A role for the organic anion transporter OAT3 in renal creatinine secretion in mice

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Creatinine is a weak organic cation with pKₐs of 4.8 and 9.2 and, thus, is monoprotonated at physiological pH. The histamine H₂ receptor antagonist cimetidine is a high-affinity substrate and competitive inhibitor of organic cation transport (OCT) (15, 40) that inhibits renal secretion of creatinine (32), and, thus, has been used to enhance the validity of creatinine clearance as a measure of GFR (28, 31, 45). OCT2 and OCT1 have been shown to be the major cation transporters in the kidney (2, 22). OCT2 is highly expressed in the basolateral membrane of the proximal tuuble, and both cimetidine and creatinine have been shown to be substrates for rat and human OCT2 when stably expressed in HEK 293 cells (16, 39). These studies indicated a potential role for OCT2 in renal creatinine secretion.

However, it is well-established that many organic substrates can interact with both the organic anion and organic cation transport systems (37, 38). In fact, renal creatinine secretion in guinea pig (3) and rat (9, 17) can be inhibited by probenecid, a prototypical inhibitor of organic anion transport (OAT). This occurred at physiological (9) as well as increased creatinine plasma levels (14, 17). Similarly, studies in humans, dogs, and chicken revealed that organic anions can inhibit renal creatinine secretion (5, 8, 27, 29). Moreover, while cimetidine inhibits OCT, it can also inhibit transport mediated by OAT1 and OAT3 (1, 6, 19), and basolateral uptake of cimetidine is additively blocked by probenecid and tetraethylammonium, blockers of organic anion and cation transporters, respectively (15, 36, 38). More recently, Eisner and colleagues (12) reported that the renal secretion of creatinine in mice was reduced by about half by both cimetidine and the competitive OAT substrate/inhibitor para-aminohippurate (PAH). Moreover, they reported that renal creatinine secretion was intact and plasma creatinine concentrations normal in mice lacking OCT1 or OCT2 (12). These findings prompted us to investigate a possible role for OAT1 and OAT3 in the tubular secretion of creatinine.

MATERIALS AND METHODS

Transport studies in Xenopus laevis oocytes. Oocyte transport assays were performed as previously described (41, 42). Briefly, capped cRNA was generated via in vitro transcription from linearized plasmid DNA (mOat1, Image clone ID 4163278; mOat3, Image clone ID 4239544) using mMessage mMachine in vitro transcription kit (Ambion, Austin, TX). Stage V and VI X. laevis oocytes were isolated, maintained in Barth’s growth medium [88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, and 10 mM HEPES, pH 7.4] supplemented with 5% fetal...
horse serum and 0.05 mg/ml gentamycin sulfate, and then injected with ~20 nl/oocyte cRNA solution (1 μg/μl) and allowed to mature for 3 days postinjection. mOAT1-expressing, mOAT3-expressing, and control uninjected oocytes were incubated in serum-free Barth’s buffer for 1 h at 25°C with various concentrations of 14C-labeled creatinine (American Radiolabeled Chemicals, St. Louis, MO) and in the presence or absence of saturating concentrations (2 mM) of the OAT inhibitor, probenecid. Our previous studies revealed that cimetidine inhibited mOAT1- and mOAT3-mediated transport with respective Ks of 1,038 and 85 μM (1), and we used ~3 times these concentrations to induce ~75% inhibition.

Transport was terminated by three washes in ice-cold Barth's buffer, and the 14C-creatine content of groups of four to six oocytes was determined by scintillation counting. The background uptake of creatinine was similar in uninjected oocytes and probenecid-inhibited, mOat1- or mOat3-injected oocytes; this background was subtracted from all uptake measurements to calculate the mOAT1- or mOAT3-mediated component of uptake. The kinetic constants Km and Vmax were determined by nonlinear regression using Prism software 5.0 (GraphPad, San Diego, CA).

Animals. The generation of Oat1 and Oat3 knockout mice (Oat1−/− and Oat3−/−) has been described (13, 33). Both lines were back-crossed to C57BL/6J mice for 10 generations. Heterozygous male mice were used in the present experiments. All experimental protocols were in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee.

Expression of Oat1 and Oat3 mRNA in the kidney. Kidneys were harvested under short-term isoflurane anesthesia. Whole kidneys (contralateral kidneys were used for protein expression analyses) were homogenized in Buffer RLT (RNeasy Mini Kit; Qiagen, Valencia, CA) using a rotator-stator homogenizer (Tissumizer; Tekmar, Cincinnati, OH). Kidney RNA was prepared with an RNeasy Mini Kit and quantified by Nanodrop. cDNA was prepared with the Superscript II First Strand Synthesis System (Invitrogen, Carlsbad, CA). For quantification of Oat1 and Oat3 mRNA, a Taqman gene expression assay (Mm00456258_m1 and Mm00459534_m1, respectively) was performed with Taqman Universal PCR Master Mix in an ABI 7300 Real-Time PCR System. Amplification efficiencies were normalized against the housekeeping gene rpl19 (forward: 5'-GCTAGAGGCTACAAGAAAGGCCTG-3'; reverse: 5'-GGAAAGTGGCATGGCGATTC-3') and relative fold increases were calculated. All PCR reactions were performed using 100 ng of cDNA (RNA equivalent) under the following conditions: 10 min at 95°C with 50 cycles of 15 s at 95°C and 1 min at 60°C. Each experiment was performed in duplicate. Kidneys of Oat1−/− and Oat3−/− mice were used as negative controls.

Expression of OAT1, OAT3, and OCT2 protein in renal membranes. Kidneys were homogenized in a buffer containing 250 mM sucrose, 10 mM triethanolamine (Sigma, St. Louis, MO), and Complete Protease Inhibitor cocktail (Roche, Indianapolis, IN) using a tissue homogenizer (Tissumizer; Tekmar). Homogenates were centrifuged at 16,000 g for 60 min to obtain a membrane pellet. Protein concentration was determined using a DC Protein Assay (Bio-Rad, Hercules, CA). Samples were diluted in 4× LDS reducing sample buffer (Invitrogen) and then heated for 10 min at 70°C for OAT1-Ab and OCT2-Ab, and 30 min at 37°C for OAT3-Ab. Samples were loaded on precast 4 to 12% Bis-Tris SDS-PAGE gels (Invitrogen) using MOPS buffer. The membranes were blocked with 5% nonfat dry milk (Bio-Rad) in PBS (pH 7.4) containing 0.1% Tween 20 for 1 h. Immunoblotting was performed at 4°C overnight with the primary OAT1-Ab, OCT2-Ab (both Alpha Diagnostic Intl, San Antonio, TX), and OAT3-Ab (26) diluted 1:1,000. Chemiluminescent detection was performed using a 1:5,000 dilution of ECL donkey anti-rabbit IgG linked to horseradish peroxidase and ECL detection reagent (GE Healthcare, Buckinghamshire, UK). To verify equal protein loading, the membrane was stripped (0.2 M NaOH for 5 min) and reprobed with monoclonal anti-β-actin Ab (Sigma).

Renal clearance experiments in Oat1−/− and Oat3−/− mice. Mice were anesthetized for terminal clearance experiments with 100 mg/kg thiobutabarbital ip and 100 mg/kg ketamine im (30, 42). The femoral artery was cannulated for blood sample withdrawal and the jugular vein for continuous maintenance infusion of 2.25 g/dl bovine serum albumin in 0.85% NaCl at a rate of 0.5 ml·h−1 ·30 g−1 body wt. GFR was determined by inulin clearance. To this end, FITC-inulin was added to establish a 0.5% infusion solution. Alternatively, [3H]inulin was added to the infusion to deliver 5 μCi h−1 ·30 g−1 body wt. A timed urine collection was performed for 45–60 min using a bladder catheter. Urinary volume was determined gravimetrically and flow rate was calculated based on collection time. Blood was withdrawn at the beginning and the end of the collection period in heparinized capillaries and centrifuged at 5,000 rpm for 3 min to obtain plasma. Plasma and urine were analyzed for FITC-inulin (Nanodrop Technologies, Wilmington, DE) or [3H]inulin (by liquid scintillation counting). Creatinine was determined by HPLC as previously described (11, 44) or by using a sensitive enzymatic assay (Crea plus; Roche Diagnostics, Indianapolis, IN), which had previously been shown to provide results similar to HPLC in plasma of rats and mice (21). Renal creatinine secretion fraction (SF) was calculated as: SF = renal creatinine net secretion/renal creatinine excretion = renal creatinine clearance − renal inulin clearance/renal creatinine clearance.

Statistical analysis. Data are shown as means ± SE. Unpaired and paired Student’s t-test was performed to analyze for statistical differences between knockout and WT mice and within groups, respectively. P < 0.05 was considered statistically significant.

RESULTS

mOAT1 and mOAT3 transport creatinine in vitro. To directly assess transport of creatinine by mouse (m) OAT1 and mOAT3, uptake of labeled creatinine into mOat1- and mOat3-mRNA microinjected X. laevis oocytes was determined. As depicted in Fig. 1A, either transporter mediated significant uptake of creatinine. At a concentration of 10 μM (0.11 mg/dl), which is similar to physiological plasma levels in the mouse, uptake into mOAT1- and mOAT3-expressing oocytes, respectively, was approximately five- and threefold greater in the absence than in the presence of saturating doses of the OAT inhibitor probenecid. (Uptake into probenecid-treated, OAT-expressing oocytes was similar to that into uninjected oocytes.) The data indicate the capacity of both mOAT1 and mOAT3 to transport creatinine in vitro.

Subsequently, the concentration dependence of creatinine transport was examined, by determining uptake across a range of concentrations from 1 μM to 10 mM and analyzing the resulting data to estimate the kinetic constants for OAT-mediated transport. The estimated Ks values of creatinine for mOAT1 and mOAT3 were 6.7 and >10 mM, respectively; and the estimated Vmax values were 311 and >440 pmol·oocyte·h−1, respectively (Fig. 1, B and C). (Given the relatively low affinity of mOAT1 and mOAT3 for creatinine, we also attempted to determine transport at concentrations greater than 10 mM. However, at these very high concentrations, we were not able to obtain reliable data, likely owing to toxic or other nonspecific effects.) In previous studies with similarly prepared X. laevis oocytes, mOAT1 Vmax values for several canonical
organic anionic substrates ranged from 0.5 to 40 pmol·ocyte\(^{-1}\)·h\(^{-1}\) (20, 35). Thus, while the apparent affinity of creatinine for the transporters is relatively low (high \(K_m\) values), this may be compensated by their relatively high transport capacity for this compound (high \(V_{\text{max}}\) values).

Since cimetidine inhibits creatinine secretion in vivo (32), we also assessed the capacity of cimetidine to inhibit creatinine transport by mOAT1 and mOAT3. In previous studies, we found cimetidine to inhibit tracer uptake by mOAT1 with \(K_i\) (inhibition constant) of \(1,038\, \mu\text{M}\) and mOAT3 with \(K_i\) of \(85\, \mu\text{M}\) (1). Accordingly, we assessed inhibition of the uptake of 10 \(\mu\text{M}\) creatinine into mOAT1-expressing oocytes by \(3.1\, \text{mM}\) cimetidine, and of mOAT3-expressing oocytes by \(250\, \text{\muM}\) cimetidine, in either case representing approximately three times the previously measured inhibition constant and thus expected to suppress transport by \(75\%\). We found that these concentrations of cimetidine decreased creatinine transport into mOAT1- and mOAT3-expressing oocytes by \(86\%\) and \(61\%\), respectively (Fig. 1D), in general agreement with expectations.

Expression of OAT1, OAT3, and OCT2 protein in renal membranes of Oat1\(^{-/-}\) and Oat3\(^{-/-}\) mice. As expected and confirming previous studies (13, 33, 42), there was no significant renal expression of Oat3 and Oat1 mRNA in the corresponding knockout mice (data not shown). Possibly as a consequence of the genes for Oat1 and Oat3 having adjacent chromosomal locations, previous studies found that the renal expression of Oat1 mRNA in Oat3\(^{-/-}\) mice was reduced to \(40\%\) of levels in WT mice (42, 43), and the renal expression of Oat3 mRNA in Oat1\(^{-/-}\) mice was similarly reduced to \(40\%\) of WT levels (42). In comparison, previous studies in Oat3\(^{-/-}\) and Oat1\(^{-/-}\) mice indicated that renal mRNA expressions of Oct1 and Oct2 were normal (42). The current...
analyses revealed that the renal expression of Oat1 mRNA in Oat3−/− mice was reduced to 64 ± 7% of levels in WT mice (n = 6–8; P < 0.05 vs. WT), and the renal expression of Oat3 mRNA in Oat1−/− mice was reduced to 22 ± 4% of WT levels (n = 5–6; P < 0.05 vs. WT). Thus, compared with previous studies performed in Oat1−/− and Oat3−/− mice (42, 43), the reduction of mRNA expression of Oat3 in Oat1−/− was stronger (22 vs. ~40% of WT) in the current study, whereas the suppression of Oat1 mRNA in Oat3−/− mice appeared lesser (64 vs. ~40% of WT) possibly reflecting environmental differences (e.g., in colony conditions).

Western blot analyses with antibodies to OAT1 and OAT3 identified protein bands in WT mice that were of the expected size and absent in the corresponding knockout mice, consistent with specific binding of the antibodies (Fig. 2, A and B). The renal membrane expression of OAT1 protein in Oat3−/− mice was reduced to ~60% of expression in WT mice (Fig. 2B), and that of OAT3 protein in Oat1−/− mice to ~6% (Fig. 2A). In comparison, renal membrane expression of OCT2 protein was not significantly affected in Oat1−/− or Oat3−/− mice (Fig. 2, A and B).

Renal creatinine secretion is reduced in Oat1−/− and Oat3−/− mice. Mice were anesthetized and prepared for terminal clearance experiments. GFR was assessed from inulin clearance, compared with endogenous creatinine clearance, and the data were used to determine renal secretion of creatinine. Endogenous creatinine clearance was consistently greater than inulin clearance in WT mice (Fig. 3); Δ clearance 113 ± 32%. This was not observed in Oat1−/− or Oat3−/− mice (Fig. 3; Δ clearance of 43 ± 28 and 11 ± 10%, respectively; each P < 0.05 vs. WT). Net urinary creatinine excretion was not different between genotypes (Fig. 4). However, whereas WT mice presented consistent net creatinine secretion that represented a renal creatinine SF of 45 ± 6%, mean values for these parameters were not significantly different from zero in both Oat3−/− and Oat1−/− mice (Fig. 4).

 Plasma creatinine concentrations in Oat1−/− and Oat3−/− mice. In a first series, mice were briefly anesthetized with isoflurane to collect blood and determine plasma creatinine concentration. Plasma creatinine concentration was similar in WT compared with Oat3−/− and Oat1−/− mice (0.08 ± 0.01 vs. 0.09 ± 0.01 and 0.10 ± 0.01 mg/dl; n = 20–25, NS). In a
second set of animals, plasma creatinine concentrations were determined during terminal clearance experiments under thiobutabarbital/ketamine anesthesia and found to be significantly increased in Oat3−/− mice vs. WT and tended to be greater in Oat1−/− vs. WT mice (Fig. 4).

**DISCUSSION**

The in vitro and in vivo data are consistent with a contribution of OAT3 and OAT1 to the renal secretion of creatinine in mice. In vitro expression studies in *X. laevis* oocytes show that both mouse proteins can transport creatinine. The studies indicate that the apparent affinity of creatinine for mouse OAT1 and OAT3 is relatively low (high K_m values), but a comparison with V_max values for several canonical organic anionic substrates, determined previously using similarly prepared *X. laevis* oocytes (20, 35), indicates that the low affinity may be compensated by a high-creatinine transport capacity of both OAT3 and OAT1.

Actual transport in vivo is influenced by multiple extraneous factors and, as a consequence, the in vivo relevance of in vitro data is difficult to predict. For example, both OAT1 and OAT3 are known to transport PAH in oocytes and kidney slices (33); however, in vivo studies in knockout mice indicated that OAT1 is primarily relevant for renal PAH secretion in vivo while renal PAH clearance is normal in Oat3−/− mice (13, 42). We therefore assessed the actual contribution of OAT1 and OAT3 to creatinine secretion in vivo, using Oat1 and Oat3 gene knockout mouse models. Since application of colorimetric creatinine analysis can overestimate plasma creatinine values and thus underestimate creatinine clearance and secretion in rodents, we determined plasma creatinine by both HPLC (11, 44) and a sensitive enzymatic assay that had previously been shown to provide results similar to HPLC in plasma of rats and mice (21). Our studies revealed consistently greater values for the clearance of creatinine vs. inulin in WT mice and net renal creatinine secretion, which represented ~45% of total creatinine excretion (Fig. 4). The values observed for creatinine clearance and secretion in WT mice were similar to previously reported data by other investigators using HPLC analysis (12). In comparison, a statistically significant difference between inulin and creatinine clearance as well as consistent net renal creatinine secretion was lacking in both Oat3−/− and Oat1−/− mice.

Impairment in renal creatinine secretion can increase creatinine plasma levels, whereas differences in creatinine formation or GFR can mask such an increase. Plasma creatinine measurements were variable with one series performed in the absence of anesthesia and surgery revealing no significant difference between WT and Oat1−/− or Oat3−/− mice, whereas measurements in clearance experiments showed significantly enhanced values for Oat3−/− mice and a tendency for higher values in Oat1−/− compared with WT. Notably, glomerular filtration of creatinine was increased in both Oat3−/− and Oat1−/− mice compared with WT mice in

![Renal clearance of inulin vs. creatinine in mice lacking Oat1 or Oat3.](http://ajprenal.physiology.org/)

**Fig. 4.** Consistent renal secretion of creatinine is observed in WT mice but not in mice lacking Oat1 or Oat3. Renal clearance experiments were performed under terminal anesthesia. Renal creatinine excretion was significantly greater than glomerular creatinine filtration in WT, indicating consistent renal creatinine net secretion (#P < 0.05), which represented 45 ± 6% of renal creatinine excretion. Mean values for renal creatinine net secretion and renal creatinine secretion fraction were not significantly different from zero in Oat1−/− and Oat3−/− mice; n = 9–13. *P < 0.05 vs. WT.

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clearance studies, whereas renal creatinine excretion was not different due to blunted net renal creatinine secretion.

In particular, mice lacking Oat1 showed variability in renal creatinine handling. While some Oat1−/− mice had substantial net creatinine secretion, others lacked it completely or even showed net creatinine reabsorption. Studies in rats have described net renal creatinine reabsorption when plasma concentrations were low but the study used a colorimetric creatinine assay (24) that may have overestimated creatinine plasma levels and the amount of creatinine filtered. Our data show that there can be OAT1-independent renal creatinine secretion. The potential candidates include OAT3 but a contribution from OCT2 is also possible, especially when OAT-mediated transport is inhibited, and the activity of these pathways may vary. Furthermore, the finding that plasma creatinine concentrations of Oat3−/− mice were greater compared with WT mice under anesthesia/surgery (with similar GFR) but not following brief isoflurane anesthesia may indicate that prolonged anesthesia/surgery enhanced the quantitative contribution of OAT3 for renal creatinine secretion, possibly due to inhibition of OAT1 and/or OCTs. Our anesthesia protocol for the clearance studies included ketamine. Ketamine has been shown to interact with both OATs and OCTs (38). Previous studies, using the same dose of ketamine in WT and Oat1/Oct2 double knockout mice, found clear evidence for prominent OCT1/OCT2-mediated renal secretion of other organic cations in WT mice under these conditions (18), indicating that ketamine-induced inhibition of OCTs may not explain the present findings.

The interpretation of studies in Oat1−/− and Oat3−/− mice is complicated by the current finding that renal membrane expression of OAT3 protein is reduced in Oat1−/− mice to ~6% of the levels in WT and, conversely, the expression of OAT1 protein in Oat3−/− mice to ~60% (while OCT2 protein expression is maintained at WT levels in both knockout models). However, our previous data suggest that the decreased expression of OAT3 protein in Oat1−/− mice, and vice versa, may not appreciably influence function at the level of the intact organism. Specifically, renal clearance of the OAT3 substrate estrone sulfate is unaffected in Oat1−/− mice (13). Conversely, secretion of PAH is unaffected in Oat3−/− mice (42) but virtually eliminated in Oat1−/− mice (13).

Moreover, different endogenous compounds accumulate in the plasma of Oat1−/− and Oat3−/− mice (13, 41), and Oat3−/− but not Oat1−/− mice have a lower blood pressure compared with WT mice, indicating the transport of an endogenous blood pressure regulator by OAT3 but not OAT1 (41). Nevertheless, a contribution of decreased OAT3 expression in Oat1−/− mice [the mRNA expression appeared lower in the current than in previous studies: 22 vs. ~40% of WT (42)] or, to a lesser extent, of decreased OAT1 expression in Oat3−/− mice [mRNA expression appeared higher in the current than in previous studies: 64 vs. ~40% of WT (42, 43)] to the observed creatinine secretion phenotypes cannot be excluded.

The current in vitro studies revealed that the organic cation cimetidine can inhibit creatinine uptake mediated by OAT3 and OAT1. This is not unexpected based on previous studies showing that cimetidine can inhibit transport mediated by OAT1 and OAT3 (1, 6, 19). The uncharged form of cimetidine may be preferentially transported by OAT1, while the charged form is translocated by OCT2 and OAT3 (34). Although hOAT1 can transport cimetidine, it may do so with a lower affinity compared with hOAT3 (34). Thus, cimetidine may lower creatinine secretion at least in part by inhibiting OAT1 and/or OAT3. This would also be consistent with a recent study showing that renal creatinine secretion was intact and plasma creatinine concentrations normal in mice lacking the major organic cation transporters OCT1 or OCT2 (12).

Renal dysfunction can increase the tubular secretion of creatinine, limiting its value as a marker of GFR (4, 7). Pathophysiological conditions can also lower the renal expression of OCT1 and OAT3 (10, 25). According to the current results (e.g., comparing data for GFR and plasma creatinine levels during clearance studies in Oat3−/− vs. WT), downregulation of OAT expression can alter plasma creatinine concentrations in the absence of changes in GFR, indicating a new confounding factor when kidney function is assessed based on plasma creatinine levels. With regard to clinical relevance, differences in the selectivity and levels of expression of OATs and OCTs are possible between mice and humans, which may affect the relative importance and impact of these transport pathways in renal creatinine secretion.

In conclusion, our results are consistent with a contribution of OAT3 and possibly OAT1 to the renal secretion of creatinine in mice. Cimetidine can inhibit OAT1- and OAT3-mediated creatinine transport, which may contribute to its inhibitory influence on tubular creatinine secretion. Given that pathophysiological conditions can change OAT expression, these findings argue for greater caution in the use of creatinine clearance as a measure of GFR.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS

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