

Ameliorative Effects of Mulberry (*Morus alba* L.) Leaves on Hyperlipidemia in Rats Fed a High-Fat Diet: Induction of Fatty Acid Oxidation, Inhibition of Lipogenesis, and Suppression of Oxidative Stress

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Received May 20, 2010; Accepted September 27, 2010; Online Publication, December 7, 2010

[doi:10.1271/bbb.100392]

To determine the effects of mulberry (*Morus alba* L.) leaves on hyperlipidemia, we performed gene expression profiling of the liver. Rats were fed a high-fat diet and administered mulberry leaves for 7 weeks. Plasma triglyceride and non-esterified fatty acid levels were significantly lower in the rats treated with mulberry leaves as compared with the untreated rats. DNA microarray analysis revealed that mulberry leaves upregulated expression of the genes involved in α -, β - and ω -oxidation of fatty acids, mainly related to the peroxisome proliferator-activated receptor signaling pathway, and downregulated the genes involved in lipogenesis. Furthermore, treatment with mulberry leaves upregulated expression of the genes involved in the response to oxidative stress. These results indicate that consumption of fatty acids and inhibition of lipogenesis are responsible for the reduction in plasma lipids caused by mulberry administration. In addition, mulberry treatment maintains the body's oxidative state at a low level despite enhancing fatty acid oxidation.

Key words: mulberry (*Morus alba* L.); hyperlipidemia; fatty acid oxidation; lipogenesis; oxidative stress

Atherosclerotic disease increases the risk of myocardial and cerebral infarctions, which are major causes of death, comparable to cancer, in developed countries. In addition to hypercholesterolemia, hypertension, and impaired glucose metabolism, hypertriglyceridemia is now recognized as a risk factor for atherosclerotic disease. Indeed, hypertriglyceridemia is an important member of a cluster of risk factors for coronary artery disease, as represented by metabolic syndrome. Therefore, measurement of plasma triglyceride is included in the criteria for diagnosis of metabolic syndrome proposed by various organizations, including the World Health Organization and the American Association of Clinical Endocrinologists.

The leaf, root bark, and fruit of mulberry (*Morus alba* L.) have a long history as a traditional Chinese medicine. These days, various food products containing mulberry leaves (e.g., mulberry tea) are easily available as functional foods in many countries. Our previous studies indicate that administration of mulberry leaves ameliorates dyslipidemia, particularly hypercholesterolemia.^{1,2} Similarly, other researchers have reported hypocholesterolemic effects of mulberry leaves.^{3,4} Addition, it was also reported that mulberry leaves can be used to treat hypertriglyceridemia,^{1,4-6} but the functional mechanisms underlying the hypotriglyceridemic effect of mulberry leaves are still unknown. In this study, we used DNA microarray analysis to investigate gene expression in the livers of hypertriglyceridemic rats treated with mulberry leaves to elucidate the mechanisms involved in the lipid-lowering effects of mulberry.

Materials and Methods

Material. Mulberry tea, a dry powder of mulberry (*Morus alba* L.) leaves, was obtained from Hekizanen (Kanagawa, Japan).

Determination of the polyphenol contents of the mulberry leaf powder. Mulberry leaf powder was added to water and heated in boiling water for 30 min. After centrifugation at 20,000 g, the supernatant (heated extract) was retrieved and filtered. The concentration of total polyphenols in the heated extract was determined by Folin-Ciocalteu assay using gallic acid as the standard. The heated extract (300 μ l) was added to 2 N Folin-Chiocalteu reagent (150 μ l). After an interval of 3 min, 2% Na₂CO₃ (900 μ l) was added, and the mixture was allowed to stand for 15 min. The absorbance at 750 nm was measured by spectrophotometer.

Mulberry powder was added to 75% methanol and this was incubated at 80 °C for 1 h with constant stirring. After centrifugation at 20,000 g, total flavonoid aglycons in the supernatant (methanol extract) were measured by the aluminum chloride colorimetric method. Quercetin, a major flavonoid in mulberry leaves, was used to make the calibration curve. The methanol extract (200 μ l) was mixed with methanol (200 μ l), 10% aluminum chloride (20 μ l), 1 M potassium acetate (20 μ l), and water (560 μ l). After incubation at room temper-

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Abbreviations: ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; GO, Gene Ontology; HPLC, high performance liquid chromatography; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAS, Micro Array Suite; PBST, Dulbecco's phosphate-buffered saline containing 0.05% Tween-20; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances

ature for 30 min, the absorbance at 415 nm was measured by spectrophotometer. The mixture without only aluminum chloride was used as a blank of each sample.

Quantification of quercetin and kaempferol, major flavonoids in mulberry leaves, was achieved by reversed-phase high performance liquid chromatography (HPLC). First, flavonoid glycosides were hydrolyzed to aglycons. Four hundred fifty ml of this methanol extract was mixed with 300 μ l of methanol containing 500 μ g/ml of *tert*-butylhydroquinone and 150 μ l of 2 N HCl, and this was heated at 90 °C for 2 h. The sample was added to 1,200 μ l of 1 M Tris-HCl (pH 7.5) and 525 μ l of dimethylsulfoxide, and this was applied to reversed-phase HPLC. The column used was an Inertsil ODS-3 (4.6 mm \times 150 mm; GL Sciences, Tokyo). Gradient elution was performed with solution A (1% tetrahydrofuran and 0.1% phosphoric acid) and solution B (acetonitrile) at a flow rate of 1 ml/min, as follows: initially 95% of solution A, a linear gradient of solution B (5–20% in 30 min), another gradient of solution B (20–50% in 30 min), and 50% of solution B for 20 min. The eluates were monitored at 260 and 370 nm with an UV detector. Quercetin and kaempferol were used to make the calibration curve.

Animals. Male Wistar/ST rats (5 weeks old) were purchased from Japan SLC (Shizuoka, Japan), and kept at 25 °C under a 12-h light/dark cycle (lights on at 8:00). The rats were given a normal diet (CE-2, crude fat 4.6%; Clea Japan, Kanagawa, Japan) for 2 weeks, and then used in the experiments. They were initially divided into five groups ($n = 6$ /group). One group was fed the normal diet (the control group), another group was fed a high-fat diet (QuickFat, crude fat 14%, beef fat; Japan SLC) without treatment with mulberry leaves (the untreated high-fat diet group), and the other three groups were fed the high-fat diet and orally administered mulberry leaf powder suspended in 0.5% carboxymethylcellulose at 250, 500, or 1,000 mg/kg body weight/d (the mulberry-treated groups). Mulberry was administered 6 times weekly for 7 weeks using a gastric probe after an overnight fast. The rats in the control and untreated high-fat diet groups were orally administered 0.5% carboxymethylcellulose alone. At week 7, the rats were anesthetized and killed by exsanguination after a 16-h fast. The liver was removed from each rat, weighed, and frozen. Parts of the liver were immediately immersed in RNAlater (Applied Biosystems, Foster City, CA) for DNA microarray analysis. All animal experiments were performed following to the Guidelines for Animal Experimentation of the Kanagawa Prefectural Institute of Public Health.

Blood biochemical examination. Blood samples were collected from tail veins into heparin-coated tubes after a 16-h fast, and plasma was prepared by centrifugation at 2,000 g. Plasma triglyceride, total cholesterol, and glucose levels were determined using a Fuji DRICHEM 7000 system (Fujifilm, Tokyo). Plasma non-esterified fatty acid levels were measured using a LabAssay NEFA kit (Wako, Osaka, Japan).

Determination of triglyceride and cholesterol in the liver. Liver samples (100–200 mg) were homogenized in 200 μ l of water. Lipids were extracted with 600 μ l of chloroform:methanol (1:2, v/v) 2 times, as described by Bligh and Dyer.⁷⁾ After centrifugation at 20,000 g for 10 min, the extracts in the lower layer were collected, evaporated under vacuum to dryness, and dissolved in isopropyl alcohol:Triton X-100 (9:1, v/v). The triglyceride and cholesterol levels in the extracts were measured using a LabAssay Triglyceride kit (Wako) and a LabAssay Cholesterol kit (Wako) respectively.

RNA isolation and microarray hybridization. Ten rats (five rats/group) were randomly selected from two high-fat diet groups (the untreated high-fat diet group and the 500 mg/kg mulberry-treated group) and used in the subsequent experiment. Total RNA was extracted from the rat liver using an RNAiso solution (Takara Bio, Shiga, Japan) and purified using an RNeasy Mini kit (Qiagen, Tokyo). RNA quality was checked using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (2 μ g) was converted to double-strand cDNA, which was washed and used to synthesize biotinylated cRNA using a GeneChip One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA), a GeneChip Sample Cleanup Module (Affymetrix), and a GeneChip IVT Labeling kit (Affymetrix) following

a standard Affymetrix protocol. The synthesized cRNA was fragmented and hybridized to a GeneChip Rat Genome 230 2.0 Array (Affymetrix), which contains 28,757 well-substantiated rat genes, with a GeneChip Hybridization, Wash and Stain kit (Affymetrix). The array was washed and labeled with phycoerythrin using this kit and a GeneChip Fluidics Station 450 system (Affymetrix). Fluorescence intensity was determined using a GeneChip Scanner 3000 7G (Affymetrix). All data have been deposited in the Gene Expression Omnibus public repository of the National Center for Biotechnology Information⁸⁾ under accession no. GSE21075 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21075>).

DNA microarray data analysis. The raw data were normalized with the Affymetrix Micro Array Suite (MAS) algorithm⁹⁾ using statistical language R¹⁰⁾ and the MAS 5.0 software in the Affy package.¹¹⁾ Of 28,757 genes, 19,270 high-quality probe sets under annotation classes A, B, and C (annotation file Rat230.2.na26.annot.csv) were used in the subsequent analysis. The relative standard deviation of the signal intensity showed very high values for a range of low signal levels (data not shown). Since the low signal data had lower reliability, the top 5,000 datasets with medium and high expression levels (signal intensity > about 2^{9.4}) were used in further analysis. To detect genes that were differentially expressed between two groups, the 5,000 normalized genes were ranked by the Weighted Average Difference method¹²⁾ using statistical language R. Then pathway enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database¹³⁾ and gene-annotation enrichment analysis based on Biological Process in the Gene Ontology (GO) database¹⁴⁾ were performed on the 600 top-ranked genes (600 upregulated genes and 600 downregulated genes) using the Database for Annotation, Visualization, and Integrated Discovery program.¹⁵⁾

Enzyme-linked immunosorbent assay. Protein expression of CPT1A (carnitine palmitoyltransferase 1A), ACOX2 (acyl-CoA oxidase 2), PHYH (phytanoyl-CoA hydroxylase), and DGAT2 (diacylglycerol *O*-acyltransferase homolog 2), as encoded by the *Cpt1a*, *Acox2*, *Phyh* and *Dgat2* genes respectively, in the liver was determined by enzyme-linked immunosorbent assay (ELISA). Rat liver samples were homogenized in 100 mM potassium phosphate buffer, pH 7.4, containing 1% Triton X-100 and a 1% protease inhibitor cocktail for use with mammalian cell and tissue extracts (Nacalai Tesque, Kyoto, Japan). After centrifugation at 500 g for 10 min, the supernatant was mixed with an equal volume of glycerol, and this was used in the subsequent experiment. A microtiter plate (Nunc MaxiSorp; Thermo Fisher Scientific, Yokohama, Japan) was coated with 100 μ l of liver extract diluted 50-fold with 50 mM carbonate buffer (pH 9.6) at 37 °C for 2 h. After washing 4 times with 400 μ l of Dulbecco's phosphate-buffered saline containing 0.05% Tween-20 (PBST), the plate was blocked with 300 μ l of 20% Blocking One solution (Nacalai Tesque) at 4 °C overnight. After washing with PBST as above, the plate was reacted with 100 μ l of goat anti-CPT1A polyclonal antibody (2 μ g/ml, CPT1, N-17; Santa Cruz Biotechnology), goat anti-ACOX2 polyclonal antibody (0.25 μ g/ml; Abnova, Taipei, Taiwan), goat anti-PHYH polyclonal antibody (2 μ g/ml, PHYH, E-19; Santa Cruz Biotechnology), or goat anti-DGAT2 polyclonal antibody (2 μ g/ml, DGAT2, A-18; Santa Cruz Biotechnology) in PBST containing 5% Blocking One at 37 °C for 2 h. After washing with PBST as above, the plate was reacted with 100 μ l of 0.1 μ g/ml of horseradish peroxidase-conjugated rabbit anti-goat IgG-heavy and light chain antibody (Bethyl Laboratories, Montgomery, TX) in PBST containing 5% Blocking One at 37 °C for 1 h. After washing again with PBST as above, the plate was incubated with 100 μ l of 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific) at 37 °C for 15 min, and then 100 μ l of 1 M sulfuric acid was added. The color developed was measured by the absorbance at 450 nm using a microplate reader.

Enzyme assay. Acyl-CoA oxidase/dehydrogenase activity in the liver extracts was measured as described by Hashimoto *et al.*¹⁶⁾ Palmitoyl-CoA (C_{16:0}) was used as the substrate. The formation of a red pigment was measured by determining the increase in absorbance at 500 nm. The molecular extinction coefficient was 6,390 liter mol⁻¹ cm⁻¹. One unit of the enzyme was defined as the amount that catalyzes the formation of 1 nmol of product/s.

Assay of thiobarbituric acid reactive substances. Lipid peroxidation in the plasma at 6 weeks was measured as thiobarbituric acid reactive substances (TBARS) by the method described by Kikugawa *et al.*¹⁷⁾

Statistical analysis. Data, except for the DNA microarray data, were evaluated by analysis of variance (ANOVA) with Dunnett's multiple comparison of means test as between the untreated high-fat diet group and the other groups.

Results

Analysis of polyphenols

The polyphenol contents of mulberry leaves are shown in Table 1. Mulberry leaf powder contains 1.24×10^3 mg polyphenols per 100 g dry weight. On the other hand, the concentration of total flavonoid aglycons in the mulberry leaf powder was 570 mg/100 g dry weight. Quercetin and kaempferol were present at concentrations of 367 and 119 mg/100 g of mulberry leaf powder.

Effects on metabolic variables

Table 2 shows the body weight, liver weight, liver weight relative to body weight, and liver and blood components, and Fig. 1 shows serial changes in plasma triglyceride levels. There were no differences in initial and final body weight, liver weight, liver weight relative to body weight, or plasma total cholesterol and glucose levels among the five groups. Feeding rats a high-fat diet induced hepatic triglyceride and cholesterol accumulation, but mulberry had no effects on liver triglyceride or cholesterol levels. As expected, the high-fat diet increased plasma triglyceride levels. Notably, this increase was significantly suppressed by mulberry administered at a 500 mg/kg dose, and was non-significantly suppressed by the two other doses of mulberry. Meanwhile, plasma non-esterified fatty acid levels were significantly lower in the rats treated with 250 and 500 mg/kg mulberry as compared with the untreated high-fat diet group.

Table 1. Polyphenol Contents of Mulberry Leaves (mg/100 g)

Total polyphenols	1.24×10^3
Total flavonoid aglycons	570
Quercetin	367
Kaempferol	119

Table 2. Effects of Mulberry Leaves on Fasting Metabolic Variables at 6 Weeks (mean \pm SD)

	Control group	High-fat diet groups			
		Untreated	Mulberry-treated (mg/kg body weight/d)		
			250	500	1000
Initial body weight (g)	191 \pm 7	188 \pm 10	186 \pm 5	187 \pm 7	190 \pm 9
Body weight (g)	330 \pm 25	347 \pm 23	358 \pm 27	348 \pm 14	352 \pm 19
Liver weight ¹ (g)	8.54 \pm 1.42	8.84 \pm 0.84	9.60 \pm 1.78	8.85 \pm 0.64	8.67 \pm 0.63
Liver weight/100 g of body weight ¹ (g/g)	2.48 \pm 0.19	2.54 \pm 0.11	2.50 \pm 0.07	2.54 \pm 0.13	2.46 \pm 0.07
Liver triglyceride ¹ (mg/g)	7.40 \pm 1.51*	23.1 \pm 4.7	28.1 \pm 10.0	29.0 \pm 16.2	20.5 \pm 8.8
Liver cholesterol ¹ (mg/g)	12.0 \pm 2.0***	19.7 \pm 2.9	20.3 \pm 2.4	19.4 \pm 4.2	17.9 \pm 3.6
Plasma triglyceride (mg/dl)	57.2 \pm 16.6**	79.2 \pm 8.2	71.0 \pm 6.1	63.2 \pm 12.1*	66.7 \pm 7.4
Plasma non-esterified fatty acid (μ Eq/dl)	39.6 \pm 11.1	45.1 \pm 9.4	31.9 \pm 9.7*	30.7 \pm 8.8*	37.6 \pm 9.0
Plasma total cholesterol (mg/dl)	67.7 \pm 9.5	60.8 \pm 7.1	57.2 \pm 4.6	55.0 \pm 10.0	52.3 \pm 7.4
Plasma glucose (mg/dl)	113 \pm 14	130 \pm 32	118 \pm 7	139 \pm 9	136 \pm 5

¹Liver weight, liver weight/100 g of body weight, liver triglyceride and liver cholesterol were measured at 7 weeks. Asterisks indicate significant differences from the untreated high-fat diet group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Effects on gene expression relating to lipid metabolism

Table 3 shows the results of pathway enrichment analysis using up- and downregulated genes. This analysis¹⁵⁾ screens about 350 biological pathways in the KEGG database.¹³⁾ The analysis extracted many pathways, in addition to lipid metabolism. Since the purpose of this study was to elucidate the mechanism involved in the hypolipidemic effects of mulberry leaves, we focused on the dynamics of the genes involved in lipid metabolism. The peroxisome proliferator-activated receptor (PPAR) signaling pathway was upregulated in the mulberry-treated group. On the other hand, mulberry treatment downregulated the androgen and estrogen metabolism pathways, the butanoate metabolism pathway, the bile acid biosynthesis pathway, and the synthesis and degradation of ketone bodies pathway. Members of the fatty acid metabolism pathway were among the up- and downregulated genes.

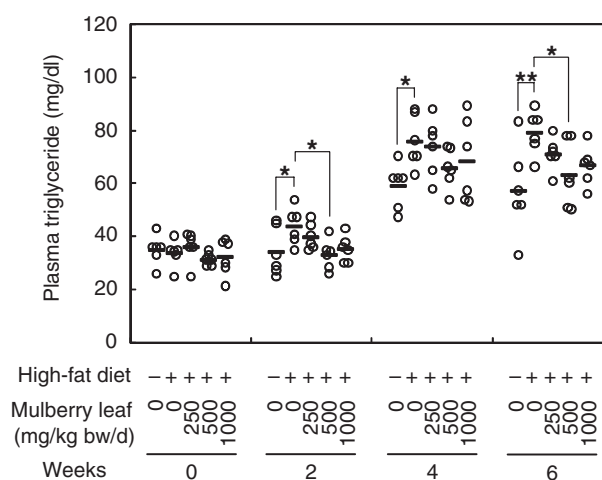


Fig. 1. Effects of the High-Fat Diet and Mulberry Administration on Fasting Plasma Triglyceride Levels.

Rats were fed a normal or a high-fat diet for 7 weeks and treated with and without mulberry leaves. Statistical analysis was performed by ANOVA with Dunnett's multiple comparison of means test as between the untreated high-fat diet group and the other groups. Circles and horizontal bars indicate individual and mean values respectively. Single and double asterisks indicate significant decreases in plasma triglyceride levels at $p < 0.05$ and $p < 0.01$ respectively.

Table 3. Pathway Enrichment Analysis Based on the KEGG Database Using 600 Upregulated and 600 Downregulated Genes

KEGG-ID	Category	Gene counts	FDR corrected <i>p</i> -value
Upregulated pathway			
map03010	Ribosome	22	7.84E-08
map04610	Complement and coagulation cascades	19	3.64E-07
map00071	Fatty acid metabolism	13	2.47E-04
map00280	Valine, leucine and isoleucine degradation	10	1.17E-02
map03320	PPAR signaling pathway	13	3.82E-02
Downregulated pathway			
map00980	Metabolism of xenobiotics by cytochrome P450	17	1.98E-06
map00150	Androgen and estrogen metabolism	14	1.04E-05
map00860	Porphyrin and chlorophyll metabolism	12	1.34E-05
map00190	Oxidative phosphorylation	22	2.87E-05
map00040	Pentose and glucuronate interconversions	10	3.39E-05
map00500	Starch and sucrose metabolism	13	7.32E-05
map00650	Butanoate metabolism	11	2.36E-04
map00071	Fatty acid metabolism	11	1.78E-03
map00280	Valine, leucine and isoleucine degradation	10	2.90E-03
map00120	Bile acid biosynthesis	8	4.75E-03
map00260	Glycine, serine and threonine metabolism	9	5.49E-03
map00310	Lysine degradation	7	2.71E-02
map00072	Synthesis and degradation of ketone bodies	4	2.71E-02

Next, we performed gene-annotation enrichment analysis¹⁵⁾ (Table 4), which screens for approximately 18,800 items corresponding to biological processes in the GO database.¹⁴⁾ In terms of lipid metabolism, fatty acid β -oxidation was significantly upregulated in the livers of the mulberry-treated rats. On the other hand, mulberry treatment downregulated lipid biosynthetic processes, including the steroid biosynthetic process. Since we focused on the hypolipidemic effects of mulberry in this study, particularly the hypotriglyceridemic effect, we describe below the up- and down-regulated genes that are involved in the metabolism of triglycerides and the degradation products, *viz.*, fatty acids and glycerol.

Expression of genes involved in the oxidation of straight-chain fatty acids

The genes involved in fatty acid oxidation that were up- or downregulated are shown in Fig. 2. Administration of mulberry upregulated expression of the *Cpt1a* gene, which is essential for the transport of long fatty acids into the mitochondria, and whose product is the rate-limiting enzyme in β -oxidation (Fig. 2(1)). The transcript levels of *Acox1* (acyl-CoA oxidase 1), *Acadl* and *Acadm* (acyl-CoA dehydrogenase, long- and medium-chain respectively) were also increased in the mulberry-treated group (Fig. 2(2) and 2(3)). These genes encode the rate-limiting enzymes in β -oxidation of saturated and unsaturated fatty acids, particularly long- and medium-chain fatty acids in the mitochondria

and the peroxisome. Furthermore, administration of mulberry increased the transcripts of the following genes, involved in the second, third, and fourth steps of the β -oxidation of saturated and unsaturated fatty acids, particularly long- and medium-chain fatty acids: *Hadhb* (hydroxyacyl-CoA dehydrogenase, β subunit), *Hsd17b4* (hydroxysteroid 17- β dehydrogenase 4) and *Acaal1* (acetyl-CoA acyltransferase 1) (Fig. 2(2) and 2(3)). Mulberry administration induced gene expression of *Dci* (dodecenoyl-CoA Δ isomerase), *Peci* (peroxisomal Δ^3, Δ^2 -enoyl-CoA isomerase), and *Echl1* (enoyl-CoA hydratase 1, peroxisomal), isomerases that are involved in the β -oxidation of unsaturated fatty acids (Fig. 2(3)). In addition, hepatic gene expression of *Cyp4a2* and *Cyp4a3* (cytochrome P450, family 4, subfamily A, polypeptides 2 and 3, respectively) genes was upregulated in the mulberry group (Fig. 2(4) and 2(5)). These genes encode the rate-limiting enzymes in the ω -oxidation of long-chain fatty acids in the endoplasmic reticulum for β -oxidation at both ends. On the other hand, the following genes, involved in the β -oxidation of short-chain fatty acids, were downregulated in the mulberry group: *Acads* (acyl-CoA dehydrogenase, short-chain), *Echs1* (enoyl-CoA hydratase, short chain, 1), *Hsd17b10* (hydroxysteroid 17- β dehydrogenase 10), and *Acaa2* (acetyl-CoA acyltransferase 2) (Fig. 2(2)). These data suggest that mulberry promotes β - and ω -oxidation of saturated and unsaturated fatty acids, particularly medium- and long-chain fatty acids. These effects of mulberry on the genes involved in fatty acid metabolism are summarized in Fig. 3(1) to 3(4).

Expression of the genes involved in the oxidation of branched fatty acids

Phyh, which encodes the rate-limiting enzyme in the α -oxidation of 3-methyl-branched fatty acids, was upregulated by mulberry treatment (Fig. 2(6)). Furthermore, administration of mulberry upregulated the expression of *Acox2*, which encodes the rate-limiting enzyme that catalyzes the first step in the β -oxidation of the 2-methyl-branched fatty acids produced by α -oxidation (Fig. 2(7)). In addition, mulberry increased the transcript levels of *Hsd17b4* and *Scp2* (sterol carrier protein 2), genes coding enzymes catalyzing the third and fourth steps in the β -oxidation of 2-methyl-branched fatty acids respectively (Fig. 2(7)). These data indicate that mulberry intake promotes the oxidation of branched fatty acids. These effects of mulberry on the genes involved in fatty acid metabolism are summarized in Fig. 3(5) and 3(6).

Effects of mulberry on expression of the genes involved in the transport of fatty acids and glycerol catabolism

The expression level of *Angptl3* (angiopoietin-like 3), the product of which inhibits hydrolysis of triglyceride, was reduced in the mulberry-treated group (Fig. 3(7)). Gene expression of *Fabp1* (fatty acid-binding protein 1) was induced in the mulberry-treated group (Fig. 3(8)). The product of this gene transports fatty acids to the mitochondria and the peroxisome. Furthermore, mulberry administration increased the mRNAs of *Bbox1* (γ -butyrobetaine, 2-oxoglutarate dioxygenase 1) and *Aldh9a1* (aldehyde dehydrogenase 9 family, member A1),

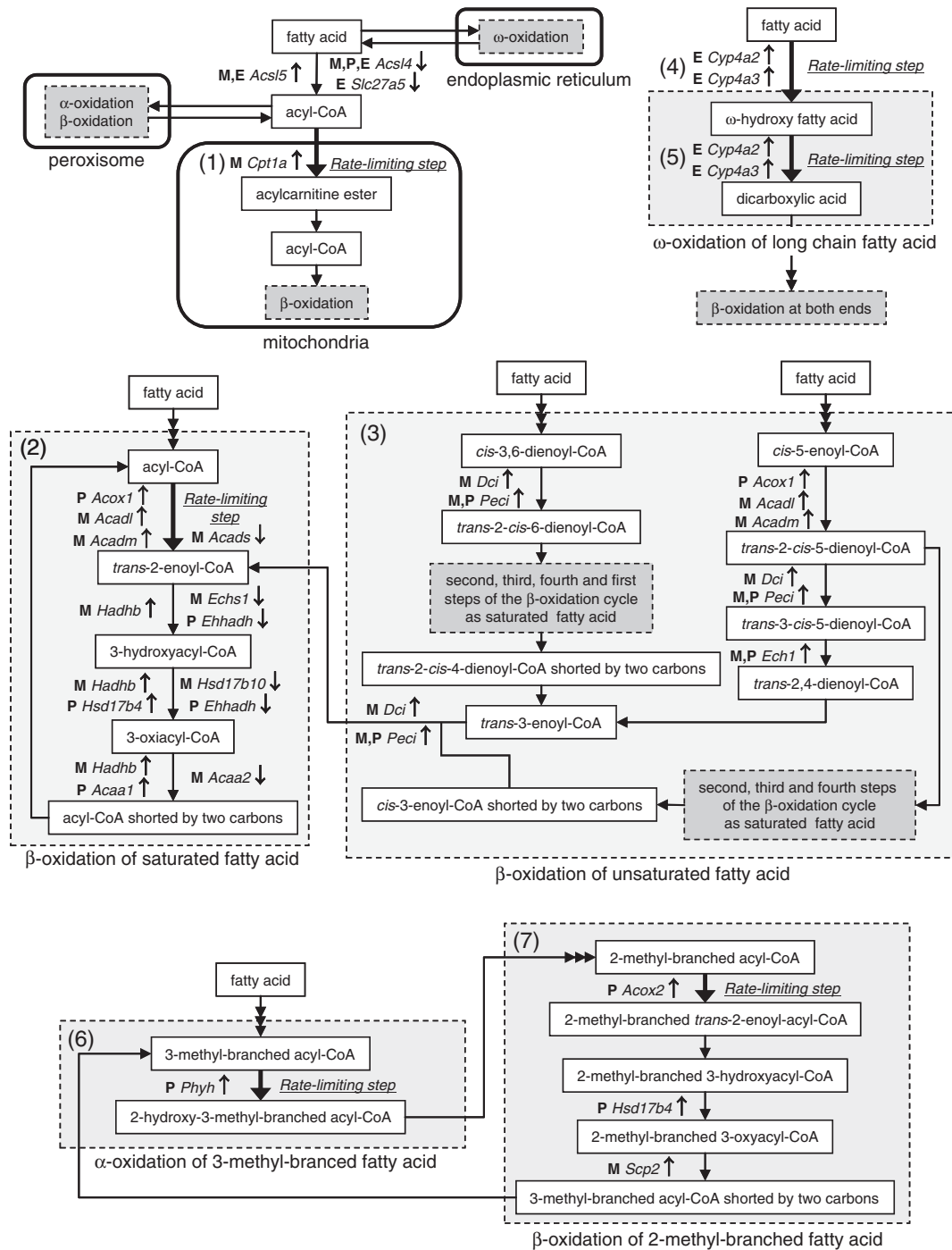


Fig. 2. Genes Involved in Hepatic Fatty Acid Metabolism That Were Up- or Downregulated by Mulberry Administration in Rats Fed a High-Fat Diet.

Up and down arrows indicate to up- and downregulated genes respectively. Bold M, P, and E indicate genes localized to the mitochondria, the peroxisome, and the endoplasmic reticulum respectively.

which synthesize the carnitine required for fatty acid influx into the mitochondria (Fig. 3(9)). In addition, *Slc22a5* (solute carrier family 22 member 5) mRNA was upregulated in the mulberry group (Fig. 3(10)). The product of this gene acts as transporter of carnitine into the cells and raises the concentration of carnitine in the liver and muscle. On the other hand, *Hagh* (hydroxyacylglutathione hydroxylase) and *Ldha* (lactate dehydrogenase A), genes involved in the catabolism of glycerol through the methylglyoxal pathway, increased in the rats of the mulberry group (Fig. 3(11)). These changes suggest that mulberry administration accelerates

the degradation of triglycerides and the transport and catabolism of the degradation products, *viz.*, fatty acids and glycerol.

Effects of mulberry on metabolism of dicarboxylic fatty acids and ketone bodies

β -Oxidation of dicarboxylic fatty acids produced by ω -oxidation results in the generation of succinyl-CoA, which is hydrolyzed to succinate by *Acot4* (acyl-CoA thioesterase 4).¹⁸⁾ In fact, the transcript level of *Acot4* increased in the rats treated with mulberry (Fig. 3(16)). Furthermore, it has been reported that dicarboxylic acids

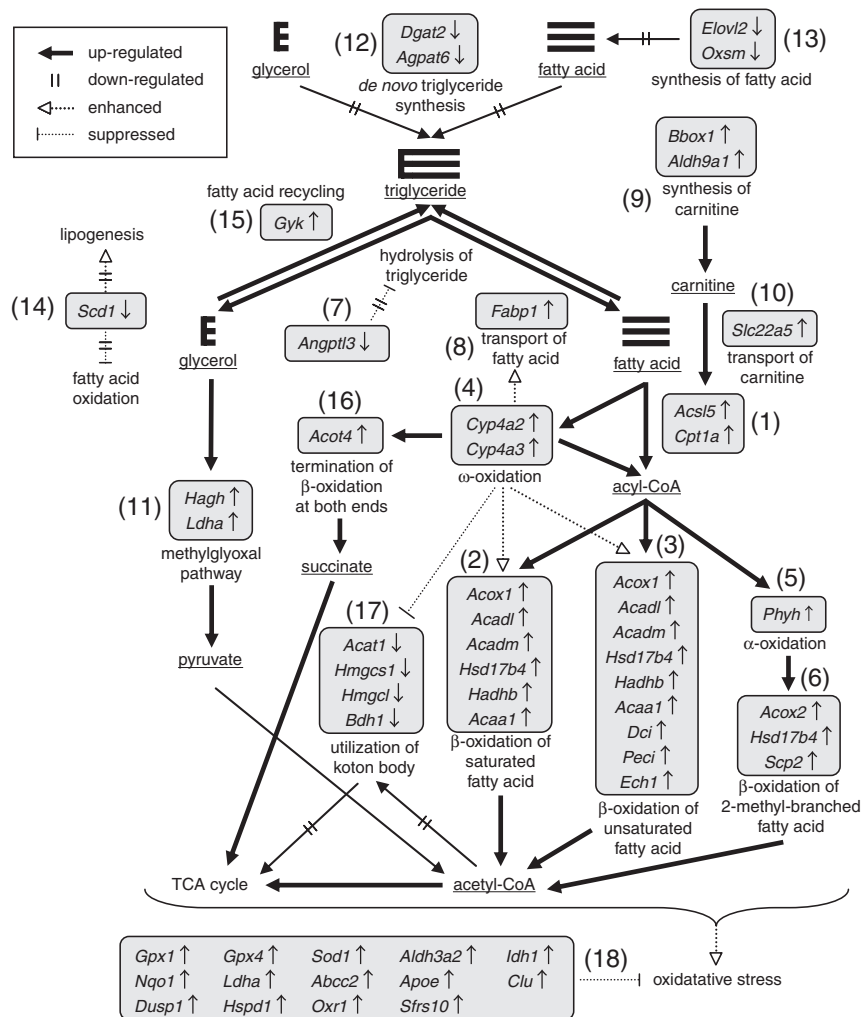


Fig. 3. Genes Involved in Hepatic Metabolic Processes That Were Up- or Downregulated by Mulberry Administration in Rats Fed a High-Fat Diet. Up and down arrows indicate to up- and downregulated genes respectively.

suppress ketogenesis.¹⁹⁾ In the mulberry-treated group, the following genes related to ketogenesis were down-regulated: *Acat1* (acetyl-CoA acetyltransferase 1), *Hmgcs1* (3-hydroxy-3-methylglutaryl-CoA synthase 1), *Hmgcl* (3-hydroxymethyl-3-methylglutaryl-CoA lyase), and *Bdh1* (3-hydroxybutyrate dehydrogenase, type 1) (Fig. 3(17)). This indicates that mulberry administration induces dicarboxylic acid catabolism and suppresses ketogenesis.

Effects of mulberry on the genes involved in lipogenesis

As for lipogenesis, mulberry administration decreased the transcript levels of *Dgat2* and *Agpat6* (1-acylglycerol-3-phosphate *O*-acyltransferase 6), which encode key enzymes involved in *de novo* triglyceride synthesis (Fig. 3(12)). In addition, mulberry downregulated the expression of *Elovl2* (an elongation of very long chain fatty acids-like 2) and *Oxsm* (3-oxoacyl-ACP synthase, mitochondrial), which are involved in the elongation of fatty acid chains (Fig. 3(13)). Furthermore, the expression level of *Scd1* (stearoyl-CoA desaturase-1) mRNA was reduced by mulberry administration (Fig. 3(14)). It has been reported that inhibition of the *Scd1* gene enhances fatty acid oxidation and inhibits lipogenesis.²⁰⁾ This suggests that mulberry administration suppresses lipogenesis. On the other hand, mulberry upregulated the

mRNA level of *Gyk* (glycerol kinase) (Fig. 3(15)), the product of which participates in the futile cycles of triglyceride breakdown and re-synthesis from free fatty acids.²¹⁾ This fatty acid recycling re-esterifies excessive free fatty acids and ultimately reduces the plasma non-esterified fatty acid level,²²⁾ but because this does not result in *de novo* triglyceride synthesis, it does not increase the plasma triglyceride level.

Measurement of CPT1A, ACOX2, PHYH, and DGAT2

The *Cpt1a*, *Acox2*, *Phyh*, and *Dgat2* genes are essential for the β -oxidation of straight chain fatty acids, the β -oxidation of branched chain fatty acids, the α -oxidation of branched chain fatty acids, and the *de novo* synthesis of triglyceride respectively. Since the transcripts of these genes were higher in the mulberry-treated group (Fig. 3), we measured the protein expression levels of the CPT1A, ACOX2, PHYH, and DGAT2 proteins by ELISA (Fig. 4A–D). The high-fat diet reduced the protein expression of CPT1A and ACOX2, which was attenuated by mulberry supplementation. The protein expression of PHYH of the 250 and 500 mg/kg mulberry-treated groups tended to be higher than that of the untreated high-fat diet group. On the contrary, the expression levels of DGAT2 were significantly lower in the 250 and 500 mg/kg mulberry-treated groups.

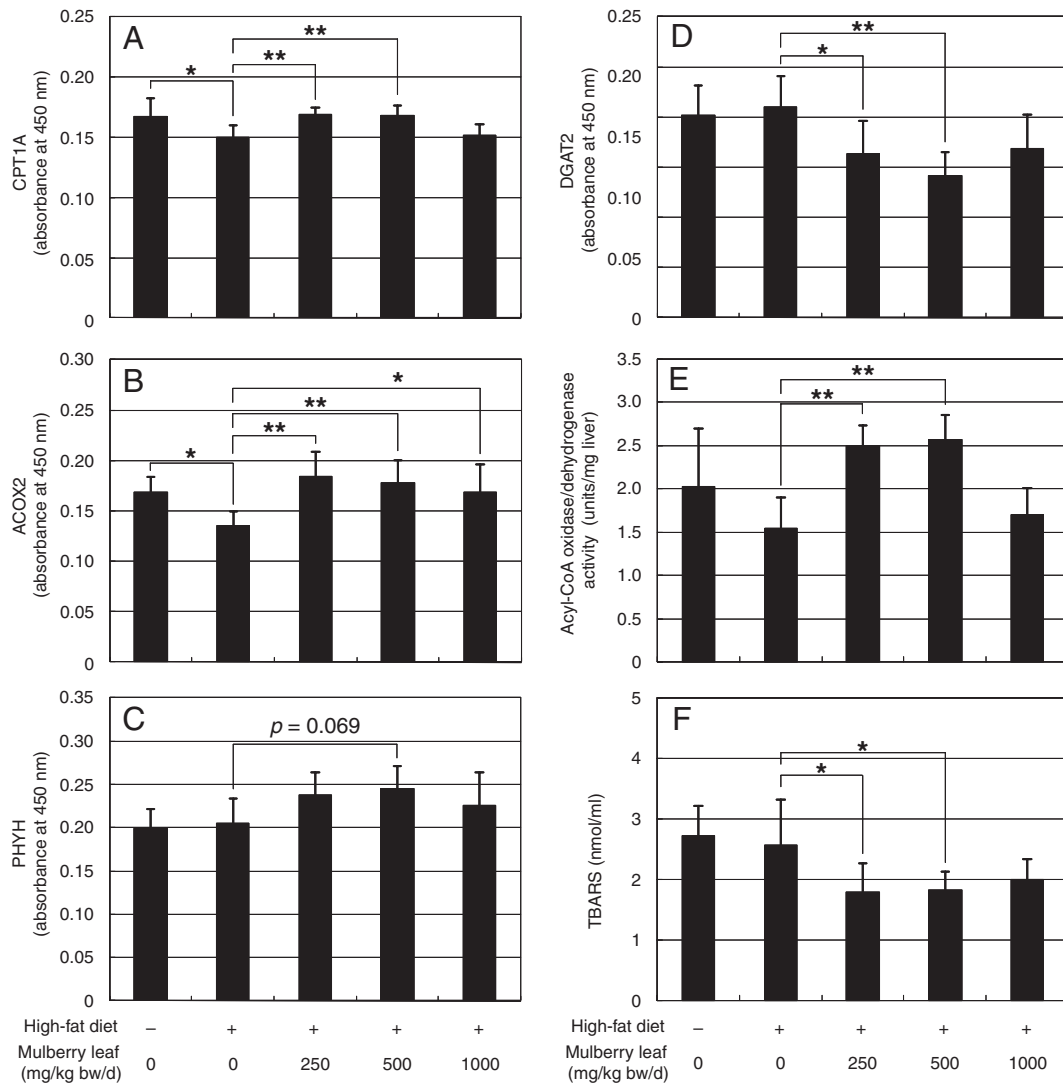


Fig. 4. Protein Expression Levels of CPT1A (A), ACOX2 (B), PHYH (C), and DGAT2 (D) in the Liver, Acyl-CoA Oxidase/Dehydrogenase Activity in the Liver (E), and Plasma TBARS Levels (F).

Statistical analysis was done by ANOVA with Dunnett's multiple comparison of means test as between the untreated high-fat diet group and the other groups. Vertical bars indicate standard deviations. Single and double asterisk values show significant differences at $p < 0.05$ and $p < 0.01$ respectively.

Measurement of acyl-CoA oxidase/dehydrogenase activity

Because dietary supplementation with mulberry increased the mRNA levels of *Acox1*, *Acadl*, and *Acadm*, the products of which catalyze the rate-limiting step in β -oxidation in the mitochondria and peroxisome (Fig. 2(2)), we measured hepatic acyl-CoA oxidase/dehydrogenase activity against a long-chain fatty acid (palmitoyl-CoA [$C_{16:0}$]) (Fig. 4E). We found no significant difference between the control group and the untreated high-fat diet group, although enzyme activity was slightly higher in the control group. Meanwhile, the enzyme activities in the high-fat fed rats treated with 250 and 500 mg/kg of mulberry were significantly higher than the untreated high-fat diet group.

Effects of mulberry on oxidative stress-related genes

Gene-annotation enrichment analysis revealed that some of the upregulated genes were involved in the response to oxidative stress (Table 4). The upregulated genes involved in this response are summarized in Fig. 3(18). The transcript levels of *Gpx1* and *Gpx4*

(glutathione peroxidases 1 and 4 respectively) were upregulated in the rats treated with mulberry. The proteins encoded by *Gpx1* and *Gpx4* are involved in the detoxification of hydrogen peroxide and lipid hydroperoxides respectively, and are the most important antioxidant enzymes. In addition, the expression levels of *Sod1* (superoxide dismutase 1) and *Aldh3a2* (aldehyde dehydrogenase family 3, subfamily A2) were higher in the mulberry-treated group. The enzyme encoded by *Sod1* destroys free superoxide radicals, while the product of the *Aldh3a2* gene is involved in aldehyde detoxification. Moreover, the administration of mulberry increased the mRNA levels of *Idh1* (isocitrate dehydrogenase 1 (NADPH), soluble), which is involved in the regeneration of NADPH for antioxidant systems, and *Nqo1* (NAD(P)H dehydrogenase, quinone 1), which supplies hydroquinone as an antioxidant. Furthermore, the following genes were upregulated by mulberry administration: *Ldha*, *Abcc2* (ATP-binding cassette, sub-family C member 2), *ApoE* (apolipoprotein E), *Clu* (clusterin), *Dusp1* (dual specificity phosphatase 1), *Hspd1* (heat shock 60kDa protein 1), *Oxr1* (oxidation

Table 4. Gene-Annotation Enrichment Analysis Based on the GO Database Using 600 Upregulated and 600 Downregulated Genes

GO-ID	Category	Gene counts	FDR corrected <i>p</i> -value
Upregulated biological process			
Metabolic process			
GO:0006631	Fatty acid metabolic process	19	4.59E-03
GO:0006635	Fatty acid beta-oxidation	8	2.35E-03
GO:0006807	Nitrogen compound metabolic process	34	5.88E-03
GO:0044270	Nitrogen compound catabolic process	11	1.17E-02
GO:0001523	Retinoid metabolic process	6	1.77E-02
GO:0006766	Vitamin metabolic process	11	4.91E-02
GO:0006412	Translation	47	1.49E-04
Response to stimulus			
GO:0007596	Blood coagulation	12	1.19E-02
GO:0010038	Response to metal ion	12	3.77E-03
GO:0006986	Response to unfolded protein	12	9.95E-03
GO:0031667	Response to nutrient levels	14	2.05E-02
GO:0048545	Response to steroid hormone stimulus	13	2.14E-02
GO:0006979	Response to oxidative stress	14	1.19E-02
GO:0006952	Defense response	41	1.07E-04
GO:0006958	Complement activation, classical pathway	12	4.37E-08
Cellular component organization			
GO:0022607	Cellular component assembly	34	3.03E-02
Establishment of localization			
GO:0006888	ER to Golgi vesicle-mediated transport	11	1.78E-03
GO:0006886	Intracellular protein transport	36	1.79E-04
Biological regulation			
GO:0051336	Regulation of hydrolase activity	16	2.05E-02
GO:0042981	Regulation of apoptosis	38	7.67E-03
Downregulated biological process			
Metabolic process			
GO:0006807	Nitrogen compound metabolic process	39	6.87E-05
GO:0006520	Amino acid metabolic process	27	7.39E-04
GO:0009063	Amino acid catabolic process	10	1.26E-02
GO:0000096	Sulfur amino acid metabolic process	8	5.01E-04
GO:0009069	Serine family amino acid metabolic process	9	7.60E-04
GO:0008610	Lipid biosynthetic process	31	1.04E-06
GO:0006694	Steroid biosynthetic process	15	7.92E-05
GO:0008203	Cholesterol metabolic process	13	1.28E-03
GO:0006120	Mitochondrial electron transport, NADH to ubiquinone	8	6.43E-03
GO:0051186	Cofactor metabolic process	33	5.22E-09
GO:0006769	Nicotinamide metabolic process	8	7.74E-03
GO:0006084	Acetyl-CoA metabolic process	8	3.84E-02
GO:0005975	Carbohydrate metabolic process	37	1.56E-03
GO:0005977	Glycogen metabolic process	9	3.27E-03
GO:0005996	Monosaccharide metabolic process	20	6.62E-03
GO:0006094	Gluconeogenesis	7	1.78E-02

resistance 1), and *Sfrs10* (splicing factor, arginine/serine-rich 10). These genes are induced by oxidative stress and alleviate the body's oxidative state. These gene expression changes suggest that mulberry administration reduces oxidative stress.

Measurement of lipid peroxidation

Because we found that mulberry upregulated the genes involved in the correction of oxidative stress, we measured the plasma levels of TBARS as a marker of lipid peroxidation (Fig. 4F). This experiment showed that dietary supplementation with 250 and 500 mg/kg of mulberry alleviated oxidative stress even though mulberry administration enhanced aerobic respiration.

Discussion

In this study, we investigated the effects of mulberry leaves in rats fed a high-fat diet. Daily oral administration of mulberry leaves reduced plasma triglyceride

and non-esterified fatty acid levels. DNA microarray analysis showed that dietary supplementation with mulberry enhanced fatty acid oxidation, mainly through β -oxidation of saturated (Fig. 3(2)) and unsaturated fatty acids (Fig. 3(3)) with long- or medium-length chains, in addition to ω -oxidation of long-chain fatty acids (Fig. 3(4)), α -oxidation of 3-methyl branched fatty acids (Fig. 3(5)), and β -oxidation of 2-methyl branched fatty acids (Fig. 3(6)). In addition, mulberry administration increased the protein expression levels of CPT1A, ACOX2, PHYH, and the activity of acyl-CoA oxidase/dehydrogenase (Fig. 4A–C and E), the rate-limiting enzymes of fatty acid oxidation. Furthermore, it suppressed lipogenesis (Fig. 3(12) to 3(14)). Indeed, mulberry reduced the protein expression of DGAT2, which is essential for *de novo* triglyceride synthesis (Fig. 4D). β -Oxidation is a source of reactive oxygen species (ROS) and thus causes oxidative stress and damage.²³⁾ Since mulberry enhances aerobic respiration using lipids as a source of energy, we considered that

ROS production and ROS-induced lipid peroxidation might be increased in rats treated with mulberry. Actually, however, mulberry administration upregulated the expression of genes involved in the response to oxidative stress (Fig. 3(18)) and thus limited oxidative stress (Fig. 4F).

Pathway enrichment analysis showed that the genes upregulated by mulberry administration included the following PPAR α and/or PPAR δ targets: *Cpt1a*, *Acox1*, *Acox2*, *Acadl*, *Acadm*, *Acaa1*, *Cyp4a3*, *Scp2*, *Fabp1*, *Gyk*, *Acsl5* (acyl-CoA synthetase long-chain family member 5), *Angptl4* (angiopoietin-like 4), and *Cyp8b1* (cytochrome p450, family 8, subfamily B, polypeptide 1). Many of these genes are involved in fatty acid oxidation (Fig. 3). In addition, PPAR α and/or PPAR δ upregulate *Acot4*, *Slc22a5*, *Bbox1*, *Aldh9a1*, and *Sod1*,^{18,24,25} and downregulate *Dgat2* and *Angptl3*.^{26,27} Such changes in gene expression were also observed in this study, suggesting that mulberry activates PPAR α and/or PPAR δ .

Mulberry leaves contain an abundance of flavonoid glycosides of quercetin, such as isoquercitrin.^{2,3} Enkhmaa *et al.* reported that their mulberry leaf powder contained 622 mg quercetin glycosides (equivalent to 352 mg quercetin aglycon) per 100 g.³ Indeed, the mulberry leaf powder used in this study contained an abundance of polyphenols (1.24×10^3 mg/100 g). Furthermore, a mass of flavonoid aglycons (570 mg/100 g) was found in this sample. Of these flavonoids, the major components were quercetin (367 mg/100 g, equivalent to about 600 mg of quercetin glycosides/100 g), and kaempferol (119 mg/100 g, equivalent to about 200 mg of kaempferol glycosides/100 g). Even onion (*Allium cepa* L.), which contains an abundance of quercetin as compared with various plant foodstuffs, contains only tens of milligrams of quercetin aglycon per 100 g of fresh edible part.^{28,29} The sample used in the present study contained from several to a dozen times more quercetin than onion.

It has been reported that quercetin exhibits hypolipidemic effects, including not only a hypocholesterolemic effect but also a hypotriglyceridemic effect.³⁰ Some of these effects have been attributed to the stimulatory effects of quercetin on the β -oxidation of fatty acids.³¹ Fatty acid oxidation occurs in three cell organelles, with the β -oxidation confined to the mitochondria and peroxisomes and ω -oxidation occurring in the endoplasmic reticulum. The key enzymes involved in these fatty acid oxidation systems are principally regulated by PPAR α and PPAR δ .^{32,33} Thus activation of these PPARs might be responsible for the upregulation of β -oxidation by quercetin. Meanwhile, quercetin was found to suppress lipogenesis.³⁴ This involves inhibition of DGAT protein,³⁵ which catalyzes the terminal step in triglyceride synthesis. It has been reported that PPAR α activation inhibits DGAT.²⁶ This study revealed upregulation of the genes involved in fatty acid oxidation, including several PPAR target genes, and downregulation of the genes involved in triglyceride synthesis, including *Dgat2*. The activation of PPAR α and/or PPAR δ by the quercetin in mulberry leaves probably participates in these changes in gene expression.

ω -Oxidation generates dicarboxylic acids, and subsequent β -oxidation of dicarboxylic acids produces

succinyl-CoA, which is hydrolyzed by ACOT4 to generate succinate. Mulberry supplementation upregulated transcription of the genes involved in ω -oxidation and elevated the expression level of *Acot4*. In addition, the catabolism of dicarboxylic acids has an anti-ketogenic effect.¹⁹ Downregulation of ketogenic genes was also observed in the mulberry-treated group. Furthermore, activation of ω -oxidation induces PPAR α -mediated β -oxidation and expression of *Fabp1*,³⁶ which encodes a carrier protein for fatty acids. In this study, these gene dynamics were confirmed in the rats treated with mulberry. The dicarboxylic acids produced by ω -oxidation also function as endogenous ligands for PPAR α ,³⁷ and expression of *Acot4* is induced by PPAR α .¹⁸ These findings provide compelling evidence for PPAR α activation by mulberry.

Angptl3 raises the plasma triglyceride level by inhibiting lipoprotein lipase,³⁸ which hydrolyzes triglyceride to glycerol and free fatty acids. Hence mulberry-induced downregulation of *Angptl3* is expected to result in activation of lipoprotein lipase and enhanced triglyceride hydrolysis. It appears that this contributes to the catabolism of fatty acids and glycerol. The free fatty acids produced in triglyceride degradation are recycled into triglycerides by the product of the *Gyk* gene.²¹ This futile cycle protects against excessive increases in blood non-esterified fatty acid levels.²² Since mulberry administration upregulated the expression of *Gyk* and caused a reduction in the plasma non-esterified fatty acid level, the futile cycle contributes to protect against the rise in the plasma free fatty acid level, in addition to increased energy expenditure through fatty acid oxidation. It is significant that *Angptl3* expression is suppressed by PPAR δ ,²⁷ while *Gyk* expression is induced by PPAR α ,²¹ further confirming the activation of PPAR δ and PPAR α by mulberry.

Scd is a gene involved in lipogenesis. It has been reported that decreased expression in the *Scd* gene inhibits lipogenesis and facilitates fatty acid oxidation, and thus suppresses triglyceride accumulation.²⁰ Since the expression of *Scd* was downregulated by mulberry administration, this downregulation appears to participate in the suppression of lipogenesis and the upregulation of fatty acid oxidation.

Mulberry administration increased the transcript levels of *Bbox1* and *Aldh9a1*, which encode carnitine synthetases, and the transcript of *Slc22a5*, the product of which raises the intercellular concentration of carnitine. In the mulberry-treated rats, the hepatic carnitine concentration might have been increased by enhanced carnitine synthesis and elevated hepatic uptake of carnitine. These activities might contribute to the accelerated rate of fatty acid oxidation. In addition, it has been reported that PPAR α upregulates the expression of these three genes, which are involved in carnitine metabolism.²⁴ Furthermore, it has been found that elevation of the carnitine concentration not only improves hyperlipidemia but also alleviates oxidative stress by the induction of antioxidant enzymes such as superoxide dismutase and glutathione peroxidases,³⁹ which were observed in this study. Therefore, increases in the hepatic carnitine concentration might improve a disease state, including hyperlipidemia and oxidative stress.

A high dosage of mulberry leaves (1,000 mg/kg) had a little effect on hypotriglyceridemic action. In our previous study, this high dosage was minimally effective for hypotriglyceridemic action.¹⁾ Dried mulberry leaf powder contains 30% and 34% soluble and insoluble dietary fibers respectively.⁴⁰⁾ Dietary fibers have an adsorbent action and attenuate the uptake of hydrophobic compounds. Therefore, the abundant dietary fibers on the high-dose mulberry treatment might have inhibited bioavailability of quercetin, which is the candidate for an active element and attenuates the lipid-lowering effect. On the other hand, the plasma and liver cholesterol levels of the mulberry-treated groups were non-significantly lower than that of the untreated high-fat diet group, especially high-dose group. The adsorption effect of the dietary fibers in mulberry leaves might reduce cholesterol levels.

Unlike the lowering effect of blood triglyceride, inhibition of triglyceride accumulation in liver could not be confirmed. The effects of the enhancement of lipolysis and fatty acid oxidation and inhibition of lipogenesis provably did not exceed the accumulation of triglyceride in the liver. On the other hand, lipolysis and fatty acid oxidation presumably increase in other tissues, for example, skeleton muscle and brown adipose tissue, and thus only the blood triglyceride level was reduced.

We also found that mulberry upregulated the expression of several genes involved in the response to oxidative stress. Quercetin, a major flavonoid in mulberry leaves, has radical-scavenging effects. In addition to this effect, quercetin can induce the expression of superoxide dismutase, glutathione peroxidases, and quinone reductase *in vitro* and *in vivo*.^{41,42)} The genes encoding those enzymes were upregulated by administration of mulberry in this study. Additionally, it has been reported that an increased expression level of *Sod* and a reduction of TBARS, which were observed in the present study, are induced by bezafibrate, an agonist of PPAR α used as a hypolipidemic drug.²⁵⁾ Regarding these antioxidative effects, the functions of quercetin and/or PPAR signaling are likely to be involved in the amelioration of oxidative stress. These effects are probably responsible for the antioxidative effects of mulberry leaves.

In this study, we confirmed that mulberry has lipid-lowering effects and elucidated the underlying mechanism. Administration of mulberry leaves induces lipolysis and fatty acid oxidation, and also inhibits lipogenesis. Presumably, the lipid-lowering effects are not due to changes in appetite, although we did not measure food intake in this study, because previous studies have revealed that mulberry leaves do not affect food intake.^{43,44)} Energy expenditure probably involves the activation of PPARs. Additionally, Park *et al.*⁵⁾ have suggested that PPAR-mediated pathways are involved in the lipid-lowering effects of mulberry. On the other hand, because mulberry enhances energy expenditure from fatty acids, the production of ROS might increase. Nevertheless, oxidative stress is limited by the induction of antioxidative enzymes. We suggest that quercetin contributes at least partly to the improvement in dyslipidemia and amelioration of oxidative stress associated with mulberry administration. As previously

described, quercetin could activate PPARs. Further studies focusing on the components of mulberry, such as quercetin, are needed to fully understand the functional mechanisms of mulberry.

Acknowledgment

This work was supported by a grant from the Kanagawa Academy of Science and Technology. We thank Ms. Junko Aso and Ms. Kyoko Shiogai of the Chemistry Division of the Kanagawa Prefectural Institute of Public Health for their assistance and support during this study.

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