

Organic Anion Transporter 3 Contributes to the Regulation of Blood Pressure

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ABSTRACT

Renal organic anion transporters (OAT) are known to mediate the excretion of many drugs, but their function in normal physiology is not well understood. In this study, mice lacking organic anion transporter 3 (Oat3) had a 10 to 15% lower BP than wild-type mice, raising the possibility that Oat3 transports an endogenous regulator of BP. The aldosterone response to a low-salt diet was blunted in Oat3-null mice, but baseline aldosterone concentration was higher in these mice, suggesting that aldosterone dysregulation does not fully explain the lower BP in the basal state; therefore, both targeted and global metabolomic analyses of plasma and urine were performed, and several potential endogenous substrates of Oat3 were found to accumulate in the plasma of Oat3-null mice. One of these substrates, thymidine, was transported by Oat3 expressed *in vitro*. *In vivo*, thymidine, as well as two of the most potent Oat3 inhibitors that were characterized, reduced BP by 10 to 15%; therefore, Oat3 seems to regulate BP, and Oat3 inhibitors might be therapeutically useful antihypertensive agents. Moreover, polymorphisms in human OAT3 might contribute to the genetic variation in susceptibility to hypertension.

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The proximal tubule of the mammalian kidney efficiently secretes a large variety of organic anions (OA) leading to the rapid urinary excretion of those compounds. Basolateral uptake from plasma into the tubular cell, the first step in this secretory process, operates *via* anion exchange, coupling the entry of OA to the exit of dicarboxylates along their concentration gradient. Subsequent apical efflux from tubular cell into urine does not seem to be rate limiting and may occur *via* efflux pumps and/or anion exchange. Substrates for this secretory process include numerous important drugs (*e.g.*, most nonsteroidal anti-inflammatory drugs, β -lactam antibiotics, diuretics, nucleoside analog antivirals). Accordingly, renal OA secretion has largely been studied from a pharmacologic perspective; however, physiologic substrates and functions are not well understood (for review, see references^{1–8}).

Organic anion transporter 1 (Oat1),^{9,10} a gene originally identified as novel kidney transporter (NKT) and proposed to function in organic ion

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transport,^{11,12} and Oat3, a gene originally identified as Roct (reduced in oc transporter),¹³ seem to account largely for the basolateral uptake step of renal OA secretion: Both transporters have been immunolocalized to the basolateral membrane of the proximal tubule of human, rat, and mouse.^{14–21} Moreover, when expressed in *Xenopus* oocytes or epithelial cell lines, both Oat1 and Oat3 function as exchangers, coupling substrate entry to dicarboxylate exit.^{10,22,23}

We, and subsequently others, recently characterized mice null for Oat1 and Oat3, finding specific defects in OA transport resulting in reduced renal excretion of diuretics and other drugs.^{24–28} In the course of performing these experiments, we discovered, unexpectedly, that the *Oat3*^{-/-} mice, although otherwise grossly physiologically normal, manifested lowered BP. (Renal blood flow [as determined by paraaminohippuric acid clearance] and glomerular filtration [inulin clearance] were unaffected,²⁶ indicating that altered renal hemodynamics do not account for the observed OA excretory defects in these mice.) BP was not affected in *Oat1*^{-/-} mice or in those null for the related proximal tubular OAT, *URAT1*.^{29–31} These findings raised the possibility that Oat3 mediates the specific transport of an endogenous compound(s) involved in the regulation of BP. Moreover, they suggested the possibility that inhibition of Oat3 might also lower BP and, therefore, that Oat3 inhibitors might be useful in the treatment of hypertension. Here we describe experiments addressing these hypotheses.

RESULTS

Oat3 Knockout Mice Manifest Reduced BP

Tail-cuff measurements consistently revealed a 10 to 15% lowering of both systolic (Figure 1) and diastolic arterial BP (data not shown) in awake *Oat3*^{-/-} compared with wild-type (WT) mice, in the absence of significant differences in heart rate (data not shown). This was confirmed when BP was directly measured *via* arterial catheterization in anesthetized mice (Figure 1); again, heart rate was not significantly different compared with WT mice (data not shown). Moreover, there were no differences between *Oat3*^{-/-} and WT mice in general aspects of renal function (GFR, renal blood flow,²⁶ and fractional excretion of fluid and electrolytes; data not shown). By contrast, BP was unaffected in mice null for the other major basolateral proximal tubular OAT, *Oat1* (Figure 1), or in those null for *mUrat1* (*RST*), an apical proximal tubular OAT involved in the renal reabsorption of urate.^{29–31} These results suggest the possibility of specific transport of an endogenous BP-regulating compound by Oat3.

Identification of Potential Endogenous Oat3 Substrates

We performed analyses of *Oat3*^{-/-} mouse urine and plasma so as to identify endogenous substrates of Oat3, among which might be included the aforementioned putative BP-regulating

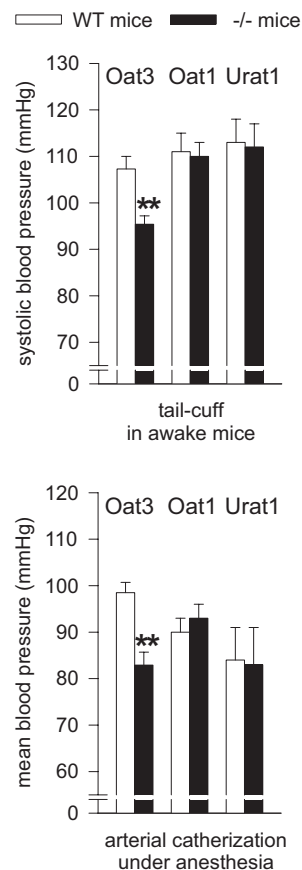
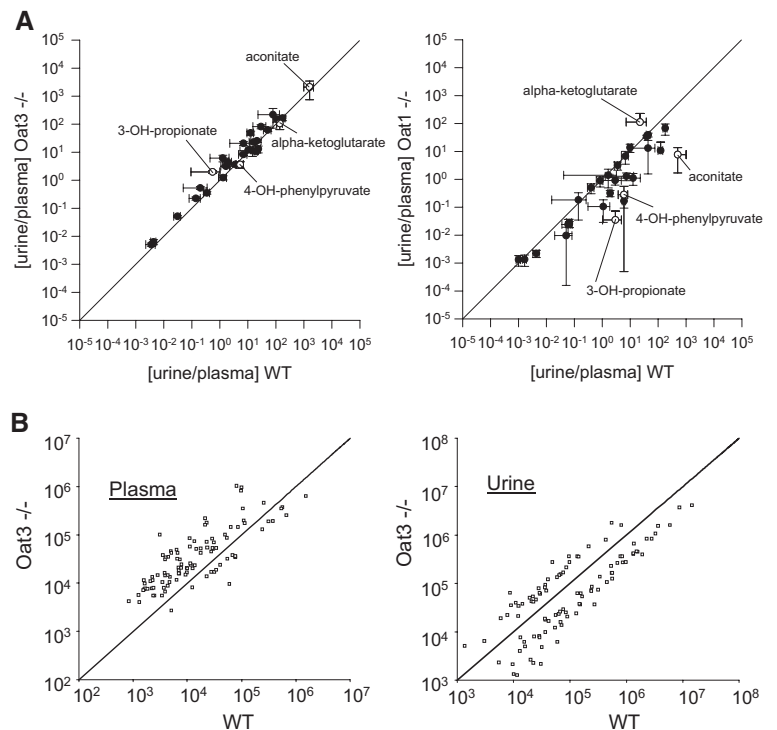


Figure 1. Low BP in Oat3 knockout mice. Systolic BP was determined by tail-cuff method in awake mice, or mice were anesthetized and mean arterial BP was directly measured via an intra-arterial catheter. Data are means \pm SEM of measurements in six to 20 mice of each genotype. BP measurements under anesthesia were previously reported for Oat1 knockout ($-/-$) mice.²⁸ Mean arterial pressure was significantly reduced in *Oat3*^{-/-} compared with WT mice (** $P < 0.01$) but not in mice lacking the related proximal tubular OAT Oat1 or *mUrat1* (*RST*), raising the possibility that an endogenous BP-regulating compound is transported by Oat3.

compound. We reasoned that such substrates should be increased in the plasma and possibly decreased in the urine of *Oat3*^{-/-} mice, owing to loss of Oat3-mediated tubular secretion. We initially performed targeted gas chromatography/mass-spectrometry (GC/MS) as described previously,²⁸ to determine the concentrations of approximately 30 of the most abundant small OA in the urine and plasma of WT and *Oat3*^{-/-} mice, including various Krebs cycle intermediates and fatty and amino acid metabolites. No significant differences between WT and null mice were noted (Figure 2A, left), indicating that this set of compounds, although including several putative novel endogenous Oat1 substrates (Figure 2A, right) does not seem to contain any authentic substrates of Oat3. Accordingly, we performed global (metabolomic) rather than targeted mass spectrometric analyses of *Oat3*^{-/-} plasma and urine, assaying the levels of several thousands of molecules

Figure 2. Detection of potential endogenous Oat3 substrates. (A) GC/MS analysis was performed to determine the levels of approximately 30 of the most abundant endogenous OA in plasma and urine. For each compound, the ratio of the concentration in urine (normalized to creatinine) to the concentration in plasma was calculated, representing a measure of the efficiency of renal excretion. Urine/plasma ratios of these compounds were largely unaffected in *Oat3*^{-/-} mice (left). In comparison, several compounds manifested markedly lower urine/plasma ratios (*i.e.*, lower apparent renal excretion) in *Oat1*^{-/-} compared with WT mice, indicating the involvement of Oat1 in their tubular secretion (data taken from reference²⁸). Notably, the urine/plasma ratio for α -ketoglutarate, which serves as a counterion for OA uptake, was greater in *Oat1*^{-/-} compared with WT mice but unaffected in *Oat3*^{-/-} mice. Data are means \pm SEM ($n = 4$ per genotype). (B) A global mass-spectrometric (metabolo-mic) analysis was performed to compare the plasma and urine metabolite composition of *Oat3*^{-/-} and WT mice. Data presented are the mean intensity values for the 100 compounds each in plasma and urine that were the most significantly different in intensity between *Oat3*^{-/-} and WT mice. The majority (81%) of the significantly varying compounds in plasma were increased in *Oat3*^{-/-} relative to WT, whereas the majority (64%) of those in urine were decreased in *Oat3*^{-/-} relative to WT, consistent in each case with diminished renal excretion of the corresponding compounds.



simultaneously. These global analyses revealed that the metabolite composition of plasma and urine of *Oat3*^{-/-} mice is indeed significantly different from that of WT mice, with 2% of the features in plasma and 14% of the features in urine significantly different in concentration between the genotypes (two-tailed *t* test, $P < 0.01$). Importantly, the observed differences were markedly skewed in the direction expected for endogenous substrates (*i.e.*, increased in concentration in plasma and decreased in urine in *Oat3*^{-/-} mice [Figure 2B]).

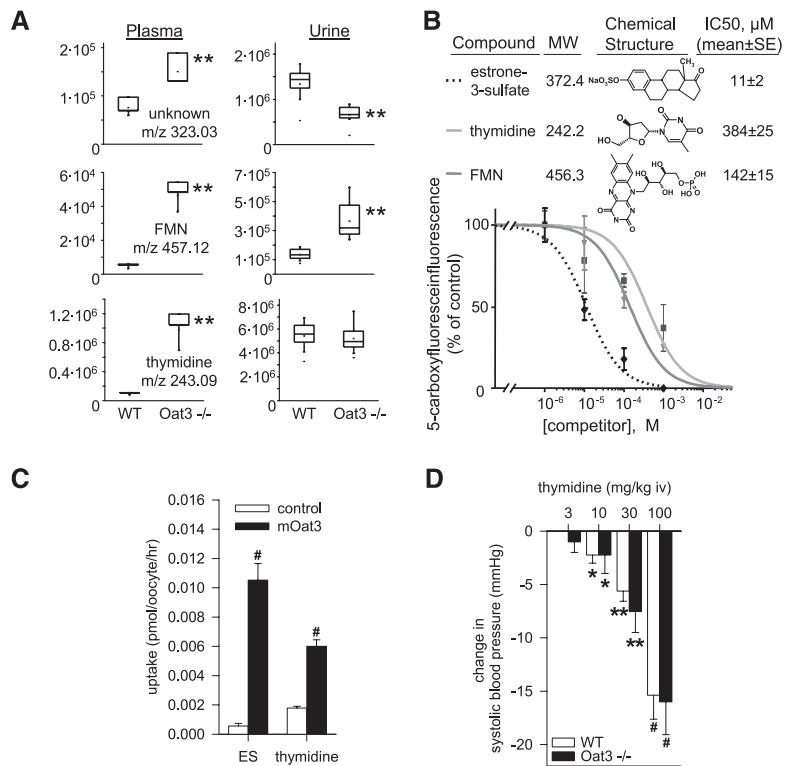
The most significantly varying features were analyzed further; however, although the masses of the corresponding compounds were determined to an accuracy of 8 ppm, only a few could be matched to known metabolites (*e.g.*, using the Metlin metabolite database). Plasma and urine intensity values in WT and *Oat3*^{-/-} mice for two of the identified compounds, flavin mononucleotide (FMN) and thymidine, are presented in Figure 3A, along with data for an unknown compound of *m/z* 323.03. We subsequently tested the identified compounds for interaction with Oat3 *in vitro* and found that both FMN and thymidine inhibited tracer uptake in Oat3-expressing oocytes in a concentration-dependent manner (Figure 3B), as would be expected in the case of genuine Oat3 substrates. Because further experiments (see next paragraph) pointed to a particular role of thymidine, we directly assessed Oat3-mediated transport of this compound. We found that labeled thymidine was taken up into Oat3-expressing oocytes to a much greater degree than into control oocytes (Figure 3C), indicating that Oat3 can mediate the transport of thymidine. We also assessed

thymidine handling by Oat1 both *in vitro* and *in vivo*. We determined that thymidine was not appreciably transported by Oat1 *in vitro* (Supplemental Figure 1A) and that plasma levels of thymidine were not elevated in the Oat1 knockout (Supplemental Figure 1B), indicating that thymidine does not undergo significant Oat1-mediated uptake/secretion *in vivo*.

Effect on BP of Administration of Endogenous Oat3 Substrates

Thymidine or FMN could be directly involved in BP regulation, and/or as apparent Oat3 substrates, they could competitively inhibit the transport of other substrates, including that of the putative BP-regulating substrate. Accordingly, exogenous administration of these compounds might reproduce the phenotype of decreased BP observed in the *Oat3*^{-/-} mice. FMN, thymidine, or vehicle (30 μ l of water) was given as an intravenous bolus in anesthetized WT mice while BP was continuously monitored. We observed that whereas FMN at dosages up to 100 mg/kg intravenously and vehicle application were ineffective (data not shown), thymidine induced a dosage-dependent reduction in BP (Figure 3D) without lowering heart rate (data not shown). The BP response peaked within approximately 30 s and lasted for 3 to 5 min. The lower potency of thymidine *versus* FMN for the inhibition of Oat3-mediated uptake (Figure 3B) suggested that lowering of BP by this compound might not have been primarily due to Oat3 inhibition. In accordance with this inference, a very similar BP response to thymidine was observed in *Oat3*^{-/-} mice (Figure 3D). Thus, the Oat3 substrate thymidine might accumulate in the

Figure 3. Identification of potential endogenous Oat3 substrates. (A) Box plots of intensity values for two of the identified compounds from the metabolomic analysis, FMN and thymidine, and of an unknown compound of *m/z* of 323.03. ***P* < 0.01. (B) Tracer uptake into Oat3-expressing oocytes was determined in the presence of the indicated concentrations of the putative substrates, thymidine and FMN, and of the well-characterized Oat3 substrate estrone-3-sulfate and in their absence (control). Data are means \pm SEM of measurements in four groups of four to five oocytes each, and the IC₅₀ values were calculated by curve-fitting the points using nonlinear regression. (C) Uptake of labeled thymidine (0.097 μ M) and ES (0.017 μ M) was determined in oocytes microinjected with Oat3 and in control (noninjected) oocytes. Data are means \pm SEM of measurements in five groups of four to eight oocytes each. Oat3-expressing oocytes manifested significantly greater uptake of both ES and thymidine than did control oocytes. #*P* < 0.001. (D) Intravenous application of thymidine induced a dosage-dependent reduction in BP in anesthetized WT and Oat3^{-/-} mice. Data are means \pm SEM in four mice. **P* < 0.05, ***P* < 0.01, #*P* < 0.001 versus vehicle.



plasma of mice lacking Oat3 and contribute to the lower BP in these mice.

Identification of Oat3 Inhibitors

Although thymidine and FMN might be insufficiently potent to inhibit Oat3 *in vivo* (see Figure 3B), more potent compounds might do so. Accordingly, we sought to identify other Oat3 inhibitors, testing the capacity for Oat3 inhibition of approximately 30 small OA of diverse chemical structures. Tested compounds encompassed fluorescein derivatives, steroid conjugates, nucleoside analog antivirals, and short-chain aliphatic dicarboxylates, among other small OA; data on the fluorescein derivatives and antivirals has previously been reported.³² Inhibition by each compound was determined as in the experiments presented in Figure 3B. The tested compounds varied widely in inhibitory potency, ranging from submicromolar to millimolar (Figure 4A). In general, the steroid conjugates were among the more potent of the compounds tested and the antivirals among those less potent

Reduced BP after Administration of the Most Potent Oat3 Inhibitors

We tested the effects on BP of a subset of the previously mentioned compounds: The well-characterized OAT inhibitor probenecid^{6,7,24,28,33} and the fluorescent OA eosin-Y, fluorescein, 5-carboxyfluorescein (5CF), and 6-carboxyfluorescein (the latter compounds were chosen because they allowed for fluorometric determination of inhibitor plasma levels). Approximately 50 to 100 mg/kg of each of these compounds was intravenously infused into mice under anesthesia as described already (resulting in initial plasma concentrations of approxi-

mately 2 to 5 mM in the case of the fluorescent compounds), followed by recording of any changes in BP. We found that administration of the two highest potency compounds (of the five tested), eosin-Y and probenecid (Figure 4B), but not of the three lower potency compounds (data not shown) consistently caused an acute decrease in BP. These results are consistent with our hypothesis that inhibition of Oat3 by sufficiently potent inhibitors can lower BP. Nevertheless, it should be noted that given the relatively high dosages used and that each of the tested compounds interacts with Oat1 with comparable potency,^{33a} nonspecific effects cannot be excluded.

Plasma Concentrations of Corticosterone, Adrenocorticotropic Hormone, Aldosterone, and Renin in Oat3 Knockout Mice

Given the high potency interaction of Oat3 with steroid conjugates demonstrated here (Figure 4A) as well as elsewhere (reviewed in reference⁶), we determined the concentrations of the BP-regulating adrenal steroids and of functionally related hormones in Oat3 knockout compared with WT plasma. We found lower BP in Oat3^{-/-} mice to be associated with greater plasma adrenocorticotropic hormone (ACTH) but unchanged corticosterone concentrations (Figure 5A). ACTH stimulation increased plasma corticosterone by a factor of approximately 2.4 in both WT and Oat3^{-/-} mice (Figure 5A), indicating intact acute ACTH-responsiveness in Oat3^{-/-} mice. These results argue against an essential role for Oat3 in corticosterone release from the adrenal gland and against a primary role for defects in corticosterone handling in causing the lower BP in Oat3^{-/-} mice.

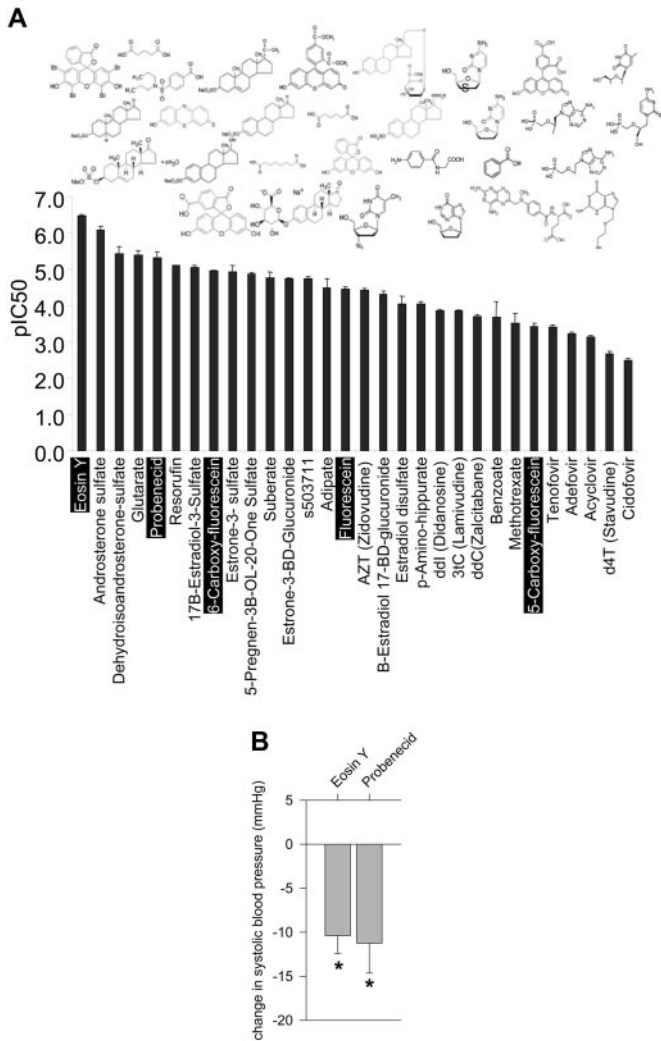


Figure 4. Reduced BP after administration of Oat3 inhibitors. (A) The inhibitory potency for Oat3 of each of approximately 30 OA was determined as in the experiments presented in Figure 3B. Among these, data on the fluorescein derivatives and antivirals has previously been reported.³² Data are means \pm SEM of determinations of pIC50 (the negative log of IC50; thus, higher values indicate greater inhibitory potency). The chemical structures of the tested compounds are depicted above their corresponding pIC50 values. (B) Selected compounds from Figure 4A (■) were intravenously infused into WT mice under anesthesia. These comprised the well-characterized OAT inhibitor probenecid and the fluorescent OA eosin-Y, fluorescein, 5CF, and 6- carboxyfluorescein, each administered at a dosage of 50 to 100 mg/kg. A consistent decrease in BP was noted after the infusion of the two higher potency compounds, eosin-Y and probenecid, but not after infusion of the three lower potency compounds (data not shown). Data are means \pm SEM of measurements in three to four independent experiments each. **P* < 0.05 versus vehicle.

Oat3^{-/-} mice had modestly greater plasma renin concentrations (Figure 5A) and greater renal expression of renin versus WT mice (data not shown). To test for a potential role of Oat3 in macula densa stimulation of renin secretion,¹⁶ we per-

formed experiments with furosemide. Furosemide (40 mg/kg intraperitoneally) stimulates renal renin secretion by inhibiting the sensing of the luminal NaCl concentration at the macula densa,³⁴ thereby increasing macula densa release of prostaglandin E₂ (PGE₂) across the basolateral membrane,³⁵ a mechanism that may involve Oat3.¹⁶ On the basis of our previous studies,²⁶ the applied dosage of furosemide is expected to induce similar maximal inhibition of NaCl reabsorption in thick ascending limb and macula densa in both genotypes; however, furosemide in fact increased renin levels to a greater degree in *Oat3*^{-/-} than in WT mice (Figure 5A), indicating enhanced rather than reduced PGE₂ signaling in the knockout; as further outlined in the Discussion, this may reflect impaired PGE₂ reuptake in the knockouts.

In addition to increased renin, *Oat3*^{-/-} mice manifested modestly greater plasma aldosterone (Figure 5A), which is expected to help stabilize BP by enhancing renal NaCl reabsorption. To test the efficiency of that system in *Oat3*^{-/-} mice, we assessed the response to a low NaCl diet given for 7 d. Under basal conditions, no significant differences were observed between WT and *Oat3*^{-/-} mice in body weight and food or fluid intake (data not shown). A low NaCl diet, however, induced a significantly greater loss of body weight in *Oat3*^{-/-} compared with WT mice (Figure 5B), which could not be explained by lower food intake. This diet increased plasma renin to similar levels in both genotypes, but aldosterone upregulation was impaired in *Oat3*^{-/-} mice (Figure 5A). It is possible that this impaired upregulation led to salt and fluid loss, lowering body weight and inducing thirst and increased fluid intake, as shown in Figure 5B.

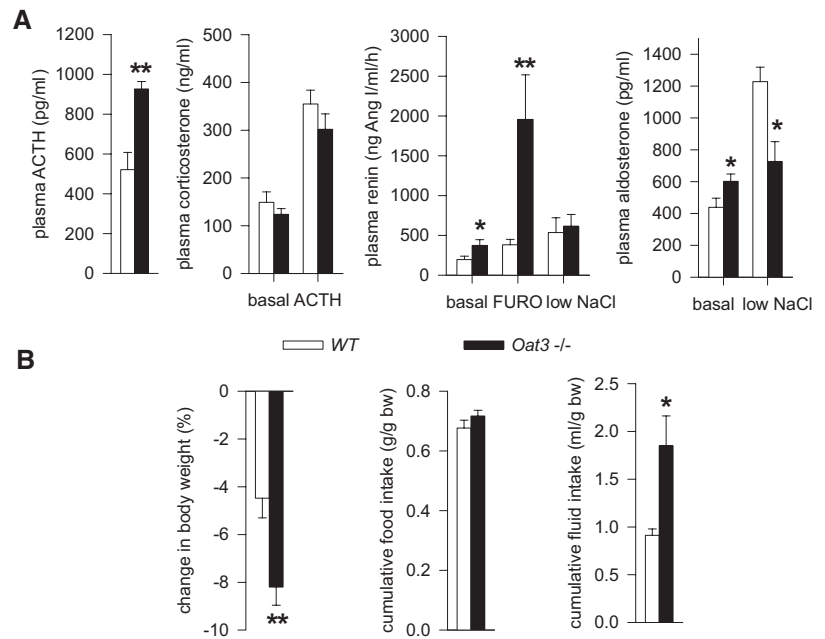
Interaction of Aldosterone and Its Potential Precursors with Oat3 in *Xenopus Oocytes*

Given that Oat3 is expressed in adrenal tissue and is able to mediate transport of cortisol,³⁶ aldosterone dysregulation in Oat3 knockouts might be due to loss of interaction of aldosterone or related compounds with Oat3. We found aldosterone and three of its potential precursors, progesterone, corticosterone, and desoxycorticosterone,^{37,38} to each dosage-dependently inhibit Oat3-mediated tracer uptake *in vitro*, with median inhibition concentration (IC50) values of 12, 29, 10, and 9 μ M, respectively (Figure 6). Although these affinities are comparable to that of the prototypical Oat3 substrate estrone-3-sulfate (Figure 3B), they are much greater than physiologic free plasma concentrations of these compounds (this study)^{39,40} (although local concentrations around the adrenal glomerulosa cells might be considerably higher). Thus, the physiologic significance of these interactions is uncertain.

DISCUSSION

Recent studies localized Oat3 to the basolateral membrane of macula densa cells, and it was proposed that Oat3 at that site might contribute to the local release or reuptake of PGE₂.^{16,41} Basolateral release of PGE₂ mediates the stimulation of renin

Figure 5. Plasma vasoactive hormones and the response to low-salt diet in *Oat3* knockout mice. (A) Lower BP in *Oat3*^{-/-} mice was associated with increased plasma concentrations of ACTH, renin, and aldosterone, whereas plasma corticosterone concentrations were not different. The ACTH-induced increase in plasma corticosterone was unaffected in the knockouts, whereas the furosemide (FURO)-induced renin response was enhanced, and the low-NaCl diet-induced aldosterone increase was blunted ($n = 6$ to 9 per genotype). (B) The blunted aldosterone response to a low-NaCl diet in *Oat3*^{-/-} compared with WT mice was associated with greater body weight loss and increased fluid intake but similar food intake ($n = 6$ per genotype). Data are means \pm SEM. * $P < 0.05$, ** $P < 0.01$ versus WT.



release (in turn leading to aldosterone activation) that occurs in response to low salt concentrations at the macula densa or to inhibition of macula densa transport by furosemide.³⁵ We find that the renin response to furosemide is enhanced in *Oat3*^{-/-} mice, suggesting that *Oat3* might contribute to the reuptake of PGE₂ (loss of such reuptake might also have contributed to greater basal renin levels in *Oat3*^{-/-} mice, helping ameliorate hypotension). Of note, the aldosterone response to salt deprivation was blunted in *Oat3*^{-/-} mice; however, aldosterone dysregulation cannot explain the observed hypotension under basal conditions because basal plasma aldosterone concentrations were *increased* in mice lacking *Oat3*.

The aforementioned results raise the possibility that *Oat3* mediates the transport of other endogenous regulators of BP. To test this possibility, we examined the effects of two of the

compounds identified *via* metabolomic analyses and subsequent transport assays as potential endogenous *Oat3* substrates: Thymidine and FMN. Whereas FMN displayed no effect, thymidine exhibited a consistent dosage-dependent lowering of BP. Normal serum concentrations of thymidine are approximately 1 μ M, and there is a thymidine pool that is in rapid equilibrium with blood thymidine and that is at least 10 times larger than the pool of extracellular thymidine.⁴² It is difficult to know what the local concentrations of the exogenously administered thymidine were; however, assuming it was rapidly distributed into the aforementioned pools, a concentration of 20 μ M would be achieved at 10 mg/kg injection, where the BP effect was first noted. Within a roughly 10-fold higher concentration, an effect comparable to that observed in the *Oat3* knockout was observed.

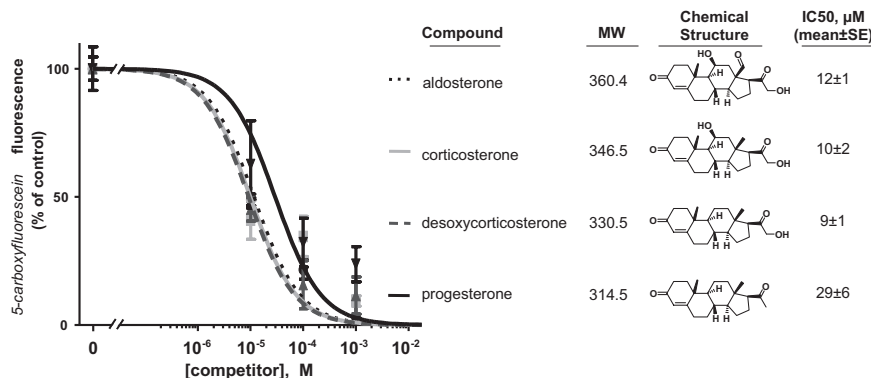


Figure 6. Interaction of aldosterone and its potential precursors, progesterone, corticosterone, and desoxycorticosterone, with *Oat3*. Uptake of 5-CF in m*Oat3*-expressing *Xenopus* oocytes was determined in the presence of the indicated concentrations of the steroid hormones to calculate the respective IC₅₀ values. Each compound caused a dosage-dependent decrease in uptake, consistent with competitive inhibition of uptake of the fluorescent tracer. Data are presented as percentage of control uptake and are the means \pm SEM of measurements in four groups of four to five oocytes each. IC₅₀ values were determined by curve-fitting the points using nonlinear regression.

Although a number of endogenous OA compounds transported by Oat3 may be involved in regulating BP, at the very least, these results suggest that some of them can, perhaps in concert, act to regulate BP under physiologic conditions. This also suggests that inhibitors of Oat3, regardless of whether they are actual substrates for the transporter, might lower BP. This could lead to a novel approach to the design of antihypertensive agents. We therefore tested a set of Oat3 inhibitors (selected from a larger screen) for effects on BP, finding that administration of two of the five tested compounds resulted in lowering of BP.

Together, the data suggest that (1) Oat3 regulates BP, (2) Oat3 affects the regulation of plasma aldosterone (although this cannot explain the hypotensive phenotype of Oat3-null mice), (3) at least some endogenous compounds that accumulate in the absence of Oat3 are substrates with the potential to regulate BP, and (4) administration of sufficiently potent inhibitors might result in lowering of BP. Although much more work needs to be done, the data suggest novel concepts regarding the physiologic role of Oat3 substrates and raise the possibilities that Oat3 inhibitors might be of use in the treatment of hypertension and that polymorphisms in human OAT3 might contribute to variability in the propensity for hypertension.

CONCISE METHODS

Animals

Oat3^{-/-} mice²⁴ were back-crossed to C57BL/6J mice for eight generations. Heterozygous mice from the last back-cross were bred to each other to generate gene knockout and WT mice. All of the animals used in the experiments described descended from those mice. Mice were genotyped by PCR as described previously.²⁴ 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.

Materials

[³H]-estrone sulfate (ES) and [³H]-thymidine were purchased from Perkin-Elmer Life Sciences (Boston, MA), and 5CF, a fluorescent OA transported by mOat3³² and other OA test compounds (all >98% pure and in the L-enantiomer) were purchased from Sigma-Aldrich (St. Louis, MO).

BP Measurements

Mice were anesthetized for terminal experiments with 100 mg/kg inactin intraperitoneally and 100 mg/kg ketamine intramuscularly, the jugular vein was cannulated for infusion of maintenance fluids and test compounds, and the femoral artery was cannulated for BP and heart rate measurement as described previously.^{43,44} In separate experiments, BP and heart rate in unanesthetized mice were determined by the tail-cuff method in trained awake mice as described previously.^{44–46}

Urine and Plasma Samples

Mice were placed in metabolic cages (Tecniplast, Hohenpeissenberg, Germany) with free access to tap water and standard diet (0.44% Na⁺,

0.97% K⁺) for quantitative collection of urine, immediately followed by collection of plasma *via* puncture of the retrobulbar plexus as described previously.⁴³

GC/MS

GC/MS was performed as described previously.²⁸ Briefly, plasma and urine samples (100 μ l or volumes equivalent to 0.25 to 1.00 μ mol of creatinine, respectively) were reacted with pentafluoro-benzylhydroxylamine to form oximes of ketones, oxo-acids, and aldehydes. The reaction products were lyophilized and extracted in 42% t-amyl alcohol:chloroform over a column of silicic acid. The dried eluate was reacted with BSTFA/TRISIL to form trimethylsilyl derivatives. The latter were injected (0.5 to 1.0 μ l) on a bonded phase (DB5) capillary column (30 m \times 0.25 mm) in an Agilent 6890/5973 GC/MS. Electron impact mass spectra were obtained in scan mode (50 to 650 amu at 2.4 cycles/s). The species were quantified using calibrated response curves of selected ions and 4-nitrophenol and 2-oxocaproic acid as internal standards.

Global Metabolomics

Urine and plasma samples were extracted in methanol and lyophilized, and the equivalents of 0.4 to 1.4 μ l of extracted urine (after normalization to the previously measured concentrations of creatinine) or 1 μ l of extracted plasma were applied to a capillary reverse-phase column with dimensions 150 \times 0.3 mm (diameter; Zorbax SB-300, Agilent) at a flow rate of 4 μ l/min. Buffer A was H₂O + 0.1% formic acid, and Buffer B was acetonitrile with 0.1% formic acid. Plasma samples were eluted with a gradient from 5 to 95% B over 45 min; for urine samples, the gradient was 5% B from 0 to 10 min then 5 to 95% from 10 to 55 min, then held at 95% to 60 min. The column was interfaced to an electrospray ionization time-of-flight mass spectrometer (Agilent). Data were collected from approximately 100 to 1000 m/z in continuum mode and converted from the instrument format (.wiff) to the common format (.cdf). Data were analyzed using the analysis and nonlinear alignment program XCMS,⁴⁷ in which the integrated intensities of all observed ions were compared between the *Oat3*^{-/-} and WT groups. These procedures resulted in the detection of approximately 4000 features in urine and approximately 5000 in plasma, which would include any metabolites with m/z values between 100 and 1000 that ionize under positive electrospray conditions and were within the detection limit of the instrument. The data were then analyzed using the recently developed software package XCMS, which performs a nonlinear alignment of the chromatograms and integration of the intensities for each ion.

Xenopus Oocyte Transport Experiments

Xenopus oocyte assays were performed as described previously.²⁸ Briefly, capped cRNA was generated *via in vitro* transcription from linearized plasmid DNA, mOat3 (Image clone ID 4239544), or mOat1 (Image clone ID 4163278), using mMessage mMachine *in vitro* transcription kit (Ambion, Austin, TX). Stage V and VI *Xenopus* oocytes were isolated, maintained in Barth's growth medium, injected with approximately 20 nl/oocyte cRNA solution (1 μ g/ μ l), and allowed to mature for 3 d after injection. Oat3-expressing and control noninjected oocytes were then incubated in Barth's buffer for 1 h at 25°C

with Oat3 tracer substrate (50 μM 5CF, 0.017 μM ^3H -ES, or 0.097 μM ^3H -thymidine) in the presence of various concentrations of test compounds or in their absence. Transport was terminated by washes in ice-cold Barth's buffer, and the tracer content of groups of four to five oocytes was determined by fluorometry or scintillation counting in the case of experiments using 5CF and the radiolabeled tracers, respectively. The background uptake of tracer was determined in noninjected oocytes and subtracted from that in Oat3-injected oocytes to calculate the Oat3-mediated component of uptake. The IC₅₀ were determined by nonlinear regression using Prism software 5.0 (GraphPad, San Diego, CA).

Plasma Concentrations of Aldosterone, Renin, Corticosterone and ACTH

Mice were placed under short-term isoflurane anesthesia, and blood was taken from the retrobulbar plexus for determination of hematocrit and basal plasma concentrations of aldosterone (Diagnostic Systems Laboratories, Webster, TX; cross-reactivity with corticosterone 0.02%), renin (see below in this paragraph), corticosterone, and ACTH (both MP Biomedicals, Orangeburg, NY). To determine a potential role of Oat3 in corticosterone release from the adrenal gland,³⁶ we performed an ACTH stimulation test. To this end, plasma corticosterone was determined 45 min after intraperitoneal application of 1 U of 1-24 ACTH (Cortrosyn®; Organon, Roseland, NJ).⁴⁸ To determine a potential role of Oat3 in macula densa stimulation of renin secretion,¹⁶ we determined plasma renin concentration 45 min after intraperitoneal application of furosemide (40 mg/kg).³⁴ In addition, the response in body weight and food and fluid intake as well as in plasma concentrations of renin and aldosterone to a low-NaCl diet for 8 d was assessed. Plasma renin concentration was measured by RIA (GammaCoat Plasma Renin Activity, CA-1533; DiaSorin, Stillwater, MN) as the generation of angiotensin I after addition of excess angiotensinogen substrate from bilaterally nephrectomized rats.⁴⁹ Concentrations of Na⁺ and K⁺ in plasma and urine were determined using a flame photometer (Cole-Parmer Instrument Co., Vernon Hills, IL).

Statistical Analysis

Data are presented as means \pm SEM. Statistical differences between gene-knockout and WT mice were analyzed by the unpaired *t* test. *P* < 0.05 was considered to be statistically significant.

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DISCLOSURES

None.

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