

RESEARCH ARTICLE

Chronic dietary intake of quercetin alleviates hepatic fat accumulation associated with consumption of a Western-style diet in C57/BL6J mice

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Scope: To determine the effect of consumption of a quercetin-rich diet on obesity and dysregulated hepatic gene expression.

Methods and results: C56BL/6J mice were fed for 20 wk on AIN93G (control) or a Western diet high in fat, cholesterol and sucrose, both with or without 0.05% quercetin. Triglyceride levels in plasma, thiobarbituric acid-reactive substances (oxidative stress marker) and glutathione levels and peroxisome proliferator-activated receptor α expression in livers of mice fed with the Western diet were all improved after 8 wk feeding with quercetin. After 20 wk, further reductions of visceral and liver fat accumulation and improved hyperglycemia, hyperinsulinemia, dyslipidemia and plasma adiponectin and TNF α levels in these mice fed with quercetin were observed. The expression of hepatic genes related to steatosis, such as peroxisome proliferator-activated receptor γ and sterol regulatory element-binding protein-1c was also normalized by quercetin. In mice fed with the control diet, quercetin did not affect body weight but reduces the plasma TNF α and hepatic thiobarbituric acid-reactive substance levels.

Conclusion: In mice fed with a Western diet, chronic dietary intake of quercetin reduces liver fat accumulation and improves systemic parameters related to metabolic syndrome, probably mainly through decreasing oxidative stress and reducing PPAR α expression, and the subsequent reduced expression in the liver of genes related to steatosis.

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Liver steatosis / Metabolic disorder / Oxidative stress / Quercetin / Western diet

1 Introduction

Consumption of a Western diet, characterized by high intake of red meat and processed meat, high-fat dairy products and sweet items, can contribute to obesity and metabolic syndrome, and increases the risk of type 2 diabetes and cardiovascular disease [1–4]. Consumption of a

diet high in fat and excessive energy is associated with obesity and the development of metabolic disorders. Currently, it is controversial whether high dietary sugar, rather than other carbohydrates, contributes to body weight gain [5, 6]. In contrast, greater consumption of vegetables, fruits and whole grains reduces risks for type 2 diabetes and cardiovascular disease [1–3]. Plant polyphenols or flavonoids, of which vegetables and fruits are a rich source, have been suggested to be the main agents reducing the risks of cardiovascular disease [7–9].

Quercetin is a major flavonoid contained in vegetables, fruits and tea, possessing antioxidant activity *in vitro* and *in vivo* [10–12]. We previously reported that feeding streptozotocin-induced diabetic mice a diet containing quercetin improved their blood glucose and plasma insulin levels, and decreased the generation of the oxidative stress marker thiobarbituric acid-reactive substances (TBARS) in the liver and pancreas [12]. Our results suggested that quercetin

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Abbreviations: AMPK, AMP-activated protein kinase; GSEA, gene set enrichment analysis; NEFA, non-esterified fatty acid; PPAR α , peroxisome proliferator-activated receptor α ; SREBP-1c, sterol regulatory element-binding protein-1c; TBARS, thiobarbituric acid-reactive substances; UCP-2, uncoupling protein 2

improved liver and pancreas functions by enabling the recovery of cell proliferation through reducing oxidative stress and blocking the expression of the cyclin-dependent kinase inhibitor *p21(WAF1/Cip1) (Cdkn1a)* [12]. Moreover, quercetin has been shown to affect various physiologically active molecules *in vitro*. It inhibits some enzymatic activities, such as protein kinase C and topoisomerase II, and suppresses expression of heat shock proteins [13, 14]. It acts as a phytoestrogen and an activator of SIRT1 [15, 16]. Favorable effects of quercetin on obesity and metabolic syndrome have been shown to a limited extent *in vivo*. Thus, Rivera *et al.* recently reported that chronic high-dose quercetin feeding reduced systolic blood pressure and plasma triglycerides, total cholesterol, non-esterified fatty acid (NEFA) and insulin in obese Zucker rats [17]. In contrast, Stewart *et al.* showed that 8 wk of feeding a diet supplemented with 1.2% quercetin did not improve insulin resistance induced by a high fat diet (45% energy) in C57/BL6J mice [18].

Dietary quercetin is metabolized in the intestine and liver, and gradually accumulates in the liver and then in the kidney and other organs [19, 20]. Thus, the liver might be a major organ affected by habitual consumption of a quercetin-rich diet. A high fat diet results in dysregulated hepatic gene expression, accompanying the induction of metabolic disorders characterized by insulin resistance, steatosis, oxidative stress and inflammation [21]. To determine the effect of its habitual dietary intake, we added 0.05% quercetin to a Western-style diet high in fat, cholesterol and sucrose, and assessed diet-induced obesity and disturbance of hepatic gene expression in mice.

2 Materials and methods

2.1 Animals and treatments

Five-week-old male C57BL/6J mice were obtained from the Institute for Animal Reproduction, Charles River Japan (Ibaraki, Japan). The mice were housed at $24 \pm 1^\circ\text{C}$, $55 \pm 5\%$ humidity and 12 h light/dark photocycles (dark period from 0800 to 2000), with free access to water and an AIN93G diet (Oriental Yeast, Tokyo, Japan), for a week prior to the experiment. The animals were treated in accordance with the basic guidelines of the Ministry of Agriculture, Forestry and Fisheries for laboratory animal studies, which were approved by the review board for animal ethics at our institute (permission number; H20-001).

The mice were divided into 10 groups of 6 mice each, housed in groups of 3 *per* cage and fed with one of the following diets: AIN93G (control), AIN93G containing 0.05% quercetin (0.05% quercetin; Funakoshi, Tokyo, Japan), a high-fat, high-cholesterol and high-sucrose Western-style diet (39.9% of energy from fat (20% unsalted butter, 1% soy oil, 0.15% cholesterol) and 34.0% of energy from sucrose) (Oriental Yeast) or this Western diet supple-

mented with 0.05% quercetin. The mice in the group fed 0.05% quercetin diets were maintained for 20 wk. The other groups were maintained for 4, 8 or 20 wk. Thereafter, animals were killed under anesthesia and blood, liver, kidney and adipose tissues were immediately collected.

2.2 Blood analysis

Blood glucose levels were measured using the glucose test meter GLUCOCARD DIAMETER- α GT-1661 (ARKRAY, Kyoto, Japan). Plasma insulin concentrations were measured using an ELISA kit (Sibayagi, Gunma, Japan). Plasma total cholesterol, triglyceride and NEFA concentrations were measured enzymatically using commercial kits (Wako Pure Chemicals Industries, Osaka, Japan).

2.3 Measurements of oxidative stress markers and mitochondrial respiratory complex I activity

Lipid peroxidation in the liver was measured as TBARS using an OXI-TEK TBARS Assay Kit (ZeptoMetrix, Buffalo, NY). Total glutathione in the liver was determined using GSSG/GSH Quantification Kits (Dojindo, Kumamoto, Japan). Liver mitochondrial respiratory complex I activity was determined spectrophotometrically according to Feillet-Coudray *et al.* [22].

2.4 Measurement of plasma and tissue levels of quercetin and metabolites

Levels of quercetin metabolites were determined as previously described [12]. Briefly, plasma samples were treated with β -glucuronidase, and extracted with ethyl acetate. The quercetin metabolites in the ethyl acetate fraction were measured as quercetin and isorhamnetin by HPLC.

2.5 Liver histology

Frozen liver sections (4 μm) were fixed with 10% buffered formalin and stained with Mayer's hematoxylin solution (Wako) and eosin (Wako).

2.6 RNA isolation and cDNA microarray analysis

Total RNA was extracted from livers using an RNeasy Midi Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. Fragmented biotin-labeled cRNA was synthesized from the total RNA of each mouse using One-Cycle Target Labeling and Control Reagents (Affymetrix Japan KK, Tokyo, Japan) and then hybridized to an array (Mouse Genome 430 2.0 array, Affymetrix) at 45°C for 16 h.

After hybridization, the gene chips were washed and stained using a GeneChip Fluidics Station 450 (Affymetrix), and then scanned (GeneChip Scanner, Affymetrix) with GeneChip Operation Software Ver. 1.4 (Affymetrix).

2.7 Quantitative RT-PCR analysis

Quantitative RT-PCR was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), according to the manufacturer's protocol. Sequences of primers used for quantitative RT-PCR were as follows: *Pparg*, 5'-gaaagacaacggacaatcacc-3' and 5'-gggggtgatgttgacttg-3'; *Ppara*, 5'-ctgagaccctcggggaac-3' and 5'-aacgtcagtcacaggggaag-3'; *Srbpflc*, 5'-atcggcgcggaagctgtcgggtagcgtc-3' and 5'-actgtcttggttgatgagctggagcat-3'; *Fasn*, 5'-gctgctgttggaagtcagc-3' and 5'-agtgtctgtctcctcggagtg-3'; *Gpx1*, 5'-ttcccgtgcaatcagtc-3' and 5'-tcggacgtacttgaggaat-3'; *Gpx2*, 5'-gttctcggcttccttgc-3' and 5'-ttcaggatctcctgttctga-3'; *Cat*, 5'-cctcaagtgttaatgcaga-3' and 5'-caagttttgatgcctgtg-3'; *Pck1*, 5'-atgtgtgggcgatgacatt-3' and 5'-aacccgtttctgggtgat-3'; *Cd36*, 5'-ttgtactatctgtggctaatgaga-3' and 5'-ctgtgtttgaacatttctgctt-3'; *Ucp2*, 5'-agcctgagacctcaagcag-3' and 5'-cctcaatcggaagacg-3'; *Gck*, 5'-ctggatgacagagcaggat-3' and 5'-ctctccaggatctgctac-3'; glyceraldehyde-3-phosphate dehydrogenase (encoded by *Gapdh*), 5'-atcccagagctgaacg-3' and 5'-gaagtcgcaggagaca-3'. The relative amount of each transcript was normalized to the amount of *Gapdh* transcript in the same cDNA.

2.8 Statistical analysis

Data are expressed as the arithmetic mean \pm SEM. The significance of differences between groups was determined by ANOVA followed by two-tailed multiple *t*-tests with the Bonferroni correction. A *p*-value of less than 0.05 was considered statistically significant.

Analysis of the DNA microarray data was performed using the Microarray Suite and GeneSpring Ver.11.0 (Agilent Technologies, Santa Clara, CA). Statistical analysis of differences in gene expression levels was performed by Welch's one-way ANOVA. Enrichment for gene ontology categories was determined using Fisher's exact test. Gene set enrichment analysis (GSEA) based on GSEA implementation by the Broad Institute (<http://www.broad.mit.edu/gsea>) was performed on GeneSpring GX. Hepatic genes that were significantly up or downregulated relative to control diet-fed mice in animals fed with a Western-style diet were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). This identified biological functions that were most significant to the data set. Right-tailed Fisher's exact testing was used to calculate a *p*-value determining the probability that each biological function for that data set was due to its change alone.

3 Results

3.1 Dietary quercetin improves Western diet-associated obesity and blood values in C57/BL6J mice

Six-week-old mice were fed for 4, 8 or 20 wk with either the AIN93G diet (control) or with a Western-style diet high in fat, cholesterol and sucrose, with or without 0.05% quercetin. In parallel, another group of mice were fed for 20 wk with the control diet containing 0.05% quercetin. Quercetin did not affect the daily food consumption of mice fed either diet (Supporting Information Fig. 1). Table 1 shows that consumption of the Western diet resulted in significantly increased body weight after 8 or 20 wk relative to controls. Quercetin significantly decreased this body weight gain in mice fed with the Western diet for 20 wk (Table 1) but did not affect body weight of mice fed with the control diet (data not shown).

Compared to the control diet, mice fed with the Western diet had significantly higher blood glucose, plasma insulin, total cholesterol, triglycerides and NEFA levels after 4 wk, and later (Table 1). Those fed with the Western diet supplemented with quercetin had significantly lower plasma triglycerides after 4 wk, and lower plasma NEFA after 8 and 20 wk (Table 1). Similarly, after 20 wk, Western diet-fed mice also receiving quercetin had significantly lower blood glucose and plasma insulin and cholesterol levels than those not supplemented (Table 1). However, quercetin did not affect blood glucose levels and plasma concentrations of insulin, total cholesterol, triglycerides and NEFA in mice fed the control diet (data not shown).

Plasma levels of the adipokine adiponectin were significantly decreased in mice fed the Western diet compared to controls, but only after 20 wk (Table 1). Quercetin supplementation prevented this decrease in plasma adiponectin in Western diet-fed mice (Table 1). Plasma levels of the inflammatory cytokine TNF α were increased after 4 wk and later in mice fed the Western diet (Table 1). The increase in plasma TNF α was also significantly inhibited by quercetin after 20 wk (Table 1). Even in mice fed the control diet, quercetin significantly reduced the plasma concentration of TNF α from 0.91 ± 0.08 to ± 0.4 ng/mL after 20 wk.

3.2 Quercetin accumulates in the liver and suppresses liver fat accumulation

The liver and kidney are reported to be the major organs, which accumulate quercetin. Thus, we determined the concentration of quercetin metabolites in plasma, liver and kidney in mice fed the Western diet containing 0.05% quercetin for 20 wk. Quercetin metabolites are hydrolyzed and can be determined as quercetin and isorhamnetin by HPLC. The concentrations of quercetin metabolites were relatively high in plasma (Table 2). A higher concentration of quercetin metabolites was found in livers than in kidneys of mice fed the Western diet containing quercetin (Table 2).

Table 1. Quercetin reduces Western diet-induced body weight gain, visceral fat accumulation and concentrations of blood constituents in C57BL/6J mice

	Wk	Control	Western	Western+0.05% Quercetin
Body weight (g)	4	25.10 ± 0.74 ^a	27.87 ± 1.45 ^a	29.46 ± 1.40 ^a
	8	30.62 ± 0.23 ^a	35.54 ± 0.46 ^b	34.23 ± 0.69 ^b
	20	40.28 ± 0.83 ^a	46.08 ± 1.09 ^b	42.00 ± 0.78^a
Visceral fat (g/mouse)	4	0.80 ± 0.04 ^a	2.07 ± 0.16 ^b	2.04 ± 0.24 ^b
	8	2.25 ± 0.08 ^a	3.32 ± 0.09 ^b	2.98 ± 0.15 ^b
	20	3.98 ± 0.10 ^a	5.56 ± 0.07 ^b	4.70 ± 0.10^c
Blood glucose (mg/dL)	4	111.2 ± 3.3 ^a	138.7 ± 5.9 ^b	139.8 ± 6.2 ^b
	8	109.5 ± 2.9 ^a	168.3 ± 10.8 ^b	149.5 ± 8.0 ^b
	20	114.0 ± 2.2 ^a	162.8 ± 5.0 ^b	134.2 ± 4.3^c
Plasma insulin (ng/mL)	4	1.28 ± 0.07 ^a	2.07 ± 0.08 ^b	1.92 ± 0.09 ^b
	8	1.76 ± 0.11 ^a	2.85 ± 0.12 ^b	2.71 ± 0.12 ^b
	20	1.73 ± 0.06 ^a	4.47 ± 0.20 ^b	3.09 ± 0.07^c
Plasma total cholesterol (mg/dL)	4	90.4 ± 8.5 ^a	143.1 ± 10.7 ^b	137.0 ± 8.1 ^b
	8	118.6 ± 14.0 ^a	190.3 ± 14.8 ^b	157.3 ± 7.0 ^{ab}
	20	124.4 ± 8.0 ^a	310.6 ± 9.7 ^b	171.8 ± 12.0^c
Plasma triglycerides (mg/dL)	4	113.0 ± 5.2 ^a	147.4 ± 11.7 ^b	107.6 ± 6.8^a
	8	121.5 ± 4.4 ^a	176.7 ± 8.8 ^b	140.2 ± 7.5^a
	20	123.2 ± 7.6 ^a	205.7 ± 10.6 ^b	155.6 ± 7.5^a
Plasma NEFA (mmol/L)	4	0.49 ± 0.02 ^a	0.69 ± 0.03 ^b	0.61 ± 0.04 ^{ab}
	8	0.53 ± 0.03 ^a	0.86 ± 0.03 ^b	0.71 ± 0.03^c
	20	0.55 ± 0.04 ^a	0.95 ± 0.04 ^b	0.71 ± 0.03^c
Plasma adiponectin (ng/mL)	4	13.5 ± 0.7 ^a	12.3 ± 0.6 ^a	12.5 ± 0.6 ^a
	8	12.4 ± 0.7 ^a	10.1 ± 0.6 ^a	11.5 ± 0.6 ^a
	20	12.5 ± 0.7 ^a	6.9 ± 0.7 ^b	10.1 ± 0.5^a
Plasma TNF α (ng/mL)	4	0.12 ± 0.02 ^a	0.29 ± 0.05 ^b	0.19 ± 0.03 ^{ab}
	8	0.23 ± 0.05 ^a	1.08 ± 0.18 ^b	0.75 ± 0.13 ^b
	20	0.91 ± 0.08 ^a	6.64 ± 0.36 ^b	3.98 ± 0.17^c

C57BL/6J mice were fed with either the control AIN93G diet (Control), a Western-style diet (Western) or the Western diet containing 0.05% quercetin (Western+0.05% Quercetin) for 4, 8 or 20 wk. Values are expressed as the mean ± SEM of 6 mice in each group. Different superscripts indicate significant differences ($p < 0.05$, (two-sided)) between the three groups at the same period by a multiple t -test with Bonferroni correction following ANOVA.

Consumption of a Western-style diet is associated with steatosis and metabolic disorders. Because the liver is a major organ accumulating quercetin metabolites, we determined the effect of quercetin on liver steatosis of mice fed with the Western diet. Figure 1A shows representative examples of liver sections from mice fed the control diet, the Western diet or the Western diet containing quercetin for 20 wk. Western diet-induced fat accumulation was suppressed by quercetin (Fig. 1A), which also prevented the increased levels of liver triglycerides and total cholesterol (Fig. 1B). Although the Western diet slightly increased liver triglyceride concentrations, quercetin did not significantly affect these, or total cholesterol levels, after only 8 wk of feeding (Fig. 1B).

3.3 Quercetin alters the expression of genes related to mitochondrial respiratory function but does not improve mitochondrial complex I activity in the livers of mice fed with a Western-style diet

Next, we determined gene expression in the liver of mice fed the control diet, the Western diet or the Western diet

Table 2. Plasma and tissue concentrations of quercetin metabolites in C57BL/6J mice fed with the Western diet containing 0.05% quercetin for 20 wk

	Quercetin	Isorhamnetin
Plasma ($\mu\text{mol/L}$)	14.16 ± 0.95	29.68 ± 2.66
Liver (nmol/g)	2.34 ± 0.15	4.89 ± 0.32
Kidney (nmol/g)	1.90 ± 0.15	2.74 ± 0.23

Values are expressed as the mean ± SEM of 6 mice in each group.

containing quercetin for 20 wk. For this, we used DNA microarrays (Mouse Genome 430 2.0 array, Affymetrix). We found that 1149 genes were differentially expressed in the liver in the three groups ($p < 0.05$ by one-way ANOVA, Supporting Information Fig. 2). Mice on a Western diet showed significantly a different expression of 1126 genes in the liver compared to controls. Ingenuity Pathway Analysis identified several biological functions and canonical gene pathways putatively affected by consumption of a Western-style diet (Table 3). Expression of the peroxisome proliferator-activated

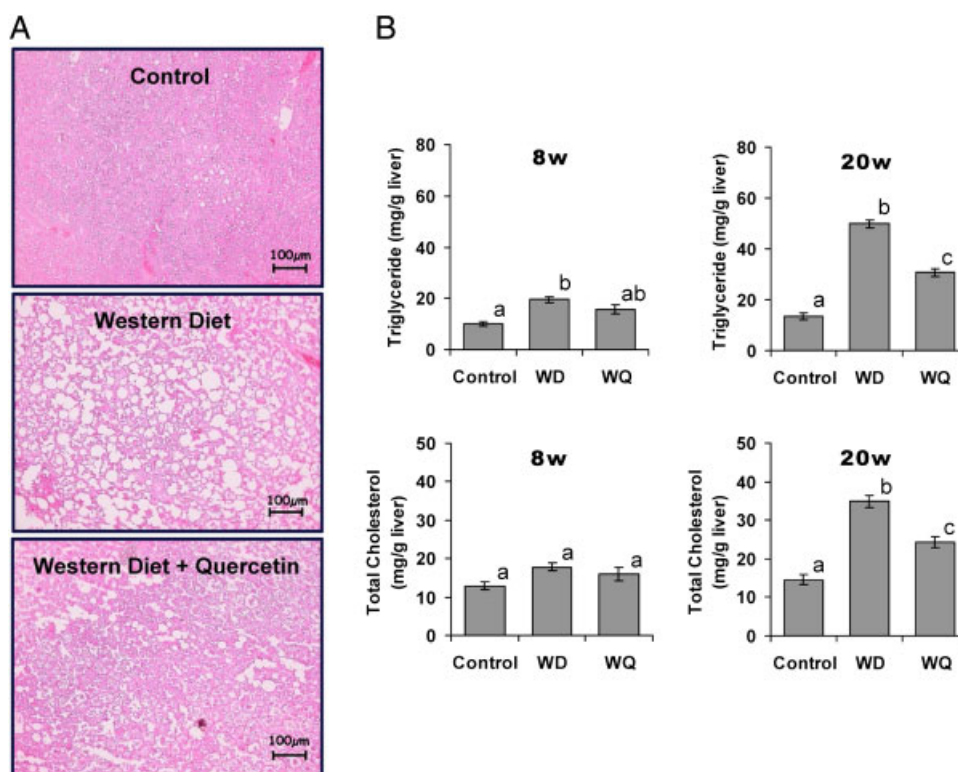


Figure 1. Quercetin alleviates Western diet-induced fat accumulation in livers of C57BL/6J mice. (A) Representative photomicrographs of H&E-stained liver sections of C57BL/6J mice. Mice were fed either a control diet (Control), a Western diet (Western diet) or a Western diet containing 0.05% quercetin (Western Diet+Quercetin) for 20 wk. (B) Hepatic total cholesterol and triglyceride concentrations in mice fed a control diet (Control), a Western diet (WD) or a Western diet containing 0.05% quercetin (WQ) for 8 or 20 wk. Values are expressed as the mean \pm SEM of 6 mice in each group. Different superscripts indicate significant differences ($p < 0.05$, two-sided) between the three groups at the same period by a multiple *t*-test with Bonferroni correction following ANOVA.

receptor γ (PPAR γ), which promotes steatosis, was higher, and networks involving PPAR γ were affected (Supporting Information Fig. 3, Network 1). Gene expression related to lipid metabolism was also significantly different in animals on a Western diet (Table 3). Furthermore, we found that canonical pathways of oxidative phosphorylation, mitochondrial dysfunction and ubiquinone biosynthesis were affected by the Western-style diet (Table 3). The network involving NADH dehydrogenases, which constitute the oxidative phosphorylation complex mitochondrial respiratory complex I, was significantly suppressed compared to controls (Supporting Information Fig. 3, Network 2).

Quercetin minimally influenced hepatic gene expression in mice fed the Western diet (Supporting Information Fig. 2). Gene set enrichment analysis (GSEA) on microarray data obtained from liver samples of mice fed the Western diet containing either 0.05% or no quercetin showed that this component altered ubiquinone biosynthesis, which was significantly different between control-fed and Western diet-fed mice (Table 4). This suggests that quercetin significantly alleviates mitochondrial dysfunction associated with consumption of a Western-style diet. However, although the Western diet reduced the activity of the mitochondrial complex I, which consists of NADH dehydrogenases, quercetin did not significantly improve this in their livers (Fig. 2).

3.4 Quercetin improves the hepatic expression of genes that regulate lipid metabolism, antioxidant status, mitochondrial transport and glucose metabolism in mice fed with the Western diet

To identify the molecular mechanisms for the suppressive effects of quercetin on Western diet-induced hepatic steatosis and metabolic disorder, we used quantitative RT-PCR to investigate hepatic expression of genes regulating lipid metabolism, antioxidant status, mitochondrial transport and glucose metabolism. Compared to controls, mice on a Western diet showed significantly different expression of the PPAR α (encoded by *Ppara*), PPAR γ (*Pparg*), CD36 (*Cd36*), sterol regulatory element-binding protein-1c (SREBP-1c) (*Srebp1*) and fatty acid synthase (*Fasn*) genes after 8 and 20 wk (Fig. 3A). Quercetin intake resulted in a significantly increased expression of *Ppara*, which relates to the β -oxidation of fatty acids, up to control levels, in the livers of Western diet-fed mice after 8 and 20 wk (Fig. 3A). Reciprocally, quercetin significantly suppressed the expression of the transcription factors *Pparg* and *Srebp1*, and their target genes *Cd36* and *Fasn*, respectively, in the liver of mice fed with the Western diet for 20 wk (Fig. 3A).

The expression of the antioxidant enzymes glutathione peroxidase 1 (*Gpx1*) and catalase (*Cat*) were reduced compared to controls after 8 or 20 wk of feeding mice on a Western-style diet, whereas expression of the mitochondrial proton carrier uncoupling protein 2 (UCP-2) (*Ucp2*) was

Table 3. Top 10 biological functions (A) and top 10 canonical pathways (B) of hepatic genes that were significantly up or downregulated by Western diet

(A) Biological functions	<i>p</i> -Value	No. of genes differentially expressed
Cellular movement	1.56E–08 – 6.29E–03	123
Cell-to-cell signaling and interaction	9.21E–08 – 6.06E–03	125
Cellular compromise	3.82E–07 – 6.29E–03	50
Inflammatory response	3.82E–07 – 6.06E–03	93
Amino acid metabolism	7.46E–07 – 6.06E–03	24
Small molecule biochemistry	7.46E–07 – 6.06E–03	130
Cellular function and maintenance	1.12E–06 – 6.06E–03	37
Tissue development	1.14E–06 – 6.06E–03	90
Lipid metabolism	1.97E–06 – 5.69E–03	91
Molecular transport	1.97E–06 – 5.21E–03	72
(B) Ingenuity canonical pathways	–log (<i>p</i> -value)	Ratio
Oxidative phosphorylation	24.8	49/155 (0.316)
Mitochondrial dysfunction	20.5	42/166 (0.253)
Coagulation system	10.6	16/35 (0.457)
Ubiquinone biosynthesis	7.85	19/114 (0.167)
Acute-phase response signaling	7.18	29/169 (0.172)
Inositol metabolism	5.80	18/95 (0.189)
LPS/IL-1-mediated inhibition of RXR function	5.04	27/202 (0.134)
Arginine and Proline metabolism	4.42	15/181 (0.083)
Butanoate metabolism	4.41	13/130 (0.100)
Tryptophan metabolism	4.22	21/244 (0.086)

The functions and canonical pathways that were most significant to the data set were identified by Ingenuity Pathway Analysis (Ingenuity Systems).

increased (Figs. 3B and C). Quercetin counteracted the decreased *Gpx1* expression associated with the Western diet after 8 and 20 wk of feeding (Fig. 3B), and significantly increased *Cat* expression while decreasing UCP-2 expression after 20 wk (Figs. 3B and C).

Phosphoenolpyruvate carboxykinase (*Pck1*) and Glucokinase (*Gck*) regulate gluconeogenesis and glycolysis, respectively. Quercetin feeding increased *Pck1* expression, which had been reduced by consuming a Western-style diet for 20 wk (Fig. 3C). However, quercetin did not significantly improve the expression of *Gck* after 20 wk of the Western diet (Fig. 3C). This result documents that prevention of the decreased expression of *Ppara* and *Gpx1* was an early event in quercetin-improved hepatic gene expression in mice fed the Western diet.

3.5 Quercetin significantly reduces lipid peroxidation and increases glutathione levels in the liver of mice fed the Western diet

Next, we quantified the oxidative stress marker TBARS and the endogenous antioxidant glutathione in the liver. Mice fed the Western diet had significantly increased levels of TBARS in their livers compared to the controls after 4 wk or at later times (Fig. 4), but addition of quercetin to this diet significantly reduced this at 8 and 20 wk (Fig. 4). Quercetin also significantly reduced hepatic TBARS levels in mice fed

Table 4. Gene sets that were significantly regulated by quercetin in livers of C57BL/6J mice fed with the Western diet for 20 wk

Gene set	FDR ^{a)}
Citrate acid cycle	0.011
Ubiquinone biosynthesis	0.011
Propanoate metabolism	0.057
Metabolism of xenobiotics by Cytochrome P450	0.071

GSEA was performed on microarray data obtained from liver samples of mice fed with either a Western diet or Western diet containing 0.05% quercetin for 20 wk using GeneSpring GX.

a) FDR, false discovery rate.

with the control AIN93G diet for 20 wk (Fig. 4). After 8 wk, liver total glutathione levels in mice fed the control diet, the Western diet or the Western diet supplemented with quercetin were 2.31 ± 0.14 , 0.84 ± 0.08 and 1.53 ± 0.12 $\mu\text{mol/g}$ tissue, respectively. Thus, quercetin also significantly restored total glutathione levels, which were decreased in mice fed a Western-style diet.

4 Discussion

Here, we show that chronic dietary intake of quercetin prevented the body weight gain and visceral and liver fat

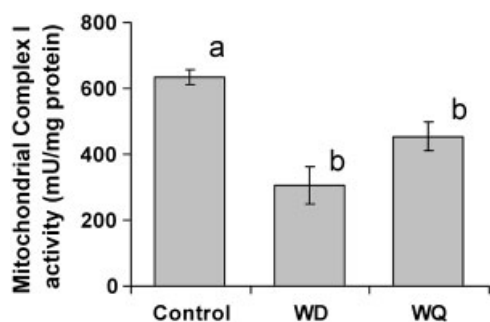


Figure 2. Effects of Western diet and quercetin supplementation on hepatic mitochondrial respiratory complex I activity in C57BL/6J mice. Mice were fed a control diet (Control), a Western diet (WD) or a Western diet containing 0.05% quercetin (WQ) for 20 wk. Mitochondrial complex I activity was determined according to the method of Feillet-Coudray *et al.* [22]. Values are expressed as the mean \pm SEM of 6 mice in each group. Different superscripts indicate significant differences ($p < 0.05$, (two-sided)) between the three groups at the same period by a multiple *t*-test with Bonferroni correction following ANOVA.

accumulation, alleviated the hyperglycemia, hyperinsulinemia, dyslipidemia, and improved plasma adiponectin and TNF α levels in mice fed a high-fat, high-cholesterol and high-sucrose Western-style diet. Dietary quercetin improved plasma triglyceride levels but not other parameters as early as after 4 wk' supplementation.

In our previous study, both 0.1 and 0.5% quercetin diet improved the blood glucose levels in streptozotocine-induced diabetic mice after 2 wk of feeding [12]. However, we also found that 0.5 and 1% quercetin diets unexpectedly reduced the expression of ubiquitin C in healthy control mice [12]. Therefore, to avoid the excessive intake of quercetin, we fed mice with a Western diet containing 0.05% quercetin. Plasma concentrations of quercetin metabolites in BALB/c mice fed a diet containing 0.1% quercetin for 2 wk were $10.55 \pm 1.48 \mu\text{mol/L}$ as quercetin and $19.53 \pm 1.96 \mu\text{mol/L}$ as isorhamnetin [12]. Thus, plasma concentrations of quercetin metabolites in C57/BL6J mice fed a Western diet containing 0.05% quercetin for 20 wk were higher than in BALB/c mice fed the AIN93G diet containing 0.1% quercetin for 2 wk.

After 20 wk, quercetin accumulated to a high level in the livers of Western diet-fed mice. The liver plays an important role in modulating Western diet-associated metabolic disorders. High-fat diets significantly alter the expression of many genes related to lipid, cholesterol and oxido-reductive metabolism, inflammation, immune responses and stress-related pathways [21]. Radonjic *et al.* showed that high-fat diets result in the early activation of inflammatory/immune pathways, including the transcription factor NF- κ B at day 3, and later the activation of lipogenesis/adipogenic pathways including PPAR γ and SREBP1 (week 12) in ApoE3Leiden mice expressing human APOE3Leiden and the apoC1 gene [21]. In our study, feeding a Western-style diet altered expression of genes related to inflammatory responses, lipid

metabolism and oxidative phosphorylation in C57BL/6J mice after 20 wk. Higher expression of the PPAR γ signaling pathway and reduced expression of NADH dehydrogenases constituting the mitochondrial respiratory complex I was observed in Western diet-fed mice. Mitochondrial dysfunction and oxidative stress are known to accelerate steatosis and initiate progression to steatohepatitis [23, 24]. Although quercetin failed to reverse the reduced mitochondrial complex I activity in mice fed a Western diet for 20 wk, the gene screening results (GSEA) were consistent with the notion that it improve mitochondrial function to some extent. Thus, chronic intake of quercetin may suppress the progression of steatohepatitis by offsetting mitochondrial dysfunction and oxidative stress.

Although the results from exhaustive gene expression analysis showed that quercetin minimally affected dysregulated hepatic gene expression associated with consumption of a Western-style diet even after 20 wk, quantitative RT-PCR analysis indicated that quercetin influences important regulators of fat accumulation and metabolic disorders. Intake of a Western diet increases oxidative stress, which enhances insulin resistance and fat accumulation in the liver [22, 25–27]. Increased generation of reactive oxygen species is thought to enhance insulin resistance by induction of serine phosphorylation of insulin receptor substrate-1 and the expression of proliferator-activating receptor γ coactivator-1 α through activating Jun NH $_2$ -terminal kinase [27–29]. It is suggested that oxidative stress induces fat accumulation directly or indirectly through the exacerbation of insulin resistance [27, 28, 30]. The antioxidants *N*-acetyl cysteine and dehydroepiandrosterone were shown to suppress the fat accumulation and lipogenic SREBP-1c expression induced by a high fat diet and a high fat plus fructose diet, respectively [31, 32]. After 8 wk' intake, quercetin improved the levels of TBARS and total glutathione, and the expression of *Gpx1*, in the livers of mice fed the Western diet. It is likely that quercetin gradually reduces fat accumulation by modulating the expression of genes related to steatosis through alleviating oxidative stress in the liver. Moreover, a polyphenol that activates AMP-activated protein kinase (AMPK) was shown to prevent lipid accumulation in the liver of diabetic LDL receptor-deficient mice [33]. AMPK inhibits the activity of acetyl-CoA carboxylase and carbohydrate response element-binding protein, and the expression of SREBP-1c [34]. Because quercetin has been reported to activate AMPK *in vitro*, it may suppress hepatic steatosis through the activation of AMPK [35, 36].

Here, we first showed that quercetin prevented the reduction of PPAR α expression in the liver of mice fed the Western diet. PPAR α mediates the expression of genes promoting fatty acid β -oxidation and its activation lowers circulating lipids [37, 38]. Quercetin probably first improves the plasma triglyceride and NEFA levels through alleviating the reduction of hepatic PPAR α expression. Elevation of plasma NEFA levels has been shown to induce insulin resistance [29, 39]. The reduction of plasma NEFA levels by

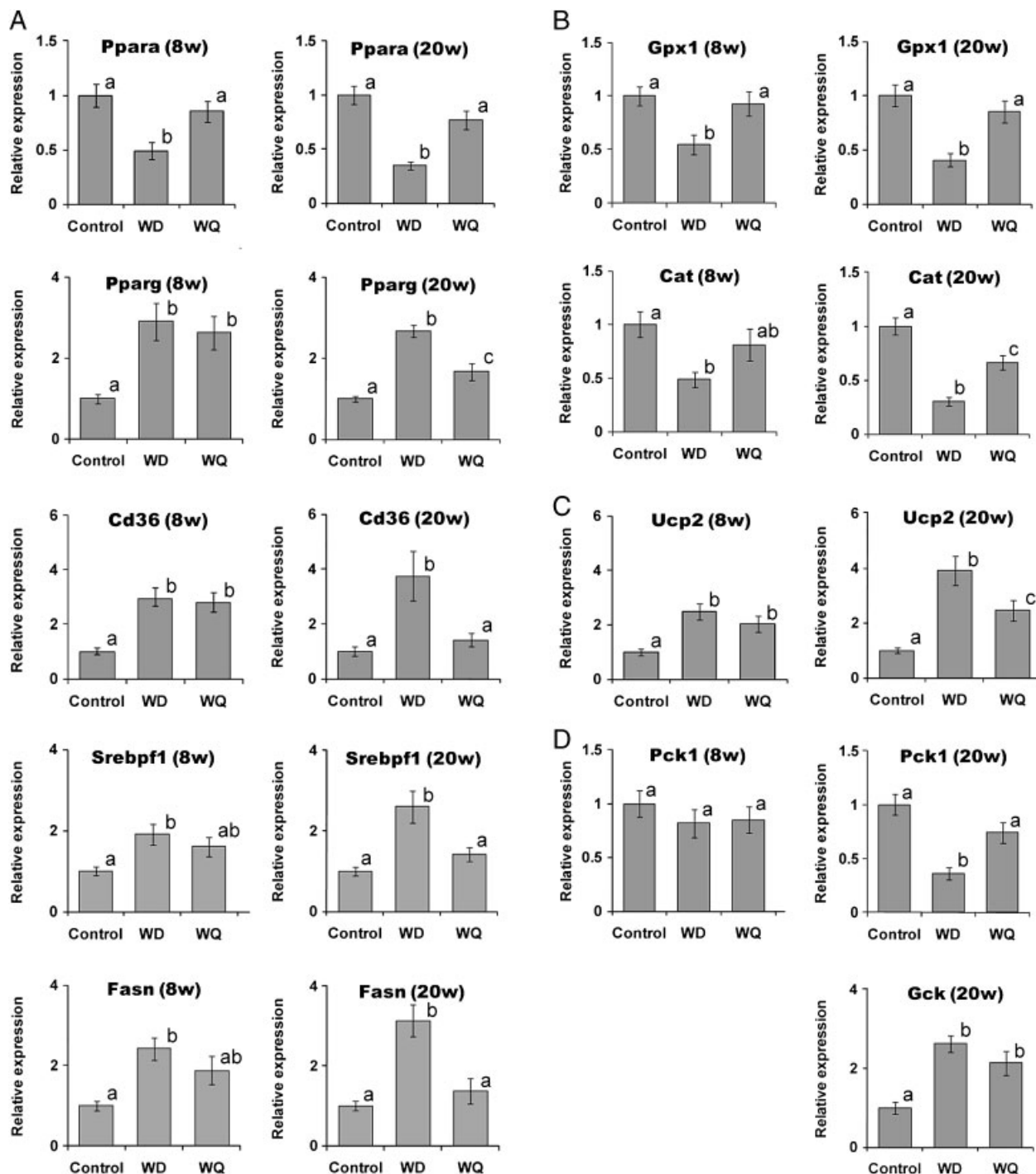


Figure 3. Effects of quercetin on the expression of hepatic genes related to lipid metabolism (A), antioxidative status (B), mitochondrial transport (C), and glucose metabolism (D). Mice were fed a control diet (Control), a Western diet (WD) or a Western diet containing 0.05% quercetin (WQ) for 8 or 20 wk. Values are expressed as the mean \pm SEM of 6 mice in each group. Different superscripts indicate significant differences ($p < 0.05$, (two-sided)) between the three groups at the same period by a multiple t -test with Bonferroni correction following ANOVA.

quercetin presumably contributes to ameliorate insulin resistance.

Quercetin first reduces oxidative stress and prevents the reduction of PPAR α expression, and then reduces fat accu-

mulation and improves the expression of other genes regulating lipid metabolism, mitochondrial transport and glucose metabolism. PPAR γ and the target gene CD36 are involved in the control of adipogenesis. PPAR γ regulates

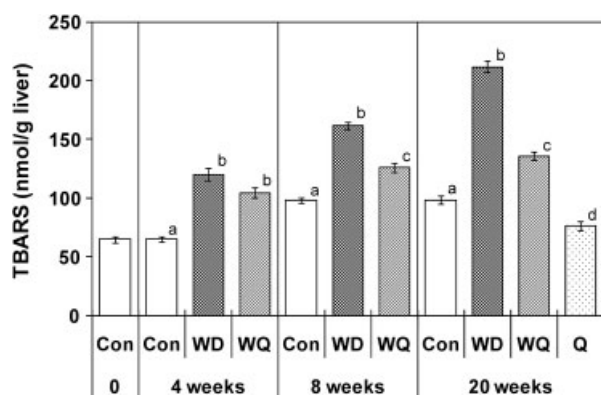


Figure 4. Quercetin alleviates the Western diet-induced lipid peroxidation in livers of C57BL/6J mice. Mice were fed control AIN93G diet (Control), Western diet (WD), Western diet containing 0.05% quercetin (WQ) or AIN93G diet containing 0.05% quercetin (Q) for 0, 4, 8 or 20 wk. Lipid peroxidation in the liver was determined by the TBARS assay. Values are expressed as the mean \pm SEM of 6 mice in each group. Different superscripts indicate significant differences ($p < 0.05$, (two-sided)) between the three groups at the same period by a multiple *t*-test with Bonferroni correction following ANOVA.

high-fat diet-induced fat accumulation in the liver [40, 41]. Inoue *et al.* reported that PPAR γ but not other genes related to fatty acid oxidation and fatty acid synthesis were specifically upregulated in their mouse model of high-fat diet-induced liver steatosis [41]. Quercetin suppresses the expression of PPAR γ and CD36 as well as SREBP-1c and the target fatty acid synthase in the liver of mice fed the Western diet.

Expression of the mitochondrial uncoupling protein UCP-2 is increased in liver steatosis associated with obesity. UCP-2 increases H⁺ leak across the inner mitochondrial membrane and decreases ATP synthesis [23]. Phosphoenolpyruvate carboxykinase regulated by insulin mediates gluconeogenesis in the liver. Partial silencing of liver *Pck1* was shown to improve glycemia, insulinemia and dyslipidemia [42]. Quercetin prevented the increase of *Ucp2* expression in Western diet-fed mice and restored expression of *Pck1*, which was reduced by the Western diet.

Adipose tissue plays an important role in metabolic syndrome [38, 43–46]. White adipose tissue produces biologically active adipokines, including adiponectin and the proinflammatory cytokine TNF α . Plasma adiponectin levels have been shown to be reduced with obesity and in metabolic syndrome [43]. In the liver, adiponectin activates AMPK and PPAR pathways through the adiponectin receptor AdipoR2 and decreases insulin resistance [43, 44, 47]. At the same time, increased secretion of TNF α from adipose tissue has been shown to induce insulin resistance [46, 48, 49]. After 20 wk of feeding quercetin, plasma adiponectin levels were significantly increased and TNF α levels were reduced. The concentrations of quercetin metabolites in mesenteric adipose tissues

were 0.43 ± 0.02 nmol/g tissue in the form of quercetin and 0.73 ± 0.04 nmol/g tissue as isorhamnetin in mice fed the Western diet for 20 wk. Although the concentrations of quercetin metabolites in adipose tissues were less than in livers and kidneys, quercetin probably has a direct affect on adipose tissues. Quercetin reduced the lipid accumulation in 3T3-L1 adipocytes (unpublished data). Effects of quercetin on adipose tissues are under investigation. The improved plasma adiponectin and TNF α levels should contribute to the reduction of fat accumulation and improvement of other blood values in mice fed the Western diet for 20 wk. Reduction of oxidative stress in adipose tissue may improve the plasma levels of adiponectin and TNF α [45].

Apple polyphenols were reported to decrease the liver and serum cholesterol levels by promoting cholesterol catabolism and inhibiting intestinal absorption of cholesterol in rats fed a diet containing 0.5% cholesterol [50]. Although quercetin has never been shown to promote the excretion of sterol, it may also affect to intestinal lipid absorption.

Chronic dietary intake of quercetin also mediated anti-oxidative and anti-inflammatory effects in mice fed the control diet for 20 wk, i.e. reduction of plasma TNF α levels, hepatic TBARS generation and the hepatic PPAR γ . In contrast, it did not affect body weight, fat accumulation or plasma levels of factors other than TNF α in mice on the control diet.

In conclusion, chronic dietary intake of quercetin reduced body weight gain and visceral and liver fat accumulation, and improved hyperglycemia, hyperinsulinemia, dyslipidemia in mice fed a Western-style diet. Quercetin reduces fat accumulation presumably through decreasing oxidative stress and increasing PPAR α expression, and the following improvement of gene expression related to steatosis in the liver. Improvement of these hepatic gene expressions probably contributes to the improvement of blood glucose and the plasma triglyceride, cholesterol and free fatty acids levels in mice fed the Western diet. Chronic dietary intake of quercetin also improved plasma adipokine levels. Our results provide evidence for a beneficial effect of habitual consumption of a quercetin-rich diet on the prevention of lifestyle-related diseases.

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