Research Article

Dietary quercetin alleviates diabetic symptoms and reduces streptozotocin-induced disturbance of hepatic gene expression in mice

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Quercetin is a food component that may ameliorate the diabetic symptoms. We examined hepatic gene expression of BALB/c mice with streptozotocin (STZ)-induced diabetes to elucidate the mechanism of the protective effect of dietary quercetin on diabetes-associated liver injury. We fed normal and STZ-induced diabetic mice with diets containing quercetin for 2 wk and compared the patterns of hepatic gene expression in these groups of mice using a DNA microarray. Diets containing 0.1 or 0.5% quercetin lowered the STZ-induced increase in blood glucose levels and improved plasma insulin levels. A cluster analysis of the hepatic gene expressions showed that 0.5% quercetin diet suppressed STZ-induced alteration of gene expression. Gene set enrichment analysis (GSEA) and quantitative RT-PCR analysis showed that the quercetin diets had greatest suppressive effect on the STZ-induced elevation of expression of cyclin-dependent kinase inhibitor *p21(WAF1/Cip1)* (*Cdkn1a*). Quercetin also suppressed STZ-induced expression of *Cdkn1a* in the pancreas. Dietary quercetin might improve liver and pancreas functions by enabling the recovery of cell proliferation through the inhibition of *Cdkn1a* expression. Unexpectedly, in healthy control mice the 0.5 and 1% quercetin diets reduced the expression of ubiquitin C (*Ubc*), which has heat-shock element (HSE) in the promoter region, in the liver.

Keywords: *Cdkn1a* / Diabetes / Mouse liver / Quercetin / Streptozotocin

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1 Introduction

Quercetin is one of the most common flavonoids and is present in a wide range of foodstuffs, such as vegetables, fruits, and tea. It shows antioxidant activity both *in vitro* and *in vivo* [1–5]. Flavonoids, including quercetin, have been suggested to prevent cardiovascular disease and other lifestyle related diseases through scavenging free radicals, inhibiting lipid peroxidation, and other antioxidative actions [5, 6]. Quercetin possibly has the potential to prevent lifestyle-related diseases, although there are concerns that an excess of the flavonoid may act as a prooxidant or a mutagen [7]. A number of studies have reported that quercetin can directly affect a range of physiologically active molecules. For example, quercetin inhibits the activities of proteins, such as *p*-glycoprotein, protein kinase C, and topoisomerase II, by binding to ATP binding sites [8]. Quercetin inhibits the expression of heat shock proteins and acts as a phytoestrogen [9, 10]. However, most of these effects have only been demonstrated *in vitro* at relatively high concentrations of quercetin.

Streptozotocin (STZ)-induced diabetes in mice is a well-established animal model of type 1 diabetes mellitus. Quercetin has been suggested to ameliorate the symptoms of STZ-induced diabetes by decreasing oxidative stress [3, 4, 11, 12]. However, quercetin has only been shown to reduce the levels of some oxidative stress markers such as thiobarbituric acid-reactive substances (TBARS) and malondialdehyde (MDA) and the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, in plasma, liver, kidney, and pancreas [3, 4, 11, 12]. To date, there has been no demonstration of the effect of quercetin on the

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Abbreviations: **Cdkn1a,** cyclin-dependent kinase inhibitor *p21(WAF1/Cip1)*; **GSEA,** gene set enrichment analysis; **Gapdh,** glyceraldehyde-3-phosphate dehydrogenase; **Gck,** glucokinase; **Gpr146,** G protein-coupled receptor 146; **Mt1,** metallothionein 1; **Nos2,** nitric oxide synthase 2; **Nqo1,** NAD(P)H:quinine oxidoreductase 1; **STZ,** streptozotocin; **TBARS,** thiobarbituric acid-reactive substances; **Ubc,** ubiquitin C

complicated changes in gene expression induced by STZ in target tissues. One possible means to investigate this problem is through a comprehensive gene expression analysis using DNA microarrays. Such an investigation will identify the beneficial effects of dietary quercetin on STZ-induced changes in gene expression and also any unexpected physiological reactions.

The liver is a major organ that is adversely affected by diabetes. Dietary quercetin is metabolized in the liver and then gradually accumulates in other organs [13]. Consequently, dietary quercetin may be particularly effective for inhibiting liver injury induced by diabetes. Here, we investigated the potential of quercetin to alleviate the symptoms of diabetes and also the safe level of intake of the flavonoid. We used DNA microarrays to identify and quantify the effects of dietary quercetin on gene expression in the livers of normal and STZ-induced diabetic mice. Our results suggest that in STZ-induced diabetic mice quercetin alleviates diabetic symptoms and liver injury by enabling cell proliferation through suppression of hepatic and pancreatic expression of *Cdkn1a*.

2 Materials and methods

2.1 Animals and treatments

Male BALB/c mice were purchased from Charles River Laboratories Japan, (Kanagawa, Japan) and maintained three *per* cage on an AIN93G diet (CLEA Japan, Tokyo, Japan). Feed and water were provided *at libitum* to the mice. The animals were housed under conditions of 24 ± 1°C, 55 ± 5% humidity, and 12 h light/dark photocycles. The animals were treated in accordance with the basic guidelines of the Ministry of Agriculture, Forestry and Fisheries for laboratory animal study. Seven-week-old male BALB/c mice were intraperitoneally injected with STZ (Wako Pure Chemical Industries, Osaka, Japan, 185 mg/kg in 0.05 M citrate buffer, pH 4.5). Mice (n = 6) in the untreated control group did not receive any treatment. Tail vein blood was sampled weekly and the blood glucose levels of the samples were immediately measured using a blood glucose test meter (Glucocard, Arkray, Kyoto, Japan). After 1 wk, 18 mice showing nonfasting blood glucose levels of 230 – 400 mg/dL were divided into 3 groups: one group was fed with AIN93G only (control group), the others with an AIN93G diet containing 0.1 or 0.5% quercetin (Funakoshi, Tokyo, Japan) for 2 wk. In a separate experiment, 7-wk-old male BALB/c mice that had not been treated with STZ (n = 18) were fed for 2 wk with AIN93G (control group) or AIN93G containing 0.1 or 0.5% quercetin. In both experiments, mice were sacrificed under anesthesia with pentobarbital; blood, liver, and pancreas tissues were immediately collected.

Plasma insulin concentrations were measured using an ELISA kit (Sibayagi, Gunma, Japan). Lipid peroxidation in the liver was measured by TBARS using an OXI-TEK TBARS Assay Kit (ZeptoMetrix, Buffalo NY).

2.2 Measurement of the plasma levels of quercetin metabolites

To determine the levels of quercetin metabolites, plasma samples were treated with β-glucoronidase, and quercetin and isorhamnetin were extracted with ethyl acetate. The ethyl acetate fraction containing the quercetin and isorhamnetin was concentrated and applied to an HPLC column (Tosoh TSK-GEL ODS-100 V, 150 mm ± 20 mm id, 3 μm, Tosoh, Tokyo, Japan) and eluted with 40% ACN in 0.5% aqueous phosphoric acid at a flow rate of 0.2 mL/min. Quercetin and isorhamnetin were detected with an electrochemical detector at 50 and 70 mV (Coulochem III, ESA Laboratories, Chelmsford, MA).

2.3 Liver histology and TUNEL staining

Frozen liver sections (9 μm) were fixed with 10% buffered formalin and stained with Mayer's hematoxylin solution and eosin (both from Wako Pure Chemical Industries). Fragmented DNA in damaged cells was detected using the DeadEnd™ colorimetric TUNEL system (Promega K.K., Tokyo, Japan).

2.4 RNA isolation and DNA microarray analysis

Total RNA was extracted from livers using an RNeasy Midi Kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. Double-stranded cDNA was synthesized from the total RNA of each mouse using the One-Cycle cDNA Synthesis Kit (Affymetrix Japan KK, Tokyo, Japan) with a T7-$(dT)_{24}$ primer. Biotin-labeled cRNA was then synthesized using an IVT Labeling Kit (Affymetrix). The biotin-labeled cRNA was further purified and fragmented using the Sample Cleanup Module (Affymetrix). Fifteen microgram aliquots of fragmented cRNA were 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 with an GeneChip Scanner (Affymetrix) with GeneChip Operation Software Ver. 1.3 (Affymetrix). DNA microarray data analysis was performed with a Microarray Suite and GeneSpring Ver.7.3.1 (Agilent Technologies, Santa Clara, CA).

2.5 Quantitative real-time reverse transcription (RT)-PCR analysis

Total RNA was extracted from livers and pancreas using an RNeasy Midi Kit (Qiagen KK) according to the manufacturer's instructions. Quantitative RT-PCR was performed with an ABI PRISM 7000 Sequence Detection System

(Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's specifications. The TaqMan primers and probes for *Cdkn1a* (assay identification number Mm00432448_m1), *Ccng1* (Mm00438084_m1), glucokinase (*Gck*) (Mm00439129_m1), *Cdkn1b* (Mm004318167_m1), ubiquitin C (*Ubc*) (Mm01201237_m1), *Grp146* (Mm01951835_s1), and metallothionein 1 (*Mt1*) (Mm004966605_g1) were TaqMan Gene Expression Assay products (Applied Biosystems). The mouse and human glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene was used as an internal control (Mouse- and Human-*GAPDH* MGB, Applied Biosystems). The thermal cycler conditions were as follows: 2 min at 50°C and then 10 min at 95°C, followed by two-step PCR for 40 cycles consisting of 95°C for 15 s followed by 60°C for 1 min. All assays were performed in triplicate. The results are expressed relative to the *Gapdh* internal control. We also carried out quantitative RT-PCR using the SYBR Green Realtime PCR Master Mix (TOYOBO) according to the manufacturer's protocol. The primer sequences used for quantitative RT-PCR were as follows [14]: Nitric oxide synthase 2 (*Nos2*), 5′-CCACAAGAGATACAAGTCTG-3′ and 5′-CCGATGCACTTTCGCTTGC-3′; NAD(P)H:quinine oxidoreductase 1 (*Nqo1*), 5′-CCATTCTGAAAGGCTGGTTTG-3′ and 5′-CTAGCTTTGATCTGGTTGTC-3′.

2.6 Statistical analysis

With the exception of the DNA microarray data, statistical analyses were performed using SYSTAT Ver.11.00 (Hulinks, Tokyo, Japan). Data are expressed as the arithmetic mean ± standard error (se). 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 Microarray Suite and GeneSpring Ver.7.3.1 (Agilent). Statistical analysis of differences in gene expression levels between the dosages was performed by Welch's one-way ANOVA. Enrichment for gene ontology categories was determined using Fisher's exact test. Cluster analysis was carried out using a Pearson correlation and average linkage for similarity measures after normalization to the median of the control samples. Gene set enrichment analysis (GSEA) was performed using GSEA software and the Molecular Signature Database (MSigDB) [15, 16] (http://www.broad.mit.edu/gsea/). The differential expression analysis used the linear modeling approach and empirical Bayes statistics as described by Smyth [17, 18].

3 Results

3.1 Dietary quercetin improves blood glucose and insulin levels, and reduces hepatic oxidative stress in STZ-induced diabetic mice

Intraperitoneal injection of STZ increased blood glucose levels in BALB/C mice after 1 wk. The mice were then fed for 2 wk with an AIN93G diet or with AIN93G containing 0.1 or 0.5% quercetin. Blood glucose levels increased further and plasma insulin levels decreased in the STZ-injected control mice fed the AIN93G diet compared to the untreated control group fed the AIN93G diet. The blood glucose levels of the group fed 0.1% quercetin were significantly lower after 2 wk than the group fed AIN93G without quercetin (Table 1). In addition, the mice receiving 0.5% dietary quercetin showed a significantly increased serum insulin level in STZ-treated mice (Table 1). STZ induces an increase in the level of the oxidative stress marker TBARS in the liver (Table 1). Both 0.1 and 0.5% quercetin diets significantly reduced the level of TBARS in the liver as compared to the control AIN93G diet (Table 1). Quercetin and

Table 1. Effect of STZ treatment and a quercetin supplemented diet on weight, blood constituents and liver lipid peroxidation in BALB/c mice[a)]

	Control		STZ		STZQuer0.1%		STZQuer0.5%	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Weight (g)	28.49	0.52	21.82^{A}	1.32	22.85^{A}	0.89	22.05^{A}	0.88
Liver (g)	1.54	0.04	1.67	0.12	1.52	0.10	1.47	0.10
Liver/weight (%)	5.39	0.08	7.65^{A}	0.17	6.63^{AB}	0.17	6.63^{AB}	0.26
Blood glucose (mg/dL)	114	4.1	479^{A}	29.3	378^{AB}	22.9	404^{A}	15.7
Plasma insulin level (ng/mL)	2.06	0.10	0.47^{A}	0.03	0.73^{A}	0.04	1.02^{AB}	0.08
TBARS (nmol/g liver)	15.21	0.46	34.70^{A}	1.38	28.92^{AB}	0.91	25.06^{AB}	0.96

a) Data from four groups of nontreated control mice (control) and STZ-treated mice are shown here: STZ-treated mice fed the control diet (STZ), and those fed with either 0.1 or 0.5% quercetin supplemented diets (STZQuer0.1% and STZQuer0.5%, respectively). Values are expressed as the means ± se of six mice in each group. The superscript alphabets indicate significant differences ($p < 0.05$, (two-sided)) by a multiple *t*-test with Bonferroni correction following ANOVA: A, compared to control group; B, compared to STZ group.

Table 2. Plasma concentrations of quercetin metabolites in STZ-treated Balb/c mice fed the quercetin diets for 2 wk[a)]

	STZQuer0.1%		STZQuer0.5%	
	Mean	SE	Mean	SE
Plasma quercetin conc. (μmol/L)	7.04	0.68	20.63	1.53
Plasma isorhamnetin conc. (μmol/L)	8.40	1.69	34.02	1.72

a) Data from two groups of STZ-treated mice fed with either 0.1 or 0.5% quercetin supplemented diets (STZQuer0.1% and STZQuer0.5%, respectively) are shown here. Values are expressed as the means ± se of six mice in each group.

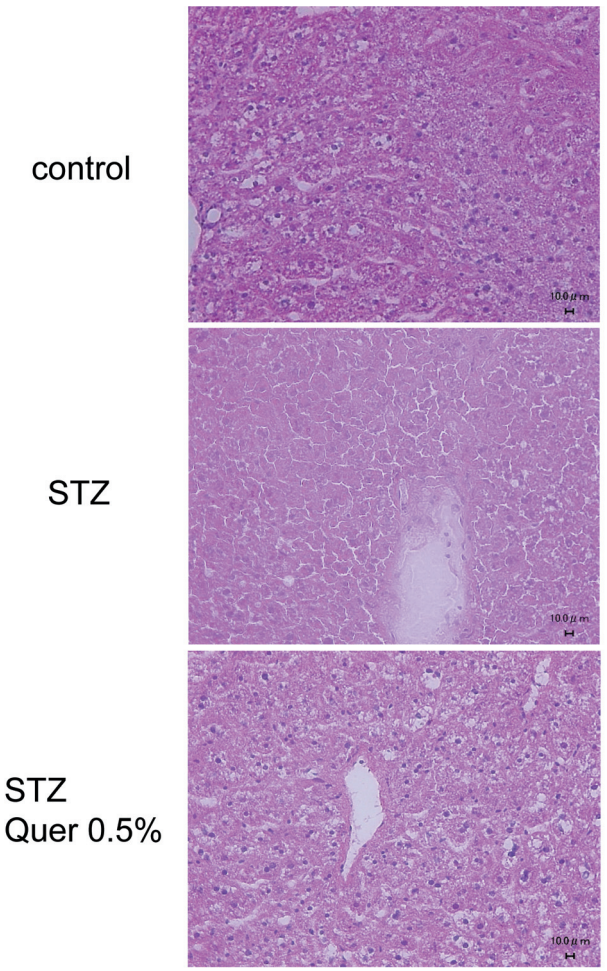

Figure 1. Representative photomicrographs of hematoxylin and eosin-stained liver sections. "Control" indicates untreated control mice. "STZ" indicates STZ-injected mice and "Quer 0.5%" indicates mice fed the 0.5% quercetin diet.

isorhamnetin conjugates are major metabolites of quercetin. We determined the plasma concentrations of quercetin and isorhamnetin by HPLC after hydrolysis of the quercetin metabolites. We found that the plasma concentrations of quercetin and isorhamnetin in mice fed the 0.5% quercetin diet were three to four times higher than those in mice fed with the 0.1% quercetin diet (Table 2).

3.2 Dietary quercetin moderates the disturbance of gene expression in livers of STZ-induced diabetic mice

To determine the extent of liver injury induced by STZ, we performed hematoxylin-eosin and TUNEL staining of liver sections [19]. Representative examples of liver sections from untreated control mice and STZ-injected mice fed 0 or 0.5% quercetin diet for 2 wk are shown in Fig. 1. Inconspicuous nuclei and decreased nuclear stainability were observed in liver sections of STZ-treated control mice compared to those of untreated control mice or STZ-treated mice fed the 0.5% quercetin diet (Fig. 1). TUNEL staining identified DNA fragmentation in damaged cells. The liver cells of STZ showed strong TUNEL labeling of their nuclei; the intensity of staining was reduced by the 0.1 and 0.5% quercetin diets (Fig. 2). These results suggest that the quercetin diets reduced liver injury in STZ-induced diabetic mice.

Hepatic gene expression of untreated control mice and STZ-injected mice fed 0, 0.1, or 0.5% quercetin diets for 2 wk were analyzed using DNA microarrays (Mouse Genome 430 2.0 array, Affymetrix). STZ significantly upregulated the expression of 276 genes ($p < 0.05$ by one-way ANOVA) and downregulated the expression of 369 genes ($p < 0.05$ by one-way ANOVA). Table 3 shows gene ontology categories that significantly enriched in the genes regulated by STZ. STZ induced the expression of genes concerned with the immune response, stress, cell cycle, and cell death (Table 3). A cluster analysis of the genes that are significantly up- or downregulated is shown in Fig. 3: the red shift signifies upregulation and the blue shift signifies downregulation. Our results showed that the 0.5% quercetin diet suppressed STZ-induced alteration of gene expression. However, the 0.1% quercetin diet had little effect on the STZ-induced changes in gene expression.

To investigate the mechanisms by which quercetin modifies expression of genes affected by STZ, we performed GSEA on microarray data obtained from liver samples of STZ-treated mice fed with either a 0 or 0.5% quercetin diet. The analysis showed that only the gene set of cell cycle regulators was significantly downregulated ($p < 0.05$ and FDR < 0.25) by quercetin. Table 4 lists the genes within the gene set of cell cycle regulator that showed decreased expression after the 0.5% quercetin diet. Downregulation of expression

Table 3. Gene Ontology classification for the hepatic genes that were significantly up- or downregulated by STZ[a)]

Category	Number of genes in category	% of genes in category
STZ upregulated genes		
GO:6955: immune response	49	22.37
GO:6950: response to stress	33	15.07
GO:9605: response to external stimulus	27	12.33
GO:7049: cell cycle	22	10.05
GO:6091: generation of precursor metabolites and energy	20	9.13
GO:6915: apoptosis	19	8.67
GO:6629: lipid metabolism	17	7.76
STZ downregulated genes		
GO:44267: cellular protein metabolism	99	35.23
GO:6412: protein biosynthesis	57	20.28
GO:6996: organelle organization and biogenesis	44	15.66
GO:6091: generation of precursor metabolites and energy	24	8.54
GO:44248: cellular catabolism	16	5.69

$p < 0.05$ by Fisher's exact test.
a) The gene ontology categories were significantly enriched in the list of STZ up- or downregulated genes.

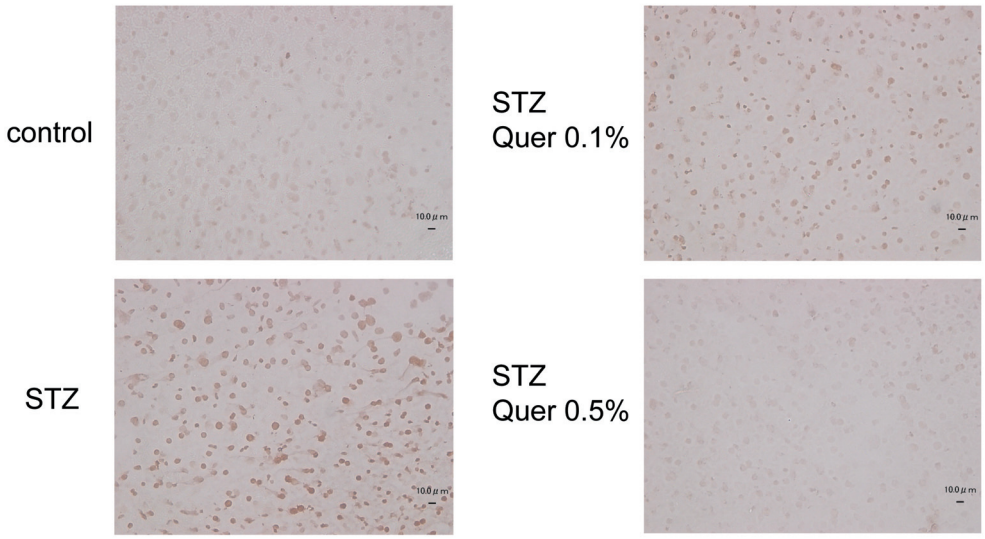

Figure 2. Representative photomicrographs of TUNEL stained liver sections. Biotinylated DNA was detected using a streptavidin–horse radish peroxidase conjugate and diaminobenzine. "Control" indicates untreated control mice. "STZ" indicates STZ-injected mice. "Quer 0.1%" and "Quer 0.5%" indicate mice fed 0.1 or 0.5% quercetin, respectively.

of the genes listed in Table 4 promotes progression of the cell cycle. A quantitative RT-PCR analysis confirmed that STZ strongly induced expression of the cyclin-dependent kinase inhibitor *p21(WAF1/Cip1)* (*Cdkn1a*) and that both 0.1 and 0.5% quercetin diets significantly reduced expression (Fig. 4). We also used quantitative RT-PCR to compare the levels of expression of some genes known to be up- or downregulated in diabetes. STZ increased expression of *Nos2* was reduced by both 0.1 and 0.5% quercetin diets (Fig. 4). The oxidative stress inducible gene *Nqo1* showed increased expression after STZ treatment, which was significantly reduced by 0.5% but not by 0.1% quercetin (Fig. 4). *Gck*, which is known to be regulated by insulin, showed a significantly reduced level of expression in STZ -treated mice (Fig. 4). However, neither the 0.1% nor the 0.5% quercetin diet significantly ameliorated the STZ-induced downregulation of *Gck* (Fig. 4).

3.3 Dietary quercetin suppresses the expression of Cdkn1a in pancreas of STZ-induced diabetic mice

We then determined the effects of quercetin on STZ-induced oxidative stress and expression of cell cycle regula-

Table 4. Reduction in the levels of expression of genes regulating the cell cycle by a quercetin supplemented diet in STZ-treated mice[a)]

GenBank Accession No.	Gene symbol	Gene name	STZ		STZQuer0.1%		STZQuer0.5%	
AK007630	*Cdkn1a*	Cyclin-dependent kinase inhibitor 1A (P21)	12.28	0.72	7.76	0.76	7.09	2.79
BG065754	*Ccng1*	Cyclin G1	3.06	0.17	2.51	0.17	1.96	0.42
NM_007570	*Btg2*	B-cell translocation gene 2, anti-proliferative	2.42	0.12	2.42	0.17	1.68	0.18
U95826	*Ccng2*	Cyclin G2	1.30	0.10	0.93	0.15	0.83	0.38
AW322026	*Btg1*	B-cell translocation gene 1, anti-proliferative	1.17	0.17	1.04	0.22	0.85	0.11
NM_009875	*Cdkn1b*	Cyclin-dependent kinase inhibitor 1B (P27)	1.07	0.08	0.63	0.05	0.86	0.08

a) Data from three groups of STZ-treated mice are shown here: those fed the control diet (STZ), and those fed with either 0.1 or 0.5% quercetin supplemented diets (STZQuer0.1% and STZQuer0.5%, respectively). GSEA showed that the gene set of cell cycle regulators containing the listed genes was significantly down-regulated ($p < 0.05$ and FDR < 0.25) by quercetin. The data show the relative changes in level of expression of the listed genes in these groups of mice compared to the median levels of expression of the genes in untreated BALB/c mice ($n = 6$).

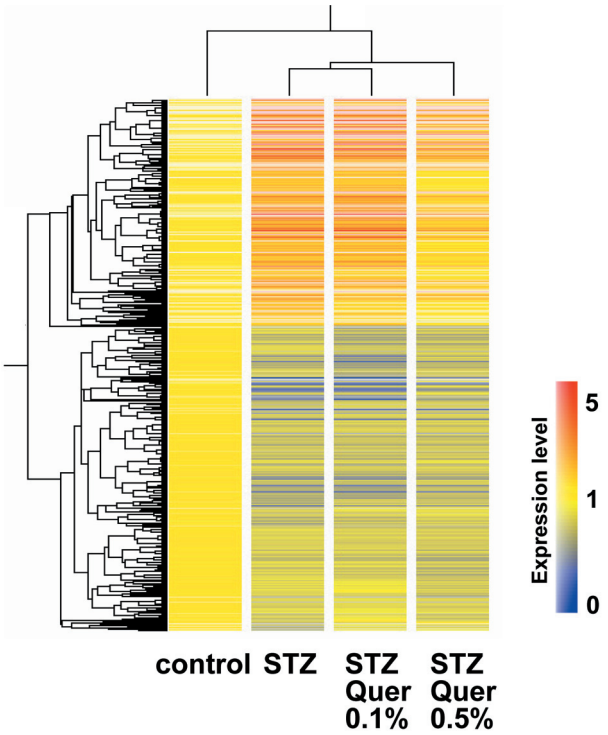

Figure 3. Cluster analysis of the effects of quercetin supplemented diets on the expression of genes that are significantly up- or downregulated by STZ treatment in BALB/c mice. Cluster analysis was performed on 645 genes significantly up- or downregulated by STZ (Welch's one-way ANOVA, $p < 0.05$) using a Pearson correlation and average linkage for similarity measures. Gene expression was normalized to the median of the untreated control samples before the analysis. Data are color coded across the spectrum according to whether the genes show upregulation (red) or downregulation (blue). "STZ" indicates STZ-injected mice. "Quer 0.1%" and "Quer 0.5%" indicate mice fed 0.1 or 0.5% quercetin, respectively.

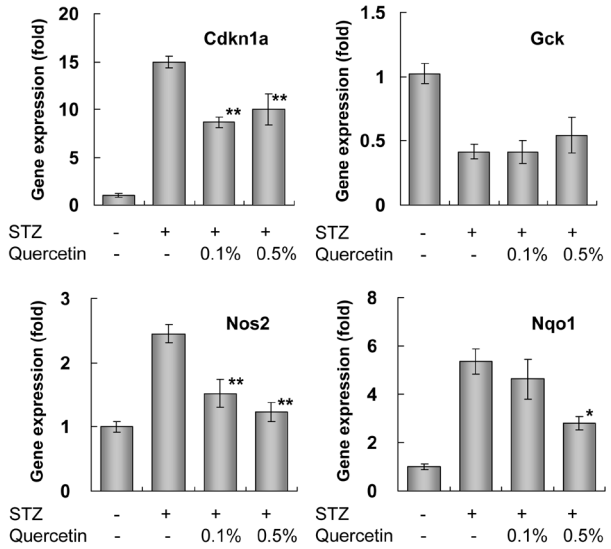

Figure 4. Effects of a quercetin supplemented diet on expression of *Cdkn1a*, *Gck*, *Nos2*, and *Nqo1* in livers of STZ-treated BALB/c mice. Expression levels were determined by quantitative RT-PCR, normalized against *Gapdh*, and plotted relative to those of control mice. Data are expressed as means ± se of six mice in each group. $*p < 0.05$, $**p < 0.01$ (two-sided), significant difference on a multiple *t*-test with Bonferroni correction following ANOVA between the STZ-injected group fed with the control AIN93G diet and that fed a quercetin supplemented diet.

tors in the pancreas. STZ increased the level of TBARS from 14.5 ± 0.53 to 43.36 ± 1.71 nmol/g in the pancreas. The 0.5% quercetin diet significantly reduced the level of TBARS to 28.3 ± 1.47 nmol/g as compared to the control AIN93G diet. Both 0.1 and 0.5% quercetin diets significantly suppressed the expression of *Cdkn1a* but not that of *Cdkn1b* (Fig. 5).

3.4 A high concentration of quercetin in the diet alters expression of Ubc and Gpr146 in mouse livers

Next, we determined the effect of quercetin on healthy control BALB/c mice that were fed the AIN93G diet containing 0, 0.1, 0.5, or 1% quercetin for 2 wk. Hepatic gene expres-

Table 5. Candidates for genes showing differential expression in livers of BALB/c mice fed a quercetin diet[a)]

GenBank Accession No.	Gene symbol	Gene name	Quer0.1%		Quer0.5%		Quer1%	
BI653033	*Ubc*	Ubiquitin C	0.92	0.02	0.61**	0.04	0.47**	0.04
AW551908	*Ubc*	Ubiquitin C	0.94	0.05	0.70**	0.04	0.55**	0.03
BQ177047	*Gpr146*	G protein-coupled receptor 146	1.04	0.09	1.72	0.09	1.92**	0.16
D50527	*Ubc*	Ubiquitin C	1.13	0.02	0.72*	0.07	0.49**	0.04
BC006680	*Ubc*	Ubiquitin C	0.94	0.09	0.74*	0.03	0.59**	0.05
AY033912	*Csad*	Cysteine sulfinic acid decarboxylase	0.93	0.02	1.12	0.11	2.00**	0.26
NM_010062	*Dnase2a*	Deoxyribonuclease II α	1.22	0.11	1.39	0.73	2.32**	0.39
BI410130	*Pnrc1*	Proline-rich nuclear receptor coactivator 1	1.08	0.05	0.92	0.06	0.62**	0.07
M58566	*Zfp36l1*	Zinc finger protein 36, C3H type-like 1	0.94	0.05	1.1	0.12	0.63**	0.06
M58566	*Zfp36l1*	Zinc finger protein 36, C3H type-like 1	0.8	0.05	1.01	0.1	0.60*	0.05
BB540543	*Slc30a10*	Solute carrier family 30, member 10	1.05	0.07	0.88	0.06	0.65*	0.05
BG071905	*Palld*	Palladin, cytoskeletal associated protein	0.86	0.04	1.09	0.07	0.63*	0.04
BB736474	*Slc30a10*	Solute carrier family 30, member 10	1.08	0.08	0.97	0.07	0.59*	0.03
AK014530	*D5Wsu178e*	DNA segment, Chr 5, Wayne State University 178, expressed	1.05	0.05	0.95	0.05	0.69*	0.03
BB079486	*Arid5b*	AT-rich interactive domain 5B (Mrf1 like)	0.81	0.09	2.76**	0.03	1.06	0.16
NM_009895	*Cish*	Cytokine-inducible SH2-containing protein	1.35	0.44	5.34**	0.47	1.95	0.61
BC022110	*Alas1*	Aminolevulinic acid synthase 1	0.58	0.07	4.93*	0.86	1.07	0.24
AV354744	*Klf9*	Kruppel-like factor 9	0.78	0.06	1.57*	0.15	1.24	0.1
AW488885	*2310051E17Rik*	RIKEN cDNA 2310051E17 gene	0.75	0.04	1.60*	0.14	1.25	0.11

a) The differential expression analysis was performed using the linear modeling approach and the empirical Bayes statistics following the method of Smyth *et al.* [16, 17]. The data show the relative changes in level of expression of the listed genes in these groups of mice compared to the median levels of expression of the genes in untreated BALB/c mice ($n = 6$). Quer0.1% represents mice fed with the 0.1% quercetin diet, Quer0.5% those fed the 0.5% diet, and Quer1% those fed the 1% diet. Significantly different from differentiated cells group, $*p < 0.05$; $**p < 0.01$.

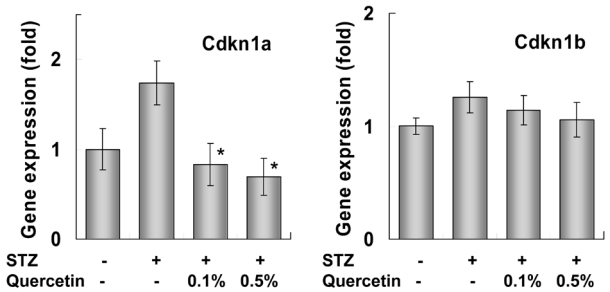

Figure 5. Effects of a quercetin supplemented diet on expression of *Cdkn1a* and *Cdkn1b* in pancreas of STZ-treated BALB/c mice. Expression levels were determined by quantitative RT-PCR, normalized against *Gapdh*, and plotted relative to those of control mice. Data are expressed as means ± se of six mice in each group. $*p < 0.05$ (two-sided), significant difference on a multiple *t*-test with Bonferroni correction following ANOVA between the STZ-injected group fed with the control AIN93G diet and that fed a quercetin supplemented diet.

sion was analyzed by DNA microarrays. A one-way ANOVA of the gene expression data indicated that mice fed with a quercetin diet did not show any significant differences in hepatic gene expression to those fed with the control AIN93G diet. We also performed a differential expression analysis using the linear modeling approach. We found that the expression of *Ubc* was significantly reduced by both the 0.5 and 1% quercetin diets (Table 5). Quantitative RT-PCR analysis confirmed that the 0.5 and 1% quercetin diets significantly reduced expression of *Ubc* (Fig. 6). The diet containing 1% quercetin induced a significant increase in the expression of the G protein-coupled receptor 146 (*Gpr146*) (Table 5, Fig. 6). Although the differences were not significant, there was an indication in some mice that the 0.5 and 1% quercetin diets strongly induced expression of stress-inducible *Mt1* (Fig. 6).

4 Discussion

In this study, we showed that dietary quercetin reduced both blood glucose levels and generation of the oxidative stress marker TBARS in the liver and pancreas, and raised plasma insulin levels in mice with STZ-induced diabetes. STZ-treated mice fed the 0.5% quercetin diet did not show a significant reduction in blood glucose levels in contrast to those on the 0.1% diet (Table 1). We therefore repeated the experiment with the same conditions to check this unexpected result. In the repeat experiment, we found that the blood glucose levels of STZ-treated mice fed diets containing 0, 0.1, and 0.5% quercetin were 413.2 ± 42.2, 333.7 ± 36.25, and 279.8 ± 29.2 mg/dL, respectively. Thus, in this instance, both the 0.1 and 0.5% quercetin diets achieved a significant reduction in blood glucose levels in STZ-induced diabetic mice. Although various studies have shown that intraperitoneally or orally administered querce-

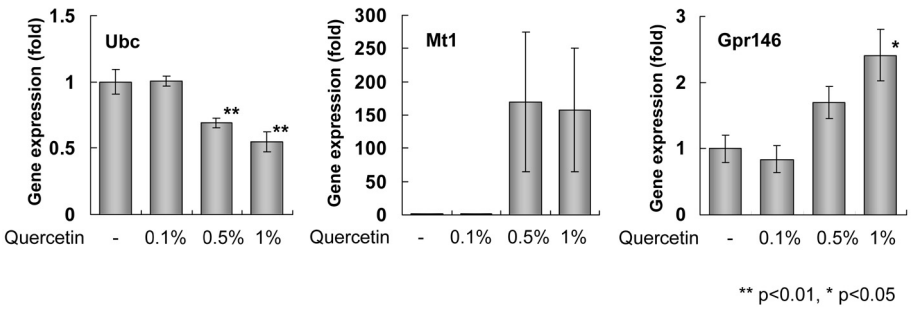

Figure 6. Effects of a quercetin supplemented diet on expression of ubiquitin (*Ubc*), *Grp146* and *Mt1* in livers of normal BALB/c mice. Expression levels were determined by quantitative RT-PCR, normalized against *Gapdh*, and plotted relative to those of control mice. Data are expressed as means ± se of six mice in each group. $*p < 0.05$; $**p < 0.01$ (two-sided), significant difference on a multiple *t*-test with Bonferroni correction following ANOVA between the STZ-injected group fed with the control AIN93G diet and that fed a quercetin supplemented diet.

tin can decrease oxidative stress induced by STZ and can moderate the symptoms of diabetes, there are few reports on the effect of diets containing quercetin [3, 11, 12, 20]. The amount of quercetin aglycon in the diet is much lower than that of quercetin glycoside but its metabolites are similar to those of the glycoside [21, 22]. Since quercetin is not readily absorbed into the body we used diets with high contents of quercetin; we then determined the metabolites in the plasma by HPLC [23].

Here, we provide the first demonstration that dietary quercetin can improve the deleterious changes in hepatic gene expression induced by STZ. Quercetin not only reduced expression of oxidative stress inducible genes but also of almost all genes that display altered patterns of expression in STZ-treated diabetic mice. The flavonoid phloridzin also alleviated diabetic symptoms of STZ-treated mice, but the reduction of STZ-induced disturbance of hepatic gene expression was not observed in mice fed with 0.1 – 1% phloridzin diet (data not shown).

STZ, an alkylating agent, induces death of insulin-producing pancreatic β-cells and causes type 1 diabetes mellitus [24]. STZ has acute toxicity effects on the liver, kidney and other organs. Moreover, the insulin deficiency and hyperglycemia that result from β-cell death further enhance liver injury [2, 25 – 28]. STZ-induced oxidative stress is also the main pathway for the development and exacerbation of induced diabetes. Kume *et al*. [25, 26] showed that STZ causes lipid peroxidation, mitochondrial swelling, peroxisome proliferation, and inhibition of hepatocyte proliferation. Then, insulin deficiency resulting from disruption of insulin producing β-cells, hyperglycemia, and the resultant oxidative stress, which enhance the autoimmune response and oxidative damage of β-cells and other tissues, cause the liver damage associated with diabetes. We assessed the extent of liver damage in STZ-induced diabetic mice using sections stained with hematoxylin-eosin and TUNEL. Analysis of the liver sections suggested that the 0.5% quercetin diet reduced liver injury in STZ-induced diabetic mice. We then detected the STZ induced changes in the expression of genes associated with the immune response, response to stress, cell cycle, and apoptotic cell death. The 0.5% quercetin suppressed the changes in gene expression that cause the liver injury associated with STZ-induced diabetes.

GSEA is a powerful analytical method to identify changes in expression of functional gene groups [15, 16]. Our GSEA of hepatic gene expression showed that the 0.5% quercetin diet had its greatest effect on the expression of the gene set associated with the cell cycle. Suppression of the expression of cell cycle regulators by the 0.5% quercetin diet might promote cell cycle progression, which is suppressed by STZ. Quantitative RT-PCR analysis confirmed that the STZ-induced increase in expression of *Cdkn1a* was significantly reduced by the 0.1 and 0.5% quercetin diets. Both 0.1 and 0.5% quercetin diets also reduced the STZ increased expression of *Nos2*, but expression level of *Nos2* induced by STZ was lower than that of *Cdkn1a*.

Cdkn1a, which regulates cell division by arresting the cell cycle at G1 and S phases, was reported to be upregulated in response to hepatic injury [29, 30]. *Cdkn1a* regulates hepatic cell growth *in vivo*, and *Cdkn1a* knockout mice show a marked acceleration of hepatocyte cell cycle progression [30, 31]. *Cdkn1a* expression is induced by oxidative stress [30, 32]. Since a high concentration of quercetin induced expression of *Cdkn1a in vitro*, quercetin probably suppressed expression of *Cdkn1a* by reducing the oxidative stress [5]. *Cdkn1a* expression is presumably the most sensitive to oxidative stress. Our results suggest that quercetin alleviates liver injury in STZ-induced diabetic mice by recovering the progression of cell proliferation as a consequence of suppressing the *Cdkn1a* expression induced by STZ and by the increased oxidative stress of diabetes. Since quercetin increased plasma insulin levels and decreased blood glucose levels in STZ-treated mice, we determined the effect of quercetin on STZ-induced oxidative stress and *Cdkn1a* expression in the pancreas. Our results suggest that

quercetin increased pancreatic insulin production by promoting cell proliferation through suppression of *Cdkn1a* expression induced by STZ. Although *Cdkn1b* also regulates pancreatic cell proliferation, quercetin did not suppress the expression of *Cdkn1b* in STZ-treated mice [33].

High concentrations of antioxidants possibly act as prooxidants that generate free radicals [7]. In addition to its antioxidative properties, quercetin is known to affect physiologically important proteins *in vitro*, such as protein kinases, topoisomerases, heat shock proteins, and estrogen receptors [8 – 10]. To make good use of dietary quercetin for the prevention of diabetes and other lifestyle related diseases we need to consider the possible effects of excessive intake of the compound under healthy normal conditions. Janssen *et al*. [34] reported that continuous feeding of 220 g onions *per* day to healthy volunteers for 7 days raised mean plasma quercetin concentrations to 1.5 μmol/L. Here, we obtained estimates of plasma concentration of quercetin in mice that had been fed a 0.1% quercetin diet for 2 wk that were approximately seven times higher than those of Janssen *et al*. [34]. A one-way ANOVA indicated that the 0.5 and 1% quercetin diets did not produce a detectable disturbance of hepatic gene expression but did cause a significant reduction in the level of expression of *Ubc* in the livers of normal mice. *Ubc* is a stress-inducible gene that provides an essential source of ubiquitin during cell proliferation and during periods of stress [35, 36]. Since quercetin has been reported to inhibit the binding of heat-shock factor (HSF) to heat-shock element (HSE), it is possible that its effect on *Ubc* expression is mediated by the HSE present in the promoter region of the gene [9, 35].

Thus, we have demonstrated that a 0.5% quercetin diet can ameliorate the disturbance to hepatic gene expression in STZ-induced diabetic mice. Quercetin not only reduced expression of oxidative stress inducible genes but also of almost all genes that display altered patterns of expression in STZ-treated diabetic mice. Additionally, we demonstrated that the quercetin diets had their greatest suppressive effect on STZ-induced elevation of expression of cell cycle regulator genes, especially *Cdkn1a*, which play an important role in the recovery from liver injury. The 0.1% quercetin diet alleviated the symptoms of STZ-induced diabetes but did not induce marked changes in gene expression, with the exception of reducing the STZ-induced elevation of *Cdkn1a* expression. Quercetin probably ameliorates liver injury in STZ-induced diabetic mice by promoting cell proliferation following the suppression of *Cdkn1a* expression. The suppression by quercetin of STZ-induced expression of *Cdkn1a* in the pancreas should elevate the plasma insulin level through increased pancreatic cell proliferation. Moreover, the reduction in oxidative stress as a result of the quercetin diets presumably reduces the oxidative damage to the liver and pancreas associated with diabetes. The changes in gene expression caused by quercetin in the livers of STZ-induced diabetic mice were not correlated with those found in untreated healthy normal mice. GSEA and one-way ANOVA did not detect any significant changes in hepatic gene expression in normal mice as a result of a quercetin diet. Using a linear modeling approach and the empirical Bayes statistics, we also found that *Ubc* were possible marker genes for an excessive intake of quercetin supplements. Our results also give a warning against an excessive intake of quercetin as a dietary supplement.

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