

Transport of the Natural Sweetener Stevioside and Its Aglycone Steviol by Human Organic Anion Transporter (hOAT1; SLC22A6) and hOAT3 (SLC22A8)

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ABSTRACT

The natural sweetening agent stevioside and its aglycone metabolite, steviol, have been shown to inhibit transepithelial transport of *para*-aminohippurate (PAH) in isolated rabbit renal proximal tubules by interfering with basolateral entry. The aim of the present study was to determine which of the cloned basolateral organic anion transporters were involved in the renal transport of stevioside and steviol. This question was addressed in *Xenopus laevis* oocytes expressing human organic anion transporter 1 (hOAT1), 3 (hOAT3), and winter flounder OAT (fOat1). The parent compound, stevioside, had no inhibitory effect on either PAH (hOAT1) or ES (estrone sulfate; hOAT3) uptake. In contrast, steviol showed significant, dose-dependent inhibition of PAH and ES uptake in hOAT1- or hOAT3-expressing oocytes, respectively. The IC_{50} of steviol for

hOAT1-mediated PAH transport was $11.1 \mu\text{M}$ compared with $62.6 \mu\text{M}$ for hOAT3-mediated ES uptake. The Michaelis-Menten inhibition constants (K_i) for steviol transport mediated by hOAT1 and hOAT3 were 2.0 ± 0.3 and $5.4 \pm 2.0 \mu\text{M}$, respectively. *Trans*-stimulation of PAH efflux by steviol was assessed to determine whether steviol itself was transported by hOAT1 or hOAT3. A low concentration of $1 \mu\text{M}$ steviol increased the efflux of [^3H]PAH (*trans*-stimulated) via both hOAT1 and hOAT3. In addition, it was shown by electrophysiology that steviol entry induced inward current in fOat1-expressing oocytes. In conclusion, stevioside had no interaction with either hOAT1 or hOAT3, whereas hOAT1, hOAT3, and fOat1 were all shown to be capable of steviol transport and thus, can play a role in its renal transport and excretion.

Stevioside is a noncaloric natural sweetener isolated from the leaves of *Stevia rebaudiana*. It is about 300 times sweeter than sucrose (Bridel and Lavielle, 1931). Therefore, it is a popular sugar substitute in a variety of foods and often used as a food supplement in Japan, China, South Korea, and Taiwan. Stevioside can be degraded to steviol, an aglycone, by the intestinal microflora from various animal species, including man (Hutapea et al., 1997; Koyama et al., 2003b). Stevioside is a large neutral molecule with both polar and hydrophobic regions, whereas steviol contains a hydrophobic ring and one negative charge on the carboxylic group. The chemical structures of stevioside and steviol are shown in

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ABBREVIATIONS: PAH, *para*-aminohippurate; hOAT, human organic anion transporter; ES, estrone sulfate; fOat1, renal organic anion transporter from winter flounder; OR-2, oocyte ringer 2; DMSO, dimethyl sulfoxide.

Fig. 1. In addition to its sweetening properties, stevioside has been shown to have potential therapeutic value as a contraceptive (Melis, 1999) and an antihypertensive agent (Chan et al., 1998; Jeppesen et al., 2002, 2003). Continued consumption of stevioside extract for 3 months reduced blood pressure in hypertensive patients (Chan et al., 2000). Both stevioside and steviol alter glucose metabolism (Suanarunsawat and Chaiyabutr, 1997) and glucose absorption (Toskulkao et al., 1995). They have also been proposed to have a potential role as antihyperglycemic agents by stimulating insulin secretion from pancreatic beta cells (Jeppesen et al., 2000, 2002, 2003). Stevioside was not effectively absorbed from everted sacs of rat intestine, whereas steviol was rapidly absorbed (Koyama et al., 2003b). Similarly, stevioside showed very limited absorption by the Caco-2 monolayers and steviol was much better absorbed (Geuns et al., 2003). In addition, limited intestinal secretion of steviol was also observed in these studies.

The renal handling of stevioside and steviol has also been evaluated. It has been reported that stevioside clearance was

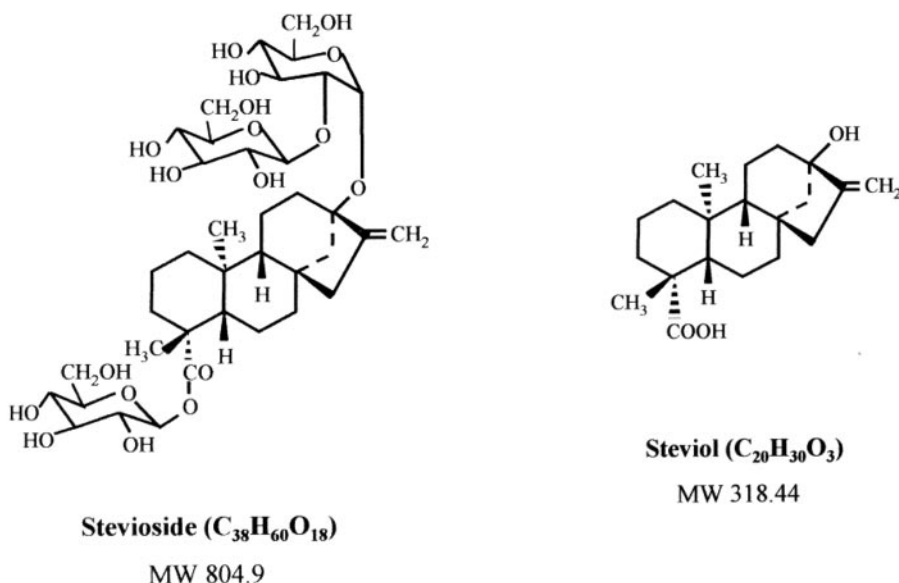


Fig. 1. Chemical structures of stevioside and its aglycone metabolite, steviol.

higher than inulin clearance, indicating that stevioside is secreted by renal tubular epithelium (Melis, 1992). After oral administration of steviol-17-[¹⁴C] into rats, its excretion was found in the feces and urine. In addition, steviol-derived radioactivity was largely found in the urine following bile duct ligation after oral or intracecal administration, indicating that steviol was absorbed from lower bowel and excreted by both renal and biliary systems (Wingard et al., 1980). However, the underlying mechanisms of stevioside and steviol excretion are still unknown. Stevioside and steviol have also been shown to induce diuresis and natriuresis without a significant change in glomerular filtration rate or renal plasma flow (Melis, 1992, 1997). Furthermore, steviol was shown to inhibit accumulation of PAH in rat renal cortical slices (Toskulkao et al., 1994). Both stevioside and steviol have shown to inhibit transepithelial transport of PAH in isolated S2 segments of rabbit renal proximal tubule by interfering with the basolateral entry step (Jutabha et al., 2000; Chatsudthipong and Jutabha, 2001). However, this experimental model did not permit the clear elucidation of the interactions of stevioside and steviol with specific basolateral organic anion transporters.

Two organic anion transporters have been cloned in man that are expressed in the basolateral membranes of the renal proximal tubule, hOAT1 and hOAT3, and both act as organic anion exchangers (Hosoyamada et al., 1999; Cha et al., 2001; Sweet et al., 2003). Thus, they mediate the energetically uphill transport of organic anions from the blood into the cells of the proximal tubule in exchange for intracellular α -ketoglutarate and drive tubular secretion of a wide variety of endogenous and exogenous anions including nucleotide antiviral agents, nonsteroidal anti-inflammatory drugs, methotrexate, and urate (Cihlar et al., 1999; Khamdang et al., 2002; Takeda et al., 2002).

In the present study, cloned hOAT1 and hOAT3 expressed in *Xenopus laevis* oocytes were used to establish whether these transporters were involved in the transport of stevioside and steviol. These data indicate that steviol, but not stevioside, is an effective inhibitor of both hOAT1 and hOAT3 transport. Furthermore, as assessed by *trans*-stimulation

and electrophysiological studies, steviol is itself a substrate for these carriers.

Materials and Methods

Chemicals. [³H]PAH (40 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [³H]ES (46 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Unlabeled PAH and ES, probenecid, furosemide, bumetanide, indomethacin, cimetidine, methotrexate, and TEA were purchased from Sigma-Aldrich (St. Louis, MO). Stevioside and steviol were kindly provided by Dr. Chaivat Toskulkao at the Department of Physiology, Faculty of Science, Mahidol University (Bangkok, Thailand). All other chemicals were analytical grade and obtained from commercial sources.

Oocyte Expression. Adult female *X. laevis* were purchased from Xenopus I (Ann Arbor, MI). The animals were anesthetized with 0.3% tricaine (Sigma-Aldrich), decapitated, and pithed. All animal procedures were conducted according to protocols approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee. Stage V and VI oocytes were selected and isolated by collagenase digestion as previously described (Cihlar et al., 1999). Capped cRNA of hOAT1, hOAT3, and fOat1 was synthesized from purified plasmid cDNA using mMESAGE mMACHINE T7 ultra kit for ultra high yields of capped RNA (Ambion, Inc., Austin, TX). Defolliculated oocytes were injected with 20 ng of cRNA for transport and electrophysiological studies.

Measurement of PAH and ES Uptake in Oocyte-Expressing hOAT1 and hOAT3. The *cis*-inhibitory effects (i.e., inhibitor and labeled substrate were presented to the same side of the membrane or cell) of stevioside, steviol, and various compounds on PAH and ES uptake mediated by hOAT1 and hOAT3 were assessed 3 days postinjection. Oocytes were incubated with 1 ml of oocyte ringer 2 (OR-2) buffer [82.5 mM NaCl, 2.5 mM KCl, 1 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM sodium-pyruvate, 5 mM HEPES/sodium hydroxide (NaOH), pH 7.6] containing either 10 μ M [³H]PAH (hOAT1) or 100 nM [³H]ES (hOAT3) in the absence or presence of stevioside, steviol, and other test compounds for 60 min at room temperature. Both stevioside and steviol were dissolved in dimethyl sulfoxide (DMSO) and diluted with OR-2 buffer. The final concentration of DMSO in the incubation medium was adjusted to 1% or less, and control measurements were made in the presence of equal DMSO concentrations. Uptake was terminated by aspiration of the incubation medium and addition of 1 ml of ice-cold OR-2 buffer. Oocytes

were washed four more times with ice-cold OR-2 and placed in individual scintillation vials, lysed with 1 N NaOH, and neutralized with 1 N hydrochloric acid (HCl). Four milliliters of scintillation fluid (Ecolume; MP Biomedicals, Irvine, CA) were added, and radioactivity was measured using a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer Life and Analytical Sciences). The uptake of PAH and ES was expressed as a mean percentage of the control.

Kinetic Analysis. Human OAT1- and hOAT3-expressing oocytes were incubated with OR-2 buffer containing either [³H]PAH (hOAT1) or [³H]ES (hOAT3) at various concentrations in the absence or presence of 50 μM steviol for 30 min (approximates initial rate of uptake for either substrate into oocytes) (data not shown). Data were plotted as double reciprocal plots. The inhibitory constants (IC₅₀) were calculated from sigmoidal dose-response analysis using GraphPad Prism version 4.00 for windows (GraphPad Software Inc., San Diego, CA). The Michaelis-Menten constants (K_m) were calculated from the interception of x-axis, whereas the maximum rate of organic anions uptake (V_{max}) was estimated from y-axis intercept. The Michaelis-Menten inhibition constants (K_i) were calculated by fitting the data to the following equation for competitive inhibition (Cheng and Prusoff, 1973).

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

Determination of PAH Efflux from hOAT1- and hOAT3-Expressing Oocytes. Three days postinjection, oocytes were loaded with OR-2 buffer containing 10 μM [³H]PAH for 120 min to generate an intracellular accumulation of [³H]PAH. Oocytes were then rinsed with room temperature OR-2 buffer. Subsequently, oocytes were incubated with OR-2 buffer in the presence or absence of 1 mM unlabeled PAH, probenecid, and various concentrations of steviol for 90 min. At the end of the efflux period, [³H]PAH in the efflux medium and intracellular accumulation were measured using liquid scintillation counter as described above. Results were expressed as a mean percentage of the total PAH that appeared in the efflux medium after 90 min of efflux.

Electrophysiology. When human or flounder Oat1 were expressed in *Xenopus* oocytes, inward currents were generated upon the presentation of substrate, indicating that both isoforms exhibit electrogenic exchange of organic anion for dicarboxylate (Burckhardt et al., 2000; Aslamkhan et al., 2003). However, the magnitudes of the currents seen were considerably greater for the flounder clone. Thus, we used fOat1-expressing oocytes to directly investigate whether steviol was itself transported by the basolateral organic anion transporters. Three to 4 days postinjection, fOat1-expressing oocytes were placed in the 1.0-mm well of a RC-1Z oocyte recording chamber (Warner Instruments, Hamden, CT) and continuously bathed at a rate of 4 ml/min with OR-2 buffer at room temperature. The conventional two-electrode (3 M KCl; resistance of 0.5–5 MΩ) voltage-clamp method (Geneclamp 500B; Axon Instruments Inc., Union City, CA) was used for measuring oocyte membrane currents. The oocyte membrane potential was held at –80 mV, which is lower than the physiological membrane potential of the proximal tubule cell in situ to increase current evoked by added organic anion substrates, e.g., PAH or steviol. OR-2 buffer containing either PAH or steviol was applied to the oocytes in a pulsed manner via solenoid-controlled valves. Recordings were sampled at 50 Hz with a 10-Hz filter frequency. After sampling, current signals were filtered with a Gaussian low band pass filter (2 Hz cut-off).

Statistics. Data were expressed as means ± S.E. Statistical differences were assessed using one-way analysis of variance with Dunnett's test. In the efflux experiment, differences in efflux were analyzed using two-way analysis of variance with randomized-block design. Differences were considered to be significant when *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001 versus control.

Results

Cis-Inhibition of hOAT1- and hOAT3-Mediated PAH and ES Uptake. To determine the relative inhibitory effectiveness of various organic compounds including stevioside and steviol on PAH and ES uptake, *cis*-inhibition studies were conducted in oocytes expressing hOAT1 and hOAT3. All compounds were tested at 100 μM. As shown in Fig. 2A, PAH uptake by hOAT1 was markedly reduced by furosemide, steviol, unlabeled PAH, probenecid, and indomethacin, whereas several compounds including TEA, stevioside, cimetidine, methotrexate, unlabeled ES, and bumetanide did not affect hOAT1-mediated PAH uptake. The inhibitory effects of these compounds on hOAT3-mediated ES uptake were also assessed (Fig. 2B). At 100 μM, stevioside and TEA had no inhibitory effect, whereas unlabeled PAH, steviol, cimetidine, methotrexate, indomethacin, unlabeled ES, bumetanide, pro-

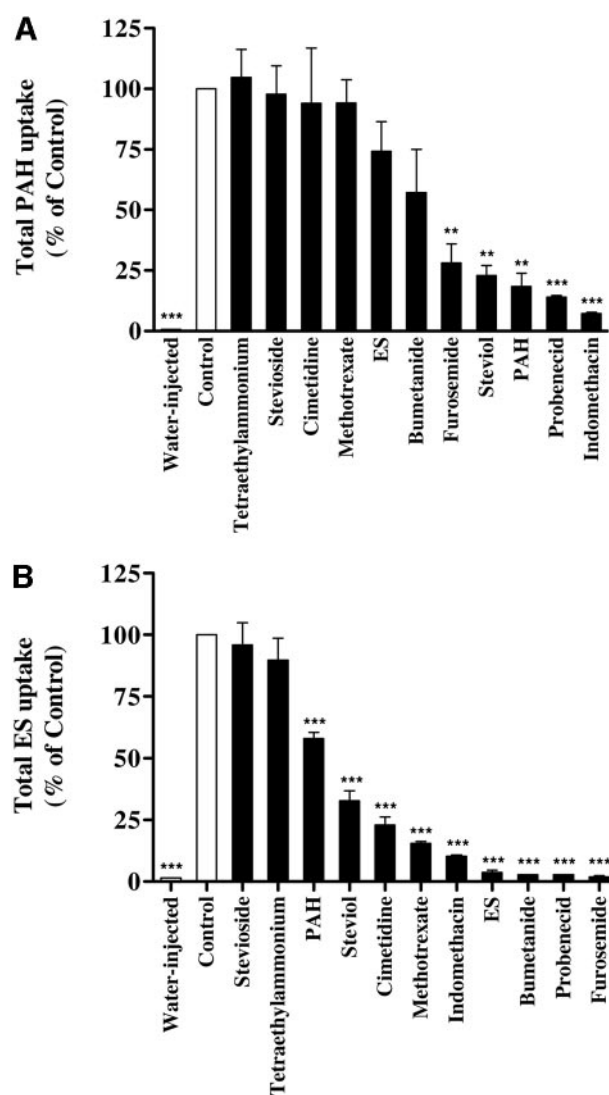


Fig. 2. Inhibition of hOAT1-mediated [³H]PAH uptake (A) and hOAT3-mediated [³H]ES uptake (B). Oocytes expressing hOAT1 (A) and hOAT3 (B) were incubated with OR-2 buffer containing either 10 μM [³H]PAH or 100 nM [³H]ES in the absence (control) or presence of 100 μM test compounds for 60 min. The results are expressed as a mean percentage of control from three separate experiments ± S.E. Mean control PAH and ES uptake were 22.1 ± 2.1 and 0.7 ± 0.1 pmol/oocyte/h, respectively. **, *p* < 0.01; ***, *p* < 0.001.

benedicid, and furosemide significantly inhibited hOAT3-mediated ES uptake.

To compare the effectiveness of stevioside and steviol with known inhibitors of hOAT1 and hOAT3, dose-response experiments were conducted. As shown in Fig. 3A, 50 μM to 1 mM stevioside had no effect on PAH uptake in hOAT1-expressing oocytes. In contrast, 10 μM to 1 mM steviol signifi-

cantly inhibited hOAT1-mediated PAH uptake in a dose-dependent manner with an IC_{50} of 11.1 μM . When the effect of stevioside on ES uptake was investigated in oocytes expressing hOAT3, no inhibition was seen, even at 1 mM stevioside. In contrast, steviol significantly inhibited hOAT3-mediated ES uptake in a dose-dependent manner (IC_{50} , 62.6 μM) (Fig. 3B).

Kinetics of Steviol Inhibition. To determine the affinity of hOAT1 and hOAT3 for steviol, kinetic analyses were performed. As shown in Fig. 4A, [^3H]PAH concentrations were varied from 3 to 30 μM . The rate of PAH uptake by the hOAT1-expressing oocytes was linear for 30 min (data not shown). Thus, this time was used to estimate the initial rate for Lineweaver-Burk analysis of hOAT1-mediated PAH uptake to determine the kinetic parameters (K_m , V_{max} , and K_i) in the presence or absence (control) of steviol (summarized in Table 1). The calculated K_m of hOAT1-mediated PAH uptake doubled in the presence of 50 μM steviol. The calculated K_i value for steviol was $2.0 \pm 0.3 \mu\text{M}$. In the hOAT3 experiments, the [^3H]ES concentration was varied from 2 to 9 μM (Fig. 4B). The estimated K_m for ES in the presence of 50 μM

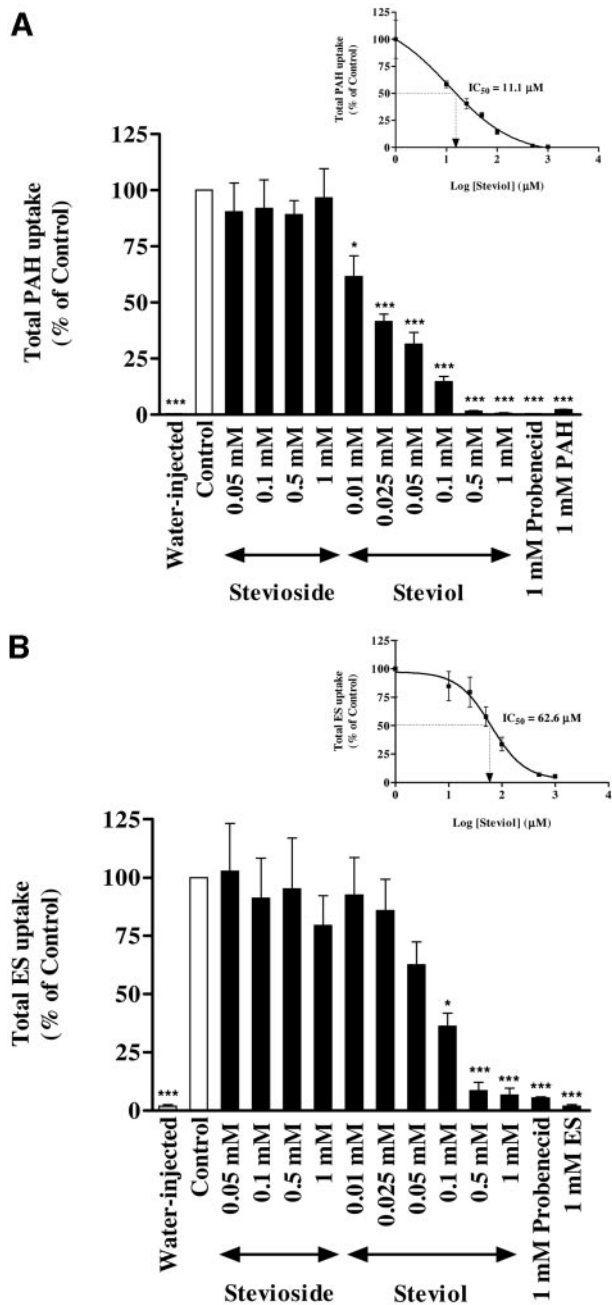


Fig. 3. Concentration dependence of stevioside and steviol inhibition of hOAT1- and hOAT3-mediated uptake. Oocytes expressing hOAT1 (A) and hOAT3 (B) were incubated for 60 min with OR-2 buffer containing either 10 μM [^3H]PAH (A) or 100 nM [^3H]ES (B) in the absence (control) or presence of various concentrations of stevioside and steviol. Unlabeled PAH (1 mM) and probenecid (1 mM) were used to illustrate complete block of mediated PAH and ES uptake. Water-injected oocytes showed minimal PAH and ES uptake. The values are expressed as a mean percentage of control from three separate experiments \pm S.E. Mean control PAH and ES uptake were 47.5 ± 8.3 and 0.6 ± 0.2 pmol/oocyte/h, respectively. *, $p < 0.05$; ***, $p < 0.001$.

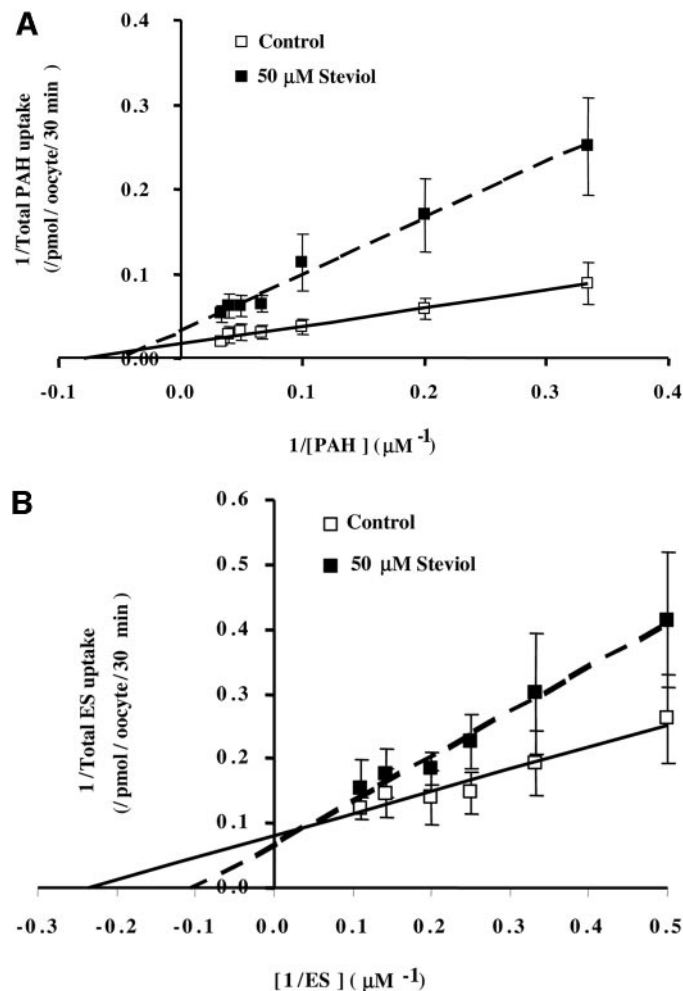


Fig. 4. Kinetic analysis of the inhibitory effect of steviol on hOAT1- and hOAT3-mediated uptake. Oocytes expressing hOAT1 (A) and hOAT3 (B) were incubated with OR-2 buffer containing either 3 to 30 μM [^3H]PAH (A) or 2 to 9 μM [^3H]ES (B) in the absence (\square) or presence of 50 μM steviol (\blacksquare) for 30 min. Each point represents mean \pm S.E. of three separate experiments.

TABLE 1

Kinetic parameters for the inhibition of hOAT1-mediated PAH transport and hOAT3-mediated ES transport by steviol in *Xenopus* oocytes

Analysis was by Lineweaver-Burke linear transformation as described under *Materials and Methods*. The experimental conditions were as described in the Fig. 4 legend, and the data are expressed as mean \pm S.E. of three separate experiments. Although the apparent K_m for hOAT1 and hOAT3 were doubled by steviol, these differences were only marginally significant by unpaired *t* test ($p < 0.10$ for hOAT1 and $p < 0.15$ for hOAT3).

Cloned	K_m		V_{max}		K_i
	Control	50 μ M Steviol	Control	50 μ M Steviol	
	μ M		pmol/oocyte/30 min		μ M
hOAT1	11.0 \pm 1.7	21.8 \pm 4.8	53.5 \pm 13.0	30.6 \pm 8.5	2.0 \pm 0.3
hOAT3	4.8 \pm 1.9	10.1 \pm 2.3	14.4 \pm 4.2	15.4 \pm 2.3	5.4 \pm 2.0

steviol was 2-fold higher than the control (Table 1), with the calculated K_i 5.4 \pm 2.0 μ M.

Trans-Stimulation of hOAT1- and hOAT3-Mediated PAH and ES Uptake. Although the above results demonstrated that steviol effectively inhibited both hOAT1 and 3, it remained uncertain whether steviol could be transported by these basolateral OATs. Therefore, *trans*-stimulation of hOAT1 and hOAT3 transport by steviol was investigated. *Trans*-stimulation takes place when the presence of one transported substrate on the opposite, or *trans*, side of the membrane increases the flux-labeled substrate. *Trans*-inhibition can also occur when an agent presented on the opposite side of the membrane reduces flux of the labeled substrate, e.g., by binding tightly to the carrier and reducing the effective number of carriers available to mediate labeled substrate transport. As shown in Fig. 5A, the efflux of [³H]PAH significantly increased by the addition of 1 mM unlabeled PAH in the incubation medium. Similarly, 1 μ M steviol significantly *trans*-stimulated [³H]PAH efflux, whereas at higher concentrations (10 μ M–1 mM), *trans*-stimulation was not seen. In fact, 100 μ M and 1 mM steviol significantly *trans*-inhibited [³H]PAH efflux. This result was comparable to that seen when 1 mM probenecid was applied. Human OAT3-mediated [³H]PAH was also significantly *trans*-stimulated when 1 mM unlabeled PAH was added to the external buffer. External addition of 1 μ M steviol also increased the efflux of [³H]PAH. However, this effect was not observed at 10 and 100 μ M steviol, and at 1 mM, steviol (like probenecid) *trans*-inhibited [³H]PAH (Fig. 5B).

Electrophysiology. Since human, rat, and flounder OAT1 have all been shown to be electrogenic, exchanging one monovalent organic anion for one divalent dicarboxylate (Burckhardt et al., 2000; Aslamkhan et al., 2003), it should be possible to directly assess whether stevioside or steviol were being transported via electrophysiology. To do this, voltage-clamp experiments using fOat1-expressing oocytes were performed. As noted above, flounder OAT1 shows a greater electrical signal than rat or human OAT1. Since the signal resulting from transport of stevioside (if any) and steviol might be modest, flounder Oat1 was chosen for these experiments. As shown in Fig. 6A, at a holding membrane potential of -80 mV, addition of 100 μ M PAH induced an inward current of -61.9 ± 2.4 nA. Addition of 100 μ M steviol evoked membrane inward current of -11.7 ± 1.0 nA, indicating that steviol is translocated by fOat1, whereas inward current was not observed upon addition of 100 μ M stevioside

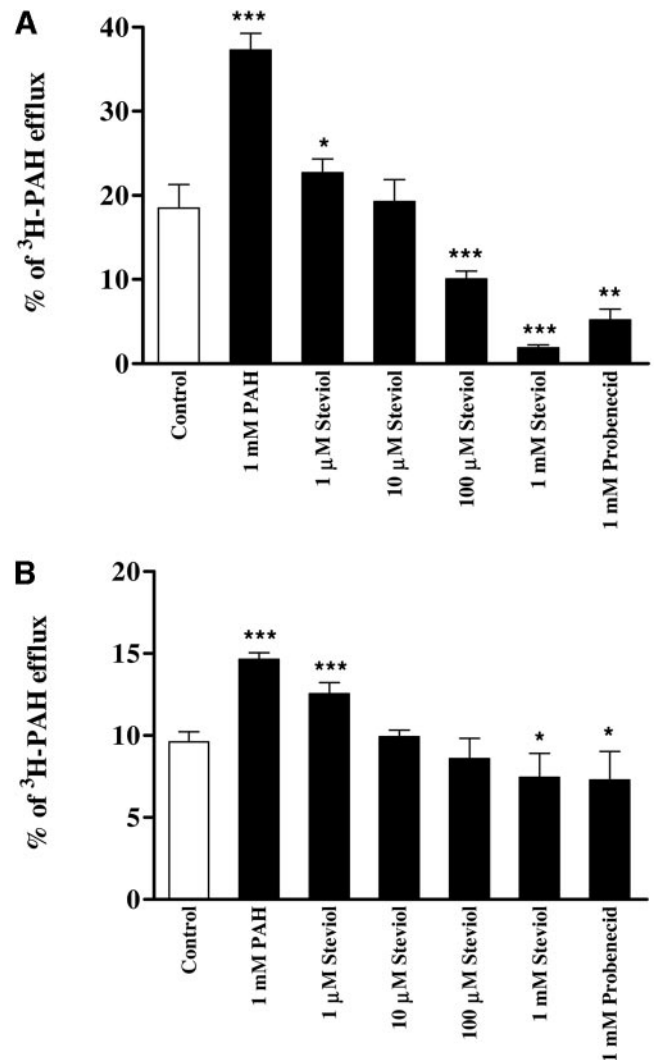
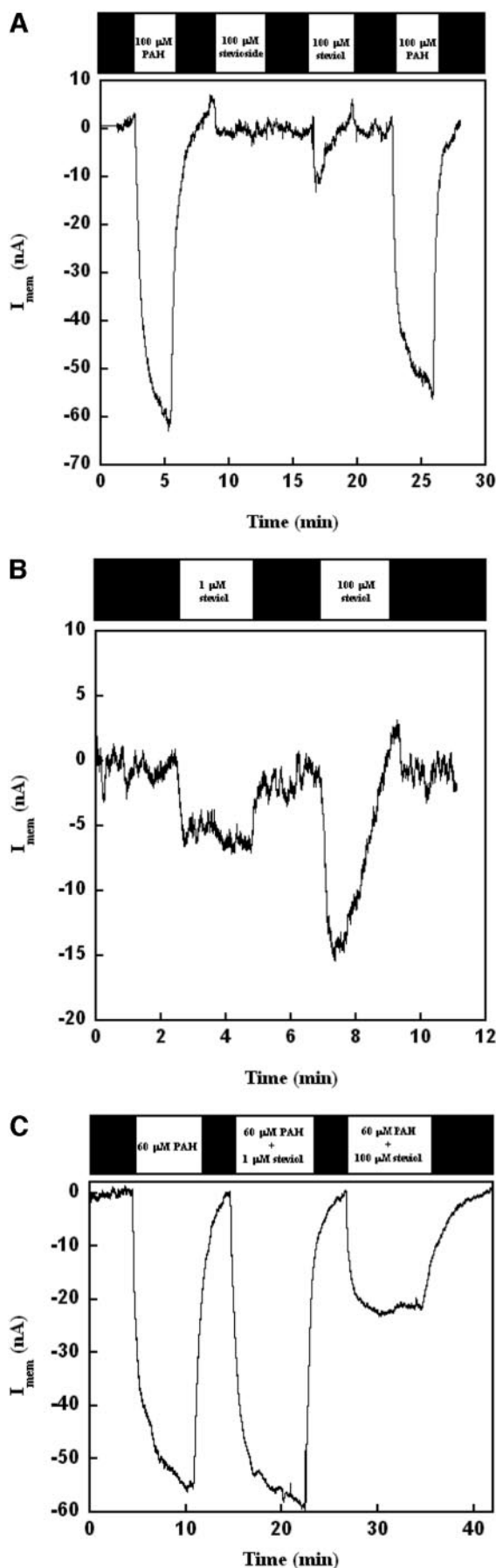


Fig. 5. Effect of steviol on [³H]PAH efflux by hOAT1- and hOAT3-expressing oocytes. Oocytes expressing hOAT1 (A) and hOAT3 (B) were loaded with OR-2 buffer containing 10 μ M [³H]PAH for 120 min and then incubated with OR-2 buffer in the presence or absence (control) of 1 mM PAH, 1 mM probenecid, or 1 μ M to 1 mM steviol for 90 min. The values are expressed as a mean percentage of the efflux \pm S.E. of three separate experiments. Total accumulation averaged 40.3 \pm 10.3 pmol/oocyte/h (hOAT1) and 4.5 \pm 1.0 pmol/oocyte/h (hOAT3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(Fig. 6A). As shown in Fig. 6B, the magnitude of the inward current induced by steviol was concentration-dependent. Control water-injected oocytes did not show inward current upon addition of PAH and steviol (data not shown). To determine whether steviol translocation is in fact slower than PAH, we assessed the effect of low (1 μ M) and high (100 μ M) steviol concentrations on the PAH-induced current. Coapplication of 1 μ M steviol with 60 μ M PAH (K_{50} for fOat1 PAH current) (Burckhardt et al., 2000) resulted in a slightly larger current than the application of 60 μ M PAH alone (103.9 \pm 1.2% of control PAH current; Fig. 6C). Moreover, coapplication of 100 μ M steviol with 60 μ M PAH resulted in a greatly reduced current (47.3 \pm 5.4% of control PAH current; Fig. 6C), paralleling its effect in the *cis*-inhibition experiments described above (Figs. 2A and 3A). Thus, it appears that although steviol is a substrate for fOat1, its rate of translocation is markedly less than that of PAH.



Discussion

Stevia extracts and stevioside have been widely used as a low-calorie sweetener in Japan, China, South Korea, and Taiwan, and this compound was approved as a food supplement in the United States (Geuns, 2003). Indeed, stevioside seems to have little or no acute toxicity (Matsui et al., 1996; Toskulkaio et al., 1997). Likewise, chronic stevioside consumption is believed to pose little human risk based on findings such as those of Yamada et al. (1985) that showed oral consumption of stevioside in amounts as high as 550 mg/kg b.wt./day (i.e., more than 200 times its likely maximum intake in man of about 2 mg/kg b.wt./day) (Xili et al., 1992) for 2 years, had no toxic or carcinogenic effects in the rat. However, pharmacological effects are suggested by other studies. For example, stevioside administration was reported to reduce blood pressure and blood glucose levels in humans (Chan et al., 1998; Jeppesen et al., 2000, 2002, 2003). Furthermore, its aglycone metabolite, steviol, was reported to be mutagenic and bactericidal in *Salmonella typhimurium* TM677 (Perzzuto et al., 1985). Thus, the spectrum of its biological effects and potential for adverse interactions with other pharmacological reagents remains uncertain.

Stevioside absorption, distribution, and metabolism have been evaluated in experimental animals. Absorption of stevioside by everted sacs of rat intestine and Caco-2 cell monolayers was very poor, whereas its aglycone, steviol, was rapidly absorbed (Geuns et al., 2003; Koyama et al., 2003b). Since stevioside can be degraded to steviol by intestinal microflora from various animal species, including man (Cardoso et al., 1996; Hutapea et al., 1997; Geuns, 2003; Koyama et al., 2003a), it has been suggested that following stevioside consumption only steviol is absorbed. Furthermore, it appears that stevioside shows nearly complete metabolic conversion to steviol in vivo. Thus, the "acceptable daily intake" of 7.9 mg/kg b.wt./day stevioside suggested by Xili et al. (1992) would yield a maximum plasma concentration of approximately 0.2 mM steviol, assuming that oral stevioside would be fully converted to steviol and fully absorbed by the gut in vivo. A recent in vivo study in rats gave plasma values in the same range. Peak plasma steviol concentration was approximately 18 μ M 2 h after single oral administration of *stevia* extract (equimolar dose of 45 mg/kg b.wt. steviol), and 15 min after oral administration of steviol itself (45 mg/kg b.wt.), plasma steviol reached a peak of 57.4 μ M (Koyama et al., 2003a). Based on these estimates, we have used concentrations of 0.05 to 1 mM for stevioside and 0.01 to 1 mM steviol to bracket the likely exposure experienced by man.

In addition, stevioside and its metabolites are very rapidly eliminated from the body. For example, following intravenous injection of [131 I]stevioside into the rat, plasma radio-

Fig. 6. Electrophysiological recording of PAH, stevioside, and steviol induced membrane current (I_{mem}) in fOat1-expressing oocytes. Oocytes expressing fOat1 were voltage-clamped at holding potential (V_h) -80 mV, and membrane currents were recorded. A, 100 μ M PAH, stevioside, or steviol were applied to the oocytes in a pulse manner, and continuous current was monitored. Inward currents were observed in response to PAH and steviol, whereas stevioside-evoked current was not observed. B, effects of 1 and 100 μ M steviol on the fOAT1-mediated positive inward current. C, PAH (60 μ M) with or without steviol (1 and 100 μ M doses) was applied to the oocytes in a pulsed manner. Experiments were repeated three times in oocytes from two to three animals. The data shown are from a representative experiment.

activity decreased rapidly to only 6% of injected dose in the first 60 min. The excreted label was equally found in urine and feces (Cardoso et al., 1996). Moreover, in the rat, stevioside renal clearance was higher than inulin clearance, suggesting active tubular secretion of stevioside (or its aglycone) by renal tubular epithelium (Melis, 1992). Similarly, the excretion of steviol-17- ^{14}C was largely found in urine of bile duct ligated rats after both oral and intracecal administration (Wingard et al., 1980). In keeping with the possibility of tubular transport and secretion, steviol was shown to inhibit PAH uptake in rat renal cortical slices (Toskulkao et al., 1994).

The two basolateral organic anion/dicarboxylate exchangers, OAT1 and OAT3, drive the critical energy-dependent uphill step in organic drug and xenobiotic secretion and, thus, control the renal elimination of endogenous and exogenous anionic drugs and metabolites from the body (Hosoyamada et al., 1999; Cha et al., 2001; Sweet et al., 2003). Together, the rapid renal clearance of stevioside/steviol from the body and the ability of steviol to inhibit PAH transport suggest that one or both agents may use these transporters as a route for the excretion into urine, reducing their systemic concentration and potential for toxicity. However, it should be noted that if they are substrates for these carriers, they may also demonstrate significant renal accumulation and increased potential for proximal tubular damage. They may also alter the handling of other organic anion substrates by these systems, changing their retention and potential for adverse effects. Hence, it is essential to gain a molecular understanding of the mechanisms by which both stevioside and steviol are transported by the kidney. The recent studies of Jutabha et al. (2000) suggest that inhibition of organic anion transport also occurs at the tubular level. They found that 0.7 mM stevioside modestly reduced PAH transport, whereas only 10 μM steviol was needed to significantly inhibit transepithelial PAH transport by the rabbit renal proximal tubule (Chatsudthipong and Jutabha, 2001). However, those studies did not assess the interactions of stevioside and steviol with specific basolateral OATs. In the studies reported above, we have demonstrated that stevioside itself did not inhibit either hOAT1 or hOAT3 (Fig. 2, A and B; Fig. 3, A and B). Likewise, our electrophysiological analysis showed that stevioside did not produce membrane currents in oocytes expressing fOat1 (Fig. 6A), suggesting that stevioside had no interaction with either hOAT1 and hOAT3 and thus, that these OATs are unlikely to mediate basolateral uptake of stevioside. However, this finding is in contrast with previous *in vivo* studies that found urinary stevioside clearance to be greater than that of inulin (Melis, 1992; Cardoso et al., 1996). In light of the present data using the cloned transporters, two explanations for the earlier data are possible. First, it is possible that the stevioside used in the earlier studies contained other stevioside derivatives or that some stevioside metabolism occurred *in vivo* after its injection. Alternatively, another transport process may be responsible for these *in vivo* findings. For example, very recently a novel organic anion transporter from human kidney (OATP4C1) has been cloned and characterized (Mikkaichi et al., 2004). This basolateral transporter appears to handle organic anions, as well as digoxin and ouabain, large neutral cardiac glycosides. Hence, OATP4C1 may play a possible role for transport stevioside. However, this possibility needs to be explored.

In contrast, the aglycone stevioside metabolite, steviol, gave very different results. Steviol very effectively inhibited hOAT1- and hOAT3-mediated transport in a dose-dependent manner (Fig. 2, A and B; Fig. 3, A and B). The inhibition of OAT1 produced by steviol was as great as that produced by equal concentrations of unlabeled PAH and furosemide (Fig. 2A), suggesting that the affinity of steviol approximates that of these compounds for hOAT1. Indeed, it was nearly as effective against hOAT1 as probenecid. Against hOAT3, it was a more effective inhibitor than PAH, but it was much less effective than estrone sulfate, bumetanide, or probenecid (Fig. 2B), all of which are known to have a high affinity for hOAT3 (Wright and Dantzer, 2004). However, these *cis*-inhibition experiments only indicate that steviol has a high affinity for basolateral OAT binding and leave open the question of whether or not steviol is itself translocated by the cloned OATs. To address this possibility, we took advantage of the observation that, like hOAT1 and rOat1, fOat1 shows a 1:1 coupling of organic anion and dicarboxylate and shows an inward positive current upon exposure to transported substrates (Burckhardt et al., 2000; Aslamkhan et al., 2003, 2004). Moreover, that current is more robust than that seen with the mammalian isoforms, in effect amplifying the signal associated with substrate transport. Using this system, we were able to demonstrate current movements upon exposure of fOat1-expressing oocytes to steviol. As shown in Fig. 6, A and B, both 1 and 100 μM steviol induced an inward current in fOat1-expressing oocytes. Additionally, consistent with its transport by hOAT1 and hOAT3, we observed that 1 μM steviol *trans*-stimulated the efflux of [^3H]PAH mediated by both hOAT1 and hOAT3 (Fig. 5, A and B). However, at higher concentrations steviol *trans*-inhibited [^3H]PAH efflux, similar to the effect of probenecid which binds very tightly to the carrier. The reason for differences in the effects of low (1 μM) and high (100 μM) steviol concentrations on PAH efflux is not clear, but may indicate that steviol translocation has a low turnover rate. Thus, at high concentrations it may mask the binding site and, like probenecid, produce *trans*-inhibition. Indeed, that rate of steviol translocation (i.e., current induction) is clearly lower than that of PAH (Fig. 6, A–C). This finding is similar to that of Groves et al. (1994), who showed that the tightly bound substrate, tetrapentylammonium, completely blocked TEA efflux from isolated rabbit proximal tubules. These investigators proposed that tetrapentylammonium binds to the organic cation transporter and markedly slows carrier turnover rate (Groves et al., 1994; Bednarczyk et al., 2003).

In conclusion, natural sweetener stevioside is not transported via basolateral organic anion transporters. In contrast, both hOAT1 and hOAT3 transport steviol, the aglycone metabolite stevioside, indicating that transport of steviol by these carriers may be responsible for the renal secretion of stevioside-derived radioactivity seen previously *in vivo*. Furthermore, steviol has a high affinity for both hOAT1 and hOAT3 and, therefore, has significant potential to diminish the renal clearance of anionic drugs and their metabolites.

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References

- Aslamkhan AG, Barros SA, Wolff NA, Miller DS, and Pritchard JB (2004) Basolateral organic anion transport in *Fundulus heteroclitus* (killifish) proximal tubules appears to be mediated by a single OAT. *FASEB J* **18**:A1250.
- Aslamkhan AG, Han YH, Walden R, Sweet DH, and Pritchard JB (2003) Stoichiometry of organic anion/dicarboxylate exchange in membrane vesicles from rat renal cortex and hOAT1-expressing cells. *Am J Physiol Renal Physiol* **285**:F775–F783.
- Bednarczyk D, Ekins S, Wikel JH, and Wright SH (2003) Influence of molecular structure on substrate binding to the human organic cation transporter, hOCT1. *Mol Pharmacol* **63**:489–498.
- Bridel M and Lavielle R (1931) The sweet principle of Kaa-he-e (*Stevia rebaudiana*). *J Pharm Chim* **14**:99–154.
- Burckhardt BC, Wolff NA, and Burckhardt G (2000) Electrophysiologic characterization of an organic anion transporter cloned from winter flounder kidney (fROAT). *J Am Soc Nephrol* **11**:9–17.
- Cardoso VN, Barbosa MF, Muramoto E, Mesquita CH, and Almeida MA (1996) Pharmacokinetic studies of ¹³¹I-stevioside and its metabolites. *Nucl Med Biol* **23**:97–100.
- Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T, and Endou H (2001) Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* **59**:1277–1286.
- Chan P, Tomlinson B, Chen YJ, Lui JC, Hsieh MH, and Cheng JT (2000) A double-blind placebo-controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. *Br J Clin Pharmacol* **50**:215–220.
- Chan P, Xu DY, Liu JC, Chen YJ, Tomlinson B, Huang WP, and Cheng JT (1998) The effect of stevioside on blood pressure and plasma catecholamines in spontaneously hypertensive rats. *Life Sci* **63**:1679–1684.
- Chatsudthipong V and Jutabha P (2001) Effect of steviol on para-aminohippurate transport by isolated perfused rabbit renal proximal tubule. *J Pharmacol Exp Ther* **298**:1120–1127.
- Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.
- Cihlar T, Lin DC, Pritchard JB, Fuller MD, Mendel DB, and Sweet DH (1999) The antiviral nucleotide analogs didofovir and adefovir are novel substrates for human and rat renal organic anion transporter 1. *Mol Pharmacol* **56**:570–580.
- Geuns JM (2003) Stevioside. *Phytochemistry (Oxford)* **64**:913–921.
- Geuns JM, Augustijns P, Mols R, Buyse JG, and Driessen B (2003) Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. *Food Chem Toxicol* **41**:1599–1607.
- Groves CE, Evans KK, Dantzer WH, and Wright SH (1994) Peritubular organic cation transport in isolated rabbit proximal tubules. *Am J Physiol* **266**:F450–F458.
- Hosoyamada M, Sekine T, Kanai Y, and Endou H (1999) Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney. *Am J Physiol* **276**:F122–F128.
- Hutapea AM, Tosulkao C, Buddhasukh D, Wilairat P, and Glinsukon T (1997) Digestion of stevioside, a natural sweetener, by various digestive enzymes. *J Clin Biochem Nutr* **23**:177–186.
- Jeppesen PB, Gregersen S, Alstrup KK, and Hermansen K (2002) Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects in vivo: studies in the diabetic Goto-Kakizaki (GK) rats. *Phytomedicine* **9**:9–14.
- Jeppesen PB, Gregersen S, Poulsen CR, and Hermansen K (2000) Stevioside acts directly on pancreatic beta cells to secrete insulin: actions independent of cyclic adenosine monophosphate and adenosine triphosphate-sensitive K⁺-channel activity. *Metabolism* **49**:208–214.
- Jeppesen PB, Gregersen S, Rolfsen SE, Jepsen M, Colombo M, Agger A, Xiao J, Kruhoffer M, Orntoft T, and Hermansen K (2003) Antihyperglycaemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. *Metabolism* **52**:372–378.
- Jutabha P, Tosulkao C, and Chatsudthipong V (2000) Effect of stevioside on PAH transport by isolated perfused rabbit renal proximal tubule. *Can J Physiol Pharmacol* **78**:737–744.
- Khamdang S, Takeda M, Noshiro R, Narikawa S, Enomoto A, Anzai N, Piyachaturawat P, and Endou H (2002) Interactions of human organic anion transporters and human organic cation transporters with nonsteroidal anti-inflammatory drugs. *J Pharmacol Exp Ther* **303**:534–539.
- Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K, Fujino A, and Ui M (2003a) In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. *Food Chem Toxicol* **41**:359–374.
- Koyama E, Sakai N, Ohori Y, Kitazawa K, Izawa O, Kakegawa K, Fujino A, and Ui M (2003b) Absorption and metabolism of glycosidic sweeteners of stevia mixture and their aglycone, steviol, in rats and humans. *Food Chem Toxicol* **41**:875–883.
- Matsui M, Matsui K, Kawasaki Y, Oda Y, Noguchi T, Kitagawa Y, Sawada M, Hayashi M, Nohmi T, Yoshihira K, et al. (1996) Evaluation of the genotoxicity of stevioside and steviol using six in vitro and one in vivo mutagenicity assays. *Mutagenesis* **11**:573–579.
- Melis MS (1992) Renal excretion of stevioside in rats. *J Nat Prod* **55**:688–690.
- Melis MS (1997) Effects of steviol on renal function and mean arterial pressure in rats. *Phytomedicine* **3**:349–352.
- Melis MS (1999) Effects of chronic administration of *Stevia rebaudiana* on fertility in rats. *J Ethnopharmacol* **67**:157–161.
- Mikkaichi T, Suzuki T, Onogawa T, Tanemoto M, Mizutamari H, Okada M, Chaki T, Masuda S, Tokui T, Eto N, et al. (2004) Isolation and characterization of a digoxin transporter and its rat homologue expressed in the kidney. *Proc Natl Acad Sci USA* **101**:3569–3574.
- Perzzuto JM, Compadre CM, Swanson SM, Nanayakkara PD, and Kinghorn AD (1985) Metabolically activated steviol, the aglycone of stevioside, is mutagenic. *Proc Natl Acad Sci USA* **82**:2478–2482.
- Suanarunsawat T and Chaiyabutr N (1997) The effect of stevioside on glucose metabolism in rat. *Can J Physiol Pharmacol* **75**:976–982.
- Sweet DH, Chan LM, Walden R, Yang XP, Miller DS, and Pritchard JB (2003) Organic anion transporter 3 (Slc22a8) is a dicarboxylate exchanger indirectly coupled to the Na⁺ gradient. *Am J Physiol Renal Physiol* **284**:F763–F769.
- Takeda M, Khamdang S, Narikawa S, Kimura H, Hosoyamada M, Cha SH, Sekine T, and Endou H (2002) Characterization of methotrexate transport and its drug interactions with human organic anion transporters. *J Pharmacol Exp Ther* **302**:666–671.
- Tosulkao C, Chaturat L, Temcharoen P, and Glinsukon T (1997) Acute toxicity of stevioside, a natural sweetener and its metabolite, steviol, in several animal species. *Drug Chem Toxicol* **20**:31–44.
- Tosulkao C, Deechakawan W, Temcharoen P, Buddhasukh D, and Glinsukon T (1994) Nephrotoxic effects of stevioside and steviol in rat renal cortical slices. *J Clin Biochem Nutr* **16**:123–131.
- Tosulkao C, Suthewattananon M, Wanichanon C, Saitongdee P, and Suttajit M (1995) Effects of stevioside and steviol on intestinal glucose absorption in hamsters. *J Nutr Sci Vitaminol* **41**:105–113.
- Wingard RE Jr, Brown JP, Enderlin FE, Dale JA, Hale RL, and Seitz CT (1980) Intestinal degradation and absorption of the glycosidic sweeteners stevioside and rebaudioside A. *Experientia (Basel)* **36**:519–520.
- Wright SH and Dantzer (2004) Molecular and cellular physiology of renal organic cation and anion transport. *Physiol Rev* **84**:987–1049.
- Xili L, Chengjian B, Eryi X, Reiming S, Yuengming W, Haodong S, and Zhiyian H (1992) Chronic oral toxicity and carcinogenicity study of stevioside in rats. *Food Chem Toxicol* **30**:957–965.
- Yamada A, Ohgaki S, Noda T, and Shimiqu M (1985) Chronic toxicity study of dietary stevia extracts in F344 rats. *J Food Hyg Soc Japan* **26**:183–196.

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