Low salt intake increases adenosine type 1 receptor expression and function in the rat proximal tubule

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Kulick A, Panico C, Gill P, Welch WJ. Low salt intake increases adenosine type 1 receptor expression and function in the rat proximal tubule. Am J Physiol Renal Physiol 295: F37–F41, 2008. First published May 14, 2008; doi:10.1152/ajprenal.00061.2008.—Adenosine mediates Na\(^+\) reabsorption in the proximal tubule (PT) and other segments by activating adenosine type 1 receptors (A1-AR). We tested the hypothesis that A1-AR in the PT is regulated by salt intake and participates in the kidney adaptation to changes in salt intake. Absolute fluid reabsorption (\(J_f\)) was measured by direct in vivo microperfusion and recollection in rats maintained on low (LS; 0.03% Na, wt/wt), normal (NS; 0.3% Na), and high-salt (HS; 3.0% Na) diets for 1 wk. The effect of microperfusion of BG9719 a highly selective inhibitor of A1-ARs or adenosine deaminase (AD), which metabolizes adenosine, was measured in each group. \(J_f\) was higher in PT from LS rats (LS: 2.8 ± 0.2 vs. NS: 2.1 ± 0.2 nl/min·mm⁻¹, \(P < 0.001\)). \(J_f\) in HS rats was not different from NS. BG9719 reduced \(J_f\) in LS rats by 66 ± 6% (LS: 2.8 ± 0.2 vs LS+CVT: 1.3 ± 0.3 nl/min·mm⁻¹, \(P < 0.001\)), which was greater than its effect in NS (45 ± 4%) or HS (41 ± 4%) rats. AD reduced \(J_f\), similarly, suggesting that A1-ARs are activated by local production of adenosine. Expression of A1-AR mRNA and protein was higher (\(P < 0.01\)) in microdissected PTs in LS rats compared with NS and HS. We conclude that A1-ARs in the PT are increased by low salt intake and that A1-AR participates in the increased PT reabsorption of solute and fluid in response to low salt intake.

Fluid reabsorption; microperfusion

ADENOSINE IS A MAJOR PARACRINE agent in several organ systems acting on four distinct receptors: type 1 (A1-AR), type 2a (A2a-AR), type 2b (A2b-AR), and type 3 (A3-AR). These receptors are widely expressed in the kidney in both vascular and tubular cells and are involved in regulation of glomerular filtration rate (GFR) by tubuloglomerular feedback (TGF), renin secretion, and tubular Na\(^+\) reabsorption (24). A1-ARs are expressed in arterioles (Af Art) and in various segments of the nephron: proximal tubule (PT), thick ascending loop of Henle (TALH), and in the cortical collecting ducts (CCD) (18, 31, 36). Activation of A1-ARs in Af Art by adenosine increases resistance and reduces renal blood flow (RBF) and, subsequently, GFR (18). Adenosine release in the renal interstitium from the renal epithelial macula densa cells mediates TGF by acting on A1-ARs in the adjacent AA. TGF is absent in A1-AR knockout mice. (33)

A1-ARs in the nephron are linked to Na\(^+\) transport in several segments. However, the natriuresis and diuresis associated with systemic inhibition of A1-ARs are due primarily to reduced PT reabsorption, based on lithium clearance and direct PT microperfusion studies (1, 2, 39). A1-AR in other segments may have additional effects on Na\(^+\) reabsorption, but these are less well understood. It is also not clear how A1-AR participates in Na\(^+\) reabsorption in the PT, but it is a critical element in normal PT function. A1-AR expression measured in renal membranes is increased by low salt intake, but no functional correlation for Na\(^+\) transport was evaluated (30). To determine the role of PT A1-ARs during high and low salt intake, we evaluated PT reabsorptive function and A1-AR expression in rats following 1 wk of various salt intake levels. Therefore, we tested the hypothesis that A1-ARs in the PT mediate Na\(^+\) fluid reabsorption during changes in dietary Na\(^+\) intake. We measured absolute fluid uptake (\(J_f\)) in S2 segments of the PT during microperfusion with artificial tubular fluid (ATF) and downstream recollection in normotensive rats exposed to low, normal, and high levels of salt. In addition, we measured \(J_f\) before and during simultaneous microperfusion of an A1-AR antagonist (BG9719) and adenosine deaminase (AD), which inactivates adenosine. To evaluate the effect of salt intake on A1-ARs, we measured protein and mRNA expression in microdissected PT S2 segments.

MATERIALS AND METHODS

Experiments were performed in adult male Sprague-Dawley rats (212–295 g). The use of rats in this study was approved by the Georgetown University Animal Care and Use Committee. Rats were anesthetized (Inactin, 80 mg/kg) and surgically prepared for in vivo micropuncture experiments. Briefly, cannulas were placed in the jugular vein to infuse maintenance fluids (0.9% NaCl solution, 1% albumin), in the femoral artery to monitor mean arterial blood pressure (MAP), in the bladder, and in the trachea. The left kidney was exposed and stabilized in a lucite cup (Vestavia, Birmingham, AL) mounted on a heated surgical table. The kidney was bathed with warm saline and secured to reduce respiratory motion.

Microperfusion. S2 segments of the PT were identified by insertion of a “finding pipette” [8-μm outer diameter (OD)] containing ATF stained with FD and C blue dye (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 (Apiezon T, Manchester, UK) was placed into the finding puncture site to stop flow. A perfusion pipette (6- to 8-μm OD) was inserted immediately downstream from the block. The perfusion pipette filled with ATF (in meq/l: 130 Na, 120 Cl, 24 HCO\(_3\), 5 SO\(_4\), 4 PO\(_4\), 80 glucose) and \(^{14}\)C-labeled inulin was connected to a nanoliter perfusion pump (Vestavia) and set at 20–24 nl/min. \(^{14}\)C-inulin (0.1 μCi/μl, Radiobiochemicals) was used to detect leakage into or out of the puncture sites. Collected samples with <95%
and >105% of the microperfused inulin were discarded. A collection pipette (10-μm OD) containing dye-stained light mineral oil was inserted two to four loops downstream from the perfusion site. A bolus of oil was inserted and maintained just distal to the insertion site by light pressure. A timed collection was started immediately after placement of the oil block. Each nephron was perfused with ATF or ATF+BG9719 (10^{-7} M, Biogen), a highly selective A1-AR antagonist. In separate nephrons, AD (5 U/ml) was perfused to test the effect of inhibition of local production of adenosine. 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.

**Calculations.** $J_v$ was calculated by the difference in the microperfusion rate ($V_{\text{perf}}$) and the collection rate ($V_{\text{coll}}$), factored by the length of the nephron: $J_v = V_{\text{perf}} \cdot \text{nl/min} - V_{\text{coll}} \cdot \text{nl/min}/\text{PT length} \cdot \text{mm}$, and expressed as nanoliters per minute per millimeter. This value was confirmed by the difference between the concentration of inulin in the perfusate and collectate, factored by the segment length. However, the data shown were calculated by the former method.

**Groups.** Sprague-Dawley rats were treated for 1 wk to a low (0.03 mg/g; LS)-, normal (0.3 mg/g; NS)-, or high (3.0 mg/g; HS)-Na^+ diet.

**Proximal tubule dissection.** Kidneys were perfused from the left kidney of Sprague-Dawley rats treated for 1 wk to a low (0.03 mg/g; LS)-, normal (0.3 mg/g; NS)-, or high (3.0 mg/g; HS)-Na^+ diet. $J_v$ was measured in PT in response to ATF, ATF+BG9719, and ATF+AD.

**RT-PCR.** RNA from microdissected PT was extracted using the guanidium method using a RNAqueous 4 PCR Kit (Ambion). The sample was DNase I treated and concentrated according to the manufacturer’s recommendations. The first-strand cDNA synthesis was done using Superscript II (Invitrogen) with oligo-dT. The cDNA was amplified using Adora1 (M64299) coding region. The gene-specific primers for rat Adora1 amplified a 200-bp fragment. The primers were designed with a Tm of 60°C: forward 5'-cattggccacagcgtcgcgt-3' and reverse 5'-ccaggagagaactcagc-3'.

**Western blot analysis.** The microdissected samples from above were placed in SDS lysis buffer (1 mM NaF, SDS 1%, 100 mM Tris-HCl, 1 mM EDTA, 1 mM DTT) containing 2X protease inhibitor Complete Mini (Roche, Genotech). The entire sample was loaded onto a 12.5% SDS-PAGE gel (Bio-Rad) after denaturation in Laemmli buffer (Bio-Rad). In this study, we used polyclonal antibody for A1-AR at a dilution of 1:1,000 (Sigma, St. Louis, MO). Membranes were washed in TBST and exposed to horseradish peroxidase-labeled secondary antibody for 30 min: rabbit anti-goat (1:10,000, Kirkegaard and Perry Laboratories, Gaithersburg, MD); after a washing with TBST, the bands were visualized with a luminol-based chemiluminescence substrate (LumiGLO, Kirkegaard and Perry Laboratories). Finally, the blots were stripped with Western blot stripping buffer (Pierce, Rockford, IL) and probed for equal loading using β-actin primary antibody (Sigma).

**RESULTS**

There were no differences in body weight, MAP, heart rate, and urine flow in the three test groups of rats. (Table 1).

$J_v$ in the PT was $2.1 \pm 0.2 \text{ nl mm}^{-1} \text{min}^{-1}$ (n = 22) in rats maintained on a NS diet, which is comparable to previous reports using similar methods (12). In rats on LS intake, PT $J_v$ was higher ($P < 0.01)$ than in NS and HS PTs (LS: $2.8 \pm 0.2$ vs. NS: $2.1 \pm 0.2$, $P < 0.01$; HS: $1.8 \pm 0.2 \text{ nl mm}^{-1} \text{min}^{-1}$, $P < 0.01$) (Fig. 1). $J_v$ was not different between NS and HS. The fractional fluid reabsorption (FR) reflected $J_v$: FR was higher in LS than in NS and HS, which were not different (LS: $68 \pm 5$ vs. NS: $52 \pm 5$ and HS: $47 \pm 6$%) (Fig. 2). Following microperfusion of BG9719 (10^{-7} M), $J_v$ was reduced in each group, by 25–40%, to similar levels: (LS: $2.8 \pm 0.2 – 1.3 \pm 0.2 \text{ nl mm}^{-1} \text{min}^{-1}$, $P < 0.001$; NS: $2.1 \pm 0.2 – 1.2 \pm 0.2 \text{ nl mm}^{-1} \text{min}^{-1}$, $P < 0.001$; HS: $1.8 \pm 0.2 – 1.0 \pm 0.2 \text{ nl mm}^{-1} \text{min}^{-1}$, $P < 0.001$). However, the decline was greater in LS PT compared with NS and HS PTs (LS: $66 \pm 5$ vs. NS: $45 \pm 4$, $P < 0.01$) (Fig. 1). BG9719 had similar

**Table 1. Body weight, mean arterial pressure, heart rate, and urine flow**

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (g)</th>
<th>MAP (mmHg)</th>
<th>HR (beats/min)</th>
<th>UoN/V (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low salt</td>
<td>254±12</td>
<td>124±6</td>
<td>366±12</td>
<td>0.12±0.03*</td>
</tr>
<tr>
<td>Normal</td>
<td>272±15</td>
<td>118±6</td>
<td>346±16</td>
<td>0.26±0.14</td>
</tr>
<tr>
<td>High salt</td>
<td>261±22</td>
<td>121±5</td>
<td>361±13</td>
<td>0.54±0.09*</td>
</tr>
</tbody>
</table>

Values are means ± SE. n. No. of rats; MAP, mean arterial pressure; HR, heart rate; UoN/V, urine flow rate. *P < 0.01 compared with normal-salt group.
These results have important implications in managing salt balance and the role of the PT. Previous studies have shown that A1-AR antagonists are powerful diuretics and a major portion of the effect is in the PT (1, 2, 12, 14, 22, 35). Conversely, adenosine agonists cause antidiuresis in rats and humans (4, 37). We showed that direct microperfusion of BG9719 reduced \( J_v \) in the PT (39). However, this is the first report to show that A1-AR can functionally adapt to changes in salt intake and participate in maintaining \( Na^+ \) balance during salt restriction. \( J_v \) was not different between HS and NS PT, suggesting that the PT might not be involved in adaptations to HS intake. Furthermore, the fractional changes in \( J_v \) to A1-AR blockade were also similar between these two groups. This suggests that A1-AR is not linked to regulation of fluid balance during HS, especially in the PT. A1-ARs expressed in downstream segments, especially in the TALH and CCD, may have been altered by salt restriction and contributed to overall \( Na^+ \) balance. However, the focus of this study was on A1-ARs in the PT, and other receptors were not evaluated.

A1-ARs are expressed in PT, TALH, and CCD (18, 31, 36) and are associated with \( Na^+ \) transport. Caffeine and theophylline are nonselective adenosine antagonists, and early reports suggest high levels of each induced diuresis (24). Indeed, moderately more selective A1-AR antagonists also produced diuresis, but these results did not clarify a specific role for A1-AR, since these agents also activated other adenosine receptors, especially at higher concentrations. Selectivity is particularly important since activation of A2-AR often has opposite physiological effects (24). BG9719, however, has 1,500-fold selectivity for A1-AR over A2-AR, which makes it ideal to test the specific role of A1-AR (32).

The PT is the site of 60–70% of \( Na^+ \) reabsorption, but despite this large contribution its role in \( Na^+ \) balance regulation has not been well established. More distal sites, such as the collecting duct, distal tubule, and TALH have been linked to regulation of \( Na^+ \) balance (10, 21, 28). In addition, FR in the PT is maintained relatively constantly by glomerulotubular balance, such that during small changes in GFR, FR does not change (23). This acts to limit the PT in the overall control of \( Na^+ \) excretion. However, recent evidence has shown several conditions that may be linked to regulation of electrolyte balance in the PT. In models of salt-sensitive hypertension, defects in PT reabsorption may contribute to excess \( Na^+ \) retention (6). Lithium clearance, which is an indicator of PT reabsorption, was higher in young (3–6 wk) spontaneously hypertensive rats (SHR) (5), saline-loaded SHR (17), and Dahl salt-sensitive rats (26). In hypertensive patients who were...
relatively salt insensitive, PT reabsorption measured by lithium clearance was appropriately elevated during LS intake, contributing to Na\(^+\) retention, and reduced during HS intake, contributing to appropriate Na\(^+\) excretion (7). However, salt-sensitive patients did not alter PT function appropriately and tended to increase PT reabsorption during HS intake and decrease PT reabsorption during LS intake, and perhaps contributing to the development of hypertension.

Whereas the mechanism of enhanced PT Na\(^+\) reabsorption is unclear, the major Na\(^+\) uptake mechanism in this segment is the Na\(^+\)/H\(^+\) exchanger-3 (NHE3). In young SHR, the expression of NHE3 is similar to normotensive Wistar-Kyoto rats, yet NHE3 activity is higher (16). This may be due to reduced expression of NHE3-regulatory factor proteins in young SHR (20). However, regulation of NHE3 is not clearly altered by salt intake. NHE3 expression and activity measured in renal cortical membrane vesicles were unchanged in response to 1 wk of LS or HS intake in rats (11). The mechanism of how A1-AR activation leads to Na\(^+\) uptake in the PT is not well known (34). However, adenosine stimulates Gi-dependent phospholipase C and protein kinase C in afferent arterioles (15), but it has not been directly linked to NHE3 in the PT. Adenosine activates NHE3 in renal cells and transcribed epithelial cells (8, 9). The role of other regulators, such as angiotensin II or nitric oxide that may interact with A1-AR-related changes in J\(_{\text{v}}\) during different salt intakes, has not yet been tested.

Our observation that AD, which metabolizes adenosine, inhibits J\(_{\text{v}}\) similarly to BG9719 suggests that locally produced adenosine contributes to transport in the PT. This raises the possibility that intracellular adenosine passes into the interstitium or the lumen via concentrating or equilibrating nucleoside transporters expressed in the PT (13, 25), which then could act on luminal A1-AR. These data further support the concept that control of transport is linked to local feedback mechanisms.

Our adenosine receptor data might have been expected based on previous studies that measured interstitial adenosine. During HS intake, interstitial adenosine was 18-fold higher than with NS intake (29). Adenosine receptors might then be downregulated during HS, and the opposite effect therefore might be observed during LS intake. We confirmed that A1-AR was increased in PT during LS intake, whereas A1-AR was not altered by HS intake, although it tended to be lower.

Blockade of A1-AR by a series of selective inhibitors increased urine and Na\(^+\) excretion in rats (1, 2, 22). Studies that measured renal lithium clearance in both rats (1, 2) and humans (3, 12, 35) showed that the major effects of A1-AR inhibition occurred in the PT. We confirmed these whole kidney analyses by more direct micropuncture analysis, which showed BG9719 increased PT flow and urine flow and decreased J\(_{\text{v}}\) (39).

The role of A1-AR in the adaptation of the kidney to salt restriction has been clarified by this study. During LS intake, the expression of A1-AR mRNA and protein in the PT was higher than in PT from NS rats. This was consistent with the increased PT fluid reabsorption shown in LS rats and the increased effect of A1-AR blockade on J\(_{\text{v}}\) compared with NS and HS rats. These results suggest that A1-AR in the PT is upregulated by salt restriction and mediates greater Na\(^+\) reabsorption, thus contributing to adaptation of the kidney to conserve and retain Na\(^+\) during salt restriction. In conclusion, A1-ARs in the PT are regulated by NaCl intake and are part of the homeostatic network in the kidney that maintains fluid and electrolyte balance.

**REFERENCES**


