Inhibition of the intestinal glucose transporter GLUT2 by flavonoids

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ABSTRACT   We tested whether the dominant intestinal sugar transporter GLUT2 was inhibited by intestinal luminal compounds that are inefficiently absorbed and naturally present in foods. Because of their abundance in fruits and vegetables, flavonoids were selected as model compounds. Robust inhibition of glucose and fructose transport by GLUT2 expressed in *Xenopus laevis* oocytes was produced by the flavonols myricetin, fisetin, the widely consumed flavonoid quercetin, and its glucoside precursor isoquercitrin. IC_{50}s for quercetin, myricetin, and isoquercitrin were ~200- to 1000-fold less than glucose or fructose concentrations, and noncompetitive inhibition was observed. The two other major intestinal sugar transporters, GLUT5 and SGLT1, were unaffected by flavonoids. Sugar transport by GLUT2 overexpressed in pituitary cells and naturally present in Caco-2E intestinal cells was similarly inhibited by quercetin. GLUT2 was detected on the apical side of Caco-2E cells, indicating that GLUT2 was in the correct orientation to be inhibited by luminal compounds. Quercetin itself was not transported by the three major intestinal glucose transporters. Because the flavonoid quercetin, a food component with an excellent pharmacology safety profile, might act as a potent luminal inhibitor of sugar absorption independent of its own transport, flavonols show promise as new pharmacologic agents in the obesity epidemic.—Kwon, O., Eck, P., Chen, S., Corpe, C. P., Lee, J-H., Kruhlak, M., Levine, M. Inhibition of the intestinal glucose transporter GLUT2 by flavonoids. *FASEB J*. 21, 366–377 (2007)

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Diabetes and obesity are emerging worldwide health problems (1, 2). New prevention and treatment options for both conditions could be based on strategies to dampen or inhibit nutrient absorption. Similar strategies are the basis of agents currently used clinically to inhibit fat absorption, cholesterol absorption, and intestinal catabolism of complex carbohydrates (3–5). A new class of agents that delayed or inhibited glucose absorption could have substantial impact in managing diabetes and obesity. Emerging evidence indicates that apical, or luminal, facing-facilitated glucose transporter 2 (GLUT2) is a major pathway of sugar absorption, and therefore an attractive target of such potential agents (6, 7).

Flavonoids are polyphenols that are widely distributed in foods, especially fruits and vegetables (8). Nutritive functions of flavonoids are unknown (9, 10). Quercetin is a commonly ingested flavonoid, and 20–100 mg daily is ingested by dietary intake (8). Peak plasma concentrations of flavonoids such as quercetin do not exceed 1–2 μM after ingestion, but intestinal luminal concentrations are believed to be ~50-fold higher (11–14). Based on these high intraluminal concentrations, we proposed that a novel action of intraluminal flavonoids may be to dampen, redistribute, or frankly inhibit intestinal absorption of candidate nutrients (15, 16). Flavonoids either as food components or coadministered with foods could potentially have these actions, and such actions would not require flavonoids themselves to be absorbed. Partial support for this proposal was provided by data showing that some flavonoids found in foods inhibited vitamin C and glucose intestinal transport and absorption (16). The findings suggested that quercetin, the dominant flavonoid ingested by humans, may modulate glucose intestinal absorption by the sodium-independent facilitative glucose transporter GLUT2.

Although these findings are promising, uncertainties remain. Some investigators suggested that flavonoids decreased glucose uptake by a sodium-dependent pathway *via* the sodium-dependent glucose transporter 1 SGLT1. This conclusion was based on experiments using intestinal cells, brush border membrane vesicles, or *Xenopus laevis* oocytes expressing SGLT1 (17–24). In cell and vesicle preparations it is difficult to distinguish which transporter(s) are inhibited, and the concentra-

tions of substrates and flavonoids used in all these experiments were not relevant to *in vivo* conditions. In systems where SGLT1 was either overexpressed in cells or in *Xenopus* oocytes, flavonoid effects on glucose transport were either modest or not directly tested (21–23). Other investigators suggested that flavonoids could be nonspecific inhibitors based on their behavior in isolated enzyme systems (25). It is uncertain which intestinal glucose transporters are inhibited by different flavonoids, which flavonoids are the most potent inhibitors of glucose transport, whether there is selectivity for inhibition of transport of different substrates, whether flavonoids must first be deglycosylated or transported for inhibition to occur, and whether the appropriate transporters are in the same location as the inhibitory concentrations of flavonoids.

Addressing these issues would provide a clear database of flavonoid action that could serve as the foundation of a pilot clinical study of flavonoid effects on sugar absorption. To test transporter specificity, we studied oocytes that were injected with cRNAs to express specific intestinal sugar transporters and incubated these oocytes with a variety of flavonoids to determine potency. To learn whether flavonoid inhibition of sugar transport was specific for glucose transporters expressed only in oocytes, cell systems were also studied. Flavonoids, flavonoid concentrations, and sugar substrate concentrations were all selected to have *in vivo* relevance. We show that several flavonoids were potent inhibitors of GLUT2-mediated glucose and fructose transport but had no effect on other major intestinal sugar transporters; that flavonoid structure and glycosylation affected inhibition; that GLUT2 overexpressed in cells and present in an intestinal cell model was inhibited by the relevant flavonoids; that GLUT2 was present in the proper location to be inhibited by luminal flavonoids; and that the potent inhibitor, quercetin, was not itself transported by GLUT2.

MATERIALS AND METHODS

Materials

[³H] 2-Deoxyglucose (25.5 Ci/mmol), [¹⁴C] fructose (300 mCi/mmol), and [¹⁴C] glucose (265 mCi/mmol) were purchased from NEN Life Science Products (Boston, MA, USA) and [¹⁴C] quercetin(53 mCi/mmol) from ChemSyn Laboratories (Lenexa, KS, USA). Quercetin, fisetin, myricetin, rutin, gossypin, apigenin, naringenin, naringen, hesperetin, genistein, luteolin, daidzein, epicatechin, catechin, phloretin, and phloridzin were purchased from Sigma (St. Louis, MO, USA). Isoquercitrin, spiraeoside, gossypetin, cyanidin, and delphinidin were purchased from Indofine Chemicals (Somerville, NJ, USA). Restriction enzymes and SP6/T7 transcription materials were obtained from Ambion (Austin, TX, USA). cDNAs encoding human GLUT2, human GLUT5, and rabbit SGLT1 were used and plasmid constructs were described previously (26). Dulbecco's modified Eagle medium (DMEM) (25 mM glucose) was obtained from Biofluids (Rockville, MD, USA); all other media supplements were from GIBCO Life Technologies (Gaithersburg, MD, USA).

AtT20/D16v-F2 (mouse pituitary adenoma cells) and AtT20ins/CGT-6 (GLUT2-overexpressing mouse pituitary adenoma cells) were purchased from American Type Culture Collection (Rockville, MD, USA). Caco-2E cells were a generous gift from Drs. David Fitzgerald and Marian McKee (National Cancer Institute, NIH, Bethesda, MD, USA). *Xenopus laevis* was purchased from Xenopus One (Ann Arbor, MI, USA).

Preparation and injection of *Xenopus laevis* oocytes

Oocytes were isolated from *Xenopus laevis* and injected with cRNAs as described (16, 26). Briefly, opened ovarian lobes were incubated with two changes of collagenase (2 mg/ml, Sigma) in OR-2 medium without calcium (5 mM HEPES, 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM Na₂HPO₄, 1 mM CaCl₂, 100 μg/ml gentamicin, pH 7.8) for 30 min each and defolliculated mature oocytes (Stages V and VI) were isolated. After a 24 h recovery period at 18°C in OR-2 medium, 36 nanograms of cRNAs coding for intestinal monosaccharide transporters GLUT2, GLUT5, or SGLT1 in 36.8 nl were injected (Nanoject II injector, Drummond Scientific, Broomall, PA, USA). After injection, oocytes were incubated at 18°C in OR-2 containing 1 mM pyruvate with daily media changes until experiments were performed.

Glucose and fructose transport in injected oocytes

Two days postinjection, oocytes were equilibrated at room temperature in OR-2. Transport was initiated by adding flavonoids and [³H] 2-deoxyglucose, [¹⁴C] glucose, or [¹⁴C] fructose together at the indicated concentrations for the times specified at room temperature. Stock solutions of flavonoids in dimethyl sulfoxide (DMSO) were diluted with OR-2 before transport experiments. The resulting final concentration of 1% DMSO did not affect the transport of 2-deoxyglucose, glucose, or fructose (data not shown). Control oocytes were incubated in substrate with 1% DMSO without flavonoids. Transport was terminated by addition of excess ice-cold PBS, followed by three washes in that solution. Individual oocytes were dissolved in 100 μl of sodium dodecyl sulfate (SDS) 1% before addition of 5 ml Cytoscint scintillation cocktail (ICN Biomedicals, Aurora, OH, USA), and internalized radioactivity was quantified by scintillation spectrometry as pmol/oocyte. [³H] 2-Deoxyglucose was used in glucose transport experiments because it is phosphorylated rapidly and completely; it is not effluxed after phosphorylation, and therefore is trapped, and is not otherwise metabolized (27, 28). In experiments comparing sugar transport by SGLT1 and GLUT2, [¹⁴C] glucose was used because SGLT1 does not transport 2-deoxyglucose. Rabbit SGLT1 was used in experiments because rabbit SGLT1 and human SGLT1 are 87% identical in amino acid sequence, their predicted secondary structures are identical, and their transport properties are virtually the same (29).

Quercetin uptake *via* intestinal sugar transporters in oocytes

Oocytes were injected to express intestinal monosaccharide transporters GLUT2, GLUT5, or SGLT1, or were sham injected. Two days later oocytes were incubated in OR-2 with 250 μM [¹⁴C] glucose (specific activity, 4.68 mCi/mM) or 250 μM [¹⁴C] quercetin (specific activity, 52.9 mCi/mM) for 10 min. Transport was terminated by four washings with excess ice-cold PBS. Five or more oocytes were used to determine total radioactivity. Remaining oocytes (always >5) were transferred individually to a wired Petri dish that contained PBS.

The oocyte injector (Nanoject II injector system, Drummond Scientific) was set to maximal withdrawal (69.8 nl) and cytosolic fractions of constant volume were withdrawn from each oocyte using a capillary needle filled with mineral oil. Each capillary containing cytosolic fractions was transferred to a scintillation vial and radioactivity was determined by scintillation spectrometry.

Cell cultures

Cells were cultured in a humidified incubator (Forma Scientific, Marietta, OH, USA) in an atmosphere of 5% CO_2-95% air (v/v; O_2 partial pressure of 150 Torr) at 37°C. Untransfected mouse pituitary adenoma cells (AtT20/D16v-F2) and GLUT2-overexpressing mouse pituitary adenoma cells (AtT2ins/CGT-6; stably transfected with GLUT2-cDNA cloned into the vector pCB-7 immediately downstream of its cytomegalovirus promoter) (30) were grown in DMEM (25 mM glucose) supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, 1% none-essential amino acids, 1.5 g sodium bicarbonate, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). Caco-2E cells were grown in DMEM (25 mM glucose), supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% nonessential amino acids, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). All cells were subcultured at confluency by trypsin treatment.

For Caco-2E cell experiments using semipermeable membranes for uptake, throughput, and confocal microscopy, Caco-2E stock cell cultures were maintained in 75-cm² plastic flasks and cultured in a 95% air, 5% CO_2 atmosphere in Dulbecco's modified Eagle's minimal essential medium containing 15 mM glucose supplemented with 10% heat-inactivated FBS, 0.1 mM non essential amino acids, and 0.1 mM glutamine. All experiments were carried out on cells of passage number 48.

Transport measurements in AtT-20 cells and Caco-2E cells

For AtT-20 and Caco-2E cell transport experiments on 12-well plates (Corning Costar, Cambridge, MA, USA), cells were seeded and grown to confluence. Cells were washed twice with PBS and preincubated with Krebs buffer (glucose 5 mM; HEPES, 30 mM; NaCl, 130 mM; KH_2PO_4, 4 mM; $MgSO_4$, 1 mM; $CaCl_2$, 1 mM; pH 7.4) for 30 min at 37°C. Transport measurements were initiated by replacing the medium with 300 μl of prewarmed Krebs buffer without glucose supplemented with [^{14}C] fructose or [^3H] 2-deoxyglucose and flavonoids together during the required time at 37°C. Flavonoids were diluted 1:100 from concentrated stock solutions prepared fresh by dissolving flavonoids in DMSO. Control experiments demonstrated that 1% DMSO had no effect on 2-deoxyglucose and fructose transport (data not shown). Transport was terminated by adding 1 ml of ice-cold PBS and cells were washed three times with the same solution before lysis with 300 μl of NaOH (0.1 M)/CHAPS (10 g/L; J.T. Baker Inc, Phillipsburg, NJ, USA) solution. Aliquots of 100 μl were added to 0.5 ml of scintillation cocktail for radioactivity determination and the remainder used for protein measurement by bichinchonic acid (BCA protein assay; Pierce, Rockford, IL, USA).

For Caco-2E cell transport experiments on semipermeable membranes (Polyester Transwell® inserts, Costar 3460, pore size 0.4 μm), cells were seeded at a density of 1×10^4 cells/cm² onto semipermeable membranes and used 30 days later. Differentiation of the monolayer was assessed by light microscopy and confluence by electrical resistance of the epithelial cells in culture using a Millicell-ERS Voltohmmeter.

Only Transwell inserts with a resistance exceeding a blank membrane by 400 Ω were utilized in the experiments. Caco-2E cells were fully differentiated at the time of experiments and demonstrated a small intestinal phenotype, as described previously (31). Medium was changed a day prior to experiments. For experiments, medium was discarded and cells were washed once with Krebs buffer. To the upper compartments was added 200 μl Krebs buffer modified as follows: without glucose and containing the indicated concentrations of [^{14}C] fructose, and 1% DMSO with or without quercetin. Krebs buffer unmodified 600 μl was added to the lower compartment. After cells were incubated for the indicated times at 37°C, 200 μl buffer was removed from the lower compartment, each membrane was washed with ice-cold PBS three times, and 1 ml NaOH (0.1 mol/L)/CHAPS (10 g/L) solution was added to lyse cells. Aliquots were taken for scintillation spectrometry (200 μl) and protein measurement (25 μl), and sugar uptake was expressed per microgram of cell protein.

Caco-2E fixation, antibody (Ab) labeling, and confocal microscopy

Caco-2E cells grown on semipermeable insert supports were fixed for 10 min with freshly prepared 2% paraformaldehyde solution in PBS. After blocking with Blocking Reagent (Chemicon, Temecula CA, USA) for 60 min, monolayers were incubated overnight at 4°C with GLUT2 (1:200) or SGLT1 (1:200) antibodies in PBS (all antibodies were rabbit-derived from Chemicon). An Alexa-fluor546 labeled goat anti-rabbit secondary Ab (Invitrogen/Molecular Probes, Carlsbad, CA, USA) was used in appropriate concentration (usually 1:500) determined for each experiment to visualize primary Ab distribution.

For confocal microscopy, Transwell® inserts were cut out and mounted on microscope slides with #1.5 coverslips (Fisher Scientific, Hampton, NH, USA) for microscopy. Images were collected with a Zeiss 510 META laser scanning confocal microscope using a 63× Plan-Apochromat (N.A. 1.4) lens, 100 nm/pixel xy sampling, and a pinhole diameter set to provide an optical slice thickness of 1.0 μ. Image Z-axis series or stacks were collected through the depth of the cell monolayer using 400 nm step size. Differential interference contrast images were collected using the 543 nm laser line. Orthogonal views of each stack were exported as Tiff files using Zeiss LSM 510 software v 3.2 and organized into figures using Adobe Photoshop V 6.0 (Adobe Systems, Inc. San Jose, CA, USA).

Statistics

All data shown are representative of at least three experiments that yielded similar results, and all error bars indicate SD. For all experiments describing glucose and fructose transport in injected *Xenopus* oocytes, each data point represents the mean value of 10~15 oocytes ± SD. For Eadie-Hofstee transformations of GLUT2 transport activity in injected oocytes, GLUT 2 is a single component low-affinity, high-capacity transporter, so that Eadie-Hofstee transformed data of GLUT2 are linear (16, 28). Negative slopes of lines from Eadie-Hofstee transformations represent K_m values, and y intercepts represent V_{max} values. Comparisons of linear regressions of Eadie-Hofstee transformed data may distort experimental error. Given this limitation, slopes of lines without and with flavonoids were calculated (Sigmaplot, Systat Software, Richmond, CA, USA). Standard deviations of slope values were <10% for quercetin and <5% for isoquercitrin. Similar parallel slopes with Eadie-Hofstee transforma-

tions indicate constant K_m and variable V_{max}, characteristic of noncompetitive inhibition. For both [³H] 2-deoxyglucose and [¹⁴C] fructose transport in oocytes, IC_{50}s were calculated for each flavonoid. Data points (each representing the mean value of 10–15 oocytes as above) were analyzed by nonlinear regression analysis by fitting to a monoexponential equation (Sigmaplot). IC_{50} indicates flavonoid concentration at which sugar uptake was decreased by half compared with control without flavonoid. For experiments describing quercetin uptake by intestinal sugar transporters in injected oocytes, five or more oocytes were used for each experimental condition. Transport measurements in AtT-20 and Caco-2E cells represent mean values ± SD of 3 replicates.

RESULTS

Flavonoid inhibition of GLUT2 expressed in cRNA-injected *Xenopus laevis* oocytes

We first investigated whether flavonoids inhibited transport of one or both of the two major substrates for GLUT2: fructose and glucose. Transport was studied using *Xenopus* oocytes injected with human GLUT2 cRNA as a specific expression system. Flavonoids are divided into at least six different structural families: flavonols, flavones, flavanones, isoflavones, catechins, and anthocyanins (32). The flavonol quercetin was initially selected as a potential inhibitor because of its safety and because it is commonly consumed by humans (8, 33). Quercetin concentrations were chosen to reflect those achievable in the intestinal lumen (11). Glucose and fructose transport was measured in *Xenopus* oocytes 2 days postinjection with GLUT2 cRNA. For both substrates at 10 mM, transport was inhibited by ~ 50% by quercetin at 10 µM, a concentration 1000-fold lower than either substrate, and nearly complete inhibition occurred with 50 µM quercetin (**Fig. 1**).

Quercetin can be ingested as such (aglycone) or in a
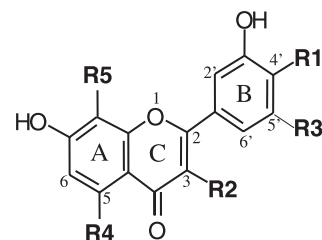
Quercetin	R1=OH, R2=OH, R3=H, R4=OH, R5=H
Rutin	R1=OH, R2=glucorhamnose, R3=H, R4=OH, R5=H
Isoquercitrin	R1=OH, R2=glucose, R3 =H, R4=OH, R5=H
Spiraeoside	R1=glucose, R2=OH, R3=H, R4=OH, R5=H
Myricetin	R1=OH, R2=OH, R3= OH, R4=OH, R5=H
Fisetin	R1=OH, R2=OH, R3=H, R4=H, R5=H
Gossypin	R1=OH, R2=OH, R3=H, R4=OH, R5=glucorhamnose
Gossypetin	R1=OH, R2=OH, R3=H, R4=OH, R5=OH

Figure 2. Flavonol and flavonol precursor structures. Flavonols have A, C, and B ring structures, with substitutions as indicated at B4′ (R1), C3 (R2), B5′ (R3), A5 (R4), and A8 (R5). Flavonol glycosides have glucorhamnose moieties, for example, at C3 (rutin) or A8 (gossypin). Flavonol glucosides have glucose moieties at C3 (termed isoquercitrin or quercetin 3-glucoside) or B4′ (termed spiraeoside or quercetin 4′glucoside). Quercetin is the aglycone form of rutin, and gossypetin is the aglycone form of gossypin. Other aglycone flavonols (myricetin, fisetin, gossypin) are shown by substitutions at R1-R5.

fully glycosylated form (rutin), which is partially or completely deglycosylated in the intestinal lumen (**Fig. 2**) (32). We investigated whether partially or completely glycosylated forms of quercetin inhibited transport of 2-deoxyglucose (**Fig. 3A**) and fructose (Fig. 3B) by GLUT2 expressed in *Xenopus* oocytes. Rutin (Fig. 2), a fully glycosylated form of quercetin found in foods, did not inhibit transport of either sugar (Fig. 3A, B). Isoquercitrin and spiraeoside, quercetin glucosides with a glucose moiety at the B4′(R1) position or C3 (R2) position, respectively, are produced as rutin is metabolized by intestinal microflora (Fig. 2) (8). Both isoquercitrin and spiraeoside inhibited 2-deoxyglucose and fructose transport by GLUT2 (Fig. 3A, B). Based on these data, we suggest that as fully glycosylated quercetin compounds are deglycosylated in the intestinal lumen, the luminal products can inhibit sugar transport by GLUT2.

In addition to quercetin and its glucosides, the efficacy of six flavonoid classes was tested upon inhibition of sugar transport by GLUT2 expressed in *Xenopus* oocytes (**Table 1**). Tested sugar substrates were 2-deoxyglucose and fructose, each at 10 mM. Flavonols and flavones were effective inhibitory flavonoid classes, and flavonols were particularly potent. For some flavonols, IC_{50} concentrations were ~1000-fold less than the sugar transporter substrate concentrations. Substitution of the 8 position hydrogen on the A ring with a hydroxyl group (gossypetin) or glucose moieties (gossypin) completely eliminated inhibition (Table 1; also refer to Fig. 2). As described above, the fully glycosylated flavonol rutin was not inhibitory. Quercetin inhi-
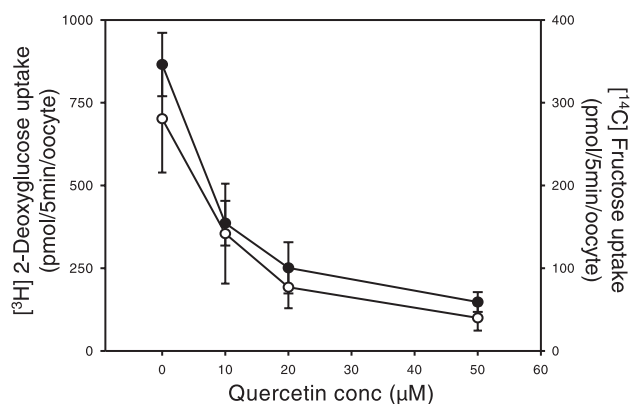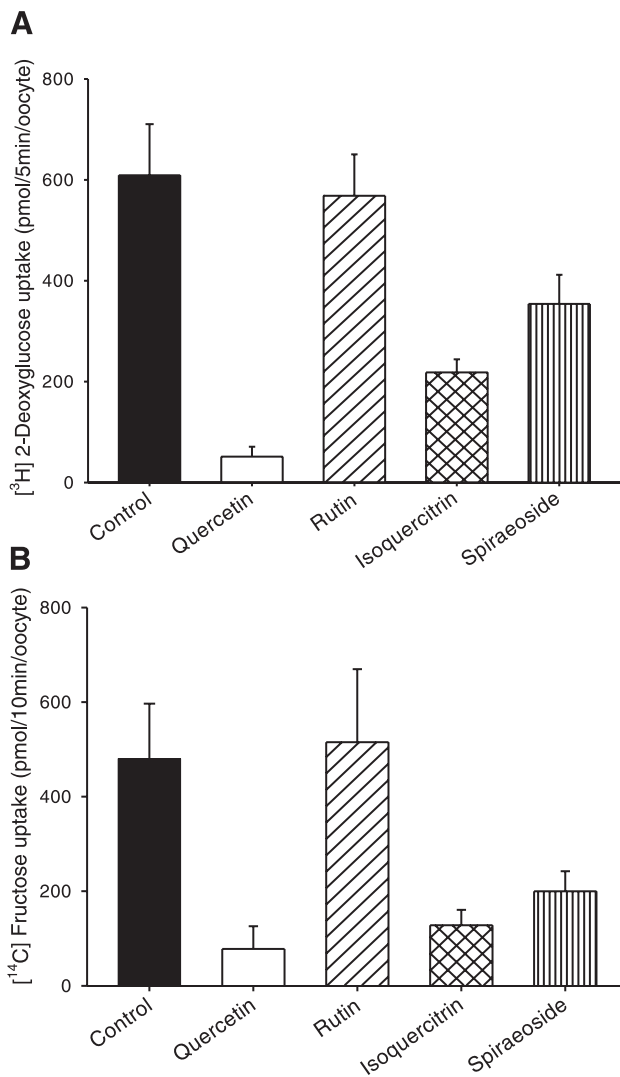
Figure 1. Quercetin inhibition of 2-deoxyglucose and fructose transport in *Xenopus laevis* oocytes injected to express GLUT2. Two days after GLUT2 cRNA injection, oocytes were incubated in OR-2 containing 10 mM of either [³H] 2-deoxyglucose (left axis, ●) or [¹⁴C] fructose (right axis, ○) with increasing concentrations of quercetin 0–50 µM for 5 min. Each data point represents mean ± SD 10–15 oocytes. Transport was determined by scintillation spectrometry.

A

B

Figure 3. Effect of quercetin precursors on 2-deoxyglucose and fructose transport in *Xenopus* oocytes injected to express GLUT2. Two days after GLUT2 cRNA injection, uptake of 10 mM [^{3}H] 2-deoxyglucose (*A*) or 10 mM [^{14}C] fructose (*B*) was measured without (■) or with 100 μM of quercetin (□), rutin (▨), isoquercitrin (▧), or spiraeoside (▥) in OR-2 for designated periods. Each bar represents mean ± SD 10–15 oocytes; transport was determined by scintillation spectrometry.

bition of sugar transport was completely reversible (data not shown).

Based on these data, the flavonol quercetin and its glucoside isoquercitrin were selected for study of inhibition mechanisms. Oocytes injected with GLUT2 were a straightforward model system useful for evaluating inhibitory mechanisms because uninjected or sham-injected *Xenopus* oocytes did not transport 2-deoxyglucose or fructose at all, and flavonol inhibition was reversible. Two days after injection of GLUT2 cRNA, *Xenopus* oocytes were tested for effects of isoquercitrin on 2-deoxyglucose transport (**Fig. 4***A*) and of quercetin on fructose transport (Fig. 4*B*). Both flavonols were noncompetitive inhibitors of sugar transport. For both flavonols, 50% inhibition of transport was observed at

flavonol concentrations that were ~1000-fold less than either sugar, confirming findings of inhibitor potency (Table 1, Fig. 1).

Specificity of flavonoid inhibition of intestinal sugar transporters

The effects of flavonoids were compared regarding the three intestinal sugar transporters GLUT2, GLUT5, and SGLT1 using transporters expressed in *Xenopus* oocytes. The bases of the experiments were that the intestinal sugars glucose and fructose are transported from the intestinal lumen into enterocytes by the following mechanisms: the facilitated transporter GLUT2 transports both glucose and fructose; the facilitated transporter GLUT5 transports fructose only; and the sodium-dependent glucose transporter SGLT1 transports glucose only (34, 35). Both glucose and fructose exit enterocytes *via* GLUT2 only (7).

For glucose, glucose transport by SGLT1 or GLUT2 was compared in the presence of no inhibitor; sodium replacement by choline (no sodium present); the flavonol quercetin; the glycosylated flavonol rutin; the flavonol glucosides isoquercitrin and spiraeoside; the GLUT2 transport inhibitor phloretin; and the SGLT1 transport inhibitor phlorizin (**Fig. 5***A*, *B*) (7). The data show that glucose transport by SGLT1 was not inhibited

TABLE 1. *IC$_{50}$s for flavonoid inhibition of 2-deoxyglucose and fructose uptake[a]*

Flavonoids		IC$_{50}$ (μM)	
		2-DG	Fructose
Flavonol	Quercetin	12.7	15.9
	Isoquercitrin	64.1	38.0
	Spriaeoside	103.3	66.1
	Fisetin	47.2	42.2
	Myricetin	17.2	11.9
	Rutin	NI	NI
	Gossypetin	NI	NI
	Gossypin	NI	NI
Flavone	Apigenin	65.7	65.3
	Luteolin	30.4	22.7
Flavanone	Hesperetin	48.6	66.5
	Naringenin	>300	>300
	Naringin	NI	NI
Isoflavone	Genistein	>300	>300
	Daidzein	NI	NI
Catechin	Catechin	NI	NI
	Epicatechin	NI	NI
Anthocyanidin	Delphinidin	NI	NI
	Cyanidin	NI	NI

[a] *Xenopus laevis* oocytes were injected to express GLUT2. Two days after cRNA injection oocytes were incubated in OR-2 containing 0–300 μM of the indicated flavonoid, and 10 mM of either 2-deoxyglucose or fructose for 5 min. IC$_{50}$ indicates the flavenoid concentration at which sugar uptake was decreased by half compared to control without flavonoid. An IC$_{50}$ for each flavonoid was calculated by nonlinear regression analysis of each inhibition curve (see Experimental Procedures, statistics). NI: No Inhibition; >300: decreased sugar uptake occurred, but that sugar uptake at 300 μM flavonoid remained above 50% compared to control without flavonoid.
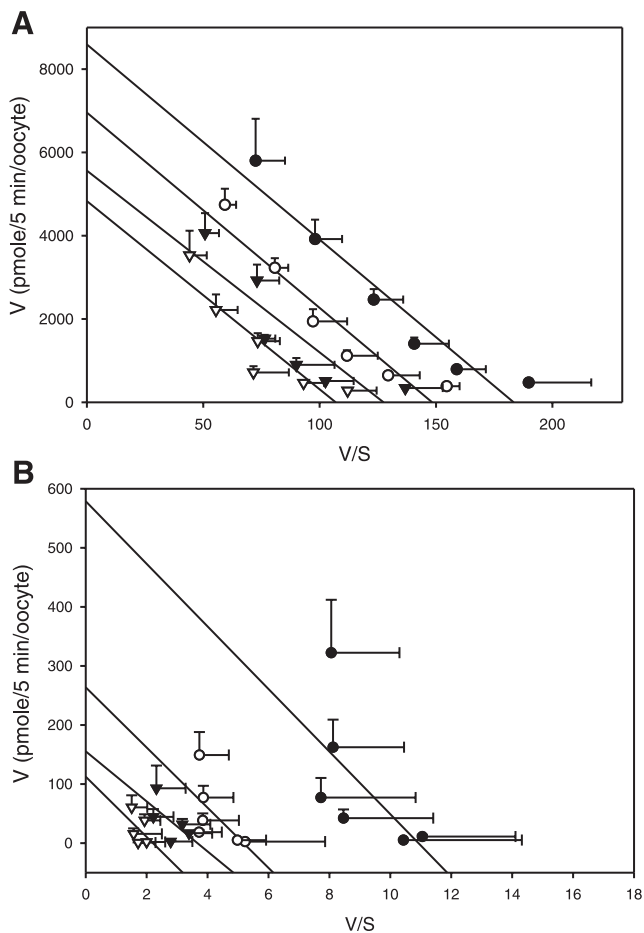
Figure 4. Kinetics of isoquercitrin inhibition of 2-deoxyglucose transport and quercetin inhibition of fructose transport in ooyctes injected to express GLUT2. Two days after GLUT2 cRNA injection, oocytes were incubated with 2.5, 5, 10, 20, 40, or 80 mM [^{3}H] 2-deoxyglucose (*A*) or 0.5, 1, 5, 10, 20, or 40 mM [^{14}C] fructose (*B*) for 5 min with or without varying concentrations of isoquercitrin (*A*) or quercetin (*B*). Transport was determined by scintillation spectrometry and kinetics was determined by Eadie-Hofstee analyses. Each point represents the mean ± sd of 10~15 oocytes. V indicates velocity; V/S indicates velocity divided by substrate concentration. *A*) Inhibition of [^{3}H] 2-deoxyglucose uptake by isoquercitrin at the following concentrations: 20 μM isoquercitrin (○), 40 μM isoquercitrin (▼), 60 μM isoquercitrin (▽), and 1% DMSO without isoquercitrin (●). *B*) Inhibition of [^{14}C] fructose uptake by quercetin at the following concentrations: 10 μM quercetin (○), 20 μM quercetin (▼), 50 μM quercetin (▽), and 1% DMSO without quercetin (●).

by quercetin, its glucosides, or rutin (Fig. 5*A*). Control data in the same panel show that SGLT1-mediated glucose transport was sodium dependent and inhibited by phlorizin but not inhibited by phloretin, a GLUT2 transport inhibitor (7, 36). The data also show that glucose transport by GLUT2 was inhibited by quercetin and its precursors as predicted and that GLUT2 was inhibited by phloretin but not phlorizin or the absence of sodium, also as predicted (Fig. 5*B*). These data confirm that flavonoids inhibit glucose transport specifically by GLUT2.

For fructose, fructose transport by GLUT2 and

GLUT5 was compared using similar conditions as for SGLT1 and GLUT2 (Fig. 5*C, D*). The data show that GLUT5 was not inhibited by quercetin, its glucosides, or rutin (Fig. 5*C*). GLUT5-mediated fructose transport was not expected to be inhibited by phloretin, phlorizin, or sodium-free media, and this was what was observed. As additional controls, GLUT2-mediated fructose transport was inhibited as predicted by quercetin, its glucoside precursors, and phloretin (Fig. 5*D*).

Flavonoid inhibition of GLUT2 in mammalian cells

Utilizing the *Xenopus* oocyte expression system, the experiments above indicated that GLUT2, but not other intestinal sugar transporters, was inhibited by quercetin, its glucosides, and flavonols. To test the relevance of the findings to cells, we investigated whether GLUT2 overexpressed in mammalian cells was similarly inhibited by flavonols, represented by quercetin. GLUT2 transport activity was studied in AtT20 GLUT2-transfected and control cells without expressed GLUT2 (30) using the substrates 2-deoxyglucose (**Fig. 6A, B**) and fructose (Fig. 6*C, D*). In GLUT2-transfected cells, 2-deoxyglucose transport was ~6-fold higher than in control cells. Transport was inhibited by quercetin in a concentration-dependent fashion and was complete at 200 μM quercetin, whereas no effect of rutin was observed (Fig. 6*A, B*). Because fructose transport is specific for GLUT2 and GLUT5, and transfected cells overexpress GLUT2, fructose transport was investigated as a further test of flavonoid inhibition of GLUT2. Fructose transport in transfected cells was 6- to 7-fold higher than in control cells, was completely inhibited by 100 μM quercetin, and was unaffected by rutin (Fig. 6*C, D*). Taken together, these data suggest that GLUT2 when overexpressed in cells is inhibited by the flavonol quercetin, similar to findings in oocytes.

To further explore the relevance of the findings, quercetin inhibition of GLUT2 was studied under conditions of GLUT2 expression in a native cell environment using Caco-2E intestinal cells. Fructose transport was investigated because it is transported only by GLUT2 and GLUT5, and because quercetin selectively inhibited GLUT2 (Fig. 5). Caco-2E cells were first studied on plates growing as polarized monolayers with the basolateral side attached (31, 37–39). Cells were incubated with 10 mM fructose and quercetin concentrations from 0 to 100 μM. Sugar transport was inhibited by progressively increasing quercetin concentrations (**Fig. 7A**). To further test quercetin inhibition, Caco-2E cells grown on semipermeable membranes (Transwell® inserts) were studied for fructose uptake and fructose throughput with and without quercetin (Fig. 7*B, C*). Fructose uptake (Fig. 7*B*, inset) and throughput (Fig. 7*B*) at 5 min was inhibited by quercetin 200 μM over a wide fructose concentration range, from 1–100 mM. Over longer incubation times, uptake of 50 mM fructose (Fig. 7*C*, inset) was delayed by 200 μM quercetin compared with control, and fructose

Figure 5. Effects of quercetin and quercetin precursors on glucose (*A, B*) or fructose (*C, D*) transport in oocytes injected to express SGLT1, GLUT5, or GLUT2. Oocytes injected to express SGLT1 (*A*) were tested for inhibition of [^{14}C] glucose transport by quercetin glycosides, quercetin, rutin, and other transport inhibitors. Oocytes injected to express GLUT 2 and incubated with the same transporter substrate (glucose) were tested as controls (*B*). Oocytes injected to express GLUT5 (*C*) were tested for inhibition of [^{14}C] fructose transport by quercetin glycosides, quercetin, and other transport inhibitors. Oocytes injected to express GLUT 2 and incubated with the same transporter substrate (fructose) were tested as controls (*D*). For all panels, medium with Na$^+$ present was OR-2 with [^{14}C] glucose or [^{14}C] fructose in the absence (■) or presence of 100 μM of the following: quercetin (□), rutin (▨), isoquercitrin (▧), spiraeoside (▥), phloretin (▦), or phlorizin (▤). Medium with Na absent (▨) was OR-2 with NaCl and Na$_2$HPO$_4$ replaced by KCl and K$_2$HPO$_4$, respectively, and contained [^{14}C] glucose or [^{14}C] fructose without flavonol. [^{14}C] Glucose concentrations; incubation times were 0.5 mM for 10 min (*A*) and 10 mM for 5 min (*B*). [^{14}C] Fructose concentration was 10 mM; incubation time was 10 min (*C, D*). Each bar represents mean ± SD 10–15 oocytes; transport was determined by scintillation spectrometry. Experiments were performed 2 days after cRNA injection.
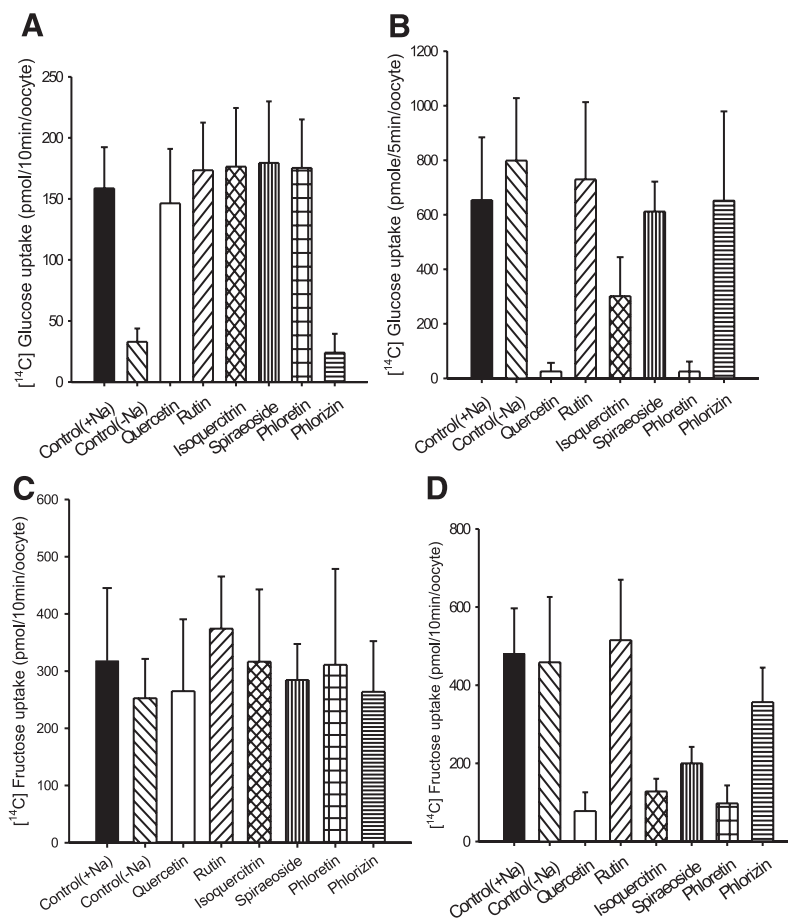
throughput (Fig. 7*C*) was inhibited over the entire 45 min time course. These data are consistent with the findings in oocytes injected with GLUT2 cRNA and pituitary cells that overexpressed GLUT2 (Figs. 1, 4, and 6).

We determined whether GLUT2 was present in the proper location to be inhibited by quercetin using Caco-2E cells grown to confluence on semipermeable membranes. GLUT2 and SGLT1 glucose transporters were visualized using antibodies to each transporter and confocal microscopy. GLUT2 was present on the apical membrane of Caco-2E cells, indicating that this transporter was in the proper location for potential inhibition by quercetin (**Fig. 8*A***). As a control, SGLT1 was also localized to the apical membrane, as predicted (Fig. 8*B*). It was not possible to visualize GLUT2 on the basolateral membrane because of interference from the membranes to which cells were adherent (not shown). These data provide evidence that inhibition of fructose uptake by quercetin in Caco-2E cells was due to inhibition of GLUT2 on the apical side.

Flavonoid transport

For transport inhibition to occur, flavonoids are predicted to interact with GLUT2. Whether flavonoids themselves are transported by GLUT2 is uncertain.

Experimental evidence suggests that some flavonoids might be transported by SGLT1 (18, 21, 23). Information concerning flavonoid transport is relevant for sugar absorption. This is because once sugars are transported into enterocytes from the apical surface by any of the three sugar transporters, sugars exit enterocytes on the basolateral surface only by GLUT2. If flavonoids were transported into enterocytes, there is the possibility they could then inhibit basolateral GLUT2. For these reasons, it was tested whether quercetin itself, as the most potent inhibitor of GLUT2, could be transported by GLUT2, GLUT5, or SGLT1.

These experiments were undertaken using [^{14}C] quercetin. Sham-injected oocytes do not transport [^{14}C] glucose or [^{14}C] fructose and have virtually no background radioactivity. However, sham-injected oocytes incubated with [^{14}C] quercetin have background radioactivity, which hinders determination of whether there is quercetin uptake. To avoid background, oocytes with or without expressed transporters were incubated with [^{14}C] substrate. After washing, transport was determined by micropuncture withdrawal of oocyte cytosol for scintillation spectrometry. As controls, glucose transport was analyzed by this method. Glucose was transported by GLUT2 and SGLT1, but no glucose transport occurred in sham-injected oocytes (**Fig. 9**). [^{14}C]Quercetin was not transported by any tested GLUT and SGLT1 (Fig. 9).
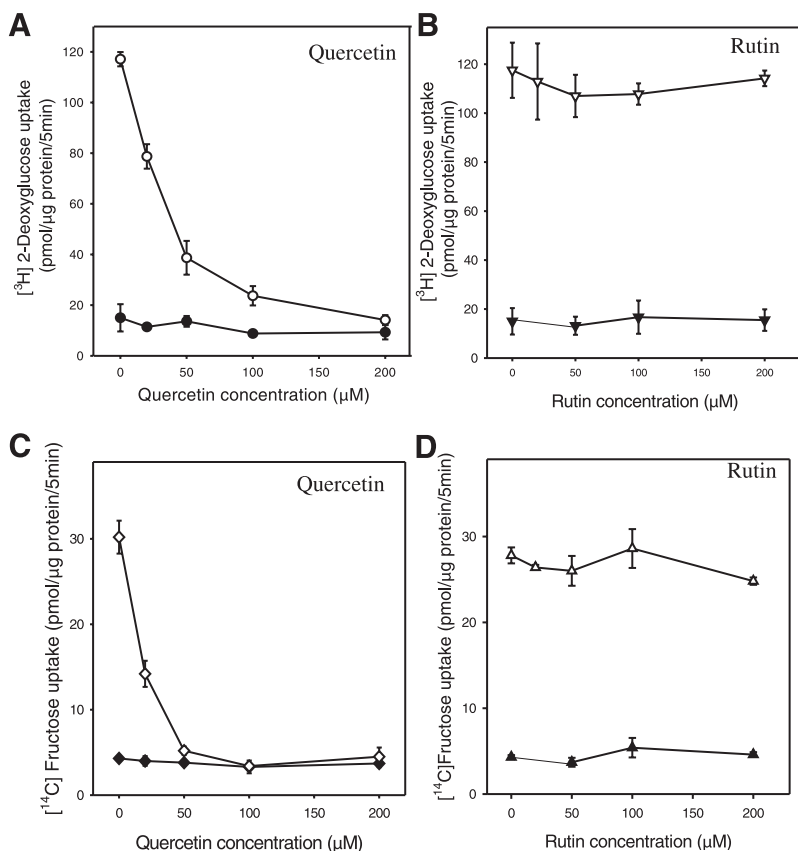
Figure 6. Quercetin and rutin inhibition of 2-deoxyglucose and fructose transport in AtT-20 cells overexpressing GLUT2. *A, B)* [^{3}H] 2-Deoxyglucose transport inhibition by increasing concentrations of quercetin *(A)* or rutin *(B)* in wild-type (WT) (filled symbols: ●, ▼) and GLUT2-overexpressing (open symbols: ○, ▽) AtT-20 cells. *C, D)* [^{14}C] Fructose transport inhibition by increasing concentrations of quercetin *(C)* or rutin *(D)* in WT (filled symbols: ◆, ▲) and GLUT2-overexpressing (open symbols: ◇, △) AtT-20 cells. Cells in 12-well plates were incubated in Krebs buffer containing 10 mM of either [^{3}H] 2-deoxyglucose *(A, B)* or [^{14}C] fructose *(C, D)* with increasing concentrations (0–200 μM) of quercetin *(A, C)* or rutin *(B, D)* for 5 min at 37°C. Data shown (mean±SD, n=3) are typical of more than three experiments with similar results.

DISCUSSION

The data here indicate that flavonols, exemplified by quercetin and a parent glucoside isoquercitrin, are potent noncompetitive inhibitors of the intestinal sugar transporter GLUT2. At flavonol concentrations 100-fold less than the sugar substrates, there was nearly complete inhibition of GLUT2 transport activity in *Xenopus* oocytes injected to express GLUT2. Quercetin, its glucoside isoquercitrin, or the related flavonol myricetin, all at concentrations 200- to 1000-fold less than sugar substrates, inhibited GLUT2 transport activity in *Xenopus* oocytes by ~50%. Quercetin and its glucosides behaved as specific inhibitors based on findings that the other major intestinal sugar transporters, GLUT5 and SGLT1, were unaffected. Quercetin inhibition of sugar transport in oocytes was reproduced in cells overexpressing GLUT2 and in Caco-2E intestinal cells where GLUT2 was present endogenously, suggesting that inhibition is independent of the membrane in which GLUT2 is expressed. GLUT2 was localized to the apical side of Caco-2E intestinal cells, indicating that GLUT2 is in the proper location to be inhibited by quercetin or its glucosides in the intestinal lumen. Although quercetin itself was not transported by any intestinal sugar transporters, other candidates have been suggested to transport quercetin or its glucosides into enterocytes (13, 23, 40). Thus, in addition to inhibition of apical GLUT2 by intraluminal flavonoids, it is possible that flavonoids transported into enterocytes are available to inhibit basolateral GLUT2 trans-port. Taken together, the data support the hypothesis that intestinal intraluminal flavonoids and their parent compounds dampen or inhibit intestinal glucose absorption (model shown in **Fig. 10**)

Our findings describing inhibition of GLUT2 by flavonoids fit well with the recent recognition that GLUT2 may be the dominant apical intestinal sugar transporter when intestinal glucose concentrations are high (7). SGLT1, the sodium-dependent glucose transporter, has long been described to be the apical intestinal transporter responsible for the majority of luminal glucose absorption (41, 42). In retrospect, this notion is inconsistent with other observations (6, 43). Kinetics properties of SGLT1 indicate that it is a high-affinity but low-capacity transporter with a K_m of $\sim \leq 2$ mM for glucose (7, 44, 45). Peak intestinal sugar concentrations, depending on glucose or fructose ingestion, might approach concentrations that are 50-fold higher. In contrast to SGLT1, GLUT2 is an attractive candidate as the dominant sugar transporter because it is a high-capacity transporter, has a much higher K_m for glucose and fructose, and transports both substrates (*vs.* glucose only for SGLT1) (7). A confounding problem was that GLUT2 was believed to localize only to the basolateral and not the apical enterocyte surface (46). More recent localization data show that GLUT2 localizes to the apical surface (6, 36); these data are confirmed here in Caco-2E cells. Inhibitor and transporter data also indicate that GLUT2 appears to play a major role in intestinal sugar absorption with high intraluminal sugar concentrations. Under these condi-
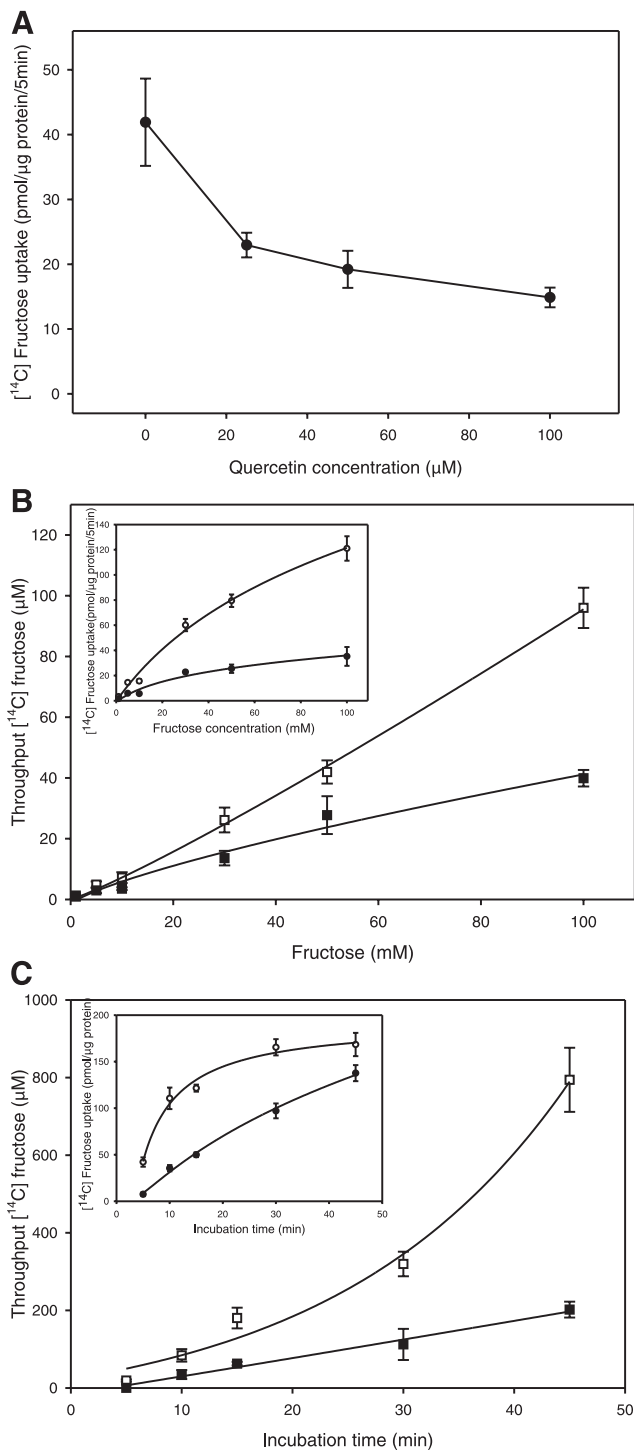
Figure 7. Quercetin inhibition of fructose transport in Caco-2E cells. *A)* Caco-2E cells grown on 12-well plates were incubated in buffer containing 10 mM of [^{14}C] fructose (●) and increasing quercetin concentrations. Fructose transport was measured after 5 min incubation. *B)* Caco-2E cells grown on semipermeable membrane supports were incubated with [^{14}C] fructose 1–100 mM for 5 min. Throughput (□, ■) and cellular content (inset) (○, ●) are displayed without (open symbols) or with (filled symbols) quercetin 200 μM. *C)* Caco-2E cells grown on semipermeable membrane supports were incubated in buffer containing 50 mM of [^{14}C] fructose from 5 to 45 min. Throughput (□, ■) and cellular content (inset) (○, ●) are displayed without (open symbols) or with (filled symbols) quercetin 200 μM.
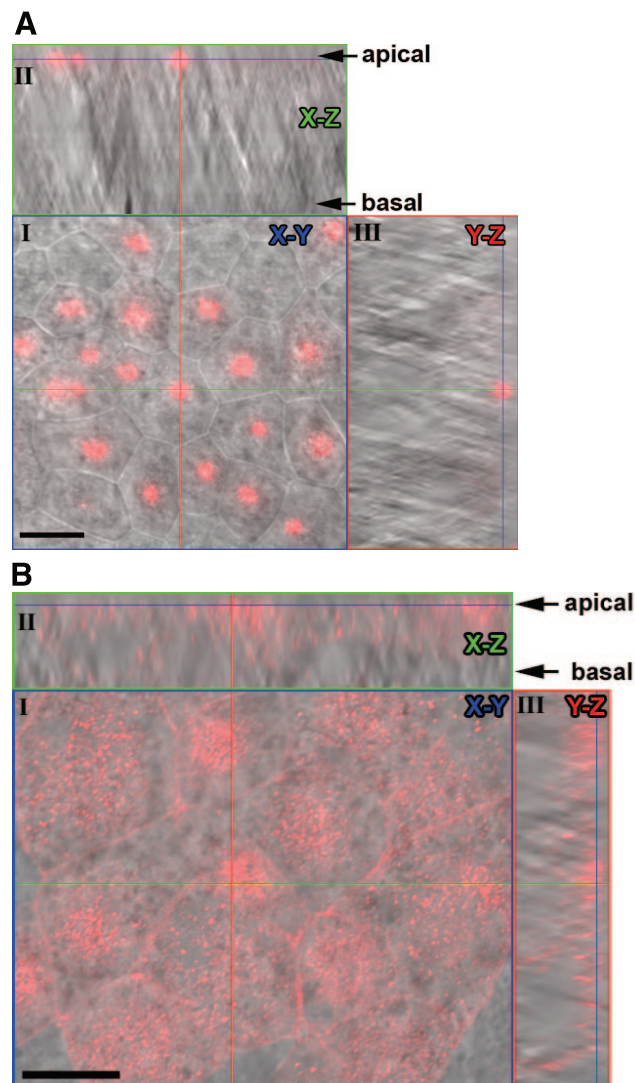

Figure 8. Distribution of GLUT2 (*A*) and SGLT1 (*B*) in Caco-2E cells. Caco-2E cells grown on semipermeable membrane supports were fixed with paraformaldehyde, incubated for 1 h with blocking reagent, and washed. After overnight incubation with GLUT2 or SGLT1 rabbit-derived antibodies in PBS, an Alexa-fluor546-labeled anti-rabbit secondary Ab was used for visualization. Represented are orthogonal views of the entire z axis series of Caco-2E cells labeled against GLUT2 (*A*) or SGLT1 (*B*). Panel I represents a single x-y plane at the apical side of the cells. GLUT2 (*A*) or SGLT1 (*B*) is shown in red fluorescence superimposed onto the differential interference contrast image. Panel II represents an x-z intersection of the x-y plane (shown by a green line in panel I), with the apical side at the top of panel II and basal side at the bottom of the panel. Panel III represents a y-z intersection of the x-y plane (shown by the red line in panel I), with the apical side shown at the far right of panel III and the basal side at the left of the panel. The blue line in panels II and III represents the position within the z axis series occupied by the single x-y plane shown in panel I. Scale bars in panels *A, B* represent ~10 μm.

tions, GLUT 2 is inserted in the apical membrane from intracellular vesicles (7, 47, 48). The findings here, that flavonoids inhibit GLUT2, specifically complement this new appreciation of GLUT2 biology.

The data here show that quercetin inhibition of

Figure 9. Quercetin uptake *via* intestinal sugar transporters in oocytes. Oocytes were injected with cRNA for GLUT2, GLUT5, or SGLT1 or were sham injected. Two days later oocytes were incubated with 250 μM [^{14}C] glucose or 250 μM [^{14}C] quercetin for 10 min. After washing, oocytes were individually transferred and fixed with phosphate buffer. Cytosolic fractions were withdrawn from each of at least 5 oocytes at each condition using the injector system at withdrawal mode (69.8 nl). Uptake was determined by quantitation of radioactivity using scintillation spectrometry.

GLUT2 is noncompetitive, indicating that binding sites on GLUT2 for sugar and quercetin are different. Similar effects of quercetin were observed in three different model systems of GLUT2, suggesting that the binding site for quercetin is a property of the transporter rather than the membrane GLUT2 inserts in. The specific binding site for quercetin on GLUT2 is unknown. Analysis of transport inhibition by flavonoids indicates that R1 and especially R2 groups (Fig. 2) of widely varying sizes on flavonols produce relatively small changes in inhibitor activity (Fig. 2, Table 1). These data suggest that some sites on the B and C flavonol rings do not interact closely with GLUT2. In contrast, hydrogen was the only R5 group found on flavonols that was inhibitory. These data imply that the A8 position might associate with a specific site on GLUT2. However, for complete structure activity relationships, a 3-dimensional structure of GLUT2 is necessary, and this remains to be determined. In depth knowledge of the specific binding site of quercetin and quercetin glucosides on GLUT2 would permit development of even more potent inhibitors, including some that would not be absorbed at all. An ideal inhibitor for future development based on the 3-dimensional structure of GLUT2 would have maximal potency with no absorption so as to minimize the possibility of adverse events.

Flavonoid inhibition of glucose transporters has been described for two other glucose transporters, but these localize to the periphery: GLUT1 and GLUT4 (49, 50). For such inhibition to occur *in vivo*, flavonoids would have to be absorbed, not undergo enterocyte or hepatic catabolism, then attain peripheral concentrations of 20–100 μM. However, intestinal absorption of quercetin is generally <10% and peak plasma concentrations are <2 μM (11, 14). Peak concentrations of flavonoid glucosides, which are better absorbed than aglycones, are 3–5 μM, and these concentrations decline rapidly. Unless additional flavonoid metabolites are found to be inhibitory and accumulate in higher concentrations, flavonoid inhibition of glucose transporters in peripheral tissues is unlikely to occur when physiology is considered (10, 12, 14).

An attractive aspect of the hypothesis presented here is *in vivo* relevance. The hypothesis is based on action of quercetin or its precursor glucosides in the intestinal lumen, independent of absorption. Depending on food choices, quercetin ingestion from foods can be as much as 100 mg daily. Quercetin or its glucosides may be available in effective amounts as intraluminal inhibitors of apical GLUT2. For plasma quercetin to be detected, some transport into enterocytes must occur. Intraenterocyte quercetin or metabolites could then further inhibit basolateral GLUT2 (see model, Fig. 10). The overall result, diminished glucose absorption, has potential benefits that can be classified in two ways: physiologically and pharmacologically.

Physiologically, inhibitory flavonoids are present and consumed in fruits and vegetables. Flavonoids present with sugars in foods could have potential benefit in dampening glucose absorption by decreasing the absorption rate. Dampening glucose absorption would be beneficial in reducing sudden increases in glucose and resulting spikes in plasma insulin concentrations. Fla-
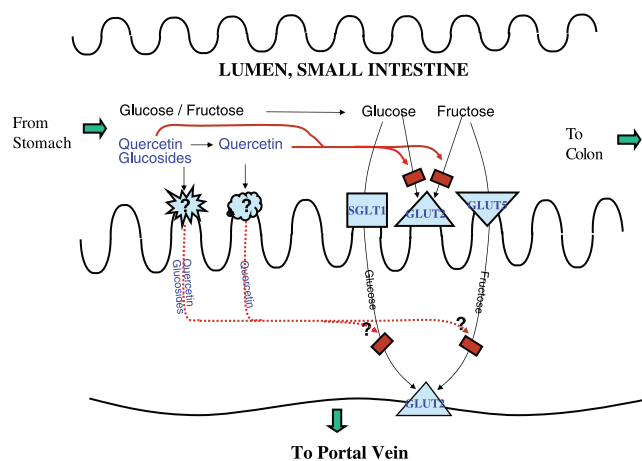

Figure 10. Model of flavonoid inhibition of sugar absorption in the small intestine. Intestinal glucose from ingested foods is transported by the apical (lumen facing) transporters SGLT1 and GLUT2, whereas fructose from foods is transported by apical transporters GLUT2 and GLUT5. Representative flavonoids are quercetin glucoside and its deglycosylated moiety quercetin. These compounds inhibit glucose and fructose transport by the dominant apical transporter GLUT2, but SGLT1 and GLUT5 are not inhibited. Identities of the apical transporters for quercetin and quercetin glucosides are uncertain, indicated by a question mark (?). Once transported, it is not known whether basolateral sugar transport (efflux) by GLUT2 is inhibited by quercetin, its glucosides, or metabolites, as indicated by a question mark (?).

vonoids in foods are both free compounds and glycosides, the latter metabolized during digestion to glucosides and free flavonoids (aglycones). As flavonoid glycosides and complex carbohydrates in foods both undergo digestion and hydrolysis, flavonoids that are increasingly potent GLUT inhibitors have the potential to be made available concurrently with glucose.

Pharmacologically, inhibitory flavonoids in principle could be used as nonabsorbed or poorly absorbed agents in the intestinal lumen to decrease either the rate or absolute amount of glucose absorption. With pharmacologic oral doses, inhibition of glucose absorption in theory could be dampened or decreased substantially by increasing the flavonoid amount above that found in foods. Quercetin is an attractive pharmacologic candidate because it has been tested extensively in animals, and in long-term studies has no toxicity (33). In humans, oral doses as high as 4 g have been given without side effects. Doses administered i.v. are toxic only when plasma concentrations are ~100-fold higher than those achieved orally. Based on the potency shown here and in animals (16), it is possible that 1 g of quercetin administered orally could inhibit absorption of 50–100 g of glucose. Two key benefits could accrue: reduction of postprandial hyperglycemia in diabetic subjects and in subjects with mild glucose intolerance; and reduction of the total amount of glucose absorbed as a caloric and weight reduction strategy.

The work presented here has as its basis the general hypothesis that pharmacologic amounts of poorly absorbed luminal compounds can inhibit intestinal absorption effectively. This hypothesis is exemplified by several compounds in clinical use, including orlistat, sucrose polyester, and acarbose (3–5). The best examples are stanol and sterol esters, natural components of some plant foods (51). In pharmacologic amounts, sterol and stanol esters decrease intestinal cholesterol absorption but are themselves poorly absorbed, especially stanol esters. Both compounds are believed to interfere with cholesterol micelle formation, a prerequisite of cholesterol absorption (51). When these agents are added to foods commonly consumed by hypercholesterolemic subjects, total and LDL cholesterol are reduced predictably and safely (5).

Although encouraging data were previously available concerning effects of flavonoids on inhibiting GLUT2 (16, 24), these findings were incomplete. Before a pilot clinical study could be considered, additional information was necessary and is now provided in this paper. This information includes transported substrate specificity for inhibition, effect of flavonoid glucoside metabolism on inhibition, effect of different flavonoids classes on inhibition, characterization of the mechanism of inhibition, determination of whether inhibition is affected by the membrane in which GLUT2 is expressed, colocalization of GLUT2 with relevant concentrations of inhibitors, and whether flavonoids themselves are transported by the affected glucose transporters. Our findings provide the essential foundation for a pilot clinical study to move forward, and this can now proceed. FJ

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