Evidence for extrarenal metabolism of 25-hydroxyvitamin D$_3$ in man

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Summary

1. Metabolites of vitamin D$_3$ were measured in the circulation of four patients on chronic haemodialysis (three of whom were surgically anephric) before and during daily ingestion of 40,000 i.u. of cholecalciferol.

2. Circulating 24,25-dihydroxyvitamin D$_3$ [24,25-(OH)$_2$D$_3$] was measurable, but abnormally low before treatment; its circulating concentration rose in a substrate dependent manner when serum 25-hydroxyvitamin D$_3$ (25-OHD$_3$) increased, but the response was reduced when compared with the normal relationship.

3. Serum 1,25-dihydroxyvitamin D$_3$ [1,25-(OH)$_2$D$_3$] and calcidiol lactone (25-OHD$_3$-lactone) were consistently unmeasurable in sera from these patients before administration of cholecalciferol. However, when serum 25-OHD$_3$ rose with treatment, 1,25-(OH)$_2$D$_3$ became detectable in the sera of three of the four patients and 25-OHD$_3$-lactone could be measured in all of them.

4. These data indicate that extrarenal sites of synthesis of 24,25-(OH)$_2$D$_3$, 25-OHD$_3$-lactone and 1,25-(OH)$_2$D$_3$ exist in chronically dialysed patients but require large amounts of substrate to be significant.

Key words: calcidiol lactone, 1,25-dihydroxycholecalciferol, 24,25-dihydroxycholecalciferol, 25,26-dihydroxycholecalciferol, renal failure, vitamin D metabolism—extrarenal synthesis of metabolites.

Introduction

Although the chemical nature of the metabolites of vitamin D has been fully characterized, their sites of production remain to be fully established. The important role of the kidney in the generation of 1,25-dihydroxyvitamin D$_3$ [1,25-(OH)$_2$D$_3$] has been shown by many laboratories [1-5]. However, there is now substantial evidence that extrarenal sites of synthesis for both 24,25-dihydroxyvitamin D$_3$ [24,25-(OH)$_2$D$_3$] and 1,25-(OH)$_2$D$_3$ also exist. In vitro, many tissues other than the kidney display hydroxylase activity [6-11], but animal experiments in vivo have been conflicting [12-14]. Early human studies indicated that 24,25-(OH)$_2$D$_3$ was not formed in patients given either 25-hydroxyvitamin D$_3$ (25-OHD$_3$) or radiolabelled vitamin D$_3$ [15], but the methods used were of limited scope and were later challenged by reports of measurement of 24,25-(OH)$_2$D$_3$ in the circulation of both untreated [16-20] and treated [21] anephric patients. 1,25-(OH)$_2$D$_3$ has been detected in the circulation of some anephric patients [22, 23] yet not in others [16, 18, 19, 21].

Here we describe studies which have relied upon the application of sensitive radioimmunoassays to determine five of the polar metabolites of vitamin D$_3$. Using these techniques we have followed the
metabolism of vitamin D<sub>3</sub> in four patients on chronic haemodialysis, three of whom had been surgically nephrectomized. They were studied both before and during treatment with large daily doses of vitamin D<sub>3</sub> for up to 6 weeks, and the findings in these subjects compared with previous studies of both animals and man.

**Methods**

**Subjects**

The four patients (Table 1) who undertook this study were all awaiting renal transplantation and had all been on routine haemodialysis for at least 3 months; all except patient no. 2 had been surgically nephrectomized. Blood samples were obtained from the patients before dialysis sessions for a period of 3-4 weeks before and 3-6 weeks during daily ingestion of 40 000 i.u. of pure cholecalciferol. The blood was separated and the sera were stored at -20°C until assayed, when all the samples from individual patients were assayed together with single samples obtained from a group of healthy British adults (five male, five female, age range 22-89, mean 52, years).

**Laboratory methods**

Serum concentrations of calcium (corrected for albumin binding), phosphate, creatinine, urea and alkaline phosphatase were measured by automated methods, and N-terminal parathyroid hormone (n-PTH, normal range < 120 pg/ml) was determined by an immunoradiometric method [24]. Biochemical details of the patients are given in Table 1.

Metabolites of vitamin D<sub>3</sub> were measured by radioassay after extraction of serum on C18 SepPaks and fractionation by high-pressure liquid chromatography (HPLC) on Zorbax-SIL (25 cm × 6.2 mm inner diameter) eluted with a ternary solvent system of hexane/methanol/isopropanol (92:4:4, by vol.) at a flow rate of 2.0 ml/min [25]. This system separates calcidiol lactone (25-OHD<sub>3</sub>-lactone) and the hydroxylated metabolites of vitamin D<sub>3</sub> to baseline and resolves putative 25-hydroxy-19-nor-10-ketovitamin D<sub>3</sub> from 1,25-(OH)<sub>2</sub>D<sub>3</sub> (unpublished work). Concentrations of 25-OHD<sub>3</sub> were measured using a competitive binding assay [26], those of 24,25-(OH)<sub>2</sub>D<sub>3</sub>, 25-OHD<sub>3</sub>-lactone and 25,26-dihydroxyvitamin D<sub>3</sub> [25,26-(OH)<sub>2</sub>D<sub>3</sub>] by radioimmunoassay using antisera S02282 [27], and 1,25-(OH)<sub>2</sub>D<sub>3</sub> by radioimmunoassay using antisera S11, which displays specificity towards 1-hydroxylated vitamin D metabolites [25]. The limits of detection of these assays were: 1.2 nmol/l for 25-OHD<sub>3</sub>, 0.096 nmol/l for both 24,25- and 25,26-(OH)<sub>2</sub>D<sub>3</sub>, 130 pmol/l for 25-OHD<sub>3</sub>-lactone and 9.6 pmol/l for 1,25-(OH)<sub>2</sub>D<sub>3</sub>. In the ten control subjects the range of concentrations of the various metabolites were: 25-OHD<sub>3</sub>, 7.5-72.5 nmol/l; 24,25-(OH)<sub>2</sub>D<sub>3</sub>, 0.24-9.86 nmol/l; 25,26-(OH)<sub>2</sub>D<sub>3</sub>, 0.48-3.13 nmol/l; 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 48-137 pmol/l. 25-OHD<sub>3</sub>-lactone was only detectable in samples from three of these subjects, the highest concentration being 160 pmol/l.

Further characterization of the metabolites was by radioimmunoassay of column eluates after fractionation of serum extracts by both straight phase (Zorbax-SIL eluted with hexane/methanol/isopropanol, 94:3:3 by vol., at 3.0 ml/min) and reverse phase (Resolve C18 eluted with methanol/water, 80/20 by vol., at 1.0 ml/min) HPLC. The effect of prior treatment of the extracts with sodium metaperiodate (5% by weight solution) was also tested.

**Statistical analysis**

The results were subjected to linear regression analysis; the significance of the relationships was also examined using the Spearman test. In addition, analysis of co-variance was also performed after logarithmic transformation of the data.

**Table 1. Initial biochemical details**

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age/sex</th>
<th>Serum calcium (mmol/l)</th>
<th>Serum phosphate (mmol/l)</th>
<th>Serum alkaline phosphatase (i.u./l)</th>
<th>Serum n-PTH (pg/ml)</th>
<th>Nephrectomy (months since)</th>
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</thead>
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<tr>
<td>1</td>
<td>54/F</td>
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<td>0.69</td>
<td>136</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
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<td>1.16</td>
<td>73</td>
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<td>—</td>
</tr>
<tr>
<td>3</td>
<td>50/F</td>
<td>2.24</td>
<td>2.00</td>
<td>77</td>
<td>140</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
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<td>2.30</td>
<td>2.04</td>
<td>60</td>
<td>640</td>
<td>3</td>
</tr>
<tr>
<td>Normal values</td>
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<td>2.20–2.55</td>
<td>0.6–1.3</td>
<td>20–85</td>
<td>&lt;120</td>
<td></td>
</tr>
</tbody>
</table>
Results
The mean pretreatment concentrations of 25-OHD₃ in the uraemic subjects were no different from those found in the ten healthy adults but both 24,25-(OH)₂D₃ (range 0.18–0.3 nmol/l) and 25,26-(OH)₂D₃ (0.26–0.5 nmol/l) were lower. Neither 25-OHD₃-lactone nor 1,25-(OH)₂D₃ could be detected in any of at least two pretreatment samples from each of the four patients (Fig. 1).

The effect of administration of cholecalciferol in all four patients is also shown in Fig. 1. During the course of the study there was a gradual increase in the circulating concentration of all of the metabolites. By the end of the study circulating 25-OHD₃ had increased sevenfold on average to range from 70 to 188 nmol/l in the four patients. 24,25-(OH)₂D₃ also increased 12-fold to attain a range of 0.89–6.98 nmol/l, and 25,26-(OH)₂D₃ increased ninefold from 1.43 to 4.16 nmol/l. 25-OHD₃-lactone became detectable in all four patients, though in one patient this was only temporary. 1,25-(OH)₂D₃ was measurable in three of the four patients, ranging from 52.9 to 62.5 nmol/l in these three; in the remaining patient it was undetectable throughout. Serum calcium fluctuated in these patients during the study but not outside previously observed concentrations for each of them, and no changes of n-PTH were observed.

Relationships between 25-OHD₃ and the other metabolites

In order to study inter-relationships between circulating 25-OHD₃ and the concentration of its metabolic products the data were divided into three sets. These were: firstly, individual measurements from the normal subjects, secondly, the data from patient no. 2 (Table 1) who still had kidney tissue in situ, and, thirdly, the pooled data from the other three, anephric patients.

There was a significant relationship between the concentration of 24,25-(OH)₂D₃ and that of 25-OHD₃ in all three sets of data tested by linear regression analysis (Fig. 2). Analysis of co-variance showed that the slope of the regression was lower (P < 0.05) in the anephric patients than in the normal subjects. The concentration of 25,26-(OH)₂D₃ was also related to that of 25-OHD₃ (Fig. 3); in this case, the slopes were not significantly different but the line was higher in the normal subjects than in those that had been nephrectomized (P < 0.05).

No relationships were found between circulating 1,25-(OH)₂D₃ and 25-OHD₃ in either the normal subjects, or in the patients when the dihydroxy metabolite was detectable in their sera. 25-OHD₃-lactone was only measurable in three of the normal subjects and so no comparison was made; however, in all those samples from the patients in which it was detectable, a positive relationship with serum 25-OHD₃ was found (r = 0.68, P = < 0.05, y = 7.18–32.53x, not shown).

Characterization of metabolites

When serum obtained from one of the anephric patients at the end of the study was fractionated and the column eluate monitored throughout for binding activity, distinct peaks corresponding to
25-OHD₃, 24,25-(OH)₂D₃, 25-OHD₃-lactone, 25,26-(OH)₂D₃ and 1,25-(OH)₂D₃ were found. Prior treatment of the extract with sodium metaperiodate removed the activity associated with 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃.

In further experiments samples were extracted, fractionated by straight phase HPLC and discrete metabolic fractions corresponding to 24,25-(OH)₂D₃, 25-OHD₃-lactone, 25,26-(OH)₂D₃ and 1,25-(OH)₂D₃ collected. Subsequent reverse phase HPLC revealed peaks of binding activity co-eluting with the respective reference compounds for all four metabolites, 1,25-(OH)₂D₃ being monitored using the specific radioimmunoassay (antiserum S11) and the other three metabolites using antisem S02282 against the relevant reference sterol (Fig. 4). Prior treatment of further aliquots treated with sodium metaperiodate after the first HPLC column and before the second reverse phase fractionation destroyed all binding activity which had been associated with peaks corresponding to both 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃ but did not affect either the putative 25-OHD₃-lactone or the 1,25-(OH)₂D₃ peak.

Discussion

An increasing amount of evidence has demonstrated that the kidney is not the only organ capable of metabolizing 25-OHD₃ to more polar derivatives. Tissues which can synthesize either 24,25-(OH)₂D₃ or 1,25-(OH)₂D₃, or both, in vitro include the rat intestine [6], yolk sac [9], endometrium [10] and placenta [10], rabbit cartilage and chondrocytes [7], chicken chondrocytes [7] and calvarial cells [11], and human placenta [8]. The physiological significance of these hydroxylases, and thus their contribution to vitamin D metabolism in vivo is, however, unclear.

Studies which are more relevant to clinical problems have relied upon measurements of vitamin D metabolites in the circulation of both nephrectomized animals and anephric humans maintained on dialysis. Although extrarenal sites of production of 25,26-(OH)₂D₃ are well documented [18-20, 28] controversy exists as to whether 24,25-(OH)₂D₃ and 1,25-(OH)₂D₃ are exclusively renal metabolites in non-pregnant humans. The first report of a direct assay for 24,25-(OH)₂D₃ applied to the study of anephric patients [15] indicated that this metabolite was not formed in significant amounts in the presence of normal or exogenously raised circulating concentrations of 25-OHD₃. However, earlier data [16] offered indirect evidence that 24,25-(OH)₂D₃ could be detected, as radiolabelled material co-migrating by silicic acid chromatography with authentic 24,25-(OH)₂D₃ was present in
Fig. 4. Separation by reverse phase HPLC on a Resolve column eluted with methanol/water (80:20, v/v) at a flow rate of 1.0 ml/min of four discrete fractions previously collected from HPLC on Zorbax-SIL of a serum extract from patient no. 2 (Table 1) taken after administration of vitamin D₃ (see text). In each panel the elution of authentic material is shown by an arrow and the shaded area represents binding activity. (a) Putative 1,25-(OH)₂D₃ monitored by antiserum S11. There is a peak immunoassayable material activity co-eluting with 1,25-(OH)₂D₃. Note in this case there is further unidentified material eluting at the void of the column, but all of the previously accountable activity from this sample was at the reference position. (b) Putative 24,25-(OH)₂D₃ monitored by antiserum S02282. (c) Putative 25-OHD₃-lactone monitored by antiserum S02282. (d) Putative 25,26-(OH)₂D₃ monitored by antiserum S02282.

The question of ectopic genesis of 1,25-(OH)₂D₃ in vivo is equally unclear. Although most reports of direct assay in the sera of anephric patients have failed to measure it [16, 18, 19], recent studies have reported low but detectable circulating concentrations [22, 23]. Similarly animal experiments give conflicting results according to the species and experimental design. Circulating 1,25-(OH)₂D₃ increases above control values in nephrectomized pigs given pharmacological doses of vitamin D₃, and maintained on peritoneal lavage over an 8 day period [13]. In contrast, in a re-evaluation of early experiments [2, 3], DeLuca's group have shown that radiolabelled 1,25-(OH)₂D₃ is not formed in nephrectomized rats after prior administration of tritiated 25-OHD₃ [14]. However, this latter type of experiment is of an acute nature (18–20 h) with administration of small physiological doses of 25-
OHD₃ and so is of a very different nature to the studies presented here which were designed to increase the available pool of vitamin D in patients adapted to chronic haemodialysis.

In our patients, 24,25-(OH)₂D₃ could be detected in the circulation before administration of vitamin D₃, but the concentrations measured were uniformly low, although the patients all had normal circulating concentrations of 25-OHD₃. When the concentration of 25-OHD₃ was raised by ingestion of large amounts of vitamin D₃, the circulating concentration of 24,25-(OH)₂D₃ rose in a substrate dependent manner, even in the anephric patients, though in them the rise was less than in normal subjects. The patients also produced 25,26-(OH)₂D₃ in a substrate dependent manner. This supports our earlier findings [28] indicating that significant extrarenal sites of synthesis for this metabolite are present in man. The kidney, however, may have some role to play as, for a given concentration of 25-OHD₃, the measured concentration of 25,26-(OH)₂D₃ in anephric patients is slightly lower than in normal subjects.

1,25-(OH)₂D₃ was undetectable in all of the samples taken from the patients before administration of vitamin D₃ and remained so in one of the anephric subjects throughout the study. However, circulating concentrations within the normal range were observed in the other three patients during the study. In a similar fashion, 24,25-(OH)₂D₃ was also formed after vitamin D₃ therapy was started. Renal synthesis of this latter metabolite has been suggested in both the pig [13] and the rat [29], although one report has indicated extrarenal production over an acute experimental period in the rat [30]. Recently, Halloran et al. [31] have shown that administration of large oral doses of 25-OHD₃ to patients on haemodialysis, but still with kidney tissue in situ, results in substrate dependent increases of 24,25-(OH)₂D₃, 25,26-(OH)₂D₃ and 1,25-(OH)₂D₃ in the circulation. They suggested that this may be due to residual renal hydroxylase activity but could not exclude extrarenal genesis of these metabolites. In the light of the results presented here it is probable that although residual renal hydroxylase activity may be present, sites outside the kidney do indeed exist.

Validation of the techniques used was provided by the finding that the putative 24,25-(OH)₂D₃ and 25,26-(OH)₂D₃ fractions co-eluted with their reference standards in three separate HPLC systems, when monitored by radioimmunassay, and were sensitive to vicinal hydroxyl function cleavage by treatment with sodium metaperiodate. In addition, the fractions collected as 25-OHD₃-lactone and 1,25-(OH)₂D₃ also displayed co-elution in a number of systems with reference preparations and resisted periodate cleavage. Extrarenal sites of synthesis of the biologically active metabolite of vitamin D are seen thus to exist in man, but require to be driven by large amounts of substrate to be significant. These findings may explain the clinical observation that anephric patients can respond to large doses of vitamin D.

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References


