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Effects of *Morus alba* L. (Mulberry) Leaf Extract in Hypercholesterolemic Mice on Suppression of Cholesterol Synthesis

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Abstract

Study background: Hypercholesterolemia causes arteriosclerosis, a risk factor for cerebral or myocardial infarctions. Prevention of hypercholesterolemia by improving dietary habits has recently attracted attention in many countries. It has been reported that the leaves of the mulberry plant, *Morus alba* L., which is commonly used for tea in Asian countries, can ameliorate hypercholesterolemic conditions.

Method: To determine its mechanism of action, we performed gene expression profiling of the liver of mice fed a high-cholesterol diet and a polyphenol-rich mulberry leaf extract containing abundant quercetin and kaempferol for 4 weeks.

Results: The levels of total cholesterol, low-density lipoprotein cholesterol, aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase in plasma, and level of total cholesterol in the liver were significantly lower in the mice treated with the mulberry leaf extract than that in the control group mice. DNA microarray analysis revealed that mulberry extract downregulated the expression of genes involved in cholesterol biosynthesis, including hydroxymethylglutaryl-CoA reductase gene, and upregulated the transcription of peroxisome proliferator-activated receptor (PPAR)α and γ, transcriptional factors known to regulate lipid metabolism or immunity, and their target genes. Additionally, the mulberry extract stimulated both innate and acquired immunity, including the induction of scavenger and Toll-like receptors and the activation of pathways in various lymphocytes, such as macrophages, eosinophils, neutrophils, natural killer cells, B cells, and T cells.

Conclusion: The results obtained in this study suggest that quercetin and kaempferol in the mulberry leaf induce the activation of PPARα and PPARγ, transcription of *Ppara* and *Pparg* genes, and stimulation of PPAR signaling pathways. These phenomena ultimately lead to the reduction of cholesterol synthesis and immunostimulation.

Keywords: Adaptive immunity; Mulberry (*Morus alba* L); Cholesterol biosynthesis; HMG-CoA reductase; Innate immunity; Kaempferol; Peroxisome proliferator-activated receptor (PPAR); Quercetin; Toll-like receptor

Introduction

In developed countries, atherosclerotic disease increases the risk of myocardial and cerebral infarctions, which are major causes of death [1]. Hypercholesterolemia, hypertriglyceridemia, obesity, hypertension, and impaired glucose metabolism are now recognized as risk factors for arteriosclerosis. Indeed, hypercholesterolemia is part of an important cluster of risk factors for coronary artery disease. Growth of atherosclerotic plaques in the coronary arteries in low-density lipoprotein (LDL)-hypercholesterolemic individuals is associated with an increase in plaque levels of oxidized LDL [2]. Higher concentration of oxidized LDL was associated with increased incidence of metabolic syndrome overall, as well as its components, abdominal obesity, hyperglycemia, and hypertriglyceridemia [3]. Since treatment results in huge health care costs, and places an enormous financial burden not only on individual patients, but also on the government, prevention of hypercholesterolemia by improving dietary habits, e.g. by increasing the consumption of functional foods, has attracted attention in many countries, especially developed ones.

Mulberry (*Morus alba* L) is a deciduous tree native of China and Korea, belonging to Moraceae family. This tree was naturalized in various Asian countries such as Japan in ancient times. The leaf, root bark, and fruit of the mulberry plant have a long history in traditional Chinese medicine. The use of the root bark of mulberry is antitussive, anti-inflammatory agent, expectorant and diuretic. The fruit of mulberry has been used as tonic and analgesic agents. On the other hand, the leaf of mulberry has been used for pyretolysis, antitussive treatment, improvement of eye problems and blood cooling. Addition to these effects, the effects of mulberry for cardiovascular disease (Chuhu in ancient Japanese) had been described in the first treatise

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on tea, Kissa-yojoki, which was published in Japan in 1211. Nowadays, mulberry leaves have become available not only as traditional Chinese medicine but also as various food products containing mulberry leaves, such as mulberry tea, in many countries.

Our previous studies indicate that administration of mulberry leaves ameliorates dyslipidemia in general, and hypercholesterolemia in particular [4,5]. Other independent researchers have also demonstrated its hypocholesterolemic effects [6]. Mulberry leaves contain an abundance of the flavonoids quercetin (PubChem CID: 5280343) and kaempferol (PubChem CID: 5280863) (Supplementary Figure S1) [5,7]. Flavonoids, especially quercetin, are inversely correlated with levels of total cholesterol or LDL cholesterol in humans [8]. Therefore, the flavonoids in mulberry leaves are expected to similarly produce hypocholesterolemic effects. A flavonoid-rich mulberry leaf extract was found to reduce plasma levels of cholesterol in rabbits fed a high-cholesterol diet [5]. However, the functional mechanisms underlying this effect are still unknown. In the previous study, we demonstrated that supplementation with mulberry downregulates the cholesterol metabolic process and steroid biosynthesis, and upregulates the peroxisome proliferator-activated receptor (PPAR) signaling pathway [7,9]. In addition, it has been reported that PPARα agonists inhibit cholesterol synthesis [10-14]. Therefore, we predicted that the mulberry leaf also contains PPARα agonists that reduce cholesterol levels in hypercholesterolemia via the PPAR signaling pathway. We used DNA microarray analysis to investigate gene expression in the livers of hypercholesterolemic mice treated with a polyphenol-rich mulberry leaf extract to elucidate the mechanisms involved in the hypocholesterolemic effects of the extract.

Materials and Method

Materials

Leaves of mulberry, *Morus alba* L., were collected at mulberry plantation in August in Aikawa, Kanagawa, Japan. A dried mulberry leaf sample was added to a 10-fold weight of 50% methanol and stirred for 2 h. The extract was filtered and centrifuged. The supernatant was passed through a column filled with a cation-exchange resin (Amberlite IR-120B, H+ form; Organo, Tokyo, Japan). The filtrate was concentrated using an evaporator to remove the methanol and was applied to a column filled with Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). After washing with water, the column was eluted with methanol. The eluent containing polyphenols was dried using the evaporator, and the residue obtained was the mulberry extract used in the following experiment.

Quantification of total polyphenols

The concentration of the total polyphenols in the mulberry extract was estimated using a Folin-Ciocalteu assay with gallic acid as a standard. The dried mulberry leaf extract was dissolved in water at a concentration of 333 μg/mL. An aliquot (300 μL) was added to 2 N Folin-Ciocalteu reagent (150 μL). At an interval of 3 min, 2% Na_2CO_3 (900 μL) was added, and the mixture was allowed to stand for 15 min. The absorbance at 750 nm was measured by spectrophotometer.

Quantification of total flavonoids

The dried mulberry leaf extract was dissolved in 50% methanol at a concentration of 1.12 mg/mL. After centrifugation at 20,000 × g for 1 min, the total amount of flavonoid glycosides in the supernatant was measured as aglycon equivalents by using an aluminum chloride colorimetric method. Quercetin, an abundant flavonoid in mulberry leaves, was used to construct a calibration curve. The supernatant (200 μL) was mixed with methanol (200 μL), 10% aluminum chloride (20 μL), 1 mol/L potassium acetate (20 μL), and water (560 μL). After incubation at room temperature for 30 min, the absorbance at 415 nm was measured by spectrophotometer. The same mixture, but without aluminum chloride, was used as a blank for each sample.

Quantification of quercetin and kaempferol

Quantification of quercetin and kaempferol, the major flavonoids in mulberry leaves, was achieved by reversed-phase high performance liquid chromatography (HPLC). First, the flavonoid glycosides were hydrolyzed to aglycons. The dried mulberry leaf extract was dissolved in 80% methanol at a concentration of 10 mg/mL. Aliquot (150 μl) was mixed with 600 μL of methanol containing 500 μg/mL tert-butylhydroquinone and 150 μL 2 mol/L HCl, before heating at 90°C for 3 h. The sample was added to 1,200 μL 1 mol/L Tris-HCl (pH 7.5) and 525 μL of dimethylsulfoxide, and the mixture was applied to reversed-phase HPLC. The column used was an Inertsil ODS-3 (4.6 mm × 150 mm; GL Sciences, Tokyo, Japan). 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, in the following order: 95% solution A, linear gradient of solution B from 5%-20% in 30 min, linear gradient of solution B from 20%-50% in 30 min, and 50% of solution B for 20 min. The eluates were monitored at 260 and 370 nm with a UV detector. Quercetin and kaempferol were used to construct the calibration curve.

Ethics statement

Animal experiments were approved by the Animal Care and Use Committee at the Kanagawa Prefectural Institute of Public Health (Permit Number: 2008-13). All animal experiments were performed according to the guidelines for animal experimentation of the Kanagawa Prefectural Institute of Public Health, which complies with Act on Welfare and Management of Animals (Ministry of the Environment, Japan) and Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Ministry of the Environment, Japan).

Animals

Male C57BL/6NCrSlc mice (5-weeks-old) were purchased from Japan Slc (Shizuoka, Japan), and kept at 25°C with a 12 h light/dark cycle (light on at 8:00). They were fed a normal powdered diet (CE-2; Clea Japan, Kanagawa, Japan) for 1 week, and then divided into the following 3 groups (n=7) based on diet: high-cholesterol, consisting of powdered QuickFat (Clea Japan) with added 2% cholesterol and 0.4% cholic acid without administration of the mulberry leaf extract (control group); high-cholesterol with administration of the mulberry leaf extract at two concentrations (mulberry groups). Mice in the two mulberry groups were orally administrated the extract solution at 0.1 or 1 mg/mL as drinking water ad libitum for 4 weeks. After 12 h of food deprivation, the mice were anesthetized by pentobarbital. Blood samples were collected via heart using heparin as an anticoagulant, and the mice were euthanized by blood removal under anesthesia. The liver of each mouse was taken, weighed, and frozen. Parts of liver were immediately immersed in RNAlater (Applied Biosystems, Foster City, California, USA) for DNA microarray analysis.

Biochemical examination of blood

Plasma was prepared from the collected blood by centrifugation at 2,000 × g. Plasma levels of triglyceride, total cholesterol, high-density

lipoprotein (HDL) cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were quantified using a Fuji DRI-CHEM 7000 system (Fujifilm, Tokyo, Japan). Plasma levels of LDL cholesterol were calculated using Friendwald's equation [15].

Determination of lipid in liver

The frozen liver (about 200 mg) was homogenized with 200 μL of water using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) with metal beads at 2,500 rpm for 20 s. Lipids were extracted with 600 μL of chloroform-methanol (1:2, v/v) twice. After centrifugation, the extracts in the lower layer were collected, evaporated in vacuo to dryness, and dissolved in isopropyl alcohol-Triton X-100 (9:1, v/v). Cholesterol, triglyceride, and non-esterified fatty acids (NEFA) in the extracts were measured using LabAssay kits (Wako, Osaka, Japan) according to the manufacturer's protocol.

RNA isolation and microarray hybridization

Ten mice (5 mice/group) were randomly selected from the control group and the 1 mg/mL mulberry group, and used in a subsequent experiment. Total RNA was extracted from the RNAlater-soaked liver of each mice in the control group and the 1 mg/mL mulberry group by using RNAiso solution (Takara Bio, Shiga, Japan) and purified using an RNeasy Mini kit (Qiagen, Tokyo, Japan). RNA qualities were checked using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, California, USA), and the average RIN score was calculated to be 8.3 ± 0.4. The total RNA (500 ng) was converted to double-strand cDNA using Agilent's Quick Amp Labeling Kit, One-Color (Agilent Technologies) according to the manufacturer's protocol. Cyanine 3-labeled cRNA was synthesized from the double-strand cDNA using the same kit. After washing using the RNeasy Mini kit, the cRNA was fragmented and hybridized to a Whole Mouse Genome Microarray 4x44K (Agilent Technologies) using a Hi-RPM Hybridization Kit (Agilent Technologies). After the array (n=5/group) was washed using Gene Expression Wash Buffer (Agilent Technologies), the fluorescence intensity was scanned using a DNA Microarray Scanner (Agilent Technologies).

DNA microarray data analysis

The scanned images were analyzed with Agilent Feature Extraction 10.1.1.1 software (Agilent Technologies) to obtain background-subtracted and spatially detrended, processed signal intensities. All data were deposited in the Gene Expression Omnibus public repository of the National Center for Biotechnology Information [16] under GEO Series accession number GSE65839. Signal intensities (gProcessedSignal) were normalized with quantile normalization [17] using statistical language R (http://www.r-project.org/) and the 'limma' version 3.6.9 package (http://www.bioconductor.org/). Probes with a gene symbol were extracted from all probes, and the signal intensities of the same gene symbol were averaged using statistical language R. The total number of probes was 21,200. The normalized, extracted, and averaged signals were transformed to a log 2 scale. To detect genes that were statistically and differentially expressed between the two groups, the probes were ranked by the Weighted Average Difference method [18] using statistical language R. The normalized, extracted, and averaged data were applied to analysis using statistical language R with R-package OCplus (http://www.bioconductor.org/), and the number of differentially expressed genes was detected to be about 3,000. Therefore, the 3,000 top-ranked probes (1,705 upregulated genes and 1,295 downregulated genes; supplementary Tables S1 and S2) in the gene ranking were subjected to further analysis. Next, pathway enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [19] was performed on the 3,000 top-ranked genes (up or downregulated genes) using the Database for Annotation, Visualization, and Integrated Discovery program [20]. Then gene-annotation enrichment analysis based on the Biological Process in Gene Ontology (GO) database [21] was performed on the 3,000 top-ranked genes (up or downregulated genes) using the Cytoscape version 2.8.1 software (http://www.cytoscape.org/) and BiNGO version 2.42 plugin [22]. Terms on the pathway enrichment analysis and the gene-annotation enrichment analysis that had FDR-corrected p-values less than 0.0001 were extracted as significantly enriched terms.

Enzyme-linked immunosorbent assay

Mouse liver samples were homogenized in 100 mmol/L potassium phosphate buffer (pH 7.4), using a Multi-Beads Shocker (Yasui Kikai) with metal beads at 2,500 rpm for 20 s. The buffer contained 1% Triton X-100 and a 1% Protease Inhibitor Cocktail for Use with Mammalian Cell and Tissue Extracts (Nacalai Tesque, Kyoto, Japan). The extracts were treated in an ultrasonic bath for 20 min. After centrifugation at 500 × g for 30 min, the supernatant was mixed with an equal volume of glycerol, and this mixture was used in the subsequent experiment. Protein levels of HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase), CYP51 (cytochrome P450, family 51), TM7SF2 (transmembrane 7 superfamily member 2), SC4MOL (sterol-C4-methyl oxidase-like), encoded by the *Hmgcr, Cyp51, Tm7sf2*, and *Sc4mol* genes, respectively, were determined by enzyme-linked immunosorbent assay (ELISA), essentially as reported previously [7]. In brief, each extract (diluted 5-30 fold) was coated on a 96-well microtiter plate and reacted successively with the primary antibodies goat anti-HMGCR polyclonal antibody (6.67 μg/mL, HMGCR, C-18; Santa Cruz Biotechnology, Santa Cruz, California, USA), rabbit anti-CYP51A1 polyclonal antibody (0.267 μg/mL, Cat. No. 13431-1-AP; ProteinTech, Chicago, Illinois, USA), goat anti-TM7SF2 polyclonal antibody (6.67 μg/mL, TM7SF2, L-12; Santa Cruz Biotechnology), or rabbit anti-SC4MOL polyclonal antibody (3.33 μg/mL, ERG25, Y-21; Santa Cruz Biotechnology), followed by the secondary antibodies: horseradish peroxidase-conjugated rabbit anti-goat IgG antibody (0.1 μg/mL, Cat. No. A50-100P; Bethyl Laboratories, Montgomery, Texas, USA) for the detection of HMGCR or TM7SF2, or horseradish peroxidase-conjugated sheep anti-rabbit IgG antibody (1:500, Cat. No. 55677; Cappel, Cochranville, Pennsylvania, USA) for the detection of CYP51 or SC4MOL. After the addition of the substrate, the color developed was measured by its absorbance at 450 nm.

Statistical analysis

Statistically significant differences between the control group and mulberry groups, except in the DNA microarray analysis, were analyzed using Dunnett's multiple comparison tests.

Results

Analysis of polyphenols

The mulberry leaf extract contained 137 mg/g polyphenols. The concentration of total flavonoid aglycons in the mulberry leaf extract was 52.3 mg/g dry weight. Quercetin and kaempferol were present at concentrations of 22.1 and 18.0 mg/g of the mulberry leaf extract, respectively.

Effects on metabolic variables

Table 1 shows the body weight, liver weight, liver weight vs. body

	Concentration of polyphenol-rich extract from mulberry (mg/ml)		
	0	0.1	1
Body weight (g)	22.0 ± 0.5	23.2 ± 0.5	22.5 ± 0.4
Liver weight (g)	1.27 ± 0.04	1.41 ± 0.09	1.34 ± 0.06
Liver weight/Body weight (mg/g)	57.6 ± 1.8	60.4 ± 2.6	56.0 ± 3.0
Liver triglyceride (mg/g)	14.9 ± 3.6	9.5 ± 1.9	24.9 ± 6.3
Liver cholesterol (mg/g)	21.0 ± 2.3	12.9 ± 1.4**	14.9 ± 2.1*
Liver NEFA (mg/g)	11.5 ± 2.1	10.6 ± 1.5	17.1 ± 2.6
Plasma triglyceride (mg/dl)	39.4 ± 6.8	57.3 ± 9.2	47.3 ± 7.5
Plasma total cholesterol (mg/dl)	214 ± 7	172 ± 16*	140 ± 18**
Plasma LDL cholesterol (mg/dl)	147 ± 10	103 ± 16*	83 ± 14**
Plasma HDL cholesterol (mg/dl)	58.7 ± 3.8	58.0 ± 6.7	47.7 ± 6.6
Plasma HDL cholesterol/ Plasma total cholesterol	27.8 ± 2.6	34.9 ± 4.1	35.4 ± 3.6
Plasma AST (U/dl)	179 ± 13	115 ± 21**	99 ± 3**
Plasma ALT (U/dl)	289 ± 67	85 ± 20**	97 ± 13**
Plasma ALP (U/dl)	532 ± 23	481 ± 21	512 ± 22
Plasma LDH (U/dl)	1041 ± 79	700 ± 54*	767 ± 114*

Asterisks indicate significant difference from the control group (*p<0.05, **p<0.01).

Table 1: Effect of polyphenol-rich extract from mulberry on metabolic variables (mean ± SE).

ID	Category	Gene counts	Fold enrichment	FDR-corrected p-value
— Up-regulated pathway —				
map03010	Ribosome	37	3.8	7.26×10^{-11}
map04650	Natural killer cell mediated cytotoxicity	41	3.1	4.73×10^{-9}
map04640	Hematopoietic cell lineage	32	3.5	1.71×10^{-8}
map04514	Cell adhesion molecules	43	2.5	3.59×10^{-7}
map04062	Chemokine signaling pathway	48	2.4	3.98×10^{-7}
map04060	Cytokine-cytokine receptor interaction	57	2.1	9.71×10^{-7}
map04612	Antigen processing and presentation	30	3	1.29×10^{-6}
map05332	Graft-versus-host disease	22	3.4	7.20×10^{-6}
map04660	T cell receptor signaling pathway	33	2.5	1.21×10^{-5}
map04662	B cell receptor signaling pathway	26	3	1.28×10^{-5}
map03320	PPAR signaling pathway	25	2.9	3.09×10^{-5}
map00071	Fatty acid metabolism	18	3.6	3.22×10^{-5}
map04670	Leukocyte transendothelial migration	32	2.4	3.55×10^{-5}
map05330	Allograft rejection	20	3.1	8.34×10^{-5}
— Down-regulated pathway —				
map04610	Complement and coagulation cascades	28	4.7	8.48×10^{-10}
map00830	Retinol metabolism	26	4.8	1.56×10^{-9}
map00140	Steroid hormone biosynthesis	21	5.9	1.92×10^{-9}
map00980	Metabolism of xenobiotics by cytochrome P450	25	4.8	2.63×10^{-9}
map00982	Drug metabolism	26	4.4	7.33×10^{-9}
map00150	Androgen and estrogen metabolism	17	6.5	1.88×10^{-8}
map00040	Pentose and glucuronate interconversions	10	7.4	3.99×10^{-5}

Table 2: Pathway enrichment analysis based on the KEGG database using the 1705 up-regulated and 1295 down-regulated genes.

weight, liver lipid contents, and blood components of the mice. There were no differences in body weight, liver weight, liver weight vs. body weight, liver triglyceride or liver NEFA between diets. As expected, the control group had high liver and plasma cholesterol levels. The mulberry extracts significantly suppressed increases of liver cholesterol as well as total and LDL cholesterol in the plasma in a concentration-dependent manner. The ratio of total cholesterol to HDL cholesterol in the plasma increased in the mulberry groups, although the differences were not significant. AST, ALT, and LDH levels in the plasma were also suppressed following mulberry administration, while plasma HDL cholesterol and plasma ALP levels were unchanged.

Effects of mulberry extract on gene expression in the liver

Table 2 shows the results of the pathway enrichment analysis using up and downregulated genes. This analysis [20] screens about 350 biological pathways in the KEGG database [19]. The PPAR signaling pathway and fatty acid metabolism were upregulated in the mulberry-treated group, while steroid hormone biosynthesis and the androgen and estrogen metabolism were downregulated. In addition to lipid metabolism, effects on the immune system were also observed in the treatment group. Mulberry supplementation upregulated natural killer cell-mediated cytotoxicity, cell adhesion molecules, the chemokine signaling pathway, cytokine-cytokine receptor interaction, antigen processing and presentation, graft-versus-host disease pathway, T cell receptor signaling pathway, B cell receptor signaling pathway, and leukocyte transendothelial migration (see Supporting manuscript and Supplementary Figure S2-S5).

Next, we performed a gene-annotation enrichment analysis for approximately 18,800 items corresponding to biological processes in the GO database [21]. Using BiNGO, the enrichment analysis [22] extracted 533 GO terms using upregulated genes and 124 GO terms using downregulated genes (supplementary Tables S3 and S4), and visualized the intricately hierarchical structures of GO categories in various biological processes. Since this study focuses on the effects of mulberry on lipid metabolism and the deepest regions in hierarchical structures are more essential in understanding the dynamics of gene expression, we further extracted the deepest GO terms related to lipid metabolism from these hierarchical structures (Figures 1 and 2). Processes relating to facilitation of fatty acid oxidation were particularly prominent among the upregulated terms of lipid metabolism, including the long-chain fatty acid metabolic process (FDR-corrected p-value=2.92×10^{-8}), long-chain fatty acid transport (3.31×10^{-7}), fatty acid β-oxidation (7.85×10^{-6}), very long-chain fatty acid metabolic process (1.19×10^{-5}), acyl-CoA metabolic process (1.73×10^{-5}), and negative regulation of lipid metabolic process (4.06×10^{-5}). Mulberry treatment downregulated the cholesterol biosynthetic process (3.69×10^{-12}) and the fatty acid biosynthetic process (4.52×10^{-7}).

Expression of genes involved in the synthesis and esterification of cholesterol

We focused our investigation on the hypocholesterolemic effect of mulberry extract in this study, and describe the up and downregulated genes that are involved in the synthesis and esterification of cholesterol below (Figure 3). Cholesterol is synthesized from acetyl-CoA and esterified to cholesterol ester in 23 steps. Only the catalysis step of Hmgcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2, mitochondrial) was upregulated in the mulberry group, whereas the following 12 genes in 14 steps of the cholesterol synthesis were downregulated: *Hmgcr, Pmvk* (phosphomevalonate kinase), *Idi1* (isopentenyl-diphosphate δ isomerase 1), *Fdps* (farnesyl diphosphate synthase), *Fdft1* (farnesyl-

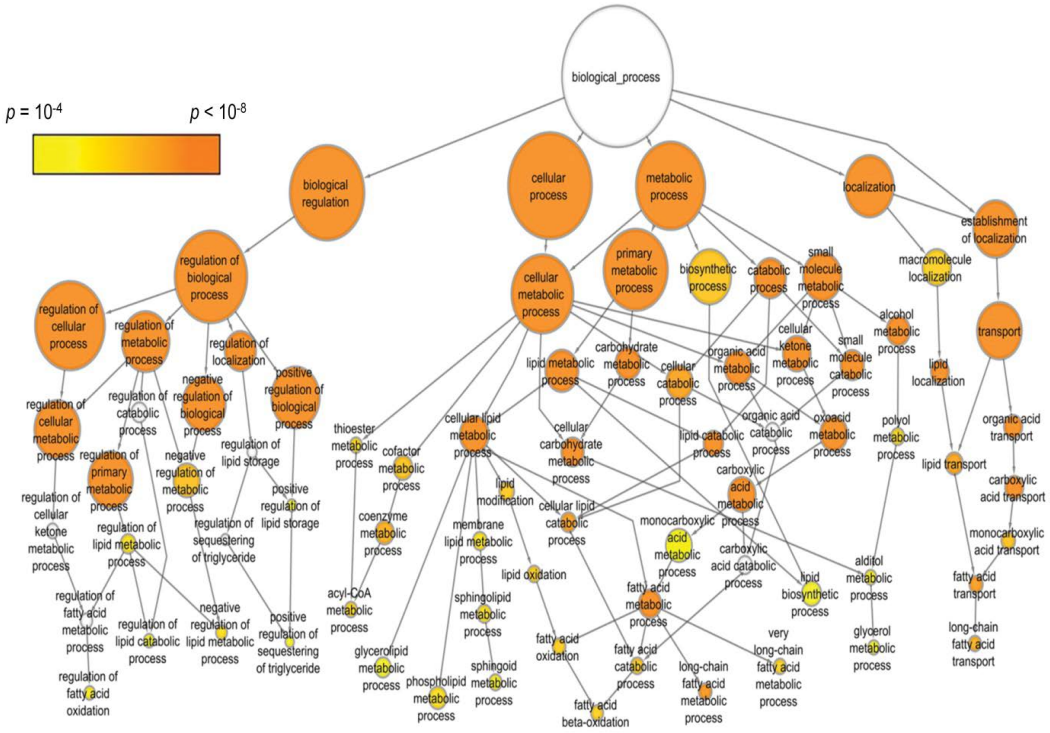

Figure 1: Hierarchical structures of GO categories involved in lipid metabolism extracted using upregulated genes. GO categories were extracted by gene enrichment analysis (p<0.0001) using 1,705 upregulated genes. Colored bar expresses significance level for categories by hypergeometric test with Benjamini and Hochberg false discovery rate (FDR) correction. Node size indicates number of genes associated with each GO term (i.e., larger nodes represent GO terms associated with many genes).

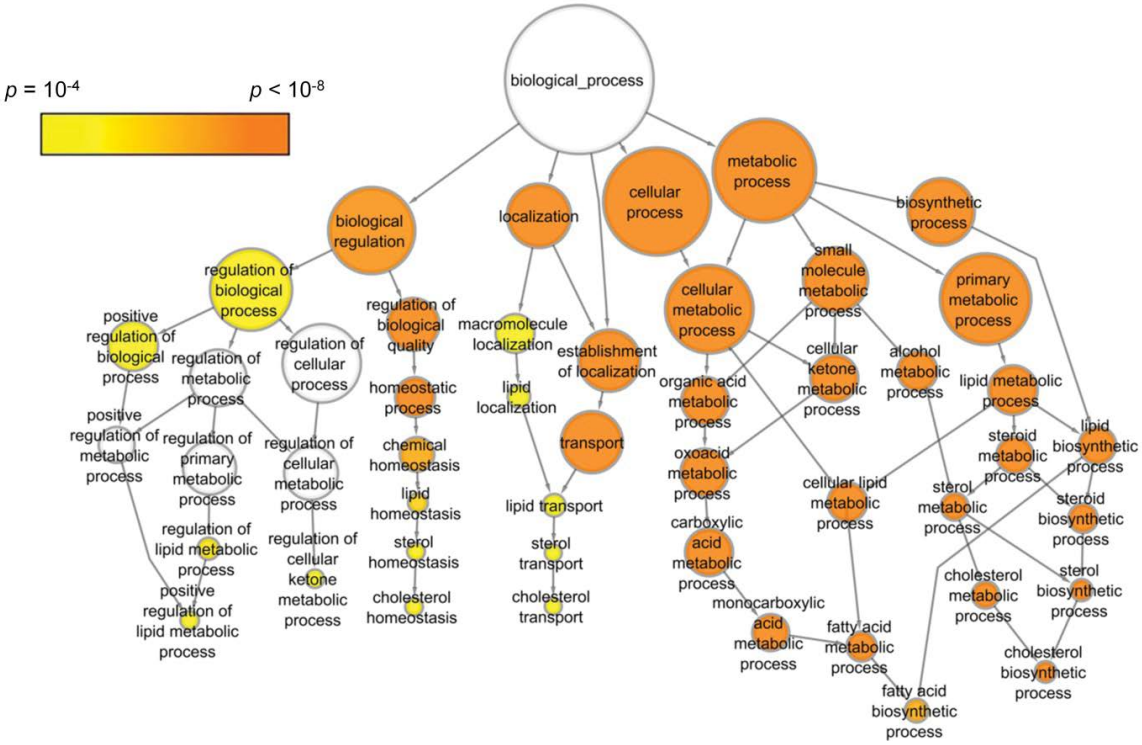

Figure 2: Hierarchical structures of GO categories involved in lipid metabolism extracted using downregulated genes. GO categories were extracted by gene enrichment analysis (p<0.0001) using 1,295 downregulated genes. Colored bar expresses significance level for categories by hypergeometric test with Benjamini and Hochberg false discovery rate (FDR) correction. Node size indicates number of genes associated with each GO term (i.e., larger nodes represent GO terms associated with many genes).

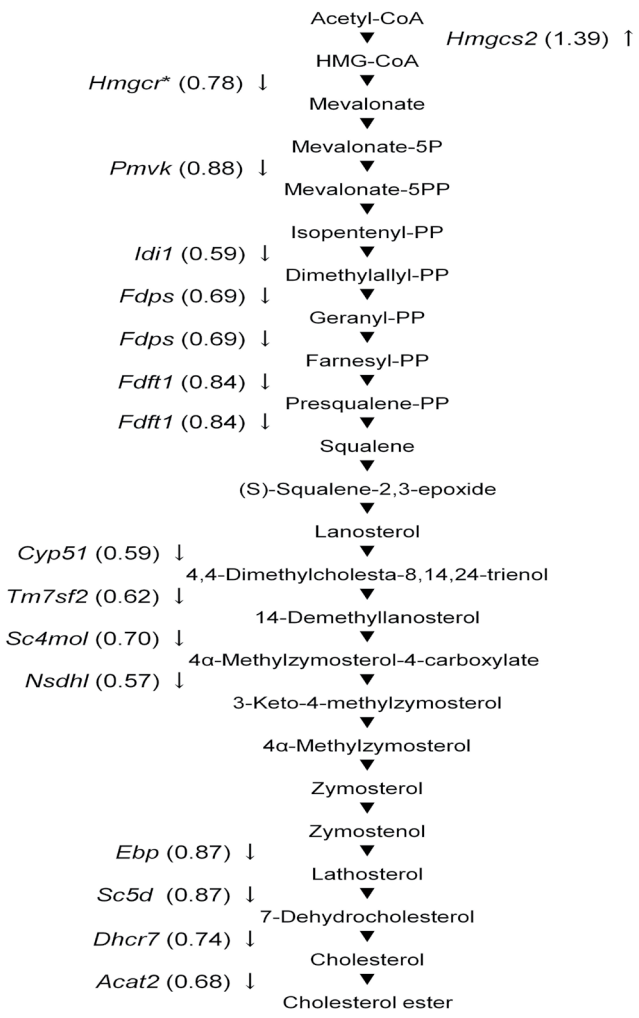

Figure 3: Genes responsible for the synthesis and esterification of cholesterol in the mulberry group. Up and down arrows indicate up and downregulated genes, respectively. Asterisk indicates gene that codes a rate-limiting enzyme. Fold changes of signal intensity is in parentheses.

diphosphate farnesyltransferase 1), *Cyp51, Tm7sf2, Sc4mol*, *Nsdhl* (NAD(P)-dependent steroid dehydrogenase-like), *Ebp* (emopamil binding protein, sterol isomerase), *Sc5d* (sterol-C5-desaturase homolog), and *Dhcr7* (7-dehydrocholesterol reductase). In these genes, *Hmgcr*, which codes for the HMGCR protein, is especially important because it catalyzes the rate-limiting step of cholesterol biosynthesis. Similarly, the *Acat2* (acetyl-Coenzyme A acetyltransferase 2) gene, which catalyzes the esterification of cholesterol, was downregulated in the mulberry-treated group. In addition, *Srebf1* (sterol regulatory element-binding transcription factor 1) coding intranuclear transcription factor, SREBP-1 (sterol regulatory element-binding protein 1), which upregulates expression of HMGCR, was downregulated in the mulberry-treated group.

Measurement of HMGCR, CYP51, TM7SF2, and SC4MOL proteins

Since the *Hmgcr, Cyp51, Tm7sf2*, and *Sc4mol* genes, which were expressed at lower levels in the mulberry group, are essential for cholesterol biosynthesis; we measured the expression levels of the HMGCR, CYP51, TM7SF2, and SC4MOL proteins by ELISA (Figure 4). Compared with the control group, the protein expression levels of HMGCR, CYP51, and TM7SF2 tended to decrease dose-dependently in the mulberry groups. The expression of these proteins in the 1 mg/mL mulberry group was significantly lower than that in the control group. There was no change in the expression of SC4MOL.

Upregulated PPAR genes and PPAR signaling pathway

Two of 3 PPAR isoform genes, *Ppara* and *Pparg*, were upregulated by intake of the mulberry polyphenol fraction (Table 3). *Ppard*, which encodes PPARδ, was not up or downregulated following mulberry consumption. Furthermore, 37 PPAR target genes (Table 3) were upregulated after mulberry supplementation. Many of these genes code important proteins that function as rate-limiting enzymes in fatty acid metabolism, in particular the β- and ω-oxidation of fatty acids. For instance, mulberry supplementation facilitated the expression of *Acadl* (acyl-CoA dehydrogenase, long-chain), *Acadm* (acyl-CoA dehydrogenase, medium chain), and *Acox1* (acyl-CoA oxidase 1, palmitoyl), which encode the rate-limiting enzyme in the β-oxidation of fatty acids. Oral administration of mulberry extract upregulated the expression of *Cpt1a* (carnitine palmitoyltransferase 1a, liver), *Cpt1b*, and *Cpt2*, which are essential for the transport of fatty acids into the mitochondria, and whose products are rate-limiting enzymes of β-oxidation. In addition, mulberry increased the transcript levels of *Cyp4a10* (cytochrome P450, family 4, subfamily a, polypeptide 10), *Cyp4a14*, and *Cyp4a31*, genes that code the rate-limiting enzymes in the ω-oxidation of long-chain fatty acids in the endoplasmic reticulum for β-oxidation at both ends. In addition to genes related to lipid metabolism, mulberry supplementation induced the expression of *Cd36*, which encodes the CD 36 antigen, which is a scavenger receptor for β-glucan and oxidized LDL.

Discussion

In this study, we investigated the effects of the polyphenol-rich extract of mulberry leaves on mice fed a high-cholesterol diet. Daily oral administration of the extract suppressed an increase in total and LDL cholesterol in the plasma, and total cholesterol in the liver. Furthermore, the supplementation of mulberry depressed the

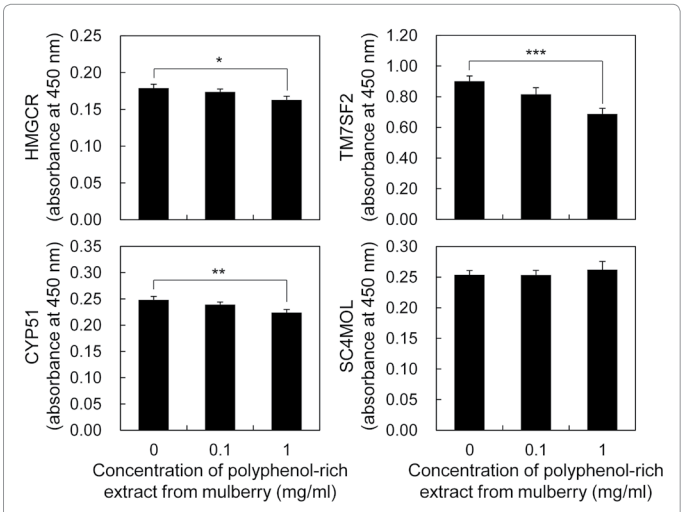

Figure 4: Protein expression levels of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), cytochrome P450, family 51 (CYP51), transmembrane 7 superfamily member 2 (TN7SF2), and sterol-C4-methyl oxidase-like (SC4MOL) in the liver. Statistical analysis was performed using Dunnett's multiple comparison test between the untreated high-cholesterol diet group and the other groups. Vertical bars indicate standard error. Single, double, and triple asterisk show significant decreases at $p<0.05$, $p<0.01$, and $p<0.001$, respectively.

Gene symbol	Gene name	Fold change
— PPAR genes —		
Ppara	peroxisome proliferator activated receptor α	1.21
Pparg	peroxisome proliferator activated receptor γ	1.33
— PPAR target genes —		
Acaa1a	acetyl-Coenzyme A acyltransferase 1A	1.49
Acaa1b	acetyl-Coenzyme A acyltransferase 1B	1.49
Acadl	acyl-Coenzyme A dehydrogenase, long-chain	1.23
Acadm	acyl-Coenzyme A dehydrogenase, medium chain	1.36
Acot1	acyl-CoA thioesterase 1	1.92
Acot8	acyl-CoA thioesterase 8	1.34
Acox1	acyl-Coenzyme A oxidase 1, palmitoyl	1.2
Acsl1	acyl-CoA synthetase long-chain family member 1	1.44
Agpat4	1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, δ)	1.72
Angptl4	angiopoietin-like 4	1.27
Cd36	CD36 antigen	1.55
Cerk	ceramide kinase	1.88
Chkb	choline kinase β	1.27
Cpt1a	carnitine palmitoyltransferase 1a, liver	1.29
Cpt1b	carnitine palmitoyltransferase 1b, muscle	1.54
Cpt2	carnitine palmitoyltransferase 2	1.18
Cyp4a10	cytochrome P450, family 4, subfamily a, polypeptide 10	1.59
Cyp4a14	cytochrome P450, family 4, subfamily a, polypeptide 14	1.77
Cyp4a31	cytochrome P450, family 4, subfamily a, polypeptide 31	1.78
Cyp8b1	cytochrome P450, family 8, subfamily b, polypeptide 1	1.7
Ehhadh	enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	1.79
Ephx2	epoxide hydrolase 2, cytoplasmic	1.1
Fabp1	fatty acid binding protein 1, liver	1.12
Fabp2	fatty acid binding protein 2, intestinal	1.22
Gpd1	glycerol-3-phosphate dehydrogenase 1 (soluble)	1.45
Gyk	glycerol kinase	1.3
Hadh	hydroxyacyl-Coenzyme A dehydrogenase	1.18
Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	1.39
Hsd17b11	hydroxysteroid (17-β) dehydrogenase 11	1.27
Hsd17b4	hydroxysteroid (17-β) dehydrogenase 4	1.17
Lipe	lipase, hormone sensitive	1.37
Pck2	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	1.43
Rps27a	ribosomal protein S27A	1.12
Slc27a1	solute carrier family 27 (fatty acid transporter), member 1	1.12
Slc27a2	solute carrier family 27 (fatty acid transporter), member 2	1.39
Ubb	ubiquitin B	1.10
Ubc	ubiquitin C	1.10

Table 3: PPAR genes and PPAR target genes which are up-regulated by mulberry supplementation.

biochemical parameters, plasma AST, ALT, and LDH. The increase in these liver function parameters, accumulation of cholesterol in the liver and high levels of plasma cholesterol in the cholesterol-feeding group without mulberry, suggests that the cholesterol crystals impaired hepatic sinusoids and bile canaliculi in the liver. Cholestasis presumably occurred in many mice fed the high-cholesterol diet. The suppressive effect of mulberry supplementation on the accumulation of cholesterol in the liver might have inhibited the progression of symptoms.

SREBP-1, which is encoded by *Srebf1*, is a nuclear factor and induces transcription of *Hmgcr* gene. Overexpression of SREBP-1c that lacks the membrane attachment site leads overproduction of cholesterol via expression of *Hmgcr* mRNA [23]. Mulberry supplementation attenuated expression of both *Srebf1* and *Hmgcr* genes. Therefore, downregulation of *Srebf1* expresson seems to suppress cholesterol synthesis through the downregulation of expression of *Hmgcr* in mulberry groups.

ACAT2 is the major ACAT enzyme in the liver. It participates in the assembly and secretion of cholesteryl esters in lipoproteins [24]. Therefore, ACAT2-deficient mice showed resistance to diet-induced and genetic hypercholesterolemia [25]. In the present study, we observed a downregulation in the *Acat2* gene. This may contribute to the reduction in serum cholesterol levels in the mulberry groups. Quercetin supplementation in rats fed a high-cholesterol diet also reduced hepatic ACAT activity [26]. Thus, the quercetin in mulberry leaves was expected to lead to suppression of cholesterol increases in the plasma and liver.

Quercetin exerts a hypocholesterolemic effect. Literatures have revealed that the addition or administration of quercetin inhibits cholesterol synthesis or reduces cholesterol levels in cultured cells [27,28], in an *in vivo* animal assay [26,29,30], and in obese human patients [31]. The mechanism is thought to be the suppression of cholesterol biosynthesis mediated by HMGCR, because the activity of HMGCR was suppressed by the administration of quercetin in hypercholesterolemic

rats [26]. Kaempferol also has a hypocholesterolemic effect. The addition of kaempferol to cultured cells [28] and oral administration in animals [32,33] led to hypocholesterolemic effects. Since the polyphenol-rich mulberry extract contains abundant quercetin and kaempferol, the functional substituents for cholesterol reduction are thought to be these flavonoids.

Issemann and Green [34] cloned PPARα for the first time and reported that clofibric acid (a metabolite of clofibrate) activates PPARα. Subsequently, many fibrates have been reported to be agonists of PPARα. Since all fibrates have a hypotriglyceridemic effect, they have been prescribed for patients with hypertriglyceridemia. However, fibrates also have a hypocholesterolemic effect and were commonly used to treat patients with hypercholesterolemia until HMGCR inhibitors, such as statin, replaced them. It has been reported that the oral administration of clofibrate in rats as well as fibrate treatments (e.g., fenofibrate and gemfibrozil) in patients with type IIa and IIb hyperlipidemia led to a reduction in cholesterol levels in the sera and liver [10,12-14,35].

Since the activation of the PPAR signaling pathway by mulberry supplementation was observed in the present study, we expected that the polyphenol-rich mulberry extract would contain a PPARα agonist. Furthermore, the mechanism of its hypocholesterolemic effect is similar to that of PPARα agonists such as fibrates. The expression of mRNAs and proteins relating to cholesterol biosynthesis in the present paper were found to be inhibited, similar to many experiments using a PPARα agonist. Fibrates not only affect the action of HMGCR, but also act downstream from the synthesis of mevalonate. Avoy et al. [10] reported that clofibrate mainly inhibited the synthesis of mevalonate from acetate, and that it somewhat suppressed the synthesis of cholesterol from mevalonate. Considering that HMGCR is the rate-limiting enzyme for cholesterol synthesis, the results reported by Avoy et al. [10] are consistent with our finding that mulberry supplementation reduced the expression of the mRNAs of HMGCR and also many genes related to cholesterol biosynthesis. Fibrates actually have been reported to reduce the activity of HMGCR in cultured cells, animals, and humans [11-14]. Reduction of HMGCR activity is attributed to the downregulation of *Hmgcr* transcription [11]. Therefore, PPARα functions in suppressing cholesterol biosynthesis. PPARα deficient mice express high cholesterol levels in the serum and liver [36].

We propose that quercetin and kaempferol in mulberry leaves are agonists of PPARα, and that these flavonoids suppress cholesterol biosynthesis via the PPARα signaling pathway. Quercetin functions as a PPARα agonist at the molecular level [9]. Kang et al. [37] used a luciferase reporter gene assay to prove the agonist activity of kaempferol for PPARα. Induction of PPARα target genes was observed after quercetin or kaempferol supplementation [38,39]. Taken collectively, these observations further support our hypothesis. Quercetin and kaempferol are nearly identical in structure except for a hydroxyl group at the 5'-position in the B ring of quercetin (Supplementary Figure S1). Thus, the hypothesis that both flavonoids are PPARα agonists is appropriate.

Quercetin and kaempferol have not only been reported to have agonist activity for PPARα, but they also induce the expression of the *Ppara* gene itself [29,32]. Mulberry extract, which is rich in quercetin and kaempferol, was observed to upregulate *Ppara* transcription in the livers of hereditary type 2 diabetic mice [39]. In our study, we also observed an induction in the expression of the *Ppara* gene after supplementation with polyphenol-rich mulberry extract, and confirmed an upregulation in the PPAR signaling pathway [7].

The *Cd36* gene was induced in the liver of rats supplemented with mulberry. PPARγ agonists have been reported to induce the expression of *Cd36* [40]. Of the 3 PPAR isoforms, only the PPARγ agonist can induce the expression of *Cd36* [41]. Mulberry administration seems to not only induce the expression of the *Pparg* gene itself, but also activate the PPARγ protein directly. CD36 functions as a receptor for oxidized LDL [42]. PPARγ protects against the progression of arteriosclerosis by suppressing foam cell formation, and this process mediates CD36. In addition, PPARγ-induced CD36 expression promotes reverse cholesterol transport [43]. Therefore, mice that overexpress CD36 have low levels of cholesterol in the blood [44]. Mulberry leaves are thought to modulate PPARγ activity and to exert inhibitory effect against arteriosclerosis via CD36.

The polyphenol-rich mulberry leaf extract, which contains abundant quercetin and kaempferol, has hypocholesterolemic and immunostimulatory effects (see supporting manuscript). DNA microarray analysis revealed that mulberry extract downregulated the expression of genes involved in cholesterol biosynthesis, including HMG-CoA reductase gene, which encodes the rate-limiting enzyme, and upregulated the transcription of PPARα and PPARγ, transcriptional factors known to regulate lipid metabolism or immunity, and their target genes. Additionally, the mulberry extract stimulated both innate and acquired immunity, including the induction of scavenger and Toll-like receptors and the activation of pathways in various lymphocytes, such as macrophages, eosinophils, neutrophils, natural killer cells, B cells, and T cells (see supporting manuscript). The data obtained in the current study indicates that quercetin and kaempferol suppress cholesterol synthesis via the PPARα signaling pathway. At the moment, the relationships between PPARα and cholesterol synthesis, and between PPARγ and immune system, are not well understood. Therefore, further studies are needed to confirm our findings. Although many vegetables and fruits contain quercetin and kaempferol [45,46], their concentrations are not very high. Therefore, because mulberry leaves contain these flavonoids at much higher levels [7], they would contain more health-promoting benefits than other foods.

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