Glomerular tubular balance is suppressed in adenosine type 1 receptor-deficient mice

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Bell TD, Luo Z, Welch WJ. Glomerular tubular balance is suppressed in adenosine type 1 receptor-deficient mice. Am J Physiol Renal Physiol 299: F1158–F1163, 2010. First published September 1, 2010; doi:10.1152/ajprenal.00202.2010.—Glomerular tubular balance maintains a stable fractional solute and fluid reabsorption in the proximal tubule over a range of glomerular filtration rates. The mediators of this process are unknown. We tested the hypothesis that adenosine, produced in proximal tubule cells acting on adenosine type 1 receptors (A1-AR) promotes Na\(^+\) and fluid uptake and mediates glomerular tubular balance. Absolute proximal fluid reabsorption (J\(_f\)) was measured by in vivo microperfusion in A1-AR knockout and wild-type mice during perfusion of the closed proximal tubule at 2–10 nl/min. J\(_f\) increased with perfuse flow from 2–4 nl/min in both strains, but the fractional increase was lower in A1-AR\(^{-/-}\) mice (A1-AR\(^{-/-}\): 114% vs. A1-AR\(^{+/+}\): 38%; P < 0.001), suggesting reduced glomerular tubular balance (GTB). At higher perfusion rates, J\(_f\) increased modestly in both strains, indicating less GTB at higher flow. The physiological effects of reduced GTB in A1-AR\(^{-/-}\) mice were assessed from the response to an acute volume load (1 ml/2 min). Na\(^+\) excretion and urine flow increased 76 and 73% more in A1-AR\(^{-/-}\) mice than A1-AR\(^{+/+}\) over the following 30 min, accompanied by a higher proximal tubule fluid (A1-AR\(^{-/-}\): 6.9 ± 0.9 vs. A1-AR\(^{+/+}\): 5.2 ± 0.6 nl/min; P < 0.05). The expression of the sodium-hydrogen exchanger 3 and sodium phosphate cotransporter-2 were similar between strains. In conclusion, GTB is dependent on adenosine acting on type 1 receptors in the proximal tubule. This may contribute to acute changes in Na\(^+\) and fluid reabsorption.

These include physical factors such as tubular flow and the balance of Starling forces, and the primary changes in transport of various ions (3, 4, 11, 30, 32, 37). We suggest that adenosine is well placed to mediate this process. Adenosine stimulates sodium and bicarbonate reabsorption in kidney cell preparations (13) and in isolated perfused PTs (36). Furthermore, microperfusion studies have shown that acute blockade of A1-ARs in rat PT segments decreased absolute reabsorption (23, 42), confirming that the diuretic and natriuretic effects of A1-AR antagonism are due to reduced PT reabsorption. Acute blockade of A1-ARs uncouples GFR and PT fluid reabsorption (42). However, the chronic effects of A1-AR antagonism on PT transport are not known. Therefore we tested the hypothesis that GTB is mediated by A1-AR activation. To test this hypothesis, the PT was perfused over a wide range of flows and the corresponding reabsorption was assessed in wild-type (WT) and A1-AR\(^{-/-}\) mice.

MATERIALS AND METHODS

Animals. Mice used in these studies were from a colony maintained at Georgetown University from the breeders provided by Dr. J. Schnermann. Mice were maintained on a standard chow (0.6 g/100 g Na content) with free access to food and water until the day of the study. The use of animals for this study was approved by the Georgetown University Animal Care and Use Committee and performed according to the National Institutes of Health guidelines for the conduct of experiments in animals.

Micropuncture. On the day of the experiment male mice were anesthetized by spontaneous inhalation of isoflurane (1.0% in room air, delivered by a continuous pump, Univentor, Malta). The mice were placed on a servo-regulated surgical table to maintain body temperature at 37°C. The trachea was cannulated with polyethylene (PE)-90 tubing to allow spontaneous breathing, and the exterior end of the cannula was placed inside a small plastic tube to maintain a continuous inhalation of isoflurane and air mixture. Heat-tapered PE-50 tubing was used to cannulate the left jugular vein and right femoral artery for infusion of isotonic saline containing 1.5% bovine albumin (Sigma Chemical, St. Louis, MO) at a rate of 0.35 ml/h and for blood pressure measurements, respectively. Blood pressure was monitored continuously throughout the animal preparation by use of the PowerLab (ADInstruments, Colorado Springs, CO) data-acquisition system. The bladder was catheterized with PE-50 tubing for urine collection. The left kidney was exposed by a flank incision, cleaned of connective tissue, stabilized in a Lucite cup, and bathed in mineral oil to prevent drying during the experiments. Micropuncture studies were initiated after 45 min to 1 h of stabilization.

Assessing GTB in S2 PT segments. S2 segments of the PT were identified by insertion of a “finding pipette” (8 μm OD) containing artificial tubular fluid (ATF) stained with Fast green FCF dye (Sigma, 0.1%). A small volume of ATF was injected to identify direction of flow and approximate location. If the bolus injection passed three or more loops, the site was selected as S2. An immobile grease block (A piezon T, Manchester, UK) was placed into the puncture site to stop tubular flow. A perfusion pipette (6–8 μm OD) was inserted imme-
diately downstream from the block. The perfusion pipeline, filled with ATF (in mmol: 125 NaCl, 20 NaHCO₃, 5 KCl, 1 MgSO₄, 2 CaCl₂, 1 Na₂HPO₄, 5 glucose, 4 urea) and ¹⁴C-labeled inulin was connected to a nonolater perfusion pump (Vestavia Scientific, Birmingham, AL) and the nephron segment was perfused at either 2, 4, 6, 8, or 10 nl/min. This range spans the physiological range of PT flow in mice. A collection pipette (8 μm OD) containing light mineral oil stained with Sudan Black B (Sigma Chemical) was inserted downstream from the perfusion site and a bolus of oil was inserted and maintained just distal to the insertion site by light pressure. A 4-min collection of the perfusate was started immediately after placement of the oil block. At the end of the perfusion, the segment was microperfused with Microfil solution (Flow Tech, Carver, MA) to measure the length of the microperfused segment. The Microfil formed a cast within 20–30 min, which was dissected at the end of the experiment. The length of the cast was measured under a dissecting microscope.

In a separate group of mice prepared as described above, late proximal free-flow fluid samples were collected to measure the clearance of inulin. Late proximal tubular loops on the kidney surface were identified by injection of small amounts of dye-stained light mineral oil. If the bolus injection immediately disappeared from the surface then an oil block was inserted and a 4-min timed fluid collection was initiated. All tubular fluid samples were transferred to 1 μl constant-bore microcaps for determination of fluid volume.

Calculations. Jₑ was calculated by the difference in the microperfusion rate (Vₑ) and the recollection rate (Vₑ), factored by the length of the nephron: Jₑ = Vₑ (nl/min) – Vₑ (nl/min)/PT length (mm). This value was confirmed by the difference between the concentrations of inulin in the perfusate and collects, factored by the segment length. If the two calculations differed by more than 10%, the data were discarded.

Whole kidney excretion and late proximal free-flow in response to an intravenous bolus of saline. Mice were prepared as described above but the maintenance infusion contained isotonic saline infused at a rate of 1.5 μl/min per gram body wt. At the end of a 1-h equilibration period urine was collected for two 5-min periods, and all mice were given an intravenous bolus of isotonic saline (1 ml delivered over 2 min). Thereafter, urine was collected every 5 min over a 30-min period. In a separate group of mice late proximal free-flow fluid samples were collected following the intravenous bolus of isotonic saline. Single-nephron GFR (SNGFR) was measured by the clearance of [¹⁴C]inulin (infused at 0.10 μCi/h) from collected PT fluid before and during acute saline loading.

SDS-PAGE gel electrophoresis and Western analysis. The kidney cortex was placed in radioimmunoprecipitation assay lysis buffer containing the following protease inhibitors: 100 μg/ml of PMSF, 5 μg/ml of leupeptin, 5 μg/ml of aprotinin, and 1 mmol/l of sodium fluoride per milliliter. The dissected sections with radioimmunoprecipitation assay lysis buffer were homogenized in FastPrep Bio101 (Thermo), and the tubes were spun at 12,500 rpm for 15 min in a cold centrifuge. The supernatant was aliquoted and frozen at −80°C for future analysis. Protein concentration was determined by Bio-Rad Protein Assay Reagent (Bio-Rad). Protein lysate (100 μg) for each sample was denatured in boiling water for 5 min. After denaturation, the lysate was placed on ice for 5 min and loaded onto a 4–20% SDS-PAGE gel (Bio-Rad). The gel was transferred to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% BLOTTO milk followed by overnight incubation (4°C) with polyclonal anti-

Analytical procedures. Urine flow was determined gravimetrically. The urinary sodium concentration was determined by flame photometry (Cole-Parmer, model 2655-10).

Statistical analyses. The significance of differences within and between groups was evaluated by an ANOVA followed by a Fisher’s post hoc test. SNGFR and absolute proximal reabsorption (APR) were analyzed by 2 × 2 ANOVA. Significance was estimated at P < 0.05.

RESULTS

As previously reported, there were no differences in mean arterial blood pressure, heart rate, and urine flow (UV) between A₁-AR⁻/⁻ and A₁-AR¹⁺⁺ mice (Table 1). However, A₁-AR⁻/⁻ mice had a decreased body weight compared with A₁-AR¹⁺⁺ mice, which may be related to the antilipolytic role of the A₁-AR (21).

To evaluate the efficiency of GTB, PT segments were perfused at 2, 4, 6, 8, or 10 nl/min. In A₁-AR¹⁺⁺ mice, Jₑ increased by 114% when the perfusion rate was increased from 2–4 nl/min (Jₑ at 2 nl/min: 0.7 ± 0.1 vs. at 4 nl/min: 1.5 ± 0.2 nl·min⁻¹·mm⁻¹, P < 0.001) demonstrating a complete match of reabsorption to perfusion and perfect GTB (Fig. 1). However, in A₁-AR⁻/⁻ mice GTB was suppressed since the increase in Jₑ from perfusion of 2–4 nl/min was increased only by 38% (APR at 2 nl/min: 1.1 ± 0.2 vs. 4 nl/min: 1.5 ± 0.2 nl·min⁻¹·mm⁻¹). This implies failure to match reabsorption to perfusion in these mice at the relevant PT flows. Jₑ continued to rise only modestly in response to increased perfusion in both groups, suggesting that GTB was less effective at the higher perfusion rates.

To test the functional role of A₁-ARs in acute Na⁺ homeostasis, we measured the excretory response to an acute volume load of 0.9% NaCl (1 ml over 2 min). Mean arterial pressure did not change significantly in either of the groups following the volume load. As shown in Fig. 2, baseline UV and Na⁺ excretion (UNaV) were not different between groups. Within the first 5 min after the volume load, UV and UNaV increased from 5.2 ± 0.6 to 14.1 ± 1.9 μl/min (P < 0.01) and from 0.9 ± 0.2 to 2.6 ± 0.2 μmol/min (P < 0.05) in A₁-AR¹⁺⁺ mice and remained stable for the remaining experimental period. In A₁-AR⁻/⁻ mice UV and UNaV increased sharply from 6.1 ± 0.7 to 34.3 ± 2.5 μl/min (P < 0.001) and from 1.2 ± 0.3 to 6.1 ± 0.7 μmol/min (P < 0.005), respectively, and remained elevated at 10 and 15 min before returning to baseline. Thirty minutes following induction of the load A₁-AR⁻/⁻ mice had excreted 74 ± 9 and 88 ± 16% of volume and Na⁺ load compared with 46 ± 6 and 53 ± 8% in A₁-AR¹⁺⁺ mice (P < 0.05).

In separate mice, we measured PT flow (VPT), SNGFR by the clearance of radiolabeled inulin, and APR in free-flow collections in the PT. SNGFR and VPT did not differ between strains measured at baseline. However, VPT was higher in

Table 1. Physiological parameters in anesthetized mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A₁-AR¹⁺⁺</th>
<th>A₁-AR⁻/⁻</th>
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<tbody>
<tr>
<td>Weight, g</td>
<td>30 ± 0.8</td>
<td>26 ± 0.6*</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>97 ± 1.7</td>
<td>93 ± 2.0</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>445 ± 13</td>
<td>450 ± 13</td>
</tr>
<tr>
<td>UV, μl/min</td>
<td>1.6 ± 0.3</td>
<td>2.1 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE. MAP, mean arterial pressure; HR, heart rate; UV, urine flow rate. *P < 0.05 compared with A₁-AR¹⁺⁺ group.
A1-AR−/−, during acute saline loading (Table 2). APR was lower in A1-AR−/− mice before and after acute saline loading. Saline loading alone had no effect in either strain.

The major Na+/H+ uptake pathways in the PT are NHE3 and NaPi2. The renal cortical expression of NHE3 and NaPi2 were not different between strains (Fig. 3) and therefore could not account for the differences in $J_v$ or UNaV in this study. Expression of A2a-AR was not different between strains.

**DISCUSSION**

The new finding in this study is that deletion of A1-AR in mice suppresses GTB in the PT. This was demonstrated by direct measurement of absolute fluid reabsorption ($J_v$) in the PT during increases in tubular flow that mimics changes in GFR. The in vivo isolated nephron method used in this study eliminates the role of TGF in maintaining GFR and focuses simply on the changes in absorption in the PT. When we increased PT flow from 2 to 4 nl/min, which is over the range of ambient flow in anesthetized mice (3), $J_v$ increased in strict proportion to perfusion in WT mice, implying excellent GTB. At higher perfusion rates GTB was not as well maintained in the WT mice. The changes in $J_v$ over PT flows of 2–4 nl/min were substantially lower in A1-AR−/− mice. (Fig. 1B). Therefore, the PT segment's ability to reabsorb the delivery was diminished without A1-ARs. The functional significance of the A1-ARs was demonstrated by the reduced ability of the kidney to reabsorb an acute volume load in A1-AR−/− mice. For 30 min after an acute volume load, UNaV and UV were more than twofold higher in the A1-AR−/− mice. This increased ability to excrete an acute saline load was localized to the PT by direct free-flow micropuncture collections, which showed that PT flow was higher in A1-AR−/− following acute volume loading. This difference in PT uptake occurred despite the lack of significant differences in SNGFR. This is partially explained by the free-flow technique, which does not control for the influence of TGF, used to measure SNGFR and GTB. Yet PT uptake by both methods was lower in A1-AR−/− mice. These data suggest that the A1-AR in the PT regulates acute changes in reabsorption and the matching of reabsorption to perfusion.

A key element in the ability of the kidney to maintain homeostasis via fluid and electrolyte balance is tight regulation of GFR during periods of stress to the system. The kidney incorporates multiple, often interlocking systems to preserve GFR. These include two closely related systems: GTB and TGF. A1-ARs have long been implicated in regulation of GFR via mediation of TGF (29, 33). TGF was suppressed by specific antagonists in rats (33, 42) and was absent in A1-AR−/− mice (8, 35). The impact of GTB on TGF in these models was not evaluated.
Table 2. Single nephron function before and during acute saline loading

<table>
<thead>
<tr>
<th></th>
<th>SNGFR</th>
<th>V_{PT}</th>
<th>APR, SNGFR-V_{PT}</th>
<th>FR, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-AR^{+/+} (n = 6)</td>
<td>14.9 ± 1.4</td>
<td>5.1 ± 0.6</td>
<td>9.9 ± 1.2</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>Acute saline</td>
<td>17.4 ± 1.2</td>
<td>5.2 ± 0.6</td>
<td>12.7 ± 0.9</td>
<td>73 ± 2</td>
</tr>
<tr>
<td>A1-AR^{-/-} (n = 6)</td>
<td>12.5 ± 1.0</td>
<td>5.2 ± 0.7</td>
<td>7.3 ± 0.8*</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>Acute saline</td>
<td>13.7 ± 1.4</td>
<td>6.9 ± 0.9*</td>
<td>7.1 ± 1.3†</td>
<td>59 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE. SNGFR, single-nephron glomerular filtration rate; V_{PT}, proximal tubular flow; APR, absolute reabsorption; FR, fractional reabsorption. *P < 0.05; †P < 0.01 compared with A1-AR^{+/+}.

The existence of GTB was first recognized in whole kidney studies that linked GFR to U_{Na}V and in initial micropuncture studies that evaluated proximal tubular function (reviewed in Ref. 41). It soon was recognized as a key component of the kidney’s ability to maintain fluid and electrolyte balance (41). Early micropuncture studies showed that fractional uptake of fluid was maintained in rat models with different GFRs (17, 27, 40) and when PT flow was controlled by microperfusion (6, 24). Subsequently, several studies suggested that isotonic reabsorption of Na^{+}, glucose, HCO_{3}⁻, and fluid in the PT reflected GTB-like maintenance of fractional reabsorption (7, 12, 15, 40). The primary event, however, has been difficult to isolate. Some studies suggested that GTB balance was regulated by extraluminal events that regulated GFR and less by intraluminal events. However, work by Tucker and Blantz (37), who evaluated both concepts, concluded that PT reabsorption correlated to nephron load. Therefore more recent work on GTB has focused on luminal signaling pathways.

Cogan (12) proposed that angiotensin II (Ang II), which promotes Na^{+} uptake in the PT and also regulates afferent and efferent arteriolar resistances, is a reasonable candidate as a mediator of GTB. Whereas a role for Ang II in regulation of GTB remains unclear, the evidence that Ang II has a biphasic effect on Na^{+} uptake in the tubule (39) diminishes that possibility. Yet, for many of these same reasons, adenosine might also be considered as a mediator of GTB. Adenosine is a likely mediator of TGF, based on its activation of vascular receptors, shown both in studies with adenosine receptor blockers and in A1-AR knockout mice (8, 35, 42). Adenosine's proposed role in GTB is based on its activation of A1-AR in the PT. Since Na^{+} uptake is the primary transport event in the epithelial cells in this segment, pathways related to Na^{+} uptake have been investigated as possible mediators of GTB (41). Glucose, phosphate, HCO_{3}⁻, and fluid transport across the PT, which

![Fig. 3. A: representative immunoblots for Na^{+}/H^{+} exchanger isoform 3 (NHE3), Na^{+}/Pi cotransporter isoform 2 (NaPi2), and A2-AR in cortex homogenates from with A1-AR^{+/+} and A1-AR^{-/-} mice. B: densitometry summary (means ± SE) expressed as % change, as labeled.](http://ajprenal.physiology.org/)
have been linked to GTB are secondary to Na⁺ transport. Further along those lines, a metabolite of Na⁺ transport, such as adenosine, that also promotes uptake may fit the role of a positive feedforward type signal. Although little is known about increased load and adenosine production, there are studies that show a link between adenosine and transport. Chronic high salt intake increases interstitial adenosine levels by four- to fivefold (34). Adenosine production is also increased by Na⁺/K⁺ ATPase activity (5). Diphosphorylating enzymes, which are expressed in PT cells and in the interstitium, generate most of the free adenosine (20). Therefore adenosine production is dependent on Na⁺ uptake. We previously showed that blockade of locally produced adenosine by PT perfusion of adenosine deaminase reduced \( J_v \) (23). This suggests that adenosine produced in cells crosses cell membranes via nucleoside transporters and acts on receptors located on the luminal membranes of epithelial cells, promoting additional Na⁺ uptake. This relationship would be consistent with the observed effect of GTB: increased stimulus of GFR generates greater Na⁺ uptake, which in turn results in greater Na⁺/K⁺ ATPase activity and ultimately more adenosine formation capable of acting on A₁-AR to regenerate more Na⁺ uptake. Conversely, reduced GFR has the reverse effect eliciting less adenosine to act on its receptors to activate Na⁺ uptake. Therefore adenosine is positioned to act as both a marker and a promoter of luminal Na⁺.

In this study we perfused artificial fluid and demonstrated GTB in a restricted range of perfusion rates in WT mice. Earlier studies in rats have had mixed results. \( J_v \) during perfusion of the PT reflects perfect GTB in some studies (27, 37), but not others (10, 11). The differences may be linked to the composition of perfusate. Indeed, in our studies, there was perfect GTB over the low physiological range of perfusion (2–4 nl/min) and less GTB at higher perfusion rates (6–10 nl/min) in WT mice using artificial PT fluid. We closed the segment to eliminate the effects of changes in GFR and artificially controlled flow. This should also remove the influence of TGF, since there was no flow to the macula densa in the perfused PT. Therefore the absolute reabsorption in our studies reflected the direct transport capability of the PT segment. This may indeed have some limitations, since the GTB and TGF systems do interact. In addition, perfusion of artificial fluid may have eliminated potentially important metabolites generated in the PT. However, we previously showed that inhibition of adenosine by perfusion of adenosine deaminase in artificial fluid lowered \( J_v \) (23).

Our study confirms some of the observations made by Vallon et al. (38), who studied A₁-AR \(-/-\) mice, but differs in other areas. GFR and SNGFR in both strains shown in Table 2 are very similar to this study. Yet these authors fail to see a difference between stains of PT Na⁺ reabsorption relative to SNGFR, unlike our findings. However, they did see an increase in volume and Na⁺ delivery to the distal tubule when they stimulate TGF by enhancing flow via the late PT in the A₁-AR \(-/-\) mice. This is entirely consistent with our data, although they did not simulate GTB by adding graded volumes to the PT. We contend that GTB is diminished and contributes to higher downstream delivery in A₁-AR \(-/-\) mice, which is most apparent during acute volume loading.

In conclusion, GTB is diminished in A₁-AR \(-/-\) mice, which are unable to sustain an acute volume load. This suggests that adenosine, via activation of type I receptors in the PT, contributes to GTB and acute sodium and fluid homeostasis. Furthermore, this also demonstrates that blockade of A₁-AR might be an effective method to eliminate acute volume overload, which would promote homeostasis in volume-challenged patients. Indeed, clinical trials have tested the efficacy of A₁-AR antagonists in treatment of severe edema in congestive heart failure patients (49). The benefits are credited to blockade of A₁-AR, but the potential loss of GTB has not been fully evaluated in those patients.

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

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