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Sex Differences in the mRNA, protein and functional Expression of Oat1, Oat3 and Oct2 in Rabbit Renal Proximal Tubules

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Abbreviations: BLMV, basolateral membrane vesicles; RPT, renal proximal tubule; OA, organic anion; OC, organic cation; PAH, *p*-aminohippurate; TEA, tetraethylammonium; ES, estrone sulfate; Oat, organic anion transporter; Oct, organic cation transporter; bDNA, branched DNA; CE, capture extender; LE, label extender; BL, blocker probe.

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ABSTRACT

Sex differences in transport of the organic anion (OA) substrate *p*-aminohippurate (PAH) and the organic cation (OC) substrate tetraethylammonium (TEA) have been recognized for some time. In the rat kidney androgens upregulate and estrogens downregulate PAH and TEA transport, which correlate with similar changes in mRNA and protein expression for the renal basolateral membrane transporters Oat1 and Oct2. However, these sex differences are not readily demonstrated in other species. The present study characterizes the kinetics of basolateral membrane PAH, estrone sulfate (ES) and TEA uptake in renal proximal tubule (RPT) suspensions isolated from female and male rabbits to compare functional expression of transport with mRNA and protein expression for rbOat1, rbOat3 and rbOct2. Although rbOat1-rbOat3 mRNA expression exhibited developmental differences, no sex differences in mRNA levels were observed. Oat1 and Oat3 protein expression in RPT suspensions also was similar between adult female and male rabbits. In contrast, rbOct1 and rbOct2 mRNA levels did not show developmental differences, but rbOct2 mRNA expression was greater in adult male than female rabbits. However, the sex difference in rbOct2 mRNA level did not translate to rbOct2 protein expression. Importantly, functional expression of Oat1, Oat3 and Oct2 transport as measured by kinetics (J_{max} and K_t) of PAH, ES and TEA uptake was similar between adult male and female rabbits, and correlated with rbOat1, rbOat3 and rbOct2 protein expression. Thus, unlike rodents, rabbit renal OA and OC transport does not exhibit sex differences, pointing to the need for caution in extrapolating transport-related sex differences between species.

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Introduction

Renal secretion plays an essential role in xenobiotic excretion by the kidney. Renal tubular secretion of xenobiotics that are organic anions (OAs) or cations (OCs) involves a two-step, carrier-mediated process occurring at basolateral and brush border membranes. OA and OC transport are influenced by numerous physiological states that may alter excretion. Sex steroid hormones have been shown to influence renal OA and OC transport, as manifested by sex differences in renal transport of PAH and TEA, respectively. Male rats exhibit greater renal PAH secretion compared to females (Reyes et al., 1998). Similarly, accumulation of PAH by renal slices or into basolateral membrane vesicles (BLMV) is greater in male rats than in female rats, suggesting that sex steroid hormones regulate basolateral uptake pathways for OAs (Huang KC and McIntosh BJ, 1955;Cerrutti et al., 2002). Sex differences in the accumulation of the OC TEA by renal slices from the rat also have been documented, again indicating the regulation of basolateral OC uptake by estrogen and testosterone (Urakami et al., 2000;Bowman and Hook, 1972).

Sex differences in renal PAH and TEA transport in the rat are correlated with expression of OAT and OCT genes. Sex differences were observed for rat renal Oat1 and Oct2 mRNA and protein expression, but not renal Oct1 mRNA levels (Urakami et al., 2000;Urakami et al., 1999;Cerrutti et al., 2002), suggesting that sex differences in basolateral PAH and TEA uptake in the rat are due to Oat1 and Oct2 expression, respectively. Sex steroid hormones also regulate the expression of other renal transporters, including the renal organic anion transporting polypeptide (oatp1) (Lu et al., 1996) for which expression of mRNA is significantly greater in male rats. Thus, in the rat, testosterone appears to consistently upregulate OA and OC transport function, whereas estrogen appears to downregulate transport.

Importantly, the sex differences in organic electrolyte transport have been demonstrated most commonly in rodent species. In fact, sex differences in renal basolateral OA or OC transport have been not been demonstrated consistently in other mammalian species, including the human. Although the renal clearance of the OA substrate urate is higher in women than in men, tubular secretion of urate is not different between the sexes (Anton et al., 1986). Furthermore, the renal excretion of urate is not influenced by estradiol administration to either oophorectomized or normal adult women (Anton et al., 1986). In contrast to the rat, humans and dogs exhibit no sex differences in the renal excretion of the OA substrate zenarestat (Tanaka et al., 1992). Whereas no sex differences in renal clearance and excretion of OA substrates have been demonstrated in the human kidney, the renal clearance of the OC substrate amantidine in humans is greater in males than females (Wong et al., 1995). However, further complicating the interpretation of clearance studies is the fact that clearance involves both basolateral and apical transport pathways, so when sex differences in secretion are noted, the identity of the processes involved are generally not clear.

The rabbit kidney has been extensively employed as a model to characterize OA and OC transport, yet the presence of sex differences in rabbit renal basolateral OA- and OC-mediated transport has not been demonstrated. The lack of evidence demonstrating sex differences in basolateral OA and OC transport in species other than the rat prompted this study to provide a more detailed characterization of the sex differences in the functional expression of the renal basolateral transporters Oat1, Oat3 and Oct2. In contrast to previous results obtained with rats, no differences were found in the functional expression of these organic electrolyte transporters in kidneys of male and female rabbits.

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Methods

Materials. [^{3}H]-Estrone sulfate ([^{3}H]-ES - 44 or 46 Ci·mmol^{-1}) and [^{3}H]-*p*-aminohippurate ([^{3}H]-PAH - 4 Ci·mmol^{-1}) were acquired from Perkin Elmer (Boston, MA). [^{3}H]-Tetraethylammonium ([^{3}H]-TEA - 20 Ci·mmol^{-1}) was synthesized by the Southwest Environmental Health Sciences Center (University of Arizona, Tucson, AZ). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or from other standard sources.

Tubule suspensions. Rabbit renal proximal tubules (RPT) were isolated and purified from adult Zealand white rabbits (15-20 weeks of age) or immature rabbits (8 weeks of age) as described previously (Groves and Schnellmann, 1998). All protocols involving rabbits were conducted in accordance with the *Guide for the Care and Use of Animals* as adopted and promulgated by the National Institutes of Health. The final tubule pellet was resuspended at a protein concentration of 2 mg/ml in a transport medium containing (in mM): 110 NaCl, 25 $NaHCO_3$, 5 KCl, 2 NaH_2PO_4, 1 $MgSO_4$, 1.8 $CaCl_2$, 10 Na-acetate, 8.3 D-glucose, 5 L-alanine, 0.9 glycine, 1.5 lactate, 1 malate, and 1 sodium citrate. Tubular protein was measured using a BioRad protein assay (Hercules, CA) with a γ-globulin standard. Crude membranes for use in western blots were prepared using a modification of the method of Ogihara et al (Ogihara et al., 1999). Tubule pellets (frozen and stored at -20^{o}C) were homogenized in a homogenization buffer containing (in mM): 230 sucrose, 5 Tris-HCl (pH 7.5), 2 EDTA, and protease inhibitor cocktail (Sigma, St. Louis, MO). The homogenate was centrifuged for 15 min at 3000 x *g* (4ºC), the supernatant removed and further centrifuged at 100,000 x *g* for 30 min. The resulting pellet was resuspended in a membrane buffer containing (in mM): 150 KCl, 300 mannitol, and 10 Hepes, pH 7.5. Protein concentrations were measured as described for tubule suspensions.

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Measurement of transport in suspensions of rabbit RPT. Tubule suspensions (2 mg/ml) were preincubated in Erlenmeyer flasks for 15 min at 37ºC and gassed with 95% O_2/5% CO_2. An aliquot of tubule suspension (0.5 ml) was transferred to a 15-ml tube containing 0.5 ml of incubation medium with radiolabeled substrate and varying concentrations of unlabeled PAH, ES or TEA. After 30 seconds, 5 ml ice-cold DME-F12 medium (Sigma Chemical Co, St. Louis, MO) was added to stop uptake, and the tubules were pelleted (~30 sec at 1480 x *g)*. The rinse was repeated; the final pellet dissolved in 0.5N NaOH/1% SDS and aliquots taken for counting radioactivity.

Branched DNA signal amplification assay. Total RNA was isolated from rabbit RPT suspensions or from the kidneys of adult C57-BL mice (14-16 weeks of age) using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) as per the manufacturer's protocol and measured using the branched DNA (bDNA) assay (Quantigene bDNA signal amplification kit) with modifications (Hartley and Klaassen, 2000). Rabbit or mouse OAT and OCT gene sequences of interest were accessed from GenBank (see Table 1). Multiple oligonucleotide probes (containing capture, label, and blocker probes) specific to a single mRNA transcript (i.e., Oat1, -2 or –3 and Oct1 or -2) were designed using Probe Designer software version 1.0, with a melting temperature of ~63°C, enabling hybridization conditions to be held constant (i.e., 53°C) during each hybridization step and for each probe set. Every probe developed in Probe Designer was submitted to the National Center for Biotechnological Information (Bethesda, MD) for nucleotide comparison by the basic logarithmic alignment search tool (BLASTn) to ensure minimal cross-reactivity with other known rabbit or mouse sequences and expressed sequence tags. The nucleotide sequence and function of these probes are given in Table 1. Total RNA (1 µg/µl - 10 µl/well) was added to each well of a 96-well plate containing 50 µl capture

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hybridization buffer and 50 µl of the desired probe set diluted in lysis buffer per the manufacturer's protocol. For each gene, total RNA was allowed to hybridize to the probe set overnight at 53°C. Subsequent hybridization steps were carried out according to the manufacturer's protocol, and luminescence was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management software version 5.02 for analysis of luminescence from the 96-well plates. The luminescence for each well is reported as relative light units per 10 micrograms total RNA.

Antibodies and Western blot. Polyclonal antibodies against rbOat1, rbOat3 and rbOct2 were raised in hens and affinity purified by Aves Laboratories (Eugene, OR). Synthetic peptides corresponding to the intracellular domains of the COOH-terminal of rabbit Oat1 (ESRRRG KPRRQQQEQQK), rabbit Oat3 (QSKKLKQEPEAEKASQR) and rabbit Oct2 (ENLQRPRKNREKVIY) were used to prepare chicken anti-rabbit antibodies. For western blotting affinity purified antibodies for rbOat1 and rbOat3 were diluted 1:2500, whereas the antibody for rbOct2 was diluted 1:1250.

Crude membranes prepared from suspensions of rabbit RPT were used for western blot analysis to determine levels of rbOat1, rbOat3 and rbOct2 between adult female and male rabbits. The protocol for electrophoresis was performed according to manufacturer's instructions for NuPAGE® Novex Tris-Acetate gels (Invitrogen Life Technologies, Carlsbad, CA). The samples were transferred onto nitrocellulose membranes by semi-dry electroblotting for 120 min. After blocking in 5% non-fat dry milk, the membranes were probed overnight (12-14 h) at 4°C with the primary antibody (1:1250 or 1:2500 as noted above). Membranes were then washed six times for 10 min in 1x PBS containing 0.05% Tween 20 (PBS-T) before being incubated with the secondary horseradish peroxidase-linked antibody diluted in 5% non-fat dry milk PBS-T for 1 h.

Detection was performed using an enhanced chemiluminescence detection system (Pierce Chemical Co., Rockford, IL). Relative protein blot intensities were determined using one-dimensional Image Analysis Software (Kodak). Actin expression was used to control for differences in protein loading among samples.

Data analysis. Uptake values are presented as means ± SE. A separate tubule preparation was used for each experiment (n = 3-6). Statistical significance of differences between mean values was calculated using the non-paired t-test. For multiple comparisons of observed differences the one-way ANOVA was used followed by the Fisher's Least Significant Difference post hoc test (ProStat, Poly Software International, Pearl River, NY). In all analyses, differences were considered statistically significant when *$p<0.05$.

Results

Sex differences in Organic Anion and Organic Cation transporter mRNA levels in rabbit RPT and mouse kidney. Current views on the influence of sex steroids on the expression of OA and OC transporters in the kidney are largely based on studies of levels of expression of mRNA for selected transporters in tissues of the rat (e.g., (Urakami et al., 2000;Buist et al., 2002;Slitt et al., 2002;Kudo et al., 2002)). Consequently, the levels of mRNA expression for Oat1, Oat2, Oat3, Oct1 and Oct2 were determined in the RPT of sexually immature and sexually mature (adult) female and male rabbits and in the renal tissues of sexually mature female and male mice (Figs. 1 and 2). Whereas significant developmental differences were observed between sexually immature (8 weeks old) and mature adult rabbits (15-20 weeks old) for Oat1, Oat2 and Oat3 (but not for Oct1 or Oct2; Fig. 1), in general, no significant sex difference in mRNA levels were observed in either immature or mature female vs. male rabbits for either OA or OC transporters. The one exception was Oct2, for which mRNA levels in adult

females were ~40% lower than adult males. Although only these limited sex differences were observed in mRNA levels for the OA and OC transporters in rabbit RPT, differences in the apparent level of mRNA expression between the different transporters were found. Levels of expression of mRNA varied in the descending order for the transporters Oat1-Oat3 and Oct1-Oct2 as follows: rbOat3>rbOat1>>rbOat2 and rbOct2>rbOct1.

In contrast to the rabbit kidney, significant sex differences were observed in mRNA expression of several OA and OC transporters in the mature mouse kidney (Fig. 2). Sex differences were observed for mOat1, mOat2 and mOct2: whereas measured mOat1 and mOct2 mRNA levels were significantly higher in kidneys from male mice, females exhibited higher mOat2 mRNA. These profiles were similar to those observed for expression of the mRNA for these transporters in male and female rat kidneys (Buist et al., 2002;Slitt et al., 2002;Kudo et al., 2002). As noted in the rabbit RPT, in female mice Oct2 levels were ~40% lower than in the male mice. Significant sex differences were not detected for expression of Oat3 and Oct1 mRNA in mouse kidney. In comparing transporter mRNA levels between the various OAs and OCs, mice differed significantly from the rabbit. Unlike the rabbit kidney, where Oat3 predominated and Oat2 mRNA expression was quite low, in the mouse Oat1 predominated, and Oat2 and Oat3 expression were similar. Expression of Oct2 mRNA was greater than Oct1 in both rabbit and mouse kidney, but the difference varied markedly: rabbit RPT displayed a 2-fold difference in Oct2 vs. Oct1 expression compared to a greater than 300-fold difference in mRNA expression for these transporters in mouse kidney.

Comparison of the signals obtained with the five probe sets used in the bDNA signal amplification method must be made cautiously because of possible differences in amplification efficiency. However, the presence of multiple hybridization sequences in the probe sets for each

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transporter reduces problems arising from variable hybridization efficiency (Hartley and Klaassen, 2000) and supports the contention that the differences in signal levels between the OA and OC transporters evident in Figs. 1 and 2 reflect real differences in the relative expression of the mRNA species.

Sex differences in protein expression for Oat1, Oat3 and Oct2 in rabbit renal RPT. Polyclonal antibodies for rbOat1, rbOat3 and rbOct2 each labeled a single 70- to 80-kDa band in crude membranes prepared from rabbit RPT and showed no cross reaction with other OAT- or OCT- transport proteins in membranes isolated from CHO cells that stably expressed rbOat1, rbOat3 and rbOct2 (data not shown). These antibodies were used to determine whether differences in the level of transporter expression differed in RPT isolated from male and female rabbits. As shown in Fig 3, no significant sex difference was detected for any of the OA or OC transport proteins examined. Densitometric analysis of these bands (normalized to the level of actin expression to correct for differences in protein loading) confirmed that there were no sex differences in the level of expression of Oat1, Oat3, or Oct2 in rabbit RPT. Particularly intriguing was the observation that sex differences in mRNA levels for Oct2 did not translate at the protein level.

Kinetics of PAH, ES, and TEA uptake across the basolateral membrane in suspensions of RPT isolated from female and male rabbits. Clearly, relevant sex differences in renal expression of Oat1, Oat3 and Oct2 would be best manifested in the expression of transport function between RPT from females and males. To this end, the kinetics of peritubular PAH, ES and TEA uptake were examined to evaluate any sex differences in the physiological characteristics of substrate transport in suspensions of native RPT comprising a population of tubules that includes both S1 and S2 segments, which were isolated from female or male rabbits.

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Figures 4-6 show the kinetic profiles of PAH (Fig. 4), ES (Fig. 5), and TEA (Fig. 6) transport in RPT suspensions from female and male rabbits. Increasing concentrations of unlabeled substrate progressively inhibited the tubular accumulation of all three labeled substrates and the kinetics of this inhibition were adequately described by the following form of the Michaelis-Menten equation (Malo and Berteloot, 1991):

$$J = \frac{J_{\max}[T^*]}{K_t + [T^*] + [S]} + C \qquad \text{eq. 1,}$$

where J is the rate of [^{3}H]-substrate transport from a concentration of labeled substrate equal to [*T]; J_{max} is the maximum rate of mediated substrate transport; K_t is the substrate concentration that results in half-maximal transport (Michaelis constant); [S] is the concentration of unlabeled substrate in the transport reaction; C is a constant that represents the component of total substrate uptake that was not saturated (over the range of substrate concentrations tested) and presumably reflects the combined influence of diffusive flux, non-specific binding and/or incomplete rinsing of the tubules. For kinetic analysis in suspensions, 30-second uptakes were measured since this incubation period provided both an adequate accumulation of labeled substrate and a reasonable estimate of the initial rate of substrate uptake. In the rabbit RPT, basolateral uptake of PAH is effectively restricted to Oat1 (Lungkaphin et al., 2004). Figures 4A and 4B show that the kinetics of [^{3}H]-PAH uptake did not differ significantly in RPT isolated from female and male rabbits. The J_{max} and K_t (1.8 ± 0.4 nmol·mg^{-1}· min^{-1} and 105 ± 20 μM, respectively, n = 6) for PAH uptake into RPT suspensions isolated from female rabbits were comparable to the J_{max} and K_t (1.25 ± 0.2 nmol·mg^{-1}·min^{-1} and 102 ± 11 μM, respectively, n = 6) measured for male rabbits. Likewise, increasing concentrations of unlabeled ES, which is primarily an Oat3 substrate (in rabbit RPT, (Lungkaphin et al., 2004)), reduced the peritubular uptake of [^{3}H]-ES in RPT suspensions (Figs. 5A and 5B). The J_{max} and K_t (2.4 ± 0.3 nmol·mg^{-1}· min^{-1} and 32 ± 7 μM,

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respectively, n = 6) for ES uptake into RPT suspensions isolated from female rabbits were not different from the J_{max} and K_t (1.7 ± 0.2 nmol·mg^{-1}·min^{-1} and 23 ± 3 μM, respectively, n = 5) measured for ES transport in male rabbits. Unlabeled TEA, a substrate for both Oct1 and Oct2 in the rabbit (Wright et al., 2004), also reduced the peritubular uptake of [^{3}H]-TEA in RPT suspensions as described by eq. 1. Kinetic analysis of TEA uptake in RPT suspensions isolated from female rabbits (n = 6) revealed a J_{max} of 0.9 ± 0.08 nmol·mg^{-1}min^{-1} and a K_t of 43 ± 9 μM which was not different fromo the J_{max} of 0.8 ± 0.08 nmol·mg^{-1}min^{-1} and a K_t of 34 ± 5 μM measured in male rabbits (Figs. 6A and 6B).

Discussion

Studies demonstrating significant sex differences in renal transport and clearance of OA and OC substrates employ the rodent model. The molecular basis of differences in the rat is correlated with sex differences in mRNA and protein expression levels for the respective transporters (Urakami et al., 2000;Urakami et al., 1999;Kudo et al., 2002;Cerrutti et al., 2002). However, sex differences in species other than the rat has received limited attention. Extensive information is available concerning the OA and OC transport in the rabbit kidney. Cloning of basolateral membrane OA and OC transporters in this species (Terashita et al., 1998;Zhang and Wright, 2002;Bahn et al., 2002;Zhang et al., 2004) allows detailed characterization of sex differences in the functional expression of transport and, should such differences occur, the molecular mechanisms responsible. In the present study the profiles of mRNA and protein expression of rbOat1, rbOat3 and rbOct2 were examined in isolated rabbit RPT and compared to transport function. In contrast to results from rats, no evidence was found for sex differences in the functional expression of these transporters.

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Developmental differences were observed in mRNA expression of rbOat1, rbOat2 and rbOat3, showing increased mRNA levels for these transporters in RPT from adult rabbits compared to immature rabbits. However, no sex differences were observed in either immature or adult rabbits. In contrast, no developmental differences were observed for either rbOct1 or rbOct2, but rbOct2 mRNA expression was significantly greater in RPT from adult male rabbits than in females. When comparing signals for mRNA expression of OA and OC transporters using bDNA signal amplification, caution should be exercised due to possible differences in amplification efficiency. However, the problem of variable hybridization efficiency is reduced by multiple hybridization sequences in the probe sets for each transporter (Hartley and Klaassen, 2000), supporting the contention that differences in signal levels between the OA and OC transporters reflect real differences in the relative mRNA expression.

Comparable to results for mRNA expression, western blots for rbOat1 and rbOat3 protein expression failed to reveal sex differences, and, in contrast to mRNA results, no significant sex differences in rabbit RPT Oct2 protein levels were observed. Of greatest significance, from a functional perspective, no differences were observed in the kinetic parameters for transport, i.e., K_t and J_{max}, (the latter a potential indicator of changes in protein levels), for basolateral transport of the rbOat1 substrate PAH, the rbOat3 substrate ES and the rbOct2 substrate TEA, in RPT isolated from female and male rabbits. These results confirm and extend results from western blots and show that sex differences do not appear to exist in the functional expression of Oat1, Oat3 and Oct2 transport proteins in the rabbit RPT.

The absence of sex differences in expression of renal OA and OC transporters in the rabbit markedly contrasts the situation observed in the rat. Significant sex differences in mRNA expression of Oat1, Oat2, and Oct2 in rat kidney have been reported (Urakami et al., 1999;Buist

et al., 2002). Unlike the rabbit, Oat1 mRNA expression is higher in male rats, and testosterone treatment of female rats, increases this expression (Kudo et al., 2002). Western blot analysis found significantly more Oat1 protein in the basolateral membrane from kidneys of male Wistar rats compared to female (Cerrutti et al., 2002;Ljubojevic et al., 2004). Interestingly, sex differences were not observed for Oat3 mRNA expression in Sprague-Dawley rats (Buist et al., 2002), a result similar to that in rabbit RPTs, but contrasts with the greater expression of rat Oat3 protein demonstrated in kidneys of male vs. female Wistar rats (Ljubojevic et al., 2004).

Further emphasizing the influence of strain differences on the apparent profile of transporter expression in rats is the observation of sex differences for rat Oct1 in Sprague-Dawley rats, as determined using branched DNA signal amplification (Slitt et al., 2002), that were not seen with Northern blot analysis of tissues from Wistar rats (Urakami et al., 1999). However, both groups noted sex differences in rat Oct2 mRNA expression, similar to those observed in the present study in rabbit RPT and mouse kidney. Consistent with mRNA data in the rat, significant sex differences have been observed for rat Oct2 protein expression. Importantly, the higher level of Oct2 protein expression in the male rat kidney is correlated with an increase in TEA transport in renal slices from males (Urakami et al., 2000;Urakami et al., 1999).

Sex differences in OA secretion in rat kidneys have been recognized for some time (Reyes et al., 1998). Renal cortical slices from males rats tend to have greater PAH uptake than slices from females, and castration or blocking the testosterone receptor in males significantly reduces PAH uptake (Huang KC and McIntosh BJ, 1955;Kleinman et al., 1966;Reyes et al., 1998). Administration of estrone (structurally estradiol is a hydroxyl while estrone is a ketone) to intact male rats also markedly reduces PAH accumulation into renal slices, whereas estrone

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treatment of females reduces PAH accumulation to a lesser extent (Huang KC and McIntosh BJ, 1955). Testosterone administration to female rats increases PAH accumulation by renal slices but has no effect on males (Braunlich H et al., 1993;Huang and McIntosh, 1955). Since transport in renal slices reflects basolateral uptake, these data examining PAH transport appear correlated with sex differences in Oat1 mRNA and protein expression. This observation is supported by results, which showed that the J_{max} for PAH uptake in rat renal BLMV was two-fold greater compared to this parameter measured in females (Cerrutti et al., 2002),. Oat1 protein levels were also greater in the male rat kidney as compared to the female, which suggests sex differences at the protein level may be responsible for differences in transport function.

Although male rats appear to express higher levels of PAH and TEA transport, females appear to have a greater capacity for transport of some xenobiotics (Hanhijarvi et al., 1982;Tanaka et al., 1991;Terashita et al., 1995). Renal clearance of the OAs zenarestat and perfluorooctanoic acid is significantly higher in female rats than in males (Kudo et al., 2002;Tanaka et al., 1991). Renal clearance of the OA perfluorooctanoic acid is 44-fold higher in females than in male Wistar rats (Kudo et al., 2002). Interestingly, ovariectomy of females, which removes the estrogen influence, further increases this clearance two-fold, (to suggest that perhaps other factors play a role in regulating the clearance of this xenobiotic), yet this increase is reduced to control levels by estradiol treatment after ovariectomy. This increase in perfluorooctanoic acid renal clearance with ovariectomy is associated with a concomitant increase in Oat3 mRNA expression, which is reduced to control levels with estradiol treatment. On the other hand, testosterone administration to intact females significantly decreases perfluorooctanoic acid clearance but Oat3 mRNA expression is no different from control. Castration or estradiol administration to intact males increases renal clearance of

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perfluorooctanoic acid, but changes in Oat2 mRNA expression are closely correlated to these alterations in perfluorooctanoic acid clearance in male rats (Kudo et al., 2002).

Whereas significant evidence suggests that sex differences play a significant role in OA excretion in the rat, the present work examining the rabbit RPT, and limited evidence available for other species, including the human, contrasts sharply with rat data (Anton et al., 1986;Tanaka et al., 1992). Consistent with our observations in the rabbit, renal OA secretion in humans has not exhibited sex differences. For example, renal secretion of the OA zenarestat shows a significant sex difference in mice and rats that exists in neither dogs nor humans (Tanaka et al., 1992). Similarly, renal secretion of urate, an OA substrate for human Oat1 and Oat3, is not different between human males and females, nor is urate secretion influenced by estradiol administration (Anton et al., 1986). These observations suggest that, in contrast to the rat but similar to the rabbit, no sex differences are present in the functional expression of Oat1 and Oat3 in the human.

When examining sex differences for OC transport, the story is more complicated. Both rat and rabbit Oct2 display significant sex differences in mRNA expression. In the rat sex differences in mRNA correlate with differences in Oct2 protein expression and TEA transport (Urakami et al., 2000;Urakami et al., 1999). In the rabbit, however, the difference in expression of Oct2 mRNA observed between females and males failed to extend to differences in either Oct2 protein or to transport of TEA into RPT. On the other hand, humans, like rats, exhibit a significant sex difference in the renal excretion of the OC substrate, amantadine (which is a substrate for Oct1, Oct2 and Oct3 (Wright et al., 2004;Goralski et al., 2002)). Importantly, renal clearance involves transport across both basolateral and apical membranes. Thus, sex

differences in renal clearance may involve apical transporters either in addition to or rather than basolateral transporters.

Although sex differences in renal OA and OC transport appear to be species specific, the extent to which this represents species-specific differences in 'physiological strategy' is not clear. It is premature to draw firm conclusions on whether species differences in transport may impart survival advantages, or if the profile of sex differences reflects broader environmental or evolutionary trends. However, significant species differences in metabolism, diet (rats are omnivorous and rabbits herbivorous), physiological cycles (e.g., rats show estrus; rabbits are induced ovulators) as well as differences in excretion within the various organ systems are some of the factors that may independently or collectively play a role in species-specific responses to sex steroid hormone regulation.

In conclusion, no sex differences were observed in rabbit RPT for mRNA, protein, or functional expression of expression Oat1 and Oat3 transport. These observations, contrast significantly with data from the rat showing significant sex differences for OA transport. The rabbit data does correlate with the limited evidence from humans that also fails to show significant sex differences in renal transport of OA substrates. Although rbOct2 did show significant sex differences in mRNA expression, rbOct2 protein expression and TEA transport, in contrast to the rat, failed to show sex differences. However, like rats, humans exhibit sex differences in transport of some OC substrates. Thus, sex steroid hormone regulation of renal OA and OC transporters displays significant species differences and, therefore, caution should be exercised in extrapolating transport-related sex differences between species.

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Footnotes:

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Figure Legends

Figure 1. Relative expression of mRNA for rabbit Oat1, Oat2, Oat3, Oct1 and Oct2 in suspensions of rabbit RPT. Total RNA was isolated from sexually immature (8 weeks of age) and sexually mature (15-20 weeks of age) female and male rabbits and analyzed by the branched DNA signal amplification assay for OAT and OCT mRNA content. Each bar represents the mean (expressed as relative light units) ± SE determined from tissues isolated from 6-7 animals of each sex. Asterisks indicate significant differences between mature and immature rabbits while superscripts indicate significant differences between adult males and female rabbits (*$p<0.05$).

Figure 2. Relative expression of mRNA for mouse Oat1, Oat2, Oat3, Oct1 and Oct2 in mouse renal cortical tissue. Cortical total RNA was isolated from 14-15 week old female and male mice and analyzed by the branched DNA signal amplification assay for OAT and OCT mRNA content. Each bar represents the mean (expressed as relative light units) ± SE determined from tissues isolated from 3 animals of each sex. Superscripts indicate significant differences between adult female and male mice (*$p<0.05$)

Figure 3. Immunoblot analysis of Oat1 (Fig. 3A), Oat3 (Fig. 3B) and Oct2 (Fig. 3C) membrane proteins isolated from suspensions of rabbit RPT. Crude membranes prepared from suspensions of RPT isolated from three female and three male rabbits were separated on SDS-PAGE and Oat1, Oat3 and Oct2 identified using polyclonal antibodies as described in Methods.

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Figure 4. A: Kinetics of [^{3}H]-PAH uptake into suspensions of RPT from female rabbits. Uptakes were measured at 30 s. Each point represents the mean ± SE of triplicate measurements from 6 separate rabbits. B: Kinetics of [^{3}H]-PAH uptake into suspensions of RPT from male rabbits. Each point represents the mean ± SE of triplicate measurements from 6 separate rabbits.

Figure 5. A: Kinetics of [^{3}H]-ES uptake into suspensions of RPT from female rabbits. Uptakes were measured at 30 s. Each point represents the mean ± SE of triplicate measurements from 5 separate rabbits. B: Kinetics of [^{3}H]-ES uptake into suspensions of RPT from male rabbits. Each point represents the mean ± SE of triplicate measurements from 6 separate rabbits.

Figure 6. A: Kinetics of [^{3}H]-TEA uptake into suspensions of RPT from female rabbits. Uptakes were measured at 30 s. Each point represents the mean ± SE of triplicate measurements from 6 separate rabbits. B: Kinetics of [^{3}H]-TEA uptake into suspensions of RPT from male rabbits. Each point represents the mean ± SE of triplicate measurements from 5 separate rabbits.

Table 1. Oligonucleotide probes generated for analysis of Oat and Oct expression by bDNA signal amplification. Target refers to the sequence of the mRNA transcript as enumerated in the GenBank file. Function refers to how the oligonucleotide probe was used in the bDNA assay.

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Table 1. Oat- and Oct- distribution in Renal tissues

Gene	GenBank ID	Target	Function	ProbeSequence
rbOat1	AJ242871	875-892	CE	gcttcccattgatccgggTTTTTctcttggaaagaaagt
		893-911	CE	aacttggccccctcttcctTTTTTctcttggaaagaaagt
		954-972	CE	ggcctggcctttgctcattTTTTTctcttggaaagaaagt
		1072-1090	CE	caaagccctgcaggtccatTTTTTctcttggaaagaaagt
		815-832	LE	gggtggagtaccagcgcgTTTTTaggcataggacccgtgtct
		833-853	LE	gggtgaggtccagtctcccagTTTTTaggcataggacccgtgtct
		854-874	LE	ccactttctgcaaggccttcaTTTTTaggcataggacccgtgtct
		912-931	LE	tccggagcacctccatactcTTTTTaggcataggacccgtgtct
		932-953	LE	gtcagttccttctgcagattcgTTTTTaggcataggacccgtgtct
		973-991	LE	gcagcaactccatggctgaTTTTTaggcataggacccgtgtct
		1032-1050	LE	gctggtggcaaaccacagcTTTTTaggcataggacccgtgtct
		1051-1071	LE	gaccaacccgtagtaggcaaaTTTTTaggcataggacccgtgtct
		1114-1131	LE	cacggcgccaaaaatcacTTTTTaggcataggacccgtgtct
		995-1009	BL	ggcggagggcagggc
		1010-1031	BL	aaggagaggcagaggaagaggt
		1091-1113	BL	ttggattaggtagatgctgaccc
rbOat2		933-952	CE	tcagggcctctctgctcagaTTTTTctcttggaaagaaagt
		1126-1143	CE	cccgaacagcagctgcgtTTTTTctcttggaaagaaagt
		1197-1212	CE	ctgcgtgaggcggcgtTTTTTctcttggaaagaaagt
		1266-1285	CE	tccaggagctcctctcggagTTTTTctcttggaaagaaagt
		916-932	LE	ccgtcctgaggcacgggTTTTTaggcataggacccgtgtct
		969-985	LE	tgcggaccacccgttccTTTTTaggcataggacccgtgtct
		986-1008	LE	gaacaggtccaagtatgagggtcTTTTTaggcataggacccgtgtct
		1025-1044	LE	gcagcacagggagatgtgccTTTTTaggcataggacccgtgtct
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		1286-1302	LE	caccgctagggcggtgcTTTTTaggcataggacccgtgtct
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		1009-1024	BL	tgagcctgggcgtgcg
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		1231-1245	BL	cagggccagggccgc
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		712-734	LE	cggatacggttaactgtagccagTTTTTaggcataggacccgtgtct
		758-781	LE	ggactctggtatccaccaggataaTTTTTaggcataggacccgtgtct
		782-800	LE	ccaggaccagccagcgtatTTTTTaggcataggacccgtgtct
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mOat3	NM031194	1315-1336	CE	cccaaatacagccagtgctgttTTTTTctcttggaaagaaagt
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		715-735	LE	ctgtagccagagccgacaaacTTTTTaggcataggacccgtgtct
		736-758	LE	ggtacaagatggctgtcgttctcTTTTTaggcataggacccgtgtct
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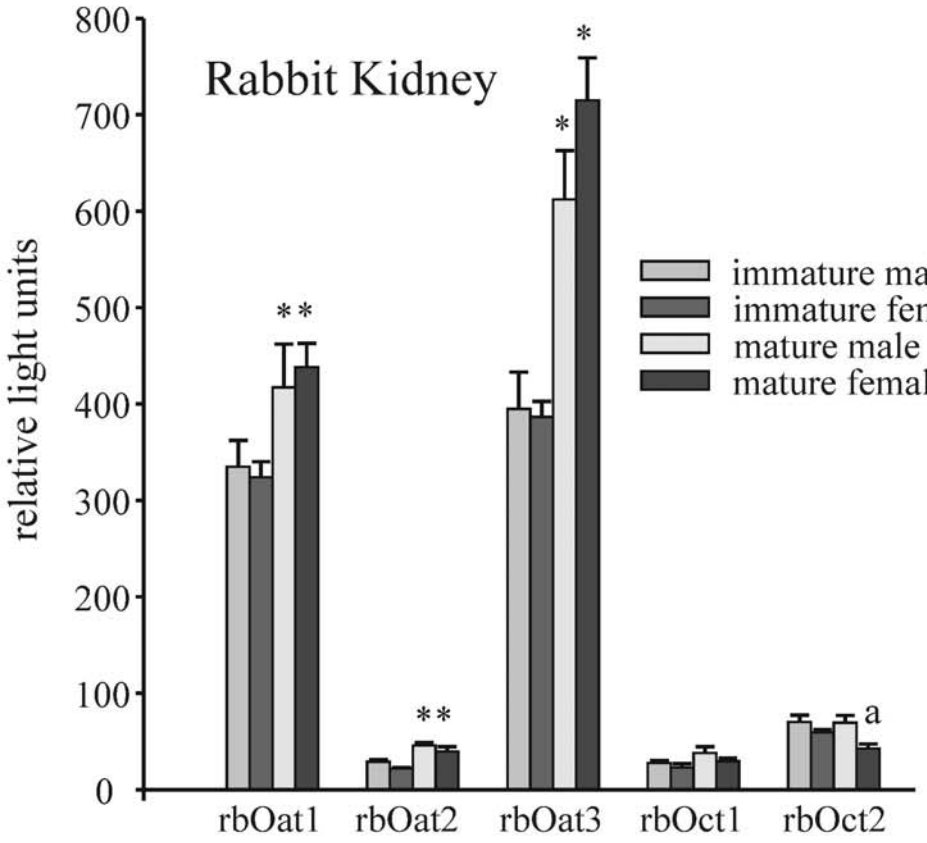

Figure 1

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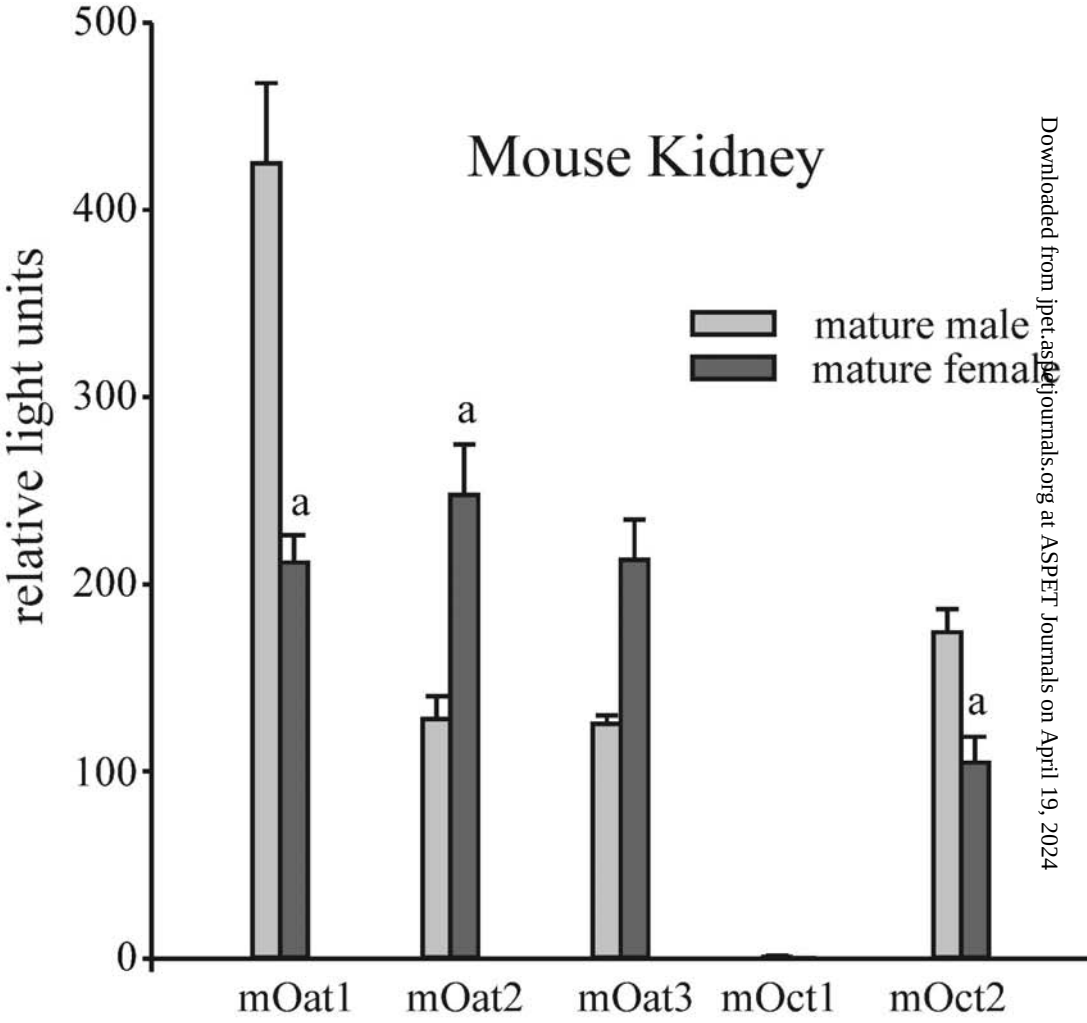

Figure 2

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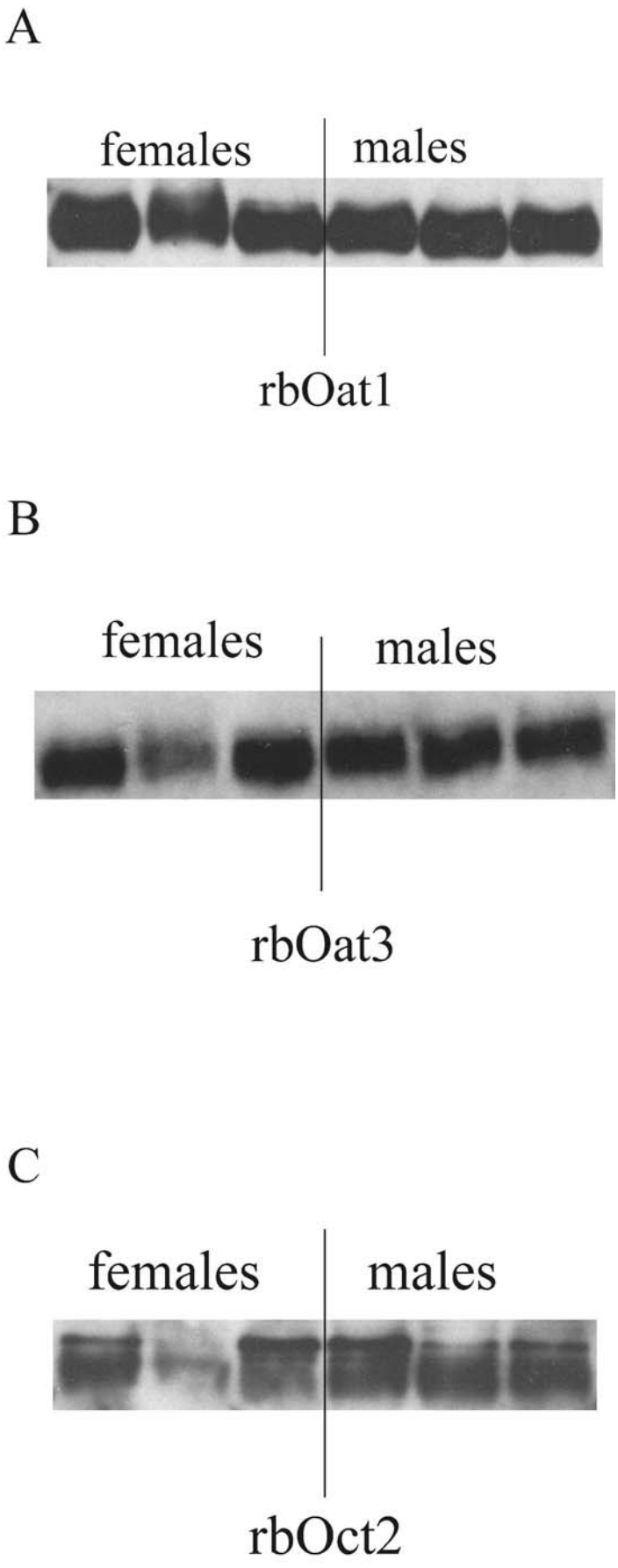

Figure 3

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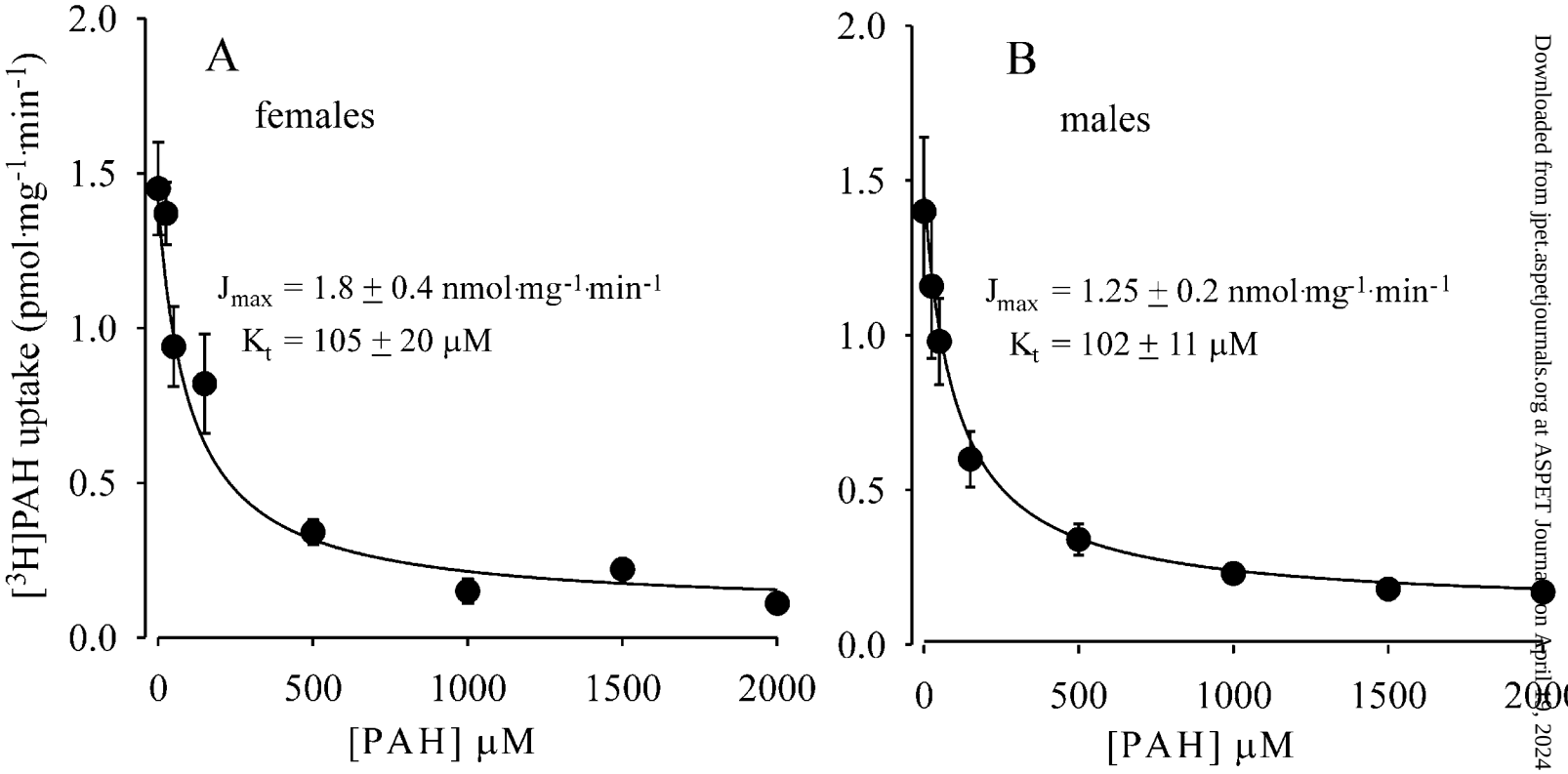

Figure 4

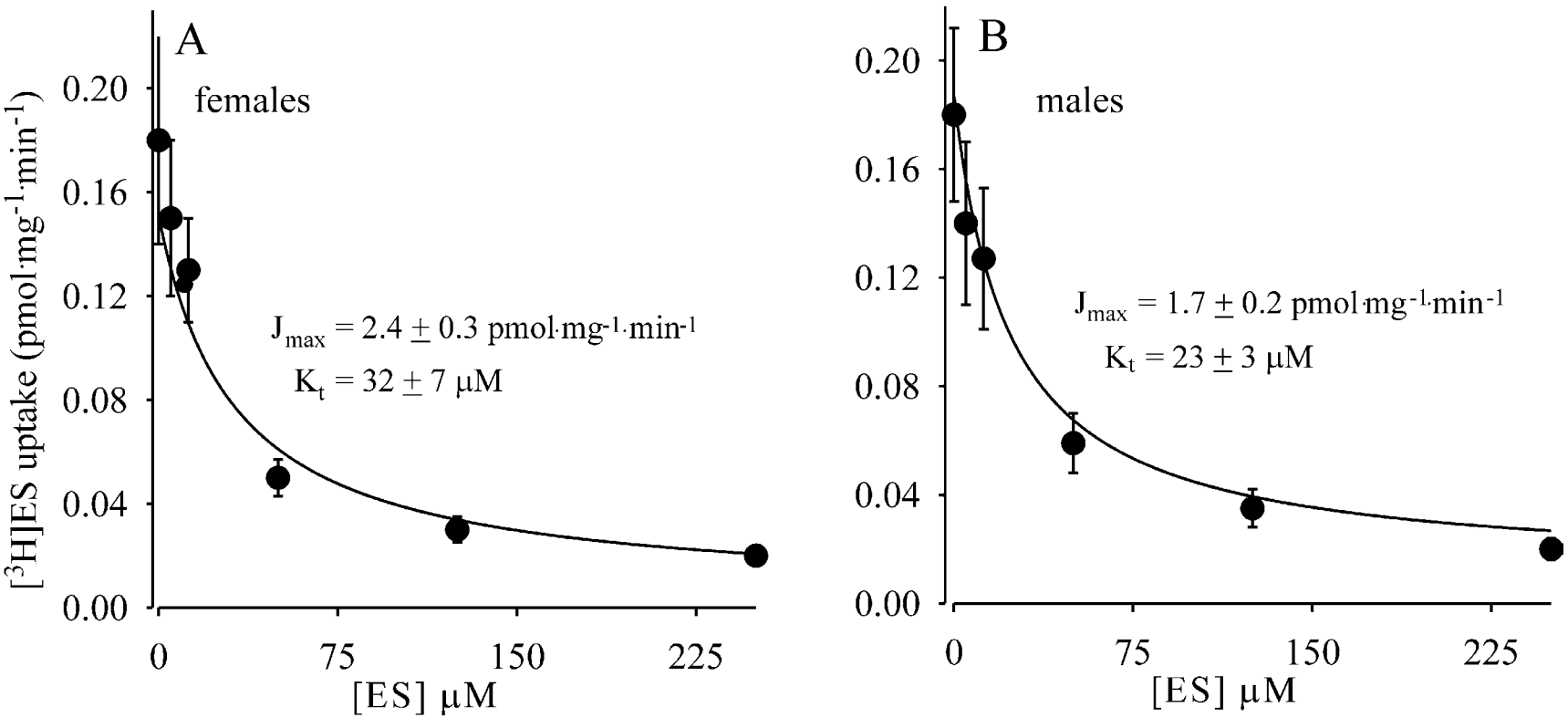

Figure 5

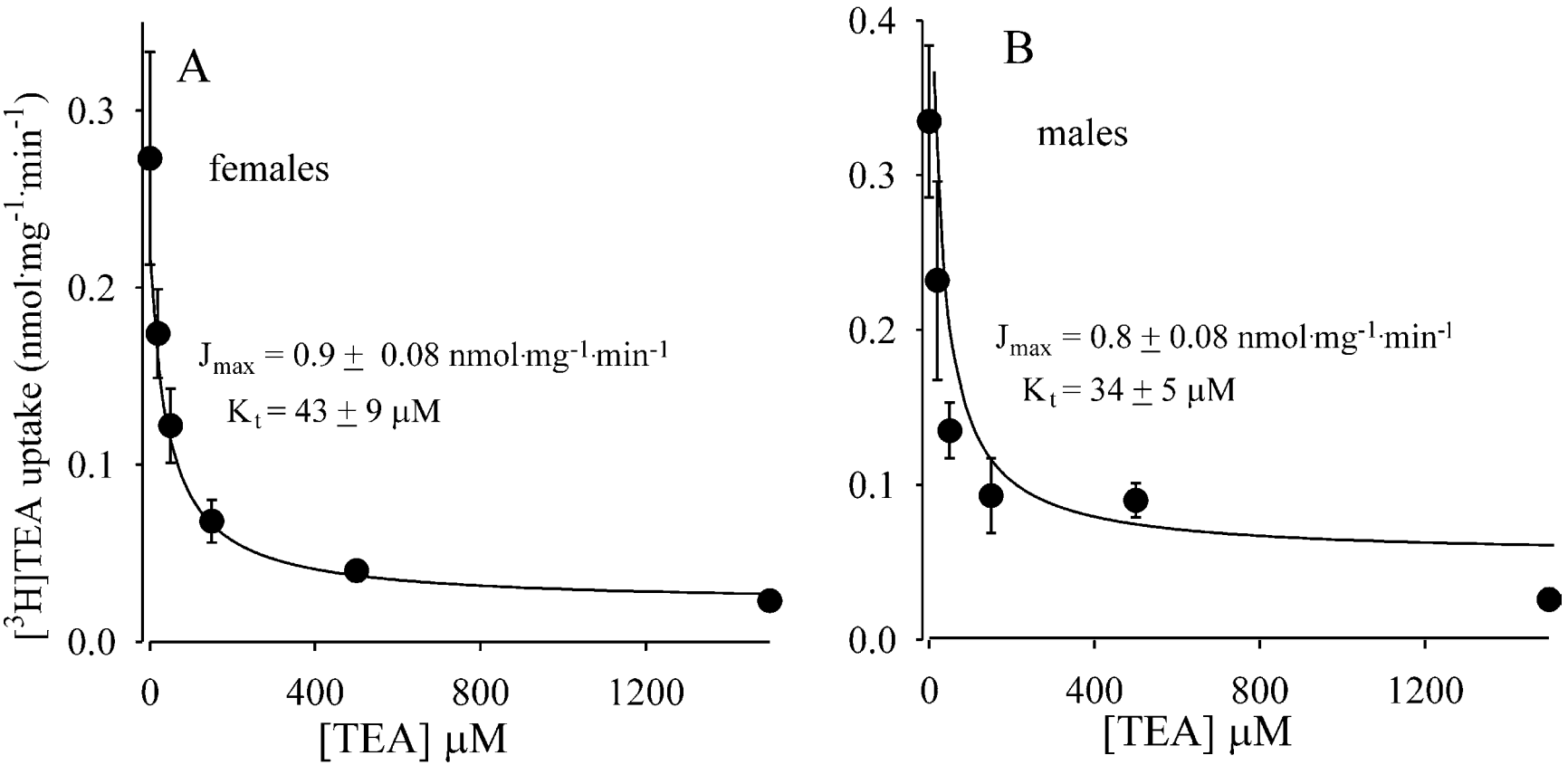

Figure 6