Flavonoid Glycosides Are Not Transported by the Human Na\textsuperscript{+}/Glucose Transporter When Expressed in Xenopus laevis Oocytes, but Effectively Inhibit Electrogenic Glucose Uptake

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ABSTRACT

There is controversy as to whether intestinal absorption of glycosylated flavonoids, and particularly quercetin glycosides, involves their uptake in intact form via the human sodium-coupled glucose transporter hSGLT1. We here describe studies using Xenopus oocytes that express hSGLT1 and the two-electrode voltage clamp technique to determine the transport characteristics of a variety of flavonoids carrying glucose residues at different positions as well as of their aglycones (altogether 27 compounds). Neither quercetin, luteolin, apigenin, naringenin, pelargonidin, daidzein, or genistein, nor any of their glycosylated derivatives generated significant transport currents. However, the inward current evoked by 1 mM of the hSGLT1 substrate α-methyl-D-glucopyranoside was potently reduced by the simultaneous application of not only various flavonoid glycosides but also by some aglycones. The inhibitory potency remained unchanged when the attached glucose was replaced by galactose, suggesting that these residues may bind to SGLT1. Kinetic analysis by Dixon plots revealed inhibitory potency remained unchanged when the attached glucose was replaced by galactose, suggesting that these residues may bind to SGLT1. Kinetic analysis by Dixon plots revealed inhibitory potency remained unchanged when the attached glucose was replaced by galactose, suggesting that these residues may bind to SGLT1. Kinetic analysis by Dixon plots revealed inhibitory potency remained unchanged when the attached glucose was replaced by galactose, suggesting that these residues may bind to SGLT1. Kinetic analysis by Dixon plots revealed inhibitory potency remained unchanged when the attached glucose was replaced by galactose, suggesting that these residues may bind to SGLT1. 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intestinal (aglycones and glucosides), and after interaction with the microflora in the colon (predominantly for glucosides with complex sugar moieties like rutinoside) (Manach et al., 2004). Regardless of the mechanism of absorption, intact glucosides of flavonoids are usually not found in the plasma and urine (for references, see Manach et al., 2004). Because most flavonoids in foods are present in glycosylated form, a considerable number of publications were devoted to the characterization of transport mainly by using quercetin glucosides, for which uptake via the intestinal sodium-coupled glucose transporter (SGLT1) was proposed.

To test whether quercetin-4′-O-glucoside (Q4′G) is a substrate for SGLT1, its cellular uptake was examined with Caco-2 cells and Chinese hamster ovary cells stably transfected with SGLT1. Fluorescent microscopy as well as high-performance liquid chromatography analysis revealed a sodium-dependent cellular accumulation of Q4′G after apical loading. Uptake was inhibited by the addition of glucose or phlorizin as a high-affinity inhibitor of SGLT1. These findings suggested that Q4′G was taken up into cells by SGLT1 (Walgren et al., 2000). In contrast, studies conducted in isolated segments of the small intestine of rats showed that the absorption of genistein-7-glucoside (genistin) was increased 2.5-fold when phlorizin was simultaneously perfused with genistin (Andlauer et al., 2004).

Using Ussing-chamber with rat jejunum, Wollfram et al. (2002) observed the disappearance of quercetin-3-O-glucoside (Q3G) from the luminal, but not from the serosal, compartment, with the aglycone quercetin appearing in the serosal compartment when Q3G was applied on the mucosal side. The addition of glucose or of phlorizin largely reduced the disappearance of Q3G from the luminal side, whereas fructose had no effect. The transport of quercetin was observed only in jejunum but not in the proximal colon. These findings led the authors conclude that SGLT1 played a role in cellular uptake of Q3G (Wollfram et al., 2002).

These conclusions were disputed in a letter to the editor (Arts et al., 2002) with the main objection being that the inhibitors chosen are not specific for SGLT1 but could also have inhibited lactase phlorizin hydrolase, an enzyme present in the brush-border membrane of the small intestine capable of hydrolyzing Q3G on the cell surface with uptake of the aglycone quercetin via passive processes. This hypothesis was finally followed experimentally to assess transport of Q3G and Q4′G in the presence and absence of phlorizin and of a specific inhibitor of lactase phlorizin hydrolase. The results suggested that Q3G was transported only as aglycone via an SGLT1-independent mechanism, whereas the transport of Q4′G may have involved both pathways (Day et al., 2003).

The rather contradictory findings from these studies on transport of quercetin glucosides and other compounds via SGLT1 prompted us to test the transport by directly measuring transport currents in Xenopus oocytes expressing the human SGLT1. By selecting flavonoids belonging to different subclasses either as aglycones or glycosylated forms, we could unequivocally demonstrate that several flavonoids interact with SGLT1 and cause inhibition of the transporter but do not serve as transported substrates.

Materials and Methods

Xenopus laevis maintenance and oocyte harvest procedures were approved by the local authority for animal care in research (Regierung von Oberbayern, approval number 211-2531.3-9/99). Oocytes were collected under anesthesia (immersion in a solution of 0.7 g/l 3-aminobenzoic acid ethyl ester; Sigma, Deisenhofen, Germany) from frogs that were killed with an anesthetic overdose after the final collection. Oocytes were treated with 1.75 mg/ml collagenase for 90 min and were separated manually thereafter. They were incubated in a Barth solution containing 88 mM NaCl, 1 mM KCl, 0.8 mM MgSO₄, 0.4 mM CaCl₂, 0.3 mM Ca(NO₃)₂, 2.4 mM NaHCO₃, and 10 mM HEPES, pH 7.5, at 17°C overnight. Thereafter, stage VI/VII oocytes were injected with 23 ng of hSGSGLT1-cRNA and incubated for 3 to 4 days at 17°C. For coexpression experiments, a mixture containing 13.5 ng each of hSGSGLT1 and the rabbit peptide transporter (rPEPT1) cRNA was injected in a 27-nl volume. α-Methyl-d-glucopyranoside (α-MDG) was used as transport substrate for hSGSGLT1 and glycyl-glutamine (GQ) as transport substrate for rPEPT1.

Electrophysiology. For two-electrode voltage clamp experiments, oocytes were placed in an open chamber and superfused (3 ml/min) with Barth solution or with solutions containing the substrates to be studied. Oocytes were voltage-clamped, and transport currents were measured at −60 mV using a TEC-05 amplifier and the CellWorks software (npi electronic, Tamm, Germany). Current-voltage relations were measured in the potential range of −160 to +80 mV, and the current generated by the transport at a given membrane potential was calculated as the difference of the currents measured in the presence and the absence of substrate. The kinetic parameters of transport Kₘ and Iₘax (nA) were calculated from least-square fits of at least three data points based on the Michaelis-Menten equation. The IC₅₀ values shown in Fig. 5 were calculated using the formula IC₅₀ (mM) = current(I) × I/(current(0) − current(I)), where current(0) and current(I) are the transport currents in the absence and the presence of inhibitor and I is the inhibitor concentration (in micromolar). Dixon-type kinetic studies were performed at a membrane potential of −60 mV. To avoid errors due to the replenishment of the oocytes with α-MDG, the application time of the substrates was limited to 15 s at an increased (7 ml/min) perfusion speed, and the substrate was added at the lowest concentration first.

Flavonoids (Table 1) were obtained from Extrasynthese (Genay, France), Axonora (Grünberg, Germany), Roth (Karlsruhe, Germany), or LC Laboratories (Woburn, MA). The dipeptide GQ was a generous gift of Degussa Rexim (Hanau, Germany). All other chemicals were obtained from Sigma. Stock solutions of flavonoids were prepared in DMSO at concentrations of 50 to 200 mM and diluted with Barth solution. The final concentration of DMSO was in most solutions not higher than 1% (v/v), but because of the low water solubility of rutelin-3′,7-diglucoside and apigenin, these substrates were applied with 2%. Because DMSO at 1% or 2% concentration slightly elevated the holding current (in the absence of substrates), the data in Fig. 4 were corrected for the DMSO-induced changes.

Data are given as the mean ± S.E.M. of n. Significant differences were determined by analysis of variance followed by post hoc multiple comparison tests, and a p value <0.05 was considered as significant.

Results

Figure 1 shows the current-voltage relationship obtained in oocytes expressing hSGSGLT1 after the addition of 1 mM α-MDG, quercetin, Q3G, or Q4′G. Whereas α-MDG (Kₘ = 0.7 mM) evoked a mean transport current of ~300 nA, neither quercetin nor its glycosylated forms induced any significant currents. To determine whether there is an interaction between hSGSGLT1 and Q4′G, current-voltage relationships were...
measured in the presence of 1 mM α-MDG and in the simultaneous presence of increasing concentrations of Q4′G. As shown in Fig. 2, the addition of 0.1 mM Q4′G reduced the α-MDG-induced current to approximately half, and the addition of 1 mM Q4′G almost completely inhibited the α-MDG-induced current. The calculated IC50 value at −60 mV membrane potential was 91 ± 2 μM (n = 7), and the inset of Fig. 2 shows that the IC50 was independent of the membrane potential.

To derive the type of inhibition, a Dixon plot was derived (Fig. 3) with α-MDG concentrations of 0.2, 0.5, and 2 mM and Q4′G added in concentrations of 0.125, 0.25, and 0.5 mM. The apparent Ki value determined from the intersection point of the three regression lines was approximately 0.04 mM, which is in good agreement with the value calculated from the IC50 value (0.037 mM). The intersection point is clearly above the x-axis, and inhibition is of therefore of competitive type.

A smaller inhibition of α-MDG-transport currents was also observed in the presence of the aglycone quercetin and by Q3G (data not shown). The IC50 value calculated for the Q3G-mediated inhibition data was 640 ± 50 μM (n = 5), and it was like that of Q4′G, nearly independent of membrane potential. The inhibition was of competitive type with an apparent Ki value of approximately 0.8 mM. The low solubility and the fast degradation of quercetin in aqueous solutions impeded Dixon-type experiments, but the addition of equal concentrations of this compound to solutions with increasing concentrations of α-MDG resulted in a decreasing relative inhibitory action, which also suggests a competitive type of interaction.

To test whether the inhibition of the current by Q3G was specific for SGLT1, we have expressed hSGLT1 and rPEPT1 simultaneously in oocytes and measured the inhibition of the transport currents evoked by the respective substrates. In the absence of Q3G, both α-MDG (2 mM) and GQ (5 mM) induced similar currents (343 ± 14 versus 388 ± 24 nA, n =...
6). The addition of 1 mM Q3G reduced the hSGLT1-mediated current reversibly by 50.3 ± 1.4%, whereas the rPEPT1-mediated current was reduced only slightly by 5.9 ± 0.7%, and this change was not reversible, i.e., the effect of Q3G was specific. Very similar inhibition was also observed when 0.1 mM luteolin instead of Q3G was used (data not shown).

The observations that Q4 and Q3G inhibited only hSGLT1 in a competitive manner suggested that the interaction these flavonoids with hSGLT1 resulted from binding of the glucose moiety to the substrate binding pocket of hSGLT1. To determine the role of the sugar residues in a more comprehensive manner, we tested a total of 27 flavonoids in their aglycone and glycosylated forms belonging to following classes: flavones, isoflavones, flavonols, flavanones, anthocyanins, and chalcones. In spite of the large number of different glycosylated forms found in plants, only a few compounds are commercially available. Special attention was given to the glycosylated forms with the glucose residues attached at different positions. Luteolin could be tested in seven variants, apigenin in five, quercetin in four, daidzein in three, and all others in only two variants.

Figure 4 shows the findings on transport current measurements. With a few exceptions due to low solubility, flavonoids were all applied at 1 mM concentration and currents were measured at membrane potential of −60 mV and are expressed as percent of current induced by α-MDG in the same oocytes. Of all compounds tested, only daidzein and pelargonidin-3-O-gluc induced currents that were statistically significant, but even these currents amounted to only approximately 4% of the α-MDG-induced current. Some substrates, however, partially inhibited the holding current of voltage-clamped oocytes (expressed as negative current). The mechanism of this effect is not known, but it seems unlikely that hSGLT1 was involved, because the holding current reduction occurred in the absence of α-MDG.

The potency for inhibition of α-MDG-induced transport currents of the same flavonoids as shown in Fig. 4 is provided in Fig. 5. The highest inhibition of α-MDG-induced currents was observed when the glucose moiety was attached to the 4'-position (luteolin-4'-O-gluc: 91 ± 0.3%, n = 8; and quercetin-4'-O-gluc: 85.4 ± 1.4%, n = 11), but also apigenin-6-C-gluc and the aglycones naringenin and luteolin (at 0.2 mM concentration) showed potent inhibition. When glucose was attached to positions 7 or 8, mostly low inhibition (luteolin, daidzein, genistein, isookanin, and apigenin) was observed with the exception of apigenin-7-O-gluc. Glucose and galactose residues were equally effective as shown by almost identical inhibition rates of Q3G and quercetin-3-O-galactoside.

The high inhibitory potencies of the aglycone luteolin and its 4'-O-glycosylated form prompted us to test whether these interactions were based on a different mechanism. Figure 6, A and B, shows the Dixon plots for luteolin and its glycosylated form. Unexpectedly, not only the glycosylated form but also the aglycone inhibited the transport of α-MDG in a

![Fig. 2. Inhibition of the α-MDG-induced transport currents in the presence of increasing concentrations of quercetin-4'-O-glucoside. The inset shows the potential dependence of the calculated K_i values. Mean values of seven oocytes.](image)

![Fig. 3. Dixon plot for inhibition of α-MDG-mediated currents by quercetin-4'-O-glucoside. Mean values of five oocytes. The crossing point of regression lines above the abscissa shows that the inhibition is of competitive type. The K_i value determined from the plot is ~0.06 mM and therefore almost identical to the one shown in Fig. 2.](image)
competitive manner, even with a higher affinity. Naringenin, another aglycone, acted as expected, in a noncompetitive way, whereas the inhibition by naringenin-7-O-gluc was also of competitive type (data not shown).

Discussion

In spite of numerous in vitro and in vivo studies, the mechanisms of the gastrointestinal absorption of flavonoids remain largely unknown. Whereas most aglycones are hydrophobic and thus may cross cell membranes by diffusion, glycosylated flavonoids are more hydrophilic, suggesting that membrane carriers are involved in their absorption in the intestine. Uptake studies using glycosylated flavonoids into cells or across epithelia demonstrated frequently that transport was reduced in the presence of glucose or phlorizin, and this was taken as an indicator for an involvement of SGLT1 in flavonoid transport (Hollman et al., 1999; Walgren et al., 2000; Wolfram et al., 2002; Day et al., 2003; Cermak et al., 2004). We here provide evidence by direct transport current measurements in *Xenopus* oocytes that none of the tested flavonoids is transported by the human intestinal sodium-coupled glucose transporter SGLT1. Our findings therefore support the interpretation that the flavonoid transport inhibition observed in various models in the presence of phlorizin may result from other mechanisms, e.g., from inhibition of lactase phlorizin hydrolase and, in consequence, from a reduced presence of the aglycone fraction, which constitutes the main transport form (Arts et al., 2002; Day et al., 2003).

Our data are also in accordance with the observation that the absorption of cyanidin-3-glucoside by mouse jejunum mounted in Ussing chambers is inhibited by Q3G but not by glucose or phlorizin (Walton et al., 2006).

Contradictory results concerning the transport and bioavailability of aglycones and the glycosylated forms of some flavonoids have not only been observed in vitro in cell models but also in studies on human volunteers. Setchell et al. (2001) showed that daidzein and genistein administered orally were absorbed to a higher degree than their corresponding 7-glucosides daidzin and genistin. In a subsequent study these authors used electrospray mass spectrometry but failed to detect even traces of daidzin or genistin in plasma, whereas plasma was enriched with the aglycone forms (Setchell et al., 2002). Based on these findings, it was concluded that isoflavone-glycosides are not absorbed intact across the epithelium of healthy adults and that the bioavailability requires initial hydrolysis of the sugar moiety by intestinal β-glucosidases.
mately 2 to 3 times less effective compared with luteolin, suggesting that the hydroxyl group in position 3 reduced binding affinity. Whereas glycosylation in position 4 increased the affinity of luteolin, glucose attached to positions 6, 7, or 8 markedly reduced it, as did the glycosylation at position 3 in the case of quercetin. On the other hand, the very low affinity of the apigenin was highly increased by the attachment of a glucose residue at positions 6 or 7, and a small increase was also observed for daidzein.

Previous analysis of the interaction of competitive and noncompetitive inhibitors with hSGLT1 suggested that the sugar binding site is located close to a broad planar vestibule that is able to interact with hydrophobic and aromatic residues attached to and coplanar with the pyranose ring (Hirayama et al., 2001). In the case of inhibitors without a sugar moiety it was assumed that the aromatic ring might bind to the same site of the transporter as the aromatic rings found in the competitive inhibitors. Based on these data, a possible explanation for our inhibition pattern might be that the flavonoids can bind to SGLT1 in two different orientations: either with their sugar moiety or with another part of the molecule, possibly ring “B.” If the aglycone form has a low affinity, then glycosylation seems to enhance binding affinity, whereas glycosylation of a high-affinity aglycone at an unfavorable position results—due to the statistical distribution of the binding between two possible sites—in an increased IC$_{50}$ value. Glycosylation at position 4 seems to be most favorable, even for high-affinity aglycones, possibly because then the sugar moiety as well as ring “B” fit best into the sugar binding site and the vestibule. In this case, the binding energy would be higher as when it comes from the sugar binding alone and this seems to be desirable for the reversible inactivation of the SGLT1 transporter (Hirayama et al., 2001).

In addition to the presence of glucose residues, the presence and the spatial positions of hydroxyl groups seem quite important for binding of the different compounds to SGLT1. The only difference between luteolin and apigenin is the presence of an -OH group in position 3’, and this seems to be essential for aglycones to allow binding. On the other hand, when the double bond between C2 and C3 of the O-heterocyclic ring of apigenin is converted into a single bond (naringenin), the affinity is high even in the absence of an -OH group in the 3’ position. The comparison of luteolin and luteolinidin shows that the anthocyanidin bearing a positive charge at position 1 and lacking the oxygen at position 4 has a several times lower affinity, whereas in case of apigenin and apigeninidin, the difference in affinities is just the opposite. Finally, isoflavonones seem to have a less favorable structure for binding to SGLT1 regardless of whether they carry a sugar moiety or not because genistein and daidzein and their glycosylated derivatives all display low affinities.

Over the last decade, convincing evidence has accumulated that the intestinal absorption of glucose and galactose is not mediated solely by SGLT1, but includes a passive, facilitated component that is dependent on a rapid, glucose-dependent activation and recruitment of the glucose uniporter GLUT2 to the brush-border membrane (for a review, see Kellett, 2001). Therefore, the question arises whether GLUT2 contributes to the bioavailability of flavonoids and whether the inhibition of SGLT1 by flavonoids and flavonoid glycosides in the gut lumen indirectly inhibits also the recruitment of
GLUT2 leading to a markedly reduced glucose absorption. In experiments on myelocytic cells, a number of aglycone flavonoids competitively inhibited glucose uptake even in the absence of sodium, suggesting an interaction with transporters of the GLUT family (Park, 1999). Similar results were observed in adipocytes expressing the insulin-dependent glucose transporter GLUT4, with the aglycone-flavonoids quercetin, myricetin, and catechin-gallate (Strobel et al., 2005).

The authors postulate—without presenting evidence—that GLUT transporters are also involved in flavonoid uptake into the cells. However, in recent experiments performed on X. nroprios oocytes expressing GLUT2, quercetin and other flavonoids of different classes were found to inhibit glucose transport without being transported itself (Kwon et al., 2007). Whether the inhibition of the glucose uptake via SGLT1 also reduces the recruitment of GLUT2 proteins to the apical membrane of enterocytes has not yet been investigated.

The question as to whether flavonoids—consumed with foods or administered in pharmacologic amounts—could be used as antidiabetic agents by decreasing small intestinal glucose absorption has been discussed in detail in a recent publication (Kwon et al., 2007). Based on the inhibitory potency of quercetin for inhibition of GLUT-2-mediated glucose absorption, the authors estimated that the ingestion of 1 g of this flavonoid could prevent absorption of 50 to 100 g of glucose. However, this value is derived solely from in vitro measurements and needs proof in clinical studies.

In summary, direct analysis of transport currents of the human sodium-dependent glucose transporter SGLT1 revealed that none of the tested flavonoids—whether bearing a sugar residue or not—serves as a transported substrate. As SGLT1 is found in high levels in brush-border membranes of intestinal epithelial cells, our findings exclude that this glucose transporter contributes significantly to the intestinal absorption of flavonoids including the quercetin-glycosides. Different flavonoids however interact—depending on their particular structure and the steric location of substituents—with high-affinity hSGLT1 and inhibit effectively electrogenic glucose uptake.

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