Supplement Material

Detailed Materials and Methods

Mice. All animal studies were approved by the Duke University Institutional Animal Care and Use Committee and conducted in 10-15 week old male C57BL/6J, Akt1$^{-/-}$ and eNOS$^{-/-}$ mice purchased from Jackson Laboratory (Bar Harbor, ME) or in genetically-engineered mitochondrial GFP (green fluorescent protein) reporter mice obtained from Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan), and bred in our vivarium [1]. Mice were injected subcutaneously with human recombinant EPO (4000 U/kg; Amgen, Thousand Oaks, CA) or an equal volume of 0.9% NaCl once daily for three consecutive days and tissues removed under general anesthesia at the indicated times.

Histology and fluorescence microscopy. Fresh tissues were fixed in 10% formalin, paraffin-embedded and sectioned at 5 µm, mounted on slides, de-paraffinized, treated with 0.1% saponin, and washed in PBS. Slides were prepared with SlowFade antifade kit (Invitrogen, Carlsbad, CA) and laser-scanning confocal microscopy was performed on a Zeiss LSM 410 microscope (MicroImaging, Inc., Thornwood, NY). Fluorescence images were acquired on a Nikon microscope through a 520 nm filter and fluorescence quantified using computer software (Nikon NIS-Element F 3.0).

Mitochondria studies and immunoblots. Cardiac proteins were resolved by SDS-PAGE, then transferred to polyvinylidene difluoride membranes, and probed with polyclonal Akt and phospho-Akt antibodies (sc-8312 and sc-135651), Erk 1,2, and phospho-Erk 1,2 (pErk), and HO-1 primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Akt1-specific antibodies were also used for negative control blots in Akt1$^{-/-}$ mice (Akt1; sc-81434 and phospho-Akt1; sc-81433; Ser 473). After application of primary antibody, the membranes were washed and incubated with appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies (Santa Cruz or Jackson) and the membranes developed in ECL (Santa Cruz) and quantified on digitized images in the mid-dynamic range. Protein loading was confirmed by stripping the membranes and probing for tubulin or β-actin (Sigma, St. Louis, MO). At least four samples were used for densitometry measurements. All proteins were checked pre and at 3, 7 and 14 days post-EPO, and where there was no response to EPO, only day 3 data were displayed in the histograms.

Pooled cardiac mitochondria were prepared by discontinuous Percoll gradient centrifugation and 1-2 mg of mitochondrial protein was suspended respiration buffer in water-jacketed chambers at 35°C [2]. States 2, 4 and 3 (ADP-supplemented) respiratory rates were measured with succinate (5 mM) or malate + glutamate (2.5 mM) using calibrated Clark mini-electrodes (Diamond General, Ann Arbor, MI) [3].

Nucleic acids. Cytoplasmic RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and cDNA synthesized using the SUPERSCRIPT System Kit (Invitrogen). Cardiac mtDNA was isolated using NaI kits (Wako, Tokyo). Mouse-specific primers and probes were designed with Primer Express (Applied Biosystems, Branchburg, NJ), and RT-PCR performed as published [4] RNA samples (1µg) were reverse-transcribed (in 20 µl) using Moloney murine leukemia virus reverse transcriptase (180 units; Promega, Madison, WI) in a buffer containing random hexamer primers, dNTPs, and ribonuclease inhibitor RNasin (Promega). The transcripts were amplified in triplicate using gene-specific primers [4, 5] and quantified by densitometry with normalization to

18S rRNA or GAPDH (Bio-Rad, Hercules, CA). MtDNA copy number was determined by PCR as reported [6].

Metabolic measurements. Steady-state O_2 consumption and CO_2 production ($\dot{V}O_2$ and $\dot{V}CO_2$) were measured in resting mice pre-acclimated to individual metabolic chambers at constant ambient temperature (25°C) at the same time of day [7]. After a stabilization period, the mouse's expired gas was collected for 6-8 min at a calibrated flow rate. The O_2 and CO_2 gas concentrations were measured on a calibrated gas chromatograph (Model 3800; Varian, Palo Alto, CA) and the $\dot{V}O_2$ and $\dot{V}CO_2$ were computed with standard formulae including temperature corrections. Resting energy expenditure (REE) was calculated using the modified Weir formula and expressed in kcal/d [8].

Voluntary exercise. Mice were supplied *ad libitum* with food and water, and housed individually in cages containing rodent exercise wheels adapted from [9]. The system consisted of an 11.5-cm diameter wheel with a 5.0-cm-wide running surface (Model 6208, Petsmart, Phoenix, AZ) equipped with a digital magnetic counter activated by wheel rotation. To minimize the variability in the time spent running at the start of the study, individual mice were initially observed for 72 hours, and then assigned randomly to groups that were well-matched for running times. One group received EPO and the other NaCl for three consecutive days, and over the next two weeks, running duration, running speed, and distance in kilometers were recorded daily for each mouse.

Transthoracic echocardiography. Mice were lightly anesthetized with 1.5% isofluorane and the measurements made with a 707B 30 MHz ultrasonic probe and recorded on a VEVO 770 System (VisualSonics, Inc., Toronto).

Statistics. Data from 4 to 6 mice per group were expressed as means ± SEM. Statistical analyses was by two-way or repeated measures ANOVA using StatView (SAS Institute, 5.0.1; Chicago, IL). $P \leq 0.05$ was considered significant.

References

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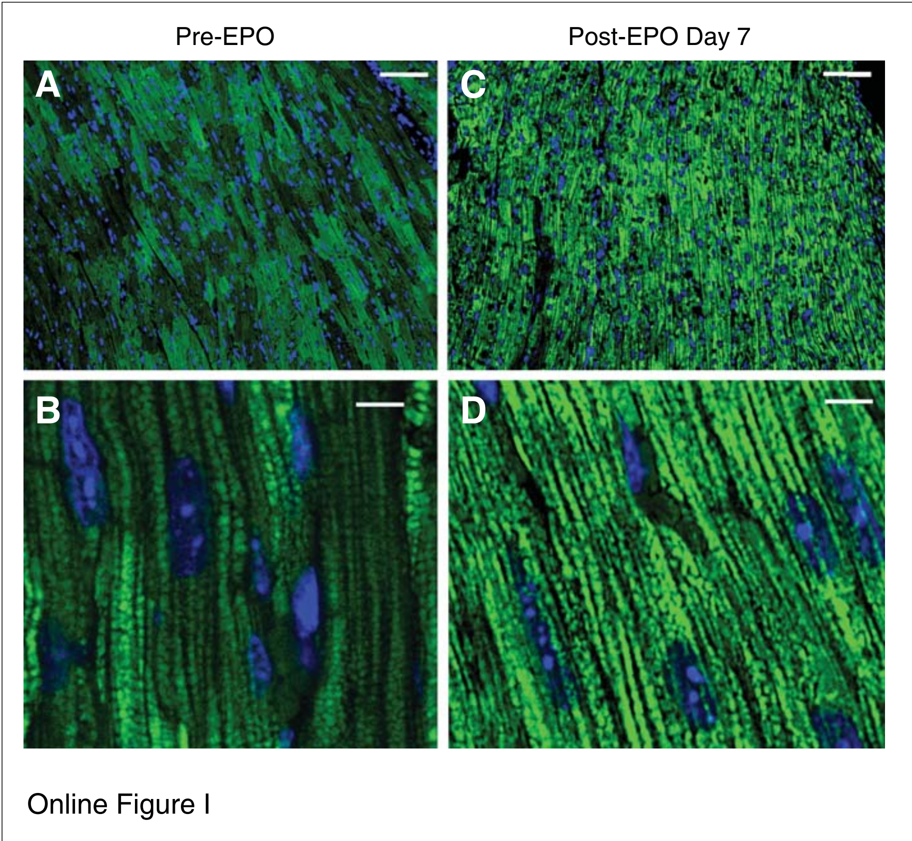
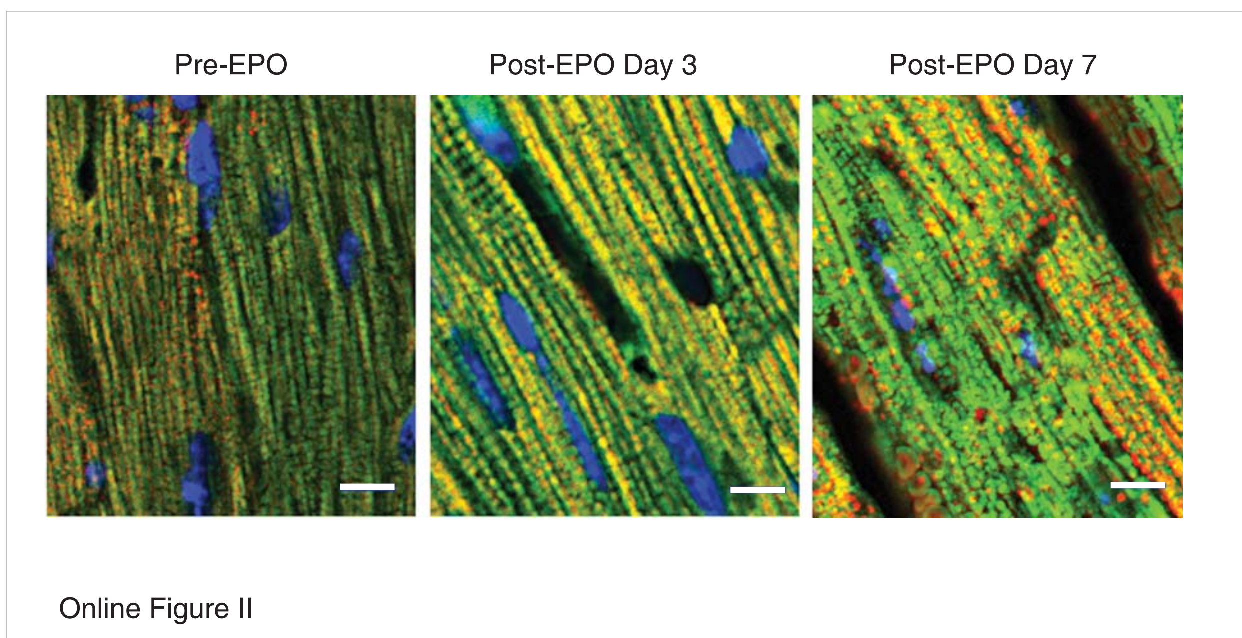


Online Figure I: Cardiac mitochondria pre- and post-EPO (day 7) in mitochondrial reporter mice. Pre-EPO sections are shown in panels **A** and **B** (100 and 1,000 magnification scale bars are 50 and 5 microns, respectively). Comparable images are shown in panels **C** and **D** at post-EPO day 7.



Online Figure II: Mitochondrial citrate synthase expression in mitochondrial reporter mice. Reporter mice expressing the GPF mitochondrial localization tag received EPO (4,000 U/kg/d) for three days and hearts were compared by fluorescence microscopy pre- and post-EPO. These tissues were then labeled with anti-citrate synthase (red fluorescence), and the images merged to demonstrate co-localization (yellow-orange). Citrate synthase staining matched the distribution of GFP, and was present with light diffuse staining pre-EPO, that increased nearly uniformly post-EPO at days 3 and 7. Scale bars are 5 microns.