Decreased Sensitivity of Distal Nephron and Collecting Duct to Parathyroid Hormone in Pseudohypoparathyroidism Type I

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Abstract. Parathyroid hormone (PTH) transiently increases urinary excretion of the lysosomal enzyme, N-acetyl-\(\beta\)-D-glucosaminidase, which is distributed mainly in proximal tubules. The response is reduced in pseudohypoparathyroidism (PHP) type I, which is characterized by target-organ resistance to PTH. Evidenced by normal calcium resorption, distal tubule sensitivity to PTH has been believed to be normal in this disorder. This hypothesis was tested through a search for another marker of distal nephron sensitivity to PTH. In the human kidney, cathepsin D was expressed predominantly in distal segments of the nephron, cortical and medullary thick ascending limbs of Henle’s loop, distal convoluted tubules, and connecting tubules and in cortical collecting ducts and medullary collecting ducts. PTH infusion transiently increased cathepsin D excretion in normal subjects. The cathepsin D response to PTH was reduced in the patients with PHP type I. The decrease in cathepsin D response in PHP type I indicates a resistance to PTH in the distal nephron (cortical thick ascending limbs of Henle’s loop, distal convoluted tubules, and connecting tubules) and cortical collecting ducts. These observations suggest that the preservation of renal tubular sensitivity to PTH in this disorder may be confined to PTH-dependent calcium resorption in distal tubules.

Lysosomes have been considered to be the final destination of unidirectionally transported soluble macromolecules. However, recent studies demonstrated the retrograde trafficking from lysosomes to a late endosome/prelysosome compartment (1,2). Moreover, \(\text{Ca}^{2+}\)-dependent direct fusion of lysosomes with the plasma membrane and lysosomal enzyme release were reported in mammalian epithelial cells (3,4).

We previously reported that parathyroid hormone (PTH) transiently increases urinary excretion of N-acetyl-\(\beta\)-D-glucosaminidase (NAG), which is distributed mainly in renal proximal tubule cells (5). The NAG response to PTH is reduced in the patients with pseudohypoparathyroidism (PHP) type I, which is characterized by target-organ resistance to PTH.

PTH type I comprises two subgroups: PHP type Ia, with heterozygous inactivating mutations in the G protein \(\text{Gs}\)-\(\alpha\) (\(\text{GNAS1}\)) gene and a 50% reduction in Gs activity (6,7), and PHP type Ib, with normal Gs activity. A recent study demonstrated the linkage of PHP-Ib to a small telomeric region on chromosome 20q (20q13.3) (8), the region that contains the \(\text{GNAS1}\) gene. A GNAS knockout mouse model demonstrated that the \(\text{G}\alpha\) gene is imprinted in the renal cortex, which contains proximal tubules and some portions of distal tubules, but not in the inner medulla (11). Heterozygous null \(\text{G}\alpha\) mutation on the maternal allele results in segment-specific resistance to tubule-targeted hormones (12).

In the rat kidney, cathepsin D, an intracellular aspartic proteinase, is found mainly in lysosomes of the distal tubule cells but not in the proximal tubule cells (13). These observations raise the possibility that PTH stimulates cathepsin D release from distal tubules and that the response would represent the sensitivity to PTH in distal segments of renal tubules.

To address this possibility, we investigated the distribution of cathepsin D in the human kidney and analyzed the effect of PTH on cathepsin D excretion. Several clinical data suggest the preservation of the sensitivity to PTH in distal tubules of the nephron in PHP type I (14,15). To address this hypothesis, we compared the cathepsin D response to PTH between patients with PHP type I and normal subjects.

Materials and Methods

Immunohistochemistry of Normal Human Kidney

Cathepsin D in the kidney was visualized by using a monoclonal antibody, Cathepsin D Ab-1 (Clone C5; NeoMarkers, Fremont, CA). Distal tubules were identified by using immunohistochemical staining for well-known segment-specific differentiation markers: calbindin D-28K for distal convoluted tubules (DCT), connecting tubules (CNT), and cortical collecting ducts (CCD) and Tamm-Horsfall glycoprotein (THP) for thick ascending limbs of Henle’s loop (TALH). Proximal tubules were visualized by immunostaining with anti-aminopeptidase N antibody.
Five human kidneys with renal cell carcinoma or transitional cell carcinoma were surgically removed, and the noninvolved organs were examined for cathepsin D, calbindin D-28K, THP, and aminopeptidase M. The tissues were fixed in 10% formaldehyde solution and embedded in paraffin, and 3-μm sections of the paraffin blocks were deparaffinized in xylene and rehydrated in ethanol. For cathepsin D and aminopeptidase N, the antigens were retrieved by microwave: 10 min at 90°C in 0.01 M citric acid buffer (pH 6.0). For calbindin D-28K, the sections were treated with 0.1% trypsin at room temperature for 10 min. No pretreatment was necessary for THP. The tissues were incubated with the primary antibodies at 4°C for 18 h. The working dilution of primary antibodies was 1:50 for mouse monoclonal anti-cathepsin D antiserum, 1:3000 for mouse monoclonal anti-calbindin D-28K antiserum (Sigma-Aldrich, Stoughton, MA), 1:500 for rabbit polyclonal anti-THP antiserum (Biomedical Technologies, Stoughton, MA), and 1:80 for aminopeptidase N (NeoMarkers). After washing in a phosphate-buffered saline, the sections were treated with the Histofine simple stain, MAX (amino acid polymer that is labeled with peroxidase and IgG Fab; Nichirei, Tokyo, Japan), for 60 min at room temperature. The sections then were colorized with diaminobenzidine solution. Positive controls for cathepsin D were breast cancer tissues.

For negative controls, normal rabbit or mouse serum was used instead of the primary antibodies. The negative controls showed no immunoreactivity.

Patients and Procedure of PTH Infusion Test

The effect of human PTH (1-34) on urinary excretion of NAG and cathepsin D was studied in 22 patients with PHP type I (7 untreated patients and 15 patients after 1.25(OH)₂D₃ treatment), 39 patients with idiopathic hypoparathyroidism (IHP; 16 untreated patients and 23 patients after 1.25(OH)₂D₃ treatment), and 12 normal subjects withoutidiopathic hypoparathyroidism (IHP and PHP type I) were infused for 5 min, beginning at 1200 h. Three consecutive 1-h urine samples were collected before and after PTH infusion for measurement of NAG and cathepsin D. After an overnight fast, 0.5 g/kg synthetic human PTH (1-34) (Asahi Chemical Industry Co., Tokyo, Japan) were infused for 5 min, beginning at 1200 h. Three consecutive 1-h urine samples were collected before and after PTH infusion for measurement of cathepsin D, NAG, adenosine 3′,5′-cyclic monophosphate (cyclic AMP), and creatinine. Informed consent was obtained from all subjects.

The diagnosis of hypoparathyroidism (IHP and PHP type I) was established by clinical symptoms along with hypocalcemia, hyperphosphatemia, serum levels of intact PTH, and urinary cyclic AMP and phosphate responses to intravenously infused 30 μg of human PTH (1-34), as described previously. Two patients with PHP type I after 1α-hydroxylated vitamin D₃ treatment had decreased levels of Gs activity and showed somatic features of Albright hereditary osteodystrophy (15). An insertion of a C was found at codon 115 in exon 5 of the GNAS1 gene. This heterozygous mutation caused frame shift with a premature stop codon at codon 139. Biochemical data and the doses of 1α-hydroxylated vitamin D₃ are presented in Tables 1 and 2.

Measurements

The concentration of cathepsin D in urine samples was determined by immunologic measurements with the Rapid Format Cathepsin D ELISA kit (Oncogene Research Products, Boston, MA). This assay uses a mouse monoclonal anti-cathepsin D IgG₂a antibody and rabbit polyclonal antibody that recognize human cathepsin D. Both antibodies were raised against mature cathepsin D that had been purified from human liver.

NAG activity in urine samples was determined by using 4-methylumbelliferyl-N-acetyl-β-d-glucosaminidase as substrate, from which NAG liberates 4-methylumbelliferone at pH 4.5. One unit of NAG activity was defined as the amount of NAG catalyzing the liberation of 1 μmol of 4-methylumbelliferone per min at 37°C. Intra- and interassay variations were <4.2%.

Cyclic AMP concentrations were measured by RIA (Yamas Shouyu Co., Choshi, Japan). Serum levels of intact PTH were determined by immunoradiometric assay with anti-PTH (1-34) and anti-PTH (39-84) antibody (Nichols Institute Diagnostics, San Juan Capistrano, CA) (normal range, 12 to 50 pg/ml). Serum ionized Ca was measured by an electrode method with the NOVA 7 analyzer (Nova Biomedical, Waltham, MA), and urine Ca was measured with an atomic absorption spectrophotometer (Perkin Elmer, Norwalk, CT).

Table 1. Descriptive characteristics and biochemical data of patients with hypoparathyroidism and of normal subjects

<table>
<thead>
<tr>
<th></th>
<th>Pseudohypoparathyroidism type I</th>
<th>Idiopathic hypoparathyroidism</th>
<th>Normal Subjects</th>
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<tbody>
<tr>
<td></td>
<td>Vitamin D₃⁻</td>
<td>Vitamin D₃+</td>
<td>Vitamin D₃⁻</td>
</tr>
<tr>
<td>No. (male:female)</td>
<td>7 (3:4)</td>
<td>15 (7:8)</td>
<td>16 (10:6)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>32 ± 4</td>
<td>38 ± 10</td>
<td>43 ± 14</td>
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<tr>
<td>Serum Ca²⁺ (mmol/L)</td>
<td>0.74 ± 0.08</td>
<td>1.16 ± 0.08</td>
<td>0.77 ± 0.13</td>
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<tr>
<td>Serum P (mmol/L)</td>
<td>1.86 ± 0.42</td>
<td>1.28 ± 0.16</td>
<td>1.78 ± 0.28</td>
</tr>
<tr>
<td>Serum PTH (pg/ml)</td>
<td>273 ± 132</td>
<td>70.2 ± 33.7</td>
<td>4.1 ± 3.3</td>
</tr>
<tr>
<td>Serum 1.25(OH)₂D₃ (pg/ml)</td>
<td>14.8 ± 3.6</td>
<td>24.2 ± 5.7</td>
<td>13.3 ± 4.9</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>103 ± 11</td>
<td>96 ± 10</td>
<td>87 ± 10</td>
</tr>
<tr>
<td>Dose of 1.25(OH)₂D₃ (μg/d)</td>
<td>1.3 ± 0.4</td>
<td>1.7 ± 0.7</td>
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* Vitamin D₃⁻, before 1.25(OH)₂D₃ treatment; vitamin D₃+, after 1.25(OH)₂D₃ treatment.
and calbindin D-28K (Figure 1, c and g) immunostainings reveals that the tubular cells that were positively stained with cathepsin D in the renal cortex were the DCT, CNT, and CCD. Cortical TALH (cTALH) and medullary TALH, which were stained with THP (Figure 1, b and f), also were positive for cathepsin D (Figure 1, e and i). Medullary collecting ducts in the outer medulla (Figure 1i) and the inner medulla (data not shown) were positively stained with anti-cathepsin D antibody. These results indicate that cathepsin D-positive cells begin within the TALH and end at the collecting ducts.

Effects of Human PTH (1-34) on Urinary Excretion of Cathepsin D and NAG

Infusion of human PTH (1-34) transiently increased urinary excretion of cathepsin D and NAG in normal subjects and patients with IHP. There was not a significant difference in

Table 2. PTH/PTHrP receptor, calbindin D-28K, and cathepsin D: distribution along the nephron

<table>
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<tr>
<th>PCT</th>
<th>PST</th>
<th>TALH</th>
<th>DCT</th>
<th>CNT</th>
<th>CCD</th>
<th>MCD</th>
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<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Calbindin D-28K (33,34)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Cathepsin D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
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</table>

a PCT, proximal convoluted tubule; PST, proximal straight tubule; TALH, thick ascending limb of loop of Henle; DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; MCD, medullary collecting duct.

Figure 1. Localization of cathepsin D in normal human kidney. Serial sections of renal cortex (a through h) and outer medulla (i through l) were stained for cathepsin D (a, e, i), Tamm-Horsfall glycoprotein (a marker of the cortical and medullary thick ascending limbs of loop of Henle: cTALH and mTALH) (b, f, j), calbindin D-28K (a marker of the distal convoluted tubules: DCT, connecting tubules: CNT, and cortical collecting ducts: CCD) (c, g, k), and aminopeptidase N (d, h, l). Cathepsin D were positively stained in the cTALH (e), mTALH (i), DCT (e), CNT (a), CCD (a, e), and MCD (i).
cathepsin D or NAG responses to PTH between normal subjects and patients with IHP. In PHP type I, cathepsin D and NAG responses were impaired. Two patients with PHP type Ia showed similar results as other patients with PHP type I.

Figure 2. The effect of human parathyroid hormone (PTH) (1-34) on urinary excretion of cathepsin D. Infusion of 0.5 μg/kg synthetic human PTH (1-34) for 5 min transiently increased cathepsin D excretion. Cathepsin D response to PTH in pseudohypoparathyroidism (PHP) type I (A) was less than in patients with idiopathic hypoparathyroidism (IHP) (B) and less than in normal subjects (C) ($P < 0.01$). Closed and open circles in A and B represent the data that were obtained from the patients before and after 1,25(OH)$_2$D$_3$ treatment, respectively. Closed triangles and squares in A represent the data that were obtained from the patients with PHP type Ia. *, $P < 0.01$, versus before PTH infusion; **, $P < 0.01$, versus the peak value in normal subjects.

Figure 3. The effect of human PTH (1-34) on urinary excretion of N-acetyl-β-D-glucosaminidase (NAG). Infusion of 0.5 μg/kg synthetic human PTH (1-34) for 5 min transiently increased NAG excretion. NAG response to PTH was less in patients with PHP type I (A) than in patients with IHP (B) and in normal subjects (C) ($P < 0.01$). The symbols represent the data that were obtained from the patients as shown in Figure 2. *, $P < 0.01$, versus before PTH infusion; **, $P < 0.01$, versus the peak value in normal subjects.

cathepsin D or NAG responses to PTH between normal subjects and patients with IHP. In PHP type I, cathepsin D and NAG responses were impaired. Two patients with PHP type Ia showed similar results as other patients with PHP type I.
The increases of cathepsin D excretion after PTH infusion were as follows: 3.4 ± 0.5 times in normal subjects; 3.6 ± 0.7 and 3.8 ± 1.0 times in patients with IHP before and after 1,25(OH)₂D₃ treatment, respectively; and 1.9 ± 0.4 and 2.2 ± 0.7 times in patients with PHP type I before and after 1,25(OH)₂D₃ treatment, respectively (Figure 2). The increases of cathepsin D excretion in patients with PHP type I were less than that in normal subjects and patients with IHP (P < 0.01). The increases of NAG excretion after PTH infusion were as follows: 5.3 ± 1.1 times in normal subjects; 6.3 ± 1.2 and 6.1 ± 1.8 times in patients with IHP before and after 1,25(OH)₂D₃ treatment, respectively; and 2.3 ± 0.6 and 2.4 ± 0.5 times in patients with PHP type I before and after 1,25(OH)₂D₃ treatment, respectively (Figure 3). The increases of NAG excretion in patients with PHP type I were less than in normal subjects and patients with IHP (P < 0.01). Urinary excretion of NAG and cathepsin D standardized with urinary excretion of creatinine showed the same results (data not shown).

The increases of cyclic AMP excretion after PTH infusion were as follows: 86.8 ± 32.9 times in normal subjects; 115.5 ± 68.7 and 104.3 ± 36.1 times in patients with IHP before and after 1,25(OH)₂D₃ treatment, respectively; and 2.6 ± 0.8 and 4.3 ± 2.0 times in patients with PHP type I before and after 1,25(OH)₂D₃ treatment, respectively.

Infusion of PTH (1-34) decreased Ca excretion in both PHP type I and IHP after 1,25(OH)₂D₃ treatment as well as in normal subjects (Figure 4).

**Discussion**

Segment-specific tubular markers in the kidney provide a method for studying the sensitivity to tubule-targeted hormones in the different segments of the nephron. Immunohistochemical study revealed that cathepsin D is expressed in the TALH, DCT, CNT, and collecting ducts within the human kidney. This finding is consistent with the previous report (13) that investigated the intrarenal localization of cathepsin D in rat kidney and provides a more detailed localization of this enzyme in the human kidney. The transient increase of urinary cathepsin D excretion after PTH infusion most likely represents the distal tubule sensitivity to PTH in the kidney, probably at cTALH, DCT, CNT, and CCD, which express PTH/PTHrP receptor and cathepsin D.

In PHP type I, PTH fails to stimulate 25-hydroxyvitamin D-1α-hydroxylase. The decreased 1α-hydroxylation of vitamin D is considered to be the main mechanism of hypocalcemia in this disorder, and it has been attributed to PTH resistance in the proximal tubules. However, recent immunocytochemistry and in situ hybridization data indicated that 25-hydroxyvitamin D-1α-hydroxylase expression is not restricted to the proximal tubules. It also is expressed at many other sites throughout the nephron (16). Therefore, nephron segments that contribute to PTH-dependent 1α-hydroxylation of vitamin D would include both proximal tubules and the more distal segments of the nephron (cTALH, DCT, CNT) and CCD, where the PTH/PTHrP receptor is expressed (17,18). These observations raise the possibility that the reduced 1α-hydroxylation of vitamin D in PHP type I may represent the resistance to PTH in both proximal and distal segments of the nephron.

The decreased cathepsin D response to PTH in PHP type I suggests a resistance to PTH in cTALH, DCT, CNT, and CCD in this disorder. Renal calcium resorption is normal in PHP type I and was taken as evidence of a normal distal tubule sensitivity to
PTH in this disorder (14,15). The DCT, CNT, and CCD are the predominant sites of PTH-dependent calcium resorption (19). Calbindin D-28K, an intracellular calcium-binding protein that is thought to participate in the shuttling of calcium from the apical to the basolateral membrane, also is expressed predominantly in these tubule segments (20–22). Therefore, the preservation of sensitivity to PTH in PHP type I may be limited to the PTH action on the calcium resorption in DCT, CNT, and CCD. Although PTH generally are believed to act through cyclic AMP to stimulate active Ca\(^{2+}\) resorption in the distal part of the nephron, PTH also act via a cyclic AMP-independent pathway, which involves a chelerythrine-inhibitable and phorbol ester-insensitive protein kinase C isotype (23,24). The latter pathway may be intact in PHP type I.

In conclusion, cathepsin D is expressed predominantly in the distal nephron (TALH, DCT, and CNT) and collecting ducts of the human kidney. PTH-induced cathepsin D excretion represents a segment-specific sensitivity to PTH at cTALH, DCT, CNT, and CCD, which express PTH/PTHrP receptor and cathepsin D. The impaired NAG and cathepsin D responses to PTH in PHP type I suggest a decreased sensitivity to PTH in the proximal and distal tubules, respectively. The preservation of sensitivity to PTH in this disorder may be confined to the PTH action on the calcium resorption in DCT, CNT, and CCD.

Acknowledgments

This study was supported by a grant from the Japanese Ministry of Health and Welfare and a grant from the Japanese Ministry of Education.

References