Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 9770–9773, December 1989
Biochemistry

1,25-Dihydroxyvitamin D_3 up-regulates the 1,25-dihydroxyvitamin D_3 receptor *in vivo*

(steroid receptor/steroid hormone/intestine/calcium)

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ABSTRACT **The level of mRNA encoding the 1,25-dihydroxyvitamin D_3 receptor in the intestine of vitamin D-deficient rats given 1,25-dihydroxyvitamin D_3 was determined by Northern blot analysis using a ^{32}P-labeled cDNA probe to the 1,25-dihydroxyvitamin D_3 receptor. mRNA levels increased 10-fold above deficiency levels at 6 and 12 hr after an intravenous dose of 1,25-dihydroxyvitamin D_3, returning to predosing levels at 24 hr. Total receptor protein level determined by an immunoradiometric assay was increased 2-fold at 12 hr. No change in unoccupied receptor levels determined by ligand-binding assay was observed during this period. These results suggest that 1,25-dihydroxyvitamin D_3 increases receptor mRNA and total receptor level to maintain constant levels of unoccupied receptor.**

1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] regulates calcium homeostasis through interaction with the 1,25-$(OH)_2D_3$ receptor (1). As proposed for steroid hormones (2), formation of the 1,25-$(OH)_2D_3$–receptor complex results in the interaction of receptor with cis-acting elements near specific genes. Thereby specific gene transcription is modulated in target organs leading to the physiological response. The best characterized response to 1,25-$(OH)_2D_3$ is the receptor-mediated regulation of gene transcription in the intestine, where 1,25-$(OH)_2D_3$ causes an increase in calcium uptake from the lumen, elevating serum calcium levels.

Previous work suggests (3–6) that 1,25-$(OH)_2D_3$ regulates its own receptor. 1,25-$(OH)_2D_3$ up-regulates its own receptor in mouse fibroblasts, as measured by radiolabeled hormone binding assay, by immunoprecipitation of ^{35}S-labeled protein from *in vitro* translated mRNA, and by Northern blot analysis (3). Treatment of pig kidney cells with 1,25-$(OH)_2D_3$ also resulted in a 2-fold increase in receptor protein levels, also measured by a radiolabeled hormone binding assay (4). Similarly, HL-60 cells show an up-regulation of receptor by 1,25-$(OH)_2D_3$ as shown by ligand binding and immunoblot analysis (5). Up-regulation of the 1,25-$(OH)_2D_3$ receptor protein has also been studied *in vivo* in rat intestine (6). Receptor levels were not affected at 24 hr after 1,25-$(OH)_2D_3$ treatment but showed a modest increase after 5 days of 1,25-$(OH)_2D_3$ treatment.

In this report we investigated 1,25-$(OH)_2D_3$ regulation of 1,25-$(OH)_2D_3$ receptor mRNA *in vivo* within 24 hr after 1,25-$(OH)_2D_3$ administration. We discovered that the 1,25-$(OH)_2D_3$ receptor mRNA was increased approximately 10-fold at 6 hr after 1,25-$(OH)_2D_3$ injection but declined to vitamin D-deficient levels by 24 hr after injection. Immunoradiometric assay showed that the 1,25-$(OH)_2D_3$ receptor protein level increased in whole cell extracts about 2-fold within 12 hr after 1,25-$(OH)_2D_3$ injection, whereas 1,25-$(OH)_2$[26,27-^{3}H]D_3 binding by these extracts did not change during this time.

MATERIALS AND METHODS

Animals. Sprague–Dawley, Holtzman strain, male rats were fed for 5 weeks on a 0.47% calcium/0.3% phosphorus purified diet supplemented with vitamins A, E, and K to produce vitamin D deficiency (7). Animals were judged vitamin D deficient when serum calcium levels fell below 6 mg/dl. The vitamin D-deficient animals were then given 500 pmol (0.2 μg) of 1,25-$(OH)_2D_3$ in 50 μl of 100% ethanol intrajugularly at various times prior to study.

Vitamins D Compounds. Nonradioactive 1,25-$(OH)_2D_3$ was a gift from the Hoffmann–La Roche. 1,25-$(OH)_2$[26,27-^{3}H]D_3 (160 Ci/mmol; 1 Ci = 37 GBq) was produced by DuPont/NEN and prepared as described (8).

Buffers. The following buffers were used: phosphate-buffered saline (PBS), 1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4, pH 8.0/137 mM NaCl/2.7 mM KCl; TE, 50 mM Tris·HCl, pH 7.4/1.5 mM EDTA; 20× SSC, 3.0 M NaCl/0.9 M sodium citrate, pH 7.0; 20× SSPE, 3.0 M NaCl/0.2 M NaH_2PO_4/20 mM EDTA, pH 7.4; 50× Denhardt's solution, 1% Ficoll/1% polyvinylpyrrolidone/1% bovine serum albumin (fraction V); 5× Mops, 0.2 M morpholinopropanesulfonic acid, pH 7.0/50 mM sodium acetate/5 mM EDTA.

RNA Isolation. Total RNA was isolated as described by Maniatis *et al.* (9). All the buffers were prepared to be RNase-free by treatment with 0.5% diethyl pyrocarbonate (9). The mucosa of the duodenum was scraped and homogenized in 4 M guanidine thiocyanate/5 mM sodium citrate, pH 7.0/0.1 M 2-mercaptoethanol/0.5% sarcosyl using a Polytron (Brinkmann Instruments). The homogenates were centrifuged at 27,000 × *g* for 15 min to remove debris, and cesium chloride was added to the supernatant at 1 g/2.5 ml. The solution was then layered onto a 5.7 M cesium chloride/0.1 M EDTA, pH 8.0 cushion. Total RNA was harvested at 100,000 × *g* in an SW 41 rotor at 18°C for 18 hr. The RNA pellet was rinsed with 1 ml of H_2O, resuspended in 10 mM Tris·HCl, pH 7.4/0.5 mM EDTA/0.1% SDS, and extracted with an equal volume $CHCl_3$/1-butanol, 4:1 (vol/vol). The organic phase was reextracted with 10 mM Tris·HCl, pH 7.4/0.5 mM EDTA/0.1% SDS. The aqueous phases were combined and precipitated with 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.2 vol of 100% ethanol at −20°C for 2 hr and centrifuged at 10,000 × *g* for 10 min, and the total RNA pellet was dissolved in 1 ml of H_2O. Polyadenylylated RNA was isolated by two passes over an oligo(dT)-Sepharose column as described (10).

Northern Blot Analysis. RNA was subjected to electrophoresis on a 1% agarose gel containing 6.6% formaldehyde in Mops buffer. The RNA from the gel was transferred by capillary action onto nitrocellulose with 10× SSC overnight.

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Abbreviation: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.
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The nitrocellulose blot was baked at 80°C for 2 hr to fix the RNA, prehybridized in 50% (vol/vol) formamide/5× Denhardt's solution/5× SSPE/0.1% SDS/denatured salmon sperm DNA (100 μg/ml)/polyuridylic acid (25 μg/ml) at 42°C for 4 hr. A 1.7-kilobase 1,25-$(OH)_2D_3$ receptor cDNA probe (11) was labeled with [^{32}P]dCTP (>3000 Ci/mmol; Amersham) by using a nick-translation kit (BRL), denatured by boiling for 10 min, and added to the filter at 10^6 cpm/ml. Hybridization was carried out for 16 hr at 42°C. The filters were washed for two 10-min periods in 2× SSC/0.1% SDS at room temperature, once at 65°C for 1 hr in 1× SSC/0.1% SDS, and another time in 0.1× SSC/0.1% SDS. The gels were visualized by autoradiography using X-OMAT AR film (Kodak) exposed for 7 days with a Cronex Lightning Plus intensifying screen (DuPont/NEN). The film was developed in an automatic film processor and subjected to densitometry on a model SL-504-XL scanning densitometer (Biomed Instruments, Fullerton, CA).

Rat Intestinal Cell Extracts. High-salt intestinal extracts were prepared as described (12). The protein concentration of each extract was determined with Bio-Rad protein stain and bovine serum albumin as a standard.

Immunoradiometric Assay. The immunoradiometric assay was carried out according to the technique developed by Sandgren and DeLuca (13). Briefly, ^{125}I-labeled monoclonal antibody IVG8C11 (14) was incubated with whole cell extracts at 4°C in minitubes (Beckman). Biotinylated monoclonal antibody (VD2F12) against a different epitope was included in the incubation. After 16 hr, avidin-Sepharose was added and the mixture was incubated for 1 hr on ice with mixing every 20 min. The avidin-Sepharose was harvested by centrifugation at 800 × *g*, the precipitate was washed three times with PBS/0.5% Triton X-100, and radioactivity was measured in a Multi-Prias Auto γ Counter (Packard Instruments). Nonspecific binding, as determined with an incubation mixture without receptor, was subtracted from all samples. The amount of receptor was determined by comparison to a standard curve by using purified receptor.

Hydroxylapatite Binding. 1,25-$(OH)_2D_3$ binding activity was determined by a hydroxylapatite binding assay as described (15).

RESULTS

1,25-$(OH)_2D_3$ Receptor mRNA Is Increased by 1,25-$(OH)_2D_3$. The administration of 1,25-$(OH)_2D_3$ to vitamin D-deficient rats caused a dramatic increase in the level of 1,25-$(OH)_2D_3$ receptor mRNA. As shown in Fig. 1, the

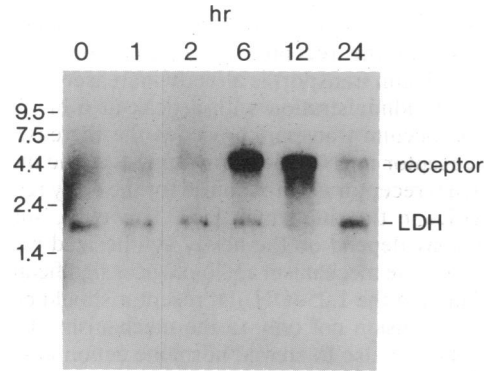

FIG. 1. Northern blot analysis. Each lane contains ≈2 μg of polyadenylylated RNA isolated from rat duodenum at the indicated times (in hr) after a single 500-pmol dose of 1,25-$(OH)_2D_3$. A ^{32}P-labeled cDNA probe for the 1,25-$(OH)_2D_3$ receptor (indicated as receptor) and a ^{32}P-labeled probe for lactate dehydrogenase (indicated as LDH) as a nonregulated control were hybridized to the filter, and the bands were visualized by autoradiography. The molecular size markers are indicated on the left in kilobases.

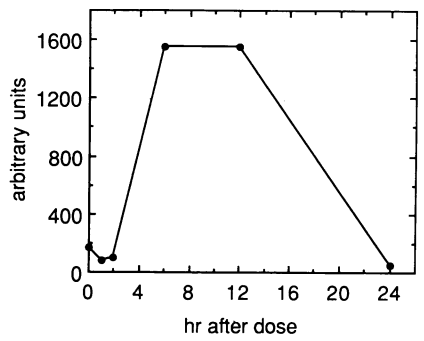

FIG. 2. Quantitation of Northern blot analysis. The autoradiogram in Fig. 1 was subjected to densitometric scanning. The integrated density of the receptor band was normalized to the integrated density of the lactate dehydrogenase band for each lane. The 6- and 12-hr time points show a 10-fold stronger intensity relative to the vitamin D-deficient time point.

1,25-$(OH)_2D_3$ receptor mRNA in rat duodena had increased significantly 6 hr after 1,25-$(OH)_2D_3$ was administered, and this increase continued at least for 12 hr. 1,25-$(OH)_2D_3$ receptor mRNA levels decreased to vitamin D-deficient levels by 24 hr. The filter was also hybridized to a probe specific for lactate dehydrogenase, a mRNA not regulated by 1,25-$(OH)_2D_3$, to control for any variations in the recovery of polyadenylylated RNA between preparations.

Densitometric scanning was used to quantitate the Northern blot (Fig. 2). Each lane was normalized to the level of lactate dehydrogenase. Densitometric scanning showed that there had been a 10-fold increase in receptor mRNA at the 6- and 12-hr time points.

1,25-$(OH)_2D_3$ Receptor Protein Is Increased by 1,25-$(OH)_2D_3$. To determine if total receptor protein levels were also changed, rat intestinal extracts were prepared at various times after 1,25-$(OH)_2D_3$ was administered, and the 1,25-$(OH)_2D_3$ receptor protein levels were analyzed with an immunoradiometric assay. Fig. 3 shows the results from the immunoradiometric assay. Total 1,25-$(OH)_2D_3$ receptor protein level had increased ≈2-fold by 12 hr after 1,25-$(OH)_2D_3$ was administered. Compared to the time course of receptor mRNA increase, total receptor protein had reached a maximum 2–4 hr later. Total receptor protein level had returned to the vitamin D-deficient level by 24 hr after hormone administration.

Unoccupied Receptor Level Is Unchanged by 1,25-$(OH)_2D_3$. There was no significant change in unoccupied receptor at any

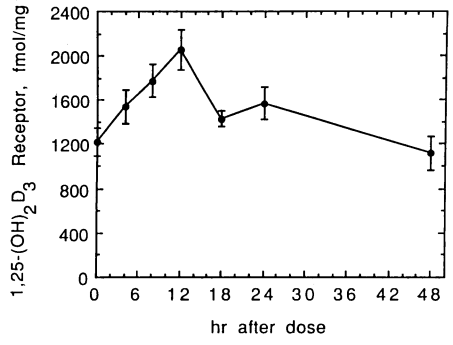

FIG. 3. Immunoradiometric assay. High-salt intestinal extracts for each time point were incubated with ^{125}I-labeled monoclonal antibody (IVG8C11) against the 1,25-$(OH)_2D_3$ receptor for 16 hr at 4°C. A biotinylated monoclonal antibody against a different epitope (VD2F12) was included in the incubation. Avidin-Sepharose was used to precipitate the antibody–receptor complex, and radioactivity was measured in a Multi-Prias Auto γ Counter (Packard). The amount of receptor precipitated was determined by comparing to a standard curve prepared from highly purified 1,25-$(OH)_2D_3$ receptor.

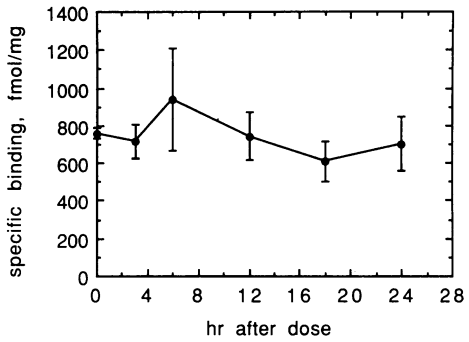

FIG. 4. Hydroxylapatite binding. High-salt intestinal extracts at each time point were incubated with 2 nM 1,25-$(OH)_2$[26,27-^{3}H]D_3 for 6 hr at 0°C to determine unoccupied receptor. Bound 1,25-$(OH)_2$[26,27-^{3}H]D_3 was separated from free hormone by using hydroxylapatite. Nonspecific binding for each sample was determined by including 200 nM nonradioactive 1,25-$(OH)_2D_3$ in the incubation mixture. Total protein was determined by a Bio-Rad assay. Specific binding is expressed as fmol of 1,25-$(OH)_2D_3$ per mg of total protein.

time tested (Fig. 4). By subtracting the unoccupied receptor (ligand binding form) from total receptor protein level (immunoradiometric assay), a value for occupied plus nonligand-binding forms was obtained. In the vitamin D-deficient animal, receptor at ≈400 fmol/mg was found in the extract that did not bind ligand. At 12 hr after 1,25-$(OH)_2D_3$ administration, occupied and other non-ligand-binding forms at 1300–1400 fmol/mg were found. Thus, at least a 4-fold increase in occupied plus non-ligand-binding forms of the receptor was found 12 hr after 1,25-$(OH)_2D_3$ was administered.

DISCUSSION

The results presented here show that the 1,25-$(OH)_2D_3$ receptor is clearly up-regulated by 1,25-$(OH)_2D_3$ in intact animals. This action by the hormone occurs rapidly, observed by 6 hr after 1,25-$(OH)_2D_3$ administration, and occurs at the level of mRNA, suggesting that it is a transcriptional event. Our findings concur with previous studies in cell culture that show up-regulation of the 1,25-$(OH)_2D_3$ receptor after 1,25-$(OH)_2D_3$ administration (3–5). In those experiments, the 1,25-$(OH)_2D_3$ receptor protein was up-regulated by 24 hr after 1,25-$(OH)_2D_3$ treatment (3, 4). Additionally, McDonnell *et al.* (3) showed an increase in the 1,25-$(OH)_2D_3$ receptor mRNA after 48 hr of 1,25-$(OH)_2D_3$ treatment in mouse fibroblasts. In animal experiments, Costa and Feldman (6) concluded that an increase in the 1,25-$(OH)_2D_3$ receptor protein is seen in rat intestine only after 5 days of 1,25-$(OH)_2D_3$ treatment. These investigators found that the 1,25-$(OH)_2D_3$ receptor protein increased 2-fold as assayed by a radiolabeled hormone binding assay. On the other hand, they found that the 1,25-$(OH)_2D_3$ receptor protein is not increased at 24 hr after hormone administration as determined by ligand binding assay. Our results are in agreement with their finding that unoccupied receptor is unchanged during the 24-hr period after the dose was administered.

By using an assay for total receptor protein and an assay for receptor mRNA, we can clearly demonstrate an up-regulation of the receptor by 1,25-$(OH)_2D_3$ itself. Northern blot analysis reveals a 10-fold increase in receptor mRNA levels by 12 hr after 1,25-$(OH)_2D_3$ administration. Immunoradiometric assay of total receptor protein shows a 2-fold increase 12 hr after 1,25-$(OH)_2D_3$ was administered. Although the 2-fold increase in total receptor protein appears modest, calculation of the amount of occupied receptor reveals a larger fold increase. By subtracting the unoccupied levels from the total receptor levels, we see a 4-fold increase in occupied plus non-ligand-binding forms of the receptor at 12 hr after 1,25-$(OH)_2D_3$ administration. In a vitamin D-deficient animal, there should be no occupied receptor in the intestine, yet total receptor measured at 0 time is 1200 fmol/mg and unoccupied receptor is 800 fmol/mg. Therefore, some 400 fmol/mg must be a non-ligand-binding form of the receptor (neither occupied nor ligand-binding forms). The data are sufficient to show that the amount of occupied receptor is increased between 2- and 4-fold after 1,25-$(OH)_2D_3$ was administered.

One interesting aspect is that neither our results nor those of Costa and Feldman (6) show a change in unoccupied receptor, as measured by a standard hydroxylapatite binding assay during the 24-hr period after 1,25-$(OH)_2D_3$ was administered. This assay measures only that receptor available for binding to exogenously added 1,25-$(OH)_2D_3$. Therefore, it does not include occupied receptor, partially degraded receptor, and nonbinding forms of the receptor. In any case, our results suggest that the unoccupied receptor concentration is maintained constant throughout the 24-hr period after 1,25-$(OH)_2D_3$ was administered. Since 1,25-$(OH)_2D_3$ up-regulates the 1,25-$(OH)_2D_3$ receptor at the mRNA level, it is reasonable to suspect that the hormone probably follows the classical mechanism of steroid action in this system. In keeping with this mechanism, the 1,25-$(OH)_2D_3$–receptor complex is expected to bind to a specific DNA sequence, the 1,25-$(OH)_2D_3$-responsive element located near or in the 1,25-$(OH)_2D_3$ receptor gene, which would induce transcription of the 1,25-$(OH)_2D_3$ receptor gene. As the amount of steroid-bound receptor increases, the more transcription activation complex is formed resulting in the increased 1,25-$(OH)_2D_3$ receptor mRNA. However, this hypothesis fails to explain why unoccupied receptor remained constant while occupied receptor increased. This mechanism would predict a large increase in the number of unoccupied and occupied receptors. An additional control at the level of translation or at the level of receptor clearance would be needed to account for the observed results. Another interesting possibility is that unoccupied receptor binds to a sequence in the receptor gene, silencing or suppressing expression of that gene. When the receptor binds ligand, it loses its ability to bind to the silencer or suppressor site. This allows expression of the receptor gene until significant amounts of unoccupied receptor again suppress receptor gene expression.

The biological relevance of the 1,25-$(OH)_2D_3$ up-regulation of the 1,25-$(OH)_2D_3$ receptor is not readily apparent. Since plasma 1,25-$(OH)_2D_3$ levels are tightly regulated, up-regulation may ensure receptor availability for responding to fluctuations in hormone concentration. It is unlikely that up-regulation of the receptor is required for target organ response. Calcium transport is already increased by 3 hr after 1,25-$(OH)_2D_3$ administration with a peak at 6 hr. Thus, the increase in calcium transport precedes the increase in 1,25-$(OH)_2D_3$ receptor mRNA, demonstrating that the increase in 1,25-$(OH)_2D_3$ receptor is not required for the early response to 1,25-$(OH)_2D_3$ in the duodenum (15). However, subsequent responses may depend on the newly synthesized receptor.

In any case, the mechanism and biological significance of the autoregulation of the 1,25-$(OH)_2D_3$ receptor should provide an important dimension not only to the mechanism of action of 1,25-$(OH)_2D_3$ but also to steroid hormone action in general.

We are grateful to Dr. R. Jungman (Northwestern University) for the lactate dehydrogenase cDNA. This work was supported by Program Project Grant DK-14881 from the National Institutes of Health and by a Harry Steenbock Research Fund of the Wisconsin Alumni Research Foundation.

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