Increasing mitochondrial membrane phospholipid content lowers enzymatic activity of electron transport complexes.

Saame Raza Shaikh, E. Madison Sullivan, Rick J. Alleman, David A. Brown and Tonya N. Zeczycki

Supporting Information

Materials and Methods.

Materials. Bovine heart cardiolipin (CL) and dioleoyl-phosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids, Inc. We ensured the fatty acyl chain composition of the CL between batches with gas chromatography. All other reagents were obtained from Sigma-Aldrich and of the highest purity available. All animal experiments were conducted in accordance with the guidelines established by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and with prior approval by East Carolina University's Animal Care and Use Committee. Male Sprauge-Dawley rats (150-250 g, obtained from Charles River) were housed on a 12:12 h light-dark cycle and had unrestricted access to standard chow and water.

Isolation of Mitochondria from Cardiac Tissue. Mitochondrial isolation was performed on ice and all instruments and buffers were chilled to 4^{o} C prior to the isolation. Rats were anesthetized with ketamine/xylazine (90 mg/kg ketamine, 10 mg/kg xylazine, i.p.). After the absence of animal eye-blink, toe-pinch, and righting reflexes, hearts were removed and immediately placed in chilled mitochondrial isolation medium (MIM) containing 20 mM HEPES (pH 7.2), sucrose (300 mM) and EGTA (1 mM). The left ventricle was dissected; the tissue was manually minced for 5-6 min and then subjected to trypsin digestion (0.125 mg/mL MIM) for 2 min. After digestion, trypsin inhibitor (0.65 mg/mL MIM, pH 7.4, supplemented with 1 mg/ml BSA) was added and the tissue was allowed to settle. Digested tissue was resuspended in MIM supplemented with BSA (1 mg/mL, pH 7.4) and subjected to homogenization with a Teflon Potter homogenizer. The homogenate was centrifuged at 600 x *g* for 10 min and the decanted supernatant was centrifuged at 8,000 x *g* for an additional 15 min. The resulting mitochondrial pellet was resuspended in MIM supplemented with 1 mg/mL BSA and centrifuged at 8,000 x *g* for 15 additional min. The final mitochondrial pellet was resupended in 300 µl of MIM (pH 7.2) and frozen at -80^{o} C. Total mitochondrial protein concentrations were determined prior to freezing using a BCA protein assay (ThermoScientific, La Jolla, CA).

Formation of CL and DOPC Small Unilamellar Vesicles and Fusion with the Mitochondrial Membrane. Phospholipids were dried down under a gentle stream of nitrogen gas and vacuum pumped as recently described.[1] Multilamellar vesicles (MLVs) were generated using CL or DOPC (1 mg) that was resuspended in MIM (2 mL, pH 7.2). The lipids were subjected to three freeze-thaw cycles (dry ice) and vortexed when thawed in the presence of glass beads to ensure MLV formation. Sonication of the MLVs on ice and under a steady stream of nitrogen gas (Branson Digital Sonifier equipped with a microtip; 10% amplitude, 15 sec; 1 min rest; 8 cycles) resulted in the formation of small unilamellar vesicles (SUVs). SUVs were added to the isolated mitochondria (1 mg total protein) and allowed to gently shake for 60 min at 4^{o} C. Samples were centrifuged at 10,000 x *g* for 10 minutes to remove excess SUVs and the fused mitochondrial pellet was resuspended in hypotonic medium (25 mM K_2PO_4, pH 7.2, 5 mM $MgCl_2$) at a final concentration of 0.8 mg of total protein/ml based on BCA assay for protein content.

Thin-Layer Chromatography (TLC). Lipids were extracted from freshly isolated and SUV-fused mitochondria (40 µg of total protein). Each sample was vortexed for ~1 min to disrupt the mitochondrial membranes. Mitochondria were suspended in a 70:30 (v:v) chloroform: methanol (0.05% BHT) solution . Samples were then vortexed for an additional 45-60 sec and prior to the addition of 0.88% KCl (2.3 ml). The mixture was centrifuged at 805 x *g* for 10 min at room temperature. The bottom layer of the sample was aspirated and dried using nitrogen gas. The extract was resuspended in chloroform and dried down two additional times prior to being resuspended in chloroform to give a final concentration of 1 µg/µL of total protein in chloroform. 5 µg of protein was then spotted on a Whatman TLC plate (10x10 cm, silica gel) and allowed to dry. Plates were developed using a chloroform:methanol:glacial:acetic acid:water (80:30:1:4; v/v/v/v) mobile phase and allowed to dry before spraying with a charring solution (4% phosphoric acid/5% copper sulfate). After 1-2 min of further drying, the TLC plates were heated in a conventional lab oven at 190^{o} C for 15 min. Charred lipid spots were quantified using an Odyssey Infrared Imager. Intensity of the charred lipids spots were determined relative to the average intensity of the background of the plate. The increase in mitochondrial lipid concentration after fusion with the individual SUVs was determined relative to the intensity of the respective charred lipid spot in the control mitochondrial sample defined as 100%.

Determination of the Relative Activities of Enzymes in Control and Phospholipid Fused-Mitochondria. Specific activities for each of the individual and oxidative phosphorylation enzymes and the multi-enzyme

reactions were determined spectrophotometrically at 37° C in either 1 mL or 1.5 mL total reaction volumes according to published procedures.[2, 3] Activities for the control mitochondrial were determined in triplicate from three separate mitochondrial isolations. Similarly, activities for the fused-mitochondria were determined in triplicate from two separate isolations/SUV-fusions. Reported activities are the specific activities of the complexes normalized to the respective citrate synthase activity.

Citrate Synthase. Activities of all complexes were normalized to the respective citrate synthase activity of the sample. The citrate synthase activity of mitochondria (40 and 80 µg total protein) in hypotonic medium was determined in reactions containing 10 mM Tris (pH 7.5), 0.2% Triton X-100, acetyl-CoA (0.2 mM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.1 mM). The reaction was initiated with the addition of freshly prepared oxaloacetate (0.5 mM) and the reduction of DTNB was monitored at 412 nm for 3 min. An ε_{412} of 14140 $M^{-1}cm^{-1}$ was used to calculate the relative citrate synthase activity. Averages of the specific activities with both 40 and 80 µg of total protein were used to normalize the specific activities of all other complexes.

Complex I (NADH: deyclubiquinone oxioreductase). Reactions containing 50 mM Tris (pH 8.0), BSA (5 mg/mL), 240 µM KCN, 4 µM Antimycin A and 50 mM decylubiquinone (DCU) and mitochondrial protein (40 µg) were initiated by the addition of 0.8 mM NADH. The rotenone-sensitive catalytic oxidation of NADH to NAD^+ was followed at 340 nm for 3 min and an ε_{340} of 6220 $M^{-1}cm^{-1}$ was used to calculate overall activity. Inhibition of the oxidation of NADH by the addition of rotenone (4 µM) confirmed that activities being determined were due solely to complex I activity.

Complex II (succinate-decylubiquinone reductase). Mitochondria (26.7 µg of total protein) were pre-incubated with succinate (10 mM) for 30 min prior to being incubated for an additional 3 min in the reaction mixture containing 10 mM KH_2PO_4 (pH 7.4), EDTA (2 mM), BSA (1 mg/mL), rotenone (4 µM), ATP (0.2 mM), succinate (10 mM) and 2,6-dichlorophenolindophenol (DCPIP, 10 µM). Reactions were initiated with the addition of decylubiquinone (DCU, 80 µM) and the catalytic reduction of DCPIP was monitored at 600 nm for 3 min. An ε_{600} of 19,100 $M^{-1}cm^{-1}$ was used to calculate activity and the addition of malonic acid (10 mM) was used to inhibit Complex II activity. No activity was observed subsequent to the addition of malonic acid.

Complex III (ubiquinol:cytochrome-c reductase). DCU (80 mM, pH ~2-3) was reduced using sodium borohydride. After complete reduction, concentrated HCl was added drop-wise until the solution became colorless. Mitochondria (40 µg total protein) in hypotonic medium were added to a reaction mixture containing

10 mM KH_2PO_4 (pH 7.4), EDTA (2 mM), BSA (1 mg/mL), KCN (240 μM), ATP (0.2 mM) and oxidized cytochrome *c* (40 μM). The reaction was initiated by the addition of the freshly prepared reduced DCU (80 mM) and the catalytic reduction of cytochrome *c* was monitored at 550 nm for 3 min and an ε_{550} of 21,840 $M^{-1}cm^{-1}$ was used to calculate activity. Antimycin A (0.4 μM) was added to the reaction to inhibit Complex III activity and the non-catalytic reduction of cytochrome *c* by endogenous quinone was measured for an additional 3 min. The non-catalytic rate of reduction was subtracted from the catalytic rate.

Complex IV (cytochrome c oxidase). Reduced cytochrome *c* was prepared by stirring oxidized cytochrome *c* in a solution of 20 mM boric acid/0.2 M sodium borohydride at room temperature until all bubbles had dissipated. The catalytic oxidation of cytochrome *c* by Complex IV was initially measured in reaction mixtures containing control mitochondria (1.6 μg total protein) or SUV-fused mitochondria (4.0 μg total protein) in hypotonic medium, 10 mM KH_2PO_4 (pH 6.5), sucrose (0.25 M), BSA (1 mg/mL) and reduced cytochrome *c* (10 μM) at 550 nm (ε_{550} of 21,840 $M^{-1}cm^{-1}$). Maltoside (2.5 mM) was then added to disrupt the outer mitochondrial membrane to allow substrates easier access to the inner mitochondrial membrane and the rate of cytochrome *c* oxidation was determined for 3 min. The reported rate of complex IV activity was determined after the addition of maltoside. KCN (240 μM) was added to the reaction mixture to inhibit Complex IV activity. No activity was observed after the addition of KCN.

Complex I+III (NADH-cytochrome c oxioreductase). Mitochondria (40 μg total protein) in hypotonic medium were added to a reaction mixture containing 50 mM Tris (pH 8.0) BSA (5 mg/mL), KCN (240 μM) and 40 μM oxidized cytochrome *c*. The reaction was initiated with the addition of NADH (0.8 mM) and the catalytic reduction of cytochrome *c* was monitored at 550 nm for 3 min. An ε_{550} of 21,840 $M^{-1}cm^{-1}$ was used to calculate activity and rotenone (4 μM) was added to inhibit complex I and the non-catalytic reduction of cytochrome *c* by endogenous quinone was measured for an additional 3 min. The non-catalytic reduction was subtracted from the catalytic rate.

Complex II+III (succinate cytochrome c reductase). Mitochondria (26.7 μg of total protein) were pre-incubated with succinate (10 mM) for 30 min. The mitochondria was then was incubated for an additional 3 min in the reaction mixture containing 10 mM KH_2PO_4 (pH 7.4), EDTA (2 mM), BSA (1 mg/mL), rotenone (4 μM), ATP (0.2 mM), succinate (10 mM) and 2,6-dichlorophenolindophenol (DCPIP, 10 μM). The reaction was initiated by the addition of oxidized cytochrome *c* (40 μM) and the catalytic reduction of cytochrome *c* was

monitored at 550 nm for 3 min. An ε_{550} of 21,840 $M^{-1}cm^{-1}$ was used to calculate activity. Malonic Acid (10 mM) was used to inhibit complex II activity and Antimycin A (0.4 μM) was added to the reaction to inhibit Complex III activity. The non-catalytic reduction of cytochrome *c* by endogenous quinone was measured for an additional 3 min and subtracted from the catalytic rate.

Statistical Analysis. All results are reported as ± SEM from several individual experiments. All statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software). Mitochondrial kinetic data was analyzed for normal distribution using a Kolmogorov-Smirnov test. If the data was not normally distributed than a Kruskal-Wallis test, followed by a Dunn's multiple comparison test, was used to establish significance. If the data was normally distributed than an ordinary one-way ANOVA was used, followed by a Turkey multiple comparison test was used to establish significance. For liposome fusion studies, data was normalized to the control group. A one-way ANOVA was used to ascertain significance. Only parametric statistics were used because the data was normally distributed. For all data *P*-values <0.05 were considered significant.

References.

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