Vitamin D reduces renal NaPi-2 in PTH-infused rats: complexity of vitamin D action on renal Pi handling

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Friedlaender, M. M., H. Wald, M. Dranitzki-Elhalel, H. K. Zajicek, M. Levi, and M. M. Popovtzer. Vitamin D reduces renal NaPi-2 in PTH-infused rats: complexity of vitamin D action on renal Pi handling. Am J Physiol Renal Physiol 281: F428–F433, 2001.—Acute administration of 1,25(OH)2D3 blunts phosphaturia and increases urinary cAMP excretion in parathyroid hormone (PTH)-infused parathyroidectomized (PTX) rats. Because chronic administration of 1,25(OH)2D3 enhances the phosphaturic response to exogenous parathyroid hormone despite blunting of urinary cAMP excretion, we have examined the expression of the renal cortex type II Na-Pi cotransporter (NaPi-2) mRNA and protein in 1) chronic PTX Sabra rats, 2) PTX rats receiving a physiological dose of 1,25(OH)2D3, 3) PTX rats receiving 35 ng/h of PTH, and 4) rats receiving both PTH and 1,25(OH)2D3, for 7 days via osmotic minipumps. Our results confirm that there is increased phosphaturia in the PTH+1,25(OH)2D3-infused animals despite blunting of urinary cAMP excretion, a reduced filtered load of phosphate, and lack of a phosphaturic effect by 1,25(OH)2D3 alone. Both PTH and 1,25(OH)2D3 significantly reduced expression of renal cortex NaPi-2 mRNA and NaPi-2 protein, and the administration of PTH together with 1,25(OH)2D3 had additive effects in further decreasing NaPi-2 mRNA and NaPi-2 protein levels. Expression of two other epithelial transporters, type 1 Na-sulfate and type 1 Na-glucose cotransporters, were not different between the groups, suggesting specificity of the effects of PTH and 1,25(OH)2D3 on phosphate transport. The effect of chronic administration of 1,25(OH)2D3 has not been noted previously, and the cellular mechanisms and signaling processes that mediate the decrease in NaPi-2 remain to be determined.

Vitamin D reduces renal NaPi-2 in PTH-infused rats: complexity of vitamin D action on renal Pi handling.

We have previously reported that the acute phosphaturic effect of parathyroid hormone (PTH) is blunted by 25 hydroxycholecalciferol [25(OH)D3], 24,25 dihydroxycholecalciferol [24,25(OH)2D3], and 1,25 dihydroxycholecalciferol [1,25(OH)2D3] (2, 4, 17). This response is associated with decreased urinary excretion of cAMP and reduced PTH-induced in vitro activation of adenylate cyclase in the renal cortex. The mechanism of this acute action of vitamin D metabolites is unclear but appears to be dependent on posttranscriptional protein synthesis (2), independent of cytoplasmic microtubule integrity (5), and probably calmodulin dependent (3). Because dibutylcAMP infusion-induced phosphaturia is not decreased by vitamin D metabolites (17) and these metabolites exert no acute antiphosphaturic effect when administered alone, it appears that vitamin D metabolites inhibit hormone-induced phosphaturia by direct interference at the receptor adenylate cyclase level.

In contrast to these acute antiphosphaturic effects of vitamin D metabolites, we have observed that vitamin D administration to hyperparathyroid rats with chronic renal failure increases phosphaturia despite blunting urinary cAMP excretion (22). Furthermore, acute 25(OH)D3 administration to parathyroidectomized (PTX) rats receiving infusion of exogenous dibutyryl cAMP causes increased phosphaturia (17). These findings suggest that 25(OH)D3 actually increases the renal tubular cell sensitivity to both endogenous and exogenous cAMP.

We have therefore reexamined the renal response to chronic vitamin D administration under controlled conditions, and, having ascertained that phosphaturia is indeed increased by continuous vitamin D administration despite blunting of urinary cAMP excretion and decreased filtered phosphate load, we have examined whether this may be due to changes in renal cortex expression of the type II sodium-phosphate cotransporter (NaPi-2) protein and mRNA.

METHODS

Metabolic studies. Male Sabra rats, weighing 200 g, were acclimatized for 5 days in individual metabolic cages with free access to tap water and standard chow containing 0.69% phosphorus and 0.97% calcium. After baseline 24-h urine collection and blood sampling from tail veins, all animals underwent acute parathyroidectomy by electrocautery under...
light ether anesthesia. Unprimed Alzet osmotic minipumps were implanted subcutaneously (model 2001, Alzet, Palo Alto, CA). They delivered bovine 1—34 PTH (Sigma, St. Louis, MO) in 2% cysteine HCl at a rate of 0.24 U (35 ng/h) and/or 1.25(OH)2D3 (Hoffmann La Roche, Basel, Switzerland) in propylene glycol at a rate of 2.5 ng (6.25 pmol)·100 g body wt−1·24 h−1.

The dose of 1.25(OH)2D3 was determined by preliminary experiments in PTX rats in which we observed that 1.25(OH)2D3 at higher doses partially corrected hypocalemia in PTX rats but that continuous coadministration of these doses of 1.25(OH)2D3 with 1—34 PTH caused undesirable hypercalcemia. We chose a dose of 1.25(OH)2D3 that did not produce hypercalcemia when administered together with PTH.

Groups studied. Experiments involved the following groups: group I [vehicle +/vehicle (n = 5)]; group II [vehicle +1.25(OH)2D3 (n = 6)]; group III [PTH +/vehicle (n = 6)]; and group IV [PTH +1.25(OH)2D3 (n = 6)].

Three rats were returned to their metabolic cages for a further 7 days. Daily body weight, water and food intake, and urine volume were noted. Urine for cAMP excretion measurements was collected under ice-cooled conditions. Urine and blood samples were analyzed spectrophotometrically for creatinine, calcium, and phosphate by using a computer-directed analysis system (Cobas-Mira Roche, Basel, Switzerland). Urinary cAMP was measured in duplicate by the protein-binding assay of Gilman (6) using a RIA [3H]cAMP kit (Amersham, Buckinghamshire, UK).

Northern blots. On the death of the animals 7 days after PTX, total RNA was immediately extracted from renal cortex of three rats from each group of rats using a Tri-reagent kit (Molecular Research Center, Cincinnati, OH). The mean biochemical values of these rats were similar to those of the group from which they were selected. Aliquots of 10–20 μg total RNA were resolved electrophoretically on 1% agarose gels under denaturating conditions (formamide/formaldehyde). Nucleic acids were transferred to nylon membranes (Gene Screen; New England Nuclear Research Products, Boston, MA) and cross-linked by ultraviolet irradiation. Membrane strips were hybridized for 16–20 h with [32P]labeled rat-specific PTH/PTH-related peptide (PTHrP) receptor cDNA under stringent conditions. Membranes were washed and autoradiographed by standard procedures. Bound cDNA probes were removed by 15 min of immersion in boiled 1× standard sodium citrate + 0.1% SDS, and the same membranes were hybridized with [32P]labeled NaPi-2 cDNA and washed and autoradiographed, the probes were removed again, and membranes were rehybridized with a control probe of 18S ribosomal cDNA.

The radioactive probes were prepared with a Rediprime DNA labeling kit (Amersham) using an EcoRI fragment of rat PTHrP receptor cDNA, a full-length cDNA probe of NaPi-2, and a cloned fragment of 18S ribosomal RNA as templates. Binding was quantified by phosphorimaging (Fuji, BAS 1000) and expressed as the ratio of intensities obtained by hybridizing the same strip with PTH/PTHrP receptor and 18S rRNA cDNAs.

Preparation of brush-border membranes. Contralateral kidneys from the three rats selected for the Northern blots were rapidly removed from the rats, and slices were cut at 4°C from the superficial cortex, homogenized in a buffer consisting of 300 mM m-nannitol, 5 mM EGTA, 16 mM HEPES, and Tris, pH 7.5, containing protease inhibitor cocktail tablets (Boehringer Mannheim). Brush-border membranes (BBM) were precipitated from this homogenate by Mg2+ precipitation and differential centrifugation as described before (13). The final pellet was resuspended in the same buffer as above. Protein concentration of the BBM preparation was determined by an automated pyrogallol red colorimetric method (Cobas-Mira Roche), and equal amounts of protein (60 μg) were added to each lane of the polyacrylamide gels.

SDS-PAGE and immunoblotting. Aliquots of BBM were denatured 1× with sample buffer containing 4% SDS, 20% glycerol, 1% β-mercaptoethanol, and 125 mM Tris-HCl, pH 6.8. Thirty micrograms of BBM protein per lane were separated on 10% polyacrylamide gels and electrotransferred onto nitrocellulose paper. Protein loading equality between the lanes was confirmed before chemiluminescence examination by staining with Ponceau S stain. After blockage with 5% fat-free milk powder, Western blotting was performed with antiserum against the COOH-terminal amino acid sequence of NaPi-2 at a dilution of 1:5,000 (10, 14). Blotting was also performed by using antibodies to the sodium-glucose cotransporter (SGLT-1; from Alpha Diagnostics, San Antonio, TX) and the sodium-sulfate cotransporter (NaSi-1) (16). The secondary antibody was goat anti-rabbit IgG at a dilution of 1:10,000. Antibody binding was visualized by using enhanced chemiluminescence, and densitometry was done by phosphorimaging.

Statistics. Results are presented as means ± SE. Analysis of variance was performed for statistical evaluation among the four groups. Results between individual groups were compared by a nonpaired Student’s t-test with a modified level of significance according to the Bonferroni method (7).

RESULTS

Metabolic data. Table 1 shows baseline data after animals were acclimatized in metabolic cages before PTX minipump insertion. The groups were similar with respect to body weight, plasma creatinine, phosphate, and calcium, and creatinine clearance and fractional excretion of phosphorus (FEphos).

Table 2 shows the metabolic data 7 days after parathyroidectomy and insertion of minipumps. Control PTX rats were significantly hyperphosphatemic and hypocalcemic. Continuous 1,25(OH)2D3 partially corrected hypocalcemia but did not significantly reduce plasma phosphate or change FEphos. Continuous PTH

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Weight, g</th>
<th>Screat, μmol/l</th>
<th>Ccr, ml/min</th>
<th>S创造出, mg/dl</th>
<th>SPhosphate, mg/dl</th>
<th>FEPhos, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PTX</td>
<td>5</td>
<td>211 ± 12</td>
<td>33.7 ± 1.2</td>
<td>1.44 ± 0.04</td>
<td>5.03 ± 0.07</td>
<td>3.16 ± 0.07</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>Vit D3</td>
<td>6</td>
<td>234 ± 10</td>
<td>36.0 ± 1.7</td>
<td>1.47 ± 0.04</td>
<td>5.11 ± 0.14</td>
<td>3.65 ± 0.18</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td>PTH</td>
<td>6</td>
<td>218 ± 6</td>
<td>35.5 ± 1.5</td>
<td>1.39 ± 0.07</td>
<td>4.97 ± 0.22</td>
<td>3.48 ± 0.17</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>Vit D3 + PTH</td>
<td>6</td>
<td>221 ± 4</td>
<td>37.3 ± 1.8</td>
<td>1.53 ± 0.12</td>
<td>4.83 ± 0.18</td>
<td>3.37 ± 0.09</td>
<td>5.4 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; Screat, S创造出, SPhosphate: serum creatinine, calcium, and phosphorus, respectively; Ccr, creatinine clearance; FEphos, fractional excretion of phosphorus; PTH, parathyroidectomized; PTH, parathyroid hormone; Vit D3, 1,25(OH)2D3.
corrected both hypocalcemia and hyperphosphatemia and increased FE\textsubscript{phos} significantly. The coadministration of continuous 1,25(OH)\textsubscript{2}D\textsubscript{3} with continuous PTH infusion further increased the FE\textsubscript{phos} significantly despite the fact that this group had the lowest filtered load of phosphate. Urinary excretion of phosphate rose significantly only in animals receiving the combination of PTH and 1,25(OH)\textsubscript{2}D\textsubscript{3} (Table 2).

The three groups of animals receiving PTH and/or 1,25(OH)\textsubscript{2}D\textsubscript{3} had similar weight gain, food intake, plasma creatinine, and creatinine clearance throughout the experiment. The PTX control rats receiving only vehicle did not gain weight and had slightly lower creatinine clearances at 7 days.

Figure 1 illustrates the plasma calcium in the four experimental groups. This was maintained within the normal range of intact rats in group IV [coadministration of PTH and 1,25(OH)\textsubscript{2}D\textsubscript{3}] and was nearly normal in group III (receiving PTH alone). 1,25(OH)\textsubscript{2}D\textsubscript{3} (group II) partially corrected PTX-induced hypocalcemia.

Figure 2A shows that both PTH-infused groups had significantly lower levels of plasma phosphate than the other two groups. Figure 2B shows that the FE\textsubscript{phos} was significantly greater in the PTH-infused groups than in groups I and II [PTX- and PTX-1,25(OH)\textsubscript{2}D\textsubscript{3}-treated animals, respectively].

Table 2. Seven-day metabolic data

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Weight, g</th>
<th>S\textsubscript{crea}, \textmu mol/l</th>
<th>C\textsubscript{crea}, ml/min</th>
<th>S\textsubscript{calcium}, meq/l</th>
<th>S\textsubscript{phos}, meq/l</th>
<th>U\textsubscript{phos}, \mu eq/24 h</th>
<th>FE\textsubscript{phos}, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PTX</td>
<td>5</td>
<td>206 ± 14</td>
<td>48.7 ± 2.7</td>
<td>1.20 ± 0.19</td>
<td>2.88 ± 0.15</td>
<td>4.64 ± 0.14</td>
<td>308 ± 27</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td>Vit D\textsubscript{3}</td>
<td>6</td>
<td>235 ± 11</td>
<td>43.1 ± 1.4</td>
<td>1.34 ± 0.06</td>
<td>3.56 ± 0.20</td>
<td>4.31 ± 0.21</td>
<td>388 ± 51</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>PTH</td>
<td>6</td>
<td>222 ± 6</td>
<td>46.9 ± 3.4</td>
<td>1.23 ± 0.10</td>
<td>4.48 ± 0.221</td>
<td>2.97 ± 0.20†</td>
<td>328 ± 42</td>
<td>6.4 ± 1.7†</td>
</tr>
<tr>
<td>Vit D\textsubscript{3} + PTH</td>
<td>6</td>
<td>232 ± 7</td>
<td>40.4 ± 1.4</td>
<td>1.46 ± 0.09</td>
<td>5.07 ± 0.16†</td>
<td>2.75 ± 0.13†</td>
<td>511 ± 53†</td>
<td>9.4 ± 1.6†</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; U\textsubscript{phos}, urinary phosphorus. *P < 0.05, †P < 0.01 vs. control.

Continuous infusion of PTH significantly increased urinary excretion of cAMP. However, coadministration of continuous 1,25(OH)\textsubscript{2}D\textsubscript{3} abolished this increase (Table 3).

NaPi-2 protein and mRNA levels. Figure 3 shows the results of renal cortex NaPi-2 mRNA levels obtained at day 7 in the four groups of animals studied. Administration of 1,25(OH)\textsubscript{2}D\textsubscript{3} or PTH both caused decreased expression of NaPi-2 mRNA. The coadministration of both continuous 1,25(OH)\textsubscript{2}D\textsubscript{3} and PTH infusions significantly decreased expression of NaPi-2 mRNA more than either alone.

Fig. 1. Serum calcium at baseline and after 7 days in parathyroidectomized (PTX) rats receiving subcutaenous minipump infusion of vehicle (control; n = 5), 1,25(OH)\textsubscript{2}D\textsubscript{3} (Vit D\textsubscript{3}; 35 ng/h, n = 6), 1–34 bovine parathyroid hormone (PTH; 2.5 ng·100 g\textsuperscript{-1}·24 h\textsuperscript{-1}, n = 6), and both 1,25(OH)\textsubscript{2}D\textsubscript{3} and PTH (n = 6).

Fig. 2. A: serum phosphate in PTX rats. B: fractional excretion of phosphate (FE\textsubscript{phos}) in PTX rats. Infusions were as described in Fig. 1.
than either agent when infused alone. Housekeeping gene expression (18S mRNA) was unaffected by any treatment. The expression of PTH receptor mRNA (Table 3) was significantly reduced by continuous infusion of PTH (group III). However, this reduction was not evident after coadministration of continuous 1,25(OH)2D3.

Figure 4 shows the NaPi-2 protein content of the renal cortex BBM obtained from the PTX animals after 7 days of minipump treatment. This was significantly reduced by both continuous PTH and continuous 1,25(OH)2D3 administration. The coadministration of both agents together decreased NaPi-2 protein content more significantly than either agent administered alone. To affirm that these changes in NaPi-2 protein content were specific, we performed Western blots for NaSi-1 and SGLT-1. These showed no significant differences among the groups. Figure 5 shows the data for SGLT-1 protein expression.

DISCUSSION

We have previously found that vitamin D metabolites have an acute antiphosphaturic effect on hormone-induced phosphaturia (2, 4, 18). This effect appears to be mediated by interference with the activation of the adenylate cyclase-cAMP receptor complex. Thus it is of great interest that in a more chronic setting, 1,25(OH)2D3 appears to increase the PTH-induced phosphaturia despite a decrease in urinary cAMP.

The obvious explanation might have been simply that in a chronic setting, vitamin D increased intestinal calcium and phosphate absorption and that the increased phosphaturia is a direct consequence of increased phosphate load. However, it may be noted that when we administered 1,25(OH)2D3 alone at a dose that has been reported to increase to normal the flow of intestinal calcium and phosphate absorption in thyroparathyroidectomized rats (20, 21), neither urinary excretion nor fractional excretion of phosphate changed significantly.

Thus explanations other than intestinal phosphate load to explain increased phosphaturia were sought. 1,25(OH)2D3 reduced urinary cAMP in PTH-infused animals after 7-day infusion, thus suggesting that a lessening of the vitamin D blunting effect on PTH-induced adenylate cyclase activity was not the explanation for the increased phosphaturia at 7 days. Notwithstanding, the possibility that increased intestinal uptake of phosphate could be involved at least partly in phosphaturia cannot be ruled out.

Of interest is our finding that the PTH receptor mRNA was downregulated from the PTX state by continuous infusion of PTH and that this was abolished by coinfusion with 1,25(OH)2D3. Others have also found that 1,25(OH)2D3 upregulates the PTH receptor in renal distal tubular cells (23). This might possibly lead to increased sensitivity to the infused PTH. However, urinary cAMP excretion was decreased by coadministration of 1,25(OH)2D3, thus ruling out increased sensitivity to PTH and activation of the adenylate cyclase-protein kinase A pathway. We cannot rule out that the lack of blunting of PTH receptor mRNA in 1,25(OH)2D3 + PTH-treated animals may have maintained phosphaturia at high levels via non-adenylate cyclase-dependent mechanisms such as protein kinase C activation.
As noted previously, we also were careful not to induce hypercalcemia in the experimental animals because this is another factor that is known to influence renal phosphate handling (1, 19).

An alternative explanation of the phosphaturic effect of 1,25(OH)₂D₃ in PTH-infused PTX animals might be via a change in the activity or amount of the major sodium-phosphorus transporter in the rat kidney cortex, i.e., NaPi-2. Our findings suggest that chronic continuous infusion of both 1,25(OH)₂D₃ and PTH decreases the NaPi-2 mRNA expression in the renal cortex of PTX rats and that their coadministration significantly decreased the NaPi-2 mRNA expression still further. Western blotting of BBM preparations from the renal cortex of the same rats showed that NaPi-2 protein levels paralleled the NaPi-2 mRNA expression. Continuous chronic infusion of PTH or 1,25(OH)₂D₃ reduced NaPi-2 protein content in the cortex of PTX rats, and their coadministration reduced NaPi-2 protein content still further. Others have found that renal NaPi-2 protein are increased two- to threefold in chronic PTX rats (10). Acute PTH infusion rapidly reduced BBM NaPi-2 protein. There was little or no effect on NaPi-2 mRNA expression, and it was determined that the rapid decrease in BBM NaPi-2 protein abundance was mediated by acute internalization (endocytosis) of NaPi-2 protein (25). In contrast to the determination of the relatively acute effects of PTH administration, the effect of chronic continuous PTH infusion on renal NaPi-2 mRNA and NaPi-2 protein content have not been previously determined.

We have found an additive effect of chronic 1,25(OH)₂D₃ infusion to further decrease renal cortex NaPi-2 expression and protein content. This has not previously been reported, and its mechanism is unknown. It appears to be specific for phosphate transport because both NaSi-1 and SGLT-1 proteins were unaltered by the PTH and 1,25(OH)₂D₃ infusions. Vitamin D stimulates intestinal Na-Pi cotransporter expression (11), whereas in some cells such as osteoblasts phosphate transport may be inhibited by 1,25(OH)₂D₃ (8). In vitamin D-deficient rats 1,25(OH)₂D₃ has been reported to increase juxtamedullary cortex expression of NaPi-2 mRNA and protein, but NaPi-2 expression was decreased in the superficial cortex (24). Our finding that continuous 1,25(OH)₂D₃ infusion given for 7 days decreases NaPi-2 mRNA and protein expression in PTX PTH-infused rats may suggest a direct effect of vitamin D in decreasing renal NaPi-2 such as has been shown for chronic glucocorticoid administration (9,14, 15), or perhaps there is secretion of an unknown phosphaturic hormone secondary to increased intestinal absorption of phosphate.

It is of mechanistic interest that chronic infusion of 1,25(OH)₂D₃ alone decreased NaPi-2 expression (Figs. 3 and 4) but that we were unable to demonstrate phosphaturia in these animals (Fig. 2B, Table 2). This is in contrast to both groups of PTH-infused animals in whom phosphaturia increased. Thus it would appear that the presence of PTH is permissive to the chronic physiological effects of vitamin D, a finding that we have previously described for the acute antiphosphaturic effects of vitamin D (4, 17, 18). Others have noted that changes in Na-Pi cotransporter expression are not always correlated well with changes in phosphate transport (12). PTH action may therefore also involve changes in Na-Pi cotransporter activity or in the activity or availability of other transporters that may influence phosphate transport.
The schema in Figure 6 shows the disparate effects of acute and chronic administration of 25(OH) vitamin D3 derivatives on PTH-induced phosphaturia. The mechanism of acute antiphosphaturia appears to be via blunting of adenylate cyclase activation. Despite blunting of adenylate cyclase activation, chronic administration of vitamin D derivatives increases phosphaturia and appears to reduce renal cortex NaPi-2 mRNA and NaPi-2 protein levels. The cellular mechanisms and the signaling processes by which this occurs remains to be determined.

The antibody to NaSi-1 was kindly provided by Dr. H. Murer.

REFERENCES