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# The effect of dietary calcium on $1,25(OH)_2D_3$ synthesis and sparing of serum $25(OH)D_3$ levels☆

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ABSTRACT

Vitamin D depletion in rats causes osteopenia in at least three skeletal sites. However it is unclear whether modulation of dietary calcium intake impacts on the relationship between the level of serum 25-hydroxyvitamin D (25D) and bone loss. Nine-month-old female Sprague–Dawley rats ($n = 5$–6/group) were pair-fed a semi-synthetic diet containing either 0 or 20 IU vitamin D3/day with either low (0.1%) or high (1%) dietary Ca for 6 months. At 15 months of age, fasting bloods were collected for biochemical analyses. Serum 25D levels were lowest in the animals fed 0 IU vitamin D and 0.1% Ca. The animals fed 1% Ca had significantly higher serum 25D levels when compared to animals fed 0.1% Ca ($P < 0.05$). The major determinants of serum 25D were dietary vitamin D and dietary calcium (Multiple $R = 0.75$, $P < 0.05$). Animals fed 0.1% Ca had higher renal CYP27B1 mRNA expression and 12–18-fold increased levels of serum 1,25D. Hence, the reported effects of low calcium diets on bone loss may be, in part, due to the subsequent effects of 25D metabolism leading to reduction in vitamin D status. Such an interaction has significant implications, given the recent evidence for local synthesis of active vitamin D in bone tissue.


## 1. Introduction

We have previously reported that vitamin D depletion in rats causes osteopenia in at least three skeletal sites including the distal and proximal femoral metaphyses and vertebrae. Furthermore, serum 25-hydroxyvitamin D (25D) levels ranging from 20 to 115 nmol/L were a positive and independent determinant of femoral trabecular bone volume [1]. It is clear that a diet containing high levels of calcium protects against bone loss, presumably by reducing PTH secretion, which in turn reduces osteoclastic activity. The question of whether, high dietary calcium also results in reduced renal vitamin D metabolism, resulting in maintenance of serum 25D levels is less certain. Such an interaction between dietary calcium and vitamin D has significant implications, given the recent evidence for local synthesis of active vitamin D in bone tissue [2–4]. We propose that the supply of 25D to the skeleton is an important factor for autocrine and paracrine activities of 1,25dihydroxyvitamin D (1,25D) via the activity of 25-hydroxyvitamin-D-1α-hydroxylase (CYP27B1) within osteoblasts [2–4] and other bone cells. A limited supply of serum 25D to the bone may impair osteoblastic 1,25D synthesis as the CYP27B1 enzyme activity does not appear to be induced to levels that have been observed in the kidney under the stimulation of PTH [2]. Previously, the role of calcium deprivation on serum 25D levels was not shown to be attributed the synthesis of picomolar levels of 1,25D as it was considered not to have the capacity to greatly influence nanomolar levels of 25D [5]. Rather, the influence of calcium deprivation on 25D catabolism within the liver implicated either PTH or 1,25D itself in the enhanced degradation of 25D by an unknown hepatic oxidative enzyme. Regardless, sustained production of 1,25D during calcium deprivation over an extended period of time, in combination with a vitamin D-deficient diet, has not been previously studied to determine its effects on serum 25D levels, particularly with regards to effects on gene expression of key vitamin D metabolising enzymes. The identification of an interaction between calcium deficiency promoting vitamin D deficiency could be important in understanding the pathogenesis of rickets and osteoporosis.

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## 2. Materials and methods

### 2.1. Animals

Nine-month-old female Sprague–Dawley rats ($n = 24$) were allocated to either vitamin D-replete (20 IU D3/day) or vitamin D-deplete diets containing either 0.1% or 1% calcium, based on the recommended semi-synthetic diet for rodents (AIN-93-VX, ICN, CA, USA). All animals were maintained on their assigned diets for 6 months, at which point they were killed. All animal procedures were approved by the Institutional Animal Ethics Committee.

### 2.2. Biochemical analyses

Fasting blood samples were collected at time of death for analyses. A chemistry analyser was used to measure serum calcium (Cobas Bio, Roche, IN, USA) and inorganic phosphate (Trace Scientific reagents, Vic, Australia; Hitachi 911 automated analyser, Roche, IN, USA). Serum 1,25D and 25D were measured by RIA (Immunodiagnostic Systems Ltd., Bolden, UK).

### 2.3. Messenger RNA analyses

The isolation of total RNA from whole rat kidney, liver and the first 10 cm of duodenum of 15-month-old rats were performed using Trizol (Invitrogen). First strand cDNA synthesis was performed as previously described [6]. Real-time RT-PCR was performed using primers designed to span an intronic sequence (Table 1) (Geneworks, SA, Australia). Target mRNAs of interest were expressed relative to the levels of β-actin mRNA using a comparative method of analysis. Multivariate statistical analysis of variance and Tukey's post hoc test analysis were performed of biochemical and mRNA measures.

## 3. Results

Low mean levels of serum 25D occurred only in animals fed 0 IU D3/day and 0.1% calcium (Table 2). Serum 25D levels were significantly higher in animals fed 1% calcium in both the 0 IU and 20 IU D3/day fed animals when compared to animals fed 0.1% calcium ($P < 0.05$), suggesting that a diet containing high levels of calcium is capable of increasing, or preserving serum 25D levels, relative to animals fed a low calcium diet. The animals fed 20 IU D3/day and 1% calcium recorded the highest mean serum 25D ($P < 0.05$). In addition to dietary vitamin D levels, dietary calcium was a major determinant of serum 25D levels ($P < 0.05$, $R^2 = 0.23$, Multiple $R = 0.75$) (data not shown). Despite the relative low serum 25D levels in animal fed 0 IU D3/day and 0.1% calcium, serum 1,25D levels were significantly increased and comparable to levels in vitamin D-replete animals fed 0.1% calcium. Mean serum calcium levels were marginally but significantly elevated in the animals fed 20 IU D/day and 1% calcium (Table 2).

Kidney levels of mRNA for CYP27B1 were significantly increased in animals fed 0.1% calcium in both the animal groups fed 0 or 20 IU D3/day when compared to animals fed 1% calcium (Fig. 1) ($P < 0.05$). In contrast, kidney CYP24 mRNA levels were highest only in animals fed 1% and 20 IU D3/day calcium ($P < 0.05$).

In the liver, there were no statistically significant differences in levels for three mRNA species, CYP27A1, CYP2R1 and CYP2J3, known to be important for the conversion of vitamin D to 25-hydroxyvitamin D in rodents (Fig. 2), suggesting that changes in serum 25D levels are not due to changes in liver 25D synthesis. Furthermore, levels of mRNA for the CYP3A isoforms, CYP3A1, CYP3A2, CYP3A9 and CYP3A11 were not determinants of serum 25D levels nor were associated with serum 1,25D levels (Fig. 3). No detectable levels of liver CYP24 mRNA levels were observed in any group (data not shown).

In the proximal intestine, the levels of TRPV6 mRNA were greater in the animals fed the 0.1% calcium diet (Fig. 3A, $P < 0.01$), consistent with the elevated serum 1,25D levels in these groups. The level of CYP24 mRNA was 4-fold higher in animals fed 0 IU D3/day and 0.1% calcium when compared to animals fed 20 IU D3/day. In contrast, CYP27B1 mRNA levels were approximately 2-fold higher in the animals fed 20 IU D3/day and 1% calcium (Fig. 4).

**Table 1**
Primer sets used for real-time RT-PCR.

| Gene | Sequence 5′ → 3′ | Genbank® accession no. |
|---|---|---|
| CYP27B1 | S: TGCAGAGACTGGAATCAGATGTTTG<br>A: CACTATGGACTGGACAGACACC | NM053763 |
| CYP24 | S: TTGAAAGCATCTGCCTTGTGT<br>A: GTCACCATCATCTTCCCAAAC | NM201635 |
| CYP27A1 | S: ATGTGGCACATCTTCTCTACC<br>A: GGGAAGGAAAGTGACATAGAC | NM178847 |
| CYP2R1 | S: CTTGGAGGCATATCAACTGTG<br>A: ATCCATCCTCTGCCATATCTG | NM001108499 |
| CYP2J3 | S: CCTGGATTTTGCTAACATTC<br>A: CTAAGCTCTTCTTTCCTAGT | NM175766 |
| CYP3A1 | S: GGAAATTCGATGTGGAGTGC<br>A: AGGTTTGCCTTTCTCTTGCC | X64401 |
| CYP3A2 | S: AGTAGTGACGATTCCAACATAT<br>A: TCAGAGGTATCTGTGTTTCCT | NM153312 |
| CYP3A9 | S: GGACGATTCTTGCTTACAGG<br>A: ATGCTGGTGGGCTTGCCTTC | NM147206 |
| CYP3A11 | S: GACAAACAAGCAGGGATGGAC<br>A: CCAAGCTGATTGCTAGGAGCA | NM007818 |
| β-Actin | S: ATCATGTTTGAGACCTTCAAC<br>A: CTTGATCTTCATGGTGCTAG | AF541940 |

## 4. Discussion

The data obtained during the present study demonstrate that reduction in serum 25D levels can be induced by feeding rats low diet calcium, regardless of the vitamin D content. The increase in serum 25D levels in animals fed high dietary calcium was not to be due to changes in the mRNA levels of known liver 25D synthetic enzymes, CYP27A1, CYP2R1 and CYP2J3. It is also unlikely that changes in serum 25D levels are due to catabolism of serum 25D via CYP24 activity, as CYP24 was undetectable in the liver and CYP24 in the kidney was only induced in the animals with the highest serum 25D levels which were vitamin D-replete animals fed high dietary calcium. It is possible however that increased expression and activity of renal CYP27B1 in response to calcium deprivation, with increased production of 1,25D may be responsible for the consumption of 25D stores providing the explanation for decreased 25D levels with low dietary calcium intake. Previously, it has been postulated that the relatively low levels of 1,25D production is unlikely to account for the reduction in the larger pool of 25D [5]. However this study was conducted over 6 months of calcium deprivation and thus the subsequent 1,25D production and its short half-life in the circulation may account for a larger fraction of the decline of 25D levels. This observation is consistent with clinical observations that during primary and secondary hyperparathyroidism, the half-life of serum 25D is inversely correlated to serum 1,25D [7].

That renal CYP27B1 activity influences the metabolic clearance of 25D does not, however, rule out the possibility that a low dietary calcium and subsequent high serum 1,25D may lead to an increased catabolism of 25(OH)D by hepatic oxidative enzymes, as

**Table 2**
Serum levels of 25D, 1.25D, PTH, calcium and phosphate.

| Vitamin D (IU/kg) | 0 | 0 | 20 | 20 |
|---|---|---|---|---|
| Dietary Ca (%) | 0.1 | 1.0 | 0.1 | 1.0 |
| 25D (nmol/L) (SEM) | 21.7 (2.4) | 84.5*(6.5) | 90.9 (11.8) | 161.3*,#(35.3) |
| 1,25D (pmol/L) (SEM) | 246.5*,#(24.7) | 19.5 (6.5) | 248.6*,#(68.1) | 13.8 (3.5) |
| Ca (mmol/L) (SEM) | 2.8 (0.1) | 2.6 (0.1) | 2.6 (0.2) | 3.2*,# (0.2) |
| Phosphate (mmol/L)(SEM) | 1.4 (0.1) | 1.6 (0.1) | 1.6 (0.1) | 1.9 (0.1) |

Values are mean (SEM), $n = 6$. 25D, 25-hydroxyvitamin $D_3$; 1,25D, 1,25dihydroxyvitamin $D_3$.
* $P < 0.05$ when compared with other 0.1% Ca fed animals.
# $P < 0.05$ when compared with other 0 IU/kg vitamin D fed animals.

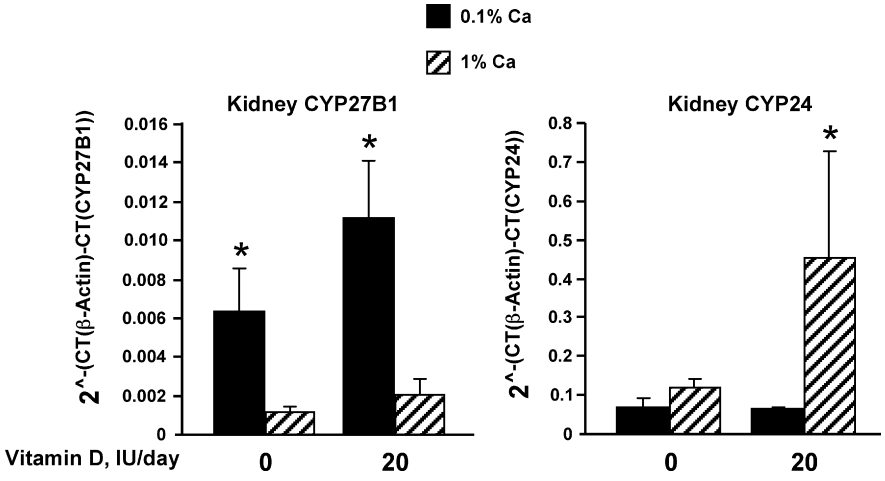


**Fig. 1.** Levels of CYP27B1 and CYP24 mRNA (relative to β-actin mRNA) in kidney tissue from each dietary treatment group. Values are mean ± SEM ($n = 6$). *$P < 0.05$ when compared with other 0.1% Ca fed animals; #$P < 0.05$ when compared with other 0 IU/kg vitamin D fed animals; CYP27B1, 25-hydroxyvitamin D-1α-hydroxylase; CYP24, 25-hydroxyvitamin-D-24-hydroxylase.

has been previously described [5]. However, the ability for 1,25D to directly stimulate the regulation of hepatic oxidative enzymes is presumably dependent on the expression of the vitamin D receptor (VDR). There are conflicting reports regarding the expression of VDR within human and rat liver tissue. Part of the confusion may be explained by general acceptance that VDR is absent in rat hepatocytes, but present in non-parenchymal cells and biliary epithelial cells [8]. Given that the major site of the target oxidative enzyme genes in hepatocytes, it appears less likely that 1,25D could directly regulate oxidative enzyme genes via a classical genomic response. There are numerous reports demonstrating that 1,25D can regulate hepatocyte cell processes. For example, Baran et al. [9] have shown that 1,25D increases intracellular calcium levels in rat hepatocytes. Furthermore, other studies have shown that the liver responds to 1,25D as indicated by its control of DNA polymerase activity as well as cytoplasmic and nuclear protein kinases. Also in human liver slices, CYP3A4 is unregulated by 1,25D, but only in liver samples that showed expression of VDR [10]. Interestingly, in separate experiments, while CYP3A4 has been shown to stimulate the 25-hydroxylation of vitamin D [11,12], CYP3A4 has also been shown to be stimulated by treatment with 1,25D and was able to generate three major metabolites of 1,25D catabolism [13], suggesting

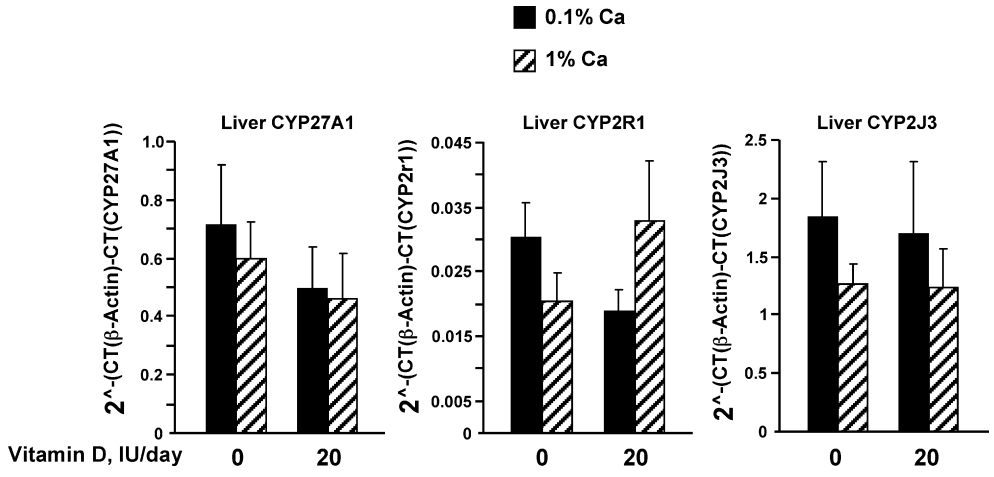


**Fig. 2.** Levels of CYP27Al, CYP2R1 and CYP2J3 mRNA (relative to β-actin mRNA) in liver tissue from each dietary treatment group. Values are mean ± SEM ($n = 6$).

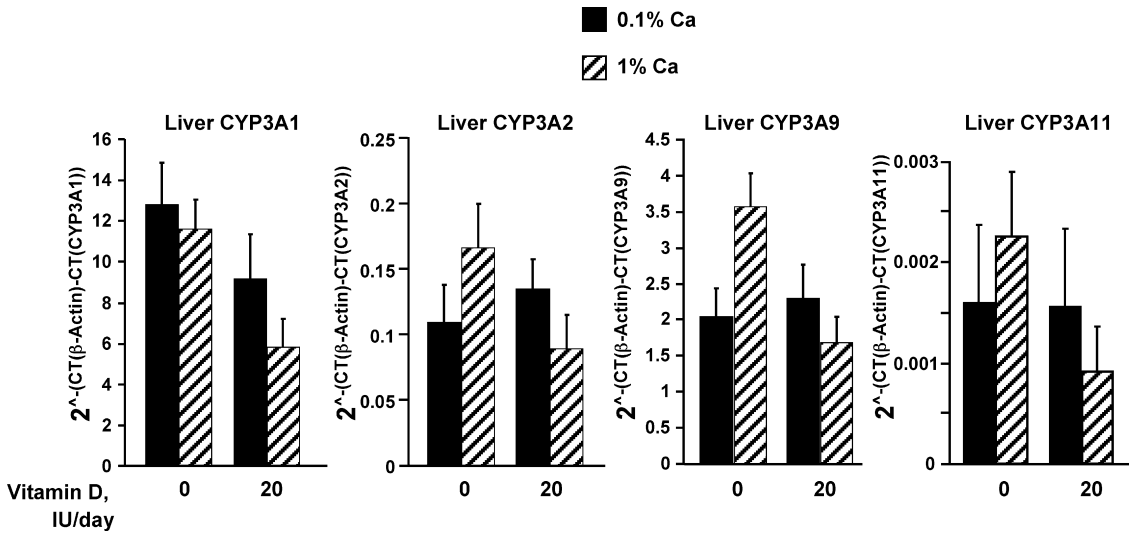


**Fig. 3.** Levels of CYP3A1, CYP3A2, CYP3A9 and CYP3A11 mRNA (relative to β-actin mRNA) in liver tissue from each dietary treatment group. Values are mean ± SEM ($n = 6$).

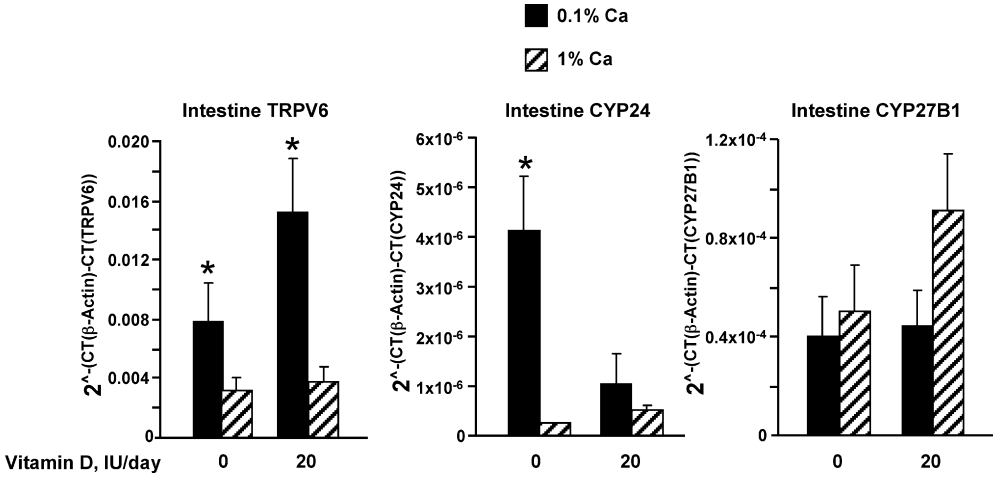


**Fig. 4.** Levels of TRPV6, CYP24 and CYP27B1 mRNA (relative to β-actin mRNA) in proximal intestinal tissue from each dietary treatment group. Values are mean ± SEM ($n = 6$). $^{*}P < 0.05$ when compared with other 0.1% Ca fed animals; $^{\#}P < 0.05$ when compared with other 0 IU/kg vitamin D fed animals.

that CYP3A4 in humans may be a target enzyme responsible for 25D catabolism as well. While rats do not possess CYP3A4, several other isoforms of CYP3A are expressed in liver tissue with reported similar actions to CYP3A4 [14–16]. In the current study, however, none of these CYP3A isoforms appear to be regulated in such a way as to explain the differences in serum 25D levels observed.

An alternative explanation for the role of high 1,25D regulating liver processes may involve the interaction of vitamin D with calcium on hepatic intracellular stores of calcium, $[Ca^{2+}]_i$. Gascon-Barre and co-workers reported that both resting and stimulated $[Ca^{2+}]_i$ are sensitive to changes in serum 25D levels as revealed in short-term primary culture of hepatocytes isolated from livers of rats depleted in 25D levels. The conclusions that hypocalcemia, secondary to low 25D levels, is a determinant of $[Ca^{2+}]_i$ levels, raises the possibility that a lower than normal $[Ca^{2+}]_i$ may be linked to changes in calcium signaling pathways, such as those shown to be involved in CYP3A induction of mouse hepatocytes [17].

Regardless of the mode of mechanism by which calcium deprivation may lead to reduced levels of 25D, the role for dietary calcium in the regulation of serum 25D levels is under-appreciated and may explain some of the beneficial effects on bone for the combination of vitamin D with calcium supplementation. Furthermore, to improve 25D status by vitamin D supplementation, one may need to consider the levels of dietary calcium, when recommending an effective regimen.

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