J Membrane Biol (2010) 234:207–215
DOI 10.1007/s00232-010-9247-4

Skeletal Muscle Type Comparison of Subsarcolemmal Mitochondrial Membrane Phospholipid Fatty Acid Composition in Rat

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Received: 23 November 2009 / Accepted: 4 March 2010 / Published online: 25 March 2010

Abstract The phospholipid composition of membranes can influence the physiological functioning of the cell or subcellular organelle. This association has been previously demonstrated in skeletal muscle, where cellular or subcellular membrane, specifically mitochondria, phospholipid composition is linked to muscle function. However, these observations are based on whole mixed skeletal muscle analysis, with little information on skeletal muscles of differing fiber-type compositions. These past approaches that used mixed muscle may have misidentified outcomes or masked differences. Thus, the purpose of this study was to compare the phospholipid fatty acid composition of subsarcolemmal (SS) mitochondria isolated from slow-twitch postural (soleus), fast-twitch highly oxidative glycolytic locomotory (red gastrocnemius), and fast-twitch oxidative glycolytic locomotory (plantaris) skeletal muscles. The main findings of the study demonstrated unique differences between SS mitochondrial membranes from postural soleus compared to the other locomotory skeletal muscles examined, specifically lower percentage mole fraction of phosphatidylcholine (PC) and significantly higher percentage mole fraction of saturated fatty acids (SFA) and lower n6 polyunsaturated fatty acids (PUFA), resulting in a lower unsaturation index. We also found that although there was no difference in the percentage mole fraction of cardiolipin (CL) between skeletal muscle types examined, CL of soleus mitochondrial membranes were approximately twofold more SFA and approximately two-thirds less PUFA, resulting in a 20–30% lower unsaturation and peroxidation indices. Thus, the results of this study indicate unique membrane lipid composition of mitochondria isolated from different skeletal muscle types, a potential consequence of their respective duty cycles.

Keywords Soleus · Plantaris · Red gastrocnemius · Fiber type · Unsaturation index

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The structural properties of membranes can influence fundamental physiological processes such as cell signaling, intracellular transport, and energy metabolism (McIntosh and Simon 2006). Membrane structure is in part dependent on lipid composition, specifically the phospholipids and their respective fatty acid tails. In turn, changes in membrane structure can influence membrane protein function (Lee 2004; McIntosh and Simon 2006). The association between membrane composition and integral membrane proteins has been described as molecular (molecular interactions between lipids and protein molecules) and/or physical (physical properties of the lipid bilayer) (Lee 2004).

Energy-producing pathways are coupled to energy demands in skeletal muscle are linked to oxidative capacity (oxidative vs. glycolytic) and muscle function (e.g., postural vs. locomotory). The predominately slow-twitch type I postural muscle soleus has a high oxidative capacity (Delp and Duan 1996), relying on lipid and carbohydrate oxidative pathways for energy production (Baldwin et al. 1972). In contrast, the fast-twitch type IIB, and IID/X locomotory muscle plantaris has a lower oxidative capacity (Delp and Duan 1996) and utilize mainly glycolytic pathways (Baldwin et al. 1972). Although intermediate in

twitch properties but still locomotory, type IIA fibers such as red gastrocnemius (RG) have a higher oxidative capacity than soleus (Delp and Duan 1996). Thus, examination of skeletal muscle provides a unique experimental model to examine membrane structure as differences in function and ranges in oxidative energy production can be seen in varying fiber types. There has been conflicting evidence in the literature for a molecular association between metabolic activity and membrane structure (e.g., higher oxidative capacity correlated with higher membrane unsaturation) in whole muscle, with one study supportive (Kriketos et al. 1995) and another showing minor differences (Blackard et al. 1997). However, oxidative energy production in the form of adenosine triphosphate (ATP) is at the level of the mitochondria, and although most lipids are synthesized in the sarcoplasmic reticulum and transported intracellularly, different subcellular organelles may have unique membrane phospholipid fatty acid composition. Thus, examination of whole tissue may result in masked differences that may exist at the level of mitochondria.

From a molecular associative perspective, a caveat to increased unsaturated membranes to increase metabolic activity is the susceptibility of unsaturated fatty acids to oxidative damage (Hulbert 2005). This is especially relevant for membranes in proximity to the electron transport chain (ETC), being one of the primary sources of reactive oxygen species (ROS) (Cadenas and Davies 2000). Within skeletal muscle, two subpopulations of mitochondria have been identified; subsarcolemmal (SS) and intermyofibrillar (Ogata and Yamasaki 1985). Because of ease of extraction, most studies have focused on SS mitochondria. In addition, it has been speculated that SS mitochondria, as a result of proximity to the region of most active respiration, are the main contributor to ROS production (Skulachev 2001), and thus their membranes may be the most susceptible to oxidative damage. However, an important feedback mechanism exists that minimizes ROS damage through ROS-mediated proton leak (Brand and Esteves 2005). Given that ROS production is increased when oxygen availability is high and ATP demand is low in the presence of high substrate concentrations, similar to locomotory muscle at rest, by-products of unsaturated fatty acid peroxidation may be necessary to contribute to regulated proton leak. Mixed muscle analysis revealed a greater saturation in mitochondria compared to whole muscle homogenate (Tsalouhidou et al. 2006). Given that these findings are based on mixed muscle, it is unclear whether this trend extends down to mitochondria of individual skeletal muscles with differing oxidative capacity and contractile duty cycles.

Phosphatidylethanolamine (PE), phosphatidylcholine (PC), and cardiolipin (CL) are the predominant mitochondrial phospholipids (Daum 1985). PC and PE are the most abundant phospholipids, but each has a unique molecular shape, independent of fatty acid composition, that alters the membrane structure. Previous research has demonstrated that membranes rich in PC are more fluid compared to membranes rich in PE (Ladbrooke and Chapman 1969). CL is of particular interest with regard to skeletal muscle mitochondrial membrane lipids as a result of its enrichment in the inner mitochondrial membrane (Daum 1985) and the fact that its content is positively correlated with the molecular association of mitochondrial enzyme activities (Wicks and Hood 1991). Thus, in addition to total fatty acid composition independent of phospholipid species, to our knowledge, no study has examined the relative proportion or fatty acid composition of these main mitochondrial phospholipid species in different skeletal muscle types, given that they may be important modulator of molecular or physical membrane properties.

The purpose of this study was to isolate SS mitochondria from specific skeletal muscles with predominant fiber-type makeup and function and to compare membrane lipid composition. To represent differing functional and metabolically active skeletal muscle types, soleus (predominately slow-twitch type I and postural), RG (predominately fast-twitch type I and IIA and locomotory), and plantaris (predominately fast-twitch type IID/X and IIB and locomotory) were used (Armstrong and Laughlin 1985; Delp and Duan 1996). It was hypothesized that compared to locomotory muscles, SS mitochondrial membrane of postural muscle will contain less fluid (more saturated and lower PC compared to PE) as a result of contractile stress and less unsaturated as a result of less need for ROS-mediated proton leak regulation; further, the positive correlation between mitochondrial membrane saturation and skeletal muscle functional characteristics will extend to the predominant mitochondrial phospholipids (PC, PE, and CL).

Methods

Animals

Twelve Long-Evans rats (465 ± 43 g, ~4 months of age) used in this study were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada). The animals were housed in a controlled environment with a 12:12 h light–dark cycle and were fed standard rat chow (27% protein, 11% fat, 63% carbohydrate; 5012 Rat Diet, Lab Diet, Oakville, Ontario) ad libitum. The study and all protocols and procedures were approved by the Brock University Animal Care and Utilization Committee and conformed to all Canadian Council on Animal Care guidelines.

Tissue Collection and Mitochondrial Isolation

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (6 mg/100 g body weight) and select hind limb skeletal muscles (soleus, plantaris, RG) were extracted. Muscles from one leg were immediately frozen in liquid nitrogen, and muscles from the other leg were kept fresh at 4°C for isolation of SS mitochondria (Makinen and Lee 1968; Peters et al. 2001). A small piece of frozen whole muscle (~100 mg) was homogenized in 10 volumes of Tris HCl buffer (pH 8.0) and the resulting homogenate was used for Western blot and lipid analysis. Briefly, after weighing, muscles were coarsely minced in 10 volumes of ice-cold buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris base, 5 mM $MgSO_2$, 5 mM Na_2EDTA, 1 mM ATP, pH 7.4) then homogenized with a glass Potter. The supernatant was retained after centrifugation at 700 × *g* for 10 min, and a crude mitochondrial pellet was extracted with centrifugation at 14,000 × *g* for 10 min. The pellet was washed, resuspended, and pelleted twice (7000 × *g,* 10 min) in 10 volumes of 100 mM KCl, 40 Tris HCl, 10 mM Tris base, 1 mM $MgSO_4$, 0.1 Na_2EDTA, and 0.25 ATP (pH 7.4). The first wash buffer included 1% bovine serum albumin, and the second was protein free. The final mitochondrial pellet was resuspended in a volume corresponding to 1 ml/1 mg fresh muscle extracted. The final buffer contained 220 mM sucrose, 70 mM mannitol, 10 mM Tris HCl, and 0.1 mM Na_2EDTA (pH 7.4). All procedures were carried out at 0–4°C.

To calculate mitochondrial recovery and quality, citrate synthase (CS) activities on total muscle homogenate (CS_{homog}) and mitochondrial suspensions were measured as previously described (Peters et al. 2001). Briefly, a small volume of mitochondrial suspension was diluted 20-fold with the final sucrose and mannitol buffer and divided into two fractions. Extramitochondrial CS (CS_{em}) was measured in intact mitochondrial preparation, and CS activity in total suspension (CS_{ts}) was measured after the preparation was frozen and thawed twice to fracture mitochondria. Triton (0.1%) was included in the cuvette for measurement of CS_{ts} and CS_{homog}. Recovery of intact mitochondria was calculated as % fractional recovery = 100 × (CS_{ts} − CS_{em})/CS_{homog}, while quality of the mitochondrial preparation was calculated as % intact mitochondria = 100 × (CS_{ts} − CS_{em})/CS_{ts}.

Before Western blot and phospholipid analyses, mitochondrial fractions were purified by use of a Percoll gradient (Benton et al. 2004). Briefly, a 60% Percoll solution was added to the top of mitochondrial fractions and spun at 20,000 × *g* for 2 h. The middle layer was removed and spun again at 20,000 × *g* for 5 h to remove the remaining Percoll. The resulting mitochondria obtained were considered highly purified.

Western Blot Analysis

Western blot analysis (LeBlanc et al. 2008) was completed on isolated SS mitochondrial fractions to determine relative contamination of other membrane fractions. Briefly, whole muscle homogenate and isolated SS mitochondria were diluted in sample buffer containing 50 mM Tris HCl (pH 6.8), 2% (wt/vol) sodium dodecyl sulfate, 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, and 0.1% (wt/vol) bromophenol blue to a final protein concentration of 2 μg/μl. Standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on 16 μg of protein with a 4% stacking and 10% separating gel. Electrophoretically separated proteins were transferred onto polyvinylidene fluoride (Immobilon-P, Millipore, Bedford, MA) membrane with a Mini Trans-Blot (Bio-Rad Laboratories, Mississauga, Ontario, Canada) with a transfer buffer containing 25 mM Tris pH 8.3 192 mM glycine 20% methanol (vol/vol), and 0.1% sodium dodecyl sulfate (wt/vol). Membranes were incubated in Tris-buffered saline–Tween (TBST) buffer (20 mM Tris base, 137 mM NaCl, 0.1% [vol/vol] Tween 20, pH 7.5) with 5% (wt/vol) nonfat dry milk for 1 h to block all nonspecific binding sites. Membranes were then incubated for 1 h in 5% milk–TBST containing monoclonal antibodies against Na^+/K^+ ATPase α-1 (Santa Cruz Biotechnologies, CA) and dihydropyridine receptor (Affinity BioReagents, CO) for sarcolemmal and t-tubule membranes, GM130 (Santa Cruz Biotechnologies, CA) for Golgi membrane, sarco(endo)plasmic reticulum Ca^{2+}-ATPase 1 (SERCA1; Santa Cruz Biotechnologies, CA) for sarcoplasmic reticulum, emerin (Santa Cruz Biotechnologies, CA) for nucleus, and adenine nucleotide translocase (ANT; Mito Science, Oregon) and cytochrome *c* oxidase subunit IV (COX IV; Mito Science, Oregon) for outer and inner mitochondrial membranes, respectively. The membranes were washed and then incubated for 1–2 h in 5% milk-TBST–containing goat antimouse IgG (peroxidase conjugated, Sigma, Ontario, Canada). Membranes were again washed, and antibody–antigen complexes were visualized with a Fluorchem 5500 imaging station (Alpha Innotech, San Leandro, CA) after addition of chemiluminescent substrate (ChemiGlow West, Alpha Innotech, San Leandro, CA). Relative densities were quantified by AlphaEase FC chemiluminescent detection software (Alpha Innotech, San Leandro, CA). Blots were washed and stained with DB-71 (Hong et al. 2000), and total protein per lane was used to normalize loading between lanes on each blot.

Phospholipid Analysis

Total lipids from both whole muscle homogenate and isolated mitochondria were extracted (Folch et al. 1957),

and thin-layer chromatography (Mahadevappa and Holub 1987) was used to separate individual phospholipids (phosphatidylcholine, PC; phosphatidylethanolamine, PE; cardiolipin, CL; phosphatidylinositol, PI; phosphatidylserine, PS; and sphingomyelin, SM) from whole-muscle homogenate and SS mitochondria. 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). A 0.1–1.0-µl sample of methyl esters from each sample was injected into a gas chromatograph (Trace GC Ultra, Thermo Electron Corp, Milan, Italy) fitted with a split/splitless injector, a fast flame ionization detector and Triplus AS autosampler (Trace GC Ultra, Thermo Electron Corp, Milan, Italy). Fatty acid methyl esters were separated on an UFM RTX-WAX analytical column (Thermo Electron Corp., Milan, Italy) with helium used 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. 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 unsaturation index (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. The peroxidation index was calculated as $\sum m_i \times p_i$ where m_i is the mole percentage and p_i is the peroxidative susceptibility of a fatty acid (monoenoics = 0.25, dienoics = 1, trenoics = 2, tetraenoics = 4, pentaenoics = 6, and hexaenoics = 8).

Statistical Analysis

All values are expressed as mean ± standard error. Differences in the phospholipid fatty acid profile of whole muscle and SS mitochondria were analyzed by one-way analysis of variance (ANOVA; Sigma Stat 3.11, Systat Software, Chicago, IL) between skeletal muscle types. All pairwise comparisons were completed by a Tukey post hoc analysis. Assumptions for normality were verified for each test. Data was transformed (log, square root, and inverse square) to meet the above assumption or nonparametric statistics were run on data that did not meet the assumption (Kruskal–Wallis ANOVA by ranks). The level of statistical significance was set at $P \leq 0.05$ for all analysis.

Results

Mitochondrial Preparation

Mitochondrial recovery (soleus, 10 ± 2%; plantaris, 18 ± 2%; RG, 9 ± 2%) and quality (soleus, 79 ± 5%; plantaris, 89 ± 3%; RG, 83 ± 3%) were similar between skeletal muscle types and comparable to previous studies (LeBlanc et al. 2007, 2008; Peters et al. 2001). Western blot analyses revealed negligible contamination from other potential membrane fractions in isolated SS mitochondria (Fig. 1).

SS Mitochondria Comparison among Skeletal Muscle Types

The hierarchy of the results presented are the phospholipid species independent of fatty acid subclasses (Fig. 2), the major fatty acid subclasses independent of phospholipid species (Fig. 3), and finally a more in-depth examination of

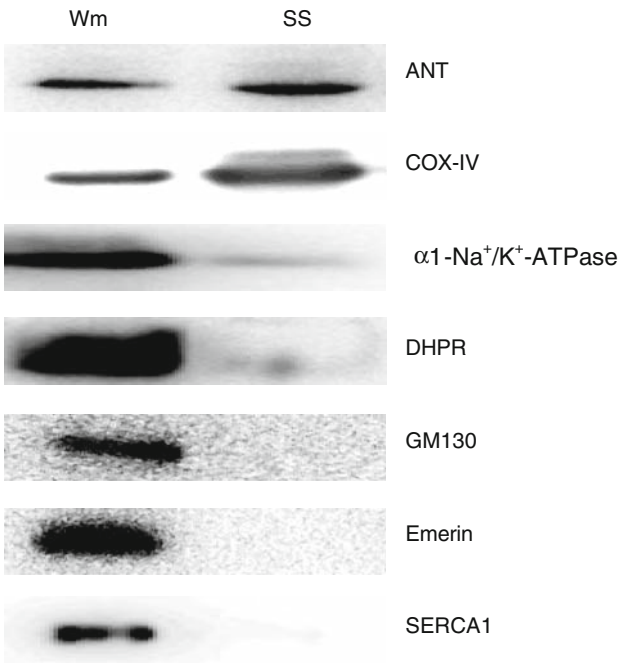

Fig. 1 Representative Western blots of whole muscle (Wm) and subsarcolemmal mitochondria (SS) revealed decreased contamination from sarcolemmal, transverse tubule, and Golgi membranes. Probed for mitochondrial, sarcolemmal/transverse tubule, Golgi apparatus, nucleus, and sarcoplasmic reticulum membrane proteins, respectively. ANT, adenine nucleotide translocase; COX-IV, cytochrome *c* oxidase subunit 4; α1-Na^+/K^+-ATPase, α1 subunit of sodium potassium ATPase; DHPR, dihydropyridine receptor; SERCA1, sarco(endo)plasmic reticulum Ca^{2+}-ATPase 1

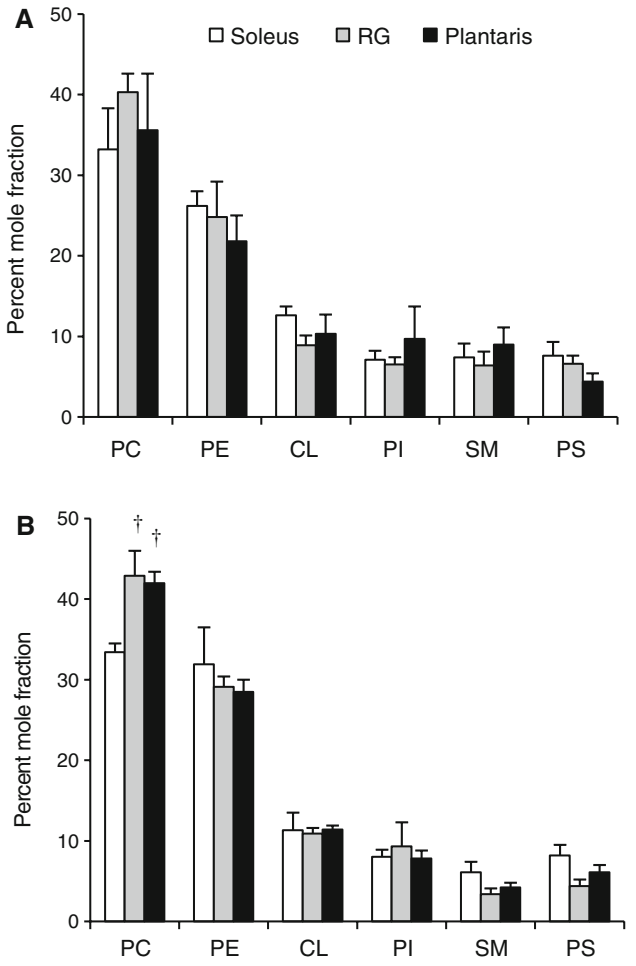

Fig. 2 Percentage mole fraction of major phospholipid species of **a** whole muscle and **b** subsarcolemmal mitochondrial membranes in rat soleus, red gastrocnemius (RG), and plantaris. Values are expressed as means ± standard error. † Significance from soleus. PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine

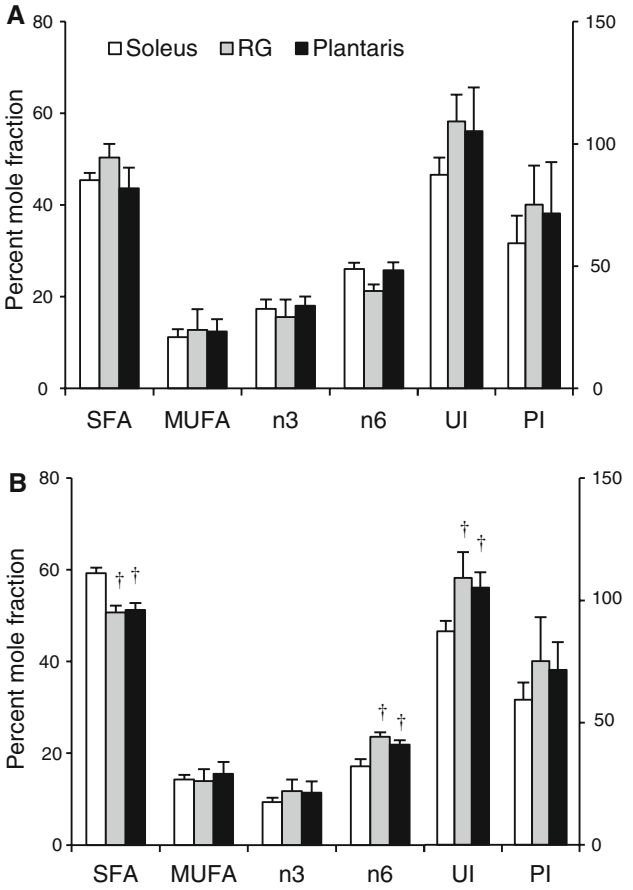

Fig. 3 Percentage mole fraction total fatty acid subclasses independent of phospholipid species of **a** whole muscle and **b** subsarcolemmal mitochondrial membranes in rat soleus, red gastrocnemius (RG), and plantaris. Values are expressed as means ± standard error with SFA, MUFA, n3, and n6 represented on the left *y*-axis and UI and PI represented on the right. †Significance from soleus. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; n3, n3 polyunsaturated fatty acid; n6, n6 polyunsaturated fatty acids; UI, unsaturation index $= \sum m_i \times n_i$, where m_i is the mole percentage and n_i is the number of carbon–carbon double bonds of the fatty acid; PI, peroxidation index $= \sum m_i \times p_i$ where m_i is the mole percentage and p_i is the peroxidative susceptibility of a fatty acid (monoenoics = 0.25, dienoics = 1, trenoics = 2, tetraenoics = 4, pentaenoics = 6, and hexaenoics = 8)

the individual fatty acids represented in the predominant mitochondrial phospholipid species (PC, PE, and CL; Table 1).

Whole muscle membrane composition did not differ between muscle types examined for both phospholipid species (Fig. 2a) and fatty acid subclasses (Fig. 3a). In contrast, SS mitochondrial membrane composition comparisons among skeletal muscle types revealed that soleus had significantly lower percentage mole fraction of PC compared to RG and plantaris (Fig. 2b). Soleus SS mitochondrial membranes were also significantly more saturated fatty acids (SFA) and contained less n6 polyunsaturated fatty acids (PUFA), resulting in a significantly lower unsaturation index compared to the other two skeletal muscle types examined (Fig. 3b).

The high SFA and low n6 PUFA seen in soleus SS mitochondrial membrane compared to the other muscles can be further examined by looking at the predominant phospholipid species, namely PC, PE, and CL (Table 1). A higher percentage mole fraction of SFA was mainly due to increased 14:0 and 16:0 in PE and CL and 18:0 in PC and CL, leading to lower unsaturation indices in PC and CL. In contrast, a lower percentage mole fraction of n6 PUFA was due to lower 18:2n6 in CL, which also contributed to a significantly lower peroxidation index in soleus SS mitochondria.

Table 1 Percentage mole fraction of the predominant phospholipid species of subsarcolemmal mitochondrial membranes in rat soleus, red gastrocnemius, and plantaris[a]

Fatty acid	PC			PE			CL		
	Soleus	RG	Plantaris	Soleus	RG	Plantaris	Soleus	RG	Plantaris
14:0	1.2 ± 0.2	0.5 ± 0.1†	0.6 ± 0.1†	2.0 ± 0.8	0.6 ± 0.1†	0.6 ± 0.1†	2.7 ± 0.8	1.4 ± 0.3†	0.9 ± 0.2†
16:0	35.0 ± 1.5	42.6 ± 1.5†	45.4 ± 1.0†	12.6 ± 0.9	8.5 ± 0.7†	8.8 ± 0.7†	17.3 ± 1.5	9.4 ± 2.3†	8.0 ± 1.6†
18:0	20.7 ± 1.1	10.4 ± 0.6†	9.9 ± 0.4†	33.4 ± 3.8	34.9 ± 2.0	34.7 ± 2.1	15.1 ± 1.6	8.7 ± 2.5†	6.8 ± 1.2†
16:1	1.9 ± 0.2	1.1 ± 0.3	1.4 ± 0.2	1.9 ± 0.2	1.0 ± 0.2†	1.0 ± 0.1†	2.8 ± 1.0	2.1 ± 0.4	2.3 ± 0.3
18:1	5.7 ± 0.4	5.4 ± 0.1	5.4 ± 0.2	4.3 ± 0.7	2.8 ± 0.2†	3.3 ± 0.5	8.2 ± 2.3	3.2 ± 0.2	6.4 ± 2.6
20:3n3	8.5 ± 0.4	10.8 ± 1.1	10.6 ± 0.4	7.9 ± 1.3	6.0 ± 0.5	6.3 ± 0.4	ND	ND	ND
22:6n3	1.8 ± 1.0	ND	2.4 ± 1.5	5.1 ± 2.6	6.5 ± 6.0	7.1 ± 3.7	ND	0.1 ± 0.1	0.1 ± 0.1
18:2n6	19.0 ± 1.9	21.7 ± 0.6	20.1 ± 0.6	7.7 ± 0.9	11.4 ± 0.5†	10.2 ± 0.4†	40.0 ± 6.1	69.2 ± 4.4†	71.1 ± 3.3†
Total saturates	59.8 ± 2.2	55.2 ± 1.9	57.4 ± 0.7	54.6 ± 1.4	45.9 ± 2.2†	46.0 ± 2.5†	40.6 ± 5.0	21.9 ± 5.0†	17.3 ± 2.9†
Total monoenes	9.2 ± 0.6	7.7 ± 0.6†	7.6 ± 0.3†	20.5 ± 3.6	25.4 ± 6.9	29.1 ± 5.5	13.9 ± 2.0	7.7 ± 1.2	10.9 ± 2.6
Total polyenes	31.0 ± 1.9	37.1 ± 2.3†	34.9 ± 0.9†	24.9 ± 2.7	28.7 ± 5.3	24.9 ± 3.2	45.5 ± 6.1	70.4 ± 4.4†	71.8 ± 3.5†
n3 polyenes	10.9 ± 1.2	14.1 ± 2.1†	13.9 ± 1.3†	14.5 ± 2.6	14.7 ± 5.6	14.1 ± 3.4	2.2 ± 1.5	0.3 ± 0.1	0.3 ± 0.2
n6 polyenes	20.0 ± 1.9	23.0 ± 0.7	21.0 ± 0.6	10.5 ± 0.8	13.9 ± 1.5	10.9 ± 0.4	43.2 ± 6.2	70.1 ± 4.3†	71.5 ± 3.4†
UI	89 ± 6	105 ± 12†	101 ± 7.3†	104 ± 13	122 ± 26	116 ± 15	111 ± 13	150 ± 9†	156 ± 6.1†
PI	57 ± 7	71 ± 16	68 ± 11	82 ± 20	99 ± 23	92 ± 27	59 ± 9	74 ± 5†	76 ± 4†

[a] Values are expressed as means ± standard error, percentage mole fractions of fatty acids below 0.1% across all phospholipid species and skeletal muscle types are not shown. SS, subsarcolemmal mitochondria; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; RG, red gastrocnemius; ND, not done; UI, unsaturation index = $\sum m_i \times n_i$ where m_i is the mole percentage and n_i is the number of carbon–carbon double bonds of the fatty acid; PI, peroxidation index = $\sum m_i \times p_i$, where m_i is the mole percentage and p_i is the peroxidative susceptibility of a fatty acid (monoenoics = 0.25, dienoics = 1, trenoics = 2, tetraenoics = 4, pentaenoics = 6, and hexaenoics = 8)

† Significance from soleus within a given phospholipid species

Discussion

To our knowledge, this represents the first study to provide a complete comparative membrane lipid analysis of SS mitochondria isolated from slow-twitch oxidative soleus, fast-twitch oxidative glycolytic RG, and fast-twitch glycolytic plantaris. This study demonstrated that despite no difference in membrane composition at the whole tissue level, unique differences existed between SS mitochondrial membranes from soleus compared to the other skeletal muscles examined. Specifically, we found an approximately one-third lower percentage mole fraction of PC and a significantly higher percent mole fraction of SFA and lower n6 PUFA, resulting in a lower unsaturation index. Further, although there was no difference in the percent mole fraction of CL between skeletal muscle types examined, CL of soleus mitochondrial membranes were approximately twofold more SFA and approximately two-thirds less PUFA, resulting in a 20–30% lower unsaturation and peroxidation indices. These findings indicate a unique relationship between membrane structure of mitochondria isolated from different muscle types and function, which are potentially explained through either molecular (molecular interactions between lipids and protein molecules) and/or physical (physical properties of the lipid bilayer) membrane factors.

Role of Phospholipid Species

The phospholipid profile of isolated SS mitochondria from skeletal muscle was composed mainly of PC, PE, and CL, which made up 76–80% of the phospholipid content. This is comparable to mitochondria from other mammalian cells (Daum 1985) and to previous studies utilizing similar methods of isolating skeletal muscle mitochondria (Fiehn et al. 1971; Hulbert et al. 2006; Tsalouhidou et al. 2006). The key difference from past studies (Fiehn et al. 1971; Hulbert et al. 2006; Tsalouhidou et al. 2006) is the current study examined specific skeletal muscle types, avoiding the use of mixed muscle. Analysis of differing skeletal muscle types has revealed proportionally less PC in SS mitochondrial membranes of slow-twitch postural soleus compared to fast-twitch locomotory RG and plantaris. This has revealed another possible level of membrane structural difference that may contribute to differences in functionality between these muscle types.

Phospholipid species have an impact on membrane structure, relative to their respective percentage molar

composition. PC and PE are the most abundant phospholipids found in mitochondrial membranes, but each has a unique molecular shape, independent of fatty acid composition, that alters membrane structure. Previous research has demonstrated that membranes rich in PC are more fluid compared to membranes rich in PE (Ladbrooke and Chapman 1969). This may be a consequence of PC possessing a cylindrical molecular shape, whereas the smaller head group of PE results in a conical shape (Cullis and de Kruijff 1979). Thus, a shift toward a lesser proportional representation of PC in soleus SS mitochondrial membranes may result in these membranes being less fluid compared to RG and plantaris. Physiologically, this may be an adaptive mechanism for mitochondria to be less fluid and less fragile in the face of chronic contractile stress in postural compared to locomotory muscles. Similar findings have been shown in the literature where acutely stressed cardiac mitochondria (Victor et al. 1985) and erythrocytes (Peuchant et al. 1988) have altered membrane composition (notably increased PC and decreased PE), making them more fragile.

Role of Fatty Acid Subclasses

In general, the distribution of the fatty acid subclasses (independent of phospholipid species) in isolated SS mitochondria in the current study are quite different compared to other studies that examined phospholipid fatty acid composition in rodent skeletal muscle mitochondria (Brand et al. 2002; Faulks et al. 2006; Fiehn et al. 1971; Hulbert et al. 2006; Tsalouhidou et al. 2006). The reason for this discrepancy may not be due to the mitochondrial population examined, because all previous studies utilized similar methods to ours of isolating mitochondria, but possibly due to the use of mixed hind limb muscle (Brand et al. 2002; Faulks et al. 2006; Fiehn et al. 1971; Hulbert et al. 2006; Tsalouhidou et al. 2006) rather than specific skeletal muscle types used in the current study. This discrepancy exemplifies the importance of looking at specific skeletal muscle types because each may have unique membrane lipid composition, and a mixed muscle analysis may mask important differences between muscles that are functionally and metabolically unique.

Individually, SS mitochondria from slow-twitch postural muscle demonstrated unique phospholipid fatty acid profiles compared to fast-twitch locomotory muscle. Specifically, soleus SS mitochondria had significantly higher percentage mole fraction of SFA and lower n6 PUFA compared to both plantaris and RG. Thus, it seems that these differences have less to do with a direct molecular association between lipids and intrinsic membrane proteins. This idea holds true even at the level of CL, an important mitochondrial phospholipid that interacts with a number of mitochondrial intrinsic membrane proteins, including those involve in oxidative phosphorylation (Claypool 2009). Alternatively, these changes may be associated with two potential consequences of a constantly active postural muscle (soleus) compared to transiently active locomotory muscles (RG and plantaris), more specifically peroxidized lipid-mediated regulation of rest-to-exercise transitional ROS production and adaptative membrane stability to contractile stress.

Given the important metabolic role of mitochondria, it is estimated that 2–4% of oxygen consumed is reduced to ROS as a result of a "leak" in the ETC (Sachdev and Davies 2008). ROS cause membrane damage, through lipid peroxidation, with PUFAs being more susceptible than monounsaturated fatty acids and SFA (Hulbert 2005). Previous research has shown mitochondria isolated from mixed muscle to be more saturated and less polyunsaturated, hypothesizing mitochondria may be more resistant to peroxidative damage (Tsalouhidou et al. 2006). This theory does not hold true in the case of soleus mitochondria. It has been observed in fish skeletal muscle that ROS production is accelerated at low respiratory rates when the ETC. complexes are reduced, resulting in fast-twitch glycolytic muscles producing more superoxides than slow-twitch oxidative skeletal muscle (Leary et al. 2003). Proton leak across the membrane can influence the efficiency of energy conversion and the tendency to produce ROS; it thus has been considered necessary to control the proton motive force so as to limit ROS damage (Brand 2005). It has been proposed that the main determinants of the proton leak may be the phospholipids and proteins in the inner membrane (Stuart et al. 2001), potentially mediated through signaling processes involving uncoupling proteins (UCP2 and -3 and ANT) and by-product of n6 PUFA peroxidation (Brand 2005; Echtay et al. 2005). Thus, when oxygen availability is high and ATP demand is low in the presence of high substrate concentrations, similar to skeletal muscle at rest, UCPs are upregulated by ROS (Brand 2005). However, during periods of high energy demand (e.g., exercise), oxidative phosphorylation would proceed with less ROS-mediated proton leak. This acute regulatory environment is characteristic of plantaris and RG as both are locomotory muscles (Armstrong and Laughlin 1985) with higher percentage PUFA compared to soleus to facilitate ROS-mediated regulation of proton leak during periods of low energy demand. In contrast, soleus is postural in nature (Armstrong and Laughlin 1985) and may not go through such drastic changes in energy demand, resulting in SS mitochondria having a lower unsaturation index.

In addition, the fatty acids associated with CL are thought to be the main target of ROS as a result of their proximity and highly unsaturated acyl chains, namely 18:2n6 (Ott et al. 2007). With an almost twofold higher

percentage mole fraction of 18:2n6 in RG and plantaris, this could potentially provide the necessary lipid substrate to regulate ROS production and proton leak during the transitions between rest and exercise. Thus, mitochondrial membrane structure as it relates to lipid peroxidation may be a parallel adaptation to facilitate the signaling process associated with proton leak.

The mitochondrial-specific phospholipid CL, despite a relatively lower proportional representation compared to PE and PC, plays a very important role as a result of its interactions with numerous mitochondrial proteins (Schlame et al. 2000). Small changes in the absolute amount of CL will greatly affect protein function (Klingenberg 2009; Schlame et al. 2000). Despite no significant difference in the percentage mole fraction of SS mitochondrial CL in all muscle types examined, the function of membrane proteins can also be influenced by fatty acid composition. Consistent with the current study, 40–80% of total CL in mammals has an atypical fatty acid profile, demonstrating fatty acid symmetry of predominantly 18:2–18:2 or 18:2–18:1 (Schlame et al. 2005). However, the significantly higher percentage mole fraction of SFA (predominately 16: and 18:0) and lower PUFA (predominately 18:2n6) in soleus SS mitochondrial CL compared to the other muscles examined may alter activity of various membrane proteins, as demonstrated in previous work (Kraffe et al. 2007; Yamaoka et al. 1988).

Comparable to the putative association between muscle contractile duty cycle and phospholipid species-mediated membrane stability, the same may be made for fatty acid composition. Thus, in addition to moderate changes in proportional representation of major phospholipid species in the postural soleus muscle, increased saturation could also contribute to membrane stability. Similar finding have been demonstrated in acute mechanically stressed erythrocytes (Peuchant et al. 1988) where more unsaturated and thus more fragile.

Perspectives and Significance

The present study analyzed whole muscle and isolated SS mitochondria from varying skeletal muscle types and compared their phospholipid fatty acid composition. This analysis has revealed a lack of difference in whole muscle membrane lipid composition between muscle types. The key differences in mitochondrial membrane structure were dependent on the skeletal muscle type examined and were not seen at the whole muscle level. These observations reveal the need to examine specific membrane fractions in isolation. In addition, given the differences between postural and locomotory muscles, this may change the view of the potential role of mitochondrial membrane composition on structural stability and metabolic regulation. This may ultimately impact the function of mitochondrial membranes under specific conditions (e.g., mitochondrial myopathic diseases) and perturbations (e.g., diet, training). Future research will need to characterize the specific lipid compositions of all subcellular organelles isolated and purified from other muscle types, including the subpopulation of mitochondria found between myofibrils (intermyofibrillar), which are thought to differ physiologically from SS mitochondria (Hood 2001). It is only through an in-depth analysis that a true structure function relationship can be examined.

Acknowledgments This study was supported by the Natural Sciences and Engineering Research Council of Canada (PJL, SJP, and BDR). LES was supported by a Master's Graduate Training Award from the Canadian Institutes of Health Research.

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