Exercise capacity and cytochrome oxidase activity in muscle mitochondria of COPD patients

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Received 18 May 2009; accepted 28 July 2009
Available online 27 August 2009

SUMMARY

Skeletal muscle dysfunction (SMD) often occurs in patients with COPD, affecting their quality of life and mitochondria is one of the cellular organelles involved in the pathogenesis of SMD in COPD.

The aim of this study was to investigate exercise capacity and mitochondria skeletal muscle oxidative processes using a pilot study, with 20 COPD patients and 10 healthy subjects, prior to and following LABA treatment.

The two groups were similar for BODE (2 – 7) and GOLD stages (2 – 3), and no one was cachectic or more symptomatic. The patients were randomized according to a distribution list.

The Cycle Ergometry test with tau evaluation was used to determine exercise capacity, while a skeletal muscle biopsy for cytochrome oxidase (CytOX) activity evaluation was used to determine mitochondria skeletal muscle oxidative processes.

In six of the COPD treated patients the individual values of tau and CytOX activity showed inversely parallel changes with a significant relationship between the tau values and the CytOX activity. No significant differences in tau values were observed in healthy subjects.

In conclusion, LABA treatment may improve skeletal muscle oxidative processes, enhancing the CytOX activity and, at least in some COPD patients, such effects could be strictly linked to the kinetic exchanges occurring at skeletal muscle level, implying an important link between

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Introduction

Chronic obstructive pulmonary disease (COPD) is a pulmonary disorder, characterised by reduced maximum expiratory flow and slow, forced emptying of the lungs, due to varying combinations of diseases of the airways and emphysema. This definition focuses exclusively on the lungs, but various recent studies have provided evidence that COPD is often associated with significant extrapulmonary abnormalities, the so-called "systemic effects of COPD", including cardiovascular disease, diabetes, osteoporosis, and skeletal muscle dysfunction (SMD). The impact of these systemic effects is clinically relevant and their analysis could contribute to a better understanding and management of the disease. Exercise limitation, an important feature of COPD that results in a reduced quality of life, has been traditionally explained by the increased effort of breathing and dynamic hyperinflation, although several recent studies have clearly shown that SMD is often a very significant contributor to exercise limitation. The precise cellular and molecular mechanisms leading to skeletal muscle dysfunction in the COPD patients are unclear. However, sedentarism, low-grade chronic systemic inflammation, and tissue hypoxia, among other factors, can contribute to SMD in these patients. These mechanisms are believed to cause mitochondrial dysfunction, enhanced skeletal muscle apoptosis and exercise-induced muscle fiber death.

Recently, Wende et al., documented an increase of cytochrome oxidase subunits (CytOX) II and IV in the skeletal muscle of β2-agonist-injected mice, while other studies indicate the formoterol as an efficient agent able to prevent muscle weight loss in tumor-bearing rats and to induce protein accretion. The β2-sympathomimetic agonists together with anti-cholinergics, are the bronchodilators currently available for use in COPD. There are few studies available on the effects of β2-agonists on the exercise capacity of COPD patients. Therefore the aim of this study was to investigate exercise capacity using the cycle ergometry test and skeletal muscle oxidative metabolism, evaluating cytochrome oxidase activity prior to and following LABA treatment in both healthy and COPD subjects.

Methods

Study subject

In this pilot study, 20 outpatients with the diagnosis of stable COPD and 10 healthy subjects were evaluated. The subjects were recruited from the outpatient clinics of the Respiratory Pathophysiology Department of Cava de’ Tirreni Hospital, with a diagnosis of COPD, made as defined by the American Thoracic Society (ATS) and European Respiratory Society (ERS) and a history of smoking >10 packets-yrs, in a stable condition of disease for at least 1 month, and matching our inclusion criteria. A complete medical history was obtained for each patient, which included a standard American Thoracic Society (ATS) symptom questionnaire (ATS-DLD 34), past medical history and evaluation of atopy. Standard posteroanterior (PA) and lateral chest roentgenograms were obtained for each patient and read to exclude any other disease. Baseline characteristics are shown in Table 1. The two groups were similar for BODE and GOLD stages, and no one was cachectic or more symptomatic. The patients were randomized according to a distribution list. Exclusion criteria were as follows: patients had evidence of pulmonary hypertension detected by eco-Doppler evaluation; chronic respiratory failure (PaCO2 > 45 mmHg or 6kpa at rest); other respiratory diseases, cardiac disease, regular use of prednisone/day, or recent respiratory tract infection; a need for therapy other than the study or reference drug and finally any patients who required more than 4 puffs per day of short-acting-β2-agonist during the run-in. On the basis of the specific limitations of the Committee of Medical Ethics, the control group was derived from subjects attending our Pulmonary Function Laboratory for preoperative evaluation: the patients were awaiting minor and elective surgery (such as orthopaedic disorders, inguinal hernia, eye and ear surgery, haemorrhoids) for which pulmonary tests are routinely performed. All patients signed their informed consent after being made fully aware of the goals, methods and risks of the study. The Institutional Committee for Ethics in Human Research of our hospital approved the muscle biopsy applied in the study (No. 177 of 18 April 2007). All the aspects of this study comply with the declaration of Helsinki.

Study design

This was a single-blind, placebo-controlled, randomized study: eligible patients were randomized to receive, before the constant CPET at day 8, inhaled formoterol (12 µg) via a single-dose, breath-activated inhaler (Aerolizer dry powder capsules for inhalation; Novartis; Basel, Switzerland), or placebo matching formoterol. Formoterol and placebo were administered in a single-blind manner: the patients were unaware of the drug delivered. The primary analysis was clinical evaluation of arterial blood gases measurements, pulmonary function (including body plethysmography) and DLCO. On day 1, the BODE index for a systemic evaluation of COPD patients in all observed subjects was obtained using a 6-min walking test. On day...
Skeletal muscle dysfunction in COPD

Table 1 Demographic and spirometric characteristics of the COPD and healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>COPD LABA (N = 10)</th>
<th>COPD control (N = 10)</th>
<th>Healthy (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>63.8 ± 3.43 (48–79)</td>
<td>63.63 ± 1.532 (57–72)</td>
<td>64.02 ± 0.924 (59–68)</td>
</tr>
<tr>
<td>BMI</td>
<td>40.7 (26.9–52.6)</td>
<td>40.4 (31.8–47.9)</td>
<td>41 (30.5–47)</td>
</tr>
<tr>
<td>Weight</td>
<td>69.3 ± 4.652 (42–92)</td>
<td>68.67 ± 3.215 (51–84)</td>
<td>69.63 ± 2.895 (49–81)</td>
</tr>
<tr>
<td>Height</td>
<td>169.40 ± 2.088 (156–176)</td>
<td>169.33 ± 1.554 (161–176)</td>
<td>169.43 ± 1.554 (159–176)</td>
</tr>
<tr>
<td>BODE</td>
<td>4.6 ± 0.77 (2–8)</td>
<td>4.5 ± 0.522 (2–7)</td>
<td>–</td>
</tr>
<tr>
<td>GOLD</td>
<td>2.6 ± 0.163 (2–3)</td>
<td>2.70 ± 0.153 (2–3)</td>
<td>–</td>
</tr>
<tr>
<td>FEV1 % pred</td>
<td>46.08 ± 4.152 (30.2–64.8)</td>
<td>45.70 ± 3.612 (33–68)</td>
<td>95.5 ± 1.462 (89–103)</td>
</tr>
<tr>
<td>FEV1/VC</td>
<td>50.55 ± 2.639 (36.5–63.8)</td>
<td>50.23 ± 2.132 (38.9–59.7)</td>
<td>77.9 ± 0.862 (74–83)</td>
</tr>
<tr>
<td>ITGV % pred</td>
<td>143.7 ± 7.449 (120–181)</td>
<td>144.7 ± 5.950 (128–184)</td>
<td>99.7 ± 1.955 (89–109)</td>
</tr>
<tr>
<td>RV % pred</td>
<td>175.90 ± 16.579 (130–270)</td>
<td>164.60 ± 8.956 (135–189)</td>
<td>99.7 ± 3.190 (87–119)</td>
</tr>
<tr>
<td>DLCO % pred</td>
<td>41.50 ± 1.327 (35–48)</td>
<td>43.30 ± 2.129 (35–50)</td>
<td>95.5 ± 2.078 (87–102)</td>
</tr>
</tbody>
</table>

The range of data is reported in parenthesis.

3, in order to verify the stability of functional status, the subjects’ repeated assessment of pulmonary function (including body pletismography) and the bronchodilator response to formoterol (12 µg) was evaluated. On day 4, each subject underwent a Cardio-Pulmonary-Exercise-Test (CPET) with ramp protocol in order to obtain the anaerobic threshold. On day 6, a 6-min CPET constant work rate protocol with a workload at 50% of anaerobic threshold was applied to calculate the tau (VO2 kinetics analysis of the “phase 2” of the response, which should more closely reflect gas exchange kinetics occurring at the skeletal muscle level). At the end of test, a muscle biopsy was performed on COPD patients. On day 8, we repeated the 6-min CPET in all subjects evaluated in the study. However, all COPD patients were randomized to receive formoterol (12 µg) or placebo 1 h before the test. At the end of the tests a muscle biopsy was repeated in all COPD patients. Healthy subjects did not receive any medication, nor was a muscle biopsy performed (Fig. 1).

Muscle biopsy

Tru-Cut biopsies were obtained from the right (day 6) and left (day 8) vastus lateralis using a 14 Ga TW6 needle. The skin and muscle were anesthetized with 2% lidocaine, and samples were taken, immediately snap-frozen in liquid nitrogen and stored at –80 °C until cytochrome oxidase activity determination.

Cytochrome oxidase activity

Samples of skeletal muscle were finely minced, diluted 1:10 w/vol and homogenized in modified Chappel–Perry medium 1 mM ATP, 50 mM Hepes buffer adjusted to pH 7.4, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA, 5 mM EGTA. The homogenate was then diluted 1:2 (v/v) in the same medium with lubrol (100 mg/g tissue) in order to unmask tissue enzyme activity. Samples were left standing in ice for 30 min and thereafter cytochrome oxidase (CytoX) activity was determined polarographically at 25 °C, using Clark oxygen and a modification of the Aulie & Grav procedure. Homogenate proteins (50–100 µg) were added to 1.5 ml of reaction medium containing 30 µM cytochrome c, 4 µM rotenone, 1.5 mM dinitrophenol (DNP), 10 mM sodium malonate and 75 mM Hepes buffer, at pH 7.4. The addition of substrate (4 mM sodium ascorbate with 0.3 nM N,N-dimethyl-p-phenylene-diamine) to the reaction medium caused oxygen consumption to commence. In order to take into account the oxygen consumption due to the auto-oxidation of ascorbate rather than the CytoX catalyzed reaction, measurements were also performed in the absence of homogenate in the reaction medium. CytoX activity was determined polarographically at 25 °C.
activity was then measured as the difference between the rate of oxygen consumption observed in the presence of the homogenate and the rate observed in its absence.

**Statistical analysis**

Group data are expressed as mean (±SD) or median (range). Since our data are based on small patient populations with data that are not normally distributed, we used the Mann–Whitney U-test for comparison between non-parametric tests. Spearman’s rank correlation coefficient test was used to examine the association between functional data and cytochrome oxidase activity. Probability values of \( p < 0.05 \) were accepted as significant. Analysis was performed using the statistical package SPSS 17.0. Additional information regarding methods is published online in Supplementary data.

**Results**

**Basal values**

The arterial gas analysis of COPD patients revealed a mean value of PaO\(_2\) of 10.00 ± 0.8 kpa and a mean value of PaCO\(_2\) of 5.56 ± 0.2 kpa. The demographic and spirometric characteristics, together with the GOLD and BODE stages of study subjects are shown in Table 1.

**Bronchodilation response and anaerobic threshold**

The bronchodilator response after inhalation of LABA did not show any significant difference in FEV\(_1\) and TGV in COPD patients according to our selection criteria. In terms of FEV\(_1\) the increase (in % of basal values and in absolute values) was 5.4 ± 6.9% (80 ± 84 ml); in terms of TGV (in % of basal values) the LABA elicited a decrease of 0.98 ± 4.54%.

The ramp CPET protocol confirmed a ventilatory limitation by a low breathing reserve (with the patient approaching anaerobic threshold). The ramp CPET protocol was stopped in two patients (No. 3 and No. 8) because of dyspnea; all other patients stopped because of fatigue.

**Tau and CytOX activity on day 6**

On day 6, after the choice of workload, the mean value of VO\(_2\) reached at the end of the constant work rate exercise was 745.2 ± 322.8 ml/min, with an oxygen pulse of 7.4 ± 3.2 ml/beat. The mean values of anaerobic threshold and tau values of all COPD patients and healthy subjects are shown in Table 2. Moreover, for COPD patients only, CytOX activity mean values are also shown in Table 2, and CytOX activity and tau individual value are shown in Table 3. The day 6 values were utilized as a starting value in order to evaluate the effects of the administration of LABA on constant CPET and CytOX activity on day 8.

**Tau and CytOX activity values on day 8**

With the cycle ergometry tests, the medians of tau and CytOX activity of skeletal muscle mitochondria in COPD LABA treated patients were lower and, respectively, higher than COPD control patients (Table 2), but the statistical analysis did not reveal significance in reason of the wider range of values of LABA-treated patients (Table 3). It is worth mentioning that six COPD LABA-treated patients showed a decrease in tau greater than the maximum decrease in COPD control patients (Fig. 2), and that the same six patients showed an increase in CytOX activity of skeletal muscle mitochondria greater than the maximum increase in COPD control patients (Fig. 3). Moreover, the decreases in tau values observed in COPD treated patients were strictly linked to the CytOX activity observed in the same patients (Fig. 4).

**Discussion**

There is evidence to suggest that the mitochondrion is one of the cellular organelles involved in the pathogenesis of SMD in COPD.\(^6\)

Our results indicate that a LABA, such as formoterol, induced, at least in some cases, an improvement in

<table>
<thead>
<tr>
<th></th>
<th>COPD LABA (N = 10)</th>
<th>COPD control (N = 10)</th>
<th>Healthy (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT (% VO(_2) max)</td>
<td>60 (20–80)</td>
<td>45 (30–80)</td>
<td>85 (70–120)</td>
</tr>
<tr>
<td>VO(_2) max6 (ml)</td>
<td>739 (388–1311)</td>
<td>748 (396–1305)</td>
<td>1090 (915–1478)</td>
</tr>
<tr>
<td>VO(_2) max8 (ml)</td>
<td>760 (357–1355)</td>
<td>828 (380–1367)</td>
<td>1078 (836–1453)</td>
</tr>
<tr>
<td>VO(_2) max%</td>
<td>96.3 (79–117)</td>
<td>106.4 (87–114)</td>
<td>98 (91–108)</td>
</tr>
<tr>
<td>CytOX6 (nmoles O(_2) min(^{-1})/mg/prot)</td>
<td>96 (66–180)</td>
<td>96.5 (48–165)</td>
<td>–</td>
</tr>
<tr>
<td>CytOX8 (nmoles O(_2) min(^{-1})/mg/prot)</td>
<td>122.3 (85–216)</td>
<td>89.7 (41–172)</td>
<td>–</td>
</tr>
<tr>
<td>CytOX% (day 8 vs. day 6)</td>
<td>128.7 (81–328)</td>
<td>96.1 (79–117)</td>
<td>–</td>
</tr>
<tr>
<td>Tau6 (s)</td>
<td>54.6 (22.5–75.6)</td>
<td>49.5 (19.9–69.4)</td>
<td>51 (23.7–66.8)</td>
</tr>
<tr>
<td>Tau8 (s)</td>
<td>44.6 (14.9–58)</td>
<td>53.3 (21.3–67.3)</td>
<td>48.4 (21.5–63)</td>
</tr>
<tr>
<td>Tau% (day 8 vs. day 6)</td>
<td>76.7 (43–110)</td>
<td>106.5 (92–109)</td>
<td>93.5 (90–109)</td>
</tr>
</tbody>
</table>
exercise capacity in COPD patients during constant load exercise, enhancing the skeletal muscle oxidative processes, with no differences found in heart rates, allowing us to speculate on the involvement of mitochondrion in the pathogenesis of SMD in COPD.

The lack of any significant changes in heart parameters rules out an effect of LABA on heart rate during exercise. Indeed, in order to unmask the contribution of the non-pulmonary response, our study was performed applying CLE according to the ATS/ACCP statement, for its clinical relevance in monitoring responses to therapeutic intervention in patients with stable COPD. The CLE allows evaluation of the cardio-respiratory response to exercise and provides an integrated approach to the evaluation of COPD symptoms and their modifications following therapy.

Furthermore, our protocol takes into account the expiratory airflow limitation in COPD that leads to air trapping (hyperinflation), whose impact on symptoms and impairment at rest and during exertion is increasingly recognized to be central to COPD.

Several authors have shown that bronchodilators that mediate improvements in hyperinflation also allow flow-limited patients to improve their exercise capacity. In our study, any patient who had a bronchodilatory response in terms of FEV₁ and air trapping to formoterol was excluded. Moreover, there was no relationship between the improvement of tau values, the scores related to the obstruction degree (GOLD) and multidimensional index (BODE) in the COPD treated group, suggesting that the above index is not a major or completely exhaustive indicator of exercise limitation.

To rule out any training effect in COPD treated patients, it is important to note that in healthy subjects and COPD control patients, no differences in tau values during the 6-min CPET protocol were observed. However a training effect is observed after weeks or months of intensive and constant training and the subjects enrolled in our study repeated the 6-min CPET after just two days. In line with our findings, Liesker et al., showed a significant effect of formoterol on exhaustion time.

### Table 3
Individual values of cytochrome oxidase activity (nmoles O₂/min mg/prot) and tau (s) at constant work rate selected as 50% of anaerobic threshold reached in ramp protocol, in COPD patients and healthy subjects.

<table>
<thead>
<tr>
<th>COPD LABA</th>
<th>COPD control</th>
<th>Healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CytOX6</td>
<td>CytOX8</td>
<td>CytOX%</td>
</tr>
<tr>
<td>1</td>
<td>81</td>
<td>103</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>216</td>
</tr>
<tr>
<td>3</td>
<td>101</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>103</td>
<td>134</td>
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<tr>
<td>5</td>
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<td>6</td>
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<td>9</td>
<td>74</td>
<td>137</td>
</tr>
<tr>
<td>10</td>
<td>83</td>
<td>111</td>
</tr>
</tbody>
</table>

![Figure 2](https://example.com/fig2.png)

**Figure 2** Relationship of tau between COPD LABA-treated patients (circles) and COPD control patients (triangles). Closed circles non responder COPD patients. Open circles responder COPD patients. Patient No. 6 of COPD LABA patients, with a slow component of kinetic, was excluded.
during an incremental work rate protocol in COPD patients. 19

Moreover, our findings showed that the activity of cytochrome oxidase, which is considered a key oxidative enzyme, was significantly enhanced following bronchodilator administration in the skeletal muscle mitochondria of COPD patients who improved exercise kinetic. Other authors have documented an upregulation of CytOX genes expression in trained COPD patients, and in a murine model Miura et al., recently showed that mRNA expression of peroxisome proliferator-activated receptor-coactivator PGC-1, which may promote mitochondrial biogenesis in skeletal muscle, was increased by \( \beta_2 \)-agonists such as clenbuterol and its increase in response to exercise was due, at least in part, to the activation of \( \beta_2 \)-adrenergic receptor. 29 This muscular effect of \( \beta \)-agonists concords with their effects on the remodeling of adipose tissue, where \( \beta \)-agonists induced mitochondrial biogenesis and increased oxidative capacity. 30 In our study, the \( \beta \)-agonist effect had a rapid onset, being evident 1 h after administration of the drug. It cannot therefore be attributed to a variation in the mitochondrial content of tissue or an enhanced concentration of mitochondria molecular components, as also described by others in COPD patients, but rather to an involvement of their kinetic properties. A question that arose at this point related to the effects of a single inhalation of formoterol on peripheral muscle biochemistry and function. On this subject, several studies have shown that the systemic absorption of inhaled formoterol is rapid; in fact the plasma peak caused by substance overflow into the systemic circulation is seen within few minutes. 32,33

Our study has certain limitations that deserve comment. Firstly, mitochondrial oxidase activity was measured in COPD patients only. For ethical reasons, muscle biopsy in healthy subjects was not permitted, but the mitochondrial oxidase activity was unaffected in the COPD control group. Secondly, we did not directly investigate the potential mechanisms that explain our observations. However, one could be linked to the effect of long-acting \( \beta \)-agonists on TNF\( \alpha \), a cytokine that is particularly important in the low-grade systemic inflammation observed in COPD, which can influence the activity of mitochondrial oxidase. 34 Overall these data suggest that the LABA effects on the CytOX activity is strictly linked to the tau value in COPD patients, probably on the basis of an "all or nothing" type mechanism. This effect should more closely reflect gas exchange kinetics occurring at the skeletal muscle level, implying an important link between the regulation of oxygen uptake, energy production and exercise capacity in these patients. However, further studies on LABA in COPD patients are required and a better understanding of the mechanism(s) underlying their effects could allow us to identify or unmask new therapeutic target(s) in these patients.

Acknowledgments

B. D’Agostino and M. Polverino are the creators of the work and have given their support by coordinating the entire study.

Figure 3 Relationship of CytOX between COPD LABA-treated patients (circles) and COPD control patients (triangles). Closed circles non responder COPD patients. Open circles responder COPD patients. Patient No. 6 of COPD LABA patients, with a slow component of kinetic, was excluded.

Figure 4 Relationship between drug-induced modifications of cytochrome oxidase activity and tau. COPD LABA-treated patients (circles) and COPD control patients (squares). Patient No. 6 of COPD treated patients, with slow component of the kinetics, was excluded, \( y = 130.4e^{-0.0037x}; R^2 = 0.706; p = 0.004 \).
Nikol Sullo, Carlo Santoriello and Francesca Polverino have performed the several functional tests and muscular biopsies.

Assunta Lombardi, Donatella Orlotti and Maria Matteis have performed the analysis for the assessment of cytochrome oxidase activity.

Bruno Grassi has provided a vital contribution to kinetic evaluations.

Giuseppe Cirino and Francesco Rossi have given an important contribution through statistical analysis and editorial support.

Conflict of interest

The authors have reported that no significant conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

Supplementary information


References


