RESEARCH ARTICLE

Ingested quercetin but not rutin increases accumulation of hepatic β-carotene in BALB/c mice

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β-Carotene is a carotenoid with a range of reported health benefits besides vitamin A activity. If the enzymatic conversion of β-carotene to retinal is suppressed in the digestive tract, residual β-carotene that reaches the tissues increases. We evaluated the function of quercetin and rutin (quercetin-3-rutinoside) to increase the accumulation of β-carotene *in vitro* and *in vivo* in BALB/c mice. When the conversion of β-carotene by a preparation of the murine small intestine was measured *in vitro*, the addition of quercetin or rutin considerably inhibited the conversion. When the levels of hepatic β-carotene and retinoids were measured among three groups of mice fed a diet supplemented with β-carotene plus quercetin or rutin or β-carotene alone (four to six mice *per* group), quercetin increased the level of β-carotene and decreased the level of retinol, whereas rutin did not. These results demonstrate that quercetin can suppress the conversion of β-carotene which develops in the cytosol of small intestinal epithelial cells, and that rutin whose rutinose-moiety prevents being absorbed in the small intestine cannot suppress the conversion *in vivo*. This study offers a novel insight into the interaction between flavonoids and carotenoids with respect to the health benefits from the latter.

Keywords:
β-Carotene / β-Carotene-15,15′-monooxygenase / Liver / Quercetin / Rutin

1 Introduction

β-Carotene is a phytochemical known to be a pro-vitamin that exerts vitamin A activity after it is metabolized to retinol. Besides vitamin A activity, many health benefits of intact β-carotene and the other carotenoids (including non-pro-vitamin A carotenoids) have been reported [1, 2]. The eliminating activity of the singlet oxygen of carotenoids is widely known. This activity is necessary to remove the oxidative stress generated during photosynthesis in plants. The anti-oxidative ability of carotenoids is therefore also expected in animals (including humans) to avoid the adverse effects triggered by reactive oxygen species. Epidemiological studies have shown that an intake of vegetables and fruits high in β-carotene and/or other carotenoids is negatively correlated with the risk of various cancers and allergic syndromes [3–8]. Intervention studies showed that long-term supplementation with β-carotene is protective against ultraviolet-induced erythema formation in human skin [9, 10].

Flavonoids are another group of phytochemicals that exhibit anti-oxidative activities. These compounds have been implicated in the prevention of various degenerative diseases triggered by reactive oxygen species [11–18]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a flavonol. In most plants, quercetin is present in the glycosylated form (*e.g.* quercetin-3-glucoside or quercetin-4′-glucoside). The ingested quercetin glucoside can be hydrolyzed by lactase-phlorizin hydrolase (which does not hydrolyze rutinoside) in the small intestine to release its aglycon, and the aglycon is absorbed in the small intestine [19]. The absorbed aglycon is modified, such as conjugation with glucuronic acid. Rutin

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Abbreviation: PLSD, protected least significant difference

(quercetin-3-rutinoside) is also a quercetin glycoside. It was originally isolated from rue, and is commonly found in buckwheat, asparagus, and figs. Rutin is absorbed more slowly than quercetin due to its requirement of hydrolysis by cecal microflora before absorption [20]. Inhibition of enzymatic activity in the digestive tract, such as inhibition of α-amylase activity [21], is proposed to be one of the health benefits of flavonoids.

One β-carotene molecule produces two retinal molecules after enzymatic conversion by β-carotene-15,15′-monooxygenase [22]. Intraluminal β-carotene incorporated into mixed micelles is absorbed by the epithelial cells of the small intestine *via* passive diffusion. Absorbed β-carotene is partially converted to vitamin A in the intestinal mucosa, and β-carotene and vitamin A are incorporated into a chylomicron and into lymphatic vessels for delivery to the blood stream [23]. With respect to the absorption of carotenoids including β-carotene, the facilitated process *via* the scavenger receptor class B type I was also reported recently [24].

An *in vitro* study revealed that flavonoids including quercetin can non-competitively inhibit the activity of β-carotene-15,15′-monooxygenase extracted from the intestine [25]. Due to the possible inhibition of intestinal β-carotene-15,15′-monooxygenase by flavonoids, increased accumulation of β-carotene in tissues can be expected *in vivo*. The elevated level of β-carotene may promise the expression of another health benefit in addition to vitamin A activity.

In this study, we evaluated the function of two flavonoids, quercetin and rutin, in enhancing the accumulation of intact β-carotene, which is expected to exert various health benefits.

2 Materials and methods

2.1 Chemicals

All-trans retinal, all-trans retinol, all-trans retinoic acid, retinyl acetate, α-tocopherol, quercetin, rutin, and β-carotene were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). ACN, methanol, and HPLC-grade dichloromethane were purchased from Kanto Chemical (Tokyo, Japan). Other solvents and organic chemical reagents were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan).

2.2 Animals

Experiments were carried out in accordance with the guidelines for the care and use of laboratory animals (University of Tokushima, 2005; Tokushima, Japan). The permission number for these experiments at the University of Tokushima is 06030.

Eight-week-old male BALB/c mice were purchased from a local breeder (SLC Japan; Hamamatsu, Japan). They were housed in an institute for animal experimentation at the University of Tokushima.

For *in vitro* experiments, mice were fed commercial chow MF (Oriental Yeast; Tokyo, Japan) for 1 wk. For *in vivo* experiments, mice were fed with a β-carotene- and flavonoid-free diet for 1 wk to eliminate β-carotene and flavonoids from the mice. They were then fed one of the experimental diets, which were supplemented with 0.05% β-carotene alone, or with 0.05% β-carotene plus 0.05% quercetin or 0.05% rutin (Table 1). The control diet (β-carotene alone) was prepared according to the methods of Umegaki *et al.* [26] with a partial modification. The supplementation of sodium cholate markedly enhances the accumulation of β-carotene in mice. Mice received experimental diets for 2 days or 7 days *ad libitum*. On the indicated days, plasma was collected and the livers isolated from the mice. Separate experiments were repeated twice using four to six mice *per* group.

2.3 Assay of β-carotene monooxygenase activity

Crude β-carotene-15,15′-monooxygenase from the small intestine was prepared according to the previously described method [27] using 8-wk-old BALB/c mice. Briefly, the small intestine was washed with cold 0.9% NaCl solution. The mucosa was then scraped and homogenized with a Potter Elvehjem homogenizer in five volumes of 50 mM HEPES-KOH buffer (pH 7.4) containing 0.154 M KCl, 1 mM EDTA, and 0.1 mM DTT. The homogenate was centrifuged at $10\,000 \times g$ for 10 min. The obtained supernatant was dialyzed against 10 mM HEPES-KOH buffer (pH 7.4) containing 0.1 mM EDTA, 0.05 M KCl and 0.1 mM DTT.

Table 1. Composition of the diets

	g/100 g of diet		
	Control group	Quercetin-fed group	Rutin-fed group
β-Carotene	0.05	0.05	0.05
Quercetin	0	0.05	0
Rutin	0	0	0.05
Sodium cholate	0.25	0.25	0.25
D,L-methionine	0.30	0.30	0.30
50% Choline chloride	0.20	0.20	0.20
Vitamin mixture	1.00	1.00	1.00
Mineral mixture	3.50	3.50	3.50
Cellulose powder	4.70	4.65	4.65
Lard	5.00	5.00	5.00
Casein	20.00	20.00	20.00
Corn oil	5.00	5.00	5.00
Sucrose	20.00	20.00	20.00
Corn starch	40.00	40.00	40.00

After centrifugation, the protein fraction was stored at −80°C until use.

The enzymatic reaction was carried out using 0.1 M *N*-[*tris*(hydroxymethyl)methyl]glycine-KOH buffer (pH 8.0) containing 0.5 mM DTT, 8 mM sodium cholate, and 0.15% Tween-20 according to the previously described method [25]. β-Carotene (as a substrate) with α-tocopherol and Tween-20 was dissolved in acetone, dried under nitrogen, and then added to the reaction mixture to give a concentration of 100 μM. To investigate the inhibitory effect of quercetin and rutin on enzymatic activity, each flavonoid was dissolved in ethanol and added to 1 mL of the reaction mixture to give a concentration of 50 μM. The reaction mixture contained 0.03% ethanol (which would not have affected the enzymatic reaction). After preincubation at 37°C for 5 min, the cleavage reaction was started by addition of the enzyme preparation. The reaction mixture was incubated at 37°C for 60 min. The reaction mixture was combined with 0.25 mL of formaldehyde and incubated for 10 min at 37°C to terminate the reaction. Retinal, a reaction product, was extracted by ACN and analyzed by HPLC under the conditions described below.

2.4 Measurement of retinal, retinol, retinoic acid, and β-carotene

β-Carotene and retinoids in the liver were analyzed using the previously described methods [27, 28], with minor modifications. β-Apo-8′-carotenal and retinyl acetate were used as internal standards for β-carotene and the retinoids, respectively. Briefly, lipid fractions containing β-carotene and retinoids were extracted from the liver homogenate with a mixture of hexane, methanol and dichloromethane (2:2:1, by volume) containing 10 μM butylhydroxytoluene. The dried residue from the extraction was dissolved in the solvent and injected into the HPLC system. β-Carotene was separated on a column of TSK gel ODS-80Ts (4.6 mm × 250 nm for β-carotene, 4.6 mm × 150 mm for retinoids; Tosoh, Japan) using a mixture consisting of ACN, methanol dichloromethane, and water (70:15:10:5, by volume). Retinol, retinal, and retinoic acid were analyzed using another mixture consisting of ACN and water (9:1, by volume) containing 0.1% ammonium acetate. β-Carotene and retinoids were monitored at 450 and 350 nm, respectively. The concentrations of β-carotene and retinoid in the liver were corrected for protein content, which was measured using a standard method with Coomassie brilliant blue.

2.5 Statistical analysis

Statistical significance was determined using one-way analysis of variance with *post-hoc* Fisher's protected least significant difference (PLSD) tests for multiple comparisons using StatView software (Hulinks, Tokyo Japan). The homogeneity of variances was confirmed by the Bartlett test. $p<0.05$ was considered statistically significant.

3 Results

Quercetin and rutin significantly inhibited the enzymatic cleavage of β-carotene to retinal by the preparation of murine small intestinal mucosa *in vitro* (Fig. 1).

In a subsequent *in vivo* experiment, data at day-0 indicated that, after a clearance period when the mice were fed the β-carotene- and flavonoid-free diet for 1 wk, hepatic β-carotene could not be detected (Fig. 2A). At day-7, hepatic β-carotene in the quercetin-fed group significantly exceeded that in the control group. Hepatic β-carotene in the rutin-fed group was not greater than that in the control group. With respect to the level of β-carotene in plasma, a higher mean value was detected in the quercetin-fed group on both days, although the differences among the three groups were not statistically significant (Fig. 2B).

In comparison with day-0, hepatic retinol and retinal were increased in all three diet groups at day-2, although there were no differences among the groups (Fig. 3). At day-7, the levels of detected retinoids in all three diet groups were reduced compared with the levels on day-2. A decrease in hepatic retinol after a transient increase occurs naturally because the increased supply of retinol to the liver induces lecithin:retinol acyltransferase, which metabolizes retinol to retinyl ester [29–31]. At day-7, the level of hepatic retinol in

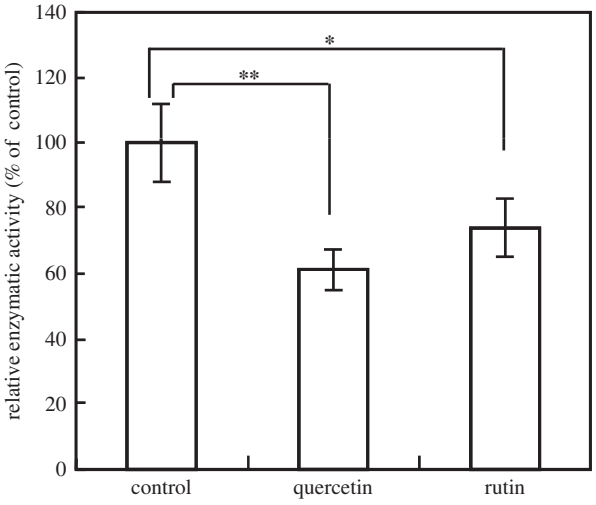

Figure 1. Effect of quercetin and rutin on β-carotene monooxygenase activity. The activity of β-carotene-15,15′-monooxygenase was determined in the presence of 50 μM quercetin or rutin as described in Section 2, comparing with that of control which did not contain flavonoids. The reaction mixture all contained 0.03% ethanol which was used as the vehicle of flavonoids. Values are mean ± SD of four experiments. An asterisk indicates a significant difference between each group (Fisher's PLSD method, $^{*}p<0.05$, $^{**}p<0.01$).

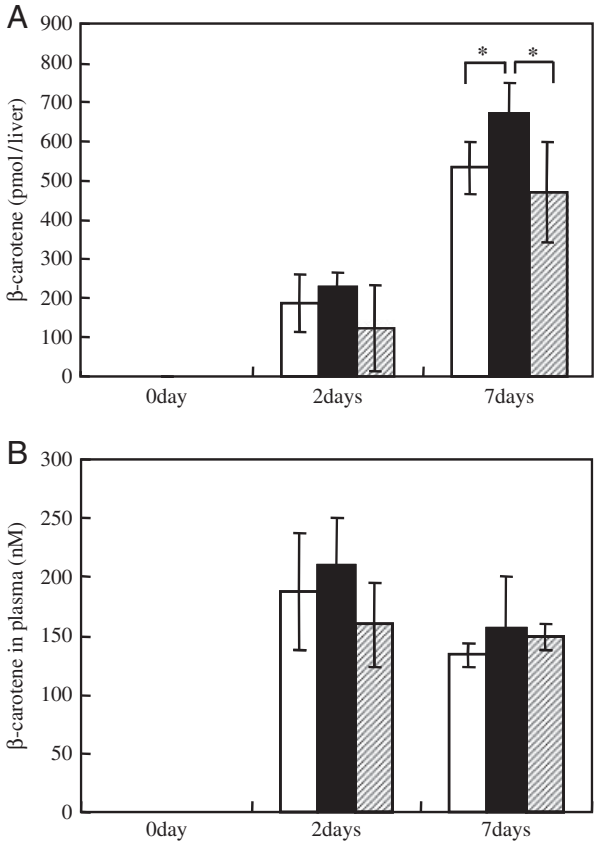

Figure 2. Concentration of β-carotene in liver and plasma in the presence of quercetin or rutin. Mice were fed a control diet containing β-carotene alone (open bar) or a test diet containing β-carotene with quercetin (filled bar) or rutin (hatched bar) for 2 days or 7 days. The concentration of β-carotene in the liver (A) and plasma (B) was determined with a HPLC system as described in Section 2. Values are mean ± SD ($n = 6$ for the diet containing quercetin- and rutin-fed groups for 2 days; $n = 5$ for the other groups). Separate experiments were repeated twice using four to six mice *per* group. An asterisk indicates a significant difference between each group (Fisher's PLSD method, $^{*}p < 0.05$).

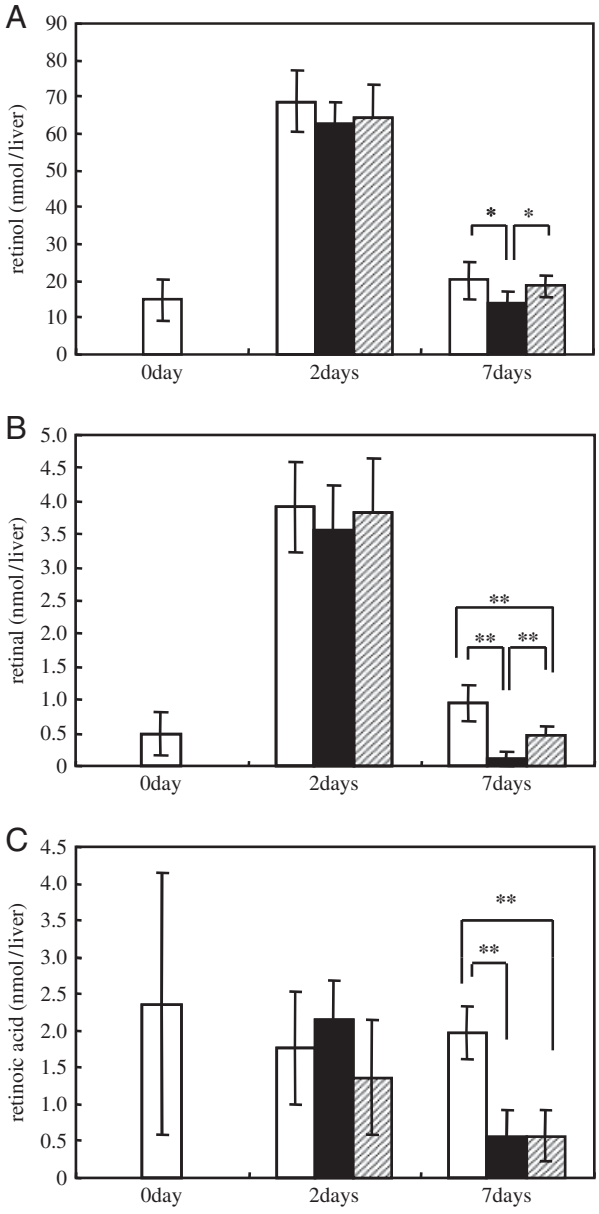

Figure 3. Effect of quercetin and rutin on the concentration of retinoids in the liver. Mice were fed a control diet containing β-carotene alone (open bar) or a test diet containing β-carotene with quercetin (filled bar) or rutin (hatched bar) for 2 days or 7 days. Concentrations of retinol (A), retinal (B), and retinoic acid (C) were determined with a HPLC system as described in Section 2. Values are mean ± SD ($n = 6$ for the diet containing quercetin- and rutin-fed groups for 2 days; $n = 4$ for the diet containing the quercetin-fed group for 7 days; $n = 5$ for the other groups). Separate experiments were repeated twice using four to six mice *per* group. An asterisk indicates a significant difference between each group (Fisher's PLSD method, $^{*}p < 0.05$, $^{**}p < 0.01$).

the quercetin-fed group was significantly lower than that in the control and rutin-fed groups. Conversely, levels of hepatic retinal and retinoic acid in the quercetin-fed group and the rutin-fed group at day-7 were significantly lower than that in the control group. The detected levels of retinal and retinoic acid were substantially lower than retinol.

4 Discussion

As with an *in vitro* study with porcine β-carotene-15,15′-monooxygenase [25], the results of this *in vitro* study with murine small intestinal epithelial homogenates showed the inhibitory activity of quercetin and rutin on the enzymatic cleavage of β-carotene. This result demonstrated that a catabolic enzyme for β-carotene present in the murine small intestine (presumably β-carotene-15,15′-monooxygenase) has sensitivity for both tested compounds. This is the first time that detection of the inhibitory effect of flavonoids against the murine enzymatic activity for β-carotene has

been reported although there has been a report for a porcine enzyme.

An increase in the level of hepatic β-carotene was detected in mice fed a diet containing β-carotene and quercetin for 7 days although the effect on the increase of hepatic β-carotene by the intake of quercetin was comparatively small for clear inhibition of β-carotene-15,15′-monooxygenase activity *in vitro*. A decrease in the level of hepatic retinol was simultaneously detected in the same mice. β-Carotene is absorbed in the small intestine; quercetin and its glucoside are also usually absorbed there. Therefore, quercetin that is simultaneously absorbed with β-carotene could inhibit the enzymatic conversion of β-carotene to retinal in small intestinal epithelial cells. Such suppressed conversion would result in an increased accumulation of β-carotene and a decreased accumulation of retinol in the liver.

Conversely, we did not observe an increase in hepatic accumulation of β-carotene in mice fed a rutin-supplemented diet, regardless of its inhibitory activity on the enzymatic conversion of β-carotene to retinal detected in the *in vitro* experiments. To explain this inconsistency, consideration of the region where β-carotene is converted to retinal is necessary. β-Carotene is converted not at the lumen but in the cytosol of small intestinal epithelial cells [32]. Luminal rutin cannot inhibit the intracellular conversion of β-carotene. Quercetin aglycon of rutin is released after rutin is digested by cecal microflora and absorbed in the large intestine [20]. Therefore, quercetin aglycon from rutin probably cannot inhibit the conversion of β-carotene in the small intestine because it is absorbed in the large intestine. This may explain the absence of an increase in the level of hepatic β-carotene in mice fed a rutin-supplemented diet. Coincidently, there was no significant decrease in hepatic retinol in mice fed a rutin-supplemented diet.

The detected levels of hepatic retinal and retinoic acid were reduced in mice fed a quercetin-supplemented diet and in those fed a rutin-supplemented diet, in comparison with mice fed a control diet. Retinal and retinoic acid are oxidized metabolites generated from retinol. The levels of these retinoids may therefore correspond to hepatic metabolic activity for retinol. The decrease in the levels of hepatic retinal and retinoic acid suggests that hepatic metabolic activity for retinol is suppressed by the intake of quercetin and rutin. To determine the effect of the oral intake of quercetin or rutin on hepatic retinoid metabolism more precisely, the level of hepatic retinyl esters should be measured in addition to the data presented here.

Beneficial health effects other than vitamin A activity of β-carotene and other carotenoids have been investigated. For example, we found that accumulation of β-carotene (but not retinol) in murine splenocytes corresponded to the up-regulation of cysteine–cathepsin activity accompanied by the increased intracellular level of reduced glutathione [33]. We also reported that β-carotene with α-tocopherol suppressed ultraviolet-A-induced activity of matrix metallopeptidase-9, *i.e.* hydrolysis of the collagen responsible for photoaging in the mouse skin [34]. Simultaneous intake of a quercetin and/or quercetin glucoside with β-carotene is expected to be effective for the acceptance of the beneficial health effects beyond pro-vitamin A activity of β-carotene. The intake of quercetin and/or quercetin glucoside may also be effective in increasing the accumulation of the other carotenoids such as α-carotene and β-cryptoxanthin because β-carotene-15,15′-monooxygenase can hydrolyze these carotenoids [35].

In large intervention studies, positive correlation between β-carotene supplementation and the risk of cancer in some populations have been reported [36, 37]. Such results are in contrast to the anticipated health benefit of β-carotene. β-Carotene can be naturally ingested with other food ingredients such as flavonoids contained in vegetables or fruits. Although our experiment was conducted only in mice, the interactive effect between β-carotene and quercetin shown in this study may underscore the importance of taking β-carotene not from isolated supplements but from vegetables and fruits in humans. In addition to our study with respect to the accumulation of β-carotene, the interaction in biological activities between carotenoids and flavonoids was reported [38–41].

We assume that the increased hepatic accumulation of β-carotene could be attributable to the inhibition of intestinal β-carotene-15,15′-monooxygenase by quercetin. However, other parameters (*e.g.* level of cellular retinol binding protein II; hepatic β-carotene-15,15′-monooxygenase activity) may also be important. Hepatic quercetin, hepatic rutin, or their metabolite(s) may affect the local metabolic enzyme activities for β-carotene and retinoids.

In conclusion, this study demonstrated that simultaneous ingestion of quercetin (but not rutin) with β-carotene increased the accumulation of β-carotene in one type of tissue in mice. Intake of quercetin with β-carotene as in whole foods may confer additional health benefits of β-carotene beyond vitamin A activity.

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The authors have declared no conflict of interest.

5 References

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