Supplementary Methods

MITOCHONDRIAL ENERGETICS IN THE HEART IN OBESITY RELATED DIABETES: DIRECT EVIDENCE FOR INCREASED UNCOUPLED RESPIRATION AND ACTIVATION OF UNCOUPLING PROTEINS

Sihem Boudina[1], Sandra Sena[1], Heather Theobald[1], Xiaoming Sheng [2], Jordan J. Wright[1] Xia Xuan Hu [1], Salwa Aziz[1], Josie I. Johnson[1], Heiko Bugger [1],Vlad G. Zaha[1] and E. Dale Abel[1].

Supplementary Methods

***Mitochondrial respirations in saponin-permeabilized fibers* –** Mitochondrial outer membrane integrity was assessed by measuring state 3 respirations in the presence of 8µmol/L cytochrome C. Augmentation of respirations under these conditions indicate damage to the outer mitochondrial membrane, and fiber preparations in which cytochrome C addition stimulated respirations were not used. This occurred in less than 5% of fiber preparations.

***Proton leak measurements* –** Mitochondria were prepared from whole hearts by differential centrifugation at 4ºC. Four mouse hearts were pooled for each experiment. Hearts were finely diced in STE medium containing (in mmol/L) 250 sucrose; 5 Tris/HCl; (pH 7.4) and 2 EGTA, digested on ice for 4 min in STE medium (to which was added 0.5% (w/v) free fatty acid BSA, 5 mmol/L $MgCl_2$, 1 mmol/L ATP and 2.5 U/mL protease (type VIII from *Bacillus licheniformis*). The mixture was then homogenized X3 by Polytron for 2 min and the homogenate centrifuged for 10 min at 8,000 g at 4°C. Pellets were suspended in STE buffer and centrifuged for 10 min at 700 g at 4°C and the

resulting supernatant centrifuged for 10 min at 8,000 g at 4°C to sediment mitochondrial pellets which were suspended in STE buffer and the protein concentration determined using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL) with BSA as a standard. Mitochondrial proton leak kinetics were measured by determining the dependence of the respiration rate required to drive proton leak (in the presence of oligomycin to inhibit ATP synthesis) on mitochondrial membrane potential, measured using an electrode sensitive to the potential-sensitive probe, triphenylmethylphosphonium (TPMP) in the presence of nigericin to dissipate the pH gradient. Briefly, mitochondria were suspended in assay medium (final concentration 0.35 mg protein/ml) containing oligomycin (1 µg/ml), nigericin (100 ng/ml) and rotenone (10 µmol/L). The TPMP-sensitive electrode was calibrated with sequential additions of TPMP up to 2 µmol/L then 4 mmol/L succinate was added to initiate respiration. Membrane potential and respiration were progressively inhibited through successive steady states with the complex II inhibitor, malonate, up to 6 mmol/L. Finally, FCCP (5 µmol/L) was added to dissipate the membrane potential and release all the $TPMP^+$ from the mitochondria, allowing correction for any small baseline drift. Respiration at each steady state was plotted against the appropriate membrane potential to display the dependence of proton leak rate on membrane potential. Proton leak measurements were performed in hearts that were pre-perfused for 20 min with 11 mmol/L glucose and 1 mmol/L palmitate prebound to 3% (w/v) BSA. The kinetics were performed in the absence of GDP or ATR (control) or in the presence of GDP (500 µmol/L) alone or combined with ATR (5 µmol/L).

Measurement of tissue triglycerides – Heart samples) were homogenized in chloroform/methanol (8:1 v/v). Samples were then let for 3-4 hours at room temperature under sacking. The phases were separated by adding 1 M H2SO4. After centrifugation at 1000 rpm for 10 min at room temperature, the organic layer (bottom) that contains triglycerides and phospholipids was collected and speed vacuumed to dry. Tryglyceride concentration was determined on the dry samples using a kit (Sigma).

Oil Red O Staining – Heart and liver samples were fixed in 10% formalin for 1hour, embedded in 20% sucrose solution for 20 min and then frozen. Sections (10 and 15 microns) were realized using a cryostat. Sections were then stained with 1% oil red O in ethanol/acetone (1:1 v/v) solution for 1 min and then washed with distilled water for 5 min. nuclei were stained in blue by Harris's Hematoxylin for 1 min and washed for 5 min with distilled waster. Sections were then mounted with a glycerin jelly.

Mitochondrial Volume Density- Mitochondrial volume density was determined using the point counting method. A 1024-point-grid was projected onto each test field, and mitochondrial area was measured. Data are presented as percent values, calculated by relating mitochondrial