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Dexamethasone Enhances Vitamin D-24-Hydroxylase Expression in Osteoblastic (UMR-106) and Renal (LLC-PK_1) Cells Treated with 1α,25-Dihydroxyvitamin D_3

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Chronic glucocorticoid therapy causes rapid bone loss and clinical osteoporosis. We previously found that dexamethasone, a potent glucocorticoid, increased renal expression of vitamin D-24-hydroxylase, which degrades such vitamin D metabolites as 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3 (1,25$[OH]_2D_3$). We therefore investigated the mechanisms of this increase in UMR-106 osteoblast-like cells and LLC-PK_1 kidney cells. To induce 24-hydroxylase expression, 1,25$(OH)_2D_3$ (10^{-7}*M*) and dexamethasone were added simultaneously to the medium of LLC-PK_1 cells, and 24 h before dexamethasone treatment, 1,25$(OH)_2D_3$ was added to the medium of UMR-106 cells. Dexamethasone dose dependently increased 24-hydroxylase mRNA and enzymatic activity in 1,25$(OH)_2D_3$-treated LLC-PK_1 and UMR-106 cells. Maximal stimulation was observed with 10^{-6} *M* dexamethasone in both cell lines. The addition of 10^{-6} *M* dexamethasone significantly increased the abundance of 24-hydroxylase mRNA by 24 and 8 h in 1,25$(OH)_2D_3$-treated LLC-PK_1 and UMR-106 cells, respectively. Stimulation for dexamethasone in UMR-106 cells persisted for up to 48 h. Dexamethasone stimulation of 24-hydroxylase mRNA expression in UMR-106 cells was abolished by pretreatment with cycloheximide, an inhibitor of protein synthesis. Northern and Western analyses indicated that 10^{-6} *M* dexamethasone markedly increased the abundance of c-*fos* mRNA at 20 min and c-*fos* protein concentration at 60 min in 1,25$(OH)_2D_3$-treated UMR-106 cells but only slightly induced the abundance of c-*jun* mRNA. The addition of phorbol 12-myristate 13-acetate increased mRNA expression for both c-*fos* and 24-hydroxylase in 1,25$(OH)_2D_3$-treated UMR-106 cells. The effect of dexamethasone on 24-hydroxylase mRNA expression was blocked by RO31-8220, a specific inhibitor of protein kinase C. Thus, dexamethasone in the presence of 1,25$(OH)_2D_3$ enhances expression of 24-hydroxylase in UMR-106 osteoblastic cells via new protein synthesis. The mechanism of this effect appears to involve activation of the AP-1 site by increased c-*fos* protein.

Key Words: Dexamethasone; osteoblast; vitamin D-24-hydroxylase.

Introduction

Glucocorticoids have marked effects on bone metabolism. Increased concentrations of glucocorticoids alter bone remodeling by decreasing bone formation and increasing bone resorption, resulting in osteoporosis *(1–3)*. However, the mechanisms underling the actions of glucocorticoids on bone are not yet fully understood. Acting via the glucocorticoid receptor, glucocorticoids promote differentiation of preosteoblasts in the bone marrow stroma into mature osteoblasts with the potential to mineralize. Actions of glucocorticoids on osteoblasts are complex and vary with the maturation stage of the osteoblastic population and animal species. By contrast, glucocorticoids have been shown to increase bone resorption in neonatal mouse calvarium *(4)* and to enhance parathyroid hormone (PTH)-stimulated bone formation and resorption by osteoblast-like cells *(5)*. While the osteoclast-activating effects of PTH initially were thought to be mediated by interleukin-6 (IL-6) and IL-11 secretion by osteoblasts, PTH-stimulated production of IL-6 and IL-11 actually was found to be decreased by dexamethasone *(6)*. Although accumulated data indicate that glucocorticoids have important effects on osteoblastic and osteoclastic activities, which effect of glucocorticoids is most crucial for inducing osteoporosis in humans still is unknown.

The metabolism of 25-hydroxyvitamin D_3 ($25OHD_3$), a circulating form of vitamin D_3, occurs mainly in the kidney, where $25OHD_3$ is converted to 1α,25-dihydroxyvitamin D_3 (1,25$[OH]_2D_3$), the active form of the vitamin, and to 24,25-dihydroxyvitmin D_3 (24,25$[OH]_2D_3$), a catabolic product and putative regulator of bone formation *(7–9)*. Formation of these metabolites is reciprocally regulated by a number of factors such as 1,25$(OH)_2D_3$ and PTH.

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While the vitamin D–endocrine system is the major regulator of mineral ion metabolism, glucocorticoids also affect mineral homeostasis. Studies attempting to demonstrate such an effect have yielded conflicting results. Long-term excess glucocorticoid has produced varied effects on serum $1,25(OH)_2D_3$ concentrations, including increases *(10,11)*, no change *(12,13)*, and decreases *(14,15)*. As for the effects of long-term excess glucocorticoid on plasma concentrations of $24,25(OH)_2D_3$ and 24-hydroxylase activity, we investigated in vivo effects of dexamethasone on vitamin D-1α-hydroxylase and vitamin D-24-hydroxylase expression in the mouse kidney. We found that excess glucocorticoid markedly stimulated renal 24-hydroxylase expression and modestly decreased renal 1α-hydroxylase expression in hypocalcemic mice without changing vitamin D receptor (VDR) number *(16)*. Vitamin D-24-hydroxylase, a member of the cytochrome P-450 enzyme system, catalyzes the 24-hydroxylation of $1,25(OH)_2D_3$ and $25OHD_3$ *(7,8)*. The hydroxylation reaction catalyzed by 24-hydroxylase has been found to be sensitive to vitamin D status and glucocorticoid administration in experimental animals *(16)*. Hydroxylation of $25OHD_3$ in the kidneys and $1,25(OH)_2D_3$ in vitamin D target tissues such as bone by 24-hydroxylase is the first step in inactivation of vitamin D metabolites. The kidneys are the major site of $25OHD_3$ conversion to produce polar metabolites such as $24,25(OH)_2D_3$ *(17)*. Bone is a major target organ of $1,25(OH)_2D_3$, where it is inactivated by 24-hydroxylase after its hormonal action. This degradative pathway first leads to the production of $1,24,25(OH)_3D_3$ followed by successive hydroxylation/oxidation reactions at carbons 24 and 23. Next, the metabolite is cleaved at the C-23/C-24 bond; calcitropic acid is produced by subsequent oxidation of the cleaved product *(18)*.

Bone and kidney, the most important sites of 24-hydroxylase activity, are also affected by glucocorticoids. The present study was undertaken to investigate the mechanism of enhanced 24-hydroxylase expression in response to a synthetic glucocorticoid, dexamethasone, in UMR-106 osteoblast-like cells from rat and LLC-PK_1 pig kidney cells.

Results

Effect of Dexamethasone on 24-Hydroxylase Expression in LLC-PK_1 Kidney Cells

Because the kidney is the major site of 24-hydroxylase expression, we first used an established renal cell line (LLC-PK_1) to investigate regulation of 24-hydroxylase expression by dexamethasone. LLC-PK_1 cells treated with $1,25(OH)_2D_3$ expressed 24-hydroxylase mRNA and enzymatic activity. Northern analysis indicated that 24-hydroxylase mRNA (length of 3.4 kb) was abundant in LLC-PK_1 cells treated with 10^{-7} *M* $1,25(OH)_2D_3$. Treatment with 10^{-6} *M* dexamethasone for 24 h significantly enhanced the abundance of 24-hydroxylase mRNA in $1,25(OH)_2D_3$-exposed cells (Fig. 1A). The stimulating effect of dexamethasone on the abundance of 24-hydroxylase mRNA was attenuated for up to 36 h. Stimulation of 24-hydroxylase mRNA expression by dexamethasone was significant at 24 h (Fig. 1B). Enzymatic activity of 24-hydroxylase after the addition of 10^{-6} *M* dexamethasone to LLC-PK_1 cells was significantly increased at 24 and 36 h (Fig. 2).

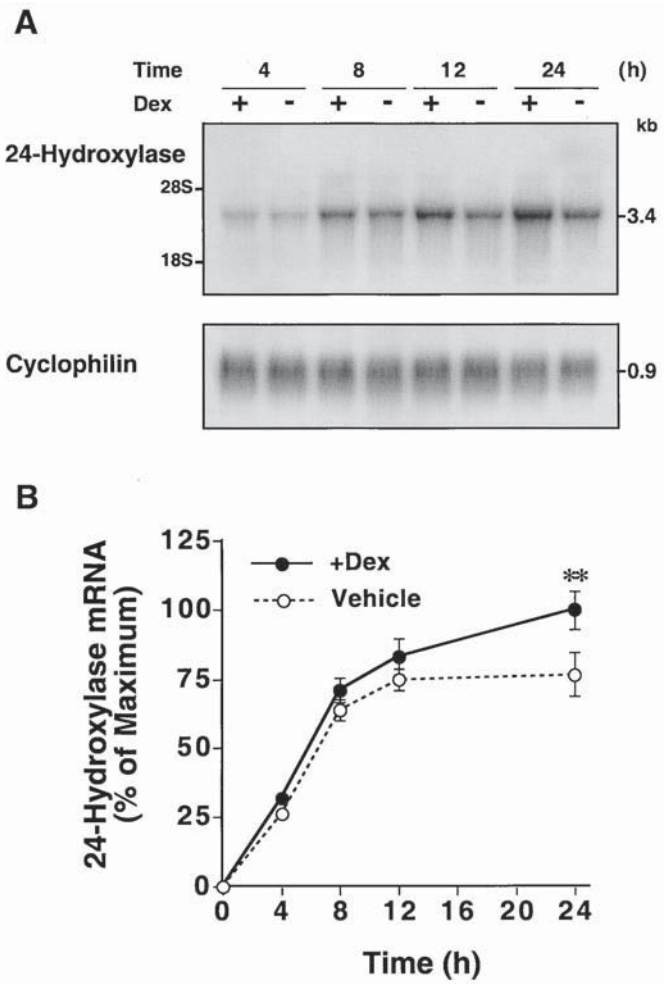

Fig. 1. Time course of effect of dexamethasone (Dex) on $1,25(OH)_2D_3$-induced 24-hydroxylase mRNA abundance in pig proximal tubular cells (LLC-PK_1). After various periods of incubation (4–24 h) with vehicle or 10^{-6} *M* dexamethasone in serum-free Dulbecco's modified Eagle's medium (DMEM) in the presence of 10^{-7} *M* $1,25(OH)_2D_3$, cells were harvested and total RNA was extracted. (**A**) Northern blots of total RNA (20 μg) for 24-hydroxylase and cyclophilin mRNA; (**B**) determination of $1,25(OH)_2D_3$-induced 24-hydroxylase mRNA abundance in cells treated with vehicle or 10^{-6} *M* dexamethasone in the presence of 10^{-7} *M* $1,25(OH)_2D_3$. 24-Hydroxylase mRNA concentration was determined densitometrically and normalized to that of cyclophilin mRNA. Data are expressed as the mean ± SEM of four determinations. $^{**}p < 0.01$ compared with vehicle control at each point.

Effect of Dexamethasone on 24-Hydroxylase Expression in UMR-106 Osteoblast-like Cells

We determined whether dexamethasone stimulated $1,25(OH)_2D_3$-induced 24-hydroxylase expression by UMR-106 osteoblast-like cells. Induction of 24-hydroxylase mRNA

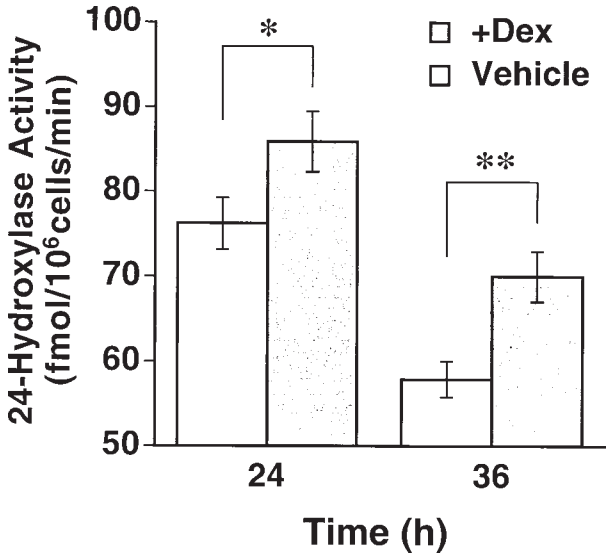

Fig. 2. Effect of dexamethasone (Dex) on $1,25(OH)_2D_3$-induced 24-hydroxylase activity in LLC-PK_1 cells. Dexamethasone or vehicle was added to the cells and treated for 24 or 36 h in the presence of 10^{-7} *M* $1,25(OH)_2D_3$. 24-Hydroxylase activity was measured by cells incubated with $1,25(OH)_2[26,27\text{-}^3H]D_3$ (125 pmol/50,000 cpm) for 45 min. The substrate and products were separated by high-performance liquid chromatography (HPLC). Data are expressed as fmol/(10^6 cells·min) and are the mean ± SEM of four determinations. *$p < 0.05$ and **$p < 0.01$ compared with vehicle control.

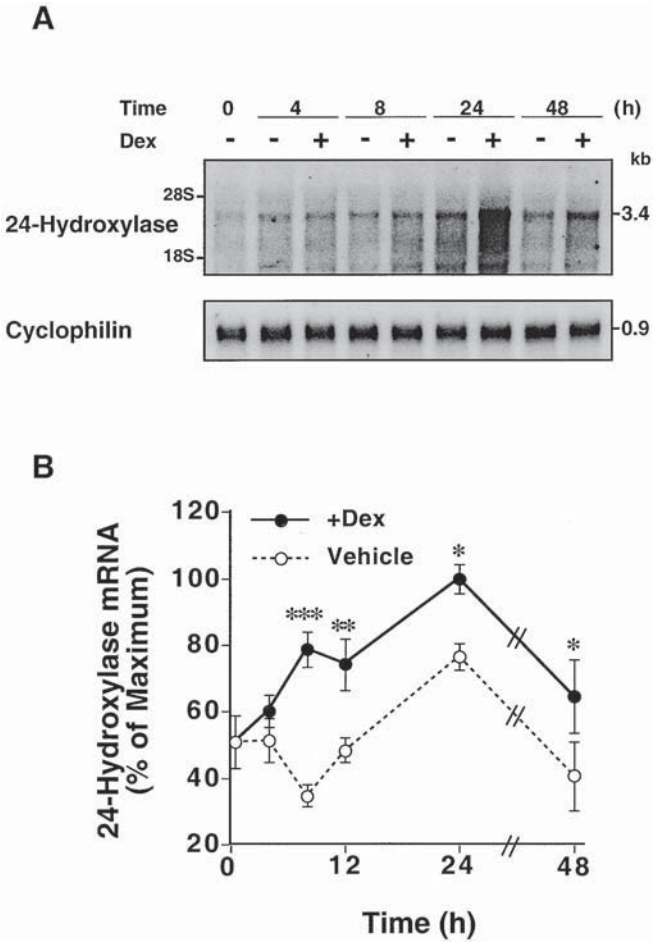

Fig. 3. Time course of stimulation of 24-hydroxylase mRNA expression by dexamethasone (Dex) in $1,25(OH)_2D_3$-treated osteoblast-like cells (UMR-106). Cells were pretreated with 10^{-7} *M* $1,25(OH)_2D_3$ for 24 h in serum-free DMEM, and thereafter 10^{-6} *M* dexamethasone or vehicle was added to the cells in the presence of 10^{-7} *M* $1,25(OH)_2D_3$ and treated for the indicated time periods. (**A**) Northern blot analysis; (**B**) densitometric determination of the Northern blots. Northern blots of total RNA (20 μg) were performed for 24-hydroxylase and cyclophilin mRNA. 24-Hydroxylase mRNA concentration was determined densitometrically and normalized to that of cyclophilin. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared with vehicle control at each point.

by dexamethasone and $1,25(OH)_2D_3$ was examined as a function of time. Northern analysis showed that the abundance of 24-hydroxylase transcript of 3.4 kb was increased in UMR-106 cells exposed to 10^{-6} *M* dexamethasone for 8 to 24 h (Fig. 3A). The increase in 24-hydroxylase mRNA already was maximal at 8 h, and significant elevation was maintained for up to 48 h (Fig. 3B). The activity of 24-hydroxylase induced in UMR-106 cells by 10^{-7} *M* $1,25(OH)_2D_3$ was significantly increased at 12 and 24 h after the addition of dexamethasone (Fig. 4). Treatment of dexamethasone for 36 h did not stimulate the 24-hydroxylase activity in the cells. Figure 5 depicts 24-hydroxylase mRNA after dexamethasone treatment for 8 h in $1,25(OH)_2D_3$-treated UMR-106 cells. Enhancement of 24-hydroxylase mRNA in the cells in response to dexamethasone was significant at a concentration of 10^{-7} *M* and maximal at 10^{-6} *M*, because a higher concentration (10^{-5} *M*) of dexamethasone decreased the abundance of mRNA (data not shown). Treatment with 10^{-6} *M* dexamethasone for 24 h significantly increased 24-hydroxylase activity induced in UMR-106 cells by 10^{-7} *M* $1,25(OH)_2D_3$, while concentrations of the glucocorticoid up to 10^{-7} *M* could not clearly stimulate activity (Fig. 6).

Effect of Cycloheximide on Dexamethasone-Stimulated 24-Hydroxylase mRNA in UMR-106 Cells

The effect of the protein synthesis inhibitor cycloheximide on the dexamethasone-induced increase in 24-hydroxylase mRNA expression was studied to determine whether the increase in osteoblast-like UMR-106 cells requires induction of transcription factors. Treatment of the cells with 3.5 μ*M* cycloheximide (1 μg/mL) 1 h before the addition of 10^{-6} *M* dexamethasone completely abolished glucocorticoid-stimulated expression of 24-hydroxylase mRNA, demonstrating that *de novo* protein synthesis was required for stimulation (Fig. 7). There was no significant difference between the cells treated and untreated with 3.5 μ*M* cycloheximide for 9 h on the cell morphology and survival using 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazalium bromide colorimetric assay.

Involvement of c-fos Gene Expression in Dexamethasone-Stimulated 24-Hydroxylase Expression in UMR-106 Cells

To determine whether dexamethasone enhanced expression of the proto-oncogene c-*fos* in UMR-106 cells, we first

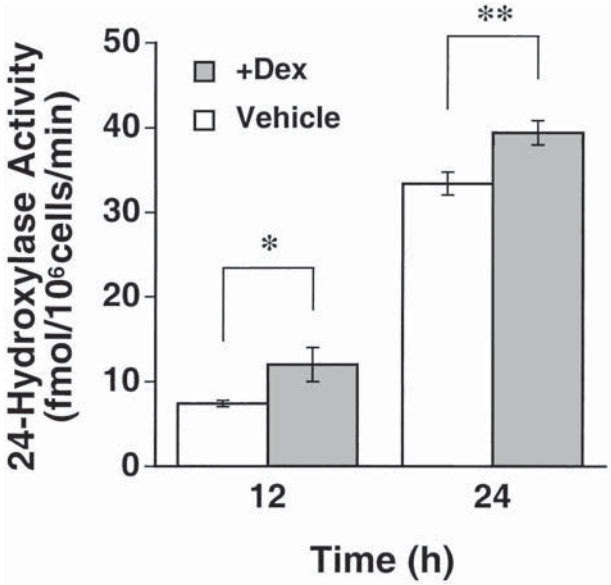

Fig. 4. Effect of dexamethasone (Dex) on $1,25(OH)_2D_3$-induced 24-hydroxylase activity in UMR-106 cells. Dexamethasone or vehicle was added to the cells that were pretreated with 10^{-7} *M* $1,25(OH)_2D_3$ for 24 h in serum-free DMEM, and was treated for 12 or 24 h in the presence of 10^{-7} *M* $1,25(OH)_2D_3$. 24-Hydroxylase activity was measured by cells incubated with $1,25(OH)_2[26,27\text{-}^3H]D_3$ (125 pmol/50,000 cpm) for 45 min. The substrate and products were separated by HPLC. Data are expressed as fmol/(10^6 cells·min) and are the mean ± SEM of four determinations. *$p < 0.05$ and **$p < 0.01$ compared with vehicle control.

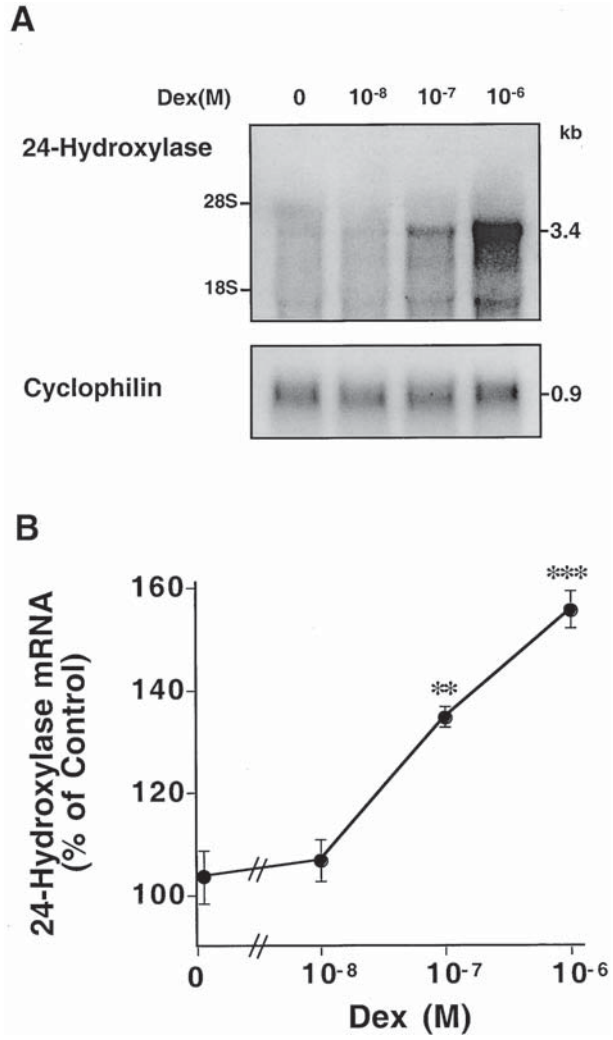

Fig. 5. Effect of dexamethasone (Dex) on abundance of 24-hydroxylase mRNA in $1,25(OH)_2D_3$-treated UMR-106 cells. At 70% confluence of UMR-106 cells, the medium was changed to serum-free DMEM for 24 h, and cells were pretreated with 10^{-7} *M* $1,25(OH)_2D_3$ for 24 h. Thereafter, cells were incubated with the indicated amounts of dexamethasone or vehicle in the presence of 10^{-7} *M* $1,25(OH)_2D_3$ for 8h. (**A**) Northern blots of total RNA (20 μg) for 24-hydroxylase mRNA and cyclophilin mRNA; (**B**) results of quantitative Northern blot analysis of 24-hydroxylase mRNA. Northern blots of 24-hydroxylase mRNA abundance were normalized to those of cyclophilin mRNA. Data are expressed as a percentage of the maximum (10^{-6} *M* dexamethasone) and are the mean ± SEM of four determinations. **$p < 0.01$ and ***$p < 0.001$ compared with vehicle control.

examined the effect of dexamethasone on c-*fos* mRNA accumulation using Northern analysis (Fig. 8). Study of the time course of c-*fos* mRNA stimulation by 10^{-6} *M* dexamethasone in the presence of 10^{-7} *M* $1,25(OH)_2D_3$ indicated that the increase in gene expression was rapid, with significant elevation evident at 20 min. Abundance of c-*fos* mRNA was maximal between 30 and 45 min after dexamethasone treatment and then decreased rapidly, returning to basal level by 60 min (Fig. 8). Next, we assessed the effect of dexamethasone on gene expression of c-*jun*, another component of the AP-1 transcription factor complex. Dexamethasone slightly increased c-*jun* mRNA abundance, whereas the stimulating effect of the steroid was much smaller in the gene expression of c-*jun* than in that of c-*fos* (Fig. 8B). Western blotting showed that dexamethasone increased the amount of c-*fos* protein. The increase was marked at 30 min and maximal at 60 min after the addition of 10^{-6} *M* dexamethasone (Fig. 9). Since phorbol 12-myristate 13-acetate (TPA) influences the expression of c-*fos*, the effect of TPA was next assessed on the regulation of c-*fos* gene expression in UMR-106 cells. In $1,25(OH)_2D_3$-treated UMR-106 cells, TPA at 200 n*M* rapidly increased c-*fos* mRNA expression, which peaked at 30 min after dexamethasone treatment and then rapidly decreased, returning to basal expression at 3 h (Fig. 10). TPA also resulted in a marked increase in c-*fos* protein synthesis in the cells (Fig. 9). The effect of TPA on 24-hydroxylase mRNA expression was studied next (Fig. 11). TPA significantly enhanced $1,25(OH)_2D_3$-induced 24-hydroxylase mRNA abundance at 4 and 6 h. Finally, to determine whether a protein kinase C (PKC) inhibitor influenced the dexamethasone-induced expression of 24-hydroxylase mRNA, we examined the effect of RO31-8220, a PKC inhibitor (Fig. 12). Treatment of the cells with 1μ*M* RO31-8220 1 h before the addition of 10^{-6} *M* dexamethasone completely abolished glucocorticoid-stimulated expression of 24-hydroxylase mRNA.

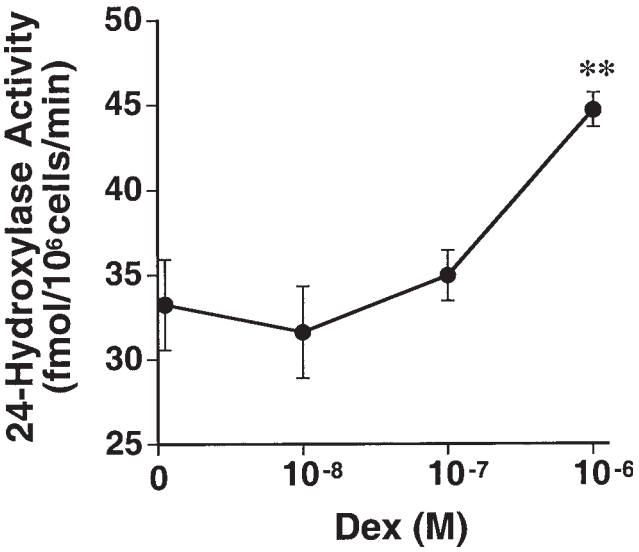

Fig. 6. Effect of dose of dexamethasone (Dex) on 24-hydroxylase activity in $1,25(OH)_2D_3$-treated UMR-106 cells. Graded doses of dexamethasone or vehicle were added to $1,25(OH)_2D_3$-treated cells, and were treated for 24 h. 24-Hydroxylase activity was measured as described in Fig. 4. Data are expressed as fmol/(10^6 cells·min) and are the mean ± SEM of four determinations. $^{**}p < 0.01$ compared with vehicle control.

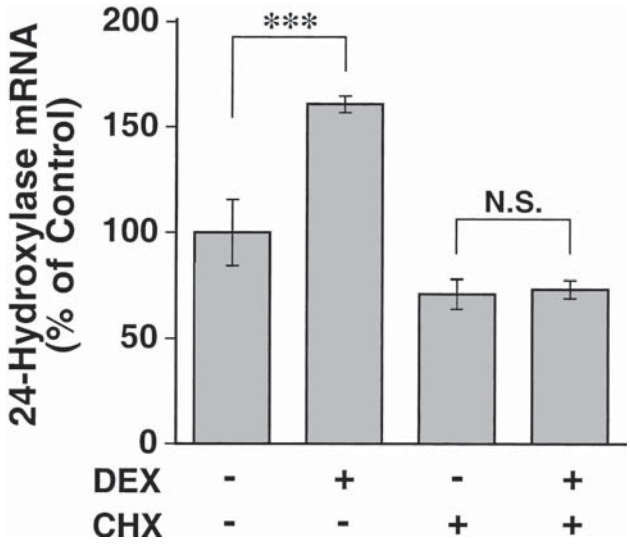

Fig. 7. Effect of dexamethasone (Dex) at 10^{-6} *M* in the presence or absence of cycloheximide (CHX) at 3.5 μ*M* on $1,25(OH)_2D_3$-induced 24-hydroxylase mRNA expression in UMR-106 cells. Serum-starved and 10^{-7} *M* $1,25(OH)_2D_3$-administered cells were treated with cycloheximide for 1 h before incubation with dexamethasone or vehicle, and thereafter cells were exposed to 10^{-6} *M* dexamethasone or vehicle in the presence or absence of cycloheximide for 8 h. Cells were harvested and total RNA was extracted for densitometric determination of the Northern blots. The abundance of 24-hydroxylase mRNA normalized relative to cyclophilin mRNA was expressed as a percentage of the vehicle control value. Each bar represents the mean ± SEM of four measurements. $^{***}p < 0.001$ compared with vehicle control; N.S., not significantly different between cycloheximide-treated groups.

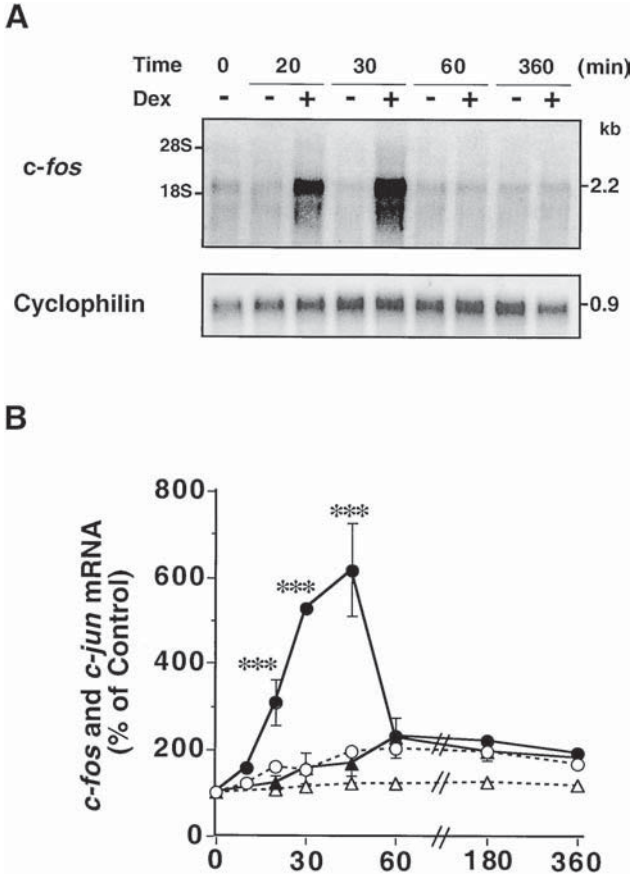

Fig. 8. Effect of dexamethasone (Dex) on $1,25(OH)_2D_3$-treated c-*fos* and c-*jun* mRNA expression in UMR-106 cells. Serum-starved and 10^{-7} *M* $1,25(OH)_2D_3$-administered cells were treated with 10^{-6} *M* dexamethasone (●, ▲) or vehicle (○, △) for the indicated time periods. (**A**) Northern blot analysis of c-*fos* and cyclophilin mRNA; (**B**) densitometric determination of the Northern blots. The abundance of c-*fos* (●, ○) and c-*jun* (▲, △) mRNA abundance normalized relative to cyclophilin mRNA was expressed as a percentage of maximum and is the mean ± SEM of four determinations. $^{***}p < 0.001$ compared with vehicle control at each time point.

Discussion

Our previous in vivo study *(16)* demonstrated that dexamethasone markedly stimulated renal 24-hydroxylase gene expression in mice. In the present study, in vitro experiments using cultured cells were conducted to evaluate the effects of dexamethasone and define the underlying mechanisms of 24-hydroxylase gene activation. Both renal cells (LLC-PK_1) and osteoblastic cells (UMR-106) were studied, since the kidney is a key metabolic site and bone is a major target tissue of $1,25(OH)_2D_3$. The results demonstrated that in the presence of $1,25(OH)_2D_3$, dexamethasone enhanced transcription of 24-hydroxylase gene via new protein synthesis.

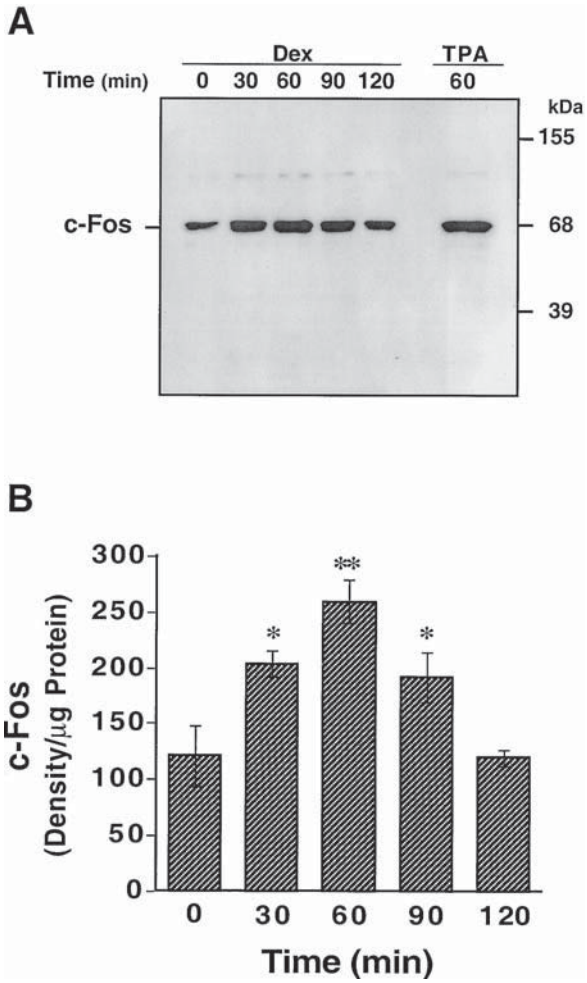

Fig. 9. Effect of dexamethasone (Dex) at 10^{-6} *M* on c-*fos* protein expression in UMR-106 cells treated with 1,25$(OH)_2D_3$ at 10^{-7} *M*. Serum-starved and 1,25$(OH)_2D_3$-administered cells were treated with vehicle or 10^{-6} *M* dexamethasone for the indicated time periods. Moreover, TPA (200 nM) for 60 min was added to the cells. The cells were harvested and subjected to Western immunoblot analysis. (**A**) Representative immunoblot; (**B**) quantification by scanning densitometry of blots from three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared with vehicle control.

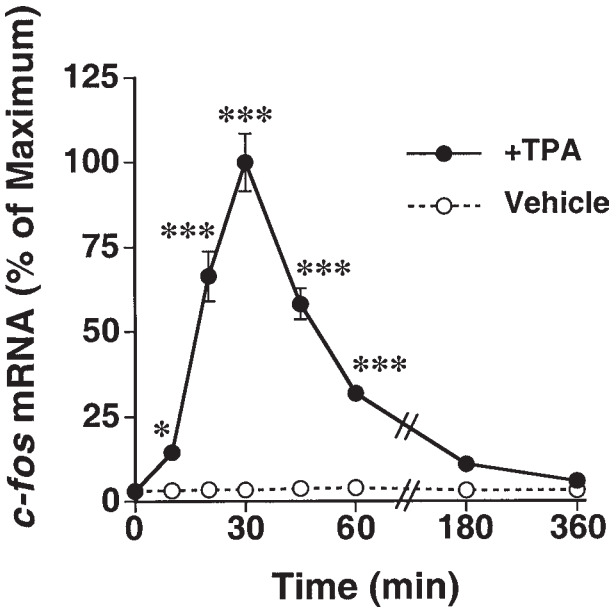

Fig. 10. Effect of TPA on abundance of 1,25$(OH)_2D_3$-treated c-*fos* mRNA in UMR-106 cells. Serum-starved and 10^{-7} *M* 1,25 $(OH)_2D_3$-administered cells were treated with vehicle or TPA (200 n*M*) for the indicated time periods. Northern blots of total RNA were performed for c-*fos* and cyclophilin mRNA. The concentration of c-*fos* mRNA was determined densitometrically and normalized to that of cyclophilin mRNA. Data are the mean ± SEM of four measurements. *$p < 0.05$ and ***$p < 0.001$ compared with vehicle control at each time point.

The kidney is the major site of catabolism of vitamin D metabolites such as 25OHD_3 by 24-hydroxylase. We first studied the effect of dexamethasone on 24-hydroxylase expression in an established porcine kidney cell line, LLC-PK_1. These cells possess VDR and show induction of 24-hydroxylase activity by 1,25$(OH)_2D_3$ *(19,20)*. The LLC-PK_1 cell monolayers showed increases in 24-hydroxylase mRNA enzymatic activity after treatment with 10^{-7} *M* 1,25$(OH)_2D_3$, and this effect was enhanced by dexamethasone, confirming our previous observation in vivo that excess glucocorticoid markedly stimulated renal 24-hydroxylase expression in mice *(16)*. The stimulating effect of dexamethasone on the abundance of 24-hydroxylase mRNA and its activity was less marked in LLC-PK_1 cells than in UMR-106 cells. Reasons for the blunted effect of dexamethasone in LLC-PK_1 cells, however, are not understood. The most likely explanation is that glucocorticoids may be less effective in renal cells than in osteoblasts of the bone, which is the target tissue for glucocorticoid-induced osteoporosis.

Glucocorticoid treatment is one of the most common causes of osteoporosis *(1,2)*. Although numerous studies have reported the effects of glucocorticoids on osteoblastic function and differentiation, little is known as to whether glucocorticoids regulate the catabolism of 1,25$(OH)_2D_3$ in osteoblasts. A major catabolic enzyme of 1,25$(OH)_2D_3$, 24-hydroxylase, also is induced by 1,25$(OH)_2D_3$ in osteoblastic cells *(21–23)*. Moreover, a recent investigation using 24-hydroxylase knockout mice showed that complete absence of 24-hydroxylase activity during development led to impaired intramembranous bone mineralization *(24)*. These studies demonstrated the physiologic importance of the hydroxylase enzyme in bone. We therefore investigated the molecular mechanism of action of dexamethasone on 1,25$(OH)_2D_3$-induced 24-hydroxylase expression in osteoblastic cells. The present results demonstrated that 1,25$(OH)_2D_3$ and dexamethasone additively increased 24-hydroxylase mRNA abundance and enzymatic activity in UMR-106 osteoblast-like cells. Expression of 24-hydroxylase mRNA was maximally induced at 8–12 h after dexamethasone exposure in 1,25 $(OH)_2D_3$-treated UMR-106 cells. As for dose, the abundance

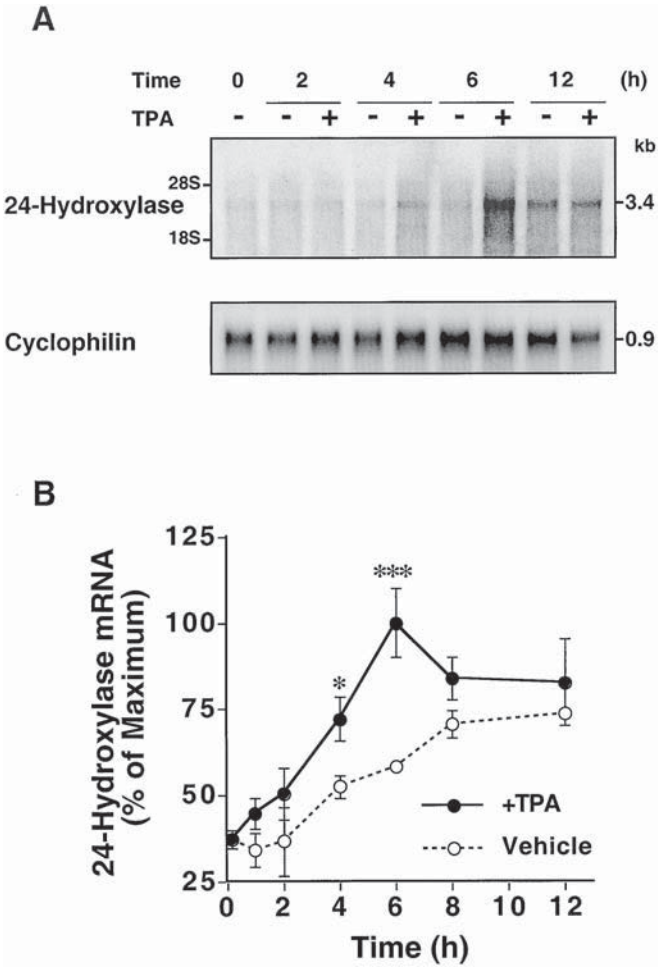

Fig. 11. Effect of TPA on $1,25(OH)_2D_3$-treated 24-hydroxylase mRNA expression in UMR-106 cells. Serum-starved and 10^{-7} *M* $1,25(OH)_2D_3$-administered cells were treated with vehicle or 200 n*M* TPA for the indicated time periods. (**A**) Northern blot analysis; (**B**) densitometric determination of the Northern blots. The abundance of 24-hydroxylase mRNA normalized relative to cyclophilin mRNA was expressed. Data are expressed as a percentage of maximum and are the mean ± SEM of four measurements. *$p < 0.05$ and ***$p < 0.001$ compared with vehicle control at each time point.

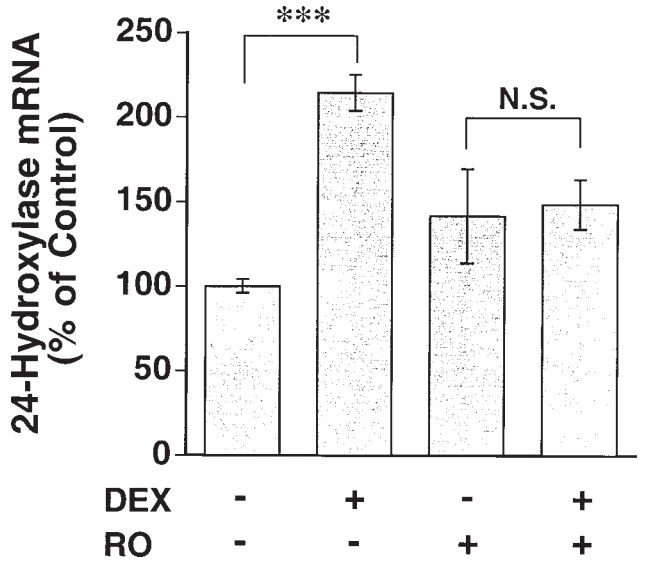

Fig. 12. Effect of PKC inhibitor on dexamethasone (Dex)-induced 24-hydroxylase mRNA expression in $1,25(OH)_2D_3$-treated UMR-106 cells. Serum-starved and 10^{-7} *M* $1,25(OH)_2D_3$-administered cells were treated with RO31-8220 (RO), a PKC inhibitor, at 10^{-6} *M* for 1 h before incubation with dexamethasone or vehicle. Thereafter, cells were exposed to 10^{-6} *M* dexamethasone or vehicle in the presence or absence of RO31-8220 for 8 h. Cells were harvested and total RNA was extracted for densitometric determination of the Northern blots. The abundance of 24-hydroxylase mRNA normalized relative to cyclophilin mRNA was expressed as a percentage of the vehicle control. Each bar represents the mean ± SEM of four measurements. ***$p < 0.001$ compared with vehicle control. N.S., not significantly different between RO31-8220-treated groups.

of 24-hydroxylase mRNA was stimulated at 0.1–1 μ*M* dexamethasone. Dexamethasone stimulation of 24-hydroxylase mRNA expression in $1,25(OH)_2D_3$-treated UMR-106 cells was abolished completely by pretreatment with cycloheximide, an inhibitor of protein synthesis, indicating that stimulation requires new protein synthesis. Taken together, these data suggest that the action of glucocorticoids on 24-hydroxylase gene expression is a receptor-mediated genomic effect.

We next sought to determine the mechanism of enhancement of 24-hydroxylase gene expression in $1,25(OH)_2D_3$-treated UMR-106 cells. Chen et al. *(25)* demonstrated that treatment with a potent PKC activator, TPA, increased 24-hydroxylase expression in renal cells treated with $1,25(OH)_2D_3$. In nontransformed rat intestinal (IEC-6) cells treated with $1,25(OH)_2D_3$, TPA rapidly increased the abundance of 24-hydroxylase mRNA, strongly suggesting that the PKC plays an important role in transcriptional activation of 24-hydroxylase gene *(26)*. Such treatment also briefly increased c-*fos* mRNA expression in MC3T3-E1 osteoblastic cells *(27)*, as in our present observation in UMR-106 osteoblastic cells.

The 24-hydroxylase gene promoter on vitamin D–responsive elements (VDREs) is activated by VDR and retinoid X receptor heterodimers. Since the rat 24-hydroxylase promoter has two VDREs *(28–30)* and synergistic interactions of the VDR with AP-1 and Sp have been demonstrated by studying an artificial rearrangement of elements in the promoter *(31)*, binding of c-*fos* protein and c-*jun* protein to AP-1-binding sites may enhance *trans*-activation of the 24-hydroxylase gene by VDR and VDRE *(32)*. Our findings indicated that dexamethasone stimulated c-*fos* protein and that TPA increased in c-*fos* protein and 24-hydroxylase expression. By contrast, dexamethasone only slightly induced the gene expression of c-*jun*, another component of transcription factor complex on an AP-1 site.

Since c-*fos* protein is phosphorylated by PKC before its binding to the AP-1 site, we assessed the effect of a PKC inhibitor, RO31-8220, in $1,25(OH)_2D_3$-treated UMR-106 cells. Pretreatment of the PKC inhibitor completely abolished the

dexamethasone-stimulated 24-hydroxylase mRNA expression in the cells, indicating that the activation of c-*fos* protein was required for transcriptional stimulation of 24-hydroxylase gene in osteoblastic cells. Collectively, dexamethasone promotes gene expression for 24-hydroxylase, at least in part, via stimulation of c-*fos* expression in UMR-106 osteoblastic cells.

Glucocorticoids such as dexamethasone have both permissive and suppressive effects on bone remodeling that vary with dose and treatment duration. Exposure to high doses of glucocorticoids or excess endogenous steroid for extended periods produces clinically significant osteopenia, termed *glucocorticoid-induced osteoporosis (1,2)*. Studies in vitro showed that exposure to high doses of glucocorticoids suppresses gene expression of insulin-like growth factor-1, a bone growth factor, by cultured osteoblasts *(33–35)*. By contrast, glucocorticoid given intermittently or at a low dose can promote osteoblast differentiation and enhance mineralized nodule formation by osteoblasts *(36,37)*. Therefore, glucocorticoids have divergent effects in osteoblasts.

The most important function of $1,25(OH)_2D_3$ is to maintain blood calcium in the physiologic range by enhancing bone resorption. The active form of vitamin D, $1,25(OH)_2D_3$, stimulates differentiation of monocytic stem cells into osteoclasts. Osteoclastic activity also is regulated through interactions with osteoblasts, whose functions are controlled by $1,25(OH)_2D_3$ *(7)*. The bone-resorptive action of $1,25(OH)_2D_3$ is mediated by receptor activator of nuclear factor-κB ligand produced by osteoblasts *(38)*. In addition, $1,25(OH)_2D_3$ directly stimulates bone resorption by osteoblasts, by inducing collagenase gene expression and protein synthesis in these cells *(39,40)*. Knockout mice with complete absence of 24-hydroxylase activity *(24)* showed elevated concentrations of $1,25(OH)_2D_3$ in serum and target tissues such as bone because of impaired degradation of the hormone. During development these mice showed severely decreased intramembranous mineralization in the calvarium, clavicle, and mandible, and in the periostium of long bones. By contrast, continuous treatment with $1,25(OH)_2D_3$ at physiologic doses reportedly has anabolic effects, such as stimulation of mineralization *(41)* and elevation of alkaline phosphatase activity in osteoblasts *(42)*. By enhancing 24-hydroxylase expression, induction by $1,25(OH)_2D_3$, glucocorticoids favors catabolism of $1,25(OH)_2D_3$ and counteracts the anabolic effect of $1,25(OH)_2D_3$ in osteoblasts. Glucocorticoids, then, may stimulate net bone resorption by increasing catabolism of this anabolically active vitamin D metabolite.

Materials and Methods

Cell Culture

Osteoblast-like rat osteosarcoma cells (UMR-106; CRL 1661; American Type Culture Collection, Manassas, VA) were grown in monolayer culture at 37ºC in an atmosphere of 95% air and 5% CO_2. DMEM (ICN, Aurora, OH), supplemented with 5% fetal bovine serum (FBS; Filtron, Brooklyn, Australia), was used. Cultures were maintained with one passage per week. At 70% confluence, the culture medium was replaced with serum-free DMEM for 24 h *(43)*. Cells were treated with 10^{-7} *M* $1,25(OH)_2D_3$ (Rossel Uclaf, Romanville, France) dissolved in ethanol vehicle for 24 h. Cells were then exposed for varying intervals to varying concentrations of dexamethasone (Sigma, St. Louis, MO) in DMEM in the continued presence of 10^{-7} *M* $1,25(OH)_2D_3$. To determine the effect of inhibitors, $1,25(OH)_2D_3$-treated cells were exposed to 3.5 μ*M* cycloheximide (Sigma), a protein synthesis inhibitor, or to 1 μ*M* bisindolylmaleimide IX methanesulfonate (RO31-8220; Alexis, San Diego, CA), a PKC inhibitor, for 1 h before the indicated duration of incubation with 10^{-6} *M* dexamethasone.

Pig kidney LLC-PK_1 cells (American Type Culture Collection; CRL 1392) were maintained at 37ºC in an atmosphere of 5% CO_2 and 95% air in DMEM supplemented with 7% FBS. At 70% confluence, the culture medium was replaced with serum-free DMEM for 24 h. Cells were exposed to test substances such as $1,25(OH)_2D_3$ and dexamethasone. LLC-PK_1 cells were incubated for the period indicated.

Northern Blot Analysis

Total cellular RNA was isolated using 4 *M* guanidine thiocyanate followed by phenol-chloroform extraction and was quantified by absorbance at 260 nm. Twenty micrograms of total RNA was separated by electrophoresis in 1.2% agarose gel with 6% formaldehyde and transferred to Hybond-N^+ membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) by capillary immobilization *(43)*. Membranes were hybridized with rat 24-hydroxylase, rat c-*fos*, rat c-*jun*, or cyclophilin cDNA probes that had been labeled with [α-^{32}P]dCTP (110 TBq/mmol; ICN, Costa Mesa, CA) using a Megaprime DNA labeling system (Amersham). Hybridization was performed for 2 d at 42ºC in 50% formamide, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 5X SSPE (150 m*M* NaCl, 10 m*M* sodium phosphate, and 1 m*M* EDTA), after which the membranes were washed in 0.1X SSPE-0.1% SDS at 65ºC for 15 min *(16)*. Amounts of mRNA were determined using an FX Molecular Imager (Bio-Rad, Hercules, CA). The abundance of 24-hydroxylase or c-*fos* mRNA was stated relative to that of cyclophilin mRNA.

Measurement of 24-Hydroxylase Activity

Serum-deprived cells were rinsed twice with warmed Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) and placed in the incubation medium (DMEM containing 25 m*M* HEPES, pH 7.4). A substrate, $1,25(OH)_2[26,27\text{-}^3H]D_3$ (125 pmol/50,000 cpm; Dupont NEN, Boston, MA), dissolved in 10 μL of ethanol was added to a flask with an area of 25 cm^2 at the base that contained confluent cells and 2 mL of incubation

medium. Cells were incubated at 37ºC for 45 min, after which the reactions were stopped by the addition of 2 mL of acetonitrile. Vitamin D metabolites were extracted with a C18/Sep-Pak (Waters, Milford, MA) and separated by HPLC *(17)*. Dependence of 24-hydroxylase activity on incubation time was observed up to 60 min, consistent with reported results in Caco-2 cells *(44)*. 24-Hydroxylase activity was expressed as femtomoles per 10^6 cells per minute.

Western Blot Analysis

For Western blotting, UMR-106 cells grown to subconfluence in T-25 flasks were cultured in modified DMEM medium with or without dexamethasone at 10^{-6} *M* for the period indicated in the presence of 10^{-7} *M* 1,25$(OH)_2D_3$. Cells were then lysed in a buffer containing 0.058 *M* Tris-HCl, 1.7% SDS, 6% glycerol, 0.8% mercaptoethanol, and 0.002% bromophenol blue. The protein concentration was determined, and 10 µg of each sample was separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to a membrane (Clear Blot Membrane-P; ATTO, Tokyo, Japan). Membranes were blocked overnight at 4ºC in 10% equine serum in PBS containing 0.1% Tween-20. Membranes were incubated for 2 h at room temperature in blocking solution containing a 1:1000 dilution of polyclonal antibody raised against c-*fos* (sc-7202; Santa Cruz Biotechnology, Santa Cruz, CA). Incubation was conducted for 45 min at room temperature with a 1:20,000 dilution of biotin-labeled antibody raised against IgG. Streptavidin-biotinated horseradish peroxidase P-labeled complex was made using a Vecstain ABC standard kit (Vector, Burlingame, CA). The antigen-antibody complexes on membranes were visualized using an ECL-Plus Western blotting detection system (Amersham). The amounts of c-*fos* protein were quantified by densitometric scanning of autoradiograms.

Statistical Analysis

Data are presented as the mean ± SEM. Statistical analysis was performed using analysis of variance followed by Fisher protected least significant difference test (Statview 4.02; Abacus, Berkeley, CA). A value of $p < 0.05$ was considered statistically significant.

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