Original Article

Dietary flaxseed oil suppresses hyperglycemia and hyperinsulinemia through increasing in α-linolenic acid content in the muscle

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Types of fats and oils affect the onset of lifestyle diseases. In this study, we investigated the relationship between the postprandial hyperglycemia and fatty acids content in the skeletal muscle of C57BL/6 mice given 20% lard, palm oil, corn oil, safflower oil, and flaxseed oil for 16 weeks. Lard increased plasma glucose and insulin levels at the end of feeding period, whereas flaxseed oil did not. It was noteworthy that there is a positive correlation between palmitic acid content in the muscle and postprandial hyperglycemia, and a negative correlation between α-linolenic acid content and hyperglycemia. Alternatively, mice were given 30% lard for 16 weeks. When lard was partially substituted with flaxseed oil (10–50% substitution), flaxseed oil dose-dependently prevented lard-induced hyperglycemia and hyperinsulinemia. In conclusion, flaxseed oil prevents the adverse effects of lard through increasing in α-linolenic acid content in the muscle.

***Key Words*: hyperglycemia, hyperinsulinemia, flaxseed oil, muscle fatty acids**

Fats and oils are essential nutrients for human as the energy sources and physiological substances. On the other hand, excess intake of fats increases the onset of lifestyle diseases, such as obesity, hyperglycemia, and type 2 diabetes. Recently, it has been reported that the types of fats and oils, in addition to their amounts, are important for preventing and ameliorating lifestyle diseases. For example, lard is known to induce abnormal glucose tolerance, whereas plant-derived oils reduce fasting insulin.[1]

Glucose uptake into muscle cells plays a central role in maintaining blood glucose. Upon binding of insulin to its receptor, glucose transporter type 4 (GLUT4) translocate to the plasma membrane and a huge amount of glucose is taken up. Inhibition of this translocation leads to insulin resistance, resulting in hyperglycemia. It is reported that saturated fatty acids (SFAs) inhibit the insulin signal by impairing phosphorylation of insulin receptor substrate (IRS) and lead to insulin resistance.[2] On the other hand, unsaturated fatty acids rich in plant-derived oils do not induce insulin resistance rather prevent it.[3] From these reports, fats and oils have different functions for glucose uptake depending on their fatty acids contents and composition in the muscle. When standard diet is taken, phospholipid in muscle tissue is rich in SFA.[4] High-fat diet causes heterotopic fat accumulation including muscle fat. Fatty acids content and composition in muscle will affect insulin signal, but relationship between content of fatty acids in muscle and glucose tolerance has not been reported.

Although many researches have been investigated to clarify the functions of fats and oils and the results have been accumulated to date, it is not fully understood to establish the evidence for anti-hyperglycemic function among various types of fats and oils. Moreover, it is unclear that influence of fatty acids content and composition of muscle tissue on hyperglycemia and hyperinsulinemia. Therefore, in this study, we investigated the relationship between the anti-hyperglycemic effect and fatty acids content and composition in the muscle using five types of fat and oils: lard and palm oil (rich in palmitic acid), corn oil (linoleic acid), oleate-rich species safflower oil (oleic acid), and flaxseed oil (α-linolenic acid). We also investigated that lard was partially substituted with flaxseed oil in high-fat diet and given to mice to estimate the effect of substituted flaxseed oil on anti-hyperglycemia and anti-hyperinsulinemia.

Materials and Methods

Materials. Fat and oils were kindly provided by J-Oil Mills inc. (Tokyo, Japan). LabAssay Glucose, Cholesterol, Triglyceride, NEFA, and LBIS Mouse Insulin ELISA kits were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). All other reagents used were of the highest grade available from commercial sources.

Animal treatments. All animal experiments were approved by the Institutional Animal Care and Use Committee of Kobe University (Permission #19-5-32) and carried out according to the guidelines for animal experiments of Kobe University. Following two animal experiments were performed using 5-weeks old male C57BL/6 mice (Japan SLC, Shizuoka, Japan). The mice were maintained at 23 ± 2°C under an automatic lighting schedule (9:00 a.m.–9:00 p.m.), allowed free access to tap water and diets for each experiment, and were acclimatized for seven days prior to each experiment.

Experiment 1. Thirty-seven mice were randomly divided into six groups of six or seven each and given a standard diet containing 5% corn oil (SD) and a high-fat diet (HF) containing 20% lard (HF-Lard), palm oil (HF-Palm), corn oil (HF-Corn), oleate-rich safflower oil (HF-Saff), and flaxseed oil (HF-Flax) for 16 weeks.

Experiment 2. Alternative thirty-one mice were randomly divided into five groups of six or seven each. Control group was given standard diet containing 5% corn oil (SD) for 16 weeks. Second group was given the high-fat diets containing 30% lard (HF-Lard). In the remaining groups, lard was partially substituted for flaxseed oil (10, 20, and 50% substitution; HF-0.1Flax, HF-0.2Flax, and HF-0.5Flax, respectively).

In both experiments, mice were fasted for 14 h and sacrificed by collecting blood from cardiac puncture using a heparinized syringe under anesthesia with medetomidine hydrochloride

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Table 1. Fatty acid composition of each fat and oil

%	Corn	Lard	Palm	Safflower	Flaxseed
12:0	0.0	0.1	0.2	0.0	0.0
14:0	0.0	1.5	0.9	0.1	0.0
16:0	10.9	24.5	44.7	4.5	4.9
16:1	0.0	2.6	0.2	0.1	0.0
18:0	1.7	12.9	4.1	1.7	3.1
18:1 (n-9)	29.0	45.5	39.1	78.5	20.4
18:2 (n-6)	55.6	7.7	9.4	13.7	16.7
18:3 (n-3)	0.8	0.8	0.3	0.1	49.1
Others	2.0	4.5	1.3	1.3	0.0

Table 2. Ingredients of diets in Experiment 1 (A) and Experiment 2 (B)

(A)

Ingredients	Standard diet	High-fat diet (20% fat or oil)				
		Lard	Palm	Corn	Saff	Flax
		(g/100 g)				
Casein	20.0	20.0	20.0	20.0	20.0	20.0
L-Cystine	0.18	0.18	0.18	0.18	0.18	0.18
Cornstarch	30.07	15.07	15.07	15.07	15.07	15.07
Sucrose	35.0	35.0	35.0	35.0	35.0	35.0
Cellulose powder	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin mix	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix	3.5	3.5	3.5	3.5	3.5	3.5
Choline bitartrate	0.25	0.25	0.25	0.25	0.25	0.25
Corn oil	5.0	—	—	20.0	—	—
Lard	—	20.0	—	—	—	—
Palm oil	—	—	20.0	—	—	—
Safflower oil	—	—	—	—	20.0	—
Flaxseed oil	—	—	—	—	—	20.0
Total energy (kcal/100 g)	406.0	478.0	478.4	477.8	479.4	480.4

(B)

Ingredients	Standard diet	High-fat diet (30% fat or oil)			
		Lard	0.1Flax	0.2Flax	0.5Flax
		(g/100 g)			
Casein	20.0	20.0	20.0	20.0	20.0
L-Cystine	0.18	0.18	0.18	0.18	0.18
Cornstarch	30.07	5.07	5.07	5.07	5.07
Sucrose	35.0	35.0	35.0	35.0	35.0
Cellulose powder	5.0	5.0	5.0	5.0	5.0
Vitamin mix	1.0	1.0	1.0	1.0	1.0
Mineral mix	3.5	3.5	3.5	3.5	3.5
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
Corn oil	5.0	—	—	—	—
Lard	—	30.0	27.0	24.0	15.0
Flaxseed oil	—	—	3.0	6.0	15.0
Total energy (kcal/100 g)	406.0	526.5	526.9	527.2	528.3

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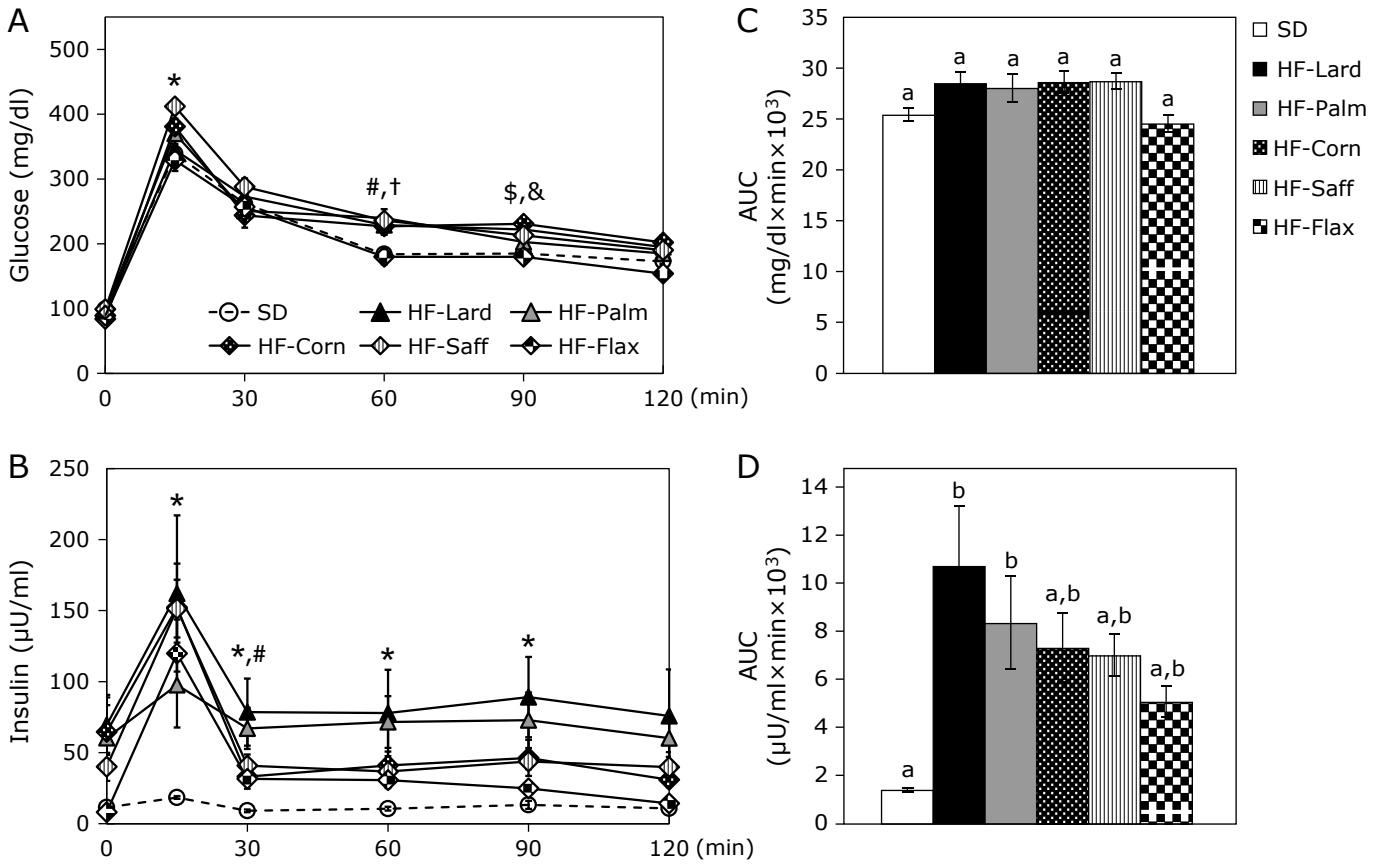

Fig. 1. Plasma glucose and insulin level after conduct of OGTT at 15 Week in Experiment 1. Time-course and area under curve of plasma glucose (A and C) and insulin (B and D). Data shown represent mean ± SE (n = 7 animals in SD and n = 6 in each HF groups). The asterisks are represented significant difference between *HF-Saff vs HF-Flax, #SD vs HF-Palm, †HF-Palm vs HF-Flax, $SD vs HF-Corn, &HF-Corn vs HF-Flax (A), and *SD vs HF-Lard, #SD vs HF-Palm (B). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test (p<0.05). AUC, area under the curve; HF-Corn, high-fat diet containing 20% corn oil; HF-Flax, high-fat diet containing 20% flaxseed oil; HF-Lard, high-fat diet containing 20% lard; HF-Palm, high-fat diet containing 20% palm oil; HF-Saff, high-fat diet containing 20% safflower oil; OGTT, oral glucose test; SD, standard diet.

(Nippon Zenyaku Kogyo Co., Ltd. Koriyama, Japan) and pentobarbital sodium salt (Tokyo Chemical Industry Co., Ltd. Tokyo, Japan). Plasma was obtained by centrifugation of blood at 3,000 × g for 10 min at 4°C. The white adipose tissue (WAT) and skeletal muscle were also collected, washed with 1.15% (w/v) KCl, weighed, and immediately frozen using liquid nitrogen. Obtained plasma and tissues were kept at −80°C.

Measurements of plasma parameters. The levels of glucose, insulin, cholesterol, triglyceride, and free fatty acids in plasma were measured using the corresponding commercial assay kit. HOMA-IR was calculated by a following formula.

HOMA-IR = glucose (mg/dl) × insulin (μU/ml)/405

Oral glucose tolerance test (OGTT). To elucidate the effect of different fats and oils on glucose tolerance, OGTT was performed 15 weeks after the feeding in both experiments. Mice were orally administered glucose at 2.0 g/kg body weight after 14 h-fasting. Blood was collected from tail vein at 0, 15, 30, 60, 90, and 120 min after glucose loading. Plasma was prepared and used for measurement of the glucose and insulin levels. For evaluation of OGTT, area under the curve (AUC) was calculated using trapezoidal method between 0–120 min.

Extraction of lipids from the muscle. Skeletal muscle (100 mg) was homogenized with 2.0 ml of 1.15% (w/v) KCl by Polytron homogenizer and filled up to 4.0 ml with KCl. Aliquot of 1.0 ml of homogenate was transferred to a glass tube, added 1.0 ml of chloroform containing methyl tricosanoate as an internal standard, and mixed vigorously to extract lipids. After adding 1.0 ml of phosphate-buffered saline, the mixture was centrifuged at 1,000 × g for 10 min at 4°C, and chloroform layer was collected. Water layer and residue were washed with 1.0 ml chloroform and centrifuged under the same conditions. Obtained chloroform layers were merged and dried up by nitrogen gas stream and used for analysis of fatty acid composition and content.

Analysis of fatty acid composition and content. Fatty acid composition and content of skeletal muscle was analyzed as follows: To hydrolyze and methyl esterified, the extracted lipids were dissolved in 2.0 ml n-hexane and 3.0 ml of 2 M KOH/methanol solution. Then, the mixture was washed with 2.0 ml saturated NaCl solution by centrifugation this solution at 1,000 × g for 2 min. Methyl esterified fatty acids were obtained under the same centrifugation conditions and dried up by nitrogen gas stream, Methyl esterified fatty acids were dissolved n-hexane and applied to a gas chromatograph (GC; GC2010, SHIMADZU Co., Kyoto, Japan) equipped with a DB23capillary column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies, Santa Clara CA). GC analysis was performed following conditions: Temperature was programmed to increase from 80 to 240°C; injector and detector temperatures were 240°C and 250°C, respectively; the split ratio is 1:50; and He was used as carrier gas. Individual fatty acids were identified with standard fatty acids methyl ester (Supelco 37

Component FAME Mix, Merck, Darmstadt, Germany). Less than 0.1% of the composition was not included because of below the limit of determination.

Statistical analysis. Data are expressed as the means ± SE (n = 6 or 7). The statistical significance of experimental observations was determined by a Tukey–Kramer multiple comparison test and correlative relationship were calculated by Pearson product-moment correlation coefficient using JMP statistical software ver. 11.2.0 (SAS Institute. Cary, NC). The level of significance was set as p<0.05.

Results

Effect of fat and oils on hyperglycemia and hyperinsulinemia in Experiment 1. Mice were fed the SD or five types of 20% HF diets for 16 weeks. Fatty acid composition of used fat and oils and ingredients of diets are shown in Table 1 and Table 2A, respectively. During the feeding period, symptoms such as skin abnormality or alopecia, which appear in the case of essential fatty acid deficiency, were not observed. When OGTT was performed 15 weeks after the feeding, changes in the plasma glucose levels of all groups including the SD group showed almost the same trend. Only the values in HF-Palm group at 60 min and HF-Corn group at 90 min significantly lowered compared with the SD group (Fig. 1A). AUC was no difference among all groups (Fig. 1C). However, AUC in HF-Flax group was the lowest among the HF groups and kept the same value as the SD group. On the other hand, the plasma insulin level of the HF-Lard group was significantly higher than that of the SD group from 15 to 90 min after the glucose loading (Fig. 1B). From the result of AUC, the HF-Lard and HF-Palm groups were significantly higher than the SD group (Fig. 1D). AUC in HF-Flax group was the lowest among the HF groups.

The plasma glucose and insulin levels were also measured at the end of experiment (Fig. 2). Fasting plasma glucose level of the HF groups, except the HF-Flax group, significantly increased compared with that of the SD group (Fig. 2A). There was no significant difference in the plasma glucose level between the HF-Flax and SD groups. Fasting plasma insulin level and HOMA-IR, which are the insulin resistance index, were significantly higher in the HF-Lard group than those in the SD group (Fig. 2B and C). In the HF-Flax group, plasma insulin level and HOMA-IR were the lowest among the HF groups, though there was no significant difference. Thus, an intake of lard and palm oil induced significant hyperinsulinemia and that of flaxseed oil was hard to induce hyperinsulinemia among used fat and oils in this study.

Effect of fat and oils on the fatty acid composition and content in the muscle of mice in Experiment 1. Composition and content of fatty acids in the tissue were altered by ingested fats and oils. This alteration in the muscle would affect glucose homeostasis and occurrence of insulin resistance because muscle is the most important tissue for consumption of postprandial hyperglycemia. The fatty acid composition and content in the muscle are shown in Table 3 and 4, respectively. Regarding the fatty acid composition and content in the muscles of SD group, oleic acid was the highest, and palmitic acid and linoleic acid were the next. After the intake of HF diets, fatty acids content increased compared with the SD group as expected. In particular HF-Lard, HF-Corn, HF-Saff groups revealed significant increase. In HF-Flax group, composition and content of α-linolenic acid were significantly higher than other groups, indicating that it was absorbed and accumulated in the muscle after the intake of flaxseed oil. In HF-Corn group, composition and content of linoleic acid were significantly higher than that in other groups. Fatty acid composition and content of polyunsaturated fatty acid (PUFA) in HF-Corn and HF-Flax were significantly higher than in that in other groups, with higher level of linoleic acid and α-linolenic acid respectively. Composition and content of arachidonic acid in HF-Flax was significantly lower than other groups except composition in HF-Lard. In HF-Saff, composition and content of oleic acid and monounsaturated fatty acid (MUFA) were the highest because of its high content in safflower oil.

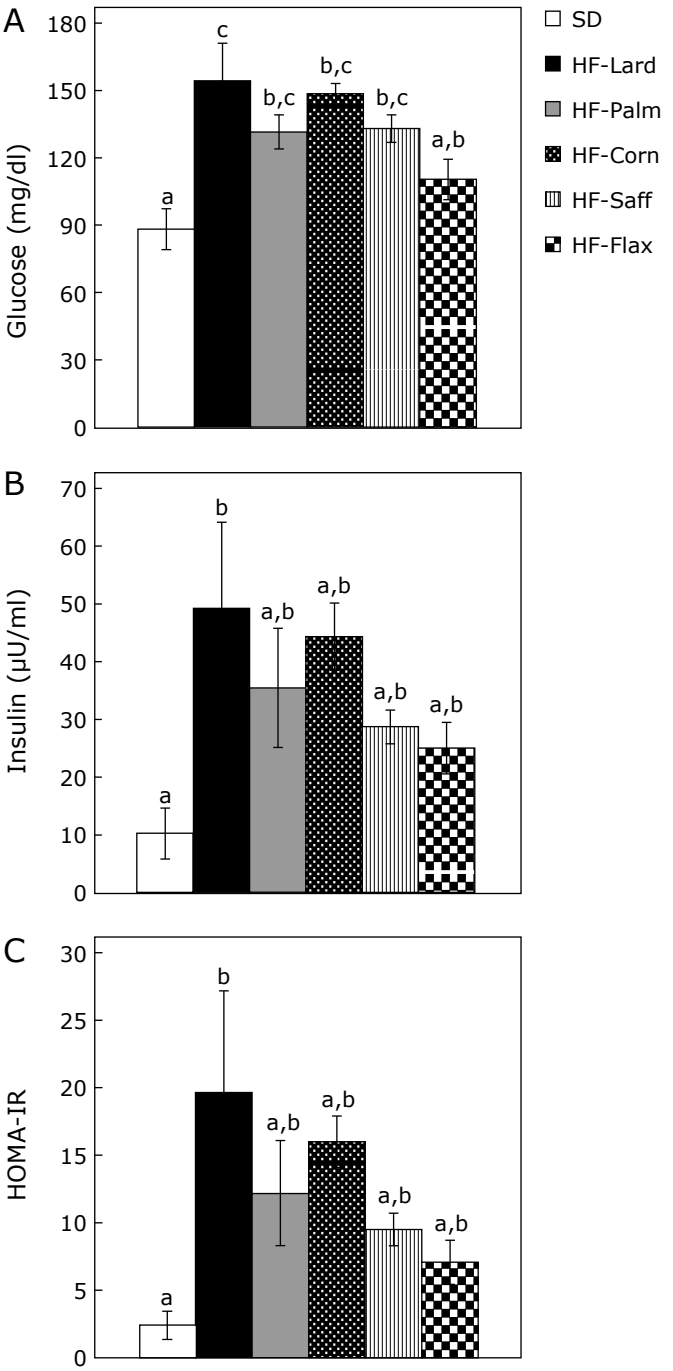

Fig. 2. Level of fasting plasma glucose (A), insulin (B), and HOMA-IR (C) in Experiment 1. Data shown represent mean ± SE (n = 7 animals in SD and n = 6 in each HF groups). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test (p<0.05). HF-Corn, high-fat diet containing 20% corn oil; HF-Flax, high-fat diet containing 20% flaxseed oil; HF-Lard, high-fat diet containing 20% lard; HF-Palm, high-fat diet containing 20% palm oil; HF-Saff, high-fat diet containing 20% safflower oil; SD, standard diet.

Correlative relationship between fatty acid content in the muscle and AUC of plasma glucose or insulin from OGTT was examined (Fig. 3 and 4) to understand whether muscle fatty acids

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Table 3. Fatty acid composition in muscle (%)

	SD	HF-Lard	HF-Palm	HF-Corn	HF-Saff	HF-Flax
14:0	1.17 ± 0.05^{a}	1.19 ± 0.03^{a}	1.03 ± 0.04^{a}	0.69 ± 0.02^{c}	0.63 ± 0.04^{c}	0.85 ± 0.03^{b}
16:0	22.89 ± 1.18^{a}	20.06 ± 0.44^{b}	25.03 ± 0.25^{a}	16.60 ± 0.41^{c}	12.97 ± 0.45^{d}	14.62 ± 0.36^{cd}
16:1	9.03 ± 0.59^{a}	10.21 ± 0.39^{a}	12.22 ± 0.29^{b}	5.85 ± 0.16^{cd}	5.29 ± 0.23^{d}	6.76 ± 0.10^{c}
18:0	5.32 ± 0.77^{a}	3.42 ± 0.15^{b}	2.86 ± 0.10^{b}	2.26 ± 0.17^{b}	2.26 ± 0.21^{b}	3.32 ± 0.09^{b}
18:1 (n-9)	34.53 ± 1.33^{a}	52.75 ± 0.81^{d}	45.21 ± 0.51^{c}	31.71 ± 0.38^{ab}	64.67 ± 1.11^{e}	30.40 ± 0.34^{b}
18:2 (n-6)	19.37 ± 1.24^{a}	8.76 ± 0.26^{c}	9.06 ± 0.12^{c}	39.07 ± 0.66^{d}	10.37 ± 0.15^{c}	15.67 ± 0.35^{b}
18:3 (n-3)	0.67 ± 0.05^{a}	0.43 ± 0.02^{a}	0.40 ± 0.03^{a}	0.69 ± 0.02^{a}	0.43 ± 0.09^{a}	24.41 ± 0.59^{b}
20:4 (n-6)	4.65 ± 0.62^{a}	1.64 ± 0.16^{bc}	2.80 ± 0.14^{b}	1.91 ± 0.18^{b}	2.22 ± 0.22^{b}	0.41 ± 0.10^{c}
22:6 (n-3)	2.48 ± 0.42^{ab}	1.54 ± 0.25^{bc}	1.38 ± 0.16^{bc}	1.22 ± 0.16^{c}	1.16 ± 0.17^{c}	3.55 ± 0.28^{a}
SFA	29.28 ± 1.94^{a}	24.66 ± 0.56^{b}	28.92 ± 0.21^{a}	19.55 ± 0.57^{c}	15.86 ± 0.66^{c}	18.80 ± 0.43^{c}
MUFA	43.55 ± 1.63^{a}	62.96 ± 0.77^{d}	57.43 ± 0.29^{c}	37.57 ± 0.29^{b}	69.96 ± 0.96^{e}	37.16 ± 0.34^{b}
PUFA	27.17 ± 1.03^{a}	12.38 ± 0.37^{b}	13.64 ± 0.39^{b}	42.88 ± 0.31^{c}	14.18 ± 0.37^{b}	44.05 ± 0.49^{c}

Data shown represent mean ± SE (n = 7 animals in SD and n = 6 in each HF groups). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test (p<0.05). HF-Corn, high-fat diet containing 20% corn oil; HF-Flax, high-fat diet containing 20% flaxseed oil; HF-Lard, high-fat diet containing 20% lard; HF-Palm, high-fat diet containing 20% palm oil; HF-Saff, high-fat diet containing 20% safflower oil; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SD, standard diet; SFA, saturated fatty acid.

Table 4. Fatty acid content in muscle (mg/g tissue)

	SD	HF-Lard	HF-Palm	HF-Corn	HF-Saff	HF-Flax
14:0	0.34 ± 0.04^{a}	1.07 ± 0.12^{b}	0.60 ± 0.06^{a}	0.60 ± 0.06^{a}	0.50 ± 0.08^{a}	0.46 ± 0.04^{a}
16:0	6.65 ± 0.62^{a}	18.42 ± 2.65^{d}	14.53 ± 1.16^{cd}	14.28 ± 1.48^{bcd}	10.16 ± 1.58^{bcd}	7.83 ± 0.47^{ab}
16:1	2.73 ± 0.45^{a}	9.21 ± 1.00^{c}	7.06 ± 0.45^{bc}	5.04 ± 0.55^{ab}	4.16 ± 0.68^{a}	3.65 ± 0.31^{a}
18:0	1.49 ± 0.14^{a}	3.11 ± 0.42^{b}	1.64 ± 0.07^{a}	1.90 ± 0.10^{a}	1.70 ± 0.18^{a}	1.78 ± 0.10^{a}
18:1 (n-9)	10.38 ± 1.42^{a}	48.67 ± 7.28^{bc}	26.28 ± 2.21^{ab}	27.63 ± 3.51^{ab}	52.62 ± 10.67^{c}	16.41 ± 1.38^{a}
18:2 (n-6)	5.86 ± 0.93^{a}	8.08 ± 1.20^{a}	5.24 ± 0.36^{a}	34.11 ± 4.46^{b}	8.41 ± 1.68^{a}	8.49 ± 0.82^{a}
18:3 (n-3)	0.20 ± 0.03^{a}	0.40 ± 0.08^{a}	0.23 ± 0.02^{a}	0.59 ± 0.05^{a}	0.30 ± 0.04^{a}	13.24 ± 1.29^{b}
20:4 (n-6)	1.31 ± 0.09^{a}	1.44 ± 0.10^{a}	1.60 ± 0.06^{a}	1.59 ± 0.09^{a}	1.67 ± 0.18^{a}	0.21 ± 0.03^{b}
22:6 (n-3)	0.69 ± 0.04^{a}	1.33 ± 0.13^{b}	0.78 ± 0.06^{a}	1.00 ± 0.05^{a}	0.84 ± 0.07^{a}	1.87 ± 0.07^{c}
SFA	8.49 ± 0.73^{a}	22.60 ± 3.19^{c}	16.78 ± 1.28^{bc}	16.77 ± 1.64^{bc}	12.35 ± 1.83^{ab}	10.07 ± 0.60^{ab}
MUFA	13.11 ± 1.84^{a}	57.88 ± 8.26^{b}	33.34 ± 2.63^{ab}	32.67 ± 4.05^{ab}	56.78 ± 11.32^{b}	20.06 ± 1.68^{a}
PUFA	8.06 ± 1.00^{a}	11.25 ± 1.41^{a}	7.85 ± 0.41^{a}	37.29 ± 4.58^{c}	11.21 ± 1.91^{a}	23.81 ± 2.08^{b}
Total	29.66 ± 3.38^{a}	91.74 ± 12.78^{b}	57.96 ± 4.30^{ab}	86.73 ± 10.25^{b}	80.35 ± 15.06^{b}	53.94 ± 4.33^{ab}

Data shown represent mean ± SE (n = 7 animals in SD and n = 6 in each HF groups). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test (p<0.05). HF-Corn, high-fat diet containing 20% corn oil; HF-Flax, high-fat diet containing 20% flaxseed oil; HF-Lard, high-fat diet containing 20% lard; HF-Palm, high-fat diet containing 20% palm oil; HF-Saff, high-fat diet containing 20% safflower oil; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SD, standard diet; SFA, saturated fatty acid.

affect glucose homeostasis and insulin resistance. The content of palmitic acid and oleic acid showed positive correlation with the AUC of glucose (Fig. 3A and B), whereas content of linoleic acid did not (Fig. 3C). The content of α-linolenic acid negatively correlated with the AUC of glucose (Fig. 3D). These results reflected that SFA and MUFA showed positive correlation (Fig. 3E and F) and PUFA showed poor negative correlation (Fig. 3G). As for correlative relationship between AUC of plasma insulin by OGTT and fatty acid content in the muscle, the content of palmitic acid and oleic acid positively correlated with the AUC (Fig. 4A and B), whereas content of linoleic acid did not correlate as the same manner as the result of AUC of plasma insulin (Fig. 4C). The content of α-linolenic acid showed poor negative correlation with the AUC of insulin (Fig. 4D). These data showed that SFA and MUFA correlated positively (Fig. 4E and F) and PUFA did not (Fig. 4G). From these results of correlative relationship, ingested fat and oils changed the fatty acid content and composition in the muscle and PUFA, especially α-linolenic acid, content related to ameliorating hyperinsulinemia.

Effect of fat and oils on body weight, adipose tissue weight, liver weight, and plasma lipids in Experiment 1. At the end of experiment, body and WAT weights in all HF groups were significantly higher than those in the SD group as expected (Table 5). Among HF groups, body and WAT weights in the HF-Flax group were the lowest, though there was no significant difference. Total WAT weight in the HF-Corn group was higher than that in the SD, HF-Lard, HF-Saff, and HF-Flax groups due to the heaviest epididymal and subcutaneous WAT weights. Liver weight in the HF-Lard and HF-Saff groups were significantly higher than that in the SD and HF-Corn groups. There was no significant difference food intake showed as g/day, but energy intake (kcal/day) of the HF groups except HF-Corn were significantly higher than that of the SD group. Feed and energy efficiency of the HF groups except HF-Flax group were also significantly higher than those of the SD group.

The plasma lipid levels (total cholesterol, triglyceride, and free fatty acid) were measured at the end of the experiment (Table 6). The plasma total cholesterol level of the HF-Lard, HF-Corn, and

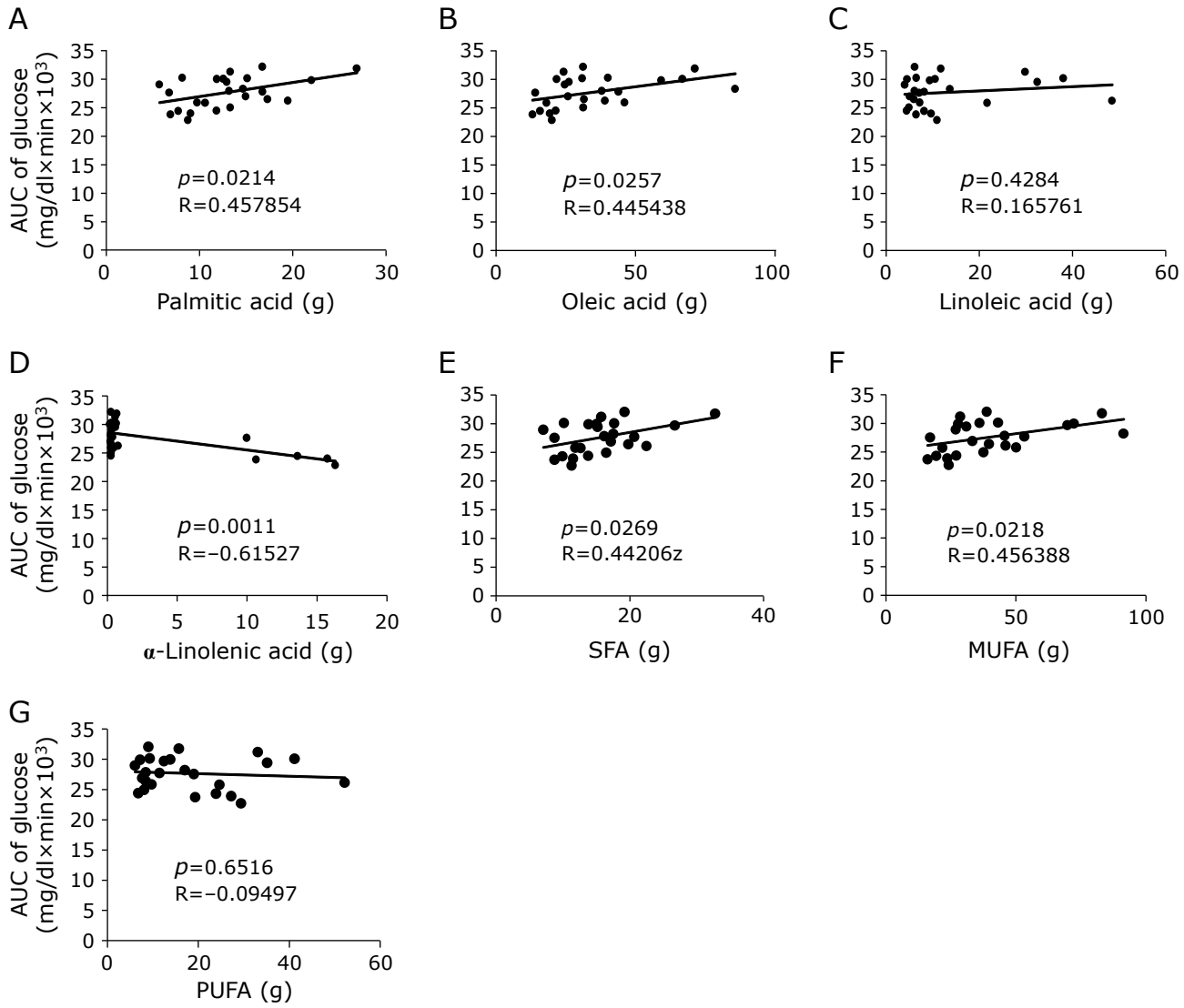

Fig. 3. Correlation between fatty acid content in muscle and AUC of plasma glucose by OGTT in Experiment 1. Relationship between AUC of glucose and content of palmitic acid (A), oleic acid (B), linoleic acid (C), α-linolenic acid (D), SFA (E), MUFA (F), and PUFA (G). AUC, area under the curve; MUFA, monounsaturated fatty acid; OGTT, oral glucose test; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

HF-Saff groups significantly increased compared with the SD group. On the other hand, the total cholesterol level of the HF-Flax group was no different from the SD group and lower than that in the HF-Lard group. The plasma triglyceride level of the HF-Saff group was the lowest among all groups and significantly lower than that of the HF-Lard and HF-Corn. Free fatty acid was no difference among the groups. From these results, the intake of flaxseed oil has been suggested to suppress the development of hypercholesterolemia.

Effect of substitution of flaxseed oil on hyperinsulinemia and hypercholesterolemia induced by lard in Experiment 2. Results from the Experiment 1 showed the excess intake of lard and palm oil induced postprandial hyperinsulinemia compared to other oils. Intake of lard also showed high level of fasting plasma insulin level. Besides, although content of palmitic acid in lard is lower than that in palm oil, intake of lard induces hyperinsulinemia to a greater or lesser extent than palm oil. On the other hand, that of flaxseed oil did not. However, these effects were not clear under the supplementation with 20% fat and oils in the diet. Thus, the 30% lard diet and part of substitution of flaxseed oil for lard were used in the Experiment 2, and suppressive effect of the substituted flaxseed oil was examined. Ingredients of diets are shown in Table 2B. Substitution of flaxseed oil did not affect the plasma glucose and plasma insulin levels when OGTT was performed (Fig. 5). Plasma glucose level of the HF groups except the HF-0.1Flax group at 15 min significantly increased compared with that in the SD group (Fig. 5A). Plasma insulin level of all flaxseed oil-substituted groups at 0 min (before the loading of glucose) and the HF-0.1Flax and HF-0.5Flax groups at 15 min did not show significant difference compared with that of the SD group (Fig. 5B). Both AUC of plasma glucose and of insulin were no significant difference among all HF groups (Fig. 5C and D).

At the end of feeding period, fasting plasma glucose level of the HF-0.5Flax group was significantly lower than that of the HF-Lard group (Fig. 6A). Fasting plasma insulin level and HOMA-IR of the HF-0.2Flax and HF-0.5Flax groups were significantly lower than that of the HF-Lard group (Fig. 6B and C).

Body and all WAT weights in the HF groups were significantly higher than that in the SD group, but there was no significant dif-

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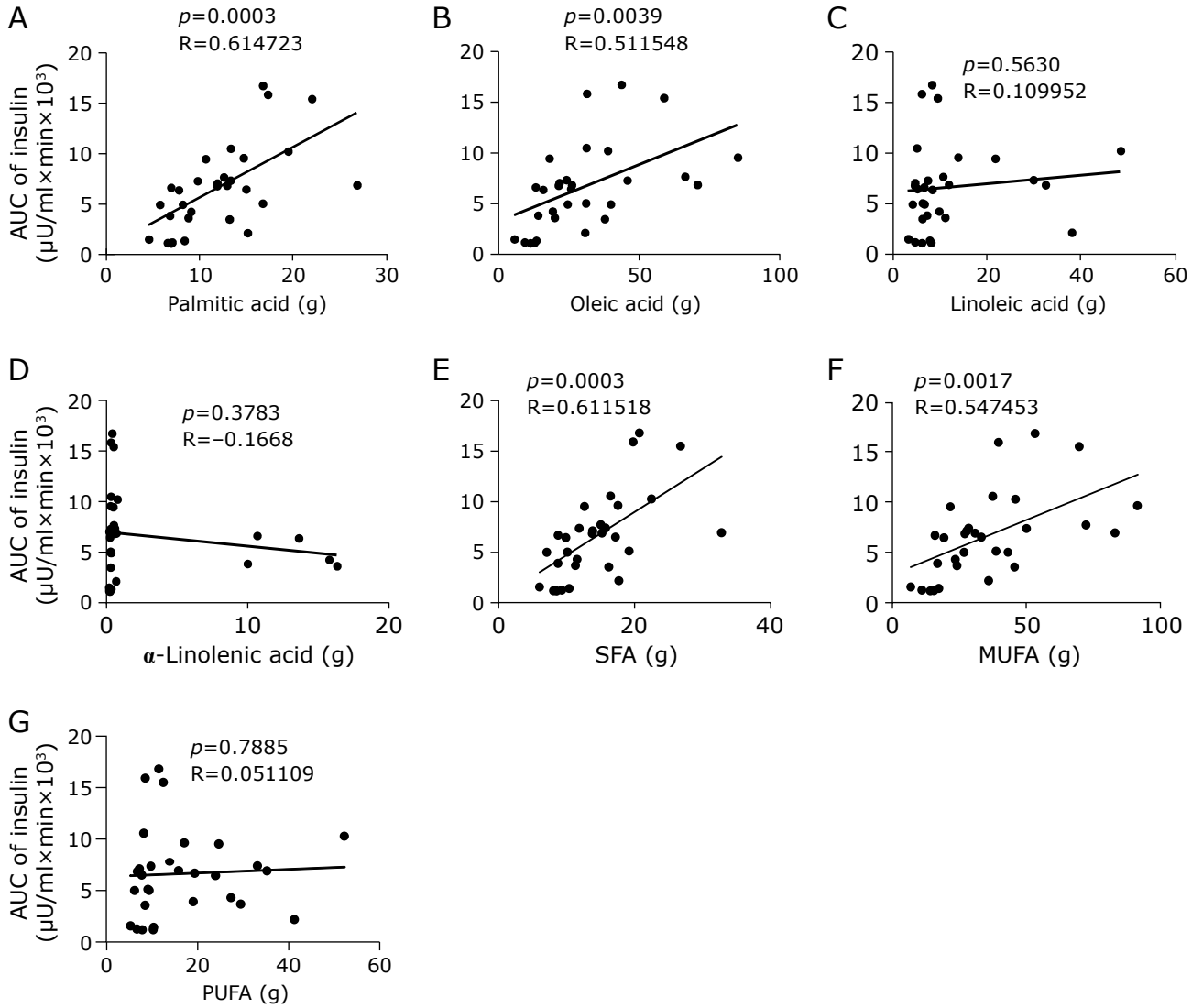

Fig. 4. Correlation between fatty acid content in muscle and AUC of plasma insulin by OGTT in Experiment 1. Relationship between AUC of insulin and content of palmitic acid (A), oleic acid (B), linoleic acid (C), α-linolenic acid (D), SFA (E), MUFA (F), and PUFA (G). AUC, area under the curve; MUFA, monounsaturated fatty acid; OGTT, oral glucose test; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 5. Final body weight, food intake, energy intake, feed efficiency, and percent of WAT and liver weight of mice in Experiment 1

	SD	HF-Lard	HF-Palm	HF-Corn	HF-Saff	HF-Flax
Final body weight (g)	25.3 ± 0.8^{a}	40.7 ± 2.1^{b}	40.0 ± 0.8^{b}	40.2 ± 0.8^{b}	39.3 ± 1.2^{b}	37.1 ± 0.7^{b}
Food intake (g/day)	2.4 ± 0.1^{a}	2.6 ± 0.1^{a}	2.7 ± 0.1^{a}	2.4 ± 0.1^{a}	2.6 ± 0.1^{a}	2.5 ± 0.1^{a}
Energy intake (kcal/day)	9.7 ± 0.6^{a}	12.3 ± 0.5^{b}	12.8 ± 0.4^{b}	11.3 ± 0.5^{ab}	12.3 ± 0.4^{b}	12.1 ± 0.3^{b}
Feed efficiency (/100 g)	2.55 ± 0.71^{a}	7.63 ± 1.15^{b}	7.05 ± 0.65^{b}	8.02 ± 0.78^{b}	7.03 ± 0.76^{b}	6.41 ± 0.96^{b}
Energy efficiency (/100 kcal)	0.63 ± 0.18^{a}	1.60 ± 0.24^{b}	1.47 ± 0.14^{b}	1.68 ± 0.16^{b}	1.47 ± 0.16^{b}	1.34 ± 0.20^{ab}
WAT weight			(% of body weight)			
Total WAT	10.01 ± 1.00^{a}	22.09 ± 1.16^{b}	23.26 ± 1.10^{bc}	27.51 ± 1.11^{c}	22.54 ± 1.03^{b}	20.26 ± 0.61^{b}
Mesenteric WAT	1.01 ± 0.13^{a}	2.53 ± 0.19^{b}	2.56 ± 0.17^{b}	2.61 ± 0.12^{b}	2.51 ± 0.18^{b}	2.38 ± 0.16^{b}
Epididymal WAT	2.68 ± 0.26^{a}	4.93 ± 0.29^{b}	5.85 ± 0.09^{bc}	6.54 ± 0.33^{c}	5.34 ± 0.42^{bc}	4.94 ± 0.12^{b}
Retroperitoneal WAT	1.29 ± 0.15^{a}	3.35 ± 0.21^{b}	3.36 ± 0.10^{b}	3.58 ± 0.19^{b}	3.44 ± 0.27^{b}	3.08 ± 0.24^{b}
Subcutaneous WAT	5.03 ± 0.51^{a}	11.27 ± 0.87^{bc}	11.49 ± 1.10^{bc}	14.77 ± 1.11^{c}	11.25 ± 1.00^{bc}	9.86 ± 0.66^{b}
Liver weight	3.33 ± 0.11^{a}	4.62 ± 0.35^{b}	4.26 ± 0.29^{ab}	3.30 ± 0.14^{a}	4.88 ± 0.23^{b}	4.07 ± 0.21^{ab}

Feed efficiency defined as ratio of weight gain (g/day) divided by food intake (g/day) and energy efficiency defined as ratio of weight gain (g/day) divided by energy intake (kcal/g) and converted to per 100 g or per 100 kcal, respectively. Data shown represent mean ± SE (n = 7 animals in SD and n = 6 in each HF groups). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test (p<0.05). HF-Corn, high-fat diet containing 20% corn oil; HF-Flax, high-fat diet containing 20% flaxseed oil; HF-Lard, high-fat diet containing 20% lard; HF-Palm, high-fat diet containing 20% palm oil; HF-Saff, high-fat diet containing 20% safflower oil; SD, standard diet; WAT, white adipose tissue.

Table 6. Levels of plasma total cholesterol, triglyceride, free fatty acid in Experiment 1

	SD	HF-Lard	HF-Palm	HF-Corn	HF-Saff	HF-Flax
Cholesterol (mg/dl)	100.5 ± 13.0[a]	224.0 ± 22.6[c]	154.9 ± 21.5[abc]	178.1 ± 14.4[bc]	180.0 ± 16.4[bc]	131.2 ± 11.5[ab]
Triglyceride (mg/dl)	66.4 ± 10.4[ab]	70.9 ± 7.8[b]	49.8 ± 5.8[ab]	71.5 ± 6.9[b]	36.4 ± 6.2[a]	54.9 ± 6.1[ab]
Free fatty acid (mEq/L)	0.94 ± 0.17[a]	0.91 ± 0.12[a]	1.01 ± 0.05[a]	0.95 ± 0.04[a]	0.84 ± 0.08[a]	1.02 ± 0.04[a]

Data shown represent mean ± SE (n = 7 animals in SD and n = 6 in each HF groups). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test (p<0.05). HF-Corn, high-fat diet containing 20% corn oil; HF-Flax, high-fat diet containing 20% flaxseed oil; HF-Lard, high-fat diet containing 20% lard; HF-Palm, high-fat diet containing 20% palm oil; HF-Saff, high-fat diet containing 20% safflower oil; SD, standard diet.

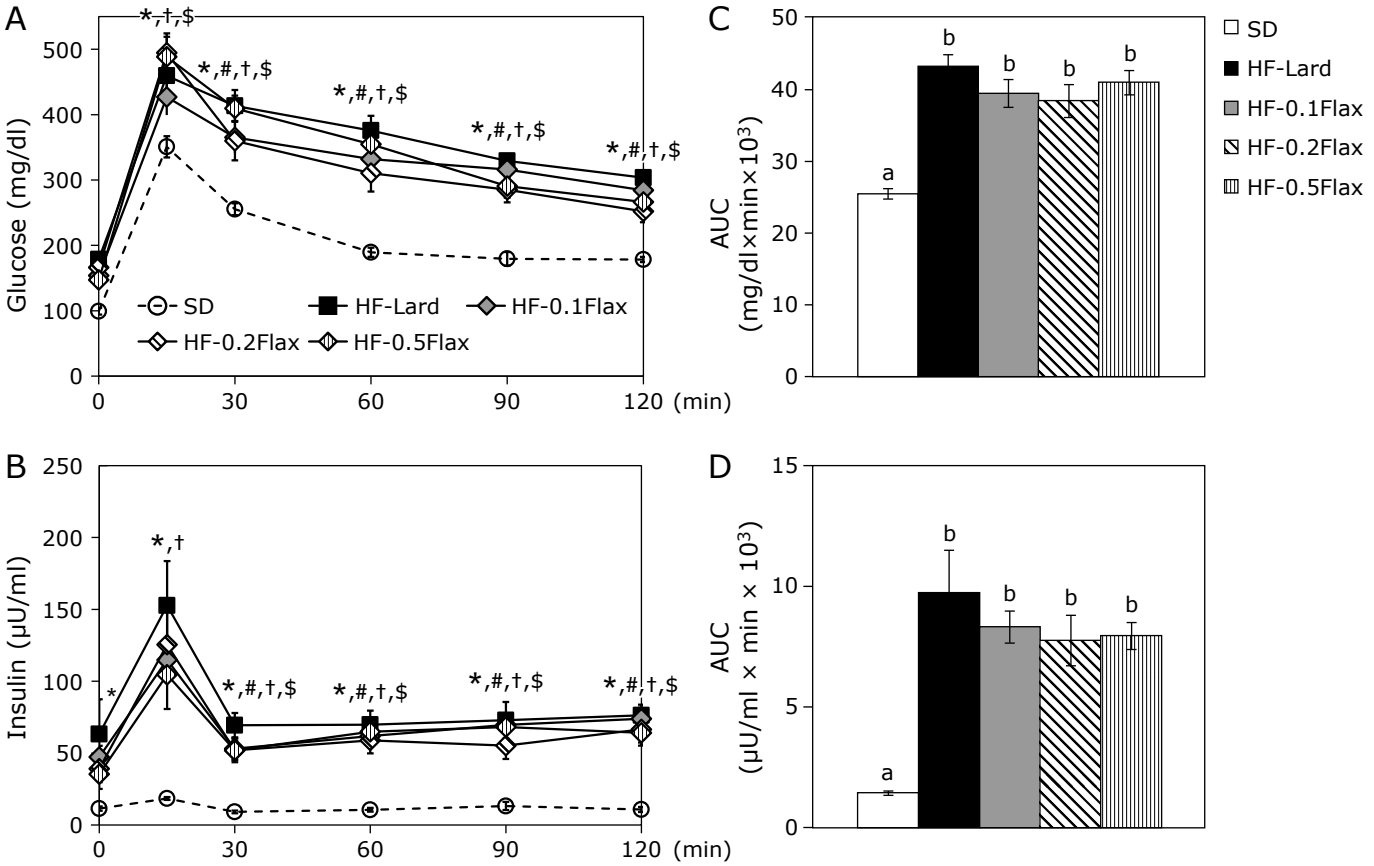

Fig. 5. Plasma glucose and insulin level after conduct of OGTT at 15 Week in Experiment 2. Time-course and area under curve of plasma glucose (A and C) and insulin (B and D). Data shown represent mean ± SE (n = 7 animals in SD and HF-Lard, n = 6 in other HF groups). The asterisks are represented significant difference between *SD vs HF-Lard, #SD vs HF-0.1Flax, †SD vs HF-0.2Flax, $SD vs HF-0.5Flax (A, B). Values with the same letters are not significantly different by Tukey Kramer multiple comparison test (p<0.05). AUC, area under the curve; HF-0.1Flax, high-fat diet containing 30% lard substituted for 10% flaxseed oil; HF-0.2Flax, high-fat diet containing 30% lard substituted for 20% flaxseed oil; HF-0.5Flax, high-fat diet containing 30% lard substituted for 50% flaxseed oil; HF-Lard, high-fat diet containing 30% lard; OGTT, oral glucose test; SD, standard diet.

ference among HF groups (Table 7). Liver weight in the HF-Lard and HF-0.2Flax were significantly higher than that in the SD group. Food and energy intake of HF-Lard was significantly higher than HF-0.1Flax, HF-0.2Flax, and HF-0.5Flax groups. There was no significant difference of feed and energy efficiency among the HF groups. Plasma total cholesterol level of the HF-Lard was higher than that of the SD group, but that of the flaxseed oil-substituted groups was lower depending on the substitution amounts (Table 8). Especially, the cholesterol level of the HF-0.5Flax group was significantly lower than that of the HF-Lard. Triglyceride and free fatty acid levels were no difference among the all groups. These results indicated that the substitution of flaxseed oil suppressed the lard-induced hyperinsulinemia and hypercholesterolemia.

Discussion

Alteration of physiological function by an excess intake of fats and oils are depending on their fatty acid composition. For example, an intake of lard rich in palmitic acid induces hyperglycemia accompanied by insulin resistance;[5] and α-linolenic acid which contains flaxseed oil is reported to ameliorate insulin

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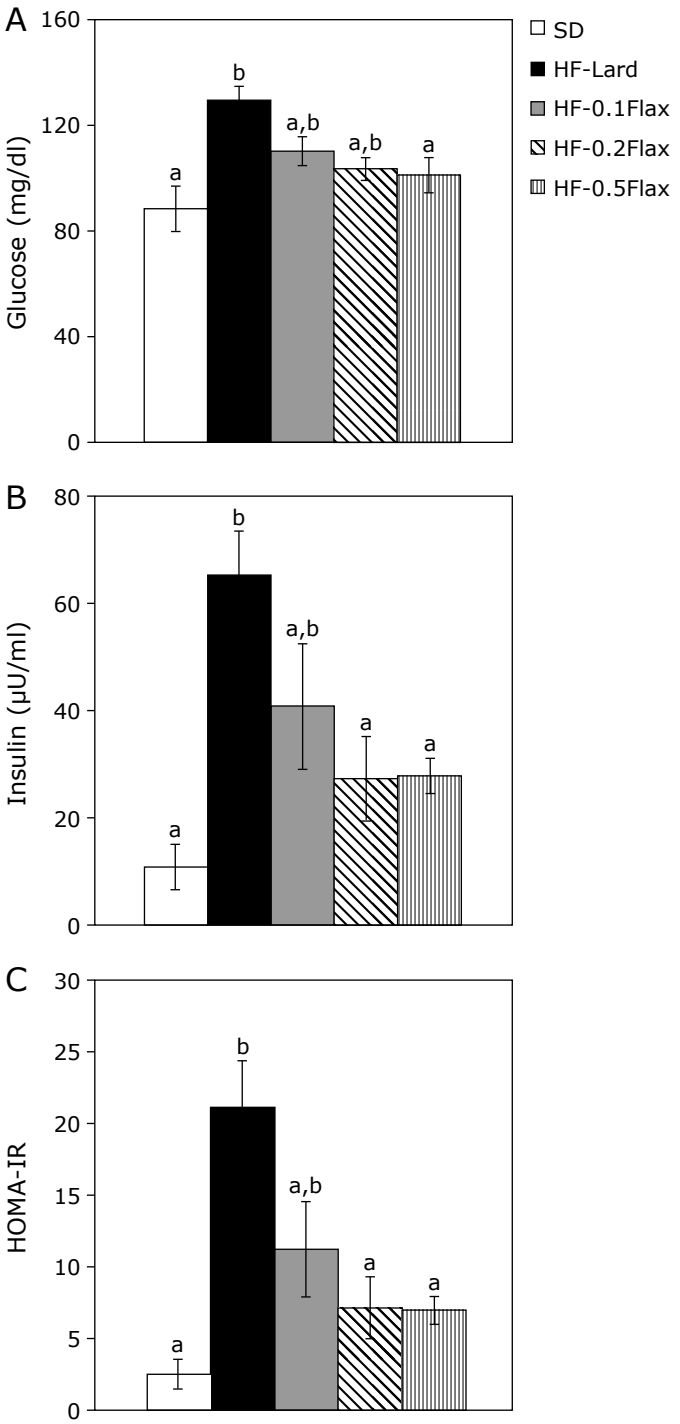

Fig. 6. Levels of fasting plasma glucose (A), insulin (B), and HOMA-IR (C) in Experiment 2. Data shown represent mean ± SE (n = 7 animals in SD and HF-Lard, n = 6 in other HF groups). Values with the same letters are not significantly different by Tukey Kramer multiple comparison test (p<0.05). HF-0.1Flax, high-fat diet containing 30% lard substituted for 10% flaxseed oil; HF-0.2Flax, high-fat diet containing 30% lard substituted for 20% flaxseed oil; HF-0.5Flax, high-fat diet containing 30% lard substituted for 50% flaxseed oil; HF-Lard, high-fat diet containing 30% lard; SD, standard diet.

resistance.[6] In this study, we demonstrated that fatty acid content and composition in the skeletal muscle affected the postprandial hyperglycemia and hyperinsulinemia: high content of palmitic acid and oleic acid was positively correlated to the postprandial hyperglycemia and hyperinsulinemia, whereas high content of α-linolenic acid was negatively correlated. Moreover, substitution of lard to flaxseed oil prevented hyperglycemia and hyperinsulinemia. It was noteworthy that only 20% flaxseed oil revealed a significant preventing effect. This result strongly suggests that α-linolenic acid contradicts the adverse function of palmitic acid. This is the first report that an intake of α-linolenic acid-rich flaxseed oil effectively prevents hyperglycemia and hyperinsulinemia. Our findings indicate that fatty acid composition in the skeletal muscle is important for glucose metabolism through the onset of insulin resistance.

Content of fatty acid alters different types of fats and oils and their amounts in diet and affects the onset of insulin resistance.[7,8] In the current study, we demonstrated that the intake of HF-Lard and HF-Palm increased palmitic acid content in the muscle with exacerbation of hyperglycemia and hyperinsulinemia, while the intake of HF-Flax increased α-linolenic acid content in the muscle and ameliorate it. It was reported that a mechanism by which the occurrence of insulin resistance is involved in the impairment of insulin signaling pathway including phosphorylation of IRS.[2,9] Since the activation of this pathway induces translocation of GLUT4 and glucose uptake, insulin resistance in the muscle leads to hyperglycemia. Our previous results demonstrate that the intake of lard-based high-fat diet down-regulated the expression of insulin receptor β, AMPK, and GLUT4 and its translocation without affecting GLUT1 expression.[10] Palmitic acid is known to induce insulin resistance through impairing the insulin pathway, whereas polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid and docosahexaenoic acid, do not cause insulin resistance.[2,11] There have been reports on the relationship between the amount of linoleic acid or other n-3 PUFAs and insulin resistance.[7,12] The current study showed PUFA in the muscle ameliorate hyperglycemia and hyperinsulinemia compared to SFA and MUFA. This effect was reflected content of α-linolenic acid, not that of linoleic acid which showed positive correlation with AUC of glucose and insulin. Tissues other than muscle, such as liver and adipose tissue, also regulate hyperglycemia, hyperinsulinemia, and insulin resistance. It was already reported that dietary flaxseed oil induce phosphorylation of AMPK and enhance insulin-stimulated phosphorylation of IRS-1 and Akt in the liver.[13,14] In addition, the another research showed composition of α-linolenic acid in adipose tissue is inversely associated with the HOMA-IR in adults.[15] Thus, the effect of dietary fat and oils on hyperglycemia and hyperinsulinemia may derived from other tissues which relate to these diseases. However, the current study has revealed the importance of fatty acid content in muscle, which plays a central role in maintaining blood glucose. Flaxseed oil contains certain active compounds such as β-carotene and tocopherol.[16] However, these compounds also contain in corn and palm oil.[17,18] Thus, these compounds might not affect the function of flaxseed oil. Therefore, α-linolenic acid, but not other active compounds, in flaxseed oil mainly contribute to prevention of hyperglycemia and hyperinsulinemia. Further research is necessary in the future to clarify the mechanism by which the intake of flaxseed oil to prevent insulin resistance through affecting insulin signal pathway. However, our findings provide evidence that α-linolenic acid also has a potency to prevent hyperglycemia and hyperinsulinemia and the content of it in the muscle is an important marker for glucose tolerance.

As to oleic and linoleic acids, they have lesser potency for the induction of insulin resistance compared with palmitic acid.[12,19] However, current result demonstrated that the content of them show significant or poor positive correlation to the AUC of

Table 7. Final body weight, food intake, energy intake, feed efficiency, and percent of WAT and liver weight of mice in Experiment 2

	SD	HF-Lard	HF-0.1Flax	HF-0.2Flax	HF-0.5Flax
Final body weight (g)	26.7 ± 1.4[a]	42.6 ± 1.3[b]	41.1 ± 0.7[b]	40.3 ± 0.8[b]	39.2 ± 0.5[b]
Food intake (g/day)	2.5 ± 0.1[ab]	2.7 ± 0.1[a]	2.2 ± 0.1[bc]	2.2 ± 0.1[bc]	2.2 ± 0.1[c]
Energy intake (kcal/day)	10.2 ± 0.3[a]	14.2 ± 0.5[b]	11.8 ± 0.4[a]	11.8 ± 0.5[a]	11.4 ± 0.5[a]
Feed efficiency (/100 g)	2.90 ± 0.54[a]	8.22 ± 1.23[b]	9.19 ± 1.23[b]	7.30 ± 1.25[ab]	8.76 ± 1.53[b]
Energy efficiency (/100 kcal)	0.72 ± 0.13[a]	1.56 ± 0.23[ab]	1.74 ± 0.23[b]	1.38 ± 0.24[ab]	1.66 ± 0.29[b]
WAT weight			(% of body weight)		
Total WAT	10.75 ± 1.03[a]	23.82 ± 0.90[b]	24.45 ± 0.60[b]	23.73 ± 0.61[b]	22.79 ± 0.49[b]
Mesenteric WAT	1.30 ± 0.16[a]	3.34 ± 0.23[b]	3.18 ± 0.24[b]	3.06 ± 0.24[b]	2.96 ± 0.15[b]
Epididymal WAT	2.92 ± 0.22[a]	4.99 ± 0.40[b]	5.29 ± 0.34[b]	5.07 ± 0.21[b]	5.43 ± 0.34[b]
Retroperitoneal WAT	1.50 ± 0.14[a]	3.71 ± 0.31[b]	3.35 ± 0.28[b]	3.35 ± 0.30[b]	3.28 ± 0.18[b]
Subcutaneous WAT	5.03 ± 0.57[a]	11.78 ± 0.56[b]	12.63 ± 0.40[b]	12.25 ± 0.54[b]	11.12 ± 0.37[b]
Liver weight	3.45 ± 0.05[a]	4.72 ± 0.22[b]	4.16 ± 0.21[ab]	4.20 ± 0.21[b]	4.04 ± 0.16[ab]

Feed efficiency defined as ratio of weight gain (g/day) divided by food intake (g/day) and energy efficiency defined as ratio of weight gain (g/day) divided by energy intake (kcal/g) and converted to per 100 g or per 100 kcal, respectively. Data shown represent mean ± SE ($n = 7$ animals in SD and HF-Lard, $n = 6$ in other HF groups). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test ($p<0.05$). HF-0.1Flax, high-fat diet containing 30% lard substituted for 10% flaxseed oil; HF-0.2Flax, high-fat diet containing 30% lard substituted for 20% flaxseed oil; HF-0.5Flax, high-fat diet containing 30% lard substituted for 50% flaxseed oil; HF-Lard, high-fat diet containing 30% lard; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SD, standard diet; SFA, saturated fatty acid.

Table 8. Levels of plasma total cholesterol, triglyceride, free fatty acid in Experiment 2

	SD	HF-Lard	HF-0.1Flax	HF-0.2Flax	HF-0.5Flax
Cholesterol (mg/dl)	118.2 ± 6.7[a]	197.5 ± 12.0[c]	184.4 ± 10.5[bc]	179.1 ± 9.7[bc]	155.6 ± 4.6[ab]
Triglyceride (mg/dl)	110.4 ± 14.0[a]	70.2 ± 10.5[a]	146.2 ± 59.2[a]	129.5 ± 13.9[a]	106.7 ± 12.6[a]
Free fatty acid (mEq/L)	1.35 ± 0.12[a]	1.03 ± 0.12[a]	1.06 ± 0.10[a]	1.08 ± 0.10[a]	1.07 ± 0.06[a]

Data shown represent mean ± SE ($n = 7$ animals in SD and HF-Lard, $n = 6$ in other HF groups). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test ($p<0.05$). HF-0.1Flax, high-fat diet containing 30% lard substituted for 10% flaxseed oil; HF-0.2Flax, high-fat diet containing 30% lard substituted for 20% flaxseed oil; HF-0.5Flax, high-fat diet containing 30% lard substituted for 50% flaxseed oil; HF-Lard, high-fat diet containing 30% lard; SD, standard diet.

plasma glucose and insulin from OGTT. This result indicated that MUFA and n-6 PUFA are not enough to prevent hyperglycemia and hyperinsulinemia. On the other hand, it is known that olive oil rich in oleic acid has preventive effect on insulin resistance, but it also contains minor active compounds such as hydroxytyrosol and oleocanthal which prevent it.[20,21] Thus, we used high-oleic safflower oil instead of olive oil to evaluate the effect of oleic acid without the minor compounds, and obtained no effect. Regarding the linoleic acid, it is known that n-6 fatty acids convert to arachidonic acid and produce inflammatory eicosanoids through the activation of c-Jun N-terminal kinase, whereas n-3 fatty acids produce anti-inflammatory eicosanoids.[22] Indeed, it was reported that a high ratio of dietary n-3/n-6 PUFAs improved both inflammation and insulin resistance through suppressing activation of TLR4 in SD rats.[23] Although we did not use fish oil in this study, long-chain n-3 PUFAs, such as eicosapentaenoic acid and docosahexaenoic acid, are well-known to prevent insulin resistance and obesity.[11,24,25] Thus, an intake of n-3 PUFA-rich oil prevents insulin resistance accompanied by suppressing inflammation. It was noteworthy that we found that only 20% substitution of flaxseed oil for lard canceled lard-caused hyperinsulinemia and HOMA-IR as the insulin resistance index. Thus, an intake of n-3 PUFA-rich oil prevents insulin resistance accompanied by suppressing inflammation. α-linolenic acid-rich flaxseed oil is one of the good sources for n-3 PUFA and it has higher stability and lesser off-flavor compared to fish oil suggesting flaxseed oil is more effective to prevent insulin resistance than corn oil. From these reasons, it is suggested oleic acid and linoleic acid show less effective to insulin resistance compared to α-linolenic acid.

Dietary lard, corn oil, and safflower oil induced hypercholesterolemia in this research. Animal fat contains cholesterol more than vegetal oil. It is known that increase of dietary cholesterol causes negative feedback regulation of cholesterol synthesis in the liver.[26–28] In addition, reports that SFAs promote the synthesis of cholesterol and that plasma cholesterol was elevated even in corn oil safflower oil,[29] which contains low cholesterol, suggest that SFAs are likely to be involved in the increase in cholesterol caused by dietary lard. Besides, intake of flaxseed oil was the lowest level of plasma cholesterol among HF groups. This result indicated that α-linolenic acid contributes to the low plasma cholesterol.

Substitution of flaxseed oil for lard is effective to prevent hyperinsulinemia and hypercholesterolemia induced by lard: By measuring the fasting plasma glucose and insulin levels, hyperglycemia and hyperinsulinemia improve by the increasing ratio of flaxseed oil. It was noteworthy that 20% substitution (6% flaxseed oil and 24% lard) canceled hyperglycemia and hyperinsulinemia. This result suggested that increasing ratio of α-linolenic acid in muscle suppressed impairing effect of palmitic acid. The food and energy intake decreased in all flaxseed oil groups, which may be due to the mice avoiding the taste and odor. Although feed and energy efficiency are same among the HF groups, the fasting insulin level only in the 10% is not at that in the SD group. Therefore, food intake has no effect on the preventive effect on hyperglycemia and hyperinsulinemia with sub-

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stitution of flaxseed oil.

In conclusion, we found that content of α-linolenic acid in skeletal muscle negatively correlated to prevention of hyperglycemia and the intake of α-linolenic acid-rich flaxseed oil canceled lard-caused hyperglycemia and hyperinsulinemia. Although it is necessary to clarify detail mechanism of insulin resistance in the future, flaxseed oil has potential to prevent and/or improve lifestyle-related diseases.

Author Contributions

HA conducted study concept and design. YM performed experiments. MS performed statistical analyses and wrote the initial draft of the manuscript. HA and YY critically revise the manuscript. All of authors read and approved the final version of manuscript and agree to submit it to the Journal of Clinical Biochemistry and Nutrition.

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Abbreviations

AUC	area under the curve
GC	gas chromatograph
GLUT4	glucose transporter type 4
HF	high-fat diet
IRS	insulin receptor substrate
MUFA	monounsaturated fatty acid
OGTT	oral glucose tolerance test
PUFA	polyunsaturated fatty acid
SD	standard diet
SFA	saturated fatty acid
WAT	white adipose tissue

Conflict of Interest

No potential conflicts of interest were disclosed.

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