Uremic Plasma Contains Factors Inhibiting 1α-Hydroxylase Activity

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ABSTRACT

The effect of uremic plasma ultrafiltrate on calcitriol synthesis was investigated. Renal 1α-hydroxylase activity was measured in normal rats infused for 20 h with 20 mL of normal or uremic plasma ultrafiltrate. Renal 1α-hydroxylase activity was determined by the generation of calcitriol measured 5, 10, 20, and 30 min after the reaction was initiated by the addition of cold 25(OH)D3. The activity was significantly lower in rats infused with uremic plasma ultrafiltrate. Kidney homogenates preincubated for 3 h with uremic plasma ultrafiltrate also had significantly lower renal 1α-hydroxylase activity than did those preincubated with normal plasma ultrafiltrate. In addition, the effect of the putative uremic toxin, guanidinosuccinic acid (GSA), on renal 1α-hydroxylase activity was studied. Normal rats infused for 20 h with 20 mL of saline solution containing 1.5 mg/dL of GSA had significantly lower renal 1α-hydroxylase activity than did rats infused with normal saline. The enzyme activity was also lower in kidney homogenates preincubated for 3 h with 4 mg/dL of GSA. Enzyme kinetic analysis revealed that the inhibition of renal 1α-hydroxylase by GSA was noncompetitive. It was concluded that uremic plasma contains substances that directly inhibit renal 1α-hydroxylase activity.

Key Words: Calcitriol synthesis, 1α-hydroxylase, uremic toxins, guanidinosuccinic acid

The vitamin D endocrine system plays a major role in normal bone and mineral homeostasis. Calcitriol, the biologically active form of vitamin D, regulates intestinal calcium absorption, renal handling of calcium, and bone flux of calcium (1). An impairment in calcium homeostasis, therefore, occurs when the production of calcitriol is subnormal. In renal failure, calcitriol synthesis is decreased. The decreased synthesis has been attributed to loss of functional renal mass (2), retention of phosphate (3,4), and metabolic acidosis (5,6). In addition, we have demonstrated that the infusion of phosphorus-free urine (7) or uremic plasma ultrafiltrate (8) into normal rats resulted in decreased calcitriol production. Thus, urine and uremic plasma appear to contain factors that inhibit calcitriol synthesis. Subsequently, we found that the putative uremic toxin, guanidinosuccinic acid (GSA), also inhibits in vivo calcitriol production rate (9). Although previous studies (8,9) suggest that the calcitriol production rate, determined in vivo by the isotope constant infusion method, is suppressed by uremic ultrafiltrate, it is not clear whether the suppression is secondary to systemic changes in calcitriol regulatory hormones or due to direct inhibition of 1α-hydroxylase activity. In this study, we examined the direct effects of uremic plasma ultrafiltrate and GSA on renal 1α-hydroxylase activity.

METHODS

Male Sprague-Dawley rats weighing 200 to 220 g were used for all experiments. Animals were fed a regular Purina rat chow (Ralston Purina, St. Louis, MO) containing 1.0% Ca, 0.8% P, and 4.5 IU/g of vitamin D. For the in vivo study, the femoral vein of the rat was cannulated under ether anesthesia with polyethylene tubing (PE 10) for fluid infusion. Animals were placed in individual metabolic cages for infusion.

Renal 1α-Hydroxylase Activity in Rats Infused With Uremic Plasma Ultrafiltrate

The uremic plasma ultrafiltrate was obtained from chronic hemodialysis patients at the initiation of dialysis with a cellulose acetate hollow fiber dialyzer (Travenol Laboratory Inc, Deerfield, IL). Normal plasma ultrafiltrate was obtained by filtering pooled normal plasma through an identical dialyzer. The ultrafiltration rate was approximately 30 mL/min at a plasma flow rate of 200 mL/min. The composition of electrolytes after adjustment was Na, 133 mEq/L; K, 2.6 mEq/L; Ca, 6.5 mg/dL; Mg, 1.63 mg/dL; P, 5.3 mg/dL; and urea nitrogen, 22 mg/dL for the normal
plasma ultrafiltrate, and Na, 134 mEq/L; K, 2.6 mEq/L; Ca, 6.4 mg/dL; Mg, 1.64 mg/dL; P, 5.6 mg/dL; and urea nitrogen, 101 mg/dL for the uremic plasma ultrafiltrate.

Rats were infused for 20 h with 20 mL of either normal plasma (N = four rats) or uremic plasma (N = 4 rats) ultrafiltrate. At the end of infusion, animals were killed by decapitation and kidneys were removed immediately for the measurement of renal 1α-hydroxylase activity. In a preliminary study, we determined the time course of calcitriol generation. The generation of calcitriol was measured at 0, 1, 3, 5, 10, 20, and 30 min after the addition of 25(OH)D3 (final concentration, 80 μM). It appears that the generation rate of calcitriol was linear for up to 5 min of incubation time (Figure 1).

For the measurement of 1α-hydroxylase activity (10), a 5% (wt/vol) cortical homogenate of kidney was prepared in an ice-cold buffer solution containing 15 mM Tris-acetate, 0.19 M sucrose, and 2 mM magnesium acetate (pH 7.4). One hundred milligrams of kidney homogenate was placed in a 25-mL Erlenmeyer flask containing 15 mM Tris-acetate, 0.19 M sucrose, 2 mM magnesium acetate, and 25 mM sodium succinate in a final volume of 3 mL (10). The flasks were oxygenated with 100% O2 and agitated at 37°C in a Dubnoff metabolic shaker at 100 cycles/min. Renal 1α-hydroxylase activity was measured as the generation of calcitriol 5, 10, 20, and 30 min after the reaction was initiated by the addition of cold 25(OH)D3. The cold 25(OH)D3, dissolved in 20 μL of 95% ethanol, was added to the incubation medium at a final concentration of 80 μM (the minimal saturating concentration of substrate [10]). The reaction was stopped by the addition of 20 mL of methanol:chloroform (2:1). [3H]calcitriol (5,000 cpm) in 20 μL of 95% ethanol was added to each flask to monitor recovery of the generated calcitriol through subsequent extraction and HPLC isolation.

Calcitriol was extracted by the method described by Lobaugh et al. (10). Briefly, the contents of each flask were mixed with 6 mL of Tris-acetate buffer and 6.5 mL of chloroform and vigorously agitated. The lower layer was removed, and the lipid extracts were dried under nitrogen and dissolved in 1.0 mL of 100% ethanol. For the purification of calcitriol, 200 μL of the lipid extracts were brought to a volume of 1.0 mL with normal saline, and 1 mL of acetonitrile was added to it. The mixture was vigorously vortexed and centrifuged at 760 × g. The supernatant was combined with 1.0 mL of KH2PO4 (0.4 M; pH 10.6), vortexed, centrifuged, and applied to a C-18 cartridge (Waters Inc., Milford, MA). The column was eluted with 5 mL of distilled water (twice), 5 mL of 70% methanol, and 5 mL of acetonitrile (11). The final fraction, containing hydroxylated vitamin D metabolites, was dried under nitrogen. The residue was dissolved in 500 μL of HPLC solvent containing 88% hexane, 10% isopropanol, and 2% methanol and was injected into an HPLC solvent delivery system (Waters Inc.) equipped with a 5-μm silica analytical column (Alltech 605 Sl; Deerfield, IL). The solvent flow rate was 4 mL/min, and the fraction containing calcitriol was collected. Calcitriol recovery averaged more than 40%. This system separated calcitriol from other vitamin D metabolites (12). Further, 19-nor-10-oxo-25(OH)D3 coeluting with calcitriol did not interfere with the assay of calcitriol by this solvent delivery system (13). We have confirmed that the concentrations of calcitriol were virtually identical in seven samples isolated by the above solvent system and with an HPLC solvent containing 5% 2-propanol: dichloromethane. The latter separates calcitriol from 19-nor-10-oxo-25(OH)D3 (14).

The Effect of Uremic Plasma Ultrafiltrate on Renal 1α-Hydroxylase Activity In Vitro

Normal Sprague Dawley rats were killed by decapitation, and kidneys were removed immediately. A 5% cortical homogenate was prepared and preincubated for 3 h in either uremic plasma (N = 4) or normal plasma (N = 4) ultrafiltrate containing 15 mM Tris-acetate, 0.19 M sucrose, and 2 mM magnesium acetate (pH 7.4). The incubation flasks were oxygenated with 100% O2 and agitated at 100 cycles/min at 37°C in a Dubnoff metabolic shaker. At the end of the 3-h preincubation, renal 1α-hydroxylase was measured as follows: 100 mg of kidney homogenate preincubated with uremic plasma or normal plasma.
ultrafiltrates was placed, respectively, in a 25-mL Erlenmeyer flask containing 3 mL of uremic or normal plasma ultrafiltrate with 15 mM Tris-acetate, 0.19 M sucrose, 2 mM magnesium acetate, and 25 mM sodium succinate. The flasks were oxygenated with 100% O2 and agitated at 100 cycles/min at 37°C in a Dubnoff metabolic shaker. Renal 1α-hydroxylase activity was measured by the generation of calcitriol 5, 10, 20, and 30 min after the reaction was initiated by the addition of cold 25(OH)D3 at a final concentration of 80 μM.

Renal 1α-Hydroxylase Activity in Rats Infused with GSA

Rats (N = 4) were infused for 20 h with 20 mL of 1.5 mg/dL of GSA in normal saline. Control rats (N = 4) were infused for 20 h with 20 mL of normal saline. At the end of infusion, rats were killed and kidneys were removed immediately for the measurement of renal 1α-hydroxylase activity as described above.

The Effect of GSA on Renal 1α-Hydroxylase Activity In Vitro

A 5% normal kidney homogenate was preincubated for 3 h in a solution containing 15 mM Tris-acetate, 0.19 mM sucrose, 2 mM magnesium acetate, and 4 mg/dL of GSA (N = 4). The flasks were oxygenated with 100% O2 and agitated at 100 cycles/min at 37°C in a Dubnoff metabolic shaker. Controls (N = 4) were preincubated in the same buffer solutions without GSA. At the end of 3 h of preincubation, 100 mg of kidney homogenate was placed in 3 mL of buffer solution containing 15 mM Tris-acetate, 0.19 M sucrose, 2 mM magnesium acetate, 25 mM sodium succinate, and 4 mg/dL of GSA. Renal 1α-hydroxylase activity was measured as the generation of calcitriol 5, 10, 20, and 30 min after the addition of 25(OH)D3. Renal 1α-hydroxylase activity of controls was determined in tissues incubated in the same buffers without GSA.

Additional experiments were carried out in which the kidney homogenates were preincubated for 3 h in the presence or absence of 4 mg/dL of GSA. After the preincubation, renal 1α-hydroxylase activity was measured in the kidney homogenates by the addition of various substrate concentrations (5, 10, 20, 30, 40, and 50 μM) of 25(OH)D3 (N = 4 for each concentration). The production of calcitriol was stopped at 1 min by the addition of 20 mL of methanol: chloroform (2:1).

For all experiments calcitriol was measured in duplicate by a radioreceptor assay by the method of Reinhardt et al. (11) and Hollis (15). The intra-assay coefficients of variation were 5.4% for low control (20 pg/mL; N = 6) and 4.7% for high control (100 pg/mL; N = 6). The interassay coefficients of variation were 7% for low (N = 12) and 4.1% for high (N = 12) control.

All data were expressed as mean ± SE. Statistical analysis was performed by t test. A P value of less than 0.05 was considered significant.

RESULTS

Renal 1α-hydroxylase activity was significantly suppressed in rats infused with 20 mL of uremic plasma ultrafiltrate when compared with that in those infused with normal plasma ultrafiltrate (Figure 2; left panel). The generations of calcitriol measured at 5, 10, 20, and 30 min were lower in rats infused with uremic plasma ultrafiltrate. Kidney homogenates preincubated with uremic plasma ultrafiltrate had significantly lower renal 1α-hydroxylase activity than did those preincubated with normal plasma ultrafiltrate (Figure 2; right panel).

Figure 3 depicts the effect of the putative uremic toxin, GSA, on renal 1α-hydroxylase activity. The activity was lower in rats infused with GSA solution (Figure 3; left panel). Kidney homogenates preincubated with GSA in vitro also had lower renal 1α-hydroxylase activity (Figure 3; right panel).

A double reciprocal plot of kinetic data for the effect of GSA on calcitriol production is presented in Figure 4. Calcitriol generation rates from kidney homogenates incubated with GSA were significantly lower for each substrate concentration (all P < 0.01, except the generation of calcitriol with substrate [5 μM], where P < 0.05). Therefore, we expect that the slopes of the regression lines are significantly different. The results suggest that the inhibition of GSA...
Figure 3. (Right panel) Renal 1α-hydroxylase activity of rats \((N=4)\) infused for 20 h with 20 mL of 1.5 mg/dL of GSA. Controls \((N=4)\) were infused with normal saline. (Left panel) Renal 1α-hydroxylase activity of kidney homogenate preincubated for 3 h with 3 mg/dL of GSA \((N=4)\). Controls \((N=4)\) were preincubated with buffer solution. was noncompetitive; the \(V_{\text{max}}\) in the presence of GSA (43.3 fmol/mg of kidney/min) appeared to be lower than that in the absence of GSA (54.7 fmol/mg of kidney/min), whereas the apparent \(K_m\) was not different (GSA, 79.4 μM versus control, 82.6 μM). Because of the presence of vitamin D–binding protein in the kidney homogenate, only an apparent \(K_m\), rather than a true \(K_m\), could be measured.

**DISCUSSION**

Altered vitamin D metabolism plays a major role in disordered mineral homeostasis in renal failure. The synthesis of calcitriol, the most biologically active form of vitamin D, is decreased in patients with moderate renal failure (8). It is generally believed that decreased calcitriol synthesis leads to the development of diminished intestinal calcium absorption, hypocalcemia, and secondary hyperparathyroidism in renal failure (16–18). Several factors appear to influence calcitriol synthesis in renal failure. Hypocalcemia (19) and elevated parathyroid hormone (20) stimulate calcitriol production, whereas hyperphosphatemia (3,4), decreased renal mass (2), and metabolic acidosis (5,6) decrease calcitriol synthesis. It appears that calcitriol production is also inhibited by uremic plasma, which contains factors, other than phosphorus, that suppress calcitriol synthesis.

Several studies support the notion that uremic toxins appear to suppress calcitriol synthesis. We have shown that the production rate of calcitriol, determined \textit{in vivo} by the isotope infusion method, fell in rats infused with phosphorus-free urine (7) or uremic plasma ultrafiltrate (8). Those infusions did not change the plasma concentrations of calcium, phosphorus, or blood pH. Because the ultrafiltration process excludes substances with a molecular weight greater than 10,000, we believe that these uremic toxins are small molecular substances. In addition, metabolic products of ingested protein also appear to suppress calcitriol synthesis, because the production rate of calcitriol fell further when partially nephrectomized rats were fed a high-protein diet (7).

The generation rate of calcitriol from kidney homogenate appeared to have two slopes in the study presented here. The slope became less steep after the initial 5 min of incubation (Figure 1), as the generated calcitriol apparently suppressed 1α-hydroxylase activity. Further, calcitriol was rapidly degraded by the kidney homogenates during the incubation, although the degradation rates were linear and stable for 90 min between kidney homogenates preincubated with normal and uremic plasma ultrafiltrates (21). The suppression of 1α-hydroxylase and calcitriol degradation during the incubation probably account for the slope change.

In this study, we have shown that uremic toxins directly inhibited renal 1α-hydroxylase activity. Enzymatic activity was significantly lower in tissues preincubated with each concentration of GSA (all \(P<0.01\), except substrate concentration (5 μM), where \(P<0.05\)).
could be mediated via alteration of plasma calcitriol regulatory hormones, we have now shown that suppression of 1α-hydroxylase occurred not only in rats infused with uremic ultrafiltrate but also in kidney homogenates incubated with uremic ultrafiltrate in vitro, indicating a direct inhibition of 1α-hydroxylase by uremic toxins. Further, the inhibition of 1α-hydroxylase activity occurred within 3 h of incubation, suggesting that the inhibition was unlikely to be mediated via inhibition of protein synthesis. It is noteworthy that the infusion of only 1 mL/h of uremic plasma ultrafiltrate, which did not change the plasma concentration of blood urea nitrogen (8), was enough to inhibit renal 1α-hydroxylase in normal rats. This finding indicates that the inhibition could occur in mild renal failure with slight elevation of uremic toxins.

We have previously demonstrated that the infusion of 10 mL of normal saline solution containing GSA (1.8 mg/dL) significantly suppresses calcitriol production rate (9). GSA could suppress calcitriol production in vivo by stimulating or converting GSA to other substances. It could also directly suppress 1α-hydroxylase activity. GSA is elevated in renal failure (22). Although the plasma concentrations of GSA were not measured in these animals, the levels were expected to be significantly lower than those of chronic renal failure patients (0.35 ± 0.03 mg/dL; serum creatinine, 4 to 6 mg/dL [22]). In this study, we have confirmed that GSA (4 mg/dL), within the plasma concentrations of hemodialysis patients [23] directly inhibited renal 1α-hydroxylase activity. The higher concentration of GSA was chosen in order to demonstrate that GSA alone without other uremic toxins could suppress 1α-hydroxylase activity. Enzyme kinetic analysis indicated that the inhibition of renal 1α-hydroxylase by GSA is noncompetitive. Thus, the inhibition by GSA can not be overcome by a high substrate concentration. It should be noted that uremic ultrafiltrate contains numerous chemical substances that could suppress 1α-hydroxylase activity by different mechanisms and kinetics.

In addition to GSA, uric acid, xanthine, and hypoxanthine are often elevated in the plasma of patients with renal failure (24). Previously, we found that both uric acid and xanthine suppress calcitriol synthesis (25).

Our studies do not establish how uremic toxins and GSA inhibit 1α-hydroxylase activity. 1α-hydroxylase is a membrane-bound multiple complex enzyme. It is composed of a flavoprotein as functions as a NADPH-ferredoxin oxidoreductase, an iron-sulfur-containing protein of the ferredoxin type that functions as a NADPH-cytochrome P-450 oxidoreductase, and a cytochrome b of P-450 type that functions as the terminal monoxygenase (26). It is important to know that the half lives of the cytochromes and ferredoxin are 3.9 and 5.1 days, respectively (27). Therefore, it is unlikely that the inhibition of 1α-hydroxylase activity by uremic toxins, which acted in few hours, involves any significant changes in the steady-state concentrations of either the mitochondrial cytochrome P-450 or the ferredoxin.

In summary, the study presented here extended our previous observations to demonstrate that uremic plasma ultrafiltrate contains factors that reduce the calcitriol production rate (8,28) via the direct inhibition of 1α-hydroxylase activity. One of the putative uremic toxins, GSA, also directly inhibits 1α-hydroxylase activity in a noncompetitive fashion. These uremic substances may accumulate in mild renal failure and contribute to the decreased calcitriol production that is characteristic of this condition.

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REFERENCES


