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Research Paper

Interactions of Stevioside and Steviol with Renal Organic Anion Transporters in S2 Cells and Mouse Renal Cortical Slices

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Purpose. Our previous studies have shown that both stevioside and steviol inhibited transepithelial transport of *para*-aminohippurate (PAH) in isolated rabbit renal proximal tubules by interfering with organic anion transport system. The current study examined the direct interactions of stevioside and steviol with specific organic anion transporters.
Methods. S2 cells expressing human organic anion transporters (hOAT1, hOAT2, hOAT3, and hOAT4) and an intact renal epithelium were used to determine the inhibitory effect of stevioside and steviol on organic anion transport.
Results. Stevioside at 0.5–1 mM showed no interaction with any OAT. In contrast, steviol markedly inhibited substrate uptake in all S2hOAT cells. Steviol had low IC_{50} values for hOAT1 (11.4 μM) and hOAT3 (36.5 μM) similar to that of probenecid, whereas IC_{50} values for hOAT2 (1000 μM) and hOAT4 (285 μM) were much higher. Results obtained in mouse renal cortical slices were very similar; that is, stevioside was without inhibitory effect and steviol was a potent inhibitor of PAH and estrone sulfate (ES) transport.
Conclusions. Stevioside has no interaction with human or mouse OATs. In contrast, steviol interacts directly with human OATs, in particular, hOAT1 and hOAT3, with a potency approximating probenecid, suggesting that the inhibition of OAT-mediated transport by steviol could alter renal drug clearance.

KEY WORDS: organic anion; organic anion transporter; renal cortical slices; steviol; stevioside.

INTRODUCTION

Stevioside is the major sweet component isolated from the leaves of *Stevia rebaudiana*. It is about 300 times sweeter than sucrose, but it is noncaloric (1). Therefore, it has become popular as a sweetener in Asia and South America and has been used as a dietary supplement in the United States (2). Stevioside can be degraded to its major metabolite, steviol, by intestinal bacterial microflora from various species including man (3–5). The chemical structures of stevioside and steviol are shown in Fig. 1. Stevioside has been shown to have therapeutic value as an antihypertensive or antihyperglycemic agent (6–10). The available data indicate that stevioside is nontoxic, nonmutagenic and noncarcinogenic in various mammalian species (11,12). In contrast, its aglycone metabolite, steviol, has been reported to be mutagenic in *Salmonella typhimurium* TM677 (13). Likewise, at doses ~6 g/kg BW, steviol was lethal to the hamster, and its LD_{50} value was ~15 g/kg BW in rats and mice (12). Thus, questions remain concerning the toxicity of stevioside and steviol that should be addressed prior to their widespread commercial use as food additives or drugs.

In particular, the renal handling of these agents is critical, as it determines the ease with which they are cleared from the body, or conversely the potential for their accumulation upon chronic consumption. Previous studies have shown that stevioside and steviol inhibited PAH uptake in rat renal cortical slices (14), suggesting that one or both compounds may be handled by the organic anion secretory system of the kidney. Indeed, our own earlier study indicated that a pharmacological concentration (0.7 mM) of stevioside inhibited transepithelial transport of PAH without changes in Na^+/K^+-ATPase activity or cellular ATP content in isolated S2 segments of rabbit renal proximal tubule (15). We also found that steviol inhibited transepithelial transport of PAH at the basolateral entry step by competitive inhibition, suggesting that steviol binds to basolateral organic anion transporter (OATs) (16). However, this *in vitro* study did not permit the clear differentiation between the interactions of stevioside and steviol with the specific organic anion transporters. Currently, several organic anion transporter isoforms have been cloned and characterized. In humans,

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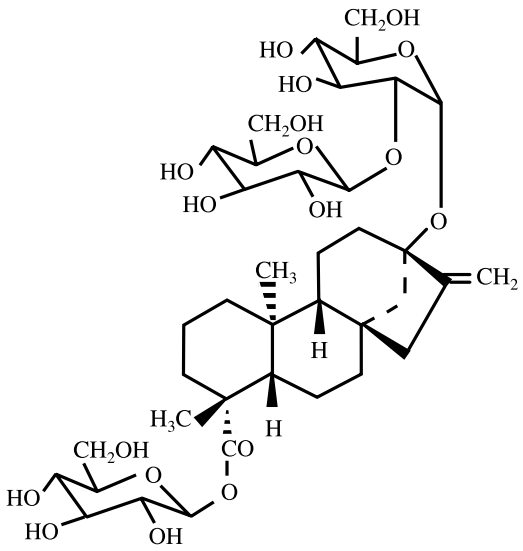
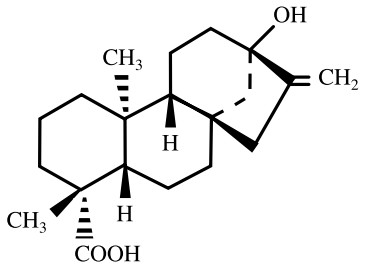
ABBREVIATIONS: D-PBS, Dulbecco's modified phosphate-buffered saline; ES, estrone sulfate; hOAT, human organic anion transporter; PAH, *para*-aminohippurate; $PGF_{2\alpha}$, prostaglandin; TEA, tetraethylammonium.



Stevioside ($C_{38}H_{60}O_{18}$)

MW 804.9



Steviol ($C_{20}H_{30}O_3$)

MW 318.4

Fig. 1. The chemical structures of stevioside and steviol.

OAT1, OAT2, and OAT3 are expressed at the basolateral membrane (17–19), whereas OAT4 is expressed at the apical membrane of the proximal tubule (20,21). These transporters play important roles for the net elimination of various organic anion compounds including therapeutic drugs. The inhibition of basolateral OATs could also reduce clearance of those therapeutic drugs transported by the OATs, potentially leading to altered therapeutic efficacy or even increased toxic side-effects of these drugs. For example, it was shown recently that methotrexate transport via hOAT1, hOAT3, and hOAT4 was inhibited by probenecid, penicillin G, and nonsteroidal inflammatory drugs (NSAIDs) (22). Therefore, these human OATs are potential sites of interactions between stevioside and steviol with anionic drugs as well.

The purpose of this study was to investigate the direct interactions of stevioside and steviol with specific renal organic anion transporters. Both S2 cells expressing specific organic anion transporters, hOAT1, hOAT2, hOAT3, hOAT4, and mouse renal cortical slices were used to determine the inhibitory effects of stevioside and steviol on organic anion transport.

MATERIALS AND METHODS

Chemicals

[^{3}H]-PAH (1480 GBq/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), and [^{14}C]-PAH (1.50 GBq/mmol), [^{3}H]-$PGF_{2\alpha}$ (6808 GBq/mmol), and [^{3}H]-ES (1861 GBq/mmol) were purchased from New England Nuclear Corp (Boston, MA, USA). Unlabeled PAH and ES, probenecid, α-ketoglutarate, glutarate, furosemide, bumetanide, indomethacin, cimetidine, methotrexate, tetraethylammonium (TEA), transferrin, and SRB were purchased from Sigma (St. Louis, MO, USA). Epidermal growth factor was purchased from Wakunaga (Hiroshima, Japan). Insulin was purchased from Shimizu (Shizuoka, Japan), RITC 80-7 culture medium was purchased from Iwaki Co. (Tokyo, Japan). Stevioside (≥98% purity) and steviol (≥98% purity) were kindly provided by Dr. Chaivat Toskulkao (Department of Physiology, Faculty of Science, Mahidol University, Thailand) (12). The purity of both compounds was analyzed by high-performance liquid chromatography (HPLC) (unpublished data). All other chemicals and reagents used were analytical grade and obtained from commercial sources.

Cell Cultures

Cells from the S2 segment of the proximal tubule transfected with human organic anion transporters hOAT1, hOAT2, hOAT3, and hOAT4 (designated as S2hOAT1, S2hOAT2, S2hOAT3, and S2hOAT4) used in this work were established previously (22,23). They were derived from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen genes. Briefly, the OAT-transfected cell lines were obtained by transfection of S2 cells with pcDNA 3.1 containing each of the human OATs 1–4 coupled with pSV2neo, a neomycin resistance gene, using TfX-50 according to the manufacturer's instructions (Promega, Madison, WI, USA). S2 cells transfected with pcDNA 3.1 lacking an insert and pSV2neo were designated as S2mock and used as control group. These cells were grown in RITC 80-7 medium containing 5% fetal bovine serum, 10 mg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor, and 400 mg/ml geneticin in a humidified incubator under 5% CO_2/95% air at 33°C, a permissive temperature for these cell lines. The cells were subcultured in the medium containing 0.05% trypsin-EDTA solution (in mM: 137 NaCl, 5.4 KCl, 5.5 glucose, 4 $NaHCO_3$, 0.5 EDTA, and 5 HEPES, pH 7.2).

Organic Anion Uptake

The transfected S2 cell lines were seeded in 24-well tissue culture plates at a cell density of 1×10^5 cells/well. After culturing for 2 days, uptake experiments were performed at 37°C. Based on the literature, the following substrates were used to determine transport by the human-transfected S2 cell lines: [^{14}C]-PAH for hOAT1 (17), [^{3}H]-prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) for hOAT2 (23), and [^{3}H]-estrone sulfate (ES) for hOAT3 and hOAT4 (18,20). For transport measurement, the cells were first washed three times with incubation

medium, Dulbecco's modified phosphate-buffered saline (D-PBS) (in mM: 137 NaCl, 3 KCl, 8 $NaHPO_4$, 1 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, and 5.6 D-glucose, pH 7.4), and preincubated in the same solution for 10 min. The cells were then incubated for 30 s for hOAT2 and 2 min for hOAT1, hOAT3, and hOAT4 (approximates initial rate of uptake for each substrate via its transporters) (data not shown) in the D-PBS solution containing specific substrates, 5 μM [^{14}C]-PAH for hOAT1, 50 nM [^{3}H]-$PGF_{2\alpha}$ for hOAT2, or 50 nM [^{3}H]-ES for hOAT3 and hOAT4 in the absence or presence of stevioside and steviol. Uptake was stopped by the addition of ice-cold D-PBS solution, and the cells were washed three times with the same solution. The cells in each well were lysed with 0.5 ml of 0.1 N NaOH, and the radioactivity was measured by liquid scintillation spectrometry (1214 Rackbeta, LKB Wallac, Sweden).

Kinetic Analysis of Steviol Inhibition of PAH

S2 cells expressing hOAT1 were preincubated in D-PBS solution at 37°C for 10 min as described above. They were then incubated in D-PBS containing [^{14}C]-PAH at concentrations from 10 to 600 μM in the absence and presence of steviol for 2 min. The inhibitory constants (IC_{50}) were calculated from sigmoidal dose-response analysis using GraphPad Prism version 4.00 for windows (GraphPad Software, San Diego, CA, USA). The data were plotted as a Lineweaver–Burk plot (1/[PAH] *vs.* 1/[PAH] uptake) and the K_m (Michaelis–Menten constant) was estimated from the *x*-axis intercept. The maximal rate of PAH uptake (V_{max}) mediated by hOAT1 was estimated from the *y*-axis intercept. The K_i of steviol for PAH transport was calculated to determine the affinity of steviol for the transporter as shown in the following equation for competitive inhibition (24):

$$K_i = \frac{IC_{50}}{\left(\frac{1 + \text{concentration of steviol}}{K_m}\right)}$$

Cell Viability

Cell viability was determined using a modified colorimetric assay with sulforhodamine B (SRB) as described previously (25). This assay measures the cellular protein content of adherent cultured cells. Briefly, the cells were seeded into 96-well microtiter plate at a cell density of 1.5×10^4 cells/well and incubated at 33°C in the medium containing various concentrations of stevioside and steviol for 3 days. After incubation, the medium containing nonviable cells and serum protein was removed, and the monolayer cells were fixed with cold 20% (w/v) trichloroacetic acid (TCA) for 30 min at 4°C. They were then washed five times with distilled water and air-dried. Subsequently, the cells were stained for 30 min at room temperature by 0.4% (w/v) SRB dissolved in 1% acetic acid. At the end of staining period, SRB was removed and quickly rinsed five times with 1% acetic acid. The cellular protein contents were extracted with 10 mM Tris–base [tris (hydroxymethyl) aminomethane] and the absorbance at 515 nm was measured using a computer-interfaced, 96-well microtiter plate reader (EL 312, Bio-Kinetics reader, Bio-Tek Instrument Inc, Finland).

Animals

Adult male C57BL/6 mice raised at the National Institute of Environmental Health Sciences, NIEHS, (Research Triangle Park, NC, USA) were used in renal cortical slice experiments. All animal procedures were approved by the NIEHS Animal Care and Use Committee.

Renal Slice Preparation and Uptake Study

Tissue slices were prepared according to published methods (26). Briefly, animals were euthanized by CO_2 inhalation and decapitated. Renal cortical slices (≤0.5 mm; 5–20 mg, wet weight) were cut with a Stadie-Riggs microtome and maintained in ice-cold oxygenated modified Cross and Taggart buffer (in mM: 95 NaCl, 80 mannitol, 5 KCl, 0.74 $CaCl_2$, and 9.5 Na_2HPO_4, pH 7.4). The slices were incubated in 1 ml of buffer containing either 10 μM [^{3}H]-PAH or 100 nM [^{3}H]-ES in the absence and presence of stevioside, steviol, and various compounds for 60 min. Uptake was stopped by the addition of ice-cold buffer. Slices were washed, blotted, weighed, dissolved in 1 ml of 1 N NaOH, and neutralized with 1 ml of 1 N HCl. Nine milliliters of scintillation fluid (Ecolume, ICN, Irvine, CA, USA) was added, and radioactivity was measured using a Tri-Carb 2900TR Liquid Scintillation Analyzer (Packard, Meriden, CT, USA). The uptake of PAH and ES were calculated as tissue to medium (T/M) ratio (i.e., dpm/mg of tissue divided by dpm/μl of medium) and then expressed as a mean percentage of the control.

Statistical Analysis

Data were expressed as means ± SE. Statistical differences were assessed using one-way analysis of variance. Differences were considered to be significant when $p < 0.05$, $p < 0.01$, $p < 0.001$ *vs.* control.

RESULTS

Interactions of Stevioside and Steviol with Specific Organic Anion Transporters

To determine the direct interactions of stevioside and steviol with organic anion transporters, *cis*-inhibition studies were conducted in S2hOAT1, S2hOAT2, S2hOAT3, and S2hOAT4 cells. Steviol markedly inhibited PAH uptake in S2hOAT1, $PGF_{2\alpha}$ uptake in S2hOAT2, and ES uptake in S2hOAT3 and S2hOAT4 cells. The calculated IC_{50} of steviol were 11.4 ± 0.3, 1000 ± 31, 36.5 ± 2.6, and 285 ± 1 μM for S2hOAT1, S2hOAT2, S2hOAT3, and S2hOAT4, respectively. In contrast, the parent compound, stevioside, at concentrations up to 1 mM did not affect substrate uptake in any of the OAT-expressing cell lines (see below). Because steviol showed higher affinity for hOAT1 and hOAT3 than for hOAT2 and hOAT4, we, therefore, focused our further study on the effects of stevioside and steviol on PAH transport by S2hOAT1 cells and ES transport by S2hOAT3 cells.

As shown in Fig. 2A, stevioside had no inhibitory effect on PAH uptake in S2hOAT1 cells at all concentrations tested; whereas 10 μM to 1 mM steviol very effectively

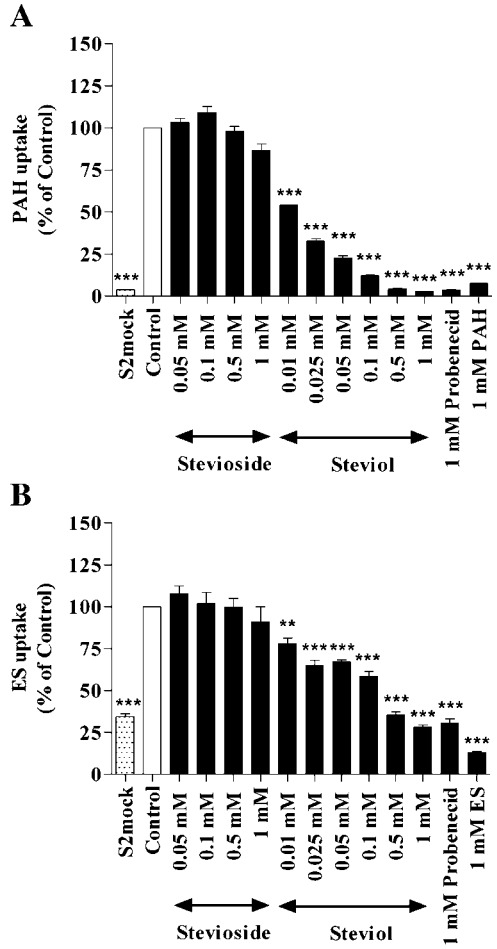


Fig. 2. The effects of stevioside and steviol on hOAT1 (A) and hOAT3 (B) mediated [^{14}C]-PAH and [^{3}H]-ES uptake in S2 cells. The uptake measurements were carried out in the presence of stevioside and steviol at various concentrations for 2 min in D-PBS containing either 5 μM of [^{14}C]-PAH for hOAT1 or 50 nM of [^{3}H]-ES for hOAT3. Unlabeled PAH, unlabeled ES, and probenecid were used to block the PAH and ES uptake in comparison with stevioside and steviol. The uptake is expressed as a mean percentage of control (mean ± SE) from three separate experiments. Mean control PAH and ES uptake were 57.2 ± 4.2 and 0.3 ± 0.1 pmol mg protein^{-1} min^{-1}, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* control.

inhibited hOAT1-mediated PAH transport in a dose-dependent manner. Stevioside at low concentrations slightly enhanced ES uptake mediated by S2hOAT3 cells, but this effect was not significant. Steviol significantly inhibited ES uptake in a dose-dependent manner at the concentrations from 10 μM to 1 mM (Fig. 2B). Importantly, 1 mM steviol inhibited PAH and ES uptake very nearly as effectively as probenecid in both S2hOAT1 and S2hOAT3 cells. For relative comparison, the IC_{50} of steviol and probenecid were 11.4 ± 0.3 and 11.9 ± 1.4 μM for S2hOAT1cells and 36.5 ± 2.6 and 4.7 ± 1.0 μM for S2hOAT3 cells, respectively.

Inhibitory Effectiveness of Steviol

The inhibitory effects of several organic compounds (100 μM) including stevioside and steviol against PAH uptake via S2hOAT1 cells were assessed (Fig. 3A). PAH uptake by S2hOAT1 cells was markedly reduced by cimetidine, unlabeled ES and PAH, bumetanide, furosemide, probenecid, steviol, and indomethacin, whereas several compounds including stevioside, TEA, and methotrexate had no effect on PAH uptake. The inhibitory effectiveness of various organic compounds on ES uptake by S2hOAT3 cells was also investigated (Fig. 3B). Stevioside 100 μM had no effect on ES uptake mediated by S2hOAT3 cells, whereas methotrexate, furosemide, TEA, unlabeled PAH, steviol, bumetanide, cimetidine, probenecid, unlabeled ES, and indomethacin all significantly inhibited ES uptake.

Kinetic Analysis of Steviol Inhibition

As shown in Fig. 4, the kinetic of PAH uptake by S2hOAT1 cells was determined in the absence and presence of 20 μM of steviol. Based on the mean values plotted in Fig. 4, the estimated K_m for PAH uptake in the presence of 20 μM of steviol was 267 μM, five times the control K_m of 52 μM. The V_{max} for PAH uptake in the presence of steviol was not markedly different from control (952 *vs.* 1030 pmol

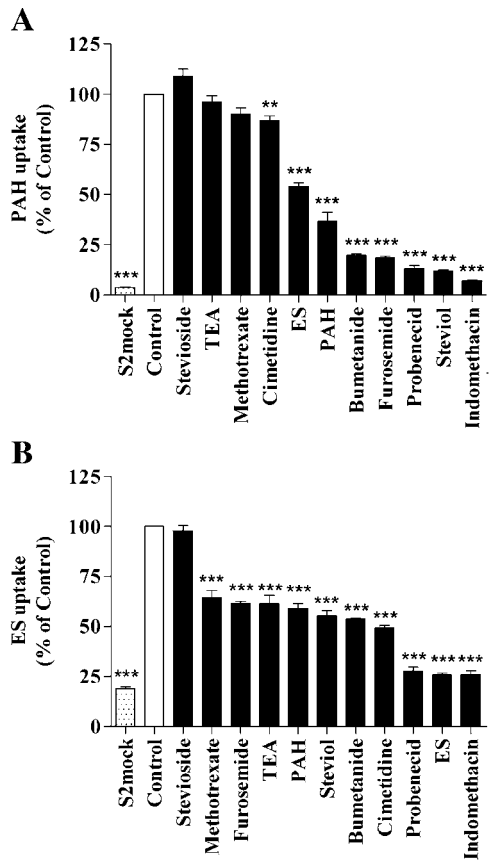


Fig. 3. The inhibitory profiles of stevioside, steviol, and various compounds on [^{14}C]-PAH and [^{3}H]-ES uptake mediated by S2 cells expressing hOAT1 (A) and hOAT3 (B). S2hOAT1 and S2hOAT3 cells were incubated for 2 min in D-PBS containing 5 μM of [^{14}C]-PAH (hOAT1) and 50 nM of [^{3}H]-ES (hOAT3) in the absence (control) or presence of 100 μM of the compounds. Each value represents the mean ± SE from three separate experiments. Mean control PAH and ES uptake were 65.0 ± 1.8 and 0.4 ± 0.1 pmol mg protein^{-1} min^{-1}, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* control.

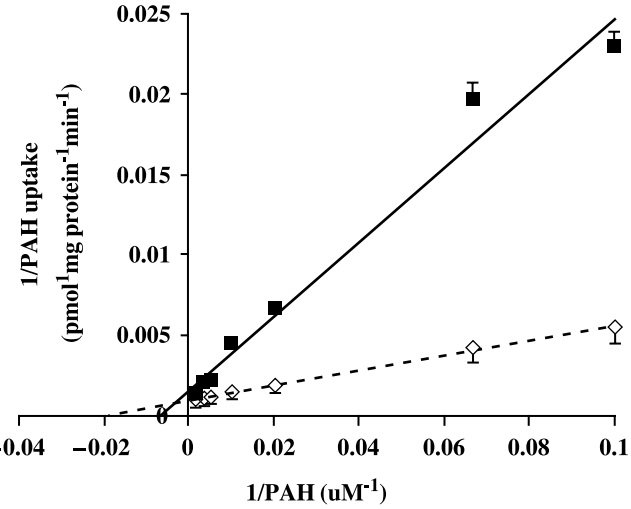


Fig. 4. Kinetic analysis of steviol inhibition. The S2 cells expressing hOAT1 were incubated with D-PBS containing [^{14}C]-PAH at concentrations from 10 to 600 μM in the absence (◇) or presence (■) of steviol (20 μM). Data are shown as the reciprocal of PAH uptake on the ordinate *vs.* the reciprocal of PAH concentrations in the medium on the abscissa. The data shown are from a representative experiment.

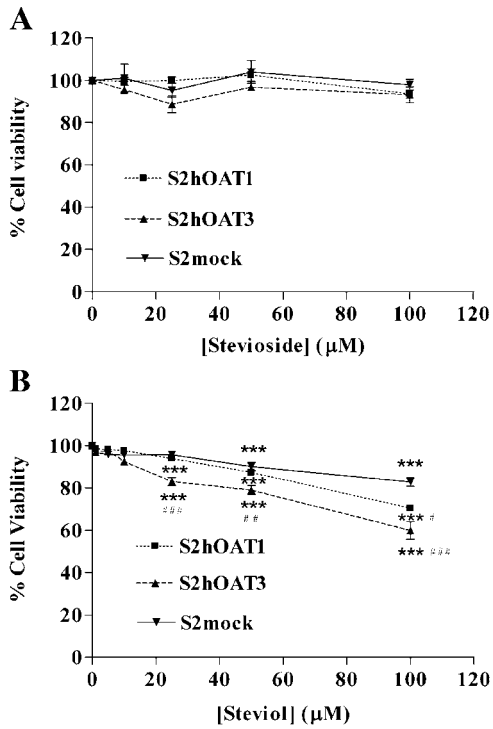


Fig. 5. Effects of stevioside (A) and steviol (B) on cell viability. The S2 cells expressing hOAT1, hOAT3, and S2mock were subcultivated in 96-well plates with various concentrations of either stevioside or steviol. The cell viability of S2hOAT1 (■), S2hOAT3 (▲), and S2mock (▼) were measured after 3 days of incubation. Each point represents the percentage of cell viability in the absence of either stevioside or steviol (mean ± SE) of three separate experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control; whereas $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* S2mock cells.

mg protein^{-1} min^{-1}), suggesting competitive inhibition. The calculated K_i of steviol on PAH uptake mediated by hOAT1 was 8.2 μM.

Effect of Stevioside and Steviol on Cell Viability

We examined cell viability after exposure to stevioside and steviol. As shown in Fig. 5A, stevioside in the range between 1 and 100 μM did not affect the cell viability of S2hOAT1, S2hOAT3, and S2mock cells. At 1 to 10 μM, steviol did not affect cell viability in S2hOAT1, S2hOAT3, or S2mock cells, but at concentrations from 25 to 100 μM, steviol reduced the cell viability of all cell types, with the greatest suppression observed in the hOAT1 and hOAT3 expressing cells (Fig. 5B). Steviol at concentrations from 25 to 100 μM significantly decreased the cell viability in S2hOAT3, whereas only 100 μM steviol significantly reduced cell viability in S2hOAT1. In addition, as a positive control, the effects of a cytotoxic drug, methotrexate, on S2hOAT1, S2hOAT3, and S2mock were examined. Methotrexate at a

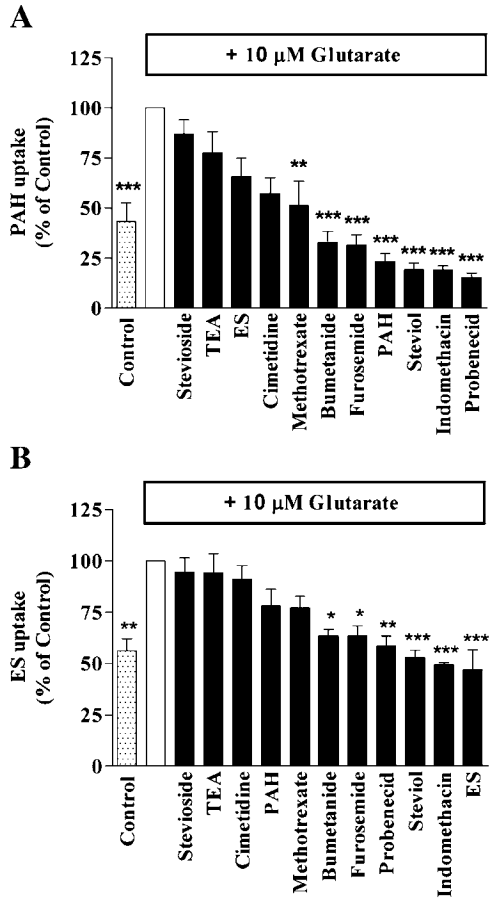


Fig. 6. The relative inhibition of stevioside, steviol, and various compounds on PAH (A) and ES (B) transport in mouse renal cortical slices. The fresh renal slices were incubated in the medium containing 10 μM of [^{3}H]-PAH and 100 nM of [^{3}H]-ES with or without 100 μM of compounds for 60 min at room temperature. The data are calculated as tissue/medium ratio and expressed as a mean percentage of control uptake (mean ± SE) of three separate experiments. Mean control T/M ratios were 8.3 ± 1.3 (PAH) and 16.1 ± 1.7 (ES), respectively. Each experiment used three to five animals. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control.

very low concentration, 1 μM, killed all S2hOAT1 cells. It was less toxic to S2hOAT3 and S2mock cells, showing 25% and 60% cell viability, respectively (data not shown).

Mouse Renal Cortical Slices

The data presented above indicate that in hOAT1 and hOAT3 expressing cell lines, steviol was an effective inhibitor, whereas stevioside was not. To assess whether these same properties are expressed in an intact renal epithelium, the effects of both agents were determined in mouse renal cortical slices. PAH is a substrate for both OAT1 and OAT3 in mouse (27), so its uptake by the mouse slice reflects the action of both carriers. As shown in Fig. 6A, control PAH uptake in renal cortical slice was increased when 10 μM glutarate was added to the medium, as previously reported (28). Therefore, 10 μM glutarate was applied in all renal cortical slice experiments. The presence of 100 μM methotrexate, bumetanide, furosemide, unlabeled PAH, steviol, indomethacin, and probenecid significantly decreased PAH uptake, whereas stevioside, TEA, unlabeled ES, and cimetidine did not affect PAH uptake by mouse renal cortical slices. To examine the effects of stevioside and steviol on the mouse OAT3 specifically, the uptake of ES was assessed, as this substrate is only transported across the basolateral membrane of the proximal tubule via OAT3 (27). As shown in Fig. 6B, the presence of 100 μM bumetanide, furosemide, probenecid, steviol, indomethacin, and unlabeled ES inhibited ES uptake, whereas stevioside, TEA, cimetidine, unlabeled PAH, and methotrexate had no effect. The effects of increasing stevioside and steviol concentrations on PAH and ES uptake by mouse renal cortical slices were also tested (Fig. 7A and B). Stevioside was without effect on either PAH or ES transport by the mouse renal slice at all tested concentrations (0.05 to 1 mM). On the other hand, steviol inhibited both PAH and ES uptake in a dose-dependent manner. The IC_{50} of steviol on PAH and ES uptake in renal cortical slices was 12.8 ± 3.0 and 67.6 ± 1.8 μM, respectively.

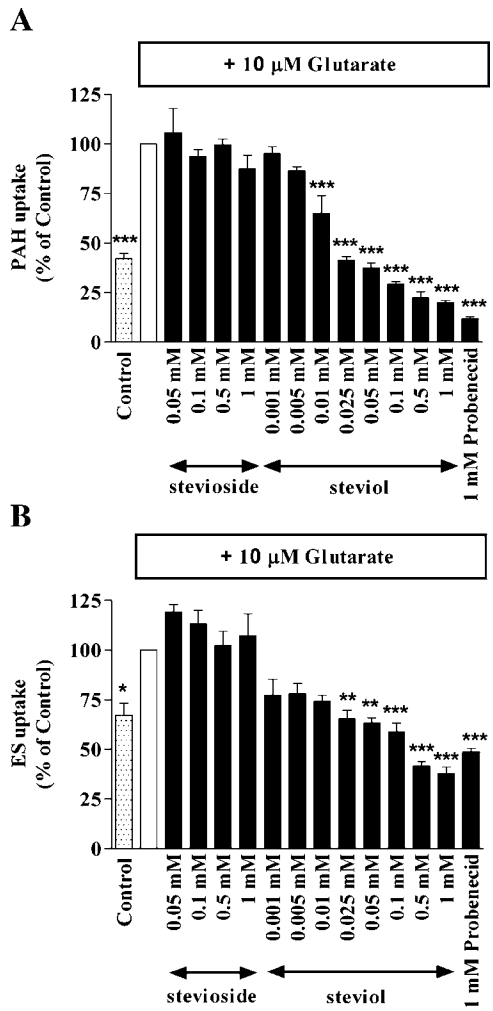


Fig. 7. The effects of stevioside and steviol on PAH (A) and ES (B) uptake in mouse renal cortical slices. Renal cortical slices were incubated in the medium containing 10 μM of [^{3}H]-PAH and 100 nM of [^{3}H]-ES with or without various concentrations of stevioside and steviol for 60 min at room temperature. The uptake was calculated as tissue/medium ratio and then transformed to a mean percentage of control. The data represent the mean ± SE of three separate experiments. Mean control T/M ratios were 5.9 ± 0.2 (PAH) and 14.3 ± 0.9 (ES), respectively. Three to five animals were used in each experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* control.

DISCUSSION

The current study examined the interactions of stevioside and steviol with specific renal organic anion transporters using renal S2 cells expressing hOAT1, hOAT2, hOAT3, and hOAT4 and mouse renal cortical slices. The use of stable cell lines allows us to be able to determine the direct interactions of both compounds with specific human OATs. It is also desirable to confirm the predictions derived from expressed transporters with the intact renal epithelium. Because intact human renal tissues were not available, we used mouse renal cortical slices to confirm the interactions of stevioside and steviol with organic anion transporters in intact tissue.

The likely human exposure to stevioside and steviol is determined by two factors: the intake of stevioside and the extent of its metabolism to steviol. It is known that stevioside should be completely converted to steviol by intestinal microflora *in vivo* (3–5). Therefore, the "acceptable daily intake" of stevioside [7.9 mg/kg BW/day (11)] would yield a maximum plasma concentration of steviol approximately 0.2 mM. It has also been shown that peak plasma steviol concentration was 18 μM 8 h after single oral administration of *Stevia* extract (equimolar dose of 45 mg/kg BW steviol), whereas 15 min after oral administration of steviol itself (45 mg/kg BW), plasma steviol reached a peak of 57.4 μM (29). Based on these estimates, we have used concentrations of 0.05 to 1 mM for stevioside and 0.01 to 1 mM for steviol to bracket the likely exposure experienced by man.

Stevioside

Previously, it was demonstrated that stevioside at a pharmacological concentration (0.7 mM) had a small and reversible inhibitory effect on rabbit renal proximal tubular transport of PAH but had no effect on cellular ATP content and Na^+/K^+-ATPase activity (15). On the other hand, its metabolite, steviol, at very low concentration demonstrated

significant inhibition of PAH transepithelial transport. Although the precise mechanisms by which stevioside and steviol inhibited transepithelial transport of PAH were still unclear, the authors proposed that stevioside and steviol might inhibit and/or interfere with the basolateral OATs (16). However, these studies examined the net transepithelial transport of PAH at the tubular level, which may involve several OATs. Thus, the current study was performed to determine which specific organic anion transporter(s) in the renal proximal tubule interacted with stevioside and steviol. We first studied the interactions of these compounds with a single organic anion transporter expressed in renal S2 cells. These results showed that stevioside at concentrations of 50 μM to 1 mM did not inhibit organic anion uptake in S2hOAT1, S2hOAT2, S2hOAT3, and S2hOAT4 cells. Similarly, stevioside showed no inhibitory effect on PAH and ES uptake in mouse renal cortical slices (Fig. 6A, B, 7A and B). Thus, there was no evidence that stevioside altered the function of any OAT. Stevioside's lack of interaction with human OATs may be due to its large size, as it is composed of one molecule of steviol and three of glucose, and its lack of charge at physiological pH (Fig. 1), making it unlikely to access the substrate binding site on the various OATs. Although our results were clear for human and mouse OATs, Jutabha *et al.* (15) found a small reversible inhibitory effect of stevioside on PAH transport of rabbit renal proximal tubule. One possible explanation for this discrepancy may be that, at the high concentration used in the rabbit study (0.7 mM), sufficient concentrations of inhibitory stevioside derivatives may have been present to reduce transport. It is also possible that these findings may reflect species differences. Certainly, differences in substrate recognition and transport properties of organic anion transporters among species have been observed (30,31). Previous studies have also reported that renal elimination of stevioside involved its net secretion *in vivo* (32,33). However, in light of our data (above) indicating complete lack of interactions between stevioside and organic anion transporters (hOAT1, hOAT2, hOAT3, and hOAT4), one must argue that stevioside secretion does not involve organic anion transporters. Once again, there are several possible explanations for the *in vivo* findings. First, as noted above, the stevioside used in the *in vivo* studies might contain other stevioside derivatives, or it is possible that some stevioside metabolism occurred after its injection. In either instance, those derivatives or metabolites may have been excreted and detected as stevioside. Another possibility is that other transporters may play a role in the renal transport of stevioside. Recently, a novel organic anion transporting polypeptide (OATP4C1) has been cloned and shown to be expressed at the basolateral membrane of the proximal tubule (34). Because it handles organic anions including digoxin, a large cardiac glycoside, it is possible that OATP4C1 may mediate stevioside uptake and secretion. However, this possibility has not yet been explored.

To directly assess the potential of basolateral transport and accumulation of stevioside and/or steviol to induce renal tubular damage, we measured the cytotoxicity of both compounds in hOAT1- and hOAT3-expressing mouse S2 cells. As shown in Fig. 5A, 1 to 100 μM stevioside did not suppress viability in any of the cell lines S2hOAT1, S2hOAT3, and S2mock cells. In the only other study of stevioside nephrotoxicity, Toskulkao *et al.* (14,35) reported that 6.25–100 μM stevioside induced toxicity in rat renal cortical slices. However, the purity of the stevioside ≥90%, as compared to ≥98% used in our study, very likely explains the lower toxicity that we observed.

Steviol

As noted above, stevioside can be degraded to steviol by the intestinal microflora from various animal species including man (3–5). Recently, steviol has been found to inhibit transepithelial transport of PAH (J_{PAH}) by isolated perfused rabbit renal proximal tubule (16). Entirely consistent with these findings, the current study showed that steviol inhibited organic anion uptake mediated by S2 cells expressing hOAT1, hOAT2, hOAT3, and hOAT4. Both hOAT1 and hOAT3 showed high affinity for steviol (IC_{50} was 11.4 and 36.5 μM for hOAT1 and hOAT3, respectively), hOAT4 (IC_{50} = 285 μM) was intermediate, and hOAT2 exhibited the lowest affinity (IC_{50} = 1 mM). Steviol inhibition of both hOAT1 and hOAT3 was dose-dependent (Fig. 2A and B). Likewise, steviol effectively inhibited PAH and ES transport in mouse renal cortical slices (IC_{50} of steviol was 12.8 ± 3.0 and 67.6 ± 1.8 μM, respectively). Thus, steviol was a potent inhibitor of hOAT1 and hOAT3 when expressed in S2 cells as well as with mouse OAT1 and OAT3 in the intact renal epithelium. The third basolateral OAT, that is, hOAT2, showed a much lower affinity for steviol (IC_{50} = 1 mM) and is unlikely to be involved in steviol secretion.

However, hOAT4, the only apical transporter studied (20), had a modest affinity for steviol (IC_{50} = 285 μM). Thus, it may not be the primary transporter that is responsible for the exit step of steviol from cells, suggesting involvement of other apical transporters in the luminal exit of steviol. Possibilities include the human multidrug resistance-associated proteins 2 (MRP2) (36), Na^+-phosphate cotransporter (NPT1) (37), and a novel voltage-driven organic anion transporter (OAT_V1) that has recently been cloned from pig renal proximal tubules (38).

Interestingly, as shown in Figs. 2A, B, 7A, and B, steviol inhibited PAH and ES uptake in both stable cell lines and renal cortical slices as effectively as several well-known inhibitors of organic anion transport, including furosemide, bumetanide, probenecid, and indomethacin. This result raises the possibility that steviol may alter the pharmacodynamics of anionic drugs, parallelling the effects of these agents. For example, probenecid is not only a potent organic anion inhibitor, but it is also widely used in combination therapy to increase half-life of the drugs in the blood circulation. Coadministration of probenecid inhibited renal excretion of furosemide, ciprofloxacin, benzylpenicillins, and acyclovir (39–41). The current study found that the IC_{50} of steviol was similar to that of probenecid on hOAT1-mediated PAH uptake (11.4 ± 0.3 μM and 11.9 ± 1.4 μM), indicating that hOAT1 has high affinity for steviol similar to probenecid. Likewise, the affinity of hOAT3 for steviol was also substantial (IC_{50} 36.5 ± 2.6 μM) but lower than that of probenecid (IC_{50} 4.7 ± 1.0 μM). Because the basolateral entry step of organic compounds is known to be a rate-limiting step in organic anion secretion, inhibition of basolateral OAT

activity would be expected to affect the plasma level of various organic anions including therapeutic drugs and could result in either enhanced therapeutic efficacy or increased toxic side effects of the drugs. In addition, steviol at high concentrations (25–100 μM) was shown to decrease cell viability in both hOAT1- and hOAT3-expressing cell lines, whereas the control S2mock cells were much more resistant (Fig. 5B). The basis for differential toxicity would appear to be the hOAT1- or hOAT3-mediated entry of steviol into these cell lines. Because steviol has previously been shown to inhibit oxidative phosphorylation in isolated rat liver mitochondria (42), it is likely that at higher concentrations (25–100 μM), OAT-mediated entry of steviol leads to decreased energy production and increased toxicity. At lower concentration (10 μM), steviol did not show any effects on cell viability in all of cell types (Figs. 2A, B, and 5B). Thus, it is possible, due to its high potency, that low concentrations of steviol might have application as an inhibitor of OAT1 and OAT3. Conversely, steviol does pose the risk of steviol-drug interactions through competition for basolateral and apical OATs taken concurrently with stevioside or steviol.

In conclusion, data from both stable cell lines expressing human organic anion transporters and in intact renal epithelium suggest that stevioside does not interact with renal organic anion transport. In contrast, steviol directly inhibited both hOAT1 and hOAT3 with high potency. HOAT2 and hOAT4 were less effectively inhibited. Stevioside had no effect on cell viability, whereas steviol at high concentrations suppressed cell viability in renal S2 cells expressing OAT transporters. The inhibitory potency of steviol toward hOAT1 and hOAT3 was similar to that of probenecid, suggesting that steviol might have potential as an inhibitor to delay the clearance or disposal of any organic anion including therapeutic drugs mediated by renal OATs from the body. On the other hand, steviol's ability to effectively inhibit organic anion transporters raises the possibility of steviol-drug interactions through competition for elimination.

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