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Effect of Polyphenol-Rich Extract from Walnut on Diet-Induced Hypertriglyceridemia in Mice via Enhancement of Fatty Acid Oxidation in the Liver

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The kernel pellicles of walnut are rich in ellagitannins with antioxidative activity. A polyphenol-rich extract from walnuts (WP, 45% polyphenol) was prepared and evaluated for its hypolipidemic effect in high fat diet fed mice. Oral administration of WP (100 and 200 mg/kg) significantly reduced liver weight and liver and serum triglycerides (TG). Hepatic β-oxidation in cytosol, including peroxisome, was enhanced by WP (50−200 mg/kg). mRNA expressions of hepatic peroxisome proliferator-activated receptor (PPAR) α and acyl coenzyme A oxidase (ACOX) 1 were enhanced by WP (50−200 mg/kg). With respect to the hypotriglyceridemic mechanism of WP, it suppressed neither olive oil induced serum TG elevation in mice nor oleic acid induced TG accumulation in HepG2 cells. On the other hand, mRNA expressions of PPARα, ACOX1, and carnitine palmitoyltransferase (CPT) 1A in HepG2 cells were significantly enhanced by addition of WP (100 μg/mL). Moreover, tellimagrandin I, a polyphenolic constituent in WP, enhanced ACOX1 expression at 1−100 μg/mL. In conclusion, WP was found to possess hypotriglyceridemic activity via enhancement of peroxisomal fatty acid β-oxidation in the liver. These results suggest that tellimagrandin I is involved in the hypotriglyceridemic mechanism of WP.

KEYWORDS: Walnut; polyphenol; hypertriglyceridemia; β-oxidation; acyl coenzyme A oxidase; tellimagrandin I

INTRODUCTION

Recently, prevention of metabolic syndrome that leads to cardiovascular disease has been recognized to be important (*1*). The dominant underlying factor for the syndrome is accumulation of abdominal fat, which causes hyperlipidemia, hypertension, and hyperglycemia. Improvements in lifestyle such as reducing high calorie intake and increasing exercise are effective for prevention of metabolic syndrome.

Walnuts, the seeds of *Juglans regia* L., are one of the most popular nuts in the world. The seeds contain unsaturated fatty acids such as oleic acid and α-linolenic acid (*2, 3*) and also are rich in vitamin E and minerals (*4*−*6*). Various epidemiological studies of walnut have revealed that ingestion of walnuts reduces the risk of lifestyle-related diseases. Recent investigations reported that a walnut diet improved arteriosclerosis (*7*), hypercholesterolemia (*5, 8, 9*), cardiovascular disease (*10, 11*), hypertriglyceridemia (*12, 13*), and diabetes mellitus (*14*). On the other hand, there are several studies that deny the effectiveness of walnuts on metabolic syndrome (*15, 16*) and obesity (*17*).

The kernel pellicle of the walnut is rich in polyphenols and contains numerous ellagitannins (*18*−*21*). Walnut polyphenol (WP) was reported to have antidiabetic (*22*) and inhibitory activity against oxidation of low-density lipoprotein (*23*). Moreover, recently we found that ellagitannins suppressed liver injury induced by carbon tetrachloride and D-galactosamine (*24*). We hypothesized that WP may improve obesity symptoms such as metabolic syndrome and hyperlipidemia. Thus, we investigated the effect of polyphenol-rich walnut extract (WP) on high fat diet induced mouse obesity models. In this study we

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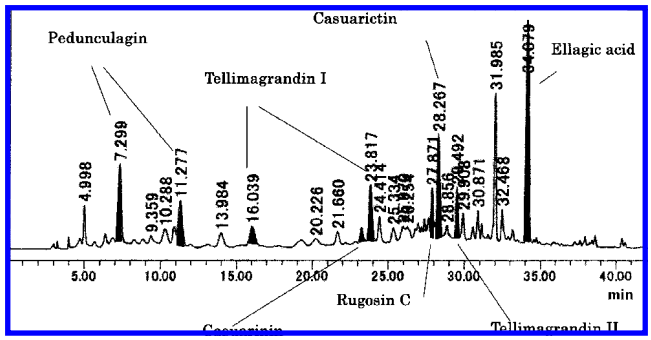

Figure 1. HPLC chromatogram of WP.

investigated the hypotriglycemic activity of WP, its mechanism, and the hypotriglycemic constituents in WP.

MATERIALS AND METHODS

Reagents. Cholesterol E Test Wako, Triglyceride E Test Wako, dithiothreitol, and Triton X-100 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A Mitochondria Isolation Kit for Tissue and BCA Protein Assay Kit were purchased from Pierce (Rockford, IL). β-Nicotinamide adenine dinucleotide (NAD) hydrate from yeast, bovine serum albumin (BSA) fraction V, coenzyme A (CoA) sodium salt hydrate from yeast, flavin adenine dinucleotide (FAD) disodium salt hydrate, palmitoyl-CoA lithium salt, Dulbecco's modified Eagle medium (D-MEM), penicillin and streptomycin mixture solution, bezafibrate and oleic acid−albumin from bovine serum were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). An RNAeasy Protect Mini Kit was purchased from Qiagen (Hilden, Germany). Random hexamers, 10 mM dNTP mixture (PCR grade), an RNase inhibitor, and fetal calf serum (FCS) were obtained from Invitrogen Co. (Carlsbad, CA). PrimeScript Reverse Transcriptase and SYBR Green I were purchased from Takara Bio Inc. (Otsu, Japan).

Preparation of WP and Polyphenolic Constituents. Dried kernel pellicles (10 kg) of walnuts cultivated in China were powdered and extracted at 80 °C for 2 h with 50 L of 50% (v/v) ethanol. The solvent was subsequently evaporated. The yield of the spray-dried WP powder was 10.5%, which contained 45% polyphenols as determined according to the Folin−Ciocalteu method (gallic acid equivalents). The contents of principal polyphenols were determined by HPLC equipped with a 250 × 4.6 mm i.d. Develosil RPAQUEOUS C30 column (Nomura Chemical Co., Ltd., Aichi, Japan). The flow rate was fixed at 1 mL/min using the following eluents with the linear gradient mode: solvent A (10 mM H_3PO_4/10 mM KH_2PO_4/CH_3CN, 45:45:10), solvent B (10 mM H_3PO_4/10 mM KH_2PO_4/CH_3CN, 30:30:40); 10 min at 100% A isocratic; from 0 to 100% B in 30 min; 10 min at 100% B isocratic. The wavelength for UV detection was 280 nm, and each compound was identified by direct comparison with authentic samples (**Figure 1**). The contents of pedunculagin (**1**), tellimagrandin I (**2**), tellimagrandin II (**3**), and ellagic acid (**4**) in the WP extract were 5.8, 2.8, 1.4, and 5.2%, respectively. Polyphenolic compounds (**Figure 2**) were isolated according to the previously reported method (*19*).

Animals and Cells. Male ddY mice (5 and 10 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in an air-conditioned room (23 ± 1 °C, 50 ± 10% RH) for 3 or more days and fed a standard CE-2 nonpurified diet (Clea Japan Inc., Shizuoka, Japan) and tap water ad libitum. The experiments were performed in accordance with the Guidelines for Animal Experimentation (Japan Association for Laboratory Animal Science, 1987). HepG2, a human hepatocellular carcinoma cell line (JCRB1054), was obtained from Health Science Research Resources Bank (Osaka, Japan).

Lipid Parameter Changes in Mice Fed a High-Fat Diet. Mice aged 10 weeks were fed a high-fat diet (HFD32) (Clea Japan Inc.) for 13 days. HFD32 contains 32% fat consisting of oleate (64.3%), palmitate (12.6%), linoleate (3.26%), and other free fatty acids. CE-2 was fed to the normal group. WP (50, 100, and 200 mg/kg) suspended in water was given orally once a day for 13 days. On the 13th day, the mice were fasted for 22 h. The mice were anesthetized with ether, and blood was collected from the abdominal aorta and the livers; epididymal

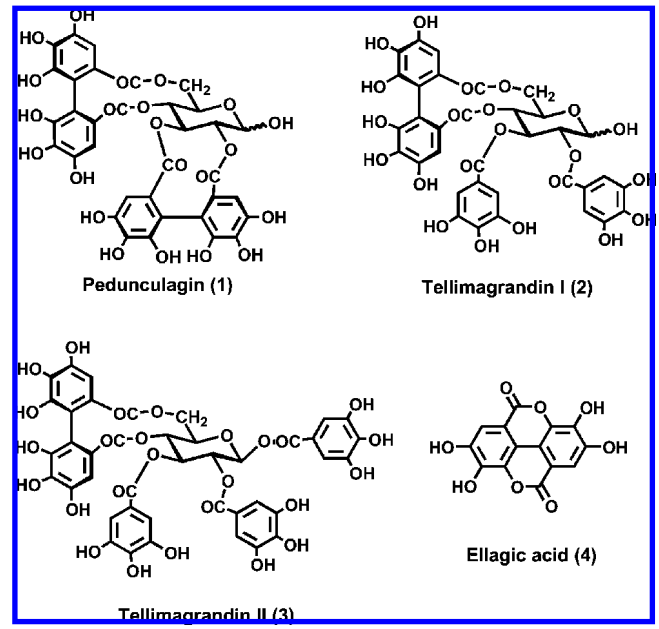

Figure 2. Major polyphenolic compounds isolated from walnuts.

fats and paranephrical fats were removed. Serum triglyceride (TG) and cholesterol were determined by a Triglyceride E Test Wako and Cholesterol E Test Wako, respectively. Liver TG and cholesterol were determined as follows (*25*). Liver segments (approximately 200 mg) were homogenized in a 19 times weight mixture of chloroform and methanol (2:1) to extract liver lipids. After centrifugation (2800*g*, 10 min) of the homogenates, supernatants (200 μL) were transferred to the other test tubes. The solvents were removed by flushing with N_2 gas, and the same volumes (200 μL) of phosphate-buffered serine without calcium and magnesium [PBS(−)] were added to the tubes. The mixtures were sonicated, and their TG and cholesterol contents were determined according to the previously mentioned kits.

Measurements of Mitochondrial and Cytosolic β-Oxidations. The mouse liver segments (approximately 200 mg) were homogenized in 800 μL of PBS(−). The homogenates were centrifuged (1000*g*, 3 min, 4 °C), and the pellets were treated with a Mitochondria Isolation Kit for Tissue. The mitochondrial fraction (precipitate) and cytosolic fraction (supernatant) were separated by centrifugation (3000*g*, 15 min, 37 °C). The mitochondrial fraction suspended in Tris-HCl buffer (pH 8, 100 μL) and the cytosolic fraction were used to measure β-oxidative abilities. The protein content in both solutions of the fraction was determined by a BCA Protein Assay Kit. β-Oxidative ability was determined according to the method of Lazarow (*26*). Tris-HCl buffer (pH 8, 940 μL) was mixed with 20 mM NAD (10 μL), 0.33 M dithiothreitol (3 μL), 1.5% BSA (5 μL), 2% Triton X-100 (5 μL), 10 mM CoA (10 μL), 1 mM FAD (10 μL), and 5 mM palmitoyl-CoA (10 μL). The mixture (993 μL) was preincubated (37 °C, 5 min) and mixed with the solution of the mitochondrial fraction (2 μL) or cytosol fraction (20 μL). The decrease in absorbance at 340 nm caused by the reduction of NAD to NADH was measured for 5 min.

Real Time RT-PCR Analysis of Liver mRNA Expression Related to Fatty Acid Metabolism. The liver segments (approximately 100 mg) of mice fed the HFD were soaked in RNAlater (RNA stabilization reagent), attached to an RNAeasy Protect Mini Kit, and stored at 4 °C. Total RNA was extracted and purified by the RNAeasy Mini Kit, and the cDNA was synthesized by random hexamers, dNTP mixture, PrimeScript Reverse Transcriptase, and an RNase inhibitor. RT-PCR was performed by a TM800 Thermal Cycler Dice Real Time System (Takara Bio Inc., Otsu, Japan) using SYBR Green I and the following primers. Primers (5′→3′) used for the mice RNA experiments were as follows: peroxisome proliferator-activated receptor (PPAR) α (forward) gagagccccatctgtcctctctc, (reverse) gagcccggacagcttcctaagta; acyl coenzyme A oxidase (ACOX) 1 (forward) agcgagccagagccccag, (reverse) tcaggcagctcactcagg; carnitine palmitoyltransferase (CPT) 1A (forward) ggatctacaattcccctctgc, (reverse) gcaaaataggtctgccgaca; and β-actin (forward) aatcgtgcgtgacatcaaag, (reverse) gaaaagagcctcagggcat.

Table 1. Effect of WP on Lipid Parameters in Mice Fed a HFD[a]

	diet				
		HFD			
	CE-2		WP		
	normal	control	50 (mg/kg)	100 (mg/kg)	200 (mg/kg)
		Body Weight			
initial (g)	40.2 ± 0.2	40.6 ± 0.7	40.0 ± 0.6	39.4 ± 0.3	38.7 ± 0.3
day 13 (g)	43.9 ± 0.4	47.7 ± 1.4	45.2 ± 1.2	44.7 ± 0.6	44.0 ± 1.4
increase (g)	3.7 ± 0.5**	7.0 ± 0.9	5.3 ± 0.9	5.2 ± 0.7	5.3 ± 1.4
		Organ Weight			
liver (g)	1.56 ± 0.03	1.60 ± 0.08	1.44 ± 0.07	1.41 ± 0.03*	1.34 ± 0.03**
paranephric fat (mg)	261 ± 48**	736 ± 88	756 ± 88	729 ± 106	773 ± 161
epididymal fat (mg)	732 ± 92**	1814 ± 282	1817 ± 188	1768 ± 150	1637 ± 227
		Liver Lipid			
TG (mg/g)	21.7 ± 3.4*	31.8 ± 3.9	32.9 ± 4.8	25.6 ± 3.2*	25.8 ± 5.5*
cholesterol (mg/g)	4.5 ± 0.7	6.2 ± 0.9	8.0 ± 1.5	5.6 ± 0.9	6.0 ± 1.2
		Serum			
TG (mg/dL)	148 ± 10	181 ± 21	98 ± 34**	82 ± 6**	98 ± 13**
cholesterol (mg/dL)	165 ± 5*	218 ± 14	225 ± 12	220 ± 14	220 ± 18

[a] Each value represents the mean with the SE ($n = 5-7$). Asterisks denote significant differences from the control group at *, $p < 0.05$, and **, $p < 0.01$, respectively.

Serum TG Elevation in Olive Oil Loaded Mice. The test was performed according to the previously reported method (*27*). WP suspended in water was given orally to fasted (20 h) mice (5 weeks old). One hour later, 5 mL/kg of olive oil was administered orally to the mice. Blood samples were collected from the infraorbital venous plexus 2, 4, and 6 h after olive oil loading. Serum TG was determined by the Triglyceride E Test Wako.

Oleic Acid Induced Fat Accumulation in HepG2 Cells. The test was performed according to the method of Okamoto et al. (*28*). HepG2 cells (4×10^4 cells/500 μL) suspended in D-MEM containing 10% FCS, penicillin (100 μg/mL), and streptomycin (100 μg/mL) were inoculated onto a 24-well culture plate and cultured for 24 h at 37 °C under a 5% CO_2 atmosphere. The medium was changed to a new one containing 0.5 mM oleic acid and WP. After 48 h of culture, the medium was removed and 200 μL of PBS(−) was added to each well. The cells were sonicated, and the TG content was determined by the Triglyceride E Test Wako.

mRNA Expressions in Hepatocytes Related to Fatty Acid Metabolism. HepG2 cells (4×10^4 cells/500 μL) suspended in D-MEM containing 10% FCS, penicillin (100 μg/mL), and streptomycin (100 μg/mL) were inoculated onto a 24-well culture plate and cultured for 24 h at 37 °C under a 5% CO_2 atmosphere. The medium was changed to a new one containing WP or its constituents. After 24 h of culture, the medium was removed and cells were collected by an attached solvent with an RNAeasey mini kit. Total RNA extraction and cDNA synthesis were performed according to a previously described method. The following primers were used for RT-PCR. Primers (5′→3′) for human were PPARα (forward) ccaacatggtgaaaccctgtctc, (reverse) aagaacaccaccattcccacaga; ACOX1 (forward) actgcctatgccttccagtttgtgggcg, (reverse) gagcttaactaccacatagtggcaatgt; CPT1A (forward) ggagaggagacagacaccatcca, (reverse) caaaataggcctgacgacacctg; and β-actin (forward) catcctcaccctgaagtaccccatcgag, (reverse) acaggactccatgcccaggaaggaaggc.

Statistics. The results were expressed as means and SE. Significance of the differences was examined by one-way ANOVA followed by Dunnett's test. Differences of $p < 0.05$ were considered to be significant.

RESULTS

Effect of WP on Lipid Parameters, β-Oxidation, and Hepatic mRNA Expression Related to Lipid Metabolism in HFD-Fed Mice. The increase in body weight of control HFD-fed mice was significantly ($p < 0.01$) higher compared to that of normal mice (**Table 1**). WP (50−200 mg/kg) slightly suppressed the increase in body weight, but the effects were not significant. Although liver weights of control mice were not significantly changed compared to normal mice, WP (100 and 200 mg/kg) significantly ($p < 0.05$ and $p < 0.01$) reduced liver weight. On the other hand, WP did not suppress the increase in either paranephric or epididymal fats. In terms of liver weight reduction, liver TG was significantly ($p < 0.05$) reduced by administration of WP (100 and 200 mg/kg). Moreover, serum TG in mice given WP (50−200 mg/kg) was noticeably reduced compared to normal and control mice. On the other hand, WP did not affect cholesterol in either the liver or serum.

Table 2. Effects of WP on Hepatic β-Oxidation in Mice Fed a HFD

		ΔOD/mg of protein/min	
	dose (mg/kg)	mitochondrial fraction	cytosolic fraction
normal		0.345 ± 0.045	0.0170 ± 0.0019
control		0.320 ± 0.072	0.0093 ± 0.0008
WP	50	0.273 ± 0.033	0.0119 ± 0.0025
	100	0.227 ± 0.006	0.0112 ± 0.0024
	200	0.263 ± 0.021	0.0133 ± 0.0030

Each value represents the mean with the SE ($n = 5-7$). No significant difference was detected.

Table 2 shows mitochondrial and cytosolic β-oxidations in HFD-fed mouse livers. Mitochondrial β-oxidations in normal and control mice were almost the same. WP (50−200 mg/kg) tended to slightly suppress mitochondrial β-oxidation, but the effects were not significant. On the other hand, cytosolic β-oxidation in control mice was almost half that of normal mice. WP (50−200 mg/kg) tended to enhance cytosolic β-oxidation compared to control mice. **Figure 3** indicates the effect of WP on hepatic mRNA expression related to fatty acid metabolism in HFD-fed mice. Expression of PPARα, a regulatory molecule in fatty acid β-oxidation (*29*), enhanced both mitochondrial and peroxisomal β-oxidation (*30*). Hepatic PPARα expressions in mice given WP (50−200 mg/kg) were enhanced. However, the effects were not dose-dependent. The expressions of CPT1A, the hepatic isoform of CPT, which is a rate-limiting enzyme of mitochondrial β-oxidation (*31*), were not enhanced by WP (50−200 mg/kg). On the other hand, expressions of ACOX1, a key enzyme in peroxisomal β-oxidation (*32*), were significantly ($p < 0.05$ and $p < 0.01$) enhanced by administration of WP (50−200 mg/kg).

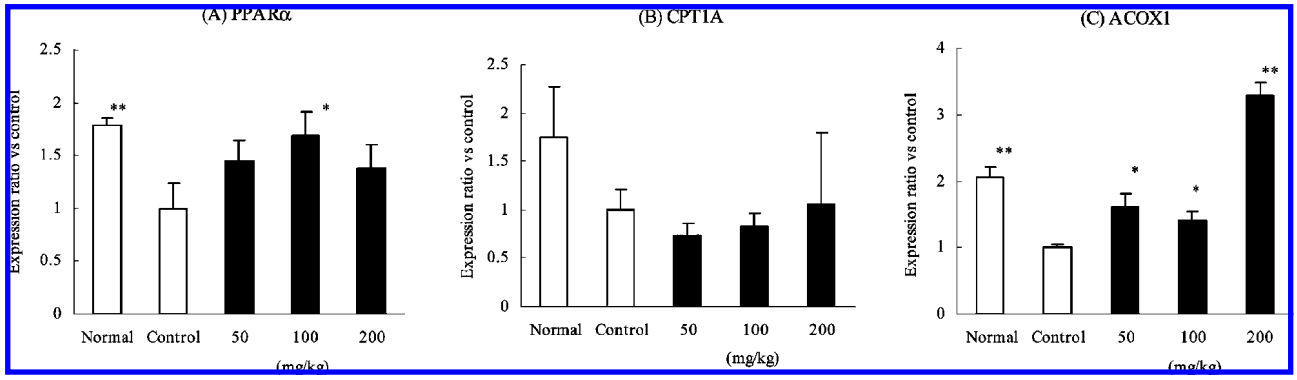

Figure 3. Effect of WP on hepatic mRNA expression of (**A**) PPARα, (**B**) CPT1A, and (**C**) ACOX1 in mice fed a high-fat diet. Each column represents the mean with the SE ($n = 5-7$). Asterisks denote significant differences from the control at *, $p < 0.05$, and **, $p < 0.01$, respectively.

Table 3. Effect of WP on Serum TG Elevation in Olive Oil Loaded Mice[a]

	dose (mg/kg)	serum TG (mg/dL) 2 h	4 h	6 h
non olive oil loaded		118 ± 13**	89 ± 17**	83 ± 4
control		477 ± 53	323 ± 47	206 ± 14
WP	50	523 ± 40	387 ± 34	228 ± 17
	100	488 ± 40	328 ± 25	295 ± 58
	200	480 ± 69	302 ± 32	207 ± 21

[a] Each value represents the mean with the SE ($n = 3-7$). Asterisks denote significant differences from the control at **, $p < 0.01$.

Table 4. Effect of WP on Oleic Acid Induced TG Accumulation in HepG2[a]

	concentration (μg/mL)	TG (μg/well)
non oleic acid treated		10.9 ± 1.5**
control		26.3 ± 0.8
WP	10	38.7 ± 1.5*
	30	37.4 ± 5.3*
	100	37.6 ± 1.9*

[a] Each value represents the mean with the SE ($n = 6$). Asterisks denote significant differences from the control at *, $p < 0.05$, and **, $p < 0.01$.

Hypotriglyceridemic Mechanism of WP. We investigated whether or not WP affected intestinal fat absorption and hepatic TG accumulation. **Table 3** reveals serum TG elevation after administration of olive oil. WP (50−200 mg/kg) did not affect serum TG. As for the oleic acid induced TG accumulation in HepG2 hepatocytes, WP significantly enhanced TG accumulation in the cells (**Table 4**). Hence, WP was not found to inhibit either intestinal fat absorption or TG accumulation in hepatocytes. On the other hand, in the treatment of HepG2 cells with WP, mRNA expression of PPARα was significantly enhanced at 1 and 100 μg/mL (**Figure 4**). At 10 μg/mL, the mRNA expression was significantly reduced. WP did not affect CPT1A mRNA expression at <10 μg/mL, but obviously enhanced the expression at 100 μg/mL. On the other hand, WP significantly and mildly enhanced ACOX1 mRNA expression from 1 to 100 μg/mL.

Effects of Polyphenolic Constituents in WP on mRNA Expression in HepG2. Pedunculagin (**1**), the major polyphenolic constituent in WP, significantly suppressed mRNA expression of PPARα at 1−10 μg/mL (**Table 5**). This compound (**1**) significantly suppressed the mRNA expressions of CPT1A and ACOX1 at concentrations of <3 μg/mL and enhanced both mRNA expressions at 10 μg/mL. Tellimagrandin I (**2**) slightly suppressed PPARα mRNA expression at 1 μg/mL and enhanced mRNA expression of CPT1A and ACOX1 at 10 μg/mL. Tellimagrandin II (**3**) did not affect PPARα mRNA expression, but, obviously enhanced CPT1A mRNA expression at 1−10 μg/mL. Ellagic acid (**4**), the second major polyphenolic constituent in WP, significantly suppressed mRNA expression of PPARα, CPT1A, and ACOX1 at 10 μg/mL.

DISCUSSION

In this study, we found that WP decreased serum and liver TG without reducing the amount of abdominal fat (**Table 1**). Zibaeenezhad et al. (*12*, *13*) reported that ingestion of walnuts improved human hypertriglyceridemia. Walnut seeds are rich in unsaturated free fatty acids, namely, linoleic acid and α-linolenic acid (*2*, *3*). The authors reported that free fatty acid composition in walnut oil was effective at preventing hyperlipidemia. On the other hand, WP contains no fatty acids and a large amount of polyphenols consisting of ellagitannins. Hence, our result reveals that polyphenols contained in kernel pellicles participate in the hypolipidemic effect of walnuts, as found by Zibaeenezhad et al. In contrast, some previous studies have denied the clinical hypolipidemic effect of walnut diets (*33*, *34*). Although various walnut diets were used for clinical trials, there were no descriptions regarding polyphenol contents. Pellicles attached to the kernel surface may influence the clinical hypolipidemic effect of walnut diets.

In dietary polyphenols, epigallocatechin gallate contained in green tea has been reported to exhibit a hypolipidemic effect in mice (*35*). Suppression of mRNA expressions of enzymes related to fatty acid synthesis (i.e., malic enzyme, stearoyl-CoA desaturase-1, and glucokinase) in the liver and enhancement of uncoupling protein (UCP) 2 expression are involved in the mechanism (*36*). A polyphenol-rich fraction from the fruit of amla (*Emblica officinalis* Gaertn.), an Indian medicinal plant, was also reported to improve age-related hypertriglyceridemia by enhancing liver PPARα expression (*37*). Moreover, licorice flavonoids enhanced hepatic mRNA expression related to β-oxidation in HFD-induced obese mice (*38*). On the other hand, several dietary polyphenols suppress fat absorption from the intestine. Oligomeric procyanidins in apple polyphenol have been reported to suppress intestinal lipid absorption by inhibiting pancreatic lipase (*39*). Polymerized polyphenols in oolong tea also suppress lipid absorption (*40*) by the same mechanism (*41*). As described above, some dietary polyphenols could affect hepatic lipid metabolism or intestinal lipid absorption. Therefore, to investigate the hypotriglyceridemic mechanism of WP, we evaluated β-oxidation in the liver of mice fed HFD and fat absorption in olive oil loaded mice. Although a significant difference was not observed, WP tended to enhance cytosolic β-oxidation, but not mitochondrial β-oxidation (**Table 2**). Cytosolic fractions contain peroxisome, which is a site of β-oxidation of very long chain and long chain free fatty acids.

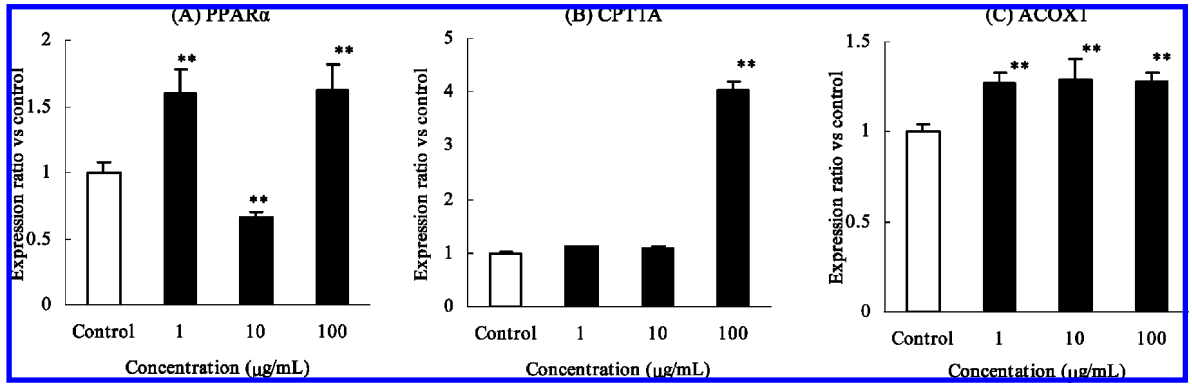

Figure 4. Effect of WP on mRNA Expression of (**A**) PPARα, (**B**) CPT1A, and (**C**) ACOX1 in HepG2. Each column represents the mean with the SE ($n = 4$). Asterisks denote significant differences from the control at **, $p < 0.01$.

Table 5. Effect of Polyphenolic Constituents in WP on mRNA Expression in HepG2 Related to Fatty Acid Metabolism[a]

		mRNA expression (vs control)		
	control	1 (μg/mL)	3 (μg/mL)	10 (μg/mL)
		PPARα		
pedunculagin (**1**)	1.00 ± 0.01	0.60 ± 0.02**	0.58 ± 0.02**	0.82 ± 0.02*
tellimagrandin I (**2**)	1.00 ± 0.08	0.84 ± 0.02*	1.08 ± 0.08	1.14 ± 0.02
tellimagrandin II (**3**)	1.00 ± 0.03	0.86 ± 0.03	1.02 ± 0.04	0.96 ± 0.02
ellagic acid (**4**)	1.00 ± 0.04	0.59 ± 0.04**	0.94 ± 0.05	0.64 ± 0.04*
bezafibrate	1.00 ± 0.05	0.47 ± 0.01**	0.75 ± 0.04*	1.00 ± 0.09
		CPT1A		
pedunculagin (**1**)	1.00 ± 0.01	0.63 ± 0.02**	0.74 ± 0.01**	1.31 ± 0.02**
tellimagrandin I (**2**)	1.00 ± 0.02	1.02 ± 0.02	1.09 ± 0.02	1.23 ± 0.02*
tellimagrandin II (**3**)	1.00 ± 0.03	1.42 ± 0.11*	1.56 ± 0.07**	1.42 ± 0.11*
ellagic acid (**4**)	1.00 ± 0.03	0.63 ± 0.04*	0.88 ± 0.06	0.69 ± 0.02*
bezafibrate	1.00 ± 0.01	0.99 ± 0.03	1.33 ± 0.06*	1.90 ± 0.08**
		ACOX1		
pedunculagin (**1**)	1.00 ± 0.02	0.63 ± 0.03**	0.82 ± 0.03*	1.20 ± 0.05*
tellimagrandin I (**2**)	1.00 ± 0.05	1.12 ± 0.02	1.33 ± 0.06*	1.69 ± 0.07**
tellimagrandin II (**3**)	1.00 ± 0.02	0.79 ± 0.04*	1.13 ± 0.05	1.13 ± 0.07
ellagic acid (**4**)	1.00 ± 0.02	0.94 ± 0.04	0.63 ± 0.03**	0.60 ± 0.02**
bezafibrate	1.00 ± 0.04	1.35 ± 0.02**	1.40 ± 0.07*	1.49 ± 0.02**

[a] Each value represents the mean with the SE ($n = 3-4$). Asterisks denote significant differences from the control at *, $p < 0.05$, and **, $p < 0.01$.

Peroxisome is well-known to be activated by fibrates, which are lipid-lowering medicines (*42*). Hence, WP was suggested to affect peroxisomal β-oxidation in the liver similar to the action of fibrates. However, WP did not suppress intestinal lipid absorption as it did not suppress serum TG elevation in olive oil loaded mice (**Table 3**). Previous papers (*39*−*41*) have shown that polymerized polyphenols with a high number of molecules appeared to inhibit lipase activity, leading to intestinal lipid absorption. The principal polyphenols of WP are ellagic acid (**4**) and ellagitannins such as pedunculagin (**1**) and tellimagrandins I (**2**) and II (**3**). The amounts of highly polymerized tannins are low (*19*, *20*). These observations are thought to be why WP did not suppress intestinal lipid absorption. WP did not suppress TG accumulation either and somewhat enhanced TG accumulation in hepatocytes induced by oleic acid in HepG2 (**Table 4**). WP may enhance the expression or activity of acyl CoA synthetase, leading to TG accumulation (*43*).

Because WP can enhance peroxisomal β-oxidation in the liver, we evaluated mRNA expression related to β-oxidation. CPT1A and ACOX1 were chosen as markers because they are rate-limiting enzymes of free fatty acid metabolism in mitochondria and peroxisome, respectively (*44*). In HFD-fed mice, WP significantly enhanced expressions of PPARα and ACOX1, whereas CPT1A was not enhanced (**Figure 3**). ACOX changes very long chain and long chain acyl CoA synthesized from acyl CoA to enoyl CoA (*44*). The reaction is a secondary step in peroxisomal β-oxidation. On the other hand, CPT1A incorporates fatty acids into the mitochondrial matrix across the inner mitochondrial membrane (*44*). The step is a prestage of mitochondrial β-oxidation. PPARα activation enhances expression of both CPT1A and ACOX1, leading to mitochondrial and peroxisomal β-oxidation (*45*). As WP enhanced PPARα gene expression, both mitochondrial and paroxysmal β-oxidations were suspected to be enhanced. However, the results of the β-oxidation study and hepatic mRNA expression in HFD-fed mice suggest that only peroxisomal lipid metabolism was upregulated. Fibrates activate PPARα and enhance both mitchondrial and peroxisomal β-oxidation (*46*). In naturally occurring compounds, eicosapentaenoic acid is well-known as a PPARα ligand (*47*). Among polyphenols, epigallocatechin gallate (*48*) activates PPARα. Resveratrol also enhances PRARα activity. The effect had a dual phase with primary enhancement followed by suppression due to activation of PPARα (*49*). Pterostilbene, a resveratrol derivative, was reported to activate PPARα in H4IIEC3 cells by luciferase assay (*50*). These compounds activate both mitochondrial and peroxisomal β-activation. The reason that WP enhanced only peroxisomal β-oxidation has not yet been clarified. Free fatty acids contained in HFD32 are long chain fatty acids, so the free acids are considered to be metabolized both in the mitochondria and in the peroxisome. Moreover, the temporal difference between mitochondrial fatty acid uptake by CPT and β-oxidation by ACOX1 is not suspected to be involved, because the livers were removed under a fasting condition. Therefore, the influence of fatty acids contained in the diet for β-oxidation can be ignored. Further investigations are required.

In an in vitro study using HepG2, WP enhanced PPARα and ACOX1 expression (**Figure 4**), but PPARα expression was not concentration-dependent. A high concentration of WP (100 μg/mL) obviously enhanced CPT1A expression despite the result in mice. In an in vitro condition, WP may enhance CPT1A expression. The difference in concentration of WP when it reached hepatocytes between in vivo and cell cultures is suspected to play a part in CPT expression. We used an in vitro assay and examined the effect of major polyphenolic compounds in WP. As a result, no compound, including bezafibrate, enhanced PPARα expression (**Table 5**). In the assay, cells were treated with the sample for 24 h. Bogdanova et al. (*51*) reported that PPARα expression in HepG2 was affected by glucose and oleate in the medium, and the expression was enhanced by 4 h of oleate treatment. Depression of glucose and oleate downregulated PPARα mRNA expression. When this finding is applied to our result, PPARα is considered to be already

expressed and activated prior to cell-harvesting. The amounts of glucose and free fatty acid in the medium were limited, and supplementation of both glucose and fatty acid to the cells was thought to be decreased. When cells were harvested, PPARα expression was finished and β-oxidation was thought to proceed in both the mitochondria and peroxisome. Actually, in the same assay, bezafibrate apparently enhanced both CPT1A and ACOX1 expression.

Among polyphenolic compounds in WP, pedunculagin (**1**) down-regulated both CPT1A and ACOX1 expression at low concentrations (1 and 3 μg/mL) and enhanced the expressions at 10 μg/mL. Tellimagrandin I (**2**) significantly enhanced CPT1A expression only at 10 μg/mL and enhanced ACOX1 expression at >3 μg/mL. This compound (**2**) is thought to more potently enhance peroxisomal β-oxidation than mitochondria. Referring to the expression pattern of WP (**Figure 4**) and contents of **2** in WP (2.8%), **2** is suggested to highly correlate with the effect of WP on CPT1A and ACOX1 expression. Moreover, **2** is considered to be a plausible polyphenolic constituent that participates in the in vivo effect of WP on ACOX1 expression. Tellimagrandin II (**3**) significantly enhanced CPT1A expression, but did not enhance ACOX1 expression. Pedunculagin (**1**), **2**, and **3** are suggested to participate in the in vitro effect of WP on CPT1A expression. However, these compounds are suspected to be not effective in vivo. As ellagic acid (**4**) tended to suppress mRNA expression of PPARα, CPT1A, and ACOX1, it may be a negative compound in the hypotriglyceridemic effect of WP. Cerda et al. (*52*) reported that ellagitannins are metabolized to urolithin B in human microflora. Further in vitro experimentation regarding the effect of urolithin B is required to speculate on the compound involved in the effect of WP in vivo.

In conclusion, we found that walnut polyphenol exhibited hypotriglyceridemic activity based on enhancement of peroxisomal β-oxidation. Tellimagrandin I, a major polyphenolic compound in walnut polyphenol, is suggested to participate in the activity. Polyphenols in the kernel pellicle may be effective for improving hyperlipidemia and metabolic syndrome.

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