PATHWAYS ASSOCIATED WITH REDUCED QUADRICEPS OXIDATIVE FIBRES AND ENDURANCE IN COPD

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Running head: Regulation of muscle oxidative fibres in COPD
**ABSTRACT**

**Rationale:** Reduced quadriceps endurance in COPD is associated with a predominance of type II glycolytic over type I oxidative fibres (fibre shift, FS) and reduced muscle energy stores. 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 FS.

**Methods:** We measured lung function, physical activity, exercise performance, quadriceps strength and endurance (non-volitionally) in 38 GOLD Stage I-IV COPD patients and 23 healthy age-matched controls. Participants had a quadriceps biopsy; type I and II fibre proportions were determined using immunohistochemistry and FS defined using published reference ranges. Calcineurin A, phosphorylated adenosine monophosphate kinase-alpha (phospho-AMPK) and protein kinase A-alpha catalytic subunits were measured by western blotting and modulators of calcineurin activity, calmodulin, 14-3-3 proteins, and myocyte-enriched calcineurin-interacting protein-1 mRNA measured by western blotting and qPCR respectively. Downstream, nuclear myocyte enhancer factor-2 capable of DNA-binding was quantified by transcription factor ELISA.
Results: Unexpectedly calcineurin expression was higher, while phospho-AMPK was lower, in COPD patients with than without FS. Phospho-AMPK levels correlated with quadriceps endurance in patients.

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

Word count: 200

Key words: AMP kinase, calcineurin, myocyte enhancer factor-2, protein kinase A
INTRODUCTION

Reduced quadriceps endurance is associated with exercise limitation in 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 as the presence of fibre shift (FS) (3). Type I fibres rely exclusively on oxidative metabolism to generate adenosine triphosphate (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 less 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 (8) (Fig. 1). Calcineurin is a phosphatase which activates type I fibre-specific gene expression in vitro and when blocked can produce FS 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-2 (MEF2) 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).

Adenosine monophosphate 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 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 a) they are all responsive to muscle activity which is decreased in COPD (17), b) they affect muscle energy production and/or type I fibre specification and c) 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 FS compared to patients without FS.

METHODS

Ethical approval

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

Participants

38 patients with COPD were recruited from clinic at the Royal Brompton Hospital. 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 American Thoracic Society (ATS) guidelines (20-22) and arterialised capillary earlobe blood gas tensions recorded. Fat-free mass index (FFMI) was calculated from bioelectrical impedance measurements (Bodystat 1500, UK) using a disease-specific regression equation (23). Physical activity was measured over 12 hours on 2 days (Dynaport accelerometer, McRoberts BV, Netherlands) as validated for COPD (24). Quadriceps strength was assessed by supine isometric maximal voluntary contraction (25). Quadriceps endurance (T80) 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-minute walk (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 Bergstrom technique (28) after subjects had rested for 20 minutes, 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 (see supplement).
Measurement of quadriceps fibre type proportions and fibre cross-sectional area (CSA)

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 CSA for each fibre type, from at least 100 fibres (29) (see supplement). FS 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-alpha, PKA-alpha 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 alpha-tubulin (see supplement). Expression of the catalytically-active subunits calcineurin A, phospho-AMPK-alpha and the predominant muscle isoform PKA-alpha was quantified.

Measurement of MEF2 in muscle nuclear extracts capable of binding DNA (DNA-binding for short)
MEF2 (all subclasses) DNA-binding was quantified using transcription factor enzyme-linked immunosorbent assays (Panomics, USA) in duplicate per sample (see supplement). Quantity was proportional to the relative light unit (RLU) 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 qPCR using SYBR green and normalised to acidic ribosomal phosphoprotein PO (RPLPO) (30) and beta-2-microglobulin transcripts using geNorm (31)(see supplement).

**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 2-tailed p-value of ≤0.05 was set as defining statistical significance.

**RESULTS**

**Characteristics of COPD patients compared to controls**
Age and gender 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). 1 (3%), 9 (23%), 14 (37%) and 14 (37%) patients had GOLD 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 (3, 32) (Table 2). 13 (34%) patients had FS. All controls had fibre proportions within the normal range.

Characteristics of COPD patients with FS compared to COPD patients without FS

Patients with FS had a significantly lower TLco, but not a lower FEV1, compared to patients without FS. In addition, patients with FS had a poorer exercise performance on the incremental cycle ergometry and a trend to a reduced 6-minute walk distance than those without FS, 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 FS fulfilled the criteria for FS 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 FS (Table 2, representative images in
supplemental Fig. E1), consistent with no difference in FFMI between the groups (Table 1).
Table 1: The physiological and quadriceps fibre characteristics of patients and controls

<table>
<thead>
<tr>
<th></th>
<th>COPD (n=38)</th>
<th>Controls (n=23)</th>
<th>p value</th>
<th>COPD without FS (n=25)</th>
<th>COPD with FS (n=13)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>68(8)</td>
<td>67(8)</td>
<td>0.38</td>
<td>68(8)</td>
<td>69[6]</td>
<td>0.64</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>63</td>
<td>57</td>
<td>0.79</td>
<td>64</td>
<td>62</td>
<td>1.00</td>
</tr>
<tr>
<td>Smoking history (pack-years)</td>
<td>45(36,60)</td>
<td>4(0,10)</td>
<td>&lt;0.0001</td>
<td>45(35,59)</td>
<td>45(37,82)</td>
<td>0.44</td>
</tr>
<tr>
<td>Smoking status (% current:ex or never)</td>
<td>18:82</td>
<td>0:100</td>
<td>0.038</td>
<td>24:76</td>
<td>8:92</td>
<td>0.39</td>
</tr>
<tr>
<td>FEV1 (L)</td>
<td>0.98(0.44)</td>
<td>2.95(0.63)</td>
<td>&lt;0.0001</td>
<td>0.94(0.64,1.17)</td>
<td>0.75(0.61,1.22)</td>
<td>0.24</td>
</tr>
<tr>
<td>FEV1 (% pred)</td>
<td>41(18)</td>
<td>110(14)</td>
<td>&lt;0.0001</td>
<td>41(27,53)</td>
<td>33(24,51)</td>
<td>0.52</td>
</tr>
<tr>
<td>FEV1 (% pred)</td>
<td>60(9)</td>
<td>38(1)</td>
<td>&lt;0.0001</td>
<td>60(9)</td>
<td>60(9)</td>
<td>0.92</td>
</tr>
<tr>
<td>Residual volume as % of TLC</td>
<td>41(17)</td>
<td>90(14)</td>
<td>&lt;0.0001</td>
<td>46(17)</td>
<td>33(15)</td>
<td>0.02</td>
</tr>
<tr>
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<td>&lt;0.0001</td>
<td>46(17)</td>
<td>33(15)</td>
<td>0.02</td>
</tr>
<tr>
<td>TLCO, lung diffusion capacity (% pred)</td>
<td>9.4(1.2)</td>
<td>10.8(1.4)</td>
<td>&lt;0.0001</td>
<td>9.5(1.3)</td>
<td>9.3(1.0)</td>
<td>0.49</td>
</tr>
<tr>
<td>PaO2 (kPa)</td>
<td>9.4(1.2)</td>
<td>10.8(1.4)</td>
<td>&lt;0.0001</td>
<td>9.5(1.3)</td>
<td>9.3(1.0)</td>
<td>0.49</td>
</tr>
<tr>
<td>PaCO2 (kPa)</td>
<td>5.2(0.7)</td>
<td>5.2(0.4)</td>
<td>0.91</td>
<td>5.2(0.4)</td>
<td>5.3(0.6)</td>
<td>0.80</td>
</tr>
<tr>
<td>Body Mass Index (kg/m^2)</td>
<td>23.8(3.6)</td>
<td>26.3(4.4)</td>
<td>0.18</td>
<td>23.8(3.7)</td>
<td>23.7(3.7)</td>
<td>0.91</td>
</tr>
<tr>
<td>Fat-free mass(kg)</td>
<td>43(8)</td>
<td>51(12)</td>
<td>0.002</td>
<td>42(7)</td>
<td>44(10)</td>
<td>0.34</td>
</tr>
<tr>
<td>Fat-free mass index (kg/m^2)</td>
<td>15.6(1.9)</td>
<td>17.4(2.4)</td>
<td>0.002</td>
<td>15.6(1.8)</td>
<td>15.7(2.1)</td>
<td>0.84</td>
</tr>
<tr>
<td>Quadriceps MVC (kg)</td>
<td>27(9)</td>
<td>36(10)</td>
<td>&lt;0.0001</td>
<td>27(22,33)</td>
<td>24(18,33)</td>
<td>0.35</td>
</tr>
<tr>
<td>Quadriceps endurance (T80; s)</td>
<td>92(41)</td>
<td>137(73)</td>
<td>0.024</td>
<td>85(78,113)</td>
<td>78(66,90)</td>
<td>0.24</td>
</tr>
<tr>
<td>Locomotion time (min/12h)</td>
<td>37(21,52)</td>
<td>95(61,131)</td>
<td>&lt;0.0001</td>
<td>38(22,66)</td>
<td>26(17,43)</td>
<td>0.27</td>
</tr>
<tr>
<td>6-minute walk test distance (m)</td>
<td>394(108)</td>
<td>617(84)</td>
<td>&lt;0.0001</td>
<td>416(110)</td>
<td>352(93)</td>
<td>0.08</td>
</tr>
<tr>
<td>Peak VO2 (ml/kg/min)</td>
<td>12.0(3.4)</td>
<td>23.9(7.3)</td>
<td>&lt;0.0001</td>
<td>12.2(10.8,14.4)</td>
<td>9.9(9.1,10.8)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Values are mean (standard deviation) or median (25th percentile, 75th percentile) depending on data distribution. Abbreviations: FEV1 Forced Expiratory Volume in 1 second, CO carbon monoxide, pred predicted, PaO2 arterialised partial pressure of oxygen.
PaCO₂ arterialised partial pressure of carbon dioxide, MVC maximal voluntary contraction, \( \dot{V}O_2 \) oxygen uptake on maximal incremental cycle ergometry.
Table 2: Fibre proportions and fibre CSA of the quadriceps muscle of COPD patients and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>Controls</th>
<th>p value</th>
<th>COPD without FS</th>
<th>COPD with FS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=38)</td>
<td>(n=23)</td>
<td></td>
<td>(n=25)</td>
<td>(n=13)</td>
<td></td>
</tr>
<tr>
<td>% type I fibres</td>
<td>29(13)</td>
<td>54(14)</td>
<td>&lt;0.0001</td>
<td>37(32,42)</td>
<td>13(7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% type I/IIa fibres</td>
<td>3(1,7)</td>
<td>0(2,6)</td>
<td>0.22</td>
<td>3(1,8)</td>
<td>3(0,7)</td>
<td>0.96</td>
</tr>
<tr>
<td>% type IIa fibres</td>
<td>60(12)</td>
<td>41(14)</td>
<td>&lt;0.0001</td>
<td>57(49,60)</td>
<td>71(12)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% type IIx fibres</td>
<td>4(1,7)</td>
<td>1(0,4)</td>
<td>0.025</td>
<td>2(1,5)</td>
<td>7(2,20)</td>
<td>0.026</td>
</tr>
<tr>
<td>Type I fibre CSA (μm²)</td>
<td>5160(1790)</td>
<td>5650(1410)</td>
<td>0.27</td>
<td>4950(1480)</td>
<td>5580(2290)</td>
<td>0.31</td>
</tr>
<tr>
<td>Type I/IIa fibre CSA (μm²)</td>
<td>5060(3600,6040)</td>
<td>5460(4620,6530)</td>
<td>0.19</td>
<td>4800(1510)</td>
<td>5030(1650)</td>
<td>0.72</td>
</tr>
<tr>
<td>Type IIa fibre CSA (μm²)</td>
<td>3870(1400)</td>
<td>4570(1600)</td>
<td>0.08</td>
<td>3730(1050)</td>
<td>4150(1930)</td>
<td>0.39</td>
</tr>
<tr>
<td>Type IIx fibre CSA (μm²)</td>
<td>2680(1740,3450)</td>
<td>5130(3600,6950)</td>
<td>&lt;0.0001</td>
<td>2870(1230)</td>
<td>2570(907)</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Values are mean (standard deviation) or median (25th percentile, 75 percentile). 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.

Abbreviations: CSA cross-sectional area, FS type I to II fibre type shift (low type I to type II fibre ratio)
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 FS than patients without FS [Table 3, Fig. 2A, representative image of blot Fig. 4A]. However, patients had significantly lower MyH7 transcripts than controls and a trend to lower MyH7 transcripts in patients with than without FS. 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 FS compared to those with FS [p=0.005, Table 3, Fig. 2B and Fig. 4B].

Expression of PKA-alpha was not significantly different in COPD patients and controls, nor between patients with and without FS [Table 3, Fig. 2C and Fig. 4C]. Alpha-tubulin levels were not significantly different between patients and controls [54.9(17.4)AU vs 48.2(20.7)AU, p=0.21] confirming reports that alpha-tubulin is a valid loading control in COPD (30, 33).
Table 3: Protein expression of calcineurin A, protein kinase A-alpha subunit and phosphorylated AMP kinase and MEF2 binding DNA in quadriceps muscle from COPD patients and healthy controls

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

Values are mean (standard deviation) or median (25th percentile, 75 percentile). Sample numbers given where analysis completed in less than full set of subjects. Figures are correct to 3 significant figures.
Abbreviations: MEF2 myocyte enhancer factor-2 (A, B, C and D), MCIP modulatory calcineurin-interacting protein 1, MyHC myosin heavy chain, phospho-AMPK phosphorylated adenosine monophosphate kinase, PKA protein kinase A
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 FS.

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

There was no correlation between calcineurin and quadriceps type I fibre proportion or quadriceps endurance in patients ($\rho=-0.17$, $p=0.33$, Fig. 3A and $\rho=-0.05$, $p=0.80$) or in patients and controls combined ($\rho=-0.18$, $p=0.21$ and $\rho=-0.05$, $p=0.75$). 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 ($\rho=0.54$, $p=0.006$, Fig. 3B) and quadriceps endurance ($\rho=0.47$, $p=0.029$, Fig. 3C) in patients.

When patients and controls were combined, MEF2 was significantly correlated with quadriceps strength ($\rho=0.43$, $p<0.0001$), FFMI ($\rho=0.27$, $p=0.033$) and exercise performance (6-minute walk $\rho=0.34$, $p=0.008$ and peak VO$_2$ on cycle ergometry ($\rho=0.32$, $p=0.012$) (see supplemental Fig. E2).
DISCUSSION

This is the first study investigating potential molecular mechanisms underlying the reduced quadriceps muscle oxidative fibres and energy stores which contribute to poor muscle endurance and exercise performance in COPD. We demonstrate that phospho-AMPK is lower in the quadriceps muscle of COPD patients with FS than patients without FS. Furthermore, levels correlated 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 FS than patients without FS 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 FS were significantly less active than those without FS (Table 1), suggesting other factors must be important in driving FS. Patients with FS had a greater degree of emphysema, but not greater airflow obstruction or resting hypoxaemia, than patients without FS, and our data do not preclude transient hypoxia, perhaps sleep or exercise-induced, as a stimulus to FS. Certainly chronic hypoxia reduces muscle oxidative fibres in animals (34). Alternatively, emphysema and FS could develop through a common mechanism, and 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 FS have poorer exercise performance than patients without FS, 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. 3), is therefore important as increasing these levels, with 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 FS, 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 FS, who showed a 3-fold increase in phospho-AMPK levels compared to controls, but not in patients with FS (Table 3). In addition, we have previously shown that downstream of AMPK, peroxisome proliferator-activated receptor delta (PPARδ), a transcription factor promoting type I fibre differentiation in animals (41), is down-regulated in the quadriceps in COPD (42).
The paradoxical increase in calcineurin expression in COPD patients, particularly those with FS (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 FS 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 peroxisome proliferator-activated receptor gamma co-activator alpha 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), that 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, supplemental Fig E2). 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 FS, who did not differ in physical activity levels, suggest that any signalling disturbances leading to FS 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) 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 \textit{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 \textit{in vivo} but still does not quantify transcriptional activity, which may be altered by presence of co-factors etc.

It could be argued that the differences in calcineurin and phospho-AMPK levels between patients with and without FS 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 FS 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 FS but is associated with muscle mass and strength, and therefore may be best explored in patients with marked quadriceps wasting and weakness.
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COMPETING INTERESTS

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REFERENCES

FIGURE LEGENDS

Figure 1: In response to muscle activity, calcineurin (and also calcium-calmodulin protein kinase IV, not shown here) signalling activates NFAT translocation to the nucleus and increases MEF2 activation to drive oxidative muscle-specific gene transcription. In response to energy depletion, phosphorylated AMPK activates peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1alpha) and peroxisome proliferator-activated receptor delta to drive oxidative muscle-specific gene transcription. Both calcineurin and phospho-AMPK upregulate GLUT4 receptors promoting muscle cell glucose uptake via MEF2. PKA promotes mobilisation of glucose production by glycogenolysis at times of increased requirement for energy production.
Figure 2: Calcineurin A expression was higher in the quadriceps of COPD patients with FS than patients without FS [0.98(0.69,1.30)AU vs 0.74(0.43,0.97)AU, p=0.030, A], and higher in COPD patients overall compared to controls [0.82(0.34)AU vs 0.60(0.37)AU, p=0.036, A]. Phospho-AMPK was six-fold lower in the COPD patients with FS than those without FS [2.07(1.73,3.24)AU vs 7.08(4.78,11.1)AU, p=0.005, B], though levels were not significantly different between COPD patients as a group compared to controls [3.93(1.32,7.95)AU vs 2.37(1.11,9.61)AU, p=0.64, B]. PKA was not significantly different between any groups (C). There was no significant difference in MEF2 capable of binding DNA between patients with and without FS [1440(329,2290)RLU vs 1750(928,2460)RLU, p=0.29], although muscle MEF2 DNA-binding was lower in COPD patients than controls [1710(696,2320)RLU vs 2660(1830,3550)RLU, p=0.004].
**Figure 3:** There was no significant correlation between type I fibre proportion and calcineurin in patients alone ($\rho=-0.17$, $p=0.33$) or when patients and controls were combined ($\rho=-0.18$, $p=0.21$, A). Phospho-AMPK was strongly correlated with quadriceps type I fibre proportion ($\rho=0.54$, $p=0.006$, B) and quadriceps endurance measured non-volitionally ($\rho=0.47$, $p=0.029$, C) in patients. Modulators of calcineurin activity, and PKA, were not correlated with muscle histology or quadriceps endurance.
**Figure 4:** The western blot images illustrate the finding that calcineurin A and phospho-AMPK-alpha protein levels were higher in COPD patients with FS than patients without FS (A and B respectively), while there was no significant difference in PKA protein between COPD patients with FS, patients without FS or healthy controls (C).