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Alteration of cellular phosphorylation state affects vitamin D receptor-mediated CYP3A4 mRNA induction in Caco-2 cells

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Abstract

Expression of cytochrome P450 3A4 (CYP3A4) is induced by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in Caco-2 cells. However, since a typical vitamin D responsive element has not been found in the 5′-flanking region of the CYP3A4 gene, the mechanism of $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression is poorly understood. In the present study, we demonstrated that vitamin D receptor (VDR) is a critical factor for the induction using the antisense oligonucleotide technique. In addition, we found that treatment of Caco-2 cells with the protein kinase C (PKC) inhibitors, staurosporine and GF109203X, and the tyrosine kinase inhibitor, genistein, but not with the protein kinase A inhibitor, H-89, suppressed CYP3A4 mRNA induction by $1,25(OH)_2D_3$. The depletion of PKC by prolonged treatment with phorbol ester abolished the induction. On the other hand, protein kinase inhibitors used had no effects on the constitutive expression of VDR mRNA. Therefore, these observations suggest that $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression might be involved in phosphorylation events in addition to transcriptional regulation via VDR. However, $1,25(OH)_2D_3$ did not rapidly activate PKC in the Caco-2 cells used, while the treatment with staurosporine and GF109203X, but not genistein, decreased basal PKC activity by ~30% of the controls. Taken together, these findings suggest that the change in the phosphorylation state via PKC and tyrosine kinase might, at least in part, modulate $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression via VDR.

Keywords: Cytochrome P450 3A4; 1,25-Dihydroxyvitamin D_3; Vitamin D receptor; Phosphorylation; Protein kinase C; Tyrosine kinase

Cytochrome P450 (CYP) enzymes are a major class of hemoproteins that participate in the oxidation of endogenous substrates such as steroid hormones and the transformation of xenobiotics. CYP3A4 is the predominant CYP isoform in the human liver and small intestinal epithelial cells [1–3] and is responsible for the metabolism of many clinically important drugs [4]. Since CYP3A accounts for approximately 50% of the total CYPs in the human intestine [2,3], intestinal CYP3A4 has been suggested to contribute to first-pass metabolism of orally administered drugs.

It is well established that 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), a major active metabolite of vitamin D, regulates calcium homeostasis in the intestine and bone. $1,25(OH)_2D_3$ is also known to play an important role in cell growth and differentiation [5]. Recent studies have shown that $1,25(OH)_2D_3$ induces CYP3A4 gene expression in human colon carcinoma Caco-2 [6,7], which is widely used in pharmaceutical research as an in vitro intestinal model. Induction of CYP3A4 in Caco-2 cells in the presence of $1,25(OH)_2D_3$ was suggested to be a good strategy to analyze the roles of intestinal CYP3A4 for determining oral bioavailability and drug/drug interactions [6,8].

CYP3A4 gene expression is known to be induced both in vivo and in cultured hepatocytes by many structurally diverse compounds such as dexamethasone, rifampicin, and clotrimazole [9,10]. Recently, it was reported that the nuclear receptor pregnane X receptor (PXR), which is classified within the nuclear receptor 1I (NR1I) subfamily [11], contributes to the induction of the CYP3A4 gene expression by these compounds [12–14]. The biological actions of $1,25(OH)_2D_3$ are attributable to the activation of the intracellular vitamin D receptor (VDR). After binding $1,25(OH)_2D_3$ to VDR,

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this receptor complex heterodimerizes with retinoid X receptor (RXR) and modulates the expression of a variety of genes [15]. Therefore, the induction of CYP3A4 by $1,25(OH)_2D_3$ is suggested to depend on transcriptional activation of the CYP3A4 gene via VDR. However, the mechanism by which $1,25(OH)_2D_3$ induces the expression of CYP3A4 was not clear. Very recently, it was reported that binding of the VDR–RXR complex to the ER6 element in the CYP3A4 promoter which is known to be a PXR responsive element caused $1,25(OH)_2D_3$-mediated CYP3A4 expression [16].

On the other hand, previous studies have shown that phosphorylation is a critical event in transcriptional control of several CYP genes. For example, the transcriptional activation of the CYP1A1 gene by aryl hydrocarbon receptor ligands was reported to be inhibited by the addition of the PKC inhibitor staurosporine or by phorbol ester-induced down-regulation of PKC [17]. Treatment with staurosporine causes a decrease in growth hormone-induced CYP2C12 expression in primary rat hepatocytes [18]. Moreover, it has been reported that other protein kinases in addition to PKC, e.g., protein kinase A (PKA), calmodulin-dependent protein kinase, and cGMP-dependent kinase, and protein phosphatases, participate in the induction of CYP gene expression by phenobarbital, a typical inducer of many CYPs [19,20].

In the present study, we addressed the contribution of VDR to $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression and determined further whether phosphorylation events are involved in the induction in Caco-2 cells using various protein kinase inhibitors.

Materials and methods

Chemicals. $1,25(OH)_2D_3$ was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Staurosporine, GF109203X, phorbol 12-myristate 13-acetate (PMA), and genistein were purchased from Wako Pure Chemical Industries (Osaka, Japan). H-89 was purchased from Seikagaku (Tokyo, Japan).

Culture and treatments of cells. Caco-2 cells were grown in DMEM supplemented with 10% fetal bovine serum (FCS), 0.1 mM non-essential amino acids, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin in a 5% CO_2/95% air incubator at 37 °C. Cells were grown to subconfluence in culture dishes. The medium was replaced with serum-free DMEM containing 100 units/ml penicillin G, 0.1 mg/ml streptomycin, and 0.1 mM non-essential amino acids and the cells were incubated for a further 24 h. To examine the effects of various protein kinase inhibitors on $1,25(OH)_2D_3$-mediated CYP3A4 mRNA expression, cells were pretreated with PKC inhibitors (staurosporine or GF109203X), a PKA inhibitor (H-89), and a tyrosine kinase inhibitor (genistein) for 30 min at the concentrations indicated in the figure legends, followed by incubation for 24 h in the presence of 100 nM $1,25(OH)_2D_3$. To test the effects of down-regulation of PKC on $1,25(OH)_2D_3$-mediated CYP3A4 mRNA expression, cells were exposed to 100 nM PMA for 16 h, prior to treatment with $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ and staurosporine were dissolved in ethanol and GF109203X, H-89, and genistein were dissolved in dimethyl sulfoxide (DMSO). The final concentration of vehicle in each culture was <0.2%.

RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the treated cells with TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was generated from 4 μg total RNA. Reverse-transcription reaction was carried out for 60 min at 37 °C in 20 μl mixture containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxynucleoside triphosphate (dNTP), 0.6 μg random primer, 0.5 units RNase inhibitor, and 200 units Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). Aliquots of the reverse-transcription reaction mixture (1 μl) were amplified with primers specific for human CYP3A4 (forward primer, 5′-CCTTACACATACACACCCTTTGGAAGT-3′; reverse primer, 5′-AGCTCAATGCATGTACAGAATCCCCGGTTA-3′), human VDR (forward primer, 5′-GAGATGATCCTGAAGCGGAA-3′; reverse primer, 5′-TCCCACCTGGAACTTGATGA-3′), and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) (forward primer, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse primer, 5′-CAAAGTTGTCATGGATGACC-3′). The 25-μl PCR mixtures contained 25 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.63 units Taq DNA polymerase (Invitrogen). For amplification of CYP3A4, VDR, and GAPDH, PCR was carried out as follows: 2 min at 94 °C, one cycle; 40 s at 94 °C, 40 s at 60 °C, and 1 min at 72 °C, 35, 28, and 27 cycles, respectively. We ascertained that there were linear correlations between the amounts of PCR products and template cDNAs under the present PCR conditions. Aliquots of the PCR mixtures were separated on 3% agarose gel (agarose:Nusieve agarose, 2:1) and stained with ethidium bromide. Densitometric analyses were performed using NIH Image. The CYP3A4 and VDR mRNA levels were normalized relative to GAPDH mRNA level in each sample.

Treatment of cells with oligonucleotides. Phosphorothioate-modified oligonucleotides against human VDR were synthesized by Takara Shuzo (Shiga, Japan). The sense and antisense oligonucleotides used in this study were as follows: sense VDR: 5′-GAGGCAATGGCGGCCAGCA-3′; antisense VDR: 5′-TGCTGGCCGCCATTGCCTC-3′. The nucleotide sequence corresponds to the first 19 bases, following the AUG sequence of human VDR mRNA. The oligonucleotides were delivered to cells using LipofectoAMINE 2000 reagents (LF2000) according to manufacturer's directions. Briefly, oligonucleotide-LF2000 complexes were made by mixing 7 μg/ml LF2000 diluted with DMEM with an equal volume of oligonucleotides and directly added to Caco-2 cells. After 3 h, the transfection medium was replaced with complete growth medium containing 10% FCS and the cells were cultured for 21 h. The medium was replaced with serum-free DMEM and the cells were maintained for a further 12 h, followed by treatment with $1,25(OH)_2D_3$ for 24 h.

PKC assay. The PKC activity was determined in Caco-2 cells in situ by the method of Heasley and Johnson [21]. Briefly, Caco-2 cells were seeded in 24-multiwell plates and cultured until 80–90% confluence. The medium was replaced with serum-free medium and then maintained for 24 h. The cells were treated with 100 nM $1,25(OH)_2D_3$ for 15 min. Protein kinase inhibitors were incubated for 1 h, prior to addition of $1,25(OH)_2D_3$. After the medium was removed, the cells were incubated for 15 min with PKC assay buffer (137 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 10 mM $MgCl_2$, 1 mg/ml glucose, 5 mM EGTA, 50 μg/ml digitonin, 25 mM β-glycerophosphate, and 20 mM HEPES at pH 7.2) containing 100 μM [γ-^{32}P]ATP and 50 μM myelin basic protein fragment 4-14, PKC substrate peptide. After the incubation for 15 min, 10 μl of 25% TCA was added and 25 μl of each reaction mixture was spotted on Whatman p-81 phosphocellulose paper. After washing with 1% phosphoric acid, radioactivity was measured using a scintillation counter.

Statistical analysis. Data were analyzed using Student's *t* test. A P value of less than 0.05 was considered statistically significant.

Results

Contribution of VDR to 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression

As shown in Fig. 1A, 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression in Caco-2 cells in a dose-dependent manner. However, typical vitamin D responsive element nucleotide sequences have yet to be found in the 5′-flanking region of the CYP3A4 gene [22]. Therefore, to confirm whether VDR is involved in 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression, we employed antisense oligonucleotide technique. As shown in Fig. 1B, the treatment with antisense VDR oligonucleotide significantly reduced 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression. In contrast, the sense VDR oligonucleotide had no apparent effect on the induction of CYP3A4 mRNA. These findings showed that VDR is an essential factor for 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression in Caco-2 cells.

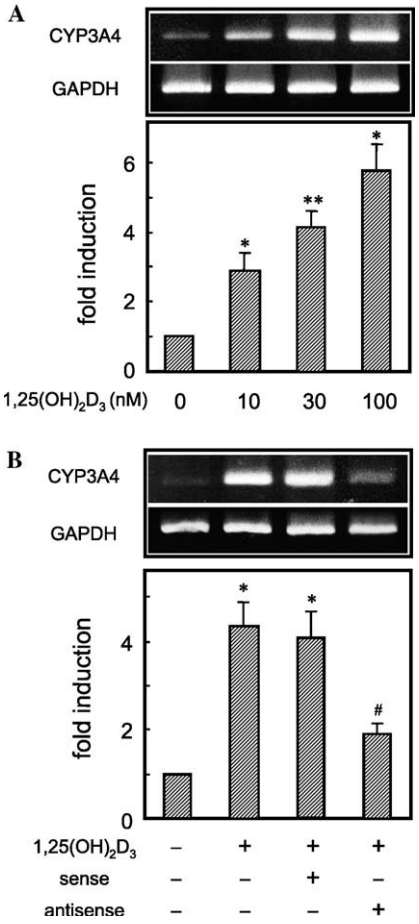

Fig. 1. Contribution of VDR in 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression. (A) Dose-dependent induction of CYP3A4 mRNA by 1,25(OH)$_2$D$_3$. Caco-2 cells were treated with 1,25(OH)$_2$D$_3$ (10, 30 or 100 nM) for 24 h. Values (means ± SE, $n = 3$) are expressed as the fold induction relative to untreated cells. * and **, Significant differences ($P < 0.05$ and $P < 0.01$, respectively) compared with untreated cells. (B) The sense or antisense oligonucleotides (2 μM) were delivered to Caco-2 cells using LF2000. After transfection for 3 h, the transfection medium was replaced with complete growth medium and the cells were cultured for 21 h. The medium was replaced with serum-free medium and the cells were maintained for a further 12 h, followed by treatment with 1,25(OH)$_2$D$_3$ (100 nM) for 24 h. Values (means ± SE, $n = 3$) are expressed as the fold induction relative to untreated cells. *, Significant differences ($P < 0.01$) compared with untreated cells; #, Significant differences ($P < 0.05$) compared with 1,25(OH)$_2$D$_3$-treated cells.

Effect of PKC inhibitors on 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression

It has been reported that the cellular phosphorylation state contributes to the 1,25(OH)$_2$D$_3$-stimulated transcriptional regulation of several genes via VDR [23,24]. Therefore, to understand the mechanism by which 1,25(OH)$_2$D$_3$ induces CYP3A4 mRNA expression in Caco-2 cells, we examined whether the PKC inhibitor, staurosporine, affects the induction. CYP3A4 mRNA expression increased 6.3-fold in the presence of 100 nM of 1,25(OH)$_2$D$_3$ (Fig. 2A). As shown in Fig. 2A, when Caco-2 cells were treated with staurosporine for 30 min, prior to the addition of 1,25(OH)$_2$D$_3$, CYP3A4 mRNA expression induced by 1,25(OH)$_2$D$_3$ was inhibited in a dose-dependent manner. To confirm this result, we also used another specific PKC inhibitor GF109203X. As shown in Fig. 2B, 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression was similarly inhibited by GF109 203X. In contrast, the PKA inhibitor H-89 showed no significant effect.

As described above, VDR plays an important role in 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression. Therefore, we confirmed the constitutive expression of VDR mRNA in the presence of the protein kinase inhibitors used. As expected, treatment with staurosporine, GF109203X or H-89 had no effect on the constitutive expression of VDR mRNA (Fig. 2).

Effect of depletion of PKC on 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression

To address further the involvement of PKC in 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression, we examined the effect of down-regulation of PKC by chronic treatment of Caco-2 cells with PMA. As shown in Fig. 3, 1,25(OH)$_2$D$_3$-induced expression of CYP3A4 mRNA was inhibited by treatment with 100 nM PMA for 16 h, prior to the addition of 1,25(OH)$_2$D$_3$. These findings suggested that PKC is involved in the induction of CYP3A4 mRNA by 1,25(OH)$_2$D$_3$ in Caco-2 cells. However, chronic treatment of Caco-2 cells with 100 nM PMA also significantly reduced VDR mRNA expression to 37% of control (Fig. 3). Therefore, in addition to the loss of PKC, the reduction of VDR appears to be attributed to the suppression of CYP3A4 mRNA induction by prolonged treatment of PMA.

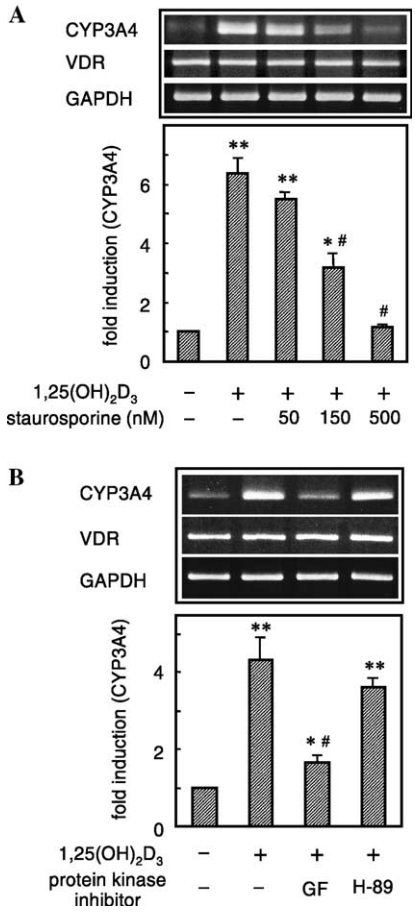

Fig. 2. Effects of protein kinases on the induction of CYP3A4 mRNA by $1,25(OH)_2D_3$. (A) Dose-dependent inhibition of $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression by staurosporine. Caco-2 cells were pretreated with the indicated concentrations of staurosporine for 30 min, followed by incubation of the cells with or without $1,25(OH)_2D_3$ (100 nM) for 24 h. Values (means ± SE, $n = 3$) are expressed as the fold induction relative to untreated cells. * and **, Significant differences ($P < 0.05$ and $P < 0.01$, respectively) compared with untreated cells; #, Significant differences ($P < 0.01$) compared with $1,25(OH)_2D_3$-treated cells. (B) Effects of GF109203X (GF) and H-89 on $1,25(OH)_2D_3$-mediated CYP3A4 mRNA. Caco-2 cells were pretreated with GF109203X (5 μM) or H-89 (10 μM) for 30 min, followed by incubation with or without $1,25(OH)_2D_3$ (100 nM) for 24 h. Values (means ± SE, $n = 5$) are expressed as the fold induction relative to untreated cells. * and **, Significant differences ($P < 0.05$ and $P < 0.01$, respectively) compared with untreated cells; #, Significant differences ($P < 0.01$) compared with $1,25(OH)_2D_3$-treated cells.

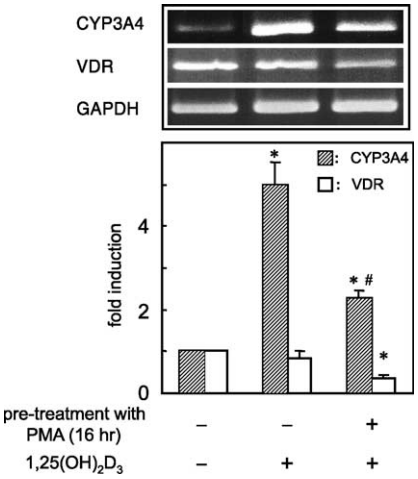

Fig. 3. PMA-induced down-regulation of PKC prevents $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression. Caco-2 cells were incubated in the presence or absence of PMA (100 nM) for 16 h, followed by treatment with or without $1,25(OH)_2D_3$ (100 nM) for 24 h. Values (means ± SE, $n = 3$ or 4) are expressed as the fold induction relative to untreated cells. *, Significant differences ($P < 0.01$) compared with untreated cells; #, Significant differences ($P < 0.05$) compared with $1,25(OH)_2D_3$-treated cells. There were no significant differences in CYP3A4 mRNA expression level between untreated and PMA-treated cells.

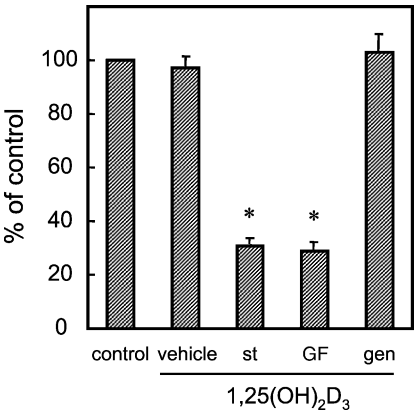

Fig. 4. $1,25(OH)_2D_3$ failed to rapidly activate PKC but reduced the basal PKC activity in the presence of PKC inhibitors. Caco-2 cells were preincubated with 150 nM staurosporine (st), 5 μM GF109203X (GF), and 50 μM genistein (gen) for 1 h, followed by the treatment with or without $1,25(OH)_2D_3$ (100 nM) for 15 min. The medium was replaced with PKC assay buffer containing 100 μM [γ-^{32}P]ATP and 50 μM PKC substrate peptide and PKC activity was determined as described in Materials and methods. Values (means ± SE, $n = 3$) are expressed as percentage of controls. *, Significant differences ($P < 0.01$) compared with untreated cells.

Inhibition of basal PKC activity by staurosporine or GF109203X

Several reports have shown that $1,25(OH)_2D_3$ rapidly activates PKC in various cell types including Caco-2 cells [25–27]. Therefore, we examined the effect of $1,25(OH)_2D_3$ on PKC activity in Caco-2 cells to confirm whether rapid PKC activation caused by $1,25(OH)_2D_3$ is involved in CYP3A4 induction. Unexpectedly, PKC activity was not changed after incubation of cells with 100 nM of $1,25(OH)_2D_3$ for 15 min (Fig. 4). On the other hand, when Caco-2 cells were treated with 150 nM staurosporine or 5 μM GF109203X, prior to the addition of $1,25(OH)_2D_3$, basal PKC activity was inhibited by ~30% of control.

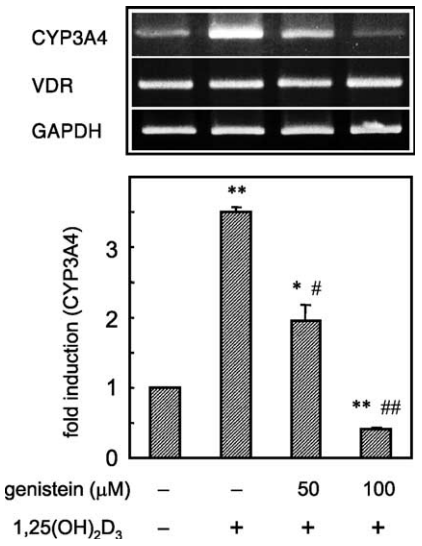

Fig. 5. Effects of genistein on $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression. Caco-2 cells were pretreated with genistein (50 and 100 μM) for 30 min, followed by incubation with or without $1,25(OH)_2D_3$ (100 nM) for 24 h. Values (means ± SE, $n = 3$) are expressed as the fold induction relative to untreated cells. * and **, Significant differences ($P < 0.05$ and $P < 0.01$, respectively) compared with untreated cells; # and ##, Significant differences ($P < 0.05$ and $P < 0.01$, respectively) compared with $1,25(OH)_2D_3$-treated cells.

Effect of genistein on 1,25(OH)$_2$D$_3$-induced CYP3A4 mRNA expression

Previous studies demonstrated that $1,25(OH)_2D_3$ activated c-Src and genistein-sensitive tyrosine kinase [28–30]. To determine the involvement of tyrosine kinase in $1,25(OH)_2D_3$-mediated CYP3A4 mRNA expression, Caco-2 cells were treated with the tyrosine kinase inhibitor genistein for 30 min, prior to the addition of $1,25(OH)_2D_3$. As shown in Fig. 5 the induction of CYP3A4 mRNA by $1,25(OH)_2D_3$ was inhibited in a dose-dependent manner and completely suppressed at the concentration of 100 μM genistein. However, genistein had no effect on constitutive VDR mRNA expression and basal PKC activity (Figs. 4 and 5). These results suggested that tyrosine kinase may also contribute to CYP3A4 mRNA expression mediated by $1,25(OH)_2D_3$.

Discussion

CYP3A4 is transcriptionally regulated by a wide range of structurally diverse compounds. Recently, it was reported that $1,25(OH)_2D_3$ induces CYP3A4 expression in Caco-2 cells. Since the biological actions of $1,25(OH)_2D_3$ involve the direct interaction of the VDR with the promoter of target genes, the transcriptional activation of the CYP3A4 gene by $1,25(OH)_2D_3$ has been suggested to be mediated via ligand-dependent activation of VDR. However, the vitamin D responsive element has yet to be found in the 5′-flanking region of the CYP3A4 gene [22]. Therefore, the mechanism by which $1,25(OH)_2D_3$ up-regulates CYP3A4 expression was not clear. In the present study, we found that the treatment of Caco-2 cells with antisense oligonucleotide against VDR abolished the induction by $1,25(OH)_2D_3$. This result suggests that VDR is essential for $1,25(OH)_2D_3$-induced CYP3A4 expression. In addition, a very recent study demonstrated the binding of VDR–RXR complex to the ER6 element in CYP3A4 promoter caused $1,25(OH)_2D_3$-mediated CYP3A4 expression [16]. Therefore, $1,25(OH)_2D_3$ is suggested to basically regulate CYP3A4 mRNA induction via VDR by a ligand-dependent mechanism.

We also demonstrated that the PKC inhibitors staurosporine and GF109203X markedly inhibited the induction of CYP3A4 mRNA expression by $1,25(OH)_2D_3$ in Caco-2 cells, whereas the PKA inhibitor H-89 showed no effect. In addition, we showed that the depletion of PKC by chronic exposure to PMA abolished $1,25(OH)_2D_3$-mediated CYP3A4 mRNA expression. These results suggested that PKC plays an important role in the expression of CYP3A4 mRNA induced by $1,25(OH)_2D_3$. It was reported that osteocalcin gene transcription mediated by VDR is inhibited by staurosporine [23]. There is evidence to suggest that $1,25(OH)_2D_3$ rapidly activates PKC in a variety of cell types including Caco-2 cells. However, $1,25(OH)_2D_3$ did not cause PKC activation within 5 min–2 h in Caco-2 cells used in this study, whereas protein kinase inhibitors inhibited basal PKC activity. These results suggest that the cellular phosphorylation state might be involved in $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression. Failure of rapid PKC activation by $1,25(OH)_2D_3$ might be due to having basal PKC activity left even in serum-starved Caco-2 cells. Since it was reported that $1,25(OH)_2D_3$ up-regulates the PKC protein level in other cell types [31], it is likely that long-term incubation of $1,25(OH)_2D_3$ may result in the elevation of the basal PKC activity in Caco-2 cells.

The tyrosine kinase inhibitor genistein decreased $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression in Caco-2 cells in a concentration-dependent manner. This finding suggested that the tyrosine kinase, in addition to PKC, might be involved in the transcriptional activation of the CYP3A4 gene by $1,25(OH)_2D_3$. However, since genistein is known as a potent ligand for estrogen receptor-β [32], which Caco-2 cells express [33], and it was present throughout the entire incubation period, we cannot exclude the possibility that CYP3A4 mRNA expression is down-regulated in an estrogen receptor-β-dependent manner. Recently, it was demonstrated that $1,25(OH)_2D_3$ elicits tyrosine phosphorylation of the VDR and VDR–Src interaction in skeletal muscle cells [34] and that VDR itself mediates tyrosine phosphory-

lation events induced by $1,25(OH)_2D_3$ [35]. Therefore, a different mechanism from that of ligand-dependent transciptional activation of VDR is also likely to participate in $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression.

In conclusion, our observations indicated that VDR is essential for the induction of CYP3A4 mRNA by $1,25(OH)_2D_3$ and that the cellular phosphorylation state mediated by PKC and tyrosine kinase may, at least in part, contribute to the induction via VDR. Since the phosphorylation of steroid hormone receptors themselves including VDR has been shown to be responsible for their transcriptional activity [36], further studies are required to find out how phosphorylational events regulate $1,25(OH)_2D_3$-induced CYP3A4 mRNA expression via VDR.

References

[1] P.B. Watkins, S.A. Wrighton, E.G. Schuetz, D.T. Molowa, P.S. Guzelian, Identification of glucocorticoid-inducible cytochromes P-450 in the intestinal mucosa of rats and man, J. Clin. Invest. 80 (1987) 1029–1036.

[2] I. de Waziers, P.H. Cugnenc, C.S. Yang, J.P. Leroux, P.H. Beaune, Cytochrome P450 isoenzymes, epoxide hydrolase and glutathione transferases in rat and human hepatic and extrahepatic tissues, J. Pharmacol. Exp. Ther. 253 (1990) 387–394.

[3] M.F. Paine, M. Khalighi, J.M. Fisher, D.D. Shen, K.L. Kunze, C.L. Marsh, J.D. Perkins, K.E. Thummel, Characterization of interintestinal and intraintestinal variations in human CYP3A-dependent metabolism, J. Pharmacol. Exp. Ther. 283 (1997) 1552–1562.

[4] A.P. Li, D.L. Kaminski, A. Rasmussen, Substrates of human hepatic cytochrome P4503A4, Toxicology 104 (1995) 1–8.

[5] M.R. Walters, Newly identified actions of the vitamin D endocrine system, Endocr. Rev. 13 (1992) 719–764.

[6] P. Schmiedlin-Ren, K.E. Thummel, J.M. Fisher, M.F. Paine, K.S. Lown, P.B. Watkins, Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1α, 25-dihydroxyvitamin D_3, Mol. Pharmacol. 51 (1997) 741–754.

[7] H. Hara, N. Mitani, T. Adachi, Inhibitory effect of nitric oxide on the induction of cytochrome P450 3A4 mRNA by 1,25-dihydroxyvitamin D_3 in Caco-2 cells, Free Rad. Res. 33 (2000) 279–285.

[8] J.M. Fisher, S.A. Wrighton, P.B. Watkins, P. Schmiedlin-Ren, J.C. Calamia, D.D. Shen, K.L. Kunze, K.E. Thummel, First-pass midazolam metabolism catalyzed by 1α, 25-dihydroxy vitamin D_3 -modified Caco-2 cell monolayers, J. Pharmacol. Exp. Ther. 289 (1999) 1134–1142.

[9] M. Daujat, L. Pichard, I. Fabre, T. Pineau, G. Fabre, C. Bonfils, P. Maurel, Induction protocols for cytochromes P450IIIA in vivo and in primary cultures of animal and human hepatocytes, Methods Enzymol. 206 (1991) 345–353.

[10] T.A. Kocarek, E.G. Schuetz, S.C. Strom, R.A. Fisher, P.S. Guzelian, Comparative analysis of cytochrome P4503A induction in primary cultures of rat, rabbit, and human hepatocytes, Drug Metab. Dispos. 23 (1995) 415–421.

[11] Nuclear Receptors Nomenclature Committee, A unified nomenclature system for the nuclear receptor superfamily, Cell 97 (1999) 161–163.

[12] G. Bertilsson, J. Heidrich, K. Svensson, M. Åsman, L. Jendeberg, M. Sydow-Bäckman, R. Ohlsson, H. Postlind, P. Blomquist, A. Berkenstam, Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction, Proc. Natl. Acad. Sci. USA 95 (1998) 12208–12213.

[13] S.A. Kliewer, J.T. Moore, L. Wade, J.L. Staudinger, M.A. Watson, S.A. Jones, D.D. McKee, B.B. Oliver, T.M. Willson, R.H. Zetterström, T. Perlmann, J.M. Lehmann, An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway, Cell 92 (1998) 73–82.

[14] J.M. Lehmann, D.D. McKee, M.A. Watson, T.M. Willson, J.T. Moore, S.A. Kliewer, The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions, J. Clin. Invest. 102 (1998) 1016–1023.

[15] S. Christakos, M. Raval-Pandya, R.P. Wernyj, W. Yang, Genomic mechanisms involved in the pleitotropic actions of1,25-dihydroxyvitamin D_3, Biochem. J. 316 (1996) 361–371.

[16] K.E. Thummel, C. Brimer, K. Yasuda, J. Thottassery, T. Senn, Y. Lin, H. Ishizuka, E. Kharasch, J. Schuetz, E. Schuetz, Transcriptional control of intestinal cytochrome P-4503A by 1α, 25-dihydroxy vitamin D_3, Mol. Pharmcol. 60 (2001) 1399–1406.

[17] Y.H. Chen, R.H. Tukey, Protein kinase C modulates regulation of the *CYP1A1* gene by the aryl hydrocarbon receptor, J. Biol. Chem. 271 (1996) 26261–26266.

[18] P. Tollet, C. Legraverend, J.Å. Gustafsson, A. Mode, A role for protein kinases in the growth hormone regulation of cytochrome P4502C12 and insulin-like growth factor-I messenger RNA expression in adult primary rat hepatocytes, Mol. Endocrinol. 5 (1991) 1351–1358.

[19] J.S. Sidhu, C.J. Omiecinski, cAMP-associated inhibition of phenobarbital-inducible cytochrome P450 gene expression in primary rat hepatocyte cultures, J. Biol. Chem. 270 (1995) 12762–12773.

[20] M. Galisteo, N. Marc, A. Fautrel, A. Guillouzo, L. Corcos, D. Lagadic-Gossmann, Involvement of cyclic nucleotide- and calcium-regulated pathways in phenobarbital-induced cytochrome P-450 3A expression in mouse primary hepatocytes, J. Pharmacol. Exp. Ther. 290 (1999) 1270–1277.

[21] L.E. Heasley, G.L. Johnson, Regulation of protein kinase C by nerve growth factor, epidermal growth factor and phorbol esters in PC12 phenochromocytoma cells, J. Biol. Chem. 264 (1989) 8646–8652.

[22] H. Hashimoto, K. Toide, R. Kitamura, M. Fujita, S. Tagawa, S. Itoh, T. Kamataki, Gene structure of CYP3A4, an adult-specific form of cytochrome P450 in human livers and its transcriptional control, Eur. J. Biochem. 218 (1993) 585–595.

[23] R.K. Desai, A.J. van Wijnen, J.L. Stein, G.S. Stein, J.B. Lian, Control of 1,25-dihydroxyvitamin D_3 receptor-mediated enhancement of osteocalcin gene transcription: effects of perturbing phosphorylation pathways by okadaic acid and staurosporine, Endocrinology 136 (1995) 5685–5693.

[24] D.W.A. Beno, L.M. Brady, M. Bissonnette, B.H. Davis, Protein kinase C and mitogen-activated protein kinase are required for 1,25-dihydroxyvitamin D_3-stimulated Egr induction, J. Biol. Chem. 270 (1995) 3642–3647.

[25] R.K. Wali, C.L. Baum, M.D. Sitrin, T.A. Brasitus, $1,25(OH)_2$ Vitamin D_3 stimulates membrane phosphoinositide turnover, activates protein kinase C, and increases cytosolic calcium in rat colonic epithelium, J. Clin. Invest. 85 (1990) 1296–1303.

[26] R. Gniadecki, B. Gajkowska, M. Hansen, 1,25-Dihydroxyvitamin D_3 stimulates the assembly of adherens junctions in keratinocytes: involvement of protein kinase C, Endocrinology 138 (1997) 2241–2248.

[27] M.D. Sitrin, M. Bissonnette, M.J.G. Bolt, R. Wali, S. Khare, B. Scaglione-Sewell, S. Skarosi, T.A. Brasitus, Rapid effects of 1,25 $(OH)_2$ Vitamin D_3 on signal transduction systems in colonic cells, Steroids 64 (1999) 137–142.

[28] S. Khare, M.J.G. Bolt, R.K. Wali, S.F. Skarosi, H.K. Roy, S. Niedziela, B. Scaglione-Sewell, B. Aquino, C. Abraham, M.D. Sitrin, T.A. Brasitus, M. Bissonnette, 1,25 Dihydroxyvitamin D_3 stimulates phospholipase C-γ in rat colonocytes: role of c-Src in PLC-γ activation, J. Clin. Invest. 99 (1997) 1831–1841.

[29] R. Gniadecki, Involvement of Src in the vitamin D signaling in human keratinocytes, Biochem. Pharmacol. 55 (1998) 499–503.

[30] S. Morelli, C. Buitrago, G. Vazquez, A.R. de Boland, R. Boland, Involvement of tyrosine kinase activity in 1α, 25$(OH)_2$-vitamin D_3 signal transduction in skeletal muscle cells, J. Biol. Chem. 275 (2000) 36021–36028.

[31] C.J. Gamard, G.C. Blobe, Y.A. Hannun, L.M. Obeid, Specific role for protein kinase C beta in cell differentiation, Cell Growth Differ. 5 (1994) 405–409.

[32] T. Barkhem, B. Carlsson, Y. Nilsson, E. Enmark, J.Å. Gustafsson, S. Nilsson, Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists, Mol. Pharmacol. 54 (1998) 105–112.

[33] M. Campbell-Thompson, I.J. Lynch, B. Bhardwaj, Expression of estrogen receptor (ER) subtypes and ERβ isoforms in colon cancer, Cancer Res. 61 (2001) 632–640.

[34] C. Buitrago, G. Vazquez, A.R. de Boland, R.L. Boland, Activation of Src kinase in skeletal muscle cells by 1,25-$(OH)_2$-vitamin D_3 correlates with tyrosine phosphorylation of the vitamin D receptor (VDR) and VDR–Src interaction, J. Cell Biochem. 79 (2000) 274–281.

[35] C. Buitrago, G. Vazquez, A.R. de Boland, R. Boland, The vitamin D receptor mediates rapid changes in muscle protein tyrosine phosphorylation induced by 1,25$(OH)_2D_3$, Biochem. Biophys. Res. Commun. 289 (2001) 1150–1156.

[36] N.L. Weigel, Steroid hormone receptors and their regulation by phosphorylation, Biochem. J. 319 (1996) 657–667.