

# Consequences of Vitamin D Receptor Regulation for the 1,25-Dihydroxyvitamin D<sub>3</sub>-Induced 24-Hydroxylase Activity in Osteoblast-like Cells: Initiation of the C24-Oxidation Pathway

A. STAAL, G. J. C. M. VAN DEN BEMD, J. C. BIRKENHÄGER, H. A. P. POLS, and J. P. T. M. VAN LEEUWEN

Department of Internal Medicine III, Erasmus University Medical School, Rotterdam, The Netherlands

A direct relationship between vitamin D receptor (VDR) level and target cell responsiveness to 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>) has been shown in osteoblast-like cell lines. However, we previously found an inverse relationship between the TGFβ-induced VDR up-regulation and subsequent 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced biological responses. A clear inhibition of the 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced stimulation of osteocalcin and osteopontin expression was observed. A biological response that has formerly been shown to be coupled to VDR level is 24-hydroxylase activity. This enzyme initiates the C24 oxidation of the side-chain, followed by cleavage and ultimate metabolic clearance of both 25-(OH)D<sub>3</sub> and its metabolite 1,25-(OH)<sub>2</sub>D<sub>3</sub>. With UMR 106 (rat) and MG 63 (human) osteoblast-like cells, we show that after preincubation with TGFβ, which causes an increase in VDR level, 1,25-(OH)<sub>2</sub>D<sub>3</sub> induction of 24-hydroxylase activity is also stimulated. In addition, we provide evidence that variations in VDR level induced by other means (PTH, EGF, medium change) are also closely associated with 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced 24-hydroxylase activity. Furthermore, we show that in MG 63 cells, but not in UMR 106 cells, TGFβ itself was able to increase the activity of the enzyme 24-hydroxylase. As 24-hydroxylation is the initial step in the further C24 oxidation of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, our results indicate a close coupling of VDR level and the degradation of its ligand, 1,25-(OH)<sub>2</sub>D<sub>3</sub>. This mechanism may provide an important regulatory feedback in the action of 1,25-(OH)<sub>2</sub>D<sub>3</sub> at target tissue/cell level. (*Bone* 20:237-243; 1997) © 1997 by Elsevier Science Inc. All rights reserved.

**Key Words:** 24-Hydroxylase; Vitamin D receptor; 1,25-Dihydroxyvitamin D<sub>3</sub>; Transforming growth factor β; Osteoblast; Metabolism.

## Introduction

For the maintenance of calcium homeostasis regulation of the formation and degradation of the biologically active form of vitamin D<sub>3</sub>, 1α,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>), is important. After 25-hydroxylation of vitamin D<sub>3</sub> in the liver, and

hydroxylation at the C1α position in the kidney, 1α,25-(OH)<sub>2</sub>D<sub>3</sub> is formed. Parathyroid hormone (PTH) and reduced plasma levels of calcium and phosphate are the most important stimulators of 1α-hydroxylase activity. In the kidney, 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibits its own synthesis, but stimulates in a number of target tissues, including the kidney, the enzyme 24-hydroxylase.<sup>1</sup> 24-Hydroxylase mediates the conversion of 1,25-(OH)<sub>2</sub>D<sub>3</sub> to 1,24,25-(OH)<sub>3</sub>D<sub>3</sub>, which is actually the initial step in a more extensive C24 and C23 oxidation of the side chain (1,24,25-(OH)<sub>3</sub>D<sub>3</sub> → 24-oxo-1,25-(OH)<sub>2</sub>D<sub>3</sub> → 24-oxo-1,23,25-(OH)<sub>3</sub>D<sub>3</sub>) and ultimately results in the production of calcitroic acid.<sup>2-4</sup> In other words, the self-induced metabolism of 1,25-(OH)<sub>2</sub>D<sub>3</sub> may provide a means to regulate its concentration at the level of its target tissues.<sup>5,6</sup> A direct relationship between vitamin D receptor (VDR) level and target cell responsiveness<sup>7</sup> to 1,25-(OH)<sub>2</sub>D<sub>3</sub>, including regulation of the C24-oxidation pathway, has been shown in osteoblast-like cell lines.<sup>8-10</sup> However, transforming growth factor-β (TGFβ), a growth factor produced by bone cells and stored in the bone matrix, which increases VDR level in osteoblast-like cells,<sup>11,12</sup> decreases the expression of osteocalcin and osteopontin in response to 1,25-(OH)<sub>2</sub>D<sub>3</sub>.<sup>12,13</sup> At the moment the biological significance of the TGFβ-induced increase in VDR level is unknown. In the present study, we examined the coupling of VDR level to 24-hydroxylase activity by studying the effect of TGFβ-induced VDR up-regulation on subsequent 1,25-(OH)<sub>2</sub>D<sub>3</sub> induction of the 24-hydroxylase activity.

## Materials and Methods

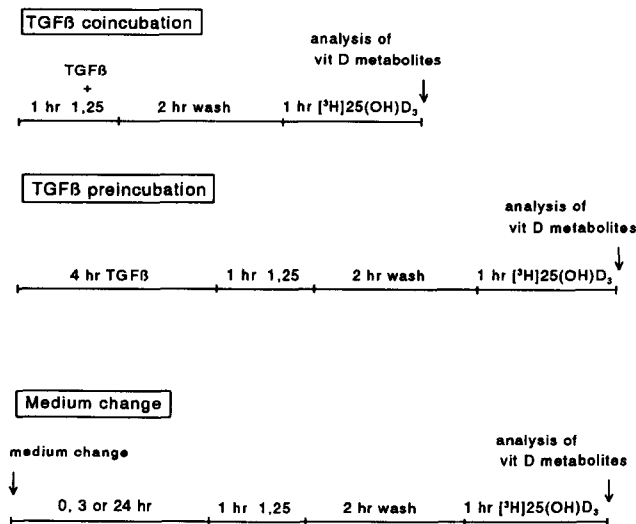
### Materials

[23,24-<sup>3</sup>H]-1,25-(OH)<sub>2</sub>D<sub>3</sub> (120 Ci/mmol) and [26,27-<sup>3</sup>H]-25-(OH)D<sub>3</sub> (28 Ci/mmol) were obtained from Amersham International (Aylesbury, Buckinghamshire, UK). Nonradioactive 1,25-(OH)<sub>2</sub>D<sub>3</sub> was generously provided by LEO Pharmaceuticals BV (Weesp, The Netherlands), and TGFβ type 2 by Dr. J. Feyen, Sandoz Pharma Ltd. (Basel, Switzerland). Fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine were purchased from Flow Laboratories (Irvine, UK). αMEM medium was purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) fraction 5 was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). All other reagents were of the best grade commercially available. The standard compounds 24,25-(OH)<sub>2</sub>D<sub>3</sub>, 24-oxo-25-(OH)D<sub>3</sub>, and 24-oxo-23,25-(OH)<sub>2</sub>D<sub>3</sub> for HPLC analyses were very generously provided by Dr. G. Jones, Queen's University, Kingston, Ontario, Canada.

Address for correspondence and reprints: Dr. J. P. T. M. van Leeuwen, Department of Internal Medicine III, Erasmus University Medical School, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. E-mail: vanleeuwen@inw3.fgg.eur.nl

Cell Culture

UMR 106 (rat) and MG-63 (human) osteoblast-like cells were maintained in  $\alpha$ MEM medium supplemented with 2 mmol/L L-glutamine, 0.1% glucose, 100 IU/mL penicillin, 100 IU/mL streptomycin, and 10% FCS at 37°C under 95% air/5% CO<sub>2</sub>. For the experiments, cells were seeded at 40,000 cells/cm<sup>2</sup> in  $\alpha$ MEM containing 10% FCS and cultured for 24 h. Next, medium was changed to  $\alpha$ MEM medium containing 2% charcoal-treated FCS (CT-FCS) and the cells were cultured for 16 h. To investigate the effect of TGF $\beta$ , and changing the medium on VDR levels, respectively, 1 ng/mL TGF $\beta$  was added to the cells cultured with 2% CT-FCS containing  $\alpha$ MEM, or medium was changed to serum free  $\alpha$ MEM containing 0.1% BSA. 1,25-(OH)<sub>2</sub>D<sub>3</sub> binding assays were performed after various incubation periods. For investigation of the effects of altered receptor levels on subsequent induction of the C24-oxidation pathway by 1,25-(OH)<sub>2</sub>D<sub>3</sub> the following incubations were chosen: (1) preincubation with 1 ng/mL TGF $\beta$  for 4 h, thereby increasing VDR level, followed by addition of vehicle or several concentrations 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h, (2) no preincubation with TGF $\beta$ , thereby maintaining a control VDR level, and subsequent coincubation of 1 ng/mL TGF $\beta$  with vehicle or several concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h (Figure 1), (3) changing the medium to serum free  $\alpha$ MEM containing 0.1% BSA, followed by either 0 h (control VDR level), 3 h (increasing VDR level), or 24 h (decreasing VDR level) of incubation and subsequent addition of vehicle or several concentrations 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h (Figure 1).



**Figure 1.** Incubation protocols for determining the effects of pre- and coincubations with several factors on 1,25-(OH)<sub>2</sub>D<sub>3</sub> induction of the C24-oxidation pathway of vitamin D<sub>3</sub>. Cells were plated at a density of 40,000/cm<sup>2</sup> and cultured for 24 h in  $\alpha$ MEM containing 10% FCS. Next, medium was changed to  $\alpha$ MEM containing 2% charcoal treated FCS and cultured for 16 h. Subsequently, cells were coincubated with 1 ng/mL TGF $\beta$  and vehicle or several concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h or cells were preincubated with 1 ng/mL TGF $\beta$  for 4 h followed by the addition of vehicle or several concentrations 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h, or medium was changed to serum free  $\alpha$ MEM containing 0.1% BSA and cells were cultured for 0, 3, or 24 h followed by the addition of vehicle or several concentrations 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h. After incubation with 1,25-(OH)<sub>2</sub>D<sub>3</sub>, cells were washed in  $\alpha$ MEM containing 2% BSA at 37°C for 2 h to remove unlabelled 1,25-(OH)<sub>2</sub>D<sub>3</sub>, followed by the incubation of 10<sup>-8</sup> mol/L [<sup>3</sup>H]-25-(OH)D<sub>3</sub> in  $\alpha$ MEM containing 0.1% BSA for 1 h. Next, the conversion of [<sup>3</sup>H]-25-(OH)D<sub>3</sub> to [<sup>3</sup>H]-24,25-(OH)<sub>2</sub>D<sub>3</sub> was determined by HPLC.

[<sup>3</sup>H]-1,25-(OH)<sub>2</sub>D<sub>3</sub> Binding Assay

1,25-(OH)<sub>2</sub>D<sub>3</sub> ligand binding assays were performed as described by Pols et al.<sup>6</sup> In brief, after incubation, the cells were washed with  $\alpha$ MEM containing 2% BSA at 37°C for 2 h to remove residual unlabelled 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Then, cells were extracted on ice in a hypertonic buffer consisting of 300 mmol/L KCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol EDTA, 5 mmol/L dithiothreitol, 10 mmol/L sodium molybdate, and 0.1% Triton X-100. High-speed supernatants were obtained, and 250  $\mu$ L aliquots were incubated overnight at 4°C for single point assays with 0.25 mmol/L [<sup>3</sup>H]-1,25-(OH)<sub>2</sub>D<sub>3</sub> in the absence or presence of a 200-fold molar excess of unlabeled hormone. After removal of the unbound ligand by charcoal absorption, receptor-bound 1,25-(OH)<sub>2</sub>D<sub>3</sub> was quantitated by liquid scintillation counting.

24-Hydroxylase Assay and Detection of Vitamin D Metabolites

Induction of the C24-oxidation pathway and 24-hydroxylase activity was determined by measuring the reduction in substrate ([<sup>3</sup>H]-25-(OH)D<sub>3</sub>) and/or the appearance of [<sup>3</sup>H]-24,25-(OH)<sub>2</sub>D<sub>3</sub> as described previously.<sup>6</sup> At the end of the incubation period, as described in the cell culture, medium was removed and cells were washed with  $\alpha$ MEM containing 2% BSA at 37°C for 2 h to remove residual unlabelled 1,25-(OH)<sub>2</sub>D<sub>3</sub> and subsequently incubated with 10<sup>-8</sup> mol/L [26,27-<sup>3</sup>H]-25-(OH)D<sub>3</sub> for 1 h. Next, medium was collected, cells were scraped in PBS-Triton X-100 and pooled with the medium. Samples were extracted three times with diethylether. The ether-extractable fraction was subjected to high pressure liquid chromatography (HPLC). Injector, pumps, and UV detector of the HPLC system were purchased from Millipore Waters (Milford, MA). HPLC analysis was performed using either two 0.3  $\times$  10 cm SI columns (Chrompack, Bergen op Zoom, The Netherlands) with hexane:isopropanol:methanol (96:3:2:0.8) as the solvent at a flow rate of 0.4 mL/min or two 0.3  $\times$  10 cm Spherisorb 5 CN columns (Chrompack, Bergen op Zoom, The Netherlands) with hexane:isopropanol:methanol (94:5:1) as the solvent at a flow rate of 0.6 mL/min. The radioactive vitamin D<sub>3</sub> metabolites were identified by their retention time after calibration with standard 25-(OH)D<sub>3</sub> and 24,25-(OH)<sub>2</sub>D<sub>3</sub>.

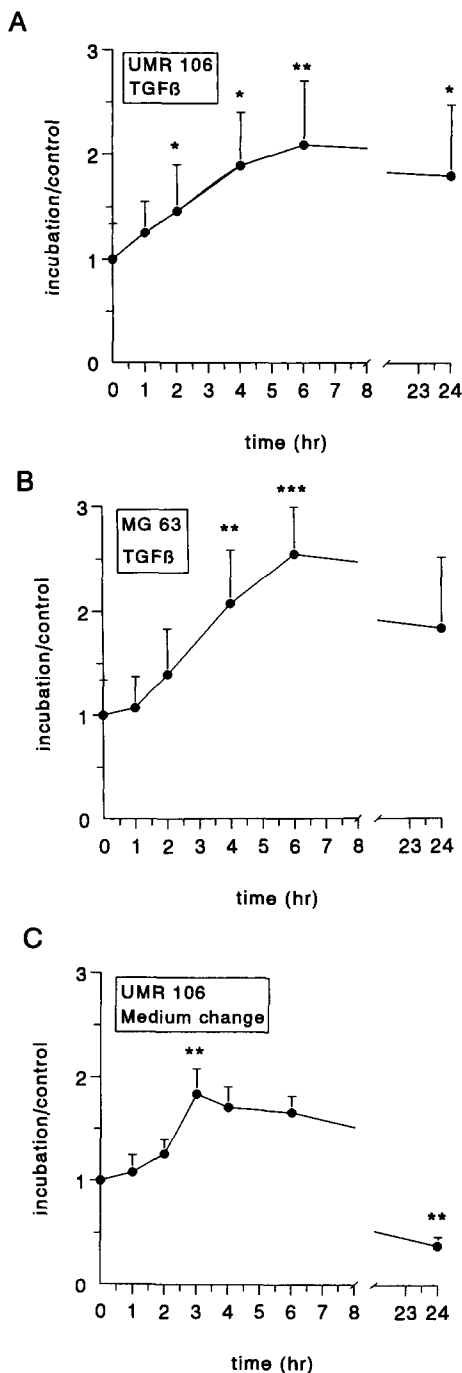
DNA and Protein Measurements

Changes in DNA content were assessed by the fluorimetric method of Johnson-Wint and Hollis.<sup>14</sup> Protein concentration was measured according to the method of Bradford.<sup>15</sup>

Results

The Effects of TGF $\beta$  and Medium Change on Vitamin D Receptor Level

Previously we have demonstrated that TGF $\beta$  increases the VDR level in osteoblasts via a transcriptional effect without affecting receptor affinity.<sup>12</sup> Figure 2 illustrates the time course of the 1 ng/mL TGF $\beta$  effect on VDR level in UMR 106 (Figure 2A) and that MG 63 (Figure 2B) cells. In both cell lines TGF $\beta$  has a maximal effect after 4-6 h of incubation, although a significant increase was already observed after 2 h in UMR 106 and 4 h in MG 63 cells. On the basis of these data, a preincubation period with TGF $\beta$  of 4 h has been selected for the 24-hydroxylase experiments. Figure 2C shows the effect of a medium change from  $\alpha$ MEM containing 2% CT-FCS to serum free  $\alpha$ MEM containing 0.1% BSA. A doubling of VDR content was observed 3 h after medium change, followed by a decline to levels significantly below control level at 24 h. The up-regulation of VDR level was blocked by the addition of actinomycin D and cycloheximide (data not shown), indicating that the observed increase



**Figure 2.** Time course of the modulation of VDR level by TGFβ treatment and medium change. (A) UMR 106 and (B) MG 63 cells were incubated with 1 ng/mL TGFβ for several incubation periods and VDR levels were determined by 1,25-(OH)<sub>2</sub>D<sub>3</sub> binding assay. (C) Medium of UMR 106 cells was changed from 2% charcoal-treated FCS to serum free αMEM containing 0.1% BSA and after several incubation periods VDR levels were determined by 1,25-(OH)<sub>2</sub>D<sub>3</sub> binding assay. Data are expressed as treatment/control ± SD as determined by single point assays in duplicate of two separate experiments. \**p* < 0.05, \*\**p* < 0.005, \*\*\**p* < 0.001 vs control.

is due to increased VDR transcription and new protein synthesis. Finally, under all conditions tested no change in receptor affinity for 1,25-(OH)<sub>2</sub>D<sub>3</sub> was observed (data not shown).

*The Effect of TGFβ Pre- and Coincubation on 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced 24-hydroxylase Activity*

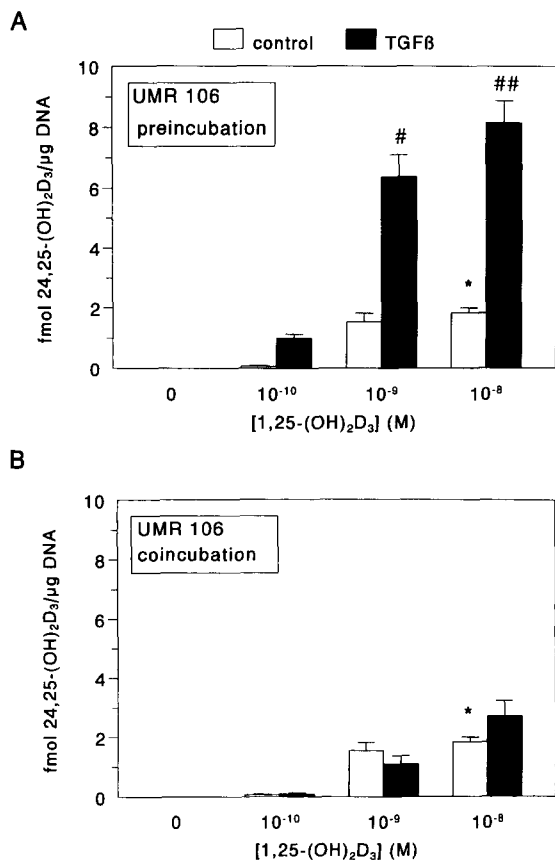
The significance of TGFβ-induced VDR up-regulation for 1,25-(OH)<sub>2</sub>D<sub>3</sub> induction of 24-hydroxylase activity was studied by preincubating UMR 106 and MG 63 cells with 1 ng/mL TGFβ for 4 h, causing an up-regulation of VDR level, followed by the addition of several concentrations 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Figure 1). To prevent interference of homologous up-regulation of the VDR, which is the increase in VDR level induced by 1,25-(OH)<sub>2</sub>D<sub>3</sub>, the incubations with 1,25-(OH)<sub>2</sub>D<sub>3</sub> were limited to 1 h. Induction of the 24-hydroxylase activity was measured by HPLC analysis as the conversion of [<sup>3</sup>H]-25-(OH)D<sub>3</sub> to [<sup>3</sup>H]-24,25-(OH)<sub>2</sub>D<sub>3</sub> during 1 h of incubation. Prior to incubation with labeled 25-(OH)D<sub>3</sub>, cell cultures were washed at 37°C for 2 h in serum free medium containing 2% BSA to remove residual unlabeled 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Figure 1). For comparison, experiments were performed in which TGFβ was coincubated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h. TGFβ incubation for 1 h does not significantly affect VDR content in UMR 106 and MG 63 cells (Figures 2A, B).

**Figures 3 and 4** illustrate the effects of pre- and coincubation with TGFβ on 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced C24 oxidation in UMR 106 and MG 63 cells, respectively. Preincubation with 1 ng/mL TGFβ strongly enhances the 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced conversion of 25-(OH)D<sub>3</sub> to 24,25-(OH)<sub>2</sub>D<sub>3</sub> (Figure 3A), concomitant with its stimulation of VDR level in UMR 106 cells (Figure 2). Also in MG 63 cells, preincubation with TGFβ resulted in increased induction of 24-hydroxylase activity (Figure 4A). However, in contrast to UMR 106 cells, it is not clear whether this increase is fully related to an increased VDR level, because a 4 h preincubation with TGFβ, followed by a control incubation (i.e., without 1,25-(OH)<sub>2</sub>D<sub>3</sub>) also induced 24,25-(OH)<sub>2</sub>D<sub>3</sub> accumulation (Figure 4A). As to the difference in effects of TGFβ in UMR 106 and MG 63 cells (cf. Figures 3A and 4A), the possibility exists that 24,25-(OH)<sub>2</sub>D<sub>3</sub> is already converted to subsequent metabolites in the C24-oxidation pathway in MG 63 cells. Therefore, after incubation with TGFβ and 10<sup>-8</sup> mol 1,25-(OH)<sub>2</sub>D<sub>3</sub> in MG 63 cells, we also examined the loss of the [<sup>3</sup>H]-25-(OH)D<sub>3</sub> substrate, which reflects the overall conversion into "catabolic" metabolites. **Figure 5** shows that preincubation with TGFβ results in a greater loss in [<sup>3</sup>H]-25-(OH)D<sub>3</sub> substrate than control incubation. This observation indicates that additional metabolites have been formed. But more importantly, it suggests that a relationship exists between TGFβ-regulated VDR level and the induction of the C24-oxidation pathway in MG 63 cells.

Coincubation with TGFβ did not significantly affect the induction of 24-hydroxylase by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in UMR 106 and MG 63 cells (Figures 3B and 4B). In MG 63 cells, we show that coincubation of TGFβ and 1,25-(OH)<sub>2</sub>D<sub>3</sub> also did not affect the loss of [<sup>3</sup>H]-25-(OH)D<sub>3</sub> substrate (Figure 5). Incubation of MG 63 cells with TGFβ alone for 1 h resulted in a small induction of 24-hydroxylase activity (Figure 4B).

*The Effects of Other Mediators on VDR Level and their Consequences for the Induction of the C24-oxidation Pathway by 1,25-(OH)<sub>2</sub>D<sub>3</sub>*

In order to address the relationship between VDR level and the C24-oxidation pathway more extensively, we examined the effect of changes in VDR by other means than TGFβ incubation on



**Figure 3.** Preincubation, but not coincubation, with TGFβ enhances the induction of 24-hydroxylase activity by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in UMR 106 cells. UMR 106 cells were preincubated for 4 h with 1 ng/mL TGFβ followed by the addition of vehicle or 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h or incubated with 1 ng/mL TGFβ and vehicle or 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h as described in Figure 1A and the effect on C24 oxidation was determined by measuring the conversion of [<sup>3</sup>H]-25-(OH)<sub>2</sub>D<sub>3</sub> to [<sup>3</sup>H]-24,25-(OH)<sub>2</sub>D<sub>3</sub>. (A) The effect of preincubation with TGFβ on the induction of C24 oxidation by 1,25-(OH)<sub>2</sub>D<sub>3</sub>. (B) The effect of coincubation of TGFβ and 1,25-(OH)<sub>2</sub>D<sub>3</sub> on C24 oxidation. \**p* < 0.05 vs. control. #*p* < 0.01, ##*p* < 0.005 calculated as significance of interaction.

the induction of the C24-oxidation pathway. Preincubation for 4 h with 10 nmol PTH, which has previously been shown to increase VDR level after 4 h of incubation in UMR 106 cells,<sup>17,18</sup> also results in increased induction of the C24-oxidation pathway by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in UMR 106 cells (data not shown).

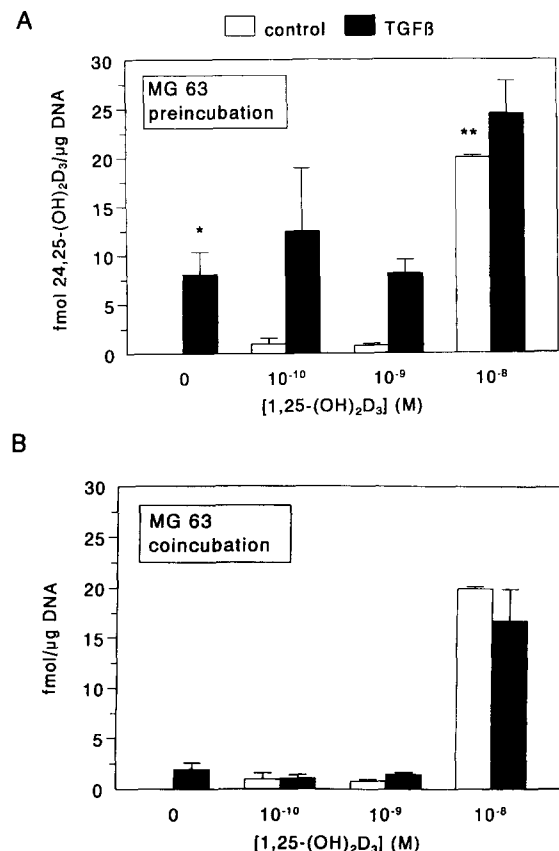
Another way to modulate VDR number in UMR 106 cells is changing cell culture medium from CT-FCS to serum free.<sup>16</sup> In this way we could time dependently either increase (3 h) or decrease (24 h) the VDR level (Figure 2C). The mechanism of and the factors involved in the medium change-induced modulation of the VDR are unknown. However, it provides an excellent tool to examine by one type of manipulation the significance of either an increased or decreased VDR level. Therefore, medium was changed from 2% CT-FCS αMEM to serum free αMEM containing 0.1% BSA and after 0, 3, or 24 h of incubation in this medium 1,25-(OH)<sub>2</sub>D<sub>3</sub> was added for 1 h. Subsequently, cells were washed for 2 h in MEM containing 2% BSA, followed by the incubation with 10<sup>-8</sup> mol/L [<sup>3</sup>H]-25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h (Figure 1C). **Figure 6A** shows a dose-dependent effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on the loss of [<sup>3</sup>H]-25-(OH)<sub>2</sub>D<sub>3</sub> substrate. The increased receptor level at 3 h after medium change is paralleled by

an increased catabolism. Comparably, decreased receptor level at 24 h after medium change is paralleled by a reduced catabolism. Figure 6B demonstrates that the differences in loss of substrate are reflected in the differences in the formation of several metabolites of the C24 oxidation pathway.

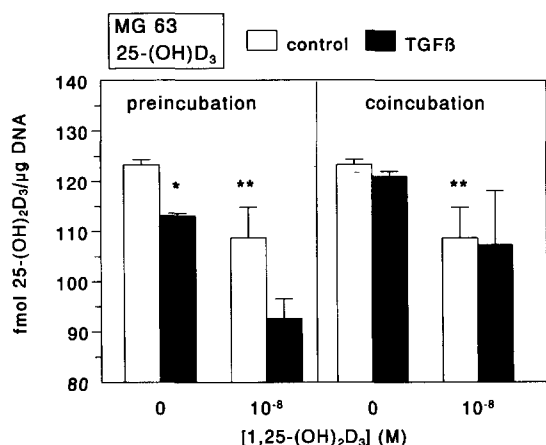
In addition, we found that preincubation for 24 h with 10 ng/mL epidermal growth factor (EGF), a growth factor that has previously been shown to decrease VDR level after 24 h of incubation in the osteoblast-like cell line UMR 106,<sup>19</sup> results in a decreased induction of the C24-oxidation pathway by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in UMR 106 cells (data not shown).

### Discussion

In general, the present study provides evidence for a close relationship between VDR regulation and the induction of 24-hydroxylase activity by 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Previous experiments in our laboratory have clearly indicated that 24-hydroxylation only represents the initial step of the C24-oxidation pathway. Furthermore, the present results underline the biological relevance of the TGFβ-induced increase in VDR level, which has previously been



**Figure 4.** The effects of pre- and co-incubation with TGFβ in the absence or presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on 24-hydroxylase activity in MG 63 cells. MG 63 cells were either preincubated with 1 ng/mL TGFβ for 4 h followed by the addition of vehicle or 1,25-(OH)<sub>2</sub>D<sub>3</sub>, or cells were co-incubated with 1 ng/mL TGFβ and vehicle or several concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h as described in Figure 1A. The effect on C24 oxidation was determined by measuring the conversion of [<sup>3</sup>H]-25-(OH)<sub>2</sub>D<sub>3</sub> to [<sup>3</sup>H]-24,25-(OH)<sub>2</sub>D<sub>3</sub>. (A) The effect on 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced formation of 24,25-(OH)<sub>2</sub>D<sub>3</sub> by preincubation with TGFβ for 4 h. (B) The effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced formation of 24,25-(OH)<sub>2</sub>D<sub>3</sub> after coincubation with TGFβ for 1 h. \**p* < 0.05, \*\**p* < 0.01 vs. control.



**Figure 5.** The effects of pre- and coincubation with TGFβ on the 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced C24-oxidation pathway. MG 63 cells were either preincubated with 1 ng/mL TGFβ for 4 h followed by the addition of vehicle or 1,25-(OH)<sub>2</sub>D<sub>3</sub>, or cells were coincubated with 1 ng/mL TGFβ and vehicle or 10<sup>-8</sup> mol/L 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h as described in Figure 1A. The effect on C24 oxidation was determined by measuring the loss of [<sup>3</sup>H]-25-(OH)D<sub>3</sub> substrate. \**p* < 0.05, \*\**p* < 0.01 vs. control.

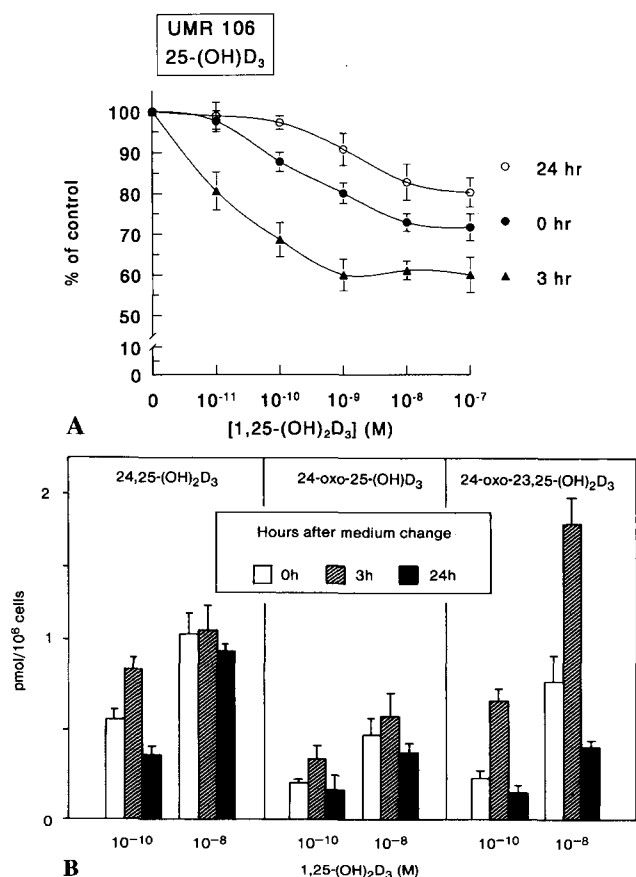
shown not to be related to two other responses to 1,25-(OH)<sub>2</sub>D<sub>3</sub>, osteocalcin, and osteopontin expression.<sup>12,13</sup> The observed relationship between VDR up-regulation and a subsequent higher 1,25-(OH)<sub>2</sub>D<sub>3</sub> induced 24-hydroxylase activity is not unique for TGFβ. We show that medium change- and PTH-induced<sup>18</sup> VDR up-regulation as well as medium change- and EGF-induced<sup>19</sup> down-regulation of the VDR is followed by enhanced or reduced 1,25-(OH)<sub>2</sub>D<sub>3</sub> induction of 24-hydroxylase activity, respectively. These observations fit with data obtained by others. Armbricht et al.<sup>20</sup> also demonstrated a synergistic increase in 24-hydroxylase activity after combined treatment of PTH and 1,25-(OH)<sub>2</sub>D<sub>3</sub> in osteoblast-like cells. Despite species differences with respect to the response to retinoic acid in VDR regulation, a close parallel was observed between the regulation of VDR level and the self-induced catabolism of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in rat and mouse osteoblast-like cells.<sup>8</sup> Dexamethasone induces an increase in VDR level and concomitantly increases 1,25-(OH)<sub>2</sub>D<sub>3</sub> induction of 24-hydroxylase activity.<sup>9</sup> Proliferation-related variances in VDR level in mouse osteoblast-like cells were also coupled to the induction of 24-hydroxylase activity by 1,25-(OH)<sub>2</sub>D<sub>3</sub>.<sup>11</sup> Also in other tissues, VDR mRNA and 24-hydroxylase mRNA expression are concomitantly induced by 1,25-(OH)<sub>2</sub>D<sub>3</sub>, which supports the relevance of the VDR for 24-hydroxylase activity.<sup>21-27</sup>

In our experiments, incubation with 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h followed by a 2 h period of washing the cells with αMEM containing 2% BSA was enough to induce the 24-hydroxylase activity. It remains unclear whether the effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on 24-hydroxylase in this short period is caused by enhancement of the expression of the gene or by enhancement of enzyme activity. Induction of gene transcription is a possibility, because two vitamin D responsive elements have been identified in the promoter of the 24-hydroxylase gene, which are involved in the transcriptional regulation of 24-hydroxylase by 1,25-(OH)<sub>2</sub>D<sub>3</sub>.<sup>28-31</sup> However, the time needed for the induction of 24-hydroxylase mRNA varies among cell lines and tissues from 1 h<sup>32</sup> to 24 h.<sup>33</sup>

An interesting observation in the present study is that in MG 63 cells TGFβ incubation alone appeared to induce 24-hydroxylase activity. MG-63 cells may, unlike UMR 106 cells, contain the mediator(s) to induce C24-oxidation by TGFβ. In-

duction of 24-hydroxylase activity by 1,25-(OH)<sub>2</sub>D<sub>3</sub> has previously been shown to be partly mediated through protein kinase C in intestinal epithelial cells<sup>34</sup> and in renal cells.<sup>35</sup> Furthermore, TGFβ responses have also been reported to be mediated by protein kinase C.<sup>36-38</sup> However, protein kinase C activity was not affected by TGFβ in UMR 106 cells.<sup>39</sup> Thus, the possibility arises that in MG 63 cells TGFβ induction of 24-hydroxylase activity may be mediated by protein kinase C. Despite the lack of knowledge on this mechanism, induction by TGFβ results in an enhanced 24-hydroxylase activity and thereby an increased degradation of biologically active 1,25-(OH)<sub>2</sub>D<sub>3</sub>. We extrapolate the data obtained with labeled 25-(OH)D<sub>3</sub> to 1,25-(OH)<sub>2</sub>D<sub>3</sub>, because up to now 24-hydroxylase is considered the initial step in the C24-oxidation pathway of both 1,25-(OH)<sub>2</sub>D<sub>3</sub> and 25-(OH)D<sub>3</sub>.

In conclusion, we and others have provided evidence for the coupling of VDR level to the magnitude of induction of the C24-oxidation pathway by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in osteoblast-like cells. This strict coupling does not exist for several other bioresponses to 1,25-(OH)<sub>2</sub>D<sub>3</sub>, including osteocalcin, osteopontin,<sup>12,13</sup> and inhibition of collagen synthesis,<sup>40</sup> indicating that additional mechanisms compensate or overrule the effect of changed receptor level in the response to 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Recently, we have identified the mechanisms by which TGFβ exerts its inhibiting ef-



**Figure 6.** The effects of medium change on the 1,25-(OH)<sub>2</sub>D<sub>3</sub> induction of the C24-oxidation pathway as measured by (A) the loss of [<sup>3</sup>H]-25-(OH)D<sub>3</sub> substrate (B) formation of several metabolites. Medium of UMR 106 cells was changed to serum free αMEM containing 0.1% BSA and cells were cultured for 0, 3, or 24 h followed by the induction of C24 oxidation by several concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> for 1 h as described in Figure 1B. Loss of [<sup>3</sup>H]-25-(OH)D<sub>3</sub> was calculated from pmol [<sup>3</sup>H]-25-(OH)D<sub>3</sub>/10<sup>6</sup> cells.

fects on 1,25-(OH)<sub>2</sub>D<sub>3</sub>-induced osteocalcin and osteopontin expression and thereby how it overrides the increase in VDR level. TGFβ directly blocks binding of the 1,25-(OH)<sub>2</sub>D<sub>3</sub>-VDR complex to its responsive element in the promoter of the osteocalcin and osteopontin genes.<sup>13</sup> This dissociation between increase in VDR level and inhibition of osteocalcin and osteopontin expression may be specific for TGFβ because in other cases a direct relation between VDR level and induction of osteocalcin expression has been demonstrated.<sup>41</sup> The present article also shows that for induction of 24-hydroxylase activity and TGFβ-induced increase in VDR level, a positive relationship exists. The coupling of VDR level to the catabolic pathway of vitamin D<sub>3</sub> may provide an important negative feedback mechanism in the regulation of biological responses to 1,25-(OH)<sub>2</sub>D<sub>3</sub> at target cell level.

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