Experimental procedures

Dual-energy X-ray absorptiometry

DXA scans were performed using a Lunar iDXA (GE Healthcare, Brøndby, Denmark) which was calibrated daily according to manufacturer's instructions. Participants rested on the scanner bed in the supine position for 10 min prior to the scanning to minimize potential effects of body fluid shifts [1]. Each scan was conducted in duplicate to minimize inter-scan variation and the mean of the two scans was used.

Muscle biopsy

Muscle biopsies were collected under local anaesthesia (20 mg/mL Xylocain without epinephrine; AstraZeneca, Cambridge, UK) through a small incision in the skin over the *vastus lateralis*. Biopsies were collected with a modified Bergström needle with suction [2]. Upon collection, the muscle biopsy piece was rinsed in saline (9 mg/mL, Fresenius Kabi, Sweden) and divided in two. One piece was put in ice cold BIOPS[3] (50 mM K^+-MES, 20 mM taurine, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, 20 mM imidazole, pH 7.1, adjusted with 5 N KOH at 0°C, 10 mM Ca–EGTA buffer) for analysis of mitochondrial respiration, and another piece was quickly frozen in liquid nitrogen and stored at -80°C for later western blot analysis and enzymatic activity assays.

Citrate synthase activity

Maximal enzyme activity of citrate synthase was determined from ≈0.5 mg d.w. muscle tissue dissected free of visible blood and connective tissue, before homogenization (1:400) in a 0.3 mol/L phosphate buffer (pH 7.7) by two rounds of 30-seconds using a TissueLyser II (QIagen, Valencia, CA, USA). Maximal enzyme activity was determined fluorometrically NAD-NADH coupled reactions (Fluoroscan Ascent, Thermo Fisher Scientific, Waltham, USA) at 25°C as previously described [4].

Immunoblotting and SDS-page

Protein contents were determined by Western blotting. Approximately 1 mg d.w. muscle tissue was homogenized for 1 min at 30 Hz on a shaking bead-mill (TissueLyser II, Qiagen, Valencia, CA, USA) in ice-cold lysis buffer containing: 10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerophosphate, 2 mM Na_3VO_4, 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8), 1 mM EGTA (pH 8) 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 3 mM benzamidine. Samples were rotated end-over-end for 30 min at 4˚C and centrifuged (18,320 × g) for 20 min at 4˚C. The protein concentration of each sample was determined in triplicate with a BSA kit (Thermo Fisher Scientific, MA, US) and samples were created in duplicate with 6× Laemmli buffer (7 mL 0.5 M Tris-base, 3 mL glycerol, 0.93 g DTT, 1 g SDS, and 1.2 mg bromophenol blue) and ddH2O to achieve equal protein concentration. Equal amounts of protein were loaded in wells of pre-cast 4-15% gels (Bio-Rad Laboratories, CA, US), except for OXPHOS complex I-V determination, which was on pre-cast 16.5% gels, with all samples for each participant loaded on the same gel. Proteins were then separated according to their molecular weight by SDS-PAGE and semi-dry transferred to a PVDF membrane (Millipore A/S, Copenhagen, Denmark). Membranes were blocked for 15 min in either 2% skim milk or 3% BSA in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) before an overnight incubation in primary antibody at 4°C and a subsequent incubation in horseradish peroxidase conjugated secondary antibody at room temperature for 1 h. Bands were visualized with ECL (Millipore) and recorded with a digital camera (ChemiDoc MP Imaging System, Bio-Rad Laboratories). Bands were quantified using Image Lab version 6.0 (Bio-Rad Laboratories) and determined as the total band intensity adjusted for background intensity. Primary antibodies used were: CD31/PECAM-1 (#AF806, R&D Systems Inc., Minneapolis, MN, United States), OXPHOS (#ab110411, Abcam, Cambridge, MA, USA), and HADHA (#ab54477, Abcam, Cambridge, MA, USA). Secondary antibodies used were HRP-conjugated Rabbit Anti-Sheep (1:5000; Dako P0163), Goat Anti-Mouse (1:5000; Dako P0447), and Goat Anti-Rabbit (1:5000; SouthernBiotech 4010-05).

High-resolution mitochondrial respirometry

Mitochondrial respiration was evaluated in vastus lateralis muscle biopsies using high-resolution mitochondrial respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) before and after the intervention. Within two hours of muscle biopsy sampling, the muscle tissue in BIOPS was prepared for high-resolution respirometry as previously described

[3]. In brief, muscle tissue was dissected free from connective tissue and fat using dissection needles and Jeweller-forceps after which fibers were permeabilized in BIOPS containing saponin and washed in mitochondrial respiration medium (MiR06) [3]. High-resolution respirometry measurements were determined in duplicates at 37°C using 1-3 mg wet weight muscle fibers per chamber in MiR06. The oxygen concentration was maintained between 200-450 μM. Hydrogen peroxide titrations were used to reoxygenate the chambers. To assess different mitochondrial respiratory states a substrate-uncoupler-inhibitor titration (SUIT) protocol was used as previously described [5]. Leak respiration (L_N) was assessed in the absence of adenylates with titration of malate (2 mM) and octanoylcarnitine (0.2 mM) to supply electrons from fatty acid β-oxidation through electron-transferring flavoproteins and complex I (CI). Fatty acid oxidation (FAO_P) capacity was determined after the addition of ADP (5 mM). CI-linked (CI_P) oxidative phosphorylation capacity was measured with the addition of pyruvate (5 mM) and glutamate (10 mM). CI+CII-linked ($CI+CII_P$) oxidative phosphorylation capacity was assessed after titration of succinate (10 mM). To test the mitochondrial outer membrane integrity cytochrome C (10 μM) was added, which changed the oxygen flux 0.8 ± 1.8%. No measurements were excluded based on outer membrane integrity. Oligomycin-induced leak respiration (L_{Omy}) was assessed after titration of oligomycin (1 μM). Lastly, rotenone (0.5 μM) and antimycin A (2.5 μM) were added to measure residual oxygen consumption (ROX). All respiratory data were corrected for ROX.

Echocardiography

For the cardiac measures participants rested in a supine left lateral position in a darkened room. Measures of left ventricular systolic and diastolic function was performed using transthoracic echocardiograms obtained using a GE Vivid E9 ultrasound machine with a 2.5-MHz transducer (GE Healthcare, Brøndby, Denmark) according to current guidelines [6]. A minimum of three consecutive cardiac cycles were collected for each series and stored offline for post hoc analysis. One investigator, blinded to the treatment of the study participants, performed the examinations, and analyzed the echocardiographic images post hoc using EchoPac software version 203 (GE Healthcare).

Global longitudinal strain (GLS) was measure using GE's automated function imaging (AFI). Left ventricular mass was measured and calculated with the linear method using the cube formula [6]. Pulsed-wave tissue Doppler images obtained in the 4-chamber view were used to

determine averages of septal and lateral mitral annular systolic (s′) and early (e′) diastole longitudinal velocities. Pulsed-wave Doppler in the same 4-chamber view was used for peak transmitral blood inflow velocities during early (E) and late (A) diastole. Left ventricular volumes and ejection fraction were calculated using the Simpson's biplane method [6]. Right ventricular tricuspid annular plane systolic excursion was determined using M-mode [6].

Total haemoglobin mass, intravascular volumes and haematological parameters

Blood volumes (*i.e.,* plasma volume and total blood volume) as well as total haemoglobin mass, were measured using the carbon monoxide (CO)-rebreathing technique [7]. All measurements were conducted at the end of the experimental trial days to avoid interference with performance tests. Briefly, participants were placed in a supine position with legs raised to avoid blood pooling in extremities. A venous blood sample was collected and gently inverted 5-8 times before an immediate complete blood count analysis on a Sysmex XN-450 (Sysmex, Kobe, Japan). Then, four capillary blood samples from a pre-heated fingertip were collected in 35µl pre heparinized tubes (safeClinitubes, Radiometer, Brønshøj, Denmark) and analysed for percent carboxyhaemoglobin on an ABL 800 blood gas analyser (Radiometer, Brønshøj, Denmark). Participants were then instructed to exhale completely to measure end-tidal CO before being connected to a custom designed spirometer (Hans Rudolph, Kansas, USA). Then, 1.0 mL × kg bw^{-1} chemically pure (99.997%) CO (CO N47; Air Liquide, Paris, France) was delivered via a 100-mL plastic syringe (Omnifix; Braun, Melsungen, Germany) to the spirometer creating a closed system. The system contained 5 L of 100% oxygen, which was rebreathed for 2 min with the applied dosage of CO. Four capillary blood samples were collected and analysed nine minutes after the inhalation of CO using the same technique used during baseline sampling. A CO analyser (Draeger, Lübeck, Germany) was used to evaluate whether a leak in the closed system occurred during the rebreathing period and to measure any leftover CO in the spirometer and end-tidal CO 3 minutes after the rebreathing period. The haematocrit, haemoglobin concentration and the difference in percent carboxyhaemoglobin were used to calculate total haemoglobin mass and intravascular volumes [7], which was adjusted for a loss of CO to myoglobin (0.3% per minute) and through ventilation (estimated alveolar ventilation of 5 L per minute).

References:

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