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ORIGINAL ARTICLES

Unsaturation of Mitochondrial Membrane Lipids is Related to Palmitate Oxidation in Subsarcolemmal and Intermyofibrillar Mitochondria

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Abstract Membrane lipid composition is thought to influence the function of integral membrane proteins; however, the potential for lipid composition to influence overall mitochondrial long-chain fatty acids (LCFA) oxidation is currently unknown. Therefore, the naturally occurring variability of LCFA oxidation rates within subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria in muscles with varying oxidative potentials (heart → red → white) was utilized to examine this relationship. To this end, SS and IMF mitochondria were isolated and palmitate oxidation rates were compared to membrane phospholipid composition. Among tissues, rates of palmitate oxidation in mitochondria displayed a 2.5-fold range, creating the required range to determine potential relationships with membrane lipid composition. In general, the percent mole fraction of phospholipid head groups and major fatty acid subclasses were similar in all mitochondria studied. However, rates of palmitate oxidation were positively correlated with both the unsaturation index and relative abundance of cardiolipin within mitochondria ($r = 0.57$ and 0.49, respectively; $p < 0.05$). Thus, these results suggest that mitochondrial LCFA oxidation may be significantly influenced by the total unsaturation and percent mole fraction of cardiolipin of the mitochondrial membrane, whereas other indices of membrane structure (e.g., percent mole fraction of other predominant membrane phospholipids, chain length, and ratio of phosphatidylcholine to phosphatidylethanolamine) were not significantly correlated.

Keywords Cardiac muscle · Cardiolipin · LCFA oxidation · Lipid composition · Mitochondrial membranes · Red muscle · Unsaturation index · White muscle

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In both skeletal and cardiac muscle, the oxidation of long-chain fatty acids (LCFA) represents a substantial contribution of the reducing equivalents required for the maintenance of adenosine triphosphate homeostasis, a process that exclusively occurs within the mitochondrial matrix. Regardless of the restricted location of LCFA oxidation, over the past two decades evidence has accumulated to show that rates of fatty acid oxidation within muscle is regulated at several sites, including the transport of LCFA across both the sarcolemmal (reviewed in Glatz et al. 2010) and mitochondrial membranes (Fritz and Yue 1963; Smith et al. 2011; Winder et al. 1989). However, the understanding of potential mechanisms regulating fatty acid oxidation at the level of the mitochondria is in its infancy.

The regulation of mitochondrial LCFA oxidation has historically been ascribed to malonyl-CoA inhibition of carnitine palmitoyltransferase I (CPTI) (Saggerson and Carpenter 1981; Winder et al. 1989). However, it is now recognized that the malonyl-CoA/CPTI axis cannot entirely account for the regulation of LCFA oxidation (Alkhateeb et al. 2011; Dzamko et al. 2008; Odland et al. 1996, 1998; Roepstorff et al. 2004). As a result, in recent years a renewed interest has emerged to unravel novel sites of regulation in mitochondrial fatty acid oxidation. Since then, several additional sites of regulation have been found, including, but not limited to, fatty acid translocase (FAT/CD36) suggested interaction with acyl-CoA synthetase

(Smith et al. 2011); NAD-dependent deacetylase sirtuin-3 deacetylation of long-chain acyl CoA dehydrogenase (Hirschey et al. 2010); and complex I of the electron transport chain (Ahn et al. 2008).

Creating another level of complexity within muscle is the fact there are two spatially distinct mitochondrial subpopulations, known as subsarcolemmal (SS) and intermyofibrillar (IMF) because they are located directly beneath the sarcolemmal membrane and between the myofibrillar apparatus, respectively. These mitochondrial subpopulations are known to have different characteristics, including size and various enzymatic activities, both of which may account for the higher rates of LCFA oxidation within IMF mitochondria (Cogswell et al. 1993; Holloway et al. 2009a, 2010; Hoppel et al. 2002; Kelley et al. 2002; Palmer et al. 1985). In addition to these reported differences between mitochondrial subpopulations, additional mechanisms that regulate LCFA oxidation within muscle mitochondria, including the unsaturation index (UI) of the mitochondrial membranes, may exist. However, to date the influence of membrane saturation in SS and IMF mitochondrial LCFA oxidation has not been examined in skeletal or cardiac muscles.

Although most of the regulation of mitochondrial LCFA oxidation studied to date involves integral mitochondrial membrane proteins, structural properties of membranes can also influence fundamental physiological processes, and changes in membrane phospholipid or fatty acid composition can influence integral membrane protein function (Lee 2004; McIntosh and Simon 2006). Specifically, lower phosphatidylcholine (PC) and cardiolipin (CL), higher phosphatidylethanolamine (PE), and fatty acid chain length and degree of saturation (Spector and Yorek 1985), and lower PC/PE ratio (Hazel and Williams 1990) are all positively correlated with membrane rigidity. In the context of LCFA oxidation, it has previously been shown that membrane UI is positively related to CPTI activity in hepatic mitochondria (Power et al. 1994). However, it remains to be determined whether these potential relationships exist in muscle mitochondria.

Past studies have successfully utilized diet manipulation (altering fat composition but not content) to alter SS and IMF cardiac mitochondrial membrane lipid composition (Khairallah et al. 2010a, b; O'Shea et al. 2009). However, this is no evidence in the literature to suggest that this approach is effective in skeletal muscle. Therefore, in the current study we took a different approach and utilized the naturally occurring variation of intrinsic rates of LCFA oxidation within SS and IMF mitochondria in muscles with varying oxidative potentials (white, red, and cardiac muscles) to examine the potential that mitochondrial membrane lipid composition is associated with rates of LCFA oxidation. Specifically, we hypothesized that greater rates of LCFA oxidation would correlate with mitochondria with higher membrane contents of PC, CL, and PC/PE, and lower PE, and shorter and more unsaturated fatty acids.

Methods

Animals

Male Sprague Dawley rats (250–300 g, $n = 5$) were used. The rats were housed in a controlled environment on a reverse 12:12 h light–dark cycle. Animals were provided with standard rat chow (Teklad Global 18 % Protein Rodent Diet; Harland Laboratories, Madison, WI; ingredients include ground wheat, ground corn, wheat middlings, dehulled soybean meal, corn gluten meal, and soybean oil fortified with essential vitamins and minerals, resulting in a percent weight contribution of 18.6 % protein, 75.2 % carbohydrates, and 6.2 % fat, which was made up predominately of 3.1 % linoleic, 1.2 % oleic, 0.7 % palmitic, 0.3 % linolenic, and 0.2 % steric) and water ad libitum. Ethical approval for this work was obtained from the Animal Care Committee at the University of Guelph and conformed to all Canadian Council on Animal Care guidelines.

Tissue Collection and Mitochondrial Isolation

Animals were euthanized [pentobarbital sodium (Somnotol), 6 mg/100 g body wt]. Heart (left ventricle) and red (red gastrocnemius and red vastus lateralis) and white (white gastrocnemius and white vastus lateralis) muscles from both hind limbs were excised and used for whole muscle determinants of citrate synthase (CS) and isolation of SS and IMF mitochondria as described below.

Citrate Synthase

Muscle samples ($\sim$10 mg wet tissue) were homogenized in 100 vol/wt of a 100 mM potassium phosphate buffer, while isolated mitochondria were diluted 20$\times$ in a sucrose mannitol buffer, for the measurements of CS. Total CS activity was assayed spectrophotometrically at 412 nm (37 °C) after ensuring intact mitochondria were lysed with 0.04 % Triton X-100 and repeated freeze-thawing (Srere 1969).

Mitochondrial Isolation

Differential centrifugation was used to obtain both SS and IMF mitochondrial fractions (Benton et al. 2008; Bezaire et al. 2004; Campbell et al. 2004). All procedures were identical to those previously published by our group

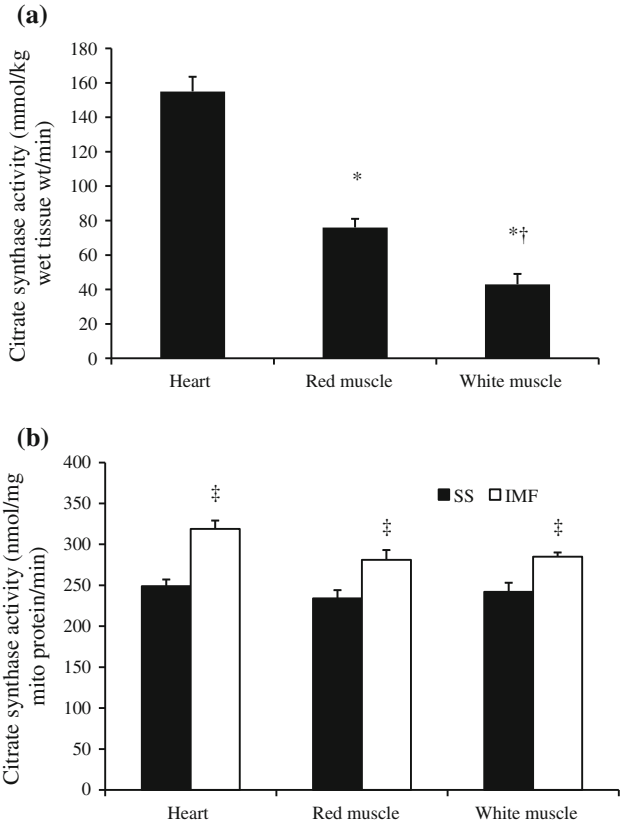

Fig. 1 Maximal citrate synthase (CS) activity in **a** whole muscle homogenates and **b** isolated subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from rat heart, red (gastrocnemius), and white (gastrocnemius) muscle. Values are means ± SE ($n = 5$); *and †denote significance from heart and red muscle, respectively; ‡ denotes significance from SS mitochondria within the same tissue

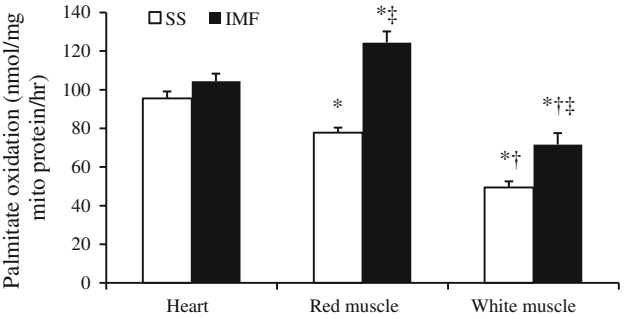

Fig. 2 In vitro palmitate oxidation rates in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle. Values are means ± SE ($n = 5$); *and †denote significance from heart and red muscle, respectively, within the same mitochondrial subpopulation; ‡denotes significance from SS mitochondria within the same tissue

(Bezaire et al. 2006; Campbell et al. 2004; Holloway et al. 2006). Briefly, muscle was homogenized with a Polytron (PT3100, Kinematica) for exactly 5 s at a setting of 7. The

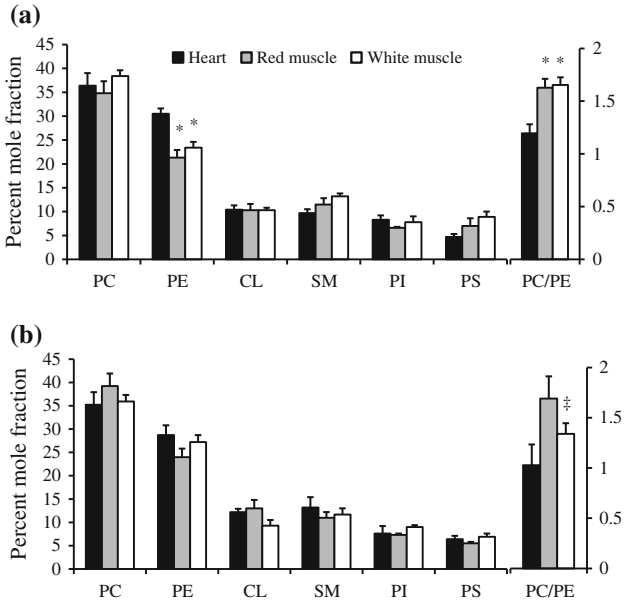

Fig. 3 Percent mole fraction of major phospholipid species of **a** subsarcolemmal and **b** intermyofibrillar mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle. Values are expressed as means ± SE ($n = 5$); *denotes significance from heart within the same mitochondrial subpopulation, ‡denotes significance from subsarcolemmal (SS) mitochondria within the same tissue. *PC* phosphatidylcholine, *PE phosphatidylethanolamine*, *CL* cardiolipin, *PI* phosphatidylinositol, *SM* sphingomyelin, *PS* phosphatidylserine

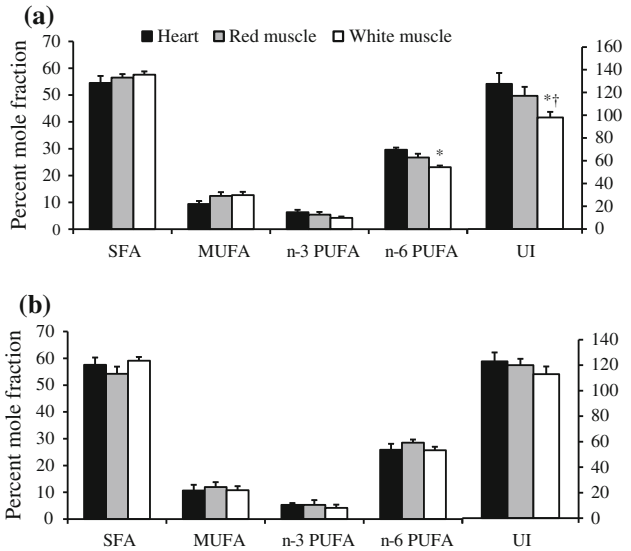

Fig. 4 Percent mole fraction of total fatty acid subclasses of total phospholipids of **a** subsarcolemmal and **b** intermyofibrillar mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle. Values are expressed as means ± SE ($n = 5$) with SFA, MUFA, n3, and n6 represented on the left y-axis and UI represented on the right. *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *UI* unsaturation index $= \sum m_i \times n_i$, where m_i is the mole percent and n_i is the number of carbon–carbon double bonds of the fatty acid

Table 1 Percent mole fraction of total phospholipid fatty acids of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle

Fatty acid	Heart		Red muscle		White muscle	
	SS	IMF	SS	IMF	SS	IMF
14:0	2.2 ± 0.3	2.6 ± 0.2	3.2 ± 0.4	2.7 ± 0.2	3.9 ± 0.8	2.6 ± 0.4
15:0	1.3 ± 0.2	1.5 ± 0.2	1.9 ± 0.3	1.5 ± 0.1	2.2 ± 0.4	1.7 ± 0.2
16:0	18.3 ± 1.2	21.0 ± 1.1	26.5 ± 1.0*	25.4 ± 1.0	24.7 ± 3.3*	24.5 ± 3.2
17:0	1.9 ± 0.2	2.4 ± 0.4	2.6 ± 0.3	2.3 ± 0.3	2.7 ± 0.3	2.7 ± 0.2
18:0	24.8 ± 0.6	27.0 ± 0.7	19.5 ± 0.3*	19.7 ± 0.7*	16.2 ± 2.0*	19.2 ± 1.8*
14:1	1.4 ± 0.4	1.3 ± 0.2	2.1 ± 0.5	1.3 ± 0.2	3.6 ± 2.0	1.3 ± 0.2
16:1	1.7 ± 0.4	2.3 ± 0.6	2.6 ± 0.3*	2.4 ± 0.3	3.4 ± 0.4*	2.4 ± 0.7
18:1	3.6 ± 0.3	4.0 ± 0.3	4.5 ± 0.7	6.3 ± 2.4	6.6 ± 1.6	4.1 ± 0.8
22:6n3	5.3 ± 0.4	4.8 ± 0.5	4.5 ± 0.6	4.5 ± 0.3	2.7 ± 0.4	3.1 ± 0.6
18:2n6	14.2 ± 1.8	11.6 ± 1.0	14.0 ± 0.7	15.4 ± 0.9	9.6 ± 1.5* †	10.6 ± 1.6
18:3n6	1.4 ± 0.3	1.7 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	1.5 ± 0.2	3.3 ± 1.4
20:4n6	11.9 ± 1.7	12.0 ± 1.1	9.1 ± 0.8	9.3 ± 0.5	7.0 ± 1.3*	8.7 ± 1.1
Chain length	16.7 ± 0.1	16.8 ± 0.1	16.5 ± 0.1	16.7 ± 0.1	16.7 ± 0.1	16.7 ± 0.1

Values are expressed as means ± SE

Fatty acids with a percent mole fractions below 1 % are not shown

SS subsarcolemmal mitochondria, *IMF* intermyofibrillar mitochondria, *UI* unsaturation index = $\sum m_i \times n_i$, where m_i is the mole percent and n_i is the number of carbon–carbon double bonds of the fatty acid

* and † denote significance from heart and red muscle, respectively, within the same mitochondrial subpopulation

Table 2 Percent mole fraction of phosphatidylcholine fatty acids of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle

Fatty acid	Heart		Red muscle		White muscle	
	SS	IMF	SS	IMF	SS	IMF
14:0	1.3 ± 0.3	1.3 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.7 ± 0.2	1.4 ± 0.1
15:0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
16:0	24.1 ± 1.0	24.2 ± 0.9	38.8 ± 0.8*	37.3 ± 1.1*	42.5 ± 1.0* †	39.7 ± 3.2*
17:0	1.1 ± 0.1	1.3 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.5 ± 0.1	1.4 ± 0.1
18:0	26.5 ± 0.7	28.0 ± 0.4	11.2 ± 0.5*	10.5 ± 0.8*	8.4 ± 0.2* †	7.1 ± 0.6* †
16:1	1.2 ± 0.3	1.6 ± 0.3	1.8 ± 0.3	1.5 ± 0.4	2.0 ± 0.6	2.3 ± 0.5
18:1	3.2 ± 0.1	3.4 ± 0.3	4.0 ± 0.2	4.8 ± 1.1	3.3 ± 0.8	3.6 ± 0.4
22:6n3	2.9 ± 0.1	2.8 ± 0.2	2.6 ± 0.3	2.8 ± 0.2	2.0 ± 0.5	1.4 ± 0.2
18:2n6	12.9 ± 0.5	13.1 ± 1.0	18.2 ± 1.0*	19.6 ± 1.0*	16.4 ± 0.2*	16.0 ± 1.4
20:4n6	20.0 ± 1.5	18.8 ± 0.8	13.7 ± 0.6*	14.4 ± 0.7*	13.5 ± 0.8*	13.3 ± 1.2*
Total saturates	55.4 ± 1.7	58.0 ± 1.7	56.2 ± 1.6	52.6 ± 1.4*	56.8 ± 1.0	56.1 ± 0.9
Total monoenes	6.5 ± 1.0	6.7 ± 0.8	7.6 ± 0.3	7.6 ± 1.2	7.0 ± 0.5	7.8 ± 0.6
n3 polyenes	3.2 ± 0.2	3.1 ± 0.3	3.1 ± 0.3	3.4 ± 0.3	3.5 ± 1.1	2.0 ± 0.2* †
n6 polyenes	34.8 ± 1.7	32.2 ± 1.6	34.3 ± 1.5	36.3 ± 1.5	32.7 ± 0.7	34.0 ± 0.7
UI	136 ± 7	130 ± 6	122 ± 6	129 ± 5	119 ± 4	128 ± 12
Chain length	17.3 ± 0.1	17.3 ± 0.1	16.9 ± 0.1*	16.9 ± 0.1*	16.9 ± 0.1*	17.0 ± 0.1*

Values are expressed as means ± SE

Fatty acids with a percent mole fraction below 1 % are not shown

SS subsarcolemmal mitochondria, *IMF* intermyofibrillar mitochondria, *UI* unsaturation index = $\sum m_i \times n_i$, where m_i is the mole percent and n_i is the number of carbon–carbon double bonds of the fatty acid

* and † denote significance from heart and red muscle, respectively, within the same mitochondrial subpopulation

Table 3 Percent mole fraction of phosphatidylethanolamine fatty acids of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle

Fatty acid	Heart		Red muscle		White muscle	
	SS	IMF	SS	IMF	SS	IMF
14:0	1.5 ± 0.1	2.0 ± 0.1‡	2.0 ± 0.2	2.0 ± 0.2	2.3 ± 0.4	2.2 ± 0.3
15:0	1.0 ± 0.2	1.0 ± 0.3	1.5 ± 0.3	1.1 ± 0.2	1.9 ± 0.2*	1.4 ± 0.2
16:0	15.2 ± 1.1	16.7 ± 0.9	14.0 ± 1.2	13.2 ± 0.6*	15.6 ± 0.5	14.2 ± 0.7
17:0	1.7 ± 0.2	1.9 ± 0.2	1.9 ± 0.2	1.7 ± 0.2	2.3 ± 0.3	1.9 ± 0.2
18:0	34.3 ± 1.2	34.5 ± 0.3	36.6 ± 1.0	38.2 ± 1.1*	34.0 ± 1.4	36.9 ± 0.6
16:1	1.1 ± 0.3	1.2 ± 0.4	1.1 ± 0.2	1.3 ± 0.3	1.8 ± 0.6	1.1 ± 0.5
17:1	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1
18:1	3.8 ± 0.1	3.8 ± 0.1	3.7 ± 0.1	5.4 ± 1.5	6.1 ± 2.3	4.1 ± 0.2
22:6n3	12.4 ± 1.0	10.7 ± 0.9	11.7 ± 1.2	11.5 ± 0.4	6.2 ± 0.4* †	8.9 ± 0.9‡
18:2n6	7.7 ± 0.3	7.4 ± 0.6	9.0 ± 0.6	8.0 ± 0.8	6.8 ± 0.2 †	7.7 ± 0.2‡
18:3n6	1.2 ± 0.7	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.2	1.0 ± 0.3	1.2 ± 0.2
20:4n6	13.1 ± 3.3	13.8 ± 0.9	11.3 ± 0.5	10.7 ± 0.8	11.4 ± 0.8	13.6 ± 0.7 †
Total saturates	57.2 ± 3.5	59.1 ± 1.3	59.0 ± 1.7	57.7 ± 1.6	59.4 ± 1.7	59.3 ± 1.0
Total monoenes	7.3 ± 0.5	8.3 ± 0.7	7.6 ± 0.1	9.0 ± 1.8	11.2 ± 2.9	7.9 ± 0.6
n3 polyenes	12.7 ± 0.9	10.5 ± 0.7	12.3 ± 1.2	12.0 ± 0.4	7.4 ± 0.2* †	9.2 ± 0.8 †
n6 polyenes	22.8 ± 2.8	22.1 ± 0.7	22.5 ± 1.2	21.4 ± 1.3	21.8 ± 1.3	23.5 ± 0.8
UI	156 ± 15	149 ± 9‡	149 ± 9	146 ± 4	121 ± 2	138 ± 6
Chain length	15.7 ± 0.1	16.0 ± 0.1	15.7 ± 0.2	15.7 ± 0.1	16.7 ± 0.1* †	16.2 ± 0.1 †

Values are expressed as means ± SE

Fatty acids with a percent mole fractions below 1 % are not shown

SS subsarcolemmal mitochondria, *IMF* intermyofibrillar mitochondria, *UI* unsaturation index = $\sum m_i \times n_i$, where m_i is the mole percent and n_i is the number of carbon–carbon double bonds of the fatty acid

‡ Denotes significance from SS within the same tissue

* and † denote significance from heart and red muscle, respectively, within the same mitochondrial subpopulation

homogenate was centrifuged at 800×*g* for 10 min to separate the SS and IMF mitochondria. The IMF mitochondria were released by treating the pellet with a protease (0.025 ml/g Subtilisin A (P-5380); Sigma, St. Louis, MO, USA) for exactly 5 min to digest the myofibrils. Further centrifugation was used to remove the myofibrils, and mitochondria were finally recovered by centrifuging twice at 10,000×*g* for 10 min and resuspending the final pellet in 500 µl of buffer (220 mM sucrose, 70 mM mannitol, 10 mM Tris HCl, and 1 mM EDTA). A portion of the mitochondria (200 µl) was used immediately for determinations of palmitate oxidation, while the remaining mitochondrial protein was purified through the use of a Percoll gradient (Benton et al. 2004) and frozen for future phospholipid analyses. Mitochondrial protein concentration was determined by bicinchoninic acid assay.

Mitochondrial Palmitate Oxidation

Palmitate oxidation was measured in a sealed system, as described previously (Benton et al. 2008; Holloway et al. 2009b). Briefly, mitochondria were added to a pregassed modified Krebs-Ringer buffer at 37 °C. The reaction (30 min) was initiated by addition of palmitate–bovine serum albumin complex (75 µM, 1-[^{14}C]palmitate [0.5 µCi/vial, 30 min, 37 °C, pH 7.4]). Oxidation was determined from trapping of the $^{14}CO_2$ produced and the ^{14}C label released from acidified buffer, as we have reported previously (Benton et al. 2008; Campbell et al. 2004; Holloway et al. 2009b; Yoshida et al. 2007).

Phospholipid Analysis

Total lipids were extracted (Folch et al. 1957), and thin-layer chromatography (Mahadevappa and Holub 1987) was used to separate individual phospholipids (PC, PE, CL, phosphatidylinositol, phosphatidylserine, phosphatidic acid, sphingomyelin) from isolated SS and IMF mitochondria. Briefly, isolated phospholipids were methylated (Mahadevappa and Holub 1987), and the fatty acid composition of each phospholipid was analyzed by gas chromatography (Bradley et al. 2008; Stefanyk et al. 2010). A 0.1–1.0 µl sample of methyl esters from each sample was injected into a gas chromatograph (Trace GC Ultra, Thermo Electron, Milan,

Table 4 Percent mole fraction of cardiolipin fatty acids of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle

Fatty acid	Heart		Red muscle		White muscle	
	SS	IMF	SS	IMF	SS	IMF
14:0	2.5 ± 0.3	4.3 ± 0.8	4.4 ± 0.8	3.1 ± 0.2	6.5 ± 1.7	3.9 ± 0.2
15:0	1.7 ± 0.4	1.9 ± 0.3	3.0 ± 0.9	1.4 ± 0.1	3.2 ± 1.1	3.2 ± 0.8
16:0	11.7 ± 1.6	20.5 ± 3.6	14.5 ± 2.5	14.5 ± 1.5	17.4 ± 3.3	17.4 ± 1.7
17:0	2.4 ± 0.5	3.1 ± 0.4	3.2 ± 0.8	3.3 ± 0.7	2.8 ± 0.5	3.9 ± 0.3
18:0	5.8 ± 0.8	11.5 ± 1.4‡	6.8 ± 1.6	6.8 ± 0.5	7.6 ± 1.9	10.7 ± 1.4
16:1	2.5 ± 0.6	3.0 ± 0.5	2.9 ± 1.0	3.1 ± 0.6	5.7 ± 1.1	3.1 ± 0.8
18:1	3.9 ± 0.7	5.0 ± 1.5	7.5 ± 4.3	7.4 ± 3.6	10.0 ± 4.2	5.6 ± 1.6
18:2n6	57.8 ± 5.4	32.1 ± 9.1	34.9 ± 7.1*	38.3 ± 3.5	25.8 ± 5.7*	29.0 ± 7.8
18:3n6	2.2 ± 0.5	3.2 ± 1.8	1.4 ± 0.6	2.2 ± 1.2	1.3 ± 0.6	4.4 ± 1.9
20:3n6	1.3 ± 0.4	1.6 ± 0.6	1.8 ± 0.5	2.2 ± 1.0	1.3 ± 0.3	1.1 ± 0.5
Total saturates	26.5 ± 3.7	39.5 ± 6.4	35.7 ± 4.8	35.8 ± 4.0	42.5 ± 3.5	45.3 ± 5.2
Total monoenes	9.5 ± 1.6	11.0 ± 1.0	21.2 ± 5.4	15.5 ± 4.3	20.1 ± 5.7	15.4 ± 1.7
n3 polyenes	1.1 ± 0.1	1.2 ± 0.3	2.1 ± 0.5	2.0 ± 0.5	1.6 ± 0.5	2.1 ± 0.2
n6 polyenes	62.7 ± 5.0	48.3 ± 6.5	40.8 ± 6.4	46.6 ± 3.0	30.3 ± 6.2*	37.1 ± 6.3
UI	144 ± 9	118 ± 13	115 ± 9*	124 ± 7	98 ± 4*	105 ± 9
Chain length	17.4 ± 0.1	17.4 ± 0.1	17.1 ± 0.2	17.6 ± 0.1‡	17.1 ± 0.1	17.1 ± 0.1* †

Values are expressed as means ± SE. Fatty acids with a percent mole fractions below 1 % are not shown

SS subsarcolemmal mitochondria, *IMF* intermyofibrillar mitochondria, *UI* unsaturation index = $\sum m_i \times n_i$, where m_i is the mole percent and n_i is the number of carbon–carbon double bonds of the fatty acid

* and † denote significance from heart and red muscle, respectively, within the same mitochondrial subpopulation; ‡ denotes significance from SS within the same tissue

Italy) fitted with a split/splitless injector, a fast flame ionization detector, and Triplus AS autosampler (Trace GC Ultra, Thermo Electron). Fatty acid methyl esters were separated on a UFM RTX-WAX analytical column (Thermo Electron) using helium as a carrier gas. Fatty acids were identified by comparison of retention times with those of a known standard (Supelco 37 component FAME mix, Supelco, Bellefonte, PA), and absolute amounts of individual fatty acids were calculated with the aid of the internal standard, tridecanoic acid (13:0), added to the samples before the methylation process (Lepage and Roy 1986). Preliminary analyses indicated no detectable endogenous 13:0 in the samples analyzed (data not shown). The molar amount of each fatty acid was then used to calculate its relative percentage. Total amounts of each phospholipid were determined from the summed amount of fatty acids in each phospholipid. The UI was calculated as $\sum m_i \times n_i$, where m_i is the mole percentage and n_i is the number of carbon–carbon double bonds of the fatty acid.

Statistical Analysis

All values are expressed as the mean ± standard error (SE). Differences in CS activity were examined by an analysis of variance (ANOVA). A two-way ANOVA was used to establish differences between tissue and mitochondrial subpopulations for palmitate oxidation rates and membrane lipid composition data. Tukey's post hoc test was used to determine significance ($p < 0.05$). Assumption for normality were verified and data transformed (log, square root, and inverse square) to meet this assumption. Pearson correlations were used to test the relationship between palmitate oxidation rates and membrane lipid components. SPSS software (Chicago, IL) was used for all statistical analyses.

Results

Total CS and Mitochondrial Palmitate Oxidation Hierarchy

Total homogenate CS enzymatic activity displayed the expected hierarchy between heart → red → white muscles (Fig. 1a), confirming the oxidative potential of muscles subsequently used to isolate SS and IMF mitochondria. Isolated mitochondrial CS values were not significantly different between muscles, but were significantly higher in IMF mitochondria within tissues (Fig. 1b). In general, mitochondrial rates of palmitate oxidation displayed the

Table 5 Percent mole fraction of sphingomyelin fatty acids of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle

Fatty acid	Heart		Red muscle		White muscle	
	SS	IMF	SS	IMF	SS	IMF
12:0	1.3 ± 0.3	1.8 ± 0.7	1.3 ± 0.3	1.6 ± 0.2	1.5 ± 0.1	1.3 ± 0.2
14:0	7.4 ± 0.6	8.3 ± 0.9	8.0 ± 0.4	7.5 ± 0.7	8.3 ± 0.4	7.7 ± 1.0
15:0	3.3 ± 0.3	4.0 ± 0.8	3.5 ± 0.3	3.7 ± 0.6	3.5 ± 0.3	4.0 ± 0.5
16:0	29.1 ± 2.6	23.0 ± 2.2	31.0 ± 1.0	26.3 ± 2.0	31.0 ± 1.0	23.9 ± 4.1
17:0	5.2 ± 0.4	5.1 ± 1.2	5.6 ± 0.5	5.0 ± 0.7	5.6 ± 0.5	4.5 ± 1.2
18:0	15.4 ± 0.6	10.5 ± 3.6	18.4 ± 0.5	17.2 ± 1.7	18.4 ± 0.5	23.5 ± 4.1
14:1	4.2 ± 0.4	4.0 ± 0.4	4.7 ± 0.4	3.8 ± 0.6	4.7 ± 0.4	6.7 ± 3.4
15:1	2.3 ± 0.2	2.2 ± 0.3	2.6 ± 0.1	2.1 ± 0.3	2.4 ± 0.2	2.1 ± 0.4
16:1	5.7 ± 1.6	6.3 ± 1.0	7.8 ± 1.3	7.1 ± 1.0	7.8 ± 1.3	5.3 ± 1.5
17:1	1.0 ± 0.2	1.8 ± 0.5	1.8 ± 0.3	1.0 ± 0.3	1.5 ± 0.2	2.0 ± 0.8
18:1	3.6 ± 1.0	10.1 ± 5.7	3.1 ± 1.1	9.9 ± 5.9	3.1 ± 1.1	4.6 ± 2.0
18:2n6	1.9 ± 0.4	1.3 ± 0.5	1.3 ± 0.4	3.2 ± 0.5‡	1.8 ± 0.5	1.9 ± 0.8
18:3n6	4.4 ± 1.9	5.7 ± 0.5	2.8 ± 1.1	2.7 ± 1.1	2.6 ± 0.7	3.5 ± 1.0
20:3n6	1.7 ± 0.6	2.1 ± 0.7	2.9 ± 0.5	2.1 ± 0.4	1.4 ± 0.4	1.5 ± 0.5
Total saturates	66.9 ± 2.9	62.0 ± 2.9	69.0 ± 2.9	63.2 ± 4.7	70.5 ± 1.9	68.5 ± 5.8
Total monoenes	17.8 ± 2.5	20.3 ± 2.7	19.4 ± 1.7	24.7 ± 5.5	20.8 ± 1.3	21.1 ± 5.3
n3 polyenes	4.6 ± 1.2	3.7 ± 1.1	2.2 ± 0.7	1.9 ± 0.5	1.1 ± 0.3	1.4 ± 0.5
n6 polyenes	10.4 ± 1.1	13.4 ± 1.1	8.7 ± 1.1	10.1 ± 1.2	7.5 ± 0.8	8.9 ± 0.8
UI	67 ± 8	69 ± 5	54 ± 7	60 ± 5	46 ± 3	51 ± 7
Chain length	16.6 ± 0.1	16.6 ± 0.1	16.6 ± 0.1	16.6 ± 0.1	16.4 ± 0.1	16.7 ± 0.2

[a] Values are expressed as means ± SE

Fatty acids with a percent mole fractions below 1 % are not shown

SS subsarcolemmal mitochondria, *IMF* intermyofibrillar mitochondria, *UI* unsaturation index = $\sum m_i \times n_i$, where m_i is the mole percent and n_i is the number of carbon–carbon double bonds of the fatty acid

* and † denote significance from heart and red muscle, respectively, within the same mitochondrial subpopulation; ‡ denotes significance from SS within the same tissue

expected hierarchy (heart → red → white), and as a result, rates of palmitate oxidation in mitochondria across all muscles displayed a 2.5-fold range (Fig. 2), creating the required continuum to determine potential relationships between membrane lipid composition and rates of mitochondrial fatty acid oxidation.

Membrane Lipid Composition

The hierarchy of the membrane lipid results presented is the phospholipid species independent of fatty acid subclasses (Fig. 3), the major fatty acids independent of phospholipid species in SS (Fig. 4a), and IMF (Fig. 4b) mitochondria, and finally a more in-depth examination of the individual fatty acids represented in individual phospholipid species.

In general, mitochondrial membrane phospholipid head group composition did not differ between tissues types or mitochondrial subpopulations, with the exception of heart SS mitochondria, which displayed higher relative abundance of PE and a lower PC/PE ratio compared to red and white muscle (Fig. 3), whereas SS mitochondria had a higher PC/PE compared to IMF mitochondria within white muscle. White muscle SS mitochondrial membranes had a lower relative abundance of n6 polyunsaturated fatty acid (Fig. 4a) compared to heart, which was mainly due to lower 18:2n6 and 20:4n6 (Table 1), and to lower UI compared to the other muscle tissues.

The fatty acid composition of the predominant (PC and PE) and mitochondrial-specific (CL) phospholipid species demonstrated unique trends between muscle types. PC of heart mitochondria demonstrated longer chain length, whereas red muscle IMF mitochondria had slightly less total saturates (mainly 18:0; Table 2). PE of white muscle mitochondria had longer chain lengths and less n3 polyunsaturates (mainly 22:6n3; Table 3), whereas CL of heart SS mitochondria had higher n6 polyunsaturates (mainly 18:2n6), resulting in a higher UI (Table 4) compared to other muscle types. Few differences were seen in the other phospholipid species (Tables 5, 6, 7).

Table 6 Percent mole fraction of phosphatidylinositol fatty acids of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle

Fatty acid	Heart		Red muscle		White muscle	
	SS	IMF	SS	IMF	SS	IMF
14:0	5.7 ± 1.0	4.5 ± 1.1	6.3 ± 0.5	5.9 ± 0.3	4.2 ± 1.3	4.5 ± 0.8
15:0	2.7 ± 0.4	3.2 ± 0.3	3.1 ± 0.3	2.5 ± 0.3	2.2 ± 0.8	2.9 ± 0.5
16:0	15.8 ± 2.4	21.4 ± 2.7	24.1 ± 2.1	22.3 ± 2.3	17.8 ± 3.8	18.7 ± 1.9
17:0	3.5 ± 0.6	4.2 ± 0.8	4.6 ± 0.3	4.1 ± 0.5	4.4 ± 0.5	5.3 ± 0.3
18:0	26.8 ± 4.6	27.7 ± 4.3	26.4 ± 2.4	27.3 ± 2.1	21.6 ± 3.0	29.2 ± 3.9
14:1	5.7 ± 2.4	2.2 ± 0.5	3.3 ± 0.3	2.8 ± 0.3	1.9 ± 0.6	2.3 ± 0.5
15:1	1.8 ± 0.2	1.7 ± 0.3	2.0 ± 0.2	1.7 ± 0.2	1.2 ± 0.4	1.8 ± 0.3
16:1	2.4 ± 1.0	4.7 ± 1.0	5.5 ± 1.3	5.7 ± 1.1	2.5 ± 1.1	3.9 ± 0.8
18:1	5.5 ± 1.5	4.1 ± 1.1	4.2 ± 0.7	9.3 ± 4.8	10.5 ± 5.3	3.4 ± 1.0
20:3n3	5.3 ± 2.3	1.9 ± 0.8	4.5 ± 1.4	4.4 ± 0.8	2.2 ± 0.9	2.7 ± 1.1
18:2n6	1.7 ± 0.6	1.4 ± 0.4	2.0 ± 0.3	1.7 ± 0.2	1.6 ± 0.5	1.2 ± 0.3
18:3n6	2.5 ± 0.9	4.6 ± 0.6	2.1 ± 0.7	1.1 ± 0.7*	1.9 ± 0.5	3.7 ± 1.0 †
20:3n6	1.3 ± 0.5	1.9 ± 0.5	2.4 ± 0.1	2.2 ± 0.3	1.4 ± 0.2	1.9 ± 0.8
Total saturates	61.2 ± 3.7	65.0 ± 3.6	68.7 ± 2.0	66.5 ± 5.2	57.3 ± 9.5	69.9 ± 3.0
Total monoenes	18.3 ± 4.4	19.3 ± 1.8	17.0 ± 2.0	21.9 ± 4.9	22.9 ± 6.2	13.3 ± 2.5
n3 polyenes	5.3 ± 3.4	3.8 ± 1.4	2.1 ± 0.3	1.4 ± 0.3	3.7 ± 1.5	2.9 ± 1.2
n6 polyenes	15.1 ± 3.1	11.7 ± 0.7	11.9 ± 2.4	10.3 ± 1.4	15.1 ± 5.1	11.6 ± 2.5
UI	83 ± 9	66 ± 11	64 ± 8	61 ± 8	63 ± 13	61 ± 12
Chain length	17.2 ± 0.2	17.1 ± 0.2	16.9 ± 0.1	17.0 ± 0.1	16.7 ± 0.1	17.4 ± 0.2‡

Values are expressed as means ± SE

Fatty acids with a percent mole fractions below 1 % are not shown

SS subsarcolemmal mitochondria, *IMF* intermyofibrillar mitochondria, *UI* unsaturation index $= \sum m_i \times n_i$, where m_i is the mole percent and n_i is the number of carbon–carbon double bonds of the fatty acid

* and † denote significance from heart and red muscle, respectively, within the same mitochondrial subpopulation, ‡ denotes significance from SS within the same tissue

Relationship between Palmitate Oxidation Rate and Membrane Lipids

In SS and IMF mitochondria from heart, red, and white muscle, there was a significant positive correlation between palmitate oxidation rates and the UI ($r = 0.57$, $p < 0.05$, Fig. 5a). Rates of palmitate oxidation were also positively correlated with the percent mole fraction of CL ($r = 0.49$, $p < 0.05$, Fig. 5b). There were no significant correlations between palmitate oxidation and the percent mole fraction of other phospholipid head groups (PC, PE, sphingomyelin, phosphatidylinositol, and phosphatidylserine), average fatty acid chain length, or PC/PE ratio of mitochondrial membranes (data not shown).

Discussion

Our primary focus was to determine the potential association between rates of LCFA oxidation and membrane lipid composition in isolated mitochondria. We examined rates of fatty acid oxidation in SS and IMF mitochondria from muscles with variable oxidative capacities (heart, and red and white skeletal muscles) and related these values to membrane lipid composition. Our data demonstrate that regardless of subpopulation or muscle origin, mitochondria are similar in lipid composition, and that rates of mitochondrial palmitate oxidation are positively related to the lipid unsaturation and CL abundance. These data suggest that mitochondrial membrane lipid composition may influence mitochondrial substrate utilization in a structure–function relationship.

Mitochondrial Membrane Lipid Composition

In the current study, PC, PE, and CL comprised the majority of the phospholipid profiles in all mitochondria, accounting for ~80 % of the phospholipid content. This is comparable to what we (Stefanyk et al. 2010) and others (Daum 1985) have previously reported. However, previous literature has exclusively examined SS mitochondrial lipid composition, and to our knowledge this is the first report

Table 7 Percent mole fraction of phosphatidylserine fatty acids of subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial membranes in rat heart, red (gastrocnemius and vastus lateralis), and white (gastrocnemius and vastus lateralis) muscle

Fatty acid	Heart		Red muscle		White muscle	
	SS	IMF	SS	IMF	SS	IMF
12:0	2.4 ± 0.5	1.8 ± 0.5	1.7 ± 0.3	1.7 ± 0.3	1.1 ± 0.2	1.2 ± 0.3
14:0	7.0 ± 0.6	7.3 ± 1.1	8.4 ± 0.7	6.7 ± 0.7	5.1 ± 1.2	4.9 ± 1.3
15:0	3.3 ± 0.3	3.6 ± 0.3	4.5 ± 0.5	3.0 ± 0.5‡	2.9 ± 0.9	2.4 ± 0.5
16:0	25.2 ± 2.1	25.6 ± 3.0	24.9 ± 1.6	26.5 ± 3.2	18.5 ± 3.7	23.4 ± 5.4
17:0	5.4 ± 0.7	5.6 ± 1.0	5.7 ± 0.3	4.6 ± 0.6*	3.7 ± 0.8	4.3 ± 0.4*
18:0	22.5 ± 3.3	22.7 ± 4.5	18.9 ± 2.1	20.4 ± 1.5	14.4 ± 3.1	21.4 ± 4.0
14:1	3.5 ± 0.7	3.5 ± 0.6	5.4 ± 0.7	3.6 ± 0.5	2.8 ± 0.6 †	2.3 ± 0.8
15:1	2.3 ± 0.2	2.0 ± 0.4	2.7 ± 0.2	2.0 ± 0.4	1.6 ± 0.3 †	1.5 ± 0.4
16:1	5.0 ± 1.0	5.4 ± 1.1	6.2 ± 1.3	4.7 ± 1.1	5.7 ± 1.5	4.0 ± 1.6
18:1	3.9 ± 1.2	3.6 ± 1.1	4.8 ± 0.7	11.0 ± 6.2	8.7 ± 5.4	7.5 ± 4.1
18:2n6	1.3 ± 0.8	0.9 ± 0.3	1.5 ± 0.1	2.0 ± 0.4	1.4 ± 0.3	1.0 ± 0.3
18:3n6	3.9 ± 0.4	3.6 ± 0.9	2.5 ± 0.9	3.1 ± 1.0	3.3 ± 0.6	5.5 ± 2.7
20:3n6	1.5 ± 0.6	2.1 ± 0.6	2.9 ± 0.5	2.2 ± 0.3	2.7 ± 0.5	5.5 ± 4.5
Total saturates	70.2 ± 3.4	67.2 ± 2.6	67.3 ± 2.7	65.5 ± 6.1	57.5 ± 5.5	73.8 ± 3.9‡
Total monoenes	16.9 ± 3.1	19.5 ± 2.5	21.1 ± 2.4	22.9 ± 6.4	22.5 ± 6.0	18.7 ± 3.5
n3 polyenes	2.3 ± 0.8	3.2 ± 0.7	3.2 ± 0.5	2.1 ± 0.6	5.1 ± 3.6	1.1 ± 0.1
n6 polyenes	10.4 ± 0.9	10.1 ± 0.8	8.1 ± 1.3	9.3 ± 1.0	15.8 ± 4.6	6.4 ± 1.3
UI	56 ± 3	54 ± 9	60 ± 5	59 ± 7	79 ± 20	60 ± 17
Chain length	16.7 ± 0.1	16.7 ± 0.1	16.5 ± 0.1	16.6 ± 0.1	17.1 ± 0.3	16.5 ± 0.3

Values are expressed as means ± SE

Fatty acids with a percent mole fractions below 1 % are not shown

SS subsarcolemmal mitochondria, *IMF* intermyofibrillar mitochondria, *UI* unsaturation index $= \sum m_i \times n_i$, where m_i is the mole percent and n_i is the number of carbon–carbon double bonds of the fatty acid

* and † denote significance from heart and red muscle, respectively, within the same mitochondrial subpopulation, ‡ denotes significance from SS within the same tissue

comparing membrane lipid analysis in SS and IMF mitochondria. In general, the lipid composition between SS and IMF mitochondria was similar across all muscles, and therefore the well-established observation that IMF mitochondria display higher rates of respiration cannot be explained by variances in lipid membrane composition. We have previously shown that the lipid composition of SS mitochondria from the soleus muscle differs from that isolated from either the red gastrocnemius or plantaris muscles (Stefanyk et al. 2010). Taken together with the current data, it appears that the soleus muscle may represent a unique muscle, as all other muscles studied from our group have similar lipid composition (current study and Stefanyk et al. 2010).

Relationships between Mitochondrial Membrane Lipid Composition and Fatty Acid Oxidation

There is controversy in the literature regarding the association between metabolic function and membrane lipids in muscle (Blackard et al. 1997; Kriketos et al. 1995). Past literature has limited membrane lipid analysis to whole muscle and does not provide a direct measure of substrate utilization. Therefore, we compared LCFA oxidation rates with various lipid composition determinants of isolated and purified mitochondria.

We have observed that the rates of fatty acid oxidation may in part be influenced by the degree of membrane lipid unsaturation, as across all muscles, the total phospholipid UI (influenced mainly by 18:2n6 and 20:4n6) was positively correlated with rates of LCFA oxidation. This observation may largely reflect the relationship between CL content and fatty acid oxidation, which mirrored that of the UI. CL is highly unsaturated, with polyunsaturated fatty acids (mainly 18:2n6) being the predominant fatty acid class. In contrast, while membranes with a higher composition of PC are more fluid than those composed primarily of PE (reviewed in Spector and Yorek 1985), PC and PE abundance, or the PC/PE ratio did not significantly correlate with LCFA oxidation rates. Therefore, tissue differences in PE and PC/PE cannot

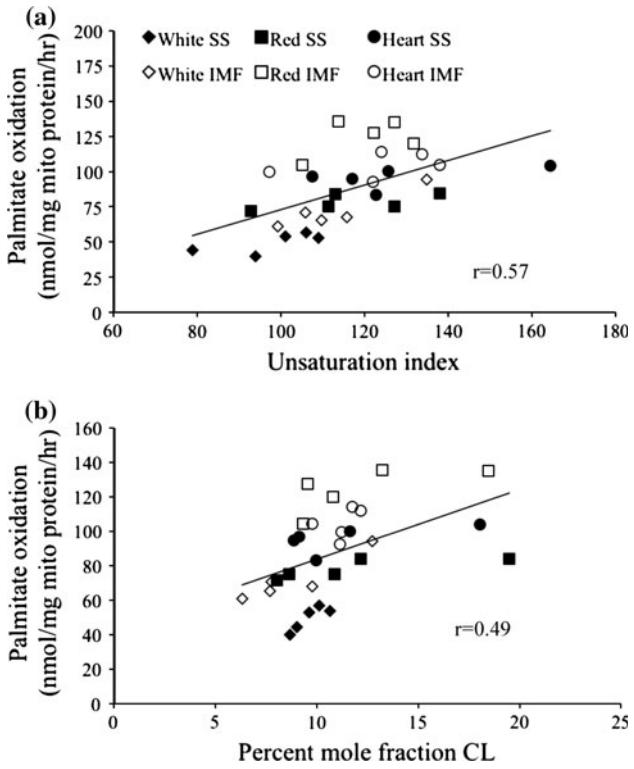

Fig. 5 Correlation between **a** palmitate oxidation rate and unsaturation index (UI) ($r = 0.57$; $p < 0.05$) and **b** palmitate oxidation rate and percent mole fraction of cardiolipin (CL) ($r = 0.49$; $p < 0.05$) in subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria isolated from heart (H), red (R; gastrocnemius and vastus lateralis), and white (W; gastrocnemius and vastus lateralis) muscle. Values are means ± SE ($n = 5$)

account for the observed variance in basal rates of LCFA oxidation in the current study. Altogether, we demonstrate and association between rates of fatty acid oxidation and the degree of membrane total phospholipid unsaturation and percent mole fraction of CL, but not percent mole fraction of PC or PE, in muscle tissue.

In the current study, the relationship between LCFA oxidation and some lipid composition surrogates of membrane fluidity (specifically percent mole fraction of CL and total phospholipid unsaturation) indicates that membrane structure may influences function of integral membrane proteins. In support of this, previous studies utilizing diet, exercise, or both to increase unsaturated lipids within mitochondrial membranes have demonstrated increased CPTI activity in liver (Karanth and Jeevaratnam 2010; Power et al. 1994). In addition, hepatic mitochondrial CL content has been shown to be positively correlated with CPTI activity (Mynatt et al. 1994) as well as the rate of accumulation palmitoylcarnitine, the CPTI reaction product (Pande et al. 1986). Therefore, previous data exist to suggest that mitochondrial membrane lipid composition can influence CPTI activity. This study is the first to examine this specific relationship in muscle tissue. In addition, CPTI is not the only site of regulation in mitochondrial LCFA oxidation, and therefore the current approach provides further support that percent mole fraction of CL and total phospholipid unsaturation can influence overall mitochondrial function. However, a limitation to this approach is that the exact location of influence cannot be determined, and it remains to be determined whether membrane lipid unsaturation and percent mole fraction of CL influences several integral proteins. In addition, only 33 % and 24 % of the variation can be attributed to CL percent mole fraction and total phospholipid unsaturation, respectively, leaving 67–76 % of the variation unexplained. Future research specifically examining the relative abundance of CL, fatty acid unsaturation, and function of integral membrane proteins located within the inner mitochondrial membrane will, we hope, rectify these issues.

In conclusion, the current data demonstrate a significant positive relationship between rates of mitochondrial palmitate oxidation, CL abundance, and unsaturation in SS and IMF mitochondria isolated from muscles with varying oxidative potential. These data suggest that mitochondrial membrane lipid composition may influence mitochondrial substrate utilization in a structure–function relationship.

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