Moderate Cholecalciferol Supplementation Depresses Intestinal Calcium Absorption in Growing Dogs

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ABSTRACT Hormonal regulation of calcium (Ca) absorption was investigated in a cholecalciferol (vitamin D₃)–supplemented group (hVitD) vs. a control group (cVitD) of growing Great Danes (100 vs. 12.5 μg vitamin D₃/kg diet). Although Ca intakes did not differ, fractional Ca absorption was significantly lower in the hVitD group than in the cVitD group. There were no differences in plasma concentrations of Ca, inorganic phosphate, parathyroid hormone, growth hormone, or insulin-like growth factor I between groups. Plasma 25-hydroxycholecalciferol [25(OH)D₃] concentrations were maintained in the hVitD dogs at the same levels as in the cVitD dogs due to increased turnover of 25(OH)D₃ into 24,25-dihydroxycholecalciferol [24,25(OH)₂D₃] and 1,25-dihydroxycholecalciferol [1,25(OH)₂D₃]. In hVitD dogs, the greater plasma 24,25(OH)₂D₃ concentration and the enhanced metabolic clearance rate (MCR) of 1,25(OH)₂D₃ indicated upregulated 24-hydroxylase activity. The increased MCR of 1,25(OH)₂D₃ decreased plasma 1,25(OH)₂D₃ concentrations. In hVitD dogs, the greater production rate of 1,25(OH)₂D₃ was consistent with the 12.9-fold greater renal 1α-hydroxylase gene expression compared with cVitD dogs and compensated to a certain extent for the accelerated MCR of 1,25(OH)₂D₃. The moderately decreased plasma 1,25(OH)₂D₃ concentration can only partially explain the decreased Ca absorption in the hVitD dogs. Intestinal vitamin D receptor concentrations did not differ between groups and did not account for the decreased Ca absorption. We suggest that 24,25(OH)₂D₃ may down regulate Ca absorption. J. Nutr. 132: 2644–2650, 2002.

KEY WORDS: • 1,25-dihydroxycholecalciferol • 24,25-dihydroxycholecalciferol • ⁴⁴Ca balance • vitamin D receptor • dogs

Intestinal calcium (Ca) absorption is absorbed by both passive and active transport processes. Passive transport is a nonsaturable paracellular process, whereas active transport is saturable and transcellular. Ca absorption is passive mainly in early life (1), whereas after weaning, passive absorption decreases and active hormonally regulated absorption becomes more prominent (1,2). Cholecalciferol (vitamin D₃) is essential for the hormonal regulation of intestinal Ca absorption. After its first hydroxylation in the liver into 25-hydroxycholecalciferol [25(OH)D₃], it is hydroxylated mainly in the kidney into 1,25-dihydroxycholecalciferol [1,25(OH)₂D₃] and 24,25-dihydroxycholecalciferol [24,25(OH)₂D₃] with the aid of 1α-hydroxylase and 24-hydroxylase, respectively. The up-regulating effects of 1,25(OH)₂D₃ on active and passive Ca absorption have been well described (3) although there is disagreement concerning the latter (4). The biological activities of 1,25(OH)₂D₃ at the intestinal level are mediated mainly by binding to a high affinity receptor (vitamin D receptor; VDR) (5). In addition, there is evidence that 1,25(OH)₂D₃ has rapid, nongenomic effects on Ca absorption (i.e., transcalcitachia) that have been reported only in chicks and are transient, modulated locally and inconsistent with the plasma 1,25(OH)₂D₃ concentrations (4). The endocrine effects of 24,25(OH)₂D₃ on Ca absorption have been demonstrated in several studies (6–8), including downregulation of VDR-related and of nongenomic effects of 1,25(OH)₂D₃ on Ca absorption (9).

The in vivo effects of vitamin D₃ toxicity are well established (10–12). The effects of vitamin D₃ supplementation have been studied extensively in the elderly and children with vitamin D₃ deficiency and/or negative Ca balance, including its effects on osteoporosis, renal failure and rickets (13–15). However, there is a lack of information concerning long-term
effects of moderate vitamin D₃ supplementation on the hormonal regulation of Ca metabolism in healthy, growing individuals in positive Ca balance.

Skeletal growth at high rates is a formidable challenge for both Ca and vitamin D₃ metabolism, and any disturbance may have important consequences. Ca metabolism in relation to skeletal growth and development has been studied in different animals, including dogs (16,17). Because interlaboratory CV for mean plasma 25(OH)D₃ concentrations may be 38% (18), absolute plasma concentrations cannot be compared. However, pathophysiologic mechanisms studied in dogs may well serve for extrapolation to humans (19,20). Dogs are convenient models with which to study the influence of vitamin D₃ on Ca metabolism because they are completely dependent on dietary intake of vitamin D₃ (21). This is attributed to the restricted availability of the vitamin D₃ precursor, 7-dehydrocholesterol, in the skin. Because vitamin D₃ status in dogs is easily controlled, seasonal variations of plasma vitamin D₃ concentration do not exist. The Great Dane breed provides a very attractive research model because it undergoes in a short time a period of gradual growth after weaning until 6 mo of age, simulating the growth curves of children. This dog breed has a body weight (BW) of ~6 kg at weaning and of ~70 kg at growth-plate closure, a stage reached in a 12- to 15-mo period. In addition, its BW allows for sequential and simultaneous sampling of material for long-term studies.

To evaluate the long-term effects of moderate dietary vitamin D₃ supplementation on the hormonal regulation of intestinal Ca absorption during growth, two groups of dogs were studied, i.e., a control group vs. an eightfold dietary-supplemented vitamin D₃ group. Dogs were studied from weaning at 6 wk until 4 mo of age and Ca absorption, and calcitropic and growth-regulating hormones were measured. In addition, the production and metabolic clearance of 1,25(OH)₂D₃ were investigated.

MATERIALS AND METHODS

**Animals and diets.** Male (n = 6) and female (n = 8) pure-bred Great Dane dogs (Utrecht University, The Netherlands), originating from three different litters, were divided into two groups at 3 wk of age: a control group (n = 7, cvitD) and a dietary vitamin D₃-supplemented group (n = 7, hvitD). Pups were fed one of two dry pellet diets (Table 1) formulated to be comparable in energy, Ca and phosphate (P) concentrations as confirmed by analysis of (Table 2). The control diet contained the recommended amount of 12.5 μg vitamin D₃/kg diet (22,23), whereas the supplemented vitamin D₃ diet contained a total of 100 μg vitamin D₃/kg diet. Diets were analyzed for their vitamin D₃ content in a reference laboratory (TNO Nutrition and Food Research, Zeist, The Netherlands). At 3 wk of age, partial weaning began and the pups received their diet as a gruel in addition to the bitch milk. Pups received their dry diet exclusively by 6 wk of age. Food intake was adapted biweekly to actual BW and provided at two times maintenance energy requirements of each dog (25) for the duration of the study. The Utrecht University Ethical Committee for Animal Care and Use approved all procedures.

**Ca absorption.** At 14 and 20 wk of age, true Ca absorption (Vₐ) was measured with the aid of one tracer, i.e., ⁴⁵Ca, by techniques described previously and validated for dogs (26,27). The use of only one tracer is justified by the following observations: 1) after intravenous (IV) administration of ⁴⁵Ca, the endogenous fecal excretion (Vₑ) of ⁴⁵Ca is measurable for a maximum of 3 d; 2) after oral administration of ⁴⁵Ca, the nonabsorbed tracer (Vₑ) is measurable for at most 4 d in the feces; and 3) the contribution of Vₑ by the tracer to Vₑ is negligible. Therefore, only one tracer can be used in the balance studies provided that the oral ⁴⁵Ca dose is given at least 3 d after the IV administration and with the assumption that the Ca balance is in a steady state during the 7-d investigation period.

In short, dogs were individually housed in metabolic cages during the ⁴⁵Ca balance studies. The actual food intake was measured and Ca intake (Vᵢ) was calculated for each investigation period. The endogenous fecal excretion of ⁴⁵Ca (Vₑ) was determined by collection of the feces for 3 consecutive days after an IV dose of 0.25 and 0.4 MBq ⁴⁵Ca as ⁴⁵CaCl₂ in water (specific activity 271.88 MBq/mg, NEN Life Science Products, Boston, MA) at 14 and 20 wk of age, respectively. Vₑ was calculated from the quotient of the part of the injected dose (Rₑ) that was excreted in the feces over 3 d and the integral of plasma specific activity C(t) according to the following formula:

$$Vₑ = \frac{Rₑ}{\int C(t)dt}$$

On d 4 of each metabolic study, a dose of ⁴⁵Ca equivalent to the IV dose was administrated orally and feces were collected for 4 consecutive days. The fecal content of ⁴⁵Ca was determined to calculate the total fecal content of Ca (Vₑ). Vₑ was calculated by the formula

$$Vₑ = Vᵢ - (Vₑ - Vₑ \cdot Vᵢ)$$

All parameters were expressed in mmol Ca/kg body d. The fractional absorption of Ca (α) was defined as α = Vₑ/Vᵢ.

Feces were collected and pooled for each part of the 3- and 4-d investigation periods, weighed and homogenized with a known quantity of distilled water with a blender. Triplicate samples were dried for 24 h at 60°C, weighed, ashed for 6 h at 550°C, suspended in 10 mL of hydrochloric acid (37% v/v) and then distilled water was added to a total volume of 100 mL. The activity of ⁴⁵Ca was determined in 0.4 mL of the supernatant mixed thoroughly with 4 mL scintillation fluid.

### TABLE 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control cholecalciferol diet</th>
<th>High cholecalciferol diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td>g/kg diet</td>
<td>g/kg diet</td>
</tr>
<tr>
<td>Corn</td>
<td>528</td>
<td>528</td>
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<tr>
<td>Chicken</td>
<td>200</td>
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<tr>
<td>Poultry fat</td>
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<td>75.5</td>
</tr>
<tr>
<td>Brewers rice</td>
<td>74.2</td>
<td>74.2</td>
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<tr>
<td>Mineral mixture¹</td>
<td>35.4</td>
<td>35.4</td>
</tr>
<tr>
<td>Beet pulp</td>
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<td>34.0</td>
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<tr>
<td>Egg</td>
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<td>25.0</td>
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<tr>
<td>Brewers yeast</td>
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<td>Menhaden oil</td>
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<td>8.0</td>
</tr>
<tr>
<td>Vitamin mixture M²</td>
<td>6.7</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin mixture H³</td>
<td>0</td>
<td>6.7</td>
</tr>
<tr>
<td>Ground flax</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

¹ Composition of the mineral mixture (amount in 35.4 g): calcium carbonate (Ca = 4.41g); monosodium phosphate (P = 2.61g); ferrous sulfate (Fe = 2.55g); zinc oxide (Zn = 2.12g); cupric sulfate (Cu = 159mg); manganese oxide (Mn = 283mg); manganese sulfate (Mn = 205mg); potassium iodide (I = 32mg); cobalt carbonate (Co = 5.3mg); with potassium chloride as the remainder.

² Composition of the vitamin mixture (amount in 6.7 g): vitamin A (13.2mg); vitamin C (367mg); cholecalciferol (22.1μg); vitamin E (226mg); thiamin (27mg); riboflavin (168mg); nicotinic acid (28mg); calcium pantothenate (52mg); pyridoxine-HCl (13mg); biotin (0.76mg); folic acid (2.5mg); inositol (17mg); cyanocobalamin (0.30mg); choline chloride (228mg); with rice hulls as the remainder.

³ Composition of the vitamin mixture (amount in 6.7 g): vitamin A (13.2mg); vitamin C (367mg); cholecalciferol (220.4μg); vitamin E (226mg); thiamin (27mg); riboflavin (168mg); nicotinic acid (28mg); calcium pantothenate (52mg); pyridoxine-HCl (13mg); biotin (0.76mg); folic acid (2.5mg); inositol (17mg); cyanocobalamin (0.30mg); choline chloride (228mg); with rice hulls as the remainder.
Endogenous metabolic clearance rate (MCR) and production rate (PR) of $1\alpha,25(OH)_{2}D_{3}$. At 19 wk of age, the MCR of $1\alpha,25(OH)_{2}D_{3}$ was determined with the aid of a bolus injection of $1\alpha,25$-dihydroxy[26,27-$^{3}$H]cholecalciferol [91,25(OH)D$_3$, specific activity 10.5 GBq/mg, Amersham Pharmacia Biotech, UK] by techniques described previously (36,37). In short, after an IV administration of $\sim3.7$ kBq $^{3}$H-$1\alpha,25$(OH)$_{2}$D$_{3}$, blood samples were drawn at 4, 6, 8, 10, 15, 20, 30, 45, 60 and 90 min and at 2, 3, 4, 5, 6, 10, 12 and 24 h after the injection and were immediately transferred into EDTA-coated tubes and put on ice until centrifuged and further processed. The plasma disappearance curve of $^{3}$H-$1\alpha,25$(OH)$_{2}$D$_{3}$ was obtained by counting plasma samples (0.5 mL with 4 mL scintillation fluid (Ultima Gold, Packard Bioscience BV)) in a liquid scintillation counter (1212 Rackbeta, LKB Wallac) for 30 min per sample. Using a computerized nonlinear least-squares fitting procedure, a biexponential function $C(t) = Ae^{-t} + Be^{-t}$ was fitted to the plasma $^{3}$H-$1\alpha,25$(OH)$_{2}$D$_{3}$ concentration. The MCR of $1\alpha,25$(OH)$_{2}$D$_{3}$ was calculated by the quotient of the injected dose D of $^{3}$H-$1\alpha,25$(OH)$_{2}$D$_{3}$ and the integral of plasma specific activity of $^{3}$H-$1\alpha,25$(OH)$_{2}$D$_{3}$ as follows:

$$\text{MCR} = \frac{D}{\int_0^\infty C(t)dt},$$

where MCR is expressed in L/(kg body $\cdot$ d). The PR $1\alpha,25$(OH)$_{2}$D$_{3}$ was derived from the formula: PR [pmol/(kg body $\cdot$ d)] = MCR [L/(kg body $\cdot$ d)] $\times$ endogenous circulating $1\alpha,25$(OH)$_{2}$D$_{3}$ (pmol/L), where these endogenous circulating $1\alpha,25$(OH)$_{2}$D$_{3}$ is the plasma $^{3}$H-$1\alpha,25$(OH)$_{2}$D$_{3}$ concentration at 19 wk of age.

1α-hydroxylase gene expression. Gene expression levels of 1α-hydroxylase were determined at the middle and the end of the study in all dogs. At 11 wk of age, two kidney biopsies were obtained by fine needle biopsy under guidance of echography with the dog under general anesthesia. At the end of the study, i.e., at 21 wk of age, the dogs were killed with an overdose of Na-panethobarbitals and biopsies from the kidney were sampled. Kidney biopsies were immediately frozen in liquid nitrogen and stored at $-70^\circ$C until required for RNA isolation.

RNA isolation and cDNA synthesis. Frozen kidney tissue sampled at 11 wk of age was resuspended in Qiagen lysis buffer, homogenized using a Polytron, and centrifuged for 3 min at 5000 $\times$ g at room temperature. Frozen kidney tissue sampled at 21 wk of age was ground in liquid nitrogen prefreeze cups of a micro-dismembrator (Micro-Dismembrator U, B. Braun Biotech International GmbH, Melsungen, Germany) using 2 cycles of 45 s at 2200 rpm. Milled tissue (30 mg) was resuspended in Qiagen lysis buffer and centrifuged for 3 min at 5000 $\times$ g at room temperature. The supernatant was applied to a Qiagen mini-column (Qiagen GmbH, Hilden, Germany) and total RNA was isolated according to the manufacturer’s protocol.

RNA was ethanol precipitated and resuspended in RNAsecure (1X; Ambion, Austin, TX), which was activated at 60°C for 10 min. After DNase I treatment (DNAfreeTM kit, Ambion), RNA was again ethanol precipitated and resuspended in 20 μL of RNase-free water. Total RNA (2 and 3 μg) was used in a cDNA-synthesis reaction with a final volume of 40 and 60 μL (Reverse Transcription System, Promega, Madison, WI) according to the manufacturer’s instructions, for the material sampled at the middle and the end of the study, respectively.

Real-time polymerase chain reaction (PCR). Real-time PCR, based on the high affinity double-stranded DNA-binding dye SYBR green I (SYBR green I, BMA, Rockland, ME), was performed in triplicate in a spectrophuorometric thermal cycler (iCycler, BioRad). Data were collected and analyzed with the application software provided. For each real-time PCR reaction, 1.67 μL (from the 40- or 60-μL stock) of cDNA was used in a reaction volume of 50 μL containing 1X PCR buffer, 2 mmol/L MgCl$_2$, 1:100,000 dilution of SYBR green I, 10 nmol/L fluorescein calibration dye (Bio-Rad, Hercules, CA), 200 μmol/L dNTP, 20 pmol forward primer, 20 pmol reverse primer, and 1.25 μL of AmpliTaQ Gold DNA polymerase (Applied Biosystems, Roche, Branchburg, NJ). Cycling conditions were optimized for the reaction of each target gene. Primer pairs were

### TABLE 2

**Analysis of the experimental diets**

<table>
<thead>
<tr>
<th>Item</th>
<th>Control cholecalciferol diet</th>
<th>High cholecalciferol diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>26.00</td>
<td>26.83</td>
</tr>
<tr>
<td>Crude lipid</td>
<td>14.65</td>
<td>15.44</td>
</tr>
<tr>
<td>Ash</td>
<td>5.10</td>
<td>5.20</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.86</td>
<td>0.87</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.74</td>
<td>0.74</td>
</tr>
<tr>
<td>Moisture</td>
<td>6.64</td>
<td>8.00</td>
</tr>
</tbody>
</table>

μg/kg dry diet

1 Moisture 6.64 g/100 g diet.
2 Moisture 8.00 g/100 g diet.

(Ultima Gold, Packard Bioscience BV, Groningen, The Netherlands) in a liquid scintillation counter (1212 Rackbeta, LKB Wallac, Turku, Finland).

**Blood measurements.** At 7, 10, 13, 16, 19 and 21 wk of age, blood samples were collected after overnight food deprivation. Blood samples for the measurement of plasma total Ca and inorganic phosphate (Pi) concentrations were transferred into heparin tubes, centrifuged at 3000 $\times$ g for 10 min at 4°C and measured according to standard procedures (Beckman Industries, Brea, CA).

Blood samples for the measurements of hormones were immediately transferred into EDTA-coated tubes and placed on ice until centrifuged. Plasma was stored at $-20^\circ$C until analysis.

25(OH)D$_3$ and 24,25(OH)$_2$D$_3$ were determined quantitatively by a modified RIA (DiaSorin, Stillwater, MN). Before processing, both labeled standards, 25-hydroxy[26,27-$^{3}$H]cholecalciferol and 24R,25-dihydroxy[26,27-methyl-$^{3}$H]cholecalciferol (specific activity 16 GBq/mg and 15.4 GBq/mg, respectively, Amersham Pharmacia Biotech, UK), were added to plasma samples and to the standards of RIA to determine individual sample recovery. Samples were extracted twice with methanol:ethylacetate:cyclohexane (4:5:5, v/v/v) (28), and extracted twice with ethylacetate:cyclohexane (1:1, v/v) and once with methanol:ethylacetate:cyclohexane (4:5:5, v/v/v) (28), and 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$ were separated by solid-phase extraction using NH$_2$ cartridges (Bakerbond spe Amino Disposable Extraction Columns, J.T. Baker, Phillipsburg, NJ) according to the method of McGraw and Hug (29). The standard curves of both stable vitamin D$_3$ metabolites paralleled the standard dilution curve of the RIA. The intra- and interassay CV for 25(OH)D$_3$ were 15.3 and 15.4%, respectively. The intra- and interassay CV for 24,25(OH)$_2$D$_3$ were 10.1 and 8.5%, respectively.

1,25(OH)$_2$D$_3$ was extracted from plasma using acetonitrile followed by a two-step solid-phase extraction (C18 and Silicagel cartridge; Waters Chromatography B.V., Etten Leur, The Netherlands) and quantitatively determined by a radioreceptor assay based on the method described by Reinhardt et al. (30) and Hollis (31) with an intra- and interassay CV of 5.7 and 6.6%, respectively.

Parathyroid hormone (PTH) was measured using an immunoradiometric assay for intact PTH (iPTH, Nichols Institute, San Juan Capistrano, CA) (32). The intra- and interassay CV were 3.4 and 5.6%, respectively. Calcitonin (CT) was measured after extraction with ethanol by a homologous RIA (33). The intra- and interassay CV were 15.3 and 15.4%, respectively.

Growth hormone (GH) was measured by a homologous RIA (34). The intra- and interassay CV were 3.8 and 7.2%, respectively. Insulin-like growth factor I (IGF-I) was measured by a heterologous RIA as described previously (35) with intra- and interassay CV of 4.7 and 15.6%, respectively. At 21 wk of age, GH and IGF-I were not determined.

Moisture 6.64 g/100 g diet.
Phosphate 0.74 g/100 g diet.
Calcium 0.86 g/100 g diet.
Ash 5.10 g/100 g diet.
Crude lipid 14.65 g/100 g diet.
designed using PrimerSelect software (DNASTAR, Madison, WI). The primer pair of β-actin based on the human sequence (M10277) distinguishes cDNA from pseudogene DNA and the sense (F) and antisense (R) primers for its amplification were 5’-GATATCGCC-
CGGCTCGTCGTG-3’ (exon 2; 1102–1122) and 5’-GGCTG-
GGTGTAGGTTACTC-3’ (exon 4; 2038–2058), respectively. Based on the human sequence of 1α-hydroxylase (AB005989) the F and R primers 5’-CCGGAAGTGGCCGAGACTC-3’ (exon 1; 103–121) and 5’-GAGGAAGAAGGCGGCGAGACTC-3’ (exon 3; 3417–440), respectively, were used for amplification of a 337-bp fragment. Based on the homologous canine sequence of this PCR product, a 134-bp fragment was designed with the F and R primers 5’-GGGTGTTGAAGGTCTC-3’ (exon 1) and 3’-5’-
CCTCGCCTTCCGCGGAGCA-3’ (exon 2, 3.3). Melt curves (iCycler, BioRad) and agarose gel electrophoresis were used to examine each sample for purity and standard sequencing procedures (ABI PRISM 310 Genetic Analyzer, Applied Biosystems) were used to verify the analytical specificity of the PCR products. Standard curves constructed by plotting the log of the starting amount vs. the threshold cycle were generated using serial 10-fold dilutions of known amounts of PCR products (from a conventional PCR). The amplification efficiency, E (%) = 10[(1/\theta) – 1] × 100 (θ = slope), of each standard curve was determined and appeared to be >90% over a large dynamic range (6–8 orders of magnitude). For each experimental sample, the amounts of 1α-hydroxylase and β-actin as endogenous references were determined from the appropriate standard curve in an autologous experiment. The amount of target was divided by the amount of endogenous reference to obtain a normalized target value. Each of the normalized target values was divided by the normalized target value of the calibrator (cVitD group) to generate relative expression levels.

VDR analysis. Nuclear extracts were prepared as described by Sandgren and DeLuca (38). Briefly, at the end of the study (i.e., at 21 wk of age) after killing and immediate laparotomy, the mucosal layer was sampled from the following defined intestinal segments: proximal and distal duodenum, beginning, middle and end of the jejunum, ileum and colon. Segments were rinsed with ice-cold TED saline (50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 5 mM/L dithiothre-
tol, 10 mg/L TPCK, 1 mg/L leupeptin, 1 mg/L pepstatin) and the mucosal layer was scraped and washed three times in TED saline by centrifugation for 10 min at 3000 × g and 4°C. Samples were homogenized in 4 volumes (wt/v) TED-300 (TED, 300 mmol/L Tris-HCl, pH 7.4, 1.5 mmol/L EDTA, 5 mmol/L dithiothre-
tol, 10 mg/L TPCK, 1 mg/L leupeptin, 1 mg/L pepstatin) and stored at –70°C until further assayed. The concentration of VDR was measured by a VDR-speci
fic ELISA (39) and expressed as fmol/mg mucosal protein.

Statistical analysis. Statistical analyses were performed using the SPSS for Windows 10.1 (SPSS Inc, Chicago, IL). Differences within groups were analyzed in a repeated measures ANOVA. Differences between groups were analyzed by the unpaired two-tailed Student’s t
 test, after testing for homogeneity of variance with Levene’s test. The significance of differences in VDR concentration between groups was tested by the nonparametric Wilcoxon-Man–Whitney U test be-
cause of the considerably high SD of the mean (i.e., in the majority >50% of the mean). Differences were considered significant when P < 0.05. Results are presented as means ± SEM.

RESULTS

The dogs were generally healthy, grew well and consumed all of the food that was administered daily. Consequently, energy and food intakes per kg metabolic BW (kg−0.75) did not differ between groups. The growth rates were 1.5 ± 0.1 and 1.5 ± 0.2 kg BW/wk of age for the cvitD and hvitD groups, respectively, and did not differ. Diets contained no detectable ergocalciferol and the vitamin D3 content was as given in the Materials and Methods.

Ca absorption. At all ages, V1 did not differ between groups (Table 3). The V1 did not differ between groups at 14 wk of age, whereas at 20 wk of age, it was significantly higher in the hvitD group than in the cvitD group. At both ages, V0 and α were significantly lower in the hvitD group than in the cvitD group.

Biochemical profiles. The hvitD group consumed approximately 8 times as much vitamin D3/kg BW as the cvitD group (Table 3). Plasma Ca concentrations did not differ between groups throughout the study, ranging from 2.70 ± 0.05 to 3.00 ± 0.02 mmol/L. Similarly, plasma P concentrations did not differ and ranged from 2.71 ± 0.05 to 2.96 ± 0.03 mmol/L. Plasma 25(OH)D3 concentrations did not significantly differ between groups for the duration of the study (Fig. 1). In the hvitD group, plasma 24,25(OH)2D3 concentrations were significantly greater than the cvitD group beginning at the age of 13 wk (Fig. 1). In the hvitD group, plasma 1,25(OH)2D3 concentrations were significantly lower at 10, 13 and 21 wk of age than in the cvitD group (Fig. 1). Plasma PTH, CT, GH and IGF-I concentrations did not differ between groups throughout the study and stayed within limits of the reference values in growing Great Dane dogs (35,40).

MCR and PR of 1,25(OH)2D3. At 19 wk of age, the MCR and PR of 1,25(OH)2D3 were significantly greater in the hvitD group than in the cvitD group (Fig. 2).

Gene expression of 1α-hydroxylase. At 11 wk of age, renal gene expression levels of 1α-hydroxylase did not differ between groups (data not presented), whereas at 21 wk of age,

| TABLE 3 |
| Effects of moderate cholecalciferol (vitamin D3) supplementation from 3 to 21 wk of age on intestinal Ca handling in growing dogs1,2 |

<table>
<thead>
<tr>
<th>Cholecalciferol, μg/kg diet</th>
<th>cvitD group</th>
<th>hvitD group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.5</td>
<td>100</td>
</tr>
<tr>
<td>Age, wk</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>16.5 ± 0.6</td>
<td>27.4 ± 0.7</td>
</tr>
<tr>
<td>Cholecalciferol intake, μg/(kg body-d)</td>
<td>0.44 ± 0.01</td>
<td>0.36 ± 0.01</td>
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<tr>
<td>V1, mmol/(kg body-d)</td>
<td>6.98 ± 0.09</td>
<td>6.02 ± 0.09</td>
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<tr>
<td>V0, mmol/(kg body-d)</td>
<td>0.88 ± 0.05</td>
<td>0.71 ± 0.03</td>
</tr>
<tr>
<td>α, %</td>
<td>82.2 ± 4.0</td>
<td>69.0 ± 3.7</td>
</tr>
</tbody>
</table>

1 Data are presented as means ± SEM, n = 7. *, P < 0.05; †, P < 0.01; and **, P = 0.069 different from the control group at the same age
2 cvitD, control group; hvitD, high vitamin D group; V1, Ca intake; V0, endogenous fecal excretion of Ca; Vf, total fecal content of Ca; Vf, true Ca absorption; α, fractional Ca absorption.

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Intestinal VDR concentration. At the end of the study, VDR concentrations did not differ between groups and decreased gradually and progressively in the distal segments of the intestine in both groups, ranging from 349 ± 35 fmol/mg protein in the proximal duodenum to 92 ± 20 fmol/mg protein in the colon.

DISCUSSION

Two groups of young dogs were raised under identical circumstances and with dietary composition differing only in vitamin D₃ content. The cVitD group was fed a diet with the amount of vitamin D₃ recommended by the international reference organization of the National Research Council (22) and had growth curves, radiological and histological skeletal development (not shown here), and Ca metabolism consistent with previous studies in healthy growing Great Danes (35). The hVitD group also grew well with growth rates similar to those of the cVitD group (41). Chronic intake of food fortified with 8 times the amount of vitamin D₃ had major influences on the formation of the biologically important vitamin D₃ metabolites and resulted in downregulation of intestinal Ca absorption. The mechanisms implicated in the depression of intestinal Ca absorption are discussed.

Fractional Ca absorption (α) was significantly lower in the hVitD compared with the cVitD group during the study and was reflected in the slightly but significantly increased Vₐ at the end of the study (42). Despite the lower α, the hVitD group retained its Ca homeostasis as indicated by maintenance of plasma PTH concentrations at control levels (43). Vitamin D₃ metabolism in relation to Ca absorption is influenced by a wide variety of minerals and hormones of which plasma Ca, Pₐ, PTH, CT, GH, IGF-I and vitamin D₃ metabolites concentrations are the most important. Because these concentrations did not differ between groups during the study, we assume that they did not differently affect vitamin D₃ metabolism in the two groups. The high growth rate and corresponding high GH and IGF-I levels may contribute to the higher plasma 1,25(OH)₂D₃ concentrations in Great Dane dogs (44) when compared with healthy children (i.e., 101 ± 31 pmol/L) (45). It remains to elucidated whether vitamin D₃ metabolism differs during growth at high and slow growth rates.

The decreased Ca absorption in the hVitD group is most likely explained by the only difference between the groups, i.e., the vitamin D₃ intake and consequently, the different plasma concentrations of the vitamin D₃ metabolites. Surprisingly, the hVitD group had plasma 25(OH)D₃ concentrations not differing from the control group, plasma 1,25(OH)₂D₃ concentrations lower than the cVitD group at 10, 13 and 21 wk of age, and plasma 24,25(OH)₂D₃ concentrations significantly higher than the cVitD group starting at 13 wk of age. Plasma 25(OH)D₃ concentrations are influenced by a variety
of factors, including vitamin D intake (46), successive hydroxylation into 1,25(OH)₂D₃ and 24,25(OH)₂D₃ (47), and negative feedback from 1,25(OH)₂D₃ on 25-hydroxylation (48). None of the factors regulating plasma 1,25(OH)₂D₃ and 24,25(OH)₂D₃ concentrations differed between groups except for vitamin D₃ intake (49). Thus, during positive Ca balance, high vitamin D₃ intake (as in the hVitD group) enhances 24-hydroxylase activity and results in enhanced production of 24,25(OH)₂D₃ (11,12) and probably competitive inhibition of the synthesis of 1,25(OH)₂D₃ from 25(OH)D₃ as has been suggested in Ca-deficient rats (50). Accordingly, between 10 and 13 wk of age in the hVitD group, plasma 24,25(OH)₂D₃ concentrations started to increase, whereas plasma 1,25(OH)₂D₃ concentrations were lower than in the cVitD group. The MCR of 1,25(OH)₂D₃ at 19 wk of age was significantly higher in the hVitD group than in the cVitD group, reflecting the upregulated 24-oxidation pathway of 1,25(OH)₂D₃ and resulting in down-regulation of plasma 1,25(OH)₂D₃ concentrations. The latter is consistent with earlier reports of in vivo studies in rats (11,51).

To counterbalance the increased MCR, the PR of 1,25(OH)₂D₃ increased in the hVitD group; this was demonstrated at 19 wk of age by using [³H]-1,25(OH)₂D₃ and by the 12.9-fold increase in the renal gene expression levels of 1α-hydroxylase at 21 wk of age compared with the cVitD group. Consequently, the combination of enhanced production of 24,25(OH)₂D₃ and 1,25(OH)₂D₃ resulted in successive hydroxylation of the substrate 25(OH)D₃ and the maintenance of the plasma 25(OH)D₃ concentrations in the hVitD group at the same levels as those in the cVitD group.

The attenuated α in the hVitD group during the in vivo ⁴⁵Ca balance studies can be attributed in part to a decreased biologic effect of the moderately decreased plasma 1,25(OH)₂D₃ concentrations. Biological activity of nuclear hormones, including 1,25(OH)₂D₃, is mediated by regulation of their receptor levels in target tissues. Intestinal VDR concentrations in the hVitD group were maintained at the same levels as in the cVitD group and thus did not account for the observed attenuation of α in the hVitD group. The most important determinant of α has been reported to be the plasma 1,25(OH)₂D₃ concentration in healthy women (52). Regression analysis reported by Morris et al. (52) predicted an 18.7% difference in α to be related to a 60% decrease in plasma 1,25(OH)₂D₃ concentrations. Consequently, at 13 wk of age in the hVitD group, it is questionable whether the 18% decreased plasma 1,25(OH)₂D₃ concentration decreased α to the same magnitude, i.e., 18.7%. Accordingly, at 20 wk of age in the hVitD group, plasma 1,25(OH)₂D₃ concentrations similar to the cVitD group were accompanied by a 15% lower α. Thus, in the hVitD group, the decrease in α is not adequately explained by the moderate decrease in plasma 1,25(OH)₂D₃ concentrations. The remainder of the difference in α between the cVitD and hVitD group may be due to a counterregulating effect of the increased plasma 24,25(OH)₂D₃ concentrations on intestinal Ca absorption. Although disputed by others (53), 24,25(OH)₂D₃ has been suggested to have endocrine effects on the intestine, in addition to the described actions on bone, cartilage and fracture healing (54). The effects of 24,25(OH)₂D₃ on intestinal Ca absorption can be executed by 1) an allosteric effect of 24,25(OH)₂D₃ on 1,25(OH)₂D₃ binding to the VDR (6), 2) a binding domain/receptor for 24,25(OH)₂D₃ independent of the VDR (5), and 3) a suppressive effect of 24,25(OH)₂D₃ on the rapid nongenomic actions of 1,25(OH)₂D₃ on P absorption (7) and transcaltachia (8) in the intestine. Transcaltachia has so far been reported only in chicks and whether it is applicable in mammalian species requires further study. Therefore, it is likely that the hVitD group a putative inhibitory effect of the elevated plasma 24,25(OH)₂D₃ concentrations on intestinal Ca transport can be expected.

In conclusion, 24-hydroxylase activity is the key to explain the changes in vitamin D₃ metabolites and in Ca absorption during chronic moderate vitamin D₃ supplementation. Further studies of 24-hydroxylase activity are necessary before all questions are answered. The results of this study indicate that chronic dietary vitamin D₃ supplementation of growing dogs in positive Ca balance has a downregulating effect on Ca absorption accompanied by moderately decreased plasma 1,25(OH)₂D₃ and increased plasma 24,25(OH)₂D₃ concentrations. The vitamin D₃-supplemented group retained its Ca homeostasis, as indicated by the maintenance of the plasma PTH concentrations within control levels. The attenuated Ca absorption is only partially due to the low plasma 1,25(OH)₂D₃, a consequence of accelerated degradation mediated by 24-hydroxylase. In addition, 24,25(OH)₂D₃ may have a downregulating effect on intestinal Ca absorption in growing dogs mediated either by its own receptor or an allosteric effect on the VDR.

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LITERATURE CITED

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