Phospholipid Fatty Acid Composition in Type I and Type II Rat Muscle

William G. Blackard[1], Jing Li, John N. Clore*, and William B. Rizzo

Departments of Medicine and Pediatrics, Medical College of Virginia/Virginia Commonwealth University, Richmond, Virginia 23298

ABSTRACT: The fatty acid composition of the membrane phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine in insulin-sensitive Type I (soleus) and insulin-resistant Type II (EDL) muscle is not known. In the present studies, soleus and EDL muscles were removed from 250–300 g Sprague-Dawley rats, and the fatty acid composition of total and individual phospholipid (PL) species was quantitated. As expected, triglyceride content was increased twofold in soleus muscle. No quantitative differences in the individual PL species or cholesterol content were found between the two muscles. However, a striking difference in PL fatty acid composition was observed in the PC fraction. An increase in 16:0 with decreases in 18:0, 18:1, 22:5n-3, and 22:6n-3 ($P < 0.001$ for each) was observed in the PC fraction of EDL compared to that from soleus, consistent with reduced elongation of PC fatty acids. Inhibition of fatty acid oxidation with the carnitine palmitoyl transferase-I inhibitor, etomoxir, did not alter the fatty acid pattern in either muscle. We conclude that an alteration in PL fatty acid composition consistent with reduced elongation of both saturated and unsaturated fatty acids is observed in Type II muscle. The restriction of these alterations to the PC fraction has important implications.

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Type I and Type II muscle fibers differ in their responsiveness to insulin. Type I muscle (slow-twitch), with its greater number of mitochondria and greater oxidative capacity, is more responsive to insulin than is Type II muscle (fast-twitch) with its greater glycolytic capacity (1–7). Differences in insulin receptor number (8–10) and receptor tyrosine kinase activity (10) have been variably reported, but no *in vitro* study has shown the expected rightward shift in the insulin dose-response curve consistent with these characteristics of Type II muscle. A more attractive explanation for the enhanced responsiveness of Type I muscle is its greater capacity for glucose transporter (Glut-4) recruitment compared to that in Type II muscle (5,7,11). Since phospholipid (PL) composition is known to influence membrane protein incorporation as well as enzyme activity and receptor function (12,13), differences in membrane PL fatty acid composition may play an important role in insulin responsiveness.

The present study examines the PL content of Type I and Type II muscle in the rat. Since the major PL in the outer layer of the sarcolemma bilayer is phosphatidylcholine (PC) (14), whereas phosphatidylethanolamine (PE) is preferentially concentrated in the inner layer, the fatty acid composition of these two predominant PL species (choline, PC and ethanolamine, PE) as well as the quantitatively less significant phosphatidylinositol (PI) in muscle have been emphasized. To our knowledge, no studies to date have examined the fatty acid composition of PC and PE in Type I and Type II muscle. To determine if differences in fatty acid oxidation in Type I and Type II muscle contribute to putative differences in the PL fatty acid composition, the carnitine palmitoyl transferase I (CPT-1) inhibitor, etomoxir, was administered in a separate series of experiments. The studies have demonstrated a striking variation in the fatty acid composition of PC in the two muscle types which was not present in either PE or the other classes of PL.

METHODS

Overnight fasted male Sprague-Dawley rats with a mean weight of 276.1 ± 7.0 g (range 250–300 g) were killed by cervical dislocation after undergoing ether anesthesia. Serum was obtained by cardiac puncture, and soleus and extensor digitorum longus (EDL) muscles were removed for PL analyses. Soleus muscle consists mainly of slow-twitch, Type I (84%) red fibers, and EDL muscle consists mainly of Type II white fibers (38% Type IIA; 59% IIB) (15).

In experiments to examine the effect of inhibition of fatty acid oxidation, etomoxir (a CPT-1 inhibitor) was administered at a dose of 15 mg/kg/d S.Q. for six weeks (16). The muscle PL composition was compared to that of litter mates fed *ad libitum*. Body weights of control and etomoxir-fed animals were measured at the end of the treatment period.

Lipid analysis [PL, triglycerides (TG), and cholesterol]. Soleus and EDL muscles (100–120 mg) were removed from the overnight-fasted male rats. The muscles were homogenized in chloroform and methanol (2:1, vol/vol) containing

[1]Deceased (June 28, 1996).

*To whom correspondence should be addressed at Medical College of Virginia, Division of Endocrinology and Metabolism, MCV, Box 908155, Richmond, VA 23298-0155.

Abbreviations: CA, cardiolipin; DAG, diacylglycerol; EDL, extensor digitorum longus; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; TG, triglycerides.

0.01% butylated hydroxytoluene using a Rockwell homogenizer, and total lipids were extracted by the method of Folch *et al.* (17). Serum samples were also treated with chloroform and methanol prior to extraction. To quantitate the various PL species, cholesterol, and TG, lipids from the Folch extraction were separated by high-performance thin-layer chromatography as described by Alvarez and Ludman (18). Following chromatography, the plates were dipped into 10% $CuSO_4$ in 8% H_3PO_4 and oven-charred at 130°C for 30 min. The plates were scanned immediately using an ultrascan XL laser densitometer (Pharmacia LKB Biotechnology, Uppsala, Sweden) and quantitated according to Macala *et al.* (19). As the charring method is only semiquantitative, the major PL species (PC and PE) were also determined by the Naphthol Blue Black staining method which has been validated for accuracy (20).

PL fatty acid composition. PL fatty acid composition was analyzed according to Storlien and coworkers (21) with minor modifications. Hemi-muscles (50–60 mg) were homogenized as described above and total lipids were extracted by the method of Folch *et al.* (17). The lipid extracts were dried under nitrogen, dissolved in 10 mL of hexane and applied to 3 mL silica gel columns (J.T. Baker, Inc., Phillipsburg, NJ). After elution of the less polar lipids with 20 mL hexane followed by 10 mL dichloromethane, PL were eluted with 10 mL methanol. The methanol eluates were dried under nitrogen and transmethylated with 1.5 mL 1 N methanolic HCl at 80°C overnight. Fatty acid methyl esters were extracted with 6 mL hexane and dried under nitrogen.

To determine the fatty acid composition of individual PL species, PC, PE, sphingomyelin (SM), PI, cardiolipin (CA), and phosphatidylserine (PS) were first separated by thin-layer chromatography on silica gel G plates (Whatman LK6D, Clifton, NJ) using a solvent system consisting of chloroform/ethanol/triethylamine/water (30:34:30:8, by vol) for the first development and hexane/dimethyl ether (50:50, by vol) for the second development. PL were visualized under ultraviolet (UV) light after spraying the plate with rhodamine G. The separated PL spots were collected by scraping and placed into glass tubes. Fatty acid methyl esters were prepared as described above by treatment with methanolic HCl.

Fatty acid methyl esters from the total PL fractions as well as the individual PL species were redissolved in 20 μL hexane and analyzed on a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) equipped with a 30 m × 0.2 mm fused silica capillary column (Omega wax 320; Supelco, Inc., Bellefonte, PA) and flame-ionization detector. The injection temperature was 250°C and detector temperature was 300°C. The initial oven temperature was 140°C. After five minutes, the oven temperature was increased from 140 to 200°C at a rate of 20°C/min, then to 280°C at 5°C/min. Fatty acids were identified by comparing their retention times with those of authentic standards.

Most of the gas chromatograph peaks were identified as specific fatty acid methyl esters. In the total PL and PC preparations, these fatty acid methyl esters accounted for 87 and 93% of the total integrated area, respectively, whereas they comprised only 79% of the integrated area in the PE fraction. In all samples, however, there were peaks immediately preceding palmitate (16:0) and stearate (18:0), which were suspected to be dimethyl acetal derivatives of fatty aldehydes released from ether PL (plasmalogens). To confirm this identification, PE from bovine brain containing 60% plasmalogens (Sigma Chemical Co., St. Louis, MO) was chromatographed before and after mild acid fume hydrolysis (22). After mild acid fume hydrolysis and separation on thin-layer chromatography plates, the peaks before 16:0 and 18:0 suspected of being derived from plasmalogens completely disappeared, confirming their identity as dimethyl acetals.

PL, TG, cholesterol, and fatty acid standards were obtained from Sigma. High-performance pre-coated silica gel Hp-K plates (10 cm × 10 cm) were purchased from Whatman. All other reagents and solvents were of analytical or HPLC-grade from Sigma or Fisher (Pittsburgh, PA).

RESULTS

Figure 1 displays the lipid composition of soleus and EDL muscles as determined by the charring method after separation by thin-layer chromatography. All six of the PL species were expressed as a percentage of total PL, whereas TG and cholesterol were expressed as a percentage of total lipids, (PL, TG, and cholesterol). There were no significant differences in PL species between the two muscles which collectively accounted for approximately 75% of total tissue lipids. The major intermuscle difference was the twofold greater TG composition in soleus muscle. Because the charring method is only semiquantitative, the two major PL species (PC and PE) were quantitated by the more accurate Naphthol Blue Black staining method. Using this technique, the concentration of PC was greater than that of PE, but no significant difference in the PC/PE ratios was noted in the two muscles (PC/PE ratio 1.41 ± 0.07 in soleus and 1.46 ± 0.07 in EDL).

The fatty acid composition of total PL and the individual PL classes was determined by gas chromatography. Approximately 90% of the chromatographic peaks were identified as

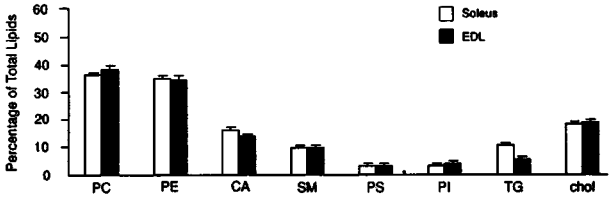

FIG. 1. Lipid composition of soleus and extensor digitorum longus (EDL) muscles. Phospholipid species are expressed as a percentage of total phospholipids, whereas triglycerides and cholesterol are expressed as a percentage of total lipids (including all phospholipids); PC, phosphatidylcholine; PE, phosphatidylethanolamine; CA, cardiolipin; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; TG, triglyceride; CHOL, cholesterol. Means ± SEM shown. $n = 12$ animals. Triglycerides are significantly increased in soleus ($P < 0.001$).

specific fatty acid methyl esters or dimethyl acetal derivatives of plasmalogen PL. Two major dimethyl acetal peaks (16:0 dimethyl acetal and 18:0 dimethyl acetal) derived from ether phospholipids (plasmalogens) were observed predominantly in the chromatograms of PE. These peaks accounted for 10.02 ± 0.28% (soleus) and 9.03 ± 0.35% (EDL) of the total methylated derivatives (fatty acids + aldehydes) on the chromatograms of PE. In contrast, these same peaks contributed only 0.69 ± 0.13% (soleus), and 0.66 ± 0.08% (EDL) of the peak area on chromatograms of PC. The plasmalogen content of PE is known to be greater than that of PC (23). These dimethyl acetal derivatives were quantitatively similar in the two muscles.

The fatty acid composition of the total PL fractions from the two muscles as well as from rat serum are shown in Table 1. The major differences in PL fatty acid composition between the two muscles were an increase in 16:0 and a decrease in 18:0 in Type II (EDL) muscle. The very different PL fatty acid composition of muscle compared to serum is indicative of local membrane remodeling (24). For example, the percentage of docosahexanoic acid (22:6n-3) was five times greater in muscle PL than in serum, whereas the percentage of oleic acid (18:1n-9) was almost twofold greater in serum.

The fatty acid compositions of individual PL classes are shown in Table 2. The increased palmitate in PC and increased stearate in PE are consistent with the known predilection of PC- and PE-synthesizing enzymes for diacylglycerol (DAG) with palmitate or stearate in the *sn*-1 position, respectively (25). The PE-synthesizing enzymes also favor synthesis from DAG with docosahexanoic acid (22:6n-3) in the *sn*-2 position (25), thus explaining the two- to threefold increase in this fatty acid in PE compared to that in PC.

The greatest difference in PL fatty acid content between the two muscles was observed in the saturated fatty acids of the PC species (Table 2). Choline PL from EDL muscle had a much higher 16:0 composition, and a significantly lower 18:0 composition than that from soleus muscle. This was reflected by a PC palmitate (16:0) to stearate (18:0) ratio of 3:1 in EDL compared to the 1:1 ratio in soleus (Fig. 2). Importantly, this ratio was minimally altered by muscle fiber type in the other PL classes (PE, CA, and SM). The longer chain saturated fatty acids (19:0, 20:0, 22:0, 24:0) of PC were also reduced in the EDL compared to the soleus, although the difference was significant only for 19:0. As shown in Table 2, oleic acid (18:1), an elongation as well as desaturation product of palmitate, was reduced in EDL, whereas linoleic (18:2n-6) and linolenic (18:3n-6) acids, two essential fatty acids, were not significantly different in the two muscles. However, the n-3 elongation products 22:6n-3 and docosapentaenoic acid (22:5n-3) were significantly reduced in the PC fraction of EDL muscle.

In contrast to PC, the differences in fatty acid composition of the PE fractions between the two muscle types were relatively trivial (Table 2). As shown in Figure 2, the 16:0 to 18:0 ratio in PE was slightly but significantly increased in EDL compared to soleus (0.16 ± 0.01 to 0.19 ± 0.01, $P < 0.003$). The ratio of 16:0 to 18:0 in the SM and CL fractions was almost identical between the two muscles (Fig. 2). Thus, the minimal intermuscle differences in fatty acid composition of these other PL classes reduced the magnitude of the difference detected when the fatty acid composition from total PL was compared in the two muscles. In fact, in the case of 22:6n-3, the significant reduction of this fatty acid in PC of EDL (Table 2) was effectively masked in the fatty acid analysis of the total PL fraction by the increase in PE 22:6n-3 in EDL (Tables 1 and 2).

Because of the putative role of PI in muscle signaling, the fatty acid composition of this species is shown (Table 3), despite its low content in rat muscle PL (2.5%). Stearate was the predominant saturated fatty acid in PI, being higher than in any of the other species. Stearate was significantly higher ($P < 0.002$) in the EDL and the 16:0 to 18:0 ratio (0.048 ± 0.006 vs 0.039 ± 0.004) was slightly but significantly lower in the EDL (opposite to the ratio observed in PC). No differences in arachadonate (20:4n-6) content in PI were found be-

TABLE 1
Phospholipid Fatty Acid Composition in Serum and Muscle

Fatty acid (%)	Serum PL	Soleus PL	EDL PL
14:0	0.93 ± 0.11[a]	0.37 ± 0.03	0.34 ± 0.04
15:0	0.46 ± 0.02[a]	0.27 ± 0.03	0.27 ± 0.02
16:0	19.21 ± 0.09[a]	11.34 ± 0.31[b]	16.90 ± 0.39
17:0	0.59 ± 0.03	0.54 ± 0.02	0.55 ± 0.01
18:0	20.90 ± 1.00[c]	19.09 ± 0.25[b]	16.15 ± 0.22
16:1n-9	1.46 ± 0.20	0.52 ± 0.02	0.53 ± 0.03
18:1n-9	10.52 ± 0.62[a]	6.89 ± 0.15[b]	5.68 ± 0.16
18:2n-6	14.71 ± 0.79	16.59 ± 0.29	16.33 ± 0.39
18:3n-6	0.60 ± 0.06[a]	0.22 ± 0.01[b]	0.15 ± 0.01
20:4n-6	12.25 ± 0.50	11.74 ± 0.22	12.12 ± 0.26
22:4n-6	0.27 ± 0.03[a]	0.59 ± 0.03[b]	0.51 ± 0.02
22:5n-3	0.69 ± 0.03	3.06 ± 0.09	2.82 ± 0.09
22:6n-3	2.74 ± 0.18[a]	13.73 ± 0.48	12.83 ± 0.51

[a] $P < 0.01$, seum vs. soleus and extensor digitorum longus (EDL).
[b] $P < 0.001$, soleus vs. EDL.
[c] $P < 0.01$, serum vs. EDL alone; $n = 12$ for soleus and EDL: $n = 6$ for serum from overnight-fasted rats. Phospholipid (PL) fatty acids accounting for less than 0.5% of the total are not shown.

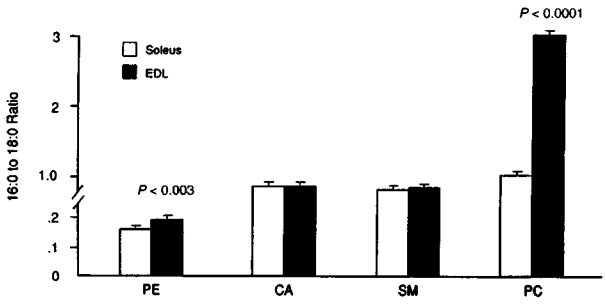

FIG. 2. Fatty acid "elongation ratio" (16:0 to 18:0) in phospholipid species from soleus and EDL muscles. See Figure 1 for abbreviations. Data are expressed as means ± SE; $n = 12$ for PC and PE, $n = 4$ for CA and SM.

TABLE 2
Phospholipid Fatty Acid Composition of Phosphatidylcholine and Phosphatidylethanolamine from Soleus and EDL Muscles

Fatty acid	Phosphatidylcholine		Phosphatidylethanolamine	
(%)	Soleus	EDL	Soleus	EDL
16:0	16.96 ± 0.72[a]	27.09 ± 0.84	3.52 ± 0.13[a]	4.35 ± 0.20
17:0	0.58 ± 0.01[a]	0.66 ± 0.02	0.55 ± 0.07	0.38 ± 0.02
18:0	16.67. ± 0.44[a]	8.83 ± 0.27	22.55 ± 1.02	22.39 ± 0.81
16:1n-9	0.95 ± 0.15	1.36 ± 0.18	0.24 ± 0.05	0.28 ± 0.02
18:1n-9	9.74 ± 0.30[a]	6.89 ± 0.27	4.13 ± 0.29	4.07 ± 0.23
18:2n-6	16.12 ± 0.34	18.15 ± 0.56	5.00 ± 0.13[a]	6.06 ± 0.15
20:4n-6	14.31 ± 0.21	14.71 ± 0.68	10.93 ± 0.29[a]	7.98 ± 0.14
20:5n-3	0.78 ± 0.15	0.77 ± 0.06	0.97 ± 0.21	0.80 ± 0.11
22:4n-6	0.35 ± 0.03	0.25 ± 0.02	0.62 ± 0.06	0.62 ± 0.05
22:5n-3	3.69 ± 0.18[a]	2.49 ± 0.09	3.97 ± 0.17	4.15 ± 0.13
22:6n-3	11.74 ± 0.60[a]	8.28 ± 0.44	25.19 ± 0.52	27.32 ± 0.59
24:1n-9	0.33 ± 0.05	0.23 ± 0.05	0.45 ± 0.08	0.47 ± 0.07

[a]$P < 0.001$, soleus compared to EDL.Phospholipid fatty acids accounting for less than 0.5 % of the total are not shown; $n = 12$ for overnight-fasted rats. See Table 1 for abbreviation.

tween soleus and EDL muscles. However, the content of 18:2n-6 was significantly increased in soleus compared to EDL (Table 3)

To determine whether differences in fatty acid oxidation rates of the two muscles might be responsible for the alteration in PC fatty acid content, the fatty acid composition of muscle PC and PE from rats treated for six weeks with the CPT-1 inhibitor etomoxir was compared to that of untreated animals. In Figure 3, it can be observed that the 16:0/18:0 ratio of PC was not altered in etomoxir-treated animals. Thus, greater rates of fatty acid oxidation in Type I (soleus) compared to Type II (EDL) muscle are unlikely to explain the divergent 16:0 to 18:0 ratios in PC. Although the mean weights of control animals and etomoxir-treated animals were not significantly different, the etomoxir-treated animals exhibited cardiac hypertrophy (0.0029 ± 0.0003 vs. 0.0025 ± 0.0002 heart/body weight ratio, $P < 0.005$) and visibly fatty livers corroborated by marked increase in TG content as assessed by thin-layer chromatography.

DISCUSSION

Since membrane PL may play a role in signal transduction (26), as well as influence membrane fluidity affecting membrane enzyme and receptor activity (12,13), we characterized the PL in membranes of the two muscle types disparate for insulin responsiveness (soleus, Type I and EDL,Type II). PL were determined on total cell lipid extracts rather than on isolated membrane fractions. However, it is generally considered that the measurement of total cell PL reflects that of cell membranes, and that the ranking of the major PL fatty acids is preserved among the various cell membranes (27). No differences in the PL species composition of soleus and EDL muscles were noted.

Analysis of the total PL fatty acid composition was not so informative as the fatty acid composition of the individual PL species. Since PC and PE contributed over 70% of the total PL, the major focus was on the analysis of the fatty acid composition in these two classes. Striking differences in the fatty acid composition of soleus and EDL were observed in the PC

TABLE 3
Fatty Acid Composition of Phosphatidylinositol from Soleus and EDL Muscles[a]

Fatty acid	Saturated fatty acids		
(%)	Soleus	EDL	*P* value
16:0	1.67 ± 0.19	1.46 ± 0.12	
18:0	35.16 ± 0.58	37.80 ± 1.06	<.05
	Unsaturated fatty acids		
18:1n-9	3.61 ± 0.013	2.99 ± 0.51	
18:2n-6	2.82 ± 0.18	1.96 ± 0.14	<.001
20:4n-6	27.62 ± 1.12	25.74 ± 0.62	
22:4n-6	2.83 ± 0.20	2.57 ± 0.17	
22:5n-3	4.48 ± 0.17	4.9 2 ± 0.19	
22:6n-3	9.77 ± 0.35	10.56 ± 0.23	

[a]Because of low phosphatidylinositol (PI) content, PI was pooled from each pair of muscles from four animals. Fatty acids accounting for more than 1% of total PI fatty acids are shown, $n = 4$. See Table 1 for other abbreviation.

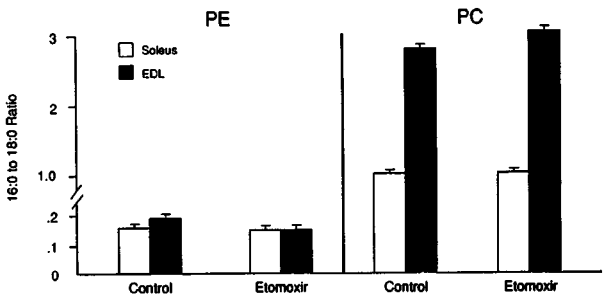

FIG. 3. Fatty acid "elongation ratio" (16:0 to 18:0) in phosphatidylethanolamine and phosphatidylcholine fractions from soleus and EDL muscles removed from control and etomoxir-treated rats. Data are expressed as means ± SEM. Ratio is significantly elevated in PC of EDL muscle from both control and etomoxir-treated animals ($P < 0.0001$); $n = 12$ overnight-fasted animals. See Figure 1 for abbreviations.

fraction. However, because of minor intermuscle differences in fatty acids from the other PL classes (PE as well as SM, CL, and PI), the dramatic differences in 16:0 and 18:0 seen in PC were partially masked when the fatty acid profiles of total PL were analyzed. In addition, the reduction in 22:6n-3 observed in the PC fraction from EDL was obscured in the total PL measurements by the increase in this fatty acid in PE.

Major differences in the fatty acid composition of PC were observed between soleus and EDL muscles. In addition to the relative increase in 16:0 and the decrease in 18:0 in EDL, oleic acid (18:1), a desaturase product of 18:0, was also reduced in EDL PC. Linoleic (18:2n-6) and linolenic (18:3n-6) acids, essential fatty acids not synthesized *in vivo*, were not decreased. Elongation products of n-3 fatty acids (22:5n-3 and 22:6n-3) were also decreased in the PC fraction from EDL compared to soleus. However, this was not the case for elongation products of n-6 fatty acids such as 20:4n-6. Since 22:6n-3 inhibits elongation of n-6 fatty acids (28), the failure to demonstrate a reduction in 20:4n-6 in EDL may be secondary to a reduced content of 22:6n-3 and loss of its inhibitory effect on elongation of n-6 fatty acids. The reciprocal changes in 22:6n-3 in EDL PL (decreased in PC and increased in PE) initially raised the possibility of reduced methylation in Type II muscles. In some tissues as much as 40% of PC is formed by methylation of PE, and PE containing 22:6n-3 is a favored substrate for this reaction. However, evidence suggests that synthesis of PC by methylation of PE occurs only in the liver and not in muscle (29).

Although reduced elongation of fatty acids destined for PL in EDL seems a reasonable explanation for the changes in fatty acid profile of the two muscles, it is not clear why such a reduction should be observed in the PC fraction only. We first considered the possibility that the lower rates of fatty acid oxidation in EDL muscle might result in preferential oxidation of elongation products of 16:0 prior to incorporation into membrane PL. However, results from experiments using the CPT-I inhibitor, etomoxir, suggest that this is not the case. Despite clear evidence for an inhibitory effect of etomoxir on fatty acid oxidation, we observed no change in the PL fatty acid profile of the two muscles compared to untreated control animals. Other possibilities for the altered fatty acid profile include differences in substrate specificity for PC biosynthesis or degradation (30). The known preference of the PC-synthesizing enzymes for substrate DAG with 16:0 in the *sn*-1 position (25) could be even more enhanced in Type II muscle such that proportionally less 18:0 is incorporated, providing a profile consistent with reduced elongation activity. This possibility not withstanding, the generalized reduction in elongation products observed in the present studies is more consistent with a global reduction in fatty acid elongation in the PC species obtained from Type II muscle rather than differential multiple substrate specificities for biosynthesis or degradation in the two muscles.

The restriction of intermuscle variations in fatty acid composition to the PC class of PL has important implications for other studies. Borkman and coworkers (31), in their important studies on PL fatty acid composition in man, may not have detected a decreased elongation profile in insulin-insensitive muscle because the entire PL fraction, rather than just PC, was used for analysis. Even so, the negative correlation of palmitate and the positive correlation of stearate with insulin sensitivity in their transformed data suggests the possibility that in man, insulin sensitivity correlates negatively with the PL fatty acid elongation profile (16:0 to 18:0 ratio). These same authors, in conjunction with scientists working with the Pima Indians, have recently reported an decrease in elongase activity in obese, insulin-resistant, Pima Indians (32) which supports this contention.

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