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CLINICAL TRIAL

Effects of eicosapentaenoic acid on serum levels of selenoprotein P and organ-specific insulin sensitivity in humans with dyslipidemia and type 2 diabetes

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ABSTRACT

Aim: Selenoprotein P (SeP, encoded by *SELENOP* in humans) is a hepatokine that causes insulin resistance in the liver and skeletal muscle. It was found that polyunsaturated fatty acid eicosapentaenoic acid (EPA) downregulates *Selenop* expression by inactivating SREBP-1c. The present study aimed to examine the effect of EPA for 12 weeks on circulating SeP levels and insulin sensitivity in humans with type 2 diabetes.

Methods: A total of 20 participants with dyslipidemia and type 2 diabetes were randomly assigned to an EPA (900 mg, twice daily) group and a control group. The primary endpoint was a change in serum SeP levels. Organ-specific insulin sensitivity in the liver (HGP and %HGP), skeletal muscle (Rd), and adipose tissue (FFA and %FFA) were assessed using a hyperinsulinemic-euglycemic clamp study with stable isotope-labeled glucose infusion.

Results: Serum SeP levels were not changed in either group at the end of the study. In the EPA group, the changes in SeP levels were positively correlated with the change in serum EPA levels ($r = 0.709$, $P = 0.022$). Treatment with EPA significantly enhanced %FFA but not %HGP and Rd. The change in serum EPA levels was significantly positively correlated with the change in %HGP, and negatively correlated with changes in Rd.

Conclusions: The change in serum EPA levels was positively correlated with serum SeP levels, hepatic insulin sensitivity, and negatively with skeletal muscle insulin sensitivity in humans with type 2 diabetes. The EPA-induced enhancement of hepatic insulin sensitivity might be associated with a mechanism independent of serum SeP levels.

INTRODUCTION

The liver plays a central role in glucose homeostasis and produces various bioactive secretory proteins, termed hepatokines[1]. We have rediscovered selenoprotein P (SeP, encoded by *SELENOP* in humans) as a hepatokine, the expression of which is correlated with post-challenge glucose levels[2] and peripheral insulin resistance[3]. SeP eliminates the reactive oxygen species required for signal transduction and thereby causes pathology to type 2 diabetes, such as insulin resistance[3], angiogenesis resistance[4], insulin secretory failure[5], exercise resistance[6], and ischemia-reperfusion injury[7]. Serum selenoprotein P levels are increased during aging[8] and in individuals with type 2 diabetes[3], nonalcoholic fatty liver disease[9], and chronic hepatitis C[10]. Elevated serum SeP levels predict the future onset of hyperglycemia[8]. Therefore, SeP might be a potential therapeutic

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target against diabetes. We found that eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid (PUFA), down-regulates *Selenop* gene expression[11].

EPA is widely used clinically as a therapeutic agent for hypertriglyceridemia. EPA supplementation exerts favorable effects on lipoprotein metabolism and inflammatory, oxidative, thrombotic, vascular, and arrhythmogenic factors implicated in cardiovascular disease[12]. In clinical trials, EPA suppresses the onset of cardiovascular events in Japanese patients with hypercholesterolemia[13,14]. The effect of EPA on insulin sensitivity remains controversial[15,16]. EPA reduces insulin resistance in Goto-Kakizaki rats and in C57BL/6J mice[17,18]. Despite the promising evidence from animal studies, currently, there is no clear consensus on the effects of PUFAs on insulin sensitivity in humans[19]. Specifically, organ-specific actions of EPA on insulin sensitivity remain unclear.

Based on these findings, we hypothesized that EPA might ameliorate insulin resistance in the liver and skeletal muscle partly by lowering serum SeP levels. To test this hypothesis, the present study aimed to examine the effect of EPA for 12 weeks on circulating SeP levels and organ-specific insulin sensitivity in humans with dyslipidemia and type 2 diabetes.

MATERIALS AND METHODS

Study overview

A randomized, controlled, parallel trial on EPA was performed in patients with dyslipidemia and type 2 diabetes at Kanazawa University Hospital following the Declaration of Helsinki. The participants provided written informed consent. This trial was registered with the University Hospital Medical Information Network (UMIN) Clinical Trials Registry (number UMIN 000015673).

Study protocol

Individuals were recruited from outpatients at Kanazawa University Hospital. Those with a definitive diagnosis of dyslipidemia and type 2 diabetes were eligible for participation. The eligibility criteria were as follows: age >20 years and diagnosis of dyslipidemia and type 2 diabetes mellitus. The exclusion criteria were as follows: (i) hypersensitivity or contraindication EPA; (ii) history of type 1 diabetes; (iii) history of ketoacidosis; (iv) symptoms of hypoglycemia; (v) treatment with EPA within 8 weeks of screening; (vi) poorly controlled unstable diabetes (state with ketoacidosis or an increase in HbA1c levels >3% at 12 weeks before screening); (vii) dialysis and severe renal function (estimated glomerular filtration rate < 30 mL/min/1.73 m^2, serum creatinine levels >2.5 mg/dL in men and >2.0 mg/dL in women); (viii) poorly controlled hypertension or systolic blood pressure >160 mmHg or diastolic blood pressure >100 mmHg; (ix) current and/or previous heart failure; (x) severe retinopathy; (xi) malignancy on an active therapeutic regimen or without complete remission or treatment; (xii) presence of a severe health problem and unsuitability for the study and inability to participate in the study (including psychiatric and psychosocial conditions); (xiii) pregnancy or lactation; and (xiv) inadequacy to participate in the study, as assessed by the investigators.

Randomization

Eligible participants were randomly assigned to the EPA group or the control group at a 1:1 ratio using a computer-generated randomization sequence. In the present parallel-group trial, those in the EPA group were prescribed EPA (Mochida Pharmaceutical Co., Ltd, Tokyo, Japan), at a dose of 1,800 mg (900 mg/pack, twice daily, under Japanese insurance coverage): after breakfast and after dinner, added to their current treatment for 12 weeks. No one (participants, investigators, and site staff) was masked to the treatment allocation.

Examination of baseline clinical features and laboratory markers, including atherosclerosis markers; euglycemic-hyperinsulinemic clamp study; bioelectrical impedance analysis; and reactive hyperemia of peripheral arterial tonometry (RH-PAT), were also performed before and 12 weeks after the initiation of the study. Venous blood samples were obtained in the morning after an overnight fast.

Participants continued to use oral hypoglycemic agents at the baseline dose throughout the study. Other, additional antihyperglycemic medications were prohibited during the study period while participants continued to use the study medication.

During the study period, all participants underwent nutritional and exercise counseling provided by experienced practitioners. Briefly, each individual was prescribed a diet to maintain or achieve a body mass index (BMI) of 22: 30 kcal/kg/day, 50–60% from carbohydrates, 20–30% from fat, and 15–20% from protein.

Efficacy endpoints

The primary endpoint was a change in serum SeP levels. The secondary endpoints were the changes in organ-specific insulin sensitivity evaluated using the euglycemic-hyperinsulinemic clamp study with the stable isotope-labeled glucose infusion, glycemic control, body composition, and laboratory markers.

Body composition (fat mass and fat-free mass) was determined by bioelectrical impedance analysis (Tanita BC118D, Tanita, Tokyo, Japan).

Serum concentrations of full length selenoprotein P were specifically measured by sol particle homogeneous immunoassay using two monoclonal antibodies, as previously established[20,21]. The serum concentrations of selenium were measured by atomic absorption spectrophotometry[8].

Endothelial function in small peripheral vessels was assessed by RH-PAT in the morning under an overnight fasting condition. RH-PAT was measured using an EndoPAT 2000 device (Itamar Medical Ltd, Caesarea, Israel) and expressed as RH-PAT index (RHI) according to previous studies[22].

A hyperinsulinemic-euglycemic clamp study was conducted as described previously[23]. The ratio of [6,6-$^2H_2$]glucose to glucose was determined by gas chromatography-mass spectrometry

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(GC-MS). In the basal state, hepatic glucose production (HGP) was calculated as the glucose rate of appearance (Ra), according to Steele's equation, as described previously[24,25]. During the clamp study, glucose Ra was calculated using Steele's equation from tracer data[24]. HGP during the clamp study was calculated as the difference between glucose Ra and the infusion rate of exogenous glucose. We calculated and defined organ-specific IR in the liver, skeletal muscle, and adipose tissue, as described previously[23,26]. Hepatic IR indices were calculated as the product of fasting HGP and fasting plasma insulin (FPI) levels (HGP * FPI [(mg/kg/min) * (mU/mL)]) and suppression of HGP by insulin during a clamp study (%HGP). The skeletal muscle IR index was calculated as insulin-stimulated glucose disposal (Rd), and the adipose tissue IR index was calculated as suppression of FFA by insulin during a clamp study (% FFA)[23,26].

Statistical analysis

All analyses were performed using SPSS software version 26.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as mean ± standard deviation. The Wilcoxon signed-rank test was used for intergroup comparisons, and the Mann–Whitney *U* test was used for intragroup comparisons. The Mann–Whitney rank-sum test was also used to compare all categorical variables of the quantity of changes between the two groups. The relationship between individual variables was assessed by Spearman's correlation. Statistical significance was considered at a value of $P < 0.05$. We defined "trending toward statistical significance" as *P* values less than 0.10[27].

RESULTS

Baseline metabolic parameters

Twenty eligible participants were screened and randomly assigned to the EPA and control groups. All participants in the EPA group were naïve to EPA at baseline. The patients were recruited between November 2014 and October 2016, with follow-up continuing for 12 weeks. Of the 20 participants enrolled in the study, one in the control group dropped out after randomization and before the intervention. We performed a completed case analysis rather than an intention-to-treat analysis because there was only one dropout. The reason for dropping out was unrelated to baseline values or their response. No adverse effects of EPA supplementation, such as liver enzyme abnormality, gastrointestinal symptoms, and hypersensitivity, were reported. There were no significant differences in any of the measured parameters between the groups before randomization, except for high-density lipoprotein (HDL) cholesterol levels. The EPA group had significantly lower mean HDL cholesterol levels than the control group.

Outcomes of EPA administration on SeP levels and metabolic parameters

Serum SeP levels were not changed in either group during the study (–0.08 ± 0.38 in the EPA group, –0.02 ± 0.39 in the control group, $P = 0.780$). Serum selenium levels were also not changed in either group during the study (–0.5 ± 18.0 in the EPA group, –2.1 ± 12.6 in the control group, $P = 0.842$) (Tables 1 and 2). Endpoint serum levels of selenium and SeP were correlated with each other. The changes in serum selenium levels were positively correlated with the changes in SeP levels in all groups (Table 3c). The changes in EPA and EPA/arachidonic acid (AA) were significantly greater in the EPA group than in the control group (138.5 ± 63.2 and 0.82 ± 0.50 in the EPA group, –6.7 ± 59.5 and –0.10 ± 0.37 in the control group, $P = 0.000$ and 0.000, respectively) (Table 1). Serum EPA levels and EPA/AA ratio significantly increased in the EPA group (73.7 ± 25.3 to 212.2 ± 79.7 and 0.34 ± 0.12 to 1.16 ± 0.55, $P = 0.002$ and 0.005, respectively), whereas these did not change in the control group (Table 2). In the EPA group, the changes in serum EPA levels were positively correlated with the changes in SeP levels ($r = 0.709$, $P = 0.022$) (Table 3, Figure 1). The change in EPA/AA ratio was not correlated with the change in SeP levels (Table 3d).

The HbA1c levels in the EPA group did not change. In contrast, it significantly increased (6.6 ± 0.8 to 7.0 ± 1.1, $P = 0.016$) in the control group, with no significant difference between the groups at the end of the study (Tables 1 and 2). The change in serum EPA levels was significantly positively correlated with the change in HbA1c ($r = 0.357$, $P = 0.039$) in the EPA group (Table 3a). The change in SeP levels was positively correlated with the changes in HbA1c ($r = 0.519$, $P = 0.023$) and total cholesterol ($r = 0.480$, $P = 0.037$) (Table 3b).

Bodyweight and BMI did not change in the EPA group, whereas these tended to increase in the control group. The fat mass and fat free mass did not change in either group. C-peptide immunoreactivity (CPR), liver enzymes (aspartate aminotransferase, alanine aminotransferase, and gamma-glutamyl transferase), lipid profiles (total cholesterol, triglycerides, HDL cholesterol, and Lp(a)), and endothelial function (RHI) did not change in either group (Tables 1 and 2).

Endpoint serum SeP levels were positively correlated with the endpoint FPG levels (Table 4b). Endpoint serum levels of EPA were not associated with SeP levels and metabolic parameters at the endpoint.

Outcomes of EPA administration on organ-specific insulin sensitivity

The glucose infusion rate did not change in either group (Tables 1 and 2).

The change in the insulin-induced suppression of HGP (% HGP) was significantly elevated in the EPA group compared with the control group (Table 1). The %HGP did not change in the EPA group, whereas it tended to decrease in the control group (Table 2). The change in serum EPA levels was significantly positively correlated with the change in %HGP ($r = 0.590$, $P = 0.013$) in all subjects (Table 3a, Figure 2a).

The increase in Rd was significantly higher in the control group than in the EPA group (Table 1). Rd did not change in

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Table 1 | Quantity of changes from baseline in the characteristics of patients

	EPA		Control		*P*
	Change	Confidence interval	Change	Confidence interval	
Selenoprotein P (mg/L)	−0.08 ± 0.38	−0.36 to 0.19	−0.02 ± 0.39	−0.32 to 0.28	0.780
Selenium (μg/L)	−0.5 ± 18.0	−13.4 to 12.4	−2.1 ± 12.6	−11.8 to 7.5	0.84
Eicosapentaenoic acid (μg/mL)	138.5 ± 63.2	93.3 to 183.6	−6.7 ± 59.5	−52.5 to 38.9	0.000
Arachidonic acid (μg/mL)	−20.8 ± 63.1	−65.9 to 24.3	6.8 ± 61.4	−40.3 to 54.0	0.447
EPA/AA ratio	0.82 ± 0.50	0.47 to 1.17	−0.10 ± 0.37	−0.38 to 0.18	0.000
Fasting plasma glucose (mg/dL)	1.4 ± 31.1	−20.8 to 23.6	5.4 ± 23.1	−12.3 to 23.2	0.905
Hemoglobin A1c (%)	−0.5 ± 1.6	−1.6 to 0.7	0.3 ± 0.5	−0.1 to 0.7	0.133
Bodyweight (kg)	−0.3 ± 2.1	−1.8 to 1.2	1.5 ± 2.8	−0.6 to 3.7	0.278
Fat mass (kg)	−0.4 ± 2.0	−1.8 to 1.0	1.6 ± 3.2	0.9 to 4.0	0.278
Fat free mass (kg)	0.6 ± 2.0	−0.8 to 2.0	0.2 ± 0.9	−0.6 to 0.9	0.968
Body mass index (kg/m^2)	0.2 ± 0.8	−0.4 to 0.8	0.6 ± 0.9	−0.1 to 1.3	0.661
Waist circumference(cm)	−0.7 ± 6.7	−5.5 to 4.0	0.3 ± 5.0	−3.5 to 4.1	0.447
Systolic blood pressure (mmHg)	2.5 ± 14.5	−7.9 to 12.9	−7.9 ± 14.9	−19.3 to 3.5	0.211
Heart rate (beat/min)	1.8 ± 10.3	−0.7 to 10.4	2.9 ± 7.0	−3.0 to 8.7	0.878
White blood cell count (/mL)	807.0 ± 106.9	−2.6 to 12.7	−213.3 ± 952.6	−1.0 to 0.5	0.182
BUN (mg/dL)	1.7 ± 5.0	−1.9 to 5.3	−0.6 ± 2.6	−2.5 to 1.4	0.182
Creatinine (mg/dL)	0.03 ± 0.05	−0.00 to 0.06	−0.01 ± 0.10	−0.09 to 0.07	0.315
Aspartate transaminase (IU/L)	−0.6 ± 12.5	−9.6 to 8.4	1.8 ± 9.8	−5.8 to 9.4	1.000
Alanine aminotransferase (IU/L)	−2.8 ± 21.9	−18.5 to 12.9	7.4 ± 19.2	−7.3 to 22.2	0.497
Gamma-glutamyl transferase (IU/L)	−5.5 ± 24.6	−23.1 to 12.1	10.1 ± 19.5	−4.9 to 25.1	0.133
Total cholesterol (mg/dL)	7.8 ± 34.2	−16.7 to 32.3	1.3 ± 29.0	−21.0 to 23.6	0.780
Triglycerides (mg/dL)	−15.1 ± 59.9	−57.9 to 27.7	24.8 ± 61.9	−22.8 to 72.4	0.182
High-density lipoprotein cholesterol	0.3 ± 6.1	−4.1 to 4.7	−3.3 ± 8.4	−9.8 to 3.1	0.400
C-peptide immunoreactivity (ng/mL)	−0.11 ± 0.46	−0.44 to 0.22	0.08 ± 0.63	−0.40 to 0.57	0.278
Lipoprotein(a) (mg/dL)	1.6 ± 10.0	−5.6 to 8.7	−1.9 ± 3.3	−4.5 to 0.7	0.156
RHI	0.02 ± 0.49	−0.36 to 0.39	−0.02 ± 0.54	−0.43 to 0.40	0.863
Evaluation of the organ-specific insulin sensitivity using the hyperinsulinemic-euglycemic clamp study					
Glucose infusion rate (mg/kg/min)	−0.13 ± 1.22	−1.00 to 0.74	0.44 ± 1.49	−0.70 to 1.59	0.356
HGP * FPI	−5.5 ± 12.7	−17.3 to 6.2	1.0 ± 28.0	−24.9 to 27.0	0.318
% HGP	17.4 ± 33.4	−8.3 to 43.0	−15.0 ± 28.6	−38.9 to 8.9	0.036
Rd (mg/kg/min)	−0.84 ± 1.92	−2.31 to 0.63	1.37 ± 1.93	−0.24 to 2.99	0.011
%FFA	6.4 ± 6.9	1.5 to 11.3	−6.3 ± 26.2	−26.4 to 13.9	0.278

All values are mean ± standard deviation. *P*-value for the intergroup comparison (change from baseline between groups). %FFA, suppression of FFA by insulin during a clamp study; %HGP, suppression of hepatic glucose production by insulin; FPI, fasting plasma insulin concentration; HGP, hepatic glucose production; Rd, insulin-stimulated glucose disposal; RHI, reactive hyperemia of peripheral arterial tonometry index.

the EPA group but tended to increase in the control group (Table 2). The change in serum EPA levels tended to be negatively correlated with the change in Rd ($r = -0.422$, $P = 0.092$) in all subjects (Table 3a, Figure 2b).

The insulin-induced suppression of FFA (%FFA) significantly increased in the EPA group (75.6 ± 14.2 to 82.0 ± 11.7, $P = 0.002$), whereas it did not change in the control group (Table 2).

Endpoint serum levels of EPA, SeP, selenium were not associated with organ-specific insulin sensitivity indices (Table 4a–c).

DISCUSSION

The present study was to test in humans our *in vitro* findings that EPA suppresses *SELENOP* promoter activity[11]. Contrary to our hypothesis, the change in serum EPA levels after EPA supplementation (1.8 g/day) for 12 weeks positively correlated with the change in SeP levels in humans with type 2 diabetes. The change in EPA was associated positively with the change in hepatic insulin sensitivity and negatively with the change in skeletal muscle insulin sensitivity. In addition, EPA supplementation was correlated with adipose tissue insulin sensitivity.

SeP causes insulin resistance in the liver and skeletal muscle[3]. *SELENOP* expression is negatively correlated with the metabolic clearance rate, a representative index for peripheral insulin sensitivity measured by hyperinsulinemic-euglycemic glucose clamp experiments, in participants with type 2 diabetes[3], suggesting that the overproduction of SeP coexists with systemic insulin resistance in type 2 diabetic conditions. The subjects in the present study were type 2 diabetic with average HbA1c 7.2%, and

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Table 2 | Changes in the characteristics of patients between baseline and 12 weeks

	EPA			Control			*P***
	Before	12 weeks	*P**	Before	12 weeks	*P**	
Selenoprotein P (mg/L)	4.30 ± 0.76	4.22 ± 0.71	0.625	4.49 ± 0.50	4.47 ± 0.84	0.820	0.563
Selenium (μg/L)	138.5 ± 26.9	138.0 ± 17.2	0.906	137.3 ± 12.4	135.2 ± 21.2	0.677	0.720
Eicosapentaenoic acid (μg/mL)	73.7 ± 25.3	212.2 ± 79.7	0.002	98.2 ± 74.6	91.4 ± 63.5	0.910	0.002
Arachidonic acid (μg/mL)	228.2 ± 61.3	207.4 ± 100.3	0.169	222.2 ± 66.5	229.1 ± 79.2	0.953	0.604
EPA/AA ratio	0.34 ± 0.12	1.16 ± 0.55	0.005	0.50 ± 0.49	0.39 ± 0.21	0.514	0.000
Fasting plasma glucose (mg/dL)	126.9 ± 20.4	128.3 ± 26.8	0.695	126.2 ± 25.1	131.7 ± 32.0	0.359	0.985
Hemoglobin A1c (%)	7.6 ± 1.7	7.2 ± 1.3	0.820	6.6 ± 0.8	7.0 ± 1.1	0.016	0.775
Bodyweight (kg)	72.8 ± 15.4	72.5 ± 14.1	0.759	79.1 ± 23.7	80.7 ± 25.7	0.093	0.838
Body mass index (kg/m^2)	25.3 ± 3.9	25.4 ± 3.3	0.475	27.7 ± 6.5	28.4 ± 7.2	0.079	0.391
Waist circumference(cm)	91.9 ± 11.5	91.2 ± 9.1	0.443	97.3 ± 19.7	97.6 ± 20.0	0.859	0.935
Fat mass (kg)	21.2 ± 7.4	20.9 ± 6.4	0.508	21.5 ± 13.6	23.1 ± 16.3	0.161	0.497
Fat free mass (kg)	51.1 ± 10.2	51.6 ± 10.0	0.813	57.6 ± 11.8	57.7 ± 11.2	0.635	0.661
Systolic blood pressure (mmHg)	115.6 ± 15.0	118.1 ± 12.7	0.646	135.6 ± 14.2	127.7 ± 18.2	0.128	0.191
Heart rate (beat/min)	79.8 ± 14.4	80.2 ± 14.1	0.446	68.4 ± 10.0	70.9 ± 14.9	0.352	0.229
White blood cell count (/mL)	6221 ± 1220	6710 ± 1330	0.169	6040 ± 1710	5820 ± 1230	0.515	0.270
BUN (mg/dL)	14.4 ± 3.2	16.1 ± 6.1	0.260	16.3 ± 8.2	15.8 ± 7.0	0.394	0.743
Creatinine (mg/dL)	0.67 ± 0.16	0.70 ± 0.18	0.065	0.79 ± 0.21	0.77 ± 0.20	0.624	0.327
Aspartate transaminase (IU/L)	30.9 ± 18.4	30.3 ± 17.4	0.859	25.2 ± 10.4	27.0 ± 11.8	0.889	0.713
Alanine aminotransferase (IU/L)	45.4 ± 33.5	42.6 ± 42.5	0.575	28.1 ± 12.3	35.6 ± 23.5	0.594	0.935
gamma-glutamyl transferase (IU/L)	58.1 ± 34.8	52.6 ± 38.4	0.314	36.9 ± 17.2	47.0 ± 31.0	0.160	0.713
Total cholesterol (mg/dL)	229.1 ± 129.4	250.7 ± 299.1	0.770	194.4 ± 39.3	195.8 ± 49.0	0.953	0.589
Triglycerides (mg/dL)	176.1 ± 33.2	183.9 ± 41.4	0.695	148.7 ± 63.4	173.4 ± 66.9	0.260	0.905
High-density lipoprotein cholesterol	41.6 ± 10.6	41.9 ± 12.3	1.000	52.1 ± 8.4	48.8 ± 9.3	0.260	0.128
C-peptide immunoreactivity (ng/mL)	2.2 ± 0.6	2.1 ± 0.5	0.760	2.4 ± 1.3	2.5 ± 1.3	0.374	0.567
Lipoprotein(a) (mg/dL)	12.5 ± 13.7	14.0 ± 19.9	0.575	8.5 ± 10.3	6.6 ± 7.5	0.128	0.120
RHI	1.67 ± 0.24	1.72 ± 0.36	0.767	1.90 ± 0.54	1.88 ± 0.46	0.953	0.289
Evaluation of the organ-specific insulin sensitivity using the hyperinsulinemic-euglycemic clamp study							
Glucose infusion rate (mg/kg/min)	3.61 ± 1.41	3.48 ± 0.97	1.000	4.11 ± 1.99	4.56 ± 1.84	0.496	0.324
HGP * FPI	30.0 ± 8.6	24.5 ± 11.4	0.250	26.4 ± 23.2	27.5 ± 12.4	0.375	0.955
% HGP	60.3 ± 39.4	77.6 ± 30.8	0.250	78.1 ± 12.2	63.1 ± 31.5	0.078	0.093
Rd (mg/kg/min)	5.43 ± 2.01	4.59 ± 0.70	0.203	4.40 ± 1.48	5.77 ± 2.05	0.055	0.589
%FFA	75.6 ± 14.2	82.0 ± 11.7	0.002	82.7 ± 11.1	76.4 ± 25.8	0.910	1.000

All values are mean ± standard deviation. %FFA, suppression of FFA by insulin during a clamp study; %HGP, suppression of hepatic glucose production by insulin; EPA/AA ratio, eicosapentaenoic acid/arachidonic acid ratio; FPI, fasting plasma insulin concentration; HGP, hepatic glucose production; Rd, insulin-stimulated glucose disposal; RHI, reactive hyperemia of peripheral arterial tonometry index. **P*-value for the intragroup comparison (baseline vs endopoint). ***P*-value for the intergroup comparison (endpoint).

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Table 3 | (a) Factors associated with changes in serum EPA levels (delta EPA)

	EPA		Control		ALL	
	r	*P*	*r*	*P*	*r*	*P*
delta SeP	0.709	0.022	0.117	0.765	0.163	0.504
delta FPG	0.239	0.506	–0.200	0.606	0.051	0.839
delta HbA1c	0.657	0.039	–0.354	0.349	–0.168	0.493
delta Glucose infusion rate	0.321	0.365	–0.050	0.898	–0.058	0.814
delta %HGP	0.517	0.154	–0.310	0.456	0.590	0.013
delta HGP*FPI	–0.234	0.614	–0.120	0.798	–0.266	0.358
delta Rd	0.083	0.831	0.262	0.531	–0.422	0.092
delta %FFA	–0.152	0.676	0.200	0.606	0.281	0.244
(b) Factors associated with changes in serum selenoprotein P levels (delta SeP)						
delta FPG	0.276	0.440	0.367	0.332	0.295	0.220
delta HbA1c	0.602	0.066	0.430	0.248	0.519	0.023
delta BW	0.304	0.393	0.050	0.898	0.258	0.286
delta ALT	0.200	0.580	0.367	0.332	0.377	0.111
delta gGTP	0.152	0.676	0.378	0.316	0.258	0.286
delta TC	0.430	0.214	0.467	0.205	0.480	0.037
delta TG	0.085	0.815	–0.067	0.865	0.098	0.689
delta HDLC	–0.232	0.518	0.160	0.682	0.086	0.725
delta Glucose infusion rate	–0.055	0.881	–0.067	0.865	–0.072	0.770
delta %HGP	0.300	0.433	–0.595	0.120	0.011	0.966
delta HGP * FPI	0.119	0.779	–0.071	0.879	–0.007	0.982
delta Rd	–0.150	0.700	0.190	0.651	–0.044	0.866
delta %FFA	–0.273	0.446	0.133	0.732	–0.131	0.594
(c) Factors associated with changes in serum selenium levels (delta selenium)						
delta EPA	0.721	0.019	0.025	0.949	0.301	0.210
delta EPA/AA	0.248	0.489	–0.385	0.306	0.056	0.819
delta SeP	0.915	0.000	0.678	0.045	0.786	0.000
delta FPG	0.239	0.506	0.167	0.667	0.213	0.380
delta HbA1c	0.511	0.132	0.284	0.459	0.424	0.070
delta Glucose infusion rate	–0.152	0.676	–0.577	0.104	–0.325	0.175
delta %HGP	0.283	0.460	0.012	0.978	0.324	0.205
delta HGP * FPI	–0.107	0.819	–0.108	0.818	–0.013	0.964
delta Rd	–0.150	0.700	–0.323	0.435	–0.309	0.227
delta %FFA	–0.442	0.200	–0.226	0.559	–0.356	0.134
(d) Factors associated with changes in EPA/AA ratio (delta EPA/AA ratio)						
delta EPA	0.564	0.090	0.030	0.717	0.874	0.000
delta SeP	0.188	0.603	0.050	0.898	–0.030	0.903
delta selenium	0.248	0.489	–0.385	0.306	0.056	0.819
delta FPG	–0.436	0.208	–0.033	0.932	–0.093	0.704
delta HbA1c	0.073	0.841	–0.051	0.897	–0.285	0.237
delta Glucose infusion rate	0.578	0.111	0.217	0.576	0.063	0.797
delta %HGP	0.133	0.732	–0.595	0.120	0.437	0.080
delta HGP * FPI	–0.179	0.702	–0.214	0.645	–0.420	0.135
delta Rd	0.167	0.116	0.524	0.183	–0.292	0.256
delta %FFA	0.030	0.934	0.550	0.125	0.402	0.088

%FFA, suppression of FFA by insulin during a clamp study; %HGP, suppression of hepatic glucose production by insulin during a clamp study; FPG, Fasting plasma glucose; FPI, fasting plasma insulin concentration; HGP, hepatic glucose production; Rd, insulin-stimulated glucose disposal; SeP, Selenoprotein P.

SeP levels were relatively high (4.39 ± 0.64 mg/L) compared with the general population studies[8,28] and a well-controlled type 2 diabetic cohort[2]. As previously reported in the general population studies[8,28], SeP levels were positively correlated with the selenium levels in the present study. The serum levels of selenium and SeP in this study were relatively high compared with randomly selected subjects from two cities in Denmark[29] and the European prospective investigation of cancer and

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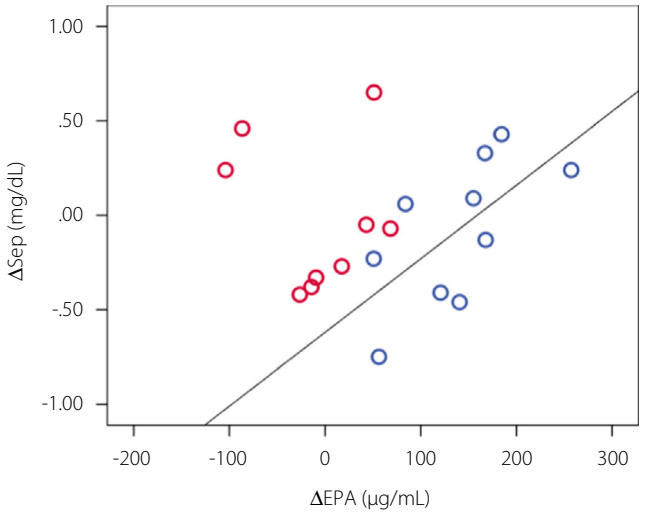

Blue circle; EPA group (r=0.709, P=0.022), Red circle; Control group (r=0.117 P=0.765)

Figure 1 | Correlation between the changes in serum EPA levels and changes in selenoprotein P levels. Blue circle, EPA group (r = 0.709, P = 0.022); red circle, control group (r = 0.117 P = 0.765).

nutrition cohort[30]. In this study, the change in SeP levels was positively correlated with the changes in HbA1c and total cholesterol. These findings suggesting that elevation of SeP is associated with worsening of HbA1c as reported previously[3]. However, there was no correlation between SeP and organ insulin sensitivity (Table 3b). Only the change in total cholesterol had a positive association with the change in SeP.

The present study used 1.8 g EPA under Japanese insurance coverage for the active arm of this trial because 1.8 g EPA changed the cumulative incidence[13,14,31], plaque components stabilization, and inflammation cytokines of major coronary events[32] in the Japanese clinical trials. EPA supplementation did not change the serum SeP levels. However, the change in serum EPA levels was positively correlated with the change in serum SeP levels, suggesting that EPA was associated with SeP in humans. This finding seems unexpected because we previously observed that EPA inhibits *SELENOP* promoter activity by inhibiting nuclear transport and promoter binding of SREBP-1c[11]. Based on the human findings in the present study, we re-examined the molecular mechanisms underlying the EPA-mediated regulation of *SELENOP* expression. The expression of *SELENOP* is regulated through the transcription factors FoxOs and SREBP-1. Studies have shown that insulin downregulated *SELENOP* by phosphorylating and inactivating FoxO1[33], whereas the antidiabetic drug metformin activated AMP-activated protein kinase, phosphorylated and inactivated FoxO3a but not FoxO1, and thereby downregulated *SELENOP* in hepatocytes[34]. Glucose and palmitate upregulate *SELENOP*[3] via unknown mechanisms. We found that EPA bidirectionally regulates *SELENOP* gene expression via FoxO1-SREBP-1c balance in hepatocytes (manuscript in preparation). In the present study, the changes in EPA levels positively correlated with A1c and SeP levels, suggesting the possibility that EPA upregulates SeP via elevating glucose levels.

In this study, EPA supplementation for 12 weeks did not change fasting plasma glucose, HbA1c, and CPR levels in participants with dyslipidemia and type 2 diabetes, which seems inconsistent with studies in rodents[17,18]. It has been long proposed that PUFA does not provide beneficial effects on glycemic control in patients with established type 2 diabetes[15,19]. As support for our study, two meta-analyses with large numbers of participants were concordant in outcomes with no effect on glycemic control or fasting insulin from PUFA supplementation[35,36]. In this study, lipid profiles including triglycerides did not change in the EPA group. The reduction of lipid profiles in EPA was relatively small compared with other lipid lowering drugs such as statin and fibrate[37]. As supported by our findings, a past RCT trial reported that the lipid profile did not change under EPA at 1.8 g for 6 months[32].

To date, only one small study reported previously the effects of PUFAs on organ-specific insulin sensitivity. Lalia *et al.* investigated the effects of 3.9 g/day EPA + DHA (n = 14) or placebo (n = 11) for 6 months on hepatic and peripheral insulin sensitivity assessed by a hyperinsulinemic-euglycemic clamp study in insulin-resistant humans[38]. In their study, EPA+DHA increased hepatic insulin sensitivity without altering peripheral insulin sensitivity. In our present study, 1.8 g/day EPA supplementation for 12 weeks significantly elevated adipose tissue insulin sensitivity (% suppression of FFA). Changes in serum EPA levels were not correlated with changes in the % suppression of FFA, suggesting that EPA may be uptaken to the adipose tissue and enhance adipose tissue insulin sensitivity independent of its serum levels. We previously reported that SeP inversely regulates adiponectin expression in the adipose tissue[39]. *Selenop*-deficient mice are protected from the diet-induced insulin resistance and adipocyte hypertrophy[3]. However, to date, there is no evidence as to whether SeP affects adipose tissue insulin sensitivity. Therefore, EPA was correlated with adipose tissue insulin sensitivity independently of the SeP actions.

The EPA supplementation did not alter hepatic insulin sensitivity. However, of note, the change in serum EPA levels was significantly correlated with the change in the suppression of hepatic glucose production, the finding of which is consistent with the above mentioned previous study[38]. To date, molecular mechanisms underlying the EPA-mediated enhancement of hepatic insulin signaling remain to be elucidated. Therefore, in the present study, we tested the role of SeP in the EPA-mediated enhancement of hepatic insulin signaling. However, contrary to our hypothesis, the change in EPA levels was positively correlated with the change in SeP levels that causes hepatic insulin resistance. Hepatic insulin sensitivity is not solely regulated by SeP. We previously found that SeP impairs hepatic

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Table 4 | (a) Factors associated with serum EPA levels at endpoint

	EPA		Control		ALL	
	r	*P*	*r*	*P*	*r*	*P*
SeP at endpoint	0.443	0.199	0.220	0.569	0.135	0.580
FPG at endpoint	–0.015	0.967	0.259	0.500	0.041	0.867
HbA1c at endpoint	–0.068	0.851	0.077	0.844	0.048	0.845
Glucose infusion rate at endpoint	0.434	0.211	–0.297	0.438	–0.232	0.339
%HGP at endpoint	0.512	0.159	0.010	0.981	0.378	0.135
HGP * FPI at endpoint	0.080	0.865	–0.286	0.534	–0.160	0.585
Rd at endpoint	–0.168	0.666	–0.210	0.618	–0.366	0.149
%FFA at endpoint	0.251	0.484	0.293	0.444	0.280	0.246
(b) Factors associated with serum SeP levels at endpoint						
FPG at endpoint	–0.018	0.960	0.833	0.005	0.483	0.036
HbA1c at endpoint	0.043	0.907	0.427	0.252	0.225	0.354
Glucose infusion rate at endpoint	0.030	0.933	0.109	0.781	0.179	0.464
%HGP at endpoint	0.067	0.864	–0.619	0.102	–0.225	0.385
HGP * FPI at endpoint	–0.321	0.482	–0.179	0.702	–0.183	0.532
Rd at endpoint	0.268	0.486	0.452	0.260	0.347	0.172
%FFA at endpoint	0.006	0.486	0.150	0.700	0.138	0.574
(c) Factors associated with serum selenium levels at endpoint						
EPA at endpoint	0.588	0.074	0.711	0.032	0.391	0.098
SeP at endpoint	0.312	0.060	0.787	0.012	0.775	0.000
FPG at endpoint	0.055	0.881	0.812	0.008	0.461	0.047
HbA1c at endpoint	0.140	0.700	0.185	0.634	0.094	0.703
Glucose infusion rate at endpoint	0.128	0.724	–0.067	0.864	0.121	0.623
%HGP at endpoint	0.586	0.097	–0.359	0.382	0.133	0.611
HGP * FPI at endpoint	–0.714	0.071	–0.180	0.699	–0.284	0.325
Rd at endpoint	–0.218	0.574	0.108	0.799	0.034	0.896
%FFA at endpoint	0.467	0.174	0.117	0.764	0.239	0.325

%FFA, suppression of FFA by insulin during a clamp study; %HGP, suppression of hepatic glucose production by insulin during a clamp study; FPG, Fasting plasma glucose; FPI, fasting plasma insulin concentration; HGP, hepatic glucose production; Rd, insulin-stimulated glucose disposal; SeP, Selenoprotein P.

insulin signaling by inactivating the energy sensor AMP-activated protein kinase (AMPK). AMPK negatively regulates the mTOR-S6 kinase axis that negatively regulates IRS-1 and insulin signaling[3]. We infer that EPA interferes with insulin signaling independently of the SeP actions. The candidate pathways involve oxidative stress and insulin signaling. We previously found that saturated fatty acid palmitate evokes insulin resistance via mitochondrial reactive oxygen species (ROS) generation in hepatocytes[40]. An animal model of nonalcoholic steatohepatitis showed that PUFAs (EPA + DHA) reduce ROS in the liver[41]. Therefore, EPA might enhance hepatic insulin sensitivity by suppressing ROS generation in the liver. This hypothesis should be tested in the future.

The EPA supplementation did not alter the skeletal muscle insulin sensitivity. However, the change in serum EPA levels tended to be negatively correlated with changes in skeletal muscle insulin sensitivity. These findings are unexpected considering the proposed beneficial effects of EPA on skeletal muscle insulin sensitivity in animals[16]. Previously unrecognized molecular mechanisms may underlie the EPA-induced skeletal muscle insulin resistance, which should be pursued in the future.

The net effects of EPA on major insulin-targeting organs, the liver, skeletal muscle, and adipose tissue, result in the previously recognized neutral effect of EPA on whole-body glucose homeostasis[19,35,36].

The strength of the present study is to evaluate organ-specific insulin sensitivity using the hyperinsulinemic-euglycemic clamp study combined with the stable isotope-labeled glucose infusion before and after the intervention. This evaluation method is not used widely because it requires complicated techniques and gas chromatography-mass spectrometry to determine the ratio of [6,6-$^2H_2$]glucose to glucose. To date, only one study[38], besides ours, has ever evaluated the effects of EPA on organ-specific insulin sensitivity in humans. Second, the present study was the first to test in humans our *in vitro* findings that EPA suppresses *SELENOP* promoter activity[11]. Unexpectedly, the change in serum EPA levels after EPA supplementation were positively correlated with the change in SeP levels, suggesting that EPA was associated with hepatic

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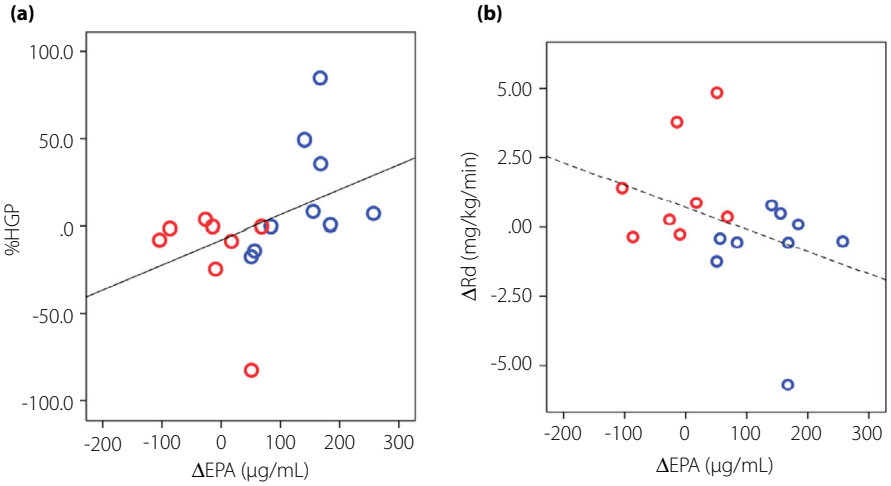

Figure 2 | (a) Correlation between the changes in serum EPA levels and changes in suppression of hepatic glucose production (HGP) by insulin during a clamp study (%HGP). Blue circle, EPA group (r = 0.517, P = 0.154); Red circle, control group (r = –0.310 P = 0.456). ALL (r = 0.590 P = 0.013). (b) Correlation between the changes in serum EPA levels and changes in insulin-stimulated glucose disposal (Rd). Blue circle, EPA group (r = 0.083, P = 0.831); red circle, control group (r = 0.262, P = 0.531). ALL (r = –0.422 P = 0.092).

SELENOP expression. Based on these findings in humans, we are further investigating the molecular mechanisms underlying the EPA-mediated regulation of *SELENOP* expression, which suggests that EPA exerts both positive and negative effects on the transcription factor responsible for the *SELENOP* expression (manuscript in submission). Third, the present study investigated the impact of EPA supplementation in Japanese subjects with relatively higher EPA levels than western subjects. Therefore, our observation here represents the add-on effects of EPA to higher serum EPA levels.

Our study has some limitations. First, this study has an exploratory design with a small number of human subjects, which may be insufficient to detect a statistically significant difference in the analyses and does not allow sub-analyses. Further large-scale clinical studies are needed to confirm the controversial findings of the present study. Second, this study was an open-label randomized trial without placebo treatment. Third, most of the participants were males (89%), which precludes the gender sub-analyses. In a large representative sample of adults from the National Health and Nutrition Examination Survey, the mean dietary EPA intakes and the plasma EPA concentration in men were higher and tended to be higher than those in women[42]. Therefore, the male-dominant cohort may be rather advantaged for the exploratory study. Fourth, the baseline EPA levels in Japanese subjects is likely to be greater than in other populations who have lower intakes of marine foods[43,44]. The range of blood EPA levels is varied both before and after the intervention in the present study and previous Japanese clinical trials[32,45,46], which might be attributable to the higher inter-individual variability in the marine fish intake and EPA absorption. Therefore, we calculated increments of serum EPA levels and analyzed their impacts on SeP levels and organ-specific insulin sensitivity.

Collectively, the present study clarified that the change in serum EPA levels is positively correlated with the change in serum SeP levels, hepatic insulin sensitivity, and negatively skeletal muscle insulin sensitivity in humans with type 2 diabetes. EPA supplementation was correlated with adipose tissue insulin sensitivity. EPA-induced enhancement of hepatic insulin sensitivity might be associated with a mechanism independent of serum SeP levels. These net effects on major insulin-targeting organs result in the previously recognized neutral effect of EPA on whole-body glucose homeostasis.

DISCLOSURE

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Approval of the research protocol: Innovative Clinical Research Center, Kanazawa University, approval number; 2014-017 (5934), Sep 5, 2014.

Informed consent: All participants provided written informed consent.

Approval date of registry and the registration no. of the study/-trial: UMIN 000015673.

Animal studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | The baseline characteristics of the study participants.

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