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Zinc Increases the Activity of Vitamin D-Dependent Promoters in Osteoblasts

Ward Lutz,* Mary F. Burritt,† David E. Nixon,† Pai Chih Kao,† and Rajiv Kumar*,‡,§
**Nephrology Research Unit, ‡Department of Medicine, §Department of Biochemistry and Molecular Biology, and †Department of Laboratory Medicine and Pathology, Mayo Clinic and Foundation, 200 First Street SW, Rochester, Minnesota 55905*

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Zinc modulates the structure and binding of the DNA binding domain of the 1α,25-dihydroxyvitamin D_3 receptor to specific vitamin D response element DNA (*Nature Biotechnology* 16, 262–266, 1998). To determine whether zinc alters 1α,25-dihydroxyvitamin D_3-regulated genes in cells, we permanently transfected rat osteoblasts with two vitamin D-dependent promoter-reporter systems and examined their responses to 1α,25-dihydroxyvitamin D_3 in the presence of increasing amounts of extracellular zinc. When extracellular zinc concentrations were increased in the presence of 1α,25-dihydroxyvitamin D_3, there was an increase in the activity of 1α,25-dihydroxyvitamin D_3-dependent promoters with increasing concentrations of zinc. The effect was specific for zinc since metals such as copper failed to increase the activity of 1α,25-dihydroxyvitamin D_3-dependent promoters. The concentration of the vitamin D receptor within the cell and the affinity of 1α,25-dihydroxyvitamin D_3 for its receptor remained unchanged with added zinc. Our results show that zinc increases the activity of 1α,25-dihydroxyvitamin D_3-dependent promoters in osteoblasts.

Zinc (Zn^{2+}) is an important regulator of biological functions. It is a constituent of various enzymes and proteins such as alkaline phosphatase, lactate dehydrogenase and carbonic anhydrase, steroid hormone receptors, and transcription factors (1, 2). In the absence of adequate dietary Zn^{2+}, growth failure, hypogonadism, impaired wound healing, and decreased taste and smell occur. Zn^{2+} deficiency is associated with bone loss and zinc supplementation is associated with increases in bone mass and osteoblast DNA synthesis (3–5).

Zn^{2+} is an important constituent of steroid hormone receptors and the metal is bound by the DNA binding domains of such receptors in two "zinc-fingers" in which Zn^{2+} is coordinated by four cysteine residues. The biologically active, hormonal form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), plays a critical role in calcium and phosphate homeostasis and ensures the deposition of bone mineral. 1α,25$(OH)_2D_3$ binds to a ligand-dependent steroid hormone nuclear transcription factor, the 1α,25$(OH)_2D_3$ receptor (vitamin D receptor or VDR) (6, 7) and the 1α,25$(OH)_2D_3$-VDR complex controls gene expression by associating with specific DNA response elements (VDREs) in target genes (8).

Using mass spectrometry and circular dichroism spectroscopy, our group recently showed changes in the conformation of the DNA-binding domain of the VDR (VDR DBD) upon the binding of Zn^{2+} (9, 10). A shift in charge states was correlated with changes observed in the far-ultraviolet circular dichroic (far-UV CD) spectrum of the protein as it was titrated with Zn^{2+}.

We also showed that the VDR DBD bound to the appropriate DNA sequence or vitamin D response element only when bound to 2 moles of Zn^{2+} per mole of protein. Further increases in Zn^{2+} sufficient to give a stoichiometry of 4 moles of Zn^{2+} per mole of protein increased protein-DNA complex formation (11). Additional binding of Zn^{2+} (>4 moles Zn^{2+}/mole protein) by the VDR caused the protein to dissociate from the dsDNA.

Given the observed alterations in the structure and DNA binding properties of the VDR by changes in its zinc content, we asked if changes in the intracellular zinc content of cells would effect their response to 1α,25$(OH)_2D_3$. Rat osteosarcoma cells (ROS 17/2.8 cells) were stably transfected with a luciferase reporter plasmid driven either by a 25-dihydroxyvitamin D_3 24-hydroxylase promoter or osteopontin promoter vitamin D response elements. Increasing the zinc content of these cells increased their responsiveness to 1α,25$(OH)_2D_3$.

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METHODS

General. Vitamin D_3, 1α-hydroxyvitamin D_3 (1α(OH)D_3), 25-hydroxyvitamin D_3 (25(OH)D_3), 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2D_3$), 24*R*,25-dihydroxyvitamin D_3, (24,25(OH)$_2D_3$), 25*S*,26-dihydroxyvitamin D_3 (25,26(OH)$_2D_3$) were obtained from Dr. Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ). UV spectra of the vitamin D analogs in ethanol were obtained using a Beckman Du70 spectrophotometer (Beckman Instruments, Fullerton, CA). Protein was measured using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Oligonucleotides were synthesized in the Mayo Molecular Biology Core Facility.

Cell culture. Rat sarcoma (ROS 17/2.8) (12) cells were grown in F12/Ham's medium (GIBCO-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) and kanamycin (100 μg/mL). Stock cultures of ROS 17/2.8 cells were maintained in T-75 culture flasks (Falcon). Cells were lifted and suspended by trypsinization.

Construction of reporter plasmids. A 158 base pair fragment of the herpes simplex virus thymidine kinase promoter (−216 to −58) was subcloned into the BglII/HindIII site of the pGL3-basic luciferase reporter vector (Promega, Madison, WI). To prepare the osteopontin vitamin D response element reporter plasmid (pPON.VDRE.tk-GL-3) a DNA fragment containing three mouse osteopontin gene VDREs AA**GGTTCA**CGA**GGTTCA**CGCATGCATGATAA**GGTTCA**CGA**GGTTCA**CGCATGCATGATAA**GGTTCA**CGA**GGTTCA** was cloned into the SacI/BglII site of the tk-GL3 plasmid. To prepare the 24-hydroxylase promoter luciferase reporter plasmid (pMAMneoLUC.24-OHPro), the rat 24-hydroxylase promoter (−1369 to −1) was obtained from rat genomic DNA by the polymerase chain reaction (Expand High Fidelity PCR System, Boehringer-Mannheim, Indianapolis, IN). The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). The 24-hydroxylase promoter insert was excised from the pCR2.1-TOPO vector and blunt ligated into the NheI site of the luciferase reporter plasmid pMAMneoLUC (Clontech, Palo Alto, CA). The identity of inserts in pPON.VDRE.tk-GL3 and pMAMneoLUC.24-OH.Pro were confirmed by dideoxy DNA sequencing (13).

Cell transfection. ROS 17/2.8 cells were seeded into a 100 mm culture dish (5.5×10^5 cells per dish) and transfected 36 h later with 4 μg pPON.VDRE.tk-GL3 DNA and 1 μg pSV2neo (Invitrogen) or 4 μg pMAMneoLUC.24-OH.Pro DNA using Lipofectamine (GIBCO-BRL, Gaithersburg, MD). Stably transfected cells were selected in the presence of the antibiotic G-418 (300 μg/mL) and tested for luciferase activity in response to 1α,25(OH)$_2D_3$.

Luciferase assay. Stably transfected cells were seeded into 24-well culture plates (18,000 cells/well) and allowed to attach for 12 to 24 h. Culture medium (F-12/Ham's medium containing 10% FBS, 100 μg/mL kanamycin and 300 μg/mL G-418) was exchanged with medium in which FBS was replaced by 0.1% ITS (Collaborative Biomedical Products, Bedford, MA) and cells were returned to the CO_2-incubator for 12 to 16 h. Medium was removed and cells were washed with zinc-free Ham's culture medium (GIBCO-BRL). Cells were treated with 1α,25(OH)$_2D_3$ or ethanol and various concentrations of $ZnCl_2$ or $CuSO_4$ in zinc-free F-12/Ham's medium containing 0.1% ITS, 100 μg/mL kanamycin and 300 μg/mL G-418 for 48 h. Cells were then washed three times with phosphate-buffered saline (1 mL/well) and lysed. Lysates were transferred to 1.5 mL microfuge tubes, frozen with dry ice and stored at −70°C.

Cell lysates were thawed, vortexed and centrifuged at 14,000*g* for 2 min. Luciferase activity was measured in 20 μL aliquots of the supernatant using Promega's Luciferase Assay System and the Turner Designs TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The protein content of the supernatant was measured. Luciferase activity was expressed as counts per second per μg protein.

To measure the response of ROS 17/2.8 cells stably transfected with the reporter plasmid pPON.VDRE.tk-GL3, to various vitamin D_3 analogs [1α,25(OH)$_2D_3$; 24*R*,25(OH)$_2D_3$; 25*S*,26(OH)$_2D_3$; vitamin D_3, 25OHD_3, 1α(OH)D_3], cells were seeded into 96-well clear bottomed microplates, and allowed to attached for 16 h. Vitamin D_3 analog standards in ethanol were added in a volume of 1 μL to wells containing 100 μL culture medium and incubated at 37°C for 20 h. Luciferase activity was measured using the LUCITE Luciferase Reporter Gene Assay Kit and the Top Count detection system (Parkard Instr. Co., Meriden, CT).

Measurement of the zinc content of cells. Zinc analyses were performed using both inductively coupled plasma-mass spectrometry (ICP-MS; Perkin-Elmer Model 6100 mass spectrometer, Wilton, CT) and inductively coupled plasma optical emission spectroscopy (ICP-OES; Perkin Elmer Model Optima 3300, Wilton, CT). The techniques were used interchangeably. Yttrium was used as the internal standard for ICP-OES while gallium was used as the internal standard for ICP-MS.

Zinc, yttrium and gallium stock standards containing 1000 μg/mL were purchased from High Purity Standards (Charleston, SC). For aqueous or liquid samples, Bio-Rad Lyphocheck Quantitative Urine Control, Level 1 (Bio-Rad ECS Division, Anaheim, CA) was used to assure correct zinc calibration. For cellular samples, National Institute of Standards and Technology (NIST, Gaithersburg, MD) Bovine Liver 1577A was assayed. A paste of the material was prepared, dried, and digested along with the cellular samples in the same type of vials as the cellular samples. This material is certified for zinc content.

Washed cell pellets were delivered to the laboratory in conical snap top tubes. Bovine liver paste was placed into a cleaned pre-weighted snap top tube. All samples were placed in an oven at 99°C for at least 4 h. Dried bovine liver samples were reweighed so that the total weight of bovine liver could be determined. All vials were treated with 100 μL of nitric acid, then digested in a microwave oven. Microwave power was applied at full power for 5 to 7 s intervals until all bovine liver was dissolved. 400 μL deionized water was added to each vial and mixed thoroughly with the digest. Blank vials were also digested so that any concomitant zinc could be subtracted from the bovine liver and cell pellet results. All cell pellet analyses were performed in duplicate. Cell pellet zinc was reported as total zinc.

Western blotting for detection of 1α,25(OH)$_2D_3$ receptor. This was performed using 1α,25(OH)$_2D_3$ (10^{-8} M) or vehicle (ethanol) treated cells grown in the presence of the indicated concentrations of $ZnCl_2$ for 48 h. The cells were rinsed twice with PBS and harvested by scraping. Cells were lysed in lysis buffer (150 mM NaCl, 50 mm HEPES, pH 7.25, 0.1% NP-40, 1% Triton X-100) containing 1 mM PMSF, 1 μg/ml pepstatin A and 5 μg/ml leupeptin. Thirty μg of lysate protein per well was electrophoresed through a 12% SDS-polyacrylamide gel and transferred to PVDF membrane. After blocking with 1% blocking solution (BM chemiluminescence blotting substrate, Boehringer Mannheim) in TBS, and treatment with human VDR anti-serum (14) for 1 h at room temperature, the blot was washed with TBS-Tween and incubated with goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase (DAKO Corp., Carpinteria, CA). The blot was developed with chemiluminescence reagents (BM chemiluminescence blotting substrate, Boehringer Mannheim) and immunoreactive bands on autoradiograms were analyzed using Kodak Digital Science ID image analysis software (Eastman Kodak Co., Rochester, NY).

1α,25(OH)$_2D_3$ binding assay. Binding of the 1α,25(OH)$_2D_3$ to the 1α,25(OH)$_2D_3$ receptor was carried out as described (15). Calf thymus was used as a source of vitamin D receptor. Dissociation constants (K_d) were determined by Scatchard analysis (16). Assay tubes were prepared in duplicate.

RESULTS

Vitamin D Response Element Activation and Extracellular Zinc

To monitor transcriptional activation by 1α,25(OH)$_2D_3$ in cells we developed two cell lines stably transfected

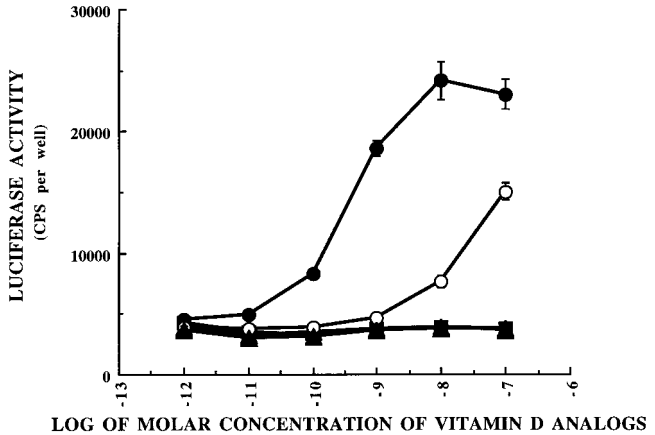

FIG. 1. Activation of the osteopontin promoter vitamin D response element in ROS 17/2.8 cells by various vitamin D analogs. ROS 17/2.8 cells stably transfected with the luciferase reporter plasmid pPON.VDRE.tk-GL3 were seeded into 96-well culture plates, allowed to attach, and exposed to various vitamin D_3 analogs (■, 24*R*,25-dihydroxyvitamin D_3; ◆, 25*S*,26-dihydroxyvitamin D_3; △, vitamin D_3; ●, 1α,25-dihydroxyvitamin D_3; ○, 1α-hydroxyvitamin D_3; ▲, 25-hydroxyvitamin D_3) at the indicated concentrations for 20 h at 37°C. Luciferase activity was measured as described under Methods. Luciferase activity is expressed as counts per second per well. Values are mean (±SD) of four observations.

with a luciferase reporter plasmid in which luciferase expression is driven by a cassette of three mouse osteopontin VDREs or the complete promoter for the human 24-hydroxylase gene. These cell lines are distinct in that the first contains VDREs in absence of other promoter elements, while the seconds contains a VDRE in the context of a complete promoter containing other regulatory elements. In these cells, VDRE activation induces synthesis of luciferase whose activity can be monitored by the production of a luminescent product. These cell lines demonstrate selective and sensitive responses to vitamin D_3 analogs. Figure 1 shows responses obtained with ROS 17/2.8 cells transfected with the osteopontin VDR-reporter plasmid. Maximal 1α,25$(OH)_2D_3$ response is apparent at 10^{-8} M and a half-maximal response is observed at $\sim 10^{-9}$ M. With the exception of 1α(OH)D_3, other vitamin D analogs showed minimal response over the dose range tested. Similar responses were seen with the 25-hydroxyvitamin D_3 24-hydroxylase promoter reporter transfected cells (data not shown).

We next used these cell lines to assess the role of zinc in modulating 1α,25$(OH)_2D_3$ transcriptional regulation. Cells grown in monolayer were exposed to a range of concentrations of $ZnCl_2$ which were above and below the normal serum levels of Zn^{2+} (10 to 15 μM). Prior to exposure to $ZnCl_2$, cells were acclimated to serum-free medium to establish a basal response. Cells were simultaneously exposed to $ZnCl_2$, and 10^{-8} M 1α,25$(OH)_2D_3$ or ethanol, and luciferase activity measured after 48 h. Exposure of cell lines to $ZnCl_2$ concentrations of 20 μM and above resulted in significantly higher 1α,25$(OH)_2D_3$ responses in both cell lines (Figs. 2 and 3). Basal luciferase activity in the absence of 1α,25$(OH)_2D_3$ increased slightly at higher concentrations of Zn^{2+}.

Effects of Copper on the Activity of the Vitamin D-Dependent 24-Hydroxylase Promoter

To assess the specificity of Zn^{2+} in modulating VDRE activation, we compared its effect to that of copper (Cu^{2+}) which has overlapping biological effects with Zn^{2+}. Cells stably transfected with the luciferase reporter plasmid driven by the 24-hydroxylase promoter were used in this experiment. Exposure of cells to 30 μM $CuSO_4$ minimally enhanced the response to 1α,25$(OH)_2D_3$; the increase was much less than that observed with 30 μM $ZnCl_2$ (Fig. 4). As noted previously, basal luciferase activity was slightly increased by exposure to extracellular $ZnCl_2$ (Figs. 2 and 3). $CuSO_4$ and $ZnCl_2$ had similar effects on basal luciferase activity, suggesting this effect is non-selective (Fig. 4). Comparison of the effects of Zn^{2+} and Cu^{2+} on VDRE activation in the absence and presence of 1α,25$(OH)_2D_3$ indicate Zn^{2+} is selectively modulating 1α,25$(OH)_2D_3$ transcriptional regulation.

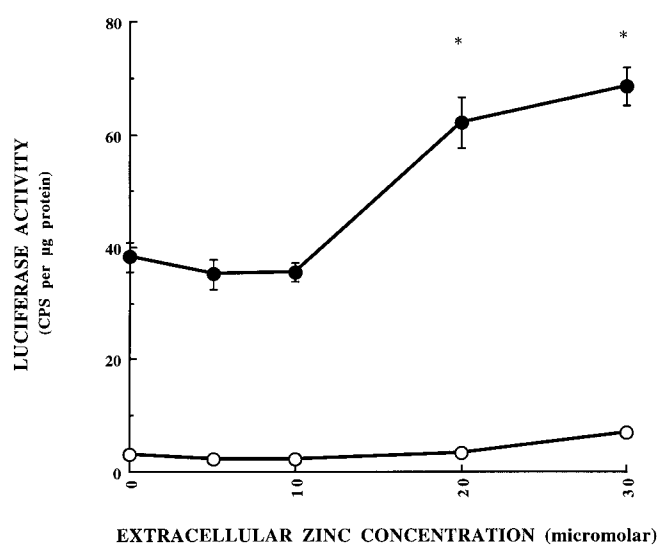

FIG. 2. Effect of extracellular zinc on osteopontin promoter vitamin D response element activity in ROS 17/2.8 cells. ROS 17/2.8 cells stably transfected with the luciferase reporter plasmid, pPON.VDRE.tk-GL3, were exposed to the indicated concentrations of $ZnCl_2$ and 1α,25$(OH)_2D_3$ (10^{-8} M, ●) or ethanol (○) for 48 h and luciferase activity measured as described under Methods. Luciferase activity is expressed as counts per second per μg protein. Values represent mean of 5 observations (±SD). The Fisher test for multiple comparisons indicated that luciferase activity at extracellular $ZnCl_2$ concentrations of 20 μM and 30 μM was significantly higher ($^*P < 0.0001$) than luciferase activity in the absence of extracellular $ZnCl_2$.

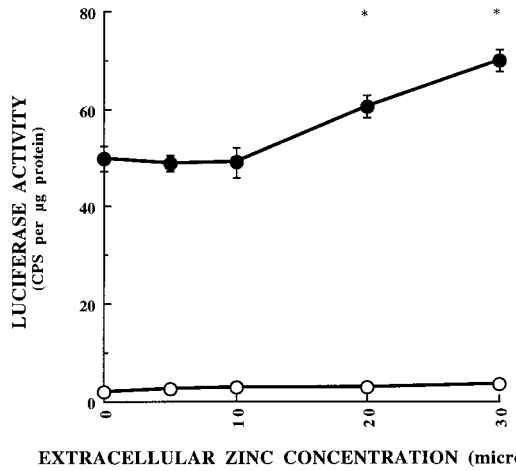

FIG. 3. Effect of extracellular zinc on 24-hydroxylase promoter activity in ROS 17/2.8 cells. ROS 17/2.8 cells stably transfected with the luciferase reporter plasmid pMAMneoLUC.24-OH.Pro were exposed to the indicated concentrations of $ZnCl_2$ and $1\alpha,25(OH)_2D_3$ (10^{-8} M, ●) or ethanol (○) for 48 h and luciferase activity measured as described under Methods. Luciferase activity was expressed as counts per second per μg protein. Values represent the mean of 5 observations (±SD). The Fisher test for multiple comparisons indicated that luciferase activity at extracellular $ZnCl_2$ concentrations of 20 μM and 30 μM was significantly higher ($^*P < 0.0001$) than luciferase activity in the absence of extracellular $ZnCl_2$.

Vitamin D Response Element Activation and Cellular Zn^{2+} Content

Given the ability of cells to resist changes in their zinc content, we measured intracellular zinc content as a function of extracellular $ZnCl_2$ concentration and correlated it with VDRE activation by $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ stimulated luciferase activity in pPON.VDRE.tk-GL3 transfected cells, correlated well with their intracellular Zn^{2+} content (Fig. 5). Cells containing the 24-hydroxylase promoter driven luciferase reporter plasmid showed a similar correlation (Fig. 6). These results indicate that Zn^{2+} content of cells is being altered in concert with the level of extracellular Zn^{2+} to which they are exposed. The increase in intracellular Zn^{2+} is associated with changes in vitamin D promoter activity.

Effect of Zn^{2+} on Cell Vitamin D Receptor Content and $1\alpha,25(OH)_2D_3$ Binding to the VDR

ROS 17/2.8 containing luciferase reporter plasmids driven by a 24-hydroxylase promoter or osteopontin vitamin D response elements were exposed to various levels of extracellular $ZnCl_2$ and $1\alpha,25(OH)_2D_3$ (10^{-8} M) or ethanol for 48 h as previously described. Vitamin D receptor content of lysates was assessed by immunoblotting. Treatment of cells with $1\alpha,25(OH)_2D_3$ clearly increased VDR levels; however, increasing extracellular Zn^{2+} concentrations had small effects on vitamin D receptor concentrations (data not shown). VDR content did not correlate with changes in Zn^{2+}-modulated, $1\alpha,25(OH)_2D_3$-driven promoter activity.

The affinity of $1\alpha,25(OH)_2D_3$ for the vitamin D receptor was determined in absence of added $ZnCl_2$ and the presence of 2 μM or 10 μm added $ZnCl_2$. The measured K_ds were 72.5, 71.6 and 67.2 pM with 0, 2 μM and 10 μM $ZnCl_2$ respectively.

DISCUSSION

Zinc is essential for all organisms. It is an integral component of numerous proteins including enzymes and other proteins (1). In some enzymes, zinc acts as an electrophilic catalyst or co-catalyst. Whereas, in other enzymes and proteins, zinc ions serve a purely structural role (1). Steroid hormone receptors (SHRs), including the VDR, are members of the latter group. In the VDR, zinc ions stabilize functional domains referred to as "zinc fingers" (6, 7). The integrity of the two zinc fingers present in the VDR is essential for the biological activity of the receptor. Mutations in these regions have been shown to result in vitamin D insensitive rickets (17, 18).

The biological activity of vitamin D is mediated through activation or repression of target genes (7, 8). Gene regulation is mediated by a ligand-dependent

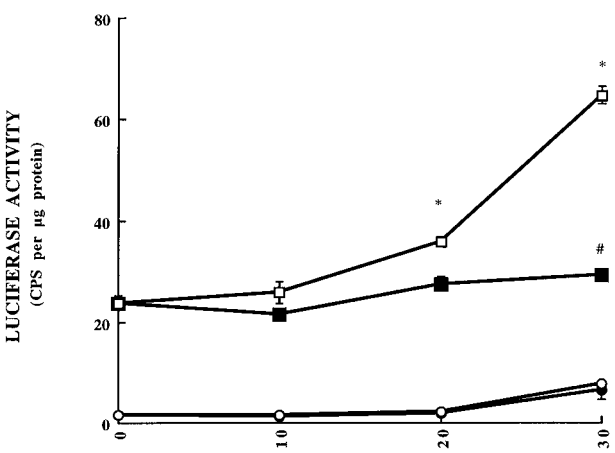

FIG. 4. Relative effect of zinc and copper on 24-hydroxylase promoter activity in ROS 17/2.8 cells. ROS 17/2.8 cells stably transfected with the luciferase reporter plasmid pMAMneoLUC.24-OH.Pro were exposed to the indicated concentrations of $ZnCl_2$ (open symbols) or $CuSO_4$ (solid symbols) and $1\alpha,25(OH)_2D_3$ (10^{-8} M, squares) or ethanol (circles) for 48 h and luciferase activity measured as described under Methods. Luciferase activity is expressed as counts per second per μg protein. Values represent the mean of four observations (±SD). The Fisher test for multiple comparisons indicated luciferase activity at an extracellular $CuSO_4$ concentration of 30 μM was significantly higher ($^{\#}P < 0.02$) than in the absence of extracellular $CuSO_4$. Similar statistical analysis indicated luciferase activities at an extracellular $ZnCl_2$ concentration of 20 μM ($^*P < 0.0002$) and 30 μM ($^*P < 0.0001$) were significantly higher than in the absence of extracellular $ZnCl_2$.

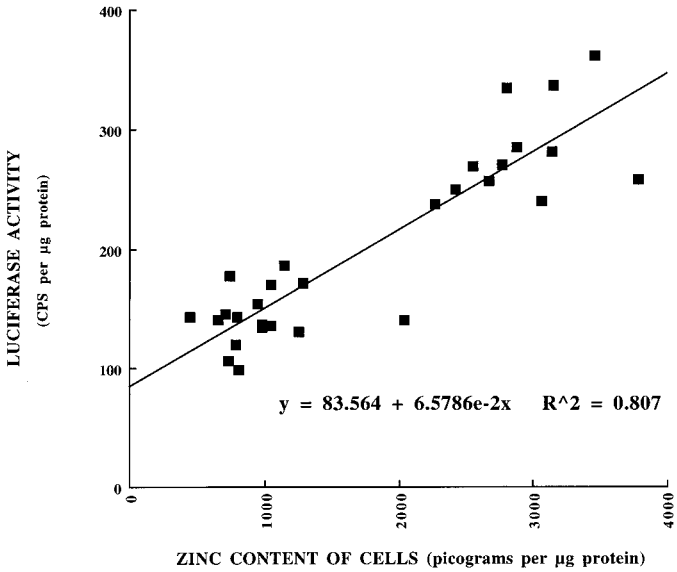

FIG. 5. Correlation of cellular zinc content and osteopontin promoter vitamin D response element activity in ROS 17/2.8 cells. ROS 17/2.8 stably transfected with the luciferase reporter plasmid pPON.VDRE.tk-G13 were exposed to 0, 5 μM, 10 μM, 20 μM, and 30 μM concentrations of $ZnCl_2$ and $1\alpha,25(OH)_2D_3$ for 48 h and luciferase activity and Zn content of cells measured as described under Methods. Six observations were made at each concentration of $ZnCl_2$. A straight line was fitted to data points by simple linear regression.

nuclear receptor, the VDR, which heterodimerizes with the retinoid X receptor and associates with DNA response elements (VDREs). Osteopontin (Op) is a noncollagenous bone matrix protein whose synthesis is regulated by $1\alpha,25(OH)_2D_3$ (19, 20). The promoter region of the Op gene has been shown to contain a 9-bp direct repeat response element that confers $1\alpha,25(OH)_2D_3$ responsiveness (21, 22). $1\alpha,25(OH)_2D_3$ is biologically inactivated by a series of reactions starting with 24-hydroxylation (23, 24). Synthesis of the 24-hydroxylase enzyme which catalyses this reaction is directly regulated by $1\alpha,25(OH)_2D_3$. The promoter region of the 24-hydroxylase gene contains a VDRE which mediates $1\alpha,25(OH)_2D_3$ based transcriptional regulation (25, 26).

The question we address in experiments described in this communication is whether the biological activity of the VDR can be effected by altering the zinc content of $1\alpha,25(OH)_2D_3$ target cells. This study was prompted by observed changes in the conformation and DNA binding properties of the VDR DNA-binding domain by changes in its zinc content (9, 10). When exposed to $ZnCl_2$ concentrations of 20 μM and above, the activity of activated vitamin D-dependent genes in ROS 17/2.8 cells was significantly higher than in the absence of Zn^{2+}. With exposure to increased amounts of $ZnCl_2$, cellular Zn^{2+} content increased, indicating that VDRs in these cells were potentially exposed to a higher Zn^{2+} concentration. The selectivity of Zn^{2+} modulation of $1\alpha,25(OH)_2D_3$ gene activation is indicated by demonstrating that Zn^{2+} is more effective than $CuSO_4$ in modulating $1\alpha,25(OH)_2D_3$ gene activation.

The mechanism by which Zn^{2+} modulates $1\alpha,25(OH)_2D_3$ gene activation in these cells is not clear. Our results indicate Zn^{2+} does not effect the affinity of $1\alpha,25(OH)_2D_3$ for the VDR. Furthermore, zinc does not appear to alter the cellular level of the VDR sufficiently to account for the enhanced $1\alpha,25(OH)_2D_3$ response; as described previously $1\alpha,25(OH)_2D_3$ increases receptor content in treated cells (27–29). We have shown by mass spectrometry that changing the Zn^{2+} content of the VDR effects its interaction with specific DNA response elements (11). While multiple mechanisms may account for our observations, one explanation may be the increase in binding of VDR to VDREs in $1\alpha,25(OH)_2D_3$-dependent promoters in the presence of increasing Zn^{2+}.

Our results expand the literature concerning steroid hormone-activated cellular activities that are regulated by Zn^{2+}. Yanaguchi and Oishi have shown that Zn^{2+} significantly enhances the effect of $1\alpha,25(OH)_2D_3$ on alkaline phosphatase activity and DNA synthesis in rat calvaria (30). Also Zn^{2+} has been shown to enhance the effect of estrogen on DNA synthesis in rat calvaria (31). It has been shown for several Zn^{2+} containing transcription factors that removal of Zn^{2+} results in reversible loss of DNA-binding and transcriptional regulation (32).

The mechanisms by which Zn^{2+} modulates biological processes has been the subject of extensive studies (2, 33–40). O'Dell *et al.* have suggested that Zn^{2+} cellular content alters sulhydryl groups in membrane proteins

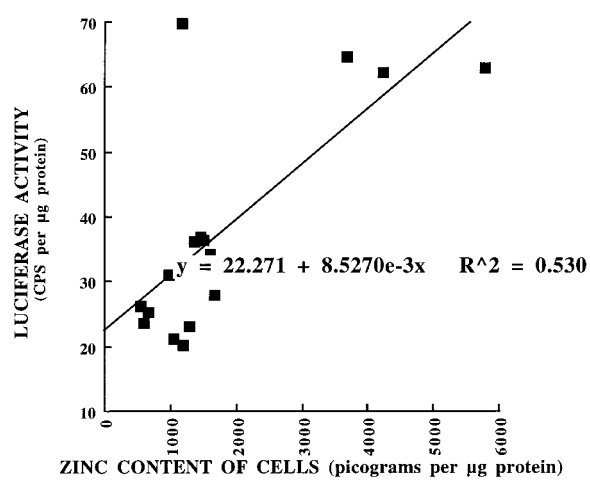

FIG. 6. Correlation of cellular zinc content and 24-hydroxylase promoter activity in ROS 17/2.8 cells. ROS 17/2.8 cells stably transfected with the luciferase reporter plasmid pMAMneoLUC.24-Pro were exposed to 0, 10 μM, 20 μM, and 30 μM concentrations of $ZnCl_2$ and $1\alpha,25(OH)_2D_3$ (10^{-8} M) for 48 h and luciferase activity and Zn content of cells measured as described under Methods. Four observations were made at each concentration of $ZnCl_2$. A straight line was fitted to data points by simple linear regression.

(34). Glutathione restores reduced platelet aggregation seen in Zn^{2+} deficiency (34, 40). Others have shown that the redox state of the cell alters the manner in which Zn^{2+} is bound by metallothionien and made available to other proteins (37, 39). Exchange of Zn^{2+} between thionein, the apo form of metallothionein, and the estrogen receptor has been shown to effect DNA binding by the receptor (38). Whether or not any of these mechanisms are responsible for our observations relating to vitamin D action in cells is unknown at present.

Zn^{2+} deficiency occurs in aging humans and is associated with an increased incidence of fractures (41). We suggest that one mechanism might involve reduced activation of genes in osteoblasts by $1\alpha,25(OH)_2D_3$. Other mechanisms might involve reduced activation of the plasma membrane calcium pump in the intestine which might, in turn, contribute to impaired calcium absorption (42, 43). In conclusion, the modulation of vitamin D action by zinc is potentially of biological and clinical significance.

ACKNOWLEDGMENTS

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