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Vitamin D Receptor mRNA Measured in Leukocytes with the TaqMan Fluorogenic Detection System: Effect of Calcitriol Administration

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Background: **The aim of the present study was to investigate the interactions between the circulating concentrations of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and the mRNA concentration of its specific nuclear receptor in human leukocytes.**

Methods: **We measured vitamin D receptor (VDR) mRNA extracted from leukocytes by use of TaqMan fluorescence analysis applied to the reverse transcription-PCR (RT-PCR) technique in 16 volunteers before and after calcitriol administration. VDR mRNA was also measured in leukocytes from calcium-stone-formers (37 hypercalciuric and 34 normocalciuric patients). The relationship between VDR mRNA concentrations and genetic VDR polymorphisms was analyzed in these patients.**

Results: **Imprecision (CV) of RT-PCR was 1.3% within assay (n = 10) and 1.7% between assays (n = 4). Oral 1,25$(OH)_2D_3$ increased mean (SE) serum 1,25$(OH)_2D_3$ 1.6 (0.3)-fold and VDR mRNA 1.6 (0.1)-fold 8 h after administration. The maximum VDR mRNA was reached 3.6 (1.3) h after 1,25$(OH)_2D_3$ ingestion. No differences in leukocyte VDR mRNA concentrations were found between normocalciuric and hypercalciuric stone-formers in the absence of stimulation. Finally, no association was found between VDR mRNA concentrations and genetic VDR polymorphisms in stone-formers.**

Conclusions: **The TaqMan RT-PCR assay is a rapid and accurate method to measure VDR mRNA, and leukocytes are a useful model to study VDR and 1,25$(OH)_2D_3$ interactions. In humans, VDR mRNA is increased by agonist 1,25$(OH)_2D_3$, a finding resembling previously reported results obtained in cellular and animal models.**

Calcitriol [1,25-dihydroxyvitamin D_3; 1,25$(OH)_2D_3$][5] is the metabolite of vitamin D with the highest activity on mineral metabolism. With parathyroid hormone (PTH) and calcitonin, it regulates intestinal calcium absorption and bone remodeling, and it influences growth, differentiation, and function of a broad range of cells *(1, 2)*. The biological actions of 1,25$(OH)_2D_3$ are mediated by interaction with its specific nuclear receptor (VDR) in target cells. Through transcriptional or posttranscriptional patterns of activity, the 1,25$(OH)_2D_3$–VDR complex can modulate the effects of vitamin D *(3)*. VDR belongs to the superfamily of steroid receptors, as do thyroid hormone and vitamin A receptors. It has a nearly ubiquitous tissue distribution, including cells of the human hematolymphopoietic tissue *(4, 5)*, where its presence allows 1,25$(OH)_2D_3$ to reduce immunologic activity of T and B lymphocytes and to activate monocyte-macrophage functions.

A feature somewhat unique is the positive effect of the ligand calcitriol on both VDR cellular production and

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[5] Nonstandard abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PTH, parathyroid hormone; SNP, single-nucleotide polymorphism; hVDR, human vitamin D receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FAM, 6-carboxyfluorescein; JOE, 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; and Ct, threshold cycle.

VDR activity. Exposure of 3T6 mouse fibroblasts to $1,25(OH)_2D_3$ was found to cause an increase of VDR cellular content, accompanied by phosphorylation of receptors and increase of its mRNA concentrations *(1)*. The up-regulation of VDR cellular production by $1,25(OH)_2D_3$ was found in isolated rat kidneys *(6)* and in intact rats *(7)*, whereas, to our knowledge, no data are available on humans.

Modified production of VDR could occur in primary hypercalciuria, a defect characterized by increased urinary calcium excretion and normal plasma calcium concentrations *(8)*. In genetically hypercalciuric stone-forming rats, the number of VDR molecules was increased in duodenal cells; the increase could be maintained by a posttranscriptional effect of $1,25(OH)_2D_3$ *(9)*. A disorder of vitamin D activity has been hypothesized in human primary hypercalciuria, which could be attributable to increased $1,25(OH)_2D_3$ synthesis or to increased sensitivity of patients' cells to $1,25(OH)_2D_3$.

Cellular production of VDR could be influenced by *VDR* gene single-nucleotide polymorphisms (SNPs) at the 3′-end region or at the translation starting site. This was suggested by results of in vitro experiments analyzing the functional effect of these SNPs in leukocytes *(10)* or in transfected HeLa or COS-7 cells *(11)*. A SNP at the 3′-end region does not cause an amino acid change in VDR protein. However, it could influence VDR activity and, thus, DNA transcription efficiency. A SNP in the translation starting codon leads to a shorter molecule lacking the first three amino acids; this variant could be more efficient in stimulating transcription of vitamin D-dependent proteins *(12)*. Cloning of the human *VDR* gene has made possible measurement of *VDR* expression in human tissues and in circulating leukocytes *(13)*.

The aim of the present study was to investigate the in vivo response of human VDR (hVDR) mRNA to $1,25(OH)_2D_3$ in human leukocytes. To measure VDR mRNA, we used the TaqMan fluorescent chemical analysis applied to reverse transcription-PCR (RT-PCR) *(14, 15)*. The dependence on exogenous $1,25(OH)_2D_3$ was evaluated in healthy volunteers after oral administration of $1,25(OH)_2D_3$. The dependence on endogenous $1,25(OH)_2D_3$ was evaluated in hypercalciuric patients with calcium kidney stones compared with normocalciuric stone-formers. These patients were genotyped for the SNPs at the 3′-end region and at the translation starting codon of the *VDR* gene; their genotypes were related to VDR mRNA production in leukocytes.

Materials and Methods

MATERIALS

All reagents used for RNA extraction were from BDH, except guanidine, which was from Boehringer-Mannheim. All reagents used for reverse transcription, PCR, and the synthesis of VDR probe and primers were from Applied Biosystems. $1,25(OH)_2D_3$ was from Roche.

VDR mRNA RESPONSE TO ACUTE ADMINISTRATION OF $1,25(OH)_2D_3$

We recruited 16 healthy adult volunteers to study the effects of acute administration of $1,25(OH)_2D_3$. The test was performed in the Outpatient Clinic of San Raffaele Hospital. A dose of $1,25(OH)_2D_3$ was ingested by volunteers after an overnight fast. The oral dose of $1,25(OH)_2D_3$ was 1 μg for individuals with a body weight of 75–100 kg, 0.75 μg for individuals with a body weight of 50–75 kg, and 0.5 μg for individuals with a body weight <50 kg. Blood was drawn to measure VDR mRNA and plasma $1,25(OH)_2D_3$ at baseline and after the $1,25(OH)_2D_3$ load. In a group of six individuals (age range, 28–45 years; three males and three females), the second blood sample was drawn 8 h after $1,25(OH)_2D_3$ ingestion. In another group of 10 individuals (age range, 24–30 years; 6 males and 4 females), blood samples were drawn 2, 3, 4, 5, and 6 h after $1,25(OH)_2D_3$ ingestion.

MEASUREMENT OF VDR mRNA UNDER BASAL CONDITIONS

We recruited 37 calcium-stone-formers with idiopathic hypercalciuria [22 males and 15 females; mean (SE) body weight, 69 (2.2) kg; mean (SE) age, 48 (2.1) years] and 34 calcium-stone-formers with normal calcium excretion [24 males and 10 females; mean (SE) body weight, 70 (1.9) kg; mean (SE) age, 47 (2.5) years] at their first visit to the Outpatient Clinic at San Raffaele Hospital during the period 1999–2002. They were required to have produced at least one calcium stone and to have no diseases other than stones. They were not taking medications for stones or other reasons and had normal kidney function, and urinary function had to be normal (plasma creatinine <12 mg/L and urinary pH <5.5 at least during one examination). No urinary tract dilatation or obstruction was shown by echographic examination. Hypercalciuria was defined as the excretion of calcium >7.5 mmol/24 h in males or >6.25 mmol/24 h in females or >100 μmol/kg of body weight for both genders. Patients were genotyped for *VDR* gene polymorphisms at the translation start codon and at the 3′-end region. Blood samples were collected from patients after a midnight fast. Calcium, creatinine, $1,25(OH)_2D_3$, and PTH were measured in plasma. Calcium was also measured in 24-h urines. VDR mRNA was measured in leukocytes.

This study was approved by the local research Ethics Committee, and informed consent was obtained from each participant (healthy volunteers and renal-stone formers).

ISOLATION OF TOTAL RNA AND cDNA SYNTHESIS

We collected 8 mL of blood from healthy donors in tubes containing 15 mL of 4 mol/L guanidine thiocyanate. Five-milliliter aliquots were frozen at −80 °C. A guanidine thiocyanate-based assay was used for the extraction of total RNA *(16)*. We mixed 2 μg of RNA with 10× reverse transcription buffer, 5.5 mM $MgCl_2$, 500 mM

deoxynucleotide triphosphates, 2.5 mM random hexamers, 0.4 U/μL RNase inhibitor, and 1.25 U/μL MultiScribe Reverse Transcriptase for the reverse transcription procedure. Reverse transcription was performed in three steps: incubation for 10 min at 25 °C, reverse transcription for 30 min at 48 °C, and reverse transcription inactivation (for protein denaturation) for 5 min at 95 °C.

PCR PROCEDURE

Real-time quantitative RT-PCR analyses of *VDR* were performed with an ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems). Oligonucleotide sequences were chosen to span the junction between exons 3 and 4 of *VDR* cDNA, using the primer design software Primer Express™ (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was chosen as an internal standard to control for variability in amplification. The sequences of primers and probes used are shown in Table 1. The *VDR* target probe was labeled at the 5′ end with the reporter dye molecule 6-carboxyfluorescein (FAM; emission at 538 nm). The *GAPDH* target probe was labeled with 6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein (JOE; emission at 546 nm). Both probes were labeled with the quencher fluor 6-carboxytetramethylrhodamine (TAMRA; emission at 582 nm) at the 3′ end. During the PCR extension cycle, the *Taq* DNA polymerase cleaves the probes and releases the reporter dye, producing an increase in fluorescent emission monitored in real time by software. Each well is screened for fluorescence every 7 s, and signal is considered positive when the fluorescence intensity exceeds 10 SD of the baseline. This is the threshold cycle (Ct) *(17, 18)*. To obtain PCR conditions with reduced variability, PCR premixes containing all reagents except for target were prepared and aliquoted into optical reaction PCR tubes in a 96-well microtiter plate format (Applied Biosystems). No-template controls, calibrators, and unknown samples were assayed in triplicate. For composition of the PCR mixture, see Table 2. The PCR was performed in three steps: AmpErase uracil-N-glycosylase activation (2 min at 50 °C), AmpliTaq Gold enzyme activation (10 min at 95 °C), and 50 cycles PCR (denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C).

Table 1. Oligonucleotide sequences.

Primer/probe	Sequence, 5′–3′
VDR probe	CCT GCC GGC TCA AAC GCT GTG
VDR forward primer	CTC ATC TGT CAG AAT GAA CTC CTT CA
VDR reverse primer	TCA CCA AGG ACA ACC GAC G
GAPDH probe	CCG ACT CTT GCC CTT CGA AAC
GAPDH forward primer	GAA GGT GAA GGT CGG AGT CA
GAPDH reverse primer	CTT TAG GGT AGT GGT AGA AG

Table 2. Composition of PCR assay.

	VDR	GAPDH
TaqMan master mixture, μL	12.5	12.5
Forward primer, nmol/L	300	
Reverse primer, nmol/L	300	
Probe, nmol/L	100	
Primers/probe, μL		1.25
cDNA, ng	100	100
H_2O[a]	Adjust to 25 μL	Adjust to 25 μL

[a] Water was added to adjust the reaction volume to 25 μL.

PCR CONDITIONS

Potential PCR product contaminants in which dTTP is substituted by dUTP were primarily digested by uracil-N-glycosylase *(19)*. The test for contaminating genomic DNA was performed in triplicate, with 100 ng of extracted total RNA processed similar to other samples on the plate. The Ct values did not reveal any amplification of genomic DNA. PCR conditions were optimized for oligonucleotide and probe concentrations.

GENOTYPING

Genomic DNA was isolated from peripheral blood cells by standard procedures. The T/C polymorphism in the first of two start codons (ATG) at the translation initiation site of the *VDR* gene was detected by restriction fragment length polymorphism analysis using the endonuclease *Fok*I. The PCR reaction was carried out in a total volume of 25 μL with the primers 5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′ (forward) and 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′ (reverse) at an annealing temperature of 60 °C for 35 cycles. PCR products were digested with *Fok*I at 37 °C for 3 h and analyzed by agarose gel electrophoresis. Depending on the digestion pattern, individuals were classified as *FF* when they were homozygous for the absence of the *Fok*I site, as *ff* when they were homozygous for the presence of the *FokI* site, or as *Ff* in the case of heterozygosity. Digestion of the amplified 265-bp PCR product gave two fragments, of 69 and 196 bp.

Polymorphism of the 3′-noncoding sequences lying in the intron between exons 7 and 8 of *VDR* was detected by restriction fragment length polymorphism analysis using the endonuclease *Bsm*I. The PCR reaction was carried out in a total volume of 25 μL with the primers 5′-CAACCAAGACTACAAGTACCGCGTCAAGTGA-3′ (forward) and 5′-AACCAGCGGGAAGAGGTCAAGGG-3′ (reverse) at an annealing temperature of 65 °C for 35 cycles. PCR products were digested with *Bsm*I at 37 °C overnight and analyzed by agarose gel electrophoresis. Depending on the digestion pattern, individuals were classified as *BB* when homozygous for the absence of the *Bsm*I site, as *bb* when homozygous for the presence of the *Bsm*I site, or as *Bb* in the case of heterozygosity. Digestion of the amplified 825-bp PCR product gave two fragments, of 650 and 175 bp, respectively.

MEASUREMENT OF PTH AND $1,25(OH)_2D_3$ IN PLASMA

Plasma $1,25(OH)_2D_3$ and intact PTH were determined by radioreceptor assay and IRMA (both from Nichols Institute), respectively. Reference intervals were 10–62 ng/L for plasma PTH and 18–62 ng/L for $1,25(OH)_2D_3$.

STATISTICAL ANALYSIS

Statistical analysis was performed with the Student *t*-test or one-way ANOVA to evaluate significant differences in the data means. Distribution of qualitative variables (means ± SE) was compared in the different groups by the Fisher exact test or the χ^2 test. Statistical analyses were conducted at the $\alpha = 0.05$ level and were two-tailed. All analyses were performed with the SPSS 10 statistical package on a Macintosh G4 computer (Apple Computer).

Results

RELATIVE QUANTIFICATION OF VDR mRNA

To quantify VDR mRNA in leukocytes, we constructed a calibration curve using *GAPDH* mRNA as an endogenous control (GAPDH being a "housekeeping" gene). Shown in Fig. 1 are amplification plots of VDR and GAPDH. Serial dilutions of cDNA from 100 ng to 6.4 pg were tested in triplicate to assess the Ct. The ΔRn value, used for construction of the amplification plots, is the emission of the reporter over the starting background fluorescence. ΔRn was calculated as the difference between Rn^+ and Rn^-. Rn is the normalized reporter, obtained by dividing the emission intensity of the reporter by the emission intensity of the passive reference; Rn^+ refers to samples with templates and Rn^- to samples without templates. Calibration curves for VDR and GAPDH mRNA are shown in Fig. 2, in which the Ct is plotted vs the logarithm of the starting quantity of RNA in pg. The equations for both lines are shown. Leukocyte VDR mRNA is reported as $2^{-\Delta\Delta Ct}$. The cell content of VDR mRNA was normalized by use of an endogenous referent, GAPDH mRNA, and a calibrator. A known quantity of VDR mRNA extracted from a human kidney was used as calibrator. The intra- and interassay CVs of this method were 1.3% (n = 10) and 1.7% (n = 4), respectively.

EFFECT OF $1,25(OH)_2D_3$ ON VDR mRNA CONCENTRATIONS IN BLOOD LEUKOCYTES

Modifications of VDR mRNA concentrations were measured in leukocytes obtained from six healthy individuals 8 h after an oral dose of $1,25(OH)_2D_3$. Results showed a similar increase in VDR mRNA in all individuals (Fig. 3). VDR mRNA concentrations increased to 1.6 (0.1)-fold above the value at time of ingestion. Correspondently, 8 h after the administration of $1,25(OH)_2D_3$, mean (SE) circulating concentrations of $1,25(OH)_2D_3$ were 1.6 (0.3)-fold higher than the concentrations at the time of ingestion (n = 6; $R = 0.645$), as shown in Fig. 3. In one individual, this test was repeated on 4 different days; the intrasubject CV was 6.9%. To determine in more detail the kinetics of the VDR mRNA increase, we measured it at different time points in another group of 10 healthy individuals. This experiment confirmed that $1,25(OH)_2D_3$ ingestion induced an increase of VDR mRNA (Fig. 4). The maximum increase was reached at different times between 1 and 5 h, with a mean (SE) of 3.6 (1.3) h. The maximum increase in VDR mRNA was also quite variable, and ranged from 1.2- to 11.1-fold of the value before $1,25(OH)_2D_3$ consumption.

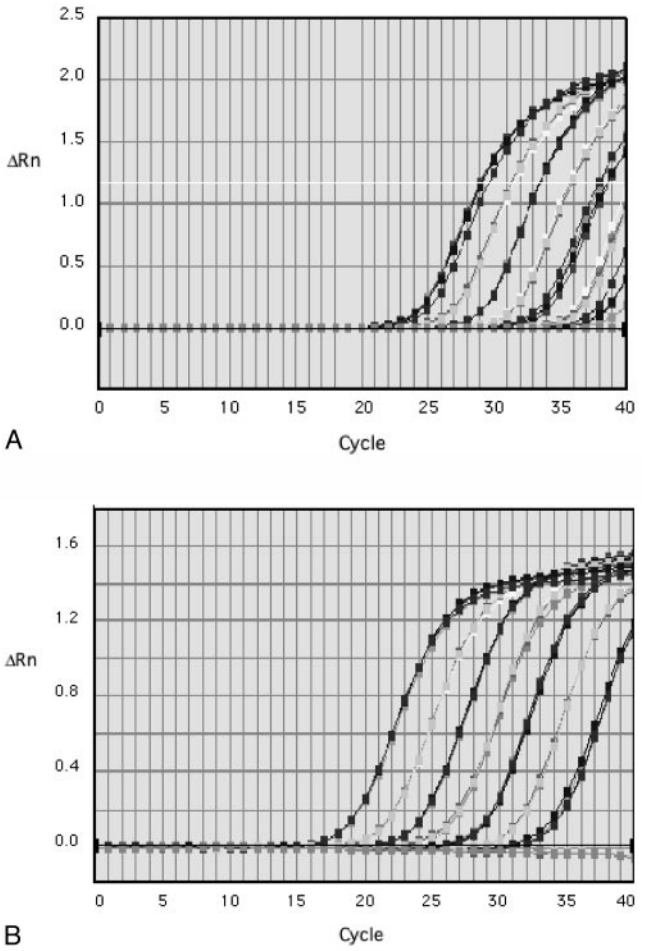

Fig. 1. Amplification plots of hVDR (*A*) and GAPDH (*B*).

Different dilutions of total RNA from leukocytes were tested in triplicate. Amplification plots were used to determine the Ct. The *x axis* represents the PCR cycle number, and the *y axis* represents the mean ΔRn value, i.e., the emission of the reporter over the starting background fluorescence.

HYPERCALCIURIC AND NORMOCALCIURIC STONE-FORMERS

Basal production of VDR mRNA was slightly higher in hypercalciuric than in normocalciuric stone-formers (Table 3). The VDR mRNA concentrations were not related to calcium excretion or to plasma calcium, plasma PTH, or $1,25(OH)_2D_3$ concentrations. Because of the wide range of values, we divided the patients in two groups, using as cutoff the mean value for leukocyte VDR mRNA in all 71 patients [0.410 (0.048) $2^{-\Delta\Delta Ct}$]. Sixteen hypercalciuric patients (43%) and 7 normocalciuric patients (21%) had VDR

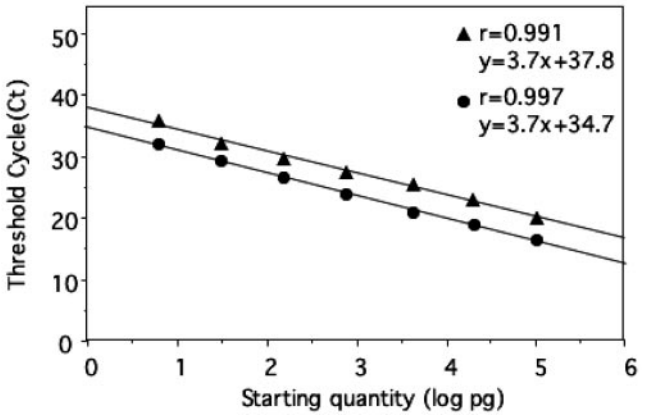

Fig. 2. Calibration curves for hVDR (▲) and GAPDH (●).

RNA was quantified by use of FAM- and JOE-labeled probes, respectively. The *x axis* represents log pg of total starting RNA, and the *y axis* represents the RT-PCR Ct number. *Equations* are derived from the lines and can be used to calculate the starting RNA concentration of an unknown sample.

mRNA above this mean value (Fisher exact test, $P = 0.047$).

The genotype distribution of the patients is shown in Table 4. The frequency of patients with high or low VDR mRNA in leukocytes was not related to the patients' genotypes. Similarly, there was no difference between the mean values for VDR mRNA in patients bearing allele variants at the two loci. No relationship was found when normocalciuric and hypercalciuric patients were analyzed separately.

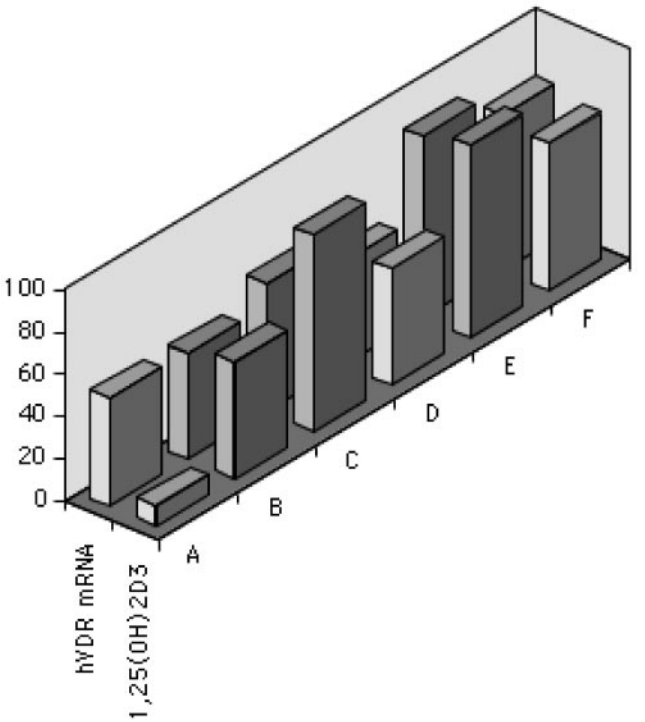

Fig. 3. Individual changes in $1,25(OH)_2D_3$ serum concentrations and hVDR mRNA measured 8 h after oral administration of $1,25(OH)_2D_3$ to six healthy volunteers (*A*–*F*), given as percentage of increase.

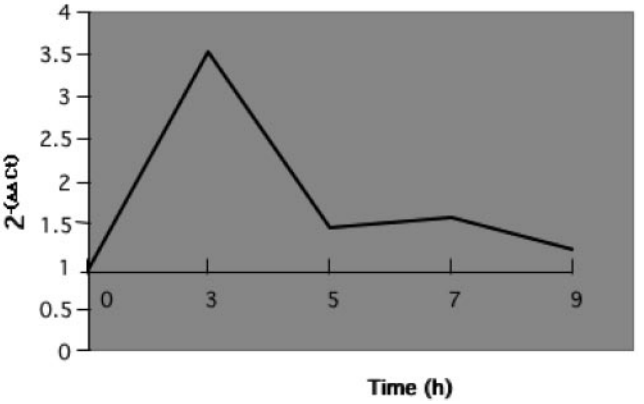

Fig. 4. Example of time course of hVDR mRNA concentrations measured before and after oral administration of $1,25(OH)_2D_3$ to healthy volunteers.

Discussion

In this study we measured the VDR mRNA in leukocytes, applying a single-tube assay for relative measurements *(13, 14)*. Stimulation of leukocyte VDR mRNA production by $1,25(OH)_2D_3$ was tested both in healthy volunteers and in hypercalciuric stone-formers, where a possible increase was investigated. Our findings suggest that VDR mRNA production is stimulated in leukocytes by $1,25(OH)_2D_3$. No endogenous activation of VDR mRNA production was found in individuals with primary hypercalciuria. The stimulation of VDR mRNA should induce an increase of VDR protein production, even if our findings do not give a direct measurement of the increase.

PCR reactions were performed in the presence of two conventional primers for the amplification of the target molecule (VDR) and of an oligonucleotide probe carrying two fluorescent dyes: FAM at the 5′ end, serving as a reporter, and the quencher TAMRA at the 3′ end. During PCR amplification, the *Taq* DNA polymerase displaces the probe from the template and separates the reporter from the quencher; as a consequence, the fluorescence signal increases. The emitted fluorescence is directly proportional to the number of template molecules in the tube. Each cycle further increases the signal, thereby allowing careful monitoring of the reaction kinetics. With this detection system we obtained highly sensitive and reproducible mRNA quantification, as demonstrated by low intra- and interassay CVs for RT-PCR. Therefore, this method can detect slight differences in mRNA production. Relative quantification is a quite common procedure used to compare differences in mRNA targets among different samples. In this case, RT-PCR was applied to the measurement of temporal variations in the concentrations of mRNA molecules (before and after calcitriol administration) and to differences between two groups (normocalciuric and hypercalciuric stone-formers), both compared with an internal control.

A single oral dose of $1,25(OH)_2D_3$ was shown to stimulate VDR mRNA production in leukocytes with a

Table 3. Data for calcium-stone formers.[a]

	Hypercalciuric	Normocalciuric
n	37	34
Plasma calcium, mmol/L	2.35 (0.02)	2.33 (0.02)
$1,25(OH)_2D_3$, ng/L	48 (4)	51 (6)
iPTH,[b] ng/L	33 (3)	37 (3)
Urinary calcium, mmol/24 h	8.98 (0.35)	4.32 (0.29)
Leukocyte VDR mRNA, $2^{-\Delta\Delta Ct}$	0.437 (0.054)	0.381 (0.081)

[a] Results are the mean (SE).
[b] iPTH, intact PTH.

constant response in the same individual. The increase in VDR mRNA production was related to circulating concentrations of $1,25(OH)_2D_3$. Marked variability of results was observed between individuals: this fact could be caused by an intrinsic variability of the $1,25(OH)_2D_3$ intestinal absorption rate. The recorded increase in blood $1,25(OH)_2D_3$ after oral ingestion is consistent with results obtained in a previous study reporting a peak in serum concentration 3 h after oral administration of $1,25(OH)_2D_3$ (1.5 $\mu g/m^2$) *(20)*. Another cause of variability among individuals could be the different degree of immunologic activation of lymphocytes and macrophages, because $1,25(OH)_2D_3$ production is antigen-activated in both cells and could possibly increase VDR production. Finally, variability could be related to genetic and dietary differences between individuals; vitamin D-sensitive cells could modulate the response to vitamin D differently.

The *VDR* gene has been considered a candidate gene for idiopathic hypercalciuria, but no firm association has been found with stone disease or primary hypercalciuria *(21)*. An up-regulation of *VDR* expression has been observed in enterocytes and bone cells of hypercalciuric rats *(9)*. $1,25(OH)_2D_3$ administration gave rise to a further activation of *VDR* expression via a posttranscriptional mechanism, causing increased sensitivity of bone and enteral cells to $1,25(OH)_2D_3$ and high urinary excretion *(9)*. An increased sensitivity to vitamin D has been hypothesized in humans and could increase *VDR* expression in all vitamin D-dependent cells, including lymphocytes and macrophages. In the present work we tested these cell lines as a models of *VDR* expression. Our results show only a slight increase in leukocyte VDR mRNA under baseline conditions and a slight increase in the number of hypercalciuric patients with values above the mean. However, because of the wide range of values both at baseline and after $1,25(OH)_2D_3$ ingestion, our findings do not allow a definite conclusion about the sensitivity to $1,25(OH)_2D_3$, although we did not observe any increase in $1,25(OH)_2D_3$ plasma concentrations in our patients with primary hypercalciuria *(22)*.

Table 4. distribution of genotypes in stone-former patients, according to low or high VDR mRNA content in leukocytes.

	No. of patients (%)	
Genotype	VDR mRNA <0.410 (0.048) $2^{-\Delta\Delta Ct}$ (n = 48)	VDR mRNA >0.410 (0.048) $2^{-\Delta\Delta Ct}$ (n = 22)
BB	11 (23%)	5 (23%)
Bb	23 (48%)	13 (59%)
bb	14 (29%)	4 (18%)
FF	23 (50%)	8 (38%)
Ff	18 (39%)	9 (43%)
ff	5 (11%)	4 (19%)

We studied the genotype distribution previously. We found no differences between normocalciuric and hypercalciuric stone-formers for polymorphisms in the starting site and in the 3′-end region of the *VDR* gene *(23)*. Genetic polymorphisms could lead to more efficient VDR activity by stimulating cellular response to vitamin D. This gain of function was suggested either for allele variants of the 3′-end region located on intron 9, which cause no amino acid change in the VDR molecule, or for the translation starting codon polymorphism, which would produce a VDR molecule three amino acids shorter. Our findings do not support this hypothesis because we did not observe any increase in VDR mRNA in individuals carrying variant alleles at the *VDR* locus. However, further experiments in which VDR production is stimulated are needed to confirm these results.

In conclusion, this research provides evidence for the use of leukocytes as a model to study interactions between $1,25(OH)_2D_3$ receptor and $1,25(OH)_2D_3$ or other calciotropic hormones, such as PTH. The technique used is sufficiently sensitive and accurate to assess increases in mRNA concentrations. We also demonstrated for the first time, to our knowledge, that nuclear receptor gene expression is regulated by the ligand in human leukocytes. Finally, we excluded differences in *VDR* expression under baseline conditions between normocalciuric and hypercalciuric stone-formers and between individuals with different *VDR* genotypes.

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