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Increased Uncoupling Protein2 mRNA in White Adipose Tissue, and Decrease in Leptin, Visceral Fat, Blood Glucose, and Cholesterol in KK-A^y Mice Fed with Eicosapentaenoic and Docosahexaenoic Acids in Addition to Linolenic Acid

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The effects of n-3 polyunsaturated fatty acids (n-3PUFA) on obesity and diabetes were examined using KK-A^y mice fed with perilla oil (P), soybean oil (S), or lard (L), and those containing 30% fish oil (PF, SF, or LF), containing eicosapentaenoic acid (EPA = 9.9%) and docosahexaenoic acid (DHA = 18.0%). Perilla oil contained the largest proportion of linolenic acid (LNA = 61.9%). Computerized tomography (CT) scans showed narrower areas of visceral fat in the abdominal cross sections of groups given fish oil (PF, SF, and LF) and lower leptin levels ($p < 0.05$-$p < 0.001$) compared with controls (P, S, and L), without significant changes in energy intake and body weight. The highest plasma n-3PUFA content (21.31 ± 0.35%) was attained with PF. This group contained 2.6-fold more plasma DHA ($p < 0.001$), and expressed 2.7-fold more UCP2 mRNA in white adipose tissue ($p < 0.01$) than in the P group. The epididymal fat pad ($p < 0.05$) weighed less, and levels of blood glucose ($p < 0.05$) and total cholesterol ($p < 0.01$) were reduced in PF compared with P.

The association between dietary fat and obesity/noninsulin-dependent diabetes mellitus (NIDDM) is controversial (1). Levels of fish-derived eicosapentaenoic acid (EPA) in the plasma of both Eskimos (2) and Japanese (3) are high and the incidence of obesity-induced disease is low in their populations. Fish oil n-3PUFA was shown to selectively limit obesity (4). To elucidate the effects of n-3PUFA, genetic, anatomical, endocrinological and mitochondrial factors should be examined.

Genetically, obesity/NIDDM is attributed to several mutations and the quantitative trait loci (QTL) responsible for human NIDDM (5) and its models, such as the KK-A^y mouse (6), have been analyzed. Linkage analysis of the KK-A^y mouse revealed a QTL on chromosome 4 (4-46.7) for the leptin receptor (5), on chromosome 6 (6-10.5) for leptin (5) and) other QTL for modification in combination (6). A mutation called yellow agouti (A^y) is related to the melanocortin receptor (7) that may prevent obesity. We used KK-A^y mice to define the genetic background of NIDDM.

Anatomically, computer tomography (CT) of patients has revealed that visceral-, but not subcutaneous-, fat accumulation is correlated with NIDDM (8). Mesenteric lipid accretion in white adipose tissue (WAT) induces high free fatty acid (FFA) in the portal circulation and causes metabolic disturbances (8). Thus, we estimated visceral fat of the KK-A^y mice under various conditions by CT.

Endocrinologicaly, leptin is a hormone secreted by adipocytes (9) that regulates the energy balance (10). A loss of WAT leads to a decrease in leptin, which increases neuropeptide Y and stimulates both food intake and energy conservation (10). Conversely, WAT accumulation leads to an increase in leptin levels and enhances energy expenditure via melanocortin receptor (7). Measurement of leptin was therefore essential in this NIDDM study.

Mitochondrial ATP synthase is driven by the electrochemical potential of protons ($\Delta\mu H^+$) (11, 12). Uncoupling proteins (UCP) regulate ATP production by

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transporting protons via FFA. The anionic form of FFA can permeate via UCPs in the mitochondrial membrane (13-15). Protonated FFA is lipophilic and can permeate the mitochondrial membrane (13-15). Thus, proton leakage caused by the cycling of protonated and anionic forms of FFA through mitochondrial membranes collapse the $\Delta\mu H^+$ (13, 15), thereby dissipating energy as heat. The three known uncoupling proteins in mitochondria are UCP1, UCP2 and UCP3 (16, 17). UCP1 is specific for brown adipose tissue (BAT), which is responsible for nonshivering thermogenesis in newborns. UCP2 (16) is ubiquitous and found in WAT, whereas UCP3 (17) is specific to skeletal muscle. Thus, mRNA of UCPs has been measured in this study.

Interactions among leptin, UCPs and FFA have been reported. Leptin overexpression increases UCP2 mRNA levels in adipocytes by over 10 fold, and mRNAs encoding the enzymes of mitochondrial oxidation by 2- to 3-fold in normal rats *in vivo* (18). All *in vivo* changes occurred in normal islets cultured with recombinant leptin, indicating a direct extraneural effect (18). Such changes were not identified in rats with defective leptin/receptor (18). In fasted rats, a 3-fold increase in the serum FFA concentration caused a significant up-regulation of mRNAs for UCP2 and UCP3 in fast-twitch fibers (19). Polyunsaturated fatty acids (PUFA) enhance uncoupling (20). Rats fed with docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) show increased cytochrome c oxidase activity in brown fat tissue (BAT) and diet induced thermogenesis (21). UCP1 levels and nonshivering thermogenesis are increased in the BAT in mice given n-3PUFA (22). However, thermogenesis was studied before the discovery of the effects of UCP2 and leptin, and BAT/UCP1 plays a very small role in adult humans. Thus, effects of n-3PUFA on leptin and other UCPs in WAT have not been reported.

Here, we show that both visceral WAT and leptin level decrease in mice fed with fish oil rich in both EPA and DHA, without a significant change in energy intake. Decreased WAT weight, blood triglyceride, blood glucose, and total cholesterol levels were explained by increased UCP2 mRNA level in the PS group.

MATERIALS AND METHODS

Lipids. Perilla oil (P; C18:1 = 12.4%, C18:2 = 14.5%, C18:3 = 61.9%), soybean oil (S; C18:1 = 20.2%, C18:2 = 54.0%, C18:3 = 7.7%), lard (L; C16:0 = 23.7%, C18:0 = 14.0%, C18:1 = 40.6%, C18:2 = 9.0%, C18:3 = 0.5%), and DHA-EPA-fish oil (F; C16:0 = 16.2%, C18:1 = 17.7%, EPA = 9.9%, DHA = 18.0%) were obtained from Nippon Yushi, Tokyo. The three control lipids (P, S and L) were free of EPA and DHA. Fish oil (F) when added, constituted 30% of P, S or L, and the lipid mixtures were called PF, SF and LF, respectively. Lipids also contained α-tocopherol (0.2% for P, S and L, and 0.4% for F).

Animal treatment. Four-week-old male KK-A^y/TaJcl mice purchased from Saitama Experimental Animals, Saitama), were fed with the synthetic diet AIN-76 (23), containing (g/kg): sucrose 300, casein 200, D-glucose 50, DL-methionine 3, α-corn starch 100, β-corn starch 150, cellulose 50, lipids 100 (P, S, L, PF, SF and LF, as described above), salt mixture 35 (AIN 76) (23), vitamin mixture 10 (AIN 76) (23) and choline bitartrate 2. The animals were randomly divided into 6 groups (n = 6) depending on the administered lipids (P, S, L, FP, FS, FL). Room temperature and humidity were maintained at 25 ± 1°C, and 65 ± 5%, respectively. The diet (5 g/mouse for the first week, and 6 g/mouse after second week) was prepared daily to prevent lipid peroxidation, and given to the mice at 17.00h every day. Twelve weeks later, the mice were killed under pentobarbital anesthesia (4 mg/kg body weight) and EDTA was added to the plasma. Organs were removed and immediately frozen at −40°C.

Chemical analyses. Blood glucose, triglycerides (TG), total cholesterol (TC) and leptin levels were measured using the Glucose B Test (glucose oxidase method, Wako, Osaka), Determiner TG-S555 (Kyowa Medicus, Tokyo), Determiner TC555 (Kyowa) and a Mouse Leptin Enzyme Assay Kit (enzyme-immuno assay, IBL, Tokyo), respectively.

Computerized tomography. The mice at 11 weeks were fixed in a chamber, and the mid-liver level was scanned using a Siemens Asahi Medic Somatome plus at Jichi Medical School (8).

Gas chromatographic analysis of lipids. Extraction with chloroform-methanol (2:1), methylation with HCl-methanol and chromatographic analysis of fatty acyl groups using Hitachi G-300 proceeded essentially as reported (24). The internal standard was the methylester of C23:0, and the fused silica capillary column DB225 (J & W Scientific Co. Inc.) was 0.25 mm × 30 m.

Quantitative RT-PCR. Total RNAs from WAT of the mice were extracted with ISOGEN and analyzed using primers for UCPs as reported previously (25). The mRNA level of UCPs was rapidly determined using TaqMan PCR (26). Relative mRNA levels were determined as an average from 6 replicates/mouse × 6 and normalized to the 18S rRNA level in each sample.

RESULTS AND DISCUSSION

Anatomical results. Here, we show that lipid accretion in visceral WAT in obese/diabetic mice was limited by feeding mice with fish oil rich in n-3PUFA, (EPA and DHA), without a significant change in energy intake. The food intake and anatomical values of the mice are summarized in Table 1. The weight of the epididymal fat pad significantly decreased in PF and SF groups (Table 1). The decrease was similarly significant ($p < 0.01$) in the parallel experiments of PF and SF under gas loading (CO_2 1%, O_2 10%, N_2 89%) (unpublished results). Visceral fat levels were estimated by CT (Fig. 1) as performed in obese patients (7). Though CT scans of visceral fat in rodents have not been reported, the retroperitoneal WAT of fish oil groups (PF, SF and LF) decreased in this , and also in the gas loading-, experiments, in accord with other studies of rats fed with fish oil (27), as well as with EPA and DHA (21, 28). The retroperitoneal WAT of the DHA group, compared to olive oil group, contained 40-75% lower levels of fatty acid synthase, lipoprotein lipase CCAAT/enhancer binding protein α and hormone-sensitive lipase (28). In contrast, levels of these proteins in subcutaneous WAT did not decrease (28).

Fatty acid composition. The fatty acid composition of mouse plasma is summarized in Table 2. The fatty acid compositions in the liver were similar to those in

TABLE 1

Effect of Dietary Fats on Daily Food Intake, Body Weight, Food Efficiency Ratio, and Organ Weight of Mice

	Experimental without fish oils groups					
	P[3]	PF	S	SF	L	LF
Food intake (g/day)	5.31 ± 0.47	5.07 ± 0.47	5.39 ± 0.42	5.06 ± 0.29	5.15 ± 0.29	5.11 ± 0.37
Body weight (g/day)	39.35 ± 0.99	40.3 ± 0.99	39.2 ± 1.29	39.2 ± 1.44	38.51 ± 1.87	40.3 ± 2.34
F.E.R.[1]	0.074 ± 0.012	0.085 ± 0.014	0.087 ± 0.016	0.078 ± 0.013	0.081 ± 0.014	0.089 ± 0.016
Liver (g)	1.73 ± 0.21	1.65 ± 0.11	1.61 ± 0.15	1.53 ± 0.24	1.92 ± 0.25	1.98 ± 0.15
E.F.P. (g)[2]	1.63 ± 0.14	1.38 ± 0.16*	1.72 ± 0.26	1.47 ± 0.14*	1.63 ± 0.19	1.51 ± 0.13

[1] F.E.R.: food efficiency ratio = body weight gain for one week (g)/food intake for one week (g).
[2] E.F.P.: epididymal fat pad.
[3] P, perilla oil; S, soy bean oil; L, Lard; F, fish oil.
Fish oil group vs. Non-fish oil group. * $P < 0.05$.

the plasma, and the total n-3PUFA and n-6PUFA levels were 18.92 ± 0.31, and 28.53 ± 0.64, respectively. Levels of oleic and arachidonic acids were consistently suppressed in the fish oil groups (Table 2). The fatty acid composition of adipocytes in rats fed with lard and fish oil has been described in detail (29).

Leptin level. Leptin levels of the fish oil groups (PF, SF and LF) were significantly lower than those of control groups (P, S and L) (Table 3). These lower leptin level in fish oil groups may reflect the size of the WAT, since leptin is mainly produced in adipocyte (10). In addition to the size decrease, lowered levels of leptin mRNA were identified in the WAT of rats fed with DHA (29).

Blood glucose, plasma TG and TC. Table 4 shows that the KK-A^y mice developed hyperglycemia, hyperlipidemia and hypercholesterolemia during weeks 4th and 8th. After 8th weeks, levels of blood glucose and TC were significantly lowered by fish oil, with the exception of blood glucose in the LF group and TC in LF. TG levels are reduced by DHA in KK-A^y mice (30), but under our conditions, a reduction was significantly reproduced only in SF (Table 4).

UCP mRNA level. An energy consuming effect of n-3PUFA on BAT has been reported (21). Adding phospholipids containing DHA to mitochondria both *in vitro* and *in vivo* reduces the respiratory control index and increases the proton permeability of mitochondrial

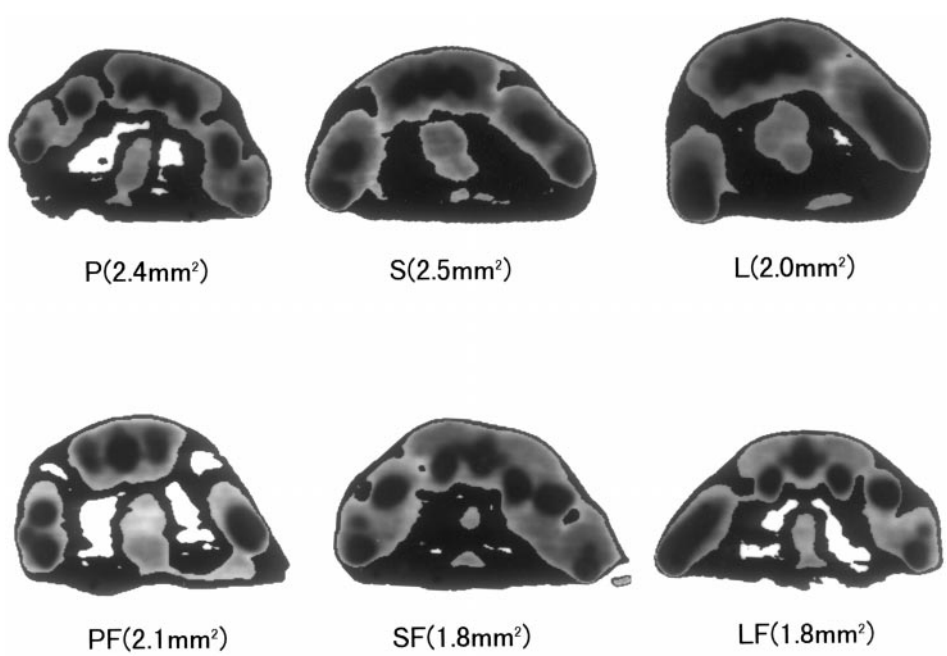

FIG. 1. Computer tomography scans of visceral fat in mice fed with various diets. Mice were anesthetized with pentobarbital (0.4 mg/100 g body weight). X-ray computer tomography scans of each of 6 mice were fixed exactly at the same level. Cross section of the fat area (black) was measured (area in parentheses).

TABLE 2

Effect of Fish[1] Oil on Fatty Acid Composition of Plasma in Mice (n = 6) (Mean ± SEM (%))

FAs		P	PF	S	SF	L	LF
SAFA	C14:0	0.21 ± 0.09	0.3 ± 0.22	0.05 ± 0.01	0.31 ± 0.04	0.3 ± 0.04	0.47 ± 0.08
	C16:0	17.79 ± 0.31	19.71 ± 0.81	17.47 ± 1.03**	21.1 ± 2.12	17.03 ± 0.35***	21.75 ± 1.88
	C18:0	7.75 ± 1.39**	10.46 ± 1.79	8.11 ± 0.98	7.89 ± 0.59	6.54 ± 0.37	6.03 ± 1.89
	C23:0	0.08 ± 0.01	0.23 ± 0.05	0.16 ± 0.02	0.19 ± 0.02	0.14 ± 0.04	0.23 ± 0.06
	Total	27.54 ± 0.16	33.29 ± 0.18	27.22 ± 0.21	31.29 ± 0.21	25.57 ± 0.11	30.43 ± 0.09
MUFA (n-7)	C16:1	2.28 ± 0.38	1.23 ± 0.18	1.22 ± 0.23	1.69 ± 0.44	2.31 ± 0.32	2.94 ± 0.57
	C18:1	2.35 ± 0.32	1.24 ± 0.09	8.21 ± 0.91***	1.43 ± 0.33	2.37 ± 0.41	2.51 ± 0.83
	Total	5.43 ± 0.36	3.28 ± 0.17	9.62 ± 0.86	3.42 ± 0.38	5.48 ± 0.39	5.67 ± 0.76
(n-9)	C18:1	23.55 ± 2.17**	16.11 ± 0.92	15.74 ± 0.92***	20.19 ± 1.91	26.4 ± 1.56*	30.03 ± 1.15
	Total	24.51 ± 1.78	16.91 ± 0.56	16.07 ± 0.68	21.45 ± 1.84	27.32 ± 1.34	30.45 ± 1.02
PUFA (n-6)	C18:2	18.93 ± 1.1**	21.87 ± 1.88	30.25 ± 0.67	28.74 ± 1.14	28.51 ± 1.31***	13.88 ± 2.27
	C20:4	1.47 ± 0.09***	3.34 ± 0.18	8.05 ± 0.45***	3.15 ± 0.44	4.93 ± 0.22**	3.54 ± 0.52
	Total	21.72 ± 0.16	25.33 ± 0.17	40.02 ± 0.23	32.97 ± 0.29	35.29 ± 0.27	19.27 ± 0.15
(n-3)	C18:3	12.67 ± 1.04	10.76 ± 1.46	2.35 ± 0.15	2.77 ± 0.41	1.73 ± 0.14***	0.64 ± 0.06
	C20:5	4.78 ± 0.58	3.62 ± 0.53	0.67 ± 0.02**	2.21 ± 0.44	0.86 ± 0.06***	5.12 ± 0.86
	C22:5	1.2 ± 0.13	0.78 ± 0.05	0.75 ± 0.44	0.62 ± 0.08	0.42 ± 0.03	1.01 ± 0.13
	C22:6	2.44 ± 0.37***	6.55 ± 0.71	3.46 ± 0.29*	5.1 ± 0.98	3.37 ± 0.15***	8.04 ± 0.79
	Total	21.06 ± 0.37	21.31 ± 0.35	7.25 ± 0.25	11.07 ± 0.42	6.37 ± 0.08	14.65 ± 0.25
	n-6/n-3	1.04 ± 0.09	1.19 ± 0.1	5.52 ± 0.1	2.98 ± 0.13	5.58 ± 0.14	1.32 ± 0.03

[1] FISH: EPA and DHA-rich fish oil.
Fish oil group vs. Non-fish oil group. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

membranes (20). This uncoupling is partly explained by the increase (270.2%, $p < 0.01$) of UCP2-mRNA in WAT of mice fed with PF compared with that fed with P (100%) (Fig. 2). Levels of mRNA for UCP1 (187.2%), UCP2 (226.8%) and UCP3 (422.4%) tended to be higher in PF and SF, than in control groups (P and F, taken as 100%) (Fig. 2). In parallel experiments under gas loading as stated above, levels of mRNAs for UCP1, UCP2 and UCP3 were also high in fish oil groups. Although UCP1 and UCP3 are ectopic in WAT, UCP1 was ectopically expressed in both WAT and skeletal muscle in KK mice during chronic administration of the β3 adrenergic agonist CL-316,243 (31). Ectopic UCP3 mRNA in rat WAT is also expressed under the influence of thyroid hormone, a β3 adrenergic agonist, dexamethasone and leptin (32). The effect of the β3 adrenergic agonist on UCP1 transcription is mediated by cyclic AMP-regulatory element (33).

The regulation of gene expression by n-3PUFA in adipocytes has also been reported (34). For example,

TABLE 3

Effect of Fish Oil Loading Plus Various Oils on Plasma Leptin in Mice (Mean ± SEM (ng/ml))

P	S	L
8.92 ± 0.13*[,1,2]	7.84 ± 1.15*	13.14 ± 0.71***
PF	SF	LF
8.01 ± 1.08	7.54 ± 1.48	10.84 ± 0.92

[1] Fish oil group:Non-fish oil group.
* $P < 0.05$. *** $P < 0.001$.
[2] Significantly different at $\alpha = 0.05$ by Student's T test.

TABLE 4

Effect of Fish Oil on Lipid and Glucose Contents of Plasma in Mice (Mean ± SEM (mg/dl))

		After 4 weeks	After 8 weeks
TG	P	119.1 ± 6.81	174.24 ± 7.36*
	S	163.53 ± 4.28	225.27 ± 17.14**
	L	113.49 ± 5.98	220.56 ± 12.54**
	PF	115.42 ± 10.19	209.57 ± 12.81
	SF	133.69 ± 14.09	176.82 ± 8.75
	LF	131.77 ± 14.42	251.37 ± 17.99
TC	P	91.9 ± 3.88	160.67 ± 12.71**
	S	67.14 ± 3.24	155.18 ± 8.53*
	L	119.52 ± 3.21	149.67 ± 13.28
	PF	80.95 ± 3.36	124.27 ± 4.59
	SF	85.47 ± 4.29	142.88 ± 2.79
	LF	95 ± 2.10	149.51 ± 6.42
Glucose	P	202.53 ± 6.61	223.59 ± 19.81*
	S	226.09 ± 14.88	274.12 ± 18.26**
	L	204.14 ± 5.75	276.41 ± 19.56***
	PF	208.11 ± 19.37	180.85 ± 19.97
	SF	219.67 ± 12.54	162.19 ± 12.70
	LF	303.98 ± 16.40	358.2 ± 28.22

Fish oil group vs. Non-fish oil group. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

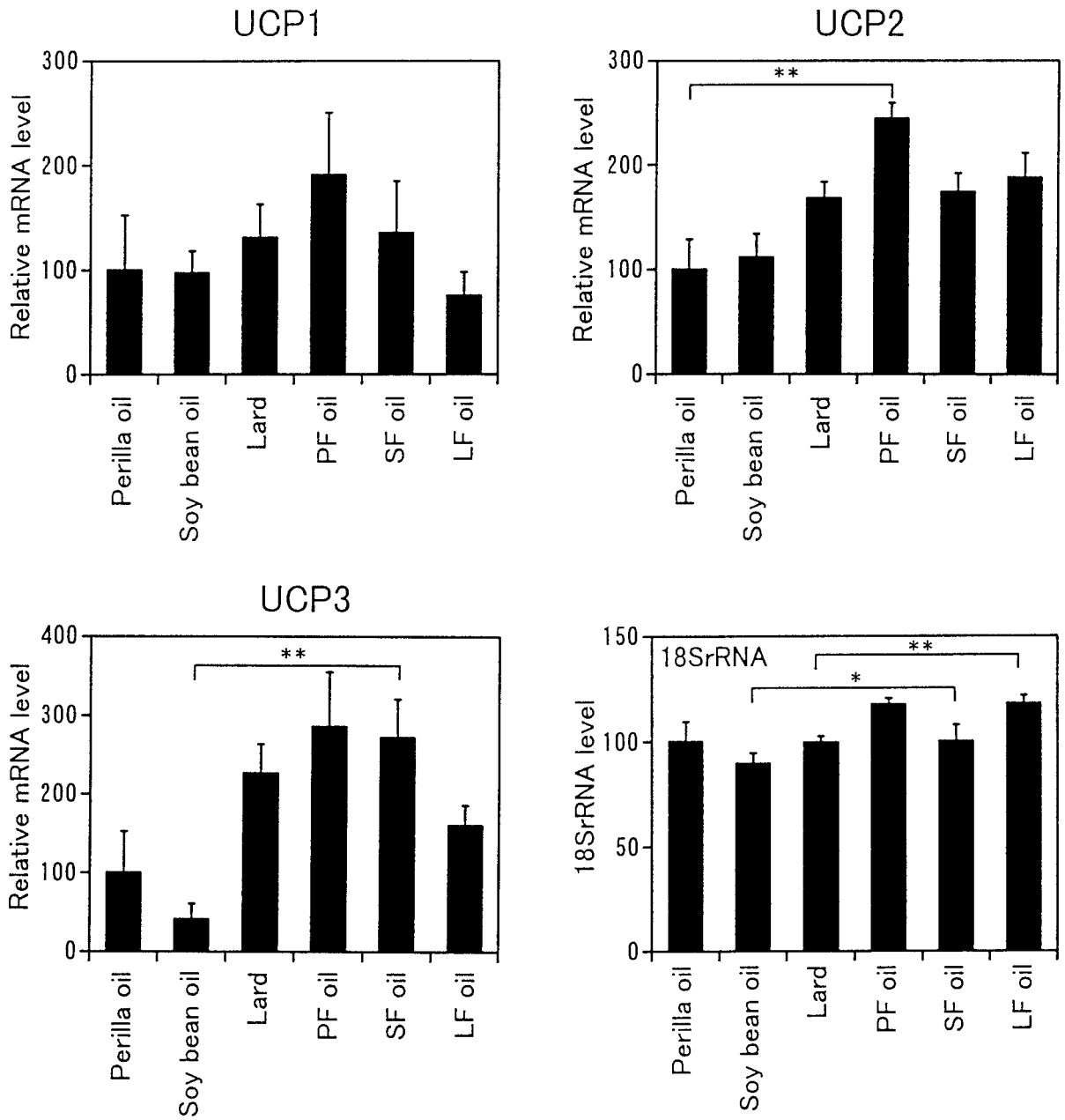

FIG. 2. Effects of lipid composition on mRNA levels of uncoupling proteins (UCP1, 2, and 3) in white adipose tissues of KK-A^y mice. Levels of mRNA were determined by real time quantitative RT-PCR as described in Materials and Methods. Relative mRNA levels (means ± S.E.) are represented in perilla oil group as 100% after an average from 6 replicates/mouse × 6 was normalized to 18S rRNA level in each sample. Statistical significance vs. control: * $P < 0.05$, ** $P < 0.01$, by Student's t-test, non-fish group vs. fish group.

Table 2 shows that oleic acid content is lowered by n-3PUFA, because the expression of stearoyl-CoA desaturase was inhibited by n-3PUFA (see ref. 35). Mitochondrial and peroxisomal fatty acid β-oxidation is stimulated by n-3PUFA (36). Rats given n-3PUFA (LNA, EPA and DHA) showed much lower levels of activities of enzymes for fatty acid synthesis, including fatty acid synthase, glucose-6-phosphatase and malic enzymes, than in those fed with LNA (37). The regulation mechanism of expression for UCP and leptin are still not clear, but peroxisome proliferator-activated receptor (PPAR) λ causes BAT to assume a WAT-like phenotype with increased UCP2 level (38). This causes a significant reduction in body weight (15%) and in WAT (58%). It is of note that UCP2 polymorphism (Ala to Val substitution) and sleeping metabolic rate are related in the NIDDM-prone Pima Indians ($p < 0.007$) (39).

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