Mitochondrial phospholipids of rat skeletal muscle are less polyunsaturated than whole tissue phospholipids: Implications for protection against oxidative stress[1]

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ABSTRACT: The fatty acid composition of phospholipids is an important determinant of membrane function. Although the mitochondria play a pivotal role in skeletal muscle function, the fatty acid composition of their individual phospholipids has not been examined. The purpose of this study was to determine the fatty acid profile of each phospholipid in rat skeletal muscle mitochondria and compare it with that of the whole muscle. Lipids were extracted from the gastrocnemius muscles of 10 Wistar rats, and phospholipids were separated by thin-layer chromatography. The fatty acid composition of each phospholipid was then determined by gas chromatography. The same procedure was applied to a mitochondrial preparation from these muscles. We found that the fatty acid composition of the individual mitochondrial phospholipids (phosphatidyl choline, phosphatidyl ethanolamine, cardiolipin, phosphatidyl inositol, phosphatidyl serine, sphingomyelin, and lysophosphatidyl choline) and of the total mitochondrial phospholipids differed markedly ($P < 0.05$) from the fatty acid composition of the corresponding whole muscle phospholipids. Notably, the mitochondrial phospholipids had higher percentages of MUFA [13.9 (2.1) vs. 10.3 (0.9)] and lower percentages of PUFA [34.8 (4.3) vs. 39.5 (5.2)] and n6 fatty acids [25.0 (2.5) vs. 27.6 (2.5)]. Overall, the mitochondrial phospholipids had a lower unsaturation index than whole muscle phospholipids [135 (20) vs. 161 (26)]. Because PUFA are susceptible to peroxidation, unlike saturated fatty acids and MUFA, we propose that the low polyunsaturation of mitochondrial phospholipids is the result of selective pressure toward membranes that are more resistant to oxidative damage by reactive oxygen species produced in their vicinity. The negative effect of the low polyunsaturation on membrane fluidity may be counterbalanced by the higher percentage of MUFA and the known low cholesterol content of mitochondrial membranes.

Key words: mitochondria, monounsaturated fatty acid, phospholipid, polyunsaturated fatty acid, rat, skeletal muscle

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INTRODUCTION

The fatty acid profile of membrane phospholipids is an important determinant of the structural and functional characteristics of cells (Spector and Yorek, 1985). The fatty acid profile of different phospholipids varies considerably (Masoro et al., 1966; Bruce, 1974b; Clore et al., 1998) and greatly influences membrane function. For example, the fatty acid profile of human muscle phosphatidyl choline (**PC**) has been correlated with insulin sensitivity (Clore et al., 1998).

Skeletal muscle mitochondria play a pivotal role in energy production during aerobic exercise, which, when repeated regularly, increases mitochondrial volume density (Hoppeler and Flück, 2003). Many studies have found significant effects of exercise on the fatty acid profile of skeletal muscle, although with conflicting data. For example, the unsaturation index (**UI**) in trained muscle has been found higher than, lower than, or not different from that in untrained muscle (Nikolaidis and Mougios, 2004). The vast majority of these studies have analyzed the whole muscle. However, if the fatty acid profile of the mitochondrial membranes differs from that of the whole tissue, the increase in mitochondrial density may create a fictitious image of

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change in the whole tissue, although no change may have occurred in the fatty acid profile of any cell membrane. Conversely, a change in the mitochondrial fatty acid profile may be concealed if it is cancelled out by opposite changes in another membrane or diluted to the effect that it is not detectable.

Although there are data on the fatty acid profile of individual phospholipids in whole muscle and on the fatty acid profile of muscle mitochondrial phospholipids as a whole, we have found no data on the fatty acid profile of the individual phospholipids in skeletal muscle mitochondria. Therefore, the purpose of the current study was to determine the fatty acid profile of each phospholipid in rat skeletal muscle mitochondria and compare it to that of the whole muscle.

MATERIALS AND METHODS

Care and treatment of the animals conformed to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The experimental protocol was approved by the Hospital Ethics Committee.

Animals

Ten healthy male Wistar rats, 3-mo-old, were supplied by the Research Center of the Theagenion Cancer Hospital in Thessaloniki, Greece. The rats lived in the facilities of the center in groups of 5 per cage at 22°C, under a 12:12-h light:dark cycle, and had free access to water and standard rodent chow.

Specimen Collection

The animals were killed by cervical dislocation after brief anesthesia with ether. The gastrocnemius muscle was surgically removed from the right hind limb within 1 min of death. The tissue was rid of visible fat, nerves, and fasciae, and was immediately immersed in liquid nitrogen. Subsequently, it was pulverized with a mortar and pestle in liquid nitrogen and was stored at –78°C until analysis.

Isolation of Mitochondria

All relevant procedures were performed at 4°C. One-half gram of muscle was homogenized with 19 volumes of 250 m*M* sucrose, 10 m*M* Tris-HCl, pH 7.3. An aliquot of the homogenate was removed for the determination of cytochrome c oxidase (**CCO**), as a specific mitochondrial membrane marker, and total protein. The remaining homogenate was centrifuged at 600 × *g* for 5 min to precipitate cell debris, myofibrils, and nuclei. The supernatant containing the mitochondria, together with the supernatants after 2 washes of the pellet with homogenization buffer, was centrifuged at 10,000 × *g* for 10 min to sediment the mitochondria. This is a commonplace procedure for isolating mitochondria. The pellet produced was washed twice with homogenization buffer and was finally suspended in homogenization buffer to a volume of 500 μL. Part of this mitochondrial preparation was removed for CCO and total protein determination, and the rest was used for lipid analysis.

Lipid Analysis

We determined the fatty acid composition of individual phospholipids by gas chromatography after separating them by 2-dimensional TLC. Lipids were extracted from both the mitochondrial preparation and the whole tissue (125 mg) with chloroform:methanol (2:1, vol/vol), according to Folch et al. (1957). The extracts were first subjected to 1-dimensional TLC to separate the phospholipid class from the other lipid classes. For this purpose, the extracts were spotted on silica gel plates (Sigma, St. Louis, MO) and were developed with petroleum ether:diethyl ether:acetic acid (50:50:1, vol/vol/vol). The spot corresponding to the phospholipids from each sample was excised and extracted with 1 mL of chloroform:methanol (2:1, vol/vol). After centrifuging briefly to sediment the silica, the upper layer was removed, condensed under a stream of nitrogen, and applied onto a new TLC plate.

The plate was developed with chloroform:methanol:acetic acid (10:5:1, vol/vol/vol) in the first dimension and chloroform:acetone:methanol:acetic acid:water (10:4:2:2:1, vol/vol/vol/vol/vol) in the second dimension (Kester et al., 1984). Phospholipids were located by brief exposure to UV light after spraying with a solution of 0.2% (wt/vol) dichlorofluorescein in ethanol and were identified by comparison with standards from Sigma. The spot corresponding to each phospholipid was excised and transferred to a test tube containing a fixed amount (5 μg) of pentadecanoic acid (Sigma) as internal standard for the determination of the percentage distribution of the phospholipids. One-half milliliter of methanolic sodium methoxide (Sigma) was added, and the test tubes were heated at 50°C for 10 min. Then, 0.5 mL of boron trifluoride (Fluka, Buchs, Switzerland) was added, and incubation was repeated as before (Kramer et al., 1997).

The fatty acid methyl esters thus produced were extracted with 1.5 mL of hexane and separated in a Hewlett Packard 5890 Series II gas chromatograph (Waldbronn, Germany) equipped with a 30-m-long AT-WAX capillary column from Alltech (Deerfield, IL) and a flame ionization detector. The column temperature was programmed from 160 to 250°C at 5°C·min^{-1}. The carrier gas was helium at a flow rate of 1 mL·min^{-1} (at 160°C). Methyl esters of individual fatty acids were identified in the chromatograms by comparing their retention times with those of pure methyl esters from Sigma and were quantified by comparing the area under their peaks to that of methyl pentadecanoate (derived from the internal standard) with the aid of the HP 3365 ChemStation software from Hewlett Packard. The molar amount of each fatty acid was then used to

calculate its percentage relative to the sum of fatty acids in each phospholipid. Additionally, this sum was divided by the number of acyl groups in the structure of each phospholipid to calculate the percentage molar distribution of the phospholipids in each preparation.

The fatty acid composition of the animals' food was determined as described above, except that no internal standard was added and the extracted lipids were not separated by TLC.

Cytochrome c Oxidase and Protein Assay

Cytochrome c oxidase activity and total protein were measured in the tissue homogenate and mitochondrial preparation to estimate the enrichment of the latter in mitochondria. The CCO was assayed spectrophotometrically with an assay kit from Sigma by monitoring the oxidation of ferrocytochrome c to ferricytochrome c at 550 nm and 25°C for 45 s (Storrie and Madden, 1990). Thirty microliters of 10-fold-diluted tissue homogenate or 20-fold-diluted mitochondrial preparation were mixed with 30 μL of homogenization buffer and 570 μL of an assay buffer containing 10 m*M* Tris-HCl, 120 m*M* KCl, pH 7.0. Then, 30 μL of 0.22 m*M* ferrocytochrome c (reduced with sodium hydrosulfite) were added to start the reaction. The CCO activity was expressed as $U \cdot g^{-1}$ of wet tissue, 1 U corresponding to 1 μmol of ferrocytochrome c oxidized per minute.

Total protein was assayed by using a Bradford reagent from Sigma. The specific activity of CCO was then calculated in the tissue homogenate and mitochondrial preparation. Finally, by dividing the specific activity in the latter to that in the former, the enrichment of the mitochondrial preparation was calculated.

Statistics

Values are expressed as the mean (SD). Differences in the fatty acid profile between the whole tissue and the mitochondria as well as among different phospholipids were examined by 2-way (preparation × phospholipid) ANOVA with repeated measures, followed by simple main effect analysis. There were 2 levels of the preparation variable (whole tissue and mitochondria) and 7 levels of the phospholipid variable (the 7 phospholipids listed below). The same analysis was applied to the percentage distribution of the phospholipids in the 2 preparations. Differences in the fatty acid profile of the total phospholipids between whole tissue and mitochondria were examined by paired Student's *t*-test. The level of statistical significance was set at $\alpha = 0.05$ for all analyses. The SPSS software (version 12.0, SPSS Inc., Chicago, IL) was used for all analyses.

RESULTS

The mitochondrial preparations were enriched, on average, 6-fold in CCO specific activity relative to the whole muscle. Seven phospholipids were detected in both the whole muscle and its mitochondria by TLC: PC, phosphatidyl ethanolamine (**PE**), cardiolipin (**CL**), phosphatidyl inositol (**PI**), phosphatidyl serine (**PS**), sphingomyelin (**SM**), and lysophosphatidyl choline (**LPC**). Fourteen fatty acids were detected in considerable amounts (above 0.1%) by gas chromatography: myristate (14:0), palmitate (16:0), palmitoleate (16:1n7), stearate (18:0), oleate (18:1n9), *cis*-vaccenate (18:1n7), linoleate (18:2n6), γ-linolenate (18:3n6), α-linolenate (18:3n3), gondoate (20:1n9), dihomo-γ-linolenate (20:3n6), arachidonate (20:4n6), docosapentaenoate (22:5n3), and docosahexaenoate (22:6n3).

Table 1. Percentage molar fatty acid composition of the rats' diet

Fatty acid	%
14:0	0.8
16:0	18.1
16:1n7	0.6
18:0	4.4
18:1n9	31.5
18:1n7	1.2
18:2n6	36.3
18:3n3	1.4
20:1n9	0.7
22:5n3	0.3
22:6n3	4.7

The fatty acid composition of the animals' diet is presented in Table 1. The major fatty acids were 16:0, 18:1n9, and 18:2n6, accounting for 86% of the total.

Fatty Acid Profile of Whole Muscle and Mitochondrial Phospholipids

Table 2 presents the fatty acid profiles of the individual phospholipids in whole muscle and its mitochondria. The statistical analysis of the data showed that the phospholipids of the whole muscle and its mitochondria differed in the percentages of most fatty acids (14:0, $P = 0.035$; 16:0, $P = 0.005$; 16:1n7, $P = 0.034$; 18:0, $P = 0.044$; 18:1n9, $P = 0.001$; 18:1n7, $P = 0.050$; 18:2n6, $P < 0.001$; 18:3n3, $P = 0.040$; 20:4n6, $P < 0.001$; and 22:6n3, $P = 0.022$). There were also significant differences among the 7 phospholipids in most fatty acids (14:0, 16:0, 16:1n7, 18:0, 18:1n9, 18:1n7, and 18:2n6, $P < 0.001$; 18:3n6, $P = 0.010$; and 18:3n3, 20:4n6, 22:5n3, and 22:6n3, $P < 0.001$). Finally, there was a significant interaction of the 2 independent variables (preparation and phospholipid) in most fatty acids (16:0, $P < 0.001$; 18:0, $P = 0.004$; 18:1n9, $P < 0.001$; 18:1n7, $P = 0.024$; 18:2n6, $P < 0.001$; 18:3n6, $P = 0.012$; 18:3n3, $P < 0.001$; 20:4n6, $P < 0.001$; and 22:6n3, $P = 0.002$). This means that the effect of one variable changes across the levels of the other variable or, in biological terms, that the percentage of a fatty acid differs between whole muscle and mitochondria in different directions across phospholipids.

To locate significant differences between percentages of each fatty acid, we performed all possible pairwise

Table 2. Percentage molar distribution of fatty acids in individual and total phospholipids of whole rat gastrocnemius muscle and its mitochondria[1]

	PC		PE		CL		PI	
Fatty acid	Whole	Mitochondria	Whole	Mitochondria	Whole	Mitochondria	Whole	Mitochondria
14:0	0.2 (0.1)	0.4 (0.1)^{cdefg*}	0.2 (0.1)	0.5 (0.1)^{cdefg*}	4.0 (3.0)	3.2 (1.5)^{ab}	0.5 (0.3)	1.5 (0.5)^{abg*}
16:0	43.3 (3.0)^{bcdeg}	42.5 (2.4)^{bcde}	8.6 (1.7)^{aefg}	11.2 (2.4)^{acefg*}	15.3 (5.6)^{afg}	19.3 (4.5)^{abfg}	7.2 (3.3)^{aefg}	17.8 (5.8)^{afg*}
16:1n7	0.3 (0.1)	0.4 (0.1)^{cdfg}	0.6 (0.3)	1.2 (0.7)^{f}	2.0 (2.1)	2.5 (0.9)^{a}	0.7 (1.1)	1.7 (0.6)^{af*}
18:0	8.3 (0.7)^{bdefg}	9.0 (1.0)^{bcdefg}	38.6 (6.7)^{acfg}	32.4 (4.0)^{acde*}	10.6 (5.1)^{bdef}	11.5 (2.3)^{abdefg}	48.0 (4.5)^{acefg}	49.7 (13.9)^{abcg}
18:1n9	6.4 (0.5)^{d}	7.5 (0.8)^{c*}	5.5 (1.3)^{de}	10.4 (3.6)*	8.5 (3.5)^{d}	13.6 (2.6)^{adfg*}	3.2 (2.1)^{abcef}	7.7 (3.8)^{c*}
18:1n7	4.2 (0.3)^{bdf}	3.7 (0.4)^{dfg*}	1.3 (0.2)^{aceg}	3.4 (3.4)	4.7 (0.7)^{bdfg}	3.3 (0.7)^{dfg*}	0.9 (0.6)^{aceg}	0.7 (0.3)^{ac}
18:2n6	15.6 (1.4)^{bcdef}	14.0 (0.9)^{bcdefg*}	7.4 (0.9)^{acdg}	6.3 (0.9)^{acef*}	45.9 (9.4)^{abdefg}	42.4 (4.9)^{abdefg}	3.9 (2.6)^{abcg}	3.7 (2.1)^{ac}
18:3n6	0.1 (0.1)^{b}	0.2 (0.0)^{bcf*}	0.3 (0.1)^{a}	0.3 (0.1)^{adf}	1.6 (1.5)	0.3 (0.1)^{af*}	0.2 (0.4)	0.1 (0.1)^{b}
18:3n3	0.1 (0.1)	0.2 (0.1)^{cdefg*}	0.2 (0.2)	1.2 (0.8)*	1.6 (1.2)^{d}	1.0 (0.3)^{af}	0.4 (0.4)^{c}	0.7 (0.3)^{af}
20:1n9	0.2 (0.1)	0.1 (0.0)^{cfg}	0.1 (0.1)	0.2 (0.1)^{fg}	0.2 (0.2)	0.3 (0.1)^{adefg}	0.1 (0.1)	0.1 (0.1)^{c}
20:3n6	0.5 (0.2)^{d}	0.6 (0.1)^{cf}	0.3 (0.1)^{d}	1.2 (3.0)	1.4 (1.0)	0.1 (0.1)^{afg*}	1.7 (0.7)^{ab}	0.5 (0.5)*
20:4n6	14.1 (2.7)^{bcdef}	14.2 (1.8)^{bcefg}	10.0 (1.9)^{acdef}	8.0 (2.8)^{acefg}	1.6 (1.3)^{abde}	1.3 (0.4)^{ab}	26.3 (5.9)^{abcefg}	8.2 (5.4)*
22:5n3	1.1 (0.4)^{bde}	1.2 (0.3)^{bc}	3.2 (1.0)^{acdfg}	2.8 (0.6)^{acfg}	0.5 (0.3)^{bde}	0.5 (0.2)^{abe}	2.2 (0.6)^{abcg}	1.5 (1.4)
22:6n3	5.5 (1.6)^{bce}	6.0 (1.4)^{bcf}	23.7 (6.9)^{acdefg}	21.0 (3.5)^{acdefg}	2.1 (0.7)^{abde}	0.7 (0.3)^{abefg*}	4.7 (0.8)^{bce}	6.0 (6.3)^{b}

	PS		SM		LPC		Total	
Fatty acid	Whole	Mitochondria	Whole	Mitochondria	Whole	Mitochondria	Whole	Mitochondria
14:0	1.2 (0.8)	1.8 (0.8)^{abf}	2.5 (2.1)	2.9 (1.2)^{abe}	1.7 (1.5)	2.8 (0.9)^{abd*}	0.5 (0.2)	1.2 (0.4)*
16:0	17.5 (5.5)^{abdfg}	21.5 (5.5)^{abfg}	40.3 (8.7)^{bcde}	35.7 (3.7)^{bcde}	35.4 (6.0)^{abcde}	37.8 (3.0)^{bcde}	31.0 (3.4)	30.7 (1.9)
16:1n7	0.5 (0.7)	2.2 (1.4)*	2.3 (2.4)	3.4 (1.0)^{abd}	1.7 (3.1)	2.7 (1.2)^{a}	0.6 (0.2)	1.2 (0.3)*
18:0	36.2 (7.0)^{acdfg}	43.8 (9.7)^{abcg}	24.0 (4.9)^{abcde}	35.5 (2.8)^{ac*}	17.7 (4.4)^{abde}	31.7 (4.8)^{acde*}	18.7 (1.9)	19.6 (3.0)
18:1n9	9.1 (2.5)^{bd}	12.7 (8.0)	8.7 (2.3)^{d}	7.8 (1.0)^{c}	6.2 (1.9)	9.1 (2.8)^{c*}	6.2 (0.7)	9.3 (1.8)*
18:1n7	4.6 (2.4)^{bd}	2.5 (3.3)	2.2 (1.2)^{ac}	0.8 (0.5)^{ac*}	3.1 (0.8)^{bcd}	1.1 (0.9)^{ac*}	3.3 (0.3)	3.2 (1.0)
18:2n6	5.9 (2.1)^{acg}	3.4 (1.0)^{abc*}	8.0 (3.9)^{ac}	4.8 (1.1)^{abc*}	14.8 (4.6)^{bcde}	5.2 (2.6)^{ac*}	14.1 (1.2)	14.9 (1.8)
18:3n6	0.2 (0.3)	0.2 (0.2)	0.2 (0.4)	0.0 (0.0)^{abc}	0.1 (0.3)	0.5 (0.4)	0.2 (0.1)	0.2 (0.0)
18:3n3	0.7 (0.7)	1.7 (0.9)^{a*}	0.9 (1.2)	1.8 (0.6)^{acd*}	0.7 (0.9)	1.3 (0.6)^{a}	0.3 (0.1)	0.7 (0.3)*
20:1n9	0.1 (0.3)	0.1 (0.2)^{c}	0.5 (0.9)	0.0 (0.0)^{abc}	0.2 (0.5)	0.0 (0.0)^{abc}	0.2 (0.1)	0.2 (0.0)
20:3n6	0.7 (0.4)	0.6 (0.5)^{f}	1.6 (1.6)	1.5 (0.6)^{ace}	1.6 (1.3)	1.2 (0.8)^{c}	0.6 (0.1)	0.7 (0.6)
20:4n6	5.7 (1.7)^{abcd}	2.2 (0.9)^{ab*}	3.9 (3.3)^{abd}	2.0 (1.5)^{ab*}	10.0 (6.9)^{d}	2.8 (2.6)^{ab*}	12.6 (2.2)	9.2 (1.9)*
22:5n3	2.8 (1.0)^{acfg}	1.8 (0.9)^{c*}	0.8 (0.9)^{be}	1.1 (0.7)^{b}	0.6 (0.8)^{bde}	0.8 (1.0)^{b}	1.7 (0.5)	1.4 (0.3)
22:6n3	14.7 (4.1)^{abcdfg}	5.4 (3.5)^{bc*}	3.9 (2.7)^{be}	2.8 (1.5)^{abc}	6.2 (3.5)^{be}	3.2 (1.7)^{bc*}	10.0 (2.8)	7.7 (2.0)*

[a–g]Different from PC, PE, CL, PI, PS, SM, and LPC, respectively, in the same preparation (whole muscle or mitochondria; $P < 0.05$).
[1]Values are mean (SD) from 10 animals. PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; CL = cardiolipin; PI = phosphatidyl inositol; PS = phosphatidyl serine; SM = sphingomyelin; LPC = lysophosphatidyl choline.
*Different from the same fatty acid in the same phospholipid of whole tissue ($P < 0.05$).

comparisons through simple main effect analysis. Starting with the phospholipids of whole muscle, the number of possible pairwise comparisons was 294 (21 pairwise comparisons of the percentages of each fatty acid in the 7 phospholipids multiplied by 14 fatty acids). One hundred five of these comparisons yielded significant differences ($P < 0.05$, denoted by superscript letters in Table 2). As a result, each phospholipid had its own fatty acid profile except for SM and LPC, which did not differ significantly in the percentage of any fatty acid. The PC had a high 16:0, 18:2n6, and 20:4n6 content. The PE had a high 18:0, 20:4n6, and the highest (P = 0.015 or less vs. all other phospholipids) 22:6n3 content. The CL had the highest ($P < 0.001$ vs. all other phospholipids) 18:2n6 content and a very low content (6%) in very long fatty acids (C20 and C22). The PI had a high 18:0 and the highest (P = 0.002 or less vs. all other phospholipids) 20:4n6 content. The PS had a high 16:0, 18:0, and 22:6n3 content. Finally, SM and LPC had high 16:0 and 18:0 contents.

As in the case of whole tissue phospholipids, a large number of the pairwise comparisons between mitochondrial phospholipids (123 out of 294) produced significant differences ($P < 0.05$, also denoted by superscript letters in Table 2), resulting in distinct fatty acid profiles. How do these profiles compare with the profiles of whole tissue phospholipids described above? On average, the percentages of 6 out of the 14 fatty acids in each phospholipid differed ($P < 0.05$, denoted by asterisks in Table 2) between whole tissue and mitochondria. However, several of these differences were quantitatively minor. As a result, the profiles of PC and CL were very similar in the 2 preparations. The major differences are presented next. Mitochondrial PE had less 18:0 (P = 0.035), although it remained the major fatty acid, and more 16:0 (P = 0.006) as well as 18:1n9 (P = 0.004) than whole tissue PE. Mitochondrial PI had 16:0 and 18:0 as its major fatty acids, with more 16:0 (P = 0.002) and 18:1n9 (P = 0.011), and less 20:4n6 ($P < 0.001$) compared with whole tissue PI. Mitochondrial PS had 16:0 and 18:0

Table 3. Indices of the fatty acid profile in individual and total phospholipids of whole rat gastrocnemius muscle and its mitochondria[1]

	PC		PE		CL		PI	
Index	Whole	Mitochondria	Whole	Mitochondria	Whole	Mitochondria	Whole	Mitochondria
MUFA (%)	11.1 (0.7)^{bd}	11.7 (1.1)^{c}	7.5 (1.7)^{acefg}	15.2 (3.7)*	15.5 (3.7)^{bd}	19.8 (2.4)^{adfg}*	4.9 (3.7)^{acefg}	10.2 (3.7)^{c}*
PUFA (%)	37.0 (4.2)^{bcf}	36.4 (3.3)^{bcefg}	45.1 (9.1)^{aef}	40.7 (4.8)^{adefg}	54.7 (9.3)^{adef}	46.2 (5.3)^{adefg}*	39.4 (3.9)^{cef}	20.7 (14.1)^{bc}*
n6 (%)	30.3 (2.5)^{bcef}	28.9 (1.9)^{bcdefg}	18.0 (1.6)^{acde}	15.8 (1.4)^{acefg}*	50.5 (9.6)^{abdefg}	44.1 (5.1)^{abdefg}	32.2 (3.4)^{bcef}	12.6 (6.9)^{ac}*
n3 (%)	6.7 (2.0)^{be}	7.4 (1.6)^{bc}	27.1 (7.8)^{acdefg}	24.9 (4.0)^{acdefg}	4.2 (1.8)^{bde}	2.2 (0.4)^{abefg}*	7.3 (1.0)^{bce}	8.1 (7.7)^{b}
n6/n3	4.9 (1.6)^{be}	0.7 (0.1)^{bcdefg}	0.7 (0.2)^{adg}	0.6 (0.1)^{acdf}	18.6 (20.8)	20.8 (3.4)^{abdefg}	4.5 (0.5)^{bef}	2.4 (1.3)^{abce}*
U/S	0.94 (0.13)^{c}	0.93 (0.11)^{bcefg}	1.16 (0.33)^{ef}	1.28 (0.19)^{acdefg}	2.60 (0.97)^{adefg}	2.04 (0.53)^{abdefg}	0.80 (0.12)^{c}	0.56 (0.53)^{bc}
UI	139 (20)^{bd}	142 (16)^{bcefg}	223 (51)^{acdefg}	208 (24)^{acdefg}	142 (19)^{bf}	121 (13)^{abefg}*	164 (18)^{abf}	98 (69)^{b}*

	PS		SM		LPC		Total	
Index	Whole	Mitochondria	Whole	Mitochondria	Whole	Mitochondria	Whole	Mitochondria
MUFA (%)	14.4 (3.5)^{bd}	17.6 (8.8)	13.7 (2.9)^{bd}	12.0 (1.7)^{c}	11.2 (2.1)^{bd}	12.9 (2.7)^{c}	10.3 (0.9)	13.9 (2.1)*
PUFA (%)	30.8 (3.9)^{bcdf}	15.4 (4.9)^{abc}*	19.4 (10.1)^{abcde}	14.0 (2.6)^{abc}	34.1 (11.9)	14.9 (4.1)^{abc}*	39.5 (5.2)	34. 8 (4.3)*
n6 (%)	12.6 (3.5)^{abcdg}	6.4 (1.9)^{abc}*	13.8 (6.8)^{acd}	8.3 (2.1)^{abc}*	26.5 (9.5)^{ce}	9.6 (4.3)^{abc}*	27.6 (2.5)	25.0 (2.5)*
n3 (%)	18.2 (4.5)^{abcdfg}	9.0 (3.6)^{bc}*	5.6 (4.0)^{be}	5.7 (2.1)^{bc}	7.6 (3.6)^{be}	5.3 (1.7)^{bc}	11.9 (3.3)	9.8 (2.3)
n6/n3	0.8 (0.4)^{adg}	0.8 (0.3)^{acd}	2.7 (1.4)^{d}	1.7 (0.8)^{abc}	3.6 (1.3)^{be}	2.1 (0.5)^{ac}	4.3 (1.1)	5.5 (1.4)
U/S	0.83 (0.15)^{bc}	0.54 (0.31)^{abc}*	0.53 (0.24)^{bc}	0.35 (0.07)^{abc}*	0.89 (0.39)^{c}	0.39 (0.09)^{abc}*	1.05 (0.18)	1.07 (0.21)
UI	156 (25)^{bf}	82 (30)^{abc}*	81 (39)^{bcde}	62 (12)^{abc}	129 (44)^{b}	66 (14)^{abc}*	161 (26)	138 (20)*

$^{a–g}$Different from PC, PE, CL, PI, PS, SM, and LPC, respectively, in the same preparation (either whole muscle or mitochondria) ($P < 0.05$).
[1]Values are mean (SD) from 10 animals. U/S, unsaturated to saturated; UI, unsaturation index. PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; CL = cardiolipin; PI = phosphatidyl inositol; PS = phosphatidyl serine; SM = sphingomyelin; LPC = lysophosphatidyl choline.
*Different from the same fatty acid in the same phospholipid of whole tissue ($P < 0.05$).

as its major fatty acids, with more 16:1n7 ($P = 0.014$) and 18:3n3 ($P = 0.005$), and less 18:2n6, 20:4n6, and 22:6n3 ($P < 0.001$ for all 3 fatty acids) compared with whole tissue PS. Remarkably, mitochondrial PI and PS did not differ significantly in the percentage of any fatty acid. Finally, the percentages of most fatty acids in mitochondrial SM and LPC changed in the same direction compared with whole tissue SM and LPC, resulting in their profiles being strikingly similar again. Both mitochondrial phospholipids had significantly more 18:0 ($P < 0.001$) and less 18:2n6 ($P = 0.029$ and $P < 0.001$, respectively), as well as 20:4n6 ($P = 0.030$ and 0.018, respectively) than the same phospholipids in whole muscle.

By adding the molar amounts of each fatty acid in the 7 phospholipids of each preparation, we calculated its amount in the total phospholipids. From that we calculated the percentage distribution of fatty acids in the total phospholipids of the whole muscle and its mitochondria. These data are presented in the last 2 columns of Table 2. Six of the 14 fatty acids had different percentages in the 2 preparations ($P < 0.05$).

Indices of the Fatty Acid Profile

We have calculated the following indices of the fatty acid profile of each phospholipid in the 2 preparations: MUFA, PUFA, n6 fatty acids, n3 fatty acids, n6/n3, unsaturated-to-saturated ratio (**U/S**), and UI (the average number of double bonds per fatty acid multiplied by 100). There were differences among phospholipids in all of these indices ($P = 0.002$ for n6/n3 and $P < 0.001$ for the other indices). The 2 preparations differed in MUFA ($P = 0.001$), PUFA, n6 fatty acids ($P < 0.001$ for both), U/S, and UI ($P = 0.002$ for both). In addition, there was an interaction of the 2 independent variables in MUFA, PUFA, n6 fatty acids ($P < 0.001$ for all 3), n3 fatty acids ($P = 0.011$), U/S ($P = 0.003$), and UI ($P = 0.007$). By analogy to the fatty acid profile, this means that an index differs between whole muscle and mitochondria in different directions across phospholipids.

Table 3 presents the indices in whole muscle and its mitochondria, along with the results of the pairwise comparisons. Whole muscle CL had the highest unsaturated fatty acid content, as evidenced by its U/S value ($P < 0.05$ vs. all other phospholipids); it had also the highest n6 fatty acid content ($P = 0.018$ or less vs. all other phospholipids). In contrast, PE had the highest n3 fatty acid content ($P = 0.035$ or less vs. all other phospholipids) and UI ($P = 0.029$ or less vs. all other phospholipids).

These observations were also true of the mitochondrial phospholipids, although several differences from the whole tissue were detected. Remarkably, most mitochondrial phospholipids had a higher MUFA and lower PUFA content, including lower n6 and n3 fatty acids ($P < 0.05$). Also, most mitochondrial phospholipids had lower n6/n3, U/S, and UI ($P < 0.05$). Most of these differences were reflected in the total phospholipids (last 2 columns in Table 3). Namely, the total mitochondrial phospholipids had more MUFA and less PUFA, n6 fatty acids, as well as UI than the total phospholipids of the whole muscle ($P < 0.05$).

Percentage Distribution of Phospholipids

Figure 1 presents the molar percentage distribution of the phospholipids in whole muscle and its mitochon-

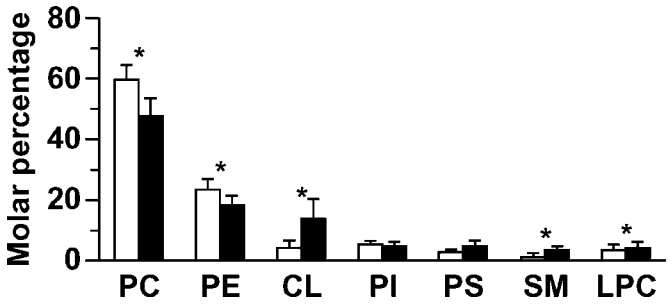

Figure 1. Percentage molar distribution of phospholipids in rat gastrocnemius muscle (open bars) and its mitochondria (solid bars). Values were derived by dividing the total molar amount of fatty acids in each phospholipid by the number of acyl groups in its structure and expressing the resulting molar amount of the phospholipid as a percentage of the sum of phospholipids. Error bars denote SD. *Different between whole tissue and mitochondria ($P < 0.01$). PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; CL = cardiolipin; PI = phosphatidyl inositol; PS = phosphatidyl serine; SM = sphingomyelin; LPC = lysophosphatidyl choline.

dria. Most of the phospholipids had significantly different percentages in the 2 preparations, the largest difference being a 3.4-fold higher abundance of CL in mitochondria.

DISCUSSION

The phospholipid composition of rat gastrocnemius muscle was similar to that reported for this and other skeletal muscles of the same or different species (Masoro et al., 1966; Masoro, 1967; Therriault et al., 1973; Bruce, 1974a; Okano et al., 1980; Górski et al., 1999; Żendzian-Piotrowska et al., 2000): PC and PE were the predominant phospholipids, by far exceeding the rest. Likewise, the phospholipid composition of our mitochondrial preparation was similar to published data on muscle mitochondrial phospholipids (Owens and Hughes, 1970; Fiehn et al., 1971; Dohm et al., 1975). Of the significant differences between whole tissue and mitochondria, the one regarding CL was expected, given its location primarily in the mitochondria (Daum, 1985), and justifies the characterization of our preparation as mitochondrial, along with its 6-fold enrichment in CCO specific activity. It is interesting that the mitochondria also had higher percentages of 2 additional phospholipids, SM and LPC, which are structurally similar (consisting of 1 acyl chain and 1 phosphoryl choline group attached to sphingosine or glycerol) and had indistinguishable fatty acid profiles. The fatty acid composition of each phospholipid in whole muscle generally agreed with published data (Masoro et al., 1966; Patriarca et al., 1969; Bruce, 1974b; Blackard et al., 1997; Clore et al., 1998; Dobrzyń and Górski, 2002; Helge et al., 2004), although wide ranges have been reported. Regarding the fatty acid profile of CL (in the whole muscle or its mitochondria), this agrees with published data on CL from skeletal muscles of primates (Masoro et al., 1966; Bruce, 1974b) and mitochondria from diverse sources (Schlame et al., 1993) in terms of the predominance of 18:2n6 and the very low percentages of very long fatty acids.

A striking finding of our study was the distinct fatty acid profile of each phospholipid in either the whole muscle or its mitochondria, with the exception of SM and LPC. These distinct patterns are apparently due to differences in substrate specificity for phospholipid biosynthesis, degradation, or remodeling (substitution of one fatty acid for another in the phospholipid molecule). It is intriguing that the cells spend considerable energy (in terms of both synthesizing specific enzymes and exchanging fatty acids on phospholipids) to ensure a different fatty acid profile for each phospholipid when one would expect that the polar head group would be sufficient to endow a phospholipid with a distinct biological role. Thus, our data strengthen the hypothesis of the importance of the role of fatty acids in membrane function.

The main focus, and novelty, of the present work was the comparison of the fatty acid profiles of the individual phospholipids in skeletal muscle mitochondria to those in the whole tissue. Individual fatty acids are known to influence fundamental regulatory processes, such as ion homeostasis, gene expression, signal transduction, and synthesis of lipid or lipid-derived messengers (Kogteva and Bezuglov, 1998), and the findings of this study seem to testify to their importance. We found many differences, which point to a distinct fatty acid profile of mitochondrial phospholipids relative to the phospholipids of other muscle compartments. The most remarkable, and unexpected, of these differences was the lower PUFA content of the mitochondrial phospholipids. Individual PUFA exhibited the most spectacular of all differences, including the 3-fold decreases, from whole tissue to mitochondria, of 20:4n6 in PI; 22:6n3 in PS; and 18:2n6 and 20:4n6 in LPC.

What would be the reason for the different fatty acid profiles of a phospholipid in the mitochondrial and other cell membranes? We can offer 4 explanations. First, the phospholipid may be synthesized from different pools of available fatty acids or from precursor molecules, such as diacylglycerol and phosphatidate, differing in fatty acid composition. Second, enzyme forms with different fatty acid specificities may catalyze the synthesis of the phospholipid at different sites. Third, there may be some selectivity in the phospholipid molecules that are transported to the mitochondria from a common pool. Fourth, differences may exist in phospholipid remodeling between the mitochondrial and other cell membranes. Because most mitochondrial phospholipids are imported from the endoplasmic reticulum and only a minor portion of them is synthesized in the organ-

elles (Hatch, 2004), the latter 2 possibilities seem more plausible.

Two particular cases deserve attention. The first is that of mitochondrial PE, which is thought to be derived mainly from the decarboxylation of PS transported to the mitochondria from the endoplasmic reticulum, in contrast to PE in other cell membranes, which is synthesized mainly from cytidine diphosphoethanolamine (Steenbergen et al., 2005). According to this distinction of biosynthetic pathways, mitochondrial PE and PS would be expected to have similar fatty acid profiles, whereas whole tissue PE and PS would not. However, the 2 phospholipids had disparate profiles and, in fact, more in the mitochondria than in the whole muscle. Two possible explanations for this finding are: 1) mitochondrial PE is extensively remodeled after its synthesis, and 2) PS molecules of a particular fatty acid profile are preferred for decarboxylation. In support of the second possibility, Heikinheimo and Somerharju (1998) found that, in mammalian cells, hydrophilic PS molecules are more readily decarboxylated to PE than hydrophobic ones. Unfortunately, the incomplete quantitation of those molecules does not permit a comparison with the fatty acid profiles of PE and PS in this study. Nevertheless, we have been able to compare our data with those of Burgermeister et al. (2004), who, also in support of the second possibility, found that in yeast the pathways involved in PE synthesis exhibit a preference for the formation of highly unsaturated molecules and that the U/S ratio was much higher in PE than in PS. This was also the case in our study, in which PE had a higher U/S ratio than PS in the whole muscle (1.16 vs. 0.83, $P = 0.040$) and even more in the mitochondria (1.28 vs. 0.54, $P < 0.001$, Table 3). Heikinheimo and Somerharju (1998) have attributed their finding to a preferential transport of hydrophilic PS molecules from the endoplasmic reticulum to the mitochondria, where PS decarboxylation takes place, and have excluded the alternative of selective decarboxylation of hydrophilic PS molecules by PS decarboxylase. In contrast, Burgermeister et al. (2004) have attributed their finding to a high selectivity of PS decarboxylase toward unsaturated molecules and have excluded the alternative of selective import of unsaturated PS molecules to the mitochondria. This difference between the studies may reside in the different biological species examined.

The second case deserving attention is that of LPC, which is derived from PC by removal of 1 acyl group. Whole muscle PC and LPC did not differ in any index of their fatty acid profile, implying no preferential removal of saturated fatty acids, MUFA, or PUFA from PC. In contrast, mitochondrial LPC had less than half the PUFA, n6, U/S, and UI values of mitochondrial PC, implying preferential removal of PUFA from PC. This could have been caused by a higher phospholipase A_2 activity, which is directed against the *sn*-2 position of glycerol that usually harbors unsaturated fatty acids (Schiller and Arnold, 2002).

Given the negative effect that a low PUFA content is known to have on membrane fluidity, we wondered what a compensatory advantage for the mitochondrial membranes might be. We propose that the low polyunsaturation of skeletal muscle mitochondria is the result of selective pressure toward membranes of lower susceptibility to oxidative stress. Individual fatty acids differ greatly in their chemical propensity for oxidative damage, the polyunsaturated ones being prone to peroxidation, unlike the saturated and monounsaturated ones (Hulbert, 2005). Reactive oxygen species are ceaselessly produced in the mitochondria and, as a result, peroxidize the adjacent phospholipids (Hulbert, 2005). Therefore, mitochondria with fewer PUFA might be more resistant to oxidative damage. This may be particularly important to skeletal muscle, which greatly increases its rate of oxygen consumption during intense exercise. It would be interesting to examine whether the differences found in the current study also exist in tissues or organs that do not display dramatic increases from basal oxygen consumption and, hence, reactive oxygen species production.

The decrease in membrane fluidity expected from the low polyunsaturation of the mitochondria could be counterbalanced by 2 factors. One is the higher MUFA content of the mitochondrial phospholipids compared with the whole muscle phospholipids found in this study. The other is the known low cholesterol content of the mitochondrial membranes (Fiehn et al., 1971; Daum, 1985).

In conclusion, we found that the fatty acid composition of the individual and total mitochondrial phospholipids from rat skeletal muscle differs markedly from that of the whole muscle phospholipids. Mitochondrial phospholipids had higher MUFA and lower PUFA, n6 fatty acids, n3 fatty acids, n6/n3, U/S, and UI. The low polyunsaturation of the mitochondrial phospholipids may mitigate the oxidative damage caused by reactive oxygen species in such a highly active tissue as muscle. Each phospholipid had a particular fatty acid profile with the exception of SM and LPC. The distinct fatty acid composition of almost each mitochondrial phospholipid relative to the other mitochondrial phospholipids and to the same phospholipid in other cell membranes underlines the importance of individual fatty acids in determining membrane functions and implies that membrane fatty acids may serve additional, as yet unknown, functions.

We believe that the results of the current study can provide a background against which the effect of various stimuli (such as diet and exercise) on the fatty acid composition of individual mitochondrial phospholipids may be set, and the comparison with the whole muscle permits the assessment of the value of fractionating mitochondria in relevant studies.

IMPLICATIONS

Biological membranes contain various phospholipids, which differ not only in their polar head groups, but

also in the fatty acids that make up their hydrophobic tails. The fatty acid composition of these phospholipids is an important determinant of membrane function. In this paper we have shown that the mitochondrial phospholipids of rat skeletal muscle have a different fatty acid composition from the phospholipids of the whole muscle. Most interestingly, the mitochondrial phospholipids have a low content in polyunsaturated fatty acids, which are known to be susceptible to peroxidation and destruction by free radicals produced in the mitochondria during respiration. Thus, we propose that the low polyunsaturation of mitochondrial phospholipids is the result of natural selection, which led to the evolution of membranes that are more resistant to oxidative damage.

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