the deltoid [34] and external intercostal muscles [35] did not differ between severe-to-moderate COPD patients and control subjects.

Several studies have already demonstrated oxidation of the cytoskeletal protein actin in different models [36, 37]. The complex modifications induced by ROS on muscle actin are characterized by severe disruption of the actin filaments, hampering their interaction with the myosin protein [38], thus suggesting that oxidation of contractile actin may contribute to muscle dysfunction in these models [36, 38]. In our study, although muscle actin levels did not differ between COPD patients and controls, disruption of the contractile actin in the diaphragms of the severe patients cannot be ruled out.

Importantly, in line with the observations of Otthenheijm *et al* [10, 11], the diaphragm of our severe COPD patients exhibited significantly lower levels (32%) of MyHC protein than the controls. The novel finding about our study is that in the diaphragms of the severe patients, carbonylation levels of MyHC protein were five times greater than in the control muscles. Although increased MyHC carbonylation and reduced MyHC content in the diaphragms of our severe patients cannot be causally related, it is likely that the greater oxidative damage exhibited by MyHC protein in these muscles may have contributed to its

higher protein breakdown [10, 11]. In fact, activity of the ubiquitin-proteasome pathway was shown to be increased in patients with mild-to-moderate COPD [11] and inflammatory cytokines and ROS are well-known triggers of muscle proteolysis [16]. Taken together, these findings suggest that in the diaphragms of severe COPD patients, highly carbonylated MyHC protein is prone to be further degraded by the ubiquitin-proteasome pathway. Clearly, a future study should be designed in order to explore the exact contribution of oxidants to increased muscle protein breakdown and myosin loss in the diaphragms of severe COPD patients.

Diaphragm superoxide anion formation

The current investigation is the first to report *in vitro* measurements of the superoxide anion being produced within distinct muscle fractions in the human diaphragm. Interestingly, superoxide anions are the initial substrates of a series of reactions resulting in the formation of more powerful ROS in tissues. In the present study, *in vitro* generation of superoxide anion by the mitochondrial and membrane compartments was significantly increased in the diaphragms of severe COPD patients compared to controls. Another important finding in this study is that rotenone, a selective mitochondrial respiratory chain inhibitor, elicited significantly greater inhibitory effects of ROS generation in the diaphragms of the patients. Importantly, in exercising muscles, oxygen utilization increases by several folds, eventually resulting in leakage of superoxide anions from the mitochondria to other muscle structures. In patients with severe COPD, the main inspiratory muscle, the diaphragm, must incessantly generate muscle contractions of relatively high intensity in order to overcome the increased inspiratory loads imposed by the mechanical alterations of their thoracic cage. This may result in the continuous generation of abnormally high levels of superoxide anion within the diaphragm mitochondria, which would not possibly revert to “control” levels in these patients.

In view of the significant increase in superoxide anion generation in the membrane compartment of diaphragms in the severe COPD patients, it may be suggested that muscle NADPH oxidase enzyme complex was a likely contributor to superoxide anion generation in these muscles, as was previously shown in septic muscles [20]. In contrast to the mitochondrial and membrane compartments, the amount of superoxide anion produced in the cytosolic fraction was relatively low in the diaphragms of both patients and controls. Furthermore, oxypurinol had no major effects on ROS generation within the diaphragm cytosolic compartment in any of the study groups, suggesting that xanthine oxidase is probably not involved in the generation of superoxide anion in the human diaphragm.

Study limitations

Although lung volume reduction surgery also makes it possible to obtain diaphragm specimens, only very severe COPD patients undergo this type of surgery. Therefore, diagnostic-therapeutic thoracotomy is the only approach available for studying moderate-to-mild COPD and normal lung function subjects. Accordingly, the population of our study shares a common morbidity: the presence of a localized lung neoplasm. Nevertheless, we do not believe that this condition has made any significant contribution to the development of oxidative stress in these diaphragms, since extremely restrictive criteria were employed to select our population. Another limitation encountered in our study is directly related to the methodologies involved in the *in vitro* measurements of muscle superoxide anion, which have been challenged on the basis of the potential production of superoxide anion by the redox recycling of lucigenin. However, as previously demonstrated [20], we do not believe that lucigenin redox recycling has made any substantial contribution to the superoxide anion concentrations detected in our study.

In the present study, both patients and controls were recruited over several years. In order to avoid unnecessary methodological concerns due to analyses conducted sequentially

rather than simultaneously, all muscle specimens were always stored frozen at -80°C until further use in the laboratory. Although we acknowledge that freezing and fractionation of the muscle samples might have somehow influenced the superoxide anion measurements, we do not believe that this has had any substantial effects in our study for the following reasons: 1) all superoxide anion analyses were conducted simultaneously for both patients and controls under identical laboratory conditions by the same investigator; 2) a fragment of the muscle specimens was always kept frozen in our -80°C freezer until muscle fractions were obtained and superoxide anion experiments were conducted; 3) fractionation of the muscle samples using specific buffers that do not destroy the different cell compartments was already demonstrated to yield reliable lucigenin-derived chemiluminescence signals [20].

(See the online supplement for additional information).

Conclusions

Our study is the first to provide evidence that in severe COPD patients, oxidation of diaphragm proteins involved in energy production and contractile performance is likely to partially contribute to the documented respiratory muscle dysfunction. Furthermore, generation of superoxide anion was increased in the diaphragms of these patients.

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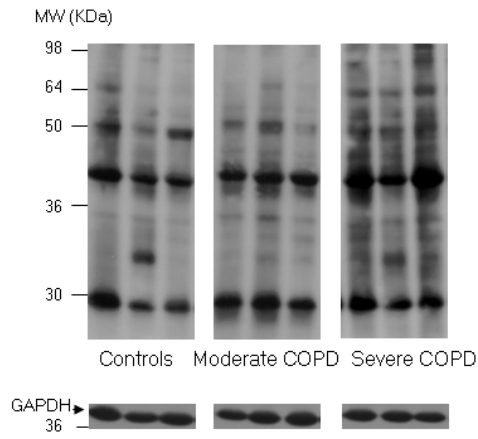
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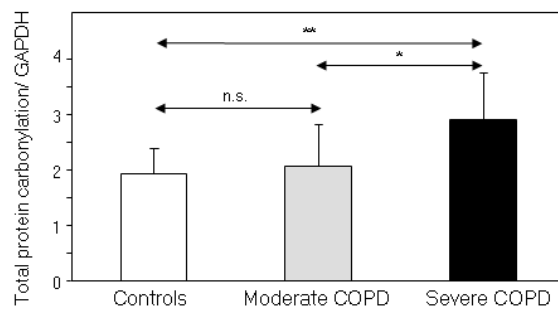
FIGURE LEGENDS

Figure 1:

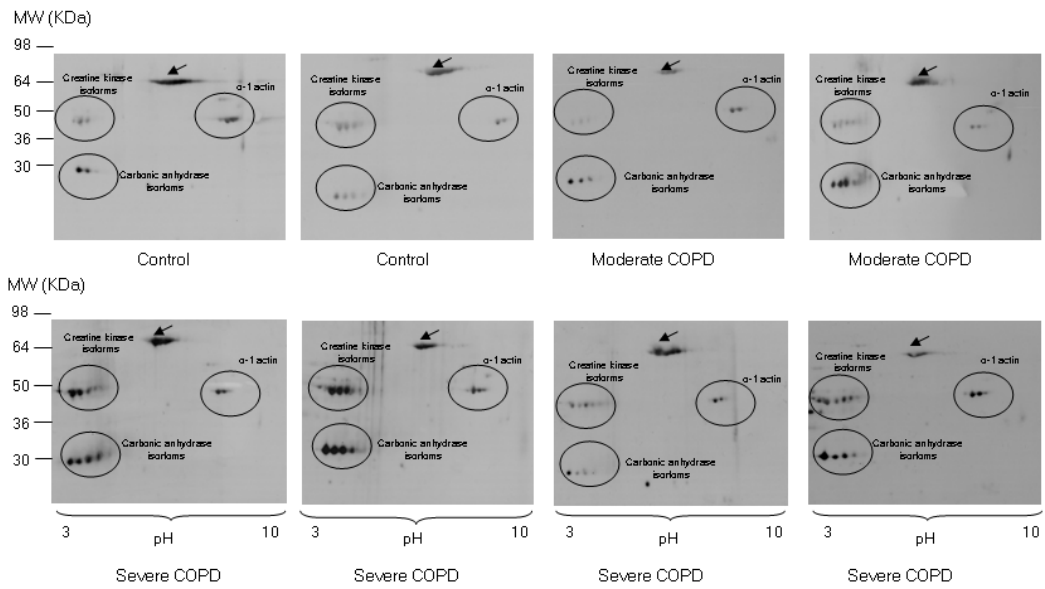
J. Marin-Corral et al. Figure 1 A



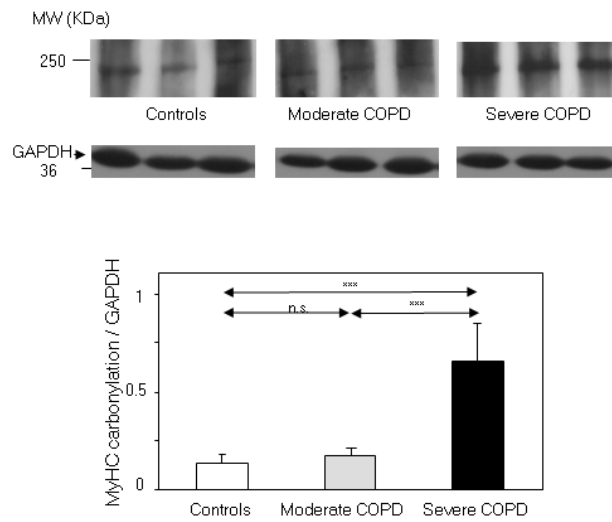
J. Marin-Corral et al. Figure 1 B



J. Marin-Corral et al. Figure 1 C



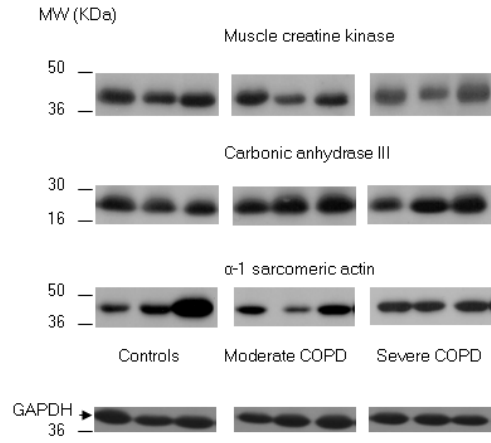
J. Marin-Corral et al. Figure 1 D



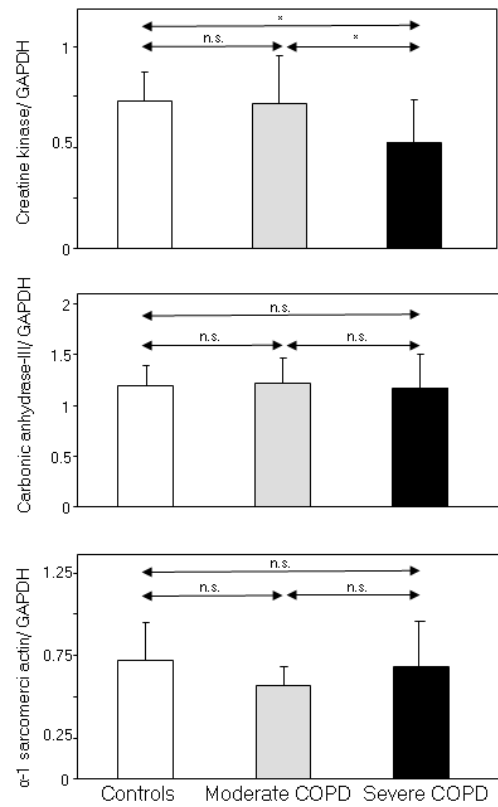
- A) Representative examples of protein oxidation (total carbonyl groups) in diaphragms of control subjects (n=10) and both moderate (n=8) and severe COPD (n=16) patients. Several protein carbonylated bands were detected. Monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) HRP conjugated antibody was used to control equal loading among various lanes.
- B) Mean values (SD) of total carbonyl formation were higher in the severe COPD (n=16) patients compared to either control subjects (n=10) (**: $p \leq 0.01$) or moderate patients (*: $p \leq 0.05$). No significant differences were found in diaphragm protein carbonylation levels between moderate COPD patients and controls (ns: non significant).
- C) Representative 2D immunoblots corresponding to the detection of carbonylated proteins in crude muscle homogenates of diaphragm muscles of two control subjects and two patients with moderate COPD (two left and two right top figures, respectively) and four patients with severe COPD (bottom figures). Muscle creatine kinase, carbonic anhydrase III, and alpha-actin were consistently oxidized in the diaphragms of both patients and controls. Albumin was also oxidized in both patients and controls (arrow in each panel).
- D) Representative examples of carbonylated MyHC protein in diaphragms of control subjects (n=9) and both moderate (n=8) and severe COPD (n=9) patients. Monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) HRP conjugated antibody was used to control equal loading among various lanes. Levels of carbonylated MyHC were greater in the diaphragms of the severe COPD patients compared to either controls or moderate patients (***: $p \leq 0.001$). Carbonylation levels of MyHC did not differ between moderate patients and the controls (ns: non significant).

Figure 2:

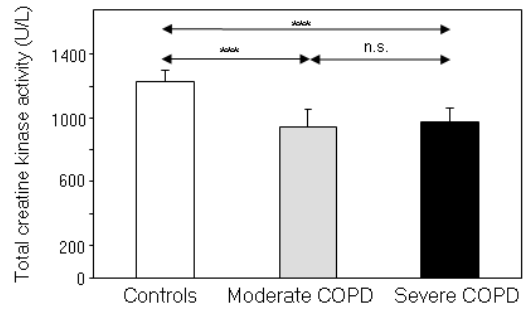
J. Marin-Corral et al. Figure 2 A



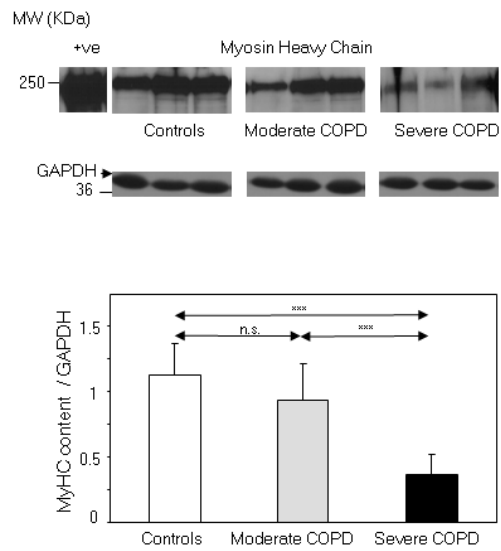
J. Marin-Corral et al. Figure 2 B



J. Marin-Corral et al. Figure 2 C



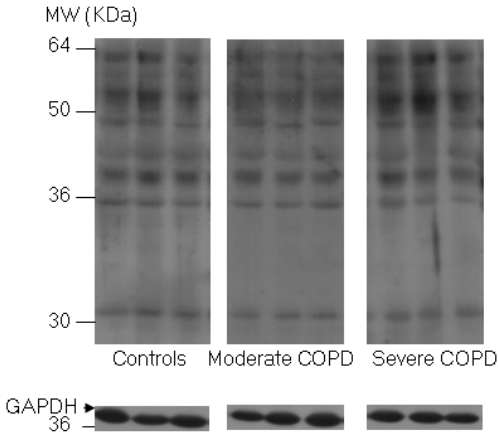
J. Marin-Corral et al. Figure 2 D



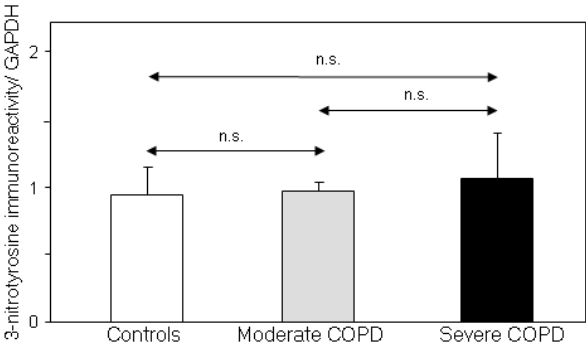
- A) Representative examples of protein expression of muscle creatine kinase, carbonic anhydrase III, and alpha-1 sarcomeric actin in the diaphragms of control subjects (n=10) and both moderate (n=8) and severe COPD patients (n=16). Monoclonal anti-GAPDH HRP conjugated antibody was used to control equal loading among various lanes.
- B) Mean (SD) values of creatine kinase, carbonic anhydrase III, and alpha-1 sarcomeric actin protein content in diaphragm muscles of control subjects (n=10) and both moderate (n=8) and severe COPD patients (n=16). Note that creatine kinase content was significantly reduced in the diaphragms of the severe COPD patients compared to protein levels in the moderate patients (*: $p \leq 0.05$) and controls (*: $p \leq 0.05$). No significant differences were found in carbonic anhydrase III or alpha-1 sarcomeric actin among the three study groups (ns: non significant).
- C) Mean (SD) values of creatine kinase activity (U/L) in diaphragm muscles of control subjects (N=10) and both moderate (n=8) and severe COPD patients (n=16). The enzyme activity of creatine kinase was significantly reduced in the diaphragms of the COPD patients, both moderate and severe, compared to activity levels in the control subjects (***: $p \leq 0.001$).
- D) Representative examples of MyHC protein content in the diaphragms of control subjects (n=9) and both moderate (n=8) and severe COPD patients (n=9). Monoclonal anti-GAPDH HRP conjugated antibody was used to control equal loading among various lanes. MyHC protein content was lower in the diaphragms of the severe COPD patients compared to either controls or moderate patients (***: $p \leq 0.001$). MyHC content did not differ between moderate patients and the controls (ns: non significant).

Figure 3:

J. Marin-Corral et al. Figure 3 A



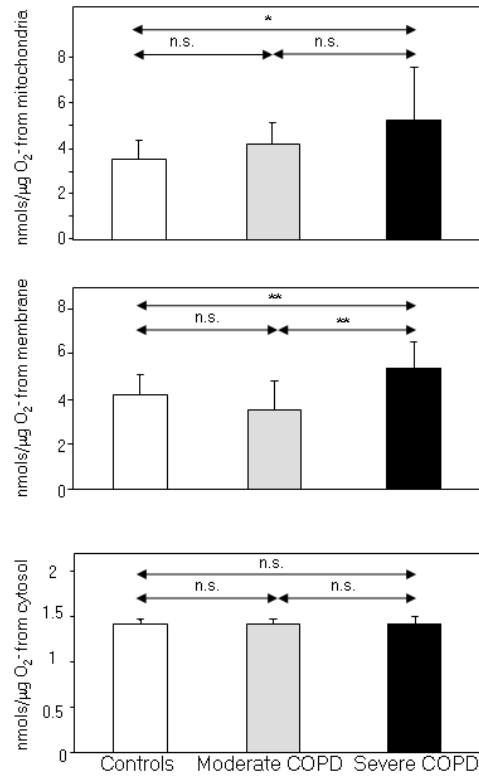
J. Marin-Corral et al. Figure 3 B



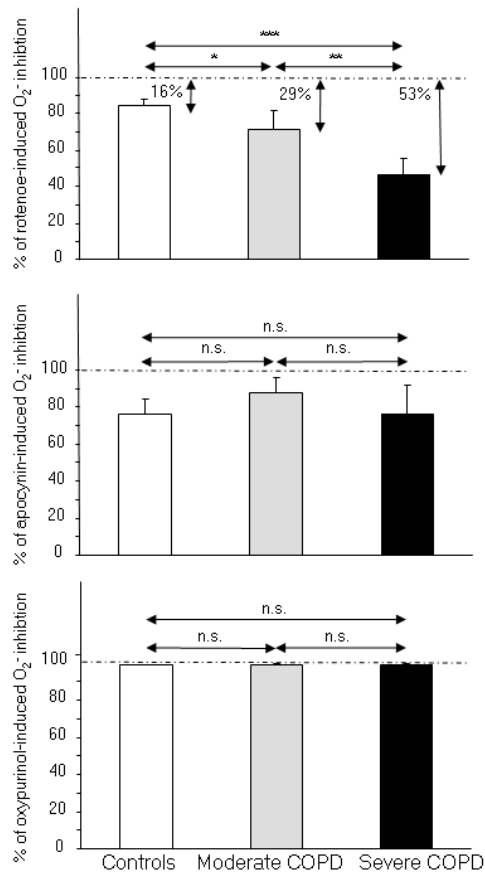
- A) Representative examples of protein tyrosine nitration in diaphragms of control subjects (n=10) and both moderate (n=8) and severe COPD (n=16) patients. Several nitrated protein bands were detected. Monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) HRP conjugated antibody was used to control equal loading among various lanes.
- B) Levels of diaphragm protein tyrosine nitration did not differ among the study groups (ns: non significant).

Figure 4:

J. Marin-Corral et al. Figure 4 A



J. Marin-Corral et al. Figure 4 B



- A) Mean (SD) values of superoxide anion production (nmol/ μ g) in the mitochondrial, membrane, and cytosolic compartments of control subjects (n=10), moderate (n=8) and severe COPD patients (n=16). Production of superoxide anion was significantly increased in the diaphragm mitochondrial fraction (top panel) of severe COPD patients compared with control subjects (*: $p \leq 0.05$) but not with moderate COPD patients (ns: non significant). Production of superoxide anion was also significantly increased in the diaphragm membrane fraction (middle panel) of severe COPD patients compared to either moderate COPD (**: $p \leq 0.01$) or control subjects (**: $p \leq 0.01$). No significant differences were observed in the production of diaphragm cytosolic superoxide anion between COPD patients and controls (ns: non significant).
- B) Mean (SD) values of the percentage of superoxide anion inhibition induced by selective enzyme inhibitors such as rotenone, apocynin, and oxypurinol. The percentage of superoxide anion inhibited by the mitochondrial respiratory chain inhibitor rotenone (top panel) was significantly greater in the diaphragms of the severe COPD patients (n=16, 53%) compared to either controls (n=10, 16%, ***: $p \leq 0.001$) or moderate patients (n=8, 29%, **: $p \leq 0.01$). Interestingly, the percentage of superoxide anion inhibited by rotenone was also significantly greater in the diaphragms of the moderate patients compared to the controls (*: $p \leq 0.05$). The percentage of superoxide anion inhibited by the NADPH oxidase inhibitor apocynin (middle panel) did not significantly differ between COPD patients and controls (ns: non significant). Likewise, the percentage of superoxide anion inhibited by the xanthine oxidase inhibitor oxypurinol (bottom panel) was not significantly different in the diaphragms of COPD patients compared to controls (ns: non significant).