Plasma levels of vitamin D metabolites in diphosphonate-treated rats

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Summary

1. Protein-binding assays have been used to measure plasma 1,25-dihydroxy-vitamin D [1,25-(OH)₂D] as well as 25-hydroxy-vitamin D [25-(OH)D] in rats given 10 mg of phosphorus (P) day⁻¹ kg⁻¹ as ethane-1-hydroxy-1,1-diphosphonate (EHDP).

2. In control animals given a normal laboratory chow plasma 25-(OH)D and 1,25-(OH)₂D were about 40 nmol/l and 300 pmol/l respectively.

3. EHDP produced a decrease of plasma 1,25-(OH)₂D to below 50 pmol/l in 2 days.

4. Both in control and in EHDP-treated rats plasma 1,25-(OH)₂D increased when dietary calcium (Ca) was restricted to 0.1%, or dietary P to 0.2%, indicating that the well-known stimulatory effect of Ca or P deprivation was at least partially effective in EHDP-treated rats.

5. In response to an increase of the oral supply of vitamin D, to 65 nmol/day the plasma level of 25-(OH)D rose in both control and EHDP groups. Plasma 1,25-(OH)₂D was not increased above the normal value in control rats. In EHDP-treated rats, however, plasma 1,25-(OH)₂D rose to a level equal to that in controls, suggesting that the effect of EHDP on plasma 1,25-(OH)₂D can be overcome at high precursor concentration.

Key words: calcium, 1,25-dihydroxy-vitamin D, diphosphonate, 25-hydroxy-vitamin D, phosphonate, vitamin D.

Introduction

In recent years diphosphonates have been recognized to be useful therapeutic agents in the management of certain disorders of bone metabolism, especially in Paget's disease [1-3]. Diphosphonates are potent inhibitors of the formation and dissolution of calcium phosphate crystals in vitro [4, 5] and of bone resorption in vivo [4]. One of the compounds, ethane-1-hydroxy-1,1-diphosphonate (EHDP), when given in large doses to animals, has been shown to inhibit bone mineralization [6-9] and to decrease the intestinal absorption of calcium [7, 9-11]. The latter effect is due to decreased 1-hydroxylation of 25-hydroxy-vitamin D [25-(OH)D] in the kidney [12-14]. 1,25-Dihydroxy-vitamin D [1,25-(OH)₂D] is the most potent known metabolite of vitamin D in promoting calcium absorption in the gut [15, 16], as well as bone resorption [17, 18], and is generally considered to be a major hormonal form of the vitamin which plays an important role in mineral homeostasis. Evidence accumulated by several authors suggests that the influence of EHDP on vitamin D metabolism is a homeostatic consequence of the effect of the diphosphonate on bone mineralization rather than a direct effect of the drug on the 25-(OH)D-hydroxylating enzymes [9, 12, 14]. Therefore, EHDP-treated animals represent an interesting model for the investigation of the regulation of vitamin D metabolism. In the studies reported in the
literature the effect of EHDP on vitamin D metabolism was assessed either by measuring the renal 1-hydroxylase of chick kidneys in vitro [13, 14] or the appearance of dihydroxy derivatives in plasma of vitamin D-deficient animals after a single injection of radioactively labelled vitamin D$_3$ [12, 14] or 25-(OH)D$_3$ [13]. No information is yet available on the circulating levels of vitamin D metabolites in diphosphonate-treated rats. Therefore we have used protein-binding assays to study the time-course of the effect of EHDP on vitamin D metabolites in EHDP-treated animals. We have studied whether the effects of EHDP on plasma 25-(OH)D and 1,25-(OH)$_2$D can be influenced by dietary calcium (Ca) and phosphate (P) as well as by the supply of vitamin D.

Methods

Animal experiments

The experiments were performed with male Wistar rats bred in this institute. EHDP (kindly provided by Procter and Gamble Co., Cincinnati, OH, U.S.A.) was dissolved in distilled water, the solution adjusted to pH 7.4 with NaOH and injected subcutaneously (s.c.) at a dose of 10 mg of P day$^{-1}$ kg$^{-1}$ in a volume of 2 ml/kg body wt. Vitamin D$_3$ supplements were given orally in 0.1 ml of arachis oil. Blood for the determination of vitamin D metabolites was obtained by aortic puncture 24 h after the last injection of EHDP. The animals were anaesthetized with a intraperitoneal (i.p.) injection of 30–40 mg of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL, U.S.A.)/kg. Heparin (kindly provided by F. Hoffmann-La Roche and Co., Basle) was used to prevent coagulation and plasma obtained by centrifugation was frozen and kept at $-20^\circ$C until analyses of vitamin D metabolites.

Time-course of effect of EHDP on plasma 25-(OH)D and 1,25-(OH)$_2$D

Rats weighing 191 ± 14 g (mean ± sd) were given a commercial diet containing 0.9% Ca/0.7% P and 39 nmol of vitamin D$_3$/kg ad libitum. They were treated with EHDP for 1, 2, 3, 4 or 7 days. Controls received an equivalent volume of NaCl solution (150 mmol/l: saline) for either 1 or 7 days.

Influence of dietary Ca and P on the effect of EHDP on plasma 1,25-(OH)$_2$D

Rats weighing 180 ± 15 g (mean ± sd) were given one of three diets containing either 1.0% Ca/1.0% P, 1.0% Ca/0.2% P or 0.2% Ca/1.0% P for 2 weeks ad libitum. The diets were made by adding calcium gluconate and a mixture of K$_2$HPO$_4$/KH$_2$PO$_4$ (3:7) to a diet containing 0.14% Ca/0.22% P (Altromin C-1034, Altromin GmbH, Lage, West Germany). Since the diet was vitamin D-poor the rats were supplemented with 1.5 nmol of vitamin D$_3$ every 2 days. After a 7 day equilibration period the animals were treated with EHDP for 7 days. Control animals received an equivalent volume of saline. The diet during EHDP-treatment was the same as the one given during the equilibration period.

Influence of vitamin D intake on the effect of EHDP on plasma 25-(OH)D and 1,25-(OH)$_2$D

Rats were housed individually and pair-fed. After an equilibration period the animals were given EHDP for 7 days. Control rats received saline.

Experiment I. Rats weighing 134 ± 5 g (mean ± sd) received 20 g of diet containing 0.9% Ca/0.7% P and about 0.6 nmol of vitamin D$_3$/day. The equilibration period was 4 days. During the experimental period half the animals received a supplement of 65 nmol of vitamin D$_3$/day.

Experiment II. Rats weighing 169 ± 6 g (mean ± sd) received 30 g of a vitamin D-poor diet containing 1.0% Ca/1.0% P per day. During the 5 day equilibration period all animals were given 0.6 nmol of vitamin D$_3$/day orally. During the experimental period they received daily supplements of 0.6, 6.5 or 65 nmol of vitamin D$_3$.

Determination of vitamin D metabolites

25-(OH)D. 25-(OH)D was measured by a modification of the method of Preece et al. [19]. The only modification was the use of 0.1% gelatin instead of 1.0% albumin to prevent the loss of 25-[$^3$H]-(OH)D from solution. Plasma samples of 0.1 ml were used. 25-(OH)-(26,27-$^3$H)D$_3$ (The Radiochemical Centre, Amersham, Bucks., U.K.; specific radioactivity 7.3 Ci/mmol) was used as a tracer.

1,25-(OH)$_2$D. 1,25-(OH)$_2$D was extracted with chloroform/methanol by the method used for 25-(OH)D [19]. The extract was processed and 1,25-(OH)$_2$D was assayed as described previously [20]. The sensitivity of the assay was 6 fmol/assay tube. Plasma samples of 1–2 ml were used, resulting in an assay sensitivity of 30–60 pmol/l. The assay procedures did not discriminate between vitamin D$_2$ and D$_3$ metabolites. However, since the vitamin D supply of...
Vitamin D metabolites in rats

1,25-[3H]-[OH]2D3 was produced biologically as described by Boyle et al. [21]. The substrate 25-(OH)-[23,24-3H]D3 (specific radioactivity 78 Ci/mmol) was provided by F. Hoffman-La Roche Inc., Nutley, NJ, U.S.A. 1,25-(OH)2D3 was supplied by F. Hoffmann-La Roche & Co. AG., Basle, Switzerland.

Results

Time-course of the effect of EHDP on plasma 25-(OH)D and 1,25-(OH)2D

As shown in Fig. 1, EHDP at a dose of 10 mg of P day-1 kg-1, during the first 3 days of treatment, produced a progressive decrease of plasma 25-(OH)D which then remained constant for the rest of the experimental period. Plasma 1,25-(OH)2D was already decreased after 1 day of treatment and fell to values below 50 pmol/l thereafter. When the limit of detection of plasma 1,25-(OH)2D in the assay was decreased to 13 pmol/l by pooling plasma of EHDP-treated rats, plasma 1,25-(OH)2D was found to be 32 pmol/l on day 4 and 20 pmol/l on day 7 (means of two plasma pools for each day from groups of six rats).

Influence of dietary Ca and P on the effect of EHDP on plasma 1,25-(OH)2D

Table 1 shows the plasma 1,25-(OH)2D in EHDP-treated and control rats on different Ca and P intakes. Lowering dietary Ca or P produced an increase in plasma 1,25-(OH)2D in control animals. EHDP-treated rats also responded to Ca or P restriction with an increase of plasma 1,25-(OH)2D, but the values remained

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![Graph](image-url)  
**Fig. 1.** Effect of EHDP on plasma 25-(OH)D and 1,25-(OH)2D. EHDP was given daily in a dose of 10 mg of P/kg subcutaneously. Values are means ± SEM. n = 4 for EHDP-treated (○) and n = 3 for control (△) animals. Significance of differences; *P < 0.05 by Student’s t-test for unpaired values.

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**TABLE 1. Plasma levels of 1,25(OH)2D in rats on different Ca and P intakes**

Plasma levels of 1,25(OH)2D are given as pmol/l. All animals received 1.5 nmol of vitamin D3 orally every 2 days. Rats were treated with EHDP for 7 days. Significance of differences between groups was evaluated with Wilcoxon’s rank test for unpaired values: *P < 0.005 vs control group on the same diet; **P < 0.005, ***P = 0.05 vs control group on 1.1% Ca/1.0% P; *P < 0.02, **P = 0.05 vs EHDP on 1.1% Ca/1.0% P.

<table>
<thead>
<tr>
<th>Dietary Ca and P</th>
<th>1.1% Ca/1.0% P</th>
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<th>1.1% Ca/0.2% P</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Control EHDP</td>
<td>Control EHDP</td>
<td>Control EHDP</td>
</tr>
<tr>
<td>210</td>
<td>113</td>
<td>887</td>
<td>212</td>
</tr>
<tr>
<td>469</td>
<td>134</td>
<td>901</td>
<td>206</td>
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<td>466</td>
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<td>436</td>
<td>119</td>
<td>881</td>
<td>155</td>
</tr>
<tr>
<td>303</td>
<td>≤58</td>
<td>793</td>
<td>295</td>
</tr>
<tr>
<td>328</td>
<td>≤60</td>
<td>1257</td>
<td></td>
</tr>
<tr>
<td><strong>Means</strong></td>
<td><strong>369</strong></td>
<td><strong>939</strong></td>
<td><strong>636</strong></td>
</tr>
</tbody>
</table>

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The rats was mostly vitamin D3, vitamin D2 metabolites were probably negligible.
below those observed in control animals on a high-Ca/high-P diet.

Influence of vitamin D intake on the effect of EHDP on plasma 25-(OH)D and 1,25-(OH)₂D

The results of two separate experiments are presented in Table 2. Plasma 25-(OH)D increased with increasing supply of vitamin D both in control and in EHDP-treated animals. EHDP produced a decrease of plasma 25-(OH)D in animals receiving 0.6 or 6.5 nmol of vitamin D₃/day, but had no effect on this in animals given 65 nmol of vitamin D₃/day. In control rats increasing dietary vitamin D₃ to 65 nmol/day produced a decrease of plasma 1,25-(OH)₂D. EHDP-treated animals responded to a supplementation with 65 nmol of vitamin D₃/day with an increase of plasma 1,25-(OH)₂D to a level equal to that of corresponding control animals.

Discussion

Our results demonstrate that the circulating levels of 1,25-(OH)₂D can be decreased to very low values with large doses of EHDP in rats maintained on ordinary, vitamin D-containing, laboratory food. This finding is important when considering previous investigations in which EHDP was used as a tool to produce 1,25-(OH)₂D deficiency in rats without vitamin D depletion [22, 23], since before the present study no information was available on the degree of 1,25-(OH)₂D deficiency obtained with EHDP treatment.

A decrease of plasma 25-(OH)D was also observed in our experiments, except at high vitamin D intake. Previous work did not provide conclusive evidence for an interference of EHDP with the formation of 25-(OH)D [12, 24]. Nevertheless further work is needed to rule out the possibility that EHDP might influence either the hepatic vitamin D-25-hydroxylase directly or the supply of vitamin D to this enzyme. Alternatively EHDP might increase the disappearance of 25-(OH)D from blood.

The well known stimulatory effect of a low-Ca or a low-P diet on plasma 1,25-(OH)₂D in rats [25], which was observed in the control animals, was also present in EHDP-treated rats, although in all the groups EHDP-treated rats had much lower plasma 1,25-(OH)₂D than corresponding control animals. These results show that the homeostatic mechanisms which mediate the response of plasma 1,25-(OH)₂D to dietary Ca or P restriction are at least partially operative in EHDP-treated rats. This should be considered when EHDP is used to produce 1,25-(OH)₂D deficiency in experiments involving low-mineral diets.

We can only speculate about the mechanism by which EHDP influences plasma 1,25-(OH)₂D. The inhibitory effect of the drug on 25-(OH)D-l-hydroxylation has been shown to depend on the animal's vitamin D status [12], as well as on dietary calcium [14]. These results suggest that EHDP influences l-hydroxylation indirectly. The absence of an effect of EHDP on the 25-(OH)D-l-hydroxylase in primary chick kidney cell culture is consistent with this hypothesis [26]. Parathyroid hormone is unlikely to be responsible for the diphosphonate-induced decrease of

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**TABLE 2. Influence of the intake of vitamin D on the effect of EHDP on plasma 25-(OH)D and 1,25-(OH)₂D**

Numbers of animals are shown in parentheses. Significance of differences: *P < 0.01 vs control; †P < 0.05; ‡P < 0.01 vs control 0.6 nmol of vitamin D₃/day by Student's t-test for unpaired values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Supply of vitamin D₃ (nmol/day)</th>
<th>25-(OH)D (nmol/l)</th>
<th>1,25-(OH)₂D (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>(5) 0.6</td>
<td>33.8 ± 3.0</td>
<td>205.5 ± 33.4</td>
</tr>
<tr>
<td>EHDP</td>
<td>(5) 0.6</td>
<td>15.6 ± 2.4*</td>
<td>&lt;58</td>
</tr>
<tr>
<td>Control</td>
<td>(5) 65</td>
<td>153.5 ± 32.3</td>
<td>93.0 ± 23.6†</td>
</tr>
<tr>
<td>EHDP</td>
<td>(5) 65</td>
<td>169.8 ± 21.5</td>
<td>95.7 ± 8.4</td>
</tr>
<tr>
<td>Expt. II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>(6) 0.6</td>
<td>75.9 ± 6.7</td>
<td>186.2 ± 22.7</td>
</tr>
<tr>
<td>EHDP</td>
<td>(5) 0.6</td>
<td>46.5 ± 2.3*</td>
<td>&lt;41</td>
</tr>
<tr>
<td>Control</td>
<td>(6) 6.5</td>
<td>97.6 ± 8.5</td>
<td>176.7 ± 30.9</td>
</tr>
<tr>
<td>EHDP</td>
<td>(6) 6.5</td>
<td>55.4 ± 8.4*</td>
<td>&lt;41</td>
</tr>
<tr>
<td>Control</td>
<td>(5) 65</td>
<td>258.7 ± 19.6</td>
<td>83.2 ± 4.5†</td>
</tr>
<tr>
<td>EHDP</td>
<td>(5) 65</td>
<td>283.5 ± 31.0</td>
<td>81.5 ± 6.6</td>
</tr>
</tbody>
</table>
plasma 1,25-(OH)\(_2\)D, since EHDP decreased intestinal calcium absorption in thyroparathyroidectomized rats and since exogenous parathyroid hormone did not reverse this effect [22]. One factor which might be involved is the increase of plasma calcium observed in rats given large doses of EHDP [9].

It is interesting to compare the relationship between plasma 25-(OH)D and plasma 1,25-(OH)\(_2\)D in control and EHDP-treated animals. In control rats increasing plasma 25-(OH)D did not lead to a rise in plasma 1,25-(OH)\(_2\)D, indicating that changes in plasma 1,25-(OH)\(_2\)D concentration were controlled by factors other than 25-(OH)D. At high plasma levels of 25-(OH)D there was on the contrary a decrease in plasma 1,25-(OH)\(_2\)D. These results are consistent with earlier findings in rats [27] and in human patients with hypervitaminosis D [28]. In EHDP-treated animals, however, plasma 1,25-(OH)\(_2\)D increased to the level observed in control animals at high plasma levels of 25-(OH)D. The finding that in EHDP-treated rats the inhibition of 1-hydroxylation can be overcome by increasing the plasma concentration of the substrate 25-(OH)D gives rise to the question whether plasma 1,25-(OH)\(_2\)D might be normalized with exogenous vitamin D also in other situations in which it has been found to be decreased, such as in chronic renal failure [29].

Acknowledgments

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References


