Dietary ω -3 and polyunsaturated fatty acids modify fatty acyl composition and insulin binding in skeletal-muscle sarcolemma

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Feeding animals with diets high in saturated fat induces insulin resistance, and replacing saturated fat isocalorically with polyunsaturated fat, especially long-chain ω -3 fatty acids, will prevent the development of insulin resistance in skeletal-muscle tissue. To investigate the mechanism, rats were fed on high-fat (20%, w/w) semipurified diets for 6 weeks. Diets containing ratios of polyunsaturated/saturated (P/S) fatty acid of 0.25 (low-P/S diet) and 1.0 (high-P/S diet) were used to study the effect of the level of saturated fat. To study the effects of ω -3 fatty acids, diets with a low-P/S ratio containing either 0 (low- ω -3 diet) or 3.3% (high- ω -3 diet) long-chain ω -3 fatty acids from fish oil were fed. Plasma membrane from skeletal muscle was purified. The content of fatty acids in sarcolemmal phospholipid was significantly related to the dietary composition. Insulin binding to intact sarcolemmal

vesicles prepared from rats fed on diets high in ω -3 fatty acids increased 14-fold compared with animals fed on the low- ω -3 diet ($P < 0.0001$). Feeding rats on a diet with a high P/S ratio increased sarcolemmal insulin binding by 2.3-fold ($P < 0.05$). Increased insulin binding was due to increased receptor number at the low-affinity high-capacity binding site. Dietary effects on insulin binding were eliminated when studies were carried out on detergent-solubilized membranes, indicating the importance of the phospholipid fatty acyl composition for insulin binding. The results suggest that dietary ω -3 and polyunsaturated fatty acids increase insulin binding to sarcolemma by changing the fatty acyl composition of phospholipid surrounding the insulin receptor, and this might be the mechanism by which dietary fatty acids modify insulin action.

INTRODUCTION

Insulin resistance is a metabolic state in which normal or high concentrations of insulin produce a less than normal biological response (De Vries et al., 1989). Defects in insulin binding, tyrosine kinase and post-kinase cascades have been postulated as the reason, but the mechanism is not completely understood (Haring, 1991). Insulin initiates its biological effects by binding to its receptors on target tissues (De Vries et al., 1989). There is evidence suggesting that impaired insulin binding is closely associated with insulin-resistant states, such as those associated with feeding high-fat diets (Olefsky and Saekow, 1978), obesity (Olefsky et al., 1976; Marchand-Brustel et al., 1978; Caro et al., 1987) and non-insulin-dependent diabetes mellitus (Trischitta et al., 1989).

Insulin binding to its receptor can be altered by changing the fatty acid composition of phospholipids in artificial membranes (Gould et al., 1982). In cultured (Ginsberg et al., 1981) and isolated cells (Field et al., 1988), membranes enriched in unsaturated fatty acids tend to bind more insulin than membranes enriched in saturated fatty acids. It has been postulated that alteration in membrane fluidity induced by enrichment of polyunsaturated fatty acids might be involved in the change observed in insulin binding (Ginsberg et al., 1981). The composition of cell membrane phospholipids can be altered by dietary fatty acids (Clandinin et al., 1991) by mechanisms that may involve altering the activity of enzymes of fatty acid desaturation (Garg et al., 1988), membrane cholesterol content and the biosynthesis of membrane phosphatidylcholine *de novo* (Hargreaves and Clandinin, 1987). In rats and humans, consumption of a high-P/S (polyunsaturated/saturated fatty acid ratio, P/S = 1.0) diet

increased the content of polyunsaturated fatty acids in adipocyte membrane phospholipids, compared with the low-P/S diet (P/S = 0.25) (Field et al., 1985, 1988). In agreement with *in vitro* studies (Ginsberg et al., 1981), the elevated content of polyunsaturated fatty acids in adipocyte membrane was associated with increased insulin binding (Field et al., 1988). By feeding a high-P/S diet, it was demonstrated in adipocytes that glucose transport was also augmented by the high-P/S diet, along with increased insulin binding (Field et al., 1990).

Skeletal muscle is an important site of insulin action. The insensitivity of this tissue to insulin plays an important role in the development of whole-body insulin resistance (Klip and Paquet, 1990). Feeding rats on a high-fat diet can lead to skeletal-muscle insulin resistance, even when polyunsaturated ω -6 fatty acids are also fed (Storlien et al., 1987, 1991). When a proportion of the ω -6 fatty acids was replaced by fish oil high in long-chain ω -3 fatty acids (22:5 ω -3 and 22:6 ω -3), the muscle insulin resistance induced by the high-fat diet was prevented (Storlien et al., 1987, 1991). Recently, we reported that maximum insulin-stimulated glucose uptake in isolated skeletal muscle was also higher in rats fed on fish oil (Sohal et al., 1992). However, the mechanism by which ω -3 fatty acids prevent insulin resistance in skeletal muscle is unknown. The membrane phospholipid content of long-chain (22:5 and 22:6) ω -3 fatty acid in skeletal muscle is apparently closely related to insulin-stimulated glucose metabolism (Storlien et al., 1991). Feeding rats on a high-fat diet also seems to decrease the amount of insulin binding to skeletal muscle (Maegawa et al., 1986). Thus we hypothesized that improved insulin binding to skeletal muscle of rats fed on a high- ω -3 fatty acid diet might be responsible for the prevention of insulin resistance. Furthermore, we hypothesized that the differences in

insulin binding may be attributable to ω -3 fatty acids *per se* or due to the high degree of fatty acid unsaturation produced in the plasma-membrane fraction.

EXPERIMENTAL

Materials

Benzamidine and iodoacetamide were purchased from Aldrich Chemical Co. (Milwaukee, WI 53233, U.S.A.). Porcine insulin, aprotinin, leupeptin, pepstatin A, phenylmethanesulphonyl fluoride, bacitracin, wheat germ agglutinin (WGA), BSA and rabbit γ -globulin were purchased from Sigma Chemical Co. (St Louis, MO 63178, U.S.A.). 125 I-insulin (porcine) was purchased from Du-Pont-New England Nuclear (Boston, MA, U.S.A.).

Animal care and treatment

Studies were carried out in accordance with the Canadian Council on Animal Care guidelines. Female Sprague-Dawley rats were obtained from a colony maintained at the University of Alberta and were fed on semipurified diets starting at 30–35 days of age. The rats were housed in individual wire mesh cages in a room that was maintained at 23 °C on a 12 h light/dark cycle. Animals were given food and water *ad libitum* and were fed on one of the experimental diets for 6 weeks.

Animals were fed on semipurified nutritionally complete diets containing (% w/w): 27% high-protein casein, 38% carbohydrates (cornstarch), 20% fat, 1% vitamin mixture (Association of Analytical Chemists), 5% mineral mixture (Bernhart-Tomerelli), 0.3% choline, 0.6% inositol, 0.3% methionine and 8% non-nutritive cellulose (Field et al., 1988). In experiments in which a different dietary level of ω -3 fatty acid was studied, 20 rats were randomly assigned to two treatment groups: low- ω -3 and high- ω -3 diets. Diets differed only in the composition of fat providing two different levels of long-chain ω -3 fatty acids. The high- ω -3 diet contained long-chain ω -3 fatty acids (2.4% 20:5 ω -3, 0.2% 22:5 ω -3 and 0.7% 22:6 ω -3) as well as α -linolenic acid (18:3 ω -3, 0.3%). The low- ω -3 fatty acid diet contained only α -

linolenic acid (C18:3 ω -3, 0.8%) as the source of ω -3 fatty acid. The fatty acid composition of the diets is illustrated in Table 1. In experiments in which the dietary P/S ratio was studied, the experimental design was similar to that described above except that the diets provided a P/S ratio of either 1.0 (high-P/S) or 0.25 (low-P/S) (Table 1) as described previously (Field et al., 1988). All four diets reflected a dietary level of ω -3, ω -6 and saturated fatty acids that is well within the range of diet fat composition consumed by humans.

Membrane preparation

Rats were killed by CO₂ asphyxiation. Plasma membrane was prepared using differential and density-gradient centrifugation as described by Ohlendieck et al. (1991). After those steps, membrane was further purified by wheat germ agglutination. Membrane isolated using this procedure is enriched in the sarcolemmal protein dystrophin, but does not contain T-tubule membrane or junctional sarcoplasmic reticulum as assessed by immunochemical methods (Ohlendieck et al., 1991). The agglutination step was incorporated into the binding protocol for membrane fractions used in insulin-binding studies. Membrane protein content was determined after solubilization with 1 M NaOH by the method of Bradford (1976) using BSA as standard protein (Sigma, fraction V). About 10 mg of membrane protein was recovered after the density-gradient-centrifugation step from 100 g of mixed hindlimb muscle dissected from rats of each treatment group. The membrane was suspended in Krebs-Ringer Hepes buffer (118 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄ and 50 mM Hepes, pH 7.6), quickly frozen in liquid nitrogen and stored at -50 °C for further analysis.

Insulin binding to skeletal-muscle sarcolemma

Insulin binding to sarcolemmal vesicles was measured as described by Grimditch et al. (1985) and Kergoat et al. (1988) with some modifications. Membrane protein (100 μ g) was incubated in a microcentrifuge tube with 0.03 nM 125 I-insulin (about 1200 Bq/tube) in Krebs-Ringer Hepes buffer containing 1% (w/v) BSA and 1 mg/ml bacitracin. The final volume was 200 μ l. After 20 h incubation at 4 °C, the binding reaction was stopped by centrifugation after agglutination of the vesicles with WGA as follows: the reaction medium was mixed with 100 μ l (1 mg/ml) of WGA; after 20 min incubation on ice, bound insulin was separated by centrifugation at 17000 *g* for 5 min using an IEC Centra-M centrifuge at 4 °C. The supernatant was discarded and the pellet was washed twice with chilled 0.25 M sucrose. The tip of the tube containing the pellet was cut off and placed in a test tube for counting of radioactivity in a Packard Cobra Auto-Gamma counter. Counts were converted into d.p.m. Non-specific binding was defined as radioactivity bound to membrane in the presence of 10 μ M unlabelled insulin and was typically about 30% of total binding. Specific binding was obtained by subtracting non-specific binding from total binding.

Insulin binding to solubilized receptor

Solubilized receptor was prepared as described by Whitson and Kaplan (1985). The receptor from 0.1 mg of membrane protein was incubated with 1200 Bq of 125 I-insulin in the buffer as described in the assay of insulin binding to sarcolemma. After a 20 h incubation at 4 °C, 100 μ l of 0.3% (w/v) γ -globulin and 300 μ l of 25% (w/v) poly(ethylene glycol) were added to the binding mixture. This mixture was placed on ice for 20 min and centrifuged at 17000 *g* for 5 min using an IEC Centra-M

Table 1 Fatty acid composition of the diets

Values are % (w/w) for the fat in the diet. SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids; P/S ratio: the ratio of polyunsaturated to saturated fatty acids; ω -6, total ω -6 fatty acids; ω -3, total ω -3 fatty acids; ND, not detectable.

	High- ω -3	Low- ω -3	High-P/S	Low-P/S
Fatty acids				
C _{14:0}	3.5	3.7	1.5	2.8
C _{16:0}	24.7	27.0	15.3	22.5
C _{18:0}	48.2	35.2	25.1	47.5
C _{18:1}	3.3	9.3	9.3	4.4
C _{18:2ω-6}	13.4	10.5	44.3	17.4
C _{18:3ω-3}	0.3	0.8	1.4	1.2
C _{20:0}	0.5	0.3	0.4	0.5
C _{20:5ω-3}	2.4	ND	ND	ND
C _{22:5ω-3}	0.2	ND	ND	ND
C _{22:6ω-3}	0.7	ND	ND	ND
SAT	77.4	68.8	43.9	75.9
MONO	4.9	10.4	9.7	5.1
PUFA	17.6	11.9	46.3	19.4
ω -6	13.5	10.9	44.8	17.6
ω -3	4.2	1.0	1.5	1.4
P/S ratio	0.23	0.18	1.05	0.25

Table 2 Effects of high- and low- ω -3 diets on the fatty acyl composition of skeletal-muscle plasma-membrane phospholipids

Values are percentages of total fatty acids in each membrane fraction. Fatty acids were determined using membranes pooled from ten rats per dietary treatment. ND, not detectable; SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; ω -6, total ω -6 fatty acids; ω -3, total ω -3 fatty acids; P/S polyunsaturated/saturated fatty acid ratio.

	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine		Phosphatidylinositol	
	High- ω -3	Low- ω -3	High- ω -3	Low- ω -3	High- ω -3	Low- ω -3	High- ω -3	Low- ω -3
C _{16:0}	36.7	33.9	7.5	4.5	5.2	6.6	16.0	13.1
C _{18:0}	14.2	15.2	20.7	20.9	47.2	46.1	32.9	33.4
C _{18:1}	8.7	7.1	5.8	5.0	9.3	8.8	12.4	9.4
C _{18:2ω-6}	22.8	24.1	19.4	20.3	4.3	4.3	8.7	7.7
C _{20:4ω-6}	5.8	13.2	4.5	16.1	3.8	9.3	10.2	15.8
C _{20:5ω-3}	0.9	0.1	2.2	0.2	0.3	0.4	2.9	1.5
C _{22:5ω-3}	1.6	0.4	2.6	2.1	5.0	1.9	2.8	ND
C _{22:6ω-3}	3.9	0.9	13.7	10.6	17.3	8.1	5.8	2.6
SAT	53.3	50.8	37.5	30.0	54.9	56.4	51.6	51.8
MONO	9.8	8.0	7.8	5.7	10.0	8.8	15.6	13.1
ω -6	30.7	39.7	32.4	49.5	11.9	24.6	21.4	31.0
ω -3	6.5	1.5	22.4	14.8	23.1	10.3	11.4	4.2
P/S	0.70	0.81	1.46	2.14	0.64	0.62	0.64	0.68

centrifuge at 4 °C. The final pellet was counted as described above.

Membrane lipid extraction, phospholipid separation and fatty acid determination

Lipid was extracted from plasma membrane obtained after WGA agglutination by a modified Folch procedure (Field et al., 1988). Individual phospholipids were separated in a one-dimensional solvent system using silica-gel H plate (ANAL-TECH, Newmark, DE, U.S.A.). Phospholipid fatty acid methyl esters were prepared using 14% (w/v) BF₃/methanol reagent (Morrison and Smith, 1964) and separated by automated g.l.c. (Vista 6010 GLC and Varian 604 chromatography data system; Varian Instruments, Georgetown, Ont., Canada). Chromatography was performed using a fused silica BP20 capillary column (25 m $\times$ 0.25 mm inner diameter; Varian Instruments) under conditions described in detail previously (Field et al., 1988).

Data analysis

The data presented were obtained from four separate feeding studies and membrane preparations. All analyses were carried out in duplicate. Rosenthal (1967) analysis was completed using the software LIGAND (Biosoft, 22 Hills Road, Cambridge, CB2 1JP, U.K.). The parameters obtained were statistically compared using Student's *t* test.

RESULTS

Methodological considerations

In preliminary experiments, membrane fractions obtained after density-gradient centrifugation were used in insulin-binding assays. In these studies, non-specific binding varied from 45 to 50% of total insulin bound to membrane fractions precipitated by ultracentrifugation at approx. 140000 *g* for 5 min. WGA was then used to precipitate membrane and it was demonstrated that WGA does not precipitate free insulin (results not shown). The non-specific binding was lowered by this procedure to approx. 30%. The absolute amount of specifically bound insulin was the same using the two precipitation methods, but both the absolute

amount of total and non-specifically bound insulin was lower using the WGA method.

Effects of diets on the fatty acyl composition of skeletal-muscle membrane phospholipids

The fatty acyl composition of individual membrane phospholipids was obtained using WGA-purified membrane prepared from a pool of ten rats per dietary treatment. The dietary content of long-chain ω -3 fatty acids exhibited profound effects on the membrane phospholipid fatty acid composition (Table 2).

Effects of high- and low- ω -3 diet

In phosphatidylcholine, the total ω -3 fatty acid content was 6.5% for rats fed on the high- ω -3 fatty acid diet compared with only 1.5% for rats fed on the low- ω -3 fatty acid diet, a more than threefold increase (Table 2). The content of 20:5 ω -3, 22:5 ω -3 and 22:6 ω -3 were 0.9, 1.6 and 3.9% for animals fed on the high- ω -3 fatty acid diet compared with 0.1, 0.4 and 0.9% for animals fed on the low- ω -3 fatty acid diet respectively. Feeding the high- ω -3 diet decreased the 20:4 ω -6 content from 13.2% observed for the low- ω -3 group to 5.8% for the high- ω -3 group.

In phosphatidylserine, the amount of total ω -3 fatty acid was higher in rats fed the high- ω -3 diet (23.1%) than rats fed the low- ω -3 diet (10.3%). This was mainly due to the increase in the contents of 22:5 ω -3 and 22:6 ω -3 fatty acids (5.0% and 17.3% for the high- ω -3 diet compared with 1.9% and 8.1% for the low- ω -3 diet respectively). The content of 20:5 ω -3 was similar in both groups (0.3% compared with 0.4%). The amount of 20:4 ω -6 was decreased from 9.3% to 3.8% by feeding the high- ω -3 diet.

In phosphatidylinositol, the total amount of ω -3 fatty acid was higher in rats fed the high- ω -3 diet than obtained for animals fed the low- ω -3 diet (11.4% compared with 4.2%). The content of all of the long-chain ω -3 fatty acids was increased by feeding the diet high in ω -3 fatty acids (Table 2). In contrast, the content of 20:4 ω -6 was 50% higher in rats fed low- ω -3 diet (10.2% compared with 15.8%).

In phosphatidylethanolamine, the total ω -3 fatty acid content was increased 51% by feeding the diet high in ω -3 fatty acids (22.3% compared with 14.8%; Table 2). This represented the smallest percentage increase obtained among all phospholipid

Table 3 Effects of high- and low-P/S diets on the fatty acyl composition of skeletal-muscle plasma-membrane phospholipids

Values are percentages of total fatty acids in each membrane fraction. Fatty acids were determined using membranes pooled from ten rats per dietary treatment. SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; P/S, ratio of polyunsaturated to saturated fatty acids; ND, not detectable; ω -6, total ω -6 fatty acids; ω -3, total ω -3 fatty acids.

	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylserine		Phosphatidylinositol	
	High- ω -3	Low- ω -3	High- ω -3	Low- ω -3	High- ω -3	Low- ω -3	High- ω -3	Low- ω -3
C _{16:0}	35.9	37.7	7.2	8.6	5.4	8.4	2.9	3.6
C _{18:0}	10.9	10.1	35.8	33.6	53.3	51.2	51.0	52.1
C _{18:1}	5.0	5.7	4.2	4.4	4.1	6.6	2.2	2.4
C _{18:2ω-6}	20.7	24.8	11.4	9.5	5.3	4.7	3.4	2.4
C _{18:3ω-3}	0.7	0.2	0.3	ND	ND	ND	0.1	0.1
C _{20:4ω-6}	20.0	17.9	23.8	23.4	9.2	10.2	35.2	33.7
C _{20:5ω-3}	0.1	0.2	0.3	ND	ND	0.8	0.1	0.1
C _{22:5ω-3}	0.4	0.5	2.5	3.7	2.1	1.1	0.6	0.8
C _{22:6ω-3}	0.8	1.1	8.3	11.2	11.0	8.7	0.7	0.8
SAT	47.3	48.5	43.2	43.2	59.8	60.3	54.4	56.2
MONO	5.5	6.1	5.5	5.4	5.2	7.5	3.0	3.1
ω -6	45.9	43.5	40.0	36.6	21.8	21.6	41.2	39.0
ω -3	1.4	1.9	11.4	14.8	13.1	10.6	1.4	1.8
P/S	1.00	0.94	1.19	1.19	0.58	0.53	0.78	0.73

Figure 1 Rosenthal plot of insulin binding to (a) membranes and (b) receptors prepared from rats fed on high- and low- ω -3 diets

B/F, Amount of bound insulin over that of free insulin. ●, Membrane or receptor prepared from rats fed on high- ω -3 diet; ▲, membrane or receptor prepared from rats fed on low- ω -3 diet.

fractions. The individual long-chain ω -3 fatty acids were slightly higher in rats fed on the diet high in ω -3 fatty acids with the exception of 20:5 ω -3, which was 10-fold higher in rats fed on the high- ω -3 diet. The content of 20:4 ω -6 was lowered by feeding the high- ω -3 diet (from 16.1% to 4.5%).

In all phospholipid fractions the content of saturated and monounsaturated fatty acids was not significantly altered by the diet (Table 2). The P/S ratios of the membrane were similar in

both dietary treatments, except for phosphatidylethanolamine in which the P/S ratio tended to be lowered for animals fed on the high- ω -3 diet.

Effects of high- and low-P/S diet

Neither the content of individual fatty acids nor the P/S ratio in phospholipids was altered by the diets with different P/S ratios (Table 3).

Effect of high- and low- ω -3 diets on insulin binding to skeletal-muscle sarcolemma

Skeletal-muscle sarcolemma from animals fed on the high- ω -3 fatty acid diet bound more insulin than that from rats fed on the low- ω -3 fatty acid diet over a wide concentration range of insulin (0.1–100 nM). This difference in binding was especially marked at high insulin concentrations (Figure 1a). Rosenthal analysis revealed two insulin-binding sites on the skeletal-muscle sarcolemma. Binding parameters were derived from Rosenthal analysis, including $B_{\max}$ and $1/K_d$ for high- and low-affinity binding sites (Table 4A). There was no significant difference caused by diet in either parameter at the high-affinity binding sites, but the $B_{\max}$ was 14-fold higher for the low-affinity high-capacity binding site in membrane of rats fed on the high- ω -3 fatty acid diet ($P < 0.0001$) (Table 4A).

When insulin receptors were solubilized, the receptors from animals fed on a high- ω -3 fatty acid diet tended to bind more insulin at high insulin concentrations than the receptors from animals fed on a low- ω -3 diet. From the Rosenthal analysis, it appears that the binding curves for the two diets were not identical (Figure 1b). The $B_{\max}$ observed was not significantly different for membrane from animals fed on either diet (Table 4A).

Effect of high- and low-P/S diets on insulin binding to skeletal-muscle sarcolemma

Skeletal-muscle sarcolemma from animals fed on high-P/S diet bound more insulin than that from animals fed on a low-P/S diet, especially at high insulin concentrations (Figure 2a). Rosenthal analysis demonstrated that the $B_{\max}$ at the low-affinity

Table 4 Insulin and insulin-like growth factor I (IGF-I) binding to membranes and solubilized receptors prepared from rats fed on high- and low- ω -3 or -P/S diets

Values are binding parameters $\pm$ S.E.M., K_d , dissociation constant expressed as M; B_{max} , total receptor concentration, expressed as sites/mg of protein; *** $P < 0.0001$, compared with the binding to membrane from rats fed on a high- ω -3 diet; ** $P < 0.05$, compared with binding to membrane prepared from rats fed on a high-P/S diet; * $P < 0.05$, compared with binding to membrane from rats fed on a high- ω -3 diet.

	High-affinity site		Low-affinity site	
	B_{max}	$1/K_d$	B_{max}	$1/K_d$
A Insulin binding				
High- ω -3, membrane	$(5.0 \pm 1.0) \times 10^{-11}$	$(17 \pm 4.8) \times 10^8$	$(75 \pm 8.5) \times 10^{-10}$	$(6.6 \pm 1.0) \times 10^6$
Low- ω -3, membrane	$(9.2 \pm 2.5) \times 10^{-11}$	$(8.5 \pm 1.6) \times 10^8$	$(5.2 \pm 2.3) \times 10^{-10***}$	$(8.2 \pm 6.2) \times 10^6$
High- ω -3, receptor	$(2.7 \pm 0.62) \times 10^{-11}$	$(23 \pm 10) \times 10^8$	$(8.6 \pm 2.5) \times 10^{-10}$	$(79 \pm 36) \times 10^6$
Low- ω -3, receptor	$(4.1 \pm 8.8) \times 10^{-11}$	$(8.3 \pm 2.2) \times 10^8$	$(3.1 \pm 0.97) \times 10^{-10}$	$(100 \pm 620) \times 10^6$
High-P/S, membrane	$(2.4 \pm 0.57) \times 10^{-11}$	$(25 \pm 12) \times 10^8$	$(2.7 \pm 0.48) \times 10^{-10}$	$(19 \pm 8) \times 10^6$
Low-P/S, membrane	$(3.0 \pm 0.43) \times 10^{-11}$	$(13 \pm 29) \times 10^8$	$(1.2 \pm 0.32) \times 10^{-10**}$	$(9.9 \pm 100) \times 10^6$
High-P/S, receptor	$(5.4 \pm 0.77) \times 10^{-11}$	$(6.9 \pm 1.2) \times 10^8$	$(5.2 \pm 2.4) \times 10^{-10}$	$(5.3 \pm 3.7) \times 10^6$
Low-P/S, receptor	$(4.6 \pm 0.4) \times 10^{-11}$	$(7.7 \pm 8.1) \times 10^8$	$(2.4 \pm 0.24) \times 10^{-10}$	$(14 \pm 3.1) \times 10^6$
B IGF-I binding to membrane				
High- ω -3	$(1.6 \pm 0.5) \times 10^{-11}$	$(7.1 \pm 4.9) \times 10^8$	$(5.0 \pm 0.5) \times 10^{-10}$	$(9.9 \pm 1.0) \times 10^6$
Low- ω -3	$(1.2 \pm 0.5) \times 10^{-11}$	$(81 \pm 67) \times 10^8$	$(2.1 \pm 0.3) \times 10^{-10*}$	$(2.7 \pm 4.0) \times 10^6$

Figure 2 Rosenthal plot of insulin binding to (a) membranes and (b) receptors prepared from rats fed on high- and low-P/S diets

B/F, the amount of bound insulin over that of free insulin. $\circ$, Membrane or receptor prepared from rats fed on high-P/S diet; $\triangle$, membrane or receptor prepared from rats fed on low-P/S diet.

binding site was 2.3-fold higher in the membrane from rats fed on a high-P/S diet than from those fed on a low-P/S diet (Table 4A). The other binding parameters were not significantly different between animals fed on the high- compared with the low-P/S diets.

Solubilized insulin receptors from animals on both high- and low-P/S diet treatments bound similar amounts of insulin at all insulin concentrations and exhibited similar binding parameters (Figure 2b and Table 4A).

Figure 3 Rosenthal plot of IGF-I binding to membranes prepared from rats fed on high- and low- ω -3 diets

B/F, the amount of bound IGF-I over that of free IGF-I. $\bullet$, Membrane prepared from rats fed on high- ω -3 diet; $\blacktriangle$, membrane prepared from rats fed on low- ω -3 diet.

Effect of high- and low- ω -3 diets on insulin-like growth factor I (IGF-I) binding to skeletal-muscle sarcolemma

Membrane from rats fed on a diet high in ω -3 fatty acids bound more IGF-I than that from rats fed on a diet low in ω -3 fatty acids (Figure 3). Rosenthal analysis revealed that the B_{max} of the high- ω -3 group was more than twofold higher than that of the low- ω -3 group at the low-affinity binding site ($P < 0.05$; Table 4B). The other binding parameters were not significantly different from each other between the two dietary groups.

DISCUSSION

Effect of dietary fat on fatty acyl composition in skeletal-muscle membrane phospholipids

In skeletal muscle, it was shown that fish oil, high in long-chain ω -3 fatty acids, changed the fatty acyl composition of muscle total phospholipids, especially the content of 22:5 ω -3 and 22:6 ω -3 (Storlien et al., 1991). However, this work was conducted on

muscle phospholipids isolated from a Folch extract of whole tissue, and would therefore represent all muscle membranes rather than sarcolemma specifically. These authors did not further differentiate the effects of dietary fat on individual skeletal-muscle membrane phospholipid fractions. This separation is critical to interpretation, as it has been demonstrated that subcellular membrane types contain different contents of phospholipid fractions which respond to dietary fat manipulation to varying degrees (Field et al., 1990).

The present study demonstrates that the fatty-acyl composition of individual phospholipids of skeletal-muscle plasma membrane is markedly sensitive to dietary long-chain ω -3 fatty acid supply, and that the content of specific fatty acids responds to dietary manipulation differently in individual phospholipids. When rats were fed on diets containing about 3.3% long-chain ω -3 fatty acids, the ω -3 fatty acids as percentage of total fatty acids were doubled in all four phospholipid classes, and the three individual long-chain ω -3 fatty acids (20:5 ω -3, 22:5 ω -3 and 22:6 ω -3) were increased by 2- to 10-fold. The increase in long-chain ω -3 fatty acids in the membrane was greatest in phosphatidylcholine, phosphatidylserine and phosphatidylinositol, and relatively smaller in phosphatidylethanolamine. In terms of degree of unsaturation, each phospholipid class had a characteristic P/S ratio, and this was not affected by ω -3 fatty acids in the diet, except for phosphatidylethanolamine, which tended to be lowered by the diet high in ω -3 fatty acids.

In addition to the increase in the content of long-chain ω -3 fatty acids, there was a concomitant decrease in the amount of 20:4 ω -6 in the rats fed on a diet high in ω -3 fatty acids (Table 2). As the content of saturated fatty acids and total polyunsaturated fatty acids was not significantly affected, the major effect of feeding animals with fish oil was the selective replacement of membrane phospholipid 20:4 ω -6 with long-chain ω -3 fatty acids. In a study in which the only source of dietary fatty acid was fish oil, the content of long-chain ω -3 fatty acids in muscle phosphatidylethanolamine was increased to 23%, and 20:4 ω -6 became undetectable (Jackson et al., 1988). These results are in agreement with the finding that ω -3 fatty acids are preferably elongated and desaturated compared with ω -6 fatty acids (Garg et al., 1989).

The P/S ratio of muscle membrane phospholipid was insensitive to the diet. The fatty acyl composition of skeletal-muscle plasma-membrane phospholipid was not different between animals fed on the diets with different P/S ratios of 0.25 and 1.05. In purified adipocyte plasma membrane, the diet high in P/S ratio (2.0 compared with 0.2) significantly changed the membrane fatty acyl composition: it increased the content of 20:4 ω -6 and decreased the amount of saturated fatty acids, hence increasing the P/S ratio (Field et al., 1990). This difference could be due to the cell type or to the more polyunsaturated diet used in that study. However, in skeletal muscle, it was shown that the content of saturated fatty acids and 20:4 ω -6 in total muscle phospholipids was not sensitive to diets with different degrees of saturation, even with a P/S ratio of up to 9.2 (Storlien et al., 1991). It seems that changing the P/S ratio is more difficult than changing the ω -3/ ω -6 ratio in skeletal-muscle plasma-membrane phospholipids. It might be possible that, for skeletal muscle, even the low-P/S (0.25 used here) diet provided enough 18:2 ω -2 to maintain an optimal level of polyunsaturated fatty acids (mainly 20:4 ω -6), and that providing a diet with higher-P/S ratio will not increase the degree of unsaturation further.

Membrane fatty acyl composition and insulin and IGF-I binding

Skeletal muscle is an important tissue for insulin action and becomes insulin-resistant under different physiological and

pathological conditions, such as obesity, high-fat diet and diabetes (Olefsky et al., 1976; Caro et al., 1987; Grimditch et al., 1987). It has been shown that dietary long-chain ω -3 fatty acids may improve insulin action in this tissue (Storlien et al., 1987; Sohal et al., 1992). Storlien et al. (1991) have proposed that there is a correlation between muscle phospholipid 22:5 ω -3 and 22:6 ω -3 levels and tissue insulin-sensitivity. When the content of 22:5 ω -3 and 22:6 ω -3 was increased from 3.1% to about 10% in skeletal muscle total phospholipid, insulin-stimulated glucose metabolism was concomitantly enhanced by more than threefold (Storlien et al., 1991). How these fatty acids improve insulin action is not clear. The present study provides an explanation for the observation that dietary polyunsaturated fatty acids, particularly long-chain ω -3 fatty acids, increase insulin-dependent glucose transport in skeletal muscle (Storlien et al., 1991; Sohal et al., 1992). Feeding rats on a diet either high in ω -3 fatty acids or of high P/S content improved insulin binding to skeletal sarcolemma (Figures 1 and 2). Insulin binding to these membranes was higher at all insulin concentrations studied, and Rosenthal analysis revealed that the receptor number at the low-affinity binding site was significantly higher. Enhanced insulin binding to skeletal-muscle sarcolemma as a result of diets high in ω -3 or ω -6 fatty acid is consistent with improvement of insulin binding to adipocytes induced by a high-P/S diet (Field et al., 1988, 1990). Together these observations suggest that dietary polyunsaturated fatty acids may have a generalized effect on insulin action via modification of hormone binding to insulin-sensitive tissues. In view of the large effect of fish oil on muscle insulin binding, it is unfortunate that the effect of ω -3 fatty acids on adipocyte insulin binding and insulin sensitivity has not been studied. In agreement with the outcome of *in vitro* studies in which membrane fatty-acyl P/S composition was manipulated (Ginsberg et al., 1981; Gould et al., 1982), the present study provides further evidence that dietary fatty acids within the range of composition consumed by humans has a profound effect on insulin action.

In terms of improving insulin binding to skeletal sarcolemma, long-chain ω -3 fatty acids seem to be a more potent factor than ω -6 polyunsaturated fatty acids. Because the P/S ratio and the content of ω -3 fatty acids in the low- ω -3 diet were similar to that in the low-P/S diet, the low- ω -3 diet and the low-P/S diet were comparable (Table 1). The high- ω -3 diet increased insulin binding 14-fold over the low- ω -3 diet, compared with an improvement of 2.7-fold in insulin binding caused by the high-P/S diet. This result is consistent with the report that ω -3 fatty acids are more effective in preventing insulin resistance induced by high-fat diets than ω -6 polyunsaturated fatty acids (Storlien et al., 1991). The lack of effect of P/S ratio on membrane phospholipid composition is in contrast with the observed changes in insulin binding. The relative amounts of different phospholipid may in this case have been a factor (Field and Clandinin, 1989), as improved insulin binding was lost after membrane solubilization.

The importance of the phospholipid environment to insulin binding is addressed further by the solubilized receptor-binding experiment. After receptors were solubilized, the effect of dietary fatty acids on insulin binding disappeared. There was no difference in insulin binding to solubilized receptors from rats fed on high- compared with those fed on low-P/S diets, and the difference between insulin binding to the solubilized receptors from rats fed on high- and low- ω -3 diets was not significant. Thus it is clear that insulin binding to its receptor is significantly affected by the lipid environment around the receptor. In light of this conclusion, it should be pointed out that caution should be exercised in interpreting results of ligand binding using solubilized receptors from muscles in different physiological and pathological

states where sarcolemmal fatty acid and/or phospholipid composition may be altered (e.g. Turinsky, 1986).

IGF-I and insulin have similar anabolic functions in skeletal muscle, especially for glucose uptake and metabolism (Dimitriadis et al., 1992). The receptors for these two hormones share functional similarity and structural homology (Abbott et al., 1992). Our results indicated that IGF-I binding to skeletal-muscle sarcolemma was altered by changes in dietary fatty acid composition. To our knowledge this is the first demonstration that IGF-I-receptor function is sensitive to dietary ω -3 fatty acids. The observation that binding activity of IGF-I, like that of insulin, is affected by dietary fatty acids implies that this might be a general phenomenon for other receptor-binding activities. It remains to be determined whether ω -3 fatty acids increase IGF-I-dependent glucose transport in skeletal muscle.

Separating insulin bound to skeletal-muscle membrane using WGA

WGA is a lectin with an affinity for *N*-acetyl- β -D-glucosamine residues (Charuk et al., 1989). It is used in purification of skeletal-muscle and cardiac membranes to separate right-side out vesicles from the inside-out vesicles and to reduce contamination by intracellular membranes (Charuk et al., 1989; Ohlendieck et al., 1991). The present study incorporated the membrane WGA purification step into insulin-binding procedures to separate bound insulin from free insulin. This procedure gave identical values for specific binding, but lowered the non-specific binding compared with identical assays using ultracentrifugation to separate bound and free insulin. This procedure could be a valuable addition to studies of insulin binding to intact membranes where the level of non-specific binding is usually reported to be high (Grimditch et al., 1985). Another advantage of this procedure is that WGA-agglutinated plasma-membrane vesicles can be pelleted at the end of binding assays by relatively low-speed centrifugation (approx. 17000 *g*), whereas non-agglutinated vesicles require ultracentrifugation (approx. 140000 *g*).

Conclusions

Feeding rats on long-chain ω -3 fatty acids significantly increased the content of 22:5 ω -3 and 22:6 ω -3 in skeletal-muscle membrane phospholipids. The fatty acyl compositions of individual phospholipids were affected differently. Feeding rats on high- and low-P/S diets did not significantly affect membrane phospholipid fatty acyl composition.

Both dietary long-chain ω -3 and polyunsaturated fatty acids can increase insulin binding to skeletal-muscle sarcolemma, as a result of the increase in the $B_{\max}$ of the low-affinity high-capacity binding site. The effect of the long-chain ω -3 fatty acids is more potent than that of ω -6 polyunsaturated fatty acids.

When receptors were solubilized from the membrane lipid, the increase in insulin binding disappeared in both dietary groups, indicating that the altered membrane fatty acyl composition is responsible for the modified insulin binding caused by dietary polyunsaturated fatty acids.

Precipitating insulin bound to skeletal-muscle sarcolemma using WGA can reduce the non-specific binding.

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