Skeletal muscle phosphatidylcholine fatty acids and insulin sensitivity in normal humans

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Clore, John N., Jing Li, Ranjodh Gill, Shona Gupta, Robert Spencer, Amin Azzam, Wilhelm Zuelzer, William B. Rizzo, and William G. Blackard. Skeletal muscle phosphatidylcholine fatty acids and insulin sensitivity in normal humans. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E665–E670, 1998.—The fatty acid composition of skeletal muscle membrane phospholipids (PL) is known to influence insulin responsiveness in humans. However, the contribution of the major PL of the outer (phosphatidylcholine, PC) and inner (phosphatidylethanolamine, PE) layers of the sarcolemma to insulin sensitivity is not known. Fatty acid composition of PC and PE from biopsies of vastus lateralis from 27 normal men and women were correlated with insulin sensitivity determined by the hyperinsulinemic euglycemic clamp technique at insulin infusion rates of 0.4, 1.0, and 10.0 mU·kg⁻¹·min⁻¹. Significant variation in the half-maximal insulin concentration (ED₅₀) was observed in the normal volunteers (range 24.0–146.0 µU/ml), which correlated directly with fasting plasma insulin (r = 0.75, P < 0.0001). ED₅₀ was inversely correlated with the degree of membrane unsaturation (C₂₀–C₂₂ polyunsaturated fatty acids; r = 0.58, P < 0.01) and directly correlated with fatty acid elongation (ratio of 16:0 to 18:0; r = 0.45, P < 0.05) in PC. However, no relationship between fatty acid composition and insulin sensitivity was observed in PE (NS). These studies suggest that the fatty acid composition of PC may be of particular importance in the relationship between fatty acids and insulin sensitivity in normal humans. Fatty acid elongation; phosphatidylethanolamine; muscle fiber type.

SKELETAL MUSCLE accounts for ~85% of insulin-stimulated glucose disposal in nondiabetic humans (7, 17, 38). Moreover, defects in skeletal muscle glucose disposal account for most of the reduction in insulin sensitivity characteristic of obesity and non-insulin-dependent diabetes mellitus (3). Recent studies have demonstrated impaired glucose transport (3) and/or phosphorylation (37) in obese diabetic and nondiabetic individuals that is associated with a reduction in the translocation of the insulin-regulated glucose transporter GLUT-4 to the sarcolemma (18). However, the mechanisms responsible for reduced glucose transport in insulin-resistant states are not known.

Insulin sensitivity is related in part to muscle fiber type (24). Increased proportion of type I (oxidative) to type II (glycolytic) fibers is associated with greater fatty acid oxidation and greater insulin responsiveness (15, 27, 39), which may be related to the known increase in GLUT-4 transporters in type I muscle (26). Characteristics of the plasma membrane bilayer, including membrane fluidity and phospholipid composition, are also associated with insulin sensitivity. Kriketos et al. (19) and Pan et al. (34) have recently shown an inverse relationship between insulin resistance and the percentage of long-chain polyunsaturated fatty acids (PUFA) in human muscle membrane phospholipids. Moreover, increased PUFA are found in membrane phospholipids of type I compared with type II fibers (19, 20), suggesting that membrane phospholipid fatty acid composition may play a significant role in the relative insulin responsiveness of muscle fiber types.

These important studies were performed on total membrane phospholipids. However, plasma membranes contain several distinct phospholipid species (40), and the fatty acid composition of these phospholipid species may have considerable variation. Of particular interest are the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which together account for >75% of the total plasma membrane phospholipids (40). In addition, it is known that the outer and inner layers of the plasma membrane bilayers contain greater proportions of PC and PE, respectively. Thus differences in the fatty acid composition of these two phospholipid species could have independent effects on insulin responsiveness. We have recently demonstrated that the fatty acid composition of PC (but not PE) in type II rat muscle is characterized by an enrichment of palmitic acid (16:0) relative to stearic acid (18:0), which suggested a defect in fatty acid elongation in the insulin-resistant muscle (2). The present studies were designed to determine whether differences in the fatty acid composition of PC and PE are observed in human skeletal muscle and are related to insulin responsiveness.

METHODS

Twenty-seven normal men and women (Table 1) were enrolled in the present studies. Body composition was determined by skinfold measurements (9), and bioimpedance was performed after an overnight fast (22). All of the subjects were in good health, with stable body weights at the time of the studies. None had a family history of diabetes mellitus. Women were studied during the early follicular phase of their menstrual cycles, and none of the women was taking exogenous estrogens or progestins. The studies were approved by the Committee for the Conduct of Human Research at the Medical College of Virginia, Virginia Commonwealth University, and informed consent was obtained from each subject before enrollment in the protocols.

Subjects were admitted to the General Clinical Research Center at the Medical College of Virginia the evening before study and consumed a regular diet while in the unit. On the
first morning of the study, a biopsy of the vastus lateralis muscle was obtained. After infiltration of the skin with 1% lidocaine, a 1-in. incision through the skin and fascia was made in the midlateral thigh, and a muscle sample was obtained by using a 14-gauge Bergstrom needle. To determine whether deep and superficial muscles were similar in muscle fiber composition and phospholipid fatty acid composition, muscle samples were obtained by both Bergstrom needle aspiration and by excisional biopsy in 19 of the subjects. A portion of the muscle sample was mounted on Tissue Tek II OCT compound (Lab-Tek Products, Naperville, IL) and then frozen in isopentane with liquid nitrogen as a coolant. All samples were then frozen at −70°C.

A three-step (0.4, 1.0, and 10.0 mU·kg⁻¹·min⁻¹) hyperinsulinenic euglycemic clamp was then performed on each subject after an overnight fast as previously described (8). The clamps were performed 2 days after the muscle biopsy to minimize the effect of the biopsy on insulin responsiveness (16). At 0700, an intravenous catheter was placed into a forearm vein for the infusion of insulin and 20% dextrose supplemented with 20 mM potassium phosphate. Another catheter was placed in a retrograde fashion in a hand vein, and the hand was placed in a Plexiglas box heated to 60°C to obtain arterialized venous blood samples (29). Both catheters were kept patent with an infusion of normal saline. Each step was performed for 120 min. During the final 30 min of each step, indirect calorimetry was performed by using a Sensormedics 2000 metabolic cart (Anaheim, CA) equipped with a plastic canopy to measure O₂ consumption (l/min) and CO₂ production (l/min).

Blood samples were obtained every 2.5–5 min during the clamps for measurement of plasma glucose (Yellow Springs Instrument). In addition, samples were obtained every 30 min for measurement of intermediary metabolites and glucoregulatory hormones. Plasma insulin (31), C-peptide (21), and glucagon (1) were determined with double-antibody RIA. Plasma free fatty acids were determined by enzymatic methods (30). Blood samples for measurement of intermediary metabolites were immediately deproteinized with ice-cold 3 M perchloric acid. The supernatant was neutralized with 3 M KOH, and the resulting supernatant was assayed for L-lactate, alanine, β-hydroxybutyrate, and acetocetate (28), citrate (33), and glycerol (42) with microfluorometric assays. Urinary nitrogen was determined by the Kjeldahl method.

Muscle fiber typing. Muscle fiber types were classified according to their myosin ATPase activity at pH 4.3, 4.6, and 10.4 as previously described (5). Fibers designated as type I were stable at acid pH but labile at pH 10.4, whereas the converse was observed for type II fibers. Type IIa fibers were determined from their lability at pH 4.6 but not 4.3. At least 300 fibers were analyzed from each sample.

Phospholipid fatty acid composition. Phospholipid fatty acid composition was analyzed according to Borkman et al. (4) with minor modifications. Muscles (50–60 mg) were homogenized and total lipids were extracted by the method of Folch et al. (10). The lipid extracts were dried under nitrogen, dissolved in 10 ml of hexane, and applied to 3-ml silica gel columns (J. T. Baker, Phillipsburg, NJ). After elution of less polar lipids with 20 ml of hexane followed by 10 ml of dichloromethane, phospholipids were eluted with 10 ml of methanol. The methanol eluates were dried under nitrogen and transmethylated with 1.5 ml of 1 N methanolic HCl at 80°C overnight. Fatty acid methyl esters were extracted with 6 ml of hexane and dried under nitrogen.

To determine the fatty acid composition of individual phospholipid species, PC, PE, sphingomyelin (SM), phosphatidylinositol (PI), cardiolipin (CL), and phosphatidylserine (PS) were first separated by TLC on silica gel G plates (Whatman LK6D) by using a solvent system consisting of chloroform-ethanol-triethylamine-water (30:34:30:8, vol/vol) for the first development (SF₁) and hexane-dimethyl ether (50:50, vol/vol) for the second development (SF₂). Phospholipids were visualized under ultraviolet light after the plate was sprayed with rhodamine G. The separated phospholipid spots were scraped and placed into glass tubes. Fatty acid methyl esters were prepared as described above by treatment with methanolic methanolic acid.

Values are means ± SE with ranges; n = 27 (18 men, 9 women).

Table 1. Demographics of subjects enrolled in metabolic studies

<table>
<thead>
<tr>
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<th>Mean ± SE</th>
<th>Range</th>
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<tr>
<td>Age, yr</td>
<td>24.3 ± 0.95</td>
<td>19–43</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.3 ± 0.65</td>
<td>17.9–31.6</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>20.7 ± 1.6</td>
<td>9.0–37.1</td>
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Calculations. Glucose disposal was determined from the glucose infusion rate for each of the insulin infusion steps during the final 30 min of each step. The glucose infusion required to maintain euglycemia at the 10 mU·kg⁻¹·min⁻¹ insulin infusion rate was assumed to be maximal insulin-stimulated glucose disposal. The insulin concentration calculated to stimulate half-maximal glucose disposal (ED₅₀) was determined from the best-fit relationship between plasma insulin concentrations and glucose infusion rates during the three-step clamps as previously described (6). Greater values of ED₅₀ reflect reduced insulin sensitivity, whereas greater glucose infusion rates at a given insulin infusion rate reflect greater insulin sensitivity. Calculation of carbohydrate and lipid oxidation was performed with the equations of Frayn.

Phospholipids, triglyceride, cholesterol, and fatty acid standards were obtained from Sigma (St. Louis, MO). High-performance precoated silica gel HPLC plates (10 × 10 cm) were purchased from Whatman (Clifton, NJ). All other reagents and solvents were of analytical or HPLC grade from Sigma or Fisher (Pittsburgh, PA).

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Despite the wide range of ED50 observed in our normal volunteers, there was no correlation between insulin sensitivity and muscle fiber composition.

The fatty acid compositions of the predominant phospholipids, PC and PE, obtained from biopsies of the vastus lateralis are shown in Table 3. A striking difference in the fatty acid composition of the two phospholipid species is readily apparent. Significant increases in the percentages of 16:0, 18:1, and 18:2 are seen in the PC fraction compared with those in PE, whereas increased percentages of 18:0, 20:4, and 22:6 were found in PE compared with PC. These differences probably reflect the fatty acid preferences for enzymes involved in the synthesis of PC and PE, and are in close agreement with previous findings (40). No differences in the fatty acid composition of muscle obtained by superficial (open) or deep (Bergstrom needle, closed) biopsy were found (data not shown). In addition, no differences in the fatty acid composition of the two phospholipids were observed between men and women.

To determine the relationship between fatty acid composition and insulin sensitivity, we analyzed the percentages of individual fatty acids, PUFA, and ratios of fatty acids that reflect the activities of enzymes of fatty acid elongation (ratio 16:0 to 18:0) and desaturase (Δ3-desaturase 20:4 to 20:3 and Δ6-desaturase 20:3 to 18:2). No association between any of these values and ED50 was observed in the fatty acid composition of PE. In contrast, ED50 was inversely related to PUFA (Fig. 1;

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>PC</th>
<th>PE</th>
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<tr>
<td>16:0</td>
<td>20.6±0.4*</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>6.5±0.3*</td>
<td>17.6±0.4</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>11.1±0.3*</td>
<td>5.5±0.4</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>36.6±0.5*</td>
<td>13.2±0.4</td>
</tr>
<tr>
<td>18:3 (n-6)</td>
<td>0.3±0.1</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>5.2±0.1*</td>
<td>24.9±0.6</td>
</tr>
<tr>
<td>22:5 (n-3)</td>
<td>0.8±0.1*</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td>0.8±0.1*</td>
<td>4.0±1.0</td>
</tr>
<tr>
<td>16:0-to-18:0 ratio</td>
<td>3.4±0.2*</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>20:4-to-20:3 ratio</td>
<td>44.5±3.4*</td>
<td>208.1±18.8</td>
</tr>
<tr>
<td>Δ (C20:4-C20:3)</td>
<td>6.8±0.2*</td>
<td>31.4±1.5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 27. PC, phosphatidylcholine; PE, phosphatidylethanolamine. *P < 0.001 for PC compared to PE.
r = -0.58, P < 0.01) and the 20:3-to-18:2 ratio (r = -0.42, P < 0.05) and directly related to the 16:0-to-18:0 ratio (Fig. 2; r = 0.45, P < 0.05) in PC. No relationship between ED50 and the 20:4-to-20:3 ratio was observed.

When insulin sensitivity was defined more conventionally as the glucose infusion rate required to maintain euglycemia during a 1.0 mU·kg⁻¹·min⁻¹ insulin infusion, insulin sensitivity was directly correlated with PUFA (r = 0.44, P < 0.05) but not with the 16:0-to-18:0 ratio or 20:3-to-18:2 ratio. Correction for obesity using the latter method decreased the relationship for C20-C22 PUFA (r = 0.44 vs. r = 0.38) and eliminated it for the 16:0-to-18:0 ratio.

To determine the impact of diet on our findings, we analyzed serum phospholipid fatty acids as an indirect measure of dietary intake (11). More than 75% of serum phospholipid fatty acids was accounted for by palmitate (17.4 ± 0.4%), stearate (7.2 ± 0.4%), oleate (21.5 ± 0.7%), linoleate (21.4 ± 0.5%), and arachidonate (10.3 ± 0.4%). These findings are consistent with diets somewhat lower in saturated fat and higher in monounsaturated fatty acids than previously reported values for normal adults (25). No correlation was found between serum phospholipid fatty acids and skeletal muscle membrane fatty acids in either PC or PE. Interestingly, we also found no correlation between serum phospholipid fatty acids and insulin sensitivity in our normal volunteers.

From our previous studies demonstrating a marked increase in the 16:0-to-18:0 ratio in type II but not type I muscle in rats (2), we reasoned that the relationship between fatty acids and insulin sensitivity might be masked by the heterogeneity of muscle fiber type in biopsies of human muscle. However, correction for the relative percentages of muscle fiber types did not increase the correlation between muscle phospholipid fatty acids and insulin sensitivity (data not shown).

DISCUSSION

The present studies were designed to examine the role of skeletal muscle fatty acid composition in the two major membrane phospholipids, PC and PE, in insulin sensitivity in humans. Using the ED50 as a measure of insulin sensitivity, our studies have shown an increase in the proportion of palmitic acid compared with stearic acid and a reduction in total PUFA content in PC in association with increasing ED50 in normal human volunteers. On the other hand, minimal association between fatty acid composition and insulin sensitivity was seen when the fatty acids contained in PE were analyzed. Our results were similar when insulin sensitivity was defined as the glucose infusion rate required to maintain euglycemia during a 1.0 mU·kg⁻¹·min⁻¹ insulin infusion. However, we have chosen the threestep clamp technique for the present studies to determine whether the putative effects of phospholipid fatty acid composition on insulin action are mediated by effects on insulin sensitivity or responsiveness. Based on the greater correlation of phospholipid fatty acids with ED50 or the 1.0 mU·kg⁻¹·min⁻¹ insulin infusion compared with the 10.0 mU·kg⁻¹·min⁻¹ insulin infusion, our studies suggest the former.

The mean distribution of fatty acids found in PC and PE from vastus lateralis samples from our normal volunteers is shown in Table 3. Our results are similar to previously reported values in human pectoral muscle samples (40) and demonstrate marked heterogeneity between the two phospholipids. In particular, increased incorporation of 16:0, 18:1, and 18:2 is found in PC, whereas greater percentages of 18:0, 20:4, and 22:6 are seen in PE. The increased proportions of 16:0 and 18:0 in PC and PE, respectively, reflect fatty acid preferences in the sn-1 position, with the other fatty acids occupying the sn-2 position, and are consistent with previous reports. Other phospholipids that contribute to overall phospholipid fatty acid composition include...
PI, PS, and CL. Together, these phospholipids make up ~13% of the total in human skeletal muscle membrane (40). Because of the limited tissue samples obtained and the relatively small contribution of each to the total, we have not determined the fatty acid composition of these phospholipids.

Membrane phospholipid fatty acids are derived from dietary fatty acids as well as de novo synthesis. With regard to dietary fatty acids, alterations in the proportion of polyunsaturates to saturates have been shown to affect membrane phospholipid fatty acids and could have contributed to the fatty acid composition observed in the present studies. Because we used plasma phospholipid fatty acids as an indicator of dietary fatty acids (25), our results suggest a diet lower in saturated fatty acids than the usual American diet. In contrast, intake of monounsaturated fatty acids was greater, whereas intake of n-3 fatty acids did not differ from previously reported values. No correlation between plasma phospholipids and skeletal muscle membrane phospholipids was observed. These data are consistent with remodeling of fatty acids by elongation and desaturation before incorporation into phospholipids.

In agreement with the findings of Borkman et al. (4), who related total phospholipid fatty acids to insulin sensitivity, increased proportions of PUFA in PC were associated with increased insulin sensitivity (lower ED50) in our nondiabetic subjects. In contrast, the content of long-chain PUFA in PE, although fivefold greater than PC, was not related to insulin sensitivity. This striking disparity suggests that the fatty acid composition of the outer portion of the lipid bilayer conveys much of the insulin sensitivity related to phospholipid fatty acids. There are several potential mechanisms by which changes in the fatty acid composition of PC might alter insulin sensitivity. Greater insulin sensitivity in membranes with increased content of PUFA (14) may be related to increased membrane fluidity and GLUT-4 translocation after insulin stimulation (36). Increased insulin receptor number and binding (13) as well as activation of insulin-receptor kinase (32) have also been observed in association with increasing membrane fluidity.

We have also observed a significant association between the relative proportions of 16:0 to 18:0 in PC and insulin sensitivity, which suggests a role for fatty acid elongation as well as total PUFA content in insulin resistance. Increased 16:0 to 18:0 ratio (decreased elongation) was associated with decreased insulin sensitivity in our cross-sectional study. Similarly, in a cross-sectional study of older men, Vessby et al. (41) demonstrated increased palmitic acid in skeletal muscle membranes of insulin-resistant subjects. Although the ratio of 16:0 to 18:0 is not provided in this report, an increase seems likely, since an increase in muscle adiposity is known to occur with aging (23). Recent studies in Pima Indians have demonstrated an increased 16:0 to 18:0 ratio in total phospholipid fatty acids in association with increased type IIb muscle in vastus lateralis biopsies (35). We have also recently demonstrated a greater 16:0 to 18:0 ratio in PC isolated from type II insulin insensitive muscle of normal rats (2). Thus the failure to demonstrate an increase in the 16:0 to 18:0 ratio in certain studies of insulin-resistant subjects may be related to the distribution of muscle fiber type in the subjects studied. Surprisingly, we were unable to increase the correlation between the ratio and insulin resistance by correcting for type IIb content in the present study. However, the content of type IIb fibers was relatively low in our subjects, and our analysis of the fatty acid composition of specific phospholipids rather than total phospholipids may have maximally amplified the differences that exist.

In conclusion, the present studies have demonstrated a major role for skeletal muscle membrane fatty acid composition of PC but not PE in insulin sensitivity in nondiabetic men and women. Failure to demonstrate a clear relationship between serum phospholipid fatty acids and insulin sensitivity suggests that fatty acid remodeling in PC by both elongation and desaturation is involved in membrane function.

We are extremely grateful to the staff of the General Clinical Research Center for their invaluable help in the performance of these studies. These studies were supported in part by National Institutes of Health Grants RO1-DK-43013, RO1-DK-18903, and RR-00065. Proof mailing address: J. N. Clore, Box 980155, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298.

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