9-*cis* Retinoic Acid Accelerates Calcitriol-Induced Osteocalcin Production and Promotes Degradation of Both Vitamin D Receptor and Retinoid X Receptor in Human Osteoblastic Cells

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Abstract   Abstract vitamin D receptor (VDR) and retinoid X receptor (RXR) heterodimerize to mediate the genomic actions of $1\alpha,25$-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$, calcitriol), the biologically active form of vitamin D_3. In this study, we show that 9-*cis* retinoic acid (9-cisRA), the ligand for RXR, accelerates calcitriol-induced expression of osteocalcin gene, the marker for mature osteoblasts. Calcitriol and its synthetic analog KH1060 (1 nM) induced osteocalcin secretion after a 96-h incubation period as detected by radioimmunoassay. When these compounds were used together with 9-cisRA, osteocalcin protein secretion was, however, detected already after 72 and 48 h, respectively. Detection of osteocalcin mRNA with quantitative PCR revealed elevated mRNA levels already after a 4-h treatment of the cells with calcitriol, KH1060, or 9-cisRA compared with untreated cells. In combination treatments, 9-cisRA rapidly stimulated osteocalcin mRNA synthesis induced by the different vitamin D_3 compounds. In MG-63 cells treated with calcitriol or KH1060, the stimulation was maximal after the first 4 h and diminished thereafter. In fact, after the 48-h incubation 9-cisRA reduced osteocalcin mRNA levels in KH1060-treated cells, the amount of mRNA being only 44% of the levels obtained with KH1060 alone. The reduction was accompanied by an increased degradation rate of both VDR and RXRβ in the presence of 9-cisRA. Furthermore, 9-cisRA increased the formation of RXRβ–VDR–VDRE complex on the osteocalcin gene VDRE. These results suggest that 9-cisRA accelerates calcitriol-induced osteocalcin production in human osteoblastic cells through increased formation of transcriptionally active chromatin complexes and, subsequently, promotes degradation of the heterodimeric complex of VDR and RXR. J. Cell. Biochem. 89: 1164–1176, 2003. © 2003 Wiley-Liss, Inc.

Key words: calcitriol; analogs; 9-*cis* retinoic acid; osteocalcin; osteoblast

Vitamins A and D exert their effects on cellular growth and differentiation via the classical steroid hormone mechanism using highly specific nuclear receptors and hormone response elements (HREs) on DNA [Chambon, 1996; Whitfield et al., 1999]. The metabolically active form of vitamin D_3, $1\alpha,25$ dihydroxyvitamin D_3 (calcitriol) binds to nuclear vitamin D_3 receptor (VDR) [Baker et al., 1988], whereas the biologically active vitamin A metabolites can use two different families of intranuclear receptors. The retinoic acid receptors (RARs, isotypes α, β, γ) [Zelent et al., 1989] bind the metabolites all-*trans* retinoic acid and 9-*cis* retinoic acid (9-cisRA) whereas the retinoid X receptors (RXRα, β, γ) [Mangelsdorf et al., 1992] are selective for 9-cisRA [Allenby et al., 1993]. All the receptor isotypes also have different isoforms

derived from altered splicing of the primary transcript or use of different promoter start sites. The isotypes are differentially expressed during development and in different organs and cell lines [Mangelsdorf et al., 1992; Chambon, 1996]. In human MG-63 osteoblastic sarcoma cells, both RXRα [Jääskeläinen et al., 1994] and RXRβ [Jääskeläinen et al., 2000] forms are expressed.

The pleiotropic actions of retinoids can be explained in part by the existence of multiple receptors and their responsiveness to the different metabolites of retinoic acid. RXRs can also form homodimers or heterodimers with RARs, which bind to HREs of the DR1 and DR5 type, respectively. In addition, RXRs can heterodimerize with numerous other nuclear receptors, such as VDR, thyroid hormone receptor (TR), and peroxisome proliferator activated receptor (PPAR) (reviewed in Mangelsdorf and Evans, 1995). These heterodimers bind to their specific HREs at the promoter region of hormone-regulated genes and mediate the actions of vitamin D_3, thyroid hormone, and fatty acid derivatives, respectively. Thus, the availability of RXR for heterodimerization is one key step regulating the effects of these ligands. The role of RXR in these heterodimers was originally believed to be a "silent" partner, a structural element needed for proper DNA binding and conformation of the heterodimer. Lately, however, it has been shown that RXR in the heterodimer can bind 9-cisRA and that this ligand can modulate the transcriptional activity of the heterodimer further diversifying the actions of retinoids [Forman et al., 1995; Willy et al., 1995; Westin et al., 1998; Li et al., 2002].

The effects of RXR ligands on calcitriol-induced gene expression have been widely studied in different cell lines and with different expression systems. The overall effect and partly the mechanism behind it have, however, remained unclear. In human keratinocytes or in mouse epidermis in vivo, 9-cisRA and the RXR-specific ligand SR11237 up-regulate calcitriol-dependent transcription [Li et al., 1997]. In rat osteoblast-like ROS 17/2.8 cells, however, 9-cisRA inhibits the calcitriol-activated expression of the endogenous osteocalcin gene and also the transfected osteocalcin VDRE–CAT construct [MacDonald et al., 1993]. It is well known that 9-cisRA induces RXR–RXR homodimer formation [Zhang et al., 1992] and thus reduces the availability of RXR for heterodimerization

with other nuclear receptors possibly resulting in reduced transcriptional activity of their ligands. On the other hand, 9-cisRA modulates conformation of RXR–RAR and RXR–PPAR heterodimers and exposes the AF-2 domain of the RXR moiety for efficient binding of coactivators resulting in increased levels of gene expression compared with the RAR or PPAR ligands alone [Westin et al., 1998]. This was also shown to be true for RXR–VDR by Li et al. [1997], who used an AF-2 domain mutant of RXR. This mutant heterodimerized with VDR and the dimer bound to VDRE. The increase obtained with 9-cisRA in calcitriol-induced gene expression with the wild type receptor was, however, abolished indicating the role for the RXR AF-2 domain in the enhanced transcriptional activity after the 9-cisRA treatment. In this study, we show that, in contrast to the inhibitory effect of 9-cisRA in rat osteoblasts [MacDonald et al., 1993], in human osteoblastic MG-63 cells 9-cisRA accelerates the calcitriol-induced expression of the osteocalcin gene through increased binding of RXR–VDR to the human osteocalcin gene VDRE. This is followed by an increased degradation rate of both VDR and RXR.

MATERIALS AND METHODS

Vitamins and Antibodies

Calcitriol and its derivatives GS1558 (23-thia-aro-1α,25-dihydroxyvitamin D_3) and KH1060 (20-epi-22-oxa-24a,26a,27a-trihomo-1α,25-dihydroxyvitamin D_3) were a kind gift from Dr. Lise Binderup, Leo Pharmaceuticals, Ltd., Ballerup, Denmark. 9-cisRA was a kind gift from Dr. Arthur Levin, Hoffman-La Roche, Inc., Nutley, NJ. The structures of these compounds are shown in Figure 1. The antibody used for VDR detection in Western immunoblotting was the C-terminal antibody sc-1008 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For RXRβ detection, the antibody sc-556 (Santa Cruz Biotechnology, Inc.), which recognizes the RXRβ1 form (previously shown to bind with VDR to the osteocalcin gene VDRE [Jääskeläinen et al., 2000]) was used.

Cell Culture and Nuclear Extracts

Human MG-63 osteoblastic sarcoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 7% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mg/ml
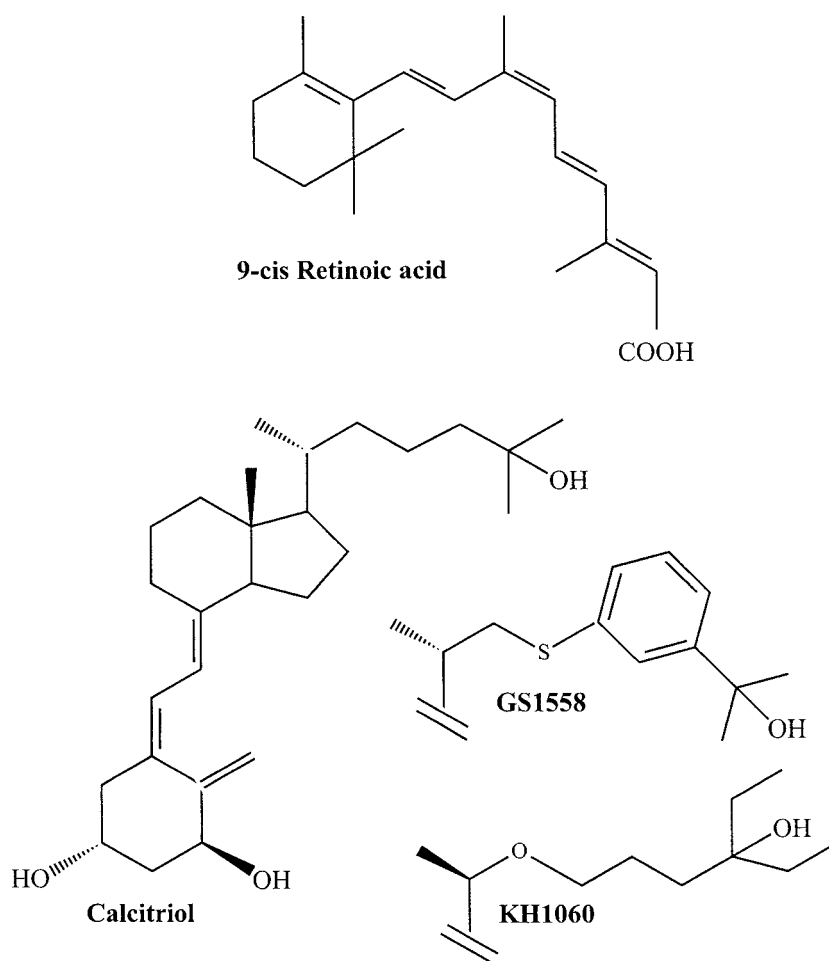
Fig. 1. The structure of 9-cisRA and calcitriol and the side chain structures of the calcitriol analogs GS1558 and KH1060.

streptomycin, and 100 U/ml penicillin at 37°C in a humidified (95% air:5% CO_2) incubator. The treatments were performed in DMEM + 2% charcoal-treated FCS. Cells were harvested at the indicated time points and nuclear extracts were prepared as indicated previously [Hurst et al., 1990] without the heat denaturation step. The lysis buffer was used for the preparation of RNA.

Determination of Cell Numbers and Measurement of Osteocalcin Protein Levels

The MG-63 cells were seeded at 5×10^4 cells/ well onto 6-well-plates 1 day before starting the treatments. Medium samples were collected and used for radioimmunoassay according to the manufacturer's instructions (CIS Bio International, Cedex, France). The cells from the respective wells were trypsinized and quanti-

fied with a Coulter Counter cell counter. The amount of osteocalcin secreted by the cells was calculated as ng/10^5 cells.

## RNA Preparation and Quantitative PCR

The lysis buffer obtained from the preparation of nuclear extracts after centrifugation of the nuclei was extracted twice with phenol:-chloroform:isoamylalcohol and RNA was precipitated with ethanol. For cDNA synthesis, 1 µg of the RNA was denatured for 5 min at 65°C in a buffer containing 50 mM Tris-HCl, pH 8.3, 50 mM KC1, 4 mM $MgCl_2$, and 10 mM dithiothreitol together with 10 U ribonuclease inhibitor, 1 mM each dNTP and 1 pmol of 16-mer oligo(dT) primer. After addition of Moloney Murine leukemia virus reverse transcriptase (10 U), the cDNA synthesis was performed for 1 h at 37°C and the products were denatured for 8 min at 70°C.

An aliquot (2 μl) of the cDNA synthesis product was amplified with quantitative PCR (iCycler, Bio-Rad, Hercules, CA). The reaction mixture contained 5 mM MgCl$_2$, 0.3 nmol each dNTP, 1:50,000 SYBR Green (Molecular Probes, OR), 1 U of TaqDNA polymerase, and 1.3 pmol of each of the specific primers for the human osteocalcin gene (5′-CTCACACTCC-TCGCCCTATT-3′; 5′-GACTGGGGCTCCCA-GCCATT-3′). The PCR protocol consisted of 40 cycles of the following sections: 95°C 30 s, 62°C 30 s, and 72°C 30 s. After completion of the PCR run with 72°C 5 min, the temperature was raised to 95°C and gradually brought down to 60°C to obtain the melt curve. These curves shown in Figure 2 verify the formation of only one product (melting point 91–92°C) during the PCR run.

The threshold cycle (Ct) values, which are the number of cycles needed to produce a certain amount of double-stranded PCR product (determined by the threshold value, see Fig. 3), were used to calculate the fold induction of osteocalcin mRNA production obtained with 9-cisRA using the following formula:

$$\frac{\text{OC mRNA with 9-cisRA}}{\text{OC mRNA without 9-cisRA}} = \frac{2^{\text{Ct without 9-cisRA}}}{2^{\text{Ct with 9-cisRA}}}$$

$$= 2^{\text{Ct without 9-cisRA} - \text{Ct with 9-cisRA}}$$

$$(1)$$
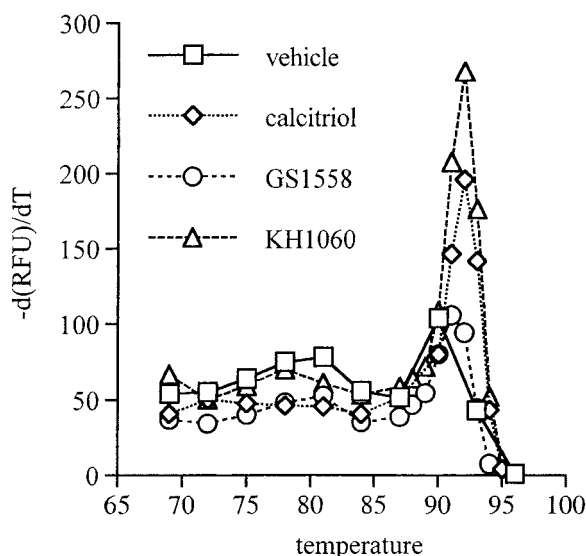
Fig. 2. The melt curve obtained from quantitative PCR. After completion of the PCR run the product was denatured at 95°C and the temperature was gradually brought down to anneal the PCR product formed during the 40-cycle run. The figure shows that there is only one PCR product with an annealing temperature of 91–92°C. RFU = relative fluorescence unit.

## Measurement of VDR and RXR Protein Levels by Western Immunoblotting

Samples (10–20 μg) of the nuclear extracts were boiled for 5 min in SDS-sample buffer containing β-mercaptoethanol and quickly chilled on ice. Proteins were separated on SDS–PAGE gels and electrotransferred onto PVDF-membranes (Roche, Mannheim, Germany). The membranes were blocked with $1 \times$ TBS-0.25% Tween-2% milk powder ($1 \times$ TBS = 140 mM NaCl, 10 mM Tris, pH 7.4) for 30 min at room temperature. The primary antibody was added in TBS-Tween-milk powder and the reaction was carried out overnight at 4°C. The membranes were washed (5×5 min) with TBS-Tween and the secondary antibody was added in TBS-Tween for the next 1 h at room temperature. After washing (4×5 min) and balancing (5 min in 5 mM MgCl$_2$, 0.1 M Tris, 0.1 M NaCl, pH 9.5) the filters, the protein pattern was developed with Sigma Fast™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (Sigma-Aldrich, Steinheim, Germany).

In Vitro Protein Production and Electrophoretic Mobility Shift Assay

Human VDR was prepared in vitro by a coupled wheat germ extract system with hVDR-cDNA inserted into plasmid pSG5 as described by the manufacturer (Promega, Madison, WI). An aliquot of the hVDR was mixed with RXRβ (Affinity Bioreagents, Golden, CO) in a buffer containing 20 mM HEPES, pH 7.6, 4.2% glycerol (vol/vol), 70 mM NaCl, 2.3 mM MgCl$_2$, 2.0 mM EDTA, 2.2 mM dithiothreitol, 80 μM phenylmethylsulphonyl fluoride, 0.3 μg/ml trasylol, 0.3 μg/ml leupeptin, and 2 μg/poly(dI-dC). The proteins were incubated with their ligands for 1 h at room temperature before addition of an end-labelled human osteocalcin gene VDRE (5′-ACCGGGTGAACGGGGGCATTGCG-3′, upper strand). After a 20-min incubation at room temperature, the RXRβ–VDR–VDRE complexes were separated from the free probe on a 5% polyacrylamide gel run in 25 mM Tris/borate, pH 8.3, 0.5 mM EDTA. The complexes were visualized by autoradiography and scanned for statistical analysis.

Statistical Analysis

Statistical analysis was performed by Student's *t*-test for two independent variables using

A

B
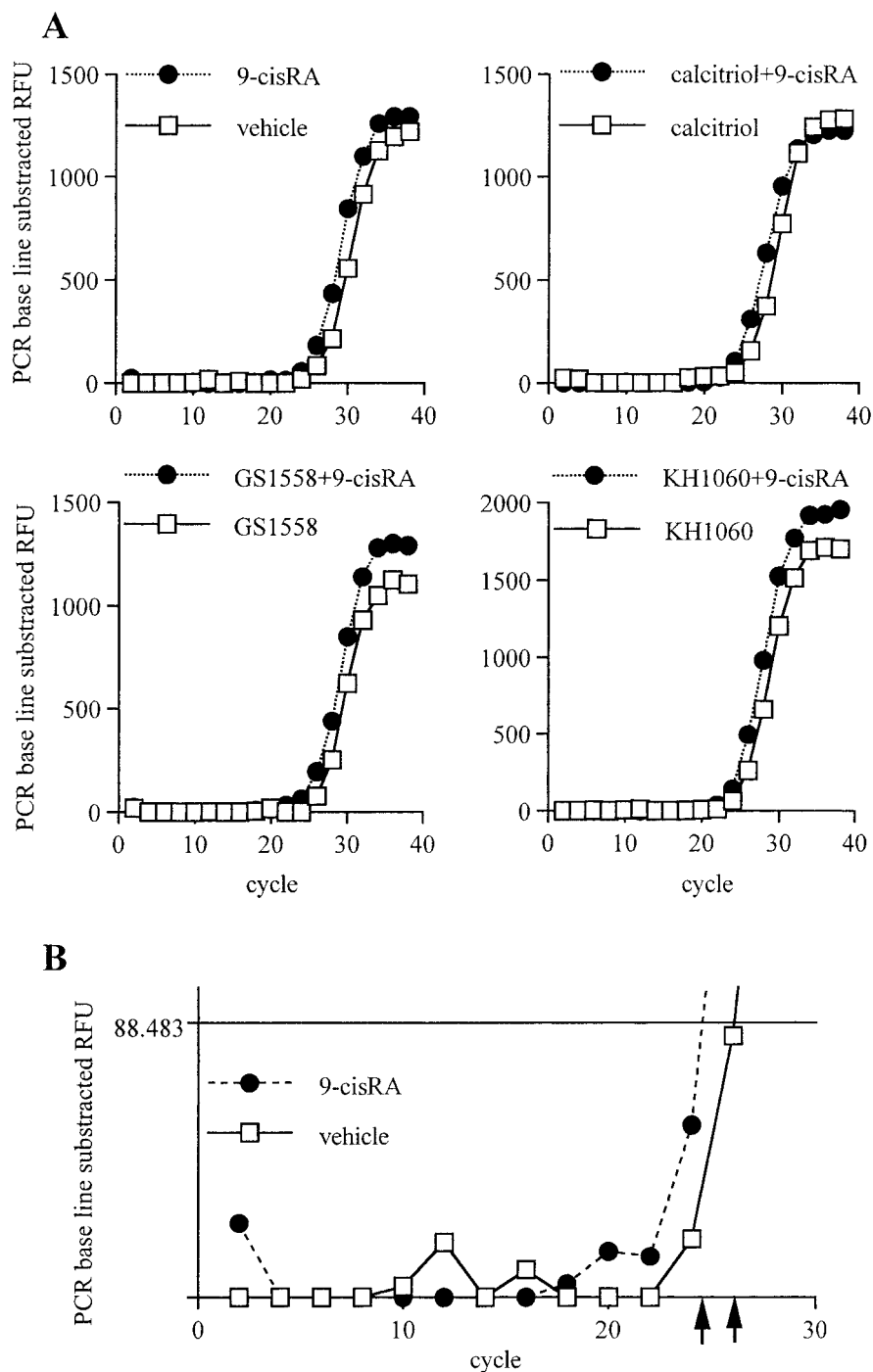
Fig. 3. Osteocalcin cDNA amplification using quantitative PCR. **A:** RNA was prepared from MG-63 cells treated for 4 h with the vehicle (**upper left**), calcitriol (**upper right**), GS1558 (**lower left**), or KH1060 (**lower right**) (1 nM) alone (open squares) or in combination with 9-cisRA (0.1 µM) (filled circles). cDNA synthesis and the quantitative PCR run were performed as indicated in Methods. Figures show the accumulation of the double-stranded PCR products during the 40-cycle amplification. RFU = relative fluorence unit. **B:** Determination of the threshold cycle (Ct). In this PCR run (same as in A, only the vehicle and 9-cisRA treatments are shown), the threshold value was 88.483 RFU. The amount of the double-stranded product detected by SYBR Green fluorescence exceeds this value at the Ct, which is indicated here by arrows for both treatments.

TABLE I. Osteocalcin Protein Production (Mean ± SD, n = 3–6) by the MG-63 Cells After Treatments With Calcitriol, GS1558, or KH1060 (1 nM) Alone or in Combination With 9-cisRA (0.1 µM)

	Osteocalcin (ng/10^5 cells)							
	48		72		96		144	
Time (h) 9-cisRA	−	+	−	+	−	+	−	+
Vehicle	0	0	0	1.4 ± 0.4**	0	1.6 ± 0.1***	0	0.8 ± 0.1***
Calcitriol	0	0	0	3.0 ± 1.4**	1.1 ± 0.4	4.8 ± 1.5*	3.7 ± 0.6	4.0 ± 0.4
GS1558	0	0	0	0	0	0	0	0
KH1060	0	3.1 ± 0.7**	0	5.9 ± 0.5**	1.5 ± 0.1	5.8 ± 1.4*	5.4 ± 1.0	4.9 ± 0.9

Statistical significance for the difference between the combination treatment vs. treatment with the different vitamin D_3 compounds alone; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

a software package (Stat-Works™, Cricket Software Inc., Philadelphia, PA).

RESULTS

9-cisRA Accelerates the Calcitriol-Induced Osteocalcin Secretion

Calcitriol and KH1060 (1 nM) induced osteocalcin secretion after a 96-h incubation period as detected by radioimmunoassay (1.1 ± 0.4 and 1.5 ± 0.1 ng osteocalcin/10^5 cells, respectively) (Table I). When these compounds were used together with 0.1 µM 9-cisRA, osteocalcin protein secretion was, however, detected already at 72 h (3.0 ± 1.4 ng/10^5 cells) and 48 h (3.1 ± 0.7 ng/10^5 cells), respectively. 9-cisRA alone also induced low levels of osteocalcin (1.4 ± 0.4 ng/10^5 cells at 72 h). With calcitriol and KH1060, the amount of osteocalcin was increased throughout the 144-h incubation the maximum values being 3.7 ± 0.6 and 5.4 ± 1.0 ng/10^5 cells, respectively. When used in combination with 9-cisRA (0.1 µM), the highest amount of osteocalcin protein with calcitriol treatment (4.8 ± 1.5 ng/10^5 cells) was obtained already at 96 h and with KH1060 (5.9 ± 0.5 ng/10^5 cells) at 72 h. The analog GS1558 did not induce detectable levels of osteocalcin secretion at 1 nM concentration and the addition of 0.1 µM 9-cisRA had no effect on osteocalcin expression with this analog (Table I).

Detection of Osteocalcin mRNA by Quantitative PCR

The observed stimulation in osteocalcin protein production after the 9-cisRA treatment was accompanied by increased osteocalcin mRNA production already at 4 h as detected by quantitative PCR (Fig. 3). During the PCR run, the accumulation of the double-stranded PCR product is detected using a fluorescent dye, which binds to double-stranded DNA. The results are expressed as relative fluorescence units (RFU) versus the amplification cycle (Fig. 3A). The earlier the product level and the amount of RFU reaches the threshold value (for this run it was 88.483, Fig. 3B), the more template cDNA is in the sample under investigation, and the lower is the Ct value for that sample. The curves obtained from the 9-cisRA-treated cells are shifted to the left compared with curves obtained with the different vitamin D_3 compounds alone indicating lower Ct values and higher osteocalcin mRNA levels in the 9-cisRA-treated samples (Figs. 3A and 4).

The highest amount of osteocalcin mRNA was produced when the cells were treated for 24 h with 9-cisRA plus either calcitriol or KH1060. The Ct values were 18.2 and 17.9 indicating 154- and 187-fold inductions compared with the 4-h vehicle treatment, respectively (Fig. 4A,B). The highest fold-induction by 9-cisRA was obtained when used for 24 h alone or in combination with GS1558 (5.2 and 10.7-fold, respectively) (Fig. 4C). Interestingly, when used in combination with KH1060 or calcitriol, the effect of 9-cisRA was maximal already at 4 h and it diminished thereafter. In fact, the osteocalcin mRNA level obtained after treating the cells with a combination of 9-cisRA and KH1060 for 48 h was only 44% of the level obtained with KH1060 alone (Fig. 4C).

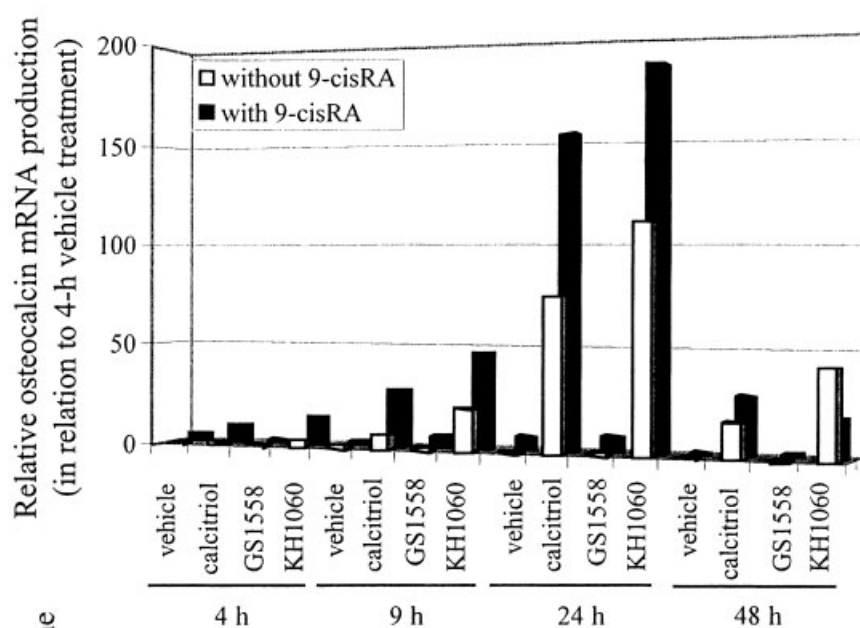### 9-cisRA Promotes Degradation of Both VDR and RXR

To reveal the mechanism of the rapid decline of the effect of 9-cisRA on the transcriptional activity via RXR–VDR we measured protein

A

Ct values (mean ± SD, n = 3-5) of osteocalcin cDNA amplification with quantitative PCR.

Time (h)	4		9		24		48	
9-cisRA	-	+	-	+	-	+	-	+
Vehicle	25.3±0.4	24.5±0.5	25.0±0.3	24.6±0.7	25.1±0.2	22.7±0.1	25.6±0.4	25.0±0.7
Calcitriol	24.5±0.0	21.8±0.4	22.5±0.0	20.9±0.1	19.2±0.2	18.2±0.5	21.2±0.2	20.5±0.2
GS1558	26.3±0.0	24.9±0.6	24.7±0.7	22.9±0.4	26.0±0.2	22.9±0.1	25.7±0.6	24.6±0.4
KH1060	23.6±0.4	21.6±0.4	21.1±0.8	20.0±0.8	18.6±0.0	17.9±0.3	19.9±0.3	21.1±0.2

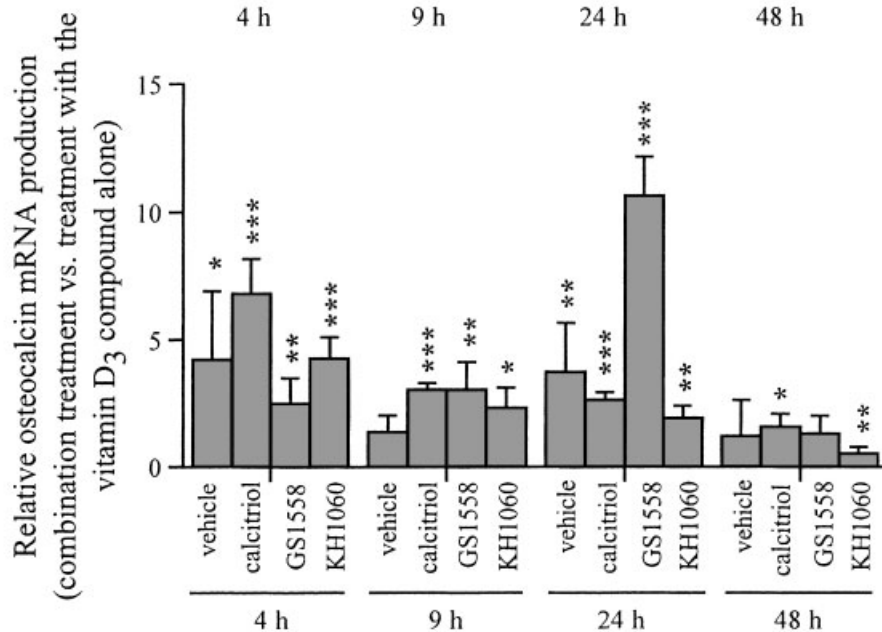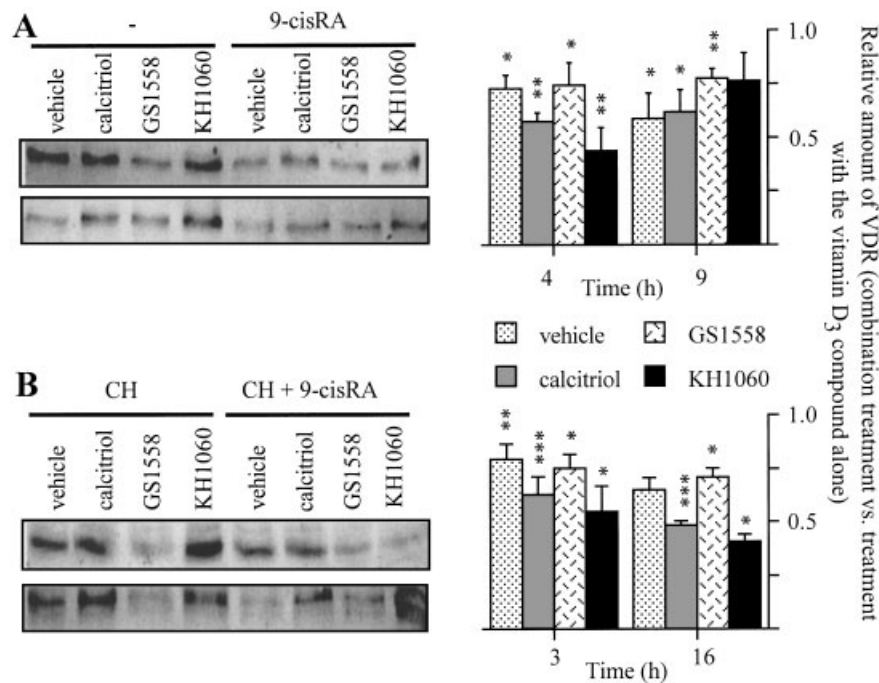(Header: **Ct values**)

B

C

Fig. 4.

Fig. 5. 9-cisRA promotes degradation of VDR. **A**: MG-63 cells were treated with the different vitamin D_3 compounds (1 nM) alone or in combination with 9-cisRA (0.1 μM). **Left**: Western immunoblots from the 4-h (upper gel) and 9-h (lower gel) treatments. **Right**: Western immunoblots shown on the left were scanned and the VDR amounts expressed as combination treatment vs. treatment with the vitamin D_3 compound alone. The bars represent means $\pm$ SD (n = 3–5). **B**: The cells were treated as in A, but in the presence of cycloheximide (10 μg/ml).

Left: Western immunoblots from the 3-h (upper gel) and the 16-h (lower gel) treatments. **Right**: Western immunoblots shown on the left were scanned and the VDR amounts expressed as combination treatment vs. treatment with the vitamin D_3 compound alone. The bars represent means $\pm$ SD (n = 2–5). Statistical significance between combination treatment vs. treatment with the vitamin D_3 compound alone, *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$.

levels of these receptors after the 9-cisRA treatment. Incubation of the cells for 4 h with 9-cisRA reduced the VDR levels to 73% of the control level (Fig. 5A). When used together with the different vitamin D_3 compounds, 9-cisRA reduced the VDR levels to 58, 72, and 40% of the levels obtained with calcitriol, GS1558, or KH1060 alone, respectively. After incubating for 9 h, the proportion was 59% for the vehicle-treated cells and 70, 78, and 81% for the calcitriol, GS1558, and KH1060-treated cells, respectively (Fig. 5A). The decline in the VDR protein levels was found to be due to increased degradation of the receptor in the presence of

9-cisRA, since addition of 9-cisRA to cell culture together with the protein synthesis inhibitor cycloheximide (CH, 10 μg/ml) and the different vitamin D_3 compounds resulted in VDR levels which were 80, 63, 75, and 55% of the levels obtained with vehicle, calcitriol, GS1558, and KH1060 alone, respectively (Fig. 5B). At 16 h, the respective levels were 65, 48, 71, and 41%.

9-cisRA treatment also reduced the RXRβ protein levels (Fig. 6). The reduction was not statistically significant at 4 h but, at 9 h, the levels were reduced to 58, 52, 38, and 45%, respectively, of the levels obtained with vehicle,

Fig. 4. 9-cisRA accelerates osteocalcin mRNA production in MG-63 human osteoblastic sarcoma cells. The cells were treated with the vehicle, calcitriol, GS1558, or KH1060 (1 nM) alone or in combination with 9-cisRA (0.1 μM) for the indicated times. RT-PCR and the quantitative PCR runs were performed as indicated in Materials and Methods. **A**: The Ct values of osteocalcin cDNA amplification with quantitative PCR. **B**: The Ct values shown in A were used to calculate osteocalcin mRNA levels in relation to the

4-h vehicle treatment according to Equation 1. **C**: The Ct values shown in A were used to calculate the fold induction in osteocalcin mRNA production obtained from cells treated with 9-cisRA plus the different vitamin D_3 compounds vs. the vitamin D_3 compounds alone according to Equation 1. Statistical significance between the combination treatment vs. treatment with the vitamin D_3 compound alone, *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$.
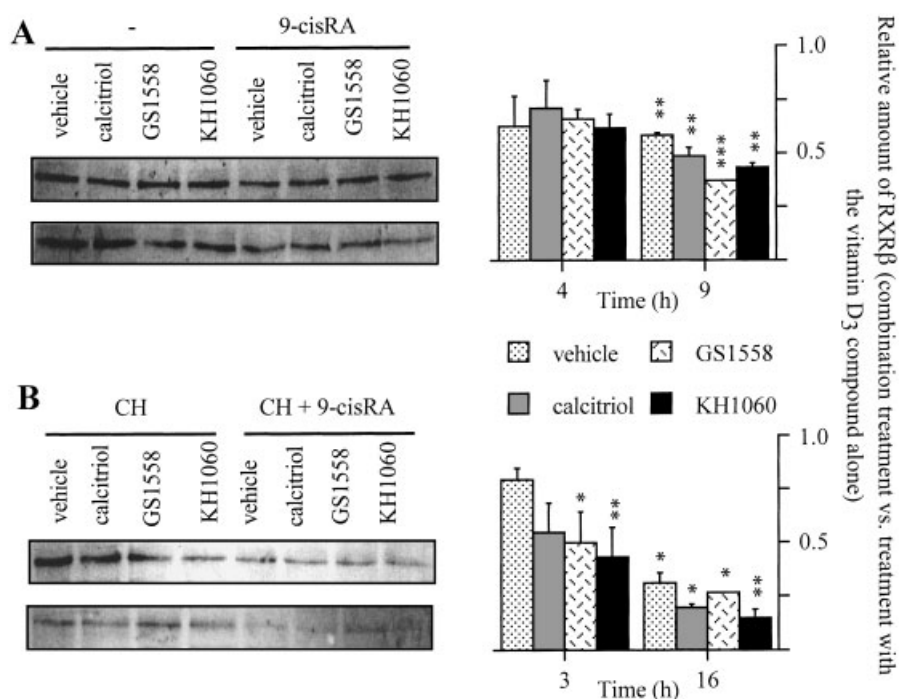
Fig. 6. 9-cisRA promotes degradation of RXRβ1. **A:** MG-63 cells were treated with the different vitamin D_3 compounds (1 nM) alone or in combination with 9-cisRA (0.1 μM). **Left:** Western immunoblots from the 4-h (upper gel) and the 9-h (lower gel) treatments. **Right:** Western immunoblots shown on the left were scanned and the RXRβ1 amounts expressed as combination treatment vs. treatment with the vitamin D_3 compound alone. The bars represent means ± SD (n = 3–5). **B:** The cells were treated as in A, but in the presence of cycloheximide (10 μg/ml).

Left: Western immunoblots from the 3-h (upper gel) and the 16-h (lower gel) treatment. **Right:** Western immunoblots shown on the left were scanned and the RXRβ1 amounts expressed as combination treatment vs. treatment with the vitamin D_3 compound alone. The bars represent means ± SD (n = 2–5). Statistical significance between combination treatment vs. treatment with the vitamin D_3 compound alone, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

calcitriol, GS1558, or KH1060 alone (Fig. 6A). As for VDR, the reduction in the RXRβ protein levels was due to an increased degradation rate of the receptor (Fig. 6B). Cells treated with CH, 9-cisRA, and the different vitamin D_3 compounds for 16 h exhibited only 31, 20, 27, and 15% of the RXRβ protein levels obtained from cells treated with CH and vehicle, calcitriol, GS1558, or KH1060, respectively.

9-cisRA Increases the Formation of RXRβ–VDR Complexes on the Human Osteocalcin Gene VDRE

To study the effects of 9-cisRA on the formation of RXRβ–VDR complexes on the human osteocalcin gene VDRE, we performed electrophoretic mobility shift assays with the VDR and RXRβ proteins. The increase in complex formation by calcitriol was 2.7-fold compared with vehicle treatment (Fig. 7). Addition of 9-cisRA (10 nM, 0.1 μM, or 1 μM) to the reaction mixture further increased complex formation resulting

in 1.3-, 1.4-, and 1.5-fold increases compared with the calcitriol treatment, respectively.

DISCUSSION

In addition to its ability to modulate transcriptional activity of different genes through homodimers, RXR is a dimerization partner for different nuclear receptors including PPAR, RAR, TR, and VDR. It is not only a silent, structural component of the heterodimer but it can also mediate vitamin A action through these heterodimers. This provides an elegant way for the RXR ligands to modulate the effects of other hormones. The regulation of calcitriol-induced gene expression by 9-cisRA through RXR–VDR is currently not quite clear. Both inhibitory [MacDonald et al., 1993; Haussler et al., 1997] and stimulatory [Kato et al., 1995; Li et al., 1997; Sneddon et al., 1998; Zhao et al., 1999] actions of 9-cisRA on calcitriol-induced gene expression have been reported. Further, in

RXRβ-VDR-VDRE
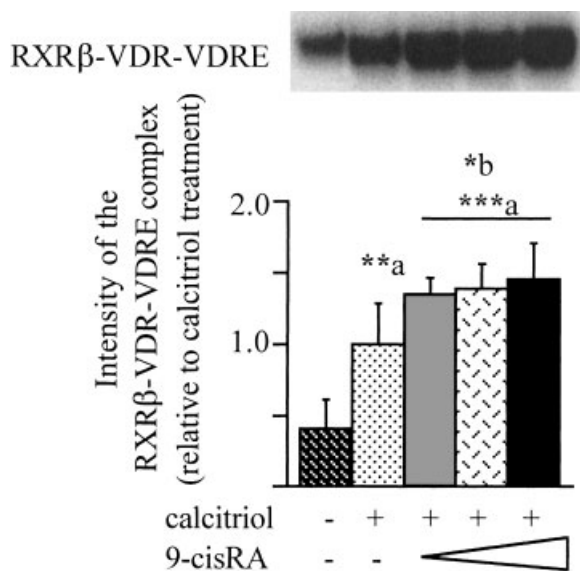
Fig. 7. 9-cisRA increases formation of the RXRβ–VDR–VDRE complex on human osteocalcin gene VDRE. In vitro-produced VDR was allowed to interact with human RXRβ protein alone or in the presence of 0.1 μM calcitriol and increasing amounts of 9-cisRA (10 nM, 0.1 μM, and 1 μM). The complexes were run on native polyacrylamide gel and visualized by autoradiography. The complexes were scanned and the results are shown in relation to calcitriol treatment. The bars represent means $\pm$ SD (n = 3–6). Statistical significance between vehicle vs. calcitriol or calcitriol plus 9-cisRA treatment (a) and between calcitriol vs. combination treatment (b), $*P \leq 0.05$, $**P \leq 0.005$, $***P \leq 0.001$.

some cases, 9-cisRA had no effect on calcitriol-induced gene expression [Ferrara et al., 1994; Quelo and Jurdic, 2000]. In this study, we used quantitative PCR method to show that, in human osteoblastic cells, 9-cisRA efficiently accelerates osteocalcin mRNA production induced by calcitriol or KH1060. When 9-cisRA was used alone or with the transcriptionally weak calcitriol analog GS1558 it also induced osteocalcin mRNA production over the levels obtained from control or GS1558-treated cultures. Osteocalcin production in cells treated with 9-cisRA alone may have resulted from residual calcitriol activity in the cell culture system.

The discrepancies in the 9-cisRA effects obtained in different cellular contexts may have resulted from different pools of coactivators expressed in different cell lines. Recently, a coactivator was recognized, which specifically and 9-cisRA-dependently binds to the RXR moiety of the heterodimer [Monden et al., 1999]. The inhibitory action of 9-cisRA on the

transcriptional activity of its heterodimeric partner can be in part explained by increased formation of RXR–RXR homodimers in the presence of 9-cisRA, leaving less free RXR available for heterodimer formation [Zhang et al., 1992]. It has also been shown that, in the absence of its ligand, RXR forms homotetramers, which are effectively disrupted by 9-cisRA. At this stage, RXR is available either for homodimer formation or, in the presence of its liganded heterodimeric partner, also for heterodimerization [Kersten et al., 1995; Dong and Noy, 1998]. Thus, maximal amounts of heterodimers are formed in the presence of both 9-cisRA, which breaks down homotetramers, and the ligand for the heterodimeric partner, which recruits RXR for heterodimers and in part inhibits RXR–RXR homodimer formation [Dong and Noy, 1998]. In addition, heterodimerization of VDR with RXR efficiently promotes nuclear localization of VDR rendering the receptor available for transcriptional events [Prüfer et al., 2000].

The first report on possible mechanisms resulting in synergistic effects of the ligands for RXR heterodimers was by Westin et al. [1998]. They showed with RXR, RAR, and PPARγ that the addition of RXR-specific ligand to the reaction mixture together with RAR or PPARγ ligand modulates the structure of the heterodimer and reveals the AF-2 domain of RXR for efficient binding of coactivators to the transactivation complex. The importance of the AF-2 domain of RXR in the synergistic transcriptional activation by 9-cisRA and calcitriol was revealed by Li et al. [1997], who used an AF-2 domain mutant form of RXR together with VDR. This mutant receptor bound VDR and the resulting heterodimer bound to DR3 DNA, but the increase in calcitriol-induced transcription obtained with an RXR-specific ligand was lost. In the present study, 9-cisRA efficiently accelerated calcitriol- and KH1060-induced expression of the human osteocalcin gene and also resulted in higher osteocalcin levels than those obtained from cultures treated with the vitamin D_3 compounds alone. Further, 9-cisRA was shown to increase the formation of the RXRβ–VDR–VDRE complex on the osteocalcin gene VDRE. Thus, the increase in osteocalcin production may be due to increased availability of RXR for RXR–VDR heterodimer formation, increased binding of the RXR–VDR to osteocalcin gene VDRE, and exposure of the RXR AF-2

domain for binding of coactivators as explained above.

The subsequent decline in the 9-cisRA response led us to explore the amounts of RXR and VDR in the presence of 9-cisRA. Ligands of RXR stimulate degradation of the receptor [Nomura et al., 1999; Osburn et al., 2001], whereas calcitriol and its transcriptionally active analogs effectively protect VDR against proteasomal degradation [van den Bemd et al., 1996; Masuyama and MacDonald, 1998; Li et al., 1999; Jääskeläinen et al., 2000]. This feature of VDR is quite unique among the nuclear receptors, since, in addition to RXR, degradation of, e.g., PPAR [Hauser et al., 2000], RAR [Kopf et al., 2000], and TR [Dace et al., 2000] is induced by their respective ligands. In this study, 9-cisRA promoted the degradation of VDR in the presence of VDR ligands. This effect was strongest with KH1060 which, in turn, resulted in highest transcriptional stimulation. The stimulation of VDR degradation in the presence of 9-cisRA suggests that the whole transcriptionally active heterodimeric complex was degraded, possibly after the transactivation event, as has been previously shown for PPAR [Hauser et al., 2000], RAR, and TR [Osburn et al., 2001]. The transcriptional activity of RXR was, however, not needed for the degradation of the receptor, since some transcriptionally weak mutant forms of the receptor were exposed to degradation by binding of the ligand, whereas some others were not [Osburn et al., 2001]. For some calcitriol analogs, a correlation has been found for prolonged transcriptional activity and VDR half-life due to stabilization of the receptor by its ligand. From the analogs used here, the transcriptionally weak calcitriol analog GS1558 induces a rapid degradation of VDR whereas the transcriptionally active KH1060 stabilizes the VDR [Jääskeläinen et al., 2000; Mäenpää et al., 2002].

Treatment of the cells with different degradation-inducing ligands of RXR heterodimeric partners, e.g., all-*trans*-retinoic acid (ligand for RAR) and thyroid hormone (ligand for TR), also leads to degradation of RXR [Osburn et al., 2001]. Similarly, treatment of the MG-63 cells with calcitriol or its synthetic analogs increased the degradation rate of RXRβ in this study. The modulation of RXR protein levels by calcitriol is most likely restricted to the RXR molecules present in the heterodimeric RXR–VDR com-

plexes. In the presence of calcitriol, the proportion of RXR in these heterodimers is apparently higher than without calcitriol [Cheskis and Freedman, 1994; Dong and Noy, 1998], and those RXR molecules are likely to be degraded more efficiently than the unliganded RXR–RXR homodimers or tetramers.

Our study shows the time-course of acceleration of calcitriol-induced gene expression by 9-cisRA in human ostoblastic bone cells. The stimulation was found to be due, in part, to increased formation of RXR–VDR on osteocalcin gene VDRE. The following attenuation of the 9-cisRA effect was accompanied by degradation of both the VDR and RXR, probably after the transcriptional event. This suggests that 9-cisRA accelerates RXR and VDR turnover through formation of transcriptionally active chromatin complexes. The exact degradation mechanisms for the different heterodimeric partners remain to be elucidated.

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