

## Reduced Expression of the Sarcoplasmic Calcium Pump SERCA2 in Skeletal Muscle From Patients With Chronic Obstructive Pulmonary Disease and Low Body Weight

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**OBJECTIVE:** To compare the concentrations and extent of nitration of sarcoplasmic-endoplasmic reticulum Ca<sup>2+</sup> adenosine triphosphatase 2 (SERCA2) in biopsies of the quadriceps femoris from patients with chronic obstructive pulmonary disease (COPD) who have normal or low body mass index (BMI).

**PATIENTS AND METHODS:** The patients were divided into 2 groups (n=7, each group), one containing individuals with normal BMI (>21 kg/m<sup>2</sup>) and the other with low BMI (<21 kg/m<sup>2</sup>). Forced spirometry and blood gas analysis were performed in both groups and percutaneous needle biopsies of the lateral portion of the quadriceps femoris muscle were performed. Western blots were used to assess the concentration of SERCA2 in the biopsy material. To determine whether or not the protein was tyrosine-nitrated, immunoprecipitation of SERCA2 was performed with an antinitrotyrosine antibody followed by Western blotting to determine the concentration of the tyrosine-nitrated protein.

**RESULTS:** Expression of SERCA2 was significantly lower in patients with low BMI (4.2 [0.5] vs 8.1 [1.2] integrated optical density units, *P*<.05). SERCA2 was also tyrosine-nitrated in the patients with low BMI. Finally, a significant negative correlation was observed between the concentration of SERCA2 and that of inducible nitric oxide synthase (determined in a previous study using the same biopsy material) in patients with COPD and low BMI (*r*=-0.89, *P*=.007), while such a correlation was not observed in patients with COPD and normal BMI (*r*=0.35, *P*=.43).

**CONCLUSIONS:** In patients with COPD, SERCA2 concentration is reduced and the protein is tyrosine-nitrated in skeletal muscle from patients with low BMI compared to those with normal BMI. These results indicate the presence of a previously unrecognized cellular alteration in skeletal muscle from patients with COPD and low muscle weight.

**Key words:** Cachexia. Chronic bronchitis. Emphysema. Smoking.

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Disminución de la expresión de la bomba de calcio sarcoplásmica (SERCA2) en el músculo esquelético de pacientes con EPOC y bajo peso corporal

**OBJETIVO:** Determinar las concentraciones y el estado de nitrosinación de SERCA2 (adenosintrifosfatasa de calcio del retículo sarcoplásmico) en biopsias del cuádriceps femoral de pacientes con enfermedad pulmonar obstructiva crónica (EPOC) e índice de masa corporal (IMC) normal o bajo.

**PACIENTES Y MÉTODOS:** Los pacientes se distribuyeron en 2 grupos (n = 7) según su IMC fuera normal (> 21 kg/m<sup>2</sup>) o bajo (< 21 kg/m<sup>2</sup>). En ambos grupos se realizó una espirometría forzada, se determinó la concentración de gases en sangre y se efectuaron biopsias percutáneas de aguja de la porción lateral del cuádriceps femoral. En estas biopsias se determinó la concentración de SERCA2 mediante inmunotransferencia y se evaluó si se encontraba nitrosinada mediante inmunoprecipitación de SERCA2 con un anticuerpo antinitrotyrosina y posterior determinación de la concentración de SERCA2 nitrosinada mediante inmunotransferencia.

**RESULTADOS:** La expresión de SERCA2 era significativamente inferior en los pacientes con IMC bajo (4,2 ± 0,5 frente a 8,1 ± 1,2 IOD; *p* < 0,05). En estos pacientes, además, SERCA2 se encontraba nitrosinada. Por último, se observó una relación negativa y significativa entre los valores de SERCA2 y las concentraciones de la óxido nítrico sintetasa inducible (tomadas de un trabajo anterior realizado con las mismas biopsias) en pacientes con EPOC y bajo peso corporal (*r* = -0,89; *p* = 0,007), relación que no existía en pacientes con EPOC e IMC normal (*r* = 0,35; *p* = 0,43).

**CONCLUSIONES:** SERCA2 se encuentra disminuida y nitrosinada en el músculo esquelético de los pacientes con EPOC y bajo peso corporal, comparados con aquéllos con peso normal. Esta observación señala una nueva alteración celular en el músculo esquelético de los pacientes con EPOC y bajo peso muscular.

**Palabras clave:** Caquexia. Bronquitis crónica. Enfisema. Tabaco.

## Introduction

Skeletal muscle dysfunction is common in patients with chronic obstructive pulmonary disease (COPD), particularly those with low body weight.<sup>1</sup> This dysfunction is clinically significant since it limits the individual's exercise capacity and worsens both prognosis and quality of life.<sup>1-3</sup> Little is known about the cellular mechanisms that underlie the development of skeletal muscle dysfunction associated with COPD<sup>4,5</sup> and understanding of those mechanisms must be improved in order to develop new treatments for the condition.

The contraction-relaxation cycle in skeletal muscle is regulated by the cytoplasmic concentration of calcium (Ca<sup>2+</sup>). Proteins of the sarcoplasmic-endoplasmic reticulum Ca<sup>2+</sup>-adenosine triphosphatase (SERCA) family are key regulators of cytoplasmic Ca<sup>2+</sup> concentration. By sequestering Ca<sup>2+</sup> in the sarcoplasmic reticulum, SERCA induces and maintains muscle relaxation.<sup>6,7</sup> At least 3 genes code for members of this protein family: *a*) *SERCA1*, which is expressed in fast-twitch skeletal muscle fibers; *b*) 2 variants of *SERCA2* messenger RNA, *SERCA2a*, and *SERCA2b*—*SERCA2a* is expressed in slow-twitch fibers, cardiac muscle, and smooth muscle, while *SERCA2b* is expressed in a variety of nonmuscle tissues; and *c*) *SERCA3*, which is also expressed in a large number of different tissues.<sup>7</sup>

The potential role of alterations in the expression and/or function of SERCA in the skeletal muscle of patients with COPD has not been investigated. In the present study, the expression of SERCA2 was analyzed by Western blot in 2 groups of patients with COPD, one in which body weight was normal and the other in which it was low. The biopsy material obtained from those patients had been used in a previous study in which the concentration of inducible nitric oxide synthase (iNOS) was analyzed.<sup>8</sup> Within this context, we investigated the possibility that SERCA2 is tyrosine nitrated in the skeletal muscle of those patients. This was based on the observation by our own group<sup>8</sup> and other authors<sup>9</sup> of nitrosative stress in skeletal muscle from patients with COPD and low body weight, and that both oxidative and nitrosative stress alter SERCA activity.<sup>10-13</sup>

## Patients and Methods

### Patients

Diagnosis of COPD was performed according to the internationally established criteria of the European Respiratory Society and the American Thoracic Society (ERS/ATS).<sup>14</sup> The patients attended our outpatient service and presented severe or very severe airflow obstruction, according to the ERS/ATS classification.<sup>14</sup> All of the patients had a sedentary lifestyle. The patients were clinically stable, defined as the absence of treatment changes and/or exacerbations during the 3 months prior to the study. All were treated with bronchodilators, which in 9 cases were combined with inhaled corticosteroids. None were treated with oral corticosteroids. To remove confounding variables in relation to the muscle tissue, only men were included in the study and patients were excluded if they presented other chronic inflammatory diseases, neuromuscular disorders, heart failure, kidney failure, liver disease, diabetes

melittus, or alcoholism. Patients were distributed into 2 groups on the basis of whether their body mass index (BMI) was greater than or less than 21 kg/m<sup>2</sup>.<sup>15</sup> The study protocol was approved by the hospital ethics committee and all patients gave signed informed consent.

### Lung Function

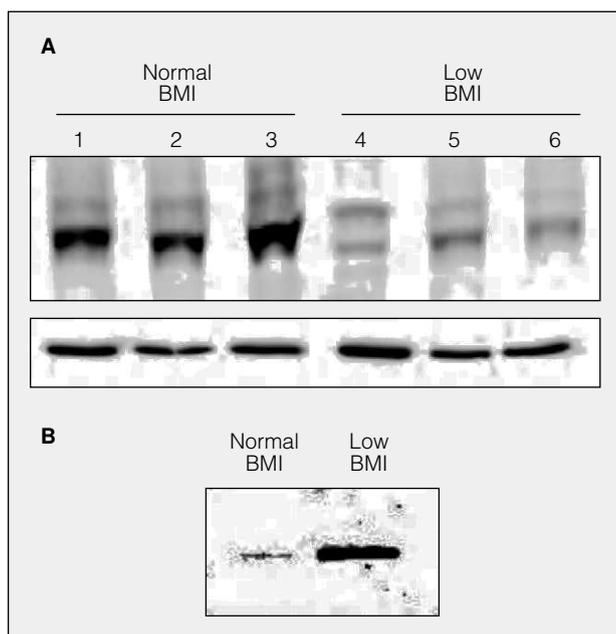
Forced spirometry (GS, Warren E. Collins, Braintree, Massachusetts, USA) was performed according to international guidelines<sup>16</sup> in all patients using reference values for the Mediterranean population.<sup>17</sup> Arterial blood gas analysis (IL BG3, Izasa, Barcelona, Spain) was performed.

### Muscle Biopsy

Percutaneous needle biopsies were performed with local anesthesia in the lateral portion of the quadriceps femoris, as described previously.<sup>18</sup> Biopsy material was immediately frozen and stored at -80°C prior to analysis. The biopsy material had been used in a previous study in which the concentration of iNOS was analyzed.<sup>8</sup>

### Immunodetection of SERCA2 and $\alpha$ -Tubulin

Western blotting was performed as described previously.<sup>8,15</sup> Briefly, frozen muscle samples were homogenized in cold Tris buffer (10 mmol/L, pH 7.5, containing 1% sodium dodecyl sulfate (SDS) and a mixture of protease inhibitors (Roche Diagnostics, Mannheim, Germany). Buffer was added at a 1:10 ratio (weight by volume) to 5-10 mg of muscle tissue prior to homogenization. Following homogenization, the samples were centrifuged at 12 000g for 10 minutes at 4°C. One hundred microliters of the resulting supernatant was mixed with an equal volume of loading buffer (125 mmol/L Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.005% bromophenol blue). The mixture was boiled for 4 minutes and stored at -80°C for subsequent analysis. Protein concentration was measured by Bradford assay.<sup>19</sup> Between 20 and 50  $\mu$ L of the resulting suspension was loaded on a 10% polyacrylamide gel and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred to nitrocellulose membranes (Western blot) and incubated in phosphate buffered saline (PBS) containing 4% nonfat dried milk as a blocking solution for 1 hour at room temperature with gentle shaking. The membranes were incubated overnight at 4°C in blocking solution containing primary antibody. After washing in PBS, they were incubated for 2 hours at room temperature with secondary antibodies. The following antibodies were used: anti-SERCA2 (Santa Cruz Biotechnology Inc, Santa Cruz, California, USA) at a dilution of 1:1000 and anti- $\alpha$ -tubulin (Sigma Chemical Co, St Louis, Missouri, USA) at a dilution of 1:2000. A horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig) G was used as a secondary antibody for SERCA2 and an HRP-conjugated anti-mouse IgG was used for  $\alpha$ -tubulin (Amersham International, Buckinghamshire, UK). Immunoreactivity was detected using a chemiluminescence system (Pierce, Rockford, Illinois, USA). Two milliliters of detection substrate (SuperSignal West Dura Extended Duration Substrate, Pierce) was added to the nitrocellulose membrane and incubated for 30 seconds. The resulting bands were then detected using the GeneGenius chemiluminescence detection system (Syngene, Cambridge, UK). The intensity of the bands was determined by densitometry with the GeneTools program (Syngene). The results were normalized in all cases for  $\alpha$ -tubulin content.



**Figure 1.** A) Representative Western blot of SERCA2 expression in the quadriceps femoris of 3 patients with COPD in whom body mass index (BMI) was normal (lanes 1-3) and 3 patients with COPD and low BMI (lanes 4-6). B) Western blot of SERCA2 immunoprecipitated from extracts of quadriceps femoris muscle from patients with low or normal BMI (n=7 in each group).

#### Immunoprecipitation of SERCA2 With Antinitrotyrosine Antibodies

The 2 groups of frozen supernatants (BMI greater than or less than 21 kg/m<sup>2</sup>) were centrifuged together at 12 000g for 2 minutes at 4°C. A mouse monoclonal antinitrotyrosine antibody (HyCult Biotech, Uden, the Netherlands) was added to both supernatants at a dilution of 1:1000 and incubated for 2 hours at 4°C. Then, 50 µL of recombinant protein-A-agarose (Sigma) was added to the suspension and incubated for a further 1 hour at 4°C. The samples were then centrifuged at 12 000g for 30 seconds. The pellets were washed 3 times in 1 mL of tris-buffered saline at 4°C for 20 minutes and then resuspended in a volume of 100 µL. This suspension was mixed with an equal volume of loading buffer (125 mmol/L Tris, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.005%

bromophenol blue) and boiled for 4 minutes. Protein concentration was measured by Bradford assay.<sup>19</sup> Fifty microliters of the suspension was loaded on a 10% SDS-PAGE gel and the protein was processed and detected as described.

#### Statistical Analysis

Data are shown as means (SD). The statistical significance of the differences observed between the groups was established with the Mann-Whitney test. Correlations between variables of interest were assessed with the Spearman correlation coefficient. A value of *P*<.05 was considered statistically significant.

#### Results

The table shows the clinical and functional characteristics of the patients. Age was similar, as was smoking habit in the 2 groups of patients (low and normal BMI). Both groups displayed severe airway obstruction (particularly in patients with low BMI) and moderate arterial hypoxemia without hypercapnia.

Figure 1A shows a representative Western blot of SERCA2 expression in biopsies of the quadriceps femoris from 3 patients with COPD and normal BMI (lanes 1-3) and 3 patients with COPD and low BMI (lanes 4-6). A clear reduction in the expression of SERCA2 is visible in the latter group. The mean levels of SERCA2, normalized for α-tubulin content, were significantly reduced in patients with low BMI (4.2 [0.5] vs 8.1 [1.2] integrated optical density [IOD] units, *P*<.05).

Immunoprecipitation with an antinitrotyrosine antibody in supernatants from the quadriceps femoris followed by detection of SERCA2 by Western blot demonstrated that SERCA2 was tyrosine nitrated in patients with COPD and low BMI compared with those in whom BMI was normal (Figure 1B).

In a previous study, we demonstrated that iNOS is overexpressed in the quadriceps femoris of patients with COPD and low body weight.<sup>8</sup> Figure 2 shows the existence of a significant negative correlation between the expression levels of SERCA2 and iNOS in patients with COPD and low BMI (*r*=-0.89, *P*=.007), while that correlation is not present in patients with COPD and normal BMI (*r*=0.35, *P*=.43).

#### Clinical and Functional Parameters of Patients Included in the Study\*

	Normal BMI (n=7)	Low BMI (n=7)
Age, y	61 (3)	60 (3)
BMI, kg/m <sup>2</sup>	27.5 (1)	18.5 (1)†
Smoking, pack-years	56 (8)	47 (5)
FEV <sub>1</sub> , % of reference	39 (2)	28 (2)†
FEV <sub>1</sub> /FVC, %	44 (4)	40 (3)†
PaO <sub>2</sub> , mm Hg	70 (3)	69 (4)
PaCO <sub>2</sub> , mm Hg	43 (3)	41 (2)

\*Data are means (SD).  
FEV<sub>1</sub> indicates forced expiratory volume in 1 second; FVC, forced vital capacity;  
BMI, body mass index.  
†*P*<.001

#### Discussion

SERCA2 is a key protein in the regulation of cytosolic Ca<sup>+</sup> concentration and, therefore, of muscle function.<sup>6,7</sup> Loss of SERCA2 expression has been described previously in other clinical conditions that are also characterized by skeletal muscle dysfunction, such as necrosis,<sup>20</sup> heart failure,<sup>21</sup> and muscle denervation.<sup>22</sup> However, to date, no changes in SERCA2 expression have been described in COPD. Consistent with those previous observations, our results show that expression of SERCA2 is reduced in patients with COPD and low body weight (Figure 1A).

Various cellular mechanisms could explain the loss of expression of SERCA2 in skeletal muscle from

patients with COPD and low body weight. One possibility is that it is due to an increase in the rate of proteolysis and degradation. In support of this hypothesis, our results show that in patients with COPD and low body weight, SERCA2 is tyrosine nitrated (Figure 1B), a structural change that facilitates degradation of the protein through the ubiquitin-proteasome pathway.<sup>10-13</sup> Furthermore, our group<sup>8</sup> and others<sup>9</sup> have previously demonstrated the presence of nitrosative stress and overexpression of iNOS in the quadriceps femoris of patients with COPD and low body weight. Figure 2 shows the presence of a significant negative correlation between the concentrations of SERCA2 and iNOS in patients with COPD and BMI ( $r=-0.89$ ,  $P=.007$ ), while this correlation is absent in patients with COPD and normal BMI ( $r=0.35$ ,  $P=.43$ ). This comparative analysis was made possible through the use of samples that were used previously by our group to study the expression of iNOS in skeletal muscle from patients with COPD.<sup>8</sup> The results are indicative of a causal relationship between iNOS induction and loss of SERCA2 through tyrosine nitration and degradation in the skeletal muscle of patients with COPD and low body weight.

In our study, we cannot rule out the possibility that processes of remodeling in the muscle fibers, such as reduction of diameter, contribute to explaining the reduction in SERCA2 levels. In this context, it is noteworthy that specific atrophy of type IIA/IIX and IIX fast-twitch fibers of the vastus lateralis muscle has been described in patients with COPD and loss of muscle mass.<sup>23</sup> Given that SERCA2 is expressed in slow-twitch fibers,<sup>7</sup> it is unlikely that the reduction in its concentration is due to that process of atrophy, suggesting that reduction of SERCA2 levels is linked to nitration of the protein and activation of proteolysis. In future studies, we will address ubiquitination of tyrosine-nitrated SERCA2 and proteasome activation.

Studies performed in humans have reported an absence of modulation of SERCA2 expression in skeletal muscle following continued exercise.<sup>24</sup> Consequently, taking into account the sedentary nature of the COPD patients in this study, it is reasonable to consider physical activity not to have played an important role in the reduction of SERCA2 levels we observed. Independently of the cause or causes of reduced SERCA2 concentration in patients with COPD and low body weight, this low concentration of SERCA2 could contribute to the pathogenesis of skeletal muscle dysfunction in these patients in a variety of ways. In vitro studies have shown that tyrosine nitration of SERCA leads to its inactivation.<sup>12</sup> Consequently, it is likely that in patients with COPD who lose weight, SERCA2 not only diminishes in concentration (Figure 1A) but is also inactivated by tyrosine nitration (Figure 1B). We can speculate that both abnormalities could alter  $Ca^{+}$  homeostasis and theoretically contribute to skeletal muscle dysfunction in at least 2 different ways: the contraction-relaxation cycles of the muscle could be negatively affected<sup>6,7</sup> and, in addition, the excess of  $Ca^{+}$  could fix calcineurin<sup>25</sup> and

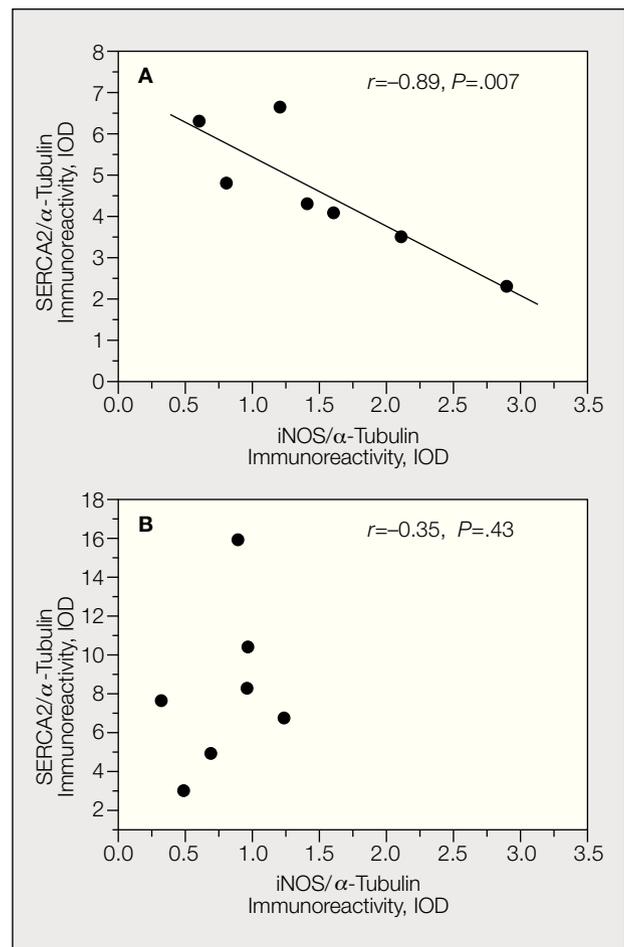


Figure 2. Correlation between concentrations of SERCA2 and expression of the inducible form of nitric oxide synthase (iNOS) in skeletal muscle from patients with COPD and low (A) or normal (B) body mass index. IOD indicates integrated optical density.

alter the expression of various  $Ca^{+}$ -dependent transcription factors, such as MEF2 and MyoD,<sup>26,27</sup> which are key to the maintenance and regeneration of skeletal muscle.

In summary, the results of this study demonstrate for the first time that patients with COPD and low body weight display reduced expression of SERCA2 in skeletal muscle, that SERCA2 is tyrosine nitrated, and that there is a negative correlation between the concentrations of SERCA2 and iNOS in patients with COPD and low BMI. Further studies will be necessary to determine the role played by these cellular alterations in the loss of muscle mass and/or skeletal muscle dysfunction in this patient group.

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MORLÀ M ET AL. REDUCED EXPRESSION OF THE SARCOPLASMIC CALCIUM PUMP SERCA2  
IN SKELETAL MUSCLE FROM PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE AND LOW BODY WEIGHT

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