Renalase regulates renal dopamine and phosphate metabolism

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THERE IS EXTENSIVE EXPERIMENTAL support for the notion that the proximal tubule generates dopamine (DA) and that endogenous intrarenal DA contributes to the regulation of renal sodium and phosphate excretion (9, 16–18, 20, 21). A physiological role of DA in P1 metabolism was strongly suggested by the findings that increased dietary phosphate intake from 0.7% to 1.8% significantly increased excretion of free dopamine by 23%, dopamine glucuronide by 32%, DA metabolite DOPAC by 30%, and total dopamine excretion by 27% (6). The effect of high P1 diet on DA metabolism did not require the presence of renal nerves, confirming that P1 intake regulates intrarenal DA synthesis by the proximal tubule (5). Additional support for intrarenal DA’s key role in P1 metabolism was provided by studies in which inhibition of aromatic amino acid decarboxylase (AADC; rate-limiting step in DA synthesis from l-DOPA) by carbidopa- or benserazide-decreased P1 excretion (12). The molecular mechanism underlying the effect of renal DA on P1 excretion has been investigated using freshly isolated mouse kidney slices, perfused proximal tubules, and cultured renal epithelial cells. Selective D1-like receptor agonists applied to the luminal membrane for 1 h induced Npt2a internalization and reduced expression of Npt2a in the brush border membrane (2).

The steady-state concentration of luminal DA depends of the rate of synthesis by the proximal tubule and breakdown by monoamine oxidases (MAO) and catechol-O-methyltransferase (COMT). MAO and COMT are intracellular enzymes and would require that luminal DA be transported into cells. The relative importance of MAO and COMT in the regulation of daily urinary excretion of dopamine and metabolites and on natriuresis and phosphaturia in 3/4 nephrectomized (3/4nX) and sham rats were examined (25, 26). Selective or combined inhibition of MAO-A and COMT for 3 days in sodium replete, partially nephrectomized (5/6 Nx), and sham-operated rats did not alter the daily urinary excretion of dopamine, sodium, or phosphate in either group. These data suggest that inhibition of MAO-A and COMT is not of major importance in regulating the dopamine-dependent natriuresis and phosphaturia in either 5/6 Nx or sham rats. These data are rather surprising and suggest that catecholamines metabolizing enzymes other than MAO and COMT might play a key role in the short-term regulation of luminal DA and, therefore, sodium and phosphate excretion.

We recently identified renalase, a novel FAD-dependent oxidase that is secreted into blood by the kidney and is hypothesized to participate in catecholamine metabolism (19, 30). Plasma renalase levels are decreased in patients with chronic kidney disease (CKD) and end-stage renal disease (ESRD), and in animals subjected to subtotal nephrectomy (5/6 Nx). Plasma catecholamines are markedly elevated in a global mouse knockout (KO) model. These animals also develop mild hypertension and increased sensitivity to cardiac ischemia (29).

Luminal catecholamines are important regulators of renal sodium and phosphate metabolism. Changes in renal dopamine concentration modulate the signaling pathways that control renal tubular P1 reabsorption. For instance, wild-type mice fed a high-phosphate (1.2%) diet for 24 h had a significant increase in renal and urinary DA and phosphate excretion (28). This was accompanied by increased DA synthesis by aromatic acid decarboxylase (AADC) and decreased activity of renalase, monoamine oxidase A, and monoamine oxidase B. A low-phosphate diet markedly decreased DA and phosphate excretion (28). These data indicate that the rapid adaptation to a high-phosphate diet involves alterations in key enzymes involved in dopamine synthesis and degradation. Micropuncture data suggest that, once DA is secreted into the proximal tubular lumen, it does not reenter PT cells (10), and it has been suggested that renalase may be particularly well suited to regulate urinary DA levels, since, unlike MAO-A, MOA-B,
and COMT, it is secreted in urine (13). In the present work, we used renalse mouse KO model to further investigate the role of renalse in renal phosphate excretion and find that renalse deficiency increases urinary DA and leads to phosphate wasting and hypophosphatemia.

MATERIALS AND METHODS

Animal genotyping, dieting, and tissue collection. All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and with protocols approved by institutional animal care and use committee of VA Connecticut Healthcare System. Renalase KO mice (29) and WT controls of the same genetic background were identified by PCR of tail genomic DNA (WT, 499-bp product, WT forward primer 5'-AAATCCCCAGTTACATTAGGCTCC-3' and WT reverse primer 5'-gagacagtgacagagagaaaccggt-3'; KO, 292-bp product, KO forward primer 5'-AGGCTATTCGGCTATGACTGGG-3' and KO reverse primer 5'-TGGATACTTCTCCAGTAGGC-3'). Age-matched animals (5–7 mo old) were fed low (Teklad custom diet, 0.1% phosphate), regular (Teklad global 0.6% phosphate, 18% protein rodent diet), or high-phosphate diet for 4 consecutive days.

Gene expression analysis. Total RNA from tissue samples was extracted using RNA-Bee solution (Tel-test, Friendswood, TX) and converted into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Levels of monoamine oxidase A (MAO-A), monoamine oxidase B (MAO-B), COMT-1, sodium-dependent phosphate transport protein 1 (Npt1), sodium-dependent phosphate transport protein 2A (Npt2a), sodium-dependent phosphate transport protein 2C (Npt2c), and Renalase mRNAs in kidney, heart, liver, and brain tissues were assessed by real-time PCR (TaqMan) and normalized to the corresponding levels in WT samples on low-phosphate diet (defined as 1.0). TaqMan assay mixtures were used: Mm00558004_m1 (MAO-A), Mm00555412_m1 (MAO-B), Mm00514377_m1 (COMT-1), Mm00436577_m1 (Npt1), Mm00441450_m1 (Npt2a), Mm00551746_m1 (Npt2c), Mm00725038_m1 (Renalase), as well as mouse GAPDH endogenous control assay, and TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, CA). Real-time PCRs were carried on Eppendorf Realplex Masterscycler machine, and the resulting Ct values were analyzed using the 2-ΔΔCT method.

Serum Pi, and urinary DA measurements. Blood samples were collected from renalse KO and WT mice fed regular, low-, or high-phosphate diet for 5 consecutive days. Mice were anesthetized using 30% isoflurane solution in 1,2-propanediol and then subjected to retro-orbital bleeding. Blood samples were incubated 45 min at room temperature, and serum supernatant was separated after 15 min at 1,500 RPM, room-temperature centrifugation. Urine samples were collected in metabolic cages (Lab Products, Seaford, DE) every 24 h for 5 consecutive days (24 h on low-phosphate diet as control). Urine creatinine as well as serum and urine phosphate levels were assessed by VA Analytical Laboratory (VAMC, West Haven, CT). DA and L-DOPA levels were assayed by HPLC with electrochemical detection (HPLC-ED) (lower limit of detection of ~350 fmol), as previously described (27).

Enzymatic activity assays. MAO-A and MAO-B activities were tested using the AmplexRed kit (Invitrogen, Grand Island, NY). This assay is based on detection of H2O2 (produced as a result of MAO-A/B activity) in a reaction producing resorufin, a stable substance with fluorescence maximum at 585 nm. Kidney tissue was homogenized in ice-cold protein extraction buffer [10% glycerol, 10 mM NaCl, 1 mM

Fig. 1. Hypophosphatemia and Pi wasting in renalse knockout (KO) mice. A: animals (KO mice, n = 5; wild-type (WT) mice, n = 5) maintained on a regular PO4 diet for 4 days. B: animals (KO mice, n = 6; WT mice, n = 6) maintained 24 h on low-phosphate diet, then 4 days on regular PO4 diet. C: animals (KO mice, n = 6; WT mice, n = 6) maintained on low PO4 diet. D: animals (KO mice, n = 6; WT mice, n = 6) maintained 24 h on regular PO4 diet, then 4 days on low PO4 diet. Filled columns are KO mice, and open bars are WT controls. *Significant difference (P < 0.05).
EDTA, 0.5 mM DTT, 25 mM BisTris, pH 6.5, Protease Inhibitor Cocktail tablet (Roche), and 500 μg of resulting kidney protein extract was used per well. MAO-A and MAO-B activities were distinguished by using different substrates (p-Tyramine for both MAO-A and MAO-B and benzylamine for MAO-B only) and specific inhibitors (clorgyline for MAO-A and pargyline for MAO-B). Five WT and five KO kidneys were used per each diet; differences with \( P < 0.05 \) (Student’s unpaired t-test) were reported as statistically significant.

COMT-1 activity was assessed by \(^{3}H\)-methyl group transfer assay (24). This test is based on organic solvent extraction that separates the radioactive product (methylated catechol) from free radioactive cofactor (\(^{3}H\)-SAM). Total protein extract was prepared as described for MAO-A/B activity test, and 100 μl of resulting extract was used per sample. Tolcapone (5 μl) (COMT-1 inhibitor) was added to control samples to detect the baseline signal. Betafluor (National Diagnostics, Charlotte, NC) scintillation fluid was used for efficient separation of organic and water phases.

AADC activity was determined as previously described by Soares-da-Silva (27) using L-DOPA as substrate (100–10,000 μM, Sigma). Fragments of renal cortex were homogenized at 4°C with a Thomas Teflon homogenizer (Poliscience) using 2 ml of resulting kidney protein (wt/vol) in the incubation medium containing 0.5 mM 3S NaH\(_2\)PO\(_4\), 0.15 mM Na\(_2\)H\(_2\)PO\(_4\), 0.11 mM Na\(_2\)B\(_4\)O\(_7\), and 0.2 mM pyridoxal phosphate (pH 7.0). The assay of dopamine was performed by HPLC with electrochemical detection.

Western blotting and immunofluorescence assay. For immunofluorescence (IF) studies, the kidney was perfusion-fixed with paraformaldehyde. IF and Western blot were performed using methods previously described (7, 8). For Western blot analysis, brush-border membrane vesicles were obtained as previously described (14). The megalin NHE3 and Npt2a polyclonal antibodies were gifts of Dr. Biemesderfer (Yale School of Medicine).

Statistical analysis. Standard unpaired Student’s t-tests were used for group comparisons at equivalent periods. All data are means ± SE, and \( P < 0.05 \) was accepted as a statistically significant difference.

RESULTS

Hypophosphatemia and phosphate wasting in renalase KO mice. The proximal tubule mediates phosphate absorption. Since the renalase gene is highly expressed in kidney, and the protein is almost exclusively found in the proximal tubule (15), we tested whether renalase deletion affected PO\(_4\)\(^{-}\) excretion. Compared with WT mice, KO animals maintained on a regular diet, containing 0.9% P\(_{i}\), have significantly lower serum PO\(_4\)\(^{-}\) (Fig. 1A) and higher urinary PO\(_4\)\(^{-}\) excretion (Fig. 1B). To test whether renalase KO animals could regulate renal P\(_{i}\) absorption, adaptation to PO\(_4\)\(^{-}\) deprivation was examined and compared with that of WT. Although KO mice fed a low-PO\(_4\)\(^{-}\) diet for 4 days had lower serum PO\(_4\)\(^{-}\) than WT mice (Fig. 1C), urinary excretion decreased in both WT and KO (Fig. 1D). The magnitude of the decrease in P\(_{i}\) excretion was greater in KO up to day 3, and at day 4 both groups were equivalent (Fig. 1D).

In KO animals on regular P\(_{i}\) diet, the daily urinary excretion of DA increased twofold compared with WT (Fig. 2A). A low-P\(_{i}\) diet markedly decreased DA excretion (357 ± 42 pmol/24 h; \( n = 4; P < 0.05 \)) at day 4 in renalase KO mice. COMT-1 expression (Fig. 2B) and activity (Fig. 2C) increased in both WT and KO maintained on a low P\(_{i}\) diet. MAO-A activity (\( V_{\text{max}} \); mmol-mg protein\(^{-1}\)-h\(^{-1}\)) was unchanged (WT = 12.09 ± 0.87; KO = 10.45 ± 0.55; \( n = 8 \)), and MAO-B activity decreased (WT = 1.77 ± 0.07; KO = 1.22 ± 0.05; \( n = 8; P < 0.05 \)). These data indicate that KO mice respond normally to PO\(_4\)\(^{-}\) deprivation by upregulating COMT-1 activity to reduce urinary DA levels and subsequently increase PO\(_4\)\(^{-}\) absorption.

Sodium/phosphate cotransporters expression unchanged in renalase KO. We next tested for differences in expression of phosphate transporters between KO and WT mice. Npt1 gene expression was decreased in KO (Fig. 3A), but there is no evidence that Npt1 contributes significantly renal PO\(_4\)\(^{-}\) metabolism (31). Npt2a, which encodes the main renal sodium/phosphate cotransporter, and Npt2c gene expression did not differ between KO and WT (Fig. 3A). Renal expression of Npt2a and NHE3 proteins were not different between WT and KO maintained on a regular P\(_{i}\) diet (Fig. 3, B and C).

Increased urinary DA and renal DA synthesis in renalase KO. Since COMT degrades DA, increased renal activity in the KO would be expected to decrease renal DA, facilitate PO\(_4\)\(^{-}\)...
absorption, and prevent hypophosphatemia. However, despite increased COMT activity, the daily urinary excretion of DA increased twofold in KO mice maintained on a regular diet (Fig. 2A). Renalase metabolizes L-DOPA and DA, with the former being the preferred substrate (15); therefore, increased urinary DA in KO mice could be the result of decreased DA metabolism and increased synthesis. Although renal tubular AADC activity was unchanged in KO mice (Fig. 4A; Table 1), there was a marked decrease in urinary excretion of L-DOPA (Fig. 4B), indicating increased L-DOPA uptake by the proximal tubule, and increased DA synthesis.

**DISCUSSION**

The regulation of phosphate transport in vivo involves short-term adaptive processes occurring within hours of changes in PO$_4^2-$ intake, and including PO$_4^2-$ sensing in the intestine, and the release or synthesis of molecules that alter the efficiency of renal PO$_4^2-$ transport (4). The intrarenal DA system is a key modulator of renal PO$_4^2-$ excretion (11). Increased PO$_4^2-$ intake stimulates renal DA synthesis, and luminal DA inhibits proximal tubular PO$_4^2-$ reabsorption (13). The proximal tubule synthesizes DA from filtered L-DOPA, and accounts for most of the urinary DA. Luminal entry of L-DOPA is mediated in part by the solute carrier family 3 member-1 (SLC3A1/SLC7A9 or rBart/b0,+AT), a high affinity L-DOPA carrier, whereas transport at the basolateral membrane occurs through the low-affinity transporter SLC7A8 (23). In PT cells, L-DOPA is converted to DA by AADC.
In the steady state, luminal DA concentration is a function of the rates of synthesis and degradation. The MAO-A, MAO-B, and COMT participate in renal DA metabolism (1). Once DA is secreted into luminal fluid, it is not reabsorbed and cannot be regulated by these intracellular enzymes. Furthermore, it is reported that the selective or combined inhibition of MAO-A and COMT does not alter the daily urinary excretion of DA, sodium, or PO$_4$ in rats (22). These results could be explained by the action of renalase in luminal fluid. Indeed, its enzymatic activity is insensitive to MAO and COMT inhibitors (30), and, in contrast to these enzymes, it is secreted by the proximal tubule into luminal fluid and is well suited to regulate luminal fluid 1-DOPA and DA levels.

In the present study, we find that renalase KO mice maintained on a regular diet excrete PO$_4$ inappropriately and develop hypophosphatemia. The increased renal PO$_4$ excretion is due to a twofold increase in urinary DA in KO mice. The changes in PO$_4$ excretion and urinary DA documented in KO mice maintained on a regular diet are similar in magnitude to those observed in WT mice fed a high-PO$_4$ diet (28). Npt2a gene and protein expression in kidney were unchanged despite high urinary DA. Since increased urinary DA is associated with internalization of Npt2a (3), one would have expected to see a decrease in plasma membrane Npt2a levels in renalase KO mice. Perhaps the reason we did not detect any significant differences is that chronic elevations of urinary DA lead to moderately severe hypophosphatemia, which in turn would stimulate proximal tubular reabsorption by upregulating Npt2a expression and activity. So the observed steady-state expression level of Npt2a in renalase KO mice reflects the balance between counteracting signals, namely elevated urinary DA and decreased serum phosphate, which would decrease and increase Npt2a expression, respectively.

Among the circulating catecholamines, renalase preferentially metabolizes epinephrine (kcat = 66.46 min$^{-1}$) (23). It does metabolizes DA (kcat = 0.11 min$^{-1}$), but it is not yet known whether the rate is fast enough to mediate a direct, in vivo action on DA levels. Renalase also metabolizes 1-DOPA (kcat = 42.32 min$^{-1}$), and, in renalase-deficient mice, 1-DOPA delivery could increase and perhaps stimulate DA synthesis. It is also possible that the renal dopaminergic system is upregulated in renalase KO mice in an attempt to maintain sodium balance and mitigate the rise in blood pressure associated with renalase deficiency (Fig. 5). The marked decrease in urinary 1-DOPA observed in the renalase KO mice strongly supports the notion of an increased renal DA synthesis. It would be interesting to investigate whether increased urinary DA in the renalase KO is associated with changes in DA receptor expression and phosphorylation, and intracellular signaling.

In summary, the renal DA system is upregulated in renalase KO mice. Increased urinary DA results in inappropriately high urinary PO$_4$ excretion, which prevents animals maintained on normal- or low-PO$_4$ diet to remain in PO$_4$ balance. The signal to increase renal DA production is unclear, and we speculate it may be part of a homeostatic mechanism designed to counteract the rise in blood pressure caused by renalase deficiency.

**REFERENCES**

F844 RENALASE REGULATES RENAL DOPAMINE AND PHOSPHATE METABOLISM


