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Flavonoid conjugates interact with organic anion transporters (OATs) and attenuate cytotoxicity of adefovir mediated by organic anion transporter 1 (OAT1/SLC22A6)

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ABSTRACT

Flavonoids are conjugated by phase II enzymes in humans to form glucuronidated and sulfated metabolites that are excreted in urine via the kidney. In this study, we examined the interaction between metabolites of quercetin and isoflavonoids found *in vivo* with human organic anion transporters 1 (OAT1) and 3 (OAT3) and their potential in attenuating OAT-induced cytotoxicity of adefovir. Accumulation of flavonoid conjugates was studied in human embryonic kidney 293H cells overexpressing OAT1 or OAT3. OAT1-overexpressing cells exhibited an increased uptake of the sulfated conjugates, genistein-4′-*O*-sulfate and quercetin-3′-*O*-sulfate. OAT3-overexpressing cells demonstrated enhanced uptake of glucuronide conjugates, such as daidzein-7-*O*-glucuronide, genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide and quercetin-3′-*O*-glucuronide. Position of conjugation was important since quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide were poorly transported. Kinetic analysis revealed high affinity uptake of quercetin-3′-*O*-sulfate by OAT1 (K_m = 1.73 μM; V_{max} = 105 pmol/min/mg). OAT3 transported isoflavone glucuronides with lower affinity (K_m = 7.9–19.1 μM) but with higher V_{max} (171–420 pmol/min/mg). Quercetin-3′-*O*-sulfate strongly inhibited OAT1-mediated *p*-aminohippuric acid uptake with an IC_{50} of 1.22 μM. Transport of 5-carboxyfluorescein by OAT3 was potently inhibited by quercetin-3-*O*-glucuronide, quercetin-3′-*O*-glucuronide and quercetin-3′-*O*-sulfate (IC_{50} = 0.43–1.31 μM). In addition, quercetin-3′-*O*-sulfate was shown to effectively reduce OAT1-mediated cytotoxicity of adefovir, an antiviral drug, in a dose-dependent manner. These data suggest that OAT1 and OAT3 are responsible for basolateral uptake of flavonoid conjugates in kidney, and flavonoid conjugates inhibit OAT1 and OAT3 activity at physiologically relevant concentrations. Interaction with OATs limits systemic availability of flavonoids and may be a mechanism of food–drug interaction via inhibition of renal uptake.

1. Introduction

Quercetin is a chemopreventive and anti-inflammatory flavonoid that interacts with nuclear factor E2-related factor 2 (Nrf2) and activates antioxidant response elements (ARE) [1,2], contributing to enhanced cellular protection against carcinogens and oxidative stress [3]. Isoflavones are phytoestrogens that bind to the estrogen receptor [4] and exert preventive effects on hormone-dependent cancer [5], cardiovascular diseases [6], and post-menopausal osteoporosis [7]. Any biological activities of flavonoids, however, are influenced by their metabolism. Dietary flavonoids are metabolized extensively in the intestine and liver, and major forms found *in vivo* are glucuronide, sulfate and/or methyl-conjugates of the parent aglycone [8]. Quercetin is metabolized into quercetin-3-*O*-glucuronide, 3′-methylquercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate following consumption of onions [9]. After soy ingestion, soy isoflavones such as daidzein and genistein are present in the circulation predominantly as their 7-*O*-glucuronide and 4′-*O*-glucuronide, and sulfates were minor metabolites [10]. Concentrations of these metabolites in human plasma are typically low after intake of 50 mg aglycone equivalent and rarely exceed 10 μM even at much higher ingested doses [11].

Interaction with uptake and efflux transporters is considered to be one of the factors that limit the bioavailability of flavonoids, by facilitating the elimination of hydrophilic conjugates into bile

Abbreviations: BCRP, breast cancer resistance protein; MRP, multidrug resistance protein; OAT, organic anion transporter.

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and/or urine [12]. Urinary excretion of flavonoid conjugates has been well documented and varies according to the individual flavonoid aglycones. For the isoflavones daidzein and glycitein, about half of the intake could be recovered in the urine as conjugated metabolites [13]. A high urinary recovery of conjugates was also observed with catechins (~25%) [14]; while for quercetin the urinary excretion is much less than 10% of the intake [15]. Many drugs and their conjugated metabolites are eliminated in the urine via tubular secretion [16]. Vectorial transport of hydrophilic substances across the renal proximal tubules involves the interplay of uptake transporters on the basolateral membrane and the efflux transporters on the apical membrane. Active efflux is carried out by multidrug resistance proteins (MRP), which transport glucuronide and sulfate conjugates of flavonoids such as quercetin, baicalein and genistein [17–19]. The molecular mechanism underlying the possible uptake of flavonoid conjugates into renal tubules via basolateral transporters is not known.

OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*) are highly expressed on the basolateral membrane of proximal tubules [20]. They play an important role in the sodium-dependent renal uptake of structurally diverse organic anions from the blood into proximal tubule cells. OAT1 and OAT3 are capable of mediating the uptake of these organic anions against their electrochemical gradients by coupling to efflux of intracellular α-ketoglutarate. OAT1 interacts with endogenous metabolites, drugs including antivirals, antibiotics, nonsteroidal anti-inflammatory agents and statins, as well as toxicants such as mercury and ochratoxin [21]. OAT3 recognizes many OAT1 substrates; in addition, it has the ability to transport corticosterone, estradiol-17β-glucuronide and taurocholate, that are not substrates of OAT1 [22]. As toxins and cytotoxic drugs are taken up by OAT1 and OAT3, these toxic substances may exert toxic effects on the proximal tubule cells and cause renal injury. *In vitro* studies with OAT1-transfected cells showed that OAT1 over-expression sensitizes the cytotoxicity caused by antiviral drugs, adefovir and cidofovir [23]. Co-administration of the inhibitors of OATs may reduce the extent of OAT-induced nephrotoxicity [24]. On the other hand, inhibition of OAT1 and OAT3 may also alter the pharmacokinetics of a variety of drugs that are OAT substrates. OAT1 inhibitors, such as probenecid, could be administered with other drugs to prolong plasma half lives and improve therapeutic effects [25].

To date, only limited information is available concerning the interaction between OATs and flavonoids. Ellagic acid is a substrate and a potent inhibitor of OAT1 [26], while several flavonoids (naringenin, morin, silybin and quercetin) were also found to inhibit OAT1 and OAT3 [27]. However, the flavonoid aglycones are mostly absent in human plasma. The aim of this work is to investigate the transport of glucuronidated and sulfated metabolites of quercetin and isoflavones, major metabolites *in vivo*, in OAT1- and OAT3-transfected human embryonic kidney 293H cells. We also examined the inhibition of OATs by flavonoid conjugates and the potential of quercetin-3′-*O*-sulfate to attenuate OAT1-induced cytotoxicity of adefovir.

2. Methods and materials

2.1. Chemicals

Genistein and quercetin were obtained from Extrasynthese (Genay, France). Quercetin-7-*O*-glucuronide, quercetin-3-*O*-glucuronide and quercetin-3′-*O*-glucuronide were synthesized from quercetin using human liver S9 (Sigma–Aldrich, St. Louis, MO) and purified by HPLC [28]. Quercetin-3′-*O*-sulfate was chemically synthesized as described and purified by gel filtration [9]. The identity of quercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate was further confirmed by comparing the retention time and the absorption spectra of the authentic standards, kind gifts from Dr. Paul Kroon, Institute of Food Research, Norwich, UK. Purities were checked by HPLC to be over 95%. Daidzein-7-*O*-glucuronide, genistein-7-*O*-glucuronide, glycitein-7-*O*-glucuronide, genistein-4′-*O*-sulfate and daidzein-7,4′-*O*-disulfate were synthesized as previously described [10,29]. Adefovir was obtained by the hydrolysis of adefovir dipivoxil (Sigma–Aldrich, St. Louis, MO) using human intestinal S9 (Xenotech LLC, Kansas City, KS) and purified by gel filtration. Human embryonic kidney cells 293H were purchased from Invitrogen (Carlsbad, CA). OAT1 (SLC22A6 transcript variant 2, Genebank Accession Number: NM_153276) and OAT3 (SLC22A8 transcript variant 1, Genebank Accession Number: NM_004254) expression plasmids were obtained from Origene (Rockville, MD). Fugene HD was purchased from Roche (Basel, Switzerland). All other chemicals, unless otherwise stated, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. HEK293 cell culture

293H cells were routinely cultured in 75 cm^2 cell culture flasks (Corning Costar Corp., Cambridge, MA) at 37 °C under a humidified 5% CO_2/O_2 atmosphere. Culture media consisted of Dulbecco's modified minimum essential medium (DMEM) high-glucose media supplemented with 10% fetal bovine serum, 1% nonessential amino acids and 50 U/mL penicillin–streptomycin (all from Sigma–Aldrich). Cells were split in a 1:4 ratio every 48 h. Antibiotics were not added in transfection experiments. All experiments were performed with 293H cells between passages 1 and 20.

2.3. Transfection and uptake transport assay

The cells were seeded into poly-L-lysine coated 24-well plates at a density of 1.2×10^5 cells/well the day prior to transient transfection. OAT1 or OAT3 plasmids, or the empty pCMV-XL6 vector, were transfected into 293H cells with the Fugene HD reagent according to the manufacturer's directions, using an optimized ratio of 3:2 (μL reagent:μg DNA). The transfection complexes were formed in Opti-MEM (Invitrogen, Carlsbad, CA) and then added to cells after incubation for 18 min. Uptake assays were performed 22–24 h after transfection. The over-expression of the OATs was confirmed by quantitative RT-PCR (Taqman assays IDs: Hs00537914_m1 (SLC22A6) and Hs01056647_m1 (SLC22A8)) (Applied Biosystems, Foster City, CA), and by the uptake of model substrates *p*-aminohippuric acid (OAT1), and estrone-3-sulfate (OAT3). Uptake experiments were carried out using HBSS buffer containing 1.8 mM $CaCl_2$ and adjusted to pH 7.4 with 1 M HCl. The media were removed and the monolayers were washed twice with 0.25 mL transport buffer and incubated for 10 min. After that, the buffer was aspirated and 0.25 mL transport buffer containing the test compounds was added. Flavonoid and isoflavone conjugates were dissolved in DMSO (final concentration $< 0.2\%$), except quercetin-3′-*O*-sulfate which was dissolved in water. After the specified incubation time, the uptake was stopped by adding 1 mL ice-cold transport buffer containing 0.2% bovine serum albumin (BSA). This was quickly aspirated and further washed twice with 0.5 mL ice-cold transport buffer with 0.2% BSA. Finally, the cells were rinsed with 1 mL ice-cold transport buffer without BSA. Cells were collected with 0.4 mL lysis solution (50% methanol) and stored at −20 °C. Extraction was performed by sonication for 5 min followed by the addition of 1 mL of ice-cold acetone. The samples were placed in a −20 °C freezer for 1 h and centrifuged at 17,000 × *g* for 5 min. The supernatant was collected and evaporated to dryness *in vaccuo* at 30 °C and stored at −20 °C until analysis. The protein pellet was re-dissolved in 0.1 N NaOH and the protein contents were determined by the Bradford assay. All the uptake values were standardized against protein content.