Ethyl Icosapentate (omega-3 fatty acid) Causes Accumulation of Lipids in Skeletal Muscle But Suppresses Insulin Resistance in OLETF Rats

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Ethyl icosapentate (EPA) is known to improve insulin resistance in non–insulin-dependent diabetes mellitus (NIDDM); however, its mechanism is unclear. In this study, we attempted to determine the mechanism of EPA's effects on insulin resistance in Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Administration of EPA caused a reduction in plasma cholesterol and triglycerides, but increased cholesterol and triglyceride contents in skeletal muscle. EPA did not have an effect on glucose or insulin levels. EPA accelerated the glucose infusion rate (GIR) and improved the endothelium-dependent relaxation of OLETF rat the thoracic aorta caused by addition of acetylcholine. However, the improvement observed in endothelium-dependent relaxation disappeared after addition of N^{w}-nitro-L-arginine (L-NA). Furthermore, when L-NA and indomethacine were added to the medium, relaxation of the aorta in EPA-treated rats was weaker than that in control rats. These actions may cause NO induction in the endothelium and an increase in prostaglandin I_2 (PGI_2) and prostaglandin I_3 (PGI_3) action, which in turn may result in improvement of insulin resistance.

REDUCED POTENCY of insulin action and/or increased insulin resistance are factors that result in non–insulin-dependent diabetes mellitus (NIDDM).[1] NIDDM causes an increased cardiovascular morbidity and mortality compared with healthy subjects.[2] Insulin resistance causes disorders of lipid metabolism and may cause development of atherosclerosis.[3]

Dietary fish oils are known to improve insulin resistance in NIDDM animal models.[4] However, the mechanism of fish oils' effects on improving insulin resistance is unclear. Dietary fish oils contain long-chain polyunsaturated ω-3 fatty acids such as ethyl icosapentate (EPA) and docosahexaenoic fatty acids (DHA). We wanted to determine the mechanism of the effects of EPA on insulin resistance. Accordingly, we performed studies where EPA was administered to Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which is a model for NIDDM.[5]

MATERIALS AND METHODS

Materials

Ethyl icosapentate (EPA) was obtained from Mochida Pharmaceutical Co, Tokyo, Japan. Acetylcholine, N^{w}-nitro-L-arginine (L-NA) and indomethacin were obtained from Sigma (St Louis, MO). All other chemicals used were high grade commercially available products.

Animal Experiments

Male OLETF rats at the age of 5 weeks were obtained from Otsuka Pharmaceutical Co (Tokushima, Japan). The animals were maintained under a 12-hour light-dark cycle at a constant temperature of 23 ± 2°C. Rats aged 10 months were used in the experiment, and were stratified by body weight and divided into 3 groups of 6 rats each. Rats were fed chow (standard laboratory chow CRF-1, Oriental Yeast Co, Tokyo, Japan) containing EPA, which was equivalent to 0.3 or 1.0 g/kg body weight EPA, for 10 weeks. Control rats were fed a standard laboratory chow. The fatty acid profile of standard laboratory chow is as follows: lauric acid, nondetectable; myristic acid, 0.4%; palmitic acid, 14.1%; palmitoleic acid, 1.4%; stearic acid, 2.5%; oleic acid, 23.2%; linoleic acid, 48.4%; linolenic acid, 4.0%; EPA, 1.6%; docosahexaenoic acid, 1.4%. However, intake of laboratory chow amounted to 35 mg/kg EPA for 11.7% of total EPA intake (EPA administration of 0.3 g/kg). When EPA administration was 1.0 g/kg, laboratory chow accounted for 3.5% of total EPA intake. The animals were given free access to food and tap water. Food consumption was measured daily and body weight was recorded weekly. The respiratory quotient of rats was measured after 10 weeks of feeding ad libitum. At the end of the experimental period, a euglycemic glucose clamp study was performed on the rats. Blood samples were collected from the posterior vena cava for lipid, glucose, and insulin measurements. Visceral fat was removed and the weights measured. Skeletal muscle (soleus) was obtained with tongs and maintained in liquid nitrogen for measurement of glycogen and lipids.

Euglycemic Glucose Clamp Studies

The euglycemic glucose clamp study was performed after a 12-hour overnight fast, which was started after the final EPA administration. Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight), and extension tubings were attached to the jugular vein by an adapter so that glucose and insulin could be infused simultaneously. The carotid catheter was used for blood sampling. After a 30-minute basal period, a continuous infusion of human regular insulin (Novolin R, Novo Nordisk, Copenhagen, Denmark) was administered at a rate of 60 pmol/kg/min throughout the study. Blood samples were drawn at 10-minute intervals for immediate determination of plasma glucose (YSI 2300 glucose analyzer, Yellow Springs Instruments, Yellow Springs, OH) and plasma concentration was kept constant at 80 mg/dL by variable infusion of 10% dextrose solution. Steady-state was generally achieved within 60 to 90 minutes. A maximal glucose clamp was then started. Insulin was infused and 20% dextrose was variably infused to maintain plasma glucose concentration at 80 mg/dL, and the glucose infusion rate (GIR, glucose mg/kg/min) was obtained.

Vasodilation Responses

A section of the thoracic aorta between the aortic arch and diaphragm was removed from the same animals following exanguination.

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These were mounted as ring preparations and normalized for measurement of isometric tension in a 5-mL organ bath (VFER Micro Easy Magnus, Medical Kishimoto Co, Kyoto, Japan) containing Krebs solution of the following composition: NaCl 118.4 mmol/L, KCl 4.9 mmol/L, $CaCl_2$ 2.5 mmol/L, $MgCl_2$ 1.2 mmol/L, $NaHCO_3$ 25.0 mmol/L, KH_2PO_4 1.2 mmol/L, glucose 11.1 mmol/L, and ascorbic acid 1.2 mmol/L. The medium was maintained at 34°C and bubbled with 95% O_2 and 5% CO_2. The arteries were contracted with phenylephrine at a concentration corresponding to EC_{80} values (10^{-6} mol/L), and acetylcholine was added at a concentration of 10^{-6} mol/L. Furthermore, 3×10^{-6} mol/L of L-NA and 1×10^{-5} molo/L of indomethacin were added to the medium. Vasodilation responses were measured with an isometric transducer (AP-5, Medical Kishimoto Co) and are expressed as percentages of the phenylephrine-induced contraction.

Analytical Methods

Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose were determined by conventional enzymatic methods. The cholesterol C-test Wako (Wako Pure Chemical Industries, Osaka, Japan) was used for the determination of cholesterol, the Nescote HDL-C kit N (Nippon Shoji, Osaka, Japan) for HDL cholesterol, the triglyceride G-test Wako (Wako Pure Chemical Industries) for triglycerides, and the glucose CII test Wako for glucose. Insulin was determined by conventional enzyme immunoassay, with the use of the Glazyme insulin-EIA test (Wako Pure Chemical Industries).

Tissue glycogen. Glycogen was measured by the method of Hassid and Abraham with slight modification.[6] Approximately 0.2 g of tissue was placed into a Pyrex centrifuge tube (Iwaki, Tokyo, Japan) containing 1 mL of 30% potassium hydroxide solution. The tissue was then digested by heating the tube in a boiling water bath for 20 minutes. After the tissue dissolved, 0.5 mL of saturated sodium sulfate was added and the glycogen was precipitated by the addition of 3 mL of 95% ethanol. The tube and contents were heated again until the mixture began to boil, then cooled and centrifuged at 3,000 rpm. The mother liquor was decanted, and the test tube was allowed to drain. The precipitated glycogen was redissolved in 1 mL of distilled water and reprecipitated with 1.5 mL of 95% ethanol, the alcoholic supernatant liquid decanted, and the tube drained as before. The purified glycogen was redissolved in 1 mL of distilled water and glycogen in this solution measured by Anthrone reagent.[6]

Tissue lipids. A tissue sample of approximately 0.2 g, together with 2 mL of chloroform-methanol (2:1) solution[7] was added to a Pyrex centrifuge tube and homogenized by Polytron (PCU-2-110, Kinematica, Luzern, Switzerland). The tube was then centrifuged at 3,000 rpm. An aliquot of chloroform-methanol extract was transferred to another Pyrex tube and dried under a stream of nitrogen gas. These samples were redissolved in 100 μL isopropylalcohol and cholesterol and triglycerides were measured in isopropylalcohol by conventional enzymatic methods.

Respiratory Quotient

The respiratory quotient (RQ) of rats was measured after 10 weeks of feeding ad libitum. Individual rats were placed in a glass metabolic chamber with sufficient food and water for 24 hours. Air was drawn through the chamber at a rate of 1.5 L/min. Outflow air composition was measured using an automated open-circuit respirometer (Datex Deltatrac II, Helsinki, Finland).

Statistical Analysis

The results are expressed as means ± SD. Comparisons among 3 groups were analyzed for statistical significance using 1-way analysis of variance followed by Dunnett's test multiple comparisons.

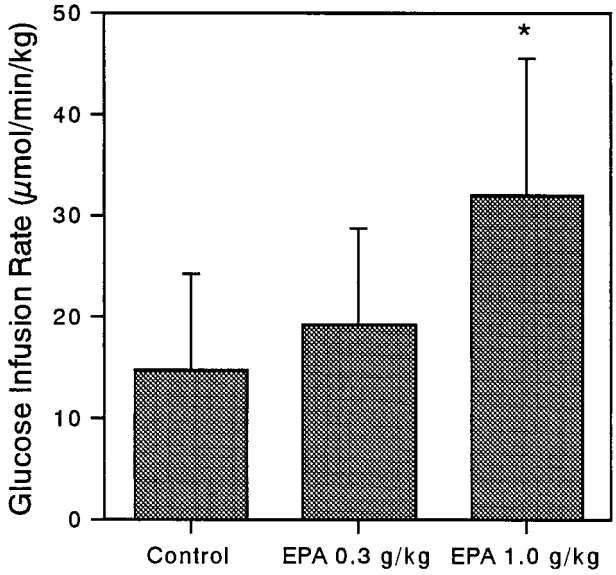

Fig 1. Effects of EPA on GIR in OLETF rats. EPA was administered to male OLETF rats (age 10 months) for 10 weeks. At the end of the experimental period, a euglycemic glucose clamp study was performed after a 12-hour overnight fast, which was started after the final EPA administration.

RESULTS

Effects of EPA on Body Weight and Food Consumption

There were no differences in body weight gain between EPA-treated rats and control rats (control: 642 ± 69; EPA 0.3-g/kg group: 685 ± 28; EPA 1.0-g/kg group: 652 ± 33 g at end of experiment).

There were no differences in food consumption between EPA-treated rats and control rats (data not shown).

Effects of EPA on Visceral Fat Weight

There were no differences in visceral fat between EPA-treated rats and control rats (control: 76 ± 12; EPA 0.3-g/kg group: 87 ± 12; EPA 1.0-g/kg group: 69.4 ± 5.0 g).

Effects of EPA on RQ

RQ was recorded at 1-minute intervals and represent mean 24-hour values. There were no differences in RQ values between EPA-treated rats and control rats (control: 0.865 ± 0.025; EPA 0.3-g/kg group: 0.877 ± 0.025; EPA 1.0-g/kg group: 0.860 ± 0.041 at end of experiment).

Effects of EPA on Glucose Infusion Rate Using a Euglycemic Glucose Clamp Study

The effects of EPA administration on glucose infusion rate (GIR) are shown in Fig 1. The GIR of 1.0 g/kg EPA-treated rats was significantly higher than control rats.

Effects of EPA on Plasma Lipids, Glucose, and Insulin

The effects of EPA administration on plasma lipids, glucose, and insulin are shown in Table 1. EPA caused a reduction in total cholesterol, HDL cholesterol, and triglycerides in a dose-

Table 1. Plasma Lipids, Glucose, and Insulin Levels in EPA-Treated OLETF Rats

Group	Total Cholesterol (mg/dL)	HDL Cholesterol (mg/dL)	Triglycerides (mg/dL)	Glucose (mg/dL)	Insulin (μU/mL)
Control					
Before dosing (n = 6)	99 ± 15	89 ± 13	219 ± 37	241 ± 34	129 ± 43
After dosing (n = 6)	113 ± 24	94 ± 11	216 ± 46	232 ± 47	114 ± 37
EPA 0.3 g/kg					
Before dosing (n = 6)	105 ± 8	90 ± 8	234 ± 21	259 ± 21	257 ± 199
After dosing (n = 6)	83 ± 8*	67 ± 6†	191 ± 38	264 ± 21	258 ± 87
EPA 1.0 g/kg					
Before dosing (n = 6)	101 ± 7	91 ± 7	220 ± 39	261 ± 22	154 ± 43
After dosing (n = 6)	61 ± 5†	53 ± 4†	122 ± 25†	243 ± 35	161 ± 34

NOTE. Data are expressed as means ± SD.
Significantly different from the values before dosing: *$P < .05$, †$P < .01$.

dependent manner. However, EPA had no effect on plasma glucose and insulin.

Effects of EPA on Skeletal Muscle Glycogen and Lipid Contents

The effects of EPA administration on skeletal muscle cholesterol, triglycerides, and glycogen contents are shown in Table 2. The cholesterol and triglyceride contents in skeletal muscle of rats treated with 1.0 g/kg EPA were significantly higher than control rats. EPA had no effect on glycogen contents.

Effects of EPA on Relaxation Responses

The endothelium-dependent relaxation of the aorta by acetylcholine in 1.0 g/kg EPA-treated rats was significantly improved compared to that of control (Fig 2A). When 3×10^{-6} mol/L of L-NA was added to the medium, endothelium-dependent relaxation did not differ among the 3 groups (Fig 2B). When 3×10^{-6} mol/L of L-NA and 1×10^{-5} mol/L of indomethacine were added, relaxation of the aorta in EPA-treated rats was weaker than that in control rats (Fig 2C).

DISCUSSION

The incidence of coronary heart disease (CHD) in diabetes mellitus is at least 3 times that of the normal population[2] and has been attributed to the presence of risk factors, such as hyperglycemia, hyperlipidemia, hypertension, and insulin resistance.[3,8,9] Long-chain polyunsaturated ω-3 fatty acids such as EPA are believed to protect against development of atherosclerosis by improvement of insulin resistance. However, the mechanism of this action has been unclear.

By means of the euglycemic glucose clamp technique, insulin resistance can be determined in experimental animals of diabetes and patients with NIDDM. In this study, we determined using the euglycemic glucose clamp technique that EPA improved insulin resistance in OLETF rats, which is a spontaneously diabetic animal. Diabetes mellitus and obesity are classic states of insulin resistance. These patients usually are hyperlipidemic, hyperglycemic, hypertensive, and hyperinsulinemic. OLETF rats are hyperlipidemic, hyperglycemic, hyperinsulinemic, and obese.[5] In this study, administration of EPA caused a reduction of plasma triglycerides, total cholesterol, and HDL cholesterol levels. However, EPA did not affect plasma glucose or insulin levels.

In this study, EPA improved the insulin resistance as measured by the euglycemic glucose clamp technique, but did not affect plasma glucose and insulin levels. This may mean that the euglycemic glucose clamp technique is highly sensitive, showing improvement in insulin resistance before any changes in plasma glucose and insulin levels. Dewailly et al[10] reported that n-3 fatty acids were positively associated with plasma HDL cholesterol and inversely associated with triglyceride. Furthermore, plasma glucose increased as n-3 fatty acid concentrations increased in Inuit of Nunavic. Effects of EPA on plasma lipids and glucose vary among reports. EPA also had no effect on body weight and visceral fat weights, and did not improve obesity in OLETF rats.

Skeletal muscle has been identified as the primary site of insulin-stimulated glucose disposal[11] and, as such, is a major site of insulin resistance. Storlien et al have reported that the amounts of triglyceride associated with skeletal muscle correlated with whole-body insulin action, and that skeletal muscle triglyceride may contribute to development of insulin resistance.[12,13] In this study, EPA improved insulin resistance, but increased triglyceride and cholesterol contents in the soleus in OLETF rats. These results conflict with previous reports; how-

Table 2. Skeletal Muscle Lipids and Glycogen Contents in EPA-Treated OLETF Rats

Group	Cholesterol (mg/g tissue)	Triglycerides (mg/g tissue)	Glycogen (mg/g tissue)
Control (n = 6)	1.65 ± 0.11	29.86 ± 8.35	3.52 ± 0.78
EPA 0.3 g/kg (n = 6)	1.97 ± 0.38	38.67 ± 15.83	3.22 ± 1.11
EPA 1.0 g/kg (n = 6)	1.80 ± 0.04*	41.96 ± 6.09*	2.95 ± 0.60

NOTE. Data are expressed as means ± SD.
Significantly different from the value in the respective control rats: *$P < .05$.

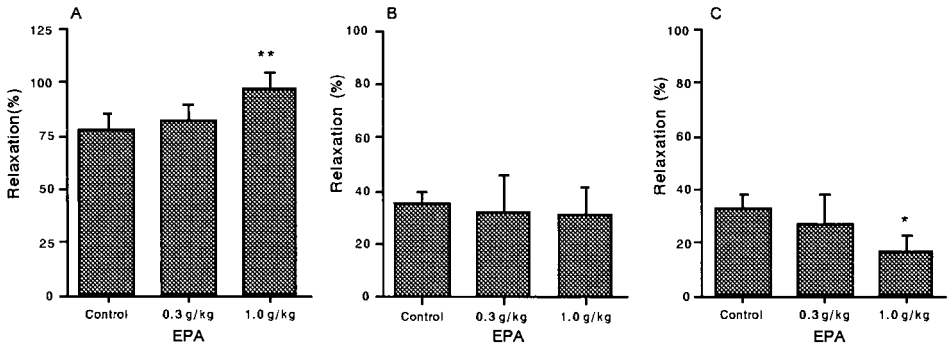

Fig 2. Effects of EPA on relaxation of aorta in OLETF rats. (A) Endothelium-dependent relaxation of the aorta by acetylcholine addition; (B) when adding 3×10^{-6} mol/L of L-NA to medium A; (C) when 3×10^{-6} mol/L of L-NA and 1×10^{-5} mol/L of indomethacin were added to B.

ever, we cannot explain why. Murphy et al[14] reported that the administration of fish oil increased triglyceride content in guinea pig heart. However, fish oil also increasesed total monounsaturated fatty acids in total phospholipid, and with decreased total saturated fatty acids in triglycerides. This shows the importance of analyzing the fatty acid profile of triglycerides.

Vasodilation responses to acetylcholine were impaired in experimental model animals of diabetes[15] and NIDDM patients.[16] Dietary fish oil supplementation has been shown to improve endothelium-dependent relaxation in atherosclerotic porcine[17] and human coronary vasculature.[18] In this study, impaired vasodilation responses to acetylcholine were improved by administration of EPA to OLETF rats. When L-NA (inhibitor of release and production of nitric oxide from the endothelium) was added to this medium, relaxation levels did not differ between control and EPA-treated rat aortae. The relaxing effect of NO was calculated by subtraction of relaxation when L-NA was added from relaxation when acetylcholine was added (control: 42.3 ± 10.9; EPA 0.3 g/kg: 50.2 ± 14.8; EPA 1.0 g/kg: 65.6 ± 14.4; $P < .05$ *v* control). These results suggest that EPA may accelerate production of NO from the endothelium in OLETF rats.

Indomethacin is a cyclooxygenase inhibitor. Cyclooxygenase produces prostaglandin I_2 (PGI_2) and prostaglandin I_3 (PGI_3,) which are vasodilatory prostaglandins. Silberbauer et al have reported that the forearm veins of patients with juvenile-onset diabetes generated significantly less prostacyclin than those of control.[19] The production of PGI_2 in the aorta of diabetic animals is lower than control.[20] When L-NA and indomethacin were added, the relaxation level of EPA-treated rat aorta was lower than control rats. The relaxing effect of vasodilatory prostaglandin was calculated by subtraction of relaxation when L-NA and indomethacin were added from relaxation when L-NA was added (control: 1.8 ± 0.5; EPA 0.3 g/kg: 5.0 ± 4.5; EPA 1.0 g/kg: 14.6 ± 4.7; $P < .05$ *v* control). These results suggest that EPA may accelerate production of vasodilatory prostaglandin.

In summary, EPA caused a reduction of plasma cholesterol and triglycerides, and improved insulin resistance in OLETF rats. However, EPA increased cholesterol and triglyceride contents in skeletal muscle. EPA improved the endothelium-dependent relaxation of thoracic aorta caused by acetylcholine. This efficacy may result from induction of NO and vasodilatory prostaglandins, but more experiments are needed to confirm this.

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