ONLINE SUPPLEMENT

METHODS

Ethical approval

The study was approved by the Royal Brompton & Harefield NHS Trust and Ealing and West London Mental Health Trust Research Ethics Committees (Studies 06/Q0404/35 and 06/Q0410/54) and subjects gave written informed consent.

Quadriceps muscle biopsy

Percutaneous needle biopsy of the *vastus lateralis* in the anterior mid-thigh of the right leg under local anaesthesia was performed according to the technique described by Bergstrom[1]. Samples for protein and gene expression analyses were immediately frozen in liquid nitrogen while specimens for histology were rested on filter paper soaked in saline for 15 minutes before mounting on cork with OCT (Tissue Tek compound), submerging in pre-cooled isopentane for 15 seconds and then kept in liquid nitrogen until storing at -80°.

Quadriceps biopsy analysis

Cryosectioning

10 µm serial muscle cross-sections with fibers in transverse section were made on a cryostat microtome (Leica CM 1900, Meyer Instruments, USA) at –20°C and mounted on slides that were stored at –80°C until analysis.

Immunohistochemistry to determine quadriceps fiber proportions and fiber CSA

Immunofluorescence for detection of myosins (to determine fiber type) and laminin (to calculate fiber CSA) was undertaken as described below.

Primary Antibodies

A4.840; Myosin heavy chain human (slow) I fibers and N2.261; Myosin heavy chain human (fast) IIA fibers, both from Developmental Studies Hybridoma Bank (DSHB), University of Iowa, USA. L-9393; polyclonal anti-laminin antibody produced in rabbit (Sigma, Zwijndrecht, the Netherlands).

Secondary Antibodies

A-21121; Alexa Fluor 488 goat anti-mouse IgG1, A-21426 (against N2.261 primary antibody); Alexa Fluor 555 goat anti-mouse IgM, A-11069 (against A4.840 primary antibody); Alexa Fluor 350 goat anti-rabbit IgG (against L-9393 primary antibody), all from Molecular Probes, Invitrogen, Breda, the Netherlands.

Transverse muscle sections were air-dried for thirty minutes at room temperature and treated for five minutes with 0.5% Triton X-100 in Phosphate-Buffered Saline

(PBS) before washing for five minutes in PBS. The sections were then incubated for one hour at room temperature with a mix of primary antibodies in PBST (A4.840 dilution 1:40, N2.261 dilution 1:40, L-9393 dilution 1:50). After washing the slides for five minutes in PBST (PBS with 0.05% Tween 20) and five minutes in PBS twice, the sections were incubated with secondary antibody mix in PBST (AlexaFluor555 dilution 1:500, AlexaFluor488 dilution 1:200, AlexaFluor350, dilution 1:130) in the dark in a humidification box for one hour at room temperature. The slides were washed (five minutes in PBST, five minutes in PBS twice) and Faramount aqueous mounting medium (Dako, USA) and a coverslip applied. Slides were stored in the dark at 4°C.

Image capture and fiber classification

Image capture was done within 3 days of staining and slides were stored in a dark room to ensure that fluorescence was clearly visible in all slides analysed. Epifluorescence signal was recorded using a Nikon Eclipse 800 microscope with a DXM 1200 camera (Nikon Instruments Europe BV, the Netherlands) under a x10 objective using three filters: Texas Red (395 to 410 nm), FITC (490 to 505nm) and DAPI UV (395 to 410 nm). Between four and nine images of fibers in transverse section were captured for each section to incorporate as many fibers as possible (see Fig. E1 for a representative image). Fibers were classified as type I, IIa (IIa and IIa/IIx, green), IIx (no staining) and hybrid I/IIa (red and green staining) and the laminin fiber border was used to measure fiber CSA using Lucia 4.81 software package (Laboratory Imaging, Czech Republic)[2, 3]. Apparent type

IIa/IIx fibers, i.e. only weakly/moderately-stained for type IIa myosin, were classified as type IIa as they were not distinguishable from pure type IIa fibers stained less strongly for a technical reason. At least 100 muscle fibers [median of 223(166, 333)] were analysed for each subject.

Calculation of fiber data

For an individual, type I, I/IIa, IIa and IIx proportions and median fiber CSA for each fiber type (data not normally distributed) was recorded. The type I and IIa fiber (as well as overall type II fiber) data from the patient and control groups were normally distributed, allowing use of parametric tests but the type I/IIa and IIx fiber data for the groups was not normally distributed, and therefore non-parametric tests were used with this.

Western blot analysis

Muscle samples were homogenised in lysis buffer [NaCl (5M), Tris-Base (0.5M), EDTA (0.5M) 2% Igepal NP40 pH 7.3 and glycerol] supplemented with protease and phosphatase inhibitors (leupeptin, PMSF). Protein levels of the supernatants were quantified by Bradford assay.

20-50µg of protein for each subject was run on SDS/PAGE (Biorad Criterion XT System using 4–12% Bis-Tris gels with XT MOPS running buffer) and transferred onto nitrocellulose membrane (Protran BA85 membrane, 0.45µm) at 0.8mA/cm^2 of membrane for 1 hour using a wet transfer technique. Membranes were blocked in 5% milk in TBS with 0.01% Tween 20 (TBST) for 1 hour at room temperature, then incubated with rabbit anti-calcineurin/mouse anti-calmodulin/goat anti-14-3-3 protein/rabbit anti-phospho AMPK/rabbit anti-PKAα/rabbit anti-NFATc3/mouse anti-MEF2/mouse anti-α-tubulin antibody (for dilutions and antibody dilution buffers see table below) overnight at 4°C. Membranes were washed 3 times in TBST then incubated with anti-mouse/rabbit/goat IgG linked to a horseradish peroxidase (Vector Laboratories, CA, USA) at a concentration of 1:5000 in 5% milk in TBST for 1 hour at room temperature. Membranes were washed 3 times in TBST and once in TBS and proteins visualised by enhanced chemiluminescence (Pierce, Rockford, IL, USA) in accordance with the manufacturers instructions. Blots were re-blotted for alpha-tubulin using mouse anti-tubulin antibody in the same way. Films were scanned using a GS-800 densitometer (Biorad, CA, USA) and target protein band density was determined with 1D densitometry analysis using Quantity One (Biorad) and normalised for alpha-tubulin band density. To compare across gels, the same sample was run on all gels as a control. The normalised result for this protein (i.e. density of

band/ density of alpha-tubulin band) was taken as a value 1 and all other sample results on the same gel were normalised to this value. This is an accepted method of correcting for gel-to-gel differences[4].

Antibodies and dilutions

Primary Antibodies	Source	Dilutions	Antibody dilution buffer
Anti-pan calcineurin (rabbit polyclonal)	Cell Signaling Technology (Cat. # 2614)	1:1000	5% milk in TBS with 0.05%
Anti-calmodulin (mouse monoclonal)	Millipore (Cat. # 05-173)	1µg/ml	3% milk in PBS
Anti-14-3-3 (goat polyclonal)	Santa Cruz Biotechnology (Cat. # sc-	1:1000	TBS with 0.05% Tween
Anti-phospho-AMPK (rabbit monoclonal)	Cell Signaling Technology (Cat. #2535)	1:1000	TBS with 0.05% Tween
Anti-protein kinase Aα (rabbit polyclonal)	Santa Cruz Biotechnology (Cat. # sc-	1:500	5% milk in TBS with 0.05%
Anti-NFATc3 (rabbit polyclonal)	Santa Cruz Biotechnology (Cat. # sc-	1:500	5% milk in TBS with 0.05%
Anti-α-tubulin (mouse monoclonal)	Santa Cruz Biotechnology (Cat. # sc-	1:500	TBS with 0.05% Tween
Anti-MEF2 (mouse monoclonal)	Santa Cruz Biotechnology (Cat. # sc-	1:5000	5% milk in TBS with 0.05%
Secondary antibodies			
Peroxidase Goat Anti-Rabbit IgG	Vector Laboratories (Cat. # PI-1000)	1:5000	5% milk in TBS with 0.05%
Peroxidase Horse Anti-Mouse IgG	Vector Laboratories (Cat. # PI-2000)	1:5000	5% milk in TBS with 0.05%
Peroxidase Horse Anti-Goat IgG	Vector Laboratories (Cat. # PI-9500)	1:5000	5% milk in TBS with 0.05%

Transcription Factor Enzyme-Linked Immunosorbent Assays (TF ELISAS) to measure quantities of MEF2 in nuclear extracts from human quadriceps muscle binding DNA

These assays were run by the US Panomics/Affymetrix Testing Service in laboratories in California, USA using the Panomics/Affymetrix TF ELISA plates, solutions and nuclear extraction kit (#EK1111, #EK1121 and EK1041).

Preparation of nuclear extracts

The frozen muscle was thawed, rinsed with ice-cold PBS twice and stored on ice. 0.5 ml of cell lysis buffer A from the nuclear extraction kit with proteinase/phosphatase inhibitors were added to the tissue and homogenised using the disposable Pellet Mixer motor-driven pestle (VWR, USA) for thirty seconds to lyse the cell membranes. The homogenate was transferred to the BioMasher column (Investigen, USA) and spun for one min at 14 000 rpm at 4^{o}C. The pass-through fraction in the tube was centrifuged for another three minutes and the supernatant (cytoplasmic fraction) was removed. The remaining pellet (nuclei) was resuspended in nuclear membrane lysis buffer and incubated for two hours at 4^{o}C with gentle agitation. After centrifugation at 14 000 rpm for five minutes at 4^{o}C, the nuclear extract (supernatant) was transferred to a fresh tube and stored at -80^{o}C. Protein concentrations of the nuclear extracts were quantified using the Bio-Rad DC protein assay kit with BSA standard (Biorad, CA, USA).

Transcription factor ELISA to quantify MEF2 binding DNA

10 ug of protein per sample were run in duplicates for MEF2 detection with a C2C12 mouse myoblast extract as a positive control and a negative control in duplicate. The nuclear extracts were thawed and kept on ice. 10 µg of each sample and positive and negative control samples were added to wells of a microplatecontaining biotinylated oligonucleotide with a MEF2 consensus binding site. After thirty minutes incubation at room temperature to allow activated TF in the nuclear extract to bind to the probe, the plates were washed thoroughly to remove any unbound proteins. Primary antibody against MEF2 (EK1080, Panomics Trancription Factor ELISA kit, Panomics/Affymetrix, USA) was added and incubated for one hour at room temperature. Then a secondary antibody linked to an alkaline phosphatase was added and incubated for one hour at room temperature. After thorough washing of the plate, substrate for the alkaline phosphatase was added and chemiluminescence read by the GENios Plate Reader (TECAN) for three seconds. Signals were detected in positive controls in the assay plates indicating that the TF-ELISA had worked. The background chemiluminescence from the average of the negative controls on that plate was subtracted from the chemiluminescence of each sample, and the resulting chemiluminescence value was directly proportional to the amount of MEF2 capable of binding DNA in the fixed quantity of muscle sample. The average of the duplicates for each sample was calculated.

Quantitative real-time PCR to determine MCIP1 mRNA

This technique was used for determination of mRNA transcripts of the myocyte-enriched inhibitory protein 1 (MCIP1) gene normalised to transcripts of acidic ribosomal phosphoprotein PO (RPLPO) and β_2-microglobulin (at Maastricht University). In summary, total RNA was extracted using the acid guanidium thiocyanate–phenol–chloroform–isoamylalcohol extraction method (Totally RNA^{TM} kit, Ambion Ltd., Foster City, CA, USA). RNA concentration was determined using a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE, USA) and the quality was verified by agarose gel electrophoresis. 400 ng of RNA per sample was reverse-transcribed into cDNA according to the manufacturer's instructions with anchored oligo ^{5} primers (Transcriptor First Strand cDNA Synthesis kit, Roche Diagnostics GmbH, Mannheim, Germany). Primers for qPCR were designed to generate a PCR amplification product of 59–278 base pairs based on Ensembl transcript sequences using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and obtained from Sigma Genosys (Haverhill, UK). The sequences are shown below. For relative quantification, standard curves were prepared from pooled cDNA aliquots by five serial five-fold dilutions. cDNA samples were diluted 1/25. 5µl diluted cDNA was loaded in a 384-well PCR plate (MicroAmp® Optical 384-Well Reaction Plate, Applied Biosystems) covered with MicroAmp® Optical Adhesive Film (Applied Biosystems). cDNA was amplified with Power SYBR® Green PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). All PCR reactions contained 300nM primers. Two-step PCR was performed with the following cycling conditions: 10 min at 95˚C, 40 cycles of 10 s at 95˚C and 1 min at 60˚C followed by a melting curve. Specificity of the

amplification was verified by melt curve analysis and efficiency of PCR amplification by the standard curve. Transcript levels for potential reference gene products RPLPO, β_2-microglobulin, RPL13 (60S ribosomal protein L13), cyclophilin A, glyceraldehyde 3-phosphate dehydrogenase, α-actin, heat shock protein 90 were measured in patient and control samples. geNorm software was applied to the data and this selected RPLPO and β_2-microglobulin as the only good housekeeping genes when comparing patients and controls and calculated a normalisation factor based on the expression levels of these. Target gene expression was therefore quantified, normalised to the geNorm normalisation factor and expressed as arbitrary units (AU).

Primer sequences

MCIP-1	Forward GAAGATGCGACCCCAGTCATAAA
MCIP-1	Reverse CGCTGCGTGCAATTCATACTTT
MyH7	Forward CCCTGGAGACTTTGTCTCATTAGG
MyH7	Reverse AGCTGATGACCAACTTGCGC
RPLPO	Forward CACCCAGGCAGAGAATGCTGAGTTC
RPLPO	Reverse GCCGCTTCACATAGCGCTGCA
B2M	Forward TGACTTTGTCACAGCCCAAGATA
B2M	Reverse AATGCGGCATCTTCAAACCT

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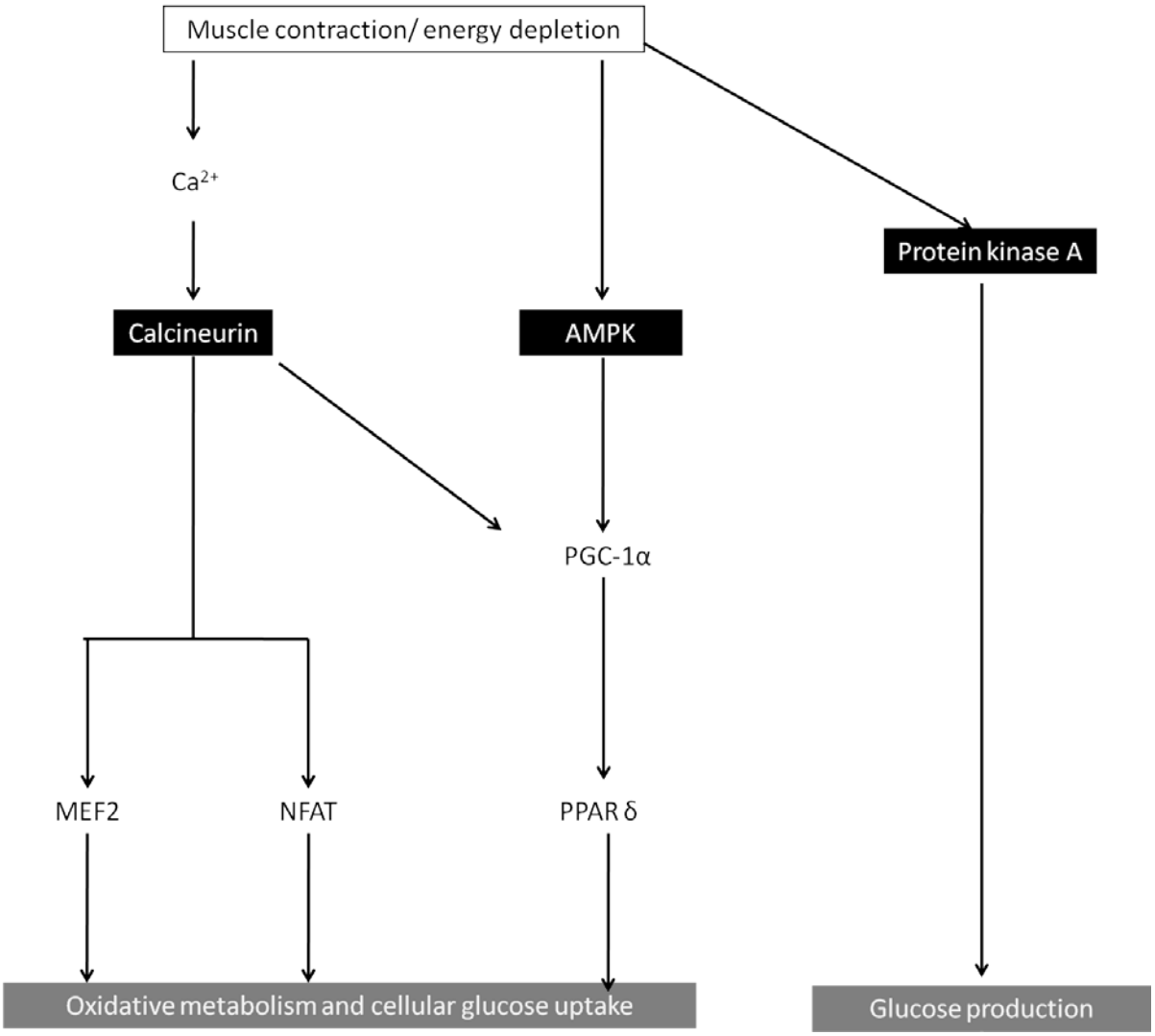

Figure 1 Schematic of pathways involved in oxidative gene expression and energy homeostasis in response to ATP depletion investigated in this study (adapted from Zierath *et al*[6])

Arrows indicate activation. Abbreviations: Ca^{2+} intracellular calcium, AMPK adenosine monophosphate kinase, PGC-1α peroxisome proliferator-activated receptor gamma 1-alpha, PPARδ peroxisome proliferator-activated receptor delta, MEF2 myocyte enhancer factor-2, NFAT nuclear factor of activated T-cells

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-1α) 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.

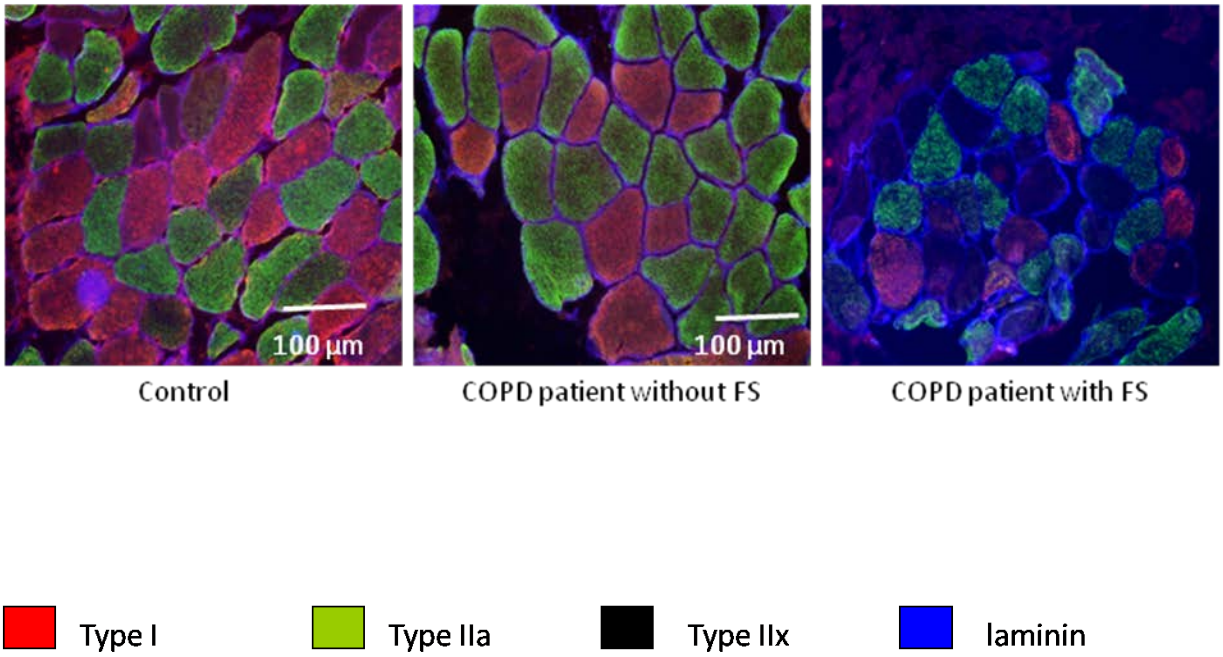

Figure E2 Representative images of transverse muscle sections from COPD patients with and without type I to type II fibre type shift and a healthy control stained for type I myosin (red), type IIa myosin (green) and laminin (blue) seen with a Nikon 800 microscope under a x10 objective