Different vitamin D substrate–product relationship after oral vitamin D supplementation in familial benign hypercalcemia, primary hyperparathyroidism, and healthy controls

Maurizio Bevilacqua, Marco Invernizzi, Velella Righini, Stefano Carda and Carlo Cisari

Endocrinology and Diabetes Unit, Department of Medicine, Luigi Sacco Hospital (Vadlha), University of Milan, 20157 Milan, Italy, 1Physical and Rehabilitation Medicine, Department of Clinical and Experimental Medicine, University of Eastern Piedmont 'A. Avogadro', AOU 'Maggiore della Carita', Viale Piazza d'Armi 1, 28100 Novara, Italy and 2Rehabilitation Center 'Mons. Luigi Novarese', SODC, 13040 Moncrivello (VC), Italy

(Correspondence should be addressed to M Invernizzi; Email: marco.invernizzi@med.unipmn.it)

Abstract

Context: In healthy subjects and in patients with primary hyperparathyroidism (PH), the administration of a low dose of 25(OH)D (25 µg/day) increases the serum levels of both 25(OH)D and 1,25(OH)2D. It is unknown whether this relationship is present in patients affected by familial benign hypocalciuric hypercalcemia (FBH).

Objective: To evaluate the different vitamin D substrate–product relationship after oral vitamin D supplementation in familial benign hypercalcemia, PH, and healthy controls.

Design: We evaluated the main physiological regulators of 1α-hydroxylase and the substrate–product relationship of 25(OH)D and 1,25(OH)2D in 20 patients with PH, 25 with FBH, and 122 healthy sex- and age-matched controls before and after administration of 25(OH)D for 2 weeks.

Results: 25(OH)D increased significantly in all subjects, whereas 1,25(OH)2D serum levels increased significantly in PH patients and healthy controls but not in patients with FBH. Therefore, a significant positive substrate–product relationship of 25(OH)D–1,25(OH)2D was found in PH and healthy controls, but not in FBH. Monomeric calcitonin (hCT-M) was significantly lower at baseline and after 25(OH)D supplementation in the FBH group compared with the other two groups.

Conclusions: The lack of 1,25(OH)2D increase in FBH may be due to a direct inhibitory effect on 1α-hydroxylase of hypercalcemia per se, increased metabolic clearance of 1,25(OH)2D, or a decreased stimulus of 1α-hydroxylase related to persistently low levels of hCT.

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Introduction

The vitamin D metabolism is the result of a complex multifactorial control mechanism, and some evidence of a substrate–product relationship between 25(OH)D and 1,25(OH)2D (1–3) exists. Chronic administration of 1,25(OH)2D increases the metabolic clearance rate of 25(OH)D (4, 5) or decreases its production (6) with consequent depletion of stored 25(OH)D. On the other hand, the administration of elevated doses of vitamin D or 25(OH)D increases the metabolic clearance rate of 1,25(OH)2D (7). This might explain the failure to observe elevations in 1,25(OH)2D after high doses of 25(OH)D (5–7).

In healthy subjects, the administration of 25(OH)D at a low dose (25 µg) produces an increase in 25(OH)D serum levels within the normal range (below 120 ng/ml) and an increase in 1,25(OH)2D (2, 8). Similarly, exogenous administration of 25(OH)D in patients affected by primary hyperparathyroidism (PH) is followed by an increase in serum levels of 1,25(OH)2D, suggesting a substrate–product relationship in these subjects (9). By contrast, monitoring of 1,25(OH)2D serum levels after administration of 25(OH)D in patients affected by familial benign hypocalciuric hypercalcemia (FBH) has never been performed.

FBH is a rare, benign cause of hypercalcemia, characterized by autosomal-dominant inheritance with high penetrance (10, 11). Affected heterozygous patients typically present with the incidental discovery of hypercalcemia, hypocalciuria, inappropriately normal or only slightly elevated PTH, and, in some subjects, mild-to-moderate hypermagnesemia (10–13). Moreover, decreased serum levels of monomeric calcitonin (hCT) in basal conditions and after oral calcium stimulation have been found in these patients (14). FBH is typically caused by inactivating mutations of the calcium-sensing receptor (CASR) gene, resulting in inappropriate secretion of PTH and in a markedly enhanced resorption of urinary calcium through
mechanisms that are both dependent on and independent of PTH (10–13, 15–20). Multiple comparisons between serum concentrations of 1,25(OH)_{2}D in FBH and PH patients at basal conditions have been performed (20–24). The results showed similar (21, 22) or significantly lower serum levels of 1,25(OH)_{2}D in FBH patients compared with PH patients (20, 23, 24).

The aim of this study was to evaluate the substrate–product relationship of 25(OH)D and 1,25(OH)_{2}D in PH patients, FBH patients, and healthy subjects after oral administration of 25(OH)D.

**Materials and methods**

**Population**

Nineteen patients with PH and 25 with FBH were recruited among those attending our clinic. Patients with PH were examined before undergoing surgery, which resulted in a histopathological diagnosis of parathyroid adenoma in 17 patients and parathyroid hyperplasia in two patients. FBH was diagnosed based on the presence of hypercalcemia, normal intact PTH, urinary calcium excretion >200 mg/day (5 mmol/day), and a Ca/Cr clearance ratio <0.01. Among the 25 patients with FBH, eight had a heterozygous missense mutation in exon 6 that substitutes a glutamic acid for glycine at codon 557 (Gly557Glu) (16), two had a P55L mutation (17), 14 had an R648X mutation (18), and one had a Y218C mutation (19). None of the FBH and PH patients had any degree of kinship. In addition to the two patient groups, we recruited 122 healthy controls. The inclusion criteria were age <75 years and normal renal function as evaluated by creatinine clearance measured with MDRD formula (25). The exclusion criteria were concomitant supplementation with calcium and/or vitamin D, renal insufficiency, liver diseases, malabsorption, hypercalciumia, Paget’s disease, diabetes mellitus, and any medical treatment that could affect calcium metabolism. Demographical characteristics of patients and controls are resumed in Table 1.

All study participants signed informed consent forms and the Institutional Review Board approved the study, which was conducted in accordance with the Declaration of Helsinki guidelines. All healthy controls and FBH and PH patients were evaluated at baseline and after 2 weeks of oral administration of 25 μg/day of 25(OH)D (Didrogol, Bruno Farmaceutici, Milan, Italy) while on an unrestricted calcium diet.

**Analytical methods**

Blood and urinary calcium, phosphate, and creatinine were measured with the Technicon Autoanalyzer SMA-12/60 (Technicon Instruments Corp., Tarrytown, NY, USA). Plasma ionized calcium was measured by StatProfile M Nova (Milan, Italy); serum immunoreactive 1–84 PTH was measured using a Nichols kit (Nichols Institute, San Juan Capistrano, CA, USA). Monomeric calcitonin was assessed as previously described (26) (Nichols Institute, San Juan Capistrano, CA, USA). Monovalent cations were measured with the Technicon Autoanalyzer SMA-12/60 (Technicon Instruments Corp., Tarrytown, NY, USA). Plasma ionized calcium was measured by StatProfile M Nova (Milan, Italy); serum immunoreactive 1–84 PTH was measured using a Nichols kit (Nichols Institute, San Juan Capistrano, CA, USA). Monomeric calcitonin was assessed as previously described (26).

### Table 1 Clinical and biochemical data of PH, FBH, and control subjects before and after 25(OH)D treatment (25 μg/day for 2 weeks). Data are presented as mean±s.d.

<table>
<thead>
<tr>
<th>PH</th>
<th>FBH</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60.4±8.3</td>
<td>58.5±10.3</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>14/6</td>
<td>16/2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.6±1.7</td>
<td>23.4±1.9</td>
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<tr>
<td>Smoking</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>MDRD (ml/min; before treatment)</td>
<td>75.1±10.2</td>
<td>80±7.3</td>
</tr>
<tr>
<td>MDRD (ml/min; after treatment)</td>
<td>73.4±8.1</td>
<td>81.2±9.3</td>
</tr>
<tr>
<td>25(OH)D (nmol/l; before treatment)</td>
<td>32.32±12.48</td>
<td>60.1±24.7</td>
</tr>
<tr>
<td>25(OH)D (nmol/l; after treatment)</td>
<td>78.49±39.36*</td>
<td>115.72±42.18*</td>
</tr>
<tr>
<td>1,25(OH)D (pmol/l; before treatment)</td>
<td>178.75±27.87</td>
<td>76.33±22.36</td>
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<tr>
<td>1,25(OH)D (pmol/l; after treatment)</td>
<td>254.15±53.09</td>
<td>72.26±39.78</td>
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<tr>
<td>Delta 25(OH)D (pmol/l)</td>
<td>46.18±13.23</td>
<td>55.61±16.97</td>
</tr>
<tr>
<td>Delta 1,25(OH)D (pmol/l)</td>
<td>75.4±15.34</td>
<td>−2.08±14.04</td>
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<tr>
<td>hCT (ng/l; before treatment)</td>
<td>7.9±0.4</td>
<td>3.7±0.2</td>
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<tr>
<td>hCT (ng/l; after treatment)</td>
<td>8.5±0.6</td>
<td>3.6±0.3</td>
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<tr>
<td>PTH (ng/l; before treatment)</td>
<td>142.08±13.11</td>
<td>44.33±12.8</td>
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<tr>
<td>PTH (ng/l; after treatment)</td>
<td>141.07±10.11</td>
<td>43.6±12.8</td>
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<td>Ca (mmol/l; before treatment)</td>
<td>1.37±0.11</td>
<td>1.24±0.1</td>
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<tr>
<td>Ca (mmol/l; after treatment)</td>
<td>1.37±0.11</td>
<td>1.24±0.1</td>
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<tr>
<td>Mg (mmol/l; before treatment)</td>
<td>0.86±0.08</td>
<td>0.97±0.04</td>
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<td>Mg (mmol/l; after treatment)</td>
<td>0.88±0.04</td>
<td>0.96±0.04</td>
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<td>Phosphate (mmol/l; before treatment)</td>
<td>0.67±0.02</td>
<td>1.09±0.06</td>
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<tr>
<td>Phosphate (mmol/l; after treatment)</td>
<td>0.71±0.02</td>
<td>1.09±0.03</td>
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<tr>
<td>Urinary Ca (mg/24 h; before treatment)</td>
<td>289.06±81</td>
<td>66.43±28.6</td>
</tr>
<tr>
<td>Urinary Ca (mg/24 h; after treatment)</td>
<td>298±81</td>
<td>66.36±32.5</td>
</tr>
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*P<0.00083 Student’s t-test before and after, †P<0.00083 PH vs controls, ‡P<0.00083 FBH vs controls, §P<0.00083 PH vs FBH.
described (26). Vitamin D metabolites were assessed with the IDS γ-B 25-hydroxy Vitamin D kit (Immunodiagnostics Systems Ltd, Boldon, UK; intra-assay coefficient of variation (CV) 6.9%, interassay CV 9%, normal range 75–300 nmol/l) and IDSγ-B 1,25-dihydroxy Vitamin D kit (Immunodiagnostics Systems Ltd; intra assay CV 9.7%, interassay CV 12%, normal range 39–169 pmol/l). According to the manufacturer, 25(OH)D has a cross-reactivity with 1,25(OH)2D lower than 0.001%.

Statistical analyses
Statistical analyses were performed using the GraphPad 4 package, version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences between single-variable measurements in each group were evaluated with Wilcoxon’s signed-rank test. Differences between single variables in different groups were evaluated with the Mann–Whitney U test. A type I error level of 0.05 was chosen. The Bonferroni correction for multiple comparisons was applied considering five variables, which resulted in a new α-error level of 0.0083. Relationships between vitamin D metabolites before and after treatment were analyzed with linear regression using Pearson’s correlation coefficients. Regression lines were compared using confidence intervals. A P value lower than 0.05 was considered statistically significant.

Results
At baseline, the serum levels of 25(OH)D in the FBH, PH, and control groups were moderately low, with PH patients showing significantly lower levels than healthy controls (Table 1). After supplementation, the levels increased significantly in all groups, and no meaningful differences were measured among groups.

Serum levels of 1,25(OH)2D at baseline were significantly higher in PH subjects than in healthy controls and in patients with FBH (Table 1). After 2 weeks of 25(OH)D supplementation, the serum 1,25(OH)2D level increased significantly in PH patients and healthy controls. By contrast, no increase in 1,25(OH)2D levels was observed in FBH patients (Table 1). Serum 1,25(OH)2D levels were, therefore, significantly different between the three groups.

As expected, PTH serum levels were significantly higher in the PH group than in the FBH and healthy control groups at baseline and after supplementation. However, no meaningful variations were observed in each group before and after treatment.

At baseline and after 25(OH)D supplementation, hCT was significantly lower in FBH patients compared with the other groups (Table 1).

Ionized calcium levels were significantly different between FBH patients and controls and between PH patients and controls at baseline and after 25(OH)D supplementation, whereas the FBH and PH groups’ ionized calcium levels did not differ at any time. Moreover, ionized calcium levels did not change significantly before and after supplementation in each group.

The 24 h urinary calcium levels were significantly different between the three groups, even if the changes after 25(OH)D supplementation were not meaningful.

A significant positive substrate–product relationship of 25(OH)D–1,25(OH)2D was found in PH and control subjects both at baseline and after supplementation, as shown in Fig. 1. No meaningful substrate–product relationship was found in FBH patients before or after 25(OH)D supplementation (Fig. 1). When we analyzed the delta changes of 25(OH)D versus the corresponding delta changes of 1,25(OH)2D in FBH, PH, and controls (Fig. 2), the slope of the 25(OH)D–1,25(OH)2D relationship was higher in PH patients than in controls, whereas no correlation at all was found in the FBH patients. Moreover, no correlation between ionized calcium and either 25(OH)D or 1,25(OH)2D was found in the three groups (data not shown).

Discussion
Our data support the hypothesis of a substrate–product relationship of 25(OH)D–1,25(OH)2D in PH patients and healthy subjects after oral administration of 25(OH)D. Conversely, in FBH patients, this relationship seems to be lacking. There have been no previous studies investigating the vitamin D substrate–product relationship of 25(OH)D–1,25(OH)2D.
relationship after vitamin D supplementation in FBH and PH patients.

Our data at baseline confirmed the findings of Law et al. (24) of a lower serum concentration of 1,25(OH)2D in FBH patients compared with PH patients. Interestingly, this difference was still present after 2 weeks of 25(OH)D supplementation. The lack of an increase in 1,25(OH)2D after supplementation could be related to reduced 1α-hydroxylase activity or enhanced metabolic clearance of 1,25(OH)2D, or both.

The activity of renal 1α-hydroxylase is under complex regulation by 25(OH)D, PTH, calcitonin (27), calcium, phosphate, magnesium (28–31), and 1,25(OH)2D itself (32). 1α-Hydroxylase has been cloned (33–35), and studies of the molecular mechanisms involved in its regulation demonstrated that administration of PTH and calcitonin, restriction of dietary calcium, and vitamin D deficiency are able to increase 1α-hydroxylase mRNA expression. On the other hand, administration of 1,25(OH)2D results in a decrease in 1α-hydroxylase expression and prevents the increased expression induced by PTH and calcitonin (36). In our study, the increased 1α-hydroxylase activity in PH patients could be related to elevated PTH serum levels, as described previously (24). Low levels of serum phosphate have been related to elevated PTH serum levels, as described recently (37). However, in our study, the serum phosphate levels were within the normal range, whereas ionized calcium was widely out of range both at baseline and after supplementation. Moreover, after supplementation, even the significant increase in 25(OH)D serum levels did not change the serum 1,25(OH)2D levels. This observation could support the hypothesis that 1α-hydroxylase activity or its induction is impaired in FBH patients, or that hypercalemia in the presence of normal PTH serum levels in FBH could suppress its activity. It is known that FBH is caused by inactivating mutations of the CASR gene and that CASR in the kidney is localized to the distal and collecting tubules but not the proximal tubules, where 1α-hydroxylase is mainly localized (10–13). Therefore, hypercalemia concomitant with normal serum PTH levels may suppress 1α-hydroxylase, regardless of the presence or absence of CASR mutations. Moreover, according to previous works in animal models (41, 42), there is an enhanced metabolic clearance rate of 1,25(OH)2D in the presence of increased serum 24,25(OH)2D concentrations, suggesting that serum 24,25(OH)2D may further enhance 1,25(OH)2D degradation (36). Moreover, Wilhelm et al. (43, 44) demonstrated that 24,25(OH)2D acts as an allosteric effector, diminishing the affinity of 1,25(OH)2D for its receptor and possibly enhancing the clearance rate of 1,25(OH)2D itself. Interestingly, these data were indirectly confirmed in a young patient with vitamin D-resistant rickets in whom the normalization of 24,25(OH)2D serum levels (to the physiological value of 2 ng/ml) brought about a marked and sustained decrease in 1,25(OH)2D serum levels (45). However, because of the high cross-reactivity of the 24,25(OH)2D measurement kit with 25(OH)D, we did not measure 24,25(OH)2D. Finally, it cannot be excluded that the low levels of hCT found in FBH may play a role in the impairment of 1α-hydroxylase (27).

The substrate–product relationship between 25(OH)D and 1,25(OH)2D has been postulated and investigated in previous studies (1, 2, 4). Some authors have reported the lack of a significant substrate–product relationship in experimental models (7). However, in these studies, high doses of 25(OH)D were used, which may have induced an increased clearance rate of 1,25(OH)2D to its final products. In experimental rat models, supplementation with lower doses of 25(OH)D produced an increase in serum 1,25(OH)2D concentrations, demonstrating a close substrate–product relationship between these vitamin D metabolites (46).
relationship between 25(OH)D and 1,25(OH)2D in a large population of postmenopausal women with 25(OH)D levels higher than 40 nmol/l. This positive relationship was subsequently confirmed in postmenopausal women with 25(OH)D levels lower than 40 nmol/l (16.03 mg/dl) (3). This positive substrate–product relationship in humans has also been recently confirmed in other works (2, 8, 47).

Our study has some limitations, the most important of which is the lack of 24,25(OH)2D measurements. However, it should be noted that the commercially available RIA kits for 24,25(OH)2D have a cross-reactivity with 25(OH)D of 100% (48). This prevented us from finding direct proof of an increased 1,25(OH)2D catabolism. Moreover, it is worth mentioning that this has been a short-term study, and lengthy studies are needed to clarify the response of 1,25(OH)2D after 25(OH)D treatment in FBH and PH subjects after a longer period, since it is likely that a new steady state has not yet been established after 2 weeks.

In conclusion, this study has been an interesting opportunity to investigate the vitamin D metabolism and in particular the substrate–product relationship between 25(OH)D and 1,25(OH)2D in patients with FBH, patients with PH, and healthy subjects. The lack of a 1,25(OH)2D increase and a substrate–product relationship in FBH after 25(OH)D supplementation may be due to three possible mechanisms: a direct inhibitory effect on 1α-hydroxylase of hypercalcemia, an increased metabolic clearance of 1,25(OH)2D, and a decreased stimulus of 1α-hydroxylase due to persistently lower levels of hCT in FBH patients compared with patients with PH and healthy subjects.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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