Enteric Excretion of Baicalein, a Flavone of Scutellariae Radix, via Glucuronidation in Rat: Involvement of Multidrug Resistance–Associated Protein 2

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Purpose. Baicalin (BG) and its aglycone, baicalein (B), are strong antioxidants and have various pharmacological actions. The purpose of this study was to evaluate efflux of BG from rat intestinal mucosal cell following glucuronidation of B absorbed after oral administration of B.

Methods. The absorption and excretion of BG and B were evaluated in rats using the *in situ* jejunal loop technique and *in vitro* jejunal everted sac experiments. BG and B levels were determined by high-performance liquid chromatography with electro-chemical detection to ensure selectivity and high sensitivity.

Results. A large amount (30.4% recovery) of BG, but no B, was detected in the intestinal lumens of germ-free rats 4 h after oral administration of B (12.1 mg/kg), in comparison with a substantial recovery (55.1%) of unabsorbed BG 4 h after its administration. During the *in situ* rat jejunal loop absorption experiment, B disappeared rapidly, and 8% of the lost B was excreted into the loop as BG 20 min after infusing 0.1 mM B. In an *in vitro* absorption experiment using everted rat jejunal sac, BG also appeared outside the sac, accompanied by the disappearance of B from the outer (mucosal) side. However, very little of B was transferred to the inner (serosal) side of the sac, and only a trace of BG was detected inside the sac. Thus, in both the loop and the everted sac systems, the efflux of BG from the mucosal surface was saturated with the concentration of B added. Moreover, the efflux rate of BG in the everted jejunal sac from Eisai hyperbilirubinemic rat (EHBR) was significantly lower by 56.4% than that from Sprague-Dawley rat.

Conclusions. These results indicate that, in rat, a large proportion of any B absorbed is retained, transformed into BG within the intestinal mucosal cells, and coordinately excreted through multidrug resistance–associated protein 2 (MRP2) into the intestinal lumen.

KEY WORDS: baicalin; baicalein; glucuronidation; intestinal excretion; MRP2.

INTRODUCTION

Scutellariae Radix, the root of *Scutellaria baicalensis* Georgi (Labiatae), is used in combination with other herbs in Oriental (Kampo) medicines, and contains baicalin (BG, 5,6,7-trihydroxyflavone-7-β-D-glucuronide; Fig. 1) as its main active constituent. BG and its aglycone, baicalein (B), have wide-ranging pharmacological effects, such as antiallergic (1), anti-inflammatory (2), antiviral (3,4), antiproliferative (5), and antitumor (6) effects, and also show strong antioxidant activity (7,8). In addition, they inhibit prostaglandin E2 production and prevent inducible nitric oxide synthase and cyclooxygenase-2 gene expression (9). However, few studies have been performed on their disposition in mammals.

BG has been detected in the plasma after oral administration to rats (10), suggesting that it is absorbed directly from the gastrointestinal tract. However, our previous *in vitro* and *in vivo* studies have demonstrated rapid absorption of B, but poor absorption of BG, from the rat intestinal tract (11). Moreover, BG, but not B, has been detected in the plasma of rats administered B orally, and B is efficiently conjugated to BG in rat hepatic and intestinal microsomes. These observations indicate that BG itself is poorly absorbed from the rat gut, but is hydrolyzed to B by intestinal bacteria and then reconverted to its original form after absorption. Although rat intestinal microsomes have a similar level of glucuronosyl transferase activity for B to hepatic microsomes (11), which seem to be the main site of metabolism for xenobiotics (including B), the contribution of the intestine to the *in vivo* glucuronidation of B in rats remains obscure.

In this study, we investigated the intestinal disposition of BG and B after oral administration using rat jejunal loop and everted sac systems. We demonstrated that B was efficiently conjugated to BG in rat intestine during absorption, and that this was followed by rapid excretion of BG into the intestinal lumen.

MATERIALS AND METHODS

Chemicals

BG and B of standard grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The internal standards used for determination of BG and B on high-performance liquid chromatography (HPLC) were 2,3-hydroxynaphthalene and quercetin, respectively, which were purchased from Aldrich (Milwaukee, WI, USA) and from Nacalai Tesque (Kyoto, Japan), respectively. The solvents used for HPLC were of special HPLC grade. All other reagents were of the best quality available commercially.

Animals and Treatment

Male Wistar, Sprague-Dawley (SD) and Eisai hyperbilirubinemic (EHBR) rats (6 weeks old) were purchased from SLC Co. (Hamamatsu, Japan), fed standard laboratory chow with water *ad libitum*, and maintained for 1 week before being subjected to the experiments. Male germ-free Wistar rats (WA/Jic; 6 weeks old) were purchased from Clea Japan Co. (Tokyo, Japan) and maintained under sterile conditions with sterile chow and water. The rats were maintained in the Laboratory Animal Research Center, and the animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Toyama Medical and Pharmaceutical University.

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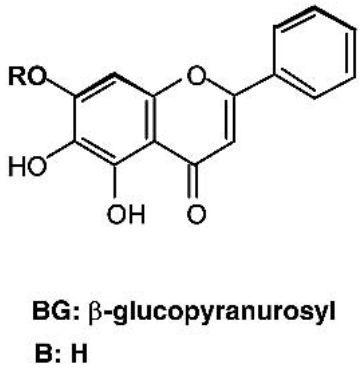

Fig. 1. Structures of baicalin (BG) and baicalein (B).

After an overnight fast, the conventional Wistar rats received oral BG at a dose of 20 mg/kg or B at a dose of 12.1 mg/kg (a dose equivalent to 20 mg BG/kg). The BG was dissolved in water (adjusted to pH 7 with phosphate buffer) to a concentration of 20 mg/ml, and the B was dissolved in dimethyl sulfoxide (final concentration 6% v/v), then diluted to 12.1 mg/ml with water just before administration. BG or B sterilized by filtration (sterile 0.45-μm pore membrane), at the same doses, were administered orally to the germ-free rats in an isolator. The gastrointestinal tracts of both types of rat were removed under anesthesia 1 or 4 h after administration and used immediately for the measurement of BG and B.

Investigation of Absorption and Excretion Using the Rat Jejunal Loop

The small intestines of conventional rats (four 7-week-old male Wistar rats for each concentration of the test compounds) that had been fasted overnight were exposed under pentobarbital anesthesia. A 10-cm loop of empty, middle-tract jejunum was prepared by ligating both ends. The loop remained *in situ* within the body cavity throughout the experiment. BG (0.1 mM) or B (0.02, 0.1, 0.5, or 1.0 mM), dissolved in 2.5 ml of aqueous solution containing 1 mg/ml bovine serum albumin (BSA), was instilled into the jejunal loop through a cannulated polyethylene tube introduced at one ligated end, and an aliquot (0.1 ml) of the solution was withdrawn from the loop at 0, 10, and 20 min after instillation. At the end of the experiment, the solution remaining in the jejunal loop was collected and its volume was measured. The amounts of BG and B in the solution were determined as described below.

Investigation of Absorption and Excretion Using the *in Vitro* Everted Rat Jejunal Sac System

The experiment was performed according to the method of Barr and Riegelman with a minor modification (11). Under pentobarbital anesthesia, a jejunal segment (about 10 cm long) was removed from a 7-week-old rat after an overnight fast. The segment was washed with saline, then a narrow plastic tube with numerous pores along its length was inserted through the lumen, and the segment was everted over the tube. The inside (serosal side) of the resulting sac was filled with 1.3 ml Krebs-Ringer bicarbonate solution containing 0.1% glucose and 1 mg/ml BSA. The intestinal sac was then placed in 20 ml of aqueous solution containing 1 mg/ml BSA and the required concentration of each drug (0.02, 0.1, 0.5, and 1 mM B or 0.1 mM BG). Aliquots (0.1 ml) of the fluids from the serosal (for transference to serosal sides) and mucosal sides (for absorption and excretion) were removed for analysis after 0, 10, 20, and 30 min of incubation at 37°C. At the end of the experiment, the solution remaining in the jejunal sac was collected and its volume was measured. In EHBRs and SD rats the mucosa was stripped off the sacs at the end and its weight was measured. The mucosa was homogenated with one volume of pure water and immediately with two volumes of methanol to extract BG and B, and then centrifuged to get the clear supernatant after acidification with 1 N HCl. The amounts of BG and B in the solution and the mucosa were determined as described below.

Inhibitory experiments on BG efflux were carried out by incubating 0.1 mM B together with agents such as probenecid (5 mM) and 1-chloro-2,4-dinitrobenzene (1 mM) in the outside (mucosal surface) of the everted sac for 30 min under the same condition as above.

Determination of BG and B

A portion of the gastrointestinal contents was sonicated and extracted with 3–4 volumes of methanol under acidic conditions as described previously (11). The determination of BG and B was carried out by HPLC on a LC-10AD Liquid Chromatograph (Shimadzu, Kyoto, Japan) equipped with an electrochemical detector (Coulochem Model 5100A, ESA, Inc., Bedford, MA, USA) at a potential of +100 mV. The column (Chemcosorb 7-ODS-H, 4.6 mm I.D. × 150 mm, Chemco Scientific Co., Ltd., Osaka, Japan) was eluted with the mobile phase described by Wakui *et al.* (10). Calibration plots of the peak areas were linear over a concentration range of 0.2–50 μM for BG ($r = 0.999$, coefficient of variation, CV = less than 3%) and of 1–50 μM for B ($r = 0.995$, CV = 3–11%). The recoveries of BG in both incubation buffer and mucosal homogenates were evaluated to be 86–102% and 76–95%, respectively, and the recoveries of B were 83–98% and 72–110%. The intra-day precision, expressed as CV, of the method for determination of BG and B at three concentrations (1, 5, and 20 μM) was less than 4% and 3–12%, respectively, in the incubation buffer, and less than 6% and 2–10% in the mucosal homogenates. The inter-day precision for BG and B at the same concentrations was 3–8% and 5–15%, respectively, in the incubation buffer, and 3–10% and 5–15% in the mucosal homogenates.

Statistical Evaluation

The results are expressed as the means ± SD of at least three independent experiments. Statistical analysis was performed using the unpaired Student's *t* test following the F test for two groups; a p value <0.05 was considered statistically significant.

RESULTS

Appearance of BG in the Rat Intestinal Lumen After Oral Administration of B

The recoveries of BG and B from the gastrointestinal tract after oral administration were estimated. When BG 20 mg/kg body weight was administered to conventional rats, a considerable amount (13.4% ± 3.1% of the dose) of BG and

an appreciable amount (21.9% ± 3.4%) of B were recovered from their intestinal tracts 4 h after administration. This was present mainly as BG in the small intestinal tract and mainly as B in the cecal and colonic tract (Fig. 2a). BG was also administered to germ-free rats to remove any influence of the intestinal bacteria. In these rats, a substantial proportion (55.1% ± 6.2%) of the BG dose, but only a trace of B, were recovered from the alimentary canals even at 4 h after administration (Fig. 3a). These results indicate that BG is poorly absorbed, but is transformed into B by the bacteria in the cecal and colonic tracts, and is then more readily absorbed.

On the other hand, in conventional rats given B orally at a dose of 12.1 mg/kg (a dose equivalent to 20 mg BG/kg), an appreciable amount (17.9% ± 3.6%) of BG, but only a small amount (3.89% ± 1.46%) of B, was recovered from the small intestinal tract 1 h after administration (Fig. 2b). At this time, 31.3% ± 18.6% of the B dose was still retained in their stomachs. Moreover, 4.90% ± 2.75% of the dose was detected as BG, not B, in the small intestinal tracts 4 h after administration; at this time, 15.7% ± 5.3% of the B was detected in the cecal and colonic tracts (Fig. 2c). The recovery pattern was similar to that seen after administration of BG (Fig. 2a). Surprisingly, when B was administered orally to germ-free rats, a large amount (30.4% ± 5.1%) of BG, but only a trace (1.11% ± 0.35%) of B, were recovered from the intestinal tract even at 4 h after administration (Fig. 3b). The recovery of only BG in the small intestine and cecum was similar to that observed after administration of BG (Fig. 3a), but with the low recovery (30.4%) in comparison with the latter recovery (55.1%). These results indicate that B, but not BG, is easily absorbed from the rat small intestine, and is effectively conjugated to BG in the body. A substantial proportion of the BG thus formed is then secreted into the intestinal lumen.

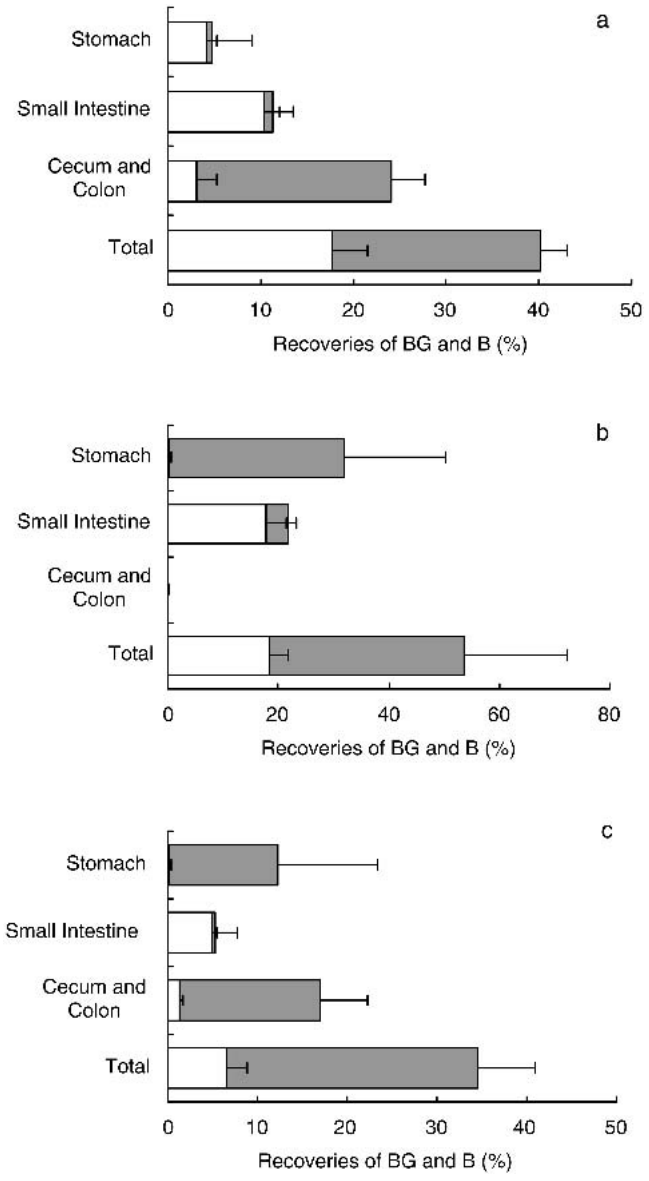

Fig. 2. Recovery (%) of BG (open column) and B (closed column) from the alimentary tract after oral administration of BG 20 mg/kg (a) or B 12.1 mg/kg (equivalent to 20 mg/kg BG) (b, c) to conventional rats (n = 3). (a) and (c) show the recovery 4 h after administration, and (b) shows recovery 1 h after administration. Each column represents the mean ± SD.

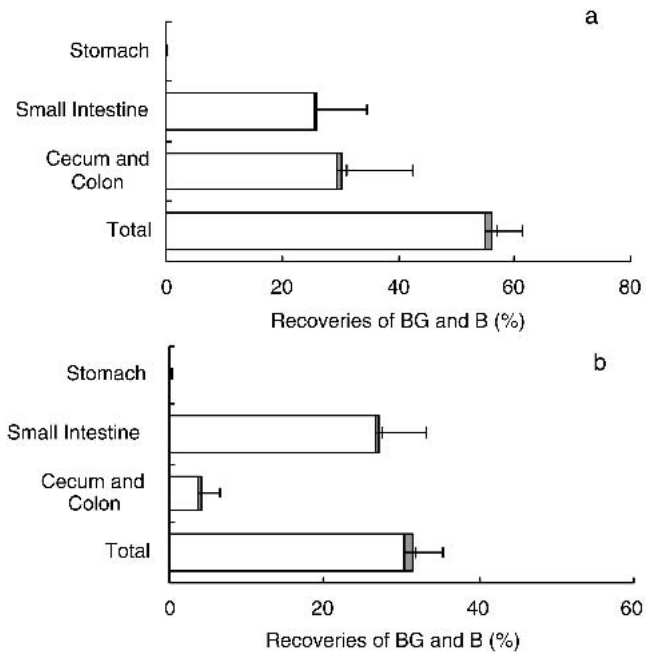

Fig. 3. Recovery (%) of BG (open column) and B (closed column) from the alimentary tracts 4 h after oral administration of BG 20 mg/kg (a) or B 12.1 mg/kg (equivalent to 20 mg/kg BG) (b) to germ-free rats (n = 3). Each column represents the mean ± SD.

Efflux of BG into the Rat Jejunal Loop After Instillation of B

The efflux of BG from the intestinal mucosal cells after the absorption of B was evaluated by the *in situ* jejunal loop method to remove any contribution of the biliary ducts to the secretion process. When BG (100 μM, 2.5 ml) was instilled into the rat jejunal loops (10-cm length), 73.2% ± 15.6% of the dose (113 ± 18.4 μM baicalin) remained in the loop after 20 min, and no B was detected (Fig. 4a).

On the other hand, when B (100 μM, 2.5 ml) was instilled into the loops, it disappeared quickly, whereas BG appeared and its concentration increased time-dependently (Fig. 4b). The B level decreased to 14.2% ± 4.1% of the dose (to a

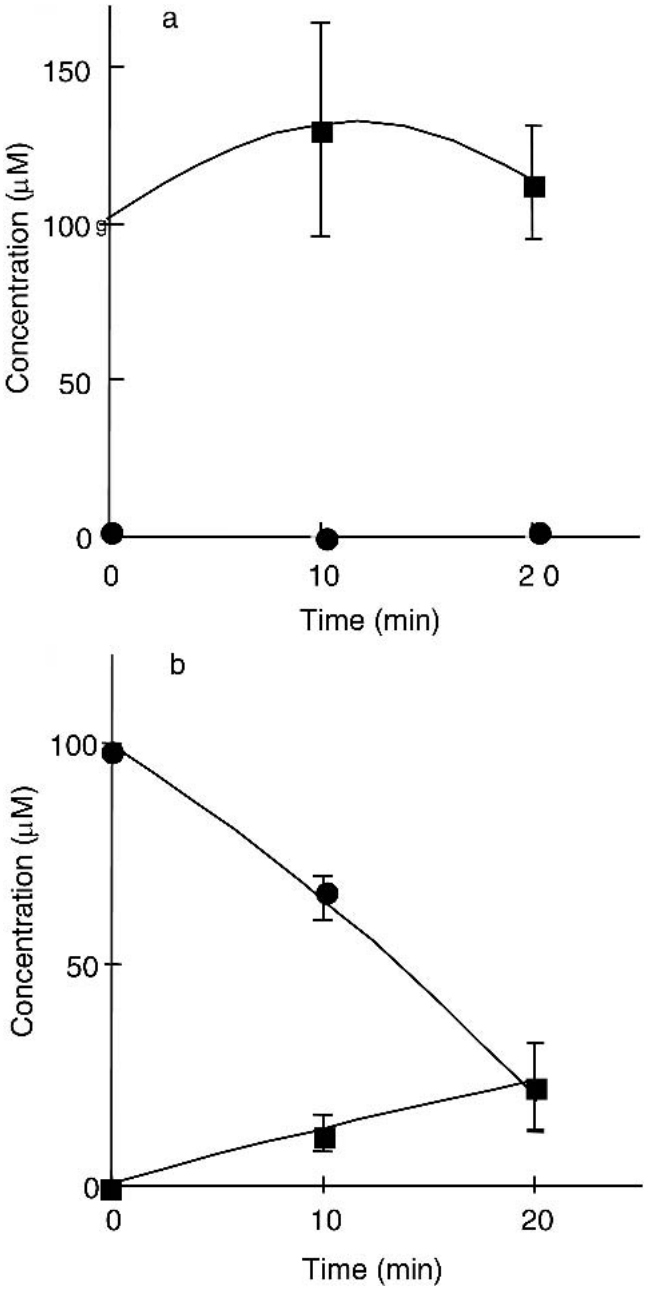

Fig. 4. Absorption of BG and B from the rat jejunal loop, and efflux of BG, after instillation of B. BG (a, upper) or B (b, lower) was instilled into 10-cm-long rat jejunal loops at a concentration of 0.1 mM in 2.5 ml. The BG (■) and B (●) levels in the loops were measured after 0, 10, and 20 min. Each point represents the mean ± SD of four different experiments.

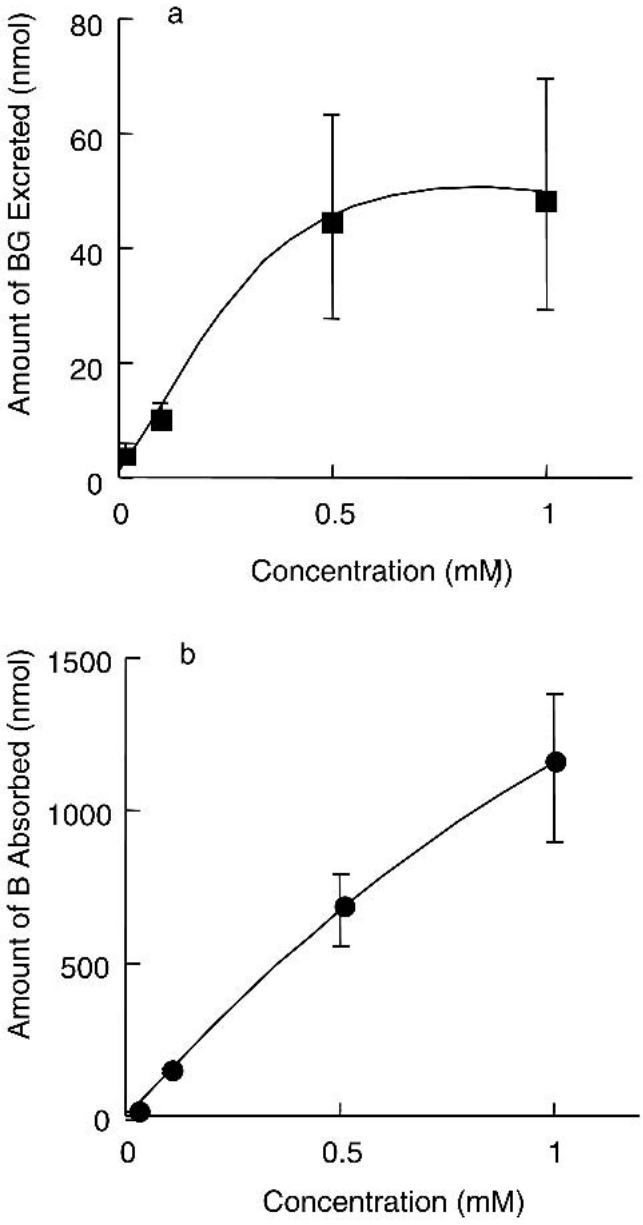

Fig. 5. Effect of the B concentration instilled into the rat jejunal loop on B absorption (b, lower) and BG efflux (a, upper). Various concentrations of B were instilled into 10-cm-long rat jejunal loops as indicated. After 20 min, the amounts of B lost (●) and the amounts of BG excreted into the loop (■) were determined. Each point represents the mean ± SD of four different experiments.

concentration of 22.0 ± 10.1 μM), whereas the BG level reached 22.3 ± 9.6 μM (about 8% of the amount of B lost) 20 min after instillation. The amount of B lost (absorbed) from the loop was directly proportional to the concentration of B instilled within the range 0.02 to 1 mM (Fig. 5b), while when 0.5 mM of B was instilled, the amount of BG excreted into the loop was saturated (Fig. 5a). Thus, any B absorbed into the jejunal mucosal cells appeared to be glucuronated, then coordinately excreted into the intestinal lumen directly from the cell, which may therefore have a BG-excreting transporter.

Efflux of BG from the Mucosal Side of the Everted Jejunal Sac After Application of B

A 10-cm-long everted rat jejunal sac was used to quantify transference of both test compounds into the intestine via the serosal surface and their efflux from the mucosal surface. BG (100 μM) applied to the mucosal surface (the outside of the sac) penetrated very poorly through the serosal side over 30-min incubation period (data not shown). B (100 μM) also penetrated very poorly through the serosal side (Fig. 6), although when it was applied to the mucosal side it decreased to almost the same extent as observed with the *in situ* jejunal loops (data not shown). Meanwhile, BG appeared on the mu-

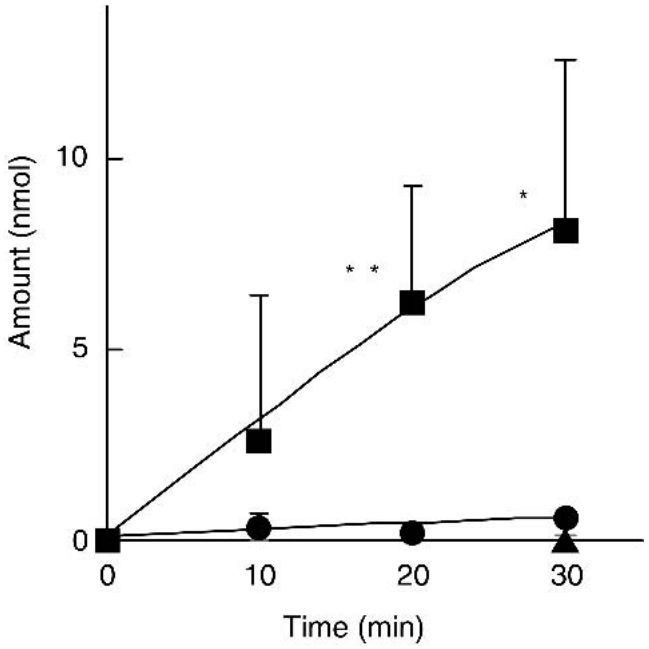

Fig. 6. Transference of B into and efflux of BG from rat everted jejunal sacs. B (0.1 mM) was applied to the outsides (mucosal surfaces) of the 10-cm-long sacs and incubated at 37°C for 30 min. The amount of B (●) transferred to the insides (serosal surfaces) of the sacs, and the amounts of BG on the outsides (■) and insides (▲), were measured at 0, 10, 20, and 30 min. Each point represents the mean ± SD of four different experiments. *$p < 0.05$, **$p < 0.01$ (vs. B concentration on the inside).

cosal side and its amount increased time-dependently (Fig. 6), whereas only a trace was apparent on the serosal side. The amount of BG excreted from the mucosal surface was significantly larger than the amount of B transferred through the serosal surface, but was only about half of that excreted by *in situ* jejunal loops of the same length.

The amount of B lost (absorbed) from the mucosal side increased in direct proportion to the concentration applied to the mucosal side within the range 0.02 to 1 mM (Fig. 7b), as observed with the jejunal loops. However, the ratio of the amount transferred through the serosal surface against the amount absorbed was extremely low at all concentrations tested (0.95% at 1 mM). Meanwhile, the efflux of BG from the mucosal surface was saturated when 0.5 mM B applied (Fig. 7a). On the other hand, the amounts of BG detected on the serosal sides were much lower (less than 7% of the amount excreted). These results demonstrate that the B absorbed by the intestinal mucosal cells penetrated to the serosal side of the intestine with great difficulty. Indeed it was transformed into BG, the major proportion of which was excreted into the intestinal lumen rather than being absorbed from the serosal surface.

Involvement of Multidrug Resistance–Associated Protein 2 (MRP2) in BG Efflux

Effects of probenecid and 1-chloro-2,4-dinitrobenzene, inhibitors of MRP2 (12), on BG efflux in the everted sacs of Wistar rats were first examined. The BG efflux from the mucosal surface was inhibited by 75.7% or 45.1% with the former (5 mM) or the latter (1 mM), respectively, but BG

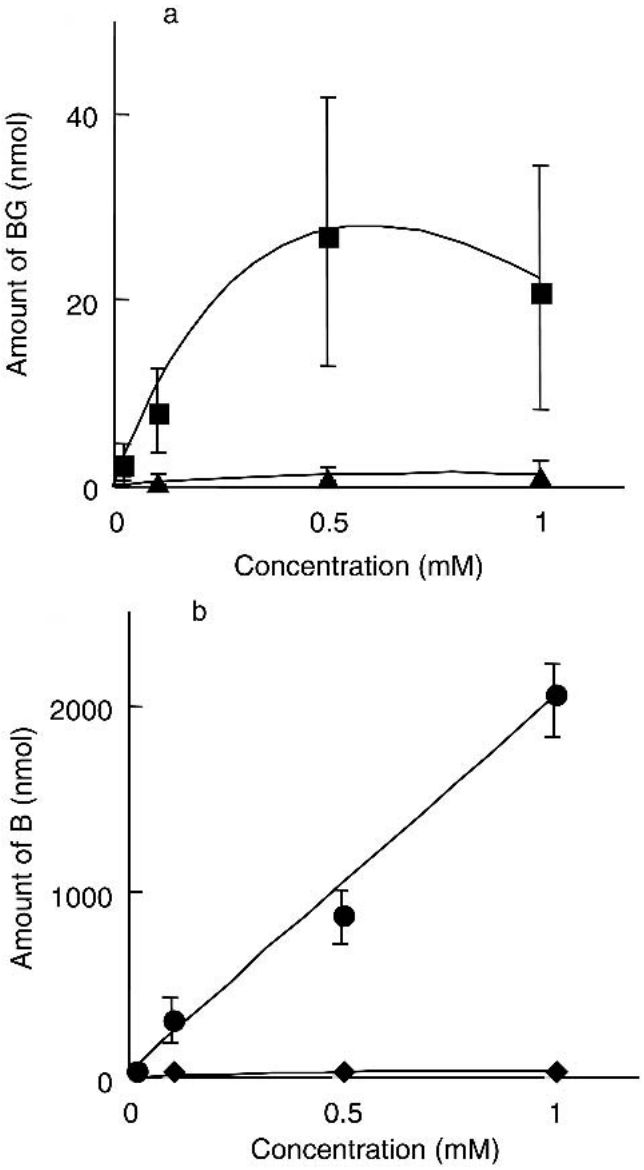

Fig. 7. Effect of the B concentration applied to the outside of the rat everted jejunal sac on absorption, transference, and efflux. Various concentrations of B were applied to the outsides of the sacs. After incubation at 37°C for 30 min, the amounts of B (b, lower) lost from the outsides (●) and transferred to the insides (♦) of the sacs were determined, and the amounts of BG (a, upper) excreted outside (■) and inside (▲) the sacs were measured. Each point represents the mean ± SD of three or four different experiments.

amount in the mucosa was also decreased by 48.4% or 62.3%, respectively, suggesting the inhibition of glucuronidation of B to BG.

BG efflux from the mucosal surface of the everted jejunal sacs in EHBRs, in which MRP-2 is hereditarily defective (13), was examined in comparison with that in SD rats. The decreasing rates of B from the mucosal sides and the amounts of B in the mucosa were almost the same in the both groups after application of B (0.1 mM) to the mucosal surfaces. However, the efflux rate of BG from the mucosal surface in EHBRs was significantly lower by 55.8% than that in SD rats (Fig. 8). On the other hand, the amount of BG recovered in the mucosa of the former was more than that of the latter

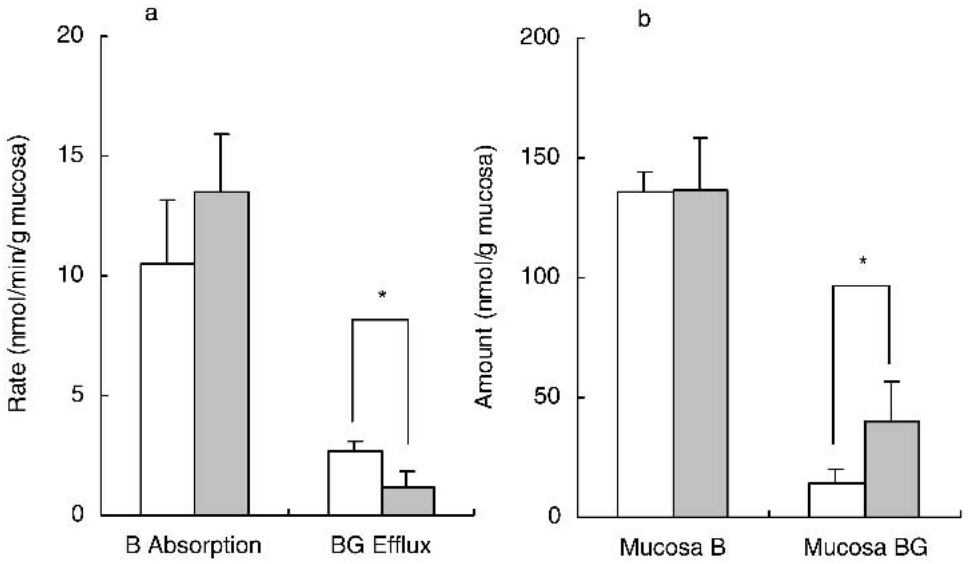

Fig. 8. B absorption and BG efflux (a, left bar graph), and mucosal B and BG amounts (b, right bar graph) in the everted jejunal sacs from EHBRs and SD rats. B (0.1 mM) was applied to the outsides (mucosal surfaces) of the 10-cm-long sacs from EHBRs (closed column) and SD (open column) rats and incubated at 37°C for 30 min. The time courses of B absorption (lost from the outside) and of BG efflux (excreted from the mucosal surface) were evaluated, and their rates (nmol min^{-1} g^{-1} mucosa) were expressed in the left graph. The amounts of BG and B in the mucosa of the sacs were measured after 30 min incubation (right graph). Each column represents the mean ± SD of four different experiments. *$p < 0.05$.

after incubating for 30 min, indicating that glucuronidation capacity for B in the jejunal mucosa was almost the same in both rats. Both the penetration of B to the serosal sides and the amount of BG detected in the serosal sides were much low and no differences between the two kinds of rats (data not shown). These results indicate that MRP2 contributes BG efflux from rat jejunal mucosal surface.

DISCUSSION

When BG, the 5,6,7-trihydroxyflavone glucuronide of Scutellariae Radix, is administered orally to conventional rats, BG (but not B, the aglycone of BG) is detected in their plasma (10). Although it has been thought that BG itself is absorbed from the rat gastrointestinal tract, our previous experiments have shown that it is poorly absorbed from the rat gastrointestinal tract, but is absorbed as B formed by the intestinal bacteria, then converted back to its original form (11). The current study demonstrates that a large proportion of the B absorbed is reconverted to BG in rat intestinal mucosal cells, and is then co-ordinately excreted into the intestinal lumen. Thus, BG has a unique metabolic fate.

When BG was administered orally to germ-free rats, 55.1% of the dose was left in their intestinal tracts and no B was detected even at 4 h after administration (Fig. 3a), confirming that the BG is indeed poorly absorbed. On the other hand, when B was administered orally to germ-free rats, high level of BG (30.4%), but not B, were recovered from their intestinal tracts (Fig. 3b). These results indicate that B is rapidly and completely absorbed, but that a large proportion is subsequently secreted into intestinal lumen as BG. The ratio of BG secretion to B absorption was estimated to be more than 55.3% based on the above recovery values (55.1% and 30.4%). When B was administered to conventional rats, an appreciable amount (6.7%) of BG was detected in their intestinal tracts (mainly the small intestinal tracts) 4 h after administration, in addition to B (16.2%), which was found mainly in the cecal and colonic tracts (Fig. 2c). As B was completely absorbed from the rat small intestine, the B (15.5%) recovered from the cecum and colon was thought to be a hydrolysate of the BG excreted into the intestinal lumen, produced by the intestinal bacteria. Accordingly, at least 22.2% of the administered B dose was recovered as BG from the intestinal lumens of conventional rats 4 h after administration. Although as much as 17.9% BG was detected in the small intestinal tracts of conventional rats 1 h after the administration of B, 29.7% of the B was still retained in their stomachs (Fig. 2b). Moreover, similar recovery profiles for BG and B (Figs. 2a and 2c) were observed after oral administration of either BG or B to conventional rats. In our previous study, similar concentration-time profiles for plasma BG were also observed after oral administration of either BG or B to conventional rats (11). These results suggest that most of an orally administered B dose is rapidly absorbed, effectively converted to BG and excreted into the intestinal lumen in rats.

One of the main routes for efflux into the intestinal lumen is the bile ducts, and biliary excretion of BG has been reported after oral administration of BG or B (14). However, as the amount of BG excreted in the bile is very small (less than 1% of a BG dose and less than 3% of a B dose, even after 12 h), and is much lower than for other metabolites, BG efflux via the biliary route is not considered significant. In the current study, it was confirmed that the BG formed from the absorbed B in the *in situ* jejunal loops was excreted into the intestinal lumen directly from the intestinal mucosal cells (Fig. 4b). When instilled at 0.1 mM, about 8% of the absorbed B had been excreted as BG from the jejunal loops 20 min

after instillation. However, as transference to the portal vein was difficult to assess quantitatively in the loop experiment, a quantitative evaluation of both the excretion of BG from the mucosal surface and the transference of B through the serosal surface was performed using the everted jejunal sac technique. Again, this showed that BG was excreted from the mucosal side of the sac after application of B (0.1 mM) to the mucosal surface (Fig. 6). Meanwhile, the amount of B transferred to the serosal side was extremely small (one fourteenth of the amount of BG excreted), and the amount of BG on the serosal side was much less. Moreover, more than 50% of the B lost from the mucosal surface was recovered in the mucosal cells of the sac after 30 min of incubation (data not shown). Thus, it was evident that a large proportion of any B absorbed into rat jejunal mucosal cells is retained, and that most of the BG formed from it is coordinately excreted into the intestinal lumen. Intestinal disposition and enteric recycling may be more important than hepatic disposition and enterohepatic cycling, respectively, in the first-pass metabolism of B, assumed from metabolism of flavonoids such as genistein and apigenin via enteric recycling (15).

The amount of B lost from the mucosal side of the sac was similar to that observed with the jejunal loop, and the amount of BG excreted from the sac was almost half of that excreted into the loop during a 20-min incubation, suggesting that the systems gave consistent results despite the lower activity of the everted sac. The rapid uptake of B into the mucosal cells depended on the concentration added (up to 1 mM) in both systems (Figs. 5b and 7b), implying passive transport. The transference of B was also proportional to the added concentration. In contrast, when 0.5 mM B was applied to both the loops and the sacs, the efflux of BG was saturated (Figs. 5a and 7a). Moreover, the BG efflux in the everted jejunal sacs from EHBRs was significantly lower than that from SD rats (Fig. 8), indicating that MRP2 contributes the efflux from rat jejunal mucosal surface. This is supported by the fact that BG is a potent inhibitor of rat MRP2 (12). MRP2 inhibitors such as probenecid and 1-chloro-2,4-dinitorbenzene suppressed BG efflux in the everted sac. However, as the inhibitors also decreased the BG amounts in the mucosa of the sac, the suppression of BG efflux might result from the decrease of B glucuronidation (BG production).

In conclusion, the results of our present *in vivo* experiments clearly show that the BG found in the intestinal lumen after oral administration of B to rats (Figs. 2 and 3) was almost all excreted directly from the intestinal mucosal cells, mainly through MRP2.

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