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Effect of pulmonary rehabilitation on muscle remodelling in cachectic patients with COPD

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Abstract

It is known that non-cachectic patients with COPD respond well to pulmonary rehabilitation, but whether cachectic COPD patients are capable of adaptive responses is both important and unknown.

Ten cachectic and nineteen non-cachectic COPD patients undertook high-intensity cycling training, at the same relative intensity, for 45 min/day, 3 days/week for 10 weeks. Before and after rehabilitation vastus lateralis muscle biopsies were analyzed morphologically and for the expression of muscle remodelling factors [IGF-I, MyoD, TNF-α, NF-κB and Myostatin) and key components of ubiquitin-mediated proteolytic systems (MURF-1 and Atrogin-1) .

Rehabilitation improved peak work-rate and the 6-min walk distance similarly in non-cachectic (18±3% and 42±13 m, respectively) and cachectic (16±2% and 53±16 m, respectively) patients, but quality of life improved only in non-cachectic COPD. Mean muscle fibre cross-sectional area increased in both groups, albeit significantly less in cachectic (7±2%) than non-cachectic (11±2%) patients. Both groups equally decreased the proportion of type IIb fibres and increased muscle capillary/fibre ratio. IGF-I mRNA expression increased in both groups, but IGF-I protein levels increased more in non-cachectic COPD. MyoD was up-regulated, whereas Myostatin was down-regulated at mRNA and protein level only in non-cachectic patients. Whilst rehabilitation had no effect on TNF-α expression, it decreased the activation of the transcription factor NF-κB in both groups by the same amount. Atrogin-1 and MURF-1 expression was increased in cachectic COPD, but it was decreased in non-cachectic patients.

Cachectic COPD patients partially retain the capacity for peripheral muscle remodeling in response to rehabilitation and are able to increase exercise capacity as much as those without cachexia, even if they exhibit both quantitative and qualitative differences in the type of muscle fibre remodeling in response to exercise training.

Introduction

Cachexia refers to severe weight loss with disproportionate muscle wasting. Muscle wasting is common in many chronic diseases including Chronic Obstructive Pulmonary Disease (COPD) [1]. In COPD, muscle wasting [defined as a fat free mass index (FFMI) below 16 kg/m^2 in males and 15 kg/m^2 in females] occurs in about 20-40% of the patient population and is highly predictive of increased mortality [2]. The causes of cachexia in COPD are probably multiple, but remain to be established. Possibilities include energy imbalance, systemic inflammation, oxidative stress, hormonal insufficiency, arterial hypoxaemia as well as physical inactivity [1-3].

Rehabilitative exercise training is recommended in many diseases associated with skeletal muscle wasting as a therapeutic strategy that could potentially attenuate muscle loss and promote muscle growth [4]. We have previously shown [5] that in non-cachectic patients with COPD, exercise training induces significant adaptations in peripheral muscle fibre size and typology that, in the absence of a decrease in systemic or local muscle inflammation, are accompanied by the up-regulation of key factors governing skeletal muscle hypertrophy and regeneration [namely local muscle expression of insulin-like growth factor-I (IGF-I) and myogenic differentiation factor D (MyoD)]. Whether skeletal muscle wasting can be mitigated by pulmonary rehabilitation in cachectic patients with COPD remains currently unknown. Several studies have shown that cachectic COPD patients have increased activity of their protein breakdown pathways, in particular the nuclear factor (NF)-κB activated ubiquitin/proteasomal pathway, leading to muscle loss [6, 7]. Inflammatory cytokines, such as TNF-α, can activate the transcription factor NF-κB by ubiquitination and degradation of the inhibitory IκB family member [8]. The interaction of IκB masks the nuclear localization sequence of NF-κB complex, thereby preventing nuclear translocation and maintaining NF-κB in an inactive state in the cytoplasmic compartment [9, 10]. Although the results of studies [11, 12] exploring the expression of local muscle TNF-α in patients with severe COPD are

inconsistent, experimental data suggest that TNF-α can interfere with the hypertrophic action of muscle IGF-I [13] and inhibit myogenic differentiation by destabilizing MyoD protein [14]. Furthermore, excess levels of Myostatin (a strong negative regulator of muscle growth) have also been proposed as another potential mediator of muscle wasting, since Myostatin inhibits myogenesis by down-regulating MyoD expression [15].

It addition, it has recently been demonstrated that in COPD patients with muscle wasting, exercise training causes up-regulation of nitric oxide synthase (iNOS) through the activation of NF-κB transcription factor, thereby inducing greater degrees of tyrosine nitration of quadriceps proteins [16] compared to COPD patients without muscle wasting. Increased protein nitrotyrosination may favor protein degradation through the ubiquitin proteasome pathway, therefore limiting the capacity for muscle remodeling in patients with muscle cachexia. Besides cachexia, the NF-κB transcription factor complex has also been implicated in muscle atrophy attributable to muscle disuse and physical inactivity [17].

Based on the above evidence it was hypothesized that cachectic patients would respond poorly to pulmonary rehabilitation in terms of muscle fibre remodelling. Accordingly, we investigated the effects of pulmonary rehabilitation on peripheral muscle fibre morphological characteristics and on the local muscle expression of a number of factors that are known [5-7, 11-17] to govern the signalling pathways for muscle remodelling [i.e.: IGF-I and its isoform mechano-growth factor (MGF), protein kinase B (Akt), MyoD, TNF-α, IκB Myostatin, Atrogin-1 and MURF-1 as well as protein nitration levels] in cachectic patients with COPD. An additional purpose of the present study was to compare the magnitude of rehabilitative-induced peripheral muscle adaptations between cachectic and non-cachectic COPD patients.

Materials and Methods

Study population

A total of 29 male patients with clinically stable COPD meeting the following criteria were recruited for this study: 1) post-bronchodilator FEV_1<50% predicted and FEV_1/FVC<70% without significant post-bronchodilator reversibility (<10% FEV_1 % predicted normal), 2) optimal medical therapy without regular use of systemic corticosteroids and 3) absence of other significant diseases that could contribute to exercise limitation. Of these, 10 patients were characterized by peripheral muscle atrophy (FFMI <17 kg/m^2 [3] as assessed by bioelectrical impedance (Bodystat 1500, Babysat Ltd, UK). Patients signed an informed consent that was approved by the University Ethics Committee.

Study design

Both cachectic and non-cachectic patient groups were admitted to a multidisciplinary pulmonary rehabilitation program as previously described [18] and detailed in the online depository. Prior to and upon completion of the program, patients were assessed for pulmonary function, exercise tolerance, and quality of life. A percutaneous muscle biopsy was also performed on all patients before and after rehabilitation (see below).

Assessment

Initial assessment included: 1) resting pulmonary function and subdivisions of lung volumes by body plethysmography (Medgraphics Autolink 1085D; MN, USA); 2) incremental cycle ergometer test (Ergoline 800; Sensor Medics, CA, USA), 3) the 6 min walk test and 4) the St' Georges Respiratory Questionnaire for assessing quality of life (QoL).

Muscle biopsy

Vastus lateralis muscle percutaneous biopsies were obtained 24 hours after the first (baseline) and 24 hours after the last (post) training session as described by Bergstrom [19]. Biopsies were analyzed blindly for fibre type classification, fibre cross-sectional area and capillary to fibre ratio as previously described [5, 19] and detailed in the online depository.

Quantitative real-time PCR

Total RNA was extracted from 30 mg of muscle biopsies using an RNeasy Fibrous Tissue (Qiagen, West Sussex, UK). Quantitative real-time PCR was performed using a Chromo4 Detector and PTC-200 Peltier Thermal Cycler and analysed with the Opticon software 2.03 (MJ Research, Massachusetts, USA). Primer sequences for TNF-α, IGF-I, MGF (that is the load sensitive splice variant of IGF-I), MyoD, Akt and Myostatin and the primer sequences for cloning partial sequences of each gene are given in Tables 1 and 2 in the online depository.

Muscle protein immunoblotting

Proteins were extracted using 10 volumes of a lysis buffer containing protease inhibitors (Complete Mini, Roche Diagnostics, Mannheim Germany) and phosphatase inhibitors (Complete PhosSTOP, Roche Diagnostics, Mannheim Germany). Western blotting techniques for measuring TNF-α, IGF-I, MyoD, Myostatin, Atrogin-1, MURF-1 and protein tyrosine nitration, as well as total and phosphorylated Akt and IκB proteins together with the antibodies and dilutions are detailed in the online depository.

Statistical analyses

The main outcome measures were the mean muscle fibre cross sectional area, and the local muscle MGF and MyoD mRNA expression in cachectic patients. The minimum sample size for the group of cachectic patients was calculated based on 80% power and a two-sided 0.05 significance level using the Statistica 8.0 program. Sample size capable of detecting a change following rehabilitation of 500 μm^2 in mean fibre cross sectional area, 0.6 units of MGF/GAPDH mRNA expression and 1.0 unit of MyoD/GAPDH mRNA expression was estimated using data obtained from a previous study of our laboratory [5] and the following standard deviations: 980 μm^2, 0.61 and 0.85 units, respectively. The critical sample size was estimated to be 10 patients.

Data are presented as mean ± SEM. SEM was chosen rather than SD because we were interested in the variance of the mean values rather that the inter-subject variance. Baseline

demographic and muscle fibre morphological data between non-cachectic and cachectic patients were compared by unpaired Student's t-tests. Differences in protein expression between patient groups at baseline were tested by one-sample t-tests after setting the mean baseline value of non-cachectic patients at 100%. For muscle fibre morphological characteristics and for mRNA expression pre- and post-training group comparisons were made by a two-way ANOVA with repeated measures followed by the LSD test for post-hoc analyses. Rehabilitation-induced changes in protein expression within each group (presented relative to the mean baseline value set at 100%) were carried out by one-sample t-tests. Between-group comparisons of rehabilitation-induced changes in protein expression were made by unpaired Student's t-tests. The level of statistical significance was set at $p<0.05$.

Results

Baseline patient characteristics

Cachectic and non-cachectic patients were well matched according to age and severity of airflow obstruction (Table 1). Cycle ergometer exercise tolerance, the 6-min walking distance and the total score of the St' Georges Questionnaire were, however, each lower ($p<0.05$) in the cachectic patients (Table 1). Vastus lateralis muscle fibre type distribution was not different between groups (Table 2). In contrast, the mean muscle fibre cross sectional area was lower ($p=0.035$) in the cachectic patients (Table 2). This difference was attributable to the lower ($p<0.05$) cross sectional areas of type IIa and IIb fibres (Table 2). Capillary: fibre ratio was not significantly different between groups (Table 2).

Effects of training on exercise tolerance and quality of life

Mean training intensity sustained during the rehabilitation program was not different between cachectic and non-cachectic (126±13 and 117±10% of baseline peak work rate, respectively) patients. Accordingly, peak work rate on the cycle ergometer was increased by a similar percentage in both cachectic patients (by 16±2%; $p=0.011$) and non-cachectic (by 18±3%; $p=0.001$). Similarly, the 6-min walk distance increased ($p< 0.05$) in both cachectic (by 53±16 m) and non-cachectic (by 42±13 m) patients with no difference between groups. However, none of the groups exhibited a clinically meaningful mean improvement, (>54 m). The total St' Georges Questionnaire score was improved (reduced) by a clinically meaningful margin (-6±2 units) only in non-cachectic patients, whereas it remained unchanged (-1±1 units) in cachectic patients.

Vastus lateralis muscle fibre morphological adaptations

Following rehabilitation, vastus lateralis mean muscle fibre cross-sectional area was increased in both cachectic (by 264±66 μm^2; $p=0.003$) and non-cachectic (by 438±66 μm^2; $p<0.001$) patients; however, the increase was relatively greater ($p=0.039$) in the non-cachectic

(11±2%) compared to cachectic (7±2%) patients (Table 3). Although both groups increased cross sectional areas of type IIa and IIb muscle fibres by a similar magnitude ($p<0.01$), only non-cachectic patients increased the cross sectional area of type I fibres (Table 3, $p=0.001$). In addition, whilst both groups equally decreased ($p<0.005$) the proportion of type IIb fibres, non-cachectic patients increased the proportion of type I fibres ($p=0.01$), whereas cachectic patients increased the proportion of type IIa fibres ($p=0.012$) (Table 3). Furthermore, capillary: fibre ratio was increased ($p<0.02$) similarly in both groups (Table 3).

Rehabilitation-induced molecular adaptations in peripheral muscle

Western blotting revealed that at baseline cachectic and non-cachectic COPD patients exhibited different levels of expression among the remodeling markers tested (Figure 1A). Accordingly, cachectic patients showed significantly lower levels of expression of both IGF-I and MyoD. In contrast, the expression of TNF-α, total IκB-α and Myostatin was similar between groups (Figure 1A). Total Akt protein expression was significantly greater in the cachectic patients (Figure 1A). The ratio of phosphorylated IκB-α to total IκB-α was higher in the cachectic group (Figure 1B). In addition at baseline, the ratio of phosphorylated Akt to total Akt was also significantly greater in cachectic patients (Figure 1C). Prior to the initiation of the pulmonary rehabilitation program there were not significant differences in protein expression of total tyrosine nitration, and MURF-1 between cachectic and non-cachectic patients. However, only in the muscle of cachectic COPD patients, Atrogin-1 protein expression was significantly increased (by 145±54% compared to non-cachectic).

Post-training, there was an increase ($p<0.02$) in the mRNA levels of IGF-I in both groups (Table 3). Similarly, there was an increase ($p<0.05$) in the mRNA levels of MGF in both cachectic (from 1.18±0.31 to 2.24±0.71 mRNA copies/10^4 GAPDH copies) and non-cachectic (from 0.82±0.14 to 1.21±0.13 mRNA copies/10^4 GAPDH copies) patients. The increase in mRNA levels of MGF was greater ($p=0.027$) in cachectic patients. In these patients

rehabilitation-induced changes in MGF mRNA expression were significantly correlated (r= 0.65, p=0.034) with the respective changes in cycle ergometry peak work rate. In non-cachectic patients rehabilitation significantly increased IGF-I protein expression two-fold (Figure 2B). In contrast, cachectic patients did not show increased IGF-I protein expression after the rehabilitation program. In addition, the post-training IGF-I protein expression levels were significantly greater in non-cachectic than cachectic patients (Figure 2B).

Non-cachectic patients exhibited a significant increase in MyoD mRNA after training (Table 3), consistent with the percentage increase in protein levels (Figure 3C). Cachectic patients, however, did not show increased post-training MyoD mRNA (Table 3) or protein (Figure 2C) expression. After training, the level of MyoD mRNA and protein expression in non-cachectic patients was significantly higher than in cachectic patients (Figure 2C).

Rehabilitation significantly reduced Myostatin mRNA (Table 3) and protein levels (Figure 3B) in non-cachectic patients. In contrast, this was not seen in cachectic patients (Figure 3B). TNF-α was not affected at either mRNA (Table 3) or protein levels (Figure 3C) by rehabilitative exercise in either group. Post-training mRNA TNF-α expression was significantly greater in cachectic compared to non-cachectic patients (Table 3).

Prior to rehabilitation, both groups of patients had the same amount of total cytoplasmic IκB-α (Figure 1A). After rehabilitation the ratio of phosphorylated IκB-α to total IκB- α, was significantly ($p<0.05$) decreased in both groups by the same amount (Figure 4B). Furthermore, prior to rehabilitation, cachectic patients had greater amounts of total cytoplasmic Akt (Figure 1A). After rehabilitation the ratio of phosphorylated Akt to total Akt did not increase in either cachectic or non-cachectic patients (Figure 4D.

Rehabilitation induced a significant increase in muscle protein nitration only in cachectic patients (Figure 5A). Interestingly, following exercise training Atrogin-1 and MURF-1 protein expression was significantly increased in cachectic patients, whereas it was significantly decreased in non cachectic patients (Figure 5 C&D)

Discussion

The present study shows that rehabilitative exercise training significantly improves exercise tolerance and induces significant adaptations in vastus lateralis muscle fibre size, typology and capillarization in cachectic patients with COPD. Whilst in these patients rehabilitation had no effect on Myostatin or TNF-α protein expression, it decreased the activation of the transcription factor NF-κB by the same amount as in non-cachectic patients. Furthermore, even if the magnitude of increase in the mean muscle fibre cross sectional area and the proportion of type I muscle fibres was greater in non-cachectic patients with COPD, those with cachexia were able to increase exercise capacity as much as those without cachexia. The observed limited training effect on muscle fibre remodeling in cachectic patients could be attributed to simultaneous activation of hypertrophy (expression of local muscle growth factors) and atrophy (ubiquitin-proteasome) signaling pathways by exercise training. As in non-cachectic patients the type of muscle fibre remodeling in terms of expression of signaling pathways (primarily hypertophic) and of phenotypical adaptations were different among cachectic and non-cachectic COPD, it is apparent that exercise training produced both quantitative and qualitative differences in muscle fibre remodeling among patients populations.

Baseline muscle remodeling markers

The biochemical pathways engaged in the development of muscle atrophy are thought to result from an imbalance between protein synthesis and breakdown; however the exact mechanisms involved are not fully understood [17]. Accordingly, molecular factors regulating muscle remodelling were analysed at baseline and compared between patient groups. Our study shows for the first time that baseline protein expression of Atrogin-1, a ligase regulating ubiquitin-mediated protein degradation [9], was significantly increased in the muscle of cachectic patients, whereas IGF-I and MyoD protein expression were lower in cachectic patients (Figure 1). Others have compared IGF-I and MyoD expression between COPD patients and healthy age-matched subjects [20] and found that the expression of these two

anabolic markers were reduced in hospitalised and clinically stable COPD patients compared to healthy controls. The study by Crul et al. [20] suggested that diminished physical activity may be the main mechanism accounting for the decreased muscle IGF-I and MyoD levels in COPD. In support of this notion, physical inactivity has been shown to be associated with decreased IGF-I mRNA levels and alterations in MyoD/myogenin expression ratio in a mouse muscle disuse model [21-22]. In contrast to IGF-I and MyoD protein expression, we found that the phosphorylated Akt to total Akt ratio was significantly increased in cachectic COPD (Figure 1C), an indication previously interpreted as a failed attempt to maintain or restore muscle mass in patients with COPD [23].

On the other hand, greater muscle fibre atrophy in cachectic compared to non-cachectic patients could also be explained by greater activation of the NF-κB transcription factor complex [6, 7]. Although at baseline cachectic and non-cachectic patients did not differ in terms of the level of expression of total IκB-α (Figure 1A), we found that the phosphorylated IκB-α to total IκB-α ratio, was two-fold greater in cachectic compared to non-cachectic patients (Figure 1B). Interpretation, however, of these findings has to be done with caution because the IκB-α ratio utilized is an indicator of the NF-kB activation and as such further experiments measuring activation of Nf-kB in the nucleus were not possible due to muscle tissue limitation.

NF-κB can be activated by a number of cachexia-associated factors including TNF-α [7]. Although we did not find significant differences in baseline local muscle mRNA or protein TNF-α expression between study groups (Figure 1A), we can not exclude the possibility that the greater degree of muscle wasting seen in cachectic COPD was instigated by increased local muscle expression of cytokines (other than TNF-α) which are involved in inflammation, such as IL-1β, IL-6, and which are thought to trigger muscle wasting via NF-κB activation [24]. Nevertheless, a recent study [24] demonstrated muscle atrophy due to systemic inflammation-dependent chronic activation of NF-κB in muscle of mice without increased TNF-α, IL-6 or IL-

8 mRNA levels. Consistent with a recent study [16] we found at baseline no differences in muscle protein tyrosine nitration between cachectic and non cachectic patients.

In muscle, NF-κB can also inhibit mRNA and protein expression of MyoD [14, 25], an essential transcription factor in myogenesis [9]. Down-regulation of MyoD expression can also be induced by over-expression of Myostatin, a strong negative regulator of muscle growth [15]. However, we found that at baseline, Myostatin protein expression was not different in cachectic compared to non-cachectic patients, thereby suggesting that the lower MyoD expression in cachectic patients is probably due to the increased activation of NF-κB [6,7].

Exercise-induced effects on exercise capacity and muscle remodeling markers

Cachectic and non-cachectic COPD patients were selected to control for differences in patient phenotype. At the outset of the study, cachectic patients were characterized by poorer exercise capacity and greater peripheral muscle fibre atrophy (Tables 1-2). Following rehabilitation, exercise capacity was increased similarly in both cachectic and non-cachectic patients most likely as a result of exercising both groups at a similar overall training load.

In addition, confirming our previous findings in non-cachectic COPD [5], we have shown for the first time that rehabilitation induces a significant increase in vastus lateralis muscle mean fibre cross sectional area and capillarization in cachectic patients. In addition, rehabilitative exercise reduced the proportion of IIb fibres in favor of the more oxidative fibres (i.e.: type IIa). These findings suggest that peripheral muscle remodeling in response to exercise training is at least partially preserved in cachectic patients with COPD. Indeed, we observed that muscle fibre hypertrophy was accompanied by significant up-regulation of the local muscle IGF-I and MGF mRNA expression that are known to play an important role in the hypertophic adaptation of muscle to overload [23, 26]. In this study the magnitude of increase in IGF-I mRNA expression after rehabilitation in cachectic COPD (by 88%) was similar to that reported for the non-cachectic patients in the present (by 79%) and previous (by 75%) studies [5]. However, the increase in IGF-I mRNA expression after rehabilitation was translated at

protein level only in non-cachectic COPD (Figure 2B); this may explain the greater degree of peripheral muscle fibre hypertrophy found in this group and highlights a potential post-transcriptional modification in cachectic patients. In addition, mRNA degradation may also act as an important mechanism in the regulation for the expression of muscle IGF-I [27].

Another potential factor that may account for the lower degree of peripheral muscle fibre hypertrophy observed in cachectic patients is their inability to up-regulate local muscle MyoD in response to rehabilitative exercise (Table 3 and Figure 2C). MyoD is expressed in muscle satellite cells and mature myofibers and has been implicated in mediating the process of cell proliferation and differentiation for subsequent muscle regeneration and hypertrophy [14].

Within the present study we investigated the effects of exercise training on two factors that are known to modulate the expression of local muscle MyoD, namely TNF-α and Myostatin [14, 15]. Myostatin down-regulation in response to exercise training in healthy humans has been shown to be essential for muscle growth [28]. In non-cachectic COPD patients, resistance training has been shown to induce a modest reduction in Myostatin mRNA abundance [29]. The results of the present study therefore expand those in non-cachectic COPD [29] by demonstrating that endurance training induced significant reductions in both mRNA and protein Myostatin expression (Table 3 and Figure 3B). Rehabilitation-induced reduction in local muscle Myostatin expression in non-cachectic patients was accompanied by a significant upregulation of both mRNA and protein expression for MyoD, thereby confirming the results of our previous study [5]. In contrast in cachectic patients, neither mRNA nor protein expression for Myostatin was significantly reduced (Table 3 and Figure 3B). This finding may explain the lack of significant training-induced up-regulation of local muscle MyoD expression in cachectic COPD and account for the lower degree of fibre hypertrophy.

Relationship between markers of inflammation and training responses

Muscle TNF-α mRNA and protein expression were not significantly affected by exercise training in cachectic patients (Table 3 and Figure 3C), thus expanding the results of

previous studies carried out in patients without peripheral muscle wasting [5, 30]. Interestingly, we found that the phosphorylated IκB-α to total IκB-α ratio was significantly down-regulated in both groups following rehabilitation, most likely indicating reduced activation of the NF-κB transcription factor (Figure 4B).

Emerging evidence suggests that NF-κB is involved in one of the most important signaling pathways linked to the loss of skeletal muscle mass in various physiological and pathophysiological conditions. A recent study using an animal model has provided unequivocal evidence that specific modulation of NF-κB activity can prevent skeletal muscle loss [31]. It has recently been recognized that NF-κB can be activated not only by cachectic factors (such as TNF-α) but also by muscle disuse [9, 32]. Muscle disuse triggers an NF-κB activation pathway that is distinctly different from that triggered by a cytokine (e.g. TNF-α) [32]. If this is correct, one would expect that exercise training would decrease the phosphorylation ratio of the inhibitory IκB-α which is in fact what the present study demonstrates (Figure 4B), thereby indicating NF-κB as a possible target for exercise training. It is thus possible that when disused, atrophic peripheral muscles of COPD patients undergo exercise training, NF-κB is progressively de-activated, as indicated by the decreased phosphorylation of IκB-α, thus allowing a number of downstream signaling pathways to be activated and promote muscle hypertrophy. On the other hand, as baseline NF-κB activity was twice as high in cachectic compared to non cachectic patients (Figure 1B) and since the phosphorylated IκB-α to total IκB-α ratio was down-regulated in both groups following rehabilitation by the same magnitude (Figure 4B), it is conceivable that NF-κB activity was still greater in cachectic patients following rehabilitation, thereby limiting the degree of muscle remodeling.

The limited degree of muscle remodeling in cachectic compared to non-cachectic patients can also be justified by our findings that exercise training caused a greater induction of muscle protein nitrotyrosination compared to non-cachectic patients [16], most likely facilitating degradation through the ubiquitin proteasome pathway [6]. The present study is the

first to provide proof for this notion since we found that two specific regulators of ubiquitin-mediated muscle proteolytic pathway (Atrogin-1 and MURF-1) were up-regulated following rehabilitation only in cachectic patients. In contrast, in non cachectic patients, Atrogin-1 and MURF-1 protein expression was down-regulated, whereas growth and regeneration factors were unregulated, thus justifying the greater degree of muscle fibre hypertrophy in these patients. The diverse response to exercise training between cachectic and non-cachectic patients resembles that recently documented [33] in COPD patients and age-matched healthy controls where the protein degradation pathway was up-regulated only in COPD. It is likely therefore that exercise training produces predominantly an anabolic influence among non cachectic COPD, while in cachectic COPD regular muscular activity simultaneously activates anabolic and catabolic pathways, thereby limiting the magnitude of the training effect.

Study limitations

The invasive nature of the muscle biopsies precluded a parallel, age-matched, healthy control, training group which would have allowed comparisons in training-induced changes in local muscle anabolic and catabolic factors between healthy subjects and COPD patients with or without muscle wasting. In addition, for the same reason a control COPD group, i.e.: not undertaking rehabilitation was not studied. Such a group would have distinguished which of the effects of rehabilitation were due to a true effect of training, and which are due to between –occasion muscle sampling differences. Furthermore, absence of quadriceps measurements of strength or mid-thigh cross sectional area is acknowledged as a limitation in the present study. Recent studies [23, 34] have, however, shown reduced mid thigh cross sectional area as well as strength in cachectic compared to non-cachectic COPD patients and healthy controls. Furthermore, both in vivo and in vitro muscle function measurements would have provided more adequate evidence to link the observed molecular/cellular changes with the clinical outcomes of pulmonary rehabilitation in cachectic COPD. Another potential limitation in this study was that cachectic patients were also those showing worse lung function. Indeed, it is

well known that weight loss and increasing severity of COPD are often associated to each other and both lead to poor prognosis of the disease [2].

It is concluded that although skeletal muscle training produces greater peripheral muscle fibre phenotypical adaptations in non-cachectic compared to cachectic patients with COPD, cachectic patients retain the capacity for peripheral muscle remodeling in response to rehabilitation and are able to increase exercise capacity as much as those without cachexia.

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References

1. Morley JE, Thomas DR, Wilson M. Cachexia: pathophysiology and clinical relevance. *Am J Clin Nutr* 2006;83(4):735-743.

2. Schols AMWJ, Broekhuizen R, Weling-Scheepers CA, Wouters EF. Body composition and mortality in chronic obstructive pulmonary disease. *Am J Clin Nutr* 2005;82(1):53-59.

3. Wagner PD. Possible mechanisms underlying the development of cachexia in COPD. *Eur Respir J* 2008;31(3):492-501.

4. Zinna EM, Yarasheski KE. Exercise treatment to counteract protein wasting of chronic diseases. *Curr Opin Clin Nutr Metab Care* 2003;6(1):87-93.

5. Vogiatzis I, Stratakos G, Simoes DCM, Terzis G, Georgiadou O, Roussos C, Zakynthinos S. Effects of rehabilitative exercise on peripheral muscle TNF{alpha}, IL-6, IGF-I and MyoD expression in patients with COPD. *Thorax* 2007;62(11):950-956.

6. Agusti A, Morla M, Sauleda J, Saus C, Busquets X. NF-{kappa}B activation and iNOS upregulation in skeletal muscle of patients with COPD and low body weight. *Thorax* 2004;59(6):483-487.

7. Rutten EPA, Franssen FME, Engelen MPKJ, Wouters EFM, Deutz NEP, Schols AMWJ. Greater whole-body myofibrillar protein breakdown in cachectic patients with chronic obstructive pulmonary disease. *Am J Clin Nutr* 2006;83(4):829-834.

8. Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, Andersen JS, Mann M, Mercurio F, Ben-Neriah Y. Identification of the receptor component of the I[kappa]B[alpha]-ubiquitin ligase. *Nature* 1998;396(6711):590-594.

9. Jackman RW, Kandarian SC. The molecular basis of skeletal muscle atrophy. *Am J Physiol Cell Physiol* 2004;287(4):C834-843.

10. Li Y-p, Schwartz RJ, Waddell ID, Holloway BR, Reid MB. Skeletal muscle myocytes undergo protein loss and reactive oxygen-mediated NF-{kappa}B activation in response to tumor necrosis factor {alpha}. *FASEB J.* 1998;12(10):871-880.

11. Barreiro E, Schols AMWJ, Polkey MI, Polkey MI, Galdiz JB, Gosker HR, Swallow EB, Coronell C, Gea J. Cytokine profile in quadriceps muscles of patients with severe COPD. *Thorax* 2008;63(2):100-107.

12. Montes de Oca M, Torres SH, De Sanctis J, Mata A, Hernandez N, Talamo C. Skeletal muscle inflammation and nitric oxide in patients with COPD. *Eur Respir J* 2005;26(3):390-397.

13. Broussard SR, McCusker RH, Novakofski JE, Strle K, Shen WH, Johnson RW, Freund GG, Dantzer R, Kelley KW. Cytokine-hormone interactions: Tumor Necrosis Factor {alpha} impairs biologic activity and downstream activation signals of the Insulin-Like Growth Factor I Receptor in myoblasts. *Endocrinology* 2003;144(7):2988-2996.

14. Langen RCJ, Van Der Velden JLJ, Schols AMWJ, Kelders MCJM, Wouters EFM, Janssen-Heininger YMW. Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization. *FASEB J.* 2004;18(2):227-237.

15. Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. Myostatin Inhibits Myoblast Differentiation by Down-regulating MyoD Expression. *J. Biol. Chem.* 2002;277(51):49831-49840.

16. Barreiro E, Rabinovich R, Marin-Corral J, Barbera JA, Gea J, Roca J. Chronic endurance exercise induces quadriceps nitrosative stress in severe COPD patients. Thorax 2009;64(1): 13-19

17. Glass DJ. Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 2005;37(10):1974-84.

18. Vogiatzis I, Terzis G, Nanas S, Stratakos G, Simoes DC, Georgiadou O, Zakynthinos S, Roussos C. Skeletal muscle adaptations to interval training in patients with advanced COPD. *Chest* 2005;128(6):3838-3845.

19. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* 1975;35(7):609-16.

20. Crul T, Spruit MA, Gayan-Ramirez G, Quarck R, Gosselink R, Troosters T, Pitta F, Decramer M. Markers of inflammation and disuse in vastus lateralis of chronic obstructive pulmonary disease patients. *Eur J Clin Invest* 2007;37(11):897-904.

21. Awede B, Thissen J, Gailly P, Lebacq J. Regulation of IGF-I, IGFBP-4 and IGFBP-5 gene expression by loading in mouse skeletal muscle. *FEBS Lett* 1999;461(3):263-267.

22. Mozdziak PE, Greaser ML, Schultz E. Myogenin, MyoD, and myosin heavy chain isoform expression following hindlimb suspension. *Aviat Space Environ Med* 1999;70(5):511-516.

23. Doucet M, Russell AP, Leger B, Debigaré R, Joanisse DR, Caron MA, LeBlanc P, Maltais F. Muscle atrophy and hypertrophy signaling in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2007;176(3):261-269.

24. Cai D, Frantz JD, Tawa NE, Jr., Melendez PA, Oh BC, Lidov HG, Hasselgren PO, Frontera WR, Lee J, Glass DJ, Shoelson SE. IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell* 2004;119(2):285-298.

25. Di Marco S, Mazroui R, Dallaire P, Chittur S, Tenenbaum SA, Radzioch D, Marette A, Gallouzi IE. NF-kappa B-mediated MyoD decay during muscle wasting requires nitric oxide synthase mRNA stabilization, HuR protein, and nitric oxide release. *Mol Cell Biol* 2005;25(15):6533-6545.

26. Kostek MC, Delmonico MJ, Reichel JB, Roth SM, Douglass L, Ferrell RE, Hurley BF. Muscle strength response to strength training is influenced by insulin-like growth factor 1 genotype in older adults. *J Appl Physiol* 2005;98(6):2147-2154.

27. Moore MJ. From birth to death: the complex lives of eukaryotic mRNAs. *Science* 2005; 309: 1514–1518,

28. Jones SW, Hill RJ, Krasney PA, O'Conner B, Peirce N, Greenhaff PL. Disuse atrophy and exercise rehabilitation in humans profoundly affects the expression of genes associated with the regulation of skeletal muscle mass. *FASEB J.* 2004:18(9): 1025-1037.

29. Lewis MI, Fournier M, Storer TW, Bhasin S, Porszasz J, Ren SG, Da X, Casaburi R. Skeletal muscle adaptations to testosterone and resistance training in men with COPD. *J Appl Physiol* 2007;103(4):1299-1310.

30. Langen RCJ, Schols AMWJ. Inflammation: friend or foe of muscle remodelling in COPD? *Eur Respir J* 2007;30(4):605-607.

31. Van Gammeren D, Damrauer JS, Jackman RW, Kandarian SC. The IkappaB kinases IKKalpha and IKKbeta are necessary and sufficient for skeletal muscle atrophy. *FASEB J* 2009;23(2):362-70.

32. Hunter RB, Stevenson EJ, Koncarevic A, Mitchell-Felton H, Essig DA, Kandarian SC. Activation of an alternative NF-{kappa}B pathway in skeletal muscle during disuse atrophy. *FASEB J.* 2002;16(6):529-538.

33. Radom-Aizik S, Kaminski N, Hayek S, Halkin H, Cooper DM, Ben-Dov I. Effects of exercise training on quadriceps muscle gene expression in chronic obstrctive pulmonary disease. *J Appl Physiol* 2007;102:1976-1984.

34. Plant PJ, Brooks D, Faughnan M, Bayley T, Bain J, Singer L, Correa J, Pearce D, Binni M, Batt J. Cellular markers of muscle atrophy in Chronic Obstructive Pulmonary Disease (COPD). *Am J Respir Cell Mol Biol* 2009 [Epub ahead of print]

Table 1. Demographic, lung function, exercise tolerance and quality of life (SGRQ) assessment of non-cachectic and cachectic COPD patients

	Non-Cachectic (n = 19)	Cachectic (n = 10)
Age (yr)	67 ± 2	63 ± 2
Weight (kg)	77 ± 3	60 ± 3 *****
BMI (kg/m^2)	27.8 ± 1.2	21.5 ± 0.7 *****
FFM Index (kg/cm^2)	19.0 ± 0.4	15.6 ± 0.4 *****
FEV_1 (L)	1.18 ± 0.12	0.98 ± 0.14
FEV_1 (% pred.)	44.1 ± 4.8	37.5 ± 6.1
FVC (L)	2.84 ± 0.19	2.49 ± 0.17
FVC (% pred.)	80.0 ± 5.2	72.9 ± 6.0
TLC (% pred.)	108.7 ± 5.5	126.2 ± 7.9
FRC (% pred.)	152.3 ± 10.5	172.6 ± 12.4
RV (% pred.)	147.6 ± 21.1	178.7 ± 40.8
TLCO (% pred)	51.3 ± 5.6	42.8 ± 4.9 *****
Peak WR (Watts)	53 ± 4	43 ± 6*****
Peak VO_2 (ml/kg/min)	15 ± 0.97	14.1 ± 1.62
6 Min Walking Distance (m)	344 ± 21	273 ± 44*****
SGRQ total score	45.22 ± 3.71	40.97 ± 4.99*****

Values are means (±SEM). Asterisks denote significant differences compared to non-cachectic patients at $p<0.05$ (unpaired t-test). BMI: Body mass index, FFM Index: Fat Free mass index, FEV_1: forced expiratory volume in one second, FVC: forced vital capacity, TLC: total lung capacity, FRC: functional residual capacity, RV: residual volume, TLCO: transfer factor for CO; WR: Work rate, VO_2: oxygen uptake and quality of life total score assessed by the SGRQ.

Table 2. Muscle fibre morphological characteristics of non-cachectic and cachectic COPD patients at baseline

	Non-Cachectic (n = 19)	Cachectic (n = 10)
Muscle fibre type I (%)	32.0 ± 3.2	33.6 ± 2.9
Muscle fibre type II (%)	67.5 ± 3.3	65.9 ± 3.0
Muscle fibre type IIa (%)	52.3 ± 3.7	50.4 ± 6.5
Muscle fibre type IIb (%)	15.2 ± 2.2	15.5 ± 3.6
Mean muscle fibre cross sectional area	4509 ± 198	3872 ± 258*****
Cross sectional area type I (μm^2)	4716 ± 271	4717 ± 215
Cross sectional area type IIa (μm^2)	4507 ± 247	3695 ± 337*****
Cross sectional area type IIb (μm^2)	3649 ± 208	2872 ± 250*****
Capillary/Fibre ratio	1.41 ± 0.10	1.44 ± 0.10

Values are means (±SEM). Asterisks denote significant differences compared to non-cachectic at $p<0.05$ (unpaired t-test).

Table 3. Effect of rehabilitation on muscle fibre morphological characteristics and on the mRNA expression of anabolic and catabolic factors

	Non-Cachectic (n = 19)			Cachectic (n = 10)		
	Pre	Post	% change	Pre	Post	% change
Muscle fibre type I (%)	32.0±3.2	38.7±3.5 †	6.6±2.3	33.6±2.9	30.0±6.6	-3.4±3.6 *
Muscle fibre type IIa (%)	52.3±3.7	51.1±3.9 †	-1.1±2.3	50.4±6.5	58.0±5.3 †	9.6±2.8 *
Muscle fibre type IIb (%)	15.2±2.2	9.3±1.4 †	-5.9±1.3	15.5±3.6	11.9±3.6 †	-5.4±2.1
Mean fibre cross sectional area	4509±198	4947± 227 †	10.9±1.7	3872±258*	4136±296 †	7.3±1.7
Cross sectional area type I (μm^2)	4716±271	5398 ±315 †	16.4±4.8	4717±215	4796±152	3.1±4.5*
Cross sectional area type IIa (μm^2)	4507±247	4954±251	11.4±3.5	3695±337*	4091±377 †	12.1±3.2
Cross sectional area type IIb (μm^2)	3649±208	3976±176 †	11.6±4.3	2872±250*	3224±388 †	12.1±3.9
Capillary / Fibre ratio	1.41±0.1	1.70±0.1 †	20.3±4.1	1.44±0.1	1.61±0.1 †	12.1±3.4
mRNA IGF-I/GAPDH expression	0.72±0.11	1.29±0.14 †	79±3	0.41±0.09	0.77±0.10 †	88±11
mRNA MGF/GAPDH expression	0.82±0.14	1.21±0.13 †	47±10	1.18±0.31	2.24±0.71 †	90±29 *
mRNA MyoD/GAPDH expression	1.30±2.83	2.20±4.16 †	69±17	3.10±1.1	2.20±8.7	-29±6 *
mRNA Myost./GAPDH expression	2.37±0.48	1.78±0.44 †	-25±11	2.02±0.48	1.96±0.27	-3±4 *
mRNA TNF-a/GAPDH expression	0.91±0.27	0.62±0.17	-32±37	1.52±0.42	1.45±0.55 †	-5±31 *

Values are mean ± S.E.M. Asterisks denote significant differences compared to non-cachectic, whereas crosses denote significant within-group differences, both at $p < 0.05$. All mRNA data were normalized against GAPDH number of copies, which were neither significantly different nor affected by exercise training in either of the two groups.

Figure Legends

Figure 1. *Factors associated with skeletal muscle hypertrophy and atrophy pathways at baseline***.** Protein expression of molecular markers in peripheral muscle homogenates from non-cachectic (white columns) and cachectic (grey columns) COPD patients were studied by Western blot at baseline for **A**: the level of TNF-α, Myostatin, IGF-I, MyoD, total IκB-α and total Akt protein expression, **B**: the ratio of phosphorylated IκB-α / Total IκB-α, and **C**: the ratio of phosphorylated Akt / Total Akt. Results of densitometric analyses (means ± SEM) for cachectic patients are presented relative to the mean baseline values of non-cachectic patients set at 100%. Crosses denote significant differences at $p<0.05$ between groups.

Figure 1

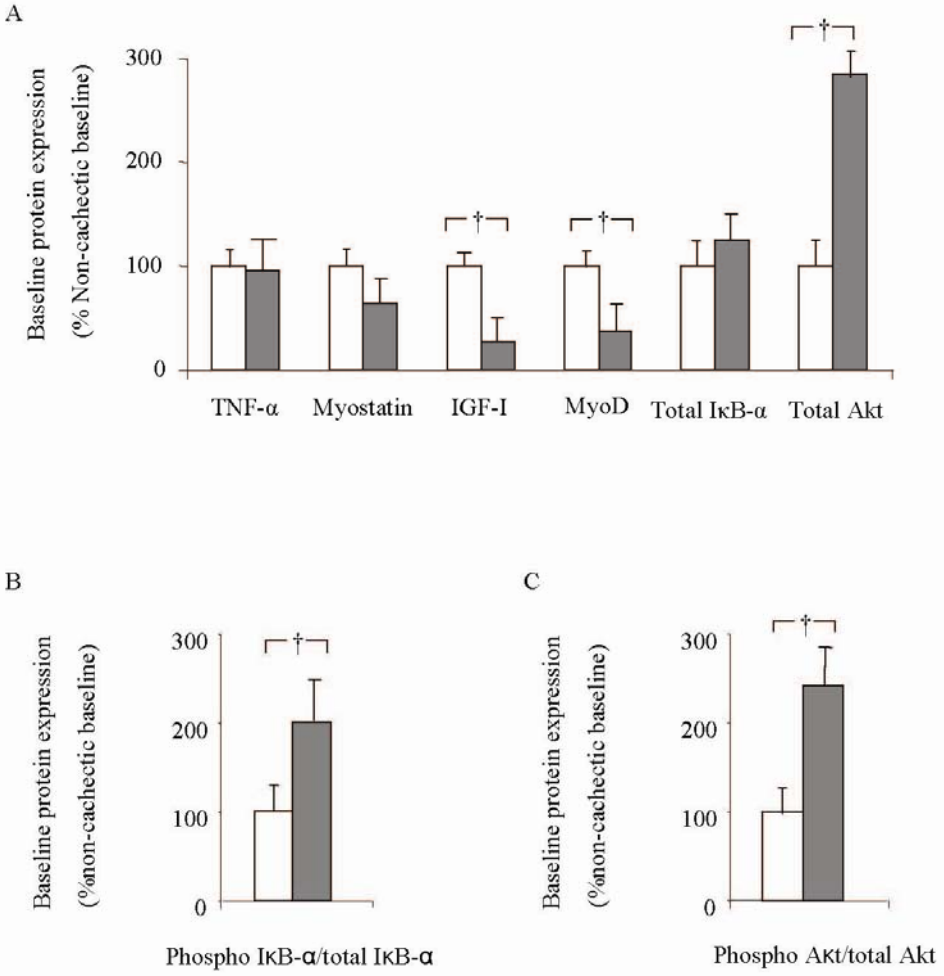


Figure 2. *IGF-I and MyoD expression following rehabilitation.* Representative Western blots for *IGF-I and MyoD* **(A)**. Densitometric analyses of Western blots from non-cachectic (white

columns) and cachectic (grey columns) COPD patients for IGF-I **(B)** and MyoD (**C)**. Rehabilitation-induced changes in protein expression within each group are presented relative to the mean baseline value set at 100%. Results are presented as mean±SEM. Asterisks denote significant differences at $p<0.05$ between baseline and post-rehabilitation for each group. Crosses denote significant differences between groups.

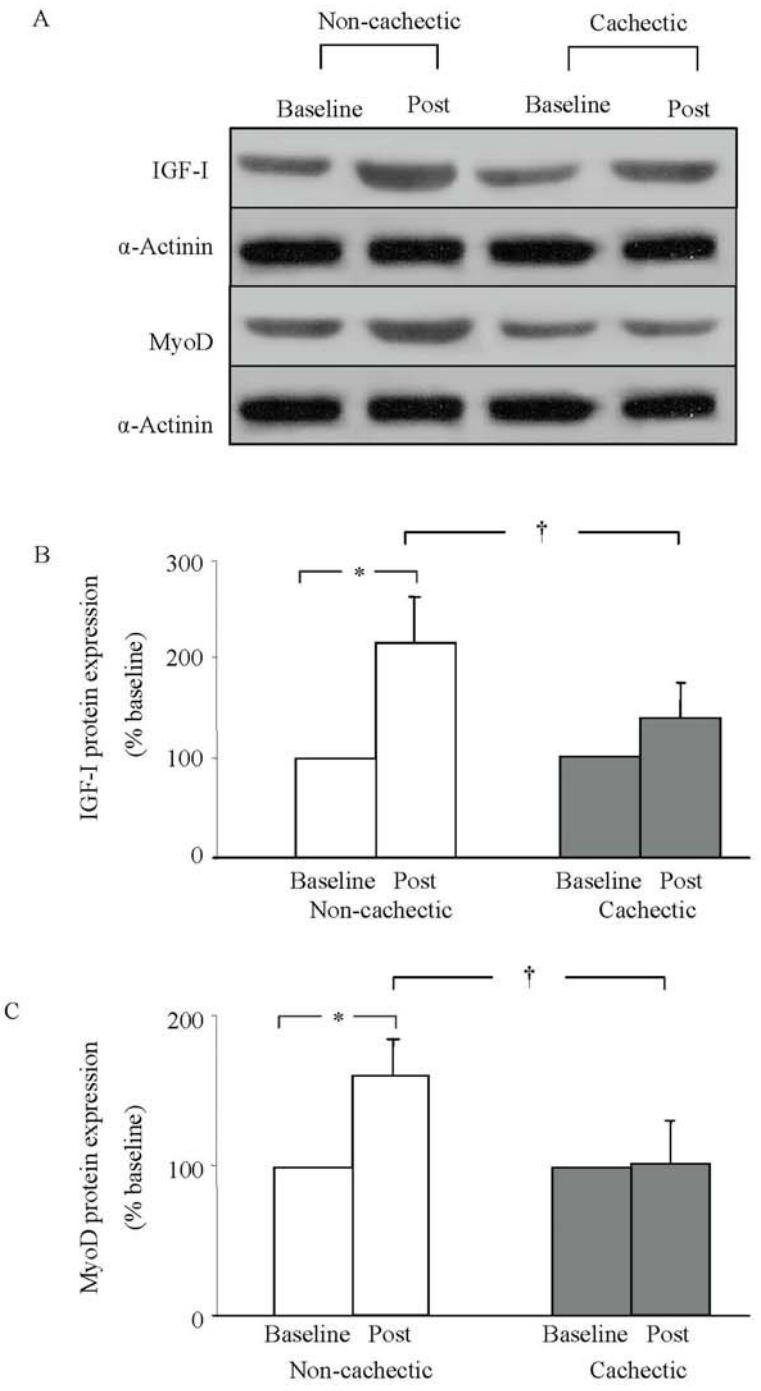


Figure 2

Figure 3. Myostatin and *TNF-α expression.* Representative Western blots for Myostatin and *TNF-α* **(A).** Densitometric analyses of Western blots from non-cachectic (white columns) and

cachectic (grey columns) COPD patients for Myostatin **(B)** and *TNF-α (***C)**. Rehabilitation-induced changes in protein expression within each group are presented relative to the mean baseline value set at 100%. Results are presented as mean ±SEM. Asterisks denote significant differences at $p<0.05$ between baseline and post-rehabilitation for each group.

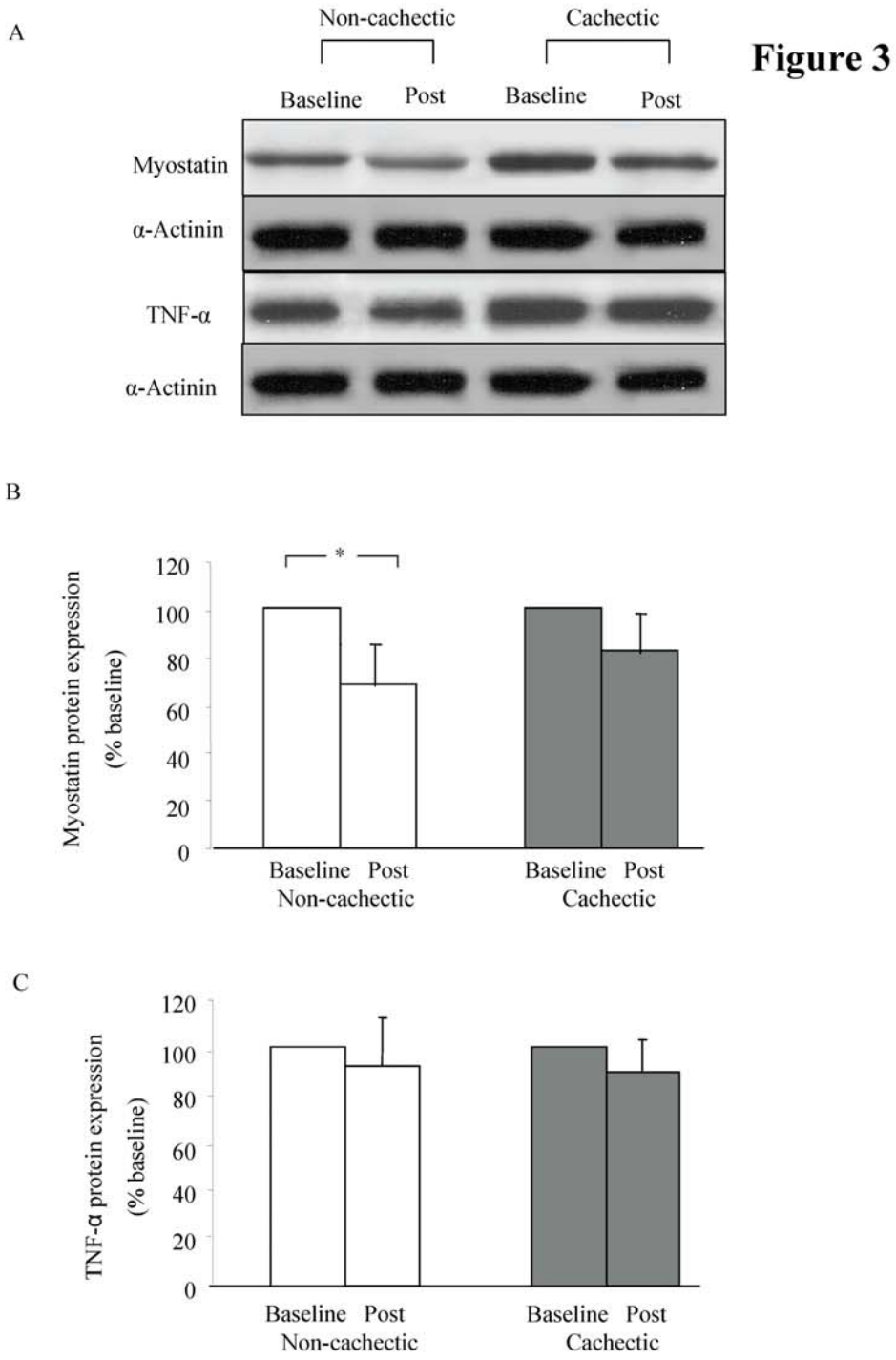


Figure 3

Figure 4. *Pulmonary rehabilitation decreases NF-κB activation and tends to increase* Akt *activation.* Representative Western blots for **A:** phosphorylated IκB-α, Total IκB-α and α-

Actinin, and **C:** phosphorylated Akt, Total Akt and α-Actinin**.** Densitometric analyses of blots studied in non-cachectic (white columns) and cachectic (grey columns) COPD patients for phosphorylated IκB-α (**B**) and for phosphorylated Akt (**D**). Rehabilitation-induced changes in protein expression within each group are presented relative to the mean baseline value set at 100%. Results are presented as mean±SEM. Asterisks denote significant differences at $p<0.05$ between baseline and post-rehabilitation for each group.

Figure 4

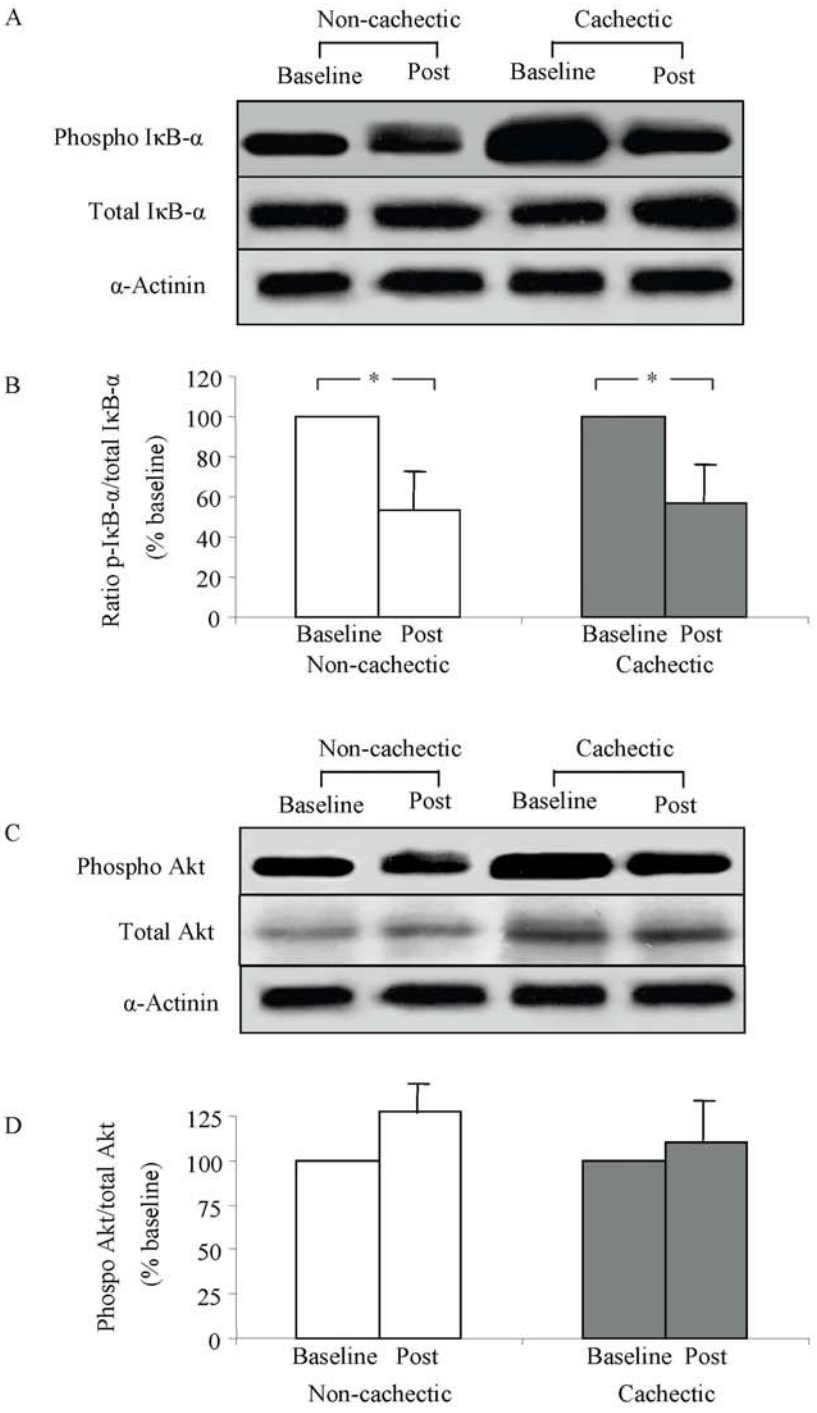
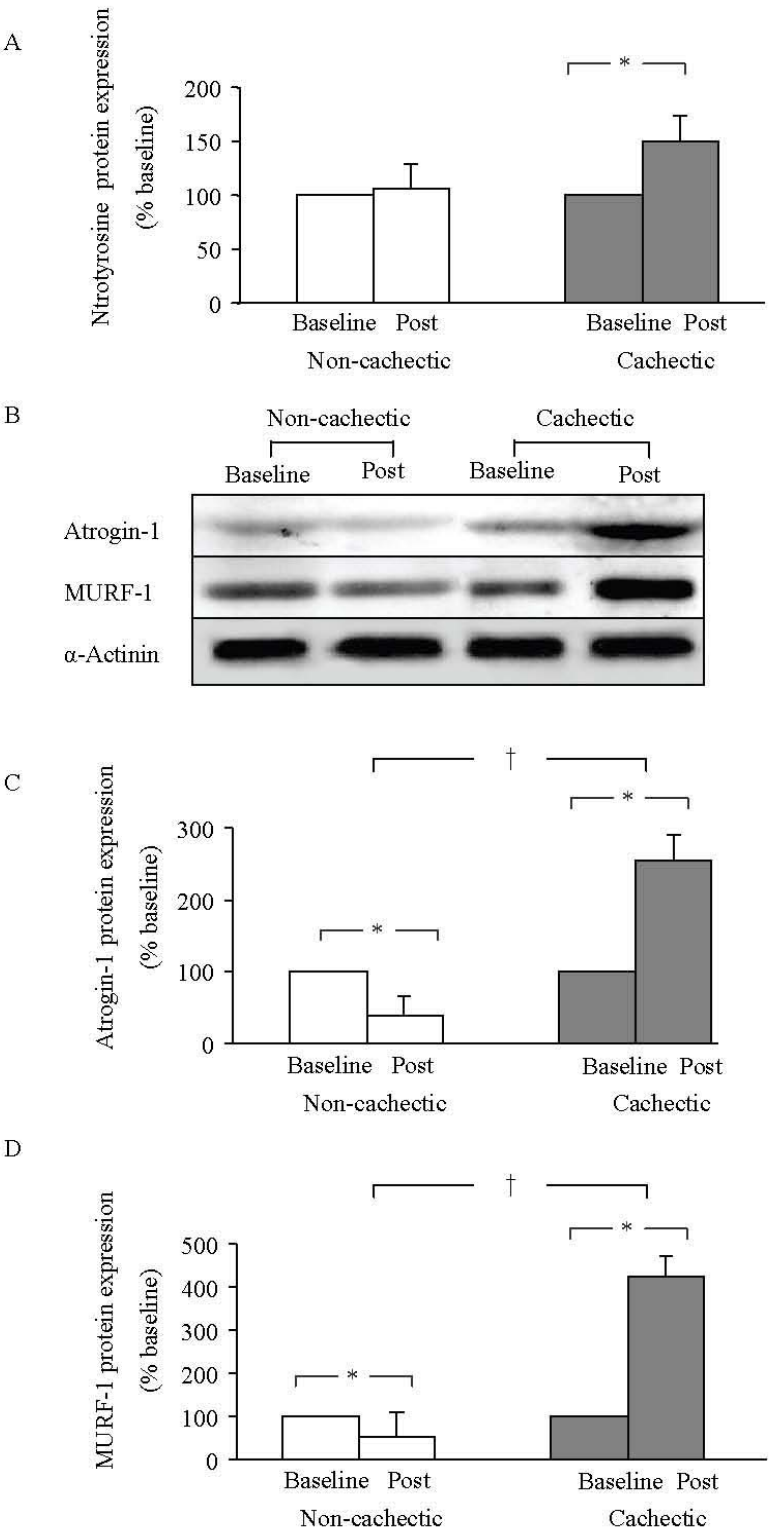


Figure 5. *Pulmonary rehabilitation increases* Ntrotyrosine, *Atrogin-1 and MURF-1 protein expression in cachectic patients.* Representative Western blots for Atrogin-1, MURF-1 and α-

Actinin **(B).** Densitometric analyses of blots studied in non-cachectic (white columns) and cachectic (grey columns) COPD patients for protein tyrosine nitration (**A**), *Atrogin-1* (**C**) and *MURF-1* (**D**). Rehabilitation-induced changes in protein expression within each group are presented relative to the mean baseline value set at 100%. Results are presented as mean±SEM. Asterisks denote significant differences at $p<0.05$ between baseline and post-rehabilitation for each group. Crosses denote significant differences between groups.

Figure 5


A
Ntrotyrosine protein expression
(% baseline)
200
150
100
50
0
*
Baseline Post
Non-cachectic
Baseline Post
Cachectic
B
Non-cachectic
Cachectic
Baseline
Post
Baseline
Post
Atrogin-1
MURF-1
α-Actinin
C
Atrogin-1 protein expression
(% baseline)
300
200
100
0
†
*
*
Baseline Post
Non-cachectic
Baseline Post
Cachectic
D
MURF-1 protein expression
(% baseline)
500
400
300
200
100
0
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Baseline Post
Non-cachectic
Baseline Post
Cachectic