Renal sodium transporter/channel expression and sodium excretion in P2Y2 receptor knockout mice fed a high-NaCl diet with/without aldosterone infusion

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Zhang Y, Listhrop R, Ecelbarger CM, Kishore BK. Renal sodium transporter/channel expression and sodium excretion in P2Y2 receptor knockout mice fed a high-NaCl diet with/without aldosterone infusion. Am J Physiol Renal Physiol 300: F657–F668, 2011. First published December 29, 2010; doi:10.1152/ajprenal.00549.2010.—The P2Y2 receptor (P2Y2-R) antagonizes sodium reabsorption in the kidney. Apart from its effect in distal nephron, hypothesetically, P2Y2-R may modulate activity/abundances of sodium transporters/channel subunits along the nephron via antagonism of aldosterone or vasopressin or interaction with mediators such as nitric oxide (NO), and prostaglandin E2 (PGE2) or oxidative stress (OS). To determine the extent of the regulatory role of P2Y2-R in renal sodium reabsorption, in study 1, we fed P2Y2-R knockout (KO; n = 5) and wild-type (WT; n = 5) mice a high (3.15%)-sodium diet (HSD) for 14 days. Western blotting revealed significantly higher protein abundances for cortical and medullary bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2), medullary α-1-subunit of Na-K-ATPase, and medullary α-subunit of the epithelial sodium channel (ENaC) in KO vs. WT mice. Molecular analysis of urine showed increased excretion of nitrates plus nitrates (NOx), PGE2, and 8-isoprostane in the KO, relative to WT mice, supporting a putative role for these molecules in determining alterations of proteins involved in sodium transport along the nephron. To determine whether genotype differences in response to aldosterone might have played a role in these differences due to HSD, in study 2 aldosterone levels were clamped (by osmotic minipump infusion). Clamping aldosterone (with HSD) led to significantly impaired natriuresis with elevated Na/H exchanger isofom 3 in the cortex, and NKCC2 in the medulla, and modest but significantly lower levels of NKCC2, and α- and β-ENaC in the cortex of KO vs. WT mice. This was associated with significantly reduced urinary NOx in the KO, although PGE2 and 8-isoprostane remained significantly elevated vs. WT mice. Taken together, our results suggest that P2Y2-R is an important regulator of sodium transporters along the nephron. Pre- or postreceptor differences in the response to aldosterone, perhaps mediated via prostaglandins or changes in NOS activity or OS, likely play a role.

purinergic signaling; extracellular nucleotides; urinary concentration; arginine vasopressin

SIGNALING VIA THE P2Y2 Receptor (P2Y2-R), by virtue of its ability to antagonize the actions of arginine vasopressin (AVP) and aldosterone, regulates water and sodium absorption in the distal nephron (20, 22, 23, 37, 46, 49, 50). Besides these distal sites, P2Y2-R is expressed to varying degrees in several segments of proximal nephron, such as the proximal convoluted tubule, and the loop of Henle (2, 6, 29, 32, 44), which play critical roles in urinary concentration. Accordingly, our recent studies and those conducted by Vallon and associates documented that signaling through P2Y2-R plays an overarching role in balancing the effect of AVP on the urinary concentration mechanism (24, 36, 54). This overarching role is uncovered in P2Y2-R knockout (KO) mice as increased sensitivity of the kidney to circulating AVP levels, resulting in significantly increased protein abundances of the aquaporin-2 (AQP2) water channel, Na-K-2Cl cotransporter (NKCC2), and UT-A isoforms of the urea transporter in the medulla.

The role of P2Y2-R-mediated signaling in antagonizing the actions of aldosterone by inhibiting amiloride-sensitive sodium transport via the apical epithelial sodium channel (ENaC) in the distal nephron is well documented (22, 23, 30, 31, 38, 50). However, a role for P2Y2-R in the regulation of thiazide-sensitive Na-Cl cotransporter (NCC), another aldosterone-stimulated protein, has not been reported. On the other hand, we (35) and others (47) have demonstrated decreased expression of NCC as a major regulatory change associated with the combination of aldosterone infusion and a high-NaCl diet (the aldosterone-escape model), compared with rats infused with aldosterone, but maintained on a low-NaCl diet. We have also demonstrated reduced medullary protein levels of NKCC2, increased eNOS (endothelial nitric oxide synthase) protein, and over a fivefold increase in urinary NOx (nitrates plus nitrates excretion) in these high-NaCl/aldosterone-infused rats (35). An increase in endothelial-derived nitric oxide (NO) production is known to inhibit expression of major sodium transporters, such as the sodium/hydrogen exchanger isofom 3 (NHE3), NKCC2, NCC, and Na-K-ATPase (19). Taken together, these findings suggest that the natriuresis of aldosterone escape is accompanied by an increase in medullary NO production, perhaps leading to decreased expression/activity of NKCC2 and NCC. Furthermore, it has been shown that cyclooxygenase (COX) inhibitors enhance urinary concentration ability by increasing the protein abundance of NKCC2 (14). Thus both NO and prostaglandin E2 (PGE2) modulate the expression and/or activity of sodium transporters/channels (14, 19). Also, interestingly, P2Y2-R is known to interact with both NO and prostanoid systems (8, 15, 20), thus potentially expanding the ability of purinergic signaling to regulate major sodium transporters/channels along the nephron by altering NO and prostanoid
production, independently of its direct regulatory effect on ENaC in the distal nephron. Finally, a growing body of evidence suggests that the damaging effects of aldosterone excess on the kidney and cardiovascular system, particularly in the presence of high salt intake, are mediated through its ability to induce “oxidative stress,” inflammatory, and fibrotic processes (4). Therefore, if P2Y2-R is involved in attenuating aldosterone actions it might also hypothetically affect renal oxidative stress.

In view of the above, we hypothesized that P2Y2-R, by virtue of its known interactions with NO and prostanoid systems, may influence the expression of sodium transporters/channels along the nephron induced by a high-sodium diet with/without aldosterone infusion. Hence, to address this hypothesis, in this study we determined the protein abundances of sodium transporters or channels in the cortex and medulla of P2Y2-R KO and wild-type (WT) mice in two different series of experiments, namely, high-sodium diet feeding for 14 days, or high-sodium diet feeding for 4 days in the background of aldosterone infusion. Furthermore, to probe the mechanisms underlying P2Y2-R-mediated effects, we evaluated the production of NO and PGE2 by assaying their urinary content and/or by determining the renal expression of enzymes involved in their biosynthesis, namely, NOS and COX isosforms. Finally, to uncover any potential link between P2Y2-R and oxidative stress, we determined the urinary excretion of 8-isoprostane (8-iso prostaglandin F2α), a marker of oxidative stress (33).

METHODS

Experimental Animals

The animal protocols describing the procedures in this study were approved by the Institutional Animal Care and Use Committees of the Veterans Affairs Salt Lake City Health Care System and the University of Utah. We obtained breeders of P2Y2-R gene KO and the corresponding WT mice from Dr. Beverly Koller (University of North Carolina at Chapel Hill, Chapel Hill, NC). These mice were generated by targeted mutagenesis of the P2Y2-R gene in mouse embryonic stem cells. Chimeric mice thus generated were bred into a genetic background of B6D2 (7, 16). Breeding and genotyping of the mice were performed as described previously (54).

Study Protocols

Two studies were conducted. In the first study, we examined the effect of high-sodium (3.51% Na) diet in WT and P2Y2-R KO mice. Groups of WT and P2Y2-R KO mice were fed a high-sodium diet (n = 5 mice/genotype) for 14 days and then euthanized. The high-sodium diet was fed as dry chow pellets (TestDiet, Richmond, IN), with ad libitum access to drinking water. In the second study, we evaluated the effect of a high-sodium diet in a background of aldosterone infusion in WT and P2Y2-R KO mice. Briefly, after collection of baseline 24-h urine samples, on day −3 under isoflurane anesthesia groups of WT and P2Y2-R KO mice (n = 5 mice/genotype) were subcutaneously implanted with osmotic minipumps (Alzet model 1002; Durect, Cupertino, CA) preloaded with aldosterone (Sigma, St. Louis, MO). Aldosterone was delivered throughout the experimental period at a rate of 20 μg/day. Immediately following the implantation of the minipumps, the mice were fed a low-sodium diet (0.03% Na; TestDiet) for 3 days (days −3 to 0). On day 0, all the mice were switched to a high-sodium diet that continued for another 4 days (days 0 to +4) before euthanasia on day +4. Throughout the experimental period, all mice had ad libitum access to drinking water.

Sample Collection and Analysis

Twenty-four-hour consumption of food and water, urine output, and urine osmolality were monitored by placing the mice in individual plastic metabolic cages. At the end of the experimental periods, the mice were euthanized and blood was collected. Kidneys were removed, and cortices and medullas were dissected out, flash frozen in liquid nitrogen, and then stored at −80°C for analysis. Aliquots of urine samples were centrifuged to obtain clear supernatants. Osmolality of the clear urine supernatants and serum were determined by the vapor pressure method (Wescor, Logan, UT). Urinary sodium levels were measured on an AVL 9180 Electrolyte Analyzer (AVL Scientific, Roswell, GA). Total nitrate/nitrite contents of urine samples were determined by a commercial colorimetric kit (Cayman Chemical, Ann Arbor, MI). Urinary excretion of PGE2 was determined using a prostaglandin E metabolite EIA kit (Cayman Chemical) as described previously (56, 57). Urinary 8-isoprostane was quantified by an EIA kit from Oxford Biomedical Research (Oxford, MI).

Western Blot Analysis of Tissue Samples

Cortical and medullary tissue samples were processed separately, and Western blotting was performed by the methods established in our laboratories (10, 43, 45). Briefly, samples were prepared by homogenizing the frozen tissues in a buffer containing protease inhibitors. After determination of the protein concentrations, the homogenates were solubilized in Laemmli sample buffer. The quality of tissue sample preparation was assessed by staining loading gels with Coomassie blue (Gelcode Blue, Pierce Endogen, Rockford, IL) and then examining the sharpness of the bands. To assess the alterations in the protein abundances of different sodium transporters, semiquantitative immunoblotting was performed. For immunoblotting, 10–30 μg of protein from each sample was loaded into individual lanes of minigel s of 7, 10, or 12% polyacrylamide (precast, Bio-Rad, Hercules, CA). Blots were probed with our own rabbit peptide-derived polyclonal antibodies against NHE3, NKCC2, NCC, sodium-phosphate cotransporter (NaPi-2), and the three subunits of ENaC (α, β, and γ), as previously described (39). We used a commercial monoclonal antibody against the α-1 subunit of Na-K-ATPase (Millipore, Temecula, CA). Loading accuracy was evaluated by reprobing nitrocellulose membranes with β-actin monoclonal antibody (Sigma).

Quantitative Real-Time RT-PCR Assay of Tissue Samples

These were performed as per the methods established in our laboratory (52, 53). Briefly, total RNA from cortical or whole medullary tissue samples was extracted by the TRIzol method (Invitrogen, Carlsbad, CA), and traces of genomic DNA were removed. Clean RNA samples were processed by SuperScript Reverse Transcriptase II (Invitrogen) to obtain cDNA samples. cDNAs for target genes were quantified by real-time amplifications in the Applied Biosystems 7500 Real-Time PCR system (Foster City, CA) with AmpliTaq gold, and SYBR Green was used for detection. cDNAs were amplified for 40 cycles. Table 1 shows the sequences of the primers, annealing temperatures, and amplicon sizes. Specificity of amplifications was assessed by sequencing the PCR products in the DNA core facility at the University of Utah, and blasting them in the National Center for Biotechnology Information (NCBI) nucleotide database. Expression of target genes was computed relative to the expression levels of the housekeeping gene β-actin.

Statistical Analysis

Quantitative data are expressed as means ± SE. Differences between the means of two groups were determined by an unpaired or paired t-test. P values <0.05 were considered significant.
RESULTS

Effect of a High-Sodium Diet

Water intake and urine parameters. The body weights of WT and P2Y2-R KO mice used in this study were 25.5 ± 0.2 vs. 22.0 ± 0.2 g, respectively, which were significantly different (P < 0.001 by unpaired t-test). Hence, the urine parameters in each mouse, except for urine osmolality, were normalized to 20 g body wt before determination of the group mean values. In both WT and P2Y2-R KO mice, water intake and urine output increased and urine osmolality decreased as a function of time (Fig. 1). Water intake on days 3, 6, and 14, and urine output on day 14, were modestly, but significantly higher in the P2Y2-R KO mice compared with the WT mice. No genotype differences in urine osmolalities were observed with a high-sodium diet.

Sodium exchanger, transporter, and channel subunit expression. Figure 2A shows immunoblots from WT and P2Y2-R KO mice fed a high-sodium diet. Figure 2B summarizes the densitometry values showing changes in sodium transporter/channel protein abundances in the cortex and medulla in P2Y2-R KO mice, relative to their respective WT controls. When fed a high-sodium diet, the protein abundances of NKCC2 in the cortex and medulla of P2Y2-R KO mice were significantly increased compared with WT mice. P2Y2-R KO mice also showed significant increases in protein abundances of α-ENaC and α-1-Na-K-ATPase in the medulla compared with the WT mice. NCC abundance was not regulated differentially between the genotypes under high-sodium intake.

Urinary excretion of NOx/NO2 and PGE2. Figure 3A shows urinary excretion of total nitrates/nitrites, a measure of NO production, in WT and P2Y2-R KO mice before (day 0) and after 14 days of high-sodium diet feeding (day 14). As shown, both WT and P2Y2-R KO mice showed marked increases in urinary nitrates/nitrites following high-sodium diet feeding. However, the increase in P2Y2-R KO mice was significantly higher than in WT mice. Figure 3B shows significant differences between WT and P2Y2-R KO mice in urinary excretion of PGE2 following 14 days of a high-sodium diet. As shown, both genotypes had significant elevations in urinary PGE2 excretion from their respective baseline (day 0) values. However, the mean urinary excretion of PGE2 in P2Y2-R KO mice on day 14 was about twofold higher compared with WT mice. Figure 3C shows urinary 8-isoprostane levels on days 0 and 14. Day 0 isoprostane levels, similar to day 0 PGE2 levels, were slightly higher in P2Y2-R KO mice vs. WT mice. However, following high-sodium diet feeding, P2Y2-R KO mice had about a threefold increase in the urinary 8-isoprostane. The modest increase in 8-isoprostane levels in WT mice on day 14 was not statistically significant from day 0 value.

Combined Effect of a High-Sodium Diet and Aldosterone Infusion

Water intake, urine parameters, and sodium. The mean body weights of WT and P2Y2-R KO mice used in this study were not significantly different (20.0 ± 0.4 vs. 19.4 ± 0.5 g). Water intake, urine output, and urine osmolality in both WT and P2Y2-R KO mice were within normal limits and did not differ during the low-sodium period (data not shown). However, when the diet was switched to high sodium, both genotypes showed time-dependent increases in water intake and urine osmolality, associated with a decrease in urine osmolality. There were no significant differences between the genotypes (data not shown). However, when daily urinary excretion of sodium was monitored, significant differences between the two genotypes were noted toward the end of the experimental period. As shown in Fig. 4A, on days +3 and +4, the urinary excretion of sodium was significantly higher in WT, compared with P2Y2-R KO mice. Figure 4B shows the differences between genotypes in sodium excretion. During the low-sodium diet feeding, the differences were very low and were close to zero, indicating that both WT and P2Y2-R KO mice were able to excrete sodium to the same extent. However, after the switch to the high-sodium diet, the P2Y2-R KO mice showed significant impairment in their ability to excrete sodium from day +2 onward. The differences on days +3 and +4 were marked and were negative, indicating that P2Y2-R KO mice were not able to excrete sodium as efficiently as the WT mice.

Sodium exchanger, transporter, and channel subunit expression. Figure 5A shows immunoblots from WT and P2Y2-R KO mice fed high-sodium diet in a background of aldosterone infusion. Figure 5B summarizes the densitometry values showing changes in sodium transporter and channel subunit protein abundances in the cortex and medulla in the P2Y2-R KO mice, relative to their respective WT controls. In the cortex, P2Y2-R KO mice had significantly higher levels of NHE3, but significantly lower levels of NKCC2 and α- and β-ENaC. In the medulla, we found significantly elevated NKCC2 in P2Y2-R KO (similar to what was observed with the high-sodium diet alone series). The protein abundances

Table 1. Nucleotide sequences of primer pairs used in PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Primer Position</th>
<th>Primer Sequence</th>
<th>Annealing Temperature, °C</th>
<th>Amplicon Size, bp</th>
<th>Reference Source</th>
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<tbody>
<tr>
<td>iNOS</td>
<td>NM_010927.2</td>
<td>2563–2582</td>
<td>ACTCTTCGTGGCTGAGTTCTCT</td>
<td>60</td>
<td>176</td>
<td>17</td>
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<tr>
<td>eNOS</td>
<td>NM_008713.3</td>
<td>513–533</td>
<td>GAGAGGAAGCTGTTGTTG</td>
<td>60</td>
<td>188</td>
<td>17</td>
</tr>
<tr>
<td>β-Actin</td>
<td>NM_007393.2</td>
<td>1034–1054</td>
<td>GCCGCTCGTCCTACAGCATTG</td>
<td>60</td>
<td>73</td>
<td>17</td>
</tr>
<tr>
<td>COX-1</td>
<td>NM_008969.3</td>
<td>1406–1429</td>
<td>GCGGGCGGGGAGAGGAGG</td>
<td>60</td>
<td>150</td>
<td>Designed by us</td>
</tr>
<tr>
<td>COX-2</td>
<td>NM_011198.3</td>
<td>1389–1408</td>
<td>TGTAAGACTGGCGAGAGAGCCC</td>
<td>60</td>
<td>282</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1692–1671</td>
<td>GAGTATTGCAGAGAACAGATG</td>
<td></td>
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</tr>
</tbody>
</table>

iNOS and eNOS, inducible and endothelial nitric oxide synthase, respectively; COX, cyclooxygenase.
of no other transporter/channel subunits shown were different between genotypes.

Urinary excretion of NO\textsubscript{3}/NO\textsubscript{2} and expression of NOS isoforms in the kidney. As shown in Fig. 6A, urinary excretion of NO\textsubscript{3}/NO\textsubscript{2} on days \(-3\) and \(0\) were low in WT and P2Y\textsubscript{2}-R KO mice and were not significantly different from each other. However, on day \(+4\), both WT and P2Y\textsubscript{2}-R KO mice had a several-fold increase in the urinary excretion of PGE\textsubscript{2}. The increase in P2Y\textsubscript{2}-R KO mice was significantly higher than that in the WT mice. To gain insight into the probable cause for this observed difference on day \(+4\), we determined the mRNA expression of iNOS and eNOS relative to the mRNA expression of \(\beta\)-actin in the cortex and medulla of these two groups of mice. As shown in Fig. 6B, relative expression of COX-2 in the cortex showed a significant twofold increase in the P2Y\textsubscript{2}-R KO mice compared with the WT mice. In addition, COX-1 expression in the cortex was modestly, but significantly lower in the P2Y\textsubscript{2}-R KO mice vs. the WT mice. No significant differences between the WT and P2Y\textsubscript{2}-R KO mice could be seen with respect to the relative expression of COX isoforms in the medulla (Fig. 6C).

Urinary excretion of 8-isoprostane. Figure 8 shows urinary levels of 8-isoprostane in WT and P2Y\textsubscript{2}-R KO mice on days \(-3\) and \(+4\). As shown, the urinary 8-isoprostane levels in both groups were low on day \(-3\), but the P2Y\textsubscript{2}-R KO mice had significantly higher levels compared with the WT mice. However, on day \(+4\) the P2Y\textsubscript{2}-R KO mice had a marked increase, whereas the corresponding increase in WT mice is lower, resulting in about a twofold higher urinary excretion of 8-isoprostane in P2Y\textsubscript{2}-R KO mice vs. WT mice on day \(+4\).

**DISCUSSION**

Several studies have already documented dietary sodium- and/or aldosterone-induced alterations in the expression and/or protein abundances of sodium transporters in the kidney (reviewed in Ref. 21). Hence, the major focus of the current study was to evaluate the role and extent of contribution of signaling through P2Y\textsubscript{2}-R for the alterations induced in the kidney by a high-sodium diet or a high-sodium diet plus aldosterone infusion. The use of P2Y\textsubscript{2}-R KO mice, with WT mice serving as controls, allowed us to evaluate the role of P2Y\textsubscript{2}-R independently of other variables, such as sodium intake or plasma aldosterone levels in the two series of experiments. Our study addressed three questions: 1) what is the effect of genetic deletion of P2Y\textsubscript{2}-R on the alterations in sodium transporter/channel expression induced by a high-sodium diet?; 2) what is the effect of genetic deletion of P2Y\textsubscript{2}-R on the alterations in natriuresis and sodium transporter/channel expression induced by a high-sodium diet plus aldosterone?; and 3) does genetic deletion of P2Y\textsubscript{2}-R influence the effect of NO and prostanoid systems or oxidative stress vis-à-vis natriuresis and sodium transporter/channel expression in the above two models? Our study revealed that genetic deletion of P2Y\textsubscript{2}-R had significant effects in both series of experiments, thus providing insights into the potential role of this receptor in maintaining sodium homeostasis. First, these two experi-
mental conditions resulted in significant alterations in the protein abundances of some, but not all, major sodium transporters/channels/exchangers along the nephron in the P2Y2-R KO mice vs. WT mice. Second, when fed a high-sodium diet in the background of aldosterone infusion, P2Y2-R KO mice had impaired natriuresis compared with WT mice. Third, further probing in both series revealed significant differences in the urinary excretion of NO, PGE2, and 8-isoprostane in P2Y2-R KO mice vs. WT mice, suggesting that these signaling factors may play an important role, and genetic deletion of P2Y2-R had a profound influence on these effects. Finally, our study provided significant mechanistic insights by documenting changes in the expression of enzymes involved in the biosynthesis of NO or PGE2 in the kidneys of P2Y2-R KO mice vs. WT mice fed a high-sodium diet in the background of aldosterone infusion. Taken together, these observations underscore the potential sodium-homeostatic value of P2Y2-R, especially in sodium-retentive states, such as a high-sodium diet with/without high aldosterone.

Fig. 2. A: immunoblots for different sodium transporter and channel proteins in WT and P2Y2-R KO mice fed a high-sodium diet for 14 days (n = 5 mice/genotype). Blots in the left column correspond to cortical tissue samples, and those in the right column to medullary tissue samples. NaPi-2, NHE3, NKCC2, NCC, ENaC: Na-Pi type 2 cotransporter; sodium/phosphate exchanger isoform 3; Na-K-2Cl cotransporter; and epithelial Na channel, respectively. B: bar graph summary of the densitometric analysis of the immunoblots shown in A. Bars show percent change in band densities of different proteins in the cortex and medulla of P2Y2-R KO mice compared with the corresponding values in WT mice (100%). *Significant change (P < 0.05) in the protein band density in P2Y2-R KO mice vs. WT mice by unpaired t-test.
We challenged P2Y2-R KO and WT mice with a high-sodium diet for 14 days. The salient finding of this study was markedly increased protein abundance of NKCC2 in the cortex and medulla, as well as $\alpha$-1-Na-K-ATPase in the medulla of P2Y2-R KO mice compared with the WT mice. In normal animals, the expression and activity of NKCC2 is regulated by AVP via its V2 receptor and the associated cAMP signaling pathway (11, 12). Agonist activation of P2Y2-R is known to antagonize the action of AVP by stimulating phospholipases and protein kinase C among other pathways (20, 37). It is possible that genetic deletion of P2Y2-R resulted in sensitization of the thick ascending limb to the action of AVP in the P2Y2-R KO mice fed a high-salt diet, resulting in increased protein abundance of NKCC2 compared with high-salt diet-fed WT mice. This, coupled with the significantly increased protein abundance of $\alpha$-1-Na-K-ATPase in the medulla, might result in increased sodium reabsorption in the medullary thick ascending limb in the P2Y2-R KO mice.

Another important finding in our high-sodium diet series was significantly increased protein abundances of the $\alpha$-ENaC subunit in the medulla of P2Y2-R KO mice. This may contribute to increased apical sodium reabsorption in the distal nephron. Aldosterone has been demonstrated to increase the abundance of this protein (27). Thus increased...
levels of this protein might be manifested as a result of altered sensitivity to aldosterone in the P2Y2-R KO mice. On the other hand, vasopressin has also been documented to increase the protein abundance and mRNA expression of the \( \alpha \)- and \( \gamma \)-subunits of ENaC (9, 10). However, we did not see significant alterations in these subunits in the high-sodium diet-fed P2Y2-R KO mice in this study.

Thus our findings are, for the most part, in agreement with those of Vallon and associates (36) using short-period (2 h) pharmacological blockade in conscious P2Y2-R P2Y2-R KO and WT mice receiving a standard NaCl diet, who showed that furosemide, but not chlorthiazide or low-dose amiloride, resulted in significantly higher urine sodium excretion in P2Y2-R KO mice compared with the WT mice. However, this does not exclude the possibility of P2Y2-R-mediated fine-tuning of amiloride-sensitive sodium absorption in the distal nephron, especially when confronted with a high-sodium diet.

**Fig. 5.** A: immunoblots for different sodium transporter and channel proteins in WT and P2Y2-R KO mice fed a high-sodium diet in a background of aldosterone infusion (3 mice/genotype). Blots in the left column correspond to cortical tissue samples, and those in the right panel to medullary tissue samples. B: bar graph summary of the densitometric analysis of the immunoblots shown in A. Bars show percent change in band densities of different proteins in the cortex and medulla of P2Y2-R KO mice compared with the corresponding values in WT mice (100%). *Significant change (\( P < 0.05 \)) in the protein band density in P2Y2-R KO mice vs. WT mice by unpaired \( t \)-test.
Since a high-sodium diet is usually accompanied by a fall in circulating levels of aldosterone, in the second series we sought to separate the effects due to differences in circulating aldosterone levels from all responses downstream of this, including differences in mineralocorticoid receptor sensitivity and/or ability to “escape” from the sodium-retaining actions of aldosterone (35, 47). This experimental protocol was designed to separate primary effects of aldosterone from the escape process by initiating the aldosterone infusion 3 days before a switch from a low-sodium diet to a relatively high-sodium intake. During the initial 3-day period, the high level of circulating aldosterone resulting from the

Fig. 6. Effect of combination of a high-sodium diet and aldosterone infusion on urinary excretion of total NO3 and NO2 and relative mRNA expression of nitric oxide synthase (NOS) isoforms in the renal cortex and medulla of WT and P2Y2-R KO mice. A: urinary excretion of total NO3/NO2 as nmol/day on days −3, 0, and +4. *Significantly different from the corresponding value in WT mice (P < 0.05 by unpaired t-test). B: mRNA expression of NOS isoforms relative to the expression of β-actin in the cortex of WT and P2Y2-R KO mice on day +4. Results are plotted as percentage of respective mean values in WT mice (100%). *Significantly different from the corresponding value in WT mice (P < 0.01 unpaired t-test).

Fig. 7. Effect of combination of a high-sodium diet and aldosterone infusion on urinary excretion of PGE2 metabolite and relative mRNA expression of cyclooxygenases (COX) in the renal cortex and medulla of WT and P2Y2-R KO mice. A: urinary excretion of PGE2 metabolite on days −3, 0, and +4 (ng/day). *Significantly different from the corresponding value in WT mice (P < 0.05 by unpaired t-test). B: mRNA expression of COX-1 relative to the expression of β-actin in the cortex of WT and P2Y2-R KO mice on day +4. Results are plotted as percentage of respective mean values in WT mice (100%). *Significantly different from the corresponding value in WT mice (P < 0.01 by unpaired t-test).

Effect of a High-Sodium Diet Plus Aldosterone

Since a high-sodium diet is usually accompanied by a fall in circulating levels of aldosterone, in the second series we sought to separate the effects due to differences in circulating aldosterone levels from all responses downstream of this, including differences in mineralocorticoid receptor sensitivity and/or ability to

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infusion was appropriate for the low level of sodium intake. Only after the sodium intake was increased at day 0 there was a stimulus for the escape process (47). We used a dose approximately equivalent to what Knepper et al. or Riazi et al. used in rats (200 μg/day) (35, 47) based on body size comparisons, but lower than what Knepper or Riazi used when translated from rat to mouse species by computation based on body surface area and $K_m$ factor for these two species (34). However, mice in general have much lower plasma aldosterone levels compared with rats (1, 13, 18, 26). Thus we felt the dose was clearly adequate to increase sodium retention via aldosterone-sensitive pathways.

After the mice were switched to a high-sodium diet, during the first 2 days both WT and P2Y2-R KO mice had comparable increases in urinary excretion of sodium. However, during the last 2 days of high-sodium diet feeding in the background of aldosterone infusion, the WT mice showed an abrupt and significant increase in urinary sodium excretion. The urination of sodium in P2Y2-R KO mice continued to increase in a linear fashion, but at a lower rate of increase than in the WT mice. We did not quantify sodium losses through feces and sweat from paw pads. Thus we could not definitively compute sodium balance.

Western blot analysis of kidney samples showed a marked (>2-fold) increase in the protein abundance of NHE3 in the cortex, and an associated significant increase in NKCC2 in the medulla of P2Y2-R KO mice vs. WT mice. However, the protein abundances of NaPi-2, NKCC2, and α- and β-ENaC in the cortex are modestly, but significantly lower in the P2Y2-R KO mice vs. WT mice. Hence, it is possible that markedly elevated cortical levels of NHE3 protein and/or modestly elevated medullary NKCC2 protein in the P2Y2-R KO mice vs. WT mice might have contributed for the impaired natriuresis observed in the P2Y2-R KO mice, a notion that was supported by the following mechanistic insights.

Alterations in NO and Prostanoid Systems or Oxidative Stress

In both series of experiments, we explored the potential influence of P2Y2-R on the NO and/or prostanoid systems or oxidative stress. The rationale for these studies is as follows. eNOS has been demonstrated to inhibit sodium transport by affecting apical membrane sodium channels in cultured collecting duct cells (41), and chronic inhibition of NO synthesis in rats has been shown to result in increased expression of major sodium transporters in the kidney. Thus endogenously derived NO may exert a tonic inhibitory effect on the expression of major sodium transporters, including Na-K-ATPase, NHE3, NKCC2, and NCC (19). Furthermore, it has been shown that extracellular nucleotides activate eNOS and increase NO generation, leading to eNOS phosphorylation at Ser-1177 in human umbilical vein endothelial cells. The latter apparently involved P2Y1-R, P2Y2-R, and P2Y4-R and was independent of adenosine, but dependent on intracellular calcium and PKC-δ (9). Thus there is a potential link between the P2Y2-R and NO system. Furthermore, COX inhibitors are known to enhance urinary concentrating ability especially by increasing the protein abundance of NKCC2 (14), one of the major sodium transporters in the kidney, and is responsible for the generation of medullary osmotic gradients. Also, we have demonstrated the interaction of P2Y2-R with the prostanoid system in the medullary collecting duct (20, 45, 52), the major site of PGE2 production in the kidney.

We observed that following high-sodium diet feeding, both WT and P2Y2-R KO mice had elevated urinary NO3/NO2 excretion, indicating increases in NO production in both genotypes. However, the elevation in P2Y2-R KO mice was significantly higher compared with the WT mice. On the other hand, when mice were fed a high-sodium diet in a background of aldosterone infusion, the urinary NO3/NO2 levels in WT mice increased even further, whereas the levels in P2Y2-R KO mice were significantly lower. This contrasting picture between the two studies suggests that in the absence of P2Y2-R signaling, with a high-sodium diet mice require greater NO production. The combination with aldosterone likely pushes them beyond the point where they can effectively compensate. This may explain the inability to excrete equivalent amounts of sodium by the two genotypes at that point. This notion was further supported by the observation of significantly lower relative expression of both iNOS and eNOS isoforms in the cortex, and iNOS in the medulla of P2Y2-R KO mice compared with WT mice. These observations were also consistent with the reported stimulatory effects of P2Y2-R, such as P2Y1-R, P2Y2-R, and P2Y4-R, on NOS isoforms (iNOS and eNOS) in the pulmonary system or human umbilical vein endothelial cells (8, 15). Furthermore, in agreement with an inhibitory effect of NO on expression of NHE3 and NKCC2, we did find reduced cortical NHE3 and medullary NKCC2 in the WT vs. P2Y2-R KO mice administered a high-sodium diet combined with aldosterone infusion.

As stated above, COX inhibitors enhance urinary concentration ability by increasing the abundance of NKCC2 (14). In this context, the enhanced production of PGE2 seen in high-sodium diet-fed plus aldosterone-infused P2Y2-R KO mice vs. high-sodium diet-fed P2Y2-R KO mice was perhaps a redundant mechanism to alleviate sodium retention, especially since NO generation may have decompensated at this point. Furthermore, increased PGE2 perhaps accounts for the lowering of cortical NKCC2 protein abundance in the P2Y2-R KO mice observed under this condition, but not seen in the high-sodium diet alone study. This notion was supported by the observed induction of COX-2 in the cortex.
but not in the medulla of P2Y2-R KO mice fed a high-sodium diet in a background of aldosterone infusion.

Strikingly, our study provides evidence that enhanced oxidative stress induced by a high-sodium diet and/or aldosterone in P2Y2-R KO mice relative to WT mice may play a potential modulatory role in the outcome of the effect of NO and PGE2 on sodium transporters. The significant increases in urinary 8-isoprostane seen in P2Y2-R KO mice vs. WT mice in both series of experiments were an indication that in the absence of P2Y2-R signaling the oxidative stress induced by a high-sodium diet and/or aldosterone was much higher. The increase in oxidative stress is apparent higher in the combined high-sodium diet and aldosterone study compared with the high-sodium diet alone study, which then might be responsible for urine NO3/NO2 levels in the combined high-sodium diet and aldosterone regimen. The increase in oxidative stress is apparently induced by a high-sodium diet and/or aldosterone, and P2Y2-R nitric oxide (NO) and oxidative stress (OS) in relation to renal sodium excretion. It is increasingly becoming clear that an integrated approach, such as the one shown in Fig. 9, is needed to understand the complex nature of renal sodium handling in health and disease. The figure also shows the potential induction of COX-2 by the combined effect of aldosterone and a high-sodium diet in the absence of P2Y2-R.

Finally, at the time of submission of this manuscript, Stockand and associates (40), using the P2Y2-R KO mice, reported that control of ENaC by purinergic signaling is necessary for aldosterone escape. A role for ENaC in aldosterone escape agrees with what we published previously (35) showing decreased abundance of the “70 kDa-band” of α-ENaC, a band thought to be due to an activating cleavage of the major band in aldosterone escape in high-NaCl-fed vs. low-NaCl-fed aldosterone-infused rats. Our current study is also in agreement with the findings of Stockand et al. (40) in that we found relatively lower levels of the major band associated with β-ENaC in the P2Y2-R KO mice, which has also been reported as an aldosterone-like pattern (28). However, no significant difference was found for γ-ENaC. In addition, our study uncovers the potential role of important signaling mediators, such as NO and prostaglandins in determining these differences in the P2Y2-R KO mice. It also demonstrates, for the first time, the likelihood for the existence of oxidative stress in these mice in the absence of P2Y2-R, especially in the high-stress environment of high salt and/or aldosterone infusion.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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