

ORIGINAL ARTICLE

Skeletal muscle molecular responses to resistance training and dietary supplementation in COPD

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ABSTRACT

Background Skeletal muscle dysfunction is a systemic feature of chronic obstructive pulmonary disease (COPD), contributing to morbidity and mortality. Physical training improves muscle mass and function in COPD, but the molecular regulation therein is poorly understood.

Methods Candidate genes and proteins regulating muscle protein breakdown (ubiquitin proteasome pathway), muscle protein synthesis (phosphatidylinositol 3 kinase/Akt/mammalian target of rapamycin pathway), myogenesis (MyoD, myogenin and myostatin) and transcription (FOXO1, FOXO3 and RUNX1) were determined in quadriceps muscle samples taken at four time points over 8 weeks of knee extensor resistance training (RT) in patients with COPD and healthy controls (HCs). Patients with COPD were randomly allocated to receive protein/carbohydrate or placebo supplements during RT.

Results 59 patients with COPD (mean (SD) age 68.0 (9.3) years, forced expiratory volume in 1 s (FEV₁) 46.9 (17.8) % predicted) and 21 HCs (66.1 (4.8) years, 105.0 (21.6) % predicted) were enrolled. RT increased lean mass (~5%) and strength (~20%) in all groups. Absolute work done during RT was lower throughout in patients with COPD compared with HCs. RT resulted in increases (from basal) in catabolic, anabolic, myogenic and transcription factor protein expression at 24 h, 4 weeks and 8 weeks of exercise in HCs. This response was blunted in patients with COPD, except for myogenic signalling, which was similar. Nutritional supplementation did not augment functional or molecular responses to RT.

Conclusions The potential for muscle rehabilitation in response to RT is preserved in COPD. Except for markers of myogenesis, molecular responses to RT are not tightly coupled to lean mass gains but reflect the lower work done during RT, suggesting some caution when identifying molecular targets for intervention. Increasing post-exercise protein and carbohydrate intake is not a prerequisite for a normal training response in COPD.

INTRODUCTION

Reduced skeletal muscle mass and function is an important clinical feature of chronic obstructive pulmonary disease (COPD) which compromises physical performance^{1–3} and predicts morbidity and mortality independently from lung function impairment.^{4–7} This is important because muscle is a potential therapeutic target in a disease in which the primary lung pathology is frequently irreversible.⁸ Proof that this approach is beneficial is demonstrated by the efficacy of physical training in

Key messages**What is the key question?**

- Impaired skeletal muscle function is a potentially remediable systemic manifestation of chronic obstructive pulmonary disease (COPD). Resistance exercise training improves muscle mass and function in patients with COPD, but molecular mechanisms underpinning these adaptations are poorly understood and the impact of post-exercise dietary supplementation aimed at increasing muscle protein accretion has not been investigated.

What is the bottom line?

- We show that thigh lean mass and strength gains with training are similar in patients with COPD and healthy controls. With the exception of myogenic proteins, molecular responses to training are uncoupled from these functional gains and more closely reflect absolute workloads performed during training. Post-exercise protein/carbohydrate supplementation does not augment molecular or functional responses.

Why read on?

- We report a detailed time-course investigation in COPD of the responses of an unprecedented range of candidate genes and proteins known to regulate muscle protein synthesis, breakdown and myogenesis to resistance training and nutritional supplementation. The findings have implications for identification of drug targets aimed at improving muscle function in COPD.

improving muscle function in COPD.^{9–10} However, the molecular and cellular events underpinning muscle dysfunction and its response to training in COPD are poorly understood and it remains unclear whether the potential for training-induced muscle adaptation is preserved in COPD compared with similar aged healthy subjects.

Cross-sectional comparative studies of patients with COPD and aged-matched healthy subjects have proposed muscle protein breakdown (MPB) as a driver of wasting in COPD.^{11–13} However, in the absence of data depicting temporal changes in

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muscle protein turnover or the expression of genes and proteins thought to regulate muscle mass in response to intervention, firm conclusions on the mechanistic and therefore clinical relevance of MPB to wasting in COPD cannot be drawn. Importantly, training-induced molecular adaptations will precede muscle mass gains and therefore may be missed if analysis is restricted to biopsy samples obtained when training is completed.

Resistance training (RT) alone or combined with post-exercise dietary protein supplementation increases muscle protein synthesis (MPS) via activation of mammalian target of rapamycin (mTOR) signalling.^{14–16} There is evidence that MPS via this signalling pathway and insulin-mediated inhibition of MPB are blunted in older volunteers,^{17–20} and that the timing of post-exercise supplementation may be important to the magnitude of the anabolic response in older people.²¹ The role of protein supplementation combined with RT in patients with COPD has not been elucidated.

We conducted a detailed investigation of genes and proteins, thought to play a role in muscle mass regulation, in response to a programme of RT in patients with stable COPD and age-matched controls. Furthermore, responses were measured during the RT intervention in a matched cohort of patients with stable COPD who ingested post-exercise protein and carbohydrate supplements. We measured a wider range of candidate genes and proteins than in previous studies and related these responses to RT-induced changes in lean mass and strength.

The study addressed these specific hypotheses:

1. RT-induced increases in lean mass and function are blunted in patients with stable COPD compared with age-matched controls.
2. This blunting in patients with COPD is mirrored by increased expression of genes and proteins associated with MPB and inhibition of muscle anabolic signalling.
3. Post-exercise dietary carbohydrate and protein supplementation inhibits muscle catabolic events and augments anabolic signalling, thereby enhancing RT functional effects.

MATERIALS AND METHODS

Detailed methods are provided in the online supplementary materials and methods.

Subjects

Patients with stable COPD meeting clinical and spirometric criteria for severe (Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage 3 or 4) COPD (forced expiratory volume in 1 s (FEV₁)/forced vital capacity (FVC) ratio <70%, FEV₁<50% predicted) with significant self-reported exercise limitation (Medical Research Council (MRC) grades 3, 4 or 5) were recruited. Age-matched healthy controls (HCs) were recruited from the local population. Leicestershire Research Ethics Committee provided ethical approval and all participants provided informed written consent. The study was registered with the UK National Research Register (reference N0123192026).

Study design

Participants undertook maximal intensity voluntary RT for 8 weeks. Patients with COPD received (randomised, double blind) a dietary protein-carbohydrate supplement (COPD (S)) or a non-nutritive placebo (COPD (P)) at the time of training. HCs received placebo. Outcome assessments and muscle sampling were performed at baseline, 24 h after the first training session (muscle biopsy and blood insulin only), after 4 weeks and post training (8 weeks). Trial design and patient flow are summarised in online supplementary figure 1S.

Interventions

Resistance training

Participants underwent 8 weeks of maximal voluntary isokinetic lower-limb RT (Cybex II Norm, CSMi, USA). Training was fully supervised and consisted of three sessions per week. Each session comprised five sets of 30 maximal knee extensions at an angular velocity of 180°/s. During training, peak torque (Nm) and total work performed (J) were recorded for each extension.

Nutritional supplementation

The supplement comprised 19 g protein and 49 g glucose polymer carbohydrate (Vitargo Gainers Gold, Swecarb, Sweden) in 500 ml of water. The placebo was an identical volume, non-caloric drink. Supplementation was supervised and took place immediately after each training session. Participants and researchers were blinded to the nutritional intervention. Healthy volunteers received only the placebo.

Outcome measurements

At baseline, spirometry was measured in the seated position (Model R; Vitalograph, UK) according to accepted standards.²² Body mass index was calculated from height and weight.

Muscle biopsies

Vastus lateralis muscle biopsies were obtained after a fast of at least 4 h and (apart from baseline samples) 24 h after the previous training session. Samples were snap frozen, stored in liquid nitrogen and analysed later as described below.

Blood sampling

Fasted venous blood was collected and analysed for serum insulin concentration (Human Insulin specific RIA kit, Millipore, USA).

Quadriceps muscle function

Maximum isometric strength was determined during maximal voluntary knee extensor contraction (Cybex II Norm, CSMi, USA). Subsequently, peak torque and total isokinetic work were recorded during two bouts of five maximal repetitions (performed at an angular velocity of 60°/s).

Lean mass

Whole body and thigh lean mass was measured using dual energy X-ray absorptiometry (Lunar Prodigy Advance, GE Healthcare, UK). Fat-free mass index (FFMI) was calculated from height and body fat free mass. Patients were deemed to have muscle wasting if FFMI <16 kg/m² in men or <15 kg/m² in women.²³

Muscle biopsy analysis

A wide range of genes (quantitative reverse transcriptase PCR) and proteins (western blotting with infrared detection) were measured at each time point. Candidate genes and proteins were selected on the basis of known association with the regulation of MPB (20S proteasome, MAFbx, MuRF1 and ZNF216, along with calpain-3), MPS (Akt, p70s6 kinase, GSK3 α , GSK3 β , 4EBP1 and Redd1), myogenesis (MyoD, myogenin and myostatin), transcription (members of the family of Forkhead transcription factors, FOXO1 and FOXO3 and RUNX1) and inflammation (tumour necrosis factor α and interleukin-6 mRNA expression).

Data analysis

We estimated from our pulmonary rehabilitation programme that a 20% increase in strength following training would be clinically and physiologically significant. To detect this strength difference (80% power, $\alpha=0.05$) we required 25 patients to complete training in each group.

We included only subjects who provided at least a baseline and 24 h time-point muscle biopsy (see online supplementary figure 1S). An independent Student *t* test was used to compare baseline clinical and functional data between groups. Two-way repeated measures analysis of variance, and when appropriate least significant difference post hoc tests, were used to compare within-group changes over time and differences between treatment groups.

For molecular data, comparison of two independent groups was performed using Mann-Whitney's non-parametric test, and within-group comparison of more than two time points was performed using Friedman's non-parametric analysis. Data in tables and figures are mean \pm SEM. Significance was set at $p < 0.05$.

RESULTS

Fifty-nine patients with COPD and 21 HCs were included in the analysis. Online supplementary figure 1S shows the flow through the study and the number of viable muscle samples at each time point.

Baseline (pretraining)

Physical characteristics

Baseline characteristics are shown in table 1. There were no differences at baseline between the COPD (P) and COPD (S) groups. Muscle function and whole body exercise performance were lower in patients with COPD than HCs. Thigh lean mass was lower in patients compared with HCs, but this difference

was not statistically significant. Seven COPD (P) and six COPD (S) subjects were deemed to have muscle wasting.

Molecular data

Baseline protein and mRNA expression levels in patients with COPD as a whole and in HCs are shown in tables 2 and online supplementary table 1S respectively. At baseline, MAFbx and MuRF1 protein expression, phosphorylation of p70s6kinase, Redd1 protein expression, Myogenin and MyoD protein expression and nuclear FOXO1 protein expression were significantly greater in patients with COPD than in HCs.

Baseline myostatin mRNA expression was greater in patients with COPD, but there was no other difference in gene expression between groups.

Training-induced changes

Functional data

Thigh lean mass increased from baseline in HCs (4.1% (0.8%) and 5.4% (0.9%) at 4 and 8 weeks of RT, respectively) and COPD (P) (4.6% (0.9%) and 6.2% (1.7%)) and COPD (S) (3.9% (1.3%) and 4.0% (1.1%)) groups (figure 1A). Isometric strength also increased relative to baseline in all groups at 4 and 8 weeks of RT (HC: 10.0% (4.1%) and 12.4% (4.2%); COPD (P): 16.9% (4.3%) and 17.7% (3.7%); COPD (S): 14.6% (2.8%) and 18.0% (3.4%)) (figure 1B).

There were no differences in training-induced gains in lean mass or strength between the HC group and the COPD (P) and COPD (S) groups. Similarly, the training-induced increase in absolute thigh lean mass was not different between groups (HC: 232 (40) g; COPD (P): 215 (49) g; COPD (S): 148 (43) g).

Table 1 Baseline physical characteristics

	Healthy controls (n=21)	COPD (P) (n=27)	COPD (S) (n=32)
Age (years)	66.1 (1.0)	66.9 (1.7)	68.9 (1.7)
Gender (M:F)	10:11	14:13	19:13
BMI (kg/m ²)	26.8 (0.6)	25.5 (1.0)	26.6 (0.8)
FFMI (kg/m ²)	17.5 (0.3)	16.9 (0.4)	17.6 (0.4)
Subjects with muscle wasting (n)	0	7	6
Smoking pack years	14.4 (5.1)* **	48.1 (5.6)	46.9 (7.9)
Physical activity score	10.3 (1.2)* **	6.1 (0.3)	6.8 (1.1)
FEV ₁ (litres)	2.5 (0.1)* **	1.1 (0.0)	1.12 (0.0)
FEV ₁ (% predicted)	105.0 (4.7)* **	45.8 (3.2)	47.7 (3.3)
FEV ₁ /FVC (%)	71.8 (1.4)* **	41.2 (2.1)	39.42.4)
Thigh lean mass (g)	4190 (189)	3700 (191)	3829 (153)
Isometric peak torque (Nm)	135.8 (9.5)* **	109.3 (9.8)	105.9 (7.4)
Isokinetic peak torque (Nm)	98.0 (7.9)**	75.8 (6.9)	79.9 (6.0)
Isokinetic peak work (J)	373.8 (33.0)**	284.5 (28.2)	307.5 (25.2)
Cycle peak VO ₂ (ml/kg/min)	22.8 (1.4)* **	14.7 (0.9)	14.4 (0.6)
Cycle peak workload (W)	119.9 (8.7)* **	51.1 (4.7)	48.9 (3.8)

Subjects who provided a minimum of a baseline and 24 h muscle biopsy were included in the analysis (see online supplementary figure 1S). Figures refer to mean (SEM).

Details of the questionnaire used to measure physical activity score is given in the online supplementary materials and methods.

* $p \leq 0.05$; HC versus COPD (S); ** $p \leq 0.05$; HC versus COPD (P).

BMI, body mass index; COPD (P), patients with COPD allocated placebo; COPD (S), patients with COPD allocated supplements; FFMI, fat-free mass index; HC, healthy control.

Table 2 Protein expression measured from muscle biopsies taken at baseline

	Healthy controls (n=21)	COPD (n=59)
Muscle protein breakdown		
20S proteasome	0.61 (0.11)	0.74 (0.07)
MAFbx	0.36 (0.05)	0.83 (0.12)*
MuRF1	0.30 (0.04)	0.69 (0.07)**
Calpain3	0.50 (0.08)	0.52 (0.04)
Muscle protein synthesis		
PAkt1/Akt1	1.27 (0.37)	1.36 (0.11)
PGSK3 α /GSK3 α	0.84 (0.26)	0.97 (0.11)
PGSK3 β /GSK3 β	1.71 (0.38)	1.28 (0.19)
PP70s6k/P70s6k	0.82 (0.10)	1.18 (0.10)*
P4EBP1/4EBP1	1.03 (0.24)	1.11 (0.10)
Redd1	0.50 (0.06)	0.85 (0.06)**
Myogenesis		
Myostatin	2.70 (0.55)	3.18 (0.51)
MyoD	0.36 (0.05)	0.80 (0.09)**
Myogenin	0.45 (0.06)	0.86 (0.11)*
Transcription factors		
PFOXO1/FOXO1	1.83 (0.39)	2.22 (0.37)
PFOXO3/FOXO3	1.42 (0.22)	2.16 (0.31)
FOXO1 (nuclear)	0.51 (0.04)	0.89 (0.07)**
FOXO3 (nuclear)	0.58 (0.10)	0.78 (0.06)

Figures refer to mean (SEM) relative optical density (normalised to either actin or laminin).

The COPD group refers to the combined placebo and supplement groups at baseline (prior to intervention).

* $p < 0.05$, ** $p < 0.01$ significantly different from control.
COPD, chronic obstructive pulmonary disease.

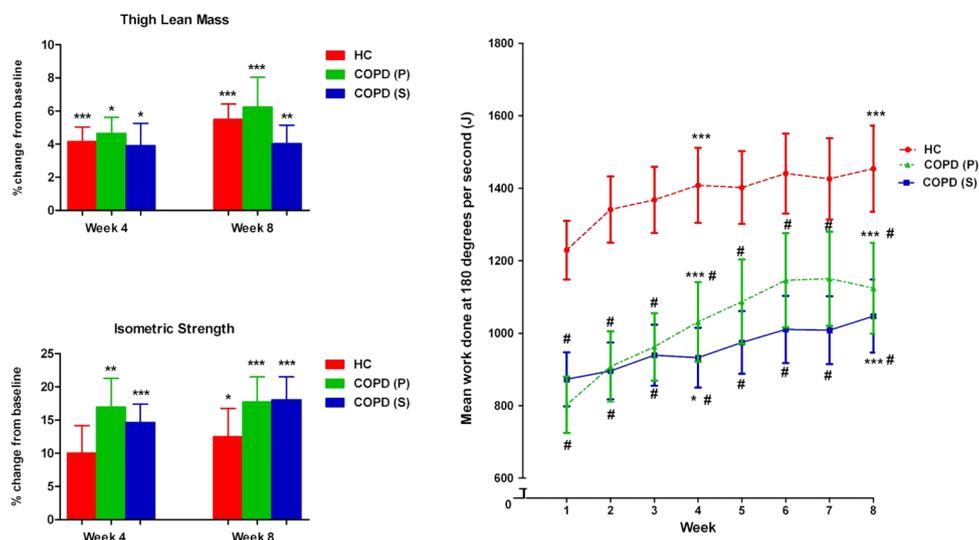


Figure 1 Training-induced changes in muscle mass and strength and progression of work. Changes (\pm SEM) from baseline in (A) thigh muscle mass, (B) isometric peak torque in the healthy control group (HC; red bars), chronic obstructive pulmonary disease (COPD) placebo group (COPD (P); green bars) and COPD supplement group (COPD (S); blue bars). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ significantly different from baseline. (C) Shows mean (\pm SEM) weekly total isokinetic work ($180^\circ/\text{s}$) performed during the 8-week training programme in the three groups. # $p < 0.05$ significant difference compared with control group.

Figure 1C shows the weekly progression of total isokinetic work performed during training (weekly average) for each group. Mean absolute work performed by HCs was greater at all stages than in both COPD groups, but there was no difference in the rate of progression of work during training between the HC and COPD groups and between the COPD (P) and COPD (S) groups.

Training-induced changes in protein expression

Muscle protein breakdown: In the HC group, expression of proteins involved in MPB was significantly increased 24 h after the first bout of training and was sustained at 4 and 8 weeks (figure 2A). In patients with COPD, the pattern of change was broadly similar, but of smaller magnitude with fewer changes being statistically significant (figure 2C,D). In particular, the expression of MURF1 and MAFbx was unchanged in both COPD groups during training. There was no difference in the response to training between COPD (P) and COPD (S) groups.

Muscle protein synthesis: In HCs, there was an increase with training in the ratio of phosphorylated protein to total protein expression for all anabolic signalling proteins with the exception of PGSK3 β /GSK3 β ratio (figure 3A). The pattern of change in COPD was similar, but training-induced changes were of substantially lower magnitude than in HC (figure 3C,D). There was no difference in the magnitude of response when comparing COPD (P) and COPD (S) groups.

Myogenesis: Myostatin protein expression did not change significantly from baseline with training in either HC or COPD groups. There was a statistically significant increase in MyoD expression after 8 weeks of training in all three groups, which was of the same magnitude. There was a tendency for myogenin protein expression to increase with training in all groups, but this did not reach statistical significance (figure 4A,C,D). There was no difference in the pattern of response to training when comparing COPD (P) and COPD (S) groups.

Transcription factors: Phosphorylated to total protein expression ratios for FOXO1 and FOXO3 transcription factors increased in all groups during training (figure 5). However, the

magnitude of change was lower in both COPD groups compared with HCs.

Training-induced changes in mRNA expression

A description of changes in mRNA expression is provided in the online supplementary materials and methods. Broadly, the pattern change in genes involved in MPB and MPS, myogenesis, transcription and inflammation was similar for HC and COPD (P) and COPD (S) groups (see online supplementary figures 2S–6S). Notably, myostatin mRNA expression was significantly reduced at 24 h, but was restored to the baseline value at 4 and 8 weeks in all groups (see online supplementary figure 4S). Similarly, inflammatory gene expression increased significantly in all groups at 24 h but was reduced to baseline at 4 and 8 weeks (see online supplementary figure 6S).

DISCUSSION

This study details the functional and molecular responses of skeletal muscle to RT and post-exercise protein/carbohydrate supplementation combined with RT in patients with COPD and aged-matched HCs. A major finding was that increases in thigh lean mass and knee-extensor strength over 8 weeks of RT in patients with COPD were similar compared with HCs. It is concluded that while baseline muscle function in patients with COPD is compromised, its responsiveness to RT is preserved.

RT increased anabolic, catabolic and transcription factor protein expression (not unexpected given exercise increases muscle protein turnover), but the magnitude of increase was blunted in patients with COPD. This was surprising given that thigh lean mass and strength gains were similar, and suggests a disconnection between changes in protein expression and lean mass gains. There appeared to be a closer association between anabolic, catabolic and transcription factor protein expression levels and work done during RT as the latter was consistently lower in patients with COPD (figure 1C). However, changes in myogenic protein expression with RT were similar in patients with COPD and HCs and may explain the similarity in lean mass gains. This is in line with the observation that testosterone-

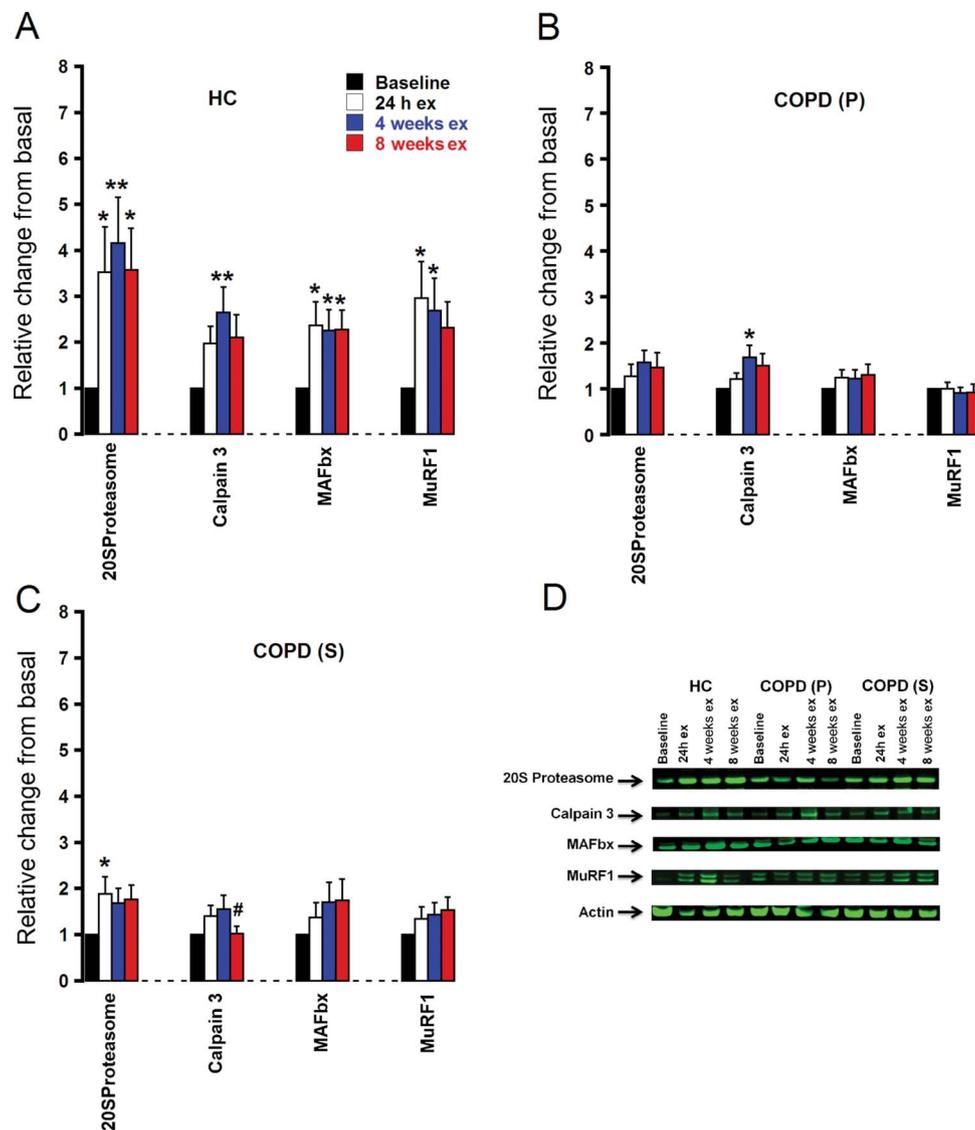


Figure 2 Expression of target proteins regulating muscle protein breakdown in response to training. Protein expression (mean±SEM) is represented as relative changes from basal and is quantified by western blotting. (A) HC, healthy control group; (B) COPD (P), patients with chronic obstructive pulmonary disease (COPD) receiving placebo; (C) COPD (S), patients with COPD receiving supplement; (D) typical western blot using IRdye 800 (green) secondary antibodies to quantify the catabolic proteins studied. * $p < 0.05$, ** $p < 0.01$ significantly different from baseline; # $p < 0.05$ significantly different from 24 h. ex, exercise.

mediated muscle hypertrophy in older people is associated with increased myogenin protein expression and satellite cell activation.²⁴ Contrary to our hypothesis, post-exercise dietary supplementation in patients with COPD did not alter target gene and protein expression or leg lean mass and functional gains compared with training alone.

Single time-point studies comparing muscle anabolic and catabolic mRNA and protein expression in patients with COPD and HCs have proposed muscle atrophy in COPD occurs as a consequence of increased ubiquitin proteasome mediated MPB, and that increased anabolic signalling may occur as a compensatory phenomenon.^{11–13} Conversely, others report little differences in anabolic gene and protein expression levels between patients with COPD and HCs.¹² In this study MuRF1, MAFbx and nuclear FOXO1 protein expression was greater in patients with COPD than in HCs at baseline (table 2). However, these traits were present even when thigh lean tissue mass and muscle inflammatory cytokine mRNA expression were similar between

patients with COPD and HCs (table 2, see online supplementary table 1S), suggesting these differences may be features of deconditioning rather than increased MPB and wasting per se, which is supported by the lack of difference in proteasome protein expression at baseline. In the absence of MPB measurements it is not possible to be more conclusive but increased muscle FOXO and MAFbx protein expression has been reported under conditions of altered muscle carbohydrate and lipid oxidation and in the absence of muscle wasting.²⁵ The greater muscle catabolic protein expression at baseline in patients with COPD was also paralleled by greater expression of selected anabolic and myogenic signalling proteins, perhaps suggesting greater basal muscle protein turnover in COPD.

To our knowledge, no COPD study has documented the time course of muscle molecular events under conditions when muscle mass has been increased by RT. Muscle cytokine mRNA expression increased transiently in response to training, but the magnitude was the same in patients with COPD and HCs (see

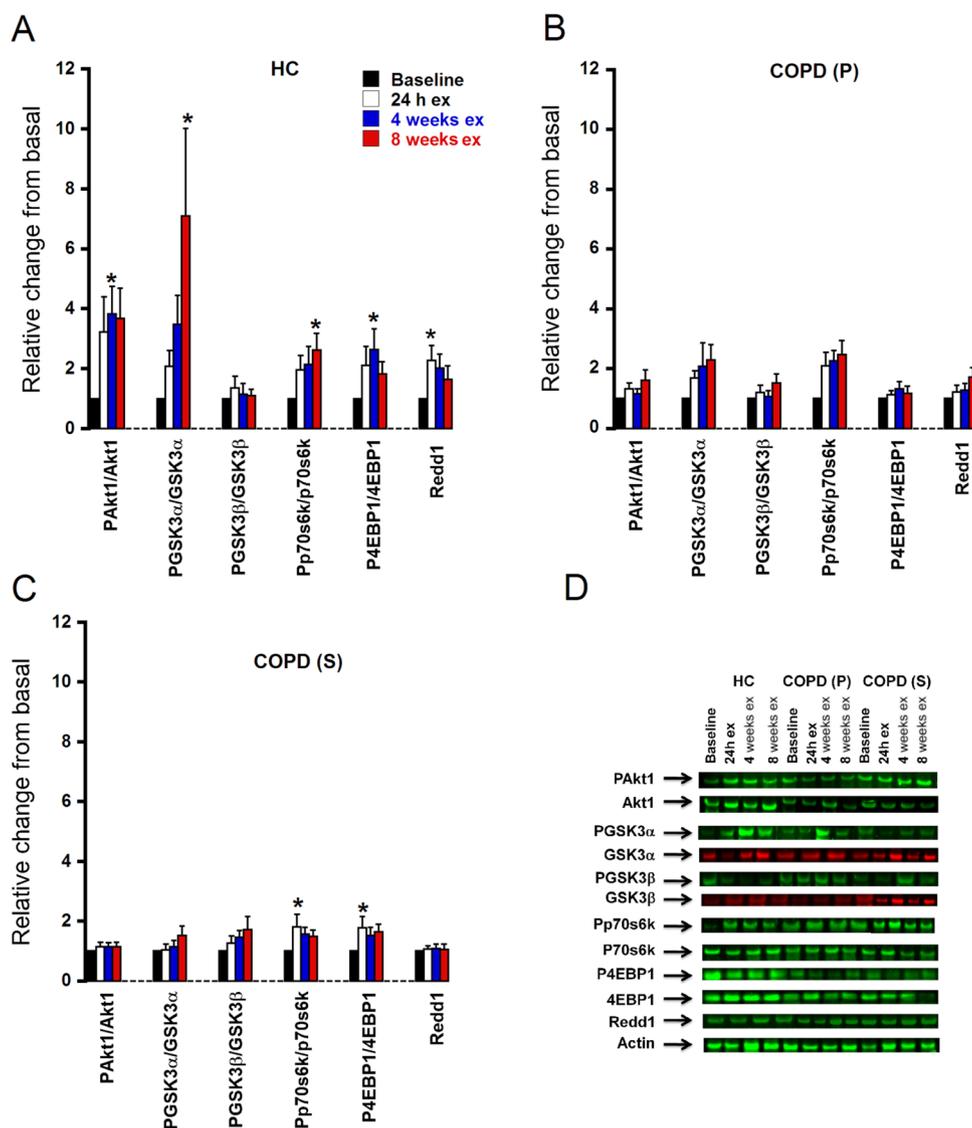


Figure 3 Expression of target proteins regulating muscle protein synthesis in response to training. Protein expression (mean \pm SEM) is represented as relative changes from basal and is quantified by western blotting. (A) HC, healthy control group; (B) COPD (P), patients with chronic obstructive pulmonary disease (COPD) receiving placebo; (C) COPD (S), patients with COPD receiving supplement; (D) typical western blot using IRDye 800 (green) and IRDye 680 (red) secondary antibodies to quantify the anabolic proteins studied. * $p < 0.05$ significantly different from baseline. ex, exercise.

online supplementary figure 6S), and likely reflected an acute inflammatory response to unaccustomed exercise. Furthermore, this response, together with the transitory decrease in myostatin mRNA expression over the same time course, demonstrates short-term changes in mRNA abundance may be of limited physiological relevance in the absence of associated protein changes. This point is further substantiated by the lack of close alliance between changes in mRNA and protein abundance with training for each molecular target in this study. The current study extends previous reports (which generally focused on relatively few mRNA and protein targets²⁶) by documenting responses of a substantially wider range of targets to a training intervention that increased lean tissue mass and strength, capturing time-course changes. The disconnection between protein expression levels and muscle mass gains with RT is supported by data showing that increasing amino acid and insulin availability (thus doubling leg protein synthesis and halving leg protein breakdown in young, healthy volunteers) did not result in

parallel changes in anabolic signalling activity (phosphorylation of the Akt/mTOR/P70S6k/eIF4F pathway), and more simply reflected changes in insulin availability.¹⁷ Additionally, the decline in MPS observed during limb immobilisation in healthy, young volunteers is not reflected by changes in expression levels of mTOR signalling proteins, which remained unchanged from basal.²⁷ It could be argued that measuring anabolic, catabolic and transcription factor protein expression in the resting fasted state (as in the present study) does not provide significant insight regarding molecular responses mediated at the time of exercise, but if this is the case it is difficult to reconcile the changes in mRNA and protein expression levels we observed with RT in the present study. A likely explanation is that closer association exists between changes in protein expression levels and work done during training than lean mass gains. In support of this, Burd *et al*²⁸ recently demonstrated that the volume (not intensity) of work done during an acute bout of resistance exercise is positively associated with the magnitude of post-exercise

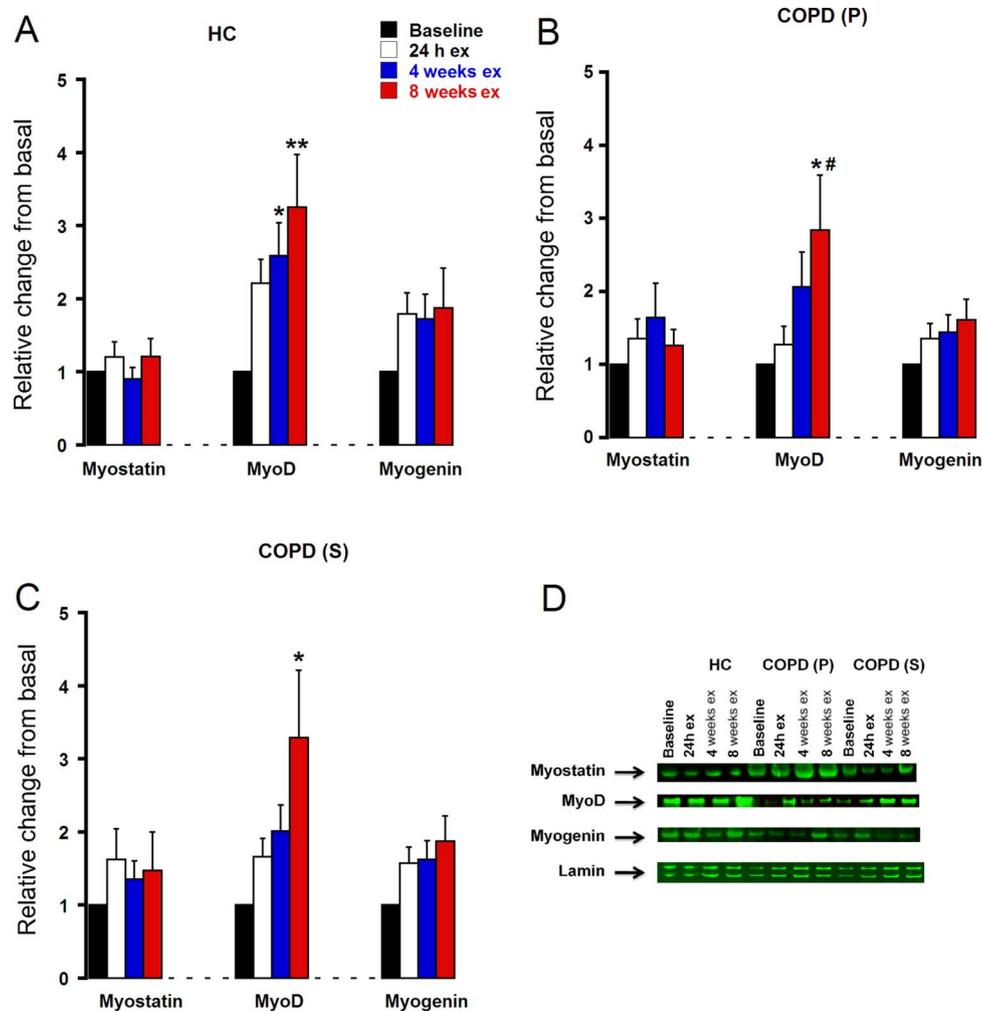


Figure 4 Expression of target proteins regulating myogenesis in response to training. Protein expression (mean±SEM) is represented as relative changes from basal and is quantified by western blotting. (A) HC, healthy control group; (B) COPD (P), patients with chronic obstructive pulmonary disease (COPD) receiving placebo; (C) COPD (S), patients with COPD receiving supplement; (D) typical western blot using IRDye 800 (green) secondary antibodies to quantify the myogenic proteins studied. * $p < 0.05$, ** $p < 0.01$ significantly different from baseline; # $p < 0.05$ significantly different from 24 h. ex, exercise.

p70S6K phosphorylation, and that this relationship exists for up to 30 h following exercise which is considerably longer than in the present study. Furthermore recent work shows the intensity of chronic RT does not determine the magnitude of training-induced muscle hypertrophy in young men.²⁹ As this study demonstrates, this has important implications for muscle rehabilitation in COPD and other wasting diseases.

Anabolic resistance of muscle to protein nutrition is a feature of ageing and has been proposed to be a causative factor in sarcopenia.^{19, 20} It has been suggested that dietary supplementation might need to be combined with muscle contraction to facilitate muscle protein accretion in ageing.^{19, 30} Despite supplementation occurring immediately after each bout of training when the anabolic response to feeding is maximised,²¹ we did not observe an effect of feeding on molecular or functional outcomes, suggesting dietary protein intake is not a major limitation to RT-induced muscle mass gains in COPD.

Controversy exists about whether impaired muscle mass and function can be ascribed to COPD-specific factors (eg, systemic inflammation, hypoxia, drug therapy) or is predominantly due to physical inactivity. Our observation that RT increased lean mass and strength in COPD, and by the same magnitude as

observed in HCs, suggests inactivity and deconditioning are key factors underpinning muscle dysfunction in COPD, although we recognise that muscle dysfunction due to disease-specific factors may also be modifiable by training. Importantly, muscle mass and function increased with RT even in the face of increased catabolic mRNA and protein expression, which probably reflects exercise-induced increases in muscle protein turnover. We recognise our observations are limited to patients with stable disease and preserved muscle mass who were able to undertake a demanding RT programme with repeated muscle biopsy sampling. However, we selected patients with significant self-reported exercise limitation (MRC grades 3–5), demonstrating markedly impaired muscle strength and aerobic capacity at baseline, suggesting the population represented those referred for pulmonary rehabilitation.

In conclusion, we demonstrated there is no disease-specific barrier to increasing lean tissue mass and function through RT in patients with COPD and lung impairment, exercise intolerance and weakness at baseline. We also showed that increasing post-exercise dietary protein and carbohydrate intake is not a prerequisite for a normal training response in COPD. Our observation that (with the exception of myogenic proteins)

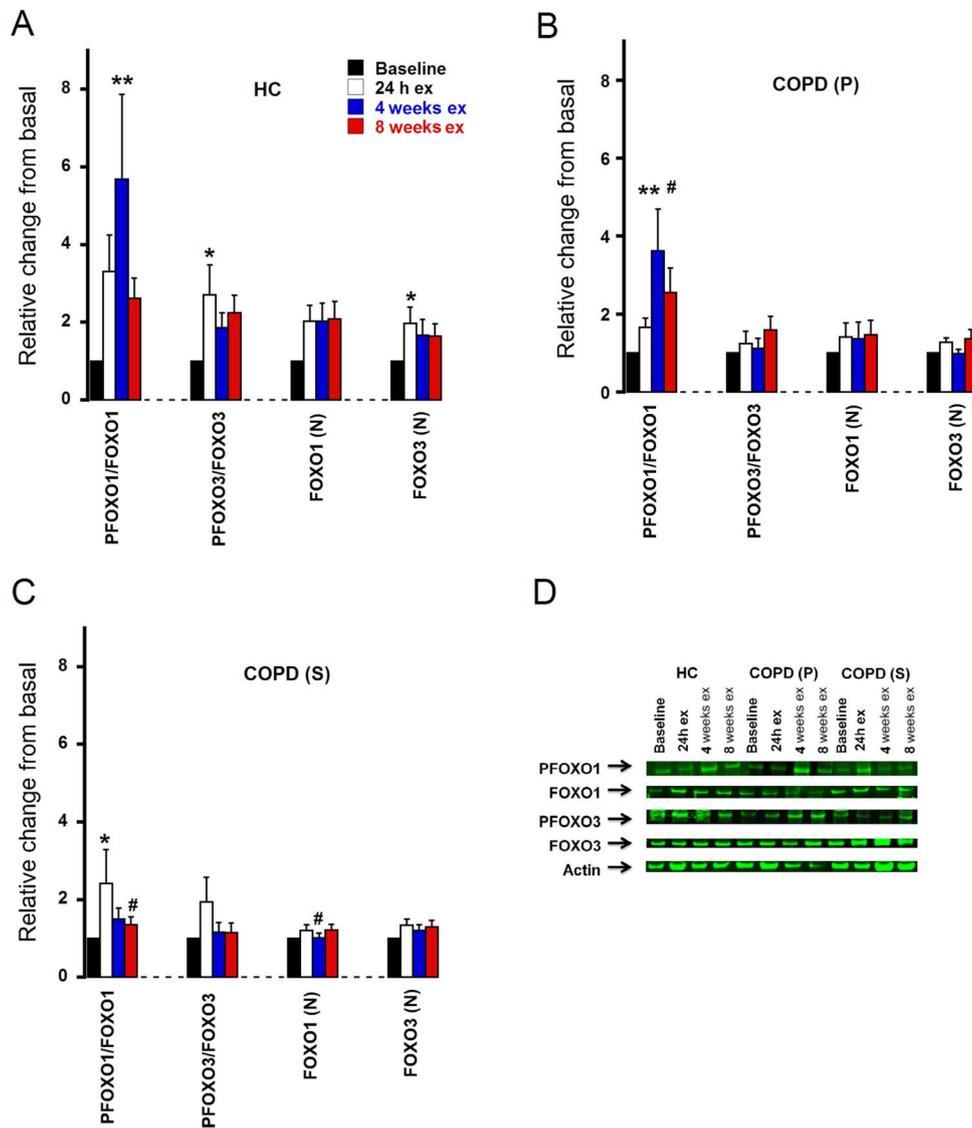


Figure 5 Expression of target transcription factor proteins in response to training. Protein expression (mean±SEM) is represented as relative changes from basal and is quantified by western blotting. (A) HC, healthy control group; (B) COPD (P), patients with chronic obstructive pulmonary disease (COPD) receiving placebo; (C) COPD (S), patients with COPD receiving supplement; (D) typical western blot using IRDye 800 (green) secondary antibodies to quantify the transcription factors studied. * $p < 0.05$, ** $p < 0.01$ significantly different from baseline; # $p < 0.05$ significantly different from 24 h. ex, exercise; (N), nuclear.

gains in lean mass are not tightly coupled to the magnitude of change in protein expression suggests some caution when identifying potential targets for intervention.

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Contributors DC conducted wet laboratory procedures and molecular data analysis. MKM recruited subjects, conducted outcome assessments (including muscle biopsies) and data analysis. LH-W supervised resistance training and nutritional supplementation and contributed to data analysis. MDM and SJS helped plan and coordinate the study and supervised staff working on the project. PG and MCS wrote the original grant application, developed the study protocol, supervised staff working on the project and contributed to data analysis. All authors contributed to manuscript writing.

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Competing interests None.

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Skeletal muscle molecular responses to resistance training and dietary supplementation in COPD

Online Supplement

Materials and Methods

Subjects

Patients with COPD were recruited from outpatient clinics and from those referred for pulmonary rehabilitation at Glenfield Hospital in Leicester (UK). Stable outpatients who met clinical and spirometric criteria for moderate to severe COPD (FEV₁/FVC ratio <70%, FEV₁<50% predicted) with significant self-reported exercise limitation (MRC Grade III, IV or V) were included in the study. Exclusion criteria were based on the use of oral corticosteroid, oral anticoagulant or long term oxygen therapy. Patients with co-morbid conditions, i.e. cardiovascular complications contributing to exercise limitation or preventing exercise training were also excluded. Healthy controls were recruited from the local population. Subjects were not taking part in regular exercise training programs and COPD patients who underwent pulmonary rehabilitation in the last 12 months were excluded. Approval was obtained from the Leicestershire and Rutland Research Ethics Committee (UK) and all participants provided informed written consent. The study was registered with the UK National Research Register [(NRR) reference: N0123192026].

Study design

All participants undertook fully supervised maximal intensity resistance exercise training for 8 weeks as described below. Patients with COPD were randomly allocated in double blind fashion to receive a dietary protein-carbohydrate supplement or a non-nutritive placebo at the time of training (see below). Healthy control subjects all received placebo. Randomisation (random varying blocks of 2, 4 and 6 volunteers) was performed by the Nottingham University Clinical Trials Unit using a web-based system. Randomisation was stratified for gender and muscle mass to ensure the treatment groups were matched at baseline. Outcome assessments were performed at baseline, 24 hrs after the first training session (muscle biopsy and plasma insulin only), 4 wks, and at the end of the training intervention (8 wks). The trial design and flow of patients is summarised in the diagram shown in the online supplement (Fig. 1S).

Interventions

Resistance training

All participants underwent 8 weeks of voluntary maximal intensity, bilateral, lower-limb resistance training on an isokinetic dynamometer (Cybex II Norm, Stoughton, MA, USA). Training was fully supervised and consisted of 3 sessions per week. Subjects performed 5 sets of 30 maximal knee extensions at a pre-set angular velocity of 180°/sec, with 1 min rest between each set. Maximal knee extension effort was chosen in an attempt to ensure a high proportion of muscle fibre recruitment and volunteers were verbally encouraged at all times to elicit maximal effort. During training, peak torque (Newton-metres; Nm) and total work done (Joules; J) was recorded for each contraction. This training programme was chosen because it does not result in the rapid fatigue associated with slower velocities of contraction (thereby stimulating training adaptation), involves recruitment of fast and slow muscle fibres,(1) and has been demonstrated to completely restore lower limb muscle mass and

increase isometric strength above baseline following 2 weeks immobilisation induced muscle wasting in young, healthy volunteers.(2)

Dietary supplementation

COPD patients were randomly allocated to receive a dietary protein-carbohydrate supplement or placebo throughout training. The supplement contained 19 g protein (Whey protein concentrate and milk protein isolate) and 49 g glucose polymer carbohydrate (Vitargo Gainers Gold, Swecarb, Sweden) made up to 500 ml of water. This is sufficient protein to saturate post-exercise muscle protein synthesis(3;4) and increase insulin above a concentration known to inhibit muscle protein breakdown in healthy, young volunteers.(5) The placebo was an identical volume non-nutritive and non-caloric drink that contained flavourings in an attempt to match the taste of the protein-carbohydrate supplement. Supplementation was provided in an unmarked package and supervised by the research team who ensured the supplement was ingested immediately after each training session. Both participants and researchers were blinded to the nutritional intervention. Healthy volunteers received only the placebo intervention.

Outcome Measurements

At baseline, spirometry was measured in the seated position (Model R; Vitalograph, Buckingham, UK) according to standards set out by the European Respiratory Society(6); the best of three attempts was taken and was recorded. Body mass index was calculated from height [measured using a wall-mounted stadiometer (SECA, Birmingham, UK to the nearest 0.1 cm] and weight [measured in light clothing to the nearest 0.1 kg] (SECA, Birmingham, UK). Physical activity was assessed at baseline using the adapted physical activity (PA) questionnaire for the elderly.(7) This questionnaire is not disease-specific and was chosen to allow comparison between patients with COPD and healthy controls. The questionnaire has previously been used to describe patients with COPD.(8;9) The questionnaire is interviewer-led and asks about household sporting and leisure activities within the last year, to produce an overall activity score of 0-35. A higher score indicates a greater level of PA.

Muscle Biopsies

Biopsies were obtained from the vastus lateralis muscle of all participants using a microbiopsy technique as previously described.(10) Several passes with the microbiopsy needle were made to harvest sufficient material, which was very well tolerated due to the less invasive nature of the procedure compared to the Bergstrom method.

Samples were taken at baseline, 24 hrs after the 1st training session, at mid point (4 wks) and at the end of the resistance training programme (8 wks). The biopsies were taken after a fast of at least 4 hrs and (apart from baseline samples) 24 hours after the previous training session. Samples were immediately snap-frozen, stored in liquid nitrogen and later analysed as described below.

Blood sampling

Venous blood was drawn in the fasted state at the same 4 time-point muscle biopsy samples were obtained. Samples were immediately added to preservative, briefly left on ice and following centrifugation plasma samples were stored at -80°C until analysed for insulin concentration, using a Human Insulin specific RIA kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol.

Isometric Quadriceps Strength

Before the first outcome assessments, subjects attended a familiarisation session for muscle strength determination. Isometric strength of the quadriceps muscle group was determined during maximal voluntary contraction of the knee extensors, with the knee fixed at an angle of 70° (Cybex II Norm, CSMi, Stoughton, USA). Subjects underwent three attempts of the manoeuvre, each separated by 30s, on 2 consecutive occasions. The highest value obtained was recorded as isometric strength.

Isokinetic Muscle Function

Isokinetic torque during knee extensor exercise was recorded at an angular velocity of $60^{\circ}/\text{s}$ to maximise motor unit recruitment. Subjects performed 2 bouts of 5 repetitions of isokinetic knee extension, with each bout being separated by 1 minute. Isokinetic peak torque and work output were recorded during each contraction. Positioning and stabilisation of the subject in the upright, seated position were standardised according to manufacturer guidelines. The chair monorail and back translation were adjusted so that the centre of the dynamometer head was in line with the subjects' knee joint line. A seatbelt, thigh strap and contra-lateral limb stabiliser were used to ensure that movement of other body parts was limited. The knee/ hip adaptor pad was then strapped to the distal part of the tibia five cm above the lateral maleolus. The subjects' leg was held out so that the knee was straight (0 degrees) and range of movement was set between 10 - 80° flexion. The weight of the limb was measured to allow the computer system to correct for gravity in its calculations. The measurements recorded were peak torque (Newton-metres: Nm) and total isokinetic work (cumulative over a set) (Joules: J) for each of the sets.

Lean Mass

Whole body and thigh (hip-to-mid patella) lean mass (LM) was measured using dual Energy X-ray Absorptiometry (DEXA) (Lunar Prodigy Advance, GE Healthcare, UK). To determine the composition of the dominant thigh, a region of interest (ROI) was traced using custom analysis software. The upper limit of this ROI was the lowest point of the ischial tuberosity, and the lower limit was the knee joint line. The pubic symphysis and the most lateral part of the thigh were used as the medial and lateral limits.⁽¹¹⁾ Whole body fat free mass (FFM) was calculated as lean mass + bone mineral mass. Fat free mass index (FFMI) was calculated from height and total body fat free mass. Patients were deemed to have muscle wasting if $\text{FFMI} < 16\text{kg}/\text{m}^2$ in men or $< 15\text{kg}/\text{m}^2$ in women. These criteria have been used previously to define muscle wasting in COPD and are predictive of poorer functional performance and quality of life in this population.⁽¹²⁻¹⁴⁾

Whole Body Exercise Performance

After a familiarisation test at baseline, subjects performed a symptom limited, exhaustive, incremental cycle ergometer test (at baseline and after completion of training only). Exercise work rate was increased by 10 W per min in the COPD group and 20 W per min in the healthy control group using a ramp protocol. Peak workrate and breath-by-breath measurements of gas exchange and ventilation were recorded (Zan-600 ErgoTest, Meßgeräte GmbH, Oberthulba, Germany).

Muscle Biopsy Analysis

A wide range of genes (Quantitative RT-PCR) and proteins (Western blotting with infrared detection) representing muscle protein breakdown, anabolic signalling, myogenesis, and transcription were measured. All have previously been associated with molecular regulation of muscle mass in cell, and less frequently, in animal based research. As far as we are aware, this is the first time such a comprehensive battery of measurements has been made over the course of an intervention aimed at increasing muscle mass in COPD patients. For the aid of clarity, genes and proteins have been grouped into the following subclasses:

Protein breakdown

Target genes and proteins included several members of the ubiquitin-proteasome pathway, namely the 20S proteasome, MAFbx, MuRF1 and ZNF216, along with calpain-3 a muscle specific member of the calpain family of calcium activated proteases.

Protein synthesis

Target genes and proteins thought to regulate translation initiation of muscle protein synthesis, namely Akt, p70s6 kinase, GSK3 α , GSK3 β , 4EBP1 and Redd1.

Myogenesis

Target genes and proteins comprising of MyoD, myogenin and myostatin.

Transcription factors

Transcription factors were targeted based on their reported involvement in muscle mass regulation, autophagy and insulin resistance and comprised members of the family of Forkhead transcription factors, FOXO1 and FOXO3, and RUNX1.

Pro-inflammatory cytokines

Tumour necrosis factor (TNF)- α and IL-6 mRNA expression.

Quantitative RT-PCR

RNA was extracted from frozen muscle biopsies using TRI Reagent (Ambion, Huntingdon, UK), according to the manufacturers protocol. First strand cDNA was then synthesised from 1 μ g RNA

using random primers (Promega) and Superscript III (Invitrogen). Additional reactions were performed, in which the reverse transcriptase was omitted to allow for assessment of genomic DNA contamination. All reactions were performed in the ABI 7900HT Fast Sequence Detection System (Applied Biosystems, Foster City, CA). Each well contained 2 μ l of cDNA, 18 μ M of each primer, 5 μ M probe, and Universal Taqman 2X PCR Mastermix for fast reaction (Applied Biosystems) in a 25 μ l final volume. Each sample was run in duplicate. Primers and MGB TaqMan probes (Applied Biosystems, Foster City, CA, USA) were designed such that probes spanned over exon-exon boundaries to avoid genomic amplification. Hydroxymethylbilane synthase (HMBS) was used as internal control, and all genes of interest were labelled with the fluorescent reporter FAM. Ct values of the target gene were normalized to Ct values of the house-keeping gene in COPD patients and healthy control volunteers, and the final results were calculated according to the $2^{-\Delta\Delta Ct}$ method. The baseline for each subject was used as the calibrator and was set at 1.

Western Blotting

Cytosolic and nuclear lysates were prepared from each muscle biopsy sample, and target protein expression was determined using Western blotting as described by Constantin *et al* (12). Total protein concentration was measured using the Bradford assay (Bradford, MM, Analytical Biochemistry, 1976). Antibodies to determine phosphorylated Akt1 (serine⁴⁷³; PAkt1) and total Akt1 (60 kDa), phosphorylated eukaryotic translation factor 4E-binding protein 1 (4E-BP1) (threonine^{37/46}; P4E-BP1) and total 4E-BP1 (15-20 kDa), phosphorylated p70 ribosomal S6 kinase (threonine³⁸⁹ Pp70S6K) (Pp70S6K) and total p70S6K (70 kDa), phosphorylated GSK3 α (serine²¹; PGSK3 α) and total GSK3 α (51 kDa), phosphorylated GSK3 β (serine⁹; PGSK3 β) and total GSK3 β (46 kDa), phosphorylated FOXO1 (serine²⁵⁶; PFOXO1; 82 kDa) and total FOXO1 (78-82 kDa) and phosphorylated FOXO3 (serine²⁵³; PFOXO3; 97 kDa) and total FOXO3 (82-97 kDa) and Redd1 (28 kDa) were obtained from Cell Signaling Technology (Danvers, MA, USA). MuRF1 (42 kDa) and MAFbx (42 kDa) antibodies, produced in-house by Pfizer Inc (USA), were provided to us as gifts. Myostatin antibody (MW 45 kDa) was obtained from Novus Biologicals (Littleton, CO, USA). PDK4 (46 kDa), calpain-3 (94 kDa), Myogenin (34 kDa) and MyoD antibodies (35 kDa) were obtained from Insight Biotechnology (Insight Biotechnology Ltd, Middlesex, UK). 20 S proteasome antibody (29kDa) was purchased from Biomol. All proteins were visualized by developing with either an IRDye 800 labelled secondary anti-rabbit antibody or an IRDye 680 labelled secondary anti-mouse antibody (used in multiplex detection) and were further quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The infrared signals have a greater dynamic linear range compared to chemiluminescence.

Data Analysis

The principle objective of the study was the investigation of the molecular signalling responses to resistance training and training combined with feeding in relation to gains in muscle function that would be clinically significant in patients with COPD. Meaningful changes in mRNA and protein

expression across the range of planned targets are difficult to quantify but most human studies have enrolled in the region of 10 subjects. Our objective was to relate changes in gene and protein expression to gains in muscle function that would be clinically significant in patients with COPD. Based on previous data from our pulmonary rehabilitation programme we estimated a 20% increase in muscle strength following training would be clinically and physiologically significant. To detect this strength difference (80% power, $\alpha = 0.05$) we required 25 patients to complete training in each group. To account for predicted dropouts in the COPD group a larger number of subjects was recruited. Our *a priori* aim was to recruit sufficient numbers of patients with low muscle mass (according to accepted criteria) to allow a subgroup analysis comparing the responses between patients with low and preserved muscle mass. However, the demanding nature of the study with the requirement for intensive physical training and repeated muscle sampling meant we could not recruit sufficient patients with low muscle mass and we therefore present data for the whole cohort.

Because observation of molecular signalling changes over time was the primary objective of the study, we included only subjects who provided at least a baseline and 24 hour time-point muscle biopsy (see Fig 1S). An independent Student t-test was used to compare baseline clinical and functional data between controls and COPD patients. Two-way repeated measures ANOVA, and when appropriate a Least Significant Difference (LSD) post-hoc test, was used to compare within group changes over time and differences between treatment groups (control vs COPD patients receiving placebo vs COPD patients receiving supplement). Training intensity progression was analysed using repeated measures ANOVA with Bonferroni corrections for multiple comparisons.

Because of the non-ordinate nature of the molecular data, comparison of two independent groups was performed using Mann-Whitney's non-parametric analysis, and comparison of more than two time related groups was performed using Friedman's non-parametric analysis. Data in Tables and Figures are expressed as mean \pm SEM. Significance was set at the $p < 0.05$ level.

Results

Seventy-one patients with COPD and 22 HC volunteers were enrolled to the study. Fig. 1S shows the flow through the study and the number of viable muscle samples available for analysis at each time point in each group.

Baseline (pre-training)

Physical Characteristics

Baseline physical characteristics are shown in Table 1. There were no significant differences at baseline, between the COPD (P) and COPD (S) groups. Compared with HC, patients had a significantly longer smoking history and worse lung function. Quadriceps muscle function and whole body exercise performance were lower in patients than HC. Thigh fat free mass was lower in patients compared with HC, but this difference was not statistically significant. Seven and six subjects in the COPD (P) and COPD (S) respectively were deemed muscle wasted according to our criteria.

Molecular data

Baseline protein and mRNA expression levels in COPD patients as a whole and in HC are shown in Tables 2 and 1S respectively. At baseline, MAFbx and MuRF1 protein expression, phosphorylation of p70s6kinase, Redd1 protein expression, myogenin and MyoD protein expression and nuclear FOXO1 protein expression were significantly greater in COPD patients than HC.

Baseline myostatin mRNA expression was greater in COPD patients but there were no other significant differences in protein expression between the groups.

Training induced changes

Functional data

Thigh lean mass increased significantly relative to baseline in the HC (4.1(0.8)% and 5.4(0.9)% at 4 and 8 wks of RT, respectively) and COPD (P) (4.6(0.9)% and 6.2(1.7)%) and COPD (S) (3.9(1.3)% and 4.0(1.1)%) groups (Fig. 1A). Isometric quadriceps strength also increased relative to baseline in all groups at 4 and 8 weeks RT (HC: 10.0(4.1)% and 12.4(4.2)%, COPD (P) 16.9(4.3) and 17.7(3.7)% and COPD (S) 14.6(2.8)% and 18.0(3.4)%); Fig. 1B.

There were no significant differences in the training induced gains in muscle mass or strength between the HC group and the COPD (P) and COPD (S) groups. Similarly, the absolute training induced increase in thigh lean mass was not different between the groups (HC: 232(40)g, COPD (P): 215(49)g and COPD (S): 148(43)g).

Whole body cycling exercise performance increased significantly relative to baseline in the HC (Δ peak VO_2 22.5(7.1)%, $p < 0.01$; Δ peak workload 12.2(2.8)%, $p < 0.001$,) and COPD (P) (Δ peak VO_2 21.2(8.7)%, $p < 0.05$; Δ peak workload 24.0 (8.6)%, $p < 0.05$) groups, but changes in the COPD(S) group

were not statistically significant (Δ peak VO_2 16.7(17.2)%, $p=0.3$; Δ peak workload 19.7(10.4)%, $p=0.07$).

The weekly progression of total isokinetic work performed during training (weekly average) by each group is shown in Fig. 1C. Mean absolute work performed by controls was significantly greater at all stages of training than in both COPD groups, but there was no significant difference in the rate of progression of work during training between the HC and COPD groups and between the COPD (P) and COPD (S) groups.

Molecular data

Figures and data depicting training induced changes in protein expression are given in the main manuscript. Data and figures depicting training induced changes in mRNA expression are given here.

Expression of genes (mRNA) involved in muscle protein breakdown

Changes in the expression of catabolic genes from baseline are shown in Fig. 2S. In HC, expression of the majority of catabolic genes increased at 4 and 8 weeks of exercise although (with the exception of calpain 3) this was not statistically significant (Fig. 2S A). The COPD patients showed up regulation in gene expression at 24 hours, 4 and 8 weeks for MuRF1, 20S proteasome and ZNF216 although statistical significance was variable (Fig. 2S B and C). There were no significant differences in the pattern of response to training between the COPD (P) and COPD (S) groups.

Expression of genes (mRNA) involved in muscle protein synthesis

Changes in mRNA expression of genes involved in the regulation of muscle protein synthesis are shown in Fig. 3S. In HC, there were increases in mRNA abundance although most were not statistically significant (Fig. 3S A). The pattern of change in expression with training was similar for the COPD groups (although statistical significance was variable) with the exception of Akt1, which was up-regulated to a greater degree in the COPD groups (Fig. 3S B and C).

There was a significant difference between the 2 COPD groups at 4 weeks ($p<0.01$) for p70s6kinase.

Expression of genes (mRNA) involved in myogenesis

In all three groups myostatin mRNA expression was significantly reduced at 24 hours but had returned to baseline levels at 4 and 8 weeks (Fig. 4S A,B,C). MyoD and myogenin mRNA expression increased during training in all groups, although statistical significance was variable.

There was also a significant treatment difference in the expression of MyoD mRNA after 4 weeks of exercise ($p<0.05$) between COPD (P) and COPD (S) groups.

Expression of muscle transcription factor mRNA.

The pattern of change in mRNA expression of FOXO1 and FOXO3 was broadly similar between the HC and COPD groups (Fig. 5S). RUNX1 was significantly up regulated after 24h exercise in all groups, decreasing significantly following 4 and 8 weeks training (Fig. 5S).

Pro-inflammatory genes

TNF- α mRNA expression increased significantly in all 3 groups after 24h exercise (Fig. 6S) In all groups however, expression declined with further training but remained significantly different from baseline at 8 wks in HC and COPD (S) groups. The pattern of change in muscle IL-6 mRNA expression was similar showing significant increases at 24 hrs and a subsequent decline after 4 and 8 weeks of training. There were no significant differences in training induced expression of inflammatory genes between the COPD groups.

Fasting plasma insulin concentration

There was no within or between group difference in fasting plasma insulin concentration at baseline or at any time point during the course of the study (Table 2S).

Associations between the molecular and functional responses to RT

Changes in protein expression, protein phosphorylation and mRNA expression were not correlated with changes in muscle mass or strength after RT.

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	Healthy Controls (n = 21)	COPD (n = 59)
Muscle Protein breakdown		
20S proteasome	3.18 (1.12)	3.92 (1.09)
MAFbx	1.45 (0.26)	1.46 (0.13)
MuRF1	1.96 (0.54)	1.93 (0.21)
ZNF216	2.28 (0.92)	1.64 (0.38)
Calpain3	3.12 (1.22)	4.89 (0.7)
Muscle Protein synthesis		
Akt1	1.44 (0.28)	2.06 (0.3)
GSK3 α	4.56 (1.67)	4.04 (0.9)
GSK3 β	1.67 (0.39)	1.73 (0.19)
P70s6kinase	1.91 (0.69)	1.20 (0.18)
4EBP1	1.34 (0.22)	1.3 (0.17)
Redd1	1.42 (0.32)	1.81 (0.23)
Myogenesis		
Myostatin	1.41 (0.22)	2.19 (0.22) *
MyoD	1.57 (0.30)	1.62 (0.26)
Myogenin	2.54 (0.79)	1.99 (0.36)
Transcription factors		
FOXO1	1.97 (0.95)	1.86 (0.24)
FOXO3	1.7 (0.35)	1.52 (0.27)
RUNX1	4.49 (1.09)	5.60 (0.73)
Inflammatory cytokines		
TNF α	1.93 (0.49)	1.29 (0.42)
IL6	2.61 (0.92)	2.08 (0.32)

Table 1S. Gene expression from muscle samples taken at baseline.

Figures refer to mean (SEM) mRNA expression relative to endogenous HMBS used as calibrator.
 HC = healthy controls.

* p < 0.05 significantly different from control

Plasma insulin (mU/L)	HC	COPD (P)	COPD (S)
Baseline	34.1 (8.9)	29.9 (7.8)	26.9 (3.7)
24 Hours	25.7 (6.0)	30.7 (6.9)	23.9 (2.7)
4 Weeks	18.7 (2.4)	30.1 (6.3)	20.0 (2.7)
8 Weeks	20.1 (3.3)	33.1 (6.2)	27.1 (4.6)

Table 2S Plasma Insulin

Figures refer to mean (SEM) values expressed in mU/L.

HC = healthy controls; COPD (P) = COPD patients receiving placebo; COPD (S) = COPD patients receiving supplement

Supplement Figure Legends

Figure 1S. Study Flow Diagram.

93 subjects were recruited to the study (71 COPD and 22 HC) but only those with at least a viable baseline and 24 hour biopsy are included in the analysis (21 HC and 59 COPD).

Non-viable biopsies at baseline (10): damaged samples/ defrosted.

Reasons for patient withdrawal at each stage, in all groups, are as follows:

COPD (S) = 1 unable to tolerate biopsy (after baseline/ before 24 hour biopsy), 2 exacerbation/ 1 back pain/ 1 family bereavement/ 1 too busy (weeks 1-4), 1 exacerbation/ 1 advised to stop by GP (weeks 4-8).

COPD (P) = 1 unable to tolerate biopsy (after baseline/ before 24 hour biopsy), 1 knee pain (weeks 1-4), 1 unable to re-start training post-operatively (weeks 4-8).

Healthy Control = 1 spouse of a COPD patient wanting to withdraw at the same time.

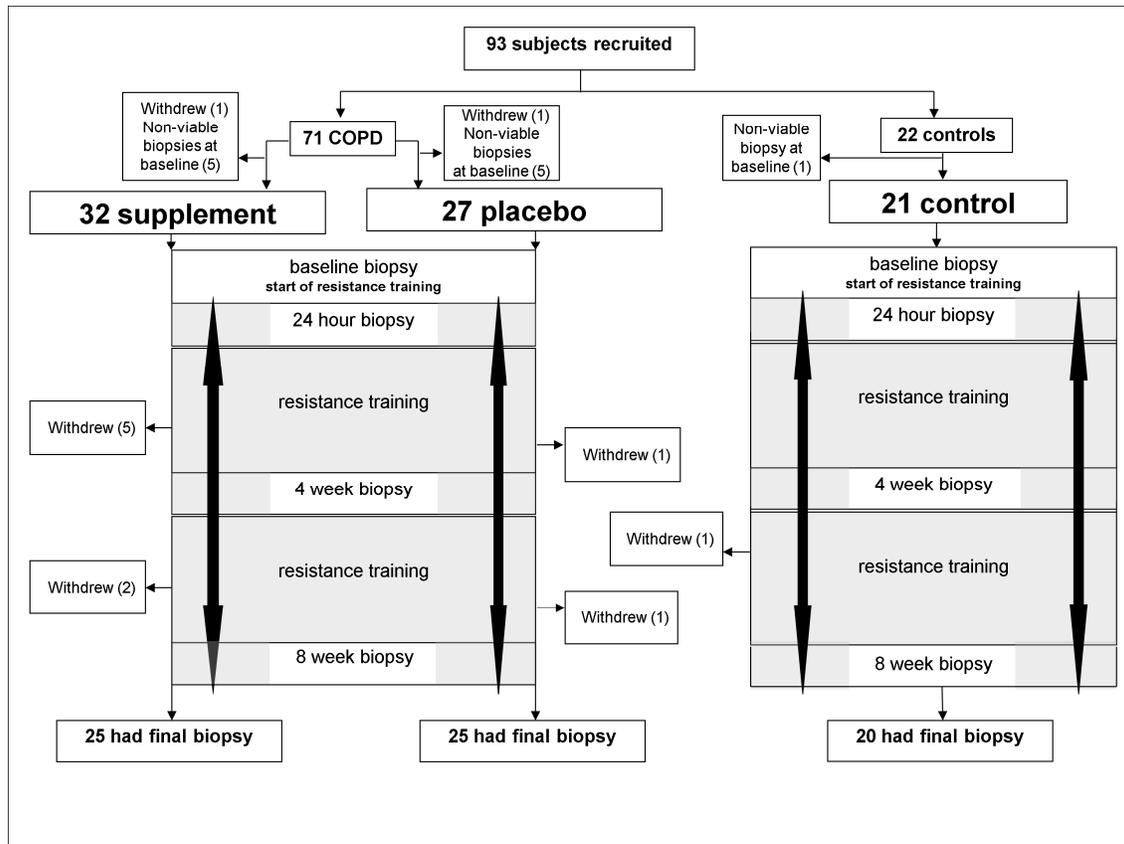


Figure 2S. Expression of target genes regulating muscle protein breakdown in response to training.

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean \pm SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement

- * Significantly different from baseline ($p < 0.05$)
- ** Significantly different from baseline ($p < 0.01$)
- *** Significantly different from baseline ($p < 0.001$)
- # Significantly different from 24 hours ($p < 0.05$)
- ## Significantly different from 24 hours ($p < 0.01$)
- ### Significantly different from 24 hours ($p < 0.001$)

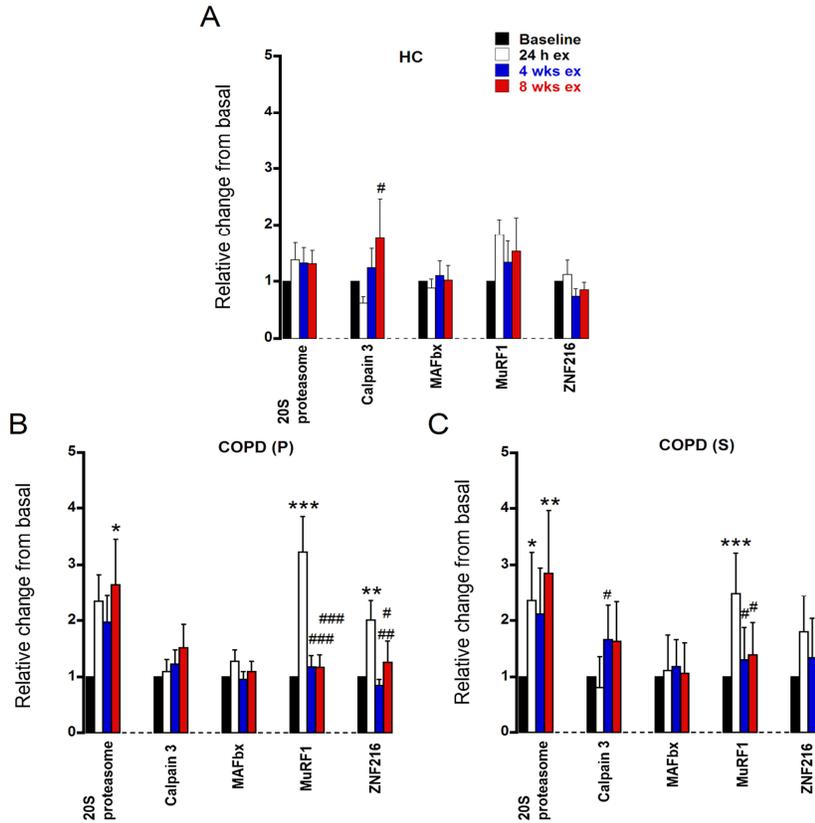


Figure 3S. Expression of target genes regulating muscle protein synthesis in response to training

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean \pm SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement.

* Significantly different from baseline ($p < 0.05$)

** Significantly different from baseline ($p < 0.01$)

Significantly different from 24 hours ($p < 0.05$)

Significantly different from 24 hours ($p < 0.01$)

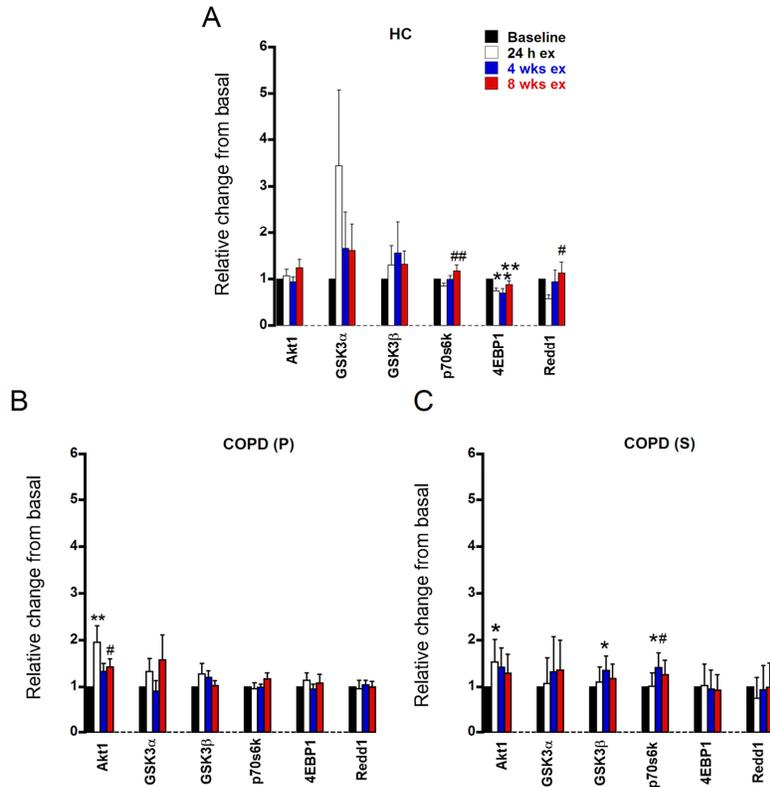


Figure 4S. Expression of target genes regulating myogenesis in response to training

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean \pm SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement.

- * Significantly different from baseline ($p < 0.05$)
- ** Significantly different from baseline ($p < 0.01$)
- # Significantly different from 24 hours ($p < 0.05$)
- ## Significantly different from 24 hours ($p < 0.01$)
- ### Significantly different from 24 hours ($p < 0.001$)

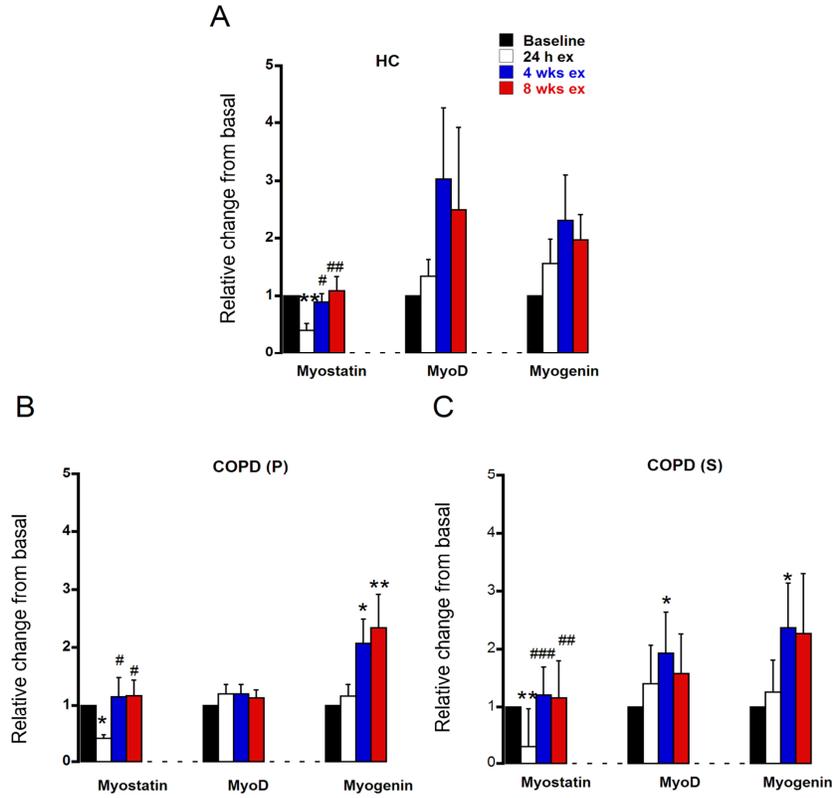


Figure 5S. Gene expression of transcription factors in response to training.

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean \pm SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement.

- * Significantly different from baseline ($p < 0.05$)
- ** Significantly different from baseline ($p < 0.01$)
- *** Significantly different from baseline ($p < 0.001$)
- # Significantly different from 24 hours ($p < 0.05$)
- ## Significantly different from 24 hours ($p < 0.01$)
- ### Significantly different from 24 hours ($p < 0.001$)

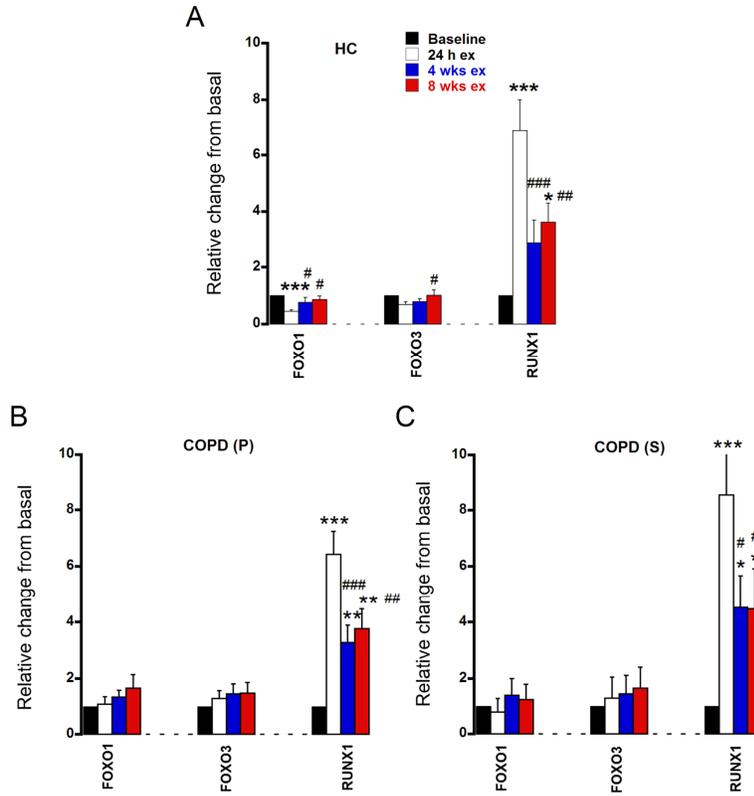


Figure 6S. Gene expression of inflammatory cytokines in response to training

Gene expression is represented as relative changes from basal. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to endogenous HMBS. Data represent mean \pm SEM.

A: HC = Healthy Control subjects; B: COPD (P) = COPD patients receiving placebo; C: COPD (S) = COPD patients receiving supplement.

* Significantly different from baseline ($p < 0.05$)

*** Significantly different from baseline ($p < 0.001$)

Significantly different from 24 hours ($p < 0.05$)

Significantly different from 24 hours ($p < 0.01$)

Significantly different from 24 hours ($p < 0.001$)

