Biochemical Pharmacology 84 (2012) 564–570

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Carrier-mediated transport of quercetin conjugates: Involvement of organic anion transporters and organic anion transporting polypeptides

Chi Chun Wong [a], Yasutoshi Akiyama [b], Takaaki Abe [b], Jonathan D. Lippiat [c], Caroline Orfila [a], Gary Williamson [a,d,*]

[a] School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK
[b] Division of Nephrology, Endocrinology, and Vascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
[c] Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK
[d] Nestle Research Center, Vers Chez les Blanc, 1000 Lausanne 26, Switzerland

ARTICLE INFO

Article history:
Received 28 March 2012
Accepted 14 May 2012
Available online 23 May 2012

Keywords:
Quercetin
HepG2
Carrier-mediated transport
Organic anion transporter
Organic anion transporting polypeptide

ABSTRACT

Flavonoids modulate cell signaling and inhibit oxidative enzymes. After oral consumption, they circulate in human plasma as amphiphilic glucuronide or sulfate conjugates, but it is unknown how these physiological metabolites permeate into cells. We examined the mechanisms of uptake of these conjugates into hepatocellular carcinoma (HepG2) cells, and found that uptake of quercetin-3′-*O*-sulfate was saturable and temperature-dependent, indicating the involvement of carrier-mediated transport. Quercetin-3-*O*-glucuronide was taken up predominantly via passive diffusion in these cells. Quantitative real-time PCR analysis showed high expression of OATP4C1, followed by OAT2, OAT4 and low expression of OATP1B1 in HepG2 cells, and addition of inhibitors of OATs and OATPs resulted in a significant reduction in quercetin-3′-*O*-sulfate uptake. The accumulation of quercetin-3′-*O*-sulfate was further evaluated in HEK293 cells expressing OAT2, OAT4 and OATP4C1. Uptake of quercetin-3′-*O*-sulfate was 2.3- and 1.4-fold higher in cells expressing OAT4 and OATP4C1 at pH 6.0, respectively, than in control HEK293 cells. siRNA knockdown of OATP4C1 expression in HepG2 cells reduced uptake of quercetin-3′-*O*-sulfate by ~40%. This study highlights a role for OATs and OATPs in the cellular uptake of biologically active flavonoid conjugates.

1. Introduction

Quercetin is a biologically active flavonoid that influences cell signaling pathways [1], inhibits the sodium-dependent vitamin C transporter [2], and modulates oxidative enzymes such as COX-2 [3] and lipoxygenase [4], thereby reducing the formation of intracellular superoxide and other reactive oxygen species. The biological activity is likely derived from the predominant forms of quercetin found in blood in vivo, the 3′-*O*-sulfated, 3-*O*-glucuronidated and 3′ or 4′-methylated derivatives. Quercetin glucuronides reduce oxidative stress via inhibition of xanthine oxidase and myeloperoxidase, enzymes that participate in free radical generation [5,6]. Some quercetin conjugates down-regulate the expression of cyclooxygenase-2 (COX-2), a key enzyme in the production of pro-inflammatory eicosanoids. Quercetin-3′-*O*-sulfate directly inhibited COX-2 activity, whereas quercetin-3-*O*-glucuronide did not [7]. Emerging evidence supports a role for quercetin metabolites in vascular health. The sulfated and glucuronidated conjugates of quercetin prevent oxidative stress-induced endothelial dysfunction in rat aorta [8]. Quercetin-3-*O*-glucuronide was also found to exert an antiatherosclerotic effect by suppressing the expression of scavenger receptors in activated macrophages [9].

In humans, quercetin is extensively metabolized via glucuronidation, sulfation, or methylation, resulting in the formation of numerous conjugated metabolites [10]. Phase II conjugates, such as quercetin-3′-*O*-sulfate, are the predominant circulating forms in plasma (<10 μM), and the aglycone is not found in vivo [10,11]. Contribution of the bile to the secretion of quercetin conjugates was estimated to be 10–30% and their concentration in the bile could reach in excess of 300 μM in mice [12].

Quercetin conjugates are markedly more hydrophilic compared to quercetin [13]. Transport of hydrophilic and ionized substances from the circulation into bile or urine involves interplay of uptake transporters on the basolateral membrane and efflux transporters on the apical side, which are present in hepatocytes and proximal

Abbreviations: COX2, cyclooxygenase-2; HBSS, hank's balanced salt solution; OATs, organic anion transporters; OATPs, organic anion transporting polypeptides.

* Corresponding author at: School of Food Science and Nutrition, University of Leeds, Leeds, LS2 9JT, UK. Tel.: +44 0 113 343 8380; fax: +44 0 113 343 2982.
E-mail address: g.williamson@leeds.ac.uk (G. Williamson).

0006-2952/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2012.05.011

tubular cells. Basolateral uptake of organic anions, such as glucuronide and sulfate conjugates, is thought to involve organic anion transporters (OATs) and/or organic anion-transporting polypeptides (OATPs) [14]. They are multispecific and mediate the transport of various amphiphilic organic anions, including endogenous compounds, structurally diverse drugs and their conjugated metabolites. Thus, OATs and OATPs are important determinants for excretion of xenobiotics.

Human liver showed a high expression of OATP1B1 and OATP1B3, as well as moderate expression of OATP1A2, OATP4C1 and OATP2B1 [15,16]. OAT2, OAT4 and OAT7 mRNA have also been detected [16,17]. OAT1, OAT3, and OATP4C1 are the major uptake transporters found in the human kidneys. Active efflux is carried out by ATP-binding cassette transporters including P-glycoprotein, multi-drug resistance protein 2 (MRP2/ABCC2) and breast cancer resistance protein (BCRP/ABCG2) [18]. OATs/OATPs and ABC transporters are believed to act in concert to affect vectorial transport of various organic anions, and thus facilitate their elimination from the body.

Recently, it has been shown that the green tea flavonoids epicatechin gallate and epigallocatechin gallate are substrates of OATP1A2 and OATP1B3 [19], and that several flavonoid and their conjugated forms can interact with OAT1, OATP1A2 and OATP1B1 [20–26]. However, no physiologically relevant flavonoid conjugates have been shown to interact with members of the OAT or OATP families. Hence, we hypothesized that conjugates of flavonoids such as quercetin may be potential substrates for OATs and OATPs. Here, we demonstrate a carrier-mediated uptake mechanism for quercetin-3′-*O*-sulfate into HepG2 cells, but not for quercetin glucuronides, and identified potential OAT and OATP transporters involved in the uptake in HepG2 cells.

2. Materials and methods

2.1. Chemicals

Sulfobromophthalein was from Acros Organics (Geel, Belgium). Quercetin-7-*O*-glucuronide, quercetin-3-*O*-glucuronide and quercetin-3′-*O*-glucuronide were synthesized enzymatically and then purified by HPLC [27]. Chemical synthesis of quercetin-3′-*O*-sulfate was performed as described [28]. The identity of the compounds was further confirmed by comparing the retention time and absorption spectra of quercetin-3-*O*-glucuronide and quercetin-3′-*O*-sulfate kindly provided by Dr. Paul Kroon, Institute of Food Research, UK. The purities were checked by HPLC to be over 95%. HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Human embryonic kidney cells HEK-293 were purchased from Invitrogen (Carlsbad, CA). OAT1 (SLC22A6 transcript variant 2, Genebank accession number: NM_153276), OAT2 (SLC22A7 transcript variant 1, Genebank accession number: NM_006672.2), OAT3 (SLC22A8 transcript variant 1, Genebank accession number: NM_004254) and OAT4 (Genebank accession number: NM_018484.2) expression plasmids were obtained from Origene (Rockville, MD). Full length OATP4C1 cDNA (Image clone 100016240) (Geneservice, Cambridge, UK) was re-cloned into pcDNA6/V5-His (forward primer: 5′-ggatccatgaagagcgccaaaggtatt-3′; reverse primer: 5′-gcggccgcacttacccttctttttactattttgttgag-3′) for transfection into mammalian cell lines. Fugene HD was purchased from Roche. Other chemicals, unless otherwise stated, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

HepG2 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. The culture media consisted of Eagle's Minimum Essential Medium media supplemented with 10% fetal bovine serum and 100 U/mL penicillin–streptomycin. All experiments were performed with cells between passages 80 and 95. HEK-293 cells were cultured in Dulbecco's modified Minimum Essential Medium high-glucose media supplemented with 10% fetal bovine serum, 1% non-essential amino acids and 50 U/mL penicillin–streptomycin, in 5% CO_2 at 37 °C. Cells were used between passages 4 and 20.

2.3. Confocal microscopy

5×10^4 HepG2 cells were plated in 12 mm poly-L-lysine-coated coverslips (BD Bioscience, Bedford, MA) within six-well plates. Cells were then cultured for 96 h before the experiment. Incubation medium used was Hank's Balanced Salt Solution (HBSS) buffer supplemented with 1.8 mM $CaCl_2$, adjusted to pH 6 with 1 M HCl. Various concentrations of quercetin, quercetin-7-*O*-glucuronide, quercetin-3-*O*-glucuronide, quercetin-3′-*O*-glucuronide and quercetin-3′-*O*-sulfate were added to the cells and then incubated for 30 min at 37 °C. Uptake was stopped by washing with ice-cold HBSS three times and then cells were fixed with 4% formaldehyde for 5 min. Coverslips were washed with water and mounted upside-down on glass slides. The images were obtained with a Leica TCS SP2 confocal laser scanning microscope (CLSM) equipped with a Linkam PE 94 heating/cooling stage. CLSM was operated in reflection mode with an Ar/ArKr laser source (488 nm) and a 40× oil-immersion objective lens [29].

2.4. Uptake of quercetin conjugates into HepG2 cells

HepG2 cells were seeded into 12-well plates (Corning Costar Corp., Cambridge, MA) at a cell density of 2×10^5 per well. After 72–96 h, uptake experiments were carried out using HBSS buffer supplemented with 1.8 mM $CaCl_2$ and adjusted to pH 6 or pH 7.4 with 1 M HCl. After removal of media, cells were washed twice with 0.4 mL transport buffer and incubated for 15 min. It was replaced with 0.4 mL transport buffer containing quercetin metabolites. Quercetin glucuronides were dissolved in DMSO (final concentration $< 0.5\%$); whereas quercetin-3′-*O*-sulfate was dissolved in water. Digoxin and inhibitors were dissolved in DMSO (final concentration $< 0.1\%$) and controls with identical concentrations of DMSO were used. After 10 min, during which uptake was linear, 2 mL ice-cold transport buffer containing 0.2% bovine serum albumin (BSA) was added. 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 0.5 mL ice-cold transport buffer.

2.5. Uptake of quercetin-3′-O-sulfate into OAT2, OAT4 and OATP4C1 expressing cells

HEK-293 cells were seeded into poly-L-lysine coated 24-well plates at a density of 1.2×10^5 cells/per well. 2 μg of OAT2, OAT4 or OATP4C1 plasmids, or the empty vector was mixed with 3 μL Fugene HD reagent, in 100 μL Opti-MEM (Invitrogen). After incubation for 18 min at room temperature, 25 μL of transfection complex was added to each well. Uptake assays were performed 22–24 h after transfection. Each was carried out in HBSS containing 1.8 mM $CaCl_2$ and 1.8 mM $MgCl_2$ at pH 7.4. Media was removed and the monolayer was washed twice with 0.25 mL transport buffer. After 10 min, buffer was replaced with transport buffer containing the test compounds. After the incubation period, uptake was stopped by adding 1 mL ice-cold transport buffer containing 0.2% BSA. This was removed and washed twice with 0.5 mL ice-cold transport buffer with 0.2% BSA, and finally washed with 1 mL ice-cold transport buffer.