Increased level of calcitonin mRNA after 1,25-dihydroxyvitamin D₃ injection in the rat


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Vitamin D metabolites are able to change plasma calcitonin (CT) levels, but nothing is known about a possible effect at the CT gene level. Here we have investigated the acute effects of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) on the CT biosynthetic activity of thyroid glands from adult rats. Plasma CT levels were significantly increased (× 2) 1 and 2 h after 1,25-(OH)₂D₃ injection in the face of unchanged plasma calcium values. The thyroidal CT content also was unchanged. A 2-fold increase in CT mRNA level measured by dot-blot hybridization occurred 1 and 2 h after 1,25-(OH)₂D₃ administration. Expression of CT gene products was examined in the rabbit reticulocyte lysate cell-free translation assay. After polyacrylamide gel electrophoresis, specific immunoprecipitates were autoradiographed and quantified by integration. A single precursor of $M_r \approx 15000$ could be specifically immunoprecipitated with CT antisera. A 3-4-fold rise in translatable CT mRNA activity was observed 1 and 2 h after 1,25-(OH)₂D₃ injection. Thus, parallel changes in CT mRNA level, CT mRNA activity and plasma CT levels were observed in adult female rats after administration of 1,25-(OH)₂D₃. These findings demonstrate for the first time that 1,25-(OH)₂D₃ enhanced CT gene expression in the face of unchanged plasma calcium levels.

mRNA level mRNA activity Calcitonin 1,25-(OH)₂D₃ Adult rat

1. INTRODUCTION

The biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), is considered to act on target cells by mechanisms similar to those of the classical steroid hormones [1,2]. Recently, it has been shown that 1,25-(OH)₂D₃ increases the relative amount of prolactin mRNA in GH₄C₁ cells and that the full expression of this effect is dependent on extracellular calcium [3]. Vitamin D₃ metabolites are known to increase the calcitonin (CT) secretion rate in pigs [4], and to decrease the thyroidal CT content before and after weaning in rats [5]. The structure of the biosynthetic precursor of CT produced in C cells has been determined [6–12], but as yet, there is no report on a possible effect of 1,25-(OH)₂D₃ at the CT gene level. This prompted us to investigate the effects of 1,25-(OH)₂D₃ on the CT mRNA level measured by dot-blot hybridization with 3²P-labeled CT cDNA, and on the CT mRNA activity evaluated by translation assay [13].

2. MATERIALS AND METHODS

2.1. Animals

Female Wistar rats weighing 211 ± 2 g were purchased from CERJ (Le Genest, France). They were fed a commercial diet (UAR 103, Usine d'Alimentation Rationnelle, Villemoisson/Orge, France) containing 0.92% calcium, 0.92% phosphorus, 0.15% magnesium and 4000 IU vitamin D/kg. After an overnight fast of 16 h, the females were subcutaneously injected with 1 μg 1,25-(OH)₂D₃/kg.
body wt (Hoffmann-La Roche, Basel). The rats were killed by aortic puncture under light ether anesthesia 1, 2, 4, 8 and 16 h after 1,25-(OH)$_2$D$_3$ injection. Thyroid glands were removed and stored in liquid nitrogen until extracted.

2.2. mRNA extraction and translation studies

RNA were extracted from batches of 10 hemithyroid glands with phenol-chloroform, purified with LiCl precipitation [14], and poly(A)-rich RNA separated by oligo(dT) cellulose [15] according to published procedures [10, 16].

1 µg mRNA (in 1 µl) was added to 10 µl rabbit reticulocyte lysate obtained from Amersham (England) containing 50 µCi $^{35}$S methionine and incubated for 60 min at 37°C. Radioactivity incorporated into synthesized proteins was estimated by precipitation with trichloroacetic acid [17]: 1-µl aliquots were spotted on 3 MM Whatman paper wetted with 20% trichloroacetic acid, boiled for 10 min in 1 N trichloroacetic acid, dried with diethyl ether, and counted. For immunoprecipitation, 5- or 6-µl aliquots were incubated for 24 h at 4°C in 300 µl of 0.1 M phosphate buffer (pH 7.4) containing 0.5% Triton X-100, 0.2% human albumin, 0.1% methionine in the presence of 0.7 µl sheep antiseraum (M 732) against human synthetic CT saturated with 10 µg human CT or without. Immunoglobulins were then precipitated by the addition of 25 µl anti-sheep (IgG) for 24 h at 4°C. Precipitates were collected and heat-denatured in 25 µl of 60 mM Tris (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol and 0.4% bromophenol blue. Aliquots were counted in scintillation mixture (Biofluor, NEN), specificity of the immunoprecipitates was checked by 0.1% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [18] and autoradiography performed using Kodak X-Omat AR5 film (8-day exposure at -80°C). Autoradiographs were quantified in an automatic densitometric scanner.

2.3. mRNA-cDNA hybridization assays

PstI-cleaved insert of pBR 3271 containing the entire coding sequence of human preprocalcitonin [12] was labeled to an activity of $10^8$ cpm/µg by nick translation using polymerase I (Kornberg) [19].

Aliquots of poly(A)-rich RNA (1 µg) were denatured by glyoxal [20] and were spotted on ‘Gene Screen’ membranes (NEN) previously washed and equilibrated with 20 × standard saline citrate (NaCl/Cit, 1 × NaCl/Cit = 0.1 M NaCl, 0.015 M sodium citrate). Membranes were dried under a lamp and baked for 2 h at 80°C. They were prehybridized in 50% formamide, 5 × NaCl/Cit, 5 × Denhart (1 × Denhart = 0.2 mg/ml bovine serum albumin, Ficoll and polyvinyl pyrrolidone), 50 mM sodium phosphate buffer pH 6.5, 100 µg/ml of denatured herring sperm DNA and 1 µg/ml of poly(A) (PL Biochemicals). Membranes were incubated 4 h at 42°C and hybridized in a modified prehybridization buffer (i.e., 1 × Denhart, 20 mM sodium phosphate buffer and 10% dextran sulfate) containing 0.3 µCi of hCT cDNA denatured by heating (100°C for 10 min). Hybridization was carried out for 18 h at 42°C. Membranes were washed by 3 changes of 2 × NaCl/Cit, 0.1% SDS for 5 min at room temperature and then 2 changes of 0.1 × NaCl/Cit, 0.1% SDS for 30 min at 55°C. Dried membranes were exposed at -80°C to Kodak Royal X-Omat AR5 film for 2 days.

2.4. Other methods

Blood samples were collected in polyethylene tubes cooled in ice water. After centrifugation at 4°C, aliquots of plasma were used immediately for calcium and phosphorus determinations, the remainder being frozen (-35°C) until CT was assayed. The thyroid glands were dissected under a microscope at 4°C and sonicated (MSE 150 W Ultrasonic Desintegrator Mk2, Crawley, England) at 4°C for 30 s in barbital buffer (0.025 M, pH 8.6) containing peptidase inhibitors [5]. The CT in plasma and sonicates was measured by a radioimmunoassay (RIA) which has been described [21]. The plasma calcium concentration was measured by flame photometry (Eppendorf, FCM 6341), and the plasma phosphate level was evaluated as in PII.

Results were expressed as means ± SE. Significance of difference between groups was determined using Student’s t-test. For plasma CT, some values were below the limits of detectability so that
a mean value could not be calculated. In this case significance of difference between groups was
determined using the non-parametric U-test of
Mann and Whitney.

3. RESULTS AND DISCUSSION

After s.c. injection of 1,25-(OH)₂D₃, plasma
calcium was unchanged until 16 h, where a slight
increase was observed (10.86 ± 0.16 vs 10.42 ± 0.09 mg/dl, P < 0.05). A trend towards lower
values occurred 2 h after injection (fig.1). Plasma
phosphate concentrations were significantly in-
creased at all times (except at 2 h) following ad-
ministration of 1,25-(OH)₂D₃ (fig.1). A peak in
plasma CT levels was apparent 1 and 2 h after in-

Fig.1. Plasma calcitonin (CT), calcium and phosphate
cconcentrations after s.c. injection of 1 µg 1,25-(OH)₂D₃/ 
kg in adult female rats. Means ± SE, and the number of
animals. Individual values were given for plasma CT.

* P < 0.05, *** P < 0.001 from time 0.

Fig.2. Dot hybridization assay. Aliquots of rat poly(A)­
rich RNA (1.25–10 µg) were spotted on Gene Screen and
hybridized with 0.3 µCi of a ³²P-labeled cDNA compe-
lementary to human CT mRNA. Autoradiograms were
scanned on a gel densitometer set at maximum gain and
results expressed as arbitrary units. The numbered
arrows denote the position of the plot of each similarly
numbered hybrid image of the autoradiography shown
in the insert. Regression curve and correlation coeffi-
cients were calculated by standard statistical techniques.

Fig.3. Autoradiographies and densitometric scans of
dot-blot hybridization of mRNA with ³²P-labeled CT
cDNA. Rat mRNA (1 µg) was immobilized on Gene
Screen (NEN) and hybridized w ³²P-labeled cDNA
excised by the restriction enzyme PstI, from pBR 3271.
jection, these plasma values returned to basal levels by 4 h, and decreased subsequently (becoming undetectable) by 8 h. A further increase was noted at 16 h (fig.1). The thyroidal CT stores remained unchanged throughout the course of the experiment (not shown). Similar patterns for CT secretion and plasma calcium have been reported following 1,25-(OH)2D3 injection [22]. To ascertain the quantities of CT related mRNAs produced in our experiments, we performed RNA/DNA hybridization assays with cloned radiolabeled human CT cDNA [131]. It was found previously that our cDNA probe to human CT mRNA crossreacts with rat CT mRNA [lo]. The amount of 32P-labeled specific cDNA probe hybridized to rat poly(A)-rich RNA increased linearly with increasing amounts of mRNA (1.25–10 μg mRNA) (fig.2). As shown in fig.3, a 2-fold increase in the CT mRNA level could be detected by the dot-blot assay 1 and 2 h after 1,25-(OH)2D3 injection. Control values were reached by 4 h. A small and slow decline was observed afterwards (fig.3). When 1 μg poly(A)-rich RNA was translated in the rabbit reticulocyte cell-free translation assay, SDS-polyacrylamide gel electrophoresis and autoradiography of the specific immunoprecipitates showed the presence of a major band (Mt ≈15000) (fig.4A). This band was absent using specific antiserum saturated with unlabeled CT (fig.4B). Translatable CT mRNA activity was clearly increased 1 and 2 h after injection (× 3–4) (figs 4,5), and then decreased to control at 4 h. A slight increase occurred 8 h after 1,25-(OH)2D3 administration followed by a decline to pre-injectional levels at 16 h (figs 4,5). Radioactivity incorporated in specific immunoprecipitates (table 1) showed a 3–4-fold increase 1 and 2 h after 1,25-(OH)2D3 administration, and declined slowly thereafter.

The present results show that the increased plasma CT level induced by 1,25-(OH)2D3 is closely related to the rise in CT mRNA. Of particular interest was the finding that the response occurred in the face of unchanged plasma calcium level since it has been shown that an acute calcium stimulation elicits a rapid increase in CT mRNA [10].

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**Table 1**

<table>
<thead>
<tr>
<th>Time after treatment with 1,25-(OH)2D3 (h)</th>
<th>cpm in specific immunoprecipitates</th>
</tr>
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<tr>
<td>0</td>
<td>431 ± 41</td>
</tr>
<tr>
<td>1</td>
<td>1669 ± 81</td>
</tr>
<tr>
<td>2</td>
<td>828 ± 57</td>
</tr>
<tr>
<td>4</td>
<td>547 ± 46</td>
</tr>
<tr>
<td>8</td>
<td>573 ± 47</td>
</tr>
<tr>
<td>16</td>
<td>401 ± 40</td>
</tr>
</tbody>
</table>

Radioactivities were estimated as the difference between two measurements (in the absence of human CT minus in the presence of an excess of human CT: 10 μg). Data represent mean ± 2 SD of radioactivity incorporated in preprocalcitonin.
good parallelism was found between the CT mRNA level measured by dot-blot hybridization and the CT mRNA activity evaluated in the translation assay.

As the present effects of 1,25-(OH)₂D₃ have been demonstrated at supra-physiological hormone concentrations, it would be premature to attempt to relate these findings to any possible physiological action of vitamin D on CT synthesis and secretion. Further studies using vitamin D-deficient rats injected with physiological doses of 1,25-(OH)₂D₃ will be required to determine the physiological relevance of these observations. Nevertheless, our data demonstrate for the first time a 1,25-(OH)₂D₃ mediated enhancement of CT gene expression independently of plasma calcium.

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REFERENCES