The effect of cardiolipin acyl chain composition on cytochrome c protein conformation and resulting peroxidase activity: Exploring the potential mechanisms that contribute to cellular apoptosis

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Submitted in partial fulfilment of the requirements for the degree of

Masters of Science

(Appplied Health Science)

Faculty of Applied Health Science, Brock University

St. Catharines, Ontario

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Master of Science (2019)

Title: The effect of cardiolipin acyl chain composition on cytochrome c protein conformation and resulting peroxidase activity: Exploring the potential mechanisms that contribute to cellular apoptosis

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Number of Pages: 110
Abstract

Skeletal muscle is a highly active tissue comprising up to 40% of total body weight. This highly dynamic tissue relies on mitochondria for calcium homeostasis, program cell death, and ATP production through mitochondrial respiration. The composition of mitochondrial phospholipids influences the functional efficiency of mitochondrial proteins, and healthy membranes include unsaturated acyl chains to promote respiration. Increasing membrane saturation has been implicated in muscle wasting, a condition caused by apoptosis and identified by the release of specific proteins from the mitochondria. It is unclear how membrane composition promotes the release of these pro-apoptotic proteins, specifically cytochrome c, to induce apoptosis. Thus, in this work synthetic membranes mimicking the composition of mitochondrial membranes from healthy and dystrophic mouse muscle were used. Cytochrome c conformation and function was measured with and without these membranes. There were no differences in protein conformation or function between the healthy and dystrophic membrane mimetics. However, cytochrome c affinity to these membranes was increased with greater unsaturation. This suggests that decreasing membrane saturation, as implicated in muscle wasting diseases, promotes the release of cytochrome c and apoptotic-mediated cell death. As such, interventions to improve or maintain membrane unsaturation may prove to be an alternative therapy for muscle wasting diseases.
Acknowledgements

Thank you to the many who have supported me throughout this journey. To my mom and Greg, thank you for pushing me well beyond what I thought were my limits. Without you, I would not be where I am today. To my father, who had supported me and encouraged me to practice and succeed, I truly appreciate it all. To John, without you, I would have lost my drive to continue long ago.

To Dr. LeBlanc, thank you for your guidance and direction. Although this project had a life of its own, your knowledge and patience helped bring it to fruition.

To Dr. Atkinson and Dr. Stuart, thank you for allowing me to tap into your vast knowledge, and helping me become the academic I am today.

To my labmates past and present, thank you for both the learning and teaching experiences you have all provided me in your own ways.

Thank you.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP-</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>AFU-</td>
<td>Arbitrary Fluorescence Units</td>
</tr>
<tr>
<td>AIF-</td>
<td>Apoptosis Inducing Factor</td>
</tr>
<tr>
<td>ANOVA-</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>Apaf-1-</td>
<td>Apoptotic Protease Activating Factor-1</td>
</tr>
<tr>
<td>ATP-</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BCL-</td>
<td>(\beta)- Cell Lymphoma</td>
</tr>
<tr>
<td>BID-</td>
<td>BH3 interacting-domain Death Agonist</td>
</tr>
<tr>
<td>Ca(^{2+})-</td>
<td>Calcium</td>
</tr>
<tr>
<td>CL-</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>Cyt C-</td>
<td>Cytochrome C</td>
</tr>
<tr>
<td>DMSO-</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>ETC-</td>
<td>Electron Transport Chain</td>
</tr>
<tr>
<td>FADH(_2)-</td>
<td>Flavin Adenine Nucleotide (reduced)</td>
</tr>
<tr>
<td>Fe-</td>
<td>Iron</td>
</tr>
<tr>
<td>GPx-</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>GSH-</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG-</td>
<td>Glutathione Disulfide</td>
</tr>
<tr>
<td>H(_2)O(_2)-</td>
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<td>His-</td>
<td>Histidine</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>IMM-</td>
<td>Inner Mitochondrial Membrane</td>
</tr>
<tr>
<td>Lys-</td>
<td>Lysine</td>
</tr>
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<td>Methionine</td>
</tr>
<tr>
<td>MUFA-</td>
<td>Monounsaturated Fatty Acid</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide Adenine Dinucleotide (oxidized)</td>
</tr>
<tr>
<td>NADH-</td>
<td>Nicotinamide Adenine Dinucleotide (reduced)</td>
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<td>Mitochondrial Nucleoside Diphosphate Kinase</td>
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<td>NADPH Oxidase</td>
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<td>OMM-</td>
<td>Outer Mitochondrial Membrane</td>
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<td>PUFA-</td>
<td>Polyunsaturated Fatty Acid</td>
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<tr>
<td>Q-</td>
<td>Ubiquinone</td>
</tr>
<tr>
<td>ROS-</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SDS-PAGE-</td>
<td>Sodium Disulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM-</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SFA-</td>
<td>Saturated Fatty Acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>SM-</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SOD-</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>SR-</td>
<td>Sarcoendoplasmic Reticulum</td>
</tr>
<tr>
<td>tBID-</td>
<td>Truncated BID</td>
</tr>
<tr>
<td>TLCL-</td>
<td>Tetralinoleoyl Cardiolipin</td>
</tr>
<tr>
<td>TMCL-</td>
<td>Tetramyristoyl Cardiolipin</td>
</tr>
<tr>
<td>TOCL-</td>
<td>Tetraoleoyl Cardiolipin</td>
</tr>
<tr>
<td>XO-</td>
<td>Xanthine Oxidase</td>
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</table>
Chapter 1: Literature Review

1.1 Overview

Mitochondria are a versatile organelle composed of two lipid membranes that mediate ionic and small molecule exchanges between the cytosol of the cell and the mitochondria, including sodium, calcium, potassium, and small proteins and enzymes. There are six primary phospholipids present within the mitochondrial membranes, however only one, cardiolipin (CL), has the ability to form the hair-pin tight cristae of the inner mitochondrial membrane (IMM) as well as alter mitochondrial function. The primary role of the mitochondria is to produce adenosine triphosphate (ATP) as the energy source for cellular functions. Mitochondrial ATP is primarily produced by the electron transport chain (ETC) contained within the IMM. In addition to its association with CL, the ETC is composed of both integral and peripheral proteins. Although all the proteins of the ETC are associated with ATP production, only one is associated with programmed cell death. This peripheral protein, cytochrome c, functions as an electron shuttle within the ETC, but when released from the interactions tethering it to the IMM can induce apoptosis. During apoptosis, CL will translocate from the inner leaflet to the outer leaflet of the IMM. This allows greater contact with peripheral proteins leading to electrostatic and hydrophobic binding to cytochrome c, altering its conformation and function from an electron shuttle to a peroxidase. Using hydrogen peroxide from the mitochondrial environment, cytochrome c peroxidizes polyunsaturated CL fatty acyl chains leading to cytochrome c release and ultimately to apoptosis. Currently, research has not examined the direct effect of CL side chain composition on cytochrome c peroxidase conformation and peroxidase activity.
1.2 Skeletal Muscle

Skeletal muscle makes up 30-40% of the human body, dependent on the balance between protein synthesis and degradation. Skeletal muscle functions to support the skeletal structure, locomotion, and regulation of metabolism. As a highly dynamic tissue, skeletal muscle is a major contributor to whole-body respiration, metabolic health, and energy expenditure, all of which depend on mitochondrial function. Loss of skeletal muscle can result in metabolic dysregulation, leading to a reduction in maximum oxygen uptake, excessive fat deposition, insulin resistance, and impaired muscle mass homeostasis leading to musculoskeletal pathologies such as sarcopenia, muscular dystrophy, or osteoporosis (McLeod et al., 2016). Thus, understanding the form and function of skeletal muscle is a necessary step in understanding these critical pathologies.

1.3 The Mitochondrion

In 1890, cytoplasmic structures identified as bioblasts were discovered by Richard Altmann who described these organelles as functional, bean-like entities that resembled bacteria (Ernster & Schatz, 1981). It wasn’t until the early 1950’s that these bioblasts, later renamed singularly as a mitochondrion, were described with great structural detail. Sequentially, between 1952 and 1953, George Emil Palade and Fritiof S. Sjostrand utilized high-resolution electron micrographs to identify the inner folding of mitochondria, later labelled the cristae mitochondriales, and the double membranes that separate the inner chamber from the outer chamber of the mitochondrion (Ernster & Schatz, 1981). Following the description of the membranes, proteins defined as the respiratory chain were localized to these inner mitochondrial membranes (Ernster & Schatz, 1981). It was this organelle that was determined to be required for oxidative phosphorylation and ATP production, an important storage source of chemical energy.
1.3.1 The Electron Transport Chain

Mitochondria are the major producer of chemical stored energy, ATP. The energy produced is used by the rest of the cell for various cellular processes. For example, ATP is used in myocytes during muscular contraction. ATP, produced within the mitochondrial membranes, is created through an aerobic process known as oxidative phosphorylation (as reviewed by Perry et al., 2013). During oxidative phosphorylation, electrons are donated from reducing equivalents, such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH$_2$) to the final electron acceptor, oxygen (Mitchell, 1996; Nath & Villadsen, 2014). Transferring an electron along the electron transport chain (ETC) results in the pumping of protons into the mitochondrial IMS (Figure 1.1; Tajeddine, 2016; (Mitchell, 1961; Mitchell, 1966; Jastroch et al., 2011; Kuksal et al., 2017). Movement of electrons creates a proton gradient that couples the resulting membrane potential to ATP synthase activation and the formation of high energy phosphate bonds by the adding P$_i$ to ADP (Mitchell, 1961; Dudkina et al., 2010).

Within the ETC, complexes I, III, IV, and ATP synthase are integral membrane proteins within the inner membrane. Complex II is a peripheral protein of the electron transport chain as the second entry point for electrons. Ubiquinone (Q) and cytochrome c, are associated with the electron transport chain which act to transfer electrons within and peripheral to the IMM, respectively. Within the membrane, complexes I-IV are connected and stabilized by the formation of supercomplexes, which function cooperatively as a single unit during respiration. Organization of these supercomplexes is dependent on the lipid environment of the mitochondrial inner membrane to promote optimal ETC function.
**Figure 1.1. The electron transport chain.** Electrons are shuttled from complex I to complex IV, where they are accepted by oxygen. The oxygen molecule is then reduced to create water. Complex V uses the proton gradient generated by the ETC to produce ATP. This protein complex is located within the cristae of the mitochondria. ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cyt c; cytochrome c; FAD\(^+\), Flavin adenine dinucleotide (oxidized); FADH, Flavin adenine dinucleotide (reduced); Q, ubiquinone; NAD\(^+\), nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced).
1.3.2 The Lipids of Mitochondrial Membranes

Structurally, mitochondria contain two lipid bilayers, the inner (IMM) and outer mitochondrial membranes (OMM). Each membrane has a specific function dictated, in part, by the phospholipid composition (Martensson et al., 2017). Based on the composition of the head group, there are six primary phospholipids found in membranes; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphoserine (PS), phosphatidylinositol (PI), sphingolipids and cardiolipin (CL) (Horvath and Daum, 2013). With the exception of cardiolipin, these phospholipids contain one glycerol backbone, two fatty acyl chains, and one head group that can influence the function of the phospholipid. Cardiolipin uniquely contains two glycerol backbones with four fatty acyl chains, making it a large phospholipid with a small head group. The percent contribution of each phospholipid species varies in biological cell membranes, however the percent contribution of each phospholipid species to the mitochondrial membranes of rat liver are presented in Table 1.1 to provide general context.
Table 1.1. Percent fraction of the major phospholipids found in the inner and outer mitochondrial membrane of rat liver.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>IMM</th>
<th>OMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiolipin</td>
<td>18</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>34</td>
<td>29</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>40</td>
<td>54</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Phosphatidic Acid</td>
<td>--</td>
<td>1</td>
</tr>
</tbody>
</table>

Data from Horvath & Daum (2013).
The composition and unsaturation of the phospholipid acyl chains is an influential component of phospholipid shape. It is the phospholipid shape, determined by both headgroup and acyl chain composition, that determines the function of the phospholipid in the membrane and the function of membrane bound proteins. Structurally, phospholipids are lamellar or non-lamellar, based on the interaction between acyl chain composition and attached head group (Goni, 2014). For example, PE is a phospholipid that preferentially adopts a non-lamellar hexagonal $H_{II}$ morphology (Guner, 1985; Lee, 2003). Although weakly connected, highly clustered concentrations of non-lamellar lipids, such as PE, facilitate negative membrane curvature, demonstrated by the ovular shape of the OMM and the inner folding of the IMM (Gruner, 1985; Lee, 2003). Peripheral proteins such as G-proteins, anchor to the membrane more efficiently in regions containing $H_{II}$ phase phospholipids where the membrane has greater curvature (Jouhet, 2013). At the same time, regions containing a high concentration of $H_{II}$ phase phospholipids may be detrimental to the activity of some proteins such as the sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase (SERCA; Lee, 2003). Thus, integral membrane proteins, such as SERCA, function at higher rates in the presence of lamellar phospholipids such as PC (Starling et al., 1996; Lee, 2003). With this in mind, it is apparent that protein function is influenced by phospholipid morphology.

The number of double bonds within the phospholipid fatty acyl chains can also influence membrane structure, and in turn protein function. Increased saturation of membranes, induced by dietary changes, has been associated with decreased cytochrome c oxidase activity in rat skeletal muscle (Fajardo et al., 2015). Membrane fluidity, thickness, and physical interaction between proteins and phospholipids not only impact protein function, but the susceptibility of acyl chains to oxidation. As polyunsaturated phospholipids are more susceptible to oxidative damage, a
highly unsaturated phospholipid environment may result in protein dysfunction due to oxidative damage. For example, greater phospholipid polyunsaturation in sarcoplasmic reticulum was associated with increased SERCA thermal inactivation (Fajardo et al., 2017). Thus, protein activity is at least in part influenced by the phospholipid environment specifically with respect to the sensitivity of fatty acyl composition to oxidation.

1.3.3 Implications of Mitochondrial Membrane Composition

The phospholipid composition, specifically CL, is an influential component of mitochondrial function. Healthy composition of mitochondrial CL in highly energetic tissues, such as skeletal muscle, are highly polyunsaturated (Daum, 1985). For example, cardiolipin fatty acyl chain composition from healthy skeletal muscle is predominately tetralinoleoyl cardiolipin (~80%) with remainder composed of a variety of other fatty acyl chains including oleic acid (Stefanyk et al., 2010; Paradies et al., 2014; Zibamanzarmofrad, 2015). However, under pathological conditions such as muscular dystrophy, mitochondrial CL shifts from primarily polyunsaturated to predominantly (~35% total lipids are monounsaturated, ~28% oleic acid) monounsaturated (Touboul et al., 2004; Tahallah et al., 2008; Benbdellah et al., 2009; Zibamanzarmodfrad, 2015). The implications of shifting from a primarily polyunsaturated cardiolipin species to an increasingly monounsaturated composition within the mitochondria are poor mitochondrial morphology (Ikon & Ryan, 2017), reduced oxidative capacity (Paradies et al., 2014; Ikon & Ryan, 2017), and higher production of reactive oxygen species (Rando et al., 1998).

1.4 Reactive Oxygen Species

Reactive oxygen species (ROS) is a classification of oxygen-based radicals and radical initiators, such as H₂O₂, that have been reduced from molecular oxygen through various
processes within cells, including myocytes. The addition of one or two extra electrons to either nitrogen or oxygen results in a reactive species. These reactive species interact with a variety of molecules, like proteins, DNA, or RNA (Dickinson & Chang, 2011; He et al., 2016). Interactions with these reactive species can produce either beneficial or detrimental events that occur quickly, dependent on the relative instability of the oxygen radical such as hydroxide which is estimated to react within $10^9$ s of production (Dickinson & Chang, 2011; He et al., 2016). Within cells, the primary reactive species are superoxide anions, hydroxyl radicals, and peroxyl radicals, specifically lipid peroxide radicals (Mason & Wadley, 2014; He et al., 2016). Hydrogen peroxide ($H_2O_2$) is another important reactive species to consider. Although a non-radical species, it has a longer half-life, is uncharged, and can pass through membranes thereby increasing the probability of interacting with various cellular components. Alternatively, $H_2O_2$ can act as an important signaling molecule within mitochondria. $H_2O_2$ signals for adaptations by increasing the transcription of various proteins, promotes transduction during insulin signaling, and redox signaling through protein thiol oxidation (Forman et al, 2010; Mason & Wadley, 2014). For example, $H_2O_2$ interacts with and activates NFκβ which regulates the transcription of superoxide dismutase 2 (Espinosa-Diez et al., 2015). Therefore, $H_2O_2$ indirectly regulates the transcription of antioxidant enzymes.

An imbalance between the quantity of ROS produced and the availability of antioxidant species to quench the produced ROS is the definition of oxidative stress (Kuksal et al., 2017). In some cases, such as ischemia, reversal of succinate dehydrogenase leads to accumulation of succinate and extensive ROS generation at complex I, with additional damage to the ETC resulting in increased superoxide production upon reperfusion, antioxidants become overwhelmed which leads to the accumulation of superoxide and $H_2O_2$ (Chen et al., 2009;
Chouchani et al., 2014; Kuksal et al., 2017). This occurs in part due to a high proton motive force and a largely reduced CoQ pool, as a result of excessive substrate availability at complexes I and II (Robb et al., 2018). Accumulation of ROS by-products and H₂O₂ can induce excessive protein modification at the amino acid level, disrupting protein conformation and function (Stadtman, 1990; Chen et al., 2017). For example, methionine groups within proteins such as cytochrome c may be converted to methionylsulfoxide, altering the physical structure of the protein (Stadtman, 1990; Birk et al., 2015). The implications of amino acid alterations include the marking of a protein for degradation and reducing or inactivating enzymes within the cell (Stadtman, 1990). Through the oxidation of protein, DNA, and lipids, intracellular redox homeostasis is disrupted (Chen et al., 2017). Should oxidation continue without correction, caspases will cleave proteins and prevent enzyme-catalyzed activity, DNA will become fragmented, and peroxidation of membrane lipids reduce membrane fluidity, ultimately perpetuating ROS production (Chen et al., 2017).

1.4.1 Sources of Reactive Oxygen Species

In skeletal muscle, a major producer of ROS is a family of proteins known as the NADPH oxidase (NOX) family. Three NOX isoforms have been found in skeletal muscle and include NOX 1 on the plasma membrane, NOX2 on the t-tubules, and NOX4 in the mitochondria and the sarcoendoplasmic reticulum (Ferreira & Laitano, 2016). In some cells NOX4 is localized in the nucleus as well (Takac et al., 2012). In skeletal muscle, NADH is the primary substrate, thus the NOX family oxidizes NADH to NAD⁺ in skeletal muscle to produce superoxide (Ferreira & Laitano, 2016). Specifically, NOX4 located on the sarcoendoplasmic reticulum is a constitutively active protein acts as an essential effector ryanodine receptor-calcium release channel (RyR1), a protein dependent on the oxidation of cysteine thiols to function (Sun et al., 2011). RyR1
colocalized with ROS produced by NOX4 on the sarcoendoplasmic reticulum exhibit significantly greater cysteine oxidation than RyR1 colocalized with other cellular fractions, including NADPH stimulated ROS production by the mitochondria in skeletal muscle samples at 20% oxygen (Sun et al., 2011). NADPH stimulated ROS production by NOX4 also induced calcium uptake by RyR1 in skeletal muscle at 20% oxygen (Sun et al., 2011). As ROS produced by NOX enzymes is dependent on the presence of adequate oxygen, ROS production by NOX4 is limited in conditions such as ischemia/reperfusion, regardless of the “ROS burst” associated with reperfusion (Cadenas, 2018). However, cellular functions such as sarcoendoplasmic reticulum calcium uptake are limited under low oxygen conditions (Sun et al, 2011). Therefore, conditions with physiological partial pressures of oxygen, such cellular growth under 20% oxygen, support significant NOX-produced ROS (Sun et al., 2011). These conditions include exercise, where rapid increases in cytosolic calcium induced by muscular contraction is followed by elevated cytosolic NOX-induced ROS, prior to mitochondrial-induced ROS production (Sakellariou et al., 2013; Pearson et al., 2014). Although a prominent producer of ROS under physiological conditions, the NOX family is not the only producer.

Xanthine dehydrogenase is a NAD+-dependent xanthine oxidoreductase that can be reversibly converted to the active xanthine oxidase (XO; Nishino et al., 2005). XO then oxidize xanthine into uric acid as part of purine catabolism within the cytosol. When reduced, XO can produce two hydrogen peroxide molecules every cycle. Although a low producer normally, XO is an important source of ROS and non-radical species during ischemia/reperfusion (IR). Despite the role XO plays during IR, the low prevalence of this enzyme in a healthy organism results in very little ROS production under physiological conditions.
Another producer of ROS are the mitochondria however, literature evidence suggests they are not the major producer in skeletal muscle (Powers et al., 2011). In part, contradictions in findings arise from mitochondrial isolation protocol and means to quantify ROS production (Nohl et al., 2005). Approximately 1-2% of all ROS produced was proposed to be produced by mitochondria, resulting from ~0.15% of electrons leaking from complexes I and III of the ETC (Mason & Wadley, 2014). There are 11 known sites of ROS production within isolated mitochondria (Brand, 2010; Kuksal et al., 2017). Some ROS is produced within the mitochondrial matrix by glycerol-3-phosphate dehydrogenase, electron-transfer flavoprotein, pyruvate dehydrogenase, and 2-α-ketoglutarate (Brand, 2010). Although each of these enzymes produce ROS through electron transfer to molecular oxygen, none produce more reactive species than the electron transport chain. Oxidative phosphorylation is highly efficient during the production of ATP, transferring electrons through the ETC to complex IV where molecular oxygen is reduced to water. However, in some cases electrons prematurely slip from the electron transport chain and reduce molecular oxygen. This inefficiency can be explained through the electron tunneling theory. Tunneling, or movement from a donor molecule such as complex I to an acceptor such as molecular oxygen, is dependent on redox center distance and difference in redox potential between the donor and acceptor (Maillaux, 2015). In the case of the electron transport chain (ETC), movement of an electron between the complexes is dependent on the proximity of the redox centers of the complexes within the chain. For example, the electron carrier cytochrome c utilizes its heme-Fe to oxidize complex III and reduce complex IV (Rovira, 1999). Thus, cytochrome c rotates along the outer leaflet of the IMM, reducing the proximity of its heme group to the redox center of either complex III or IV. If, however, molecular oxygen is in closer proximity to the redox center of complex III than the heme group of cytochrome c,
molecular oxygen will extract the free electron from complex III, producing a free radical, superoxide anion. Complex I can produce superoxide on both the matrix and the IMS side of the IMM if coenzyme Q is in further proximity than molecular oxygen (Brand, 2010). However, it is important to note that superoxide produced at complex I is primarily a result of reverse electron flow from complex II and contributes little to overall ROS production in healthy mitochondria (Brand, 2010). In contrast, St-Pierre suggested mitochondria are unlikely to produce significant amounts of reactive oxygen species under physiological conditions (St-Pierre et al., 2002). In part some mitochondrial isolation methods result in a high membrane potential, which results in falsely elevated ROS measurements (Nohl et al., 2005). Alternatively, mitochondrial isolation may damage and fragment the mitochondria, also resulting in falsely elevated ROS measures (Nohl et al., 2005). For these reasons, extrapolating ROS production from isolated mitochondria to in vivo mitochondria is incredibly difficult, if not impossible. Despite mitochondrial ROS being predominantly produced by the ETC of the IMM, mitochondrial ROS production appears to be in myocytes under resting physiological conditions (Figure 1.2).

ROS production from mitochondria is dependent on the mitochondrial membrane potential across the inner mitochondrial membrane. Under exercise conditions when ADP and mitochondrial substrates such as succinate increase, the proton motive force decreases, respiration resulting in ATP production increases, and mitochondrial rates of ROS production decrease (Goncalves et al., 2015; Wong et al., 2017). Based on ex vivo experimentation, mitochondrial rates of ROS production negatively correlate with exercise intensity due to the progressively diminishing proton motive force and progressively increasing oxidation of the ubiquinone pool (Wong et al., 2017). Upon the cessation of exercise, where ATP production is no longer necessary, and the proton motive force builds resulting in increased electron leak from
the ETC and theoretically, a greater rate of ROS production. To combat this, ROS produced because of a high membrane potential stimulates mitochondrial uncoupling which reduces the membrane potential and subsequent reduction of ROS (Brookes et al., 2004). Mitochondrial uncoupling acts as a regulator of ROS production specifically in the mitochondria, producing heat rather than ROS. In addition to uncoupling, an intricate system of antioxidants is synthesized endogenously to maintain the homeostatic ROS production.

1.4.2 Removal of ROS from the cell

The cell’s innate protective system against oxidative stress involves antioxidants in the cell to neutralize and offset the production of ROS and the consequences of excessive ROS, creating homeostasis. Antioxidants are defined as “any substance that, when present in low concentration compared to those of an oxidizable substrate, significantly delay or inhibit oxidation of that substrate” (Halliwell & Gutteridge, 1990). Non-enzymatic antioxidant species provide single electrons to oxidant species, neutralizing free radicals from within or around the periphery of the cell (Birben et al., 2012). The oxidized antioxidant species can then oxidize a reactive species, neutralizing the radical and regenerating the reducing antioxidant species (Birben et al., 2012). Alternatively, enzymatic antioxidant species catalyze oxido-reduction reactions, utilizing non-enzymatic antioxidants in the removal of free radicals (Birben et al., 2012). Ideally, a combination of antioxidants and antioxidant enzymes are in balance with ROS production that allows for the production of H$_2$O$_2$ in quantities that allow for signal transduction but prevent oxidative stress. These non-enzymatic antioxidants and enzymatic antioxidants include but are not limited to superoxide dismutase, catalase, glutathione peroxidase, and tocopherols.

Superoxide dismutase (SOD) is present as three major isoforms. SOD1, SOD2, and SOD3, found in the IMS, mitochondrial matrix, and extracellular compartment respectively. SOD
performs a dismutation or disproportionation reaction, reducing superoxide to H$_2$O$_2$ and oxidizing an additional superoxide molecule to molecular oxygen (Powers et al., 2014). From this point, catalase, a heme-based antioxidant, reduces H$_2$O$_2$ to water (Alfonso-Prieto et al., 2009). The most abundant antioxidant systems contained within the cell is the glutathione peroxidase system, creating redundancy and protection against oxidative stress (Brigelius-Flohe & Maiorino, 2012). Glutathione peroxidase catalyzes the oxidation of monomeric glutathione by reducing H$_2$O$_2$ or lipid hydroperoxides that occur in the membrane to produce water and glutathione (Brigelius-Flohe & Maiorino, 2012; Kuksal et al., 2014). Glutathione reductase then utilizes NADPH to reduce glutathione disulphide (GSSG) to two molecules of glutathione (GSH)(Kuksal et al., 2014). Therefore, the glutathione system relies on NADPH as a reducer to reactivate their antioxidant abilities located within both the cytosol as well as in the mitochondria. Regulation of this antioxidant system is based on the glutathione to glutathione disulfide ratio- as more ROS is produced, more glutathione is oxidized by hydrogen peroxide to glutathione disulfide (Steinbacher & Eckl, 2015). Glutathione peroxidase is ROS-dependent, therefore as amounts of ROS produced increase, there is a compensative increase in the action of the glutathione system. This antioxidant system works in concert with the $\alpha$-tocopherol/ ascorbic acid system whereby the $\alpha$-tocopheryloxy radical is reduced by ascorbate, which is in turn reduced by glutathione (Espinosa-Diez et al., 2015). As a versatile antioxidant system, the glutathione system is effective in maintaining redox homeostasis within the cell.

In a lipophilic environment, such as within the mitochondrial membranes, tocopherols and carotenoids are the primary antioxidants. With respect to tocopherols, D-$\alpha$-tocopherol (vitamin E) is the major antioxidant species contained in phospholipids of most membranes (Pamplona, 2008). This antioxidant works cyclically with ascorbate or ubiquinone where vitamin E reduces
the oxidized species, chiefly phospholipid peroxyl radicals within the lipophilic environment and ascorbate or ubiquinone regenerates vitamin E from the tocopheroxyl radical using an ascorbate radical or hydroquinone (Pamplona, 2008). α-Tocopherol radical interacts with acyl chains of the lipid species contain within a membrane, scavenging the terminal lipid peroxyl radical and preventing further lipid radical formation (Espinoasa-Diez et al., 2015). Water-soluble ascorbic acid reduces the tocopheroxyl radical to restore α-tocopherol (Buettner, 1993). A such, α-tocopherol is present in the membranes to reduce peroxyl radicals and reduce membrane damage where ascorbic acid is used to maintain functional α-tocopherol.

The production and activity of antioxidant species within a cell is mediated, in part, by ROS production to support homeostatic balance between ROS signaling and ROS damage (Figure 1.2). Without this balance, ROS production may quickly become oxidative stress. If this oxidative stress event is uncorrected, apoptosis may occur.
Xanthine Oxidase

NOX

Extracellular Space

Catalase

Plasma membrane

Cytosol

H₂O + O₂ → H₂O₂

NADPH

NADP⁺

Fe²⁺

GPx

GSSG

2GSH

·OH

SOD1

SOD2

SOD3

O₂⁻ → H₂O₂

SOD

H₂O₂ → H₂O + O₂

ATP

ADP + Pᵢ

NAD⁺ + H⁺

NADH

FADH₂

FAD

H₂O₂

H₂O

2H⁺ + 1/2 O₂ → 2e⁻

2H₂O + O₂ → 2H₂O₂

H₂O₂ → H₂O + O₂

SOD

GPx

GSSG

2GSH

IMS

OMM

IMM

Matrix

ER

PLA-dependent processes

NOX

NOX

17

Page

Extracellular Space
Figure 1.2. Cellular map of ROS production and ROS removal. ADP, adenosine diphosphate; ATP, adenosine triphosphate; ER, endoplasmic reticulum; FADH, flavin adenine nucleotide (reduced); FAD+, Flavin adenine nucleotide (oxidized); Fe, iron; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulfide; H$_2$O$_2$, hydrogen peroxide; IMM, inner mitochondrial membrane; IMS, intermembrane space; NADPH, nicotinamide adenine dinucleotide (reduced), NADP$^+$, nicotinamide adenine dinucleotide (oxidized); NOX, NADPH Oxidase; OH$, hydroxide; OMM, outer mitochondrial membrane; PLA, phospholipase A; SOD, superoxide dismutase. Adapted from Espinoasa-Diez et al. 2015 and Kuksal et al, 2017.
1.5 Apoptosis

Apoptosis is a form of programmed cell death that is implicated in the maintenance of cellular health during such events as embryogenesis, aging, and the pathology of disease progression unique to metazoans (Green & Fitzgerald, 2016). This form of cell death can be identified by cell shrinkage, membrane blebbing into apoptotic bodies, and ultimately phagocytosis of these bodies without swelling or inflammation (Kerr et al., 1972; Elmore, 2007; Green & Fitzgerald, 2016). Progression through apoptosis is tightly controlled to develop the typical shapes associated with organs and extremities during fetal development and to act as a balance to the development of new cells through the aging process (Renehan et al., 2001). In addition to acting as a homeostatic mechanism, apoptosis can be initiated to remove cells that engage in tumor suppressor mechanisms, senescent, stressed, and dysfunctional cells such as those exerting consistently excessive quantities of ROS (Reed, 2000; Tower, 2015; Tummers & Green, 2017). However, cell death can result from uncontrolled apoptosis as seen in conditions such as muscular dystrophy or ischemia/reperfusion. Therefore, apoptosis is an essential process for the cell that requires tight regulation to promote growth and development of embryos, and the maintenance of cellular health in the aging individual.

Apoptosis can proceed through extrinsic or intrinsic pathways, depending on the initial source of the apoptotic signal. Extrinsic apoptosis is a result of an external stimuli, such as trauma, leading to a cascade of chemical signaling events ending in programed cell death. Extrinsic apoptosis is also known as the tumor necrosis factor (TNF) family receptor stimulated pathway (Reed, 2000; Elmore, 2007). This pathway is initiated through a complex network of proteins linking the outside of the cell to the mitochondria leading to cell death through the stimulation of proteolytic enzymes known as caspases (Reed, 2000; Elmore, 2007). This
pathway is primarily used by the cell as a defense mechanism for the protection of the cell against invading pathogens.

Intrinsic apoptosis is mitochondrially mediated, stimulated by intracellular stressors such as oxidative stress or excessive calcium uptake by the mitochondria (Campo, 2009). Intrinsic apoptosis proceeds through the activation of BCL-2 family proteins, OMM permeabilization, dissipation of the inner mitochondrial potential, and release of pro-apoptotic proteins sequestered in the intermembrane space, such as Endo-G, Smac/Diablo, and cytochrome c (Petrosillo et al., 2003; Green & Kroemer, 2004; Green & Fitzgerald, 2015; Figure 1.3). The released cytochrome c interacts with apoptotic protease-activating factor-1 (APAF-1) and dATP to activate caspases associated with the end phase of apoptosis (Reed, 2000; Zhou et al., 2015; Figure 1.3). As intrinsic apoptosis is the most commonly activated pathway in vertebrates, it is imperative to understand the mechanisms leading to this form of apoptosis.
Figure 1.3. Mitochondrially mediated apoptotic cascade. BID, BH interacting-domain death antagonist; SMAC/DIABLO, second mitochondrial derived activator of capases/direct IAP-binding protein with low PI; tBID, truncated BID. Adapted from Reed et al., 2000.
1.5.1 BCL-2 proteins in apoptosis

Healthy mitochondria are dynamic organelles, constantly undergoing fission and fusion of the membranes to determine the shape and function of mitochondria, dependent on the energy requirements of the cell at any given moment (Westermann, 2012). A delicate coordination of fusion events during periods of high aerobic ATP production or mitochondrial repair, and fission of mitochondrial sections with low membrane potential and poor ATP generating capacity is required. Dysregulation of fusion and fission events may lead to poorly functioning, extended mitochondrial tubules or fragmented, dysfunctional, and permeable mitochondria respectively, ultimately leading to apoptosis.

The balance of mitochondrial fusion and fission is regulated by the β-cell lymphoma-2 (BCL-2) protein family. This protein family can be divided into three different components; anti-apoptotic proteins (BCL-2, BCL-xL), pro-apoptotic proteins (Bak, Bax), and BH3-only proteins (Bid, Bim). The anti-apoptotic BCL-2 proteins serve to inhibit the activation of anti-apoptotic BCL-2 proteins (Cosentino & Garcia-Saez, 2014). In contrast, the pro-apoptotic proteins which form a pore to permeabilize the OMM upon activation by the BH3-only “activators”, dimerize and insert into the OMM to induce this permeabilization (Cosentino & Garcia-Saez, 2014). In healthy cells, the pro-apoptotic protein Bak is maintained in an inactive state on the OMM, sequestered away from its pore-forming counterpart, Bax (Breckenridge & Xue, 2004; Edlich et al., 2011; Todt et al., 2013; Cosentino & Garcia-Saez, 2014). Bax is also inactive and remains free in the cytosol until activation by BH3-only proteins (Breckenridge & Xue, 2004). Once activated, Bax inserts into the OMM, dimerizing with Bak, leading to the permeabilization of the OMM and fragmentation of the mitochondria, creating a gateway for IMS protein release and ultimately apoptosis (Frank et al., 2001; Breckenridge & Xue, 2004; Green & Fitzgerald, 2016).
One such Bax and Bak activator protein, Bid, is a BH3-only protein located in the cytosol. Caspase-8 activated Bid, also known as tBid, induces conformational changes within the Bax and Bak proteins under enhanced cellular stress, such as excessive reactive oxygen species production (Li et al., 1998; Wei et al., 2000; Raemy & Martinou, 2014). These induced structural modifications allow Bak and Bax to insert into and oligomerize within the OMM, permeabilizing the membrane and releasing cytochrome c from the IMS. Importantly, the activation of Bak and Bax by tBid is dependent on the presence of cardiolipin in the OMM.

1.5.2 Cardiolipin in OMM permeabilization

Cardiolipin is a critical component of healthy mitochondrial membranes with a role in both the structural integrity of the mitochondrial cristae as well as the function of the mitochondrial ETC. This unique phospholipid, synthesized on the inner leaflet of the inner mitochondrial membrane, contains two glycerol groups and four predominantly polyunsaturated fatty acyl chains (Macfarlane, 1958; Paradies et al., 2014; Petrosillo et al., 2016). Specifically, these acyl groups contain up to 73% linoleic acid (18:2n6) in healthy membranes (Pangborn, 1947). With predominantly unsaturated acyl chains, cardiolipin forms a non-bilayer hexagonal shape, creating the iconic rod-like tubules identified as the mitochondrial cristae (Wallis et al., 2002; Claypool, 2009; Horvath & Daum, 2013; Basu Ball et al., 2017; Ikon & Ryan, 2017). As a major site for ATP production through oxidative phosphorylation, the tight hair-pin like bends supported by cardiolipin increase the surface area of the IMM and decrease the distance between ETC proteins, improving ETC efficiency and potentially reduce ROS production (Acehan et al., 2011; Vartak et al., 2013; Basu Ball et al., 2017). Decreases in total cardiolipin content are associated with decreases in ATP production efficiency. To some extent, lower ATP production is due to poor ATP synthase activity which occurs as a result of decreased total cardiolipin content or less
unsaturation of the acyl chains (Acehan et al., 2011; Vartak et al., 2013). Similarly, diminishing cardiolipin content or reducing acyl chain unsaturation decreases ETC function as a result of cristae tubule broadening (Acehan et al., 2011). Because of cristae broadening, the distance between ETC proteins increase, contributing to poor ATP production, electron leakage, and enhanced ROS production.

1.5.3 Cardiolipin Peroxidation During Apoptosis

The exact sequence of events regarding changes in the lipid membrane during apoptosis is unclear, but it has been demonstrated that peroxidation and subsequent translocation of cardiolipin to the OMM is a critical initial signal. Evidence supports that peroxidation of cardiolipin occurs prior to PS externalization, cytochrome c release, and outer membrane permeabilization under staurosporine-induced apoptosis (Kagan et al., 2005). Once peroxidized, cardiolipin is transferred by NDPK-D to the OMM where it acts as a docking site for tBid, Bax, and Bak (Lutter et al., 2000; Gonzalvez & Gottlieb, 2007; Maguire et al., 2016). After docking, tBid activates Bax and Bak, inducing oligomerization, and membrane permeability. Kagan et al. used cytochrome c deficient mouse embryonic cells to confirm the positive correlation between cytochrome c and Smac/Diablo release with cardiolipin peroxidation (2005). Prevention of cardiolipin peroxidation through the use of antioxidants or attrition of lipid peroxidases reduces both peroxidation and translocation of cardiolipin (Fernandez et al., 2002; Kagan et al., 2005). Under both circumstances, minimizing cardiolipin peroxidation resulted in a reflective decrease in apoptotic markers (Fernandez et al., 2002; Kagan et al., 2005). Therefore, cardiolipin peroxidation occurs prior to pro-apoptotic protein release, and is an important component of OMM permeabilization.
Cardiolipin peroxidation has a role both in increasing the permeability of the OMM and cytochrome c release. As peroxidized cardiolipin has a lower affinity for cytochrome c than unoxidized cardiolipin, peroxidation contributes to cytochrome c dissociation from the IMM, resulting in cytochrome c release (Ott et al., 2001; Iverson & Orrenius, 2004; Kagan et al., 2005; Yin & Zhu, 2012). Adding exogenous antioxidant enzymes, superoxide dismutase and catalase specifically, to antimycin a-incubated beef heart submitochondrial particles prevented cardiolipin peroxidation and cytochrome c release altogether (Petrosillo et al., 2001). Taken together, this data suggests that cardiolipin peroxidation is a critical event occurring prior to cytochrome c detachment, OMM permeabilization, and cytochrome c release.

There are two known means through which cardiolipin can become peroxidized: i) ROS-induced lipid peroxidation, and ii) complexation of cardiolipin and cardiolipin-specific cytochrome c peroxidase (Ow et al., 2008). As cardiolipin contains predominantly polyunsaturated fatty chains (18:2n6) and is in close proximity to the ETC, cardiolipin is a target for peroxidation. Polyunsaturated acyl chains are more likely to become peroxidized by superoxide due to its readily reactive nature and the increased number of double bonds within polyunsaturated acyl chains in comparison to monounsaturated species (Holman et al., 1954; Bielski et al., 1983; Pamplona, 2008; Kim et al., 2011). However, due to its fleeting nature and short half-life, the concentration of singlet oxygen is relatively low in comparison to H2O2. Despite composition and location, cardiolipin is relatively resistant to ROS-induced damage and undergoes rapid deacylation-reacylation cycles to remove any peroxidized fatty acyl chains (Ma et al., 1999; Wiswedel et al., 2010). Once peroxidized, calcium-independent mitochondrial calcium-independent phospholipase A2-γ hydrolyzes the acyl chain (Buland et al., 2016), and a
new acyl chain is added predominantly by taffazin (Schlame, 2013). More effectively, cardioli- 
lin can be peroxidized through complexation of a cardioli- lin-specific peroxidase.

1.6 The Cardiolipin/ Cytochrome C Complex

1.6.1 Cytochrome c peroxidase transition

Cytochrome c is an important 12 kDa globular hemoprotein as a component of the ETC
(Kagan et al., 2009a; Jiang et al., 2013). Conformational and functional stability of this protein is
controlled by many factors including hydrogen bonding, electrostatic interactions, hydrophobic
interactions, van der Waals interactions, and the presence of disulfide bonds (Zaidi et al., 2014).
The functional redox capabilities of this protein are influenced by axial ligation, heme solvent
accessibility, and electrostatic interactions, similar to the factors regulating protein confor-
modation (Zaidi et al., 2014). To date, the most influential factors are associated with the heme group
located within the center of cytochrome c.

Cytochrome c has a tightly controlled hexa-coordinated heme-iron (Fe) and a large surface
area available for binding to anionic lipids (Aluri et al., 2014). Both cysteine 14 and 17
covalently attach the heme, contributing to the electrostatic environment of the heme-Fe crevice
(Zaidi et al., 2014). These bonds assist in maintaining redox potential of the protein and
ultimately contribute to increased strength of axial ligand binding (Zaidi et al., 2014). The most
conserved and functional axial ligands include histidine 18 (His18) and methionine 80 (Met80).
His18 is the fifth ligand of heme-Fe and is used to stabilize cytochrome c protein conformation
and promote electron transfer and ascorbate oxidation (Ziadi et al., 2014). Met80, a highly labile
and weak axial ligand, is a critical component in maintaining protein redox potential, structure,
stability, and electron transport (Ziadi et al., 2014). This coordination, specifically the Met80-Fe
axial bond present in cytochrome c’s native form is an important blockade, preventing Fe
interactions with ROS such as singlet oxygen or reactive oxygen containing species, specifically hydrogen peroxide (Abe et al., 2011; Aluri et al., 2014).

In healthy mitochondria, natively folded cytochrome c functions as an electron transfer protein. However, under circumstances that disrupt the covalent bonds maintaining the heme group structure, cytochrome c will partially unfold to expose its Fe group to the IMS milieu. Unfolding negatively shifts cytochrome c’s redox potential by 400mV which prevents it from oxidizing ascorbate, interrupting the ETC, and disturbing oxidative phosphorylation and ATP production (Basova et al., 2007). Cytochrome c unfolding promotes free radical production from the ETC (Iverson & Orrenius, 2004; Kagan et al., 2009a) and a new peroxidase-like function emerges from unfolded cytochrome c.

Cytochrome c can unfold in multiple ways culminating in a variety of different tertiary structures, only some of which exhibit peroxidase activity (Pandiscia & Schweitzer-Stenner, 2015). It is important to note that conformational change resulting in the removal of the Met80 ligand through oxidation shifts the heme-Fe group from a hexa-coordinated to a high-spin pentacoordinated state; one that is conducive to peroxidase activity (Aluri et al., 2014; Pandiscia & Schweitzer-Stenner, 2015). This specific shift is indicative of an opening known as the ‘heme crevice’ which further exposes the active heme-Fe to ROS and non-radical species (Kagan et al., 2006; Abe et al., 2011; Maguire et al., 2016). ROS impacts cytochrome c structure, however from these studies it is unclear whether ROS is the inducing factor for cytochrome c transition.

Dissociation of the Met80-Fe bond within cytochrome c was previously associated with oxidative stress, and access of the exposed heme-Fe group with substrates used in peroxidation reactions. It has been shown that Met80-Fe dissociation can occur without the presence of ROS, suggesting that excess ROS is not the only trigger for cytochrome c unfolding (Birk et al., 2015).
It has also been proposed that Met80-Fe dissociation leading to cytochrome c unfolding and peroxidase activity precedes and can induce oxidative stress through the disruption of the ETC resulting in increased ROS production from complex III (Birk et al., 2015). As an example, the ETC is damaged during ischemia which results in decreased mitochondrial ATP production, substrate availability, and access to oxygen (Chen et al., 2008; Aluri et al., 2014; Birk et al., 2015). Mitochondria become hypoxic and accumulate reducing equivalents, subsequently followed by interruption of electron flow through the ETC (Chen et al., 2008). ROS production rates are lower during ischemia and remains lower until oxygen is reintroduced to the mitochondria during reperfusion (Chen et al., 2008). During reperfusion, ROS production from complexes I and III increases, suggesting electron leakage from these complexes and additional damage of an upstream component of the ETC, ultimately reducing ATP production (Chen et al., 2008; Birk et al., 2015). Therefore, although ROS has a role in the transformation of cytochrome c by oxidizing the Met80 residue, it is not required to induce the structural transition from cytochrome c to cytochrome c peroxidase. As an alternative to ROS-induced changes in cytochrome c, cytochrome c interactions with cardiolipin have also been shown to disrupt this Met80-Fe bond in mitochondria.

### 1.6.2 Cardiolipin-induced cytochrome c structural transition

There are two sites within cytochrome c, termed A-site and L-site, which are responsible for weak electrostatic interactions binding cytochrome c to the anionic region of the membrane (Rytomaa & Kinnunen, 1994; Rytomma & Kinnunen, 1995; Kagan et al., 2009). Interactions at these sites are influenced by the cellular environment and serve different functions in relation to cytochrome c protein conformation (Pletneva et al., 2004; Hanske et al., 2011; Figure 1.4). A transient electrostatic interaction between cardiolipin and cytochrome c at the A-site facilitates
movement of cytochrome c from complexes III to IV within the ETC, where L-site interaction only occurs in acidic conditions. The A-site is a high affinity site on cytochrome c that readily binds cardiolipin without apparent alterations to the Met80-Fe bond (Mandal et al., 2015; Pandiscia & Schweitzer-Stenner, 2015). Due to the low molar concentration of cardiolipin in the outer leaflet of the IMM, cardiolipin binds to 90% of cytochrome c through electrostatic interactions at the A-site (Kalanxhi & Wallace, 2007; Paradies et al., 2014). This specific site occurs at low cardiolipin concentrations and bonds formed in this location can be reversed with increasing ionic concentration (Nicholls, 1974; Paradies et al., 2014; Pandiscia & Schweitzer-Stenner, 2015). As the milieu of the IMS in healthy mitochondria has a high ionic strength and the outer leaflet of the IMM contains a relatively small quantity of cardiolipin, it is likely electrostatic interactions are brief to support oxidative phosphorylation.

Although most cytochrome c is electrostatically bound, a second pool consisting of about 10% of available cytochrome c is strongly bound to the mitochondrial membrane (Kalanxhi & Wallace, 2007). It has been proposed that one acyl chain of cardiolipin extends outwards from the bilayer and inserts it into the hydrophobic core, known as the heme crevice, of cytochrome c (Tuominen et al., 2002; Kalanxhi & Wallace, 2007; Ziadi et al., 2014). Insertion of cardiolipin creates an opening for oxidative substrates and increases the proximity of the acyl chain to the heme-Fe. Under physiological conditions, the 10% of cytochrome c which is strongly bound to the membrane uses the inserted unsaturation of the acyl chain as a means to remove \( \text{H}_2\text{O}_2 \), creating a lipid peroxyl radical (Kagan et al., 2005; Huttermann et al., 2011; Figure 1.5). The peroxyl radical is terminated by \( \alpha \)-tocopherol and reduced by glutathione peroxidase (Kagan et al., 2005; Brigelius-Flohe & Maiorino, 2012). Removal of \( \text{H}_2\text{O}_2 \) in the membrane results in the prevention of acyl chain peroxide formation which prevents translocation and accumulation of...
lipid peroxides on the OMM, thereby impeding BCL-2 pro-apoptotic protein activation, OMM permeabilization, and apoptosis.

Upon failure of the mitochondrially located reductant species, or increased cardiolipin presence in the outer leaflet of the IMM, accumulation of cardiolipin peroxides on the OMM may result. Although it is theoretically cardiolipin movement from the outer leaflet of the IMM to the OMM is a result of NDPK-D assisted translocation, the mechanism for cardiolipin translocation from the inner leaflet to the outer leaflet of the IMM is currently unknown, however it has been suggested to be dependent on the transmembrane redox potential associated with the IMM (Gallet et al., 1997; Claypool, 2009; Maguire et al., 2016). Once located in the outer leaflet, cytochrome c interaction with the outer leaflet induces cardiolipin clustering which increases the number of possible cardiolipin binding partners (Thong & Touskanova, 2018). Theoretically, by increasing the number of cardiolipin binding partners, the second pool of strongly membrane bound cytochrome c also increases, resulting in greater cardiolipin peroxidation. It has been speculated that cytochrome c interaction and mutation of the membrane, leading to cardiolipin peroxidation, facilitates cardiolipin removal from the model IMM (Thong & Touskanova, 2018). Therefore, it is apparent that cytochrome c interaction with cardiolipin induces a conformational change to cytochrome c, inducing cardiolipin peroxidation, cytochrome c membrane detachment, and cardiolipin translocation to the OMM (Figure 1.4).
a) Complex III

Complex IV

H+ + H+ → IMS

Matrix

b) Complex III

Complex IV

H+ + H+ → IMS

Matrix
Figure 1.4. Cytochrome c shape and function as influenced by electrostatic and hydrophobic bonding. a) When bound to cardiolipin electrostatically, cytochrome c rolls along the membrane to transfer an electron from complex III to complex IV. b) When bound to cardiolipin hydrophobically, cytochrome c unfolds, penetrating the membrane, resulting in cytochrome c release from the membrane. IMS, intermembrane space. Adapted from Hanske et al., 2012.
Figure 1.5. Peroxidation of cardiolipin acyl chains. a) Cytochrome c binds to cardiolipin. b) Cytochrome c then unfolds, allowing H$_2$O$_2$ access to the heme-Fe. c) Cytochrome c uses this H$_2$O$_2$ to peroxidize cardiolipin acyl chains. Adapted from Lokhmatikov et al., 2014.
1.6.3 Effect of Cardiolipin Side Chain on the Cardiolipin/ Cytochrome C Complex

In healthy membranes, cardiolipin is primarily unsaturated (tetralinoleoyl CL; TLCL). A significant proportion of current research examining the cardiolipin/cytochrome c complex in liposomes use cardiolipin that has been isolated from mitochondria and contains primarily 18:2n6 chains. However, in some pathological states such as muscular dystrophy, the cardiolipin composition in mitochondrial membranes differ compared to healthy controls. Specifically, cardiolipin composition of mitochondria isolated from patients with muscular dystrophy contained more monounsaturated cardiolipin species and less polyunsaturated cardiolipin species than mitochondria isolated from healthy individuals (tetraoleic CL; TOCL; Kunze et al., 1975; Onopiuk et al., 2009; Srivastava et al., 2017). Should cardiolipin side chains become less polyunsaturated, as in muscular dystrophy, the mitochondria experience structural changes, which may include alterations to the cardiolipin/ cytochrome c relationship.

Based on the relevance of cardiolipin fatty acyl chain on hydrophobic interactions with cytochrome c, and peroxidation of cardiolipin leading to cytochrome c release, it is imperative to understand the contribution of acyl chain composition to cardiolipin-cytochrome c complex formation (Tuominen et al., 2002; Kalanxhi & Wallace, 2007; Ziadi et al., 2014). Using single layer liposomes containing 50% phosphatidylcholine and 50% cardiolipin species, Belikova et al. determined that concentrations of cardiolipin fatty acyl chains containing at least one double bond positively correlated with cytochrome c affinity (2006). Cytochrome c had the greatest affinity for cardiolipin isolated from bovine heart (TLCL), whereas synthesized polyunsaturated, tetralinoleoyl cardiolipin (TLCL) and monounsaturated, tetraoleoyl cardiolipin (TOCL) exhibited equal affinity for cytochrome c (Belikova et al, 2006). In addition, TOCL-induced cytochrome c peroxidase activity, measured through etoposide production, positively correlated...
with cardiolipin concentration similar to the pattern shown in cytochrome c binding affinity (Belikova et al., 2006). As confirmation, TMCL-induced cytochrome c peroxidase activity did not correlate with cardiolipin concentration, nor did TMCL concentration correlate with binding affinity (Belikova et al., 2006). Therefore, it is apparent from this study that cardiolipin acyl chain having at least one double bond, influences the characteristics of membrane-bound cytochrome c.

As mentioned above, characteristics exhibited by membrane-bound cytochrome c depend on more than cardiolipin side chain composition. Research using single layer liposomes that were 10% cardiolipin species and 90% phosphatidylcholine, determined that the rate at which cytochrome c peroxidizes cardiolipin is unaffected by cardiolipin side chain composition in this model (Abe et al., 2011). TLCL-based liposomes had greater affinity for cytochrome c TOCL (Abe et al., 2011). In contrast to Belikova et al., there is no difference in binding affinity between TOCL and TMCL species (Belikova et al., 2006; Abe et al., 2011). Taken with the previous group’s results, this suggests cardiolipin side chain composition and liposome composition can influence the characteristics of membrane-bound cytochrome c. Despite these differences, both groups confirm an apparent positive relationship between cytochrome c membrane affinity and peroxidase activity. This suggests that cardiolipin acyl chain composition may influence cytochrome c’s conformation and function.

In conclusion, while previous studies have determined the importance of cardiolipin in altering membrane-bound cytochrome c to display characteristics conducive to apoptosis, it is unclear whether these effects are due to the composition of the cardiolipin acyl chain or a result of the high cardiolipin concentration within the liposome, regardless of acyl chain composition,
Therefore, increasing membrane saturation of cardiolipin in the mitochondrial membrane may have a role in regulating mitochondrial-mediated apoptosis.

1.7 Statement of the Problem

It is currently understood that changes in the phospholipid environment of mitochondrial membranes alter membrane-associated protein function. These changes to mitochondrial associated protein function can be highly detrimental to mitochondrial function, leading to poor mitochondrial respiration and increased rates of apoptosis. Variation in fatty acid composition of mitochondrial specific phospholipid, cardiolipin, away from primarily polyunsaturated chains has many implications including decreased cristae density, poor organization and destabilization of ETC supercomplexes, and increased permeability of the outer mitochondrial membrane. The effect of fatty acyl chain composition on the apoptotic complexation between cardiolipin and cytochrome c has recently been under scrutiny. Data collected using synthetic membranes, specifically single layer liposomes, have been inconsistent. This inconsistency may be due to a lower cardiolipin to phosphatidylcholine ratio in the liposomes (Birk et al., 2006; Abe et al., 2011). Previous scientists have constructed liposomes with a mixture of phosphatidylcholine and a single cardiolipin species. However, due to the variety of cardiolipin species within mitochondrial membranes, and a cardiolipin clustering effect induced by cytochrome c binding, cytochrome c may interact with several cardiolipin species at one time. Thus, it is currently unclear how membrane composition effects cytochrome c, or the cardiolipin/cytochrome c complex.
1.8 Project Objectives

The following are the objectives used to guide the experimental design:

1) Cytochrome c conformation
   a. To confirm the effect of cardiolipin fatty acyl chain saturation on the ligand occupancy at the porphyrin of cytochrome c as a measure of the cytochrome c to cytochrome c peroxidase transition.
   b. To determine the effect of liposomes that broadly reflect the membrane composition of healthy and dystrophic mitochondria on the ligand occupancy at the porphyrin of cytochrome c as a measure of the cytochrome c to cytochrome c peroxidase transition.

2) Cytochrome c peroxidase activity
   a. To confirm the effect of cardiolipin fatty acyl chain saturation on cytochrome c’s peroxidase activity in comparison to previous literature using similar liposome compositions.
   b. To determine the effect of liposomes that broadly reflect the membrane composition of healthy and dystrophic mitochondria on cytochrome c’s peroxidase activity.
   c. To determine the effect of cardiolipin peroxidation prior to cardiolipin/cytochrome c complex formation on cytochrome c’s peroxidase activity.

3) Cytochrome c’s membrane affinity
   a. To confirm the effect of cardiolipin fatty acyl chain composition on cytochrome c affinity for the liposome.
b. To determine the effect of liposomes that broadly reflect the membrane composition of healthy and dystrophic mitochondria on cytochrome c binding affinity for the liposome.

1.9 Project Hypotheses

Ultimately, we wish to determine the role of membrane composition and contribution of acyl chain peroxidation on the structural and functional changes in cytochrome c using membrane mimetic liposomes. It is hypothesized that:

1) In relation to cytochrome c conformation:
   a. All cardiolipin acyl chain compositions will induce critical conformational changes within cytochrome c equally.
   b. There will be no differences between the liposomes that broadly reflect the membrane compositions of healthy and dystrophic mitochondria in inducing changes in cytochrome c conformation.

2) In relation to cytochrome c peroxidase activity:
   a. Cytochrome c peroxidase activity will positively correlate with the number of double bonds on the cardiolipin side chains in liposomes containing one cardiolipin composition.
   b. Cytochrome c peroxidase activity will be higher in the membrane mimetic liposome with greater polyunsaturated lipid.
   c. Cytochrome c peroxidase activity will be lower when incubated with cardiolipin containing peroxidized side chains.

3) In relation to cytochrome c binding affinity:
a. Cytochrome c affinity will positively correlate with the number of double bonds in the cardiolipin side chains of liposomes containing one cardiolipin species.

b. Cytochrome c affinity will positively correlate with increasing polyunsaturated species within the membrane mimetic liposomes.

Chapter 2: The effect of cardiolipin side chain composition on cytochrome c protein conformation and resulting peroxidase activity: Exploring the potential mechanisms that contribute to cellular apoptosis

2.1 Introduction

Apoptosis, or programmed cell death, is a highly regulated process used to maintain tissue homeostasis through the removal of senescent, dysfunctional, and infected cells. Apoptosis can be triggered through an internal or external pathway, both of which end in the same final phase of apoptosis whereby executioner caspases are activated resulting in cytoplasmic endonuclease activation, externalization of phosphatidylserine (PS), and engulfment by phagocytes (Elmore, 2007). Cell death primarily proceeds through the internal pathway, known as the mitochondrially mediated pathway, identified by outer mitochondrial membrane (OMM) permeabilization, dissipation of the inner mitochondrial membrane potential, and release of pro-apoptotic proteins such as Endo G, SMAC/DIABLO, and cytochrome c (Green & Kroemer, 2004). The cascade of events following OMM permeabilization is dependent on the release of the pro-apoptotic protein, cytochrome c.

Cytochrome c is a small electron transferring heme-protein located within the intermembrane space (IMS) which is positively charged around pH 7.0, and loosely associated with the outer leaflet of the inner mitochondrial membrane (IMM; Kawai et al., 2009). As a
heme-protein, cytochrome c contains a tightly regulated hexa-coordinated heme-iron (Fe) primarily used to transport electrons between respiratory complexes during oxidative phosphorylation. Under these conditions, cytochrome c associates with anionic phospholipids, namely cardiolipin, within the IMM through electrostatic bonds conducive to electron transfer (Huttermann et al., 2011). However, by disrupting this interaction with the IMM, cytochrome is mobilized which makes it susceptible to release into the cytosol (Gonzalvez & Gottlieb, 2007; Mohammadyani et al., 2018). In addition to release, conformational and functional changes to cytochrome c resulting from the disruption of its interaction with the IMM promote cytochrome c-dependent activation of the apoptotic cascade, thus irreversibly committing the cell to apoptosis (Gonzalvez & Gottlieb, 2007; Mohammadyani et al., 2018). Understanding the molecular events leading to the dissociation of cytochrome c from cardiolipin is critical to understanding the initial phases of apoptosis.

Cardiolipin is an anionic phospholipid predominantly located on the inner leaflet of the IMM with a minor presence on the outer leaflet, representing ~20% of the total IMM phospholipids (Daum, 1985; Gonzalvez & Gottlieb, 2007). This phospholipid contains four primarily polyunsaturated acyl chains causing an inverted cone-like shape, important in the negative curvature, specifically used to form the hairpin tight bends of the IMM cristae. Within the cristae, cardiolipin interacts with the electron transport chain (ETC) to promote energy production through oxidative phosphorylation. Cardiolipin also docks up to 85% of cytochrome c in the IMS to the IMM through both weak electrostatic and strong hydrophobic interactions (Gonzalvez & Gottlieb, 2007). The weak electrostatic interactions occur between the negatively charged phosphate groups of the cardiolipin head group and the positively charged lysine residues on cytochrome c, located opposite to the heme-Fe, at a site known as the A-site.
(Paradies et al., 2013). Electrostatic interactions occurring at the A-site are reversible and can be displaced by either adenosine nucleotides or salts located in the IMS (Huttermann et al., 2011; Paradies et al., 2013). Using cytochrome c isolated from horse heart and cardiolipin-containing vesicles, adenosine triphosphate (ATP) was most effective at displacing cytochrome c from the vesicle requiring concentrations six times lower than sodium salts (Rytomaa et al., 1992). The displacement of cytochrome c from cardiolipin may be a feedback mechanism resulting in suppression of ATP production when ATP demand is low, such as during rest. Alternatively, cardiolipin will bind to cytochrome c through strong, hydrophobic interactions by inserting one or two fatty acyl chains into the hydrophobic cleft of cytochrome c (Ott et al., 2001; Tuominen et al., 2002; Iverson & Orrenius, 2003; Kalanxhi & Wallace, 2007). Consequently, these hydrophobic interactions have a significant physiological impact on the structure and function of cytochrome c resulting in peroxidation of cardiolipin, cytochrome c’s reduced membrane affinity, and dissociation of cytochrome c from the IMM (Ott et al., 2001; Iverson & Orrenius, 2003).

In 2005, a seminal discovery determined that cytochrome c not only interacts with cardiolipin prior to release during apoptosis, but also acts as a specific cardiolipin oxygenase leading to cytochrome c release from the IMM in vitro (Kagan et al., 2005). Upon hydrophobic interactions with cardiolipin, cytochrome c undergoes partial unfolding resulting in a transition from a hexa-coordinated to penta-coordinated state (Kagan et al., 2005; Belikova et al., 2006; Abe et al., 2011). In contrast, some studies suggest that hydrophobic interactions occur through the direct insertion of a cardiolipin acyl chain into the heme crevice of cytochrome c, however the extent of this interaction remains uncertain (Kalanxhi & Wallace, 2007; Rajagopal et al., 2012). Thus, it is believed that cytochrome c unfolding exposes the heme-Fe to the surrounding
environment, allowing one or two cardiolipin acyl chains and hydrogen peroxide access to the Fe centre, thus creating cytochrome c peroxidase (Belikova et al., 2006). This newly realized structure leads to cardiolipin peroxidation, permeabilization of the outer mitochondrial membrane, and cytochrome c release (Kagan et al., 2005; Abe et al., 2011).

Subsequent investigations into the critical cardiolipin-cytochrome c complex phenomenon, through which cardiolipin is peroxidized by cytochrome c, delved into understanding the importance of cardiolipin acyl chain compositions on this complex. Based on the findings of Belikova et al. (2006) and Abe et al. (2011), the presence of a double bond in each of the cardiolipin fatty acyl chains is important in inducing peroxidase activity when measuring the cardiolipin/cytochrome c complex with liposomes containing ~50% cardiolipin species. However, it is noted that physiochemical properties of the liposomes created, such as microdomain formation and hydrophobicity, are dependent on the acyl chain composition of cardiolipin used in these studies (Abe et al., 2011; Pennington et al., 2017). Clustering of cardiolipin into microdomains, dependent on cardiolipin acyl chain saturation, influences cardiolipin-protein interactions such that a reduction in microdomain results in decreased protein association (Pennington et al., 2017). Interaction of cytochrome c with the membrane induces the clustering of anionic phospholipids resulting in large domains containing only cardiolipin in situ and in vitro (Haverstick & Glaser, 1989; Pennington et al., 2019). The acyl chain composition of cardiolipin in mitochondrial membranes is dependent on the function of the cell such that the greater the reliance on the mitochondria for oxidative phosphorylation, the more polyunsaturated the fatty acyl chains of cardiolipin will be (Schlame, 2013). Thus, cardiolipin composition of the clusters would be cell and tissue dependent.
As healthy mitochondrial membranes in highly energetic tissues, such as skeletal and cardiac muscle, are composed of primarily tetralinoleoyl cardiolipin species, these cardiolipin clusters would contain primarily tetralinoleoyl cardiolipin species. Cardiolipin fatty acyl chain composition from healthy skeletal muscle is ~80% 18:2n6, primarily from tetralinoleoyl cardiolipin, with the final 20% composed of a variety of other fatty acyl chains including oleic acid. Therefore, it is likely that cytochrome c interacts with multiple fatty acyl chains of varying composition within the IMM of healthy skeletal muscle mitochondria. Under pathological conditions such as muscular dystrophy, the mitochondrial membrane shifts from a primarily polyunsaturated cardiolipin species (~80% total polyunsaturated, ~75% linoleic acid; Zibamaraford, 2015) to a predominantly monounsaturated cardiolipin species (~35% total monounsaturated, ~28% oleic acid; Touboul et al., 2004; Tahallah et al., 2008; Benabdellah et al., 2009). As such, cytochrome c becomes increasingly likely to interact with cardiolipin species containing primarily monounsaturated fatty acids, like oleic acid, through the pathological progression of muscular dystrophy. Therefore, the current study examined the influence of mixtures of liposomes composed of 50% pure cardiolipin (TLCL, TOCL, or TMCL) and liposomes broadly reflecting the cardiolipin composition of healthy and dystrophic membranes on cytochrome c conformation, binding affinity, and peroxidase activity.

2.2 Methods

2.2.1 Liposome Formation

Liposomes were created from commercially available CL species (TMCL; 1’,3’-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol from Avanti Polar Lipids, TOCL; 1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol from Avanti Polar Lipids, TLCL; bovine heart cardiolipin (>80% TLCL) from Sigma-Aldrich Canada) and L-α-phosphatidylcholine (PC;
Sigma-Aldrich Canada). Liposomes were created using a 1:1 (2.5mM: 2.5mM) ratio of PC to CL species as per Birk et al., 2015. In addition to liposomes containing one type of cardiolipin species, liposome membrane mimetics were created to broadly reflect the composition of skeletal muscle mitochondria found in healthy (Con; 39% TLCL, 6% TOCL, 5% TMCL, 50% PC) and dystrophic (Dys; 28% TLCL, 17% TOCL, 4% TMCL, 50% PC) (Kunze et al., 1975; Pearce & Kakulas, 1980; Tahallah, et al., 2008; Benabdellah, et al., 2009; Zibamanzarmofrad, 2015) skeletal muscle mitochondria.

The commercially available lipid species in chloroform were mixed into a small glass vial. These mixtures were dried under a steady stream of nitrogen gas until a lipid film formed on the bottom of the vial. Once dried, 32mM HEPES buffer (Sigma-Aldrich Canada) was added, and the solution was vortexed until milky white without a visual lipid film within the vial. Each sample was placed on ice and sonicated using a tip sonicator (Sonic Dismembrator Model 10, Fisher Scientific, Ottawa, Ontario, Canada). Two 30 second sonication intervals were completed under two watts at which point the solution changed from an opaque, milky white to transparent.

Once sonicated, cardiolipin species were added to a plastic cuvette at a 50µM concentration. This plastic cuvette was placed into a ProteinSolutions DynaPro-99-E-50 Dynamic Light Scattering Module (Santa Barbara, California, USA) to measure liposome size. The dynamics program read the size of the liposomes in solution every 20 seconds, with an average at six minutes.

2.2.2 Cytochrome C Peroxidase Assay

Cytochrome c peroxidase activity was measured using 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP; Cayman Chemicals), as per Birk et al (2015). Cytochrome c (1 µM; Sigma-Aldrich) was added to a mixture containing the previously mentioned liposomes of
varying species (15µM CL). Cytochrome c peroxidase activity was measured using 50µM H₂O₂ as per Birk et al. (2015) and Mandal et al., (2015). Cytochrome c peroxidase activity was identified by the production of the oxidized ADHP byproduct, resorufin, over five minutes. Resorufin is fluorescent, so peroxidase activity was measured fluorescence (excitation at 530 nm and emission at 590 nm) using a plate reader (Synergy HT-1 Plate Reader, Bio-Tek, Winooski, Vermont, USA). Rate of resorufin production was calculated by dividing the arbitrary fluorescent units by the five-minute time course. Enzyme kinetics were measured using H₂O₂ concentrations ranging from 0-500µM.

2.2.3 Ultraviolet-Visible Absorption Spectrophotometry for Cytochrome C Unfolding

Samples containing CL (750µM) and cytochrome c (50µM) were incubated in quartz cuvettes for 15 minutes at room temperature and away from light to induce the CL/ cytochrome c complex without the presence of H₂O₂, as per Gouterman (1959) and Birk et al. (2013). Absorption across the 200nm-900nm spectrum was performed (Ultraspec 2100 Pro, UV/Visible spectrophotometer, Biochrom, Massachusetts, USA) to determine the influence of cardiolipin on cytochrome c protein unfolding, with a focus on the Soret band located around 408nm and the Q-band located around 530-560nm (Pettigrew & Moore, 1987). Correlations of Soret band and Q-band absorbance values with cytochrome c peroxidase activity were calculated. Cytochrome c peroxidase activity measured without H₂O₂ were used.

2.2.4 Ultraviolet-Visible Absorption Spectrophotometry for Cardiolipin Peroxidation

Two lots of commercially available TLCL samples suspended in chloroform:methanol which were purchased from Sigma, with the purchase separated by >1 year. Both remained stored at -20°C. Differences in cytochrome c peroxidase activity between lots raised concerns about possible peroxidation. Thus, both TLCL lots were suspended in HEPES buffer in a quartz
cuvette for a final concentration of 100µM. Absorbance at 234nm was measured without the presence of H$_2$O$_2$ (Ultraspec 2100 Pro, UV/Visible spectrophotometer, Biochrom, Massachusetts, USA) to assess the formation of conjugated dienes as an indirect measure of peroxidation (Lokhmatikov et al., 2014).

2.2.5 Cardiolipin/ Cytochrome c Binding Strength

Samples of 500µM cytochrome c in HEPES buffer (pH 7.4) were incubated with or without 350µM of respective CL liposomes for 30 minutes with intermittent shaking. Opening of cytochrome c’s heme crevice is in part dependent on temperature, such that as temperature increases, the heme crevice progressively opens (Yuan & Hawkridge, 1993). Therefore, the cardiolipin-cytochrome c samples were incubated at room temperature. Samples were then incubated with 0, 0.7mM, 1.4mM, 2.1mM, 2.8mM, 3.5mM, or 4.2mM nonyl-acridine orange for one hour at room temperature, as per Petit et al. (1992). Finally, samples were filled with equivalent volume of sample buffer (25% v/v, 62.5mM Tris HCl, pH=6.8) before loading onto an 8% native polyacrylamide gel. Samples were run for one hour at 150V at room temperature. Gels were removed and stained for one hour with 0.2% Coomassie blue R-250, with consistent rocking at room temperature followed by washing with a destaining solution (20% ethanol v/v, 5% acetic acid v/v, 1% glycerol v/v, distilled water) for two hours and finally in distilled water over night on the rocker at room temperature, followed by one hour of washing in destaining solution the following day to ensure the pores of the gel were completely rid of the staining solution, as per Belikova et al. (2006). Gels were imaged using iScanner Pro and imported to Image Studio (Li-Cor) for analysis, where the density of bands with cytochrome c and CL were compared to the sample containing cytochrome c only. Migration patterns of cytochrome c were similar between the CL species, creating streaks down the gel (Figure A1, Appendix I).
Correlations between cytochrome c binding affinity to the cardiolipin-containing liposomes and cytochrome c peroxidase activity were calculated. Cytochrome c peroxidase activity values measured in the absence of H$_2$O$_2$ was used.

2.2.6 Statistics

All values are expressed as the mean ± standard error (SE). All statistical analyses were performed using GraphPad Prism 5 software (San Diego, USA). One-way ANOVAs were performed on all data sets. A p value <0.05 was considered significant for all tests. When interactive effects were observed, Tukey’s post-hoc analysis was used to confirm true significance between groups.

2.3 Results

2.3.1 Liposome Size

To ensure differences between cardiolipin species composed of differing acyl chains was not an artifact of differing liposomes size, cardiolipin-containing liposomes were measured for size. Liposome size was not different between liposomes of differing CL composition (Figure 2.1).
Figure 2.1. Liposome size measured immediately after sonication. Values are mean ± SEM, n=3 for each condition. TLCL, bovine heart cardiolipin; TOCL, 1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]sn-glycerol cardiolipin; TMCL, 1’,3’-bis[1,2-dimyristoyl-sn-glycero-3-phospho]sn-glycerol cardiolipin; Con, mimetic liposome to broadly reflect the cardiolipin composition of normal skeletal muscle mitochondria; Dys, mimetic liposome to broadly reflect the cardiolipin composition of dystrophic skeletal muscle mitochondria.
2.3.2 Cytochrome c Peroxidase activity is enhanced with unsaturated cardiolipin side chains

Cytochrome c peroxidase activity at 50µM H₂O₂ with TLCL liposomes, labelled Lot 1, was identified to be significantly lower than cytochrome c peroxidase activity with TLCL liposomes in the literature (Figure 2.2a; Belikova et al., 2006, Abe et al., 2011). Additionally, a second lot of TLCL, labelled Lot 2, incubated with cytochrome c induced significantly higher cytochrome c peroxidase activity (Figure 2.2a). As the lot integrity was in question, diene conjugation was measured (Figure 2.2b). As cardiolipin lot 1 was, confirmed through presence of conjugated dienes (Figure 2.2b), and cytochrome c peroxidase activity was lower than cardiolipin from lot 2, it is possible that peroxidation of cardiolipin species prior to incubation with cytochrome negatively impacts cytochrome c peroxidase activity at 50µM H₂O₂.

Cytochrome c peroxidase activity at 50µM H₂O₂ increased 50% with polyunsaturated (TLCL) and monounsaturated (TOCL) cardiolipin species compared to cytochrome c alone (Figure 2.2c). Incubating cytochrome c with TMCL did not alter the measured peroxidase activity in comparison to cytochrome c alone (Figure 2.2c). Cytochrome c peroxidase activity increased equally between Con and Dys liposomes compared to TLCL or TOCL (Figure 2.2c). Therefore, under these conditions, cytochrome c peroxidase activity increased in the presence of liposomes containing cardiolipin with at least one double bond.
Figure 2.2. Cytochrome c peroxidase activity is enhanced in the presence of cardiolipin with non-oxidized unsaturated acyl side chains at 50µM H₂O₂. A) Cytochrome c peroxidase activity with and without polyunsaturated cardiolipin species from two separate lots at 50µM H₂O₂. B) Spectrophotometric measures of conjugated dienes within the side chains of polyunsaturated cardiolipin species without cytochrome c from two separate lots as a surrogate measure of cardiolipin peroxidation. C) Cytochrome c peroxidase activity with and without cardiolipin of varying species at 50µM H₂O₂. Values are mean ± SEM, n=3 for each condition; * indicates significant difference compared to cytochrome c alone (p<0.05). AU, arbitrary units; Cyt C, cytochrome c; TLCL, bovine heart cardiolipin; TOCL, 1’,3’-bis[1,2-dioleoyl-sn-glycerol-3-phospho]-sn-glycerol cardiolipin; TMCL, 1’,3’-bis[1,2-dimyristoyl-sn-glycerol-3-phospho]-sn-glycerol cardiolipin; Con, mimetic liposome to broadly reflect the cardiolipin composition of normal skeletal muscle mitochondria; Dys, mimetic liposome to broadly reflect the cardiolipin composition of dystrophic skeletal muscle mitochondria; AU, arbitrary units; TLCL oxidation.
2.3.3 Effect of CL species on cytochrome c peroxidase activity

To identify the effect of cardiolipin fatty acyl chain on cytochrome c peroxidase’s maximal activity and sensitivity to H$_2$O$_2$, cytochrome c with and without cardiolipin-containing liposomes was incubated with H$_2$O$_2$ concentrations incrementally increasing from 0-500µM where observed peroxidase activity plateaued. The calculated rate of conversion of ADHP to resorufin by cytochrome c alone using H$_2$O$_2$ as a substrate reached a maximal velocity (V$_{max}$) at 379±34 µM H$_2$O$_2$ (Figure 2.3a). Compared to cytochrome c alone, TOCL and TMCL V$_{max}$ values were not significantly different (Figure 2.3b). However, TLCL significantly increased V$_{max}$ of cytochrome c peroxidase in comparison to cytochrome c alone (Figure 2.3b). Although V$_{max}$ values are likely supraphysiological, this indicates that cytochrome c is catalyzing more peroxidative events in a given period of time when incubated with TLCL-based liposomes compared to TMCL, TOCL, or cytochrome c alone. K$_m$, or the H$_2$O$_2$ concentration at half V$_{max}$, was unchanged with TOCL and TMCL compared to cytochrome c alone (Figure 2.3c). However, K$_m$ increased with TLCL, suggesting that TLCL decreases the affinity of cytochrome c peroxidase to H$_2$O$_2$ compared to TOCL, TMCL, or cytochrome c alone.

The peroxidase activity of cytochrome c with membrane mimetic liposomes reached a V$_{max}$ at 593±22µM H$_2$O$_2$ (Figure 2.3b). There were no significant differences between Con and Dys liposomes, which were not different from TLCL, however they were both significantly higher than TMCL, TOCL, and cytochrome c alone. Cytochrome c peroxidase maximal activity and affinity to H$_2$O$_2$ was not affected by the presence of either TMCL or TOCL within the liposomes but was affected by the presence of TLCL (Figure 2.3b; Figure 2.3c). Therefore, provided TLCL composes at least 28% of the liposome, it appears the percent contribution of TMCL or TOCL do not significantly influence cytochrome c peroxidase kinetics.
Figure 2.3. Presence of tetralinoleyl cardiolipin enhances maximal cytochrome c peroxidase activity and decreases H$_2$O$_2$ affinity. A) Michaelis-Menten kinetics of cytochrome c peroxidase alone. Values are mean ± SEM, n=3. AU, arbitrary units; [H$_2$O$_2$], hydrogen peroxide concentration. B) Vmax and C) Km of cytochrome c with and without cardiolipin of varying species. D) Michaelis-Menten kinetics of cytochrome c peroxidase with membrane mimetic liposomes. Values are mean ± SEM, n= 3 for each condition; * indicates significant difference from cytochrome c alone. Cyt C, cytochrome c; TLCL, bovine heart cardiolipin; TOCL, 1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycero cardiolipin; TMCL, 1’,3’-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; AU, arbitrary units; H$_2$O$_2$, hydrogen peroxide; Con, mimetic liposome to broadly reflect the cardiolipin composition of normal skeletal muscle mitochondria; Dys, mimetic liposome to broadly reflect the cardiolipin composition of dystrophic skeletal muscle mitochondria.
2.3.4 Incubating cytochrome c with cardiolipin species influences the folded and unfolded states of cytochrome c

The Soret band, located in the 350-450nm region, is associated with both the ligand coordination and oxidation state of the porphyrin group of the cytochrome c protein (Figure 2.4a). Change in the ligand environment around the iron of the porphyrin ring, such that the Met80 group is replaced with an alternative amino acid, shifted away from the heme-Fe, or a change in the oxidization state of the heme-Fe, can contribute to differences in this region which is indicative of either electron transfer or a peroxidase-like porphyrin ring. For cytochrome c, the peak absorbance was at 408nm, indicating a hexa-coordinated ferric species (Pettigrew & Moore, 1987) (Figure 2.4a). The Q-band, located in the 530-560nm region is associated with a structurally altered porphyrin group of a non-native cytochrome c conformation, a conformation that may facilitate electron transport or peroxidase reactions (Figure 2.4a).

In comparison to cytochrome c alone, cytochrome c with TMCL or TLCL decreased the maximal absorbance of the Soret peak but did not shift the peak wavelength (Figure 2.4b). This suggests that cytochrome c remained in a hexa-coordinated ferric state, however it is possible that the ligand either deviated from Met80 when mixed with TMCL or TLCL liposomes or Met80 moved further away from the heme group after incubation with these cardiolipin species, likely through insertion of a single acyl chain. The measured absorption of the Q-bands increased, suggesting the altered ligation influenced the electrochemical properties of the heme group. When incubated with TOCL, neither maximal absorbance nor peak Soret band wavelength absorption were significantly different from cytochrome c alone (Figure 2.4b). This data suggests that TOCL does not change the spin state of the heme-Fe nor the heme ligation. The Q-band of cytochrome c incubated with TOCL was significantly higher than cytochrome c
alone (Figure 2.4b), suggesting that TOCL had some effect on the porphyrin ring, however it is unclear of the significance of this change. Furthermore, TMCL decreased the maximal absorbance of the Soret peak of cytochrome c compared to TLCL (Figure 2.4b), suggesting that cytochrome c structure was affected greatest by saturated cardiolipin species.

The maximal absorbance at the Soret peak of cytochrome c was decreased in the presence of Con or Dys membrane mimetic liposome compared to cytochrome c alone (Figure 2.4b). Similarly, the measured decrease in the Q-band confirmed electrochemical changes in the porphyrin ring of cytochrome c in the presence of membrane mimetic liposomes (Figure 2.4b). It can be suggested that both membrane mimetic liposomes support a non-native cytochrome c conformation, analogous to cytochrome c incubated with TLCL or TMCL. Therefore, cytochrome c adopts some non-native conformation in the presence of cardiolipin liposomes, regardless of liposome composition. However, it is still unclear what the exact changes are.

Soret and Q-bands are quantitative regions contributing to the identification of cytochrome c conformation that may influence peroxidase activity. The peak absorbance of the Soret band or the Q-band did not correlate with cytochrome c peroxidase activity (Figure 2.4c-d). As such, the identified structural changes within cytochrome c with cardiolipin are not directly related to cytochrome c peroxidase activity.
Figure 2.4. Incubation of cytochrome c with cardiolipin species containing different acyl side chains influences the folded and unfolded states of cytochrome c but these changes do not correlate with cytochrome c peroxidase activity at 50 µM H₂O₂. A) Representative ultraviolet-visible spectrum of cytochrome c alone identifying the Soret band and Q-band. B) Peak of the Soret and Q-bands of cytochrome c with or without cardiolipin of varying acyl side chain composition. C) Correlation between cytochrome c peroxidase activity using ADHP and Soret band absorbance intensity using UV-Vis. D) Correlation between cytochrome c peroxidase activity using ADHP and Q-band absorbance intensity using UV-Vis. Values are mean ± SEM, n=4 for each condition; no statistical difference between values with the same letter (p<0.05) within a given absorption intensity band. Cyt C, cytochrome c; TMCL, 1’,3’-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; TOCL, 1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; TLCL, bovine heart cardiolipin; Con, mimetic liposome to broadly reflect the cardiolipin composition of normal skeletal muscle mitochondria; Dys, mimetic liposome to broadly reflect the cardiolipin composition of dystrophic skeletal muscle mitochondria.
2.3.5 Binding affinity of cytochrome c is influenced by the presence of polyunsaturated cardiolipin species

The dissociation constant was dependent on cardiolipin acyl composition such that cytochrome c had the greatest affinity for TLCL and TOCL-containing membranes compared to TMCL (Figure 2.5a). When comparing cytochrome c’s binding affinity for mimetic membranes, cytochrome c binding affinity was unchanged between Con and Dys liposomes (Figure 2.5a). Although there were no significant differences between mimetic membrane liposomes, TLCL affinity was similar to Con but greater than Dys (Figure 2.5a). The affinity of cytochrome c to the liposome was dependent on the concentration of TLCL in the membrane, which was not seen with TOCL or TMCL (Figure 2.5b). As cytochrome c affinity is positively influenced by the presence of TLCL, lessening the TLCL concentration in a membrane reduces cytochrome c’s affinity for the membrane. With a lower affinity for the membrane, cytochrome c is less likely to interact with the cardiolipin species within the membrane, and thus reducing the likelihood of cytochrome c’s structural and functional transition to cytochrome c peroxidase. However, cytochrome c peroxidase activity did not significantly correlate with $K_d$ for each cardiolipin species (Figure 2.5c).
Figure 2.5. Binding affinity of cytochrome c is highest to tetralinoleoyl cardiolipin liposomes and positively correlated with the percent polyunsaturated fatty acid composition of the acyl side chain. A) Dissociation constant between cytochrome c and cardiolipin of varying composition or with membrane mimetic liposomes. B) Correlation between K_d (dissociation constant) and percent contribution of TLCL, TOCL, and TMCL in the liposome. C) Correlation between cytochrome c peroxidase activity at without H_2O_2 with cardiolipin of varying compositions or membrane mimetic liposomes and their respective K_d dissociation constants. Values are mean ± SEM, n=3 for each condition; values with the same letter are not significantly different from each other (p<0.05); * indicates significant correlation (p<0.05). TLCL, bovine heart cardiolipin; TOCL, 1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol; TMCL, 1’,3’-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; Con, mimetic liposome to broadly reflect the cardiolipin composition of normal skeletal muscle mitochondria; Dys, mimetic liposome to broadly reflect the cardiolipin composition of dystrophic skeletal muscle mitochondria; K_d, dissociation constant x10^8 M^-1 @ 90%.
2.4 Discussion

The present study investigated the relationship between liposomes containing cardiolipin species with differing side chain compositions and cytochrome c form and function as a peroxidase. As an important heme-containing protein in the mitochondria, cytochrome c has many functions, including electron transfer, peroxidase activity, and antioxidant-like properties. Perturbations to cytochrome c’s native form, specifically those resulting in the exposure of the heme-Fe to the IMS environment, can act as an initiator for cytochrome c peroxidase activity. This peroxidase activity results in the release of cytochrome c into the cytosol, which leads to apoptosis and cell death. This is the first study to compare cytochrome c peroxidase activity, conformational changes to cytochrome c monitored spectrophotometrically, and binding affinity with liposomes containing 50% TLCL, TOCL, or TMCL. To our knowledge, this is also the first study to examine the effect of liposomes modeled after the cardiolipin composition of both healthy and dystrophic mitochondrial membranes, on cytochrome c conformation and function.

The major findings of this study are 1) cytochrome c peroxidase activity positively correlates with liposomes containing at least one double bond, provided cardiolipin species are non-oxidized, 2) the presence of polyunsaturated cardiolipin increased maximal peroxidase activity and decreased cytochrome c’s affinity to hydrogen peroxide with equal cytochrome c peroxidase activity at 50µM H₂O₂, suggesting cytochrome c with TLCL performs more peroxidative events at a lower amount of H₂O₂ 3) presence of cardiolipin, regardless of acyl chain composition, influences the folding kinetics of cytochrome c without correlation to cytochrome c peroxidase activity, and 4) cytochrome c binding affinity to cardiolipin liposomes is side chain dependent such that binding affinity increases with the number of double bonds on the cardiolipin side chain.
2.4.1 Cytochrome c peroxidase depends on the presence of at least one double bond

2.4.1.1 Cytochrome c peroxidase activity increases in the presence of cardiolipin species with at least one double bond

Our results suggest the importance of at least one double bond in cardiolipin-containing liposomes during the transition of cytochrome c to a peroxidase. Specifically, cytochrome c peroxidase activity increased in the presence of liposomes containing 50% TLCL with 50µM of \( \text{H}_2\text{O}_2 \). Both maximal peroxidase activity and \( K_m \) of cytochrome c incubated with TLCL were higher than cytochrome c in the absence of cardiolipin, suggesting higher potential for activity but reduced affinity for \( \text{H}_2\text{O}_2 \). Cytochrome c’s reduced affinity for \( \text{H}_2\text{O}_2 \) in comparison to TOCL or TMCL is likely a result of comparatively high affinity of cytochrome c for TLCL. With more TLCL bound, less cytochrome c is readily available to interact with \( \text{H}_2\text{O}_2 \), resulting in a high \( K_m \) value. Increases in cytochrome c peroxidase activity with TLCL has previously been identified with liposomes containing 20% and 50% TLCL (Belikova et al., 2006). Cytochrome c peroxidase activity is dependent on cardiolipin concentration, thus Cytochrome c electrostatically and hydrophobically interacts with TLCL, inducing cytochrome c unfolding and promoting peroxidase activity (Figure 2.5a; Li et al., 2019).

The measured shift from cytochrome c electron transferring properties to a peroxidase may be explained by the conformational transition induced by TLCL as identified by reduction of the Soret peak absorbance and amplification of cytochrome c’s Q-peak. These spectrophotometric changes suggest that TLCL modifies at least one of the extraplanar ligands bound to the heme-Fe, which is suggestive of partial protein unfolding (Mugnol et al., 2008; Tomaskova et al., 2010). As it has previously been identified that the His18 ligand remains throughout conformational perturbations like unfolding in the presence of guanidine chloride, it is likely that
Met80 had been predominately replaced by an alternative ligand, such as Lys72-3, Lys79, His26, His33, or a hydroxide (Elove et al., 1994; Gu et al., 2015; Figure 2.5b). As cytochrome c peroxidase activity did not correlate with change in cytochrome c conformation, it is unlikely that the alternative ligand is a hydroxide. Specifically, Met80 is most likely replaced with a His based on previous resonance Raman measures (Milazzo et al., 2017). Therefore, with the presence of His as an alternative extraplanar ligand and the bulkiness of polyunsaturated cytochrome c chains, it is hypothesized that H$_2$O$_2$ is hindered from interacting with the heme-Fe, reducing cytochrome c’s sensitivity to H$_2$O$_2$ (Zucchi et al., 2003). Regardless, cytochrome c incubated with polyunsaturated side chains likely undergoes modifications that result in Met80 removal and replacement with a weak field ligand, likely a His, however it is currently unclear what modifications occurred.

TLCL also had the largest K$_d$, thus had the greatest affinity for cytochrome c in comparison to all other cardiolipin species, similar to previous research (Belikova et al., 2006; Abe et al., 2011). It is likely that the identified alteration of the extraplanar ligand induced unfolding, creating a large enough heme crevice, to promote hydrophobic interactions with the bulky polyunsaturated side chain of TLCL resulting in a greater affinity for TLCL (Zucchi et al., 2003). As such, with greater affinity, cytochrome c is more likely to interact with the membrane, thus more likely to induce cytochrome c peroxidase activity.

Similar to TLCL, TOCL increased cytochrome c peroxidase activity with 50µM H$_2$O$_2$ but did not change maximal peroxidase activity nor the K$_m$ compared to cytochrome c in the absence of cardiolipin. Previous literature has demonstrated increased cytochrome c peroxidase activity with TOCL-containing liposomes (Belikova et al., 2006; Abe et al., 2011). Cytochrome c’s affinity for TOCL is lower than that for TLCL, thus increasing the amount of cytochrome c
readily available to interact with H2O2. Thus, reducing the K_m value in comparison to TLCL-incubated cytochrome c. The Soret band of cytochrome c with TOCL was unaltered, but the Q-band absorbance peak increased in comparison to the absence of cardiolipin. Taken together, it is possible that the Met80 had been replaced by an alternate ligand with comparable spectrophotometric properties. Spectral measures of the charge transfer band at 695nm showed a reduced absorbance in cytochrome c samples incubated with TOCL, suggesting a dissociation of Met80 from the heme-Fe (Belikova et al., 2006, Mugnol et al., 2008). Based on the spectral differences between cytochrome c incubated with TLCL in comparison to TOCL, it is unlikely that a His residue replaces Met80. It is likely that the Met80 group of TOCL-incubated cytochrome c is replaced in the heme coordination with a hydroxyl group, a strong field ligand, namely H2O2, which would be readily available for use in the peroxidation of cardiolipin (Pettigrew & Moore, 1987). Despite these conformational changes, cytochrome c’s affinity for TOCL is the same as TLCL. It is likely that with TOCL, the heme crevice of cytochrome c opens, similar to cytochrome c incubated with TLCL, supporting hydrophobic interactions in addition to hydrogen peroxide access to the heme group. It is hypothesized that these observations do not translate into increased maximal peroxidase activity or H2O2 sensitivity due to the number of double bonds present in the monounsaturated side chains of cardiolipin. A lack of bulk in the side chains allows easy access for H2O2 to reach the heme-Fe and lack of substrate availability produced by the double bonds reduces maximal activity at concentrations of H2O2 above 100µM.
Figure 2.6. Cytochrome c interacts electrostatically and hydrophobically with polyunsaturated cardiolipin, resulting in cytochrome c unfolding, cytochrome c peroxidase activity, and peroxidation of cardiolipin acyl chains.
Figure 2.7. Cytochrome c interacts electrostatically and hydrophobically with monounsaturated cardiolipin, resulting in cytochrome c unfolding and cytochrome c peroxidase activity but likely does not peroxidize cardiolipin acyl chains.
TMCL does not increase cytochrome c peroxidase activity, maximal enzyme activity, or 
$K_m$ in comparison to cytochrome c incubated without cardiolipin. Cytochrome c has little affinity 
for TMCL, thus it is logical that cytochrome c’s availability to interact with $H_2O_2$ would be 
unaffected, as confirmed by an unaltered $K_m$ value in comparison to cytochrome c alone. 
Cytochrome c also has a lower affinity for TMCL than TLCL or TOCL. However, TMCL 
reduces cytochrome c Soret peak and increases absorbance around the Q-band. Although a study 
using 50% TMCL liposomes agrees that TMCL does not promote cytochrome c peroxidase 
activity comparable to TLCL or TOCL (Belikova et al., 2006), liposomes using 20% TMCL 
contradict these findings. Liposomes using 20% TMCL induced peroxidase activity equal to that 
of liposomes containing 20% TLCL or TOCL (Abe et al., 2011). This discrepancy may be due to 
the differences in amount of cardiolipin used in the liposomes. At higher concentrations, 
cardiolipin is more likely to form hydrophobic interactions with cytochrome c rather than 
interact electrostatically, possibly due to the increased number of acyl chains available for 
hydrophobic interactions (Rytomaa & Kinnunen, 1994; Gorbenko et al., 2006). Hydrophobic 
interactions without a peroxidizable lipids, such as TMCL, are less likely to induce peroxidase 
activity than hydrophobic interactions with peroxidizable fatty acyl chains, like TLCL. 
Consequently, liposomes with high concentrations of TLCL or TOCL (ie. 50%) will induce 
higher peroxidase activity than those with high concentrations of TMCL. The saturated chains of 
TMCL induce extraplanar ligand modifications, removing or replacing Met80, supporting 
cytochrome c unfolding and the presence of a heme crevice. However, based on the measured 
peroxidase activity, it is unlikely that cytochrome c unfolding induced by TMCL supports the 
cytochrome c to peroxidase transition. Therefore, it is unlikely that Met80 is replaced by His as 
in TLCL-incubated cytochrome c (Milazzo et al., 2017). In part, this may be due to a lack of
hydrophobic bonds between TMCL and cytochrome c as identified by the calculated $K_d$. This suggests that conformational transitions alone, without the presence of a substrate such as a double bond within the acyl chains, is unlikely to result in peroxidase activity (Figure 2.6).

Ultimately, TMCL-based liposomes will weakly bind to cytochrome c, initiating conformational changes within the heme group, but this interaction does not facilitate functional changes resulting in enhanced peroxidase activity.

The current evidence suggests the importance of a double bond in the fatty acyl chain of cardiolipin in the transition from cytochrome c to a peroxidase. Provided the cardiolipin acyl chains contain at least one double bond, cytochrome c will strongly bind to cardiolipin, prompting functional shifts to promote peroxidase activity. Without a double bond, short fatty acyl chains of cardiolipin are unlikely to form meaningful hydrophobic interactions that enhance peroxidase activity.
Figure 2.8. Cytochrome c interacts electrostatically with saturated cardiolipin species, inducing transitions in cytochrome c conformation that do not stimulate cytochrome c peroxidase activity.
2.4.2 Characteristics of cytochrome c peroxidase in the presence of healthy and dystrophic mitochondrial mimetic liposomes

Mitochondrial membranes are composed of a multitude of cardiolipin species rather than a single species with homogeneous acyl chain composition. Thus, we measured cytochrome c peroxidase activity with mimetic liposomes that broadly reflect the cardiolipin composition of healthy (Con) and dystrophic (Dys) skeletal muscle mitochondria. Healthy liposomes contained 6% TOCL and 39% TLCL, where Dys liposomes contained 18% TOCL and 28% TLCL, mimicking the shift from polyunsaturated to monounsaturated cardiolipin seen with muscular dystrophy (Touboul et al., 2004; Tahallah et al, 2008; Benabdellah et al., 2009; Zibamanzarmofrad et al, 2015).

Cytochrome c peroxidase activity at 50µM H₂O₂, maximal enzyme activity, and Km were higher in Con compared to cytochrome c without cardiolipin. Although Con contained a mixture of cardiolipin species, cytochrome c peroxidase activity at 50µM H₂O₂ was increased similarly to both TOCL and TLCL, but higher than TMCL. This suggests that provided the liposome contains at least 45% cardiolipin with at least one double bond, cardiolipin containing liposomes will induce cytochrome c peroxidase activity. These findings are similar to previous literature where cytochrome c peroxidase activity increased when associated with liposomes composed of 50% cardiolipin species with at least one double bond (Belikova et al., 2006). Alternatively, with liposomes containing 20% cardiolipin species, Abe et al. (2011) demonstrated that cytochrome c peroxidase activity did not differ when incubated with liposomes containing TLCL, TOCL, or TMCL. Based on the current evidence, it is possible that the ability of cardiolipin to complex with cytochrome c becomes more efficient when the liposome contains 50% cardiolipin species. As such, the more cardiolipin species easily accessible for complexation and the greater substrate
availability, supplied by double bonds within cardiolipin acyl chains, the greater induction of cytochrome c peroxidase activity. Con liposomes increased maximal enzyme activity and $K_m$, similar to TLCL-based liposomes, but not TOCL. It is thus likely that, under experimental conditions where cardiolipin makes up 50% of the liposome, liposomes composed of at least 28% TLCL will increase cytochrome c maximal peroxidase activity, and that this effect is due to the increased presence of double bonds through which cytochrome c peroxidase activity is enhanced (Figure 2.7a). Previous research has suggested liposomes vesicles containing 50% cardiolipin species loosely reflects the interaction of cytochrome c and cardiolipin during the early stages of apoptosis, such that one cytochrome c protein interacts with six cardiolipin species during the initiation of apoptosis (Li et al., 2019). Based on the experimental methods in this study, it is possible that maximal cytochrome c peroxidase activity during the early stages of apoptosis can be induced provided at least 1.5 of the six cardiolipin species interacting with cytochrome c contains 18:2n6 acyl chains. These liposomes reduce the amount of access $H_2O_2$ has to cytochrome c’s active heme-Fe, thus reducing cytochrome c’s ability to use $H_2O_2$ and its sensitivity to $H_2O_2$. Reduced cytochrome c sensitivity to $H_2O_2$ is likely due to the increased flexibility of the heme loop covering the active site of the heme-Fe with polyunsaturated cardiolipin species (Benga & Holmes, 1984; Zucchi et al., 2003; Li et al., 2019) and comparatively greater affinity for Con liposomes than TOCL or TMCL-based liposomes. The disruption of extraplanar ligands shown in the Soret band similar to cytochrome c incubated with TLCL support an increase in cytochrome c heme loop flexibility to induce cytochrome c peroxidase activity. Cytochrome c incubation with the Con liposome increased absorbance of the Q-band, suggesting removal and replacement of the Met80 extraplanar ligand (Mugnol et al., 2008; Tomaskova, Varinska, & Sedlak, 2010). In addition, cytochrome c affinity for the Con
mimetic liposome is equivalent to that of TLCL, TOCL, and TMCL. Taken together, the existence of polyunsaturated cardiolipin species may be the dominant influence in cytochrome c peroxidase activity, but the presence of both TOCL and TMCL in the liposome may influence cytochrome c binding. As less cytochrome c is bound to the membrane, but peroxidase activity remains the same relative to TLCL, it is likely that there is a threshold TLCL concentration for promoting cytochrome c peroxidase activity (Figure 2.7a).

Incubating cytochrome c with Dys liposomes led to increased cytochrome c peroxidase activity with 50µM H₂O₂, increased maximal peroxidase activity, and decreased cytochrome c H₂O₂ sensitivity, similar to that of cytochrome c incubated with TLCL. Although the Dys liposome is only 28% TLCL, it appears that TLCL remained the most influential cardiolipin species with respect to cytochrome c peroxidase activity. Also, as TOCL-based liposome induce peroxidase activity equivalent to TLCL liposomes, TOCL likely contributes to cytochrome c peroxidase activity at 50µM H₂O₂. However, maximal activity and H₂O₂ sensitivity was unhindered compared to TLCL-based liposomes, suggesting TOCL’s influence in the Dys liposome was minimal. As cytochrome c peroxidase activity induced by the Dys liposome is dependent on TLCL, TLCL concentration that induces cytochrome c peroxidase activity lies between Abe’s (2011) published concentration of 20% and our measured concentration at 28%. This peroxidase activity is reflected in the conformational changes induced in cytochrome c. As such, both the Soret and Q-bands suggest cytochrome c unfolding, thus supporting cytochrome c peroxidase activity. Contrary to our hypothesis, cytochrome c affinity for the Dys mimetic liposome is equal to that of the Con mimetic liposome. However, to confirm our hypothesis, cytochrome c’s affinity for the Dys mimetic liposome is less than TLCL liposomes. Overall, it is evident that increasing TOCL concentration in the membrane reduces cytochrome c’s affinity for
dystrophic mimetic liposomes without reducing cytochrome c peroxidase activity (Figure 2.7b). This suggests that the Dys liposome environment is conducive to cytochrome c release which results in apoptosis. Cardiolipin has been shown to cluster together away from other phospholipid species, creating the intrinsic curvature of the IMM (Schlame & Ren, 2010). Although it is currently unknown whether similar cardiolipin species cluster together, cytochrome c affinity positively correlates with TLCL suggesting TLCL clustering and sequestration of cytochrome c to these regions. It is thus likely that cytochrome c is specifically attracted to TLCL regions resulting in strong hydrophobic bonds, thereby inducing protein conformational changes resulting in peroxidase activity (Figure 2.7b). However, within the limitations of the study, it is unclear whether cytochrome c is sequestered to a specific region.

Taken together, it appears that liposomes broadly reflecting the cardiolipin composition of healthy and dystrophic mitochondrial membranes can induce cytochrome c peroxidase activity, a critical initiating event leading to apoptosis. The greater the affinity of cytochrome c to these membrane mimetic liposomes, the less likely cytochrome c will dissociate from the membrane. In vitro, cytochrome c that is unbound to the membrane can release through the outer mitochondrial membrane to induce apoptosis, thus the greater the affinity cytochrome c has for the membrane, the less likely cytochrome c is to induce apoptosis (Ott et al., 2001; Iverson & Orrenius, 2004; Kagan et al., 2005). Therefore, it is possible that membranes that exhibit greater saturation, such as dystrophic mitochondrial membranes mimetic liposome, are less likely to associate with cytochrome c and more likely to induce cytochrome c peroxidase activity. It is also probable that the greater the saturation of the membrane, the more likely cytochrome c will induce apoptosis.
Figure 2.9. Dystrophic mitochondrial membrane mimetics are more likely to release cytochrome c. **a)** Cytochrome c preferentially interacts with polyunsaturated cardiolipin in the Con membrane mimetic liposome, resulting in cytochrome c unfolding and cytochrome c peroxidase activity. **b)** Cytochrome c preferentially interacts with polyunsaturated cardiolipin in the Dys membrane mimetic liposomes, resulting in cytochrome c unfolding and cytochrome c peroxidase activity. However, cytochrome c is less likely to interact with the Dys liposome than the Con liposome due to the increased concentration of TOCL, leading to increased cytochrome c release from the liposomes.
2.5 Conclusion

This was the first study to describe the effect of TLCL, TOCL, and TMCL-based liposomes on the conformation, function, and binding affinity of cytochrome c as individual biochemical properties, in addition to identifying the relationship between these properties as they relate to the transition from cytochrome c to cytochrome c peroxidase. It was also the first to determine the effects of liposomes mimicking the membranes of healthy and dystrophic mitochondria on these aforementioned biochemical properties. Our findings suggest the presence of a cardiolipin species with at least one double bond increases cytochrome c’s binding affinity to the liposome. Increased binding affinity likely contributes to conformational and functional changes in cytochrome c that promote peroxidase activity to possibly remove excess intracellular H$_2$O$_2$. However, by increasing the saturation of the membrane environment, cytochrome c becomes more sensitive to H$_2$O$_2$ and is more likely to dissociate from the membrane, ultimately increasing the likelihood of inducing apoptosis. As such, increasing mitochondrial membrane saturation may be a trigger for the induction of apoptosis. Although future studies should confirm this hypothesis in mitochondria isolated from healthy and dystrophic skeletal muscle, this study suggests the mitochondrial membrane to be a viable target in therapies aimed at reducing cellular apoptosis in muscle wasting conditions such as muscular dystrophy.

2.6 Importance of Research

Apoptosis, or programmed cell death, is an important and highly regulated phenomenon used to remove toxic, damaged, senescent or dysfunctional cells in addition to balancing the development of new cells (Renehan et al., 2001). This process is highly regulated by the mitochondria, through the cleavage and activation of pro-apoptotic BCL-2 proteins which serve to permeabilize the outer mitochondrial membrane (Green & Kroemer, 2004; Gonzalvez &
Prior to the events resulting in OMM permeabilization, mitochondrial-specific cardiolipin must be peroxidized. Once peroxidized, cardiolipin is recruited to the outer membrane, the BCL-2 proteins dock, increasing the permeability of the membrane and allowing the movement of small, pro-apoptotic proteins such as cytochrome c to the cytosol (Kagan et al., 2005; Ren et al., 2014; Li et al., 2015). Oxidized cardiolipin not only increases membrane affinity for the BCL-2 proteins to the outer mitochondrial membrane but is essential for the release of cytochrome c (Kagan et al., 2005). Thus, oxidation of cardiolipin is a critical event in the early stages of apoptosis, supporting the necessity for further in-depth research.

In a healthy mitochondrial population, 18:2n6 makes up ~80% of the cardiolipin species in the inner mitochondrial membrane. This polyunsaturated cardiolipin species acts as an anchor for cytochrome c that improves the efficiency of the electron transport chain through the formation of tight cristae, decreasing the distance between the ETC proteins, thus reducing the likelihood of electron leakage (Paradies et al., 2013). However, it is currently understood that altering the CL species, from predominantly 18:2n6 to 18:1, results in increasingly disorganized cristae, reduced ETC efficiency, increased ROS production, and overall mitochondrial dysfunction (Paradies et al., 2013). Remodeling cardiolipin from primarily 18:2n6 to 18:1 and peroxidation of cardiolipin side chains has been associated with various pathologies such as heart failure, ischemia reperfusion, and muscular dystrophy (Pennington et al., 2017). Due to the importance of cardiolipin in the membrane, these changes have dire consequences for the mitochondria, such that peroxidation of acyl chain or remodeling away from 18:2n6 acyl chain serves to reduce ETC function, induce mitochondrial dysmorphia, and triggers apoptosis (Abe et al., 2011; Kagan et al., 2014; Martensson et al., 2017). Here, we have focused on how the peroxidation and remodeling of cardiolipin in membranes supports the triggering events leading to apoptosis.
To our knowledge, this study is the first to identify the effect of liposomes mimicking the membranes of healthy and dystrophic mitochondria on cytochrome c conformation and function. As a novel approach to understanding the cardiolipin/cytochrome c complex, this is an important addition to the current understanding of the influence of membrane composition on lipid-protein interactions and promotion of the mitochondrial membrane as a target for future therapies.

2.7 Strengths & Limitations

2.7.1 Strengths

The primary goal of this study was to determine the influence of cardiolipin side chain composition, in isolation and within a membrane-like environment, on the transition from cytochrome c to cytochrome c peroxidase. This study used purified materials such as cytochrome c and cardiolipin isolated from bovine heart, as well as mono unsaturated and saturated cardiolipin species to achieve this goal. Using isolated species allows us to manipulate the material into liposomes with some control over the physiochemical properties such as size and composition of the liposomes used for a variety of measures.

Due to the *in vitro* synthetic nature of this study, there is limited variability between the groups. As each liposome was created under the same conditions with the same protocols, there theoretically should be very little variability between the liposomes. Due to this limited variability, confirmed by statistical analyses, we can be confident in the values recorded with smaller samples.

Finally, with liposomes, a variety of measures between protein and membrane compositions can be directly correlated without the influence of external factors such as contamination from alternative subcellular membranes. Thus, these correlations allow us to fully
understand the influence of cardiolipin acyl chain composition on the transition of cytochrome c to a peroxidase. In addition, this was the first study, to our knowledge, that compared the effect of peroxidized TLCL to TLCL species on cytochrome c peroxidase activity. As such, this study adds to the growing body of knowledge suggesting the importance of membrane composition as an influencing factor of apoptosis which may act as a regulatory mechanism for the health of the mitochondria.

2.7.2 Limitations

As with any study design, this study has a few limitations. Although the liposomes were created under the same conditions, we are unable to measure phospholipid distribution throughout individual liposomes. Previous literature has demonstrated that cardiolipin clusters in parts of biomimetic membranes when interacting with peripheral membrane proteins, a property that has also been proposed to occur at the contact sites of mitochondrial membranes (Epand et al., 2007). However, it is unclear how cardiolipin segregates, specifically in our liposomes. It is therefore possible this segregation is based on the headgroup only, creating a heterogeneous mixture of cardiolipin species within clustered regions, thus also segregating the cytochrome c protein to distinct regions of cardiolipin but interacting with a variety of fatty acyl chains. Alternatively, it is possible that cardiolipin may cluster based on acyl chain composition such that like species cluster with like species. In this situation, cytochrome c could be isolated to distinct regions of cardiolipin species based on fatty acyl chain composition. Therefore, future studies should investigate using unique fluorescently labelled cardiolipin species in this in situ synthetic model to identify the regions with which cytochrome c associates.

Commercially available TLCL used in this study was isolated from bovine heart. Although the acyl chains of this sample are primarily 18:2n6 purified to >80%, there may be
alternative acyl chains, differing in saturation and chain lengths. Similarly, because the product used is isolated rather than synthesized in lab, it contains up to 3% of a variety of other species of which includes phosphatidylethanolamine. Therefore, it is possible the effects of acyl chain composition are not truly reflective of TLCL possibly due to contamination.

Finally, the preferential peroxidation of cardiolipin side chain can influence cytochrome c peroxidase activity, binding affinity, and conformation of cytochrome c. As cytochrome c preferentially peroxidizes cardiolipin acyl chains, it is likely that these acyl chain may be peroxidized prior to the substrate (ADRP) used to identify peroxidase activity (Kagan et al., 2006). It is possible that the measured cytochrome c peroxidase activity was lower than the theoretical maximal peroxidase activity of cytochrome c incubated with TLCL due to preferential peroxidation of the acyl chains. Peroxidation of acyl chains may influence the shape and reactivity of cardiolipin. As cytochrome c’s affinity for cardiolipin is significantly diminished when interacting with peroxidized side chains, it is possible that the measured cytochrome c peroxidase activity and binding affinity values appear lower with cardiolipin species containing at least one double bond than theoretical values.

2.8 Future Directions

Apoptosis can be triggered by a variety of events including excessive ROS, calcium uptake, damage to DNA, and dysfunction of the electron transport chain. Future experiments should work to elucidate the interactions between cytochrome c and different cardiolipin species in a variety of environments in further detail. These environments include altered pH, calcium concentration, and varying ATP concentrations in the isolated membrane mimetic model used in this study. Furthermore, to supplement this in situ data, it is important to understand how altering
the membrane affects both the function of the mitochondria and the lipid-protein interactions \textit{in vitro} utilizing isolated mitochondria.

2.8.1 Membrane Mimetic Model

The pH of the IMS is influenced by the pH of the cytosol. As the concentration of hydrogen ions increase, similar to exercise, the pH of the cytosol decreases (Rytomaa et al., 1995). As the degree of protonation of cardiolipin is influenced by the hydrogen ion concentration, it is understood that the lower the pH of the IMS, the more cardiolipin will be protonated. The more cardiolipin species are protonated, the more likely the cardiolipin/cytochrome c complex will occur (Rytomaa et al., 1995). Although this theory has been tested and confirmed previously, only liposomes that contain PUFA cardiolipin have been used to measure this (Sinibaldi et al., 2008). Molecular dynamic stimulation studies have determined the effect of fatty acyl chain removal (Sathappa & Alder, 2016) and tetramyristitol chains (Lemmin et al., 2013) on the protonation state of CL, but none have determined protonation state of CL with physiologically predominant acyl chains like tetralinoleoyl and tetraoleoyl CL. It is unclear if the fatty acyl composition of cardiolipin influences this protonation event and whether the cardiolipin composition of the liposomes has an impact on cardiolipin protonation. By extension it is unclear if protonation of the differing cardiolipin species changes the dynamics of the cardiolipin/cytochrome c complex.

It is known that the ionic strength, or the sum of the molalities of each type of ions in solution multiplied by the square of their charge representative of the electrical field in a solution, of the IMS can affect the cardiolipin/cytochrome c complex and it has previously been shown that the initial electrostatic bond is critical to the cardiolipin/cytochrome c complex (Kalanxhi & Wallace, 2007). In healthy mitochondria the ionic strength of the IMS was
estimated to reside between 100-150 mM, allowing cytochrome c to interact with cardiolipin electrostatically for a brief period of time to allow for protein movement between complex III and IV (Cortese et al., 1991). Under pathological conditions through which calcium regulation becomes dysfunctional and the transient increase in calcium becomes sustained, apoptosis can be triggered (Nicotera & Orrenius, 1998; Brookes et al., 2004). Alternatively, increases in intracellular sodium have been identified as an initial event in the early phases of apoptosis (Bortner & Cidlowski, 2006). Under these conditions, the ionic strength can measure above 150 mM (Cortese et al., 1991), compromising the protein-lipid interaction between cardiolipin and cytochrome c, likely leading to cytochrome c release from the membrane and disruption of the ETC. Although the effect of ionic strength, specifically the influence of NaCl on ion concentration, has been measured using liposomes composed of PUFA cardiolipin, it has neither been measured with calcium as the ion nor with varying cardiolipin species. As such, it is important to understand how altering the cardiolipin species effects the protein-lipid interactions in an environment high in calcium, similar to an environment that supports apoptosis.

Finally, ATP is in competition with cardiolipin for the positively charged binding sites located on cytochrome c. As an anionic molecule, ATP competes with cardiolipin for the A-site located on cytochrome c, decreasing the affinity of cytochrome c for cardiolipin (Ahluwalia et al., 2011; Snider et al., 2013). In situ, it has been shown that 10mM ATP weakens cytochrome c interaction with cardiolipin (Snider et al., 2013) and has been confirmed to occur at 3mM ATP concentrations as well (Patriarca et al., 2007). Due to skeletal muscle demand for ATP, it has been estimated that [ATP] ranges from 1-10mM depending on cell type, function, and work capacity (Patriarca et al., 2009), but is unlikely to reach these concentrations within the mitochondria. With the use of genetically introduced fluorescent probes, Imamura et al.
estimates mitochondrial ATP concentration to be 60% of cytosolic ATP, thus mitochondrial ATP concentration is theoretically 0.6-6mM (2009). Therefore, it is possible that as the concentration of ATP in the IMS increases the less cytochrome c will be bound to cardiolipin. This reduces the likelihood of the presence of hydrophobic bonds, resulting structural transition, and peroxidase activity. Consequently, ATP influences not only the cardiolipin/ cytochrome c complex, but cytochrome c folding as well (Sinbadli et al., 2005; Ahluwalia et al., 2011). In millimolar concentrations, ATP can promote protein refolding to a more globular structure once bound to cardiolipin, thus preventing the structural transition from cytochrome c to cytochrome c peroxidase (Snider et al., 2013). Despite this connection, the influence of ATP has not been measured across a variety of cardiolipin species, nor under the influence of mixed cardiolipin liposomes.

2.8.2 In Vitro

Physiologically, healthy mitochondrial membranes are composed of 10-20% cardiolipin, which supports electrostatic interactions between cardiolipin and cytochrome c for the purpose of electron transfer through the electron transport chain (Belikova et al., 2006; Abe et al., 2011). However, during apoptotic conditions, like pathological calcium uptake, it is evident that cardiolipin will translocate from the inner leaflet of the IMM to the outer leaflet supporting complexation between cardiolipin and cytochrome c (Fernandez et al., 2002). This movement of cardiolipin from the inner to the outer leaflets of the mitochondrial membranes is poorly characterized. It is unclear whether all cardiolipin species translocate equally or if this event is controlled in part by fatty acyl chain composition. Understanding the membrane environment during the initial phases of apoptosis is critical to identifying how cardiolipin/ cytochrome c complex acts as a trigger for apoptosis.
Cardiolipin has an important role in both mitochondrial structure and function. The presence of tetralinoleoyl-cardiolipin is critical for the function of a variety of proteins within the inner mitochondrial membrane. For example, no other phospholipid or cardiolipin species can support ADP/ATP carrier function (Paradies et al., 2014). Under pathological conditions where the cardiolipin composition of the membrane shifts from predominantly 18:2n6 to 18:1 (Kunze et al., 1975; Touboul et al., 2004; Tahallah et al., 2008; Benabdellah et al., 2009; Zibamanzarmofrad, 2015), ADP/ATP carrier function is reduced (Paradies et al., 2014), thereby reducing ATP concentration in the IMS and increasing membrane potential, both of which are suggested to promote the cardiolipin/ cytochrome c complex (Sinbaldi et al., 2004; Snider et al., 2013). Furthermore, cardiolipin maintains stability of the electron transport chain supercomplexes, and thus maintains electron transport through the electron transport system (Paradies et al., 2014). Optimization of electron transport through this series of proteins reduces the likelihood of electron slippage from the supercomplexes to molecular oxygen. Without superoxide produced through ETC electron slippage, less superoxide is available for reduction to H$_2$O$_2$, thereby reducing total concentration of H$_2$O$_2$ within the mitochondria. With a poor substrate pool, in this case hydrogen peroxide, cytochrome c is less likely to act as a peroxidase, and thus less likely to peroxidize cardiolipin. Therefore, it is important to understand how the changes of the protein-lipid interaction affect mitochondrial function including oxidative phosphorylation and reactive oxygen species production in its relation to stimulation of apoptosis.

These experiments, both in the membrane mimetic model and in vitro, will shed some light on the influence of the cardiolipin composition on the cardiolipin-cytochrome c interactions
within the mitochondria. A better understanding of these interactions will provide insight on how to prevent apoptosis in pathological conditions such as muscular dystrophy.
2.9 References


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Figure A 1.1. Spectra of cardiolipin from A) Lot 1 exhibits a large absorbance at 234nm than cardiolipin from B) Lot 2. Absorbance at 234nm indicates presence of conjugated dienes, which occurs during cardiolipin peroxidation. Therefore, cardiolipin from Lot 1 was peroxidized and was not used in future experimental measures, n=3.
Michaelis-Menten kinetics of cytochrome c peroxidase with cardiolipin based liposomes. A) Michaelis-Menten kinetics of cytochrome c incubated with TLCL-specific liposomes. B) Michaelis-Menten kinetics of cytochrome c incubated with TOCL-specific liposomes. C) Michaelis-Menten kinetics of cytochrome c incubated with TMCL-specific liposomes. D) Michaelis-Menten kinetics of cytochrome c incubated with Con liposomes. E) Michaelis-Menten kinetics of cytochrome c incubated with Dys liposomes. Values are mean ± SEM, n=3. AU, arbitrary units; [H₂O₂], hydrogen peroxide concentration. TMCL, 1’,3’-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; TOCL, 1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; TLCL, bovine heart cardiolipin; Con, mimetic liposome to broadly reflect the cardiolipin composition of normal skeletal muscle mitochondria; Dys, mimetic liposome to broadly reflect the cardiolipin composition of dystrophic skeletal muscle mitochondria.
Figure A 1.3. Incubation of cytochrome c with cardiolipin species containing different acyl side chains influences the folded and unfolded states of cytochrome c. A) Representative ultraviolet-visible spectrum of cytochrome c incubated with TLCL-specific liposomes. B) Representative ultraviolet-visible spectrum of cytochrome c incubated with TOCL-specific liposomes. C) Representative ultraviolet-visible spectrum of cytochrome c incubated with TMCL-specific liposomes. D) Representative ultraviolet-visible spectrum of cytochrome c incubated with Con liposomes. E) Representative ultraviolet-visible spectrum of cytochrome c incubated with Dys liposomes. Values are mean ± SEM, n=4 for each condition. TMCL, 1’,3’-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; TOCL, 1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; TLCL, bovine heart cardiolipin; Con, mimetic liposome to broadly reflect the cardiolipin composition of normal skeletal muscle mitochondria; Dys, mimetic liposome to broadly reflect the cardiolipin composition of dystrophic skeletal muscle mitochondria.
Figure A. 1.4 Migration patterns of cytochrome c incubated with cardiolipin. A) Cytochrome c incubated with TLCL and 0, 0.7mM, 1.4mM, 2.1mM, 2.8mM, 3.5mM, or 4.2mM nonyl-acridine orange. B) 500µM cytochrome c incubated with 350µM TOCL with 0, 0.7mM, 1.4mM, 2.1mM, 2.8mM, 3.5mM, or 4.2mM nonyl-acridine orange. C) 500µM cytochrome c incubated with 350µM with TMCL and 0, 0.7mM, 1.4mM, 2.1mM, 2.8mM, 3.5mM, or 4.2mM nonyl-acridine orange. D) 500µM cytochrome c with 350µM Con cardiolipin with 0, 0.7mM, 1.4mM, 2.1mM, 2.8mM, 3.5mM, or 4.2mM nonyl-acridine orange. E) 500µM cytochrome c incubated with 350µM DMD cardiolipin with 0, 0.7mM, 1.4mM, 2.1mM, 2.8mM, 3.5mM, or 4.2mM nonyl-acridine orange. TLCL, bovine heart cardiolipin; TOCL, 1’,3’-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol; TMCL, 1’,3’-bis[1,2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol cardiolipin; Con, mimetic liposome to broadly reflect the cardiolipin composition of normal skeletal muscle mitochondria; Dys, mimetic liposome to broadly reflect the cardiolipin composition of dystrophic skeletal muscle mitochondria.