Tubular handling of phosphate along the nephron of thyroparathyroidectomized rats injected with ethane-1-hydroxy-1,1-diphosphonate

R. C. MÜHLBAUER, J.-P. BONJOUR AND H. FLEISCH
Department of Pathophysiology, University of Berne, Berne, Switzerland

(Received 19 March 1980; accepted 9 September 1980)

Summary

1. Previous studies have shown that in thyroparathyroidectomized rats injection of disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) at doses that inhibit bone mineral retention (0.16 mmol = 10 mg of phosphorus/kg body wt. per day subcutaneously) leads to a decrease in the net tubular reabsorption of phosphate.

2. In the present work the tubular response to EHDP (0.16 mmol/kg body wt.) injected subcutaneously for 9 days has been localized by free-flow micropuncture in thyroparathyroidectomized rats.

3. The results show that the net tubular reabsorption of phosphate along the first portion of the (early) proximal tubule was markedly depressed in the EHDP-injected thyroparathyroidectomized rats compared with that in the pair-fed thyroparathyroidectomized control animals. In this latter group the delivery of phosphate to the distal tubule was larger than in the final urine, confirming previous reports. In the EHDP-injected thyroparathyroidectomized rats no difference in delivery of phosphate to the distal tubule was found between the distal tubule and the final urine, suggesting that diphosphonate inhibited net reabsorption of phosphate in the terminal nephron.

4. The sites of the EHDP-induced changes in the tubular handling of phosphate were similar to those previously determined for the adaptive response to an increase in the supply of phosphate.

Key words: adaptation, diphosphonate, distal tubule, phosphate, renal transport, micropuncture, proximal tubule, thyroparathyroidectomy.

Introduction

The renal handling of phosphate has been shown to be selectively altered in man or animals given the compound disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP). In normal man or in patients with Paget's disease the chronic administration of EHDP leads to an increase in the tubular reabsorptive capacity for phosphate, which can account for the hyperphosphataemia observed with diphosphonate treatment (Recker, Hassing, Lau & Saville, 1973; Russell, Smith, Preston, Walton & Woods, 1974; Walton, Russell & Smith, 1975). The increased reabsorptive capacity observed in man is probably independent of parathyroid hormone (Recker et al., 1973; Walton et al., 1975). In experimental animals EHDP can also alter the tubular reabsorptive capacity for phosphate through a parathyroid hormone-independent mechanism. However, the effect so far observed has been opposite to that described in man, since EHDP administration decreased the tubular reabsorptive capacity for phosphate in thyroparathyroidectomized rats (Bonjour, Tröhler, Preston & Fleisch, 1978). The reason for these opposing responses has not yet been clarified. In both man and rats the renal response of the transport system of phosphate to EHDP is associated with extrarenal alterations in phosphate and/or calcium metabolism (Walton et al., 1975; Bonjour et al., 1978). Therefore, it is possible that the change in the renal handling of phosphate is secondary to these extrarenal al-
terations. In thyroparathyroidectomized rats, EHDP leads to a decrease in the tubular reabsorptive capacity for phosphate when given in doses large enough to inhibit bone mineral retention. It has been suggested (Bonjour et al., 1978) that the renal change represents an adaptation to a reduced demand for phosphate similar to that observed in response to an increase in the phosphate supply (Steele & DeLuca, 1976; Tröhler, Bonjour & Fleisch, 1976). The adaptive response to variations in the phosphate supply in intact and thyroparathyroidectomized rats has been localized by free-flow micropunctures in the first portion of the proximal tubule and probably also along the terminal nephron (Mühlbauer, Bonjour & Fleisch, 1977; Mühlbauer, Bonjour & Fleisch, 1979). The aim of the present work was to determine by free-flow micropuncture where, along the nephron, the EHDP-induced inhibition of phosphate reabsorption takes place in thyroparathyroidectomized rats.

Materials and methods
Preparation of the animals
Male Wistar rats of 232 ± 19 g (mean ± SD) from our breeding colony were used. They were raised in standard conditions and fed a commercial chow (Altromin 1314) containing 22.5 mmol (0.9 g) of calcium and 22.6 mmol (0.7 g) of phosphorus/100 g dry weight. During the 10 days preceding the micropuncture experiments, diphosphonate-injected and control rats were pair-fed with a diet containing 30 mmol (1.2 g) of calcium and 35.5 mmol (1.1 g) of phosphorus/100 g dry weight. This diet consisted of a basic regimen (Altromin C1034), containing a low amount of calcium and phosphorus to which calcium gluconate and sodium phosphate were added. All rats had free access to distilled water. Since the basic diet was poor in vitamin D, a supplement of 25 i.u. of vitamin D₃ in 0.25 ml of vegetable oil was added to the food portion three times weekly. The test animals received daily subcutaneous injections of EHDP during the 10 days preceding the micropuncture study. The daily dose of EHDP was 0.16 mmol (=10 mg of phosphorus)/kg body weight dissolved in distilled water, injected in a volume of 2 ml/kg body weight. The solution was iso-osmotic and its pH was adjusted to 7.4. The control rats were injected with the same volume of sodium chloride solution (0.15 mol/l, saline). All rats were thyroparathyroidectomized 48–72 h before the micropuncture experiments. Since EHDP, given at this dose, tends to normalize the plasma concentration of both calcium and phosphate in thyroparathyroidectomized rats (Bonjour et al., 1978), it was not possible to check the adequacy of the removal of the parathyroid glands in the test animals. However, in the present study, a marked decrease in plasma calcium was observed in all control rats 2 days after the operation.

Micropuncture study
During the night preceding the study, the pair-feeding was maintained and water was given ad libitum. On the experimental day, i.e. 24 h after the last injection of EHDP or saline, the rats were anaesthetized with pentobarbital (Nembutal) as previously described (Mühlbauer et al., 1977). The animals were placed on an operating table heated at 37°C. A tracheostomy was made, and one jugular vein and the left femoral artery were catheterized. The rats were then placed on the right side and the left kidney was exposed and prepared for micropuncture as previously described (Mühlbauer et al., 1977). The ureter of the exposed kidney was catheterized with slightly pulled PE-50 tubing. A first arterial blood sample was taken to assess the effect of this treatment on plasma calcium, then all rats received a priming injection (1.6 ml/100 g body wt.) followed by a constant infusion (7.5 ml/h) of a solution containing inulin (2.1 g/100 ml), sodium chloride and neutral phosphate. Since the control rats displayed a higher initial plasma phosphate concentration, they received a solution containing less phosphate than that given to the EHDP-injected group. The solution given to the control rats contained monosodium dihydrogen phosphate (1.57 mmol/l), disodium hydrogen phosphate (6.43 mmol/l) and sodium chloride (143.3 mmol/l). The solution given to the EHDP-injected rats contained monosodium dihydrogen phosphate (3.92 mmol/l), disodium hydrogen phosphate (16.08 mmol/l) and sodium chloride (143.3 mmol/l). The osmolality of the two solutions was 300–310 mmol/kg of water and the pH varied between 7.35 and 7.45. Thus phosphate was delivered at 60 and 150 μmol/h in the control and EHDP-injected rats respectively.

After an equilibration period of 75 min, ureteral urine of the experimental kidney was collected during two periods of clearance of 30 min each. Blood samples (about 80 μl) were taken at the beginning and at the end of each period, and after each micropuncture. Tubular fluid samples were collected from superficial tubules randomly chosen and standard techniques for free-flow micropunctures were used as previously described (Mühlbauer et al., 1977). Each rat
Tubular handling of phosphate in diphosphonate-injected rats

underwent 1–6 micropunctures. The blood, urine and micropuncture specimens were analysed for phosphate and inulin. In the first blood sample, plasma calcium concentration was also determined. As described by Mühlbauer et al. (1977), on the day of the experiment all samples were diluted in acid and kept frozen or cooled as appropriate. Calcium in undiluted plasma was determined by titration with ethylenebis(oxyethylenenitro)tetra-acetic acid (Corning calcium analyser, model 940). Inulin in plasma, urine and tubular fluid was determined with diphenylamine (Harrison, 1942; Mühlbauer et al., 1977). Inorganic phosphate concentration in plasma, urine and tubular fluid was determined with a molybdate/malachite green reagent (Altmann, Fürstenau, Gielewski & Scholz, 1971; Mühlbauer et al., 1977).

Statistics

Results are expressed as means ± SEM. Significance of difference was calculated by the two-tailed Student's t-test (Table 1). The statistical significance of the EHDP-induced decrease in net phosphate reabsorption observed at various sites along the nephron by free-flow micropuncture (Table 2, Fig. 1) was evaluated by the one-tailed Student's t-test. The inhibitory effect of EHDP on net phosphate reabsorption as determined by the overall renal clearance (see Table 1) can be considered as ‘prior knowledge’ (Sachs, 1974) of the direction of the tubular effect to be localized by free-flow micropuncture, thus allowing the use of the one-tailed test.

Results

Clearance data during micropuncture

Table 1 shows that the clearance of inulin and the urine flow of the whole kidney was similar in control and EHDP-injected rats. During acute phosphate infusion, plasma phosphate increased from a basal value before infusion of 2.97 ± 0.08 to 3.51 ± 0.05 mmol/l in the control group and from 2.00 ± 0.06 to 2.90 ± 0.08 mmol/l in the EHDP-injected animals. The urinary excretion of phosphate was significantly increased in the group injected with EHDP despite a significantly lower plasma phosphate and filtered load of phosphate. As previously described EHDP significantly increased plasma calcium in thyro-parathyroidectomized rats. Just before starting the infusion of phosphate, plasma calcium was found to be 2.09 ± 0.05 mmol/l (n = 12) and 2.65 ± 0.05 mmol/l (n = 12) in control and EHDP-injected rats respectively (P < 0.001).

Micropuncture data

The single nephron glomerular filtration rate (mean ± SEM) was 43.5 ± 4.2 (n = 39) and 48.2 ± 4.3 (n = 50) nl/min in the control and EHDP-injected animals respectively. This difference was not statistically significant. The ratio of tubular fluid to plasma concentration of phosphate is presented in Table 2. For better analysis, the data have been pooled by their localization with respect to water reabsorption [(tubular fluid/plasma)inulin]. EHDP significantly increased (tubular fluid/plasma)phosphate in all six ranges of (tubular fluid/plasma)inulin.

Table 1. Influence of EHDP on the renal handling of phosphate in thyro-parathyroidectomized rats: clearance data during micropuncture (experimental kidney)

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 19)</th>
<th>EHDP (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clearance of inulin (ml/min)</td>
<td>0.93 ± 0.08</td>
<td>0.87 ± 0.05</td>
</tr>
<tr>
<td>Urine flow (ml/min)</td>
<td>0.0283 ± 0.0030</td>
<td>0.0357 ± 0.0033</td>
</tr>
<tr>
<td>Plasma concentration of phosphate (mmol/l)</td>
<td>3.51 ± 0.05</td>
<td>2.90 ± 0.08**</td>
</tr>
<tr>
<td>Filtered load of phosphate (μmol/min)</td>
<td>3.24 ± 0.28</td>
<td>2.54 ± 0.14*</td>
</tr>
<tr>
<td>Urinary phosphate excretion per ml of glomerular filtrate (μmol/ml)</td>
<td>0.47 ± 0.07</td>
<td>1.61 ± 0.11**</td>
</tr>
</tbody>
</table>

*2P < 0.05; **2P < 0.001 compared with the controls.
TABLE 2. Influence of EHDP on (tubular fluid/plasma)phosphate in thyroparathyroidectomized rats

Results are means ± sem. The numbers of observations are given in parentheses. The mean (tubular fluid/plasma)inulin ratio within each range was not significantly different between the two groups. Significance of differences: *P < 0.05; **P < 0.01; ***P < 0.001 compared with the controls.

<table>
<thead>
<tr>
<th>Range of (tubular fluid/plasma)inulin</th>
<th>(Tubular fluid/plasma)phosphate</th>
<th>Control</th>
<th>EHDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 1.49</td>
<td>1.03 ± 0.09 (5)</td>
<td>1.62 ± 0.22 (6)*</td>
<td></td>
</tr>
<tr>
<td>1.50–1.99</td>
<td>0.90 ± 0.10 (4)</td>
<td>1.38 ± 0.12 (8)*</td>
<td></td>
</tr>
<tr>
<td>2.00–2.49</td>
<td>0.99 ± 0.17 (3)</td>
<td>2.00 ± 0.17 (11)**</td>
<td></td>
</tr>
<tr>
<td>2.50–2.99</td>
<td>1.04 ± 0.14 (5)</td>
<td>2.19 ± 0.18 (3)**</td>
<td></td>
</tr>
<tr>
<td>3.00–3.49</td>
<td>1.04 ± 0.16 (5)</td>
<td>1.99 ± 0.33 (7)*</td>
<td></td>
</tr>
<tr>
<td>above 3.50</td>
<td>1.32 ± 0.23 (17)</td>
<td>2.79 ± 0.23 (15)**</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Influence of EHDP on the tubular handling of phosphate in thyroparathyroidectomized rats. The fraction of filtered phosphate remaining at the puncture site is plotted against the (tubular fluid/plasma)inulin ratio. The fraction of filtered phosphate excreted in ureteral urine is presented on the right side of the diagram. ▲, Control rats; ○, EHDP-injected rats. Values are means ± sem. The corresponding (tubular fluid/plasma)phosphate ratios for the same ranges of (tubular fluid/plasma)inulin are presented in Table 2, where the number of observations for each point is indicated. *P < 0.05; **P < 0.01; ***P < 0.005 compared with the corresponding value for the control group.

phosphate reabsorption was observed in the control group, since at a (tubular fluid/plasma)inulin ratio of 1.36 ± 0.04, only 0.74 ± 0.07 of the filtered load of phosphate was recovered.

To assess more accurately the influence of EHDP on phosphate transport along the nephron, the net and fractional reabsorptions were calculated for four tubular segments. The assumption was made that the punctured tubules were representative of the whole population of nephrons. The calculated values listed in Table 3 indicate that EHDP markedly inhibited the net and fractional reabsorption of phosphate in the first convolutions of the proximal tubule. In the second segment, which should correspond to the accessible proximal convoluted tubule, the effect of EHDP appeared to be much less pronounced. This is also true for the third segment that probably includes the pars recta of the proximal tubule, the loop of Henle and part of the accessible distal tubule. Finally, between the distal tubule and the final urine, there is again, as in the first portion of the nephron, a conspicuous difference between the control and EHDP-injected rats in the fractional reabsorption of phosphate. In both the first and last segment the values of reabsorption for the EHDP group were negative, suggesting net addition of phosphate to the tubular fluid.

Discussion

As mentioned in the Introduction, EHDP can produce opposing effects on the tubular capacity to reabsorb phosphate. Administration of EHDP to man has been reported to increase the tubular reabsorption of phosphate (Recker et al., 1973; Walton et al., 1975). These results are contrary to those reported in thyroparathyroidectomized rats (Bonjour et al., 1978) and further documented in this study. Several factors could account for these contradictory effects. The first one to consider is the amount of EHDP given; in man the dose (0.08–0.12 mmol/kg orally = 0.0025–0.004 mmol/kg subcutaneously) that increases the tubular reabsorption of phosphate is about fifty times smaller than that which causes the opposite
**Table 3. Influence of EHDP on segmental tubular transport of phosphate in thyroparathyroidectomized rats**

The absolute and fractional contribution of each segment was calculated from the mean filtered or segmental load and the mean fractional delivery. Early proximal: punctures with (tubular fluid/plasma) inulin up to 1·49 were considered. The mean (tubular fluid/plasma) inulin ratio was 2·14 ± 0·78. Late proximal: punctures with a (tubular fluid/plasma) inulin ratio ranging from 2·0 to 2·49 were considered. The mean (tubular fluid/plasma) inulin ratio was 5·77 ± 0·57 (n = 17) and 5·86 ± 0·78 (n = 15) for the control and EHDP-injected group respectively.

<table>
<thead>
<tr>
<th>Segmental phosphate delivery</th>
<th>Net phosphate reabsorption</th>
<th>10^2 × Fractional phosphate reabsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control EHDP Control EHDP Control EHDP Control EHDP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomerulus to early proximal</td>
<td>3·24 2·54</td>
<td>0·84 -0·41</td>
</tr>
<tr>
<td>Early proximal to late proximal</td>
<td>2·40 2·95</td>
<td>0·91 0·69</td>
</tr>
<tr>
<td>Late proximal to distal</td>
<td>1·49 2·26</td>
<td>0·78 0·94</td>
</tr>
<tr>
<td>Distal to final urine</td>
<td>0·71 1·32</td>
<td>0·26 -0·08</td>
</tr>
</tbody>
</table>

The possibility that EHDP could affect the tubular transport of phosphate directly, for instance by competition for a common pathway, has been postulated in previous publications (Tröhler, Bonjour & Fleisch, 1975; Walton et al., 1975; Bonjour et al., 1978). So far, however, no evidence for such a direct effect has been reported. The effect of EHDP on the tubular handling of phosphate cannot be elicited acutely and it lasts for several days after stopping administration (Walton et al., 1975; Bonjour et al., 1978). The present micropuncture study was made 24 h after the last injection of EHDP, at a time when the diphosphonate was no longer detectable in the plasma of the animals (unpublished data). These findings do not support the idea of a simple direct interaction of EHDP with the tubular transport of phosphate. The EHDP-induced changes in the tubular transport of phosphate in man, as in the rat, are associated with alterations in the extrarenal handling of phosphate (Walton et al., 1975) and/or in bone mineral retention (Bonjour et al., 1978). The renal response may be secondary to these alterations, which can be expected to be different according to the dose of the diphosphonate and the phosphate status of the organism. Therefore it would be premature to conclude that a species difference between man and rat exists before excluding all other possibilities.

The effect of EHDP on the kidney in man, as in the rat, is particularly intriguing because it appears to be confined to the transport of phosphate. So far no other tubular transport function has been shown to be altered significantly in man or animals given EHDP. In rats, for instance, 0·16 mmol of EHDP/kg body weight given subcutaneously for several days did not change the urinary excretion of water and sodium (Von Herrath, Schaefer, Bonjour & Fleisch, 1972), the tubular reabsorption of glucose or the ability of the kidney to concentrate the urine (Bonjour et al., 1978). Likewise, EHDP given at the same dose did not modify the tubular handling of calcium in EHDP-injected thyroparathyroidectomized rats (Hugi, Bonjour & Fleisch, 1979). In rats with intact renal mass, EHDP (0·121 mmol/kg body wt. subcutaneously) did not alter the extracellular acid–base parameters (Goulding & Broom, 1979). The EHDP-induced alteration in the tubular handling of phosphate in rats cannot be ascribed to the effect of the diphosphonate on the production of 1,25-dihydroxyvitamin D₃ (Bonjour et al., 1978). It has not been found to be accompanied by any change in other humoral or hormonal factors known to affect, directly or indirectly, the renal transport of phosphate. The possibility that in thyroparathyroidectomized rats the change is secondary to the increased plasma calcium could be suggested (Bonjour et al., 1978, and present study). We have, however, presented evidence that the EHDP-induced change in the tubular handling of phosphate is also present in thyroparathyroidectomized rats in the absence of a rise in plasma calcium (Bonjour et al., 1978).
response to EHDP takes place mainly in the early proximal convoluted tubule. In addition, EHDP abolishes the difference in phosphate delivery between the distal tubule and the final urine. This latter effect may correspond entirely to an inhibitory action of the diphosphonate on net phosphate reabsorption along the terminal nephron. Alternatively, it may be a result, at least in part, of a greater EHDP-induced inhibition of the fractional phosphate reabsorption along the deep nephrons compared with those accessible for micropuncture at the kidney surface.

Thus the influence of EHDP on net phosphate reabsorption along the nephron is quite similar to that observed in rats fed a diet rich in phosphate. Indeed it has been reported that a high phosphate diet inhibits net phosphate reabsorption in the early proximal tubule and also affects the difference in phosphate delivery between the distal tubule and the final urine (Mühlbauer et al., 1977; Ullrich, Rumrich & Klöss, 1977; Mühlbauer et al., 1979).

The difference in phosphate delivery between distal and final urine can vary according to the amount of phosphate acutely infused during the micropuncture study. Thus under moderate phosphate infusion the apparent effect of a high phosphate diet along the terminal nephron has been shown to abolish net phosphate reabsorption (Mühlbauer et al., 1979). During high phosphate infusion, however, a condition where animals fed a low phosphate diet display no net movement of phosphate along the terminal nephron, a high phosphate diet leads to an apparent net secretion of phosphate (Mühlbauer et al., 1977).

In tubular samples in which the fractional recovery of water was about 0.72, that of phosphate was 1.16 and 0.74 (P < 0.05) in the EHDP-injected and control group respectively. Such a result means that the effect of EHDP on the tubular concentration of phosphate does not merely result from an increased reabsorption of the glomerular filtrate. Furthermore if the difference in the fractional recovery of phosphate (Fig. 1) at the highest (tubular fluid/plasma) inulin concentration ratios (3-5) were just due to an effect of EHDP on water reabsorption, this effect should be quite dramatic and thus be detectable in the final urine. The clearance study (Table 1) does not indicate that EHDP affects the overall net tubular reabsorption of water. Therefore it is very unlikely that EHDP significantly alters fluid reabsorption along the renal tubule. Accordingly, the change in the relationship between the fractional recovery of phosphate and the (tubular fluid/plasma) inulin ratio should mainly reflect the alteration in the transport of phosphate.

The present study reveals a striking similarity with respect to the tubular sites of action for the response to an increment in the supply of phosphate (Mühlbauer et al., 1977, 1979) and to EHDP administration. This certainly does not prove but does, however, add further support to the hypothesis that both factors may act through a common mechanism (Bonjour et al., 1978; Bonjour & Fleisch, 1980). The finding that in thyroparathyroidectomized rats EHDP (Stoll, Murer, Fleisch & Bonjour, 1980), like dietary phosphate (Stoll, Kinne, Murer, Fleisch & Bonjour, 1979), affects the sodium-dependent transport of phosphate in the brush border membranes of proximal tubules would also be consistent with such a common mechanism. As a result of the present work and previous micropuncture studies (Mühlbauer et al, 1977, 1979) the question arises as to whether the phosphate transport system of luminal or contraluminal membranes of more distal segments might also be involved in the overall tubular response to variation in dietary phosphate or to EHDP.

Acknowledgments

We acknowledge the excellent technical assistance of Ruth Rubli and Christine Marti. This work was supported by the Swiss National Science Foundation (3.725.76), by the Procter and Gamble Co., U.S.A., and by the Ausbildungs- und Förderungsfonds der Arbeitsgemeinschaft für Osteosynthese (AO), Switzerland. We thank Mrs B. Gyger for preparation of the manuscript and Mrs C. Stiegler for assistance with the illustrations.

References


