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Metabolic Conversion of Dietary Quercetin from Its Conjugate to Active Aglycone Following the Induction of Hepatocarcinogenesis in Fisher 344 Rats

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Quercetin exhibits a potent anticarcinogenic activity. However, ingested quercetin circulates as the glucuronide/sulfate conjugates, which are less active compared to the aglycone in healthy individuals. This study aimed to develop further understandings of the cancer-preventing mechanism with dietary quercetin. According to a two-stage hepatocarcinogenesis model with *N*-diethylnitrosamine (DEN) and phenobarbital (PB), preneoplasms were induced specifically in the liver of Fisher 344 rats. In the liver, glutathione *S*-transferase placental form (GST-P) positive foci were produced 14 weeks later. β-Glucuronidase activity increased significantly in the liver by 1.2-fold in the DEN/PB group compared to the activity in a saline group. In the kidney, thymus, lung, heart, and plasma, the activities were similar between both groups. When quercetin was dosed intragastrically 15 min before sacrifice, the aglycone level of quercetin in liver was significantly 1.9-fold higher in the DEN/PB group than in the saline group. On the other hand, quercetin was dosed to rats 3 times a week for 14 weeks. The treatment kept the aglycone level of quercetin at a significantly higher level and tended to suppress the formation of GST-P positive foci. The increase in β-glucuronidase activity with carcinogenesis induction became insignificant following the frequent doses of quercetin. It was considered that quercetin aglycone played a preventative role and, thus, the conjugates were converted to the active aglycone by β-glucuronidase that was induced by the generation of preneoplasms.

KEYWORDS: Quercetin; *N*-diethylnitrosamine; phenobarbital; cancer prevention; rats

INTRODUCTION

Flavonoids such as quercetin widely occur in plant foods. They are well recognized to possess antioxidative activity and protein function-modulating activity. Antioxidative activity is an action eliminating reactive oxygen species (*1–3*). Protein function-modulating activity modifies metabolic activity through interaction with enzymes, membranous transporters, cellular or intracellular receptors, or signaling proteins such as Nrf2-Keap1 (*4*), arylhydrocarbon receptor (*5*), estrogen receptor (*6*), cyclooxygenase-2, and inducible NO synthase (*7*). It is believed that these activities contribute to the prevention of degenerative diseases including cancers (*8–10*).

The average daily intake of flavonoids is estimated to be 25 mg in humans (*11*). Dietary flavonoids have been found to be mostly as glucoside forms (*12*). They are incorporated into intestinal cells by sodium-dependent glucose transporter-1 and simultaneously hydrolyzed to the aglycones by β-glucosidase or are absorbed by simple diffusion after hydrolysis to the aglycones by lactase-phlorizine hydrolase on the membrane of intestinal cells (*13*). The aglycones in the intestinal cells are immediately conjugated to glucuronides and/or sulfates by UDP-glucuronosyltransferase (UDPGT) and/or sulfotransferase (*14, 15*). Most conjugates are excreted into the intestinal lumen, and only a small portion is transported into the blood stream and circulates at concentrations of <1.5 μM (*16*) and then is excreted through the urine within 25 h after ingestion (*17*). The conjugations mainly occur on functional groups of flavonoids such as hydroxyls, and their biological activities are changed, approximately lower than the aglycone by conjugation with glucuronic acid and/or sultate (*18–22*). Epidemiological studies, however, showed that the daily intake of flavonoids is inversely associated with the risk of degenerative diseases including several cancers (*23–26*). The less active endogenous forms of dietary flavonoids are contradictory to their beneficial effects. It is, thus, suggested that the flavonoids circulating as conjugates may convert to active aglycones under a generating condition of degenerative diseases.

β-Glucuronidase is one of the deconjugation enzymes that hydrolyzes flavonoid glucuronides to aglycones (*27, 28*) and expresses in various tissues and organs (*29*). It has been reported that β-glucuronidase activity increases in several cancers and

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in inflammation (*30–33*). The activity has been found to be higher in tumor tissues than in surrounding normal tissues (*34*). This information suggests that flavonoids circulating as the conjugates can be deconjugated to aglycones by β-glucuronidase in developing cancer. This allows us to assume that dietary flavonoids convert to active aglycones under a generating condition of cancer and subsequently play preventive roles against the diseases.

In the present study, we adopted liver as the target tissue to verify the hypothesis because the liver is an important tissue for the metabolism of flavonoids along with the small intestine (*35*), and an increase in β-glucuronidase activity had been reported in liver cancer (*36*). We employed quercetin as a dietary flavonoid because it is the most abundant flavonoid in daily food (*12*). Quercetin was dosed to Fisher 344 rats with induced preneoplasms specifically in the livers according to a two-stage hepatocarcinogenesis model with *N*-diethylnitrosoamine (DEN) and phenobarbital (PB). It was determined whether the formation of preneoplasms influenced β-glucuronidase activity and the chemical forms of quercetin. Additionally, the effect of frequent doses of quercetin on the formation of preneoplasms was investigated.

MATERIALS AND METHODS

Chemicals. Quercetin, isorhamnetin, and hesperetin were purchased from Extrasynthèse (Germy, France). DEN and 4-methylumbelliferyl-β-D-glucuronide (MUG) were from Sigma Chemical Co. (St. Louis, MO). PB, propylene glycol (PG), and 4-methylumbelliferone (MU) were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Animals. This study was approved by the Institutional Animal Care and Use Committee (permission no. 17-03-02) and carried out according to the Kobe University Animal Experimentation Regulations. Male Fisher 344 rats (5 weeks old, 50–70 g in body weight) were supplied from Clea Japan (Tokyo, Japan). After acclimatization for 1 week, they were housed in an animal facility, which was maintained on a 12 h light/dark cycle at a constant temperature of 23 ± 1 °C. The animals were allowed free access to diet (Oriental MF diet, Oriental Yeast, Tokyo, Japan) and drinking water, and their consumptions were monitored.

Experimental Design. Preneoplasms were induced using a two-stage hepatocarcinogenesis model induced with DEN and PB for 14 weeks (*37, 38*). In experiment 1, 50 rats were divided into DEN/PB and saline groups. After 1 week on basal diet and water, the DEN/PB group was injected intraperitoneally with 100 mg/kg of body weight of DEN in saline once a week for 3 weeks. One week later, they were given drinking water containing 500 ppm of PB for 10 weeks, whereas the saline group received drinking water after an intraperitoneal injection with saline instead of DEN. At the end of the experiment, 15 min before sacrifice, both groups were administered quercetin intragastrically at 20 mg/kg of body weight dissolved in 200 μL of PG.

In experiment 2, 20 rats were divided into 4 groups; DEN/PB + PG, DEN/PB + Q, saline + PG, and saline + Q. The DEN/PB + PG and DEN/PB + Q groups underwent the induction of hepatocarcinogenesis with DEN and PB, whereas the saline + PG and saline + Q groups were treated with saline in the same manner as in experiment 1. In the DEN/PB + Q and saline + Q groups, the rats were dosed intragastrically with 20 mg/kg of body weight of quercetin in 200 μL of PG 3 times a week for 14 weeks, whereas the rats of the DEN/PB + PG and saline + PG groups were dosed with PG instead of quercetin. Fifteen minutes following the final administration of quercetin or PG, all rats were killed.

At death, the rats were anesthetized with 5% pentobarbital. Blood was collected by a cardiac puncture in heparinized tubes, and plasma was obtained by centrifugation at 2000*g* for 15 min. Livers were perfused with ice-cold 0.1 M phosphate-buffered saline (PBS, pH 7.5) and then divided into two portions. One portion was immediately frozen in liquid nitrogen, and the other portion was fixed in PBS containing 4% paraformaldehyde at 4 °C and then embedded in an optimal cutting temperature compound (Sakura Finetech, Tokyo, Japan) to be used for immunohistochemical staining. Kidney, lung, heart, and thymus were frozen immediately in liquid nitrogen. All samples were stored at –80 °C until use.

Immunohistochemical Staining of Glutathione *S*-Transferase Placental Form (GST-P). The fixed liver was cut into 40 μm sections, and eight sections each per rat were treated with a primary rabbit anti-rat GST-P antibody (Medical and Biological Laboratories, Tokyo, Japan) diluted to 1:50, peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulins in Tris-HCl buffer (EnVision Plus, Dako, Kyoto, Japan) and 3,3′-diaminobenzidine (DAB) in Tris-HCl containing 0.01% hydrogen peroxide and 0.08% ammonium nickel sulfate as the substrate. The numbers and areas of GST-P positive foci >0.2 mm in diameter were measured using Scion Image beta version 4.03 developed at the National Institutes of Health.

Determination of β-Glucuronidase Activity. The activity of β-glucuronidase was determined using a modified version of a described previously (*39*). Tissue samples were homogenized in 20 mM Tris-HCl, pH 7.4, and a 10 μL aliquot of the homogenates or plasma was incubated for 10 min at 37 °C with 50 μL of assay buffer [0.5 mM substrate, MUG, 0.2 M sodium acetate, pH 5.0, 10 mM EDTA, 0.01% (w/v) bovine serum albumin, and 0.1% (v/v) Triton X-100]. The enzymatic reaction was stopped by adding 150 μL of 0.2 M sodium carbonate. The mixture was centrifuged at 10000*g* for 5 min at 4 °C after the addition of 2.5 nmol of 9-chloromethylanthracene (Tokyo Kasei Kogyo, Tokyo, Japan) as the internal standard. The concentration of hydrolyzed product of MUG, MU, in the supernatant was determined by HPLC analysis with fluorescence detection (excitation at 355 nm and emission at 460 nm). Calibration curves with MU were linear over a concentration range of 1−20 pmol. Protein contents were determined according to the Lowry method (*40*).

Measurement of UDPGT Activity in Liver. Microsomal fractions in the liver were prepared according to the method described previously (*41*), and the protein content was determined according to the Bradford method (*42*). UDPGT activity was measured according to the method of Collier et al. (*43*) with slight modification. Briefly, 60 μg of microsomal protein was mixed with 550 μL of 50 μM MU in 0.1 M Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$ and 0.05% BSA. The cofactor UDP-glucuronic acid (2 mM final concentration) was added to initiate the enzymatic reaction and incubated for 37 °C. Consumption of substrate MU was determined by fluorescence (excitation at 355 nm and emission at 460 nm) at 2 min intervals for 10 min. UDPGT activity was calculated with a standard curve constructed with 0–200 μM MU.

Extraction of Quercetin and Its Metabolites from Liver and Plasma. The liver was homogenized in 0.1 M sodium acetate, pH 5.0, containing 6 g/L ascorbic acid and divided into two parts of 300 μL each. Conjugates with glucuronic acid and/or sulfate of quercetin and isorhamnetin were determined, distinguishing them from their aglycone forms as follows: the homogenates were incubated in the presence or absence of 15 units of sulfatase type H-5 (Sigma Chemical) for 30 min at 37 °C and centrifuged at 10000*g* for 10 min after the addition of 1.2 mL of methanol and 1 pmol of hesperetin as the internal standard. The supernatant (1.2 mL) was mixed with 1 mL of 0.1 M sodium phosphate buffer, pH 3.0, and applied to an Oasis HLB cartridge (Waters, Milford, MA) conditioned with methanol and 0.5 M EDTA. Quercetin, isothamnetin, and hesperetin were recovered from the cartridge with 1 mL of methanol after washing with 60% aqueous methanol and then submitted to HPLC analysis.

Plasma samples (50 μL) were acidified with 50 μL of 0.1 M sodium acetate, pH 5.0, containing 20 mM ascorbic acid, incubated similarly to the liver samples, and then extracted with 900 μL of ethyl acetate three times after the addition of hesperetin as the internal standard. The ethyl acetate extracts were dried under a nitrogen stream, dissolved in 200 μL of HPLC mobile phase, and then submitted to HPLC analysis.

HPLC. The HPLC conditions were as follows: column, Capcell pak C18 MG (3.0 × 250 mm, 5 μm, Shiseido, Tokyo, Japan); column temperature, 35 °C; mobile phase, 0.1 M sodium phosphate buffer, pH

Table 1. Changes in Weights of Body and Liver and Formation of GST-P Positive Foci with DEN/PB Treatment in Experiment 1[a]

		liver wt		GST-P positive foci	
group	body wt (g)	absolute (g)	relative (%)	number (no./cm^2)	area (mm^2/cm^2)
saline	319 ± 16	9.6 ± 0.9	3.0 ± 0.2	9 ± 12	0.01 ± 0.01
DEN/PB	304 ± 24	12.0 ± 1.2[b]	4.0 ± 0.4[b]	448 ± 311[b]	0.99 ± 1.16[b]

[a] Values are mean ± SD, $n = 25$. [b] Significantly different from the saline group, $P < 0.05$.

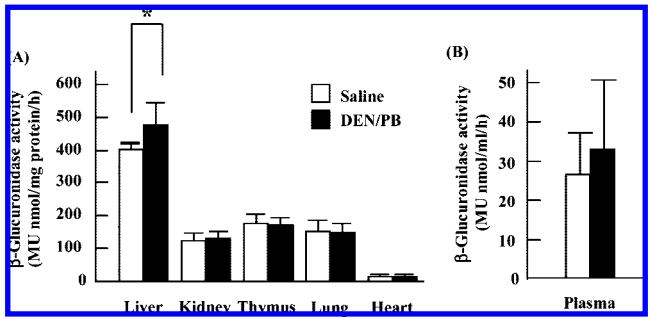

Figure 1. Changes in β-glucuronidase activity with induced preneoplasms in experiment 1: β-glucuronidase activity in liver, kidney, thymus, lung, and heart; (**A**); β-glucuronidase activity in plasma (**B**). The homogenate or plasma was incubated with substrate MUG for 10 min at 37 °C, and the enzymatic reaction was stopped with sodium carbonate. The MU produced from MUG was analyzed by HPLC with detection of fluorescence with excitation at 355 nm and emission at 460 nm. The values are mean ± SD ($n = 25$; $*$, $P < 0.05$).

3.0; methanol/acetonitrile = 55:35:10 (v/v/v); flow rate, 0.45 mL/min; detector, coulometric electrochemical detector (Coulochem III with a model 5010 analytical cell; ESA, Chelmsford, MA) with an analysis voltage of +600 mV. The detection limits for quercetin were 1 nmol/L.

Statistical Analysis. Student's *t* test and two-way ANOVA were employed for statistical analysis of experiments 1 and 2, respectively. The data are expressed as the mean ± standard deviation (SD). A value of $P < 0.05$ was judged as being statistically significant.

RESULTS

Changes in β-Glucuronidase Activity with Formation of GST-P Positive Foci. In the animal experiment, there were no significant differences among all groups in experiments 1 and 2 in food and water consumption (data not shown). **Table 1** shows the body weights and liver weights in experiment 1. The treatment with DEN and PB significantly increased the liver weights without changes in the body weight compared to the treatment with saline.

The expression of GST-P positive foci, liver-specific markers for preneoplasms (*44*), increased to 448 foci/cm^2 and 0.99 mm^2/cm^2 in the DEN/PB group, although they were rarely observed in the saline group [9 foci/cm^2 and 0.01 mm^2/cm^2 (**Table 1**)]. **Figure 1** shows the β-glucuronidase activity in liver, kidney, thymus, lung, and heart (**A**) and plasma (**B**). In the liver, the carcinogenesis-target tissue, the enzyme activity in the DEN/PB group was 475 ± 63 MU nmol/mg of protein/h, which was significantly, 1.2-fold, higher compared to 410 ± 20 MU nmol/mg of protein/h of the saline group. In kidney, thymus, lung, heart, and plasma, the activities were similar between both groups. The results suggest that the β-glucuronidase activity increases specifically in carcinogenic tissue.

Conversion of the Form of Quercetin from Conjugates to Aglycone. The rats were dosed intragastrically with 20 mg/kg of body weight of quercetin 15 min before sacrifice. **Table 2** shows the levels of quercetin and its metabolites in liver and plasma. The aglycone level of quercetin in liver was 21.2 pmol/g of liver in the DEN/PB group, whereas the level in the saline group was 11.0 pmol/g of liver. In plasma, the aglycone level tended to increase but not significantly. The levels of conjugates with glucuronic acid/sulfate of quercetin showed a tendency to decrease in liver and plasma in the DEN/PB group compared to the saline group. Isorhamnetin (quercetin-3′-*O*-methyl) is one of the metabolites of quercetin and is produced by methylation with a catechol-*O*-methyl transferase (COMT) expressed mainly in the liver (*45*). Isorhamnetin is shown as one of the aglycones in **Table 2** because it possesses a biological activity similar to that of quercetin aglycone (*21, 22*). The liver content of isorhamnetin was 30.5 pmol/g of liver in the DEN/PB group and was significantly, 1.4-fold, higher than 22.5 pmol/g of liver in the saline group. The contents of isorhamnetin conjugates with glucuronic acid/sulfate remained unchanged. These increases in aglycone levels of quercetin coincided with the increasing activity of hepatic β-glucuronidase in **Figure 1**.

Table 2. Levels of Quercetin and Its Metabolites in Liver and Plasma in Experiment 1[a]

	aglycones		conjugates with glucuronic acid/sulfate	
group	quercetin	isorhamnetin	quercetin	isorhamnetin
	Picomoles per Gram of Liver			
saline	11.0 ± 5.2	22.5 ± 7.3	307 ± 88	349 ± 87
DEN/PB	21.2 ± 7.9[b]	30.5 ± 10.7[c]	248 ± 11	358 ± 215
	Picomoles per Milliliter of Plasma			
saline	12.4 ± 4.7	ND[c]	1660 ± 1170	688 ± 383
DEN/PB	15.5 ± 6.8	ND	1370 ± 640	593 ± 270

[a] Values are mean ± SD, $n = 25$. [b] Significantly different from corresponding saline group, $P < 0.05$. [c] ND, not detected in this study.

Table 3. Change in UGT Activity in the Liver Following with Induction of Preneoplasms in Experiment 1[a]

group	UGT activity (MU nmol/mg of protein/h)
saline	145 ± 55
DEN/PB	549 ± 177[b]

[a] Values are mean ± SD, $n = 25$. [b] Significantly different from the Saline group, $P < 0.05$.

Changes in Hepatic UDPGT Activity with Induction of Preneoplasms. The hepatic UDPGT activity is shown in **Table 3**. The activity in the DEN/PB group was 549 nmol/mg of protein/h and significantly, 3.8-fold, higher than 145 nmol/mg of protein/h in the saline group. The UDPGT activity, thus, increased with the induction of preneoplasms, but no decrease was observed. The results suggest that aglycone forms of quercetin and isorhamnetin are converted from the conjugates with glucuronic acid/sulfate to aglycones by β-glucuronidase in the liver following the induction of carcinogenesis.

Effects of Frequent Doses of Quercetin on β-Glucuronidase Activity. An interesting point remains: when quercetin is ingested frequently, does β-glucuronidase activity increase and can dietary quercetin prevent carcinogenesis?

The rats were dosed intragastrically with quercetin or PG, 3 times a week for 14 weeks. There were no significant differences between any of the groups in terms of body weight (**Table 4**). Liver weights and liver weights relative to the body weight were significantly increased in the DEN/PB + PG group to 14.3 g and 5.3% compared to 10.3 g and 3.6% in the saline + PG group, respectively, similar to the results of **Table 1**. The

Table 4. Body and Liver Weights and Formation of GST-P Positive Foci in Rats of Experiment 2[a]

group	body wt (g)	liver wt absolute (g)	liver wt relative (%)	GST-P positive foci number (no./cm^2)	GST-P positive foci area (mm^2/cm^2)
saline + PG	286 ± 6	10.3 ± 0.8	3.6 ± 0.3	22 ± 18	0.01 ± 0.01
saline + Q	265 ± 20	9.2 ± 0.9	3.5 ± 0.1	24 ± 12	0.02 ± 0.01
DEN/PB + PG	272 ± 14	14.3 ± 0.7[b]	5.3 ± 0.3[b]	434 ± 189[b]	1.10 ± 0.38[b]
DEN/PB + Q	255 ± 12	12.4 ± 1.0[c,d]	4.9 ± 0.2[c,d]	331 ± 43[c]	0.85 ± 0.25[c]

[a] Values are mean ± SD, $n = 5$. [b] Significantly different from the saline + PG group, $P < 0.05$. [c] Significantly different from the saline + Q group, $P < 0.05$. [d] Significantly different from the DEN/PB + PG group, $P < 0.05$.

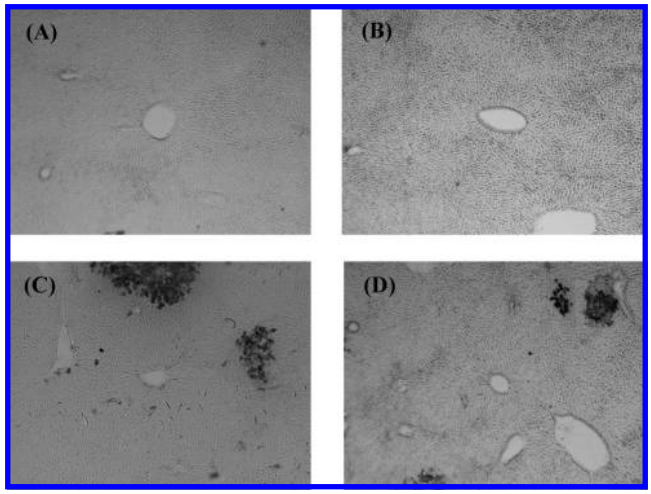

Figure 2. Immunohistochemical staining of GST-P in experiment 2: Fisher 344 rats were treated according to the experimental schedule. The GST-P positive foci in the livers of rats were stained with DAB as described under Materials and Methods. The black dots are DAB-stained GST-P positive foci, and representative images are shown: (**A**) saline + PG; (**B**) saline + Q; (**C**) DEN/PB + PG; (**D**) DEN/PB + Q. Magnification ×210.

frequent doses of quercetin to the rats treated with DEN and PB significantly reduced the liver weights, 12.4 g and 4.9%, compared to the weights in PG-dosed rats. **Table 4** also shows the formation of GST-P positive foci. The positive foci were observed rarely in the saline + Q and saline + PG groups (**Figure 2A**,**B**), but were clearly observed at rates of 434 foci/cm^2 and 1.10 mm^2/cm^2 in the DEN/PB + PG group (**Figure 2C**). The frequent doses of quercetin apparently suppress the formation to 331 foci/cm^2 and 0.85 mm^2/cm^2 (**Figure 2D**). These were 76 and 77% of those in the DEN/PB + PG group, but were not significantly different between the groups. These results indicate that frequent doses of quercetin suppressed the increase in liver weight and potentially inhibited the formation of GST-P positive foci induced by DEN and PB.

The β-glucuronidase activity and formation of aglycones were compared similarly in experiment 1. Hepatic β-glucuronidase activity increased in the DEN/PB + PG group significantly at $P < 0.05$ compared to the activity in the saline + PG group (**Figure 1A**). The frequent doses of the quercetin group (DEN/PB + Q) also led to increased β-glucuronidase activity, but it was not significantly different from that of the saline + Q group at $P < 0.08$ (**Figure 3A**). Increases in β-glucuronidase activity were not observed in kidney and plasma (**Figure 3**). **Table 5** shows the contents of quercetin and its metabolites in liver and plasma. The frequent doses gave higher concentrations of quercetin in the DEN/PB + Q and saline + Q groups compared to the single dose in **Table 2**. In the DEN/PB + Q group, the

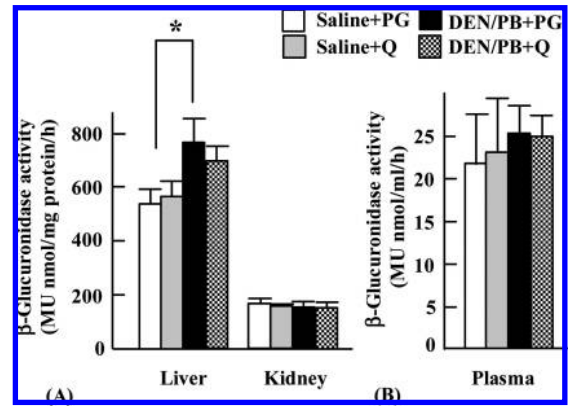

Figure 3. β-Glucuronidase activity following frequent doses of quercetin in experiment 2: homogenate or plasma was incubated with substrate MUG for 10 min at 37 °C, and the enzymatic reaction was stopped with sodium carbonate. The MU produced from MUG was analyzed by HPLC with detection of fluorescence with excitation at 355 nm and emission at 460 nm. Values are mean ± SD ($n = 5$; *, $P < 0.05$).

Table 5. Levels of Quercetin and Its Metabolites in Liver and Plasma in Experiment 2[a]

group	aglycones quercetin	aglycones isorhamnetin	conjugates with glucuronic acid/sulfate quercetin	conjugates with glucuronic acid/sulfate isorhamnetin
Nanomoles per Gram of Liver				
saline + Q	2.62 ± 1.06	6.03 ± 2.89	12.2 ± 5.2	12.0 ± 5.0
DEN/PB + Q	5.77 ± 1.95[b]	6.49 ± 2.58	7.7 ± 5.5	4.8 ± 3.2
Nanomoles per Milliliter of Plasma				
saline + Q	2.42 ± 1.75	ND[c]	49.9 ± 9.8	11.6 ± 1.5
DEN/PB + Q	2.46 ± 0.91	ND	33.7 ± 17.3	9.3 ± 1.8

[a] Values are mean ± SD, $n = 5$. [b] Significantly different from the saline + Q group, $P < 0.05$. [c] ND, not detected in this study.

liver content of quercetin aglycone was 5.77 nmol/g of liver and was significantly, 2.2-fold, higher than the 2.62 nmol/g of liver in the saline + Q group. Thus, the frequent doses of quercetin tended to suppress the formation of GST-P positive foci and kept the aglycone level of quercetin higher in carcinogenesis-induced rats.

DISCUSSION

Quercetin ubiquitously occurs in daily food (*12*) and is well recognized to exhibit anticarcinogenic activities such as induction of cell cycle arrest and apoptosis in tumor cells (*46, 47*) and inhibition of DNA adduct formation (*48*). Dietary quercetin, however, has been detected mostly as conjugates in the blood of healthy humans (*16, 17*). The conjugates with glucuronic acid/sulfate are less active compared to the aglycones, because the conjugates are difficult to incorporate into the body cells and are easily excreted through the urine (*21*). To express conspicuous activity, the conjugates should undergo a deconjugation to the active aglycones. To improve the understanding of the preventative role of dietary quercetin it is important to know which conditions facilitate the conversion of the conjugates to the aglycones. In the present study, we demonstrated that β-glucuronidase activity and the levels of quercetin aglycone increased in carcinogenic tissue and that frequent doses of quercetin suppressed the formation of preneoplasms.

A two-stage hepatocarcinogenesis model with DEN and PB employed in the present study has been considered to generate preneoplasms specifically in the liver (*36*), and markers for

preneoplasms, GST-P positive foci, were detected in the liver (**Table 1**). β-Glucuronidase activity increased specifically in the carcinogenesis-target tissue (**Figure 1**). Simultaneously, levels of quercetin aglycone and its methoxyl metabolite, isorhamnetin, significantly increased in the liver by induction of preneoplasms (**Table 2**). However, if the activity of UDPGT, a glucuronidation enzyme, decreases with induction of preneoplasms, aglycone levels of quercetin and isorhamnetin also increase in liver and plasma. In the present study, UDPGT activity increased significantly in the liver by induction of preneoplasms (**Table 3**). These results suggest that an increase in aglycone levels of quercetin is associated with conversion from the conjugates with glucuronic acid to aglycones by an increase in β-glucuronidase activity in the liver following cancer generation and the differentiation degree of tumor cells.

On the increasing mechanisms of β-glucuronidase activity, Mürdter et al. reported that β-glucuronidase activity was 10 times higher at acidic pH than at neutral pH and that the extracellular pH in tumorigenic tissue was pH 6.5 and was lower than the pH 7.4 in healthy tissue (*49*). Bosslet et al. demonstrated that β-glucuronidase released from lysosome in necrotic cells of tumor tissue and exhibited the enzymic activity but did not in normal tissue (*50*). In the present study, the increase in β-glucuronidase activity is recognized to have correlation with morphological alterations (*36*). The necrotic cells in preneoplasms probably leaked β-glucuronidase, and the acidic pH in the cells facilitated increased enzyme activity. On the other hand, the increase in the β-glucuronidase activity was 1.2-fold higher than the activity in saline group (**Figure 1**) and was lower compared to the increase in the level of quercetin aglycone (1.9-fold) shown in **Table 2**. The β-glucuronidase activity was determined using the liver that included both necrotic cells and normal cells. The β-glucuronidase activity in the normal cells probably diluted the total activity and then showed the lower increasing rate of enzyme activity.

The increase in β-glucuronidase activity is a key factor in cancer prevention by dietary flavonoids such as quercetin. The effects of frequent doses of quercetin on β-glucuronidase activity and formation of GST-P positive foci were examined. The frequent doses suppressed the formation of GST-P positive foci, but these were not significant (**Table 4**; **Figure 2**). The increase in β-glucuronidase activity was also not significant (**Figure 3**), whereas the amounts of quercetin aglycone increased significantly in the liver (**Table 5**). Kohno et al. (*31*) demonstrated that oral doses of silymarin suppressed the formation of precarcinoma by observing no increase in β-glucuronidase activity in azoxymethane-induced colon carcinogenesis in rats. Manju et al. (*51*) also reported that β-glucuronidase activity increased in colon cancer induced by dimethylhydrazine in rats, but the increase in enzyme activity was not associated with the prevention of precarcinoma by oral doses of luteolin. These findings suggest that the aglycones produced by β-glucuronidase in preneoplasms may suppress the further formation of neoplasm and result in holding down the β-glucuronidase activity.

In the present study, 20 mg/kg was used as the dosing amount of quercetin, and the amount is much higher than the normal dietary intake. Tapiero et al. demonstrated that when high-dose flavonoids were administered, metabolism occurred primarily in the liver, whereas when smaller doses were administered, metabolism took place first at the intestinal mucosa, the liver playing a secondary role to further modify the conjugated flavonoids (*35*). In saline groups of the single dose (experiment 1) shown in **Table 2**, a ratio of glucuronide/sulfate conjugation, conjugates/sum of quercetin and isorhamnetin, was 2.8, and a methylation ratio, isorhamnetin/quercetin, was 2.3. In the frequent dose in **Table 5**, the ratio of conjugate formations increased dramatically to 19.6 and the ratio of isorhamnetin/quercetin remained unchanged. These results suggest that 20 mg/kg was the tolerant amount that was able to detoxify with the conjugation enzymes and showed no excess amount for rats. Additionally, Piskula and Terao had used about 50 mg/kg for the oral doses to rats, and they indicated that the dose amount of flavonoids was metabolized sufficiently in the intestinal cells (*52*). We believe that the present amount 20 mg/kg is not excessive for the oral dose experiment and can give results useful in the evaluation of a role of dietary quercetin on carcinogenesis.

In conclusion, flavonoids such as quercetin are immediately conjugated during intestinal absorption and circulate in the blood stream in healthy individuals. The deconjugating enzyme, β-glucuronidase, increases in abnormal conditions such as generation of cancer, and the conjugates are converted to active aglycones. The produced aglycones play a preventative role in the formation of preneoplasms, and then the β-glucuronidase activity decreases with the recovery from preneoplasms. Thus, flavonoids possess no effects and no side effects in healthy individuals, and they are transformed to active forms as the need arises. We think this is the general mechanism of the beneficial effect on health of flavonoids.

ABBREVIATIONS USED

DEN, *N*-diethylnitrosamine; PB, phenobarbital; PG, propylene glycol; GST-P, glutathione *S*-transferase placental form; MUG, 4-methylumbelliferyl-β-D-glucuronide; MU, 4-methylumbelliferone; DAB, 3,3′-diaminobenzidine; COMT, catechol-*O*-methyl transferase; UDPGT, UDP-glucuronosyl transferase.

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