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Intestinal absorption and metabolism of a soluble flavonoid, αG-rutin, in portal cannulated rats

MGUMI MATSUMOTO[1,2], HIDEYUKI CHIJI[1], & HIROSHI HARA[2]

[1]*Faculty of Human Life Science, Department of Food Science and Human Nutrition, Fuji Women's University, Ishikari, Hokkaido 061-3204, Japan, and* [2]*Laboratory of Nutritional Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan*

Abstract
A highly soluble quercetin glycoside, αG-rutin, is a glucose adduct of insoluble rutin, and intestinal absorption and metabolism of αG-rutin has not been known. We investigated the intestinal absorption and metabolism of αG-rutin by using portal and duodenal cannulated rats and the isolated rat intestinal mucosa. After a duodenal instillation of αG-rutin (150 μmol), intact αG-rutin, rutin and quercetin were appeared in the portal blood and these concentrations were similarly increased at 15 min. Portal quercetin reached a peak value at 60 min, and the value was higher than those of αG-rutin and rutin at that time. Quercetin-conjugates were also increased 30 min after the instillation. The remaining of αG-rutin metabolites, mainly rutin, in the intestine were 58% of instilled αG-rutin after 150 min. In the experiment by using the isolated mucosa of the jejunum, ileum and cecum, αG-rutin and rutin, but not quercetin, appeared in the serosal sides of all segments, and they were increased linearly from 10 to 100 mmol/l of mucosal αG-rutin. We also showed portal injected αG-rutin was very rapidly cleared from the blood, and appeared a large amount of conjugates. In conclusion, a soluble flavonoid-glycoside, αG-rutin, was absorbed as glycosides into the portal blood. A part of αG-rutin was hydrolyzed to rutin, but not to aglycone, through the intestine.

Keywords: *Flavonoid, net absorption, metabolism, portal cannula, Ussing chamber, rat*

Introduction

Polyphenols are widely distributed throughout the plant world, especially in fruits and vegetables as secondary metabolites [1]. These compounds act as antioxidants in foods and the body [2]. Flavonoids, a kind of polyphenol, are used as natural pigments in foods, however, the absorption and metabolisms of polyphenolic compounds have not been fully understood. Recently, many findings on catechin, anthocyanin and quercetin have been reported due to the development of analytical instruments. Quercetin is a wellknown natural flavonoid contained in onions, green tea and sophora [3–5], and has beneficial effects for human health as an antioxidant [6,7]. Quercetin is usually present in glycosylated forms, mainly as β-glucosides, in plant foods [8]. The nature of glycosylation probably influences the efficiency of quercetin absorption. Absorbed quercetin is rapidly conjugated in both the small intestine and the liver [9]. There have been no reports on the existence of quercetin aglycone or glycosides in the systemic circulation. Quercetin-3-*O*-glucoside did not appear in systemic blood after the administration of quercetin-3-*O*-glucoside, which is the most abundant glycoside in plant foods [10].

Correspondence: H. Hara, Kita-9, Nishi-9, Kitaku, Sapporo, Hokkaido 060-8589, Japan. Tel: 81 11 706 2504. Fax: 81 11 706 3352. E-mail: hara@chem.agr.hokudai.ac.jp

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Recently, a new quercetin glycoside, αG-rutin, has been developed and manufactually be available (Figure 1). This is a very water-soluble D-glucose adduct of rutin (quercetin-3-O-glucosyl-rhamnose). Previous reports have shown that rutin was hardly absorbable and metabolizable in the small intestine with *in vivo* experiments [11–13]. Rutin is slightly soluble in water and other solvents, which is a reason for its low absorbability in the small intestine [14–16]. Therefore, improvement of water solubility of rutin by conversion to αG-rutin possibly enhances the intestinal absorption.

The aims of the present study were to examine the intestinal absorption and metabolism of the quercetin glycoside, αG-rutin, by *in vivo* and *in vitro* studies of rats. We observed intestinal absorption and metabolism of αG-rutin by using portal and jugular cannulated rats to collect portal and jugular blood diachronically under unstrained conscious conditions, and by using the isolated mucosa of the intestine. We measured αG-rutin and its metabolites concentration by LC/MS analyses, which allow to isolate and quantify αG-rutin and its metabolites.

Materials and methods

Chemicals

αG-rutin was kindly donated by Toyo Sugar Refining Co. Ltd. (Tokyo, Japan). Quercetin and rutin were obtained from Wako Pure Chemical Industries Co., LTD. (Osaka, Japan). All other reagents and chemicals were of the highest-grade commercially available extra-pure grade products.

Animals and diets

Male Wistar rats (200 g, Japan SLC, Shizuoka, Japan) were housed in individual wire-bottomed cages in a temperature-controlled room at 22°C throughout the experiments. Rats were fed a 25% casein-sucrose diet; 60.25% sucrose, 25% casein, 5% cellulose, 3.5% mineral mixture (AIN 93G), 1% vitamin mixture (AIN 93G), 5% corn oil, 0.25% choline chloride for a week before experiments.

This study was approved by the Hokkaido University Animal Committee and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Experiment 1: Portal absorption of αG-rutin

Ten acclimated rats, weighing 230–250 g, were implanted with portal and duodenal canulae under sodium pentobarbital anesthesia (40 mg/kg body weight, Nembutal, Abbott, North Chicago, IL, USA). The portal cannula (polyethylene tube, sp 28; I. D. 20.4 mm, O. D. 0.8 mm, Natsume Seisakusho, Tokyo, Japan) was directly inserted into the portal vein [17], and the duodenal cannula (silicon tube, Silascon No.00) was inserted through an intestine fistula at 1 cm distal from the pylorus. After a 24-h fast, 1 ml of αG-rutin solution (150 μmol) was instillated into the duodenum, and the portal blood (0.3 ml each) was collected before and at 15, 30, 60, 90 and 120 min after an instillation. The abdominal aorta blood was collected 150 min after the instillation of αG-rutin under sodium pentobarbital anesthesia, and the rats were killed. The whole small intestine and the cecum were removed after ligation of both ends of the segment, and collected their contents [18]. The contents were frozen and stored at $-80°C$ until subsequent analyses.

Experiment 2: Absorption and metabolism by isolated mucosa

The small intestine from the ligament of Treitz to the ileocecal junction and cecum were removed from six acclimated rats, weighing 230–250 g, under a pentobarbital anesthesia. The outside and inside surfaces of the isolated intestine were washed with ice-cold (4°C) saline (154 mmol/l NaCl). The jejunum (15-cm segment distal from the Trietz ligament), ileum (15-cm segment proximal from the ileocecal junction) and cecum (whole sac) were collected, these segments were cut open along the mesenteric border to be a flat sheet, and rinsed with an ice-cold balanced salt solution buffered by HEPES (HBS); 125 mmol/l NaCl, 4 mmol/l KCl, 10 mmol/l D-glucose, 30 mmol/l HEPES and 1.25 mmol/l $CaCl_2$, gassed with 100% O_2, pH 7.4. The serosa and muscle layers were removed from the each segment, and the stripped preparations consisting of the mucosa and the submucosal tissue were mounted onto Ussing chambers (diffusion chamber system, Corning Costar Co., Cambridge, UK) that exposed a circular area of the epithelium of 0.64 cm^2. The serosal and mucosal sides of the segments were bathed in 1 ml of HBS continuously exposed to 100% O_2 gas. After a 30-min stabilization period, the medium on the serosal
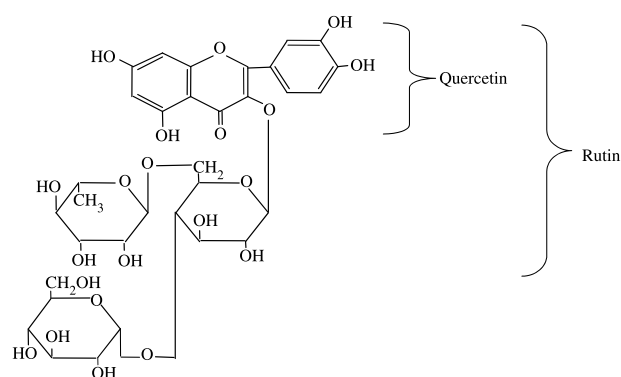

Figure 1.   Structure of αG-rutin.

sides was replaced with a fresh HBS, and that on the mucosal sides was replaced with HBS containing 0.01, 1, 10 or 100 mmol/l αG-rutin. After incubation for 30 min at 37°C, the serosal solution was collected and analyzed. The integrity and viability of the tissue was checked by transepithelial electrical resistance (TEER, Millicell-ERS, Millipore, Billerica, Massachusetts, USA). The TEER value of the epithelial preparation in the jejunum, ileum and cecum was measured before and after the 30-min incubation period with 100 mmol/l αG-rutin added to the mucosal chamber. The TEER value was indicated in terms of Ωcm^{-2}.

Experiment 3: Injected into the portal vein

Nine acclimated rats, weighing 230–250 g, were implanted with portal and jugular canulae under pentobarbital anesthesia. The portal cannula was directly inserted into the portal vein, as stated above and the jugular cannula was inserted in the jugular vein. After a 24-h fast, 0.5 ml of αG-rutin solution (5 μmol) was instilled into the portal vein, and the jugular blood (0.3 ml each) was collected before and at 2, 5, 10, 15, 20, 30, 60 and 150 min after an instillation.

Analytical method

Plasma sample treatment

Plasma samples (100 μl) obtained by centrifugation from portal and aortic blood were acidified (to pH 4.9) with 10 μl of acetic acid (0.58 mol/l), then treated for 30 min at 37°C in the absence (to measure unconjugated forms of flavonoids) or presence (to measure total flavonoid) of 10 μl of *Helix pomatia* extract (Sigma G-0876, 5, 106 U/L, β-glucuronidase and 2.5, 105 U/L sulfatase). The reaction mixture was then added to 100 μl of MeOH, heated at 100°C for 1 min, centrifuged for 3 min at 9,000g, and the supernatant was collected. This extraction procedure was repeated 3 times without heating. The combined supernatant was applied to oasis HLB cartridges (Waters Co. LTD, Milford, MA, USA), the eluent was dried, and dissolved in a 100 μl of 50% MeOH solution (sample solution). The sample solution was analyzed by LC/MS.

LC/MS analysis

αG-rutin and its metabolites were identified and quantified by a ZQ 2000 Waters mass spectrometer-computer system through the positive ion at an electric spray ionization (ESI)-interface (Waters Co. LTD, Milford, MA, USA). The temperature of the capillary heater and the vaporization heater was maintained at 100°C and 300°C, respectively. The flow rate of the sheath gas (nitrogen) was 70 arb. LC/ESI-MS was carried out in scan mode from m/z: 50 to 2000 [M + H] + and in selected ion monitoring (SIM) mode m/z: 303

[M + H] + for quercetin, m/z: 611 [M + H] + for rutin and m/z: 773 [M + H] + for αG-rutin, respectively. The HPLC system was fitted with a 5 μm C-18 Waters Puresil TM column (150 × 4.6 mm², Waters Co. LTD, Milford, MA, USA) and the temperature maintained by the column oven set at 40°C. Solvent A (water: methanol: trifluoroacetic acid, 70:30:0.1) and B (methanol: trifluoroacetic acid, 100:0.1) were run at a flow rate of 1 ml/min using a linear gradient up to 30% solvent B from 10% until 20 min, back to 10% solvent B linearly for next 5 min and held at the condition for a further 5 min. UV chromatograms were recorded at 360 nm. Concentrations of flavonoids were estimated by using calibration curves of quercetin, rutin and αG-rutin standard solution.

Calculates and statistics

Concentrations of αG-rutin, rutin and quercetin were calculated from the peak area of each mass spectra and calibration curves. The concentrations of conjugated derivatives were estimated as the difference between quercetin concentrations before and after a β-glucuronidase/sulfatase treatment. Statistical analyses were performed by oneway ANOVA. The differences among treatment groups were analyzed with Duncan's multiple range test and were considered significant at $P < 0.05$.

Results

Portal absorption of **αG-rutin**

Figure 2. shows the LC/MS chromatograms of the standard solution (αG-rutin, rutin and quercetin). Peaks for αG-rutin and rutin were detected at 7 min, and that for quercetin was detected at 15 min. Recovery rates of standard αG-rutin, rutin and quercetin added to the portal blood were over 90% with the same treatment of plasma samples.

On the chromatograms at 360 nm absorbance in the portal blood 30 min after instillation of 150 μmol of αG-rutin (Figure 3A), intact αG-rutin, quercetin, rutin and several unidentified broad peaks were detected. The unidentified peaks disappeared while the quercetin peak was increased by β-glucuronidase/sulfatase treatment, indicating that the three unidentified peaks correspond to conjugated derivatives of quercetin (Figure 3B).

Intact αG-rutin concentration in the portal blood quantified by LC/MS increased and reached a peak value at 15 min. Concentrations of quercetin and rutin peaked at 60 min, though the rutin concentration was much lower than that of quercetin (Figure 4A). Portal concentrations of αG-rutin, rutin and quercetin after an instillation of 150 μmol/ml αG-rutin at 60 min were 2.56, 1.14 and 16.1 μmol/l, respectively. Quercetin-conjugate concentration (sum of three peaks) in the portal plasma was markedly increased up to 30 min after the instillation of αG-rutin and remained at a high level until 120 min (Figure 4B). The highest concentration was 43.0 μmol/l
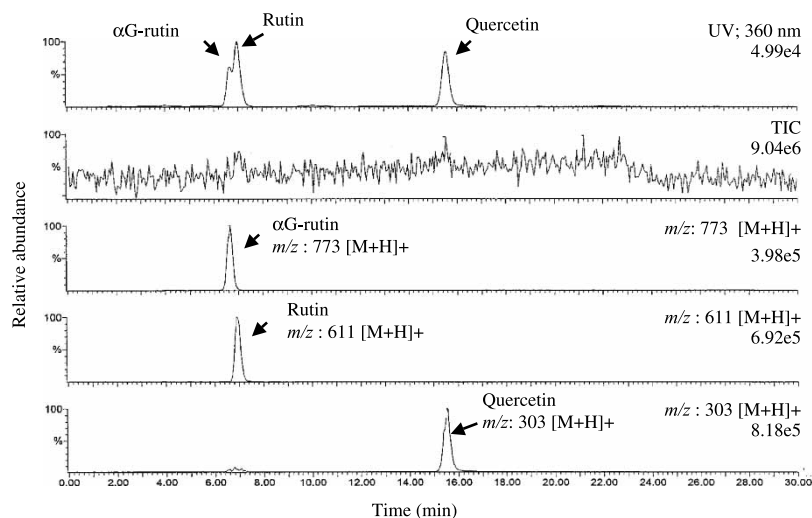
Figure 2.   LC/MS chromatogram monitoring relative absorption at 360 nm and selected ion monitoring (SIM) mode *m/z:* 303 [M + H] + for quercetin, *m/z*: 611 [M + H] + for rutin and *m/z*: 773 [M + H] + for αG-rutin.

at 90 min, which was 180% higher than the concentrations of the unconjugated forms of quercetin. The concentration of quercetin-conjugates in the abdominal aortic plasma was 30.4 ± 5.3 μmol/l (*n* = 10) 150 min after an instillation, which was a similar to the portal concentration of the conjugates at 120 min (Figure 4B). αG-rutin, rutin and quercetin were not detected in the aortic blood. We obtained to a similar result by using a half concentration, 75 μmol αG-rutin. It results that portal concentrations of αG-rutin, rutin, quercetin and
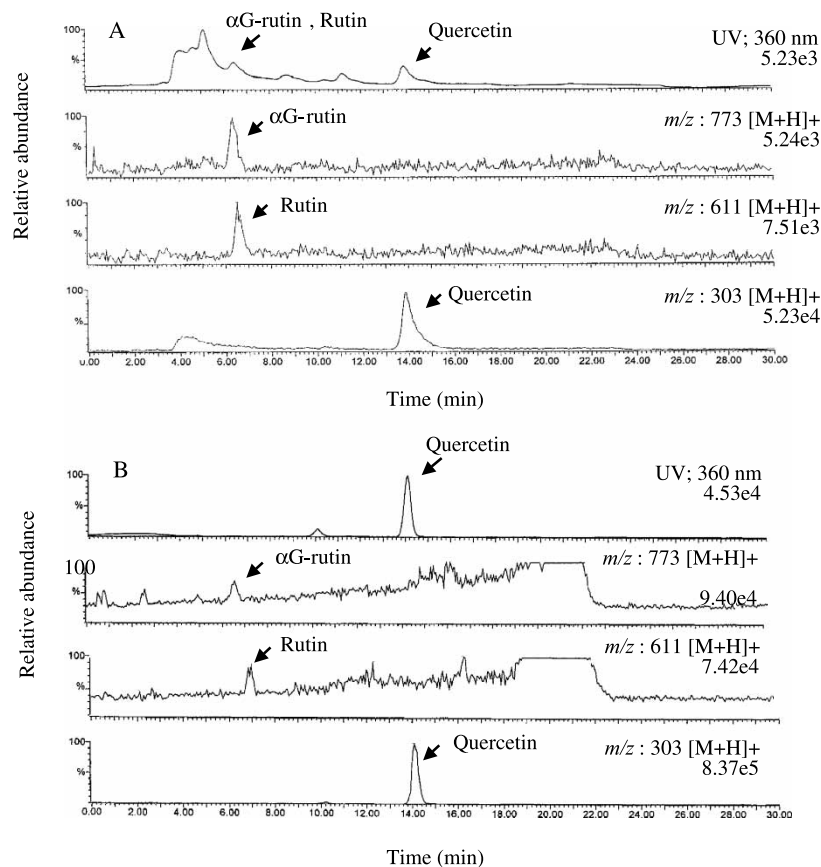

Figure 3.   LC/MS analysis of αG-rutin, rutin, quercetin and metabolites of αGrutin in portal plasma after instillation of αG-rutin. LC/MS chromatogram monitored relative absorption at 360 nm. (A) Non-treated portal vein plasma 30 min after the instillation of αG-rutin. (B) Portal plasma treated with by β-glucuronidase and sulfatase 30 min after the instillation of αG-rutin.
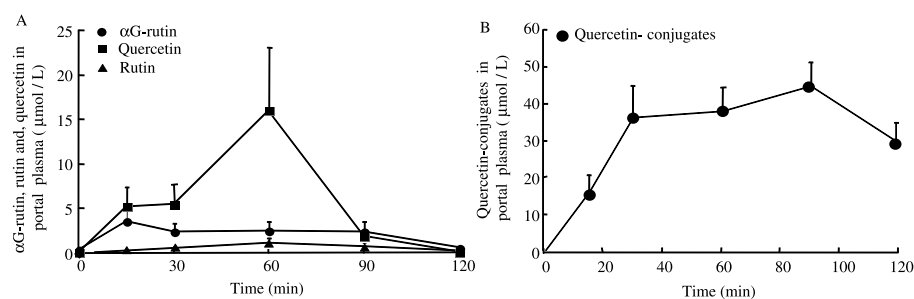
Figure 4. Concentrations of αG-rutin, quercetin and rutin in the portal plasma of rats instillated with αG-rutin. (A) Portal blood was collected at 15, 30, 60, 90 and 120 min after instillation of the αG-rutin solution (150 μmol/ml/rat). Concentration of quercetin-conjugates in the portal vein plasma of rats administered αG-rutin. (B) Portal blood was collected at 15, 30, 60, 90 and 120 min after the instillation of the αG-rutin solution (150 μmol/ml/rat) and treatment with β-glucuronidase and sulfatase. Values are means ± SEM, $n = 10$.

quercetin-conjugate after an instillation of 75 μmol/ml ($n = 3$) αG-rutin at 60 min were 0.15 ± 0.11, 0.09 ± 0.07, 0.25 ± 0.10 and 4.57 ± 0.58 μmol/l, respectively.

Degraded products of αG-rutin remaining in the small intestinal contents were mainly rutin 150 min after the instillation of αG-rutin (150 μmol). Amounts of αG-rutin and rutin in the contents were 1.08 and 61.9 μmol in the small intestine (Figure 5A). Quercetin and quercetin-glucuronides were 0.26 and 4.19 μmol in content of the small intestine. Amounts of αG-rutin, rutin, quercetin and quercetin-conjugates in the cecal contents were 0.52, 19.4, 0.09 and 1.13 μmol, respectively (Figure 5B). Sum of αG-rutin and its metabolites in the whole intestine was 88.6 ± 9.58 μmol.

Absorption and metabolism by isolated mucosa

The transport and hydrolysis of αG-rutin for 30 min by the isolated mucosa of the rat jejunum, ileum and cecum are shown in Figure 6A, B and C. The TEER in the three intestinal portions before and after incubation for 30 min are shown in Table I. The initial values of TEER were 140–200 Ω cm^{-2}, and 30-min incubation with 100 mmol/l αG-rutin did not affect the TEER for any portion of the intestine. αG-rutin and rutin, but not quercetin, were detected in the serosal fluid from the all

intestinal segments 30 min after incubation with addition of αG-rutin in the mucosal fluid. The transport of these glycosides into the serosal fluid was linearly increased from 10 to 100 mmol/l of αG-rutin in the mucosal fluid. Increases in rutin concentration of the serosal fluid were similar to those in αG-rutin up to 10 mmol/l αG-rutin the mucosal fluid of the jejunum and ileum. However, the αG-rutin concentration was much higher than that of rutin, when the mucosal αG-rutin was 100 mmol/l. Quercetin glycosides appeared in the serosal fluid of the cecal mucosa was almost entirely αG-rutin. The changes in αG-rutin and rutin transport with increases in the mucosal concentration of αG-rutin were very similar between the jejunal and ileal mucosa. Sum of transported αG-rutin and rutin was similar among the three different parts of the intestines. Quercetin and quercetin conjugates were not detected in both the serosal and mucosal fluid.

Injected into the portal vein

Intact αG-rutin concentration in the jugular plasma was a very low level 2 min after the instillation of αG-rutin to the portal vein (Figure 7). Remaining αG-rutin in the blood is less than 1% of the amount of αG-rutin injected into the portal vein. At this time, quercetin-conjugate was found a much higher levels,
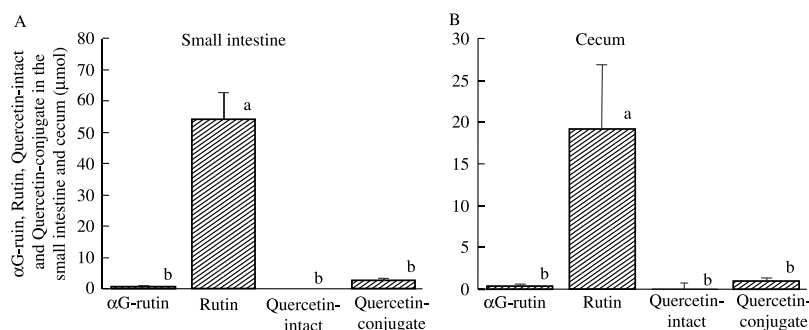

Figure 5. Remaining αG-rutin and rutin in the small intestine (A) and cecum (B). Rats were instillated with a αG-rutin solution (150 μmol/ml/rat), and the intestinal contents were collected after 150 min. Each value is the mean for ten rats. Value are means ± SEM, $n = 10$. Statistical analyses were performed by one-way ANOVA. The differences among treatment groups were analyzed with Duncan's multiple range test and were considered significant at $P < 0.05$.
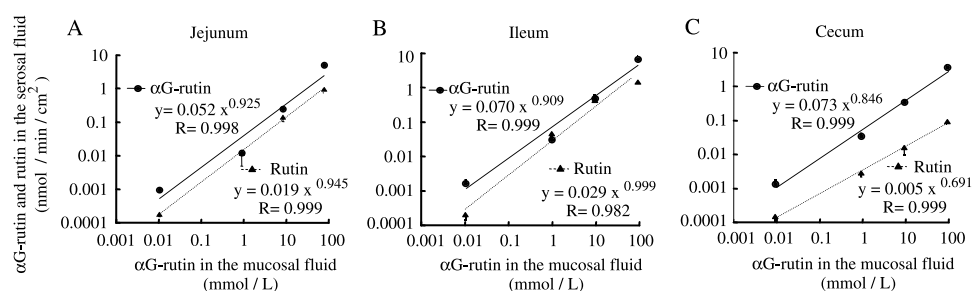
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Figure 6.   The transport and hydrolysis of αG-rutin for 30 min by the isolated mucosa of the rat jejunum (A), ileum (B) and cecum (C). Fresh HBS was applied to the serosal bath and 0.01, 1, 10 or 100 mmol/l of αG-rutin-HBS was applied to the mucosal bath and incubation for 30 min at 37°C. Value are means ± SEM, $n = 7$.

11.8 μmol/l than the level of intact αG-rutin in the systemic plasma.

Discussion

In this study, we examined intestinal absorption and metabolism of αG-rutin in *in vivo* (portal and duodenal cannulated rats) and *in vitro* (isolated mucosa of the intestines) experiments. Firstly, we established an analytical method for αG-rutin and rutin by using an LC/MS system because the separation of these compounds by HPLC is very difficult. The results of the LC/MS analysis clearly demonstrated that a considerable amount of intact αG-rutin was released into the portal blood after an administration of αG-rutin. To our knowledge, this is the first report that quercetin glycoside is transported through the intestinal mucosa as the intact form in *in vivo* study under unrestrained physiological conditions. Morand et al. [10] showed that only conjugated form of quercetin, but not the intact form, was released, into the abdominal aortic blood in rats after administration of quercetin-3-*O*-glucoside, another soluble quercetin glycoside. These two compounds are difference nature, that is, quercetin-3-*O*-glucoside has just one glucose moiety, however, αG-rutin has three different sugar moieties. We detected quercetin glycosides in the portal blood, and did not detect glycosides in the aortic blood, which agrees with the result of Morand et al. observed in the systemic circulation.

In *in vitro* study by using the isolated mucosa of the intestines, we also observed that both of αG-rutin and rutin were transported from the mucosal side to the serosal side without any hydrolysis to quercetin aglycone and conversion to quercetin conjugates. We validated the preparations by the initial TEER and no significant changes in TEER during experiments. Spencer et al. [19] also demonstrated that most of quercetin-3-*O*-glucoside and rutin were absorbed as intact forms in the perfusion study with the use of isolated rat jejunum and ileum, which supports our present results. The mechanism for intact αG-rutin transport through the intestinal mucosa is not known. It has been shown that quercetin-3-*O*-glucoside interacts with a sodium-dependent glucose transporter and is absorbed as a glycoside into the mucosal cells [20,21]. However, it is unlikely that αG-rutin, a glycoside with three sugar moieties is transported across the brush border membrane as the intact form. αG-rutin may be transported via the tight junction between the intestinal epithelial cells by diffusion. It has been reported that fluorescein isothiocyanate-dextran-4 (MW 4400) transported via the tight junction. This compound used as a paracellular passage marker and has much higher molecular weight than αG-rutin [22]. Also, we showed linear increases in αG-rutin transport from the mucosal side to the serosal side of the isolated mucosa with the application of αG-rutin from a low and physiological concentration, 10 μmol/l, up to a very high concentration, 100 mmol/l by graphs with logarithmic scales for both X and Y axes (Figure 6A, B and C). The linear and non-saturable increases dependent on mucosal αG-rutin demonstrates that the glycoside was transported through a simple diffusional pathway, which may be the paracellular route via the tight junction [23].

Table I.   TEER in the rat Intestinal epithelium before and after incubation for 30 min with αG-rutin.

	TEER (Ω cm^{-2})	
	Pre-incubation	Post-incubation (100 mmol/l αG-rutin)
Jejunum	160 ± 5.4[b]	160 ± 10.0[b]
Ileum	140 ± 5.5[a]	148 ± 7.3[a]
Cecum	170 ± 11.8[a]	208 ± 22.0[a]
ANOVA		
P-values		
Portions (*P*)	0.0015	
Pre- or	0.1520	
post-incubation (*I*)		
$P \times I$	0.1095	

Values are means ± SEM, $n = 6$. Values in two columns not sharing a superscript letter are significantly different ($P < 0.05$) by Duncan's multiple-range test. TEER, transepithelial electrical resistance.
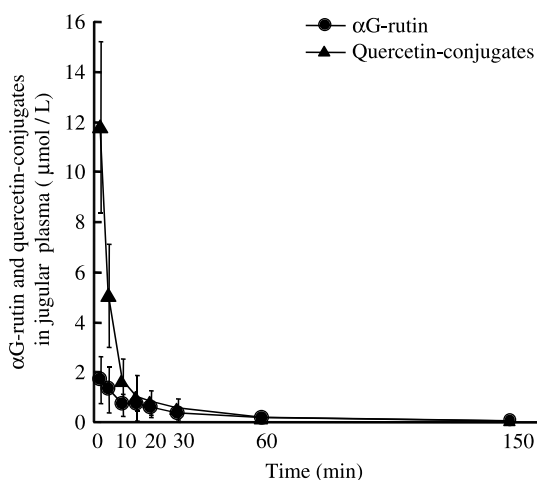
Figure 7.   Concentrations of αG-rutin and quercetin-conjugates in the jugular plasma of rats instillated with αG-rutin. Jugular blood was collected at 2, 5, 10, 15, 20, 30, 60 and 150 min after instillation of the αG-rutin solution (5 μmol/0.5 ml/rat) to the portal vein. Value are means ± SEM, $n = 9$.

We detected very low, but significant amounts of rutin in the portal plasma. αG-rutin is stable for the analytical procedure used in this study, and αG-rutin products contain less than 1% rutin (data not shown). We found a large amount of rutin with αG-rutin in the small intestinal lumen 150 min after an instillation of αG-rutin, and found that a comparable amount of rutin appeared in the serosal fluid in the *in vitro* experiment using the isolated mucosa. These results indicate that rutin is produced in the intestinal lumen from αG-rutin before absorption.

The quercetin aglycone and conjugate levels were much higher than that of αG-rutin and rutin in the portal plasma. The finding indicates that the major part of αG-rutin was hydrolyzed into quercetin during or after absorption. However, in the experiment by using the isolated intestinal mucosa, we did not find any aglycone of quercetin in the serosal fluid. It has been shown that β-glucosidase in the rat small intestine efficiently hydrolyzes quercetin glucosides, however rutin is a poor substrate for this enzyme [24,25]. Sheep lactose-phloridzin hydrolase (LPH) in the small intestine was able to hydrolyse some quercetin glucosides but not rutin [26]. These previous reports and our present results suggest that aglycone or conjugates appeared in the portal blood after administration of αG-rutin are not produced by the intestinal mucosal cells, but converted after absorption into the portal blood. We investigated that a large amount of αG-rutin instilled into the portal vein was very rapidly disappeared and high level of the quercetin-conjugates appeared into the jugular blood, which indicates efficient hydrolysis of αG-rutin to aglycone and conversion to conjugates by tissues other than the intestine. It has been reported that flavonoid compound is deglycosylated in the pig liver

and conjugated in the human liver cell [27,28,29]. These results reveal that intact αG-rutin absorbed into the portal blood very rapidly converted to aglycone and to form conjugate. This finding and results with the Ussing chamber study suggest that considerable part of αG-rutin is absorbed as intact form from the intestine and rapidly converted to quercetin conjugate in tissues other than the intestine, which maybe the liver. No conversion of αG-rutin into aglycone or conjugates in the intestine should be evidenced also by the *in vivo* study in future.

We measured remaining luminal quercetin-related compounds derived from αG-rutin 150 min after αG-rutin instillation, and the amount of these compounds remaining in the lumen was 88.6 μmol, which corresponds to 58% of the administered αG-rutin (150 μmol). It has been reported in *in vivo* studies that rutin is scarcely absorbed in the stomach or small intestine, but hydrolyzed by enterobacterial enzymes and absorbed from the colon [13,16,30]. However, we found no hydrolytic activity of rutin and also degradation activity of quercetin aglycone in the small intestinal and cecal contents in a preliminary experiment. These findings indicate that considerable part of the αG-rutin was absorbed from the intestine to the portal blood without degradation of its aglycone structure in the lumen. We also detected small amounts of quercetin and quercetin-conjugates in the intestinal lumen. These metabolites may be diffused from the portal blood to the intestinal lumen.

Quercetin and rutin are strong antioxidants in human [6,7,31]. Dose of αG-rutin using in *in vivo* present study is rather high, 150 μmol. This dose is comparable to 1/3 of 0.5% quercetin diet (rat fed 25–30 g/day), in which quercetin acts as an anticarcinogen. [32] Also, it is possible that αG-rutin is supplied in a high dose as a supplement because, αG-rutin is already manufactured and used as safety food additives. Efficient and rapid absorption of αG-rutin and metabolized to quercetin shown in the present study, may be true in human. αG-rutin will be an effective source of quercetin and expected to be beneficial as antioxidant for human health. It is, however, still necessary to elucidate the mechanism for the absorption of naturally occurring, water soluble flavonoid compounds in future.

We conclude that a considerable part of αG-rutin was absorbed as the intact form from the intestine via the paracellular pathway, and the major part of absorbed glycosides was rapidly converted to its conjugates in an organ other than the intestine.

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