24-Hydroxylase: potential key regulator in hypervitaminosis D₃ in growing dogs


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IT IS WELL ESTABLISHED THAT the diet and the skin are sources of cholecalciferol (vitamin D₃; see Ref. 27). Vitamin D₃ is converted in the liver to 25-hydroxycholecalciferol [25(OH)D₃] and with a sequential hydroxylation primarily in the kidney to 1,25-dihydroxycholecalciferol [1,25(OH)₂D₃] with aid of 1α-hydroxylase and to 24,25-dihydroxycholecalciferol [24,25(OH)₂D₃] with the aid of 24-hydroxylase. 1,25(OH)₂D₃ is the most biologically active vitamin D₃ metabolite with regard to Ca metabolism and skeletal growth with a variety of target organs, including the skeleton, intestine, and kidney (7). 24,25(OH)₂D₃ is a biologically active metabolite mainly directed to the skeleton (6, 56) but also with putative actions at the intestine (39, 40, 59, 60).

The rate of renal synthesis of 1,25(OH)₂D₃ is directly responsive to plasma levels of P₃, growth hormone (GH), insulin-like growth factor I (IGF-I), parathyroid hormone (PTH), and calcitonin (CT; see Refs. 24, 36, and 41). Regulatory feedback on 1α-hydroxylation is provided by 1,25(OH)₂D₃ by induction of 24-hydroxylase activity and thus conversion of 1,25(OH)₂D₃ into less biologically active metabolites in its target tissues, including intestine, kidney, and bone (7, 52). In the kidney, 24-hydroxylase activity is enhanced by 1,25(OH)₂D₃ and downregulated by PTH (48, 62, 63), whereas in the intestine, 24-hydroxylase is enhanced by 1,25(OH)₂D₃ and downregulated by CT (3).

The period of rapid growth is a formidable challenge for vitamin D₃ metabolism in preserving skeletal mineralization. There are few investigations in young intact animals that have studied the hormonal regulation of excessive vitamin D₃ with respect to the activity of 1α-hydroxylase and 24-hydroxylase (4, 47, 57, 58). However, these studies confined their measurements to single-moment observations, possibly because of technical limitations. Therefore, there is insufficient knowledge concerning the time-dependent changes of vitamin D₃ metabolism during elevated dietary vitamin D₃ intake. Obtaining insight into the complexity of vitamin D₃ homeostasis in relation to its regulating hormones and enzymes requires large research animals for long-term studies on the effect of dietary vitamin D₃ supplementation. Dogs are of adequate size to allow for simultaneous and sequential sampling of blood and tissue material during the rapid growth period. Additional advantages are complete dependence on the dietary intake of vitamin D₃ (29) and thus easy regulation of the vitamin D₃ status without interpretation problems caused by seasonal variation of plasma vitamin D₃ metabolite concentrations.


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To study the time-dependent changes and interactions of hormones implicated in Ca homeostasis during long-term dietary vitamin D₃ supplementation and their effect on in vivo regulation of 1α-hydroxylase and 24-hydroxylase, a control group of dogs was investigated vs. an ample 100-fold vitamin D₃-supplemented group for 18 wk immediately after partial weaning. Calcitropic and growth-regulating hormones were measured throughout the study. The influence of long-term vitamin D₃ supplementation on parathyroid chief cell and thyroid C cell function was evaluated by dynamic tests. Furthermore, the gene expression of renal 1α-hydroxylase and 24-hydroxylase, and intestinal 24-hydroxylase, was determined, and the production and clearance rates of 1,25(OH)₂D₃ were investigated.

MATERIALS AND METHODS

Animals and diets. The Utrecht University Ethical Committee for Animal Care and Use approved all procedures. Sixteen Great Danes, originating from three different litters, were divided into the following two groups at 3 wk of age: a control group (n = 9, CVitD) and a dietary vitamin D₃-supplemented group (n = 7, HVitD). Pups were raised on an extruded diet formulated to be comparable in energy, Ca, and phosphate content (450 kcal, 9.5 g, and 7.5 g/100 g dry matter diet, respectively). The control diet was formulated to contain the recommended amount of 500 IU vitamin D₃/kg diet (1, 38), whereas the supplemented vitamin D₃ diet was formulated to contain a total of 50,000 IU vitamin D₃/kg diet. Diets were analyzed for their vitamin D₃ content (55) by a reference laboratory (TNO Nutrition and Food Research, Zeist, The Netherlands), and the analyzed vitamin D₃ content was 470 and 54,000 IU vitamin D₃/kg diet for the CVitD and HVitD, respectively, with no detectable traces of ergocalciferol. From 3 until 6 wk of age, pups received their diet as a gruel in addition to the bitch milk and received dry diet exclusively later on. Body weight was measured biweekly, and food was provided at two times maintenance energy requirements of each dog (31) for the duration of the study.

Blood measurements. At 7, 10, 13, 16, and 19 wk of age, blood samples were collected after an overnight fast. Blood samples for the measurement of plasma total Ca and P levels were transferred to heparin tubes, centrifuged, and measured according to standard procedures (Beckman Industries). Blood samples for hormone analysis were immediately transferred to EDTA-coated tubes and placed on ice until centrifuged. Plasma was stored at −20°C until analysis. Quantitative determination of 25(OH)D₃ and 24,25(OH)₂D₃ was by a modified RIA (DiaSorin, Stillwater, MN). Before processing, both labeled standards (25-hydroxy[26,27-methyl-³H]cholecalciferol and 24R,25-dihydroxy[26,27-methyl-³H]cholecalciferol (sp act 16 and 15.4 GBq/mg, respectively; Amersham Pharmacia Biotech) were added to plasma samples and to the standards of the RIA to determine individual sample recovery. Samples were extracted two times with ethylacetate-cyclohexane (1:1 vol/vol) and one time with methanol-ethylacetate-cyclohexane (4:5:5 vol/vol/vol; see Ref. 5), and 25(OH)D₃ and 24,25(OH)₂D₃ were separated by solidphase extraction using NH₂ cartridges (Bakerbond spe Amino Disposable Extraction Columns, J. T. Baker) according to the method described by McGraw and Hug (34). The standard curves of both stable vitamin D₃ metabolites showed good parallel dilution to the standard curve of the RIA. The intra- and interassay coefficients of variation (CV) for 25(OH)D₃ were 15.2 and 6.1%, respectively. The intra- and interassay CV for 24,25(OH)₂D₃ were 10.1 and 8.5%, respectively. 1,25(OH)₂D₃ was extracted from plasma using acetonitrile followed by a two-step solid-phase extraction (C₁₈ and silica gel 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. (44) and Hollis (28) with intra- and interassay CV of 5.7 and 6.6%, respectively. PTH was measured using an immunoradiometric assay for intact PTH (Nichols Institute, San Juan Capistrano, CA; see Ref. 54). The detection limit was 1 ng/l. The intra- and interassay CV were at 40 ng/l for 3.4 and 5.6%, respectively, and at 266 ng/l for 1.8 and 6.1%, respectively. GH was measured after extraction with ethanol by a homologous RIA as described before (25) with a detection limit of 25 ng/l. The intra- and interassay CV were at 40 ng/l for 4.5 and 9.2%, respectively. GH was measured by a homologous RIA, as described previously (20). The intra- and interassay CV were 3.8 and 7.2%, respectively. Total IGF-I concentrations were measured by a heterologous RIA, as described previously (37), with intra- and interassay CV of 4.7 and 15.6%, respectively.

Dynamic tests on C cell and chief cell function. Tests were performed after overnight food deprivation with a week between tests to avoid reciprocal influences. Ca infusion tests were performed at 6, 12, and 18 wk of age, whereas EDTA infusion tests were performed at 7, 13, and 19 wk of age in all dogs.

In short, during the Ca stimulation tests, dogs were kept in a sitting position, and calcium gluconate (13.75 mg calcium gluconate/ml; Sandoz Pharma, Basel, Switzerland) was administered for 3–5 s through an indwelling catheter in the cephalic vein. The total dose was 0.28 ml calcium gluconate/kg body wt, equivalent to a dose of 2.52 mg Ca²⁺/kg body wt. Blood samples were taken by jugular venipuncture at −5, 0, 1, 2, 4, 8, and 16 min after the initiation of the Ca infusion.

During the EDTA infusion tests, dogs were kept in right lateral recumbency, and 30 mg Na₂EDTA/kg body wt in 1 ml of 0.9% NaCl were administered at a constant infusion rate of 0.25 ml·kg body wt⁻¹·min⁻¹ through an indwelling catheter in the cephalic vein. Blood samples were obtained through an indwelling catheter in the jugular vein at −5, 0, 1, 2, 4, 8, and 16 min after the initiation of the EDTA infusion. Dogs were monitored closely during the infusion for the occurrence of clinical and electrocardiographic signs of hypocalcemia.

Plasma PTH, CT, and Ca²⁺ levels were determined at all time points. Blood samples for the analysis of Ca²⁺ were collected anaerobically in heparinized syringes (PICO 50; Radiometer, Copenhagen, Denmark), placed in melting ice, and analyzed within 2 h after collection with the aid of an ionized calcium analyzer (ABL 605; Radiometer).

Preinfusion plasma levels of Ca²⁺, PTH, and CT were measured at −5 and 0 min served as the baseline and were defined as CBaselinity, PTHBaselinity, and CTBaselinity, respectively. Postinfusion maximal-response plasma levels were defined in the Ca infusion tests as Cmax, PTHmin, and CTmax, and in the EDTA infusion tests as Cmin, PTHmax, and CTmin, respectively. In each group, the areas under the curve above zero and above baseline were calculated from the stimulated values. The mean response levels above zero (RLₐ) and above baseline (RLₐ Baseline) were derived from the corresponding AUC corrected for the duration of the response (t, min), i.e., AUC/t.

The Ca set point for PTH release (Sₚ for PTH), i.e., the plasma Ca²⁺ level that inhibits PTH secretion to 50% of its maximal stimulated value, was calculated according to the
model described by Brown (9). The parathyroid gland function was analyzed with a logistic model containing the four parameters equation: \( \gamma = (A - D)x/(1 + (x/C)^\alpha) + D \), in which \( y \) is the plasma PTH level, \( A \) is the maximal plasma PTH level during hypocalcemic stimulation (i.e., \( \text{PTH}_{\text{max}} \)), \( D \) is the nonsuppressible plasma PTH level during hypercalcemic inhibition (i.e., \( \text{PTH}_{\text{min}} \)), \( x \) is the plasma Ca\(^{2+} \) level, \( C \) is the \( \text{CaS}_{\text{thr}} \), \( \alpha \) is the slope of the log-logarithically centered mathematical function described below. The equation can be rewritten as \( z = (y - D)/(A - D) = 1/(1 + (x/C)^\alpha) \) and thus \( z^* = (1/z) - 1 = (x/C)^\alpha \). Using the \( \text{PTH}_{\text{max}} \) and \( \text{PTH}_{\text{min}} \) values from the data, \( x \) and thus \( z^* \) can be calculated and by applying linear regression on the log-logarithically transformed variables \( z^* \) and \( x \), the values of \( C \) and \( \alpha \) can be determined. Plasma levels obtained after the completion of the EDTA infusion were excluded from the analysis, since this would cause hysteresis (10). Because of the time delay in Ca\(^{2+} \)-driven PTH secretion, values also obtained at \( t = 1 \) min were not used.

After euthanasia, at 21 wk of age, both left and right thyroid and parathyroid glands were removed in total and fixed in 10% buffered formalin. After fixation, a longitudinal section was made, and the material was embedded routinely in paraffin. Sections were stained with hematoxylin and eosin for routine histological investigation and were examined blindly. Activity of the parathyroid glands was estimated from the cellular and nuclear size, aspect of the cytoplasm, and number of mitotic figures. The activity of the C cells was estimated histologically from the number of C cells, the cellular and nuclear size, and the aspect of cytoplasm.

1α-Hydroxylase and 24-hydroxylase gene expression. Gene expression of 1α-hydroxylase and 24-hydroxylase was determined at the middle and the end of the study in all dogs. At 10 wk of age, five duodenal forceps biopsies were taken under endoscopic guidance with the dog under general anesthesia. In addition, at 11 wk of age, two kidney biopsies were obtained with the aid of 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 animals were killed with an overdose of pentobarbital sodium, and biopsies from the kidney and mucosa of the proximal duodenum were sampled. Kidney and intestinal biopsies were frozen immediately in liquid nitrogen and stored at −70°C until required for RNA isolation.

Frozen intestinal and kidney tissue sampled at the middle of the study was resuspended in QIagen lysis buffer, homogenized using a Polytron, and centrifuged for 3 min at 5,000 g at room temperature. Frozen tissue sampled at 21 wk of age was ground in liquid nitrogen prefrozen cups of a microdisembitter (Micro-Dismembrator U; B. Braun Biotech International, Melsungen, Germany) using two cycles of 45 s at 2,200 rpm. Thirty milligrams of milled tissue (kidney or intestine) were resuspended in QIagen lysis buffer and centrifuged for 3 min at 5,000 g at room temperature. The supernatant was applied to a QIagen minicolumn (Qiagen, Hilden, Germany), and total RNA was isolated according to the manufacturer’s protocol.

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

Real-time PCR based on the high-affinity double-stranded DNA-binding dye SYBR green I (BMA, Rockland, ME) was performed in triplicate in a spectrophotofluorimetric thermal cycler (iCycler; Bio-Rad, Hercules, CA). Data were collected and analyzed with the provided application software. For each real-time PCR reaction, 1.67 μl (of the 60-μl stock) of cDNA were used in a reaction volume of 50 μl containing 1× PCR buffer, 2 mM MgCl₂, 1:100,000 dilution of SYBR green I, 10 nM fluorescein calibration dye (Bio-Rad), 200 μM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Roche, Branchburg, NJ). Cycling conditions were optimized for the reaction of each target gene. Primer pairs (Table 1) were designed using PrimerSelect software (DNASTAR, Madison, WI).

Melt curves (iCycler; Bio-Rad) 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, \( E(\%) = [10^{(1/C(Tm) - 1)}] \times 100 \) (s = 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 target (1α-hydroxylase and 24-hydroxylase) and β-actin as endogenous reference 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 (i.e., CVitD) to generate n-fold relative expression levels.

Table 1. Sense and antisense primer pairs used in real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers 5′-3′</th>
<th>Amplified Fragment, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>Sense: 5′-GATACTGCCGGCGCTCGTCGTC-3′ (exon 2, 1102–1122)</td>
<td>384</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-GGCTGGGAGGTTGAGGTC-3′ (exon 4, 2038–2058)</td>
<td></td>
</tr>
<tr>
<td>1α-Hydroxylase</td>
<td>Sense: 5′-GGGGCGGCTCGGCGCTCGGTC-3′ (exon 2)</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-CTCTGGCTTCCGGCGGCGT-3′ (exon 4)</td>
<td></td>
</tr>
<tr>
<td>24-Hydroxylase</td>
<td>Sense: 5′-GAGCTCAGAAAGAAGAGGCTAGCC-3′ (exon 1, 658–681)</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-GCCCCATACCCCTGTTGGAGATGTC-3′ (exon 2, 842–865)</td>
<td></td>
</tr>
</tbody>
</table>

Primer pair for β-actin distinguishes cDNA from pseudogene DNA and is based on human sequence M10277. Data for 1α-hydroxylase based on the homologous canine sequence within a 337-bp fragment amplified by the primers 5′-CCGCCGCTCGGAGCATC-3′ (sense; exon 1, 103–121) and 5′-GAGGGAGGAGGGGCAGCGATC-3′ (antisense; exon 3, 417–440) based on the human sequence AB005989. Data for 24-hydroxylase based on porcine sequence AF-245504.
Endogenous metabolic clearance rate and production rate of 1,25(OH)\textsubscript{2}D\textsubscript{3}. At 19 wk of age, the metabolic clearance rate (MCR) of 1,25(OH)\textsubscript{2}D\textsubscript{3} was determined in eight CVitD and seven HVitD dogs with the aid of a bolus injection with \textsuperscript{1}H\textsuperscript{9251}25-dihydroxy[23,24(n)-3H]cholecalciferol ([\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3}, sp act 10.5 GBq/mg; Amersham Pharmacia Biotech, UK) by techniques described previously (19, 23). In short, after an intravenous administration of \textsuperscript{1}H\textsuperscript{11011}3.7 KBq [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{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; transferred immediately to EDTA-coated tubes; and placed on melting ice until centrifuged and processed further. The plasma disappearance curve of [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} was obtained by counting plasma samples (0.5 ml) with 4 ml scintillation fluid (Ultima Gold; Packard Bioscience, Groningen, The Netherlands) in a liquid scintillation counter (1212 Rackbeta; LKB Wallac, Turku, Finland) for 30 min/sample. By means of a computerized nonlinear least-squares fitting procedure, a biexponential function $C(t) = Ae^{-at} + Be^{-bt}$ was fitted to the plasma [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} concentrations. The MCR of 1,25(OH)\textsubscript{2}D\textsubscript{3} was calculated by the quotient of the injected dose (D) of [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} and the integral of plasma specific activity of [\textsuperscript{3}H]1,25(OH)\textsubscript{2}D\textsubscript{3} as follows: $MCR = \frac{D}{\int C(t)dt} = 25(\frac{A}{a} + \frac{B}{b})$, where MCR is given in liters per kilogram body weight per day. The production rate (PR) of 1,25(OH)\textsubscript{2}D\textsubscript{3} (expressed in pmol kg\textsuperscript{-1} day\textsuperscript{-1}) was derived from the formula $PR = MCR \times \text{endogenous circulating 1,25(OH)}_2\text{D}_3$, where the endogenous circulating 1,25(OH)\textsubscript{2}D\textsubscript{3} is the plasma 1,25(OH)\textsubscript{2}D\textsubscript{3} level at 19 wk of age.

Statistical analysis. Statistical analyses were performed using SPSS for Windows 10.1 (SPSS). Homogeneity of variance was tested according to Levene. Differences between groups were analyzed by the two-sided Student’s $t$-test. The AUC of the basal plasma PTH and CT levels were calculated for the duration of the study for both groups. Values were considered to be significant at $P < 0.05$. Results are presented as means ± SE. For the analysis of the pre- and postinfusion plasma levels within the group, baseline values were compared with the corresponding maximal response values by a one-sided Student’s $t$-test for paired data. Differences in $RL_{0,RL_{Baseline}}$, and $CS_{for\ PTH}$ between groups were analyzed by the two-sided Student’s $t$-test.

RESULTS

Dogs had good general health, grew well, and consumed the total amount of food that was offered daily. Consequently, energy and food intake per kilogram metabolic body weight (kg\textsuperscript{0.75}) did not differ between groups. HVitD consumed ~35 times more vitamin D\textsubscript{3}/kg body wt compared with CVitD. The mean growth rate per week for the entire study period was 1.5 ± 0.4 and 1.6 ± 0.3 kg body wt/wk of age for CVitD and HVitD, respectively, and was not significantly different between groups.

Blood measurements. Plasma Ca levels did not differ between groups for the duration of the study (Fig. 1). Plasma Pi levels did not differ between groups and ranged from 2.68 ± 0.03 to 2.95 ± 0.03 mmol/l for the duration of the study. Plasma levels of 25(OH)D\textsubscript{3} were 30- to 70-fold increased, and levels of 24,25(OH)\textsubscript{2}D\textsubscript{3} were 10.220.33.6 on July 8, 2017 http://ajpendo.physiology.org/ Downloaded from by

![Fig. 1. Plasma levels of total Ca (A), parathyroid hormone (PTH; B), calcitonin (CT; C), 25-hydroxycholecalciferol [25(OH)D\textsubscript{3}; D], 24,25-dihydroxycholecalciferol [24,25(OH)\textsubscript{2}D\textsubscript{3}; E], and 1,25-dihydroxycholecalciferol [1,25(OH)\textsubscript{2}D\textsubscript{3}; F] in two groups of dogs raised on a diet with different vitamin D\textsubscript{3} content (CVitD: 470 IU vitamin D\textsubscript{3}/kg diet, n = 9 and HVitD: 54,000 IU vitamin D\textsubscript{3}/kg diet, n = 7) from 3 to 21 wk of age. Data are presented as means ± SE.

* $P < 0.05$ and † $P < 0.01$ from CVitD, as analyzed by the 2-sided Student’s $t$-test.

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were 12- to 16-fold increased in HVitD vs. CVitD for the duration of the study (Fig. 1). Plasma 1,25(OH)2D3 levels did not differ between groups at 7 wk of age, whereas for the remainder of the study they were decreased significantly in HVitD vs. CVitD (Fig. 1). Most basal plasma PTH levels and the PTH AUC were significantly lower in HVitD vs. CVitD for the duration of the study (Fig. 1). Basal plasma CT levels were highly variable, being mainly increased in the beginning period of the study in HVitD vs. CVitD (Fig. 1). The CT AUC for the duration of the study was increased significantly in HVitD vs. CVitD. Plasma GH and IGF-I levels did not differ between groups and increased and decreased with age from 19.7 ± 2.4 to 6.6 ± 1.1 µg/l and from 216 ± 24 to 360 ± 27 µg/l, respectively.

**Ca and EDTA infusion tests.** Plasma Ca2+Baseline levels did not differ between groups in the beginning (6 and 7 wk) and middle (12 and 13 wk) of the study (on average 1.52 ± 0.03 mmol/l), whereas they were significantly increased at the end of the study (18 and 19 wk) in HVitD vs. CVitD (i.e., 1.54 ± 0.01 vs. 1.48 ± 0.04 mmol/l, respectively). Plasma PTHBaseline levels were significantly lower in all tests and at all ages in HVitD vs. CVitD (Fig. 1). Plasma CTBaseline levels were increased significantly at 6 and 7 wk of age and were variably higher later on in the study in HVitD vs. CVitD (Fig. 1).

During the Ca infusion tests at all ages and in both groups, plasma levels of Ca2+max, PTHmin, and CTmax were significantly different with respect to the preinfusion levels. The RL0 of PTH was at all ages significantly lower in HVitD vs. CVitD, whereas the RLBaseline of PTH did not differ between groups (Table 2). The RL0 and RLBaseline of CT were significantly higher in HVitD vs. CVitD only at 6 and 12 wk of age (Table 2).

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>Infusion, ng/l</th>
<th>CVitD (n = 9)</th>
<th>HVitD (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RL0</td>
<td>RLBaseline</td>
</tr>
<tr>
<td>6–7</td>
<td>Ca</td>
<td>PTH 48.6 ± 3.8</td>
<td>−13.1 ± 6.4</td>
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<td></td>
<td></td>
<td>CT 277.2 ± 51.3</td>
<td>199 ± 42</td>
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<tr>
<td></td>
<td></td>
<td>EDTA PTH 140.2 ± 11.0</td>
<td>73.9 ± 11.2</td>
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<td></td>
<td></td>
<td>CT 81.2 ± 21.6</td>
<td>−31.2 ± 16.5</td>
</tr>
<tr>
<td>12–13</td>
<td>Ca</td>
<td>PTH 42.7 ± 3.3</td>
<td>−4.6 ± 3.3</td>
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<td></td>
<td></td>
<td>CT 432.4 ± 54.6</td>
<td>283 ± 58</td>
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<tr>
<td></td>
<td></td>
<td>EDTA PTH 138.8 ± 10.6</td>
<td>71.1 ± 8.6</td>
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<td></td>
<td></td>
<td>CT 90.0 ± 17.3</td>
<td>−45.8 ± 15.5</td>
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<tr>
<td>18–19</td>
<td>Ca</td>
<td>PTH 28.8 ± 1.4</td>
<td>−6.0 ± 4.5</td>
</tr>
<tr>
<td></td>
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<td>CT 536.7 ± 125.4</td>
<td>424 ± 97</td>
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<td>EDTA PTH 142.2 ± 15.6</td>
<td>91.1 ± 12.1</td>
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<tr>
<td></td>
<td></td>
<td>CT 63.7 ± 13.6</td>
<td>−25.9 ± 7.1</td>
</tr>
</tbody>
</table>

Data are given as means ± SE; n, no. of dogs. PTH, parathyroid hormone; CT, corticotropin. CVitD, 470 IU vitamin D3/kg diet; HVitD, 54,000 IU vitamin D3/kg diet. The mean response levels above zero (RL0) and above baseline (RLBaseline) were derived from the corresponding areas under the curve (AUC) through division by the duration of the response (t, min), i.e., AUCl/T. Significant differences from CVitD determined by the two-sided Student’s t-test are given with *P < 0.01; †P < 0.001, and ‡P < 0.05.
main regulating factors in vitamin D₃ metabolism for
the total duration of the study.

**Effects of hypervitaminosis D₃ on the main vitamin
D₃ metabolites.** At states of excessive vitamin D₃ as in
HVitD, the abundant substrate is metabolized by the
loosely regulated 25-hydroxylase (53), and plasma
25(OH)D₃ levels increase (47). In HVitD, the 12- to
16-fold increased plasma 24,25(OH)₂D₃ levels were at-
tributed to the increased renal 24-hydroxylase activity
indicated by the striking 10.5- and 6-fold increase of
the renal 24-hydroxylase gene expression levels at both
measure points compared with CVitD. Plasma
25(OH)D₃ and 24,25(OH)₂D₃ levels may rise far above
the binding capacity of the vitamin D-binding protein
(DBP) and displace 1,25(OH)₂D₃ from DBP, resulting
in an increase of the free plasma 1,25(OH)₂D₃ levels
(43). The latter may have resulted in increased biolog-
ical activity of 1,25(OH)₂D₃ without a concomitant in-
crease in the total plasma 1,25(OH)₂D₃ levels. The
lowered total plasma 1,25(OH)₂D₃ levels in HVitD
were mainly a consequence of increased MCR of
1,25(OH)₂D₃ in HVitD vs. CVitD with conversion of
1,25(OH)₂D₃ into less biologically active products (33),
including 1,24,25-trihydroxycholecalciferol (14). In
support, the significantly increased renal and intesti-
nal 24-hydroxylase gene expression indicated upregu-
lation of the 24-oxidation pathway. At the same time,
in HVitD, the PR of 1,25(OH)₂D₃ was increased signif-
icantly, being in accordance with the 9.3- and 8-fold
increases in the renal 1α-hydroxylase gene expression
at both measure points compared with CVitD. Renal
synthesis of 1,25(OH)₂D₃ is regulated tightly by Ca,
1,25(OH)₂D₃, PTH, and CT (7, 35, 36). Increased
plasma Ca²⁺ levels at the end of the study did not seem
to affect the production of 1,25(OH)₂D₃ in HVitD,
whereas the decreased plasma 1,25(OH)₂D₃ levels in-
dicated withdrawal of homologous negative feedback
on the production of 1,25(OH)₂D₃. Plasma PTH levels

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Fig. 2. The Ca set point for PTH release (CaS for PTH in mmol/l) presented as mean ± SE and the sigmoid
relationship between fractional PTH release and plasma Ca²⁺ levels (in mmol/l) during hypervitaminosis D₃ in
growing dogs from partial weaning at 3 wk until 21 wk of age. *P < 0.05 and †P < 0.01 from CVitD, as analyzed
by 2-sided Student's t-test.

Fig. 3. Gene expression levels of renal 1α-hydroxylase (A), 24-hydroxylase (B), and intestinal 24-hydroxylase (C)
during hypervitaminosis D₃ in growing dogs from 3 to 21 wk of age. Data are expressed as mean n-fold relative
expression levels ± SE; *P < 0.05 and †P < 0.01 vs. CVitD at the same age, as analyzed by 2-sided Student's t-test.
There were no differences within groups, as analyzed with the paired Student's t-test.

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Effects of hypervitaminosis $D_3$ on PTH secretion and production. Although basal plasma PTH levels and RL$_0$ of PTH induced by an elevation or decrease of $Ca^{2+}$ were lower in HVitD vs. CVitD, the RL$_{Baseline}$ of PTH induced by elevation of $Ca^{2+}$ did not differ between groups. This indicated that, in HVitD, regardless of the lower basal PTH secretion levels, the chief cells retained their responsiveness to an elevation or decrease of $Ca^{2+}$. Exceptionally, at the end of the study, RL$_{Baseline}$ of PTH induced by a decrease of $Ca^{2+}$ was significantly lower in HVitD vs. CVitD, indicating a decrease in the maximal secretion rate of PTH and a probable initiation of hypoparathyroidism. The decreased PTH secretion rate resulting in decreased basal plasma PTH levels may be attributed either to increased sensitivity of the parathyroid gland to any given plasma $Ca^{2+}$ level, to a decrease in PTH production, to an adjustment of the degradation of the newly synthesized PTH at the chief cell level, or any combination. This consideration is rather tentative, since PTH gene expression levels and the secretory profile of the parathyroid gland are not available (i.e., intact PTH in relation to carbon-terminal PTH fragments). Increased sensitivity of the parathyroid gland in the beginning and middle of the study is indicated by the significantly lower $Ca_{SForPTH}$ in HVitD vs. CVitD. The shift of the $Ca_{SForPTH}$ to the left can be attributed to induction of the chief cell Ca-sensing receptor (CaR; see Ref. 11). Accordingly, $1,25(OH)_2D_3$ administration has been reported to result in a shift of the $Ca_{SForPTH}$ to the left (17, 18, 32) and in increased CaR gene expression of the parathyroid gland in vitamin D-deplete and -replete rats (8, 13). A lower $Ca_{SForPTH}$, the production of PTH is not necessarily decreased, although increased free plasma $1,25(OH)_2D_3$, the abundantly circulating $1,25(OH)_2D_3$ at the beginning and middle of the study is indicated by the significantly lower $Ca_{SForPTH}$ in HVitD vs. CVitD. The shift of the $Ca_{SForPTH}$ to the left can be attributed to induction of the chief cell Ca-sensing receptor (CaR; see Ref. 11). Accordingly, $1,25(OH)_2D_3$ administration has been reported to result in a shift of the $Ca_{SForPTH}$ to the left (17, 18, 32) and in increased CaR gene expression of the parathyroid gland in vitamin D-deplete and -replete rats (8, 13). A lower $Ca_{SForPTH}$, the production of PTH is not necessarily decreased, although increased free plasma $1,25(OH)_2D_3$, the abundantly circulating $25(OH)D_3$, and increased plasma $Ca^{2+}$ levels have been reported to have an inhibiting effect on the production of PTH (50, 51). Histological evaluation of the activity of the parathyroid glands at the end of the study did not show any obvious differences between HVitD and CVitD dogs. A modified demand for intact biologically active PTH may also have been achieved by adjustment of the parathyroid secretion profile toward an increased secretion of carbon-terminal fragments of PTH, as reported in hypercalcemic states or treatment with $1,25(OH)_2D_3$ (15) without necessarily a decrease in production of PTH.

Effects of hypervitaminosis $D_3$ on CT secretion. The principal regulator of CT secretion is the increase in plasma $Ca^{2+}$ levels (2). Only in the beginning and middle of the study were the increased CT secretion and increased responsiveness of C cells to stimuli indicated by the significantly higher RL$_0$ and RL$_{Baseline}$ of CT in HVitD vs. CVitD during stimulation or depression of CT secretion. These findings were independent of the basal plasma $Ca^{2+}$ levels and indicated increased CT production with or without C cell hyperplasia (22, 30, 42). However, C cell hyperresponsiveness seemed to be elapsing with the duration of the study, since the differences in CT secretion and responsiveness between groups were diminished at the end of the
study. The latter was also verified by histological evalu-

ation at the same time point. Responsiveness of the C
cells to changes of the plasma Ca$^{2+}$ levels and the
production of CT may be mediated by the extracellular
CaR of C cells (11, 21). It remains to be elucidated
which positive regulator or mediator may have re-
sulted in increased secretion of CT and responsiveness
of the C cells in HVitD, including endocrine systems
directly or indirectly connected to bone metabolism
(45). The biological significance of an increase in CT
during states of positive Ca balance, as during growth,
is mainly directed at avoiding hypercalcemia by de-
creasing osteoclastic resorption and enhancing deposi-
tion of Ca in bone (46).

Conclusively, during 135-fold vitamin D$_3$ supple-
mentation in growing dogs despite the increase in
1,25(OH)$_2$D$_3$ production, plasma 1,25(OH)$_2$D$_3$ levels
were decreased as a result of an apparently even

greater catabolism of 1,25(OH)$_2$D$_3$ by 24-hydroxylase.
Downregulation of the PTH secretion and hypercalc-
toninemia provided extra protection against hypercal-
celmia by cessation of bone resorption and thus reduc-
tion of the liberation of Ca. There was only a slight increase
in plasma Ca$^{2+}$ levels at the end of the study, suggest-
ing that the total burden of vitamin D$_3$ might have
reached a critical stage. However, there was no clear
shift toward a vitamin D$_3$ toxic state with the typical
clinical signs of impaired growth, hypercalcemia and
hyperphosphatemia, and kidney failure. In spite of the
135-fold vitamin D$_3$ supplementation, HVitD dogs
grew well and retained normophosphatemia and nor-
mal plasma total Ca levels for most of the study.
Efficient hormonal counteraction with a key role for
24-hydroxylation prevented the development of vitamin
D$_3$ toxicosis during the course of the study.

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