Up-regulation of the Vitamin D Receptor in Response to 1,25-Dihydroxyvitamin D₃ Results from Ligand-induced Stabilization*

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Several studies have shown that the 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) receptor protein levels increase in response to 1,25-(OH)₂D₃. We have studied the mechanism of this regulation in both mouse fibroblasts and rat intestinal epithelial cells. Cell extracts and total RNA were prepared at varying times after addition of 1,25-(OH)₂D₃. The 1,25-(OH)₂D₃ receptor protein levels, measured using an immunoradiometric assay, rose significantly 2–3 h posttreatment and had risen 3-fold at 8 h. Concurrently, the 1,25-(OH)₂D₃ receptor mRNA content, measured using a ribonuclease protection assay, was not altered by 1,25-(OH)₂D₃ during this time. In cycloheximide-blocked cells, the administration of 1,25-(OH)₂D₃ markedly reduced the degradation rate of previously formed receptor. The 1,25-(OH)₂D₃ receptor protein half-life was determined as 4 h in the absence of 1,25-(OH)₂D₃ and increased to at least 8 h in the presence of 1,25-(OH)₂D₃. We also measured the 1,25-(OH)₂D₃ receptor mRNA levels in the duodena and kidney of vitamin D-deficient rats after a single 150-pmol injection of 1,25-(OH)₂D₃. Again, we found that 1,25-(OH)₂D₃ receptor mRNA levels were not changed in these tissues after 1,25-(OH)₂D₃ treatment. Therefore, the elevation of the 1,25-(OH)₂D₃ receptor protein following 1,25-(OH)₂D₃ administration is apparently the result of increased receptor protein lifetime and not increased transcription.

The regulation of gene transcription by 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), the active form of vitamin D₃, mediated by its intracellular receptor proceeds in a manner common to the steroid/thyroid hormone family (1, 2). According to this model, 1,25-(OH)₂D₃ binding to its receptor facilitates an interaction between this complex and specific DNA sequences which can initiate the transcription of responsive genes (3). Identification of vitamin D-responsive elements in the genes coding for human (4, 5) and rat (6) osteocalcin, mouse osteopontin (7), and rat intestinal Ca²⁺ binding protein (8) supports this model. The parathyroid hormone has been reported to be transcriptionally down-regulated by 1,25-(OH)₂D₃ (9). Other proteins shown to be regulated by 1,25-(OH)₂D₃ are avian Ca²⁺ binding protein (10), osteonectin (11), metallothionien (12), and cellular retinol binding protein type II (13).

The 1,25-(OH)₂D₃ receptor has been the subject of many studies, because it is up-regulated by its own ligand. It has been demonstrated that the 1,25-(OH)₂D₃ receptor protein levels are increased in vitro in pig kidney cells (LLC-PK1) (14), mouse fibroblasts (3T6) (15, 16), rat osteosarcoma cells (ROS 17/2) (17), and human promyelocytic leukemia cells (HL-60) (18) and in vivo in rat intestine (19) shortly after addition of 1,25-(OH)₂D₃. Longer term studies have also shown elevated 1,25-(OH)₂D₃ receptor protein levels in the rat kidney after administration of vitamin D (20, 21). However, the mechanism of the accumulation of 1,25-(OH)₂D₃ receptor has not been defined. Most studies have focused on transcriptional regulation of the 1,25-(OH)₂D₃ receptor gene. The mouse 3T6 cells grown in the presence of 1,25-(OH)₂D₃ for 24–72 h have higher 1,25-(OH)₂D₃ receptor mRNA content than controls (15). The intestinal 1,25-(OH)₂D₃ receptor mRNA levels showed a large increase by 6 h but returned to control levels by 24 h in vitamin D-deficient rats after a single 150-pmol dose of 1,25-(OH)₂D₃ (19). Dosing daily for 5 days did not alter the level of 1,25-(OH)₂D₃ receptor mRNA in either the intestine or kidney (22). Up-regulation of the 1,25-(OH)₂D₃ receptor mRNA in response to 1,25-(OH)₂D₃ has also been shown in the human osteosarcoma cell line (MG-63) (23), HL-60 cells (24), and rat parathyroid gland (25).

Increased transcription has not been the only mechanism studied. Stabilization of the 1,25-(OH)₂D₃ receptor protein by its ligand has been suggested in pig LLC-PK1 cells (26), whereas in the rat osteosarcoma cell line ROS 17/2, there was no stabilization of the receptor by 1,25-(OH)₂D₃ (17).

Our present study focuses on short-term regulation of 1,25-(OH)₂D₃ receptor protein and its mRNA by 1,25-(OH)₂D₃ in the 3T6 mouse fibroblasts and IEC-6 rat intestinal epithelial cells and the rat intestine and kidney. The results of this study provide evidence that the up-regulation of the 1,25-(OH)₂D₃ receptor protein by 1,25-(OH)₂D₃ is largely the result of increased lifetime or stability of the receptor rather than an increase in transcription.

EXPERIMENTAL PROCEDURES

Materials—1,25-(OH)₂D₃ was a gift from Hoffmann-La Roche Co. (Nutley, NJ). [³²P]UTP (~3000 Ci/nmol, 370 MBq/ml) was from Amersham Corp. Dulbecco’s modified Eagle’s medium (DMEM) containing 0.45% glucose was from GIBCO or Hyclone Laboratories (Logan, UT). Ribonuclease protection assay kit (RPA or RPA II) and mouse β-actin cDNA were from Ambion (Austin, TX).

Animals—Weanling male Holtzman rats were purchased from...
Time Course of 1,25-(OH)\textsubscript{2}D\textsubscript{3} Receptor Protein Levels after 1,25-(OH)\textsubscript{2}D\textsubscript{3} Treatment in the 3T6 and IEC-6 Cells—Cells were grown to confluence, and 1,25-(OH)\textsubscript{2}D\textsubscript{3} was added to a final concentration of 10 nM at varying times prior to preparation of cell extracts. The 3T6 cells and some IEC-6 cells were washed and harvested in PBS and collected by centrifugation at 500 \( \times \) g. The cells were resuspended in TED + DFP and homogenized using a stainless steel Dounce homogenizer. The mixture was rehomogenized. The homogenate was spun in a Beckman Ti70.1 ultracentrifuge rotor at 150,000 \( \times \) g for 1 h. The supernatant was frozen at \(-80^\circ\)C until receptor content was measured. The remaining IEC-6 cells were collected as above and the cells resuspended in TEDK\textsubscript{sec} + DFP. The cells were lysed by three cycles of freezing and thawing and frozen and resuspended as described above.

The protein concentration of the extracts was determined using the BioRad dye assay, and 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor levels measured using the immunoradiometric assay of Sandgren and DeLuca (27).

Time Course of 1,25-(OH)\textsubscript{2}D\textsubscript{3} Receptor mRNA Levels after 1,25-(OH)\textsubscript{2}D\textsubscript{3} Treatment of Cells in Culture—The cells were grown to confluence and dosed with 1,25-(OH)\textsubscript{2}D\textsubscript{3} (10 nM final concentration) at varying times prior to harvest. The cells were washed with PBS, harvested by scraping, and centrifuged at 500 \( \times \) g. The pellets were resuspended in 4 M guanidine thiocyanate solution and vortexed to lyse the cells. Total RNA was prepared using the guanidine/CsCl method of Chirgwin et al. (31) as modified by Maniatis et al. (32). Levels of 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor mRNA were determined using a ribonuclease protection assay (RPA) as described below.

Ribonuclease Protection Assay—Five or thirty micrograms of total RNA were hybridized simultaneously with the indicated 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor and \( \beta \)-actin probes. The samples were processed according to the procedure described in the RPA or RPA II kits (Ambion Inc., Austin, TX). The protected fragments were separated on a 6% polyacrylamide, 42% urea, TBE gel. The gels were dried and exposed to x-ray film with intensifying screen. The radioactivity of the bands was determined with a Betascope 603 blot counter (Betagen Corp., Waltham, MA), and the ratio of radioactivity in the 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor band to that in the actin band was determined.

1,25-(OH)\textsubscript{2}D\textsubscript{3} Receptor Protein Stability Study—When 3T6 or IEC-6 cells had grown to confluence, cycloheximide was added to a final concentration of 10 \( \mu \)M in the presence of 1,25-(OH)\textsubscript{2}D\textsubscript{3} (10 nM final concentration) or the ethanol vehicle. Cell extracts were prepared at various time points and the receptor protein content determined using the immunoradiometric assay (27).
Effects of 1,25-(OH)\textsubscript{2}D\textsubscript{3} Treatment on 1,25-(OH)\textsubscript{2}D\textsubscript{3} Receptor mRNA in Vivo—Next, the response of 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor mRNA levels were measured in the intestine and kidney of vitamin D-deficient rats after a single dose of 1,25-(OH)\textsubscript{2}D\textsubscript{3} (Fig. 3). No significant alteration of the 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor mRNA levels in either the intestine (Fig. 3A) or the kidney (Fig. 3B) was detected up to 24 h after dosing. Therefore, it is clear that 1,25-(OH)\textsubscript{2}D\textsubscript{3} did not cause transcriptional up-regulation of the 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor mRNA in these tissues of vitamin D-deficient rats.

Stability of 1,25-(OH)\textsubscript{2}D\textsubscript{3} Receptor in the Presence or Absence of 1,25-(OH)\textsubscript{2}D\textsubscript{3} in the 3T6 or IEC-6 Cells—In order to study whether the accumulation of the 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor protein in 3T6 or IEC-6 cells in response to 1,25-(OH)\textsubscript{2}D\textsubscript{3} treatment was due to increased stability of the receptor or some other mechanism, the 1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor protein levels were measured in cells whose protein synthesis was blocked with cycloheximide. The cycloheximide was able to inhibit more than 75% of the translation and maintain this
level of inhibition through 24 h without any change in cell viability (data not shown). When treated with 1,25-(OH)2D3, the 1,25-(OH)2D3 receptor protein concentration remained constant for the 8 h studied, whereas these levels decreased significantly within 2 h and reached half the initial levels by 4 h in both 3T6 cells (Fig. 4A) and IEC-6 cells (Fig. 4B) when treated with vehicle. The results of these experiments demonstrate that the presence of 1,25-(OH)2D3 increases the stability of the 1,25-(OH)2D3 receptor protein in these cell lines.

**DISCUSSION**

We report in this study that accumulation of the 1,25-(OH)2D3 receptor protein in response to 1,25-(OH)2D3 results from stabilization of the receptor protein by its ligand in 3T6 and IEC-6 cells and not increased transcription either in vitro or in vivo. It has been reported previously that the 1,25-(OH)2D3 receptor protein levels increase in response to 1,25-(OH)2D3 in LLC-PK1 (14), 3T6 (15, 16), ROS 17/2 (17) and HL-60 (18) cells, rat intestine (19), and kidney (20, 21). While it is clear that the 1,25-(OH)2D3 receptor protein is increased in response to 1,25-(OH)2D3, the mechanism of this increase has not been clearly defined.

In 3T6 cells, an accumulation of 1,25-(OH)2D3 receptor mRNA was reported after 24–72 h exposure to 1,25-(OH)2D3 (16). Although we observe a slight increase in 1,25-(OH)2D3 receptor mRNA levels in response to 1,25-(OH)2D3 in 3T6 cells, this clearly occurs after the elevation in 1,25-(OH)2D3 receptor protein has taken place (compare Figs. 1A and 4A). The 1,25-(OH)2D3 receptor protein content rose significantly within 2 h and reached three times the control levels by 8 h after 1,25-(OH)2D3 treatment at which time no significant increase in 1,25-(OH)2D3 receptor mRNA had occurred. The increase in 1,25-(OH)2D3 receptor mRNA was first seen at 12 h, reaching a maximum of less than 2-fold for the 24 h studied. Therefore, it is unlikely that the 1,25-(OH)2D3 receptor protein accumulation is the result of changes in 1,25-(OH)2D3 receptor mRNA. Also, similar results were obtained with the IEC-6 cells, where the 1,25-(OH)2D3 receptor protein concentration rose 3-fold in 8 h (Fig. 1B) with only a 20% increase in the 1,25-(OH)2D3 receptor mRNA (Fig. 2B). This slight rise in 1,25-(OH)2D3 receptor mRNA takes place after most of the increase in the 1,25-(OH)2D3 receptor protein had occurred. Again this indicates that the elevated 1,25-(OH)2D3 receptor protein content does not result from increased transcription of the 1,25-(OH)2D3 receptor gene.

We studied the stability of the 1,25-(OH)2D3 receptor protein in cycloheximide-treated cells in the presence or absence of 1,25-(OH)2D3. The inhibition of protein synthesis allowed the measurement of the degradation rate of the 1,25-(OH)2D3 receptor protein. It is evident in both cell lines that the 1,25-(OH)2D3 receptor has a half-life of 4 h in the absence of 1,25-(OH)2D3 while stable for at least 8 h in the presence of 1,25-(OH)2D3 (Fig. 4, A and B). This increase in protein lifetime would lead to a doubling of the 1,25-(OH)2D3 receptor protein level in 4 h and a 3-fold elevation at 8 h, which has been demonstrated for both cell lines studied (Fig. 1, A and B). A 4 h half-life for the receptor in the absence of 1,25-(OH)2D3 has also been demonstrated in LLC-PK1 cells (26). Although stabilization of the receptor by the ligand was suggested in this study, it was not demonstrated, because 24,25-(OH)2D3 and not 1,25-(OH)2D3 (the hormonal form of vitamin D3) was used in those experiments. Since 24,25-(OH)2D3 binds poorly (2000 times less than 1,25-(OH)2D3) to the receptor, conclusions regarding 1,25-(OH)2D3 in this regard are not possible.

The results obtained with the cells of the present study are consistent with the results reported for HL-60 cells in which 1,25-(OH)2D3 receptor protein levels have been shown to increase approximately 3-fold in 8 h (18). Also in this study, the 1,25-(OH)2D3 receptor protein concentration remained constant for 12 h in the presence of both cycloheximide and 1,25-(OH)2D3 (18), indicating stability in the ligand-receptor complex similar to that demonstrated for the 3T6 and IEC-6 cells (Fig. 4, A and B). Two groups have reported on the regulation of 1,25-(OH)2D3 receptor mRNA in response to 1,25-(OH)2D3 in HL-60 cells. The first reported that there is no regulation of the 1,25-(OH)2D3 receptor mRNA (34), whereas the second claims a transient or biphasic up-regulation of the 1,25-(OH)2D3 receptor mRNA depending on HL-60 cell subtype (24). However, if accelerated transcription leads to a proportionate increase in protein and the protein were also stabilized, 1,25-(OH)2D3 receptor protein content in these cells should be much greater than reported (18).

Two previous studies showed increases in 1,25-(OH)2D3
receptor mRNA in rat intestine (19, 25). Our data clearly show that there is little or no up-regulation of the 1,25-(OH)2D3 receptor mRNA in response to 1,25-(OH)2D3 in vivo or in vitro. In one report it was suggested that in vitamin D-deficient rats, the intestinal 1,25-(OH)2D3 receptor mRNA sharply increased between 2 and 6 h before falling to control levels by 24 h (19). One possible explanation is that the authors examined a limited number of RNA samples at each time point which resulted in the observation of a spurious result. In the present studies, we have tested several mRNA samples for each time point, each prepared independent from rat intestine, rat kidney, and the two cell lines. We found virtually no increase in the receptor transcript in response to 1,25-(OH)2D3. A second possible explanation is that those authors examined poly(A)+ RNA, while we have used total RNA. The discrepancy between these observations may suggest that the processing of the 1,25-(OH)2D3 receptor RNA is altered in response to 1,25-(OH)2D3 treatment. In the other study, 1,25-(OH)2D3 receptor mRNA was not quantitated so magnitude, significance, or correlation of up-regulation compared with that of 1,25-(OH)2D3 receptor protein concentration is not possible (25). Our data are also consistent with long-term dosing studies that showed no increase in 1,25-(OH)2D3 receptor mRNA due to 1,25-(OH)2D3 in rat intestine or kidney (22).

An increase in 1,25-(OH)2D3 receptor mRNA in response to 1,25-(OH)2D3 has also been reported in the parathyroid gland (25). While this study suggests significantly increased levels of 1,25-(OH)2D3 receptor mRNA, the less than 2-fold increase occurred only after 24 h. In the studies done to date including this one, the rise in 1,25-(OH)2D3 receptor protein in response to 1,25-(OH)2D3 treatment occurs much earlier than 24 h. Therefore, the physiological significance of the elevated 1,25-(OH)2D3 receptor mRNA in the parathyroid gland is unknown.

It is interesting that 1,25-(OH)2D3 receptor levels increase in rat osteosarcoma cells in response to 1,25-(OH)2D3 (17), but in that study, 1,25-(OH)2D3 did not appear to stabilize the receptor. This coupled with results obtained in the human osteosarcoma cell line MG-63 that 1,25-(OH)2D3 increases its own receptor's mRNA suggests that the mechanism of 1,25-(OH)2D3 receptor protein increase in bone cells may be different from that of the 3T6 and IEC-6 cells.

It is reasonable that 1,25-(OH)2D3 stabilizes the receptor when bound. It has been demonstrated that 1,25-(OH)2D3 prevents inactivation of its receptor during purification (35). This could be due to the ligand protecting the 1,25-(OH)2D3 receptor from proteolytic attack from endogenous proteases present in the extracts. These enzymes may also function in intact cells. Ligand-induced stabilization is a simple and reasonable explanation for accumulation of 1,25-(OH)2D3 receptor protein induced by treatment with 1,25-(OH)2D3.

REFERENCES