Renal actions of vitamin D in D-deficient rats

LINDA S. COSTANZO, PAUL R. SHEEHE, AND I. M. WEINER

Department of Pharmacology and Department of Preventive Medicine, State University of New York, Upstate Medical Center, Syracuse, New York 13210

Renal actions of vitamin D in D-deficient rats. Am. J. Physiol. 226(6): 1490-1495. 1974.--The renal clearances of calcium and phosphate were studied in vitamin D-deficient rats and similar rats repleted with 100 U of vitamin D 48 h prior to the experiments. Thyroparathyroidectomy (T-PTX) was performed on all animals immediately before the experiments. A statistical method was devised to estimate the effect of vitamin D repletion on multiple observations of C&GFR at several levels of C&GFR. The calcium clearance ratios at comparable levels of sodium clearance ratios were significantly lower in D-repleted than in D-deficient animals over the range of sodium excretion encountered. In these treatment groups phosphate excretion was not measurable. The effect of vitamin D on phosphate excretion was studied in similar groups of animals under conditions of phosphate loading. There was virtually no phosphate excretion in D-repleted T-PTX rats at filtered loads as high as 5 μmol/min. D-deficient T-PTX rats excreted significant quantities of phosphate especially at filtered loads above 2 μmol/min.

IN NORMAL ANIMALS total calcium balance and the concentration of calcium in plasma are closely regulated by a system of controls operating on the turnover of bone salts, the absorption of calcium from the gut, and the excretion of calcium by the kidney. Vitamin D and its metabolites constitute major components of this system. The direct effects of vitamin D deficiency or repletion with vitamin D (or its metabolites) on bone and gut are well documented, although the underlying biochemical mechanisms are incompletely understood (6, 21, 35). However, a direct effect of the vitamin on renal calcium transport has not been firmly established. In some studies, vitamin D was found to increase the tubular reabsorption of calcium (10, 24), whereas in another, net calcium transport was reported to decrease under the influence of the vitamin (2). Report of a vitamin D-induced calcium binding protein in chicken kidney tissue (29, 30) suggests strongly that the vitamin does have a direct effect on renal calcium transport. Except for the latter discovery, the vitamin D-dependent protein had been found only in known target tissues of vitamin D (29).

Among the factors which might complicate the resolution of this question are: 1) the interdependence of calcium and sodium excretions (34), 2) changes in plasma or ultrafiltrable calcium (or both) and plasma phosphate concentrations after vitamin D repletion (5), 3) alteration of the parathyroid status of experimental animals (8), and 4) hyperparathyroidism, if exogenous vitamin D is given to a non-deficient animal. Consequently, it seemed appropriate to compare the renal clearances of calcium in D-deficient and D-repleted animals under conditions where: 1) the influence of sodium excretion on calcium excretion could be assessed; 2) the plasma concentrations of calcium were comparable in the two groups; 3) the rates of glomerular filtration were comparable; 4) the influence of the parathyroids could be minimized; and 5) the dose of vitamin D was in the range known to be necessary for restoration of another calcium transport system. The experiments were performed in rats since vitamin D deficiency can be produced in these animals in a comparatively short time, and because a normocalcemic state can be maintained in spite of D deficiency (25). The results are consistent with a direct effect of vitamin D to enhance calcium reabsorption relative to sodium reabsorption. In addition, other experiments suggest that vitamin D may enhance the tubular reabsorption of phosphate.

METHODS

Animals. Sprague-Dawley male rats were housed in hanging wire cages. Rats designated as vitamin D deficient were obtained as weanlings (50-60 g), fed a normal calcium-low phosphorus vitamin D-deficient diet essentially as described by DeLuca et al. (7) but containing 0.44 % calcium and 0.05 % phosphorus. They were housed in the absence of daylight or fluorescent lighting for a period of 3.5-6 wk prior to use. Food and water were allowed ad libitum up to the time of the clearance experiments. These animals maintained normocalcemia as long as they had access to food (see below). However, they had the characteristic appearance of D-deficient rats as described by Steenbock and Herting (28). In preliminary experiments we ascertained that rats maintained on this diet for 35 days no longer gained weight; the mean weight at this time was 150 g. Rats fed the same diet supplemented with 75 U of vitamin D, every 3 days reached a mean weight of 177 g at 35 days and continued to grow thereafter. Hypocalcemia could be produced in the D-deficient rats by withholding food for 12 h; plasma calcium concentration fell to 67% of the prefasting value. Those rats described as vitamin D repleted in the subsequent text were D-deficient animals treated with a single dose (100 U) of vitamin D₃, p.o., 48 h prior to experiments. This is the dose shown to restore calcium transport in rat intestine (16).

Clearance experiments. Rats were anesthetized with Inactin (Promonta, Hamburg), intraperitoneally, 80 mg/kg for 85- to 120-g rats and 100 mg/kg for larger rats. A trache-
otomy was performed and a thyroparathyroidectomy (T-PTX) was performed by electrocautery. (In control experiments with normal rats, we demonstrated that this technique resulted in the virtual disappearance of phosphate from the urine within 2 h when plasma phosphate concentration was normal.) The right femoral vein and artery were cannulated for infusion and blood sampling, respectively. For studies on calcium excretion, the intravenous infusion contained 0.9% NaCl and 5% mannitol and inulin. The infusion was delivered at 0.1 or 0.2 ml/min. In experiments on the tubular titration of phosphate, a 1:1 mixture of 0.9% NaCl and 65 mM NaH₂PO₄ (adjusted to pH 7.4) containing 5% mannitol and inulin was infused into one femoral vein while a second infusion of CaCl₂ in 0.9% NaCl was infused via a second vein, the combined infusion rates were the same as in experiments in which only one infusion was used. Both ureters were cannulated near the renal pelvices. At least 2 h were allowed to elapse from the time of thyroparathyroidectomy. The infusion was delivered for at least 1 h for stabilization of initial urine flow and plasma inulin concentration. Six to eight clearance periods of 15 min duration were taken. Urine was collected in preweighed tubes, and volumes were determined by weight, assuming a specific gravity of 1.0. Blood samples of 0.8 ml were drawn at the beginning of alternate clearance periods with an additional terminal sample. These blood losses were replaced by equal volumes of heparinized whole blood from donor animals whose treatment was identical to that of the experimental animals. Interpolated values were used for those clearance periods in which plasma was not taken for analysis.

Analytical techniques. Inulin (13), phosphate (3), and calcium (32) concentrations were determined by modifications (scaled down) of published methods. A small inulin "blank" in rat plasma, approximately 4% of average readings, was ignored. Sodium was determined by flame photometry. Ultrafiltrates of 8-ml samples of pooled rat plasma were prepared at 37°C in an atmosphere of 5% CO₂-95% O₂ according to the method of Toribara et al. (31).

RESULTS

Effect of vitamin D repletion on calcium excretion. Saline was infused in 11 vitamin D-deficient and 10 vitamin D-repleted rats in order to study the relationship between calcium and sodium clearances. The infusion rate was increased from 0.1 to 0.2 ml/min during the experiments in order to obtain a wide range of urine flows and sodium clearances. The increase in infusion rate was made at the beginning of the first, third, or fourth clearance period in the various experiments. Plasma calcium and phosphate concentrations and GFR were relatively stable throughout the experiments (Table 1). The mean plasma calcium concentration was 4% higher during the first clearance period in vitamin D-repleted rats than in depleted rats; in no other period was there a statistically significant difference in the plasma concentrations of calcium or phosphate. Plasma ultrafilterable calcium was determined in samples from 14 depleted rats and 14 repleted rats, treated as were those used in clearance experiments. Within each group the plasma from four to five rats was pooled to give three separate samples of adequate size. The fraction of total calcium which was ultrafilterable was 67% (range 65.5-67.5%) in depleted rats and 68% (range 66.5-69.0%) in repleted rats. In both groups of animals plasma phosphate was lower than in normal animals on a chow diet, 3.15 mM. Values for GFR were relatively steady and not significantly different in the two groups. In both groups of animals, phosphate excretion was below the limit of detection.

The details of two experiments, one with a D-deficient and one with a D-repleted rat, are given in Table 2. Urine flow did not equal infusion rate in any of the clearance periods, and thus the rats were becoming progressively volume expanded. Urine flow and the sodium clearance ratio were higher in later clearance periods, presumably the result of this volume expansion and the mannitol.

<table>
<thead>
<tr>
<th>TABLE 1. Plasma concentrations of Ca and P0₄ and glomerular filtration rates in D-deficient and D-repleted rats</th>
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<tr>
<td>PₐCa, mM</td>
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<tr>
<td>2.71±0.07</td>
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<tr>
<td>PₐP₀₄, mM</td>
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<td>GFR, ml/min</td>
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Values are means ± SE. *Significantly different from n-repleted group, P < 0.05.

<table>
<thead>
<tr>
<th>TABLE 2. Illustrative clearance experiments in rats on D-deficient diet, with acute thyroparathyroidectomy, without (R₃), and with (R₄) vitamin D repletion</th>
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<tr>
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<td>Experiment R₃, 150-g rat, D-deficient diet for 20 days</td>
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<td>1.50 g NaCl at 0.1 ml/min</td>
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<td>0-15</td>
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Experiment R₄, 150-g rat, D deficient diet for 30 days, 100 IU of vitamin D 48 h prior to experiment

|                  | Time, min | GFR, ml/min | V, ml/min | PₐCa, mM | PₐP₀₄, mM | CₚₐGFR | Cₚₐ/V, ml/min |
|                  |           |             |           |          |           |        |              |
| 0-15             | 0.49      | 0.059       | 146       | 2.58     | 1.76      | 0.027  | 0.069        |
| 15-30            | 0.50      | 0.053       | 138       | 2.50     | 1.62      | 0.016  | 0.056        |
| 30-45            | 0.54      | 0.054       | 132       | 2.50     | 1.62      | 0.016  | 0.056        |
| 45-60            | 0.57      | 0.074       | 132       | 2.57     | 1.33      | 0.038  | 0.066        |
| 60-75            | 0.61      | 0.111       | 132       | 2.57     | 1.33      | 0.038  | 0.066        |
| 75-90            | 0.56      | 0.120       | 133       | 2.57     | 1.33      | 0.038  | 0.066        |

GFR = glomerular filtration rate. V = urinary flow rate. P = plasma concentrations. Subscripts refer to the specific ions. C = clearance. Concentrations of electrolytes for periods in which blood was not taken were estimated by interpolation.
induced diuresis. These experimental manipulations and spontaneous variations gave a range of sodium clearance ratios from less than 0.001 to about 0.10 (Fig. 1). In the experiments given in detail (Table 1) and in general (Fig. 1), there was a tendency for the calcium clearance ratio to increase with increasing sodium clearance ratio.

Although there is considerable overlap, there is a perceptible tendency for the clearance ratio of calcium to be lower in D-repleted rats than in D-deficient rats at the various levels of sodium clearance ratio. Statistical analysis by the method described in the Appendix yielded \( P < 0.05 \). The calculated \( \Delta \) was 0.0128 and suggests the magnitude of the decrease in calcium clearance ratio attributable to the vitamin.

Effect of vitamin D-repletion on phosphate excretion. Experiments were performed in eight vitamin D-deficient and five vitamin D-repleted rats. After two 15-min control periods of saline infusion, plasma phosphate concentration was gradually elevated by infusion. Rats received small amounts of calcium intravenously to prevent precipitous falls in plasma calcium. Experiments in these two groups of rats were associated with a falling GFR. Data from periods in which GFR fell to less than 30% of control were discarded. The mean fall in GFR at the last period included from each experiment was 15% in deficient rats and 20% in repleted rats. Correction of the decreasing plasma calcium to normal concentrations did not prevent the fall in GFR in most experiments. (It is of interest that plasma calcium concentration does not fall significantly during the infusion of similar quantities of phosphate in T-PTX rats fed a normal diet (unpublished observations).)

Figure 2 shows the urinary excretion of phosphate as a function of the filtered load. Deficient rats receiving a single dose of vitamin \( \text{D}_3 \) had no appreciable urinary phosphate excretion even at the highest levels of filtered load encountered, i.e., reabsorption was virtually complete. Rats deficient in vitamin D did excrete phosphate especially at filtered loads exceeding 2 \( \mu \text{mol/min} \). In D-deficient animals, the degree of phosphaturia resulting from the increased filtered loads seemed not to depend on whether or not normocalcemia was maintained; at least one-half of the data points in which phosphate excretion was significant were obtained from periods characterized by normal plasma calcium levels.

DISCUSSION

The parallelism between renal calcium and sodium clearances has been most extensively studied in dogs (18, 33). The two clearances are approximately equal when calcium clearance is based on the ultrafilterable moiety and the ratio of clearances, calcium/sodium, is about 0.6 in terms of total plasma calcium concentration. The interdependence is not altered under a variety of different experimental conditions, although interventions which disturb distal tubular function characteristically dissociate the clearances of the two ions (34). Our data from vitamin D-deficient and repleted rats are consistent with a positive relationship between calcium and sodium excretion, but they do not conform to a linear relationship.

The present results constitute the first demonstration of a specific enhancement by vitamin D of calcium transport relative to sodium transport in the kidney. Our results cannot be explained on the basis of differences in the concentrations of plasma or ultrafilterable calcium, plasma phosphate, or GFR. Although thyroparathyroidectomy was performed acutely to obviate changes in circulating parathyroid hormone (PTH) during the experiments, we cannot be confident that the plasma was completely cleared of PTH. Based on a half-life of 20 min for the hormone (19, 27), it seems likely that PTH remaining after 2 h would be insignificant. However, it remains possible that if
vitamin D repletion caused a substantial decrease in circulating PTH and if some PTH remained at the time of experimentation that the amount of hormone remaining might be different in the two treatment groups. Such a phenomenon could not explain our results on calcium, since a lower circulating PTH (as might exist in the vitamin-repleted group) would be expected to reduce calcium relative to sodium reabsorption (1, 17, 18); our findings were the opposite.

Puschett et al. (24), who found that vitamin D caused a decrease in phosphate excretion in dogs, also noted decreases in sodium and calcium excretion of proportional magnitude. The present results indicate that vitamin D may increase phosphate reabsorption, the vitamin D action on calcium was observed under conditions in which phosphate reabsorption was complete and therefore not susceptible to enhancement.

From the foregoing we suspect that the site of action of vitamin D on calcium transport may be the distal convoluted tubule. The vitamin D-induced calcium binding protein has not been localized to particular nephron segments. However, calcium binding protein (not known to be vitamin D dependent) has been found in human (22) and dog (26) kidney cortex in four to five times the amount as in the medulla. This finding may not have bearing on the vitamin D effect on calcium transport, but is certainly not inconsistent with a site of action in the distal tubule. The fact that radioactive vitamin D is localized in the first third of the proximal tubule (15) may not be related to its effect on renal calcium and phosphate transport but rather to the site of conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol in the kidney (6).

The present work sheds no light on the question of which of the metabolites of vitamin D is responsible for the enhanced calcium reabsorption. The vitamin was administered while the animals had intact parathyroid glands, and there was more than sufficient time for any necessary metabolic conversions.

Despite earlier controversy over the effect of vitamin D on phosphate reabsorption (4, 11, 14, 20), at present there seems to be a consensus that it increases renal phosphate transport (9, 23, 24); our results are consistent with this idea. The present experiments were complicated by the extreme sensitivity of the rats on the special diet to intravenous infusion of phosphate. The resultant depression of GFR severely limited the range of filtered loads which could be obtained in the two groups. In spite of the uncertainty about the overall performance of kidneys whose GFR is falling, the differences in phosphate handling are quite apparent. In addition, it seems that vitamin D-deficient rats have defective phosphate reabsorption as compared to chow-fed T-PTX rats (unpublished observations). Unfortunately, this comparison is not entirely satisfactory because phosphate loading in normal T-PTX rats did not cause lowering of glomerular filtration rate. There is evidence that a positive correlation exists between GFR and the reabsorption of phosphate (12).

A point of some concern is that the effect of vitamin D repletion on phosphate excretion may be due to a difference in the residual circulating parathyroid hormone (see above). The greater phosphate excretion upon phosphate loading in D-deficient animals might be explained by a relatively higher level of residual PTH than in the D-repleted group.

APPENDIX

As is apparent from Fig. 1, the relationships between the clearance ratios for calcium and sodium are not necessarily described by straight lines. It is also apparent from the experimental design that each rat provided data at several levels of fractional sodium excretion. Consequently, it was desirable to develop a statistical test which neither required an exact description of the sodium-calcium relationship nor treated individual observations on a given rat as independent of each other. The hypothesis was made that at any given clearance ratio for sodium that the prior administration of vitamin D would result in a change, Δ, in the calcium clearance ratio. Conceivably, Δ could vary with the level of sodium clearance, but this refinement was not necessary in testing the null hypothesis that Δ = 0. The range of sodium clearance ratios (abscissa in Fig. 1) from 0 to 0.10 was divided into six equal parts, and the individual observations of calcium clearance ratios were classified according to the six levels, designated h.

The notations used are as follows:

- \( i = 1, 2 \): treatment group, vitamin D-deficient or repleted, respectively
- \( j = 1, n \): individual rat
- \( m = 1, p \): observation on a given rat. Individual observations were not identified as to whether they were first, last, etc. In each experiment \( p = 6 \), except one in which \( p = 4 \).
- \( k = 1, 6 \): level of sodium clearance ratio
- \( r_{hk} \): number of observations in treatment group \( i \) at sodium level \( h \), where \( r_{1h} + r_{2h} = r_h \).
- \( k = 1, 6 \): level of sodium level treatment group \( i \) is designated: \( y_{hk} \).

The general structure which will quantify the effect of vitamin D on the calcium clearance ratio is given by:

\[
y_{ma} - y_{1a} + \Delta
\]

If all the rats had received treatment 1, the mean at level \( h \) would have been:

\[
f_{1h} = \left( \frac{\sum_{k=1}^{6} y_{hk} + \sum_{k=1}^{6} y_{1hk}}{r_{1h}} \right)
\]

Substituting from Eq. 1 and rearranging terms, this becomes:

\[
f_{1h} = f_{1h} - \frac{r_{2h} \Delta}{r_{2h}}
\]

where

\[
f_{h} = \frac{\sum_{k=1}^{6} y_{hk} + \sum_{k=1}^{6} y_{2hk}}{r_{2h}}
\]

Similarly, the mean value at level \( h \), if all the rats had received treatment 2, would be:

\[
f_{2h} = f_{2h} + \Delta = f_{2h} + \frac{r_{1h} \Delta}{r_{2h}}
\]

The measurement of the average deviation of a given rat in treatment
group i from the respective values of \( \tilde{y}_{ih} \) is given by:

\[
d_i = \sum_{m=1}^{n_i} \frac{Y_{ijm} - \tilde{y}_{ih(jm)}}{p_{ij}}
\]

where \( \tilde{y}_{ih(jm)} \) is the value of \( y_{ih} \) at the level of h which corresponds to the mth observation of the jth rat in treatment i.

The least-squares estimate of \( \Delta \) is derived as follows:

The sum of squared average deviations (S) is

\[
S = \sum_{j=1}^{n_1} d_{i1} + \sum_{j=1}^{n_2} d_{i2} + \sum_{j=1}^{n_3} d_{i3} + \sum_{j=1}^{n_4} d_{i4} + \sum_{j=1}^{n_5} d_{i5} + \sum_{j=1}^{n_6} d_{i6}
\]

substituting, differentiating with respect to \( \Delta \), setting equal to 0, and rearranging gives:

\[
\hat{\Delta} = \frac{\sum_{j=1}^{n_1} \left( \sum_{m=1}^{r_1} \frac{(Y_{ijm} - \tilde{y}_{ih(jm)})}{p_{ij}} \right) - \sum_{j=1}^{n_2} \left( \sum_{m=1}^{r_2} \frac{(Y_{ijm} - \tilde{y}_{ih(jm)})}{p_{ij}} \right)}{\sum_{j=1}^{n_1} \frac{r_1}{p_{ij}} - \sum_{j=1}^{n_2} \frac{r_2}{p_{ij}}}
\]

where \( \hat{\Delta} \) is the least-squares estimate of \( \Delta \).

REFERENCES


The significance of \( \Delta \) is determined by applying the Student t test as an approximation to the randomization test:

\[
s^2 = \frac{S}{n_1 + n_2 - 2}
\]

with S evaluated at \( \hat{\Delta} \)

\[
t = \frac{\hat{\Delta}}{\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}
\]

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