Muscle Tissue Ancillary Study

In the Year 16 clinic visit of Health ABC, muscle tissue samples were collected from 41 Pittsburgh clinic participants. Two ancillary study datasets were created and provided to the Coordinating Center. The first was a small dataset of five participants that contained single muscle fiber data. The second dataset contains mitochondrial respiratory capacity (pmol/sec/mg) and immunohistochemistry information on all 41 participants.

The single fiber data file is a Microsoft Excel file, "*MuscleTissueSingleFiber.xlsx*". To download this Excel file, please click on "Datasets/Documentation" in the banner on the Health ABC Keeptrack website, then click on "Current Datasets" and select "MTSingleFiber" under the "Substudies" section of the downloadable datasets. The first sheet, "Single Fiber Data Explanation" in the Excel file provides variable names and descriptions. Below is an explanation of data collection procedures for these data.

Single Muscle Fiber Experimental Set-Up

On the day of an experiment, a 2.5-3.0 mm muscle fiber segment was isolated from a muscle bundle and transferred to an experimental chamber filled with relaxing solution where the ends were securely fastened between a force transducer (model 400A, Cambridge Technology, Lexington, MA, USA) and a direct current torque motor (model 308B, Cambridge Technology, Lexington, MA, USA) as described by Moss (1).

The force transducer and torque motor were calibrated before each experiment. Instrumentation was arranged so a muscle fiber could be rapidly transferred back and forth between experimental chambers filled with relaxing (pCa 9.0) or activating (pCa 4.5) solutions. The apparatus was mounted on a microscope (Olympus BH-2, Japan) to view the fiber (x800) during an experiment. Using an eyepiece micrometer, sarcomere length along the isolated muscle was adjusted to 2.5 µm, and the fiber length (FL) was measured (2). All single fiber experiments were performed at 15°C.

Unamplified force and length signals were sent to a digital oscilloscope (Nicolet 310, Madison, WI, USA), enabling monitoring of muscle fiber performance throughout data collection. Analog force and position signals were amplified (Positron Development, Dual Differential Amplifier, 300-DIF2, Inglewood, CA, USA), converted to digital signals (National Instruments, Austin, TX, USA), and transferred to a computer (Micron Electronics, Nampa, ID, USA) for analysis using customized software.

Servomotor arm and isotonic force clamps were controlled using a computer-interfaced force-position controller (Positron Development, Force Controller, 300-FC1, Inglewood, CA, USA).

For each single muscle fiber experiment, a fiber with a compliance (calculated as FL divided by y-intercept) >10% and/or a decrease in peak force (Po) of >10% was discarded and not used for analysis. The within-fiber test/retest of a single muscle fiber in our lab for the measurements of size, force-power relationships, Po, and contractile velocity were <1%. The coefficients of variation for the force transducer and servomechanical lever mechanism during the timeframe of this investigation was <1%.

Following completion of single muscle fiber physiology experiments, each fiber was solubilized in 80 µl of 1% SDS sample buffer and stored at -20°C until assayed for MHC fiber type.

Single Muscle Fiber Analysis

Individual muscle fibers were analyzed for force-velocity relationships and power, peak force (Po), maximal unloaded shortening velocity (Vo), diameter, and fiber type. Experimental procedures were identical to those previously used in our human studies (2,3).

Single muscle fiber power.

Submaximal isotonic load clamps were performed on each fiber for determination of force-velocity parameters and power. Each fiber segment was fully activated in pCa 4.5 solution and subjected to a series of three isotonic load steps. This procedure was performed at various loads so that each fiber underwent a total of 15-18 isotonic contractions.

For the resultant force-velocity relationships, load was expressed as P/Po (P =force during load clamping, Po = peak isometric force developed before submaximal load clamps). Force and shortening velocity data points were derived from the isotonic contractions and fit by the hyperbolic Hill equation (4). Only individual experiments in which R^2 was ≥ 0.98 were included for analysis.

Fiber peak power was calculated from the fitted force-velocity parameters (Po, Vmax, and a/Po, where α is a force constant and Vmax is the y-intercept). Absolute power ($\mu N \bullet FL \bullet s^{-1}$) was defined as the product of force (μN) and shortening velocity ($FL \bullet s^{-1}$). Normalized power ($W \bullet L^{-1}$) was defined as the product of normalized force and shortening velocity.

Single muscle fiber Po

Force and position transducer outputs were amplified and sent to a microcomputer via a Lab-PC+ 16-bit data acquisition board (National Instruments, Austin, TX, USA). Resting force was monitored and then the fiber was maximally activated in pCa 4.5 solution. Peak active force (Po) was determined in each fiber by computer subtraction of the baseline force from the peak force in the pCa 4.5 solution.

Single muscle fiber Vo

Fiber unloaded shortening velocity (Vo) was measured by the slack-test technique as described by Edman (5). The fiber was fully activated in pCa 4.5 solution and rapidly released to a shorter length, such that force fell to baseline. The fiber shortened, taking up slack, after which force began to redevelop. Then, the fiber was placed in pCa 9.0 solution and returned to original length. Computer analysis determined the duration of unloaded shortening, or time between onset of slack and redevelopment of force. Four different activation and length steps (150, 200, 250, and 300 μm; ≤15% of FL) were used for each fiber, with the slack distance plotted as a function of the duration of unloaded shortening. Fiber Vo ($FL \bullet s^{-1}$) was calculated by dividing the slope of the fitted-line by the fiber segment length (data were normalized to a sarcomere length of 2.5 μm).

Single muscle fiber diameter

A video camera (CCD-IRIS, DXC-107A; Sony, Japan) connected to the microscope and computer interface allowed for viewing and storage of single muscle fiber digital images. Fiber diameter was determined from an image taken with the fiber briefly suspended in air (<5 s). Fiber width (diameter) was determined at three points along the segment length of the captured image using NIH public domain software (Scion Image, release Beta 4.0.2, for Windows). For the fiber size-dependent variables (i.e. Po/CSA and normalized power), CSA was determined with the assumption that the fiber forms a cylindrical shape while suspended in air.

Fiber Type Analysis

Following the single fiber contractile measurements, the MHC isoform profile was analyzed for each fiber segment using SDS-PAGE. Briefly, samples were run overnight at 4°C on a Hoefer SE 600 gel electrophoresis unit (San Francisco, CA, USA) utilizing a 3.5% (wt/vol) acrylamide stacking gel with a 5% separating gel (6). After electrophoresis, gels were silver stained as described by Giulian et al. (7). MHC isoforms (I, I/IIa, IIa, IIa/IIx, IIx, I/IIa/IIx) of each

single muscle fiber were identified according to migration rate as we have previously described (6).

References

1. **Moss RL**. Sarcomere length-tension relations of frog skinned muscle fibres during calcium activation at short lengths. *J Physiol* 292: 177-192, 1979.
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The mitochondrial respiratory capacity (pmol/sec/mg) and immunohistochemistry data file is a Microsoft Excel file, "*MuscleTissueMitochondrialRespiratoryData.xlsx*". To download this Excel file, please click on "Datasets/Documentation" in the banner on the Health ABC Keeptrack website, then click on "Current Datasets" and select "MTMitoResp" under the "Substudies" section of the downloadable datasets. A separate Excel file is downloaded with the data, "*MuscleTissueMitochondrialRespiratoryDataDictionary.xlsx*", that provides variable names, descriptions and explanations. Below is an explanation of data collection procedures for these data.

MUSCLE BIOPSY

Percutaneous muscle biopsies were obtained at the University of Pittsburgh's Clinical Translational Research Center on a morning after an overnight fast. Participants were instructed not to perform physical exercise 48 hours prior to the biopsy procedure. Biopsy samples were obtained from the middle region of the vastus lateralis under local anesthesia (2% buffered lidocaine). A portion of the biopsy specimen (~10-15 mg) was placed in ice-cold preservation buffer (BIOPS) (1) for analysis of mitochondrial respiration. The remaining muscle tissue were processed for histochemistry (~20-30 mg) or frozen in liquid nitrogen (20-130 mg) and stored at −80°C.

Mitochondrial respiratory capacity

Immediately after the biopsy procedure, permeabilized myofiber bundles were prepared. Briefly, myofiber bundles were gently teased apart in a petri dish containing ice-cold BIOPS solution using 2 sharp tweezers and a dissecting microscope (Leica Microsystems, Heerbrugg, Switzerland). The myofiber bundles were then permeabilized with saponin (2 mL of 50 ug/mL saponin in BIOPS solution) for 20 minutes at 4°C on an orbital shaker, and then washed twice for 10 minutes at 4°C with MIR05 respiration medium (Protocol 1) (1) or Buffer Z (Protocols 2 and 3).

Mitochondrial respiratory capacity in permeabilized myofibers was evaluated by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Measurements were performed in duplicate, at 37°C, in the range of 230-150 nmol O_2/ml. Three different protocols were performed. Protocol 1: LEAK (L_I) respiration was determined through the addition of pyruvate (5mM), malate (2mM) and glutamate (10mM). ADP (5mM) was added to elicit complex I supported oxidative phosphorylation (OXPHOS) (P_I). Cytochrome c (10µM) was added to check the quality of the muscle fiber preparation and assess the integrity of the outer mitochondrial membrane. Succinate (10mM) was then added to elicit complex I+II supported OXPHOS (P_{I+II}). FCCP (1uM) was added to determine electron transfer system (E_{I+II}) capacity. Finally, rotenone (1µM) was added to inhibit complex I supported OXPHOS respiration, and the remaining respiration revealed the maximal ETS capacity with complex II substrates only (E_{II}). Protocols 2: Fatty acid oxidation (FAO) supported LEAK respiration (FAO_L) was determined through the addition of palmitoylcarnitine (25uM), malate (2mM), glutamate (5mM), and succinate (10mM). ADP titration (37.5 to 4000 uM) was performed to elicit FAO supported OXPHOS (FAO_P). The integrity of the outer mitochondrial membrane was assessed by the addition of cytochrome c (10 uM). Electron transfer system (E) capacity was assessed after addition of the uncoupler FCCP (2µM). Protocols 3: LEAK (L_{I+II}) respiration was measured following the addition of glutamate (5mM), malate (2mM), and succinate (10mM). Maximal OXPHOS respiration was assessed using an ADP titration from 37.5 to 4000 uM (P_{I+II}). The integrity of the outer mitochondrial membrane was assessed by the addition of cytochrome c (10 uM). The electron transfer system capacity (E_{I+II}) was assessed after addition of the uncoupler FCCP (2µM). Following the assay, myofiber bundles were recovered, dried, and weighted on an analytical balance (Mettler Toledo, XS105). Steady state O_2 flux for each respiratory state was

determined and normalized to myofiber bundle dry weight using Datlab 4 software (Oroboros Instruments, Innsbruck, Austria).

Immunohistochemistry

Histochemical analyses were performed on serial sections using methods previously used in our laboratory (2). Briefly, biopsy samples were sectioned (10 μm) on a cryostat (Cryotome E; Thermo Shandon, Pittsburgh, PA) at -20 °C and placed on individual pre-cleaned glass slides (Fisherfinest, Fischer Scientific, Pittsburgh, PA). Intramyocellular triglyceride (IMTG) content was determined by Oil Red O and fiber type costain, allowing fiber specific IMTG measurements and cross-sectional area (3). Succinate dehydrogenase (SDH) (complex II of the electron transport chain) staining was used as a marker of oxidative capacity (2). Images were visualized using a Leica microscope (Leica DM 4000B; Leica Microsystems, Bannockburn, IL), digitally captured (Retiga 2000R camera; Q Imaging, Surrey, Canada), and analyzed using specialized software (Northern Eclipse, v6.0; Empix Imaging, Cheektowaga, NY).

Bibliography

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