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Breast Cancer Resistance Protein (Bcrp1/Abcg2) Limits Net Intestinal Uptake of Quercetin in Rats by Facilitating Apical Efflux of Glucuronides

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ABSTRACT

The intestinal absorption of the flavonoid quercetin in rats is limited by the secretion of glucuronidated metabolites back into the gut lumen. The objective of this study was to determine the role of the intestinal efflux transporters breast cancer resistance protein (Bcrp1)/Abcg2 and multidrug resistance-associated protein 2 (Mrp2)/Abcc2. To study the possible involvement of Mrp2, we compared intestinal uptake of quercetin-3-glucoside between control and Mrp2-deficient rats, using an in situ intestinal perfusion system. The contribution of Bcrp1 was determined using the specific inhibitor fumitremorgin C (FTC) in Mrp2-deficient rats. Furthermore, vectorial transport of quercetin was studied in Madin-Darby canine kidney (MDCK)II cells transfected with either human MRP2 or murine Bcrp1. In these MDCKII cells, we showed an efficient efflux-directed transport of quercetin by mouse Bcrp1, whereas in control and MRP2-transfected cells no vectorial transport of quercetin was observed. In Mrp2-deficient rats, intestinal uptake of quercetin from quercetin-3-glucoside, efflux of quercetin glucuronides to the gut lumen, and plasma concentration of quercetin were similar to that in control rats. When intestinal Bcrp1 was inhibited by FTC in Mrp2-deficient rats, total plasma concentrations of quercetin and its methylated metabolite isorhamnetin after 30 min of perfusion were more than twice that of controls (12.3 ± 1.5 versus 5.6 ± 1.3 μM; $p < 0.01$), whereas uptake of free quercetin from the intestinal lumen was not affected. Instead, inhibition of Bcrp1 lowered the efflux of quercetin glucuronides into the perfusion fluid by approximately 4-fold. In conclusion, Bcrp1 limits net intestinal absorption of quercetin by pumping quercetin glucuronides back into the lumen.

Flavonoids are currently being recognized as bioactive compounds from plant food with potential beneficial effects on public health. Important sources of flavonoids are fruits and beverages and to a lesser extent, vegetables (Scalbert and Williamson, 2000). For some of these flavonoid and flavonoid-rich products, a protective effect on cancer and particularly cardiovascular diseases is reported (Law and Morris, 1998; van't Veer et al., 2000; Arts and Hollman, 2005). In experimental studies, flavonoids have been shown to beneficially affect several aspects of carcinogenesis and the development of cardiovascular diseases (Middleton et al., 2000). Uptake of flavonoids from the intestinal lumen into plasma is limited because of incomplete intestinal absorption or rapid biliary efflux (Crespy et al., 2003; Arts et al., 2004). In food products, flavonoids are largely present as glycosides. Recent experimental in vivo studies suggest that extracellular hydrolysis of the glycosides by intestinal lactase phlorizin hydrolase plays a major role in flavonoid absorption (Day et al., 2003; Sesink et al., 2003). After being released from the sugar moiety, the flavonoid aglycone enters the enterocyte, is partly methylated to isorhamnetin, and both compounds are conju-

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ABBREVIATIONS: Pgp, P-glycoprotein; MRP2/Mrp2, multidrug resistance-associated protein 2; BCRP/Bcrp1, breast cancer resistance protein; MK-571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid; MDCK, Madin-Darby canine kidney; FTC, fumitremorgin C; PSC833, 3-oxo-4-butenyl-4-methyl-(Thr1)-(Val2)-cyclosporin; HPLC, high-performance liquid chromatography; SN-38, 7-ethyl-10-hydroxycamptothecin; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridymethyl) benzothiazole.

gated with glucuronic acid by UDP glucuronosyltransferase or with sulfate by sulfotransferase (Crespy et al., 1999). A part of these conjugates is subsequently transported back into the intestinal lumen via the apical membrane of the enterocyte (Crespy et al., 2003; Day et al., 2003; Sesink et al., 2003). Crespy et al. (2003) showed that apical efflux of conjugated flavonoid metabolites depended on the structure of the flavonoid and varied from zero for catechin to 52% for quercetin (Crespy et al., 2003). Intestinal secretion of conjugates can be quantitatively similar to biliary secretion (Jia et al., 2004), indicating that this efflux pathway may significantly contribute to the low oral bioavailability of flavonoids. The mechanism of apical secretion is currently unknown. In the apical membrane of the small intestine, three transport proteins have been identified [i.e., P-glycoprotein (Pgp/multidrug resistance protein 1), multidrug resistance-associated protein 2 (MRP2/ABCC2), and breast cancer resistance protein (BCRP/ABCG2)]. Pgp is considered to be an efflux pump for large, uncharged, or cationic hydrophobic compounds, whereas MRP2 and BCRP can transport negatively charged drug conjugates (Jonker et al., 2000; Nakatomi et al., 2001; Chen et al., 2003). Considering the anionic nature of flavonoid conjugates, it seems likely that MRP2 and/or BCRP contribute to the efflux of flavonoid conjugates across the intestinal apical membrane. Both transporters have been shown to be able to modulate oral bioavailability of natural food components by limiting intestinal uptake of these components (Dietrich et al., 2003). In Caco-2 (Vaidyanathan and Walle, 2001) and HepG2 cells (O'Leary et al., 2003), the nonspecific MRP-inhibitor MK-571 reduced efflux of conjugated flavonoid metabolites. MRPs were also shown to mediate efflux of nonanionic flavonoid conjugates, such as quercetin-4′-glucoside (Walgren et al., 2000), phloretin-5-glucoside (Walle and Walle, 2003), and (−)-epicatechin-3-gallate (Vaidyanathan and Walle, 2003). For the anticancer drug flavopiridol, a conjugated flavonoid that is extensively glucuronidated in the body, it was shown that Mrp2 mediated biliary efflux of its glucuronide conjugates (Jager et al., 2003).

Flavonoids have been shown to be effective inhibitors of BCRP-mediated transport in vitro (Yoshikawa et al., 2004; Zhang et al., 2004). Moreover, the isoflavone genistein seemed to be a substrate for BCRP, as demonstrated by preferential basolateral-to-apical transport of this compound in BCRP-transduced renal kidney cells (LLC-PK1) (Imai et al., 2004). Therefore, in the present study, we explored the role of Mrp2 and Bcrp1 in intestinal absorption of the dietary flavonoid quercetin in rats. To investigate this, we performed in situ intestinal perfusion experiments using control Wistar Hannover rats and Mrp2-deficient TR^- rats and determined vectorial transport of quercetin in MRP2- or Bcrp1-transfected MDCKII cells.

Materials and Methods

Materials. Quercetin-3-glucoside was purchased from Extrasynthese (Genay, France). Quercetin and β-glucuronidase/sulfatase were obtained from Sigma-Aldrich (St. Louis, MO). Isorhamnetin (3′-methoxy-quercetin) was purchased from Roth (Karlsruhe, Germany). Fumitremorgin C (FTC) was a gift from Wyeth Research (Pearl River, NY). A mix of quercetin glucuronides used in this study was kindly provided by Karen O'Leary (Institute of Food Research, Norwich, UK), and all other chemicals were of analytical grade. The Mrp2-deficient Groningen Yellow/transport mutant rat strain (GY/TR^-:TR^- rats) were obtained from the group of Jansen (University of Groningen, Groningen, The Netherlands) (de Vries et al., 1989) and bred in Nijmegen.

Animals and Diets. The experimental protocol was approved by the Animal Welfare Committee of Wageningen University (Wageningen, The Netherlands). Male Wistar Hannover and TR^- rats were housed individually in a room with controlled temperature (22–24°C), relative humidity (50–60%), and light/dark cycle (lights on from 6:00 AM to 6:00 PM). During three consecutive days before the experiment, all rats were fed a commercially available soy-free semi-purified diet (54 g/kg dextrose, 5 g/kg cellulose, 10 g/kg corn starch, 20 g/kg casein, 5 g/kg corn oil, standard American Institute of Nutrition-76 vitamin, and mineral mix; Hope Farms, Woerden, The Netherlands). No quercetin could be detected in plasma of the rats before start of the experiments (limit of detection 11 nM).

In Situ Intestinal Perfusion Studies. We explored the role of Mrp2 by comparing intestinal uptake of quercetin-3-glucoside (a naturally occurring easily absorbable form of quercetin) between control Wistar Hannover rats ($n = 6$) and Mrp2 deficient rats ($n = 4$) (body weight 217 ± 2 g). To study the role of Bcrp1, a second study was conducted in which intestinal uptake of quercetin aglycone was measured in Mrp2-deficient rats (body weight 246 ± 6 g; $n = 12$) in the absence or presence of the specific Bcrp1-inhibitor FTC (10 μM) (Rabindran et al., 1998; van der Kolk et al., 2002). In situ perfusion of the cannulated small intestinal segment of Mrp2-deficient rats and control rats was performed as described previously (Arts et al., 2004). In brief, after anesthetizing the rat, the abdominal cavity was opened, and the portal vein was cannulated. Then, a cannula was inserted in the small intestine, distal of the entrance of the bile duct. The contents of the small intestine were removed by gently flushing with 60 ml of saline and a second cannula was then inserted proximal to the caecum. Perfusion was started by injecting 10 ml of the buffer containing the test compounds and connecting the intestine to a single-pass perfusion system. Blood samples were taken from the portal vein, and samples of the perfusion fluid were taken at the end of the cannulated segment of the small intestine. In the second study, the portal vein was not cannulated and one blood sample was taken from the vena cava inferior at the end of the experiment. Samples were treated as described previously (Arts et al., 2004). Volume changes in perfusion buffer caused by passage of the buffer through the cannulated intestinal segment were recorded by weighing. The amount of buffer recovered after intestinal perfusion was not significantly different in the absence or presence of FTC (control, 39 ± 1 ml; FTC, 36 ± 1 ml; $p = 0.08$).

In Vitro Transport Assays. For determining transcellular transport of quercetin aglycone, we used MDCKII cells transfected with human MRP2 or murine Bcrp1 (Evers et al., 1998; Jonker et al., 2000). Cells were seeded on Transwell polycarbonate membrane filters (3.0-μm pore size, 24.5 mm in diameter, Transwell 3414; Costar, Cambridge, MA) at a density of 2×10^6 cells per well. Cells were grown for 3 days in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum , with one change of medium. A 50 mM stock solution of quercetin was prepared in dimethyl sulfoxide and diluted to 50 μM in Opti-MEM with L-glutamine, containing 1 mM ascorbic acid (to prevent oxidation of quercetin) and 5 μM PSC833 (for specific inhibition of endogenous P-glycoprotein activity; van der Kolk et al., 2002). The experiment was started by replacing the medium on either the apical or basolateral side of the cells (donor compartment) with 2 ml of the quercetin solution (50 μM) in Opti-MEM. The opposite compartment (receiver compartment) was filled with 2 ml of Opti-MEM with ascorbic acid and PSC833, but without quercetin. At 2, 4, or 6 h, 75-μl samples were taken from the receiver compartment. Samples were acidified with 10 mM sodium acetate/5.7 mM ascorbic acid (final concentrations) and immediately frozen at −20°C. Samples were analyzed by HPLC. Apparent permeability coefficients (P_{app}) were calculated as follows:

$$P_{app} = \frac{V}{AC_0} \times \frac{dC}{dt} \quad (cm\ s^{-1})$$

In this equation, V is milliliters of medium in receiver compartment (2 ml = 2 cm^3), A is membrane surface area of Transwell filter (4.714 cm^2), C_0 is initial quercetin concentration in donor compartment (50 μM), and dC/dt is concentration quercetin in receiver compartment after 6-h incubation (Artursson and Karlsson, 1991). Transport ratio is defined as $P_{app,\ B\text{-}A}/P_{app,\ A\text{-}B}$, where $P_{app,\ B\text{-}A}$ is the apparent permeability coefficient for the basolateral-to-apical direction, and $P_{app,\ A\text{-}B}$ is the apparent permeability coefficient for the apical-to-basolateral direction.

In control experiments, paracellular flux of [^{3}H]inulin (0.15 μCi/ml) through the monolayer was always less than 1.5%, indicating intactness of the monolayer.

Preparation of Samples and HPLC Analyses. Samples were analyzed as described previously (Arts et al., 2004). In short, quercetin metabolites in plasma were deconjugated by treatment with β-glucuronidase/sulfatase. Enzyme-treated plasma and samples from the perfusion fluid and from the in vitro experiments were deproteinized with acetonitrile/20% *o*-phosphoric acid and analyzed by HPLC. For this, separation was performed using an acetonitrile gradient in citrate buffer on a reversed-phase HPLC column (Chromolith RP-18e; Merck, Darmstadt, Germany), coupled to a coulometric array detector (ESA Inc., Chelmsford, MA). Relative standard deviations for duplicate analyses of standards of quercetin-3-glucoside, quercetin aglycone, and isorhamnetin aglycone were 0.4% (n = 8), 0.06% (n = 17), and 0.2% (n = 13), respectively. The relative standard deviation of duplicate analyses of quercetin in plasma was 4%. Recovery of quercetin from plasma with enzymatic hydrolysis was 76 to 85% and that of isorhamnetin was 71 to 78%. Recovery of quercetin-3-glucoside and quercetin glucuronides, added to plasma without subsequent enzymatic hydrolysis, ranged from 83 to 115%. All standards and metabolites extracted from samples were stable during storage overnight in the autosampler (Arts et al., 2003). Because no glucuronides of quercetin could be detected in samples from the in vitro transport experiments, a UV detector set at 380 nm was used for detection of quercetin aglycone in these samples.

Statistics. Results are given as mean values ± S.E.M. A commercially available package (Prism version 4.02; GraphPad Software Inc., San Diego, CA) was used for all statistics. Analysis of variance followed by Bonferroni multiple comparison tests were used to test for significant differences. Differences were considered statistically significant when $p < 0.05$ (two-sided).

Results

The Effect of Mrp2 Deficiency on Net Intestinal Absorption of Quercetin from Quercetin-3-glucoside in Rats. The concentration of quercetin-3-glucoside in perfusion fluid before the experiment in the control group (54.8 ± 1.5 μM) did not differ from that in the group of Mrp2-deficient rats (53.1 ± 0.8 μM). Under the experimental conditions, the initial concentration of quercetin-3-glucoside did not decline and no free quercetin was formed in the tube containing the perfusion fluid, indicating stability of quercetin-3-glucoside during the 30-min perfusion. After passage through the cannulated intestinal segment, the concentration of quercetin-3-glucoside in the perfusion fluid of the control group had dropped to 38.8 ± 1.0 μM (disappearance of approximately 29%) and to 39.5 ± 1.0 μM (disappearance of approximately 26%) for the Mrp2-deficient rats. The difference was not statistically significant, indicating that intestinal hydrolysis of quercetin-3-glucoside was similar in both groups. The concentration of quercetin aglycone in the effluent after intestinal passage was also similar in both groups (2.6 ± 0.3 and 2.1 ± 0.3 μM for control and Mrp2-deficient rats, respectively; not significant). These results suggest that the amount of quercetin available for entering the intestinal cell does not differ between the experimental groups. Figure 1, with trace a representing a HPLC chromatogram of a mixture of quercetin glucuronides, shows that compounds with retention times similar to these standard quercetin glucuronides (indicated by an asterisk) were present in the perfusion fluid of both control rats (trace b) and Mrp2-deficient rats (trace c). These peaks were not present in the perfusion fluid before intestinal passage, suggesting that these peaks represent quercetin glucuronides that were removed from the small intestinal cells at the apical membrane into the perfusion medium. Semiquantification of these compounds by calculating mean peak heights showed that concentrations of these compounds in the perfusion fluid were comparable in both groups (data not shown). These results suggest that Mrp2 is not involved in the apical efflux of quercetin glucuronides from intestinal cells.

In plasma from portal vein, quercetin and its methylated metabolite isorhamnetin could only be measured after enzymatic hydrolysis by β-glucuronidase/sulfatase, indicating that transfer of free aglycones to the blood does not take place. In plasma from control rats, quercetin was the major form, reaching almost 7 μM after 30 min of intestinal perfusion, whereas the concentration of isorhamnetin was more than 4-fold lower than that of quercetin (Fig. 2). In accordance with measurements from the perfusion fluid, quercetin and isorhamnetin levels in plasma from Mrp2-deficient rats

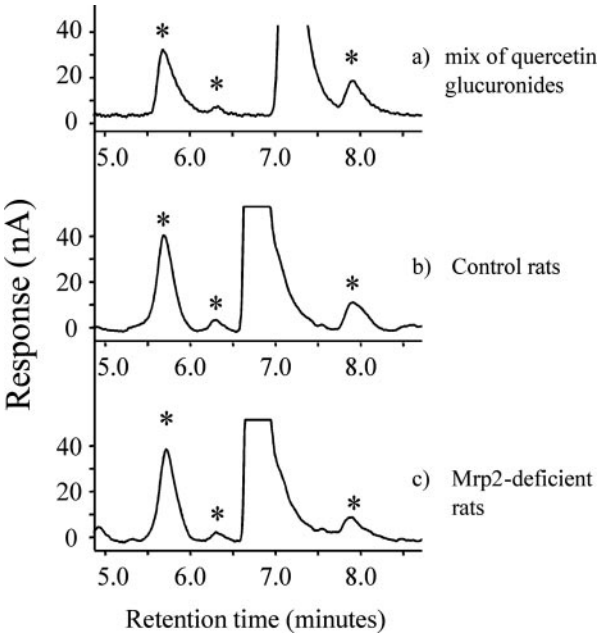

Fig. 1. HPLC-chromatograms of perfusion fluid from in situ rat intestinal perfusion experiments in control and Mrp2-deficient rats. Chromatogram of a mixture of quercetin glucuronides is shown in trace a (the peak just after 7.0 min also represents a glucuronide that is not present in perfusion fluid). The small intestine of control and Mrp2-deficient rats was perfused with 54 μM quercetin-3-glucoside, and samples were taken at the outlet of the perfused intestinal segment at 30 min. Samples of control (trace b) and Mrp2-deficient rats (trace c) were analyzed with coulometric array detection. Asterisks in the chromatograms represent glucuronides in the different samples with similar retention times. The large peak at approximately 6.9 min represents quercetin-3-glucoside.

did not differ from those in control rats, showing that Mrp2 is not limiting net absorption of quercetin from quercetin-3-glucoside in rat small intestine.

Transport of Quercetin across MDCKII Monolayers Transfected with Bcrp1 or MRP2. Because our first rat study showed that Mrp2 was not essential for the apical efflux of quercetin glucuronides from enterocytes, we hypothesized that the efflux transporter Bcrp1 might be involved. To study this, we measured transport of quercetin aglycone using MDCKII cells transfected with mouse Bcrp1 and compared this with parental and MRP2-transfected MDCKII cells as described under *Materials and Methods*. No conjugates of quercetin could be detected in the apical or basolateral compartments of either parental or transfected cells during the course of the experiment. In the Bcrp1-transfected cells, we found efficient basolateral-to-apical transport of quercetin, whereas transport of quercetin in the opposite direction was almost negligible (Fig. 3, top). Only a minor fraction of apically applied quercetin (0.6%) could be detected in the basolateral compartment after 6 h of incubation. The apparent permeability coefficient for the basolateral-to-apical direction ($P_{app,\ B\text{-}A}$) was $11.1 \pm 0.2 \times 10^{-6}$ cm s^{-1} ($n = 3$), which was significantly higher than P_{app} for the apical-to-basolateral direction ($P_{app,\ A\text{-}B} = 0.07 \pm 0.01 \times 10^{-6}$ ($n = 3$, $p < 0.001$ versus $P_{app,\ B\text{-}A}$). The high efficiency of this apically directed (efflux) transport of quercetin is reflected by a transport ratio of approximately 160. In contrast to the efficient quercetin efflux by Bcrp1, no directional transport was detected in either MRP2-transfected MDCKII cells (Fig. 3, middle) or parental MDCKII cells (Fig. 3, bottom).

Effect of Inhibition of Bcrp1 on Net Transfer of Quercetin across the Intestinal Wall in Mrp2-Deficient Rats. To study the role of Bcrp1 in the intestinal uptake of quercetin, we performed intestinal perfusion experiments in Mrp2-deficient rats to circumvent the possibility that Mrp2 compensated for Bcrp1 in transporting flavonoid conjugates when the latter is inhibited. Based on our in vitro studies showing that quercetin itself is efficiently transported by human BCRP, we choose to perfuse the intestine with quercetin aglycone itself instead of with its glucoside. Quercetin concentration in the perfusion fluid at the start of the experiment was similar in the control (43.7 ± 1.4 μM) and the FTC group (43.0 ± 2.8 μM). After 10 min of perfusion, concentration of quercetin in the perfusion fluid had dropped to 29.0 ± 2.0 μM in the control group and to 24.1 ± 2.6 μM in the FTC group ($p = 0.09$). This difference was smaller after 30 min of perfusion (28.1 ± 1.0 and 26.3 ± 0.7 μM for control and FTC group, respectively; not significant), indicating that uptake of quercetin from the perfusion buffer was not affected by Bcrp1-inhibition. Apart from quercetin, several other compounds were detected in the perfusion fluid after intestinal passage. Figure 4, with trace a representing an HPLC chromatogram of a mixture of quercetin glucuronides, shows that compounds with retention times similar to these standard quercetin glucuronides (indicated by an asterisk) were present in the perfusion fluid of both control rats (trace b)

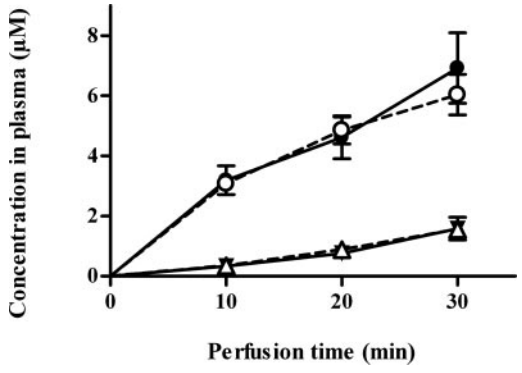

Fig. 2. Concentration of quercetin (●, ○) and its methylated metabolite isorhamnetin (▼, △) in portal vein plasma during in situ perfusion of small intestine with 54 μM quercetin-3-glucoside, comparing control Wistar Hannover rats (●, ▼) and Mrp2-deficient rats (○, △). Plasma samples were enzymatically hydrolyzed before analysis by HPLC. Values are means ± S.E.M. ($n = 6$ for control, $n = 4$ for Mrp2-deficient rats). There were no statistically significant differences between controls and Mrp2-deficient rats.

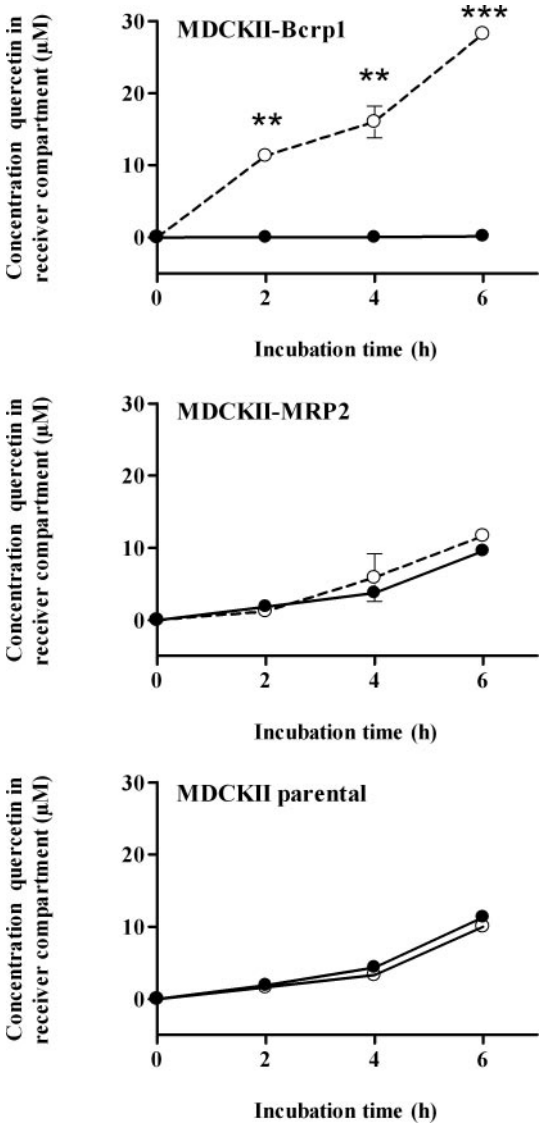

Fig. 3. Transport of quercetin in MDCKII cells transfected with murine Bcrp1 (top) or human MRP2 (middle) and in parental MDCKII (bottom). Cell lines were preincubated for 2 h with 5 μM PSC833 (in 2 ml OptiMEM in each compartment). At the start of the experiment, 50 μM quercetin was applied to the donor compartment and appearance of quercetin in the receiver compartment was determined at times indicated in the figure. ●, apical-to-basolateral transport; ○, basolateral-to-apical transport. Values are means ± S.E.M. ($n = 3$ for all groups). Significance levels, basolateral-to-apical transport of Bcrp1-transfected cells, compared with apical-to-basolateral transport, are **, $p < 0.01$; ***, $p < 0.001$.

and rats from the FTC group (trace d). These compounds in the perfusion fluid coeluted with a spiked mixture of quercetin glucuronides (not shown) and were refractory to β-glucuronidase/sulfatase treatment [trace c (control) and trace e (FTC group)], indicating that these compounds represented apically excreted glucuronides of quercetin. In a comparison of trace d (FTC) and trace b (control) of Fig. 4, it is clear that secretion of quercetin glucuronides into the perfusion fluid was lower when Bcrp1 was inhibited by FTC. Mean peak height of the major glucuronide (with retention time of 6.3 min) in the perfusion fluid of the FTC group was significantly lower than that of controls (20 ± 8 versus 78 ± 18 nA; $p < 0.05$). The other glucuronides present in plasma (with retention times of 6.9 and 8.5 min) were also less secreted into the perfusion fluid (data not shown). In plasma, quercetin and isorhamnetin could only be detected after enzymatic hydrolysis of the samples, indicating that only conjugated forms were present. Figure 5 shows that plasma levels of quercetin and its metabolite isorhamnetin were significantly higher in the FTC group compared with that in controls ($p < 0.05$). Thus, inhibition of Bcrp1 in small intestine decreases efflux of quercetin glucuronides across the apical membrane into the gut lumen, leading to increased efflux across the basolateral membrane, which results in higher levels of glucuronides in the plasma. This is visualized for the individual rats in Fig. 6.

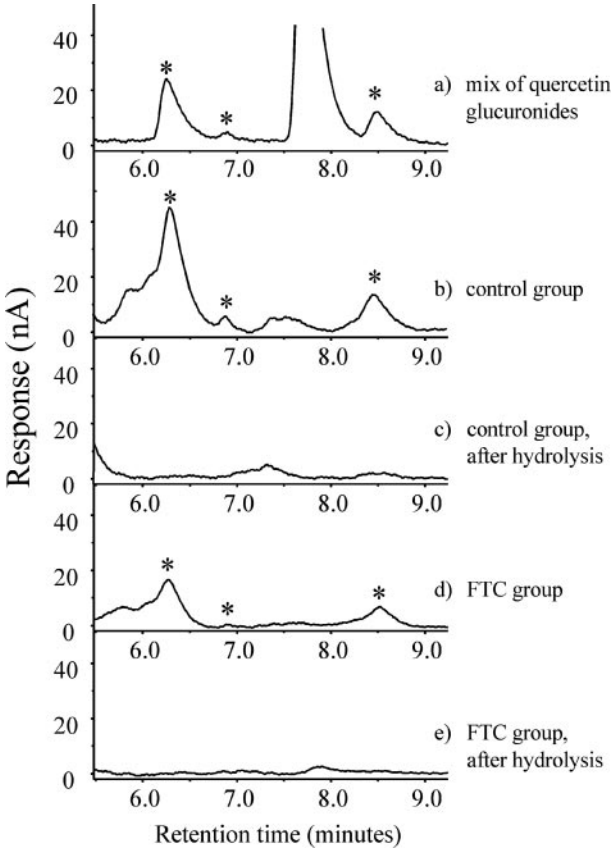

Fig. 4. HPLC chromatograms of perfusion fluid from in situ rat intestinal perfusion experiments. Note that the peaks have different retention times compared with Fig. 1 due to the use of a different pump system and injector. Chromatogram of a mixture of quercetin glucuronides is shown in trace a (the peak just before 8.0 min also represents a glucuronide that is not present in perfusion fluid). The small intestine of Mrp2-deficient rats was perfused with 43 μM quercetin aglycone in the absence or presence of the Bcrp1-inhibitor FTC, and samples were taken at the outlet of the perfused intestinal segment at 30 min. Samples of control (traces b and c) and FTC rats (traces d and e) were analyzed with coulometric array detection before (traces b and d) and after treatment with β-glucuronidase/sulfatase (traces c and e). Asterisks in the chromatograms represent glucuronides in the different samples with similar retention times.

Discussion

It has been shown previously that reflux of quercetin conjugates into the gut lumen contributes to the limited intestinal absorption of flavonoids, but the mechanism of this apical secretion was still unknown. In this study, we show that when the intestinal efflux transporter Bcrp1 was inhibited by the specific inhibitor FTC, plasma concentrations of quercetin in rats increased more than 2-fold. We demonstrated that Bcrp1 limited net transfer of quercetin across the intestinal wall by pumping intracellularly formed glucuronide conjugates of quercetin across the apical membrane back into the gut lumen. To our knowledge, this is the first study showing a role for a transport protein (i.e., Bcrp1) in the in vivo disposition of a flavonoid. ABC transporters, including the breast cancer resistance protein, are strongly conserved

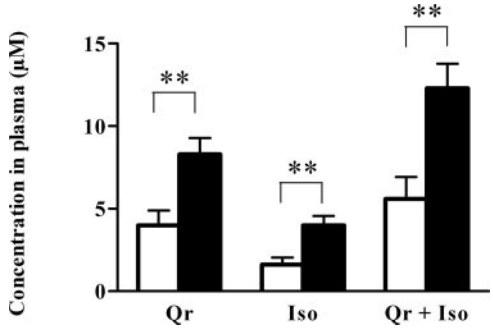

Fig. 5. Concentration of quercetin (Qr) and its methylated metabolite isorhamnetin (Iso) in peripheral plasma after in situ perfusion of small intestine of Mrp2-deficient rats for 30 min with quercetin in the absence (open columns) or presence (closed columns) of the Bcrp-inhibitor FTC. Blood samples were taken from the vena cava inferior and plasma samples were enzymatically hydrolyzed before analysis by HPLC. Values are means ± S.E.M. ($n = 6$ for both groups). **, $p < 0.01$, Student's t test.

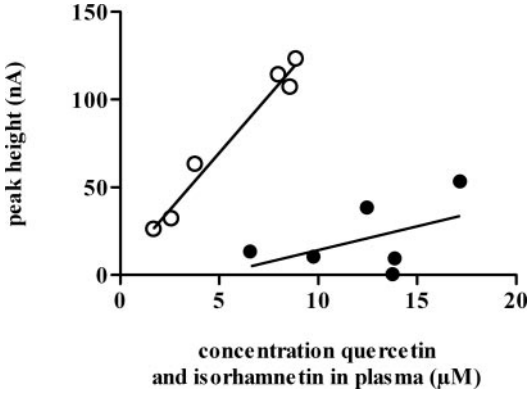

Fig. 6. Peak height of the major quercetin glucuronide in the perfusion buffer (retention time 6.3 min; see Fig. 4) as a measure of apical glucuronide efflux (y-axis) plotted against the total concentration of quercetin and isorhamnetin in plasma samples (x-axis) of the individual Mrp2-deficient rats after in situ intestinal perfusion for 30 min with quercetin in the absence (○) or presence (●) of the Bcrp1-inhibitor FTC. Given an equal quercetin uptake from the perfusion buffer, inhibition of intestinal Bcrp1 clearly leads to a lower efflux of glucuronides into the perfusion fluid, concomitant with increased efflux to the plasma.

among species (Sparreboom et al., 2003), and human BCRP and mouse Bcrp1 seem to have a very similar cellular localization and substrate specificity (Maliepaard et al., 2001; Allen et al., 2002; Van Herwaarden et al., 2003). Furthermore, because intestinal absorption of quercetin has been shown to be comparable in rats (this study) and humans (Walle et al., 2000) (i.e., quercetin is conjugated within the small intestinal cell and excreted at both the basolateral and apical membrane), we argue that our results are relevant to the intestinal absorption of dietary flavonoids in humans.

Based on our in vitro studies, showing that murine Bcrp1 efficiently transported quercetin aglycone in the basolateral-to-apical direction, we expected that in our perfusion experiments, inhibition of Bcrp1 of rat small intestine would favor uptake of unconjugated quercetin from the perfusion fluid. However, disappearance of quercetin from the perfusion fluid was not significantly higher when Bcrp1 was inhibited. This discrepancy between the results from our in vitro and in vivo studies may be accounted for by a difference in metabolism: quercetin is conjugated by the rat small intestinal cells (and thus may be prevented from efflux by Bcrp1), whereas quercetin glucuronides could not be detected in the MDCKII cells and free quercetin may be available for efflux by Bcrp1. In vivo, coupling of conjugating enzymes to apical efflux in gut epithelium has been shown to significantly contribute to the low bioavailability of flavonoids (Jia et al., 2004).

It was recently hypothesized that flavonoids could be substrates for BCRP, based on their reported interaction with the human BCRP protein. In BCRP-overexpressing cell lines, flavonoids, including quercetin, stimulated cellular accumulation of several BCRP substrates (Yoshikawa et al., 2004; Zhang et al., 2004), indicating an inhibitory interaction with BCRP. In BCRP-enriched membrane vesicles, quercetin competitively inhibited transport of the anticancer agent SN-38 with an inhibition constant in the nanomolar range (K_i = 280 nM) (Yoshikawa et al., 2004). However, attachment of a sugar moiety attenuated or even abolished BCRP inhibitory activity of flavonoids (Imai et al., 2004; Zhang et al., 2004). In these studies, physiologically relevant conjugates such as glucuronides or sulfates were not tested. The effect of anionic conjugation of flavonoids on its interaction with BCRP needs to be established to better predict the in vivo relevance of the BCRP inhibitory capacity of flavonoids. For example, glucuronidation of SN-38 decreased its affinity toward BCRP approximately 6-fold (Nakatomi et al., 2001). One study on the interaction of flavonoids with another efflux transporter (i.e., Pgp) exemplifies this problem (Hsiu et al., 2002). Based on in vitro studies, quercetin was reported to be an inhibitor of intestinal Pgp and was thus expected to enhance intestinal uptake of the Pgp substrate cyclosporin. However, coadministration with quercetin significantly decreased oral cyclosporin bioavailability. The authors speculated that differential interaction of either quercetin or its conjugates with Pgp could account for their unexpected results.

Bcrp1 may be involved in transport of quercetin (conjugates) not only in the small intestine but in other organs as well. The ability of flavonoid aglycones to cross the blood-brain barrier has been studied in in situ rat brain perfusion experiments (Youdim et al., 2004). It was shown that the flavonoid naringenin could easily cross the blood-brain barrier, whereas quercetin was strongly retained in the perfusion medium. Coincubation with GF120918, an inhibitor of both Pgp and Bcrp1, drastically enhanced accumulation of quercetin in brain tissue, whereas the specific Pgp-inhibitor PSC833 was without effect. This suggests that also at the blood-brain barrier Bcrp1 may limit passage of quercetin. Because transfer of naringenin into the brain was not affected by the Pgp and Bcrp1 inhibitors, it should be noted that our results regarding the role of Bcrp1 for uptake of quercetin in the small intestine cannot be extended to the entire class of flavonoids. In the liver, Bcrp1 is present in the bile canicular membranes. This raises the possibility that also in the liver Bcrp1 serves as the excretion transporter of quercetin conjugates. Flavonoid conjugates are efficiently removed from plasma by active biliary secretion (Manach et al., 1996; Arts et al., 2004). In bile, the total concentration of quercetin and its metabolites was more than 60 times higher than in plasma (Arts et al., 2004).

Until now, it was speculated that Mrp2 was responsible for the apical efflux of flavonoid conjugates in small intestine. The salient finding of our study was that intestinal absorption of quercetin and apical efflux of its conjugates was not

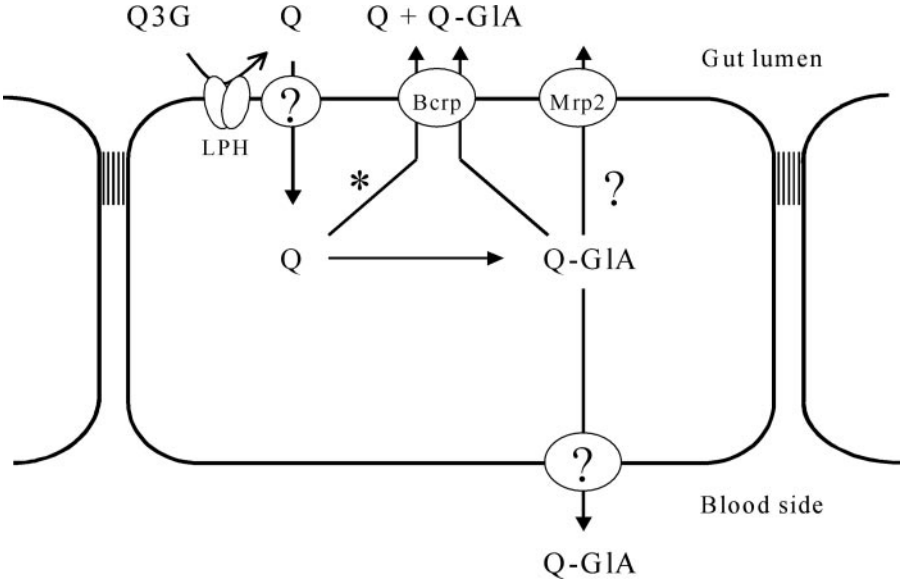

Fig. 7. Proposed scheme for absorption of quercetin-3-glucoside by small intestinal cells. Quercetin-3-glucoside (Q3G) is hydrolyzed by lactase phlorizin hydrolase (LPH) and free quercetin (Q) enters the cell by passive diffusion or is taken up by a yet unknown transporter. Once in the cell, quercetin is conjugated, for example, with glucuronic acid (glA), and this conjugate is partly transported to the blood by a yet unknown transporter at the basolateral membrane. At the apical membrane, Bcrp1 pumps quercetin glucuronides back into the lumen. The asterisk (*) denotes that in vitro, murine Bcrp1 is also able to transport free quercetin. However, in the rat small intestine, this phenomenon apparently did not contribute to the absorption of quercetin. The role of Mrp2 still needs to be evaluated (see *Discussion* details).

affected by the absence of Mrp2, as shown by similar intestinal uptake and plasma levels of quercetin in Mrp2-deficient and control rats. Nevertheless, this does not rule out a role for Mrp2 in the apical efflux of quercetin conjugates. Several in vitro studies clearly show that the Mrp-inhibitor MK-571 reduces cellular efflux of flavonoids conjugates (Vaidyanathan and Walle, 2001; O'Leary et al., 2003). In addition, compounds such as methotrexate and estradiol-17-β-glucuronide are transported by both Mrp2 (Zelcer et al., 2003) and Bcrp1 (Chen et al., 2003), indicating substrate overlap between the two transporters. Based on these observations, it can be speculated that efflux of quercetin conjugates in control rats is mediated by both Mrp2 and Bcrp1. In the Mrp2-deficient rat, Bcrp1 may compensate for the lack of transport function of Mrp2. During the preparation of the revised version of this manuscript, a study was published on the intestinal excretion of glucuronide and sulfate conjugates of 4-methylumbelliferone and E3040 formed in enterocytes of Mrp2-deficient rats and Bcrp1(−/−) mice (Adachi et al., 2004). In line with our findings, Bcrp1 had an important role in extruding glucuronide conjugates of 4-methylumbelliferone and E3040 into the intestinal lumen, whereas Mrp2 seemed only responsible for the efflux of E3040 glucuronide. These findings emphasize that further research is needed to elucidate the specific role of Mrp2 in the intestinal handling of flavonoid conjugates.

In conclusion, we have shown that both quercetin and its glucuronide conjugates are substrates for Bcrp1. In vitro quercetin aglycone was efficiently transported by murine Bcrp1, but not by human MRP2. In Mrp2-deficient rats, intestinal absorption of quercetin and plasma concentration of quercetin was not affected compared with control rats. Inhibition of intestinal Bcrp1 in the Mrp2-deficient rats also did not influence the net amount of quercetin absorbed from the intestinal lumen, but it clearly diminished apical efflux of quercetin glucuronides, leading to significantly elevated plasma quercetin levels (Fig. 7). Thus, from our present data and data from the literature, it is clear that rat intestinal Bcrp1 is likely to limit oral bioavailability of dietary flavonoids. Given the strong interaction of flavonoids with intestinal transporters such as Bcrp1 and Pgp, and its potential effect on the bioavailability of drugs, further understanding of the effect of dietary flavonoids on intestinal metabolism and transporter activity is of utmost importance.

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