

Acute Homeostatic Changes Following Vitamin D₂ Supplementation

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Context: Changes in vitamin D binding protein (DBP) concentrations and catabolism of 25-hydroxyvitamin D to 24,25-dihydroxyvitamin D (24,25D) after vitamin D₂ supplementation may alter concentrations and bioavailability of circulating 25-hydroxyvitamin D (25D).

Objective: Examine acute changes in vitamin D metabolism and bioavailability after vitamin D₂ supplementation.

Methods: Study design was secondary analysis of a single-arm interventional study. Thirty consenting volunteers were treated with five 50,000 IU oral doses of ergocalciferol over 2 weeks. Main outcome measures included concentrations of DBP, vitamin D metabolites, and bioavailable 25-hydroxyvitamin D (25D) in pre- and posttreatment serum samples.

Results: After supplementation, 25D₂ (mean ± standard deviation) increased from 1.4 ± 0.9 ng/mL to 45.3 ± 16.5 ng/mL ($P < 0.0001$), and 25D₃ levels decreased from 26.8 ± 9.9 ng/mL to 19.7 ± 8.2 ng/mL ($P < 0.0001$). Total 25D (25D₂ plus 25D₃) increased from 28.2 ± 10.0 ng/mL to 65.0 ± 21.1 ng/mL (152.2% ± 102.5%; $P < 0.0001$). DBP and total 24,25D concentrations increased 39.1% ± 39.4% (165.6 ± 53.8 μg/mL to 222.0 ± 61.1 μg/mL; $P < 0.0001$) and 31.3% ± 48.9% (3.9 ± 2.0 ng/mL to 4.7 ± 2.1 ng/mL; $P = 0.0147$), respectively. In contrast to total 25D, bioavailable 25D increased by 104.4% ± 99.6% (from 5.0 ± 2.0 ng/mL to 8.7 ± 2.7 ng/mL; $P < 0.001$), and 1,25D increased by 32.3% ± 38.8% (from 45.5 ± 10.7 pg/mL to 58.1 ± 13.0 pg/mL; $P = 0.0006$). There were no changes in calcium or parathyroid hormone ($P > 0.05$ for both).

Conclusion: Changes after vitamin D₂ supplementation involve acute rise in serum DBP and 24,25D, both of which may attenuate the rise in bioavailable 25D and 1,25D.

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Freeform/Key Words: 24,25-dihydroxyvitamin D, 25-hydroxyvitamin D, ergocalciferol supplementation, feedback regulation, vitamin D binding protein

Vitamin D deficiency has been associated with several adverse health outcomes, including abnormal bone mineralization, heart disease, and premature mortality [1–8]. Conversely, hypervitaminosis D has been linked with hypercalcemia, tissue calcinosis, and renal injury

Abbreviations: 1,25D, 1,25-dihydroxyvitamin D₃ plus 1,25-dihydroxyvitamin D₂; 24,25D, 24,25-dihydroxyvitamin D; 24,25D₂, 24,25-dihydroxyvitamin D₂; 24,25D₃, 24,25-dihydroxyvitamin D₃; 25D, 25-dihydroxyvitamin D; 25D₂, 25-hydroxyvitamin D₂; 25D₃, 25-hydroxyvitamin D₃; CYP24A1, 25-hydroxyvitamin D 24-hydroxylase; DBP, vitamin D binding protein; FGF23, fibroblast growth factor-23; LC-MS/MS, liquid chromatography–tandem mass spectrometry; PTH, parathyroid hormone.

[9]. In response to variations in exposure (*e.g.*, sunlight, diet) and exogenous supplementation, counter regulatory mechanisms are in place that maintain appropriate concentrations of 1,25-hydroxyvitamin D (1,25D) and its precursor 25-dihydroxyvitamin D (25D) [6, 10, 11]. As with other hormones, feedback loops involve anabolic and catabolic pathways [12–19] that modify levels of bioavailable forms of the hormone [15, 20–25].

Changes in levels of binding proteins and, consequently, changes in bioavailable levels of hormones are well-known endocrine regulatory mechanisms [22, 26–28]. Vitamin D binding protein (DBP) is a circulating binding protein for both 25D and 1,25D [20, 29, 30] and an important determinant of 25D concentrations in the circulation that likely regulates the bioavailability of 25D and 1,25D to target tissues [16]. The determinants of DBP concentrations in humans, however, are incompletely understood. Both 25D and DBP are synthesized and secreted by the liver, and because of the high binding affinity of DBP for 25D, most circulating 25D is tightly bound to DBP [17, 20, 30]. Humans with liver disease demonstrate low blood levels of DBP, and accordingly low total 25D levels, but exhibit normal bioavailable or free serum 25D levels [31]. In contrast, excess DBP results in lower bioavailable levels. For example, in tissue culture models of vitamin D receptor signaling, exogenous addition of DBP to culture media dramatically reduces bioavailability of both 25D and 1,25D [16]. It is unknown, however, whether and to what extent vitamin D supplementation affects DBP levels following routine supplementation.

A second mechanism that allows vitamin D target tissue to modulate vitamin D signaling is by regulating expression of enzymes that convert 25D to its active form (1,25D) and to its inactive catabolite [24,25-dihydroxyvitamin D (24,25D)]. Conversion of circulating 25D to 1,25D occurs primarily in the kidneys and is upregulated during states of vitamin D deficiency [32]. In contrast, 25-hydroxyvitamin D 24-hydroxylase (CYP24A1) is highly expressed in the kidney and converts 1,25D and 25D to the inactive metabolites 1,24,25-trihydroxyvitamin D and 24,25D, respectively [32]. CYP24A1 is thus an efficient suppressor of vitamin D signaling by depleting both the active form of vitamin D (1,25D) and its precursor (25D).

We hypothesized that acute administration of high doses of vitamin D₂ would lead to counter regulatory changes in circulating DBP and expression of CYP24A1 and CYP27B1 that, in turn, would lead to sequential alterations in the bioavailability of 25D and its conversion to the active 1,25-dihydroxy form (Fig 1). Given the evidence of multiple mechanisms regulating vitamin D signaling, including the influence of DBP on concentrations of bioavailable 25D, it is likely that there is reciprocal regulation of blood concentrations of DBP after administration of vitamin D supplements, which may be an important feedback mechanism for maintaining appropriate concentrations of 1,25 D. Similar feedback regulatory

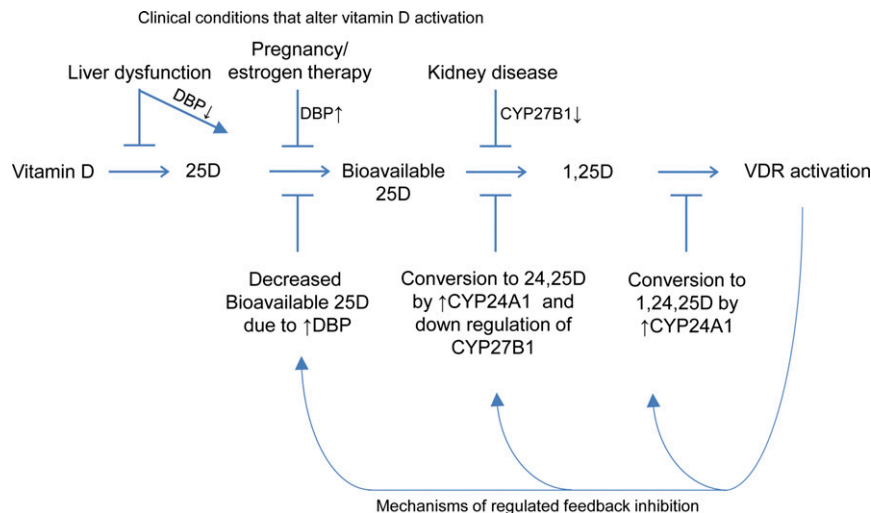


Figure 1. Model for regulated sequential conversion of vitamin D to its active form, and clinical conditions known to alter these steps. ↑, increased; VDR, vitamin D receptor.

changes in binding proteins have been described in other hormonal systems (*e.g.*, increases in sex hormone-binding globulin after administration of estrogen) [33, 34]. In this study, we examined the short-term changes of circulating concentrations of DBP, 24,25D, and bioavailable 25D after acute treatment of subjects with high doses of ergocalciferol (vitamin D₂).

1. Materials and Methods

A. Study Design and Participants

One hundred healthy adults (age ≥ 18 years) were recruited using both hard-copy and online advertisements. The study was conducted at the Massachusetts General Hospital from July 2007 to January 2012. Subjects were excluded if they had a known history of kidney disease, nephrolithiasis, or hypercalcemia. Exclusion criteria also included exposure to an active vitamin D analog (*e.g.*, calcitriol, doxercalciferol, or paricalcitol) within 30 days, use of immunomodulatory or immunosuppressive medications, pregnancy, known or suspected active infectious processes, or known granulomatous diseases. Candidates who signed written informed consent underwent screening laboratory testing including serum 25D, calcium, phosphorus, and serum creatinine levels; and urine pregnancy testing. Estimated glomerular filtration rate was calculated using the simplified Modification of Diet in Renal Disease equation [35]. Subjects with serum levels of calcium >10.5 mg/dL and phosphorus >5 mg/dL, a positive urine pregnancy test, or an estimated glomerular filtration rate <60 mL/min were excluded from further participation. All eligible participants with serum 25D level ≤ 32 ng/mL were offered treatment with 50,000 IU ergocalciferol taken orally every other day for five total doses (250,000 IU) over 2 weeks.

This study represents a secondary analysis of the original aims, which were intended to examine the relationship of vitamin D levels and cathelicidin [36]. The study was conducted in compliance with the Declaration of Helsinki principles, approved by the institutional review boards of Massachusetts General Hospital and the Massachusetts Institute of Technology, and registered with ClinicalTrials.gov (NCT00742235).

B. Data Collection

Subjects were screened for eligibility and, after consent and enrollment, a blood sample was collected before their first dose of ergocalciferol. Subjects were seen again 2 weeks later, after their final dose of ergocalciferol, when a final follow-up blood sample was obtained. For this study, baseline and posttreatment blood samples were analyzed for DBP, 25-hydroxyvitamin D₂ (25D₂), 25-hydroxyvitamin D₃ (25D₃), 1,25D, 24,25-dihydroxyvitamin D₂ (24,25D₂), 24,25-dihydroxyvitamin D₃ (24,25D₃), parathyroid hormone (PTH), calcium, phosphorus, and albumin concentrations. Vital signs including heart rate, temperature, and blood pressure were measured and recorded at both visits.

C. Biochemical Analyses

Blood samples were drawn into serum separator tubes without anticoagulant, centrifuged at 1430g for 15 minutes, and stored at -80°C for future analysis. To screen subjects for eligibility, baseline blood samples were tested for total 25D using a US Food and Drug Administration-approved immunoassay within a Clinical Laboratory Improvement Amendments-certified hospital laboratory.

To measure pre- and postsupplementation serum concentrations of 25D₃, 24,25D₃, 25D₂, and 24,25D₂, serum samples were subsequently tested using liquid chromatography-tandem mass spectrometry (LC-MS/MS). For these analyses, 100 μL of serum was mixed with 25D₃-[²H₆] and 24,25-(OH)₂D₃-[²H₆] isotopic internal standards dissolved in 5% bovine serum albumin (IsoSciences, King of Prussia, PA), and 25D₃, 25D₂, 24,25D₃, and 24,25D₂ were isolated by solid-phase extraction (Strata C-18E 96-well SPE plates; Phenomenex, Torrance,

CA), eluted with acetonitrile, and derivatized with 4-phenyl-1,2,4-triazole-3,5-dione. Samples were vacuum lyophilized and redissolved with 100 μ L of 50% ethanol. Samples were then analyzed for vitamin D metabolites using reverse-phase chromatography coupled to tandem mass spectrometry in multiple reaction monitoring mode [intra-assay coefficients of variation (CVs): 1.1%, 1.3%, 3.5%, and 4.2% for 25D₃, 25D₂, 24,25D₃, and 24,25D₂, respectively]. Assays were calibrated using 25D3 and 25D2 certified reference standards (Cerilliant, Round Rock, TX; accuracy traceable to National Institute of Standards and Technology SRM 2972 reference material), 24R,25-(OH)₂D₃ standard from Santa Cruz Biotechnology (Santa Cruz, CA), 24R,25-(OH)₂D₂ standard from Sigma-Aldrich (St. Louis, MO). Complete descriptions of chromatography and mass spectrometer settings have been previously described [37]. Intact serum PTH was measured using the Cobas electrochemiluminescence immunoassay on the Modular Analytics E170 automated analyzer (Roche Diagnostics, Indianapolis, IN; inter-assay CV, 2.5%).

Serum levels of 1,25D were measured in the 28 subjects with remaining sample by immunoassay on the Diasorin platform (Stillwater, MN; inter- and intra-assay CVs, <7.5%, validated against multiple clinical assay methods as previously described in [38]). Serum albumin and calcium levels were measured by colorimetric assay on automated platforms in a Clinical Laboratory Improvement Amendments-certified hospital laboratory. Serum fibroblast growth factor-23 (FGF23) was measured in pre- and posttreatment samples from the 24 subjects with remaining sample using the second generation human FGF23 (C-term) enzyme-linked immunosorbent assay kit (catalog no. 60-6100) from Immutopics (San Clemente, CA), using the manufacturer's instructions.

Calculation of bioavailable 25D (defined as the sum of free and albumin-bound 25D) was performed using methods we have previously described [39]. Briefly, total 25D is calculated from the sum of 25D₃ and 25D₂ concentrations; concentrations of free, DBP-bound, and albumin-bound 25D are calculated based on previously estimated binding affinity constants [20]; and concentrations of bioavailable 25D are calculated by summing the concentrations of free and albumin-bound 25D. To corroborate results obtained by calculated bioavailable 25D measurements, bioavailable 25D was also measured using a direct assay method described in Supplemental Materials. The direct assay for bioavailable 25D was calibrated using standards composed of buffered saline, 5% bovine serum albumin, and varying amounts purified vitamin D binding protein (Sigma-Aldrich). Calculated and direct bioavailable assay values were highly correlated with each other ($R^2 = 0.6901$; $P < 0.0001$; Supplemental Fig 1).

C-1. Mass spectrometric measurement of DBP

DBP was measured using two different assays, both involving high-performance liquid chromatography and tandem mass spectrometry. Serum from each subject (3 μ L) was first mixed with 3 μ L of DBP internal standard. The internal standard was composed of bovine DBP purified from fetal calf serum using Cohn ethanol fractionation followed by anion-exchange chromatography.

In the first LC-MS/MS assay method, premixed subject serum and DBP internal standard were diluted into 50 μ L of protease digestion mixture composed of 50 μ L of 25 mM Tris-HCl, pH 7.4, 10 mM dithiothreitol, and 0.1 mg/mL glutamyl endoproteinase (Worthington Biochemical, Lakewood, NJ), and digested overnight at 37°C. Human and bovine DBPs were then analyzed and quantified by LC-MS/MS. In the second LC-MS/MS assay, which was very similar to a recently published method [40], 3 μ L of serum was mixed with DBP internal standard, denatured and reduced with trifluoroethanol and dithiothreitol, and alkylated with iodoacetamide. Denatured samples were then digested overnight at 37°C in 25 mM Tris-HCl, pH 7.4, with 0.1 mg/mL tosyl phenylalanyl chloromethyl ketone-modified trypsin (Worthington Biochemical). After digestion with either Glu-C or trypsin, 10 μ L of the resulting digested serum peptides were injected and resolved by reverse-phase chromatography on a Kinetex C18 column, 50 \times 3 mm, 5- μ m bead diameter (Phenomenex)

eluted with 0.1% formic acid and 0% to 100% acetonitrile gradient. Digested peptides specific for the human and bovine DBP were measured using multiple reaction monitoring using settings shown in Supplemental Table 1. Assay characteristics are described in Supplemental Figs 2–4.

The peptides used to quantify DBP for both assay methods are digested peptides common to DBP protein variants Gc1S, Gc1F, and Gc2, and thus quantify total serum DBP concentrations including all major variants. The peptides used to quantify the internal standard for both methods are the homologous peptides liberated by digestion within bovine DBP. Purified DBP calibrators were purchased from Sigma-Aldrich (Gc-globulin, catalog no. G8764). Aliquots of pooled serum frozen at -80°C were used to monitor interassay quality control; the CV of the assay was 6.2%.

All LC-MS/MS assays were performed using an API 5000 triple quadrupole mass spectrometer (SCIEX, Framingham, MA) interfaced with a Shimadzu ultra-high-pressure liquid chromatography system with autosampler (Shimadzu USA, Columbia, MD). Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich.

D. Statistical Analysis

Baseline data were summarized using means, standard deviations, medians, and interquartile ranges. Frequencies were computed for all categorical and ordinal variables. Laboratory parameters were compared using pairwise *t* tests or Wilcoxon tests, depending upon the normality of the data. Correlation analyses were reported using the Pearson correlation coefficient. The statistical significance was set at a two-sided $P < 0.05$. All analyses were performed with R version 3.2.2 (<https://www.r-project.org/>).

2. Results

A. Subject Characteristics

Of the 36 subjects offered oral ergocalciferol supplementation, 30 completed the study; three were excluded due to inadequate sample, and three were excluded due to non-compliance based on the absence of detectable 25D_2 in their posttreatment samples (Fig 2). Baseline characteristics of the 30 subjects are shown in Table 1. Most were white, normotensive, and had normal renal function. Baseline levels of serum calcium, parathyroid hormone, and albumin were also within normal limits. As shown in Table 2, the mean baseline serum concentration of vitamin 25D_3 was 26.8 ± 9.9 ng/mL (range, 9.6–44.7 ng/mL), mean baseline serum concentration of 25D_2 was 1.4 ± 0.9 ng/mL (range, 0.4–4.2 ng/mL), and mean concentration of $24,25\text{D}_3$ was 3.9 ± 2.0 ng/mL (range, 0.81–8.17 ng/mL). The subjects included in the study all had baseline total 25D level < 32 ng/mL when tested by immunoassay during screening for eligibility, but a few were found to have baseline values > 32 ng/mL when subsequently tested by LC-MS/MS. This is most likely due to the poorer precision and negative bias that have been previously described for clinical immunoassays [41–44]. The mean baseline concentration of serum DBP among subjects was 165.6 ± 53.8 $\mu\text{g/mL}$ (range, 105.7–323.0 $\mu\text{g/mL}$). Correlations between baseline PTH and total 25D_3 and baseline PTH and bioavailable 25D_3 were -0.411 ($P = 0.02$) and -0.413 ($P = 0.02$), respectively.

B. Acute Homeostatic Changes in Vitamin D Metabolites

Table 2 summarizes the changes in levels of standard vitamin D measures, compensatory changes in the vitamin D metabolites, and calcium, phosphate, albumin, PTH, and FGF23 levels after a cumulative dose of 250,000 units of ergocalciferol over 2 weeks (22.3 times the recommended dose of 800 IU per day [45]). The marked rise in levels of 25D_2 was reflected in a significant rise in total 25D levels (152%). Changes in concentrations of total 25D within

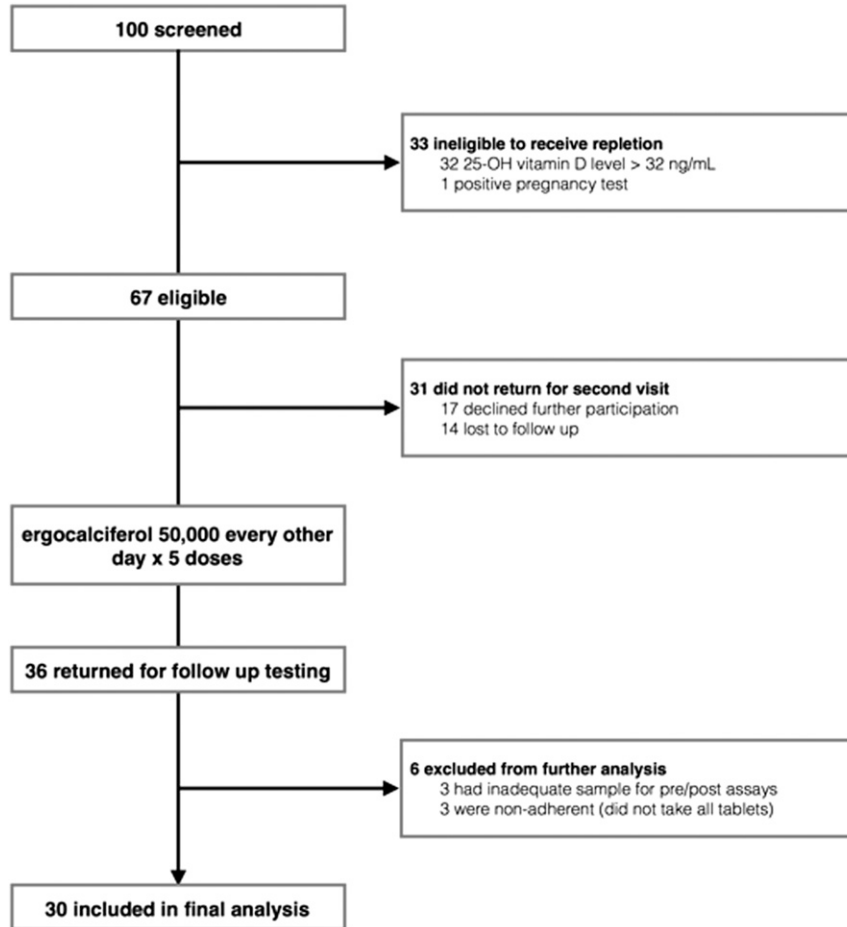


Figure 2. Consort diagram.

individual subjects are depicted in Supplemental Fig 5. When concentrations of bioavailable 25D were calculated based on measured total 25D, albumin, and DBP concentrations, bioavailable 25D was found to have increased by 104%. When we measured concentrations of bioavailable 25D using a newly developed direct binding assay, bioavailable 25D was found to have increased by 99%, corroborating the results of the calculated bioavailable 25D measurements. Last, concentrations of 1,25D measured by immunoassay (D_2 and D_3 combined) increased by only 32%.

Table 1. Subjects' Baseline Characteristics

Characteristic	Data (N = 30 subjects)
Age, y	30.7 (25.9 – 49.4)
Male sex	53 (16)
Race	
White	60 (18)
Black	13.3 (4)
Asian	13.3 (4)
Other	13.3 (4)
Hispanic ethnicity	23.3 (7)
Never smoked	46.7 (14)
Body mass index, kg/m ²	24.3 (22.7–28.4)

Data reported as median (interquartile range) or percentage of total (no.).

Table 2. Changes in Laboratory Parameters Pre- and Posttreatment With High-Dose Oral Ergocalciferol

Standard Vitamin D Parameters	Pretreatment*	Posttreatment*	<i>P</i> ^a	Average % Change
25D ₂ , ng/mL	1.4 ± 0.9	45.3 ± 16.5	<0.0001	4926 ± 4622
25D ₃ , ng/mL	26.8 ± 9.9	19.7 ± 8.2	<0.0001	-25.0 ± 25.2
Total 25D, ng/mL	28.2 ± 10.0	65.0 ± 21.1	<0.0001	152.2 ± 102.5
Bioavailable 25D, calculated, ng/mL	5.0 ± 2.0	8.7 ± 2.7	<0.0001	104.4 ± 99.6
Bioavailable 25D, direct assay, ng/mL	3.4 ± 0.2	5.6 ± 0.4	<0.0001	99 ± 21
1,25D, pg/mL	45.5 ± 10.7	58.1 ± 13.0	0.0006	32.3 ± 38.8
Compensatory responses				
DBP (GluC method), μg/mL	165.6 ± 53.8	222.0 ± 61.1	<0.0001	39.1 ± 39.4
DBP (trypsin method), μg/mL	156.2 ± 54.0	189.3 ± 52.6	0.015	26.3 ± 38.9
24,25D ₂ , ng/mL	0.05 ± 0.03	0.91 ± 0.38	<0.0001	2835.5 ± 2557.7
24,25D ₃ , ng/mL	3.8 ± 2.0	3.8 ± 1.9	0.8042	3.3 ± 40.0
Total 24,25D, ng/mL	3.9 ± 2.0	4.7 ± 2.1	0.0147	31.3 ± 48.9
24,25D ₃ :25D ₃ ratio	0.14 ± 0.04	0.19 ± 0.04	<0.0001	40.6 ± 41.7
24,25D ₂ :25D ₂ ratio		0.021 ± 0.001		
Minerals and hormones				
Calcium, mg/dL	9.4 ± 0.3	9.4 ± 0.3	0.76	-0.1 ± 3.2
Phosphate, mg/dL	3.5 ± 0.5	3.6 ± 0.6	0.20	4.1 ± 14.8
Albumin, g/dL	4.6 ± 0.2	4.6 ± 0.2	0.45	-0.6 ± 5.2
Parathyroid hormone, pg/mL	47.3 ± 16.7	47.6 ± 19.5	0.91	5.4 ± 40.7
Fibroblast growth factor-23, IU/mL	36.1 ± 34.4	26.5 ± 25.3	0.10	-25 ± 170

Data reported as mean ± standard deviation.

^a*P* values indicate statistical significance of difference between pre- and posttreatment average values by pairwise *t* test or pairwise Wilcoxon test, whichever was appropriate.

After 2 weeks of supplementation with ergocalciferol, mean circulating DBP concentrations increased approximately 39%, from 165.6 ± 53.8 μg/mL to 222.0 ± 61.1 μg/mL (*P* < 0.0001). Changes in concentrations of DBP within individual subjects are depicted in Supplemental Fig 6. DBP measured using the second LC-MS/MS assay method also demonstrated a significant increase in DBP concentrations after ergocalciferol supplementation, from 156.2 ± 54.0 μg/mL to 189.3 ± 52.6 μg/mL (*P* = 0.015). Note that DBP measurements between the two assays were strongly correlated and in close agreement (*R*² = 0.8088; Supplemental Fig 4). There was a significant correlation between serum DBP and 1,25D concentrations after vitamin D₂ supplementation, suggesting a relationship between the rise in 1,25D and DBP concentrations (Fig 3). After supplementation, there was a corresponding increase in serum concentrations of the breakdown metabolite 24,25D₂ and an overall increase of 24,25D by approximately 31% (Table 2). Furthermore, a strong correlation between 24,25D₂ and 25D₂ was observed (Fig 4).

Although there was no change in subjects' diets or sun exposure during the 2-week study period, concentrations of 25D₃ decreased by 25% after supplementation with ergocalciferol. Despite the decrease in concentrations of 25D₃, concentrations of its metabolite 24,25D₃ did not decrease and, as a consequence, there was a 41% increase in the ratio of 24,25D₃ to 25D₃. The decrease in 25D₃ concentrations relative to 24,25D₃ was also reflected by a shift to the left in the linear relationship between 24,25D₃ and 25D₃ after supplementation (Fig 5). Together, these findings are highly suggestive of an increase in CYP24A1 activity, which would result in increased production of 24,25D₃ despite the simultaneous catabolism (and loss) of 25D₃.

B-1. Minerals, PTH, and FGF23

Serum levels of calcium, PTH, and albumin were normal at baseline and did not significantly change after supplementation with ergocalciferol (*P* > 0.05 for all comparisons). Concentrations of FGF23 also did not show any significant changes after ergocalciferol treatment (Table 2).

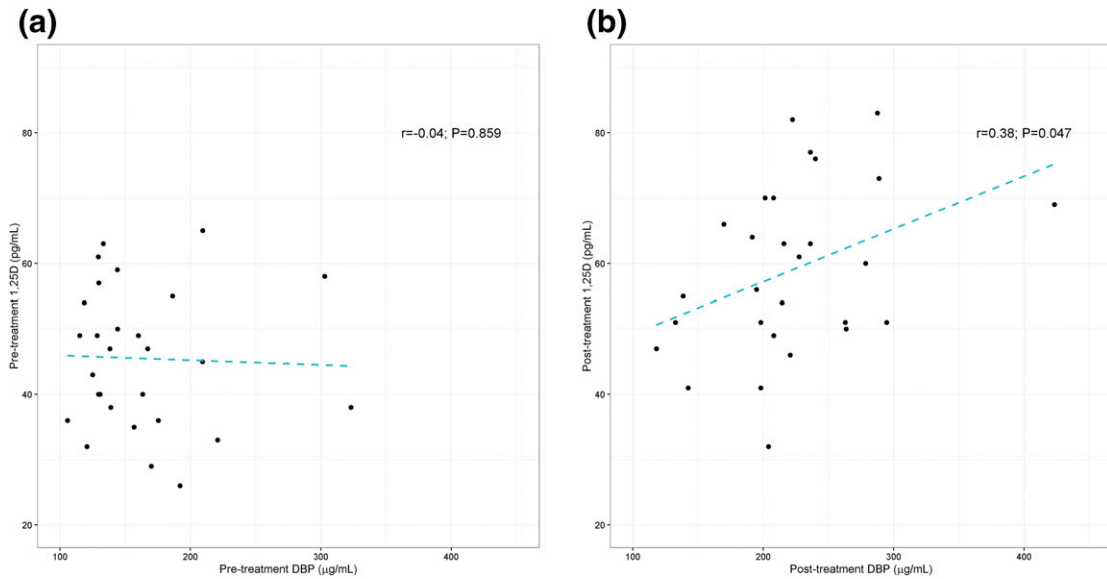


Figure 3. Correlations between serum DBP and 1,25D concentrations before and after vitamin D₂ supplementation. (a) Pretreatment. (b) Posttreatment. Pearson correlation coefficient and *P* values are shown.

3. Discussion

Our results suggest that physiologic changes after oral vitamin D₂ supplementation involve an acute increase in circulating DBP levels that may attenuate the rise in bioavailable 25D levels, as well as a parallel rise in 24,25D levels, whose production reflects an increased catabolism of 25D by CYP24A1. Upregulation of CYP24A1 would also be predicted to attenuate the rise of the active metabolite 1,25D. This may explain why healthy subjects with

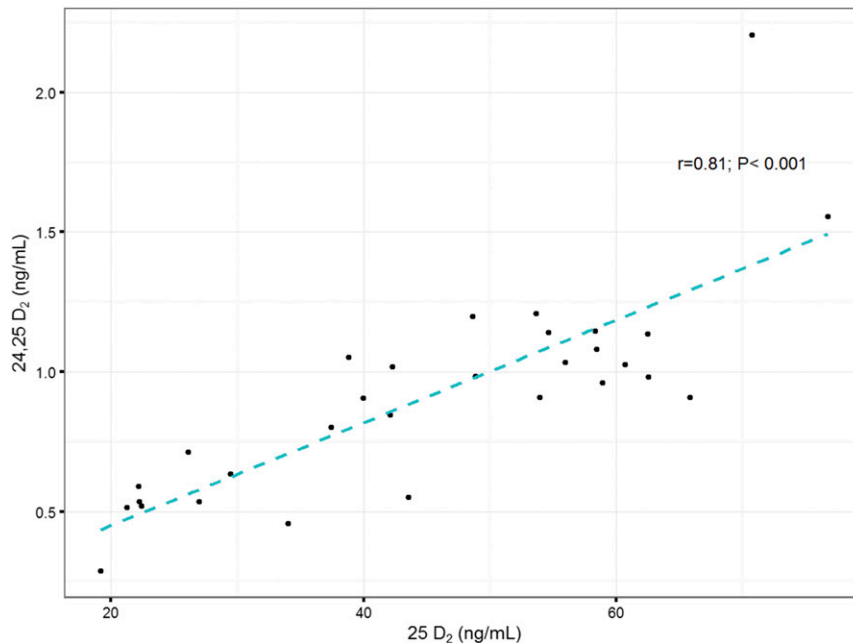


Figure 4. Linear relationships between serum concentrations of 24,25D₂ and 25D₂ after vitamin D₂ supplementation. Linear regression trendlines and Pearson correlation coefficients are shown.

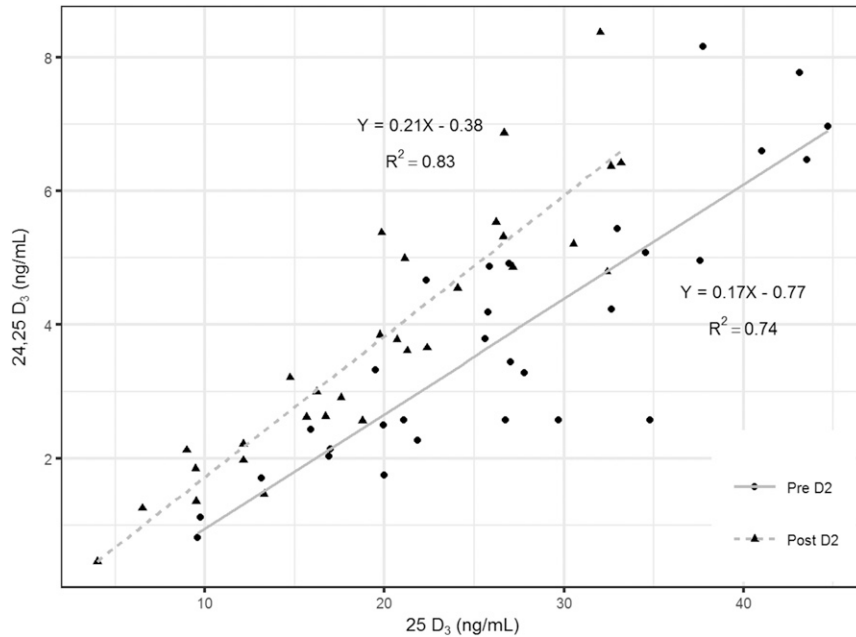


Figure 5. Linear relationships between serum concentrations of 24,25D₃ and 25D₃ before (circles) and after (triangles) vitamin D₂ supplementation. Linear regression trendlines and Pearson correlation coefficients are shown.

relatively normal levels of vitamin D and normal levels of serum calcium do not become hypercalcemic after receiving high doses of vitamin D₂.

DBP is the major carrier protein for circulating 25D, with estimated dissociation constants in the low nanomolar range [20, 30, 46, 47]. The high binding affinity of DBP makes it the major carrier for 25D but also results in reduction of the concentrations of free and bioavailable 25D [16, 24], analogous to the effects of thyroid hormone-binding globulin on thyroid hormone bioavailability [22, 26–28]. It has been hypothesized that DBP is essential for delivery of 25D to the kidney via megalin-mediated uptake in the proximal tubules [48]. Cell culture studies, however, confirm that DBP limits uptake of 25D and 1,25D by cells, thus acting as a key regulator of bioavailability of 25D for conversion and bioavailability of 1,25D for signaling [16]. Furthermore, mice genetically deficient for DBP develop profoundly decreased circulating 25D concentrations but are free from any ill effects of vitamin D deficiency while receiving a vitamin D-replete diet, presumably because of adequate bioavailable 25D levels due to the absence of DBP [24]. As a corollary, patients with DBP deficiency secondary to liver disease, as well as women with low DBP concentrations associated with estrogen deficiency after menopause, have low total 25D levels but normal bioavailable or free 25D levels [49, 50]. Our data support the cell culture studies in that the rise in DBP after vitamin D supplementation attenuated the increase in bioavailable 25D and 1,25D levels, compared with the dramatic rise of total 25D (104% and 32%, vs 152%).

After treatment with ergocalciferol, DBP concentrations increased significantly and were also correlated with concentrations of 1,25D. Increases in levels of circulating DBP in patients after vitamin D supplementation have been reported by others. In one study of patients with both hip fracture and vitamin D deficiency, it was found that 3 months of treatment with either cholecalciferol or ergocalciferol produced significant increases in serum DBP concentrations [51]. A similar pattern has been seen in women given estrogens or women during pregnancy; following increases in estrogen, the concentrations of 1,25D increased, and concentrations of DBP increased in parallel with 1,25D [49, 52, 53]. The increase in DBP levels after introduction of exogenous vitamin D and increased 1,25D signaling is analogous to the increase in sex hormone-binding globulin levels in patients treated with exogenous estrogen [33, 34]. Together, these observations suggest that production of DBP may be directly induced

by increased activation of vitamin D receptor by 1,25D (Fig 1). Although it may be premature to conclude that our observations offer definitive evidence of a physiologic mechanism for regulation of vitamin D activity, our data suggest that the observed rise in DBP after vitamin D supplementation may be acting to attenuate the rise in 1,25D levels.

In addition to the effects of vitamin D₂ supplementation on serum concentrations of DBP, we also observed coordinated changes in 25D catabolism. Although there were no changes in the diet or sun exposure of the subjects, average concentrations of 25D₃ decreased significantly among the subjects, while average concentrations of its downstream metabolite 24,25D₃ did not decrease. Because production of 24,25D depend on the concentration of its precursor 25D, we would normally expect 24,25D concentrations to decrease proportionally to the decrease in 25D level (this decrease in 24,25D relative to 25D should be further amplified by the fact that the half-life of 25D₃ is approximately 55.7-fold longer than that of 24,25D₃ [54, 55]). As a consequence of these changes, we observed that the average ratio of 24,25D₃ to 25D₃ and the linear relationship between 24,25D₃ and 25D₃ (Fig 5) increased significantly after vitamin D₂ supplementation. All these findings suggest a change in the equilibrium between 24,25D₃ and its precursor 25D₃ after the subjects received vitamin D₂ supplements. Together, these findings are most easily explained by an increase in the conversion of 25D to 24,25D by CYP24A1. If we accept this explanation, then the increase in catabolism of 25D₃ to 24,25D₃ further provides an explanation of why concentrations of 25D₃ decreased despite no change in subjects' sunlight exposure or diet.

These findings have considerable precedence; for example, decreased 25D₃ concentration after 4 weeks of supplementation with vitamin D₂ have been reported [56]. Furthermore, several studies from our group and others have shown that expression of CYP24A1 is increased by 1,25 D [32, 57–59], that the ratio of 24,25D₃ to 25D₃ increases disproportionately in subjects with higher 25D₃ concentrations [37, 60–65], and that supplementing patients with vitamin D deficiency with vitamin D₃ results in increases in the ratio of 24,25D₃ to 25D₃ similar to the effects we observed in response to vitamin D₂ supplementation [62, 45, 66]. One note of significance: A previous study found that 12-week treatment with high-dose ergocalciferol resulted in significant increases in subjects' FGF23 levels [67], and previous studies have suggested that FGF23 plays a role in regulation of CYP24A1. In our short-term treatment study, however, we observed no statistically significant changes in subjects' FGF23 levels, thus the changes in 24,25D₃ metabolism and 24,25D₃ to 25D₃ ratio after ergocalciferol treatment could not be explained by changes in FGF23 [68, 69].

This study has several limitations. In contrast to our results (and those of Glendenning *et al.* [51]), two previous studies of subjects receiving 15 weeks or 1 year of vitamin D supplementation did not report significant changes in DBP concentrations [66, 70]. Reasons for the discrepancy may be due to the fact that our study explored the acute effects of 2 weeks of vitamin D supplementation, whereas the other studies examined changes after longer periods of supplementation, after which feedback inhibitory mechanisms may have already normalized 1,25D signaling. Alternatively, differences may be due to our use of an LC-MS/MS assay to measure DBP, whereas these previous reports used various immunoassays. Second, after supplementation with vitamin D₂, there were increases in concentrations of 1,25D, 25D, and 24,25D. Although we hypothesize that the changes in DBP are in response to increased activation of vitamin D receptor by 1,25D, it is possible that other vitamin D metabolites (*e.g.*, 25D or 24,25D) may be influencing DBP expression, because there is growing evidence that 24,25D may have its own physiologic activities [71]. It is also possible that a portion of the increase in 1,25D is due to the increase in DBP concentrations and not the reverse. To clarify these issues, future studies are needed to investigate whether supplementation with 1,25D directly causes similar changes in DBP expression. Third, the number of subjects in our study was relatively small, and the study design did not include placebo controls. Thus, although the effects seen in our subjects were substantial, the strength of our conclusions would be fortified by additional studies with larger numbers of subjects and the inclusion of placebo controls. It should also be noted that baseline average concentrations of DBP in our healthy subjects were lower than the DBP concentrations reported by others [40, 72, 73]. The fact that we used a

different calibrator for DBP than these past studies may explain these differences; furthermore, these previous studies used a polyclonal immunoassay that has been shown to produce measurements significantly higher than other validated assay methods [73]. Furthermore, because our study focused on changes in DBP concentrations within individuals after treatment, and not on differences in absolute DBP concentrations, differences in DBP concentrations compared with other assay methods should not alter the interpretation or significance of our results.

In conclusion, after supplementation with high doses of ergocalciferol (29.7 times the daily recommended supplement of 600 IU) among otherwise healthy subjects, a marked rise in 25D₂ concentrations and a 152% increase in total 25D concentrations were accompanied by a 39% rise in blood levels of DBP and a 36% rise in 24,25D levels. Together, the increases in DBP levels and CYP24A1 activity may serve as regulatory mechanisms meant to actively prevent excessive signaling and vitamin D receptor activation, and maintain minerals and respective hormones in homeostatic balance.

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