Supplemental Digital Content 1, which describes the procedures for mitochondrial respiration measurements

A detailed description of the procedures is also available in our previous report ([2](#_ENREF_2)).

After collection, muscle samples for respirometric measurements were immediately placed in ice-cold biopsy preservation solution (BIOPS) containing 2.77 mM CaK2EGTA buffer, 7.23 mM K2EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM 2-(N-Morpholino)ethanesulfonic acid hydrate (K-MES), 0.5 mM dithiothreitol (DTT), 6.56 mM MgCl2•6H2O, 5.77 mM ATP, and 15 mM phosphocreatine (pH 7.1). Muscle samples were then gently dissected with the tips of two 18 gauge needles, followed by chemical permeabilization via incubation in 2 mL of BIOPS with saponin (50 μg/mL) for 30 minutes in 4°C. Lastly, samples were washed with a mitochondrial respiration medium containing 0.5 mM EGTA, 3 mM MgCl2•6H2O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2P04, 20 mM HEPES, 110 mM sucrose, and 1 g/L bovine serum albumin (pH 7.1) for 10 minutes in 4°C ([5](#_ENREF_5)). Following the rinse, muscle preparations were blotted dry and measured for wet weight in a balance-controlled scale (XS205 DualRange Analytical Balance, Mettler-Toledo AG, Switzerland). Respirometric measurements were performed in mitochondrial respiration medium 06 (MiR06; MiR05 + catalase 280 IU/mL). Measurements of oxygen consumption were performed at 37°C using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria) with all additions in each substrate, uncoupler, and inhibitor titration protocol added in series. Standardized instrumental calibrations were performed to correct for back-diffusion of oxygen into the chamber from the various components, leak from the exterior, oxygen consumption by the chemical medium, and sensor oxygen consumption.

*Cytochrome c oxidase measurements.* Chemical calibrations were performed to determine, and later control for, the amount of non-mitochondrial auto-oxidation that occurs in the respiratory chambers during cytochrome c oxidase (COX) analysis, as previously described ([1](#_ENREF_1)). For this we measure oxygen consumption over time in the respiratory chamber using MiR06, free of any biological sample, with the additions cytochrome c (10 μM), ascorbate (2 mM) and N,N,N',N'-tetramethyl-1,4-benzenediamine, dihydrochloride (TMPD, 500 μM) titrated into the chamber. This establishes the linear relationship between the degree of non-biological oxygen consumption via auto-oxidation of chemicals added to the medium across different oxygen concentrations within the chamber.

Oxygen flux was resolved by software allowing nonlinear changes in the negative time derivative of the oxygen concentration signal (DatLab, Oroboros, Innsbruck, Austria). All respirometric analyses were done in duplicate and were carried out in a hyperoxygenated environment to prevent any potential oxygen diffusion limitation with oxygen concentration within the chamber ranging between 250 – 420 nmol⋅mL-1.

The titration protocol was specific to the examination of individual aspects of respiratory control through a sequence of coupling and substrate states induced via separate titrations. This titration protocol was modified from previous protocols where they are described in detail ([1](#_ENREF_1),[3](#_ENREF_3)). All titrations were added in series as presented.

Leak respiration in absence of adenylates (LN) was induced with the addition of malate (2 mM) and octanoyl carnitine (0.2 mM). The LN state represents the resting oxygen consumption of an unaltered and intact electron transport system free of adenylates. Maximal electron flow through electron transferring-flavoprotein (ETF) and fatty acid oxidative capacity (PETF) was determined following the addition of ADP (5 mM). Lactate-stimulated respiration was induced with the additions of nicotinamide adenine dinucleotide (NAD+; 5 mM) and lactate (60 mM). Prior to the titration of lactate, however, the respiratory flux was allowed to equilibrate following the addition of NAD+ to assess and control for any potential influence of NAD+ on respiration via mammalian ortholog of Sir2, sirtuin 1 ([3](#_ENREF_3)). There were no measureable alterations in respiration from PETF versus that after NAD+ titration during baseline measurements or during respirometric analysis following six weeks of endurance training (data not shown). Submaximal state 3 respiratory capacity specific to CI (PCI) was induced following the additions of pyruvate (5 mM) and glutamate (10 mM). Maximal state 3 respiration, oxidative phosphorylation capacity (P), was then induced with the addition of succinate (10 mM). As an internal control for compromised integrity of the mitochondrial preparation, the mitochondrial outer membrane was assessed with the addition of cytochrome c (10 μM). There was no evidence of any compromised mitochondrial membrane integrity across samples measured at baseline with the titration of exogenous cytochrome c (102.6 ± 22.0 to 94.4 ± 26.6 pmol O2⋅min-1 ⋅mg ww-1, *p* = n.s.) or following six weeks of endurance training (103.2 ± 20.2 to 106.1 ± 22.4 pmol O2⋅min-1⋅mg ww-1, *p* = n.s.). Oligomycin was added inhibiting ATP synthase to achieve oligomycin-induced leak respiration (LOMY). Phosphorylative restraint of electron transport was assessed by uncoupling ATP synthase (complex V) from the electron transport system with the titration of the proton ionophore, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP; steps of 0.5 μM) reaching electron transport system (ETS) capacity. Rotenone (0.5 μM) and antimycin a (2.5 μM) were added, in sequence, to terminate respiration by inhibiting CI and complex III (cytochrome bc1 complex), respectively. With CI inhibited, electron flow specific to CII (PCII) can be measured. Prior uncoupling with FCCP has no effect on PCII ([1](#_ENREF_1)), as individual electron input to CII does not saturate the Q-cycle. Inhibition of respiration with antimycin a then allows for the determination and correction of residual oxygen consumption, indicative of non-mitochondrial oxygen consumption in the chamber.

Finally, ascorbate (2 mM) and TMPD (0.5 mM) were simultaneously titrated into the chambers to assess COX, complex IV activity. TMPD and ascorbate are redox substrates that donate electrons directly to COX and activity was measured by pmol O2⋅min-1⋅mg ww-1. COX activity has been shown to strongly correlate with mitochondrial volume density and total cristae area (both measured via transmission electron microscopy) in addition to respiratory capacity ([4](#_ENREF_4)).

The sequential respiratory capacities PETF and that following titration of NAD+ to the respiration medium, testing the influence of SIRT activity on respiratory control, as well as P and respiration following the titration of exogenous cytochrome c, testing mitochondrial membrane integrity, were analyzed by a one-way ANOVA on repeated measurements.

**References**

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