Tubuloglomerular feedback and renal function in mice with targeted deletion of the type 1 equilibrative nucleoside transporter

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Li L, Mizel D, Huang Y, Eiiser C, Hoerl M, Thiel M, Schnermann J. Tubuloglomerular feedback and renal function in mice with targeted deletion of the type 1 equilibrative nucleoside transporter. Am J Physiol Renal Physiol 304: F382–F389, 2013. First published December 26, 2012; doi:10.1152/ajprenal.00581.2012.—A1 adenosine receptors (A1AR) are required for the modulation of afferent arteriolar tone by changes in luminal NaCl concentration implying that extracellular adenosine concentrations need to change in synchrony with NaCl. The present experiments were performed in mice with a null mutation in the gene for the major equilibrative nucleoside transporter ENT1 to test whether interference with adenosine disposition by cellular uptake of adenosine may modify TGF characteristics. Responses of stop flow pressure (Psf) to maximum flow stimulation were measured in mice with either C57Bl/6 or SWR/J genetic backgrounds. Maximum flow stimulation reduced Psf in ENT1−/− compared with wild-type (WT) mice by 1.6 ± 0.4 mmHg (n = 28) and 5.8 ± 1.1 mmHg (n = 17; P < 0.001) in C57Bl/6 and by 1.4 ± 0.4 mmHg (n = 15) and 9 ± 1.5 mmHg (n = 9; P < 0.001) in SWR/J. Plasma concentrations of adenosine and inosine were markedly higher in ENT1−/− than WT mice (ado: 1,179 ± 78 and 225 ± 48 pmol/ml; ino: 179 ± 24 and 47.5 ± 9 pmol/ml). Renal mRNA expressions of the four adenosine receptors, ENT2, and adenosine deaminase were not significantly different between WT and ENT1−/− mice. No significant differences of glomerular filtration rate or mean arterial blood pressure were found while plasma renin concentration, and heart rates were significantly lower in ENT1−/− animals. In conclusion, TGF responsiveness is significantly attenuated in the absence of ENT1, pointing to a role of nucleoside transport in the NaCl-synchronous changes of extracellular adenosine levels in the juxtaglomerular apparatus interstitium.

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quence of reducing the dynamics of the acute vascular response to increased macula densa NaCl.

METHODS

Animals. Experiments were performed in mice with null mutations of the gene for the ENT1 originally generated by Choi et al. (4). Mice used in this study had been bred into a C57Bl/6 background for more than 10 generations and into a SWR/J background for more than 6 generations at the National Institutes of Health animal facility. Mice were genotyped by PCR of tail DNA using the following primers: 5’-AAGTGGTGTCATCACAAGTCCC-3’, and 5’-ATGAGATC-CACAACTT GGTTCCTG-3’ as originally described (4). Animals ranged in weight from 20–31 g and in age from 3–5 mo. Animals were kept on a standard diet (NaCl content 0.2%) and tap water. Animal care and experimentation were approved and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

mRNA expression studies. Kneys were removed from anesthetized mice. Twenty milligrams of sliced kidney cortex (or in some experiments of cortex, outer and inner medulla) were immediately soaked in 600 μl of lysis buffer (Qiagen kit; Qiagen, Valencia, CA) and homogenized. The homogenates cleaned by centrifugation were used to purify total RNAs by using RNAeasy Mini kit (Qiagen, Valencia, CA). One microgram of RNA was synthesized to 20 μl of cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). One microliter of cDNA synthesized from 50 ng of RNA was used to detect adenosine receptor mRNA expression by real-time PCR. Primers were purchased from the Taqman gene expression inventory (Applied Biosystems, Foster City, CA) under the following codes: A1AR Mm01308023_m1, A2aAR Mm00802075_m1, A2bAR Mm00839292_m1, A3AR Mm00802076_m1, ENT1 Mm01270582_m1, ENT2 Mm00432817_m1, adenosine deaminase Mm00545720_m1, and hypoxanthine phosphoribosyltransferase 1 (HPRT1) Mm03024075_m1. HPRT1 RNA was used as endogenous control throughout. The difference in expression of target cDNA was calculated using the comparative cycle threshold (CT) method.

Measurement of adenosine and inosine in plasma. ENT1 knockout mice (C57Bl/6; n = 6) and background-matched wild-type (WT) mice (n = 5) were anesthetized with isoflurane (induction: 4%, maintenance: 2%) delivered with 100% oxygen over a nose mask. Immediately after sufficient anesthesia was achieved blood was drawn from the carotid artery into a syringe prefilled with a stop solution to prevent metabolism of adenosine (19). The ratio of blood to stop solution was 1:1. After centrifugation, the plasma was separated, de-proteinated with 70% perchloric acid, and shock-frozen in liquid nitrogen. The denatured proteins were later removed by centrifugation, and the sample was neutralized by an equal amount of KOH. The concentrations of adenosine and inosine were measured using a dual column switching high-performance liquid affinity chromatography (HPLAC)/reversed-phase HPLC technique (11).

Micropuncture experiments. Mice were anesthetized with 100 mg/kg thiobutabarbital (inactin) intraperitoneally and 100 mg/kg ketamine subcutaneously. Body temperature was maintained at 37.5°C by placing the animals on an operating table with a servo-controlled heating plate. The trachea was cannulated, and a stream of 100% oxygen was blown towards the tracheal tube throughout the experiment. The left carotid artery was catheterized with hand-drawn polyethylene tubing for continuous measurement of arterial blood pressure and blood withdrawal. A catheter connected to an infusion pump was inserted into the right jugular vein for an intravenous maintenance infusion of saline at 300 μl/h. The left kidney was approached from a flank incision, freed of fat and tissue connections, and placed in a lucite holding cup. Measurements of stop flow pressure (Psf) during perfusion of loop of Henle were done as described previously (32, 37). When Psf had stabilized, the perfusion rate of the loop of Henle was increased to 30 nl/min and maximum Psf responses were determined. The perfusion rate was then reduced to 0 nl/min and maintained until steady states were achieved at each flow rate. Two such responses were determined successively in each nephron. The perfusion fluid contained the following (in mM/l) 136 NaCl, 4 NaHCO3, 4 KCl, 2 CaCl2, 7.5 urea, and 100 mg/100 ml FD&C green (Keystone, Bellefonte, PA).

Glomerular filtration rate. The glomerular filtration rate (GFR) was measured by single injection FITC inulin clearance as described by Qi and colleagues (8, 27) modified to minimize blood collections. During brief isoflurane anesthesia from which the mice recovered within ~20 s, FITC-sinistrin dissolved in saline at a concentration of 5 g% was injected at 3.74 μl/g body wt into the retroorbital plexus (FITC-sinistrin was kindly supplied by Dr. Norbert Grett, Medical Faculty Mannheim, Germany). At 3, 7, 10, 15, 35, 55, and 75 min, mice were placed in a restrainer, and ~4 μl blood were collected into heparinized 5-μl microcaps by nicking the tail vein (Drummond Scientific, Broomall, PA). One microliter of plasma was diluted in 9 μl of 500 mmol HEPES buffer (pH 7.4), and fluorescence was determined using a NanoDrop ND-3300 fluorospectrometer. A standard curve was generated by determining fluorescence in 1 μl of 5%-FITC-sinistrin diluted 1:50, 1:100, 1:500, and 1:1,000 in 500 mmol HEPES buffer. Fluorescence was determined in 1.7 μl in a Nanodrop-ND-3300 spectrometer (Nanodrop Technologies, Wilmington, DE). GFR was calculated using a two-compartment model of two-phase exponential decay (27).

Plasma renin concentration. Blood was collected from conscious mice by puncture of the mandibular vein and collection of ~20 μl of the emerging blood into an EDTA-containing microhematocrit tube. Plasma renin concentration was determined by incubating plasma in the presence of an excess of rat renin substrate and measuring the generated angiotensin I with a RIA kit (Diasorin, Stillwater, MN) as described in detail earlier (3).

Blood pressure and heart rate. Arterial blood pressure and heart rate were measured with radiotransmitters (model TA11PA-C10; Data Sciences International, St. Paul, MN) implanted during ketamine and xylazine anesthesia (90 and 10 mg/kg, respectively) in the carotid artery as previously described (21). Seven to ten days after surgery data sampling was done for 10 s every 2 min over a period of at least 5 days. Radiosignals were processed using a model RPC-1 receiver, a 20-channel data exchange matrix, APR-1 ambient pressure monitor, and a Data Quest ART Silver 2.3 acquisition system. The recording room was maintained at 21–22 °C with a 12:12-h light-dark cycle.

Fig. 1. Regional distribution of equilibrative nucleoside transporter 1 (ENT1) mRNA in kidneys of male C57Bl/6 wild-type (WT) mice. Values are means ± SE, and measurements in outer medulla (OM) and inner medulla (IM) are expressed as percentage of kidney cortex. *P < 0.05, by one-way ANOVA with Bonferroni post hoc test for comparison with cortex.
RESULTS

*ENT mRNA expression.* As shown in Fig. 1, measurements of intrarenal distribution of ENT1 expression showed a corticomedullary expression gradient with highest expression levels in the inner medulla. Expression levels of ENT1 and ENT2 mRNA are shown in Fig. 2. ENT1 expression in the kidneys of female WT mice was markedly higher than expression of ENT2. ENT1 deletion reduced ENT 1 expression to undetermined levels as expected, but it did not cause significant changes in ENT2 expression.

Adenosine receptor mRNA and circulating adenosine. Measurements of adenosine receptor mRNA by quantitative RT-PCR are summarized in Figs. 3 and 4. Renal expression of all adenosine receptors was higher in female than in male mice with statistical significance being reached for A1AR and A2bAR, the receptor subtypes with the highest renal expression levels (Fig. 3). The main conclusion in the context of the present studies is that the impaired nucleoside uptake in ENT1⁻/⁻ mice is not associated with significant changes in the renal expression of any of the adenosine receptor subtypes (Fig. 4). Likewise, adenosine deaminase mRNA expression was not found to be different in kidneys of WT or ENT1⁻/⁻ mice while it was significantly downregulated in the brain (Fig. 5). As shown in Fig. 6, adenosine levels in the plasma of ENT1-deficient mice were significantly higher than WT mice by a factor of ~6. There was also a significantly higher plasma inosine concentration by a factor of ~5 in the ENT1-deficient mice.

*TGF.* Measurements of Psf responses to a saturating increase of loop of Henle flow rate (0–30 nl/min) were done in male WT and ENT1⁻/⁻ mice on both C57Bl/6 (WT: 7 mice, ENT1⁻/⁻: 8 mice) and SWR/J backgrounds (WT: 3 mice, ENT1⁻/⁻: 5 mice). Data are summarized in Fig. 7. In the C57Bl/6 strains Psf fell from 33.7 ± 1.5 to 27.9 ± 1.6 mmHg in WT mice (n = 17) and from 37.5 ± 2 to 35.8 ± 1.9 mmHg in the ENT1⁻/⁻ mice (n = 28). Mean arterial blood pressure during micropuncture averaged 87 ± 3.6 mmHg in WT and 85 ± 3 mmHg in ENT1⁻/⁻ mice. An attenuated TGF response was also observed in the SWR/J strain in which Psf fell from 39.9 ± 1.9 to 30.9 ± 2 mmHg in WT (n = 9) and from 30.2 ± 1.5 to 28.9 ± 1.6 mmHg in ENT1⁻/⁻ animals (n = 15). In these mice, mean arterial pressure was 89.6 ± 5.5 mmHg in WT and 93.5 ± 4.6 mmHg in ENT1⁻/⁻ mice. As shown in Fig. 8, the average magnitude of the on-response was 5.8 ± 1.1 mmHg in WT and 1.6 ± 0.4 mmHg in ENT1⁻/⁻ of the C57Bl/6 back-
ground \( (P < 0.001) \), and 9 ± 1.5 mmHg in WT and 1.4 ± 0.4 mmHg in ENT1/−/− of the SWR/J background \( (P < 0.001) \). The difference in TGF responses between C57Bl/6 and SWR/J WT mice was not significant \( (P = 0.12) \).

**Renal function and plasma renin.** Measurements of GFR in conscious female C57Bl/6 WT and ENT1/−/− revealed no significant differences between genotypes \( (345.8 \pm 26.5 \text{ vs. } 347.5 \pm 54 \text{ ml/min}; n = 8 \text{ and } 11, \text{ respectively}) \). However, osmolarity and chloride concentrations of spontaneously voided urine were significantly lower in ENT1-deficient animals \( \text{(Fig. 9)} \). Although we did not rigorously explore the causes for the reduced urine osmolarity, we observed in a subset of mice that 24 h of water withdrawal markedly augmented urine osmolarity in ENT1/−/− mice virtually eliminating the difference between genotypes. The decrease in urinary concentration in the ENT1/−/− mice was accompanied by a significant increase in water intake by 25\% \( \text{(Fig. 9)} \). Plasma renin concentration measured with the standard radioactive assay was significantly lower in ENT1-deficient mice \( \text{(Fig. 10)} \).

**Blood pressure and heart rate.** Telemetric measurements in C57Bl/6 WT (4 male and 5 female) and ENT1/−/− mice (4 male and 6 female) are summarized in Fig. 11. Since there were no discernible gender differences data from male and female were combined. While mean arterial blood pressure did not reveal significant differences between genotypes, heart rates were significantly lower during both day and night time periods. As shown in Fig. 12, daily variations of blood pressure and heart rate indicate that circadian rhythms were normal and not different between genotypes although heart rates cycled at a lower level in the ENT1/−/− animals.

**DISCUSSION**

The present experiments were performed with the aim of investigating the effects of chronic deficiency of ENT1-mediated nucleoside transport on the regulation of glomerular arteriolar tone by changes in tubular NaCl concentration in the juxtaglomerular region of the nephron. The relevance of this question lies in the well-recognized role of adenosine as a vascular mediator of this regulatory pathway and the possibility that transmembrane transport of adenosine may be part of the mechanisms that allow mediator concentrations in the juxtaglomerular interstitium to track changes in luminal NaCl concentration. Our data show that TGF responses are signifi-
cantly diminished in mice with a null mutation in ENT1, the major equilibrative nucleoside transporter in the kidney.

Nucleoside transport across cell membranes is mediated by a family of membrane proteins that act either as facilitative carriers or as Na-dependent cotransporters (15). Facilitation of transport by ENT proteins is a downhill movement according to prevailing concentration gradients thereby permitting equilibration between intra- and extracellular levels of nucleosides (1). The main equilibrative nucleoside transporter ENT1 is widely expressed across tissues and species, and it is found in the kidney in both tubular and extratubular locations including glomeruli and vascular smooth muscle and endothelial cells (5, 9). The present studies show some prevalence of medullary compared with cortical ENT1 expression, and this may be due to ENT1 expression in collecting ducts and thick ascending limbs as previously reported (5). ENT2 expression in the kidney was found to be rather low comparatively, and its abundance was not affected by deletion of ENT1. The prevalence of ENT1 in renal nucleoside transport has been demonstrated previously in isolated tubule preparations that showed a marked reduction of adenosine uptake in tissue from ENT1−/− mice while uptake in tissue from ENT2−/− mice was not different from controls (10). Thus although it is conceivable that ENT2 contributes to adenosine release, these results indicate that this effect is unlikely to be different between WT and ENT1−/− animals.

The clear increase in circulating adenosine and inosine levels in ENT1-deficient mice indicates that the bulk of extracellular adenosine is derived from breakdown of released adenine nucleotides. An increase of plasma adenosine levels has previously been observed in ENT1−/− mice (28) as well as...
in humans or rabbits treated with the ENT inhibitors dipyridamole or dilazep (18, 33). The important question of whether an increase of extracellular adenosine through nucleotide metabolism also occurs in the JGA interstitium cannot be answered directly. Nevertheless, evidence for the release of ATP across the basal membranes of macula densa cells and functional observations supporting a role of nucleotidases in the adenosine-dependent TGF response would appear to support this notion. Furthermore, recent in vivo observations with fluorescent markers are consistent with the proposal that afferent arterioles close to the glomerulus are permeable and that this may support substance transfer from plasma to the JGA interstitium by convective fluid flow (29). Direct activation of A1AR in JG cells by elevated adenosine may be responsible for the inhibition of renin secretion and the decrease of plasma renin concentration observed in ENT1−/− mice. A consequence of A1AR activation by elevated levels of adenosine outside the kidney may be the fall in heart rate in ENT1-deficient mice that was found despite a small reduction in arterial blood pressure. Studies in A1AR-deficient mice have suggested that this may reflect a direct effect of adenosine on cardiac pacemaker activity (38). On the other hand, an increase in heart rate without changes in arterial blood pressure has recently also been observed in mice with selective deletion of A1AR in neuronal cells induced by nestin-driven cre-recombinase indicating that inhibition of central nervous cardiovascular pathways may contribute to the heart rate effect (16).

The reasons for the reduction of TGF responsiveness by chronic ENT1 deletion are not entirely clear. Assuming that ENT1 deficiency does indeed increase JGA adenosine levels, one would have to conclude that a chronic elevation of local adenosine levels renders the generation of a TGF signal less efficient. It is possible that persistently high adenosine concentrations lead to A1AR receptor desensitization and subsequent nonresponsiveness. Receptor desensitization is a characteristic of most G protein-coupled receptors including A1AR although expressed native or recombinant A1ARs desensitize relatively slowly, over a time frame of hours to days rather than minutes (25, 26). Desensitized A1AR in ENT1−/− mice could explain the absence of changes in GFR that have previously been reported to result from an acute infusion of adenosine (24). In addition, we have demonstrated earlier that a number of vaso-dilators reduce TGF responses in an apparently nonspecific fashion (30, 31). While direct evidence for a reduced renal vascular resistance in the ENT1-deficient mice is lacking, it is conceivable that the increased adenosine levels exert a dilatory influence that might reduce TGF efficiency nonspecifically. Administration of dilazep has been noted to cause a lasting decrease of renal blood flow accompanied by vasodilatation of both afferent and efferent arterioles, and these changes were prevented by inhibition of adenosine receptors (18, 39). Vasodilatation is also the net effect of prolonged elevations of circulating adenosine levels although it is not clear whether the distal afferent arteriolar TGF effector site participates in this relaxation (12). Lastly, it is appropriate to consider the original proposal that has linked an increased luminal NaCl concentration and the subsequently enhanced NaCl transport with increased cellular adenosine levels since transport-dependent increases in energy expenditure would lead to augmented ATP utilization and adenosine formation (22, 23). In this proposal, ENT-mediated nucleoside transport from intra- to extracellular space would obviously play a central part that could not be filled in the ENT1-deficient mice. This possibility cannot be refuted definitively without measurements of adenosine in the juxtaglomerular interstitium. The direct evidence in support of a role of extracellular adenosine formation by ATPases and 5′-nucleotidase would seem to argue against this interesting hypothesis although the isolated absence of either CD39 or CD73 did not fully eliminate TGF responsiveness (3, 13, 20). Thus a transient reversal of the transmembrane adenosine flux from the NaCl activated macula densa cells could potentially contribute to local extracellular adenosine levels. A comparable example may be the recent evidence showing that the increase of extracellular adenosine during enhanced neuronal activity is the result of neuronal adenosine release rather than ATP dephosphorylation (17). Independent of the exact causes for the reduced TGF responsiveness the present findings suggest that ENT1 expression levels may contribute to setting TGF efficiency. For example, the downregulation of ENT1 by hypoxia-inducible factor-1α may protect GFR in hypoxic conditions through attenuation of the GFR-restraining actions of TGF (6).

To reduce the chances that differences in the genetic background may confound the phenotype under study, the ENT1 null mutation was bred into C57Bl/6 and SWR/J backgrounds. No major modification of the TGF phenotype associated with ENT1 deficiency was detected although maximum TGF responses were numerically greater in SWR/J than C57Bl/6 WT mice. Thus we conclude that the altered TGF phenotype is genuinely due to the absence of ENT1. Arterial blood pressure has been found to affect TGF responses, but there were not detectable differences in systemic hemodynamics between WT and ENT1-deficient mice. Downregulation of A1AR or up-
regulation of dilatory A2AR in ENT1-deficient mice could have explained our findings, but no changes of adenosine receptor expression, at least at the mRNA level, were found in these animals. This is consistent with earlier studies in guinea pigs showing that a 2-wk administration of dipyridamole did not change adenosine receptor binding activities in the kidney (36). It would also seem conceivable that A1AR are mostly occupied in ENT1-deficient mice so that the TGF signal-induced adenosine does not find enough free receptors for acute binding. However, acute exposure of A1AR to an excess of the A1AR agonist CHA did not reduce TGF strength unless endogenous adenosine formation was inhibited at the same time (34). Nevertheless, the effect of prolonged activation of A1AR by CHA or adenosine on TGF responsiveness has not been examined. Finally, accelerated degradation of adenosine may affect TGF responses since an upregulation of adenosine deaminase mRNA in kidney tissue.

In summary, our data show that the chronic absence of ENT1-mediated nucleoside transport and the resulting elevation of extracellular adenosine levels are associated with a marked reduction of the TGF response to changes in luminal NaCl concentration. This may be another example where interference with NaCl-dependent cycling of interstitial mediator concentrations leads to an altered ability of afferent arterioles to respond normally to changes of luminal NaCl concentration.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.L., D.M., Y.H., C.E., M.H., and J.S. performed experiments; L.L., D.M., C.E., M.H., and J.S. analyzed data; L.L., M.T., and J.S. interpreted results of experiments; L.L. and J.S. prepared figures; L.L., M.T. and J.B.S. edited and revised manuscript; J.S. conceived and designed the experiments; J.S. drafted manuscript.

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