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Sesame lignans reduce LDL oxidative susceptibility by downregulating the platelet-activating factor acetylhydrolase

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Abstract. – OBJECTIVE: Low-density lipoprotein (LDL) oxidative susceptibility is recognized as a risk factor for atherosclerosis. We previously reported that the ingestion of a supplement containing sesame lignans (sesamin/episesamin) for 4 weeks reduced LDL oxidative susceptibility in humans.

MATERIALS AND METHODS: To elucidate the mechanisms underlying this observation, 12-week-old New Zealand White rabbits were fed a fat/cholesterol-enriched diet (100 g/day) for 6 weeks followed by oral administration of vehicle (control) or sesame lignans (50 mg/kg) for 4 weeks with the fat/cholesterol-enriched diet.

RESULTS: The results showed that the ingestion of sesame lignans prolonged LDL oxidation lag time, regardless of the existence of the anti-oxidative catechol metabolite of sesamin/episesamin in LDL. Plasma platelet-activating factor acetylhydrolase (PAF-AH) activity was significantly reduced by sesame lignans. The prolongation of LDL oxidation lag time was abolished by the addition of a PAF-AH inhibitor. The expression level of pro-inflammatory cytokines and macrophage infiltration observed in the liver following the feeding of the fat/cholesterol-enriched diet were also significantly reduced by sesame lignans.

CONCLUSIONS: These results indicate that sesame lignans reduce LDL oxidative susceptibility by downregulating plasma PAF-AH activity *via* the reduction of inflammation in the liver induced by fat/cholesterol-enriched diets.

Key Words:

LDL oxidation, Sesame lignans, Sesamin, Episesamin, Platelet-activating factor acetylhydrolase, Cardiovascular diseases.

Abbreviations

CTL: control; EC1: mono-catechol metabolites of episesamin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HNE: hydroxynonenal; IL-1β: interleukin-1 beta; LCAT: lecithin-cholesterol acyltransferase; LDL: low-density lipoprotein; LPO: lipid hydroperoxides; LPS: lipopolysaccharide; LysoPC: lysophosphatidylcholine; MAPK: mitogen-activated protein kinase; NOX: NADPH oxidase; oxLDL: oxidized low-density lipoprotein; oxPC: oxidized phosphatidylcholines; PAF: platelet-activating factor; PAF-AH: platelet-activating factor acetylhydrolase; PON1: paraoxonase 1; SC1: mono-catechol metabolite of sesamin; sdLDL: small-dense LDL; SE: sesame lignans; TC: total cholesterol; TNF-α: tumor necrosis factor-alfa.

Introduction

Oxidative stress is associated with many age-related diseases, such as cardiovascular diseases[1,2]. Oxidized low-density lipoprotein (oxLDL), which induces cytotoxicity in endothelial cells, secretion of adhesion molecules, monocyte migration, and foam-cell formation, plays a crucial role in the pathogenesis of atherosclerosis[2]. Stewart et al[3] have suggested that oxLDL is recognized by macrophage pattern-recognition receptors and functions to directly modulate inflammatory signaling pathways. It is, therefore, hypothesized that reducing low-density lipoprotein (LDL) oxidative susceptibility will lower the risk of cardiovascular disease, as well as reducing the inflammatory response evoked by oxLDL.

Multiple factors affect LDL oxidative susceptibility, including the concentration of free radical scavenging compounds[4], fatty acid composition[5], the concentration of lipid hydroperoxides (LPO) in LDL[6], and the size of LDL proteins[7]. LDL oxidative susceptibility is also affected by enzyme activities that eliminate LPO, such as paraoxonase 1 (PON1), lecithin-cholesterol acyltransferase (LCAT), and platelet-activating factor acetylhydrolase (PAF-AH)[8-10].

PAF-AH is an arylesterase, produced primarily in the liver, which catalyzes the hydrolysis of acetyl ester at the sn2 position of platelet-activating factor (PAF), as well as the hydrolysis of truncated oxi-

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dized phosphatidylcholines[11]. The ability of PAF-AH to decompose LPO has been shown to protect LDL against oxidation; however, a recent study[12] has also revealed that high PAF-AH activity is a risk factor for atherosclerosis. Although PAF-AH reduces oxidized phosphatidylcholines (oxPC) in LDL, the decomposition products from this process, lysophosphatidylcholine (LysoPC), and oxidized acyl chain (core aldehyde) are released[13]. LysoPC is recognized by macrophages and subsequently induces inflammatory signaling more strongly than oxPC[14], while aldehyde compounds function to modify amino-acid residues in the lipoprotein[15]. Within Kupffer cells, expression of PAF-AH and activation of the mitogen-activated protein kinase (MAPK) signaling pathway following lipopolysaccharide (LPS) treatment[16,17] serve to shorten LDL oxidation lag time[18]. PAF-AH may, therefore, be one of the key factors responsible for the exacerbation of LDL oxidative susceptibility through the initiation of inflammation.

Sesame (*Sesamum indicum*) is a traditional health food that contains lignans as biologically active compounds. Sesamin is the most abundant lignan in sesame seed. It is partly isomerized to episesamin during the refining of sesame oil, resulting in a mixture of approximately equal proportions of sesamin and episesamin. These lignans elicit multiple beneficial effects, including anti-oxidative and anti-inflammatory activities[19-23]. The main metabolites of sesamin/episesamin (SC1/EC1, respectively) exhibit potent radical scavenging properties[19,20]. Sesame lignans (sesamin/episesamin) activate liver anti-oxidative enzymes, resulting in decreasing of the concentration of LPO increased by exercise[21]. Sesamin suppresses LPS-induced inflammatory signaling[22,23]. Previously, we reported a clinical trial[24] that showed that consuming sesame lignans with α-tocopherol for 4 weeks prolongs LDL oxidation lag time. This effect is thought to be due to the anti-oxidative and anti-inflammatory effects of sesame lignans; however, the underlying mechanism is not clear.

The aim of this study was to elucidate the mechanisms by which sesame lignans reduce LDL oxidative susceptibility. Using rabbits fed a fat/cholesterol-enriched diet, we first investigated the effect of consecutive intake of sesame lignans on LDL oxidation lag time and its relationship with the concentrations of SC1 and EC1 in LDL. We also examined the effect of sesame lignans on multiple factors that affect LDL oxidation lag time, including antioxidants and LPO concentrations, compositions of fatty acids, LDL size, and enzyme activities that eliminate LPO.

Materials and Methods

Animal Experiments

The animal experiment protocol was designed according to the atherosclerosis model[25,26]. Sesame lignans (sesamin/episesamin = 1:1) were purchased from Takemoto Oil & Fat (Aichi, Japan). New Zealand white rabbits (Kitayama Labes, Nagano, Japan) were kept at 18 to 25°C and 40-70% humidity with a 12-h dark/light cycle. Following more than 1-week acclimatization on a basal diet (LRC4; Oriental Yeast, Tokyo, Japan), 12-week-old animals were acclimated stepwise to a fat/cholesterol-enriched diet through oral administration of oil to prevent poor feeding outcomes. In a preliminary study, we found that the administration of refined olive oil, commonly used as a vehicle, prolonged LDL oxidation lag time. Therefore, in this study, coconut oil contained in the diet utilized in the atherosclerosis model[26] was used as a vehicle. First, animals were fed a fat/cholesterol-enriched diet containing 10% coconut oil (Nisshin Oillio Group, Tokyo, Japan) and 0.25% cholesterol (Riken Vitamin, Tokyo, Japan) added to the basal diet at 100 g/head/day for 3 weeks. The animals were then fed a diet containing 5% coconut oil and 0.25% cholesterol added to the basal diet of 100 g/head/day and orally administered 5% coconut oil (w/w; 5 mL/head/day) for 3 weeks. At 18 weeks of age, the animals were allocated to either the control (CTL) group (n = 10) or sesame lignans (SE) group (n = 11) to equalize the plasma total cholesterol (TC) and LDL oxidation lag time, which were both measured 1 week before the administration began. Beginning at 18 weeks of age (week 0), the CTL control group was orally administered coconut oil (5 mL/head/day) and the SE group was orally administered coconut oil (5 mL/head/day) containing SE (50 mg/kg) at the same time every day for 4 weeks. Both groups were fed diets containing 5% coconut oil (w/w) and 0.25% cholesterol diets (100 g/head/day). Weekly blood samples were collected from the marginal ear vein from -6 to 4 weeks. For estimation of the relationship between the concentration of sesamin/episesamin metabolites and LDL oxidation lag time, the blood samples were collected just before and 3 and 24 h after the first SE administration on week 0, as well as 24 h after the last administration in week 4. An additional blood

sample was obtained under butorphanol and pentobarbital sodium anesthesia, 24 hours after administration on week 4 (22 weeks of age) from the carotid artery (***Supplementary Figure 1***), and livers were harvested. Blood samples were separated into tubes containing heparin (Nipro, Osaka, Japan) and ethylenediaminetetraacetic acid (EDTA)-2Na (FUJIFILM Wako Pure Chemical, Osaka, Japan) and were centrifuged at 2,200 × g for 5 min at 4°C. Supernatants and liver samples were stored at -80°C. Food intake was measured daily, and body weight was recorded weekly.

To evaluate the effect of consumption of a fat/cholesterol-enriched diet for 10 weeks on LDL oxidation lag time and the inflammatory response, 12-week-old New Zealand white rabbits were fed only basal diet (LRC4) for 10 weeks under the same conditions, and multiple markers were assayed. The sampling protocol followed what described above.

Measurement of LDL Oxidation Lag Time

LDL oxidative susceptibility was measured as previously described[27]. The specific gravity of the EDTA plasma was adjusted with potassium bromide (Nacalai Tesque, Kyoto, Japan), and the LDL fraction was obtained by ultracentrifugation at 410,000 × g for 40 min at 4°C. The protein concentrations in the LDL fraction were measured with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to 70 μg/mL with phosphate-buffered saline (PBS). To initiate LDL oxidation, 2,2'-azobis (4-methoxy-2,4-dimethyl-valeronitrile) (FUJIFILM Wako Pure Chemical, Osaka, Japan) dissolved in acetonitrile was added to the sample followed by incubation at 37°C (final concentration, 400 μM). Absorbance at 234 nm was measured every 2 min for 150 min using a spectrophotometer (UV-2600, Shimadzu, Kyoto, Japan). The LDL oxidation lag time, defined as the amount of time to the start of conjugated diene formation, was calculated based on the absorbance curve.

Analysis of Plasma Lipids

Lipids concentrations in heparin plasma were measured by L-type Wako CHO M, L-type Wako PL, and NEFA-HA test Wako (FUJIFILM Wako Pure Chemical, Osaka, Japan), and Determiner L LDL-C and MetaboLead L HDL-C (Hitachi Chemical Diagnostics Systems, Tokyo, Japan) using a Hitachi 7180 clinical analyzer (Hitachi High-Technologies, Tokyo, Japan).

Measurement of Sesamin and Episesamin Metabolites in LDL

The concentration of these metabolites in LDL was measured by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The standards, including furosemide (Nacalai Tesque, Kyoto, Japan) as an internal standard (100 μL) or test samples (100 μL) were incubated for 1 h at 37°C with β-glucuronidase/arylsulfatase (Roche Biomedical, Mannheim, Germany) in 140 mM acetate buffer (100 μL, pH 5.0). The samples were then mixed with 2% (v/v) phosphoric acid solution (200 μL) and applied to Oasis HLB 1 cc (30 mg) extraction cartridges (Waters, Milford, MA, USA). Columns were washed with 30% methanol and eluted with methanol/acetonitrile (1:1). The collected fractions were evaporated. Residues were reconstituted with methanol/water (1:1) and purified using membrane filters (0.45 μm, Merck Millipore, Darmstadt, Germany). HPLC (Agilent 1290 infinity, Agilent Technologies, Waldbronn, Germany) was connected to the mass spectrometer (4000 Q Trap, AB SCIEX, Foster City, CA, USA). The column (Cadenza CD-C18, 150 mm × 2 mm, 1.7 μm, Intakt, Kyoto, Japan) was maintained at 40°C. The mobile phase was composed of solvent A (10 mM ammonium formate water/formic acid, 2000:1) and solvent B (methanol/formic acid, 5000:1), and the flow rate was set to 300 μL/min. Metabolites were detected using the following gradient: 0-3 min, B 35%; 3-4 min, B 35-50%; 4-7 min, B 50%; 7-11.5 min, B 50-70%. In the negative mode, the transition (*m/z*) of the precursor ion to the product ion was detected as follows: SC1 and EC1, 341.1 to 175.8; internal standard, 329.2 to 285.3. Calibration curves were operated using least-squares linear regression with $1/x^2$ weighting.

Measurement of Enzyme Activity in Plasma

EDTA plasma was used to examine enzymatic activity. PAF-AH activity was measured using a PAF-AH activity assay kit (Cayman Chemical, Ann Arbor, MI, USA). LCAT activity was determined using an LCAT activity assay kit (Sigma-Aldrich Japan, Tokyo, Japan). Since PON1 is a calcium-dependent enzyme, the EDTA in the plasma was replaced with PBS using Amicon Ultra (Merck Millipore, Darmstadt, Germany). PON1 activity was then measured using a PON1 activity assay kit (BioVision, Milpitas, CA, USA). All assays were conducted according to the manufacturers' instructions. PAF-AH and PON1 ac-

tivities were normalized to the concentration of plasma protein, and LCAT activity was compared among samples using the ratio of hydrolyzed to intact substrate.

The Effect of PAF-AH Inhibition on LDL Oxidative Lag Time

LDL was incubated with 0.1 mM Pefabloc (Sigma-Aldrich, Tokyo, Japan) or PBS at 37°C for 30 min. LDL oxidation lag time was then measured as described above.

Measurement of α-Tocopherol Concentrations in LDL

The concentration of α-tocopherol was measured by HPLC method (NIKKEN SEIL, Tokyo, Japan). Briefly, diluted LDL with PBS (1:4, 200 μL) was added α-tocopherol acetate as an internal standard (200 μL) and was extracted by n-hexane. Collected fractions were evaporated. The residues were reconstituted with ethyl acetate (20 μL) and ethanol (10^5 μL), then, analyzed by HPLC (Chromaster, Hitachi High-Tech Science, Tokyo, Japan). The column (Wakosil 5C18 column, FUJIFILM Wako Pure Chemical, Osaka, Japan) was maintained at 27°C. The mobile phase was composed of solvent A (acetonitrile/dichloromethane/methanol, 85:5:10) and solvent B (6:3:1), and the flow rate was set to 1 mL/min. α-tocopherol were detected by using the following gradient: 0-0.5 min, B 0%; 0.5-30 min, B 0-100% with a fluorescence detector (excitation wavelength 295 nm, emission wavelength 335 nm). Concentrations were normalized to the amount of total protein of LDL.

Analysis of LDL Density

Lipoprotein density was analyzed by HPLC using a gel filtration column (Lipo SEARCH, Skylight Biotech, Tokyo, Japan). The proteins were separated into 20 fractions based on size, of which fractions 8-13 were determined to contain LDL, whereas fractions 10-13 contained small-dense LDL (sdLDL). The composition ratio was calculated and normalized to the amount of cholesterol.

Analysis of Fatty Acid Composition in LDL

Lipids in LDL were extracted and purified using a method described by Bligh and Dyer[28]. Fatty acid in total lipids was transmethylated by methanolic HCl. Fatty acid methyl esters were extracted by n-hexane and analyzed with the gas-liquid chromatography as previously described[29].

Measurement of LPO in LDL

LPO were extracted from LDL into chloroform and measured using a lipid hydroperoxides assay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. The results were normalized to the amount of total protein of LDL.

Measurement of Enzymatic Activity in the Liver

Liver tissues were homogenized in ice-cold PBS with a protease and phosphatase inhibitor cocktail (100 x) (Thermo Fisher Scientific, Waltham, MA, USA). The homogenates were then centrifuged at 10,000 × g for 20 min, and extracellular PAF-AH activity in the supernatants was measured using a PAF-AH activity assay kit. The results normalized to the amount of total protein.

Quantitative Real Time-PCR

Total RNA was extracted from the liver using the RNeasy kit (QIAGEN, Hilden, Germany) and cDNA was synthesized from 2 μg of total RNA using a high-capacity cDNA reverse transcription kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Gene expression was then measured using StepOnePlus (Applied Biosystems, Foster City, CA, USA). TaqMan fast universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and the TaqMan probes were added to cDNA; or, primers (Table I) and iTaq universal SYBR green supermix (Bio-Rad Laboratories, Hercules, CA, USA) were added. Gene expression was normalized to GAPDH expression. TaqMan probes were purchased from Applied Biosystems (Foster City, CA, USA); Oc03397715_m1 for tumor necrosis factor-alfa (TNF-α), Oc03823250_s1 for interleukin-1 beta (IL1-β) and Oc03823402_g1 for GAPDH.

Immunohistochemistry

Macrophages recognized by the RAM11 antibody in the liver were identified by immunohistochemical staining. Livers were fixed in paraformaldehyde, and the tissue sections were subsequently dewaxed in xylene and rehydrated in alcohol. Autoclave antigen retrieval and endogenous peroxidase quenching with 1% hydrogen peroxide were then carried out. The sections were then incubated with skim milk to block nonspecific antibody binding, and the slides were incubated overnight with RAM-11 antibody (M0633, DAKO Japan, Tokyo, Japan) at a 1:100 dilution at 4°C followed by incubation with biotin secondary antibody (K0609,

Table I. Primer sequences.

Gene	Forward	Reverse
PAF-AH	5'-CCACCCAAATTGCATGTGC-3'	5'-GCCAGTCAAAAGGATAAACCACAG-3'
GAPDH	5'-GCCAAGGTCATCCATGACAAC-3'	5'-GGGGCCATCCACAGTCTTC-3'

DAKO Japan, Tokyo, Japan). The sections were stained with DAB substrate (343-00901, FUJIFILM Wako Pure Chemical, Osaka, Japan), and counterstained with Mayer's hematoxylin (Muto pure chemicals, Tokyo, Japan). Macrophages were counted with a light microscope (BX52, Olympus, Tokyo, Japan) using Lumina Vision software (Mitani, Tokyo, Japan) from 5 random fields for each animal. The results are expressed as numbers of macrophages per 1 mm^2.

NADPH Oxidase (NOX) Activity Assay

NOX activity was measured using a lucigenin-enhanced chemiluminescence assay, as previously described[30]. Briefly, the liver tissues were homogenized in Krebs buffer (20 mM HEPES, 119 mM NaCl, 4.7 mM KCl, 1 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 0.15 mM Na_2HPO_4 and 1.25 mM $CaCl_2$). The membrane fractions were obtained from liver tissue by ultracentrifugation at 100,000 × g for 60 min at 4°C and resuspended in Krebs buffer. Aliquots of the membrane fractions (4.5 µg of protein) were added together with 50 µM lucigenin (Sigma-Aldrich Japan, Tokyo, Japan), 200 µM NADPH (Sigma-Aldrich Japan, Tokyo, Japan), 1 µL DMSO or VAS-2870 (NADPH oxidase inhibitor, final concentration 2.5 µM, Sigma-Aldrich Japan, Tokyo, Japan). The reaction was followed under temperature-controlled conditions (37°C). Light emission was measured every 5 min for 60 min using an Infinite M200 PRO (Tecan Japan, Kanagawa, Japan) plate reader in the chemiluminescence mode. The results are expressed as the difference between the areas under the curve in the absence and presence of VAS-2870.

Immunoblotting

Oxidized proteins were quantified, as previously described[31]. Briefly, liver tissue was homogenized in RIPA buffer with protease and phosphatase inhibitor and 2x sample buffer solution (Bio-Rad Laboratories, Hercules, CA, USA) with 0.01% (w/v) β-mercaptoethanol and was heated at 95°C for 5 min. Aliquots containing 1 µg of protein per well were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted on polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Colored molecular weight standards (ATTO, Tokyo, Japan) were run simultaneously. The membranes were incubated for 1 h in PVDF blocking buffer (Toyobo Co., Ltd., Osaka, Japan) and incubated overnight in the presence of either anti-4 hydroxynonenal (ab46545, Abcam, Cambridge, UK) or anti-GAPDH (ab181602, Abcam, Cambridge, UK) primary antibodies (1:1000). After a subsequent incubation for 60 min at 25°C in the presence of the HRP-conjugated secondary antibody (NA934, 1:5000, GE Healthcare Japan, Tokyo, Japan), the complexes were visualized by chemiluminescence. Films were scanned, and densitometric analysis was performed using Fusion FX (Vilber Lourmat, Collégien, France). Total band densities were normalized to the GADPH content.

Statistical Analysis

All data are expressed as means ± SEM. For statistical analysis, Student's *t*-tests were performed to compare the CTL and SE group at 4 weeks and repeated measured two-way ANOVA was performed to analyze the effect of single ingestion using Statistical Package for the Social Sciences (SPSS) version 25 (International Business Machines Corporation, Armonk, NY, USA). Differences were considered significant when $p < 0.05$.

Results

Effect of Sesame Lignans on Body Weight, Food Intake, and Plasma Lipids

Body weights gradually increased throughout the acclimation and the experimental period (-6 to 4 weeks) in both groups (***Supplementary Figure 2A***, ***Table II***). Consumption of a fat/cholesterol-enriched diet served to increase TC from -6 weeks until 0 weeks (***Supplementary Figure 2B***) and stabilized from 0-4 weeks (Table II). No significant differences were observed between the CTL and SE groups in body weight and TC at any of the time-points (Table II). At the end of the experiment, there were no differences in the plasma levels of LDL cholesterol,

Table II. Bodyweight, food intake, and total plasma cholesterol in rabbits treated with or without SE.

	0 w		4 w		*p*-value	
	CTL	SE	CTL	SE	0 w	4 w
Body weight (g)	2920 ± 121	2925 ± 146	3069 ±125	3053 ±178	*0.93*	*0.82*
Food intake (g)	68.3 ± 3.3	67.9 ± 1.9	58.9 ± 2.8	64.1 ± 3.0	*0.74*	*0.23*
TC (mmol/L)	24.5 ± 5.5	25.3 ± 7.5	26.1 ± 6.4	25.2 ± 6.5	*0.62*	*0.74*

Data are shown as means ± SEM. No significant differences were observed between the two groups at each timepoint by Student's *t*-test, $n = 10$ (control group, CTL), $n = 11$ (sesame lignans group, SE). Food intake is shown as a weekly average, 0 to 1st week, 3rd to 4th weeks. TC: total cholesterol.

high-density lipoprotein cholesterol, phospholipids, and non-esterified fatty acid between the 2 groups. The amount of food intake did not decrease following the administration of a fat/cholesterol-enriched diet. Oral administration of coconut oil tended to reduce food intake; however, there was no significant difference between the 2 groups.

Effect of Sesamin and Episesamin Intake on LDL Oxidation Lag Time and the Concentration of Metabolites in LDL

No significant differences were observed in LDL oxidation lag time at 0 weeks between the two study groups (***Supplementary Table I***). In the SE group, following 4 weeks of SE administration, the LDL oxidation lag time was significantly longer compared to the CTL group at 4 weeks (Figure 1). The concentrations of SC1 and EC1 were low following 24 hours administration of SE at 4 weeks (SC1; 0.4 ± 0.1 ng/mg LDL protein, EC1; 0.3 ± 0.1 ng/mg LDL protein).

The relationship between the concentration of SC1/EC1 and LDL oxidation lag time was evaluated after the first administration of SE at 0 weeks. LDL oxidation lag time did not change at 3 nor at 24 hours after the first administration at 0 weeks in both groups (***Supplementary Table I***). Sesame lignans metabolites SC1 and EC1 were detected at relatively high concentrations in the LDL fraction obtained 3 hours after the first administration of SE; however, both SC1 and EC1 were only detected at low levels following 24 hours administration of SE at 0 weeks. Of note, the increase in LDL oxidation lag time was not correlated with the concentrations of sesamin and episesamin metabolites.

PAF-AH Inhibition on LDL Oxidation Lag Time

PAF-AH activity was significantly lower in the SE group following 4 weeks of SE administration than that of the CTL group (Figure 2A).

Since PAF-AH activity in LDL was significantly different between the CTL and SE groups, we next evaluated the effect of a PAF-AH inhibitor (pefabloc) on LDL oxidation lag time. Treatment of the LDL fraction with the PAF-AH inhibitor eliminated the difference between LDL oxidation lag times (Figure 2B).

Phospholipase Activity in Plasma, the Concentration of Antioxidants, LPO, Fatty Acids in LDL, and the Proportion of sdLDL

There were no significant differences in the activities of PON1 (CTL; 0.38 ± 0.08 mU/mg protein, SE; 0.36 ± 0.06 mU/mg protein) and LCAT (relative activity CTL; 1.00 ± 0.01, SE; 1.04 ±

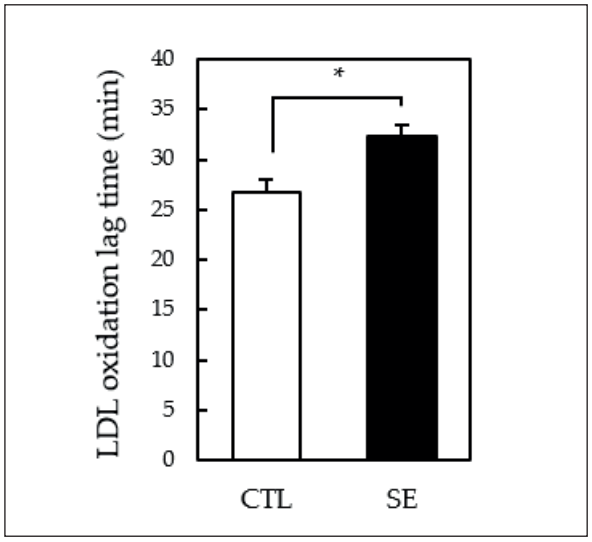

Figure 1. Effects of sesame lignans on LDL oxidation lag time. Animals were administrated vehicle (CTL) or 50 mg/kg of sesame lignans (SE) for 4 weeks. LDL was fractionated from plasma collected 24 hours after the last administration. Values are shown as means ± SEM. There was a significant difference between the 2 groups: *$p<0.05$, Student's *t*-test, $n=10$ (CTL), 11 (SE).

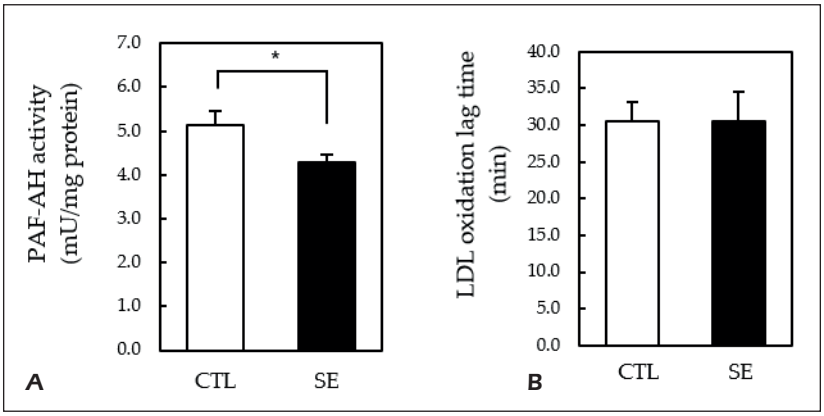

Figure 2. Effects of sesame lignans on PAF-AH activity and LDL oxidation lag time. (**A**) PAF-AH activity in plasma and (**B**) LDL oxidation lag time after incubation with the PAF-AH inhibitor pefabloc. Values are shown as means ± SEM. There was a significant difference between the control and SE groups: *, $p<0.05$, Student's *t*-test, $n=10$ (CTL), 11 (SE).

0.01) at 4 weeks between the CTL and SE groups.

No differences were observed in the concentrations of α-tocopherol and LPO in LDL, the fatty acid composition, and the sdLDL ratio between groups (***Supplementary Table II***).

Inflammation and Oxidation Status in the Liver

When inflammation occurs in the liver, local PAF-AH activity increases, which subsequently leads to increased PAF-AH activity in the plasma. We, therefore, evaluated PAF-AH activity and inflammatory state in the liver. Activity and gene expression of PAF-AH were significantly lower in the livers of animals in the SE group at 4 weeks relative to controls (Figure 3A, 3B). The expression of inflammatory cytokine IL-1β was significantly decreased in the SE group; however, the results for TNF-α were not statistically significant (Figure 3B). The number of RAM-11$^+$ macrophages was significantly lower in the SE group compared to the CTL group (Figure 3C, 3D). The level of NOX, which stimulates the differentiation of inflammatory macrophages, was significantly decreased (***Supplementary Figure 3A***). The level of 4-hydroxynonenal (4-HNE) adduct protein, served as a marker of oxidative stress, was also decreased in the SE group (***Supplementary Figure 3B, 3C***).

Parameters of Animals Fed the Basal Diet

To evaluate whether intake of a fat/cholesterol-enriched diet for 10 weeks (from -6 weeks to 4 weeks) affected LDL oxidation lag time and inflammatory conditions, several markers were measured using the same aged rabbits fed a basal diet. TC, LDL oxidation lag time, plasma and liver PAF-AH activity, and the number of liver macrophages were all markedly lower in animals fed the basal diet than in animals fed fat/cholesterol-enriched diets (***Supplementary Table III***).

Discussion

We previously reported that sesame lignans with α-tocopherol ingestion for 4 weeks reduced LDL oxidative susceptibility in humans[24]; however, the underlying mechanism was not clear. In the present study, we investigated how sesame lignans reduce LDL oxidative susceptibility in a rabbit atherosclerosis model. We detected that sesame lignans prolonged LDL oxidation lag time independent from the anti-oxidative metabolites SC1 and EC1 concentration in LDL. PAF-AH activity was significantly lower in the plasma compared to that in the control group, while the addition of a PAF-AH inhibitor completely suppressed the difference of LDL oxidation lag time between the two groups. Sesame lignans have no effects on various other factors that affect LDL oxidation lag time. These results suggest that the downregulation of PAF-AH activity may contribute to the reduction of LDL oxidation susceptibility.

Ingested sesamin and episesamin were metabolized by P450 to the mono-catechol metabolites SC1 and EC1 in rabbits, in accordance with the previous study in humans[32]. Since SC1 and EC1 have potent radical scavenging properties[19,20], the relationship between the concentration of SC1/EC1 and LDL oxidation lag time was investigat-

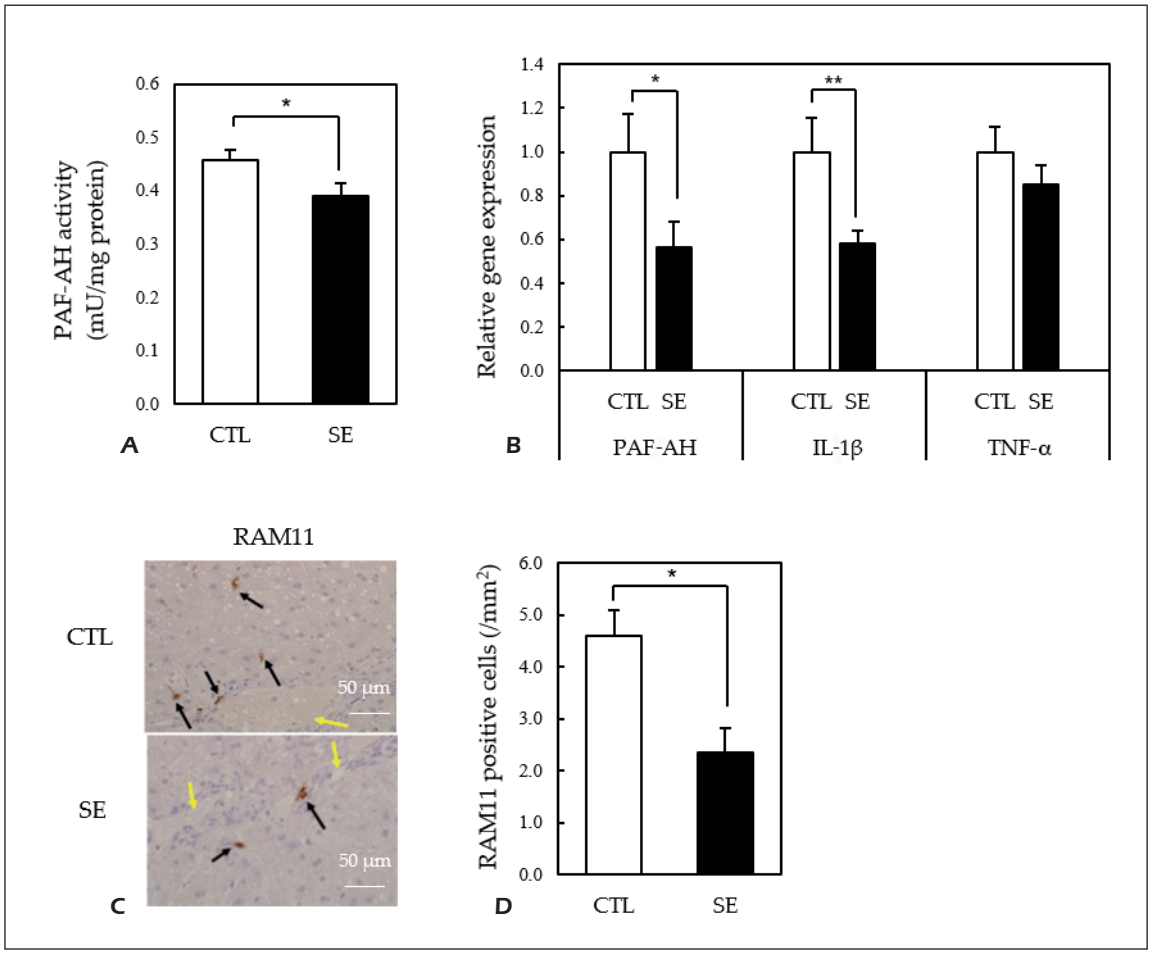

Figure 3. Effects of sesame lignans on the inflammatory state of the liver. (**A**) PAF-AH activity and (**B**) relative gene expression of PAF-AH, IL1-β, and TNF-α. Gene expression was normalized to GAPDH expression. (**C**) Representative photos and (**D**) average numbers of macrophages in the liver. Macrophages were counted as RAM-11 positive cells (black arrows) per area. Yellow arrows indicate the presence of vessels. Values are shown as means ± SEM. There are significant differences between the CTL and SE groups: **p*<0.05, ***p*<0.01, Student's *t*-tests, *n*=10 (CTL), 11 (SE).

ed on the first day of sesame lignans ingestion. The concentration of these metabolites clearly increased 3 hours after ingestion; however, during this period, the LDL oxidation lag time did not change. Although the concentration of these metabolites was barely detectable 24 hours after the last ingestion of sesame lignans for 4 weeks, LDL oxidative susceptibility was significantly reduced, suggesting that the effect of sesamin and episesamin on LDL oxidative susceptibility was not dependent on the direct radical scavenging properties of their metabolites.

Sesame lignans have been reported to increase the bioavailability of vitamin E in rodents[33], prolonging LDL oxidation lag time. However, in this study, 4 weeks of sesame lignans ingestion did not significantly affect the concentration of α-tocopherol (a type of vitamin E) in LDL. Ikeda et al[21] reported that sesame lignans increased the activity of glutathione peroxidase, an enzyme that reduces LPO, consequently suppressing LPO in the plasma induced by exercise. Although LPO in LDL has been shown to exacerbate LDL oxidative susceptibility[4], in our study, the ingestion of sesame lignans did not significantly affect the concentration of LPO in LDL. Small dense LDL is known to be a risk factor for cardiovascular disease since it is easily oxidized. Polyunsaturated fatty acids are also vulnerable to oxidative stress[5,7]. However, in this study, no significant changes were observed in the proportion of sdLDL in LDL or in the fatty acid composition of LDL following 4 weeks of sesame lignans ingestion. These results suggest that neither the concentration of antiox-

idants, nor of LPO in LDL, nor the size or fatty acid composition of LDL contributed to reducing LDL oxidative susceptibility by sesame lignans.

Enzymes that decompose LPO, such as PON1 and LCAT, protect LDL from oxidation under high oxidative stress conditions[8]. Sesame lignans did significantly decrease the activity of PAF-AH, but not those of PON1 and LCAT (Figure 2). PAF-AH decomposes LPO and formerly has been recognized to exert an anti-inflammatory function by degrading PAF, a known pro-inflammatory molecule. However, recent studies have revealed that PAF-AH also produces LysoPC as a byproduct of the decomposition of truncated oxidized phosphatidylcholines. LysoPC is a chemical mediator that is highly associated with the development of arteriosclerosis[14], and as such, is attracting attention as an inflammation-inducing factor[34,35]. LDL with highly activated PAF-AH exhibited short LDL oxidation lag times[9,10], and treatment with phospholipase A2, a type of PAF-AH, increased the level of negatively-charged LDL[36], which is easily oxidized[37]. It, therefore, appears that the activation of PAF-AH in plasma exacerbates LDL oxidative susceptibility. Together, these results suggest that sesame lignans may reduce LDL oxidative susceptibility through downregulation of plasma PAF-AH activity.

Plasma PAH-AH is synthesized in hematopoietic cells and hepatocytes; hence, the liver is its primary source[13]. LPS induces the expression and activity of PAF-AH through activation of the p38 MAPK signaling pathway in Kupffer cells[16,17] and also exacerbates LDL oxidative susceptibility[18]. In this study, fat/cholesterol-enriched diets induced inflammatory responses, which is in agreement with a previous study[25]. We showed that these diets shortened LDL oxidation lag time compared to control animals (***Figure 1, Supplementary Table III***). In previous studies, sesamin suppressed inflammatory responses, including LPS-induction of TNF-α and IL-1β, *via* inhibition of the p38 MAPK signaling pathway *in vitro* and *in vivo*[22,23]. We, therefore, hypothesize that sesame lignans reduce plasma PAF-AH activation by suppressing the macrophage activation in the liver *via* inhibition of the p38 MAPK signaling pathway, thereby reducing LDL oxidation susceptibility. Sesame lignans reduced the activity of NOX, which produces reactive oxygen species and stimulates macrophage differentiation[38], and the level of 4-HNE, a oxidative stress marker in the liver. Oxidative stress is strongly associated with inflammation. However, further investigation is required to clarify the contribution of the anti-oxidative properties of sesame lignans to reduced LDL oxidative susceptibility and inflammation.

These study findings were obtained under experimental conditions in animals that were fed fat/cholesterol-enriched diets to induce inflammation. However, plasma PAF-AH activity increases with age in active, PAF-AH-genotyped, healthy Japanese subjects[39]. The LDL oxidation lag time has been shown to be shorter for individuals in their 30s compared to those in their 20s[27]. Since LDL oxidative susceptibility and PAF-AH activation are recognized as risk factors for cardiovascular disease[40], it is possible that consecutive ingestion of sesame lignans will reduce the risk of cardiovascular disease with aging through reduction of LDL oxidative susceptibility, especially in individuals with high PAF-AH activity.

Conclusions

This study revealed that sesame lignans reduce LDL oxidative susceptibility through the downregulation of PAF-AH activity by suppressing inflammation in the liver induced by a fat/cholesterol-enriched diet. Therefore, the consecutive ingestion of sesame lignans may be effective in the maintenance of health by reducing LDL oxidative susceptibility.

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Author Contributions

Conceptualization, YN, HO, YO and TR; Data curation, YN and YO; Formal analysis, YN and HO; Investigation, YN and HO; Project administration, YO; Supervision, YK and TR; Writing – original draft, YN; Writing – review & editing, YO, YK, TR and HS.

Ethics Approval

All protocols for animal procedures were approved by the Ethics Committee of Animal Experiment at the Suntory Holdings and Kitayama Labes in accordance with the Internal Regulations on Animal Experiments established by the Suntory and Kitayama Labes, which are based on the Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, amended 2 June 2017).

Conflict of Interests

YN, HO, YO, YK, TR, and HS are employees of Suntory Wellness Limited, which is a manufacturer of foods that contain sesame lignans and vitamin E. The authors have no conflicts of interest to declare. This research was conducted with a fund from Suntory Wellness Limited, to which the authors belong.

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