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Levels of plasma insulin, leptin and adiponectin, and activities of key enzymes in carbohydrate metabolism in skeletal muscle and liver in fasted ICR mice fed dietary n-3 polyunsaturated fatty acids

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Abstract

The aim of this study was to clarify the mechanisms related to plasma glucose concentration in mice fed a diet rich in n-3 polyunsaturated fatty acids (n-3 PUFAs). Male Crlj:CD-1 (ICR) mice were fed experimental diets containing 6% lard (LD), 6% fish oil (FO) or 4.1% lard plus 1.5% docosahexaenoic acid ethyl ester and 0.4% eicosapentaenoic acid ethyl ester (DE) for 12 weeks. There were no marked differences in plasma glucose and insulin concentration changes on glucose tolerance test between the three dietary groups. At the end of the feeding trial, plasma glucose concentration was significantly lower in fasted mice in the FO group than in those in the LD group ($P<.005$). Plasma adiponectin concentration was significantly higher in the FO group than in the LD group ($P<.05$). Hexokinase, phosphofructokinase, glucose-6-phosphate dehydrogenase and glycerophosphate dehydrogenase activities in skeletal muscle tended to be lower in the FO group than in the LD group, while there were no differences in glucokinase and phosphofructokinase activities in liver between the three dietary groups. However, hepatic glycerophosphate dehydrogenase activity was 53-fold and 4.2-fold higher in the FO group than in the LD and DE groups, respectively ($P<.0005$ and $P<.05$, respectively). These results suggest that the reduction in plasma glucose concentration in mice fed n-3 PUFAs is mainly caused by acceleration of glucose uptake and glycerol synthesis in the liver rather than in the skeletal muscle.

Keywords: Fish oil; n-3 polyunsaturated fatty acid; Glucose; Insulin; Adiponectin; Mice

1. Introduction

Type 2 diabetes is related to obesity, hypertension and hyperlipidemia. It is thought that changes in lifestyle, such as increased fat intake and/or physical inactivity, are responsible for the currently increasing incidence of type 2 diabetes mellitus [1–3]. Type 2 diabetes is accompanied by insulin resistance, which is the reduced efficacy of insulin in vivo, and this characteristic is common to type 2 diabetes, obesity and metabolic syndrome.

Epidemiological studies have indicated that the risk of developing type 2 diabetes mellitus is decreased by fish intake [4,5]. Fish oil contains large amounts of n-3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). For example, the incidence of diabetes mellitus among Eskimos is low, despite the traditional fat-enriched diet consisting primarily of fish and seal [6,7].

Experimental studies using animals fed high-fat n-3 PUFA-containing diets have shown an improvement in insulin resistance in skeletal muscle [8,9]. The effects of adipocytokines, such as adiponectin, on glucose uptake into peripheral tissues have also recently been noted. A strong

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; DHA, docosahexaenoic acid; DHA-EE, docosahexaenoic acid ethyl ester; EPA, eicosapentaenoic acid; EPA-EE, eicosapentaenoic acid ethyl ester; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GK, glucokinase; GPDH, glycerophosphate dehydrogenase; HK, hexokinase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; PFK-2, fructose 6-phosphate 2-kinase; PK, pyruvate kinase; PPARα, peroxisome proliferator-activated receptors alpha; SREBP-1, sterol regulatory element binding protein-1.

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negative correlation was observed between plasma adiponectin concentration and body mass index [10,11]. These data suggest that adiponectin is an important adipocytokine for preventing the development of type 2 diabetes with obesity and vascular disease.

Glucose is converted into energy by various enzymes related to glucose catabolism. Key enzyme activities in carbohydrate catabolism decrease during glucose uptake to liver and muscle, and tend to decrease during insulin deficiency and/or resistance. Decreases in the enzymatic activities of hexokinase and phosphofructokinase have also been reported in diabetic animals, resulting in depletion of liver and muscle glycogen [12,13]. Thus, it is important to better understand the relationship between changes in key catabolic enzyme activities and development of diabetes.

We previously reported that the reduction in plasma glucose concentration in mice fed fish oil takes place over a longer period of time when compared with similar decreases in plasma lipid concentration and may be related to changes in lipid metabolism [14]. However, the mechanism of glucose uptake into peripheral tissues and the changes in carbohydrate and lipid metabolism by supplementation with dietary n-3 PUFA have not been clarified. It is necessary to determine the changes in various key enzymatic activities in order to determine the relationship between the reduction of plasma glucose concentration and the carbohydrate and/or lipid metabolism. The aim of this study was to clarify the mechanisms of reductions in plasma glucose concentration in mice fed dietary n-3 PUFAs.

2. Materials and methods

2.1. Animals

Male Crlj:CD1 (ICR) mice (age, 4 weeks) were obtained from Charles River Japan (Atsugi, Kanagawa, Japan). All animals were switched from laboratory chow, MF (Oriental Yeast, Tokyo, Japan), to experimental diets at 27 weeks of age. Sixty mice were randomly divided into six groups of 7–11 animals each. Mice were housed in suspended stainless-steel cages with wire mesh bottoms, and three groups (n=7–9) were used for glucose tolerance test. The animal room was kept at a temperature of 24±0.5°C and a relative humidity of 65±5%. Room lighting consisted of 12-h periods of light and dark. Diet and water were provided ad libitum. The diets given to each group had similar energy content, and all mice were fed experimental diets for 12 weeks. Body weight was measured once every 2 weeks. All mice were maintained according to the guidelines for experimental animals of the National Food Research Institute, Japan.

At the end of the feeding trials (Week 12), all mice were fasted for 24 h before being anesthetized with diethyl ether. Blood was collected from the inferior vena cava with a heparinized syringe and was then put into ice-cold tubes. After blood collection, livers and leg skeletal muscles were removed. The obtained livers and skeletal muscles were rapidly frozen using liquid nitrogen. Liver and skeletal muscle were homogenized with 0.15 M NaCl and 20 mM Tris-HCl (pH 7.5) using a Teflon-glass homogenizer. Plasma was separated by centrifugation at 900×g for 20 min at 4°C. Plasma, liver and skeletal muscle were stored at −80°C until analysis.

2.2. Diets

Lard was supplied by NOF (Tokyo, Japan), fish oil was supplied by Nippon Chemical Feed (Hakodate, Japan), DHA-ethyl ester (DHA-EE; DHA-95E, ethyl ester derivative of all *cis*-4,7,10,13,16,19-docosahexaenoic acid, 95% pure) was purchased from Harima Chemicals (Tsukuba, Japan) and EPA ethyl ester (EPA-EE; Epadel, ethyl ester derivative of all *cis*-5,8,11,14,17-eicosapentaenoic acid, 93.5% pure) was supplied by Mochida Pharmaceutical (Tokyo, Japan). Experimental diets contained 6% lard (LD), 6% fish oil (FO) or 4.1% lard+1.5% DHA-EE+0.4% EPA-EE (DE). Diet compositions are presented in Table 1. The percentages of DHA-EE and EPA-EE used in this study were almost the same as those of DHA and EPA in fish oil. The same amounts of experimental diet were given to each group. In order to prevent oxidative loss of n-3 PUFAs during storage, experimental diets were stored below −40°C.

The fatty acid composition of each experimental diet is shown in Table 2. The predominant fatty acids in the experimental diets were 16:0, 18:0 and 18:1n-9. The percentages of 18:0 and 18:1n-9 in the FO and DE diets

Table 1
Composition of experimental diets (%)

	Dietary group		
	LD	FO	DE
Corn starch	47.8	47.8	47.8
Casein	20	20	20
Granulated sugar	15	15	15
Cellulose powder	5	5	5
Mineral mixture [a]	4	4	4
Vitamin mixture [b]	2	2	2
L-Methionine	0.2	0.2	0.2
LD	6	–	4.1
FO	–	6	–
DHA-EE	–	–	1.5
EPA-EE	–	–	0.4

Mineral and vitamin mixture were purchased from Oriental Yeast (Tokyo, Japan).

[a] Mineral mixture contained (per kg) the following: $CaHPO_4 \bullet 2H_2O$, 14.56 g; KH_2PO_4, 25.72 g; NaH_2PO_4, 9.35 g; NaCl, 4.66 g; Ca-lactate, 35.09 g; Fe-citrate, 3.18 g; $MgSO_4$, 7.17 g; $ZnCO_3$, 0.11 g; $MnSO_4 \bullet 4H_2O$, 0.12 g; $CuSO_4 \bullet 5H_2O$, 0.03 g; KI, 0.01 g.

[b] Vitamin mixture contained (per kg) the following: retinyl acetate, 0.1 g; cholecalciferol, 0.00025 g; α-tocopheryl acetate, 0.5 g; menadione, 0.52 g; thiamin•HCl, 0.12 g; riboflavin, 0.4 g; pyridoxine•HCl, 0.08 g; cyanocobalamine, 0.00005 g; ascorbic acid, 3 g; biotin, 0.002 g; folic acid, 0.02 g; calcium pantothenate, 0.5 g; *p*-aminobenzoic acid, 0.5 g; niacin, 0.6 g; inositol, 0.6 g; choline chloride, 20 g; cellulose powder, 73.1 g.

Table 2
Percentage of main fatty acids in experimental diet (%)

	Dietary group		
	LD	FO	DE
SFA			
14:0	2.3	4.4	1.6
16:0	25.1	21.6	17.6
18:0	11.7	4.7	9.6
MUFA			
16:1n-7	3.5	6.3	1.9
18:1n-9	41.6	18.7	29.7
18:1n-7	2.9	2.7	2.3
20:1n-9	–	1.7	–
PUFA			
18:2n-6	8.1	1.4	5.7
20:4n-6	–	1.8	–
20:5n-3	–	6.5	6.2
22:5n-6	–	1.1	–
22:5n-3	–	1.2	–
22:6n-3	–	21.2	23.2
n-3/n-6	0	6.7	5.2

Values are given as mean. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

were lower than in the LD diet, while the n-3/n-6 ratio of the FO diet was higher than that of the LD and DE diets.

2.3. Measurement of plasma glucose, lipids, insulin, leptin and adiponectin, and hepatic lipid levels

Plasma glucose was measured using the method of Trinder [15] with an oxidase–peroxidase system. Total cholesterol, triacylglycerol, phospholipid and nonesterified fatty acid (NEFA) concentrations in plasma samples and liver homogenates were determined by the enzymatic methods of Allain et al. [16], Spayd et al. [17], Takayama et al. [18] and Shimizu et al. [19], respectively. Plasma insulin, leptin and adiponectin were measured with a mouse insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan), a mouse leptin ELISA kit (Morinaga Institute of Biological Science) and a mouse/rat adiponectin ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan), respectively.

2.4. Intraperitoneal glucose tolerance test

Experimental animals were raised under the conditions described above. Glucose tolerance test was performed at the end of the feeding trials. Blood samples were collected from the caudal vein at 0, 15, 30, 60 and 120 min after intraperitoneal injection of 2 mg/g body weight glucose following 24 h of fasting. Plasma separation and measurement of plasma glucose and insulin were carried out as described above.

2.5. Measurement of glycogen, glucose-6-phosphate and fructose-6-phosphate content

Glycogen content was measured using the method of Keppler and Decke [20]. In brief, tissue pieces (>100 mg) were homogenized with 5 volumes of 0.6N perchloric acid. Homogenates were neutralized by adding 0.5 volumes of potassium hydrogen carbonate solution and incubated with 10 U of amyloglycosidase (EC 3.2.1.3) suspension at 40°C for 2 h. The reaction was stopped with 0.3N perchloric acid. After centrifugation at 3000×*g* for 10 min, supernatant was separated and used for measurement of glucose concentration. Five microliters of supernatant was mixed with 1 ml of 0.3 M triethanolamine (pH 7.5), 1 mM ATP, 0.9 mM NADP, 4 mM $MgSO_4$, 0.7 U/ml glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and 0.4 mM glucose, followed by incubation at 37°C for 5 min, and absorbance was then measured at 340 nm (E_1). Next, 1.4 U/ml hexokinase (HK, EC 2.7.1.1) suspension was added, and the absorbance at 340 nm (E_2) was measured after incubation at 37°C for 10 min. Glycogen content was calculated by subtracting E_2 from E_1.

Glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) content were analyzed using the method of Lang and Michal [21]. Homogenates in 0.6N perchloric acid were centrifuged at 3000×*g* for 10 min. The obtained supernatants were adjusted to pH 3.5 (as measured by indicator paper) by addition of potassium carbonate solution. Samples were allowed to stand in an ice bath for about 15 min, and supernatant was then decanted or pipetted from the precipitate. Next, 0.2 M triethanolamine (pH 7.5), 0.2 mM NADP and 5 mM $MgCl_2$ were added to the supernatant, followed by incubation at 37°C for 3 min, and absorbance was measured at 340 nm. After addition of 170 U/L G6PDH suspension, absorbance at 340 nm was measured and G6P content was calculated. Moreover, 700 U/L phosphoglucose isomerase (EC 5.3.1.9) suspension was added to the solution and absorbance at 340 nm was measured for calculation of F6P content.

2.6. Enzymatic activity assay

Skeletal muscle and liver homogenates were prepared in 10 ml/mg tissue of 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 1 μg/ml aprotinin. Homogenates were centrifuged at 700×*g* for 10 min, and the supernatant was centrifuged at 10,000×*g* for 10 min. Supernatant was used for assay of various enzymatic activities. Measurement of protein concentration was performed according to the Lowry et al. method [22]. All enzymatic activities were coupled to either NADP reduction or NADH oxidation and followed by measurement of absorption at 340 nm in a total reaction volume of 1 ml at 25°C. One unit of enzymatic activity corresponds to the reduction of 1 nmol NADP/min or oxidation of 1 nmol of NADH/min. The measurement method for each enzymatic activity was as follows.

2.6.1. Hexokinase

This assay was carried out by the method of Dentin et al. [23] with the following modifications: assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 2 mM

dithiothreitol (DTT), 5 mM $MgCl_2$, 5 mM ATP, 1 mM NADP and 0.3 U/ml of G6PDH. The phosphorylating capacity obtained at 0.5 mM glucose was considered to be the HK activity, while the difference between the activity measured at 100 mM glucose and the activity measured at 0.5 mM glucose was considered to be the glucokinase (GK) activity of the extract. The reaction was initiated by adding ATP.

2.6.2. Phosphofructokinase

This assay was carried out using the method described by Raïs et al. [24] with the following modifications: phosphofructokinase (PFK) activity was assayed in 50 mM glycylglycine (pH 8.2) containing 3 mM $MgCl_2$, 3 mM DTT, 0.1 mM EGTA, 1 mM ATP, 2.4 mM F6P, 0.3 mM NADH, 0.4 U/ml aldolase (EC 4.1.2.13), 2.4 U/ml triosephosphate isomerase (EC 5.3.1.1) and 0.8 U/ml glycerophosphate dehydrogenase (GPDH, EC 1.1.1.8). The reaction was initiated by adding ATP.

2.6.3. Pyruvate kinase

Pyruvate kinase (PK) activity was assayed using the method of Miyanaga et al. [25] with the following modifications: assay was carried out in a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 150 mM KCl, 5 mM $MgCl_2$, 0.25 mM NADH, 5 U/ml lactate dehydrogenase (LDH, EC 1.1.1.27), 2 mM ADP and 1 mM phosphoenolpyruvate. The reaction was initiated by adding phosphoenolpyruvate.

2.6.4. Glucose-6-phosphate dehydrogenase

This assay was carried out by the method of Delplanque et al. [26], with some modification. G6PDH was reacted in a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM DTT, 5 mM $MgCl_2$, 250 μM NADP and 250 μM G6P. The reaction was initiated by adding G6P.

2.6.5. Lactate dehydrogenase

LDH activity was measured in 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 300 μM NADH and 1 mM pyruvate, as described by Kaczor et al. [27], with some modification. The reaction was initiated by adding pyruvate.

2.6.6. Glycerophosphate dehydrogenase

GPDH activity was measured in 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 2.5 mM EDTA, 0.12 mM NADH, 0.1 mM DTT and 0.1 mM dihydroxyacetone phosphate, as described by Wise and Green [28], with some modification. The reaction was initiated by adding dihydroxyacetone phosphate.

2.7. Statistical analyses

All results are expressed as means±S.E.M. The statistical significance of differences in glucose, lipid components, insulin, adipocytokine concentrations and enzymatic activities between dietary groups was determined by one-way analysis of variance (ANOVA), and Spjotvoll and Stoline tests using the Statistica statistical software package (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Food intake, and weights of body, liver and visceral adipose tissue

There were no significant differences in mean food consumption between the three dietary groups. Food intake (mean±S.E.M.) in each group was as follows: LD group, 4.4±0.0 g/day; FO group, 4.5±0.0 g/day; DE group, 4.5±0.0 g/day. Weights of body, liver and visceral adipose tissues are shown in Table 3. Although body and visceral adipose tissue weights tended to be greater in the LD group than in the FO and DE groups, there were no statistically significant differences between any of the dietary groups.

3.2. Changes in plasma glucose and insulin concentrations on intraperitoneal glucose tolerance test

Changes in plasma glucose and insulin concentrations during glucose tolerance test are presented in Fig. 1. There were no differences between any of the dietary groups in glucose concentration at 0, 15, 30 and 60 min after injection. However, plasma glucose concentration was significantly higher in the FO group than in the DE group at 120 min ($P<.05$). The differences in plasma insulin concentration were not statistically significant at any time point between the three dietary groups, although plasma insulin concentration in the FO group tended to be higher at 60 and 120 min when compared to the LD and DE groups.

3.3. Concentrations of plasma glucose, total cholesterol, triacylglycerol, phospholipid and NEFA

Concentrations of plasma glucose, total cholesterol, triacylglycerol, phospholipid and NEFA are shown in Table 4. Plasma glucose concentrations in the FO and DE groups were lower when compared to the LD group, and the differences in plasma glucose concentration between the LD and FO groups were statistically significant ($P<.005$). Total cholesterol and phospholipid concentrations in plasma were significantly lower in the FO and DE groups than in the LD group ($P<.0005$ and $P<.0005$, respectively). Plasma triacylglycerol concentrations tended to be lower in the FO and DE groups than in the LD group, but there were no significant differences between the three dietary groups.

Table 3
Body, liver and visceral adipose tissue weights of mice (g)

	Dietary group		
	LD	FO	DE
Body	47.4±2.5	47.0±1.1	46.7±1.4
Liver	1.73±0.14	1.73±0.05	1.66±0.06
Visceral adipose tissue	2.03±0.36	1.91±0.18	1.67±0.27

Weights of body, liver and visceral adipose tissue in mice fed experimental diets for 12 weeks (n=9–11/group). Each value represent the mean±S.E.M., and significant differences were analyzed by Spjotvoll and Stoline test. There were no significant differences in weights of body, liver and visceral adipose tissue between the three dietary groups.

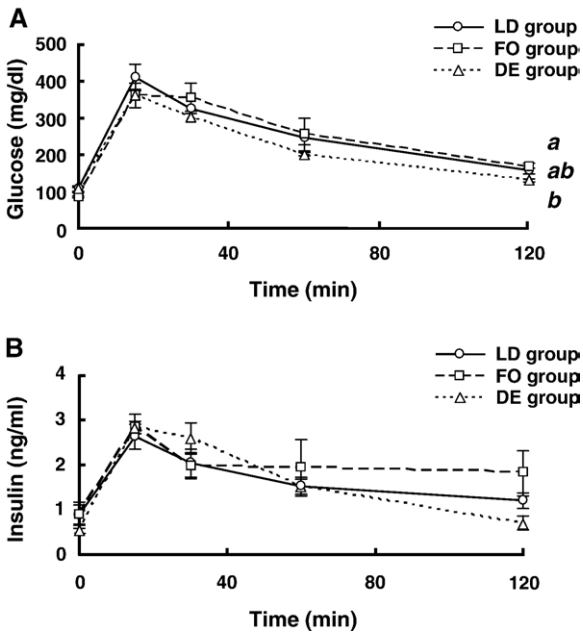

Fig. 1. Time courses of plasma glucose (A) and insulin (B) concentrations during intraperitoneal glucose tolerance test (2 mg/g body weight) in mice fed the LD (circle), FO (square) and DE (triangle) diets (n=7–9/group). Blood samples were obtained from the caudal vein at the indicated time points. Values (means±S.E.M.) indicated by italic letters were significantly different at $P<.05$ by Spjotvoll and Stoline test.

There were no differences in plasma NEFA concentrations between the three dietary groups.

3.4. Hepatic total cholesterol, triacylglycerol and phospholipid contents

Hepatic total cholesterol, triacylglycerol and phospholipid contents in mice are shown in Table 4. Hepatic total cholesterol contents tended to be lower in the FO and DE groups than in the LD group, and were significantly lower in the DE group than in the LD group ($P<.05$). There were no significant differences in triacylglycerol and phospholipid contents between the three dietary groups.

Table 4
Plasma glucose, and plasma and hepatic lipid levels of mice

	Dietary group		
	LD	FO	DE
Plasma			
Glucose (mg/dl)	198.6±24.7	102.6±12.5 **	153.6±11.3
Total cholesterol (mg/dl)	131.4±6.7	70.9±3.8 ***	63.8±5.5 ***
Triacylglycerol (mg/dl)	93.5±4.8	77.5±5.1	80.3±8.2
Phospholipid (mg/dl)	221.8±10.8	138.8±6.0 ***	120.6±8.4 ***
NEFA (mEq/L)	0.68±0.06	0.54±0.06	0.69±0.07
Liver			
Total cholesterol (mg/g)	5.0±0.4	4.3±0.2	4.0±0.1 *
Triacylglycerol (mg/g)	34.9±3.3	31.6±4.1	34.9±3.2
Phospholipid (mg/g)	19.6±0.7	21.2±0.7	21.7±0.6

Plasma glucose, and plasma and hepatic lipid levels of mice fed experimental diets for 12 weeks (n=9–11/group). Blood samples and liver were obtained from the inferior vena cava after fasting for 24 h. Values (means±S.E.M.) marked with asterisks were significantly different by Spjotvoll and Stoline test.

* $P<.05$ vs. LD group

** $P<.005$ vs. LD group

*** $P<.0005$ vs. LD group.

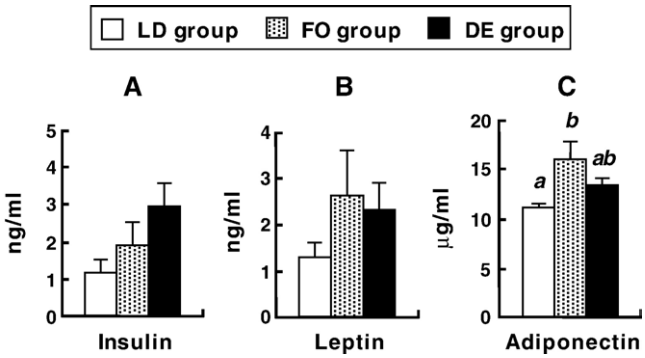

Fig. 2. Plasma insulin (A), leptin (B) and adiponectin (C) concentrations in mice fed experimental diets for 12 weeks (n=9–11/group). Blood samples were obtained from the inferior vena cava after fasting for 24 h. Values (means±S.E.M.) indicated by italics were significantly different at $P<.05$ by Spjotvoll and Stoline test.

3.5. Concentrations of plasma insulin, leptin and adiponectin

Plasma insulin, leptin and adiponectin concentrations in each dietary group are shown in Fig. 2. Plasma insulin and leptin concentrations tended to be higher with the FO or DE diets than with the LD diet, but there were no significant differences between the diet groups. Plasma adiponectin concentrations in the FO and DE groups tended to be higher than in the LD group, and there was a significant difference between the LD and FO groups ($P<.05$).

3.6. Skeletal muscle glycogen, G6P and F6P contents

Glycogen, G6P and F6P contents in skeletal muscle are shown in Fig. 3. There were no marked differences in

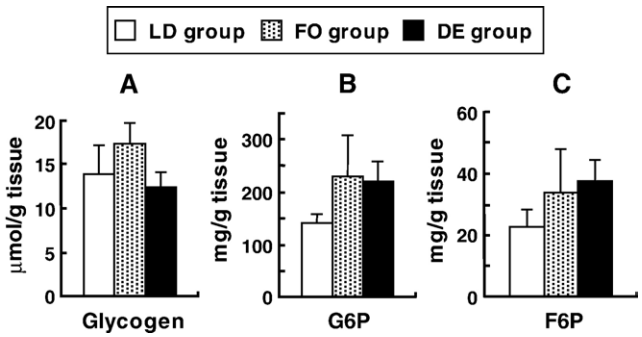

Fig. 3. Skeletal muscle glycogen (A), G6P (B) and F6P (C) contents in mice fed experimental diets for 12 weeks (n=9–11/group). Skeletal muscle samples were obtained from the leg after fasting for 24 h. Each value represents the mean±S.E.M., and significant differences were analyzed by Spjotvoll and Stoline test. Results are expressed as millimoles per gram and milligrams per gram skeletal muscle. There were no significant differences in glycogen, G6P or F6P contents between the three dietary groups.

glycogen contents between the dietary groups. The G6P and F6P contents tended to be higher in the FO and DE groups than in the LD group; however, these differences were not significant. Hepatic glycogen, G6P and F6P were not detected, as mice were fasted for 24 h.

3.7. Enzymatic activities in skeletal muscle and liver

The enzymatic activities related to carbohydrate metabolism in skeletal muscle are shown in Fig. 4. Activities of HK, G6PDH and GPDH tended to be lower in the FO and DE groups than in the LD group, and were significantly lower in the FO group than in the LD group ($P<.0005$, $P<.0005$, and $P<.05$, respectively). PFK activity in the FO group tended to be lower than in the LD group, but the difference was not significant. In contrast, activities of PK and LDH were significantly higher in the FO group than in the LD group ($P<.0005$ and $P<.05$, respectively).

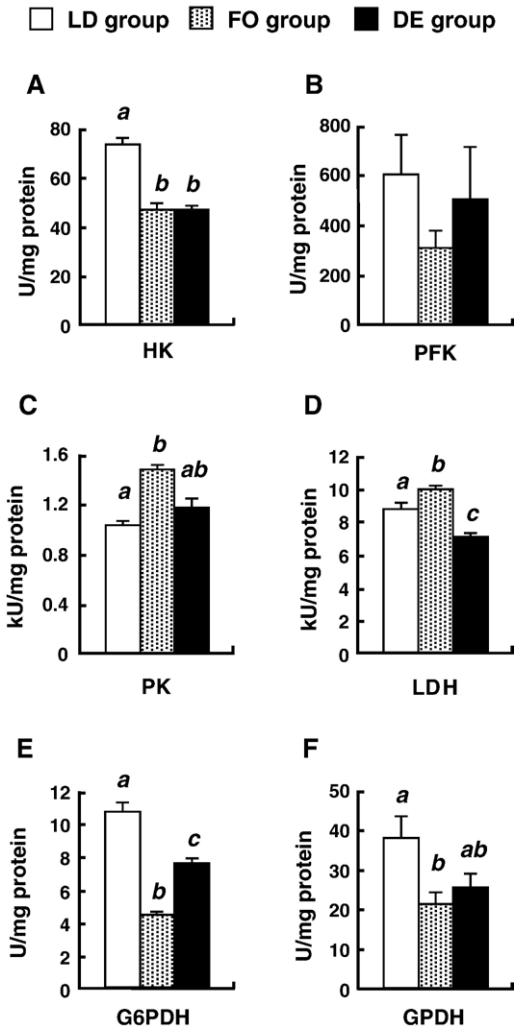

Fig. 4. Activities of enzymes [HK(A), PFK(B), PK(C), LDH(D), G6PDH(E) and GPDH(F)] in skeletal muscle in mice fed experimental diets for 12 weeks (n=9–11/group). Skeletal muscle samples were obtained from the leg after fasting for 24 h. Muscle homogenates were prepared and enzymatic activities were assayed, as described in the Materials and Methods section. Results are expressed as unit per milligram muscle protein. One unit corresponds to the oxidation of 1 nmol of NADH/min or reduction of 1 nmol NADP/min. Values (means±S.E.M.) indicated by italics were significantly different at $P<.05$ by Spjotvoll and Stoline test.

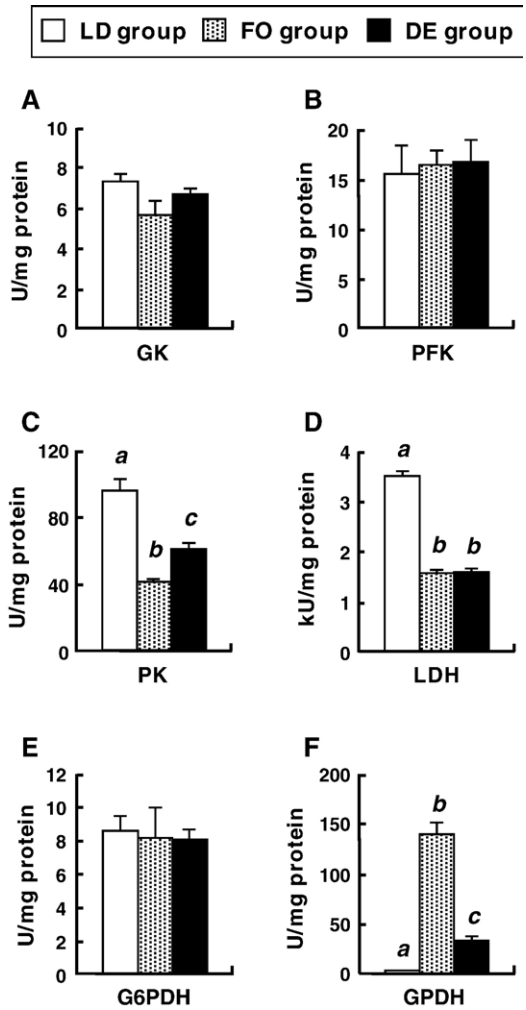

Fig. 5. Enzyme activities [GK(A), PFK(B), PK(C), LDH(D), G6PDH(E) and GPDH(F)] in livers of mice fed experimental diets for 12 weeks (n=9–11/group). Liver samples were obtained after fasting for 24 h. Liver homogenates were prepared and enzymatic activities were assayed, as described in the Materials and Methods section. Results are expressed as unit per milligram liver protein. One unit corresponds to the oxidation of 1 nmol of NADH/min or reduction of 1 nmol NADP/min. Values (means±S.E.M.) indicated by italics were significantly different at $P<.05$ by Spjotvoll and Stoline test.

Hepatic enzymatic activities are shown in Fig. 5. There were no marked differences in GK, PFK and G6PDH activities between the three dietary groups. The activities of PK and LDH were significantly lower in the FO (PK, $P<.0005$; LDH, $P<.0005$) and DE (PK, $P<.0005$; LDH, $P<.0005$) groups than in the LD group. GPDH activity in the FO and DE groups was significantly higher than in the LD group ($P<.0005$ and $P<.05$, respectively). Furthermore, GPDH activity was 53-fold and 4.2-fold higher in the FO group when compared to the LD and DE groups.

4. Discussion

We previously reported that plasma glucose concentration after fasting for 24 h was reduced by supplementation with dietary n-3 PUFA [14]. First, we investigated the effects of dietary n-3 PUFA on the insulin-induced decrease in plasma glucose concentration. As shown in Fig. 1, the changes in plasma glucose and insulin concentrations did not differ significantly between the three dietary groups during glucose tolerance test, nor did the differences in fasting plasma insulin concentration at the end of the trial (Fig. 2A). This suggests that n-3 PUFA has no effect on insulin secretion. However, it has been reported that insulin secretion in diabetic model mice increases with dietary FO supplementation [29,30]. Normal mice are thus relatively insensitive to the effects of dietary n-3 PUFAs on insulin secretion and/or enhancement of glucose uptake when compared to diabetic model mice. Plasma insulin concentration during glucose tolerance test at 60 and 120 min and fasting plasma insulin concentration at the end of the trial in mice fed the FO diet tended to be higher than in mice fed the LD diet (Figs. 1 and 2A). It has been reported that plasma NEFA concentration increases when insulin resistance develops [31]. This suggests that insulin resistance did not occur, as there were no differences in plasma NEFA concentrations between the three dietary groups (Table 4).

Although there were no significant differences in plasma leptin concentration between the three dietary groups, leptin concentrations tended to be higher in the FO and DE groups than in the LD group (Fig. 2B). It has been reported that plasma leptin is reduced in rodents by dietary FO intake, and decreases in visceral adipose tissue weight are related to the reductions in plasma leptin [32,33]. However, mice fed the FO or DE diets did not show any increase in visceral adipose tissue weight when compared to animals fed the LD diet (Table 3). Thus, in the present study, as the plasma leptin data conflict with the visceral adipose tissue weight, it is possible that the effects of plasma leptin on lipid and/or carbohydrate metabolism in the FO and DE groups are similar to those in the LD group.

Plasma adiponectin levels in the FO group were significantly higher than in the LD group (Fig. 2C). It was recently reported that supplementation with dietary FO increases plasma adiponectin concentration in mice [34,35]. The amount of adiponectin secretion is thought to be negatively related to increases in visceral adipose tissue weight [36]. In the present study, visceral adipose tissue weight in the FO and DE groups tended to be lower than in the LD group. Thus, the increases in plasma adiponectin in the FO and DE groups may be related to decreases in visceral adipose tissue weight.

Adiponectin enhances glucose utilization and fatty acid oxidation via activation of adenosine monophosphate-activated protein kinase (AMPK) [37,38]. It has been reported that dietary n-3 PUFA supplementation increases hepatic AMPK activity in rats [39]. However, Dobrzyn et al. [40] reported that AMPK in the mouse liver, skeletal muscle and heart muscle was not activated by 14-day supplementation with dietary n-3 PUFA. In a previous study, we showed that n-3 PUFA intake only reduced plasma glucose concentration in mice after 8 weeks [14]. Moreover, Dobrzyn et al. did not investigate the effects of dietary n-3 PUFA on adiponectin secretion and/or signaling. Therefore, it is possible that the reduction in plasma glucose concentration in mice fed dietary n-3 PUFA is partially mediated by adiponectin-induced AMPK activation.

The muscle and liver utilize large amounts of glucose as a source of energy, as well as in lipid synthesis. It is thus important to investigate the detailed changes in glucose metabolism in muscle and liver with n-3 PUFA intake. Insulin stimulation induces glucose uptake into muscle and adipose tissues, accelerates glycogen synthesis and glucose phosphorylation, and suppresses glucose discharge from liver [41–43]. Although, as shown in Fig. 3, mice fed the FO and DE diets tended to have higher glycogen, G6P and F6P contents in skeletal muscle when compared to animals fed the LD diet, there were no marked differences between the three dietary groups. Moreover, the activity of HK in skeletal muscle was lower in the FO and DE groups than in the LD group (Fig. 4A). It has been reported that insulin stimulates HK gene transcription [44,45], and thus HK activity would increase in vitro. However, the data have contradicted this hypothesis. It therefore appears that supplementation with dietary n-3 PUFA scarcely influences insulin-stimulated glucose uptake into skeletal muscle.

The activity of skeletal muscle PFK in the FO group tended to be lower than that in the LD group (Fig. 4B). Fructose-2,6-bisphosphate, an activator of PFK, is synthesized by fructose 6-phosphate 2-kinase (PFK-2) from fructose-6-phosphate and Mg-ATP [46]. It is known that activated AMPK activates PFK-2 and enhances glycolysis [47]. Receptors of adiponectin, which transmit the AMPK activation signal, are present in liver, adipose tissue and skeletal muscle [48]. It has been reported that the expression of adiponectin receptors in skeletal muscle is reduced by increases in insulin secretion [49]. Although plasma insulin concentrations tended to be higher in the FO and DE groups than in the LD group, there were no significant differences between the three dietary groups (Fig. 2A). It is possible that the AMPK-induced activation of PFK and/or PFK-2 in skeletal muscle does not occur via adiponectin stimulation. Therefore, we believe that adiponectin has a greater influence in liver than in skeletal muscle with ingestion of FO diet.

The activity of PK in skeletal muscle in the FO and DE groups was higher than that in the LD group (Fig. 4C). However, it is possible that the reduction in plasma glucose does not occur via accelerated glucose oxidation in skeletal muscle, as the activities of HK and PFK were lower in the FO and DE groups than in the LD group. It is necessary to assay pyruvate dehydrogenase activity in skeletal muscle in order to evaluate the effects of dietary n-3 PUFAs on

glucose oxidation. The activity of G6PDH in skeletal muscle of the FO and DE groups was lower than that in the LD group (Fig. 4E). Glucose uptake into skeletal muscle and synthesis of fatty acids derived from glucose are apparently reduced in the FO and DE groups in comparison to the LD group.

Hepatic GPDH activity in the FO group showed the most marked elevation of all assayed enzymes in the liver in the three dietary groups (Fig. 5F). GPDH plays an important role in the mediation of glycolysis, lipogenesis and glycerol metabolism. It has been reported that dietary n-3 PUFA supplementation suppresses triacylglycerol synthesis in the rodent liver by reducing the expression of sterol regulatory element binding protein-1 (SREBP-1) [50,51]. Nakatani et al. [50] reported that FO feeding decreased body weight and fat mass in a dose-dependent manner, in parallel with SREBP-1 mRNA. In the present study, some suppression of triacylglycerol synthesis may have occurred in the liver of mice fed the FO and DE diets, as the weight of visceral adipose tissue tended to decrease when compared to mice fed the LD diet (Table 3).

On the other hand, Brown et al. [52] reported that phospholipid synthesis increased in cultured hepatocytes from rats fed dietary FO. As the phospholipid contents of the liver in the FO and DE groups were slightly higher than in the LD group (Table 4), hepatic phospholipid synthesis may have been accelerated by supplementation with dietary n-3 PUFA. We previously reported that plasma phospholipid concentrations were significantly lower in mice fed the FO and DE diets than in those fed the LD diet at Week 2; concentrations in the FO and DE groups remained at 151–185 and 175–195 mg/dl, respectively, until the end of the trial [14]. This acceleration in phospholipid synthesis may compensate for the reduction in plasma phospholipids.

Hepatic PK activity in the FO and DE groups was significantly lower than in the LD group (Fig. 5C). Inhibition of PK gene expression and reduction of PK activity occurs during supplementation with dietary FO [53]. It is likely that hepatic glucose catabolization to pyruvate is difficult. Moreover, there were no differences in hepatic G6PDH activity between the three dietary groups (Fig. 5E). This suggests that ingestion of FO scarcely influences fatty acid synthesis. These results suggest that the reductions of plasma glucose by supplementation with dietary n-3 PUFA are caused by the acceleration of glucose uptake and phospholipid synthesis in liver.

The effects of dietary EPA-EE and DHA-EE on concentrations of plasma glucose and adiponectin, and key enzyme activities related to carbohydrate metabolism in skeletal muscle and liver were smaller than those of dietary FO. Hong et al. [54] reported that EPA-EE and DHA-EE could not mimic the physiological activity of fish oil, at least with regard to affecting hepatic fatty acid oxidation in rats. EPA-EE and DHA-EE may have a similar effect on glucose metabolism, as well as hepatic fatty acid oxidation. However, the reasons for the weak effects of EPA-EE and DHA-EE on glucose metabolism are unknown.

In summary, although there were no differences in plasma glucose between the three dietary groups after glucose tolerance test, plasma glucose concentration in fasted mice fed the FO diet was significantly lower than in those fed the LD diet. Plasma insulin concentrations did not show any marked differences between the three dietary groups on glucose tolerance test. We do not believe that the reductions in plasma glucose in mice fed n-3 PUFAs were dependent on insulin stimulation. Increases in plasma adiponectin concentration and hepatic GPDH activity in mice fed n-3 PUFAs were confirmed in the present study, and these results suggest that plasma glucose uptake occurs in the liver rather than in skeletal muscle. We previously reported that the reductions in plasma glucose in mice fed the FO diet takes place over a longer period of time than similar decreases in plasma lipids and may be related to changes in lipid metabolism [14]. We believe that the decrease in plasma glucose may be initiated via changes in phospholipid metabolism and/or increases in adiponectin stimulation in the liver. However, the detailed changes in phospholipid metabolism and the effects of adiponectin stimulation during supplementation with dietary n-3 PUFA remain unknown. Further studies are needed to clarify the effects of dietary n-3 PUFA on hepatic phospholipid synthesis and adiponectin signaling.

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