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Regulation of 25-Hydroxyvitamin D_3-24-Hydroxylase mRNA by 1,25-Dihydroxyvitamin D_3 and Parathyroid Hormone

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Abstract The 25-hydroxyvitamin D_3-24-hydroxylase mRNA is tightly and reciprocally regulated by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and parathyroid hormone (PTH). The upregulation of the 24-hydroxylase by $1,25(OH)_2D_3$ is well established and occurs at the transcriptional level through two vitamin D response elements in the promoter of the gene. However, this induction is blocked by the protein synthesis inhibitor cycloheximide (CHX) indicating a protein component in the regulation pathway. CHX treatment reduced total vitamin D receptor (VDR) protein levels in cells, but reintroduction of VDR and/or retinoid X receptor protein into cells by transfection did not reduce the inhibition by CHX. This indicates that production of another transcription factor or mRNA-stabilizing protein synthesized in response to $1,25(OH)_2D_3$ is required for optimal accumulation of 24-hydroxylase mRNA. PTH downregulates the 24-hydroxylase mRNA by affecting its stability. The half-life of 24-hydroxylase mRNA is reduced 4.2-fold in AOK-B50 cells by PTH. Untranslated regions of the 24-hydroxylase mRNA in reporter gene assays did not confer PTH responsiveness. Further analysis of the coding region of the rat 24-hydroxylase may reveal sites of action of PTH. J. Cell. Biochem. 88: 234–237, 2003.

Key words: vitamin D receptor; AOK-B50; half-life; cycloheximide; actinomycin D

Vitamin D synthesized in the skin or ingested in the diet is sequentially hydroxylated at the C25 position in the liver and at the C1α in the kidney to form the active metabolite 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) [Jones et al., 1998]. 24-Hydroxylase is the enzyme responsible for the catabolism of $1,25(OH)_2D_3$ that ultimately leads to the excretion of the hormone as calcitroic acid [Jones et al., 1998]. The 25-hydroxyvitamin D_3-1α-hydroxylase and the 24-hydroxylase are very tightly and reciprocally regulated by $1,25(OH)_2D_3$ itself and parathyroid hormone (PTH) [Tanaka and DeLuca, 1981]. $1,25(OH)_2D_3$ activates its own breakdown by strongly inducing 24-hydroxylase expression while at the same time downregulating the 1α-hydroxylase expression. PTH induces the 1α-hydroxylase while downregulating the 24-hydroxylase. These pathways act synergistically to maintain optimal levels of $1,25(OH)_2D_3$ in serum to maintain calcium homeostasis.

$1,25(OH)_2D_3$ upregulates 24-hydroxylase mRNA by binding to the vitamin D receptor (VDR) which binds to two vitamin D response elements (VDRE) with its partner retinoid X receptor (RXR) and various coactivators in the promoter region of the gene [Jones et al., 1998]. The distal VDRE is located at positions −262 to −238 from the transcriptional start site, while the more proximal VDRE is located at position −154 to −125. Both consensus sequences are located on the antisense strand. Each VDRE alone confers only limited responsiveness to $1,25(OH)_2D_3$ in a reporter assay, but the fragment of promoter containing both VDREs and its intervening sequence can account for most but not all of the $1,25(OH)_2D_3$-induced activity in a reporter assay [Zierold et al., 1995].

Though it has been known for many years that PTH downregulates the 24-hydroxylase activity [Tanaka and DeLuca, 1984] and its mRNA [Shinki et al., 1992], the mechanisms of

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PTH action have only recently been investigated. One of the problems with this inquiry was to find a cell model that would be responsive to PTH because cells in culture loose their PTH receptors. AOK-B50 cells are LLC-PK1 cells with stably transfected opossum PTH receptors [Bringhurst et al., 1993]. In this system, though somewhat artificial, the 24-hydroxylase responds to $1,25(OH)_2D_3$ and PTH like the whole animal model [Zierold et al., 2000]. In AOK-B50 cells, PTH downregulates 24-hydroxylase mRNA by affecting its stability [Zierold et al., 2001]. However, further studies are needed to elucidate the mechanisms of action of PTH, including the site of action on the 24-hydroxylase mRNA and proteins involved.

PROTEIN SYNTHESIS IS REQUIRED FOR OPTIMAL INDUCTION OF 24-HYDROXYLASE mRNA BY $1,25(OH)_2D_3$

In AOK-B50 cells, 24-hydroxylase mRNA was markedly induced in response to $1,25(OH)_2D_3$ as expected. When the protein synthesis inhibitor cycloheximide (CHX) was added together with $1,25(OH)_2D_3$, there was a 76% reduction in expected 24-hydroxylase mRNA levels (Fig. 1A) [Zierold et al., 2002]. This was observed not only in cells but also in animal studies. In rats, intraperitoneal administration of CHX together with $1,25(OH)_2D_3$ caused a 72.3% reduction in duodenal 24-hydroxylase mRNA compared to rats treated with $1,25(OH)_2D_3$ alone (Fig. 1B). This observation may suggest an important step in the mechanism of action of $1,25(OH)_2D_3$ that has so far been overlooked, i.e., the need for a transcription factor, mRNA-stabilizing protein, or modifying enzyme that is rapidly induced by $1,25(OH)_2D_3$.

Other $1,25(OH)_2D_3$ target genes require protein synthesis for maximal induction of mRNA. Osteocalcin and osteopontin are two such genes. Unlike the 24-hydroxylase gene, these genes are transcribed at significant levels in the absence of $1,25(OH)_2D_3$. This characteristic response was used to rule out the possibility that a rapidly turned-over protein not induced by $1,25(OH)_2D_3$ is, in fact, responsible for reduced mRNA production in the presence of CHX. We reasoned that if the effects are attributable to the rapid turn-over of a transcription factor, then CHX would affect mRNA synthesis even in the absence of $1,25(OH)_2D_3$ because CHX would block production of this factor.

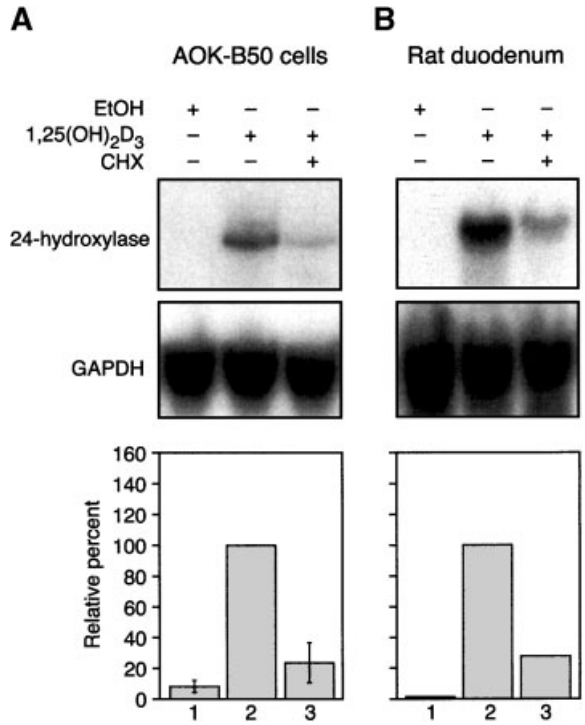

Fig. 1. **A**: AOK-B50 cells were dosed with vehicle (ethanol) or $1,25(OH)_2D_3$ (10^{-8} M) or $1,25(OH)_2D_3$ + CHX (10 μg/ml) as shown. Cells were collected 7 h after the $1,25(OH)_2D_3$ or vehicle dose, RNA was extracted, and Northern analysis was performed using 24-hydroxylase and GAPDH as probes. A representative Northern blot is shown as well as quantitations on multiple experiments expressed as the mean ± SD. All data was standardized to the amount of 24-hydroxylase/GAPDH expressed in $1,25(OH)_2D_3$-dosed cells and plotted as relative percentage. **B**: Sprague–Dawley rats were dosed with vehicle (ethanol) or $1,25(OH)_2D_3$ (650 pmoles) or $1,25(OH)_2D_3$ + CHX (2 mg) as shown. The animals were sacrificed 7 h after the $1,25(OH)_2D_3$ or vehicle dose and the duodenal mucosa collected. RNA was extracted and Northern analysis was performed using 24-hydroxylase and GAPDH as probes. All data was standardized to the amount of 24-hydroxylase/GAPDH expressed in $1,25(OH)_2D_3$-dosed cells and plotted as relative percentage.

Osteocalcin mRNA levels were not reduced by CHX in the absence of $1,25(OH)_2D_3$ as they were in the presence of $1,25(OH)_2D_3$, indicating that the needed protein(s) must be induced by $1,25(OH)_2D_3$ [Zierold et al., 2002].

Since $1,25(OH)_2D_3$ acts through the VDR, the effects of CHX on the levels of VDR in AOK-B50 cells were also analyzed. AOK-B50 cells treated with ethanol had VDR levels of 38.7 ± 5.0 fmoles/mg protein, and cells treated with $1,25(OH)_2D_3$ had VDR levels of 90.2 ± 4.6 fmoles/mg protein. Concomitant treatment of $1,25(OH)_2D_3$ with CHX reduced VDR levels to 41.2 ± 8.3 fmoles/mg protein indicating that

CHX treatment may reduce VDR levels leading to a decrease in 24-hydroxylase mRNA [Zierold et al., 2002]. To rule out the possibility that reduced levels of VDR or RXR are responsible for the effects of CHX, we used a transfection agent for proteins (Chariot, Active Motif, Carlsbad, CA) to reintroduce functional VDR and/or RXR into AOK-B50. Transfected cells were treated with vehicle, $1,25(OH)_2D_3$, or $1,25(OH)_2D_3 + CHX$ and their 24-hydroxylase mRNA levels were measured using quantitative real time PCR. If the effects of CHX were due to the reduced levels of receptor, the protein transfection would act to rescue the 24-hydroxylase mRNA in the presence of CHX to near normal levels. As can be seen in Figure 2, transfections of VDR, RXR, or VDR + RXR were unable to rescue the suppression by CHX. This indicates that the effects observed in the presence of CHX were not due to reduced levels of VDR or RXR but to another unidentified $1,25(OH)_2D_3$-induced product. This product may be a transcription factor, mRNA-stabilizing protein, or modifying protein required for optimal production of 24-hydroxylase mRNA.

PTH DECREASES THE HALF-LIFE OF 24-HYDROXYLASE mRNA

Previously, Northern analysis of cells dosed with PTH at different time points following induction with $1,25(OH)_2D_3$ suggested that the mechanism by which PTH acts must be by decreasing 24-hydroxylase mRNA stability rather than by blocking transcription [Zierold et al., 2001]. Treatment with actinomycin D, an mRNA synthesis inhibitor, at the time of PTH treatment blocked the effect of PTH. In addition, the cascade of events triggered by PTH with the final effect of 24-hydroxylase mRNA destabilization was shown to take at least 1 h [Zierold et al., 2001]. To determine the half-life of 24-hydroxylase mRNA, the above-mentioned characteristics needed to be considered. The experiment represented in Figure 3 was designed to determine 24-hydroxylase half-life in AOK-B50 cells, using actinomycin D to block new mRNA production. AOK-B50 cells were first induced with $1,25(OH)_2D_3$ for 16 h. After that time, some cells were dosed with PTH and some with vehicle (water) for 1.5 h. PTH was allowed to carry out its actions for 1.5 h before actinomycin D was added. 17.5 h after the $1,25(OH)_2D_3$ dose, actinomycin D was added to block mRNA synthesis. Reference cells ($1,25(OH)_2D_3$ and $1,25(OH)_2D_3 + PTH$) were collected at this time point, while other cells were harvested at the indicated times after the addition of actinomycin D. RNA was isolated from all cells and mRNA was quantified using real time quantitative PCR. Figure 3A shows a representative graph of the experiment described above. In the presence of PTH, the 24-hydroxylase mRNA was more rapidly destroyed. Figure 3B shows the average calculated half-life

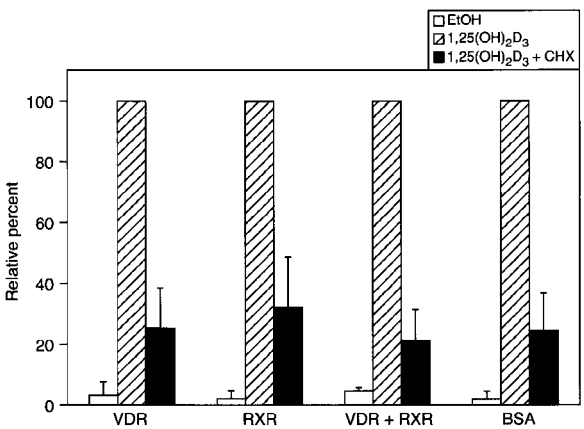

Fig. 2. VDR and/or RXR protein (0.5 μg) were transfected into AOK-B50 cells in 24-well plates using Chariot (Active Motif, Carlsbad, CA) according to manufacturer's instructions. Cells were dosed with vehicle, $1,25(OH)_2D_3$ (10^{-8} M), or $1,25(OH)_2D_3 + CHX$ (10 μg/ml) as shown. Cells were harvested 6 h after the dose and RNA was extracted. 24-Hydroxylase and GAPDH mRNA were quantified using quantitative real time PCR. 24-Hydroxylase/GAPDH ratios were expressed as relative percent of the $1,25(OH)_2D_3$ dosed cells to standardize for interassay variability. The data are expressed as mean ± SD.

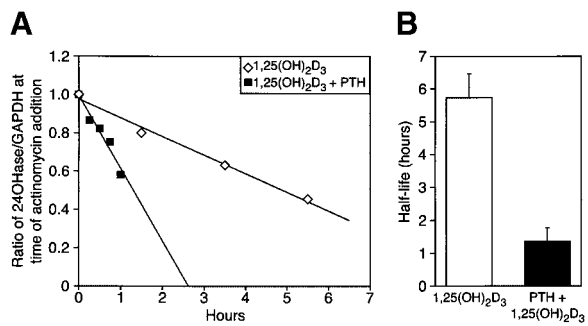

Fig. 3. **A**: AOK-B50 cells were dosed with $1,25(OH)_2D_3$ (10^{-8} M) for 16 h before PTH (150 nM) or vehicle addition. Actinomycin D was added 17.5 h after the addition of $1,25(OH)_2D_3$ (0 h time point), and cells were collected at various time points after actinomycin D addition (0.25–5.5 h). RNA was extracted and 24-hydroxylase and GAPDH mRNA were quantified using quantitative real time PCR. 24-Hydroxylase/GAPDH ratio of cells collected at time of actinomycin D addition (0 h) treated with vehicle or PTH were used as standard (= 1) and all other time points were expressed relative to these levels.

from multiple experiments. The half-life of 24-hydroxylase mRNA in the presence of $1,25(OH)_2D_3$ alone was 5.7 ± 0.7 h, while in the presence of $1,25(OH)_2D_3$ and PTH, it was 1.4 ± 0.4 h, a 4.2-fold reduction.

The effect on mRNA stability is usually mediated through 3′ or 5′ untranslated regions (UTR). With this in mind, we tested the 3′UTR and 5′UTR of the rat 24-hydroxylase cDNA in a reporter gene assay. Regions responsive to PTH were not found; the 3′ UTR had an effect on the stability of luciferase mRNA but not in a PTH-dependent manner [Zierold et al., 2001].

A previous report showed that the coding region of a gene can contain sequences that influence turnover of mRNA: *c-myc*, a proto-oncogene, was shown to have regions in exon 2 and 3 that influence mRNA stability [Yeilding and Lee, 1997]. We hypothesized that a similar relationship may exist within the 24-hydroxylase gene. Preliminary data on analysis of two fragments of rat 24-hydroxylase mRNA (base pairs 247–900 and 900–3,166) in a reporter gene assay did not show reduction of luciferase in response to PTH (data not shown). Confounding possibilities that need to be explored include: (1) the necessity for two or more regions of mRNA, now in different constructs, to confer instability to PTH; or (2) that the reporter system used to measure decreases in mRNA through luciferase activity may inaccurately reflect what is occurring in the cells.

CONCLUSIONS

Although the upregulation of the 24-hydroxylase mRNA has been extensively studied and was shown to occur largely through VDR, RXR, and coactivators binding to two VDREs in the promoter of the 24-hydroxylase gene, an important step of the mechanism has been overlooked. Recent data suggests that a $1,25(OH)_2D_3$-induced transcription factor, mRNA-stabilizing protein, or modifying enzyme is required early on for optimal production of target gene mRNA.

The mechanisms by which PTH acts to decrease 24-hydroxylase mRNA are only beginning to be elucidated. We found that PTH acts to decrease the stability of 24-hydroxylase mRNA by reducing its half-life 4.2-fold in AOK-B50 cells when PTH is present in the medium. Much more work needs to be done to locate the sites of action and to identify the proteins involved.

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