Pathways associated with reduced quadriceps oxidative fibres and endurance in COPD.

Eur Respir J 2013; 41: 1275–1283
DOI: 10.1183/09031936.00098412

Pathways associated with reduced quadriceps oxidative fibres and endurance in COPD

Samantha A. Natanek*,#,¶, Harry R. Gosker¶, Ilse G.M. Slot¶, Gemma S. Marsh*, Nicholas S. Hopkinson*, John Moxham+, Paul R. Kemp#, Annemie M.W.J. Schols¶,§ and Michael I. Polkey*,§

ABSTRACT: Reduced quadriceps endurance in chronic obstructive pulmonary disease (COPD) is associated with a predominance of type II glycolytic fibres over type I oxidative fibres (fibre shift) and reduced muscle energy stores. The molecular mechanisms responsible for this remain unknown. We hypothesised that expression of known regulators of type I fibres and energy production in quadriceps muscle would differ in COPD patients with and without fibre shift.

We measured lung function, physical activity, exercise performance, quadriceps strength and endurance (nonvolitionally) in 38 Global Initiative for Chronic Obstructive Lung Disease stage I–IV COPD patients and 23 healthy age-matched controls. Participants underwent a quadriceps biopsy: type I and II fibre proportions were determined using immunohistochemistry and fibre shift defined using published reference ranges. Calcineurin A, phosphorylated AMP kinase (phospho-AMPK)-α, protein kinase A-α catalytic subunits, modulators of calcineurin activity and calmodulin, 14-3-3 proteins were measured by Western blotting, and myocyte-enriched calcineurin-interacting protein-1 mRNA measured by quantitative PCR. Downstream, nuclear myocyte enhancer factor-2 capable of DNA binding was quantified by transcription factor ELISA.

Unexpectedly, calcineurin expression was higher, while phospho-AMPK was lower, in COPD patients with fibre shift compared to COPD patients without fibre shift. Phospho-AMPK levels correlated with quadriceps endurance in patients.

Reduced phospho-AMPK may contribute to reduced quadriceps oxidative capacity and endurance in COPD.

KEYWORDS: AMP kinase, calcineurin, myocyte enhancer factor-2, protein kinase A

Reduced quadriceps endurance is associated with exercise limitation in chronic obstructive pulmonary disease (COPD) [1]. Underlying the loss of endurance is a reduction in type I myosin and oxidative enzymes [2], the hallmarks of the reduced oxidative type I to glycolytic type II fibre ratio observed in the quadriceps of COPD patients, which we will refer to in this study as the presence of fibre shift [3]. Type I fibres rely exclusively on oxidative metabolism to generate ATP, type IIx fibres depend on glycolysis, and type IIa fibres utilise both oxidative and glycolytic metabolism [4]. Since oxidative metabolism generates several times more ATP than glycolysis per molecule of glucose [5], type I fibres are fatigue-resistant compared to type II fibres. COPD patients, with their fewer oxidative fibres, have fewer muscle energy stores and exhibit metabolic stress from ATP depletion at rest and at low workloads, unlike controls. Reduced energy turnover may be exacerbated by reduced insulin sensitivity in COPD [6]; insulin driving cellular glucose uptake *via* the GLUT-4 receptor [7]. Understanding mechanisms underlying these changes may lead to treatments to improve exercise capacity in patients with COPD.

Pathways influencing muscle type I fibre differentiation during development, muscle oxidative enzymes and energy production/glucose uptake have been described in animal models (fig. 1) [8]. Calcineurin is a phosphatase which activates type I fibre-specific gene expression *in vitro* and, when

AFFILIATIONS
*Dept of Respiratory Medicine, National Institute for Health Research Respiratory Biomedical Research Unit of the Royal Brompton and Harefield NHS Foundation Trust and National Heart and Lung Institute, Imperial College London, London, and
#Molecular Medicine Section, National Heart and Lung Institute, Imperial College London, London,
+Dept of Respiratory Medicine, Guy's King's and St Thomas' School of Medicine, London, UK.
¶Dept of Respiratory Medicine, NUTRIM School for Nutrition, Toxicology and Metabolism, Maastricht University Medical Centre, Maastricht, The Netherlands.
§Both authors contributed equally.

CORRESPONDENCE
S.A. Natanek
National Heart and Lung Institute
Sir Alexander Fleming Building
South Kensington Campus
Exhibition Rd
London
SW7 2AZ
UK
E-mail: a.natanek@imperial.ac.uk

Received:
June 22 2012
Accepted after revision:
Aug 24 2012
First published online:
Dec 20 2012

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

This article has supplementary material available from www.erj.ersjournals.com

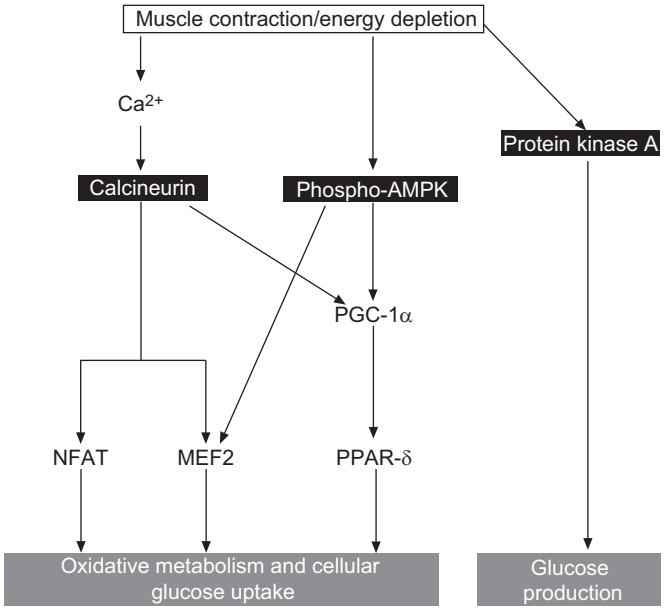

FIGURE 1. In response to muscle activity, calcineurin (and also calcium-calmodulin protein kinase IV, not shown here) signalling activates nuclear factor of activated T-cells (NFAT) translocation to the nucleus and increases myocyte enhancer factor (MEF)2 activation to drive oxidative muscle-specific gene transcription. In response to energy depletion, phosphorylated AMP kinase (phospho-AMPK) activates peroxisome proliferator-activated receptor γ co-activator (PGC)-1α and peroxisome proliferator-activated receptor (PPAR)-δ to drive oxidative muscle-specific gene transcription. Both calcineurin and phospho-AMPK upregulate GLUT-4 receptors, promoting muscle cell glucose uptake *via* MEF2. Protein kinase A promotes mobilisation of glucose production by glycogenolysis at times of increased requirement for energy production. Modified from [8].

blocked, can produce type I to type II fibre shift in animals, for example [9]. Calcineurin is activated by calcium bound to calmodulin in response to muscle activity, and is inhibited by endogenous protein inhibitors, particularly myocyte-enriched calcineurin-interacting protein-1 (MCIP1) [10]. Calcineurin stimulates targets including the myocyte enhancer factor (MEF)2 and nuclear factor of activated T-cells (NFAT) transcription factors. MEF2D and NFATc1 activate type I fibre gene expression [11]. 14-3-3 proteins influence calcineurin signalling by binding MCIP1 [12], releasing its inhibition of calcineurin activity, and by inhibiting NFAT [13]. In addition, calcineurin promotes cellular glucose uptake *via* the GLUT-4 receptor [9].

AMP kinase (AMPK) activates genes enhancing muscle oxidative metabolism to increase exercise endurance in animals [14], and induces muscle glucose uptake *via* GLUT-4 receptor transcription through a MEF2-dependent mechanism [15]. It is activated by phosphorylation under conditions of metabolic stress including exercise. Protein kinase A (PKA) is an enzyme that mobilises glucose (*e.g.* by glycogenolysis) to provide muscle with ATP during exercise [16].

This is the first study investigating the expression of calcineurin, phospho-AMPK and PKA, and MEF2 DNA binding in quadriceps muscle from COPD patients and healthy controls. The rationale for investigating the three enzymes was: 1) they are all responsive to muscle activity which is decreased in COPD [17]; 2) they affect muscle energy production and/or type I fibre specification; and 3) calcineurin and AMPK both activate MEF2. We hypothesised that calcineurin and MEF2 expression would be reduced, but phospho-AMPK and PKA expression would be increased in response to metabolic stress, in the quadriceps muscle of COPD patients with fibre shift compared to patients without fibre shift.

METHODS

Ethical approval

Ethical approval was received from the Royal Brompton and Harefield NHS Trust (06/Q0404/35) and the Ealing and West London Mental Health Trust Ethics Committees (06/Q0410/54). Patients gave written, informed consent.

Participants

38 patients with COPD were recruited from clinics at the Royal Brompton Hospital, London, UK. 23 healthy controls were recruited by advertisement. Exclusion criteria were: diagnoses of heart, renal or liver failure; systemic inflammatory, metabolic or neuromuscular disorders (independently associated with skeletal muscle abnormalities); warfarin therapy (bleeding risk from biopsy); or a moderate/severe exacerbation (*i.e.* requiring intervention) within the preceding 4 weeks. Specimens from these participants have been used previously [18, 19].

Physiological measurements

Post-bronchodilator spirometry, lung volumes (plethysmography) and diffusion capacity were measured according to European Respiratory Society/American Thoracic Society (ATS) guidelines [20–22], and arterialised capillary earlobe blood gas tensions were recorded. Fat-free mass index (FFMI) was calculated from bioelectrical impedance measurements (Bodystat 1500; Bodystat, Douglas, UK) using a disease-specific regression equation [23]. Physical activity was measured over 12 h on 2 days (Dynaport accelerometer; McRoberts, The Hague, the Netherlands) as validated for COPD [24]. Quadriceps strength was assessed by supine isometric maximal voluntary contraction [25]. Quadriceps endurance was assessed by timing the force decline to 80% of initial response during trains of magnetic femoral nerve stimulation as described previously [2]. Exercise performance was assessed with a 6-min walk test [26] and symptom-limited incremental cycle ergometry with measurement of peak oxygen uptake [27].

Quadriceps sampling

Percutaneous biopsy of the vastus lateralis was performed using the technique of BERGSTROM [28] after subjects had rested for 20 min, on a day without strenuous physical activity. Samples for histology and mRNA/protein analysis were frozen in melting isopentane and liquid nitrogen, respectively, prior to storing at -80°C (online supplementary material).

Measurement of quadriceps fibre type proportions and fibre cross-sectional area

Immunohistochemistry using antibodies against type I and IIa myosin and laminin was performed on transverse muscle sections to calculate type I, IIa (both pure IIa and hybrid IIa/IIx fibres, which could not be differentiated), IIx and hybrid I/IIa fibre proportions and the median cross-sectional area (CSA) for each fibre type, from ⩾100 fibres (online supplementary

material) [29]. Fibre shift was defined by type I fibre proportions falling below and/or type IIx fibre proportions falling above the cut-off taken from healthy 60–70-year-olds [3].

Measurement of calcineurin A, phospho-AMPK-α, PKA-α subunits, calmodulin and 14-3-3 proteins

Western blotting was performed with 20–50 μg protein from 38 patients and 23 controls (34 patients and 19 controls for calcineurin, 24 patients and 23 controls for phospho-AMPK) and results normalised by immunoblotting for α-tubulin (online supplementary material). Expression of the catalytically active subunits calcineurin A, phospho-AMPK-α and the predominant muscle isoform PKA-α was quantified.

Measurement of MEF2 in muscle nuclear extracts capable of binding DNA

MEF2 (all subclasses) DNA-binding was quantified using transcription factor ELISA (Panomics, Santa Clara, CA, USA) in duplicate per sample (online supplementary material). Quantity was proportional to the relative light unit fluorescence emitted.

Measurement of MCIP1 and myosin heavy chain I mRNA

Both MCIP1 and the myosin heavy chain I gene (*MyH7*) are target genes of calcineurin. *MyH7* transcripts indicate current transcription supporting type I fibres, while type I fibre proportion reflects transcription several weeks prior; hence both were measured. mRNA was measured by quantitative (q)PCR using SYBR green and normalised to acidic ribosomal phosphoprotein PO [30] and β-2-microglobulin transcripts using geNorm (online supplementary material) [31].

Statistics

Group differences in normally distributed, non-normally distributed and categorical data were tested with the t-test, Mann–Whitney U-test and Fisher's exact test, respectively. Spearman's rank (ρ) correlation was calculated to assess correlations. A two-tailed p-value of $\leqslant 0.05$ was set as defining statistical significance.

RESULTS

Characteristics of COPD patients compared to controls

Age and sex were not significantly different between patients and controls. Patients had significantly reduced lung function, arterialised oxygen tensions, FFMI, quadriceps strength and endurance, physical activity and exercise performance compared to controls (table 1). One (3%), nine (23%), 14 (37%) and 14 (37%) patients had Global Initiative for Chronic Obstructive Lung Disease stage I, II, III and IV disease, respectively. As a group, COPD patients had a significantly lower type I and higher type IIa and IIx fibre proportion, and a reduced type IIx fibre CSA compared with controls, consistent with previous observations (table 2) [3, 32]. 13 (34%) patients had fibre shift. All controls had fibre proportions within the normal range.

TABLE 1 The physiological and quadriceps fibre characteristics of patients and controls

	COPD	Controls	p-value	COPD without fibre shift	COPD with fibre shift	p-value
Subjects n	38	23		25	13	
Age years	68±8	67±8	0.38	68±8	69±6	0.64
Male	63	57	0.79	64	62	1.00
Smoking history pack-years	45 (36–60)	4 (0–10)	<0.0001	45 (35–59)	45 (37–82)	0.44
Smoking status			0.038			0.39
Current	18	0		24	8	
Ex or never	82	100		76	92	
FEV_1 L	0.98±0.44	2.95±0.63	<0.0001	0.94 (0.64–1.17)	0.75 (0.61–1.22)	0.24
FEV_1 % pred	41±18	110±14	<0.0001	41 (27–53)	33 (24–51)	0.52
Residual volume % of TLC	60±9	38±1	<0.0001	60±9	60±9	0.92
***T*LCO % pred**	41±17	90±14	<0.0001	46±17	33±15	0.02
***P*aO$_2$ kPa**	9.4±1.2	10.8±1.4	<0.0001	9.5±1.3	9.3±1.0	0.49
***P*aCO$_2$ kPa**	5.2±0.7	5.2±0.4	0.91	5.2±0.6	5.3±0.6	0.80
Body mass index kg·m^{-2}	23.8±3.6	26.3±4.4	0.18	23.8±3.7	23.7±3.7	0.91
Fat-free mass kg	43±8	51±12	0.002	42±7	44±10	0.34
Fat-free mass index kg·m^{-2}	15.6±1.9	17.4±2.4	0.002	15.6±1.8	15.7±2.1	0.84
Quadriceps MVC kg	27±9	36±10	<0.0001	27 (22–33)	24 (18–33)	0.35
Quadriceps endurance T_{80} s	92±41	137±73	0.024	85 (78–113)	78 (66–90)	0.24
Locomotion time min·12 h^{-1}	37 (21–52)	95 (61–131)	<0.0001	38 (22–66)	26 (17–43)	0.27
6-min walk test distance m	394±108	617±84	<0.0001	416±110	352±93	0.08
Peak *V*′O$_2$ mL·kg^{-1}·min^{-1}	12.0±3.4	23.9±7.3	<0.0001	12.2 (10.8–14.4)	9.9(9.1–10.8)	0.02

Data are presented as %, mean±SD or median (interquartile range), unless otherwise stated. COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; TLC: total lung capacity; *T*LCO: transfer factor of the lung for carbon monoxide; *P*aO$_2$: arterial oxygen tension; *P*aCO$_2$: arterial carbon dioxide tension; MVC: maximal voluntary contraction; T_{80}: force decline to 80% of initial response; *V*′O$_2$: oxygen uptake.

TABLE 2 Fibre proportions and fibre cross-sectional area (CSA) of the quadriceps muscle of chronic obstructive pulmonary disease (COPD) patients and healthy controls

	COPD	Controls	p-value	COPD without fibre shift	COPD with fibre shift	p-value
Subjects n	38	23		25	13	
Type I fibres %	29±13	54±14	<0.0001	37 (32–42)	13±7	<0.00011
Type I/IIa fibres %	3 (1–7)	0 (2–6)	0.22	3 (1–8)	3 (0–7)	0.96
Type IIa fibres %	60±12	41±14	<0.0001	57 (49–60)	71±12	<0.0001
Type IIx fibres %	4 (1–7)	1 (0–4)	0.025	2 (1–5)	7 (2–20)	0.026
Type I fibre CSA μm^2	5160±1790	5650±1410	0.27	4950±1480	5580±2290	0.31
Type I/IIa fibre CSA μm^2	5060 (3600–6040)	5460 (4620–6530)	0.19	4800±1510	5030±1650	0.72
Type IIa fibre CSA μm^2	3870±1400	4570±1600	0.08	3730±1050	4150±1930	0.39
Type IIx fibre CSA μm^2	2680 (1740–3450)	5130 (3600–6950)	<0.0001	2870±1230	2570±907	0.47

Data are presented as mean±SD or median (interquartile range), unless otherwise state. Fibre proportions are correct but do not add up to 100% as median values for each fibre type may not be from the same individual.

Characteristics of COPD patients with fibre shift compared with COPD patients without fibre shift

Patients with fibre shift had a significantly lower transfer factor of the lung for carbon monoxide, but not a lower forced expiratory volume in 1 s, compared with patients without fibre shift. In addition, patients with fibre shift had a poorer exercise performance on the incremental cycle ergometry and a trend to a reduced 6-min walking distance than those without fibre shift, without group differences in FFMI or quadriceps strength to account for this. Physical activity levels were not significantly different between these groups (table 1).

All patients with fibre shift fulfilled the criteria for fibre shift by having a low type I fibre proportion, with an increase in the type IIa population without the type IIx fibre proportion exceeding the normal range. Type I and II fibre CSA were not different between patients with and without fibre shift (table 2 and fig. 1), consistent with no difference in FFMI between the groups (table 1).

Expression of calcineurin and calcineurin modulators, phospho-AMPK and PKA in COPD patients and controls

Calcineurin A expression was higher in quadriceps of COPD patients than controls, and furthermore, was significantly higher in patients with fibre shift than patients without fibre shift (table 3, figs 2a and 3a). However, patients had significantly lower *MyH7* transcripts than controls and there was a trend to lower *MyH7* transcripts in patients with fibre shift than without. There were no significant differences in expression of calmodulin, 14-3-3 proteins, or MCIP1 mRNA.

Phospho-AMPK was not significantly different between COPD patients and controls but the median amount was three-fold higher in the COPD patients without fibre shift compared with those with fibre shift (p=0.005) (table 3, figs 2b and 3b).

Expression of PKA-α was not significantly different in COPD patients and controls, or between patients with and without fibre shift (table 3, figs 2c and 3c). α-tubulin levels were not significantly different between patients and controls

TABLE 3 Protein expression of calcineurin A, protein kinase A-α subunit and phosphorylated AMP kinase and MEF2 DNA-binding in quadriceps muscle from chronic obstructive pulmonary disease (COPD) patients and healthy controls

	COPD	Controls	p- value	COPD without fibre shift	COPD with fibre shift	p-value
Subjects n	38	23		25	13	
Calcineurin A protein AU	0.82±0.34[#]	0.60±0.37[¶]	0.036	0.74 (0.43–0.97) [+]	0.98 (0.69–1.30)[§]	0.03
MEF2 DNA-binding RLU	1710 (696–2320)	2660 (1830–3550)	0.004	1750 (928–2460)	1440 (329–2290)	0.29
Calmodulin AU	1.09±0.66	1.05±0.70	0.81	0.71 (0.56–1.38)	1.32 (0.63–1.67)[ƒ]	0.19
MCIP mRNA AU	0.03 (0.03–0.05)	0.04 (0.03–0.06)	0.66	0.03 (0.03–0.05)	0.05 (0.03–0.08)	0.14
14-3-3 proteins AU	0.94 (0.64–1.48)	0.89 (0.43–1.11)	0.30	0.94 (0.72–1.48)	0.94 (0.38–2.02)	0.78
MyHC I mRNA AU	0.05 (0.03–0.06)	0.08 (0.04–0.10)	0.004	0.05 (0.04–0.07)	0.03 (0.02–0.05)	0.06
Phospho-AMPK-α protein AU	3.93 (1.82–7.95)[##]	2.37 (1.11–9.61)	0.64	7.08 (4.78–11.1)[ƒ]	2.07 (1.73–3.24)[ƒ]	0.005
PKA-α protein AU	1.28±0.51	1.48±0.58	0.18	1.17 (0.99–1.43)	2.1 (1.7–3.2)	0.93

Data are presented as mean±SD or median (interquartile range), unless otherwise stated. Sample numbers are given where analysis was completed in less than a full set of subjects. Figures are correct to 3 significant figures. MEF: myocyte enhancer factor; RLU: relative light unit; MCIP: modulatory calcineurin-interacting protein 1; MyHC myosin heavy chain; phospho-AMPK: phosphorylated AMP kinase; PKA: protein kinase A. [#]: n=34; [¶]: n=19; [+]: n=23; [§]: n=11; [ƒ]: n=12; [##]: n=24.

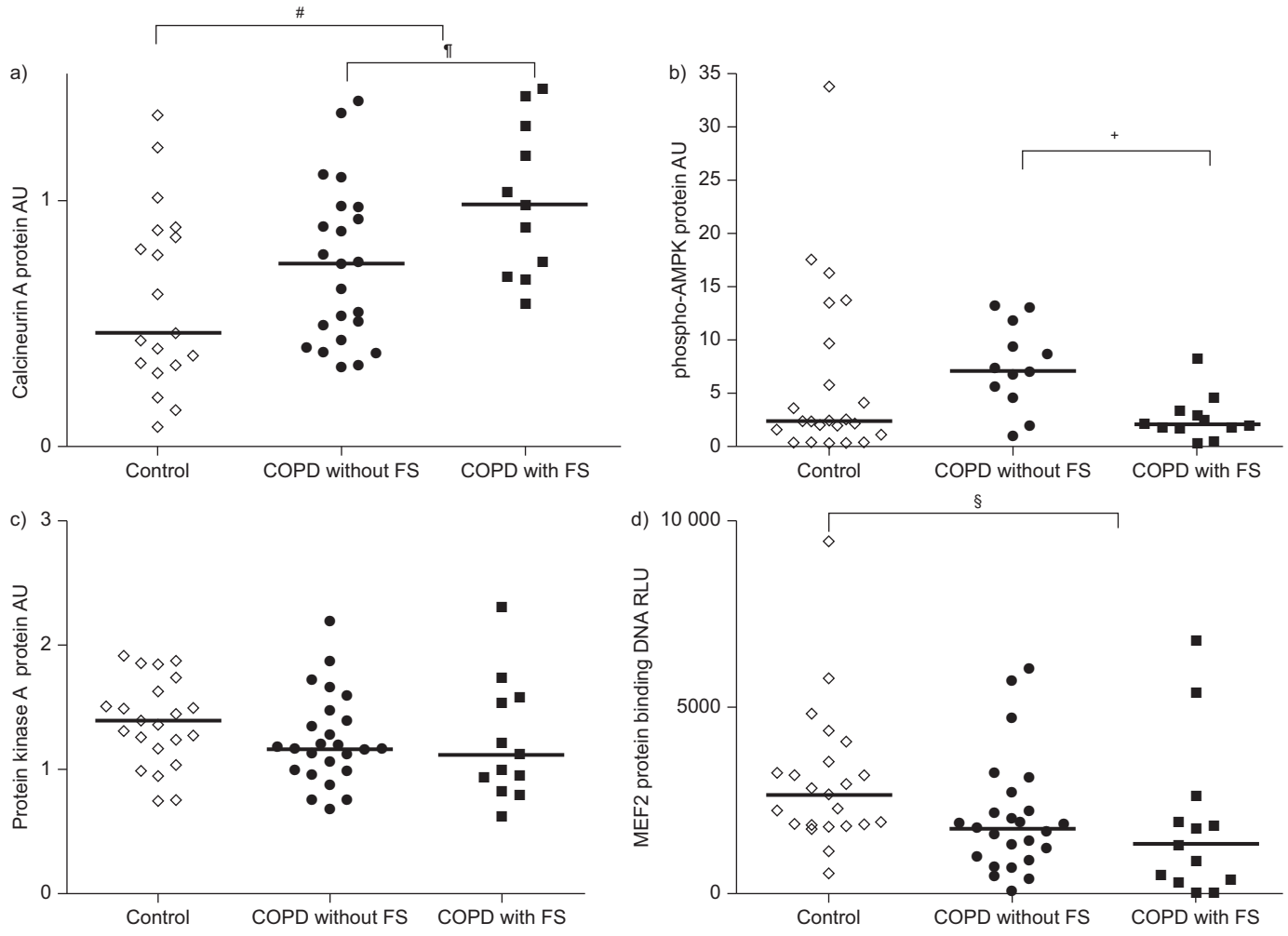

FIGURE 2. a) Calcineurin protein A expression was higher in the quadriceps of chronic obstructive pulmonary disease (COPD) patients with fibre shift (FS) than patients without fibre shift (0.98 (0.69–1.30) AU *versus* 0.74 (0.43–0.97) AU, p=0.030), and higher in COPD patients overall compared to controls (0.82±0.34 AU *versus* 0.60±0.37 AU, p=0.036). b) Phospho-AMP kinase protein (phospho-AMPK) was three-fold lower in the COPD patients with fibre shift than those without fibre shift (2.07 (1.73–3.24) AU *versus* 7.08 (4.78–11.1) AU, p=0.005), although levels were not significantly different between COPD patients overall compared to controls (3.93 (1.32–7.95) AU *versus* 2.37 (1.11–9.61) AU, p=0.64). c) Protein kinase A protein was not significantly different between any group. d) There was no significant difference in myocyte enhancer factor (MEF)2 capable of binding DNA between patients with and without fibre shift (1440 (329–2290) relative light units (RLU) *versus* 1750 (928–2460) RLU, p=0.29), although muscle MEF2 DNA-binding was lower in COPD patients than in controls (1710 (696–2320) RLU *versus* 2660 (1830–3550) RLU, p=0.004). #: p=0.036; ¶: p=0.03; +: p=0.005; §: p=0.04.

(54.9±17.4 AU *versus* 48.2±20.7 AU, p=0.21), confirming reports that α-tubulin is a valid loading control in COPD [30, 33].

MEF2 DNA-binding in nuclear extracts of quadriceps muscle

MEF2 DNA-binding was significantly lower in muscle from COPD patients compared to controls (table 3, fig. 2d), but there was no difference between patients with and without fibre shift.

Correlations between mediators, quadriceps oxidative fibre proportion and quadriceps endurance in COPD

There was no correlation between calcineurin and quadriceps type I fibre proportion (ρ= -0.17, p=0.33) (fig. 4a) or quadriceps endurance (ρ= -0.05, p=0.80) in patients or in patients and controls combined (ρ= -0.18, p=0.21 and ρ= -0.05, p=0.75, respectively). Modulators of calcineurin activity, PKA and MEF2 were not correlated with muscle histology or muscle endurance. Phospho-AMPK, however, was strongly correlated with quadriceps type I fibre proportion (ρ=0.54, p=0.006) (fig. 4b) and quadriceps endurance (ρ=0.47, p=0.029) (fig. 4c) in patients.

When patients and controls were combined, MEF2 was significantly correlated with quadriceps strength (ρ=0.43, p<0.0001), FFMI (ρ=0.27, p=0.033) and exercise performance (6-min walk distance ρ=0.34, p=0.008 and peak oxygen uptake on cycle ergometry (ρ=0.32, p=0.012)) (fig. E1).

DISCUSSION

This is the first study investigating potential molecular mechanisms underlying the reduced quadriceps muscle oxidative fibres and energy stores that contribute to poor muscle endurance and exercise performance in COPD. We

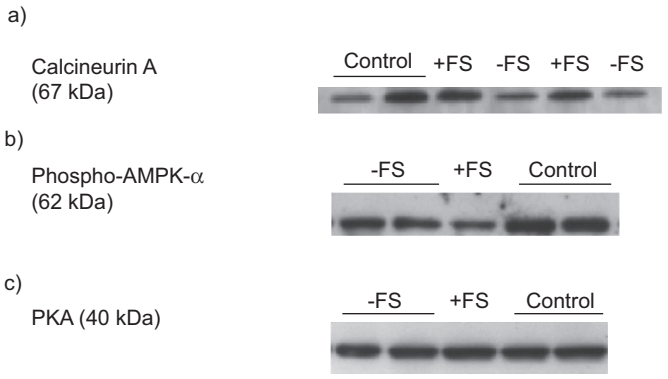

FIGURE 3. Western blot images illustrating the finding that a) calcineurin levels were higher while b) phosphorylated AMP kinase (phospho-AMPK)-α protein levels were lower in chronic obstructive pulmonary disease (COPD) patients with fibre shift (+FS) than patients without fibre shift (-FS), while there was no significant difference in c) protein kinase A (PKA) protein between COPD patients with fibre shift, patients without fibre shift or healthy controls.

demonstrated that phospho-AMPK is lower in the quadriceps muscle of COPD patients with fibre shift than patients without fibre shift. Furthermore, levels correlate with quadriceps endurance in patients. Reduced muscle AMPK expression may, therefore, contribute to reduced quadriceps endurance in COPD. Unexpectedly, calcineurin expression was increased in COPD patients with fibre shift compared to patients without fibre shift and healthy controls. Despite this adaptation, downstream nuclear MEF2 capable of binding to DNA and myosin heavy chain I transcription were reduced in patients compared to controls.

We did not find that COPD patients with fibre shift were significantly less active than those without fibre shift (table 1), suggesting that other factors must be important in driving fibre shift. Patients with fibre shift had a greater degree of emphysema, but not greater airflow obstruction or resting hypoxaemia, than patients without fibre shift, although our data do not preclude transient hypoxia, perhaps sleep or exercise induced, as a stimulus to fibre shift. Certainly chronic hypoxia reduces muscle oxidative fibres in animals [34]. Alternatively, emphysema and fibre shift could develop through a common mechanism, and may not be linked through muscle hypoxia.

Significance of the findings

We have consolidated previous data highlighting the importance of muscle oxidative phenotype to exercise endurance in COPD [35] and in health [36], by demonstrating that patients with fibre shift have poorer exercise performance than patients without fibre shift, independent of differences in muscle mass or quadriceps strength (table 1). Quadriceps fatigue during exercise has been reported in a significant proportion of patients with COPD, who are poorly responsive to bronchodilators during exercise [1] presumably because performance is limited by muscle endurance rather than ventilatory limitation. Our finding that phospho-AMPK levels correlated not just with quadriceps type I fibre proportion, but also with quadriceps endurance in COPD patients (fig. 4), is therefore important, as increasing these levels using a pharmacological agent such as metformin [37] or a nutritional supplement such as resveratrol [38], for example, could potentially enhance exercise performance in patients with fibre shift, in whom muscle fatigue is likely to pose a limitation.

We had expected an adaptive increase in phospho-AMPK in the muscle of patients, as an appropriate response to the metabolic and oxidative stress that occurs in COPD [39, 40]. This was observed in patients without fibre shift, who showed a three-fold increase in phospho-AMPK levels compared to controls, but not in patients with fibre shift (table 3). In addition, we have previously shown that downstream of AMPK, peroxisome proliferator-activated receptor (PPAR)-δ, a transcription factor promoting type I fibre differentiation in animals [41], is downregulated in the quadriceps in COPD [42].

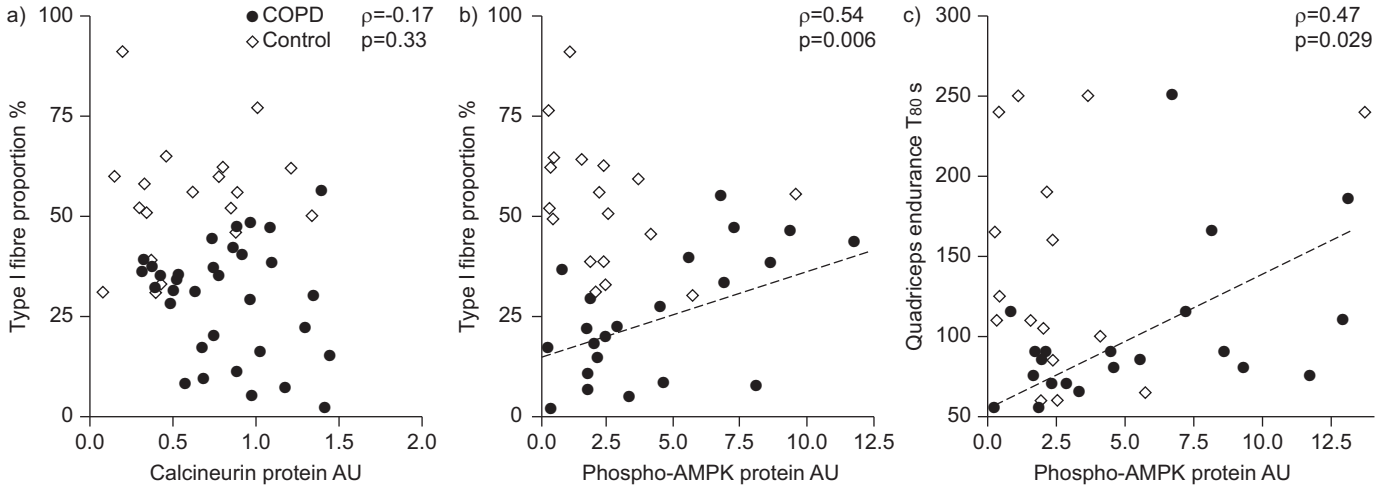

FIGURE 4. a) There was no significant correlation between type I fibre proportion and calcineurin in patients alone (ρ= -0.17, p=0.33) or when patients and controls were combined (ρ= -0.18, p=0.21). Phospho-AMP kinase (phospho-AMPK) was strongly correlated with b) quadriceps type I fibre proportion (ρ=0.54, p=0.006) and c) quadriceps endurance measured nonvolitionally (ρ=0.47, p=0.029) in patients. Modulators of calcineurin activity and protein kinase A were not correlated with muscle histology or quadriceps endurance. COPD: chronic obstructive pulmonary disease. T_{80}: force decline to 80% of initial response.

The paradoxical increase in calcineurin expression in COPD patients, particularly those with fibre shift (table 3), has a number of possible explanations. It could be the result of feedback in response to reduced calcineurin activity, from reduced muscle activity [8] (though we did not find an association between physical activity and calcineurin) or resistance to calcineurin activity downstream. The finding of increased phosphorylated AKT in COPD muscle by DOUCET *et al*. [30] supports a resistance to calcineurin activity, since AKT is dephosphorylated by activated calcineurin [43]. Alternatively, the increase in calcineurin expression could be a compensatory response to fibre shift generated due to disruption of an alternative signalling pathway. The enhanced expression could also be a response to muscle atrophy in view of calcineurin's possible, but debated, role in skeletal muscle regeneration and hypertrophy [44, 45]. Interestingly, calcineurin may have different effects depending on the disease context: in the *mdx* mouse model of Duchenne muscular dystrophy, calcineurin activation has a protective effect [46], whereas in limb-girdle muscular dystrophy models calcineurin disruption is beneficial [47]. The influence of calcineurin on muscle phenotype is clearly complex and further work on downstream signalling in patient muscle may indicate whether augmenting this pathway could be of therapeutic benefit to COPD patients.

MEF2 is a key regulator of muscle-specific gene expression, and is co-activated by PPAR-γ co-activator 1-α when involved in type I fibre-specific gene expression [48]. Although DNA-binding is a prerequisite for transcriptional activity, additional factors influence MEF2 activity such as sumoylation and acetylation [49] (increase activity) and interaction with class II histone deacetylases, HDAC4 and HDAC5 [50], which are activated in muscle by inactivity and decrease MEF2 activity. We have previously shown increased HDAC4 in the quadriceps muscle of COPD patients compared to controls [18], and here MEF2 DNA-binding was lower in patients than controls and associated with quadriceps strength, muscle mass and exercise performance (table 3 and fig. E1). These findings may have been the result of lower physical activity in patients than controls; we were unable to match these groups for activity since reduced physical activity is so intrinsic to the disease, occurring even in mild COPD [17], not only the moderate to very severe group we studied. The lack of difference in patients with and without fibre shift, who did not differ in physical activity levels, suggest that any signalling disturbances leading to fibre shift occur independently of a reduction in nuclear MEF2 able to bind DNA.

In stable COPD patients at rest, PKA expression was not chronically altered (table 3). However, this does not exclude differences in PKA activity or PKA transcription/post-translational modification during exercise since patients experience greater metabolic stress than controls [39].

Critique of the method

As is common with human work, the study is descriptive and observational; causality cannot be confirmed without selective manipulation of each signalling factor in patients. We also report expression of the various enzymes and modulators and not activity, which is influenced by factors in addition to expression, as we have alluded to. We attempted to measure calcineurin activity using a phosphatase testing kit (Profluor kit 5, Promega, Madison, WI, USA) and found it impossible to isolate the effects of calcineurin from phosphatases 2A and 2C, even utilising inhibitors such as okadaic acid. To our knowledge, calcineurin activity in human skeletal muscle has not been reported previously.

There are limitations to most accepted techniques for measuring transcription factor activity. Transcription factor ELISAs, like electrophoretic mobility shift assays, measure the amount of transcription factor translocated to the nucleus in an appropriate configuration and affinity for DNA to bind, but not the amount bound to DNA *in vivo* at the time of the biopsy. The technique of chromatin immunoprecipitation, not previously performed on human muscle, can quantify transcription factor bound to DNA *in vivo* but still does not quantify transcriptional activity, which may be altered by presence of cofactors, *etc.*

It could be argued that the differences in calcineurin and phospho-AMPK levels between patients with and without fibre shift are simply the result of differential expression between type I and type II fibres. Were this the case, we would have found significant differences in phospho-AMPK between patients and controls, and correlations between phospho-AMPK and calcineurin and type I fibre proportion in controls, which we did not. Also, to our knowledge, there are no published data on fibre type differences in calcineurin and AMPK protein expression in human muscle, although in human skeletal muscle, mRNA levels of the inhibitory AMPK-γ subunit [51] are higher in glycolytic fibres.

In summary, phospho-AMPK was reduced in the quadriceps of stable patients with COPD who have oxidative to glycolytic fibre type shift, reduced muscle endurance and reduced exercise performance. Therefore, increasing phospho-AMPK in skeletal muscle of COPD patients with fibre shift may be a viable therapeutic approach. Conversely, calcineurin expression was increased in patients with fibre shift in the quadriceps, which we suggest is the result of resistance to calcineurin signalling, in which case increasing expression further may not be an appropriate therapeutic strategy. MEF2 DNA-binding does not appear to differentiate patients with and without fibre shift, but is associated with muscle mass and strength, and therefore may be best explored in patients with marked quadriceps wasting and weakness.

SUPPORT STATEMENT

S.A. Natanek was funded by a Wellcome Clinical Fellowship (grant number 079686). H.R. Gosker was funded by a Netherlands Asthma Foundation grant (grant number NAF 3.4.05.038). G.S. Marsh was funded by an award from GSK to Imperial College. N.S. Hopkinson is a Higher Education Funding Council for England (HEFCE) clinical senior lecturer. The study was carried out within the National Institute for Health Research Respiratory Biomedical Research Unit at the Royal Brompton Hospital and Imperial College (London, UK), which part funds M.I. Polkey's salary.

STATEMENT OF INTEREST

Conflict of interest information can be found alongside the online version of this article at www.erj.ersjournals.com

ACKNOWLEDGEMENTS

We would like to thank Winston Banya, in the Statistics Department of the Royal Brompton Hospital (London, UK), for his help with the statistical analysis, Derek Cramer, Mark Unstead and the lung function

department at the Royal Brompton Hospital, London, for performing the pulmonary function tests, and Kathleen Daenen at The University Hospital Maastricht (Maastricht, the Netherlands) for the cryosectioning.

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