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Increasing Mitochondrial Membrane Phospholipid Content Lowers the Enzymatic Activity of Electron Transport Complexes

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Supporting Information

ABSTRACT: Activities of the enzymes involved in cellular respiration are markedly influenced by the composition of the phospholipid environment of the inner mitochondrial membrane. Contrary to previous suppositions, we show that fusion of mitochondria isolated from healthy cardiac muscle with cardiolipin or dioleoylphosphatidylcholine results in a 2−6-fold reduction in the activity of complexes I, II, and IV. The activity of complex III was unaffected by increased phospholipid levels. Phospholipid content had an indiscriminate yet detrimental effect on the combined activities of complexes I+III and II+III. These results have strong implications for therapeutic lipid replacement strategies, in which phospholipid modification of the mitochondria is proposed to enhance mitochondrial function.

Cellular respiration in the mitochondria of higher organisms is paramount to supplying the ATP needed to sustain biological processes.[1] The redox reactions of the mitochondrial respiratory chain, which consists of four transmembrane enzyme complexes, a lipid-soluble electron carrier (ubiquinone), and a water-soluble electron carrier (cytochrome *c*), generate the electrochemical proton gradient necessary to drive ATP synthesis. NADH and succinate are initially reduced by complex I (NADH:ubiquinone oxidoreductase) and complex II (succinate dehydrogenase), respectively. Electrons are then transferred through the inner mitochondrial membrane (IMM) to complex III (cytochrome *c* reductase), via ubiquinone, where the catalytic reduction of cytochrome *c* in the intermembrane space allows for the transfer of an electron from complex III to complex IV [cytochrome *c* oxidase (Figure 1)]. Cellular respiration culminates in the electrochemically driven synthesis of ATP by complex V.[2]

Considering that the electron transport complexes are imbedded within the phospholipid rich environment of the IMM, it is not surprising that the composition of the lipids surrounding the complexes has a marked effect on protein activity.[3] Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL) comprise the bulk of the membrane phospholipids in the mitochondria (~44% PC, 34% PE, and 14% CL).[4] CL is of particular interest because of its unique structure and essential role in facilitating electron transport.[3g,h] Structural studies of purified respiratory complexes from prokaryotes and eukaryotes have revealed numerous protein−CL interactions, specifically with complexes I, III, and IV. The contributions of the protein-bound CL to the structure and function of the individual respiratory complexes vary. For example, delipidation of isolated prokaryotic complex III reversibly inactivated the enzyme without affecting structural stability,[5] while the structural integrity of complex I is contingent on CL occupying two high-affinity phospholipid binding sites.[3d] Even though specific CL/phospholipid binding sites in complex II have yet to be identified, CL promoted dimerization and activity of prokaryotic complex II reconstituted in lipid bilayers mimicking the natural lipid composition of the IMM.[3f] Additionally, CL is proposed to have a broader effect on cellular respiration by facilitating the organization of the individual electron transport complexes into higher-order assemblies, or supercomplexes, which allows for an increased rate and efficiency of metabolite transfer and electron flux.[6] CL has arguably the most influential effect on cellular respiration. Decreases in mitochondrial levels of CL concomitant with the progression of cardiovascular disease[3h,7] and type 2 diabetes have been directly correlated to a decrease in the overall activity and efficiency of cellular respiration.[6b,8] Replenishment of mitochondria depleted of cardiolipin can restore protein and cellular respiration activities to near normal levels. For instance, the fusion of PC/CL (4:1 molar ratio) vesicles restored the decrement in complex I activity in response to ischemia-reperfusion injury. Notably, the fusion of PC/CL vesicles to healthy mitochondria had no effect.[3d]

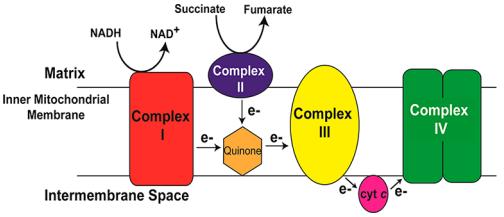

Figure 1. Electron flow among complexes I−IV of the oxidative phosphorylation system in the inner mitochondrial membrane. Cardiolipin (CL) directly interacts with complexes I, III, and IV.

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Surprisingly, the consequences of phospholipid content modification on the individual specific activities of these complexes in the context of intact mitochondria isolated from healthy tissue have not been extensively studied. Furthermore, the individual influence of each phospholipid component has not been addressed. In light of the increasing level of support for recently developed therapeutic lipid replacement strategies for treating damaged mitochondria in various metabolic diseases,[8] we investigated the effects of increased phospholipid content on the relative enzymatic activities of the complexes in the mitochondrial respiratory chain in mitochondria isolated from healthy cardiac tissue. Contrary to our expectations, the results reported here indicate that an increased level of lipidation of the isolated mitochondria selectively reduces the enzymatic activities of complexes I, II, and IV and has a detrimental impact on the rate of metabolite transfer (ubiquinone) and electron flux through complexes I+III and complexes II+III.

The three main phospholipids comprising >90% of the membranes in the mitochondria in the control and phospholipid-fused mitochondria were isolated from intact rat heart mitochondria and visualized by thin-layer chromatography (TLC) (Figure 2A). Comparison to commercially available

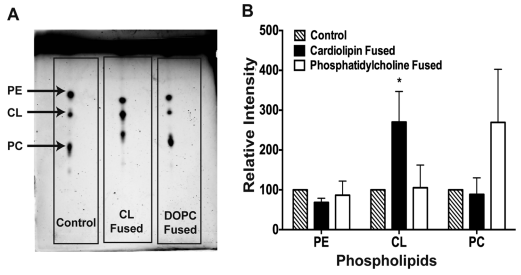

Figure 2. Fusion of CL and PC vesicles with rat heart mitochondria. (A) Representative TLC plate showing the relative increase in phospholipid composition after fusion with CL and DOPC small unilamaller vesicles. (B) Quantification of phospholipid composition using densitometry. Phospholipid concentrations in fused mitochondria were normalized to the content of control mitochondria. Data are averages ± the standard error of the mean from three independent experiments. The asterisk indicates significance from control ($p < 0.05$).

phospholipids established the identities of the isolated lipids as PC, PE, and CL (see Supporting Information). The phospholipid content of cardiac mitochondria isolated from healthy rats was increased by fusing the isolated mitochondria with small unilamaller vesicles (SUVs) composed of either bovine heart cardiolipin, which is highly enriched tetralinoleoyl-cardiolipin, or dioleoylphosphatidylcholine (DOPC). The relative lipid composition of the fused mitochondria was then quantified using densitometry and normalized to control lipid levels (Figure 2B). A marked increase in the relative concentrations of CL (2.7-fold increase) and PC (2.7-fold increase) was observed in mitochondria treated with the CL SUVs and DOPC SUVs, respectively. While the relative amount of enrichment for both PC and CL was the same, the absolute amount of PC present in the DOPC SUV-treated mitochondria is appreciably higher than that of CL in the CL SUV-fused mitochondria considering the native lipid composition of the mitochondrial membrane.[4] The increase in DOPC concentration was determined not to be significant but is attributed to a single outlier in the data set. Fusion of mitochondria with either CL or DOPC- SUVs had no influence on the endogenous PE levels.

To determine if SUV fusion had an effect on the overall integrity of the mitochondria, citrate synthase (CS) activity, which is routinely used as a biomarker for mitochondrial content and relative vitality,[9] was determined in the control, CL-fused, and DOPC-fused mitochondria (Figure 3A). CS activity in the

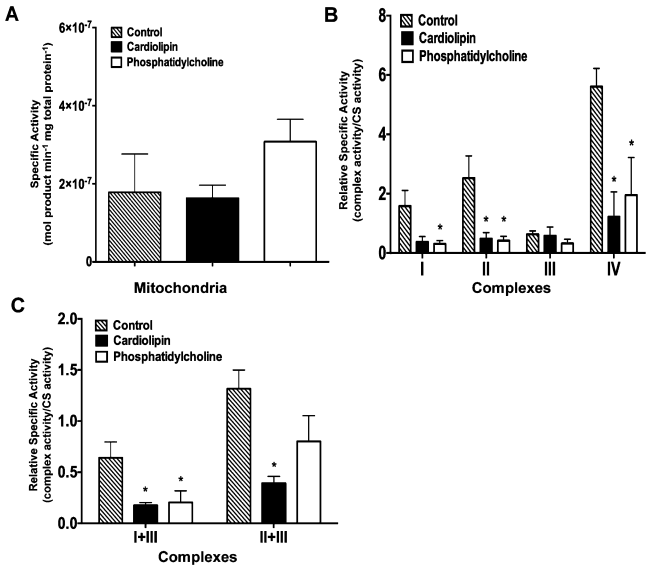

Figure 3. (A) Citrate synthase activity in control, CL-fused, and PC-fused mitochondria. (B) Relative specific activities of complexes I–IV and (C) complexes I+III and II+III in control and phospholipid-fused mitochondria. Data are averages ± the standard error of the mean from three independent mitochondrial isolations determined in triplicate. Activities were determined relative to total protein content (moles of product per minute per milligram of total protein) and then normalized to the respective citrate synthase (CS) activity of each sample. Asterisks indicate significance from control ($p < 0.05$).

lipid-fused mitochondria remained remarkably unchanged relative to the control, signifying that the mitochondria remained intact after fusion. The specific activities of each of the individual complexes in control mitochondria, CL SUV-fused mitochondria, and DOPC SUV-fused mitochondria were subsequently determined spectrophotometrically[10] and normalized to the CS activity of the sample (Figure 3B).

Addition of CL and DOPC caused a statistically significant decrease in the extent of catalytic oxidation of NADH by complex I, and similar decreases in the extent of complex II-catalyzed oxidation of succinate were observed in CL- and DOPC-fused mitochondria. When fused with CL-SUVs, complex I exhibited a 4-fold decrease in activity compared to the control while complex II exhibited a 5-fold decrease in activity. When fused with DOPC, complex I exhibited a 5-fold reduction in activity while the activity was reduced 6-fold in the CL-fused mitochondria. CL fusion had no discernible effect on complex III activity, which agrees with previous studies showing that the enzymatic activity of the isolated enzyme was fairly insensitive to mild (2–4-fold) changes in CL concentration.[11] In contrast, DOPC vesicles marginally reduce complex III activity nearly 2-fold ($p < 0.08$). Kinetic models of complex III–lipid interactions derived from the reconstitution of eukaryotic complex III in a phosphatidylcholine bilayer indicate that the protein has an indefinite number of unspecific and independent PC binding sites that, when saturated, could have an effect on the relative enzyme activity.[12]

Similarly, the rate of NADH oxidation and succinate oxidation coupled to cytochrome *c* reduction, complex I+II and II+III

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activities, respectively, was determined (Figure 3C). In control mitochondria, there was no observable difference in the rate of cytochrome *c* reduction by complex III when it was coupled to NADH oxidation (complex I), but the rate of cytochrome *c* reduction doubled when it was coupled to succinate oxidation (complex II). Because succinate has a redox potential significantly lower than that of NADH,[13] it is unlikely that the rate enhancement is energetic in origin. It is possible that the increased rate of complex II+III activity is partially due to the unrestricted movements of complex II. Complexes I+III and II +III were similarly affected by an increase in CL content with an approximately 3-fold decrease in activity. When fused with DOPC, complex I+III showed a 3-fold decrease compared to the control. Complex II+III had an average 1.6-fold decrease when fused with DOPC when compared to the control. On the basis of these data, we can conceive three plausible explanations for the detrimental effects of increasing lipid concentrations on the activities of the electron transport complexes: (1) excessive concentrations of lipids result in the disruption of the overall quaternary structure of the individual enzymes by overloading subunit interfaces with nonspecific phospholipid binding, (2) phospholipids have an undefined role in allosterically activating or inhibiting enzymatic activity,[5b] or (3) increasing lipid concentrations markedly affect the formation of cardiolipin microdomains of the membrane,[3a] resulting in a weakened ability to transfer metabolites or form supercomplexes.[3c,6c]

Our results were not in agreement with the aforementioned study that showed the complex I activity of healthy mitochondria was unaffected upon fusion with PC/CL vesicles.[3d] The discrepancy may be driven by differences between studies, most notably the previous use of mixed PC/CL (4:1) vesicles which more closely mimics the native PC/CL ratio. Further investigation, including determination of relative k_{cat}/K_m values for each of the complexes and extending the studies to include different combinations of phospholipids, is needed to discern whether the decreased activity is due to the proposed structural or functional roles of the phospholipids and is ongoing in our laboratories. The data also reveal no significant differences in the effects of CL and DOPC on enzymatic activity. While we had anticipated some potential differences would arise given that CL and DOPC have different structures and thereby phase behaviors, it is possible that fusion of the mitochondria with fluid-phase lipids may result in a similar decrease in membrane microviscosity regardless of structure. Overall, our results show that an increase in CL or DOPC content can selectively lower the activities of complexes I, III, and IV and complexes I+III and II+III. These results are of biological significance given that emerging therapies[3h,12] aim to boost mitochondrial enzymatic activity with specific combinations of lipids. In particular, lipid replacement therapy,[8] in which oral phospholipid supplements are taken, is proposed to rescue cellular membrane damage incurred in response to oxidative stress associated with aging, cardiovascular disease, and type II diabetes. This type of damage is particularly pronounced in the IMM, which leads to loss of CL.[14] Therefore, these data are highly relevant because they raise the possibility that healthy mitochondria in select tissues may also be impacted in response to lipid replacement therapy.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

S.R.S. and E.M.S. contributed equally to this work.

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Notes

The authors declare no competing financial interests.

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