Research Article

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Effect of flavonol glycoside in mulberry (*Morus alba* L.) leaf on glucose metabolism and oxidative stress in liver in diet-induced obese mice

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Abstract

BACKGROUND: Mulberry therapies on type 2 diabetic patients or streptozotocin-induced diabetic rats have been reported to improve fasting blood glucose levels. We investigated the effects of dietary consumption of mulberry-leaf powder and purified quercetin 3-(6-malonylglucoside), the quantitatively major flavonol glycoside in mulberry leaves, on glucose and lipid metabolism in high-fat diet-induced obese mice. Male C57BL/6J mice aged 8 weeks were assigned to three groups (control, mulberry leaf powder (MLP), and quercetin 3-(6-malonylglucoside) (Q3MG)) and treated with their respective diets for 8 weeks.

RESULTS: We found that dietary supplementation of 10 g MLP kg^{-1} or 1 g Q3MG kg^{-1} in high-fat diet effectively suppressed blood glucose levels. We also noted increased expression of glycolysis-related genes and suppression of thiobarbituric acid reactive substances concentrations in the liver of Q3MG group compared to control mice.

CONCLUSION: Dietary consumption of Q3MG, the quantitatively major flavonol glycoside in mulberry leaves, improved hyperglycemia in obese mice and reduced oxidative stress in the liver.
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Keywords: antioxidant; quercetin 3-(6-malonylglucoside); mulberry; *Morus alba* L.; DNA microarray; anti-hyperglycemia

INTRODUCTION

Mulberry (*Morus alba* L.) leaves, bark and branches have long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, strengthen joints, facilitate discharge of urine and lower blood pressure. We have previously reported that mulberry leaves attenuated atherosclerotic lesion development in low-density lipoprotein (LDL) receptor-deficient mice through enhancement of LDL resistance to oxidative modification.[1] We also reported that these anti-oxidative and anti-atherogenic protective effects were mainly attributed to quercetin 3-(6-malonylglucoside) (Q3MG), the quantitatively major flavonol glycoside in mulberry leaves.[1,2] Moreover, mulberry therapies on type 2 diabetic patients have been reported to improve fasting blood glucose levels.[3] One of the mechanisms of mulberry therapy on anti-hyperglycemia is thought to be mainly caused by 1-deoxynojirimycin (DNJ), which is indigenous to mulberry leaves and plants and known to be a potent α-glucosidase inhibitor.[4] For comprehensive understanding of the effects of mulberry leaves on the improvement in blood glucose levels in diabetic models, it is important to determine whether antioxidant components in mulberry leaves affect metabolic disorders in diabetic models, as it is known that antioxidant compounds such as tea catechins affect glucose and lipid metabolism in the liver,[5] and oxidative stress in the liver has been implicated as an etiological factor in many progressive liver diseases.[6]

We investigated the effects of dietary consumption of mulberry-leaf powder (MLP) and purified Q3MG on glucose and lipid metabolism in high-fat diet-induced obese mice. We used high-fat diet-fed C57BL/6J mice in this study, as they are considered to model many of the features of human obesity and metabolic syndrome.[7] Additionally, we conducted DNA microarray analysis and screened genes that had changed in expression upon ingestion of Q3MG to ascertain the mechanism by which Q3MG exerts an anti-hyperglycemic effect on the liver.

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Table 1. Composition of the experimental diets

	Control	MLP	Q3MG
Crude protein (g kg^{-1})	206	204	206
Crude fat (g kg^{-1})	219	217	219
Crude carbohydrate (g kg^{-1})	410	406	410
Moisture (g kg^{-1})	76	75	76
Crude fiber (g kg^{-1})	34	34	34
Crude ash (g kg^{-1})	55	54	55
Mulberry leaf (g kg^{-1})	0	10	0
Q3MG (g kg^{-1})	0	0	1
Calories (kcal kg^{-1})	4440	4400	4440

MATERIALS AND METHODS

Materials and reagents

Mulberry leaves were harvested in Gotsu City, Shimane Prefecture, Japan, in 2006, air-dried and ground to powder using a vibrating sample mill (Heiko Seisakusho Ltd, Tokyo, Japan). The flavonol compounds of the leaves were extracted by suspending the dried powder in 60% (v/v) ethanol aqueous solution, and then analyzed by quantitative HPLC system (LaChrom, Hitachi Ltd, Tokyo, Japan), by comparisons with standard compounds using an ODS 80 Ts column (4.6 × 250 mm) (Tosoh Corporation, Tokyo, Japan); solvent, acetonitrile–0.1% formic acid (20 : 80); UV detection, 280 nm (0–7.5 min) for detection of chlorogenic acid and 370 nm (7.5–30 min) for detection of flavonols, velocity of fluid, 1 mL min^{-1}.[2] MLP contained 8.3 g flavonol glycosides kg^{-1} (equivalent to 3.5 g quercetin aglycone and 1.2 g kaempferol aglycone) including 4.0 g Q3MG, 1.3 g quercetin 3-glucoside (Q3G), 0.93 g rutin, 0.47 g kaempferol 3-glucoside, and 1.6 g kaempferol 3-(6-malonylglucoside). MLP also contained 0.96 g DNJ kg^{-1}. Q3MG was purified from mulberry leaves as described previously.[2]

Chemicals were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) unless otherwise stated.

Animals and study design

Male C57BL/6J mice (7 weeks of age) were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Mice were maintained under controlled environmental conditions (temperature 23 ± 2 °C, relative humidity 55% ± 10%, 12/12 h light–dark cycle, air change 13–15 times h^{-1}) and given food and water ad libitum. All mice were acclimated for 1 week prior to the experiment. Three groups of 11 mice each, all 8 weeks of age, were treated with their respective group diet for 8 weeks. The control group was fed a high fat-diet containing 30 g cholesterol kg^{-1}, 150 g cocoa butter kg^{-1} and 820 g rodent diet kg^{-1}, CE-2, obtained from CLEA Japan, Inc. (Tokyo, Japan). The other two experimental groups were fed the same high fat-diet with a supplement of 10 g dry MLP kg^{-1} for the MLP group, and 1 g Q3MG kg^{-1} for the Q3MG group. Composition of the experimental diets is shown in Table 1. Food intake and body weight were measured twice a week. This study and all procedures were approved by the Animal Care and Use Committee of Shimane University School of Medicine, Japan.

Biochemical analysis

After 16 h fasting, the mice were anesthetized by intraperitoneal injection of sodium pentobarbital solution (50 mg kg^{-1} body weight). At sacrifice, blood was collected from the main abdominal vein into a tube containing 1 mg mL^{-1} ethylenediaminetetraacetic acid (EDTA) and centrifuged immediately at 1000 × *g* for 15 min at 4 °C. Plasma was stored at −80 °C before analysis. The livers and epididymal white adipose tissue (WAT) were excised and weighed, and each organ was stored at −80 °C until use.

Concentrations of glucose, total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglyceride (TG), and free fatty acid (FFA), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) in plasma were measured using an enzymatic assay kit (Glucose C II test, Cholesterol E test, HDL cholesterol test, Triglyceride G test, NEFA test, GOT · GPT C II test; Wako Pure Chemical Industries, Osaka, Japan). LDL cholesterol was calculated by the Friedewald formula.[8] Plasma insulin was measured using a mouse insulin ELISA kit (Shibayagi, Gunma, Japan) according to the manufacturer's protocol.

Lipids were extracted from the liver by the method as described.[9] Commercial kits were used to measure concentrations of TG and total cholesterol in the liver lipid extracts as well as in the plasma. To measure liver thiobarbituric acid reactive substances (TBARS) levels,[10] a 50 mg aliquot of the liver was homogenized in 800 µL of 11.5 mg mL^{-1} KCl solution containing 0.1 mg mL^{-1} butylated hydroxytoluene to prevent the endogenous peroxidation of lipids during the procedure. 50 µL homogenate, 100 µL of 30 mg mL^{-1} sodium dodecyl sulfate, 800 µL of 0.1 mol L^{-1} HCl, 100 µL of 100 mg mL^{-1} phosphotungstic acid and 400 µL of 3.5 mg mL^{-1} 2-thiobarbituric acid were combined and placed at 90 °C for 30 min. Samples were cooled on ice and mixed with 400 µL 1-butanol. After centrifugation (1800 × *g*, 10 min, 4 °C), a 200 µL aliquot of the butanol phase was separated and analyzed spectrofluorometrically[11] (excitation 515 nm and emission 555 nm) using a fluorescence meter (FP-6500, JASCO Corporation, Tokyo, Japan). TBARS were expressed as nanomoles of malondialdehyde (MDA) equivalents per gram of liver.

RNA isolation and DNA microarray analysis

To determine gene expression differences in the liver between the control and Q3MG groups, we conducted DNA microarray analysis using four mice selected from each group. The selection was based on the three parameters which showed significant differences between two groups (Table 2): blood glucose, FFA, and LDL cholesterol levels. We selected four particular mice with the three parameter levels that were close to the median for each group. Total RNA was extracted from the liver using RNeasy Mini Kit (QIAGEN KK, Tokyo, Japan) and checked for quality and quantity by agarose gel electrophoresis and spectrophotometry. DNA microarray analysis was conducted according to the manufacturer's protocol using the Affymetrix mouse genome 430 2.0 array (Affymetrix, Santa Clara, CA, USA) with 43 000 genes. Briefly, 4 µg of the total RNA was used to synthesize cDNA, and the resultant cDNA was used as a template for synthesis of biotinylated cRNA by T7 DNA polymerase. After fragmentation, the cRNA was added, hybridized, and stained to the Affymetrix mouse genome 430 2.0 array. The fluorescence signals were scanned by the Affymetrix GeneChip system. The global normalization method was used to correct for minor differences in the amount of each cRNA added to the microarrays. In each microarray, gene expression values, calculated from fluorescence signals by the Affymetrix GeneChip operating software, were classified with detection calls indicating whether or not the transcript of the gene was reliably detected (Present), marginally detected (Marginal), or not detected (Absent), using Affymetrix statistical algorithms. In our study, we selected genes for use in the evaluation of the effects of Q3MG administration using

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Table 2. Growth parameters and plasma chemistries of C57BL/6J mice fed either a high-fat diet or high-fat diet with 1% (w/w) MLP, or 0.1% (w/w) Q3MG for 8 weeks[a]

Parameters	Diet			ANOVA
	Control	MLP	Q3MG	*P*-value
Growth parameters				
Food intake (kcal/mouse d^{-1})	18.8 ± 0.7a	22.3 ± 1.1b	19.0 ± 0.7a	0.011
Flavonol intake (mg d^{-1})[b]	NC[c]	0.07 ± 0.01	0.70 ± 0.08	
DNJ intake (mg d^{-1})	NC	0.02 ± 0.00	NC	
Final body weight (g)	31.1 ± 0.5	30.9 ± 0.7	30.1 ± 0.6	0.498
Liver weight (g)	1.56 ± 0.13	1.54 ± 0.08	1.38 ± 0.05	0.337
Liver TG (g kg^{-1})	63.5 ± 10.2	70.9 ± 6.5	48.5 ± 7.1	0.153
Liver cholesterol (g kg^{-1})	13.1 ± 0.8	13.4 ± 1.0	12.3 ± 1.0	0.687
Plasma chemistries				
Glucose (mg dL^{-1})	210.8 ± 13.7a	167.8 ± 8.8b	144.9 ± 10.5b	0.001
Triglyceride (mg dL^{-1})	24.3 ± 0.3	23.9 ± 0.4	24.7 ± 0.4	0.374
FFA (mg dL^{-1})	11.7 ± 1.0a	11.8 ± 1.2a	6.5 ± 0.8b	0.001
LDL cholesterol (mg dL^{-1})	93.9 ± 13.7a	98.2 ± 27.2a	28.7 ± 6.6b	0.017
HDL cholesterol (mg dL^{-1})	57.3 ± 4.4a	59.9 ± 1.5ab	68.4 ± 2.3b	0.034
Insulin (pg dL^{-1})	46.7 ± 10.4	26.9 ± 3.9	54.9 ± 11.5	0.149
AST (IU L^{-1})	64.1 ± 5.1	58.1 ± 3.9	63.0 ± 3.8	0.587
ALT (IU L^{-1})	25.4 ± 3.6	21.3 ± 2.7	19.6 ± 1.5	0.318

[a] Data are means ± SEM ($n = 11$). Means in a row without a common letter differ, $P < 0.05$.
[b] Represented as sum of quercetin and kaempferol aglycon.
[c] Not contained.

GeneSpring software (Agilent Technologies Japan, Ltd, Tokyo, Japan) by the following steps. First, we selected genes detected as 'present' or 'marginal' in three or four microarrays in one test group. Second, we selected genes exhibiting more than a 2.0-fold difference in the mean signal between the control and Q3MG groups. Finally, differential expression was tested by Student's *t*-test, and Benjamin–Hoechberg multiple testing correction was applied to obtain the false discovery rates (FDA). We defined the genes with significant changes in expression levels (FDR < 0.1) as the genes with biologically significant changes, and 136 genes were selected.

Quantitative real-time polymerase chain reaction (PCR)

To determine gene expression differences in the liver among the three groups, we conducted quantitative real-time PCR using all mice from each group ($n = 11$). The sequences of the primers were as follows: glucokinase forward, 5′-TACGACCGGATGGTGGATGA, reverse, 5′-ACCAGCTCGCCCATGTACTTTC; Srebf-1, 5′-ACAGAGCTTCCGGCCTGCTA, reverse, 5′-CCGAGCTGTGGCCTCATGTA; Cyp7a1, 5′-GCTGAGAGCTTGAAGCACAAGA, reverse, 5′-TTGAGATGCCCAGAGGATCAC; Cdkn1a, 5′-CTGTCTTGCACTCTGGTGTCTGA, reverse, 5′-CCAATCTGCGCTTGGAGTGA; Hspa1ba, 5′-CGCTCGAGTCCTATGCCTTCA, reverse, 5′-TCCTGGCACTTGTCCAGCAC; p47phox, 5′-CTGGGTTAATCTCCACCGGTATTG, reverse, 5′-AGGGCGTTCCTAAGGCACTTG; gp91phox, 5′-GCACTCAAGGCTGGTTCTGGTAA, reverse, 5′-GCAACACGAAGGTCTGTCTGGA; β-actin forward, 5′-CATCCGTAAAGACCTCTATGCCAAC, reverse, 5′-ATGGAGCCACCGATCCACA. One microgram of total RNA was reverse-transcribed according to the manufacturer's protocol using commercially available kit, ReverTra Ace-α-® (Toyobo Co., Ltd, Osaka, Japan), in 20 µL of reaction mixture, containing reverse transcriptase, 1 unit RNase inhibitor, 5 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} KCl, 10 mmol L^{-1} Tris-HCl, (pH 8.3), 2.5 µmol L^{-1} oligo (dT) primer, and 1 mmol L^{-1} dNTPs. The reaction mixture was heated to 42 °C for 20 min and then denatured at 99 °C for 5 min. The PCR reaction mixture containing primers, RNase/DNase-free water, and the reverse transcriptase products was added to the SYBR® Premix Ex TaqTM II (Takara Bio Inc., Ohtsu, Japan) to obtain a final volume of 25 µL per reaction. The reaction mixture was incubated at 95 °C for 10 min and then run for 40 cycles at 95 °C for 5 s and 60 °C for 31 s in the ABI Prism 7000 sequence detection system (Applied Biosystems Japan Ltd, Tokyo, Japan). The PCR results were analyzed with ABI SDS software (Applied Biosystems). The relative expression levels of each sample were calibrated by the standard curve method, and the expression of β-actin was used for normalization.

Statistical analysis

Statistical analyses, apart from microarray analyses, were done with SPSS statistical analysis software (Version 16.0J, SPSS Japan Inc., Tokyo, Japan). One-way analysis of variance (ANOVA) was used to assess the differences among three groups, and post hoc analyses were performed using the Tukey test for two independent variables. Unless otherwise noted, a nominal two-sided *P*-value of <0.05 was used to assess significance.

RESULTS

Body composition, food and quercetin intakes

Growth parameters are summarized in Table 2. The daily energy intake in the MLP group was 21% more than that of the control group, while no significant differences were found in the Q3MG group. The mean daily intakes of flavonol, the sum of quercetin and kaempferol in mice as aglycons based on food intake, were 0.07 mg d^{-1} in the MLP group and 0.7 mg d^{-1} in the Q3MG group.

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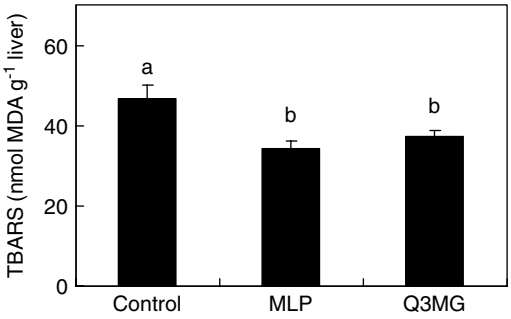

Figure 1. Effects of Q3MG and MLP ingestion for 8 weeks on oxidative stress in the liver. Levels of TBARS in the liver from each group of mice are shown. Data are expressed as means ± SEM, $n = 11$. Means without a common letter differ, $P < 0.05$.

Table 3. Functional classification of genes induced by Q3MG ingestion

Molecular function	Increase	Decrease	Total
Metabolism	16	7	24
Defense/stress responses	1	5	6
Signal transduction/apoptosis/cell cycle	12	9	21
Transcription regulation	8	3	11
Protein synthesis and modification	12	3	15
Transport	4	2	5
Unclassified	40	14	54
Total	93	43	136

The mean daily intake of DNJ in mice was 0.02 mg d^{-1} in the MLP group. Naturally, the diet of the Q3MG group contained no DNJ. The final body, liver and epididymal WAT weights in the MLP and Q3MG groups showed no significant differences from those of each control group.

Effects of MLP and Q3MG on plasma biochemistry

Plasma glucose levels in the MLP and Q3MG groups were significantly lower than those of the control group (Table 2). Moreover, FFA and LDL cholesterol levels in the Q3MG group mice were significantly lower than those of control mice, while HDL cholesterol levels in the Q3MG group were higher than those of the control mice (Table 2).

Effects of MLP and Q3MG on lipid levels and oxidative stress in the liver

Compared with the control group, liver triglyceride and cholesterol in the MLP and Q3MG groups did not changed significantly. We next measured TBARS concentrations in the liver to assess the effects of mulberry leaf on oxidative stress. Treatment with MLP and Q3MG significantly decreased TBARS concentrations by 26% and 20%, respectively (Fig. 1).

DNA microarray analysis

To characterize the mechanism of the effects of Q3MG in C57BL/6J mice fed a high-fat diet, gene expression levels in the livers of four mice each from the control group and the Q3MG group were analyzed using a DNA microarray. One hundred and thirty-six genes showed more than a 2.0-fold change in expression level with significant differences (FDR < 0.1); Q3MG ingestion upregulated the expression of 93 genes and downregulated 43 genes. These genes were categorized into six groups; however, 57 genes could not be categorized (Table 3). The greatest number of genes affected differentially by Q3MG ingestion were those categorized as having metabolism function (Table 4). These included genes encoding glucose metabolism-related enzymes such as glucokinase and ATP citrate lyase, which increased 2.8- and 2.3-fold, respectively, relative to the control group. The sterol regulatory element binding transcription factor 1 (Srebf1) gene, which in transcription factors plays a central role in energy homeostasis by promoting glycolysis and lipogenesis-related genes, including glucokinase,[12] increased 4.0-fold in the Q3MG mice. The pyruvate dehydrogenase kinase isoenzyme 4 (Pdk4), which inhibits glycolysis via the inactivation of pyruvate dehydrogenase by its phosphorylation,[13] decreased 5.5-fold. In the genes encoding lipid metabolisms, elovl family member 6, elongation of long chain fatty acids (Elovl6), a component of the mammalian elongation system that converts palmitic to stearic acid, increased 2.1-fold. The genes encoding enzymes of cholesterol metabolism, cytochrome P450 7a1 (Cyp7a1) and StAR-related lipid transfer domain containing 4 (Stard4), increased 3.1- and 2.1-fold, respectively, in the Q3MG mice. The genes for steroidogenic enzymes encoding hydroxysteroid dehydrogenase-3, delta<5>-3-beta, hydroxysteroid dehydrogenase-2, delta<5>-3-beta, and hydroxysteroid dehydrogenase-5, delta<5>-3-beta increased 2.1-, 2.0- and 7.3-fold, respectively.

Another interesting result was that most genes in the Q3MG group related to defense or stress responses showed decreased expression patterns: cyclin-dependent kinase inhibitor 1A (P21) (Cdkn1a), heat shock protein 1B (Hspa1b), defensin beta 1 (Defb1), spondin 2, extracellular matrix protein (Spon2), extracellular matrix protein, and transformation-related protein 53 inducible nuclear protein 1 (Trp53inp1) (Table 5).

Quantitative real-time PCR

To validate the DNA microarray analysis and compare the effect of MLP, we measured the representative genes changed by Q3MG ingestion in DNA microarray analysis using quantitative real-time PCR in every liver sample of the control, Q3MG and MLP groups ($n = 11$) (Fig. 2). The representative genes were selected according to metabolic functions in terms of glucose metabolism-related genes (glucokinase, Srebf-1 and Pdk4), cholesterol metabolism-related genes (Cyp7a1), and defense or stress response-related genes (Cdkn1a and Hspa1b). Additionally, NADPH oxidase family proteins, p47phox and gp91phox, were selected as genes for quantitative real-time PCR; in the DNA microarray analysis, these two genes decreased 1.7- and 1.9-fold, respectively, compared to the control group, although the changes were not significant.

In glucose metabolism-related genes, the expression of glucokinase and Srebf-1 increased 1.9- and 2.0-fold, respectively, by Q3MG ingestion, compared to control group. The expression of Pdk4 decreased 2.1-fold in the Q3MG group, though not significantly. In cholesterol metabolism-related genes, Cyp7a1 increased 2.1-fold. No significant changes were observed in these latter four genes as a result of MLP ingestion. As for the defense or stress response-related genes, expressions of Cdkn1a and Hspa1b decreased 3.3- and 3.5-fold, respectively, with Q3MG ingestion. The expression of Hspa1b was also affected by MLP ingestion: a 1.9-fold decrease. Additionally, the expression of p47phox in the Q3MG group decreased 1.6-fold.

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Table 4. Metabolism related hepatic genes induced by Q3MG ingestion

Gene name	Gene symbol	Fold change	Accession number[a]
Glucose metabolism			
Glucokinase	Gck	2.81	NM_010292
ATP citrate lyase	Acly	2.27	NM_134037
Lipid metabolism			
Sterol regulatory element binding factor 1	Srebf1	4.00	NM_011480
Pyruvate dehydrogenase kinase, isoenzyme 4	Pdk4	−5.46	NM_013743
Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	Ppargc1a	−2.19	NM_008904
6, elongation of long chain fatty acids	Elovl6	2.11	NM_130450
Elongation of very-long-chain fatty acid-like 3	Elovl3	−2.07	NM_007703
Thyroid hormone responsive spot14 homolog	Thrsp	3.13	NM_009381
Choline kinase alpha	Chka	−2.11	NM_013490
Solute carrier family 27, member 1	Slc27a1	−2.31	NM_011977
Lipase, endothelial	Lipg	−2.20	NM_010720
Cholesterol and steroid metabolism			
Cytochrome P450, family 7, subfamily A, polypeptide 1	Cyp7a1	3.10	NM_007824
StAR-related lipid transfer domain containing 4	Stard4	2.14	NM_133774
Hydroxysteroid dehydrogenase-3, delta<5>-3-beta	Hsd3b3	2.08	NM_001012306
Hydroxysteroid dehydrogenase-2, delta<5>-3-beta	Hsd3b2	2.04	NM_008295
Hydroxysteroid dehydrogenase-5, delta<5>-3-beta	Hsd3b5	7.30	NM_153193
Other			
Cytochrome P450, family 2, subfamily C, polypeptide 55	Cyp2c55	6.43	NM_028089
Carbonic anhydrase 3	Car3	6.11	NM_007606
Cytochrome P450, family 2, subfamily b, polypeptide 10	Cyp2b10	5.27	NM_009998
cDNA sequence BC016495	BC016495	2.33	NM_145497
Nudix-type motif 7	Nudt7	2.06	NM_024437
Uridine phosphorylase 2	Upp2	2.00	NM_029692
5,10-methylenetetrahydrofolate reductase	Mthfr	−4.72	NM_010840

[a] GenBank ID.

Table 5. Defense and stress related hepatic genes induced by Q3MG ingestion

Gene name	Gene symbol	Fold change	Accession number[a]
Chemokine (c-c motif) ligand 9	Ccl9	2.03	NM_011338
Cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1A	−10.32	NM_007669
Heat shock protein 1B	Hspa1b	−9.53	NM_010478
Defensin beta 1	Defb1	−2.71	NM_005218
Spondin 2, extracellular matrix protein	Spon2	−2.63	NM_133903
Transformation-related protein 53 inducible nuclear protein 1	Trp53inp1	−2.11	NM_021897

[a] GenBank ID.

DISCUSSION

In the present study, we have shown that dietary supplementation with Q3MG, a major flavonol glycoside in mulberry leaf, effectively improves blood glucose levels in high-fat diet-induced obese mice. It has previously been believed that anti-hyperglycemic effects of mulberry leaf are mainly attributable to DNJ, a potent α-glucosidase inhibitor.[14] However, the present study suggests that Q3MG also suppresses hyperglycemia in obese mice through improved glucose metabolism in the liver.

DNA microarray study suggested that the lessening of the metabolic disorders in high-fat diet-induced obese mice by Q3MG resulted from regulation of expression of metabolism-related genes (Table 4). Glucokinase, which is a member of the hexokinase family that catalyzes the first committed step in glycolysis, was upregulated in the Q3MG group (Table 4), as confirmed by the quantitative real-time PCR (Fig. 2). A previous study demonstrated that overexpression of glucokinase in diabetic mice treated with streptozotocin results in normalization of blood glucose and FFA.[15] Several studies have reported that antioxidant compounds affect glucose metabolism in rodent models via expression of glucokinase.[16,17] Supplementation of epigallocatechin gallate, a main and potent antioxidant in green tea, improved blood glucose in food-deprived *db/db* mice, and mRNA expression of glucokinase was upregulated.[17] Dietary phenolic compounds such as chlorogenic acid, caffeic acid and rosmarinic acid induced glucokinase mRNA in rat liver cells *in vitro*.[16] The expression of

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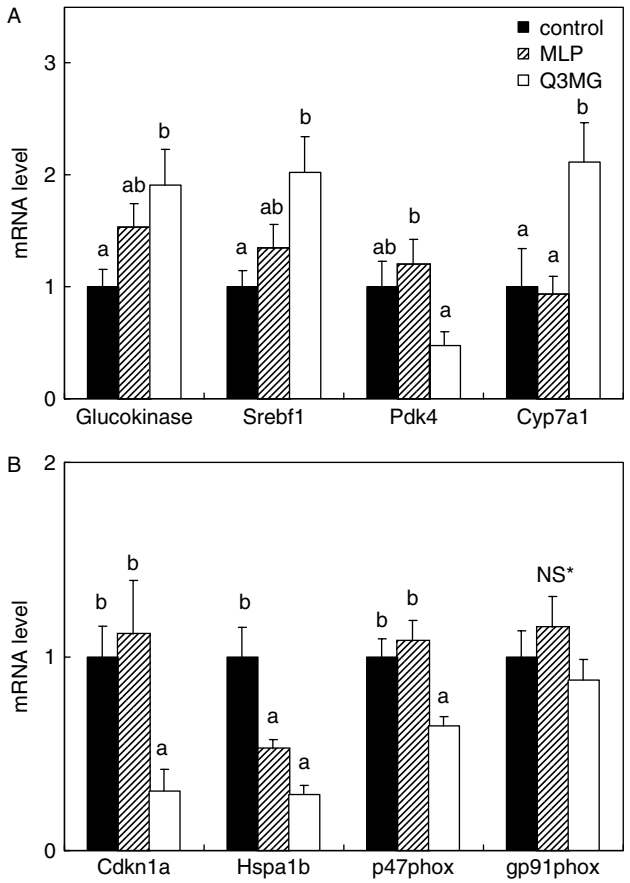

Figure 2. Effects of Q3MG and MLP ingestion for 8 weeks on gene expression in the liver. Expressions of each mRNA level in the liver in each group of mice are shown. Data are expressed as means ± SEM, $n = 11$. Means without a common letter differ, $P < 0.05$. *Not significant.

Srebf-1, which in transcription factors plays a central role in energy homeostasis by promoting glycolysis-related genes including glucokinase,[12] was also upregulated in the Q3MG group (Table 4 and Fig. 2). Pdk4, which inactivates pruvate dehydrogenase activity by its phosphorylation,[13] was markedly downregulated in the Q3MG group in the microarray study (Table 4). The expression of Pdk4 in the Q3MG group also decreased 2.1-fold in the real-time PCR study using every sample (Fig. 2). The expression of Pdk4 is noticeably induced in most tissues by high-fat diet, and Pdk4 deficiency lowers blood glucose and improves glucose tolerance and insulin sensitivity in high-fat diet-induced obese mice.[18] Therefore, we speculated that suppression of the increase in blood glucose levels by Q3MG ingestion was attributable mainly to the activation of glycolysis in the liver. Q3MG ingestion also upregulated genes related to cholesterol metabolism. Of these, Cyp7a1, a key enzyme in cholesterol catabolism (bile acid synthesis),[19] was upregulated (Table 4 and Fig. 2). Curcumin or turmeric ingestion increases the activity of Cyp7a1, resulting in lower plasma and hepatic cholesterol levels in streptozotocin-induced diabetic rats.[20] The expression of StarD4, which is thought to be implicated in cholesterol transport,[21] was also upregulated in the Q3MG group (Table 4). These results indicate that suppression of the increase in blood LDL cholesterol levels by Q3MG ingestion resulted from activation of cholesterol catabolism.

DNA microarray study also suggested that Q3MG treatment affects expression of oxidative stress-related genes. Q3MG suppressed expression of five of six genes related to defense and stress responses categorized in the present study: Cdkn1a, Hspa1b, Defb1, Spon2, and Trp53inp1 (Table 5). These suppressive effects were also confirmed by quantitative real-time PCR (Fig. 2). A previous study demonstrated that a long-term high-fat diet in C57BL/6J mice induces expression of genes related to defense and stress responses such as heat shock proteins,[22] suggesting that there appear to be transcriptional regulations of biological defense responses to oxidative stress elevated by long-term high-fat diet. It has been reported that oxidative stress is increased in obesity via NADPH oxidase activation, and NADPH oxidase is a major source of reactive oxygen species (ROS) in various organs.[23] We confirmed by quantitative real-time PCR that Q3MG suppressed expression of p47phox, a subfamily of NADPH oxidase (Fig. 2). The decreased expression of genes implicated in defense and stress responses by supplementation with Q3MG may strengthen relaxation of the oxidative state in the liver through its antioxidant effect. This was further confirmed by the measurement of TBARS (Fig. 1), an indicator of lipid oxidation state. Our prior study showed that mulberry leaf treatment attenuates atherosclerotic lesion development in LDL receptor-deficient mice through enhancement of LDL resistance to oxidative modification via the potent antioxidant effect of Q3MG.[1] It has also been demonstrated that increased oxidative stress in the liver precedes high-fat diet-induced obesity and insulin resistance.[24] A genetic model of type 2 diabetes, *db/db* mice, showed high ROS in the liver, although overexpression of superoxide disumutase 1 in the liver by adenoviral injection reduced hepatic ROS and blood glucose level.[25] These data may strengthen the suggestion that Q3MG improves glucose metabolism through relaxation of oxidative stress in the liver derived from its antioxidant activities. Further studies are needed on the relationship between antioxidant activity and glucose metabolism in the liver.

Suppressive effects of blood glucose levels and TBARS concentrations in the liver were observed in the MLP group as compared to the control group (Table 2 and Fig. 1). The gene expression of Hspa1b decreased significantly compared to the control group (Fig. 2). Heat shock proteins, known as molecular chaperones, are postulated to be responsible for repairs of misfolded or damaged proteins under highly oxidative and lipotoxic environment.[22] These results suggested that antioxidant compounds in MLP attenuated the oxidation state in the liver induced by high-fat diet. On the other hand, expression of metabolism-related genes, glucokinase, Srebf1 and Cyp7a1, increased significantly in the Q3MG group relative to the control group, although not in the MLP group (Fig. 2). In the present study, flavonol intake in the MLP group was approximately 10 times lower than that in the Q3MG group (Table 2), sufficient to attenuate the oxidative state in the liver (Fig. 1); however, a larger dose of Q3MG may be required to affect gene expression. Further, the extract of mulberry leaves may be more effective than the mulberry leaf itself on intake, insofar as the absorption of Q3MG in the intestine is concerned.

The mulberry leaf contains DNJ,[14] and mulberry leaf powder enriched with DNJ suppresses the elevation of postprandial blood glucose and secretion of insulin in humans,[4] suggesting that the effect of MLP on plasma glucose levels can be attributed to additional or cooperative effects of DNJ with flavonol glycosides such as Q3MG. Further studies are needed on the dose response of Q3MG and its interactional effects with other compounds in mulberry leaves such as DNJ. We are currently investigating the effects of extract of mulberry leaves having greater concentrations of flavonol glycosides, with and without DNJ.

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The dose of Q3MG used in this study was 1000 mg kg^{-1} diet. Q3MG intake calculated using final body weight in the present study was 43 mg Q3MG kg^{-1} body weight d^{-1}; this is equivalent to 2150 mg Q3MG per day for a 50 kg human. Further studies are needed on the effect and dose of Q3MG for human health. We are conducting a preparation of Q3MG-rich extract of mulberry leaves for human study.

In conclusion, dietary consumption of Q3MG, the quantitatively major flavonol glycoside in mulberry leaves, improved plasma glucose levels in high-fat-induced obese mice and reduced oxidative stress in the liver. The data are significant in that they show that Q3MG in mulberry leaves possesses anti-hyperglycemic effects as well as suppressive effects on oxidative stress in the liver, and help in better understanding the mechanisms of anti-hyperglycemic effects of mulberry leaves.

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