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Prenylation Enhances Quercetin Uptake and Reduces Efflux in Caco-2 Cells and Enhances Tissue Accumulation in Mice Fed Long-Term[1–3]

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Abstract

Prenyl flavonoids are widely distributed in plant foods and have attracted appreciable attention in relation to their potential benefits for human health. Prenylation may enhance the biological functions of flavonoids by introducing hydrophobic properties in their basic structures. Previously, we found that 8-prenyl naringenin exerted a greater preventive effect on muscle atrophy than nonprenylated naringenin in a mouse model. Here, we aimed to estimate the effect of prenylation on the bioavailability of dietary quercetin (Q). The cellular uptake of 8-prenyl quercetin (PQ) and Q in Caco-2 cells and C2C12 myotube cells was examined. Prenylation significantly enhanced the cellular uptake by increasing the lipophilicity in both cell types. In Caco-2 cells, efflux of PQ to the basolateral side was <15% of that of Q, suggesting that prenylation attenuates transport from the intestine to the circulation. After intragastric administration of PQ or Q to mice or rats, the area under the concentration-time curve for PQ in plasma and lymph was 52.5% and 37.5% lower than that of Q, respectively. PQ and its *O*-methylated form (MePQ) accumulated at much higher amounts than Q and *O*-methylated Q in the liver (Q: 3400%; MePQ: 7570%) and kidney (Q: 385%; MePQ: 736%) of mice after 18 d of feeding. These data suggest that prenylation enhances the accumulation of Q in tissues during long-term feeding, even though prenylation per se lowers its intestinal absorption from the diet. J. Nutr. 143: 1558–1564, 2013.

Introduction

Prenyl flavonoids possess a C5 isoprenoid unit in a diphenylpropane structure. Prenyl flavonoids are present in Leguminosae, Moraceae, and Asteraceae (1) and are mainly distributed in the roots, leaves, and seeds (1,2). Recent studies have suggested that prenyl flavonoids exert biological functions. For example, the prenyl flavonoid icaritin has been shown to prevent growth of carcinoma cells by inducing cell-cycle arrest (3). Prenyl flavanones extracted from the roots of *Sophora flavescens* were found to possess antibacterial and antiandrogen activities (4). Prenyl flavones from *Psoralea corylifolia* have been demonstrated to suppress the production of NO in nerve cells (5). Prenylation has been shown to enhance the estrogenic activity and tyrosinase activity of naringenin (6) and luteolin (7), respectively. These observations imply unique properties of prenyl flavonoids in in vitro cell culture systems.

Our previous study using 8-prenyl naringenin (PN)[8] (8) suggested that the biological potential of prenyl flavonoids could be due to their greater absorption by the body and efficient accumulation in target tissues. However, it remains unknown whether prenylation enhances the bioavailability of flavonoids for intestinal absorption and tissue accumulation in vivo.

Prenylation at the 8-position in flavonoids is found in plants and an enzyme that introduces a prenyl group to flavonoids at the 8-position was recently identified (9). In fact, in the plant kingdom, the 8-position is prenylated in naringenin (10), kaempferol (11), and isoflavones (12). PN is present in hops and beer (10). Recently, 8-prenyl quercetin (PQ) was found in *Desmodium caudatum* (13), which is used as an ingredient in traditional foods in Japan. Therefore, we selected PQ to clarify the effect of prenylation on the bioavailability of quercetin (Q)

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[3] Supplemental Figure 1 and Table 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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[8] Abbreviations used: ABC transporter, ATP-binding cassette transporter; C_{max}, maximum concentration; I, isorhamnetin; MePQ, *O*-methylated prenyl quercetin; MeQ, *O*-methylated quercetin; PN, 8-prenyl naringenin; $P_{o/w}$, partition coefficient in octanol/water phases; PQ, 8-prenyl quercetin; Q, quercetin.

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(**Supplemental Fig. 1**). Q is a common flavonoid relevant to studies of intestinal absorption and metabolic conversion. The mechanism of intestinal transport for dietary Q present as Q glycosides in plant foods has been well documented in intestinal epithelial cell lines and animal models (14). It is also known that glucuronide-/sulfate-conjugated metabolites with or without *O*-methylation are exclusively detected in human plasma after intake of Q-rich foods (15,16). Q metabolites are also present in the lymph in rats (17). Therefore, it is likely that blood and lymph can deliver Q metabolites to target tissues (18,19). Previous reports have suggested that dietary icaritin and PN are transported in human plasma (20,21) as conjugated metabolites in a similar way to nonprenyl flavonoids (22,23). Nevertheless, studies focusing on the mechanism of the intestinal absorption and tissue accumulation of prenyl flavonoids are lacking.

We examined how prenylation affects the intestinal absorption and tissue accumulation of Q using monolayer cultures of Caco-2 cells and C2C12 myotube cells. We also measured the PQ concentrations in plasma and lymph of mice and rats. In addition, the amounts of PQ, Q, and their metabolites in various tissues were determined after mice were fed a diet containing Q or PQ for 18 d. The combination of these in vitro and in vivo studies may provide clues to clarify the effects of prenylation on the modulation of the bioavailability of dietary Q.

Materials and Methods

Materials. Q (3,3′,4′,5,7-pentahydroxyflavone) was purchased from Nacalai. Kaempferol, isorhamnetin (I), and 3,3′,4′,5,7-pentamethoxyflavone were obtained from Extrasynthase. PQ, 8-prenyl I, and PN were synthesized by our research team (24). Other reagents were of the highest grade available from commercial sources.

Measurement of the partition coefficient in the octanol/water phase. The method for measurement of the partition coefficient in the octanol/water phase was carried out as previously described (25) with slight modifications. A methanol solution (100 μL) of 300 mmol/L PQ or 1 mmol/L Q was placed in a test tube and the solvent was removed with a nitrogen stream. The residue was dispersed in 100 μL of 50 mmol/L Tris-HCl (pH 7.4) and 100 μL of 1-octanol. The solution was vortex-mixed for 1 min, followed by centrifugation (16,000 × *g*, 4°C, 5 min). The concentrations of each flavonoid in the water and octanol phases were measured by HPLC as described below. The log partition coefficient in octanol/water phases ($P_{o/w}$) value was calculated using the following equation:

$$\log P_{o/w} = \log_{10}[\text{flavonoid in octanol layer (mM)}/\text{flavonoid in Tris-HCl layer (mM)}].$$

Cellular uptake and permeability of PQ and Q in Caco-2 cells. The cellular uptake and permeability in Caco-2 cells were ascertained as previously described (26). Briefly, Caco-2 cells at passages 42–56 were seeded at 1×10^5 cells/cm^2 and cultured for 20–22 d until the experiments were performed. The inserts were washed with HBSS (pH 7.4) for 30 min in a CO_2 incubator. Subsequently, 50 mmol/L PQ or Q in DMSO solution was diluted with HBSS and the final concentration was adjusted to 50 μmol/L. The test solutions were added to the apical side of Caco-2 monolayers and incubated for 0.25, 1, 2, 4, or 6 h at 37°C. After the incubation, the apical and basolateral solutions were collected. Cellular extracts were prepared by incubating the whole inserts with methanol containing 0.5% acetic acid for 30 min. The apical and basolateral solutions and the cellular extracts were evaporated using a Centrifugal Vaporizer (CVE-100; Tokyo Rikakikai). The residues were mixed with 0.1 mL of 100 mmol/L acetate buffer (pH 5.0), 0.22 mL of 50 mmol/L ascorbic acid solution, and 100 U of β-glucuronidase type H-1 solution in 100 mmol/L acetate buffer (0.1 mL, pH 5.0) and incubated at 37°C for 30 min. After centrifugation (9000 × *g* for 10 min at 4°C), aglycones were extracted 3 times in ethyl acetate by sonication for 1 min using an XL2020 Ultrasonic Processor (Astrason) at level 10. Before the extraction, 14 pmol of 3,3′,4′,5,7-pentamethoxyflavone (for PQ) or kaempferol (for Q) was added to the suspensions as an internal standard. After centrifugation (9000 × *g* for 10 min at 4°C), the supernatants were collected, evaporated under nitrogen gas, and dissolved in 70 μL of methanol containing 0.5% phosphoric acid. Flavonoids were determined by HPLC as described below.

Uptake of PQ and Q in myotube C2C12 cells. We determined the uptake of PQ or Q in the mouse myoblast cell line C2C12 (American Type Culture Collection). The cell culture and differentiation method were described in a previous study (8). PQ or Q in DMSO solution (10 mmol/L) was diluted with culture medium (2% horse serum in DMEM) and the final concentration was adjusted to 10 μmol/L . To estimate the effect of inhibition of ATP production on efflux transportation, 10 mmol/L sodium azide was added at 15 min before flavonoid treatment. The protein concentration of the cell suspensions was measured using the Bradford assay (27). The extraction and analyses were performed according to the uptake and permeability experiment using Caco-2 cells as described above.

Animal experiments. All experimental protocols were in accordance with the Guidelines for the Care and Use of Laboratory Animals set by the Graduate School of the Institute of Health Biosciences of the University of Tokushima. The protocol was approved by the Committee on Animal Experiments of the University of Tokushima (permit no. 11013). All surgery was performed under anesthesia using sodium pentobarbital or diethyl ether. All efforts were made to minimize the suffering of the animals.

Administration of PQ or Q to mouse stomachs and determination of the plasma concentration of their metabolites: single dose. Administration of PQ or Q to the stomach and blood collection was completed as previously described (8). Seven-week-old male C57/BL6 mice (Japan SLC) were housed in a room maintained at 23 ± 1°C on a 12-h-light/-dark cycle. They were allowed free access to AIN-93M diet (8) with no modification (Oriental Yeast) and water for 1 wk. Before administration, the mice were deprived of food for 18 h but had free access to water. PQ or Q dissolved in propylene glycol (5 g/L) was administered (50 mg/kg body weight) to mice by a gastric feeding tube. Sample preparation, deconjugation, and flavonoid extraction were performed as previously described (8). PN [for PQ and *O*-methylated prenyl quercetin (MePQ)] or kaempferol [for Q and *O*-methylated quercetin (MeQ)] was added to the sample as an internal standard. Flavonoids were determined by HPLC as described below.

Administration of PQ or Q to rat stomachs and determination of their metabolites in lymph fluid: single dose. Male Wistar rats (10–14 wk) were purchased from Japan SLC. The rats were allowed free access to drinking water and AIN-93M diet with no modification (Oriental Yeast). Under anesthesia, cannulation of the thoracic lymph duct, along with introduction of a gastric tube for sample administration, was undertaken as previously described (17). Rats were infused with 1 mL of PQ solution or Q solution in propylene glycol at 50 mg/kg body weight via the gastric tube, followed by continuous infusion of glucose-NaCl solution. Lymph fluid was collected in tubes containing 10 mmol/L sodium-EDTA solution to avoid coagulation. The resulting aglycones were determined by HPLC analyses employing the same method described for the plasma analyses.

Determination of PQ, Q, and their O-methylated metabolites in the plasma and tissues of mice: long-term feeding. PQ or Q (0.2% wt:wt) was mixed with AIN-93M diet (Oriental Yeast Co.). The cellulose content was reduced to adjust the composition of the other nutrients. The diets were given to 7-wk-old male C57/BL6 mice (Japan SLC) for 18 d. Tissue samples were collected and stored at −80°C under nitrogen gas until HPLC analyses. Sample preparation, deconjugation, and flavonoid extraction were performed as previously described (8). PN (for PQ and MePQ) or kaempferol (for Q and MeQ) was added to the samples as an internal standard.

HPLC analyses for determination of Q, PQ, and their O-methylated forms. PQ and Q were determined by reference to their respective standard curves. MePQ and MeQ were determined using the standard curve for 8-prenyl isorhamnetin and I, respectively. A Cadenza 3-μm CD-C18 HPLC column (150 × 4.6 mm; Imtakt) was employed. The flow rate was set at 1.0 mL/min. Because each sample had specific interference peaks in the HPLC chromatogram, we selected an optimum detection condition for each experiment, as shown in **Supplemental Table 1**. The analytical conditions were defined as follows. By monitoring the chromatogram from each sample (e.g., plasma, lymph, cell, tissues) without Q or PQ treatment, we confirmed the retention time and wavelength of potentially interfering peaks. Subsequently, we developed analytical conditions for separating the interfering peaks from the peaks of Q (MeQ) and PQ (MePQ). It was confirmed that no interference peaks overlapped with the peaks for the determination.

Statistical analyses. Data are presented as the means ± SEMs of at least 3 independent determinations for each experiment. We analyzed the equality of variance by Levene's test. If there was a significant difference ($P < 0.05$), each value was converted to the logarithmic value. Data were analyzed by 2-way (methylation × prenylation) or 3-way (methylation × prenylation × time) ANOVA as appropriate. When an interaction was significant, means were compared using Tukey's test. Differences were considered significant at $P < 0.05$. In the time course experiments (Caco-2, C2C12, plasma, and lymph), means were compared at each time point. The analyses were performed by PASS Statistics 17.0 (IBM).

Results

Effect of prenylation of Q on hydrophobicity. Hydrophobicity was measured using the $P_{o/w}$. The log$P_{o/w}$ values of PQ and Q were calculated to be 3.73 and 2.53, respectively. Prenylation increased the hydrophobicity of Q.

Accumulation and permeability of PQ in Caco-2 cells. We compared the permeability of PQ with that of Q in Caco-2 cells (**Fig. 1**). The amounts of PQ and Q in the apical solutions were decreased within 1 h after addition and thereafter the efflux of Q to the apical side was higher than that of PQ (Fig. 1*A*) ($P < 0.05$). PQ and Q were incorporated into Caco-2 cells and the amount of PQ incorporated into the cells was higher than that of Q from 1 to 6 h of incubation ($P < 0.05$) (Fig. 1*B*). Figure 1*C* shows the amounts of PQ and Q in the solution in which a small amount of PQ appeared during incubation for 6 h, and its concentration was lower than that of Q from 1 to 6 h of incubation ($P < 0.05$). This phenomenon indicated that the efflux of PQ from the cells to the basolateral side was less than that of Q. The amounts of MePQ and MeQ did not differ from or were lower than those of the respective non-*O*–methylated PQ and Q, respectively. These findings suggested that *O*-methylation was a minor pathway in the metabolism of PQ and Q in this enterocyte model. Taken together, prenylation increased the cellular uptake of Q from the apical side and decreased the efflux of Q to the basolateral side, resulting in higher accumulation in epithelial cells.

Uptake of PQ in mouse C2C12 myotube cells. We measured the uptake of PQ in C2C12 myotube cells as a potential cellular target of prenylated flavonoids (8). The conjugation reaction of flavonoids was not found in C2C12 cells (data not shown), so we did not employ a deconjugation treatment for this cell culture experiment. The results of the cellular uptake are shown in **Figure 2***A*. PQ was incorporated into cells and/or associated with cellular membranes at a much higher amount than Q after 0.25, 1, and 2 h of incubation ($P < 0.05$) (Fig. 2*A*). PQ and Q were converted to their *O*-methylated forms and the amount of

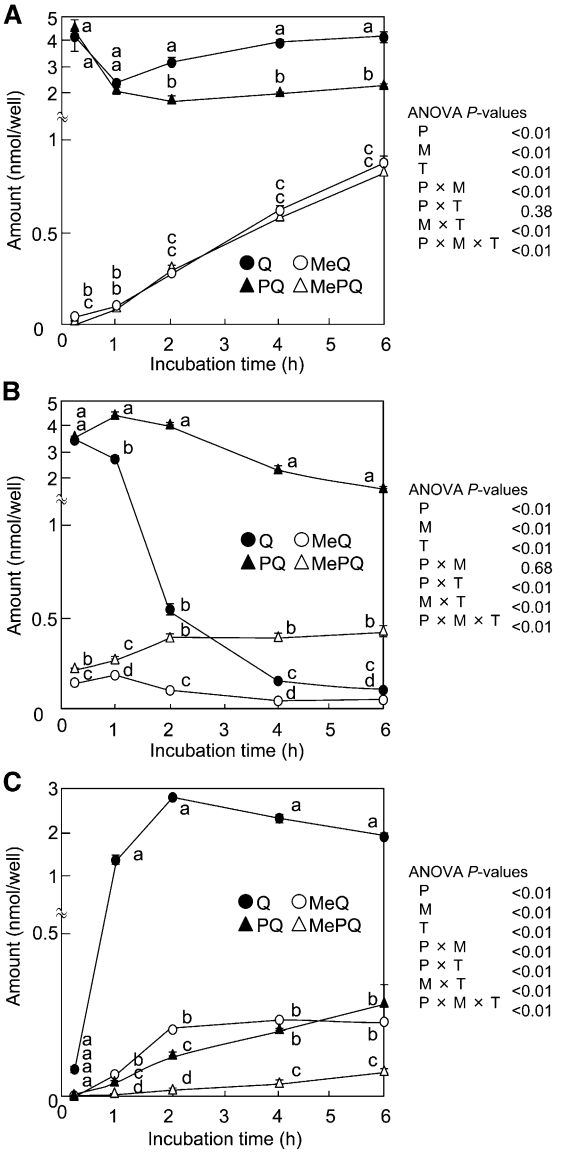

FIGURE 1 Permeability of PQ and Q in Caco-2 cells. PQ or Q was incubated for 0.25, 1, 2, 4, or 6 h in apical solutions (*A*), cells (*B*), and basolateral solutions (*C*). Data are means ± SEMs, $n = 5$. Data were log-transformed before ANOVA; prenylation (P), methylation (M), and time (T). Means at a time without a common letter differ, $P < 0.05$. MePQ, O-methylated prenyl quercetin; MeQ, O-methylated quercetin; PQ, 8-prenyl quercetin; Q, quercetin.

MePQ in cells was higher than that of MeQ from 1 to 24 h of incubation ($P < 0.05$). *O*-methylation was a major pathway for the cellular metabolism of PQ in myotubes, because *O*-methylated products accumulated at a higher amount than intact PQ after incubation of cells with PQ from 4 to 24 h ($P < 0.05$). Next, we confirmed the contribution of ATP to the efflux of PQ using sodium azide (Fig. 2*B*). The sum of Q and MeQ in C2C12 cells was increased by sodium azide ($P < 0.05$), which was a different pattern from that seen with PQ treatment. These findings suggested that PQ was less susceptible to elimination than Q via an energy requirement pathway.

Transport of PQ into the blood and lymph circulation after administration in rodent stomachs at a single dose. PQ or Q was administered to mice and the plasma concentration was analyzed by HPLC (**Fig. 3***A*). Pharmacokinetics were calculated

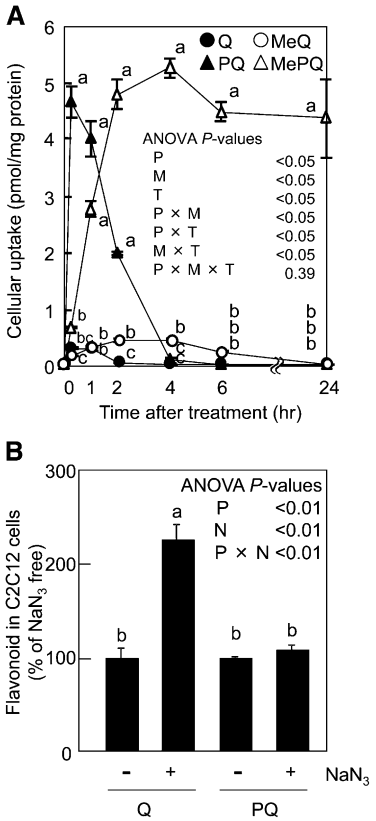

FIGURE 2 Uptake of PQ and Q in mouse C2C12 myotubes. (*A*) Q or PQ (10 μmol/L) was administered to cells for the times indicated. Data are means ± SEMs, $n = 3$. Data were log-transformed before ANOVA. Means at a time without a common letter differ, $P < 0.05$. (*B*) Effect of NaN_3 on the efflux of PQ from C2C12 cells was determined. The amount of cellular uptake was calculated as the sum of Q and MeQ or PQ and MePQ. Data are shown as the percent of NaN_3-free condition of the each flavonoid and are means ± SEMs, $n = 3$. Data were log-transformed before ANOVA; prenylation (P), methylation (M), NaN_3 treatment (N), and time (T). Means without a common letter differ, $P < 0.05$. MePQ, *O*-methylated prenyl quercetin; MeQ, *O*-methylated quercetin; PQ, 8-prenyl quercetin; Q, quercetin.

and are summarized in **Table 1**. At 30 min after administration, the plasma concentrations of PQ, MePQ, Q, and MeQ reached the highest amount (termed C_{max}). Prenylation decreased the mean C_{max} values in plasma ($P < 0.001$). The amount of transport into the blood circulation was estimated by the area under the concentration-time curve (AUC). The AUC of MePQ (7.63 μmol/L · h) was lower than that of MeQ (229 μmol/L · h) ($P < 0.05$). The MeQ concentration was higher than that of MePQ during the experiment ($P < 0.05$).

To determine the amount of flavonoids in lymph, PQ or Q was injected into rat stomachs (Fig. 3*B*). At 30 min after administration, PQ and MePQ as well as Q and MeQ appeared in the lymph circulation. The lymph concentration of MePQ was lower than that of MeQ during the experiment ($P < 0.05$). Pharmacokinetics were calculated and are summarized in **Table 2**. Prenylation and methylation affected the C_{max} in lymph ($P < 0.001$). The AUC values of PQ and MePQ were also lower than those of their nonprenyl forms, Q and MeQ, respectively ($P < 0.01$). These results demonstrated that prenylation reduced the transport from the digestive tract to the blood and/or lymph circulation. In other words, prenylation reduced the *O*-methylation of Q in metabolic pathways during absorption from enterocytes.

Plasma concentration and tissue accumulation of PQ after long-term feeding. The plasma total concentrations of Q, MeQ, PQ, and MePQ after deconjugation treatment were 2.40, 3.96, 1.63, and 1.16 μmol/L, respectively (**Table 3**). The plasma concentration of MePQ was lower than that of MeQ after long-term feeding ($P < 0.05$). Prenylation increased tissue accumulation in liver and kidney ($P < 0.001$). Prenylation and methylation did not affect tissue accumulation in muscle and heart ($P > 0.10$). Tissue accumulation in brain was affected by methylation as well as the interaction (prenylation × methylation) ($P < 0.0001$). In brain, the amounts of MQ and MePQ were higher than those of their respective nonmethylated forms ($P < 0.05$). Contrary to the concentrations in plasma and lymph, prenylation was likely to elevate tissue accumulation of Q.

Discussion

We found that prenylation at the 8-position affected the intestinal absorption, internal circulation, and tissue distribution of Q. PQ is found in *D. caudatum* (13), and 8-prenylflavonoids

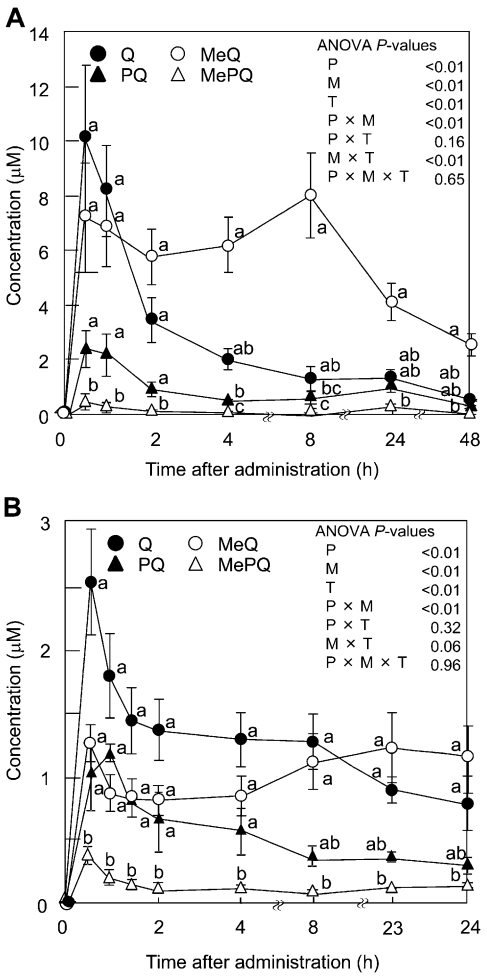

FIGURE 3 Concentration of PQ and Q in plasma and lymph after their administration into rodent stomachs as a single dose. PQ or Q was administered to mice (*A*, plasma) and rats (B, lymph). Data are means ± SEMs, $n = 5$. Data were log-transformed before ANOVA; prenylation (P), methylation (M), and time (T). Means at a time without a common letter differ, $P < 0.05$. MePQ, O-methylated prenyl quercetin; MeQ, O-methylated quercetin; PQ, 8-prenyl quercetin; Q, quercetin.

TABLE 1 Pharmacokinetic parameters in plasma after intragastric administration of PQ or Q to mice at 50 mg/kg body weight[1]

Variable	C_{max}	T_{max}	AUC
	μmol/L	*h*	*μmol/L · h*
Q	10.2 ± 2.58	0.25 ± 0.00^{b}	68.7 ± 10.5^{ab}
MeQ	7.18 ± 2.05	5.10 ± 1.78^{a}	229 ± 38.5^{a}
PQ	2.43 ± 0.67	0.80 ± 0.12^{ab}	36.1 ± 8.33^{b}
MePQ	0.43 ± 0.29	0.90 ± 0.29^{ab}	7.63 ± 3.64^{c}
ANOVA *P* value			
Prenylation	<0.01	0.18	<0.01
Methylation	0.15	<0.05	0.26
Prenylation × methylation	0.76	<0.05	<0.01

[1] Data are means ± SEMs, $n = 5$. Data from Tmax, and AUC were log transformed before ANOVA. Labeled means in a column without a common letter differ, $P < 0.05$. C_{max}, maximum concentration; MePQ, *O*-methylated prenyl quercetin; MeQ, *O*-methylated quercetin; PQ, 8-prenyl quercetin; Q, quercetin; T_{max}, time to maximum concentration.

TABLE 2 Pharmacokinetic parameters in lymph after intragastric administration of PQ or Q to rat at 50 mg/kg body weight[1]

Variable	C_{max}	T_{max}	AUC
	μmol/L	*h*	*μmol/L · h*
Q	2.51 ± 0.41	0.50 ± 0.00^{b}	27.5 ± 4.01^{a}
MeQ	0.92 ± 0.15	15.7 ± 4.83^{a}	25.3 ± 4.80^{a}
PQ	1.18 ± 0.37	1.20 ± 0.12^{b}	10.3 ± 1.92^{b}
MePQ	0.20 ± 0.07	0.80 ± 0.12^{b}	2.25 ± 0.32^{c}
ANOVA *P* value			
Prenylation	<0.01	0.06	<0.01
Methylation	<0.01	<0.01	<0.01
Prenylation × methylation	0.15	<0.01	<0.01

[1] Data are means ± SEMs, $n = 5$. Data were log transformed before ANOVA. Labeled means in a column without a common letter differ, $P < 0.05$. C_{max}, maximum concentration; MePQ, *O*-methylated prenyl quercetin; MeQ, *O*-methylated quercetin; PQ, 8-prenyl quercetin; Q, quercetin; T_{max}, time to maximum concentration.

(including PQ) are known to be distributed in plants used as food ingredients (10–12). Therefore, we investigated the effect of prenylation on the bioavailability of Q by comparing the characteristics of PQ with those of intact Q.

Prenylation increases the hydrophobicity of Q as evaluated by $\log P_{o/w}$. Flavonoid aglycones have been suggested to be incorporated into cells by simple diffusion controlled by the hydrophobicity of substances and their affinity for hydrophobic phospholipid bilayer membranes (28). Therefore, prenylation may enhance the cellular uptake of flavonoids, resulting in their biological activities in in vitro model systems (6,7). Prenylation enhanced the uptake of Q in myotube cells (Fig. 2*A*).

In the Caco-2 cell line, the amount of PQ in the epithelial cells was higher than that of Q, whereas a lower amount of PQ existed on the basolateral side compared with Q (Fig. 1). Although the lifespan of enterocytes is only a few days, the time to maximum concentration of each flavonoid in blood plasma as well as lymph is <24 h. These observations suggest that the shedding of intestinal cells has little effect on the transport of flavonoids to the circulation. Although the lipid condition on the basolateral side would attenuate efflux of PQ from intestinal cells, we considered that efflux of PQ to the basolateral side was lower than that of Q, even if lipids existed in the fluid, because the PQ concentration in lymph and plasma after a single dose was lower than that of Q (Fig. 3). Q is subjected to the actions of phase-II enzymes in epithelial cells during intestinal absorption (26,29). The resulting glucuronide and/or sulfate conjugates are released to the basolateral side through ATP-binding cassette transporters (ABC transporters) (30–32). ATP inhibition increased the amount of Q, but not PQ, in C2C12 cells (Fig. 2*B*). Therefore, it is likely that PQ is not well transported through ATP-dependent transportation systems. Similar to the case for C2C12 cells, intestinal cells may not transport PQ well from enterocytes to the internal circulation (blood and lymph) via ATP-dependent transportation systems (e.g., ABC transporters).

PQ was circulated as its conjugated metabolites in blood and lymph at a lower amount than Q (Fig. 3). These data are in accordance with our previous study using PN (8). In general, flavonoids undergo conjugation in enterocytes and hepatocytes before entering the circulation. Although studies have demonstrated that prenyl flavonoids are metabolized by conjugation enzymes (20,21,23), substitution of the prenyl group in the 8-position might prevent the activity of conjugation enzymes. However, the effect of prenylation on conjugation enzymes was not clarified in the present study, because we introduced a deconjugation treatment in the quantitative analyses.

Previously, we discovered that orally administered Q can enter the lymph circulation (17). Although we were draining

TABLE 3 Tissue distribution of PQ or Q in mice after 18 d of feeding of each flavonoid-containing diet[1]

Variable	Liver	Kidney	Muscle	Heart	Brain	Plasma
	nmol/g wet tissue	*nmol/g wet tissue*	*nmol/g wet tissue*	*nmol/g wet tissue*	*nmol/g wet tissue*	*μmol/L*
Q fed						
Q	0.29 ± 0.07	0.20 ± 0.07	0.23 ± 0.01	0.12 ± 0.01	0.04 ± 0.00^{a}	2.40 ± 0.21^{ab}
MeQ	0.23 ± 0.08	0.11 ± 0.04	0.18 ± 0.01	0.14 ± 0.02	0.002 ± 0.00^{d}	3.97 ± 0.34^{a}
PQ fed						
PQ	9.86 ± 2.33	0.77 ± 0.21	0.32 ± 0.16	0.10 ± 0.04	0.01 ± 0.00^{c}	1.63 ± 0.06^{bc}
MePQ	17.4 ± 3.88	0.81 ± 0.23	0.16 ± 0.07	0.17 ± 0.06	0.02 ± 0.00^{b}	1.16 ± 0.28^{c}
ANOVA *P* value						
Prenylation	<0.01	<0.01	0.45	1.00	0.10	<0.01
Methylation	0.13	0.45	0.26	0.25	<0.01	0.83
Prenylation × methylation	0.12	0.39	0.59	0.57	<0.01	<0.01

[1] Data are means ± SEMs, $n = 4$. Data from kidney, muscle, and plasma were log transformed before ANOVA. Labeled means in a column without a common letter differ, $P < 0.05$. MePQ, *O*-methylated prenyl quercetin; MeQ, *O*-methylated quercetin; PQ, 8-prenyl quercetin; Q, quercetin.

lymph fluid from rats via a thoracic lymph duct during the experiment, PQ was detected in lymph fluid until 24 h after injection (Fig. 3*B*). Because it was considered that PQ might pass through the small intestine at 24 h after injection, PQ in the lymph circulation came not only from intestinal absorption but also from body fluids. Therefore, one must take into account the contribution of the circulation of blood and lymph to the tissue distribution of PQ.

The plasma total concentration reached 1.63 μmol/L (PQ) and 1.16 μmol/L (MePQ) after long-term feeding of PQ (Table 3). MePQ may accumulate gradually in plasma by continuous daily intake even though the intestinal absorption is limited by a prenyl group (Fig. 1; Table 3). Although the concentrations of PQ and MePQ in the blood circulation were lower than those of Q and MeQ, simple diffusion into cells and association with the surface membranes of cells were enhanced by prenylation because of the higher hydrophobicity. The cellular uptake of PQ was higher than that of Q in C2C12 cells (Fig. 2*A*). This finding and our previous report on PN (8) confirm the elevation of cellular uptake of flavonoids by the introduction of a prenyl group. PQ seemed to efflux only slightly through ABC transporters (which was different from the effect seen with Q), because cellular accumulation of PQ was not appreciably affected by an ATP inhibitor (Fig. 2*B*). Continuous intake for a long time may be advantageous for effective accumulation of prenyl flavonoids in target tissues. In fact, the amounts of PQ and MePQ accumulated in the liver and kidney were much higher than those of their nonmethylated forms (Table 3). Food components are transported to the liver via the portal vein and to the kidney via afferent arterioles. Q has been suggested to serve as a substrate for a transporter, bilitranslocase (33). Certain flavonoids (e.g., Q or anthocyanins) have been found to be transported into the liver or kidney through bilitranslocase (34,35). Taken together, it is likely that prenylation of Q promotes its uptake into the liver and kidney by increasing its affinity for a transporter (e.g., bilitranslocase). Some flavonoid metabolites transferred to the liver are moved into the bile for the enterohepatic circulation (36) and finally excreted in the feces (37,38). Flavonoid metabolites are also excreted in urine (37,38). It seems that limitation of the excretion causes accumulation in those tissues. Interestingly, ABC transporters [multidrug resistance-associated protein-2 and/or multidrug resistance-associated protein-3] are expressed in the liver and kidney (39) in a similar way to enterocytes (30,32). Therefore, prenylation may lower efflux from the liver and kidney by attenuating the transport activity of these ABC transporters for flavonoids.

Q produces I (3′-*O*-methylated quercetin) and tamarixetin (4′-*O*-methylated quercetin) by the action with catechol *O*-methyltransferase (40). This reaction is regarded as detoxification of catechol-type flavonoids, because the catechol group can yield reactive oxygen species by a redox reaction to act as a cytotoxic prooxidant (40,41). PQ also seems to produce 3′-*O*-methylated PQ and 4′-*O*-methylated PQ. We could not distinguish between these 2 *O*-methylated metabolites, because the *O*-methylated metabolites were simultaneously eluted in the HPLC chromatograms. *O*-methylated PQ was present in a greater amount than non-*O*–methylated PQ in the brain (Table 3). Therefore, *O*-methylation probably occurs preferentially during PQ accumulation in the brain. In contrast, the occurrence of *O*-methylation was not remarkable during Q accumulation compared with PQ accumulation (Table 3). It seems that rapid *O*-methylation is required to avoid the unfavorable prooxidant effect of prenyl flavonoids, because prenyl flavonoids possess greater biological potential than nonprenyl flavonoids.

In conclusion, we found that prenylation lowers the transport of Q from enterocytes to the internal circulation, elevates its incorporation from the circulation to tissues, and slows its efflux from cells. Despite the lower bioavailability of this flavonoid in a single dose, prenylation facilitates its accumulation in target tissues in the case of continuous, long-term dietary intake. Thus, substitution of the prenyl group may modulate the fate of flavonoids from their absorption to excretion in the body.

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Literature Cited

1. Barron D, Ibrahim RK. Isoprenylated flavonoids: a survey. Phytochemistry. 1996;43:921–82.
2. Botta B, Vitali A, Menendez P, Misiti D, Delle Monache G. Prenylated flavonoids: pharmacology and biotechnology. Curr Med Chem. 2005; 12:717–39.
3. Huang X, Zhu D, Lou Y. A novel anticancer agent, icaritin, induced cell growth inhibition, G1 arrest and mitochondrial transmembrane potential drop in human prostate carcinoma PC-3 cells. Eur J Pharmacol. 2007;564:26–36.
4. Kuroyanagi M, Arakawa T, Hirayama Y, Hayashi T. Antibacterial and antiandrogen flavonoids from Sophora flavescens. J Nat Prod. 1999;62:1595–9.
5. Lee MH, Kim JY, Ryu JH. Prenylflavones from Psoralea corylifolia inhibit nitric oxide synthase expression through the inhibition of I-kappaB-alpha degradation in activated microglial cells. Biol Pharm Bull. 2005;28:2253–7.
6. Kretzschmar G, Zierau O, Wober J, Tischer S, Metz P, Vollmer G. Prenylation has a compound specific effect on the estrogenicity of naringenin and genistein. J Steroid Biochem Mol Biol. 2010;118:1–6.
7. Arung ET, Shimizu K, Tanaka H, Kondo R. 3-Prenyl luteolin, a new prenylated flavone with melanin biosynthesis inhibitory activity from wood of Artocarpus heterophyllus. Fitoterapia. 2010;81:640–3.
8. Mukai R, Horikawa H, Fujikura Y, Kawamura T, Nemoto H, Nikawa T, Terao J. Prevention of disuse muscle atrophy by dietary ingestion of 8-prenylnaringenin in denervated mice. PLoS ONE. 2012;7:e45048.
9. Sasaki K, Mito K, Ohara K, Yamamoto H, Yazaki K. Cloning and characterization of naringenin 8-prenyltransferase, a flavonoid-specific prenyltransferase of Sophora flavescens. Plant Physiol. 2008;146:1075–84.
10. Stevens JF, Taylor AW, Deinzer ML. Quantitative analysis of xanthohumol and related prenylflavonoids in hops and beer by liquid chromatography tandem mass spectrometry. J Chromatogr A. 1999; 832:97–107.
11. Jung HA, Moon HE, Oh SH, Kim BW, Sohn HS, Choi JS. Kinetics and molecular docking studies of kaempferol and its prenylated derivatives as aldose reductase inhibitors. Chem Biol Interact. 2012;197:110–8.
12. Chen SG, Chen JJ, Gao K. Prenylisoflavone derivatives from the roots of Hedysarum scoparium. Chem Pharm Bull (Tokyo). 2007;55:1181–4.
13. Wu Y, Luo Q, Sun C, Wang G, Chen Q, Guo Z, Zou X, Chen H. [Chemical constituents contained in Desmodium caudatum]. Zhongguo Zhong Yao Za Zhi. 2012;37:1788–92.
14. Murota K, Terao J. Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. Arch Biochem Biophys. 2003; 417:12–7.
15. Moon JH, Nakata R, Oshima S, Inakuma T, Terao J. Accumulation of quercetin conjugates in blood plasma after the short-term ingestion of onion by women. Am J Physiol Regul Integr Comp Physiol. 2000;279: R461–7.

16. Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr. 2000;130:S2073–85.
17. Murota K, Terao J. Quercetin appears in the lymph of unanesthetized rats as its phase II metabolites after administered into the stomach. FEBS Lett. 2005;579:5343–6.
18. Bieger J, Cermak R, Blank R, de Boer VC, Hollman PC, Kamphues J, Wolffram S. Tissue distribution of quercetin in pigs after long-term dietary supplementation. J Nutr. 2008;138:1417–20.
19. de Boer VC, Dihal AA, van der Woude H, Arts IC, Wolffram S, Alink GM, Rietjens IM, Keijer J, Hollman PC. Tissue distribution of quercetin in rats and pigs. J Nutr. 2005;135:1718–25.
20. Shen P, Wong SP, Yong EL. Sensitive and rapid method to quantify icaritin and desmethylicaritin in human serum using gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;857:47–52.
21. Rad M, Humpel M, Schaefer O, Schoemaker RC, Schleuning WD, Cohen AF, Burggraaf J. Pharmacokinetics and systemic endocrine effects of the phyto-oestrogen 8-prenylnaringenin after single oral doses to postmenopausal women. Br J Clin Pharmacol. 2006;62:288–96.
22. Pang Y, Nikolic D, Zhu D, Chadwick LR, Pauli GF, Farnsworth NR, van Breemen RB. Binding of the hop (Humulus lupulus L.) chalcone xanthohumol to cytosolic proteins in Caco-2 intestinal epithelial cells. Mol Nutr Food Res. 2007;51:872–9.
23. Yilmazer M, Stevens JF, Deinzer ML, Buhler DR. In vitro biotransformation of xanthohumol, a flavonoid from hops (Humulus lupulus), by rat liver microsomes. Drug Metab Dispos. 2001;29:223–31.
24. Kawamura T, Hayashi M, Mukai R, Terao J, Nemoto H. An efficient method for c8-prenylation of flavonols and flavanones. Synthesis. 2012;44:1308–14.
25. Shirai M, Moon JH, Tsushida T, Terao J. Inhibitory effect of a quercetin metabolite, quercetin 3-O-beta-D-glucuronide, on lipid peroxidation in liposomal membranes. J Agric Food Chem. 2001;49:5602–8.
26. Murota K, Shimizu S, Chujo H, Moon JH, Terao J. Efficiency of absorption and metabolic conversion of quercetin and its glucosides in human intestinal cell line Caco-2. Arch Biochem Biophys. 2000; 384:391–7.
27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
28. Tammela P, Laitinen L, Galkin A, Wennberg T, Heczko R, Vuorela H, Slotte JP, Vuorela P. Permeability characteristics and membrane affinity of flavonoids and alkyl gallates in Caco-2 cells and in phospholipid vesicles. Arch Biochem Biophys. 2004;425:193–9.
29. Mullen W, Edwards CA, Crozier A. Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. Br J Nutr. 2006;96:107–16.
30. O'Leary KA, Day AJ, Needs PW, Mellon FA, O'Brien NM, Williamson G. Metabolism of quercetin-7- and quercetin-3-glucuronides by an in vitro hepatic model: the role of human beta-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. Biochem Pharmacol. 2003;65:479–91.
31. Brand W, van der Wel PA, Rein MJ, Barron D, Williamson G, van Bladeren PJ, Rietjens IM. Metabolism and transport of the citrus flavonoid hesperetin in Caco-2 cell monolayers. Drug Metab Dispos. 2008;36:1794–802.
32. Zhang L, Lin G, Kovacs B, Jani M, Krajcsi P, Zuo Z. Mechanistic study on the intestinal absorption and disposition of baicalein. Eur J Pharm Sci. 2007;31:221–31.
33. Karawajczyk A, Drgan V, Medic N, Oboh G, Passamonti S, Novic M. Properties of flavonoids influencing the binding to bilitranslocase investigated by neural network modelling. Biochem Pharmacol. 2007;73: 308–20.
34. Passamonti S, Terdoslavich M, Franca R, Vanzo A, Tramer F, Braidot E, Petrussa E, Vianello A. Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organisms. Curr Drug Metab. 2009;10:369–94.
35. Passamonti S, Vrhovsek U, Vanzo A, Mattivi F. The stomach as a site for anthocyanins absorption from food. FEBS Lett. 2003;544:210–3.
36. Vanzo A, Vrhovsek U, Tramer F, Mattivi F, Passamonti S. Exceptionally fast uptake and metabolism of cyanidin 3-glucoside by rat kidneys and liver. J Nat Prod. 2011;74:1049–54.
37. Mullen W, Rouanet JM, Auger C, Teissedre PL, Caldwell ST, Hartley RC, Lean ME, Edwards CA, Crozier A. Bioavailability of [2-(14)C] quercetin-4′-glucoside in rats. J Agric Food Chem. 2008;56:12127–37.
38. Ueno I, Nakano N, Hirono I. Metabolic fate of [^{14}C] quercetin in the ACI rat. Jpn J Exp Med. 1983;53:41–50.
39. Dietrich CG, Geier A, Oude Elferink RP. ABC of oral bioavailability: transporters as gatekeepers in the gut. Gut. 2003;52:1788–95.
40. Zhu BT, Ezell EL, Liehr JG. Catechol-O-methyltransferase-catalyzed rapid O-methylation of mutagenic flavonoids. Metabolic inactivation as a possible reason for their lack of carcinogenicity in vivo. J Biol Chem. 1994;269:292–9.
41. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free Radic Biol Med. 1997; 22:749–60.