Decrease in calcitonin and parathyroid hormone mRNA levels and hormone secretion under long-term hypervitaminosis D₃ in rats

J.M. Fernández-Santos¹*, J.C. Utrilla¹*, E. Conde¹, A. Hevia¹, M. Loda² and I. Martín-Lacave¹

¹Department of Normal and Pathological Citology and Histology, School of Medicine, University of Seville, Spain and ²Department of Adult Oncology, Dana Farber Cancer Institute, and Department of Pathology, Brigham and Women Hospital, Boston, MA, USA

*Co-first authors

**Summary.** In calcium homeostasis, vitamin D₃ is a potent serum calcium-raising agent which in vivo regulates both calcitonin (CT) and parathyroid hormone (PTH) gene expression. Serum calcium is the major secretagogue for CT, a hormone whose biosynthesis is the main biological activity of thyroid C-cells. Taking advantage of this regulatory mechanism, long-term vitamin D₃-induced hypercalcemia has been extensively used as a model to produce hyperactivation, hyperplasia and even proliferative lesions of C-cells, supposedly to reduce the sustained high calcium serum concentrations. We have recently demonstrated that CT serum levels did not rise after long-term hypervitaminosis D₃. Moreover, C-cells did not have a proliferative response, rather a decrease in CT-producing C-cell number was observed. In order to confirm the inhibitory effect of vitamin D₃ on C-cells, Wistar rats were administered vitamin D₃ chronically (25,000 IU/d) with or without calcium chloride (CaCl₂). Under these long-term vitamin D₃-hypercalcemic conditions, calcium, active metabolites of vitamin D₃, CT and PTH serum concentrations were determined by RIA; CT and PTH mRNA levels were analysed by Northern blot and in situ hybridization; and, finally, the ultrastructure of calcitrophic hormone-producing cells was analysed by electron microscopy. Our results show, that, in rats, long term administration of vitamin D₃ results in a decrease in hormone biosynthetic activities of both PTH and CT-producing cells, albeit at different magnitudes. Based upon these results, we conclude that hypervitaminosis D₃-based methods do not stimulate C-cell activity and can not be used to induce proliferative lesions of calcitonin-producing cells.

**Key words:** Vitamin D₃, Calcitonin, PTH, C cells, Parathyroid cells, Hypercalcemia

**Introduction**

Calcitonin (CT), parathyroid hormone (PTH) and 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃), the active metabolite of vitamin D₃, are the main hormones involved in regulating calcium homeostasis. CT is produced mainly in the C-cells, which represent about 5% of all the endocrine cells in the rat thyroid gland (Martín-Lacave et al., 1992), while PTH is synthesized in the chief cells of the parathyroid gland. A high calcium stimulates CT secretion whereas a low calcium stimulates PTH secretion (Naveh-Many et al., 1992).

Expression of the PTH gene is regulated by 1,25-(OH)₂D₃ and estrogens (Silver et al., 1985; Naveh-Many and Silver, 1990; Naveh-Many et al., 1992). PTH mRNA levels are reduced over 90% 24 hours after 1,25-(OH)₂D₃ administration (Silver et al., 1985, 1986; Naveh-Many and Silver, 1990). Moreover, 1,25-(OH)₂D₃ is a powerful inhibitor of CT gene transcription that reduces CT expression to 4% of basal within 24 hours (Silver and Naveh-Many, 1993). In addition, estrogens, as in the case of PTH, induce CT gene expression. It has also been reported that chronic hypercalcemia induced by large doses of vitamin D₃ produces hypertrophic and hyperplastic changes of C-cells and has even been used as a model to induce proliferative lesions derived from them (Thurston and Williams, 1982; Capen and Martin, 1989; Okada et al., 1991). However, in those studies, plasma calcitonin levels were not measured to verify the supposed increasing hormonal synthesis by C-cells.

We have recently demonstrated that during a long-term hypercalcemia induced by vitamin D₃, calcitonin serum levels, and, also, the number of calcitonin immunopositive cells of the thyroid gland, slightly decreased (Martín-Lacave et al., 1998). Those last results are in sharp contrast to previous papers reporting a hypertrophic and hyperplastic effect on C-cells after vitamin-D treatment (Zabel, 1976; Okada et al., 1991).

Nevertheless, the effect of prolonged treatment with vitamin D₃ metabolites on CT and PTH transcription...
and secretion by specialized endocrine cells has not been examined yet. In this study, we present the different behaviour of C-cells and chief cells in the synthesis and release of calcitrophic hormones under a long-term treatment with pharmacological doses of oral vitamin D$_3$. We demonstrate that vitamin D$_3$-induced hypercalcemia does not stimulate biosynthetic CT activity. Moreover, vitamin D$_3$ long-term administration leads to a decrease in CT mRNA and CT serum levels and, also, in PTH mRNA and seric PTH; results which are not compatible with hyperactivation-mediated proliferative changes.

**Materials and methods**

**Experimental model**

Male Wistar rats (n=90), aged 2 months, were divided into groups. Group O (n=10) was the no treatment control. Group I (n=20) was the control utilized at each timepoint. Group II consisted of 20 animals receiving 50,000 IU of vitamin D$_3$ (previously diluted in an alcoholic vehicle) per 100 ml of drinking water for either 1 or 2 months. On average, each subject drank over 50 ml of this solution every day. Group III consisted of 20 animals receiving the same treatment as group II plus 0.1% of CaCl$_2$. Finally, group IV: 20 animals which received only 0.1% CaCl$_2$. In the course of the experiment, the rats were housed and handled according to approved guidelines, maintained under a standard laboratory diet and allowed water ad libitum. Animals were sacrificed under ether anaesthesia. Blood samples were taken by aortic puncture and thyro-parathyroid complexes were extracted and immediately either fixed or frozen in liquid nitrogen.

**Serum analysis**

Total serum calcium was determined using a calcium analyzer (Merck Vitalab Selectra). The level of serum CT was determined using commercially available kits (Nichols Institute Diagnostics, CA, USA). The levels of serum PTH were determined using the Rat PTH (IRMA) Kit (Nichols Institute Diagnostics, CA, USA). Serum concentrations of 25-OHD$_3$ were measured by RIA using a 25-hydroxyvitamin D, $^{125}$I RIA Kit (INeSTAR Corporation-Stillwater, Minnesota, USA). The measurement of 1,25-(OH)$_2$D$_3$ levels was carried out using a RIA for the determination of 1,25-dihydroxyvitamin D (Nichols Institute Diagnostics CA, USA). Results were expressed as pg 1,25-(OH)$_2$D$_3$/ml rat serum.

**Probes**

For CT mRNA, a 45 base antisense oligodeoxy-nucleotide probe, complementary to 3' end of exon 4 (Jacobs et al., 1981) was used. For PTH mRNA, a cocktail of two 45-base oligodeoxy-nucleotide antisense probes, corresponding to 3' and 5' end coding regions, respectively, was used (Heinrich et al., 1984). For GAPDH mRNA, a cocktail of two 45-base oligodeoxy-nucleotide antisense probes corresponding to NH$_2$ and COOH ends of mature protein, respectively, was used. Sequences were chosen using the ggc Wisconsin Package and showed no homology with any other known sequence of Genebank Database. Antisense probes and complementary sense chains, used as negative controls, were 3'-OH end-tailed with Digoxigenin-11-dUTP/dATP, according to the manufacturer's instruction (Oligonucleotide Tailing Kit, Roche).

**Northern blot analysis**

Total thyro-parathyroid RNA was isolated as previously described (Chomczynski and Sacchi, 1987). Twenty µg of total RNA was size fractionated by electrophoresis through 1% agarose/2% formaldehyde gel and transferred to nylon membrane (Bionylon Z*, Bioprobe). For hybridization, 30 ng/ml of specific probe was diluted in 5xSSC, 0.1% N-Laurylsarcosyne, 0.02% SDS, 1% Blocking Reagent (Roche) and incubated overnight at 60 °C. After stringency washes, digoxigenin-labelled nucleotides were detected with alkaline phosphatase-linked anti-digoxigenin antibodies which were finally revealed by quimioluminescence. CT and PTH hybrid signals were normalized by subsequent hybridization of membranes with the GAPDH mRNA specific probe. Analysis of autoradiographs was performed using an image analysis program (NIH Image 6.01). mRNA levels (the ratio of integrated intensities CT/GAPDH and PTH/GAPDH) were expressed in arbitrary units. CT and PTH mRNA levels in treated groups were expressed as % of control values.

**In situ hybridization**

After the isolation, the thyro-parathyroid complex was fixed in 4% paraformaldehyde and paraffin-embedded. Paraffin-embedded 5 µm sections were deparaffinized, rehydrated and permeabilised with Proteinase K (Roche). For hybridization, 2 ng/µl of specific antisense, or control sense oligonucleotide probe, was diluted in 50% deionized formamide, 10% dextran sulphate, 2xSSC, 0.1xTris-EDTA, 1 µg/µl sonicated sperm salmon DNA, 5xDenhardt's solution. Sections were incubated overnight with 40 µl of this solution, under coverslip, at 42 °C in humidity chamber. After extensive stringency washes, digoxigenin was detected using an anti-digoxigenin alkaline phosphatase-conjugated antibody which was subsequently detected by a standard histochemical color reaction. Finally, sections were methyl green-counterstained and mounted with glycerol-gelatin.

**Electron microscopy**

Small fragments of thyroid-parathyroid complexes
Calcitrophic hormones synthesis under hypervitaminosis D₃

Table 1. Body weight (g) and total serum calcium (mg/dl) in control and experimental animals.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>BODY WEIGHT (g)</th>
<th>SERUM CALCIUM (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Months</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>339±33</td>
</tr>
<tr>
<td>Vit D₃</td>
<td></td>
<td>371±41</td>
</tr>
<tr>
<td>Vit D₃ + CaCl₂</td>
<td></td>
<td>347±45</td>
</tr>
<tr>
<td>CaCl₂</td>
<td></td>
<td>369±57</td>
</tr>
</tbody>
</table>

Mean±SD. *: P<0.05; **: P<0.01; ***: P<0.001. Statistical differences versus control rats of same age.

were fixed in 2.5% glutaradehyde and 2% para-formaldehyde in 0.1M phosphate buffer, pH 7.4, for 4 h, postfixed in OsO₄ in the same buffer, dehydrated through graded alcohols, and embedded via propylene oxide in Spur. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

Statistical analysis

Data were subjected to ANOVA. The mean values in each experiment were compared with the control group using Student’s test. Results were given as mean ±SEM. P values of less than 0.05 were accepted as significant.

Results

Effects on serum calcium and rat body weight

The administration of vitamin D₃ produced a marked increase in serum calcium concentration as compared with controls. Calcium concentration one month after treatment with vitamin D₃ raised from 10.54 mg/ml (normocalcemia) to 14.81 mg/ml (Table 1), a state that can be characterized as severe sustained hypercalcemia. There was no significant change in calcium levels after two months of treatment. There was a significant decrease in the weight of the treated rats in comparison with the control rats. This decrease was more pronounced in rats without calcium chloride supplement in the drinking water. These rats also showed generalized weakness and increasing signs of cachexia following the treatment. In contrast, animals treated with calcium chloride alone showed stable serum calcium levels and body weights. Therefore, further studies were limited to animals treated with vitamin D₃.

Effects on serum concentration of vitamin D₃ metabolites

The hypervitaminosis D₃ state was mainly due to the second main active metabolite of vitamin D₃, the 25-OHD₃. The administration of vitamin D₃, combined or not with CaCl₂, produced an approximately 10-fold increase of 25-OHD₃ serum levels in all treated rats, as compared with control rats, whose basal 25-OHD₃ serum levels did not vary at the different time-points of the experiment (Fig. 1B). In contrast with 25-OHD₃ data, the administration of vitamin D₃ did not raise the serum levels of the most active metabolite of vitamin D₃.
Calcitrophic hormones synthesis under hypervitaminosis D3

1,25-(OH)2D3 (Fig. 1C). As a result of the treatment, decreased serum 1,25-(OH)2D3 values were found in all experimental animals. This effect was more evident after the first month of treatment. In relation to this, a significant decrease in basal levels of 1,25-(OH)2D3 was seen in 2-, 3- and 4-month-old control rats, which diminished more than 60% during this period of their lives (Fig. 1A).

Table 2. Changes of serum calcitonin and PTH levels under long-term hypervitaminosis D3.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>SERUM CALCITONIN (pg/ml)</th>
<th>SERUM PTH (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>months</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Vit D3</td>
<td>21.94±2.95</td>
<td>49.30±10.05</td>
</tr>
<tr>
<td>Vit D3 + CaCl2</td>
<td>25.16±9.19</td>
<td>26.79±6.09***</td>
</tr>
</tbody>
</table>

Means±SD. *: P<0.05; ***: P<0.001. Statistical differences versus control rats of same age.

A

Time of treatment

1 month | 2 months

Calcitonin mRNA 1.0 kb

GAPDH mRNA 1.27 kb

B

Time of treatment

1 month | 2 months

PTH mRNA 0.8 kb

GAPDH mRNA 1.27 kb

Fig. 2. Detection of CT and PTH mRNAs by Northern blot analysis after 1 and 2 months of treatment with vitamin D3 and vitamin D3 with calcium chloride. A. Gel blot of total RNA from thyroid-parathyroid tissue hybridized sequentially for CT mRNA and GAPDH mRNA. Twenty μg were applied on each lane. Lanes: 1, control group; 2, vitamin D3-treated group; 3, vitamin D3+CaCl2-treated group. Figure represents the results of one representative of five equivalent experiments. Results are shown as the mean±SEM for 5 different experiments. B. Gel blot of total RNA from thyroid-parathyroid tissue hybridized sequentially for PTH mRNA and GAPDH mRNA. Twenty μg were applied on each lane. Lanes: 1, control group; 2, vitamin D3-treated group; 3, vitamin D3+CaCl2-treated group. Figure represents the results of one representative of five equivalent experiments. Relative CT mRNA levels of control and experimental rats. Results are shown as the mean±SEM for 5 different experiments.
Effects on CT expression and CT serum concentration

A moderate decrease in the steady-state CT mRNA levels was found by Northern blot analysis after one month of vitamin D₃ treatment (Fig. 2). This decrease was fairly small in the group treated with vitamin D₃ and calcium chloride. CT gene expression decreased more significantly in both groups after two months of

![Image of in situ hybridization on rat thyro-parathyroid gland](image-url)

**Fig. 3.** Detection of CT (A-C) and PTH mRNAs (D-F) by in situ hybridization on rat thyro-parathyroid gland after 2 months of treatment. A. Control rat: There is a clear localization of CT mRNA in all C cells. B. Vitamin D₃-treated rats: There is a reduction in the amount of CT mRNA which makes the identification of the C cells difficult in comparison with control glands. C. Vitamin D₃+CaCl₂-treated rats: the staining of C cells with the CT probe is more reduced than in control glands but slightly higher in comparison with vitamin D₃-treated rats. x 400. Bar: 25 μm. D. Control rat: There is a clear localization of PTH mRNA in all chief cells. E. Vitamin D₃-treated rats: There is a complete reduction in the amount of PTH mRNA which makes the parathyroid gland negatively stained. F. Vitamin D₃+CaCl₂-treated rats. P: parathyroid gland; T: thyroid gland. x 200. Bar :50 μm.
treatment. Thyroid sections stained by in situ hybridization corroborate the decrease in the amount of CT mRNA in C cells after vitamin-D treatment, however the proportion of CT mRNA positive cells seemed not to vary in comparison to controls (Fig. 3). Similarly, the decrease in serum CT concentration was more pronounced in the group treated with vitamin D$_3$, and, also, more evident after the second month of treatment (Table 2).

Effects on PTH expression and PTH serum concentration

The administration of vitamin D$_3$ strongly inhibited the expression of the PTH gene. As can be seen in Figs. 2, 3, the amount of PTH mRNA became undetectable by Northern blot analysis of 10 µg of total thryoparathyroid RNA and by in situ hybridization. No differences were observed with the addition of calcium chloride. As a result, PTH levels in serum were markedly decreased (Table 2), becoming undetectable after one month of treatment.

Effects on the ultrastructure of C cells and chief cells

In comparison with control rats, secretory granules of C-cells in all treated animals were markedly reduced in number but they did not disappear completely. The Golgi apparatus was moderately developed and the endoplasmic reticulum was mostly vesicular in shape.
and somewhat decreased in volume. Therefore, the cytoplasm of the degranulated cells was much clearer than that of the normally granulated cells (Fig. 4A,B). In the parathyroid glands of treated rats, the majority of chief cells were characterized by the presence of numerous empty vesicles of different sizes in the cytoplasm (Fig. 4C,D). Furthermore, the experimental rats presented chief cells with less complex infoldings of the cellular membrane than in control animals. Different organelles such as rough endoplasmic reticulum and free ribosomes, appeared to be similar to those of non-treated rats.

**Discussion**

The results of our investigations, together with some literature data, support our first hypothesis that C-cells, in a hypercalcemic state due to an intoxication with vitamin D₃, do not respond with hypertrophic and hyperplastic changes, but rather in these conditions, the main biosynthetic activity of C-cells is inhibited (Martín-Lacave et al., 1998). The hypervitaminosis D₃ state obtained in our experimental model was mainly due to the rising of 25-OHD₃ serum levels. 25-OHD₃ is the first active and most abundant metabolite in the pathway of vitamin D₃ activation (Parfitt, 1999). Conversion of vitamin D₃ in 25-OHD₃ is not subjected to strict regulation and basically depends on vitamin D₃ availability (Reichel et al., 1989). In our experiment, non-treated experimental rats, aged 2, 3 and 4 months, showed similar serum 25-OHD₃ values, whereas the oral administration of vitamin D₃ led to a ten-fold increase in serum levels of 25-OHD₃. In contrast, synthesis and degradation of 1,25-(OH)₂D₃ (the most active vitamin D₃ metabolite) are strictly down regulated by serum calcium, and up regulated by calcitonin and PTH (Kawashima et al., 1981; Shigematsu et al., 1986; Sinki et al., 1992). Based on this, as a result of the hypercalcemia and decreased levels of serum calcitonin and PTH, lower levels of 1,25-(OH)₂D₃ were observed in both treatment groups. In our study we have also observed a decrease in 1,25-(OH)₂D₃ serum levels with age in control animals. A similar decrease has also been described in humans with aging (Fujisawa et al., 1984; Quesada et al., 1994). This observation, in our experimental model, might be related to developmental calcium requirements rather than changes caused by the prolongation of the treatment.

In chronic hypervitaminosis D₃, such as in the experimental model we have presented, parathyroid cells must respond to both Ca²⁺ and vitamin D₃, which act synergistically on PTH expression and secretion. Steady-state levels of PTH mRNA dramatically decreased (as assessed by either Northern blotting or in situ hybridization). This, in turn, results in undetectable levels of serum PTH. These patterns of PTH expression and secretion are similar to those elicited by short-term treatments with 1,25-(OH)₂D₃ (Karmali et al., 1989; Naveh-Many et al., 1989, 1992; Naveh-Many and Silver 1990; Moallem et al., 1998). Ultrastructural appearance of the parathyroid gland in long-term vitamin-treated rats also coincides with that seen in chief cells under short-term hypercalcemia or hypervitaminosis D (Setoguti et al., 1981, 1995; Wild et al., 1985). The apparent degradation of storage granules by hydrolysis, and the decrease of the cell surface area, seems to be closely associated with an intracellular regulatory mechanism for PTH secretion.

Hypervitaminosis D₃ affects CT-producing cells in a distinctly different manner. C-cell population may respond to the increase in extracellular calcium by releasing CT stored in granules through exocytosis. However, vitamin D₃ reduces the transcription of the CT gene. CT secretion gives the cells an almost completely degranulated appearance under electron microscopy. Nonetheless, they still conserve morphological signs of the persistence of some hormonal synthesis. These ultrastructural findings concur with those obtained by in situ hybridization and Northern blot analysis. Based upon these, C-cells, still have a moderate rate of CT synthesis which is not observable in short-term treatments, where CT mRNA decreased 4% of baseline level at 48 h (Naveh-Many and Silver, 1988; Silver and Naveh-Many, 1993). Importantly, in these studies, 1,25-(OH)₂D₃ did not raise serum calcium. These results indicate that in the case of C-cells, where hypercalcemia and hypervitaminosis D₃ are antagonic stimuli, a second hormone synthesis regulatory mechanism, besides the vitamin D₃-mediated transcriptional inhibition or mRNA stability, would be present.

In the group treated with vitamin D₃ and CaCl₂, CT mRNA levels were higher than those elicited by vitamin D₃ alone. In this case, the effect of calcium ingestion on the release of different mediators at the gastrointestinal level, such as gastrin, cholecystokinin and glucagon, might be a decisive factor. These hormones are well-known CT secretagogues and are also involved in regulating calcitonin gene transcription (Naveh-Many and Silver, 1988; Raue et al., 1991, 1993). The administration of CaCl₂ with vitamin D₃ in the drinking water may thus act as a "buffer" against the 25-OHD₃-induced blockade of CT synthesis.

In numerous studies in which vitamin D₃ has been used as a serum-calcium-raising agent, to investigate the reaction of C cells to long-term hypercalcemia, C-cell hypertrophy and hyperplasia has been described (Zabel, 1976; Thurston and Williams, 1982; Kameda et al., 1985; Capen and Martin, 1989; Okada et al., 1991). We have recently described (Martín-Lacave et al., 1998) the effect of long-term administration of vitamin D₃ at hypercalcemic doses on the thyroid gland of the rat. In contrast to previous reports, we have found that C cells did not respond with hypertrophic and hyperplastic changes. In addition, the number of C cells that were immunopositive for CT decreased. This observation concurs with the findings of our current study in which vitamin D₃ does not stimulate the C-cell population, but rather inhibits CT synthesis. Based upon these results it is difficult to accept that hypervitaminosis D₃, which inhibits the main biosynthetic activity of C-cells, could
induce proliferative changes in this endocrine population. We conclude that the oral administration of vitamin D₃ at serum-calcium-raising doses for prolonged periods reduces the rate of biosynthesis and secretion of both PTH and CT. Therefore, supporting our previous data (Martín-Lacave et al., 1998), hypervitaminosis D₃-based methods are not reliable models to induce proliferative lesions of calcitonin-producing cells, as has been extensively described in the literature.

Acknowledgments. This work was supported by the National Fund for Medical Research of the Social Security (F.I.S.S.S.), Spain (0571).

References


