Dysregulation of renal vitamin D metabolism in the uremic rat

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The progressive decline in kidney function and concomitant loss of renal 1α-hydroxylase (CYP27B1) in chronic kidney disease (CKD) are associated with a gradual loss of circulating 25-hydroxyvitamin D3 (25(OH)D3) and 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3). However, only the decrease in 1α,25(OH)2D3 can be explained by the decline of CYP27B1, suggesting that insufficiency of both metabolites may reflect their accelerated degradation by the key catabolic enzyme 24-hydroxylase (CYP24). To determine whether CYP24 is involved in causing vitamin D insufficiency and/or resistance to vitamin D therapy in CKD, we determined the regulation of CYP24 and CYP27B1 in normal rats and rats treated with adenine to induce CKD. As expected, CYP24 decreased whereas CYP27B1 increased when normal animals were rendered vitamin D deficient. Unexpectedly, renal CYP24 mRNA and protein expression were markedly elevated, irrespective of the vitamin D status of the rats. A significant decrease in serum 1α,25(OH)2D3 levels was found in uremic rats; however, we did not find a coincident decline in CYP27B1. Analysis in human kidney biopsies confirmed the association of elevated CYP24 with kidney disease. Thus, our findings suggest that dysregulation of CYP24 may be a significant mechanism contributing to vitamin D insufficiency and resistance to vitamin D therapy in CKD.

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KEYWORDS: adenine rat model; chronic kidney disease; 1α-hydroxylase; 24-hydroxylase; vitamin D metabolites

Vitamin D insufficiency is commonly observed in patients with chronic kidney disease (CKD) and is causally related to secondary hyperparathyroidism, a disorder characterized by elevated serum-intact parathyroid hormone (iPTH) levels, parathyroid gland hyperplasia and imbalances in bone and mineral metabolism.¹⁻³ Low vitamin D levels have also been linked to the pathogenesis of other diseases related to CKD, including diabetes,⁴ hypertension,⁵ and obesity.⁶,⁷ External factors, such as lack of sunlight and inadequate vitamin D intake, are recognized as important factors contributing to vitamin D insufficiency in CKD patients;⁸ however, disturbances in the regulation of key cytochrome P450 enzymes involved in the synthesis (1α-hydroxylase; CYP27B1) and catabolism (24-hydroxylase; CYP24) of vitamin D metabolites may also be implicated.

Vitamin D₃ is synthesized in human skin from 7-dehydrocholesterol after ultraviolet light exposure and is metabolized in the liver to form the prohormone, 25-hydroxyvitamin D₃ (25(OH)D₃). Circulating 25(OH)D₃ provides substrate for conversion to the biologically active hormone 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) by 1α-hydroxylase CYP27B1 primarily expressed in renal proximal and distal convoluted tubules.⁹,¹⁰ Although kidneys produce the bulk of circulating hormones, extra-renal expression of CYP27B1 is thought to be important for localized production of 1α,25(OH)₂D₃.¹¹,¹² The effects of 1α,25(OH)₂D₃ are mediated by the vitamin D receptor expressed in target organs, including those involved in the maintenance of calcium/phosphate homeostasis and normal bone mineralization, immunomodulation, as well as the regulation of cell growth and differentiation, insulin secretion, cardiovascular function, and blood pressure regulation.¹³ Vitamin D insufficiency observed in CKD is associated with morbidity, which extends well beyond compromised bone and mineral metabolism,¹⁴⁻¹⁶ and contributes to increased mortality.¹⁷⁻²⁰

Declining renal mass and concomitant loss of renal CYP27B1 capacity in CKD are commonly associated with reductions in circulating levels of both 1α,25(OH)₂D₃ and 25(OH)D₃.²¹,²² However, observations of low serum
1α,25(OH)2D3 have not been consistently linked with decreases in renal CYP27B1 expression, as levels of CYP27B1 mRNA may in some cases remain unchanged in CKD patients deficient in 1α,25(OH)2D3.18,19 Moreover, diminishing CYP27B1 expression levels cannot directly account for the progressive loss of 25(OH)D3. These findings suggest that additional intrinsic mechanisms may underlie declining vitamin D metabolites, namely, 25(OH)D3 and 1α,25D(OH)2D3, in renal disease.

Apart from disturbances in 1α,25(OH)2D3 synthesis, accelerated catabolism may also have a role in lowering circulating 1α,25(OH)2D3 and 25(OH)D3 levels in CKD patients. The mitochondrial cytochrome P450 enzyme CYP24 has a unique role in the catabolism of both 1α,25(OH)2D3 and 25(OH)D3.20–22 Deletion of the CYP24 gene significantly increases the half-lives of circulating 1α,25(OH)2D3 and 25(OH)D3 and renders CYP24-null animals hypersensitive to vitamin D, thus confirming the importance of CYP24 in vitamin D homeostasis.22,23 Normally, CYP24 protein seems to be most abundant in the proximal tubule of the kidney, with lower expression observed in distal segments.24,25 However, CYP24 is also ubiquitously expressed in vitamin D target tissues external to the kidney.26 In some disease states, such as genetically linked hypophosphatemia27–29 and certain types of cancer,30–35 CYP24 expression and activity is enhanced and may be linked to both vitamin D insufficiency, as well as increased resistance to vitamin D treatment often associated with these pathologies. Given the important functional role of CYP24 in tightly regulating the biological activity of 1α,25(OH)2D3 and 25(OH)D3, overexpression of this enzyme in kidney can also have a significant impact on vitamin D status.

To determine whether CYP24 and CYP27B1 expression is altered in uremia, we investigated the regulation of these enzymes in normal and adenine-induced uremic rats, as well as in renal biopsy tissue from patients with kidney disease. Our findings suggest that dysregulation of CYP24 may be a significant mechanism contributing to vitamin D insufficiency and resistance to vitamin D therapy in CKD.

RESULTS

Renal CYP24 and CYP27B1 gene and protein expression in uremic vs normal rats

The effects of uremia on the expression of renal CYP24 and CYP27B1 mRNA and protein were examined using the adenine rat model of CKD. Previous studies using adenine-treated rats have shown that this model exhibits all key features of CKD pathiology, including elevated creatinine, iPTH and fibroblast growth factor 23 (FGF23), hypocalcemia, hyperphosphatemia, and reduced serum 1α,25(OH)2D3.40–42 Uremia in adenine-treated rats was evident from elevated mean serum creatinine levels of 1.86 ± 0.20 mg/dl compared with 0.39 ± 0.17 mg/dl in normal rats (P<0.001). Plasma iPTH and serum FGF23 levels were elevated in uremic rats, serum calcium was decreased, serum phosphorus was increased (Table 1), and serum 1α,25(OH)2D3 declined (59.20 ± 9.80 pg/ml nonuremic vs 15.20 ± 3.16 pg/ml uremic; P<0.01; Figure 1d). Although serum 25(OH)D3 levels remained unchanged (23.90 ± 2.09 ng/ml nonuremic vs 25.90 ± 1.90 ng/ml uremic; Figure 1e) 1 week after adenine treatment, a decline of about 20% in 25(OH)D3 levels was observed at 6 and 8 weeks after treatment compared with normal control animals (Figure 1f). These findings are consistent with the accelerated elimination of 25(OH)D3, raising the possibility that elevated CYP24 may have a role in declining vitamin D status in CKD patients.

Examination of renal mRNA revealed a greater than fivefold increase in CYP24 expression after adenine treatment (P<0.001; Figure 1a). Consistent with this finding, an increased CYP24 protein expression was also observed in uremic kidneys (Figure 1b). CYP27B1 mRNA expression increased nearly twofold in uremic kidney (Figure 1a; P<0.01). Concordant with mRNA, CYP27B1 protein expression was clearly elevated in uremic kidney (Figure 1b), indicating that translation of CYP27B1 mRNA was not impaired in this model.43

Renal CYP24 and CYP27B1 mRNA and protein expression in uremic rats treated with 1α,25(OH)2D3

We next investigated the regulatory effect of 1α,25(OH)2D3 on these enzymes in uremia. It is well established that 1α,25(OH)2D3 treatment induces CYP24 and attenuates CYP27B1 expression in vitamin D target tissues, including kidney.9,28,44 In the uremic kidney, CYP27B1 mRNA levels were approximately threefold greater than levels of CYP27B1 (Figure 1c). Administration of 1α,25(OH)2D3 markedly increased the expression of CYP24 by approximately 12-fold relative to CYP27B1 expression, which increased only slightly (Figure 1c). Administration of 1α,25(OH)2D3 (0.50 μg/kg) to uremic rats increased mean serum

<table>
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<tr>
<th>Table 1</th>
<th>Biochemical parameters measured in normal vs uremic rats</th>
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<tr>
<td></td>
<td>Nonuremic vehicle</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>0.39 ± 0.17 (8)</td>
</tr>
<tr>
<td>iPTH (pg/ml)</td>
<td>199 ± 91.3 (10)</td>
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<tr>
<td>FGF23 (ng/ml)</td>
<td>0.41 ± 0.02 (10)</td>
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<tr>
<td>Calcium (mg/dl)</td>
<td>11.04 ± 0.24 (10)</td>
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<tr>
<td>Phosphorus (mg/dl)</td>
<td>10.41 ± 0.16 (10)</td>
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Abbreviations: FGF23, fibroblast growth factor 23; iPTH, intact parathyroid hormone; 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3.

Significant from nonuremic vehicle *P<0.05) **P<0.01) ***P<0.001; significant from uremic vehicle (P<0.05) (P<0.01) (P<0.001). Data are presented as mean ± s.e.m.; (n) denotes sample size.
Uremia induces a strong elevation in basal CYP24 mRNA levels in adenine-treated rats with vitamin D deficiency. (a-e) Rats were fed a standard or adenine diet (0.75% adenine) for 4 weeks and intravenously administered a daily dose of vehicle (nonuremic and uremic) or 0.50 μg/kg 1α,25-dihydroxyvitamin D3 (1α,25(OH)2D3) (uremic only) for 7 days beginning at week 4. Serum vitamin D metabolites and kidneys were collected 24 h after the last dosing. (a) Summary plot of CYP24 and CYP27B1 mRNA expression levels for nonuremic vehicle (NU+Veh; n = 10) and uremic vehicle (U+Veh; n = 9) rats. Relative mRNA values are normalized to NU+Veh (relative expression = 1). (b) Kidney lysates prepared from representative nonuremic and uremic vehicle rats were subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot with antibodies against CYP24 and CYP27B1. Each lane is representative of an individual rat kidney fraction. Human kidney lysate was run as a control for CYP27B1. Molecular weights of recognized bands are indicated. (c) CYP24 and CYP27B1 mRNA levels are presented for U+Veh and uremic + 1α,25(OH)2D3 (U+1,25D3: n = 7 CYP24; n = 6 CYP27B1) groups. Relative mRNA values are normalized to NU+Veh (relative expression = 1) in (a). (d) Serum 1α,25(OH)2D3 and (e) 25-hydroxyvitamin D3 (25(OH)D3) levels in nonuremic and uremic rats treated with vehicle or 1α,25(OH)2D3 are shown. U+Veh (n = 10) was compared with NU+Veh (n = 10), whereas U+1,25D3 (n = 7 1α,25(OH)2D3; n = 6 25(OH)D3) was compared against U+Veh. (f) Rats were fed a standard or adenine diet (0.75% adenine) for 4 weeks, followed by 8 weeks with a standard diet. Blood was sampled at 2, 4, 6, and 8 weeks after adenine diet. Average percentage change in 25(OH)D3 levels for NU+Veh (n = 8–10) and U+Veh (n = 7–10) at 2, 4, 6, and 8 weeks after treatment is shown. Values are normalized to 100% at 2 weeks for each group. (*) P < 0.05 (**) P < 0.01 (+ + +) P < 0.001 represents significant difference. Vitamin D metabolite and CYP24 mRNA values are reported as mean ± s.e.m.

1α,25(OH)2D3 (15.20 ± 3.16 pg/ml uremic to 78.0 ± 12.09 pg/ml uremic + 1α,25(OH)2D3; P < 0.001; Figure 1d), reduced serum 25(OH)D3 (25.90 ± 1.90 ng/ml uremic to 18.10 ± 2.53 ng/ml uremic + 1α,25(OH)2D3; P < 0.05; Figure 1e), improved creatinine and iPTH, and elevated calcium, FGF23 and phosphorus (Table 1).
Effect of vitamin D status on renal CYP24 and CYP27B1 gene and protein expression in uremic vs normal rats

To examine the effect of vitamin D status and uremia on CYP24 and CYP27B1 expression, rats were fed either a normal or vitamin D-deficient diet and treated with adenine or vehicle through oral gavage. Adenine-gavaged animals exhibited serum chemistries comparable to those observed in adenine-diet treated animals (data not shown). Serum 1α,25(OH)2D3 and 25(OH)D3 levels fell below the limit of detection in rats fed a vitamin D-deficient diet independent of renal status (Table 2). As expected, uremic rats fed a normal diet exhibited elevated levels of renal CYP24 mRNA (Figure 2a; ***P < 0.01). Renal CYP24 mRNA levels in nonuremic vitamin D-deficient rats dropped to approximately 25% of those observed in nonuremic rats fed a normal diet, whereas CYP27B1 mRNA levels more than doubled (Figure 2a and b). Unexpectedly, CYP24 mRNA remained significantly elevated in vitamin D-deficient uremic rats (Figure 2b; **P < 0.01). Moreover, CYP24 protein expression was augmented in vitamin D-deficient renal tissue from uremic animals (Figure 2c). Changes in vitamin D status did not induce any appreciable changes in CYP27B1 mRNA levels in uremic rats (Figure 2b).

Histological examination of kidney tissue showed no pathological abnormalities in periodic acid-Schiff- (Figure 3a), hematoxylin and eosin- (Figure 3b), and trichrome-stained sections analyzed using light microscopy in nonuremic rats, irrespective of vitamin D status (Figure 3A and B). However, uremic (Figure 3aC and bC) and uremic vitamin D-deficient (Figure 3aD and bD) rats showed marked intraluminal tubular deposition of brown adenine crystals. This deposition was accompanied by marked interstitial inflammation, interstitial fibrosis, and acute tubular injury on periodic acid-Schiff stain (Figure 3aC and aD). Glomeruli seemed diffusely ischemic, with shrunken glomerular tufts and thickened corrugated glomerular capillary walls. Hematoxylin and eosin staining showed that the interstitial inflammatory infiltrate consists of mixed infiltrating leukocytes, including mononuclear cells and

Table 2 | Serum levels of vitamin D metabolites in nonuremic or uremic rats fed a normal or vitamin D-deficient diet

<table>
<thead>
<tr>
<th>Nonuremic vehicle</th>
<th>Uremic vehicle</th>
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<tr>
<td>Normal VDD</td>
<td>Normal VDD</td>
</tr>
<tr>
<td>1α,25(OH)2D3 (pg/ml)</td>
<td>250 ± 49.5 (8) BLD (7) 140 ± 65.0 (4) BLD (9)</td>
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<tr>
<td>25(OH)D3 (ng/ml)</td>
<td>14.4 ± 2.92 (10) BLD (10) 19.2 ± 2.99 (4) BLD (9)</td>
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Abbreviations: BLD, below the limit of detection; VDD, vitamin D-deficient diet; 1α,25(OH)2D3, 1α,25-dihydroxyvitamin D3; 25(OH)D3, 25-hydroxyvitamin D3.

Data are presented as mean ± s.e.m.; (n) denotes sample size.

Figure 2 | Upregulation of basal CYP24 mRNA expression is independent of vitamin D status in uremic rats. (a-c) Rats were fed a normal or vitamin D-deficient diet for 6 weeks, with both groups dosed orally with adenine (uremic) or vehicle (nonuremic) from week 4 to 6. Vitamin D status was measured at 1 week and kidneys were harvested 2 weeks after adenine treatment. (a) Summary plot of CYP24 and CYP27B1 mRNA expression in nonuremic (NU + Veh: n = 7 CYP24; n = 10 CYP27B1) and uremic (U + Veh: n = 4) rats fed a normal diet or (b) vitamin D-deficient diet (NU + Veh: n = 7 CYP24; n = 10 CYP27B1 and U + Veh: n = 9). Relative mRNA values for (a) and (b) are normalized to NU + Veh rats fed a normal diet (relative expression = 1). (c) Kidney lysates prepared from representative NU + Veh and U + Veh vitamin D-deficient (Vit D-deficient) rats were subsequently analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot with antibodies against CYP24. Each lane is representative of an individual rat kidney fraction. Molecular weight of the recognized band is indicated. (*) P < 0.05 (**) P < 0.01 denotes statistical difference. Values are presented as mean ± s.e.m.
occasional neutrophils (Figure 3bC and bD). On trichrome staining, there was marked interstitial fibrosis in uremic and uremic vitamin D-deficient rats (data not shown). This was seen in parallel to severe tubular dilatation, microcystic change, and foci of tubular atrophy.

Normal control and nonuremic vitamin D-deficient rats showed proximal tubular staining of CYP24 (2+) that was predominantly located along the cell membrane at the apical (luminal) aspect of epithelial cells. There was no significant glomerular or vascular staining for CYP24. In contrast, kidneys from uremic and uremic vitamin D-deficient rats showed not only apical staining of proximal tubular epithelial cells (2+) but also cytoplasmic staining of the same cells (2+ to 3+) (Figure 4c and d). In addition, thick ascending distal tubular epithelial cells showed immunoperoxidase staining (2+) in these groups of animals.

Renal CYP24 protein expression in CKD patients and normal age-matched controls

Kidney biopsy samples from patients with CKD and normal age-matched controls were examined for CYP24 renal expression by immunohistochemistry. CYP24 staining was highly localized to the apical membrane (1+ to 2+) in proximal tubules in control tissue (Figure 5aA), whereas CKD tissue showed marked (2+ to 3+) and diffuse cytoplasmic staining in the proximal tubular (Figure 5aB), as well as cortical (Figure 5bB) and medullary (Figure 5cB) distal tubules in six of eight biopsy samples. In most biopsy samples, mural staining in the interlobular arteries was evident and seemed to be intracytoplasmic in arterial wall smooth muscle cells. Interstitial staining in three of eight biopsy samples was focally present (data not shown). The intensity of immunostaining of CYP24 was not associated with the clinical parameters outlined in Table 3.

DISCUSSION

Vitamin D insufficiency is highly prevalent in CKD patients and may contribute significantly to the morbidity and mortality associated with this disease. Although reduced exposure to sunlight and vitamin D intake are important factors contributing to insufficiency, disruptions in the
Table 3 | Biochemical parameters measured in patients with CKD

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Control</th>
<th>CKD</th>
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<tr>
<td>Phosphorus (mg/dl)</td>
<td>3.37 ± 0.34 (8)</td>
<td>3.78 ± 0.28 (8)</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>9.64 ± 0.32 (8)</td>
<td>9.30 ± 0.48 (8)</td>
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<tr>
<td>Creatinine (mg/dl)</td>
<td>1.20 ± 0.2 (8)</td>
<td>2.40 ± 0.40 (8)*</td>
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Abbreviations: CKD, chronic kidney disease. Measured in serum; significant from control *(P<0.05).

Data are presented as mean ± s.e.m.; (n) denotes sample size.

Synthesis and catabolism of 25(OH)D₃ and 1α,25(OH)₂D₃ may also have considerable etiological roles. Altered expression or activity of CYP27B1 has previously been implicated in reduced blood levels of 1α,25(OH)₂D₃ in patients and animal models of CKD; however, recent evidence suggests that aberrant CYP27B1 expression cannot entirely account for low 1α,25(OH)₂D₃ levels or explain 25(OH)D₃ depletion in kidney disease. Our studies show that elevated renal expression of CYP24 induced by factors associated with the uremic state may have an important effect on vitamin D status and may possibly affect tissue responsiveness to vitamin D therapy.

The adenine rat model is a recently established animal model of CKD that closely mimics several clinical features of secondary hyperparathyroidism, a serious complication arising from declining renal function in CKD patients. Adenine-treated rats in our study showed pathology consistent with secondary hyperparathyroidism, including elevated iPTH and FGF23 levels, hypocalcemia and hyperphosphatemia, as well as a comparative decline in both serum levels of 1α,25(OH)₂D₃ and 25(OH)D₃ over time.

Depletion of 1α,25(OH)₂D₃ in CKD patients has long been ascribed to reduced CYP27B1 activity caused by decreased renal mass, inadequate 25(OH)D₃ availability, suppression and/or dysregulation of CYP27B1 expression. Interestingly, in our study, both renal CYP27B1 mRNA and protein levels were elevated in adenine-fed rats, suggesting that observed decreases in blood levels of 1α,25(OH)₂D₃ were not due to changes in CYP27B1 expression. Previous studies using a kidney remnant model of early-stage CKD showed that CYP27B1 levels rose in conjunction with plasma iPTH levels. In this model, however, 1α,25(OH)₂D₃ levels remained unchanged, indicating that the increase in CYP27B1 could effectively compensate for the loss of total CYP27B1 associated with the reduction in kidney mass. The observed increase in CYP27B1 expression in adenine-treated animals may similarly reflect a compensatory response to reduced blood levels of 1α,25(OH)₂D₃, although an unsuccessful one.

Zehnder et al. recently reported increased CYP27B1 mRNA expression in renal biopsy tissue from patients with kidney disease and significantly depleted circulating 1α,25(OH)₂D₃ levels, possibly also reflecting unsuccessful compensatory responses to low vitamin D status. Given the usual strong correlation between expression of renal CYP27B1 mRNA and synthesis of 1α,25(OH)₂D₃, these findings suggest that CYP27B1 may be adequately expressed in the uremic state, but its effectiveness to generate ample 1α,25(OH)₂D₃ is compromised.

Elevated CYP24 expression levels in the uremic kidney in combination with low vitamin D status may reflect accelerated catabolism of 1α,25(OH)₂D₃ and 25(OH)D₃ by CYP24. Although CYP24 activity was not directly evaluated in our study, previous studies have shown an inverse relationship between renal CYP24 mRNA and 1α,25(OH)₂D₃ concentration, as well as increased renal side chain oxidation of 1α,25(OH)₂D₃ in tissues expressing abnormally high levels of CYP24. The importance of CYP24 in regulating vitamin D status has been further demonstrated in the CYP24 knockout mouse, in which null
animals exhibit high ambient levels of 1α,25(OH)2D3. Given the strong inverse relationship between CYP24 expression levels and vitamin D status, reduced blood levels of 1α,25(OH)2D3 in certain diseases may represent vitamin D insufficiency as a consequence of aberrant expression levels of CYP24. For example, although suppression of CYP27B1 caused by abnormally high levels of circulating FGF23 has been proposed to be a potential factor delineating vitamin D status in genetic hypophosphatemia, additional elevation in the renal expression of CYP24 may also contribute to reduced 1α,25(OH)2D3 and 25(OH)D3. In the Hyp mouse, which is a rodent model of human X-linked hypophosphatemic rickets, high kidney levels of CYP24 mRNA and immunoreactive protein have been observed and are proposed to be major mechanisms underlying accelerated degradation of 1α,25(OH)2D3. Growing evidence indicates that basal CYP24 expression can also be abnormally high in various forms of cancer and may be a primary determinant of vitamin D deficiency and decreased responsiveness of tumor cells to 1α,25(OH)2D3 treatment.

Our findings, in particular, the strong expression of CYP24 in kidney tissue from uremic patients, suggest that CYP24 may have a greater role in causing vitamin D insufficiency in CKD patients than has been previously suspected. Interestingly, immunohistochemical analyses indicate that CYP24 protein seems more widely expressed in the uremic kidney, showing apical staining of both proximal and distal tubule epithelial cells with a stronger apparent cytoplasmic component. Cytoplasmic expression of elevated CYP24 protein has also been reported in human colorectal cancer tissue after malignant transformation, as well as in breast cancer tissue. The widespread expression of CYP24 in uremic kidney may have a direct impact on kidney exposure to vitamin D hormone. If elevated CYP24 functions to devoid the kidney tissue of both 25(OH)D3 and 1α,25(OH)2D3, progression of kidney disease may be accelerated through the promotion of pathological processes, such as fibrosis and inflammation, which can normally be suppressed, at least to some extent, by vitamin D hormones. It is unexpected that adenine-treated animals exhibited similar renal CYP24 mRNA and protein expression irrespective of vitamin D status. This is in marked contrast to the very low levels of CYP24 expression seen in otherwise normal rats fed a vitamin D-deficient diet. These findings indicate that mechanisms other than 1α,25(OH)2D3 are also involved in the regulation of CYP24 in the uremic kidney. One possible candidate is the phosphaturic hormone FGF23, which can indirectly modulate 1α,25(OH)2D3 levels by controlling the expression of CYP27B1 and CYP24.

Previous studies have shown that disease models characterized by elevated circulating FGF23 levels, such as X-linked hypophosphatemic rickets and autosomal-dominant hypophosphatemic rickets, as well as animals overexpressing FGF23 or injected with a bolus dose of FGF23 exhibited increased basal levels of renal CYP24 coincident with reduced serum levels of 1α,25(OH)2D3. FGF23 levels are also significantly elevated in serum of adenine-treated animals, and can be, in part, responsible for elevated basal CYP24 expression in this model.

The present study shows that kidney tissue from either rats exposed to adenine or biopsy samples from human CKD patients exhibit significant upregulation in basal CYP24 mRNA and protein expression, which may have an impact on vitamin D status, as well as deprive kidneys of local exposure to vitamin D hormone. These findings suggest that the vitamin D-degrading catabolic enzyme CYP24 may be an important determinant of vitamin D status, kidney tissue resistance to hormone therapy and progression of kidney disease. Furthermore, they suggest that use of CYP24 inhibitors alone or in combination with 1α,25(OH)2D3 or other vitamin D analogs may provide a means of overcoming such resistance and maintaining optimal levels of vitamin D metabolites in CKD patients.

**MATERIALS AND METHODS**

**Animals and diet**

Adult male Sprague-Dawley rats (6–8 weeks of age) were purchased from Hilltop Lab Animals Inc. (Scottsdale, PA, USA). All animals had access to food and water ad libitum. The standard diet contained 0.80% calcium and 0.60% phosphorus (PMI Certified Rodent Diet 5002; PMI Nutrition International Inc., St Louis, MO, USA). Rats were fed a standard diet containing 0.75% adenine to induce uremia, a purified diet devoid of vitamin D3 to induce vitamin D deficiency (TestDiet, Richmond, IN, USA; AIN-93M/No Vitamin D), or the same vitamin D-deficient diet supplemented with 1 IU/g vitamin D3 (TestDiet; AIN-93M Maintenance). Diets with altered vitamin D content contained 0.50% calcium and 0.30% phosphorus.

**Experimental procedures in vivo**

In one study, rats were fed a standard diet alone or containing 0.75% adenine for a total of 4 weeks to determine enzyme expression in uremic and normal tissue. After 3 weeks on these diets, uremic and nonuremic rats were treated intravenously daily with 0.30 μg/kg 1α,25(OH)2D3 (Sigma–Aldrich/Fluka, St Louis, MO, USA) or vehicle (propylene glycol/saline/ethanol (30:50:20 v/v/v)) for 7 days. Serum or plasma was sampled from the tail-vein and organs were collected in RNAlater (Ambion Inc., Austin, TX, USA) at 24 h after final injection.

In a second study, rats were fed a standard diet alone or containing 0.75% adenine for a total of 4 weeks, then returned to standard diet for up to 8 weeks. Serum was sampled from the tail vein at 2, 4, 6, and 8 weeks after adenine diet to investigate changes in 25(OH)D3 levels over time.

To explore the effects of vitamin D deficiency on enzyme expression in uremic and nonuremic kidney, rats were fed a purified diet devoid of vitamin D3 for 6 weeks or maintained on a matching diet supplemented with vitamin D3. After 4 weeks, some vitamin D-deficient and -sufficient rats were administered an adenine diet supplemented with 1 IU/g vitamin D3 (Sigma–Aldrich/Fluka, St Louis, MO, USA) orally once per day to induce uremia or given vehicle (100% H2O) by oral gavage for 2 weeks. Vitamin D status was measured at 1 week and kidneys were harvested at 2 weeks after adenine treatment.
Real-time polymerase chain reaction (PCR)
Aliquots of RNAs were reverse transcribed using random hexamers and Thermostart Reverse Transcription according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems (ABI), Foster City, CA, USA) using Taqman Universal PCR Master Mix (ABI #4304437) according to the manufacturer’s instructions. Taqman gene expression assays (ABI) with the following ID numbers were used: human glyceraldehyde 3-phosphate dehydrogenase, Hs99999905_m1; human CYP24, Hs00167999_m1; rat CYP24 Rn01423141_g1; rat CYP27B1 Rn00587137_m1; mouse glyceraldehyde 3-phosphate dehydrogenase, Mm99999915_g1; mouse CYP24, Mm00487244_m1; mouse CYP27B1, Mm01165919_g1. PCR reaction volumes of 20 μl were used with 50 cycles of amplification. Each cDNA sample was tested in duplicate or triplicate. Quantitative real-time-PCR results were analyzed using sequence detection system software V1.0 (ABI). Gene expression levels were calculated using the comparative cycle threshold method, and normalized to glyceraldehyde 3-phosphate dehydrogenase expression levels.

Preparation of rat kidney lysate and western blot analysis
Rat kidneys stored at ~20°C in RNAlater were rinsed with phosphate-buffered saline and subsequently homogenized in a prechilled buffer composed of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.8), 150 mM KCl, 2 mM ethylenediaminetetraacetic acid, 10% glycerol, 1.0% Triton X-100, and 0.1% sodium dodecyl sulfate. Protease inhibitor cocktail set III (EMD Biosciences, San Diego, CA, USA) was diluted to a ratio of 1:100 and added to the buffer immediately before homogenization. The resultant homogenates were centrifuged for 30 min at 18,000 g to remove insoluble materials. The protein concentration of the clarified lysates was determined using a detergent-compatible Lowry assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of kidney lysates (15 μg) from representative animals were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After incubation with the monoclonal antibody to CYP24 (Abnova Corp., Taipei, Taiwan) or with the rabbit polyclonal antibody to CYP27B1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), appropriate horseradish peroxidase-conjugated secondary antibodies were applied and recognized proteins were subsequently detected by chemiluminescence (GE Healthcare, Piscataway, NJ, USA). Human kidney lysate (Biochain, Hayward, CA, USA) was used as a control for CYP27B1 experiments. Molecular weights were estimated using Precision Plus Protein WesternC standards (Bio-Rad Laboratories).

Histopathology and immunohistochemistry of rat tissue
Rat kidneys were bisected and immediately placed in 4% neutral-buffered formalin for 24 h, followed by 70% ethanol. All formalin-fixed paraffin-embedded kidneys were stained with periodic acid-Schiff reaction, hematoxylin and eosin, and Masson’s trichrome stains and examined by light microscopy. Formalin-fixed, paraffin-embedded sections from rat kidneys were used for all immunohistochemical analysis. Heat-induced antigen retrieval was used. Endogenous peroxidase activity was prevented by pretreating all sections with 3% hydrogen peroxide. Anti-CYP24 primary antibody (M02) (Abnova) was used at a dilution of 1:2000. Negative controls, with no primary antibody added, were used for all immunohistochemical staining. Immunoperoxidase staining for CYP24 was scored using a compartmental schema with staining intensity graded from 0 (no staining), 1+ (mild), 2+ (moderate), to 3+ (marked).

Human renal tissue from normal individuals and from patients with renal disease
Institutional ethics board approval was granted for the use of archived biopsy tissue from the University Health Network (Toronto, Ontario, Canada). Renal tissue was obtained from patients with diabetic nephropathy (Table 3) and from normal age-matched controls. Patients were not taking active 1,25(OH)2D3, additional vitamin D analoges and/or bisphosphonates, whereas five of eight diabetic patients and one of eight age-matched controls were currently taking angiotensin-converting enzyme inhibitors and/or angiotensin II receptor blockers. Formalin-fixed paraffin-embedded sections of renal biopsy samples were cut at 3 μm, followed by heat-induced antigen retrieval. Sections were incubated with monoclonal anti-CYP24 (Abnova Corp.). To eliminate any potential nonspecific biotin activity, slides were stained with a secondary antimouse antibody (Dako, Glostrup, Denmark) using the EnVision system (Dako). Endogenous peroxidase activity was prevented by pretreating with 3% hydrogen peroxide. Negative controls with irrelevant primary antibody and no primary antibody were used. Immunoperoxidase staining intensity was graded as shown above.

Blood biochemistry
Serum samples of 25(OH)D3 and 1α,25(OH)2D3 were spiked with [26,27-2H6] 25(OH)D3 or [25,26-2H6] 1α,25(OH)2D3 and dissolved in acetonitrile to serve as an internal standard. 1α,25(OH)2D3 or 25(OH)D3 and internal standards were extracted from serum using Accubond II ODS-C18 100 mg, 1 ml SPE cartridges (Agilent Technologies, Palo Alto, CA, USA). The collected fractions were dried under a steady stream of nitrogen gas, residues reconstituted in 50 μl of methanol/H2O (80/20; v/v) and analyzed using LC-MS/MS (Waters Alliance HPLC-Waters Quattro Ultima Mass Spectrometer, Milford, MA, USA). Creatinine levels were measured using a sarcosine oxidase-based enzymatic assay (Roche Diagnostics, Laval, QC, Canada). Plasma iPTH levels were determined using the rat iPTH ELISA kit (Immutopics, San Clemente, CA, USA). Serum FGF23 was measured using an FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan) as per the manufacturer’s instructions. Serum phosphorus and calcium levels were determined using an o-cresolphthalein complexone-based assay and an ammonium molybdate-based assay (Pointe Scientific, Canton, MI, USA), respectively.

Statistical analysis
To calculate fold induction, nonuremic vehicle values were normalized to 1 unless otherwise specified. Student’s independent t-test was used to assess the difference between two groups, whereas multiple groups were assessed using one-way ANOVA, with Bonferroni’s multiple group comparison after test. The level of significance was set at P<0.05 (Graphpad Prism 5, La Jolla, CA, USA). n denotes sample size and data are presented as mean ± s.e.m.

DISCLOSURE
All the authors declared no competing interests.

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REFERENCES


