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Cardiolipin linoleic acid content and mitochondrial cytochrome c oxidase activity are associated in rat skeletal muscle

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ABSTRACT

Cardiolipin (CL) is an inner-mitochondrial membrane phospholipid that is important for optimal mitochondrial function. Specifically, CL and CL linoleic (18:2ω6) content are known to be positively associated with cytochrome c oxidase (COX) activity. However, this association has not been examined in skeletal muscle. In this study, rats were fed high-fat diets with a naturally occurring gradient in linoleic acid (coconut oil [CO], 5.8%; flaxseed oil [FO], 13.2%; safflower oil [SO], 75.1%) in an attempt to alter both mitochondrial CL fatty acyl composition and COX activity in rat mixed hind-limb muscle. In general, mitochondrial membrane lipid composition was fairly resistant to dietary treatments as only modest changes in fatty acyl composition were detected in CL and other major mitochondrial phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE). As a result of this resistance, CL 18:2ω6 content was not different between the dietary groups. Consistent with the lack of changes in CL 18:2ω6 content, mitochondrial COX activity was also not different between the dietary groups. However, correlational analysis using data obtained from rats across the dietary groups showed a significant relationship ($p = 0.009$, $R^2 = 0.21$). Specifically, our results suggest that CL 18:2ω6 content may positively influence mitochondrial COX activity thereby making this lipid molecule a potential factor related to mitochondrial health and function in skeletal muscle.

1. Introduction

Cardiolipin (CL) is a unique acidic membrane phospholipid found predominantly in the inner mitochondrial membrane (Frick et al., 2010; Hemmingsen et al., 1983; Robinson, 1993). Structurally, CL is comprised of three glycerols, two phosphates, and four unsaturated fatty acyl tails of which are mainly linoleic acid (18:2ω6) (Robinson, 1993). Functionally, CL is an important phospholipid in mitochondrial electron transport activity and is known to be required for maximal cytochrome c oxidase (COX, EC 1.9.3.1) activity (for review see (Robinson, 1993). Lipid-depletion studies have shown that there are four to five CL molecules that are strongly bound to the COX enzyme (Robinson and Capaldi, 1977; Vik and Capaldi, 1977; Vik et al., 1981). Removal of these boundary CL molecules leads to reductions in maximal COX activity which can be restored with exogenous CL phospholipids but not with other major mitochondrial phospholipids such as phosphatidylcholine (PC) or phosphatidylethanolamine (PE) (Fry and Green, 1980, 1981; Robinson et al., 1980; Vik et al., 1981).

Apart from the apparent requirement of CL head groups, the fatty acyl composition of CL may be another major determinant of COX activity. Studies in rat cardiac muscle demonstrate that CL is comprised of roughly 80% linoleic acid and reductions in this fatty acid accomplished through feeding dietary oils deficient in linoleic acid were followed with reductions in COX activity (Holloway et al., 2012; Yamaoka et al., 1988, 1990). Similar to that in heart, previous studies have shown that linoleic acid is a dominant fatty acid found in CL molecules obtained from rat skeletal muscle ranging from 25 to 70% mol fraction depending on muscle type and mitochondrial population (subsarcolemmal, SS or intermyofibrillar, IMF) (Holloway et al., 2012; Stefanyk et al., 2010; Tsalouhidou et al., 2006). Whether CL in skeletal muscle requires linoleic acid to maximize COX activity similar to that previously observed in rat heart muscle (Yamaoka et al., 1988, 1990) remains unknown.

In a previous study from our laboratory, rats fed dietary coconut oil (CO), safflower oil (SO), or flaxseed oil (FO) (20% by weight) for 65 days resulted in changes in femur lipid composition that reflected their respective diets (Lau et al., 2010). Fatty acid analysis of these diets revealed a gradient in the percent mole fraction of

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linoleic acid (CO, 5.8%; FO, 13.2%; SO, 75.1%) that was also reflected in the fatty acyl composition of both the proximal and distal epiphysis of the rat femurs (Lau et al., 2010). Thus, the purpose of this study was to examine the impact of 65 days of various high fat diets on mitochondrial membrane lipid composition of mixed hind-limb muscle and if these changes would relate to changes in COX activity. It was hypothesized that the lower the linoleic acid content of the diet, the lower the mole fraction of linoleic acid would be found in mitochondrial membranes, specifically CL, resulting in lower COX activity.

2. Materials and methods

2.1. Animals and diets

Forty-day old Sprague-Dawley rats (Charles River Laboratories, Saint-Constant, Quebec) were housed in standard environmental conditions, with a 12 h light:12 h dark cycle and ad libitum access to water and their assigned diet for 65 days: an 18% coconut oil and 2% soybean oil diet (% by weight) (no. TD.0.8023; Harlan Teklad, Madison, Wisconsin) for the CO group ($n = 7$); a 20% flaxseed oil diet (% by weight) (no. TD.07777; Harlan Teklad) for the FO group ($n = 7$); or a 20% safflower oil diet (% by weight) (no. TD.07776; Harlan Teklad) for the SO group ($n = 7$). The content of macro- and micronutrients, vitamins and minerals were the same across all diets (Lau et al., 2010). Fatty acid compositions of each high-fat diet is presented in Table 1 as previously reported (Lau et al., 2010). The study and all protocols and procedures were approved by the Brock University (St. Catharines, ON) Animal Care and Utilization Committee, and conformed to the guidelines of the Canadian Council on Animal Care (1993).

2.2. Tissue collection and mitochondrial isolation

On day 65 of treatment, all rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (6 mg × 100 g^{-1} body weight) and whole hind-limb muscles were excised for isolation of subsarcolemmal (SS) and intramyofibrillar (IMF) mitochondria (Benton et al., 2008; Bezaire et al., 2004; Campbell et al., 2004). 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 homogenate was centrifuged at 700 × g for 10 min to separate SS (supernatant) and IMF (pellet) mitochondria. IMF mitochondria were released by treating the pellet with a protease (0.25 mg/ml nagarse (P80038); Sigma, St. Louis, MO, USA) for 5 min to digest myofibrils. Mitochondria from both SS and IMF samples were extracted with centrifugation at 14,000 × g for 10 min. The pellets were 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 pellets were 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}.

2.3. Phospholipid analysis

Total lipids from SS and IMF mitochondria ($n = 6$ per group) were extracted (Folch et al., 1957), and thin-layer chromatography (Mahadevappa and Holub, 1987) was used to separate individual phospholipids (PC, PE, CL, phosphatidylinositol [PI], phosphatidylserine [PS], and sphingomyelin [SM]). 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; Holloway et al., 2012; 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, 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 an 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. 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. For correlational analysis, the absolute amount of 18:2ω6 was calculated relative to protein content. 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.

2.4. Cytochrome c oxidase assay

Cytochrome c oxidase (COX; EC 1.9.3.1) activity was determined in isolated mitochondria as previously described (Cooperstein and Lazarow, 1951). Enzyme reaction rates were determined at room temperature (25 °C) by measuring the change in absorbance of reduced to oxidized cytochrome c at 550 nm (millimolar extinction coefficient $\varepsilon_{550} = 29.5$) using an Ultrospec 2100 pro

Table 1
Fatty acid composition of treatment diets (as previously published in Lau et al., 2010).

Fatty acid	CO	FO	SO
12:0	55.76	0.72	0.19
14:0	18.45	0.49	0.43
16:0	8.80	5.56	8.04
18:0	2.42	2.35	2.43
16:1	0.06	0.07	0.17
18:1	7.51	14.97	11.84
18:3n3	0.78	61.39	0.60
18:2n6	5.80	13.17	75.08
Total saturates	85.68	9.58	11.80
Total monoenes	7.68	15.32	12.29
n3 polyenes	0.81	61.67	0.73
n6 polyenes	5.81	13.43	75.19

Values are expressed as percent mole fraction of total fatty acids; CO: coconut oil; FO: flaxseed oil; SO: safflower oil; SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids.