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Increased nuclear expression and transactivation of vitamin D receptor by the cardiotonic steroid bufalin in human myeloid leukemia cells

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ABSTRACT

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], is a potent ligand for the nuclear receptor vitamin D receptor (VDR) and induces myeloid leukemia cell differentiation. The cardiotonic steroid bufalin enhances vitamin D-induced differentiation of leukemia cells and VDR transactivation activity. In this study, we examined the combined effects of 1,25$(OH)_2D_3$ and bufalin on differentiation and VDR target gene expression in human leukemia cells. Bufalin in combination with 1,25$(OH)_2D_3$ enhanced the expression of VDR target genes, such as CYP24A1 and cathelicidin antimicrobial peptide, and effectively induced differentiation phenotypes. An inhibitor of the Erk mitogen-activated protein (MAP) kinase pathway partially inhibited bufalin induction of VDR target gene expression. 1,25$(OH)_2D_3$ treatment induced transient nuclear expression of VDR in HL60 cells. Interestingly, bufalin enhanced 1,25$(OH)_2D_3$-induced nuclear VDR expression. The MAP kinase pathway inhibitor increased nuclear VDR expression induced by 1,25$(OH)_2D_3$ and did not change that by 1,25$(OH)_2D_3$ plus bufalin. A proteasome inhibitor also enhanced 1,25$(OH)_2D_3$-induced CYP24A1 expression and nuclear VDR expression. Bufalin-induced nuclear VDR expression was associated with histone acetylation and VDR recruitment to the CYP24A1 promoter in HL60 cells. Thus, the Na^+,K^+-ATPase inhibitor bufalin modulates VDR function through several mechanisms, including Erk MAP kinase activation and increased nuclear VDR expression.

1. Introduction

The active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], regulates calcium and bone homeostasis, immunity, and cellular growth and differentiation through binding to the vitamin D receptor (VDR) [1]. VDR is a member of the nuclear receptor superfamily of ligand-activated transcription factors that regulate many physiological processes including cell growth and differentiation, embryogenic development, and metabolic homeostasis [2]. Upon ligand binding, nuclear receptors undergo a conformational change that induces nuclear translocation and formation of an active transcription factor complex, resulting in transcription of specific target genes. Natural and synthetic VDR ligands, such as 1,25$(OH)_2D_3$ and its derivatives, inhibit the proliferation and/or induce the differentiation of various types of malignant cells, including myeloid leukemia, breast, and colon cancer cells [3]. The administration of 1,25$(OH)_2D_3$, or 1α-hydroxyvitamin D_3, which is rapidly metabolized to 1,25$(OH)_2D_3$, prolongs survival in a mouse model of myeloid leukemia [4]. Although vitamin D_3 and its synthetic derivatives are utilized in the treatment of bone and skin disorders, adverse effects, especially hypercalcemia, limit their use in the treatment of leukemia and cancer. The development of synthetic vitamin D analogs with low calcemic activity and identification of VDR ligands with selective action offer promising strategies in overcoming the adverse effects [5,6]. Another potential solution is combined administration of 1,25$(OH)_2D_3$ with other drugs, such as differentiation inducers [7]. Investigation of VDR-regulated mechanisms of leukemia differentiation should be helpful in incorporating vitamin D_3 into cancer therapy.

Bufalin is a major active component of the toad venom preparation *Chan Su* or *Senso*, which has been used as a cardiotonic and local anesthetic agent in China and Japan for centuries and has been shown to be a potent inducer of human leukemia cell differentiation [8,9]. Like other cardiotonic steroids including ouabain and digoxin, bufalin inhibits the membrane Na^+,K^+-ATPase and activates various intracellular signaling pathways presumably by a Na^+,K^+-ATPase-dependent mechanism [10]. While high concentrations of bufalin induce apoptosis, bufalin at low concentrations dramatically enhances the differentiation of myeloid leukemia cells induced by tumor necrosis factor-α and 1,25$(OH)_2D_3$ [9]. We previously reported that bufalin enhances VDR transactivation in a cell transfection assay and activates VDR-mediated endogenous gene expression [11]. Since bufalin does not bind to VDR or change the binding affinity of 1,25$(OH)_2D_3$ for VDR, and the

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α subunit of Na^+,K^+-ATPase is the only established receptor for bufalin, a Na^+,K^+-ATPase-mediated mechanism is likely involved in the VDR-modulating effects of bufalin. In this study, we investigated the effects of bufalin on VDR-mediated differentiation of human leukemia cells and found that bufalin modulates VDR function by multiple mechanisms, including increased nuclear expression of VDR protein.

2. Materials and methods

2.1. Compounds

Bufalin, ouabain, digitoxigenin, cinobufagin, and SP600125 were purchased from Sigma–Aldrich (St. Louis, MO), $1,25(OH)_2D_3$ was from Wako (Osaka, Japan), and PD98059, SB203580 and MG-132 were from EMD Chemicals (San Diego, CA).

2.2. Cell culture, cell growth, and nitroblue tetrazolium-reducing activity

Human myeloid leukemia HL60, THP-1 and U937 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in RPMI1640 medium containing 10% fetal bovine serum, 100 unit/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were counted in a Z1S Coulter Counter (Beckman Coulter, Fullerton, CA). Nitroblue tetrazolium (NBT) reduction was assayed colorimetrically [12]. Cells were incubated with 1 mg/ml NBT (Sigma–Aldrich) and 100 ng/ml 12-*O*-tetradecanoyl phorbol-13-acetate (Sigma–Aldrich) in RPMI1640 medium at 37 °C for 30 min, and the reaction was stopped by adding HCl. Formazan deposits were solubilized in DMSO, and the absorption at 570 nm was measured in a spectrophotometer (Molecular Devices, Sunnyvale, CA). NBT-reducing activity data were normalized to the cell numbers.

2.3. Real-time quantitative reverse transcription-polymerase chain reaction

Total RNAs from samples were prepared by the acid guanidine thiocyanate-phenol/chloroform method [13]. cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega, Madison, WI) [14]. Real-time PCR was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems). Primers were as follows: CYP24A1, 5′-TGA ACG TTG GCT TCA GGA GAA-3′ and 5′-AGG GTG CCT GAG TGT AGC ATC T-3′; CD14, 5′-AAC TGA CGC TCG AGG ACC TAA A-3′ and 5′-CGC AAG CTG GAA AGT GCA A-3′; cathelicidin antimicrobial peptide (CAMP), 5′-GCT AAC CTC TAC CGC CTC CT-3′ and 5′-GGT CAC TGT CCC CAT ACA CC-3′; prostaglandin-endoperoxide synthase 1 (PTGS1), 5′-CAA CAG TGT GAAG TGC GTG GTA T-3′ and 5′-TCT TTG ACC CTG AGC CAG ACA-3′; arachidonate 5-lipoxygenase (ALOX5), 5′-TCC TCC CTT CGG ATG CAA AA-3′ and 5′-CAG ACA CCA GAT GTG TTC GCA G-3′; CD11b, 5′-CTG TCT GCC AGA GAA TCC AGT G-3′ and 5′-GAG GTG GTT ATG CGA GGT CTT G-3′; cytochrome b-245, β polypeptide (CYBB), 5′-TTG CTG GAA ACC CTC CTA TGA-3′ and 5′-AAA ACC GCA CCA ACC TCT CAC-3′; cyclin-dependent kinase inhibitor 1A (CDKN1A), 5′-CAA GCT CTA CCT TCC CAC GG-3′ and 5′-TTG GAG AAG ATC AGC CGG C-3′; VDR, 5′-GCT GAC CTG GTC AGT TAC AGC A-3′ and 5′-CAC GTC ACT GAC GCG GTA CTT-3′; retinoid X receptor α (RXRα); 5′-TTC TCC ACC CAG GTG AAC TC-3′ and 5′-GAG CTG ATG ACC GAG AAA GG-3′; β-actin, 5′-GAC AGG ATG CAG AAG GAG AT-3′ and 5′-GAA GCA TTT GCG GTG GAC GAT-3′. The RNA values were normalized to the amount of β-actin mRNA.

2.4. Western blotting

Nuclear and cytosolic proteins were prepared as described previously [15]. They were subjected to SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, probed with antibodies (anti-VDR antibody, anti-RXRα antibody, anti-lamin B antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-β-actin antibody (Sigma–Aldrich)) and detected with an alkaline phosphatase conjugate substrate system [14].

2.5. Chromatin immunoprecipitation

After nuclear proteins were cross-linked to DNA in 1% formaldehyde for 15 min, cells were washed and lysed in lysis buffer (50 mM Tris–HCl, pH 7.8, 1% SDS, 10 mM EDTA) [16]. The lysates were sonicated to 300–1000 bp in DNA length. After cellular debris was removed by centrifugation, the lysates were diluted in chromatin immunoprecipitation (ChIP) dilution buffer (16.7 mM Tris–HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl). ChIP was performed with Acetyl-Histone H4 ChIP Assay Kit (Upstate, Lake Placid, NY) and anti-VDR antibody (Santa Cruz Biotechnology). DNA was purified with MonoFas DNA Purification Kit (GL Sciences, Torrance, CA). PCR was performed using GoTaq Master Mix (Promega) with the following primers: 5′-TTT TCT GGG CCC GCA CTC GGG G-3′ and 5′-TGG AGT CAG CGA GGT GAG CG-3′, detecting the region −405 to −135 in CYP24 promoter. The PCR products were separated by electrophoresis in 2% agarose gel.

3. Results

3.1. Differentiation and VDR target gene induction in HL60 cells

Bufalin stimulates the differentiation of human myeloid leukemia cells and enhances the effects of other inducers, including $1,25(OH)_2D_3$, on leukemia differentiation [9,17]. We examined the combined effects of $1,25(OH)_2D_3$ and bufalin on the proliferation and differentiation of myeloid leukemia HL60 cells. Bufalin inhibited proliferation of HL60 cells at a concentration-dependent range of 7.5–12.5 nM, and 12.5 nM bufalin induced NBT-reducing activity, a marker for differentiation [12] (Fig. 1A). $1,25(OH)_2D_3$, at concentrations of 1–100 nM, weakly suppressed proliferation of HL60 cells but did induce NBT-reducing activity. Although $1,25(OH)_2D_3$ binds to VDR with a K_d value of ∼0.1 nM [14,18], $1,25(OH)_2D_3$ exhibits biological effects at two-log higher concentrations when experiments are conducted in the presence of serum [19]. The decreased $1,25(OH)_2D_3$ potency may be due to serum vitamin D-binding protein. Combined treatment with bufalin and $1,25(OH)_2D_3$ dramatically increased NBT-reducing activity in HL60 cells. Cell viabilities after all of the treatment were more than 90%, as determined by exclusion of trypan blue.

Bufalin enhances VDR transactivation of a transfected expression vector and on the endogenous CYP24A1 gene by $1,25(OH)_2D_3$ in kidney-derived HEK293 cells and monocyte-derived THP-1 cells [11]. We examined CYP24A1 mRNA expression in HL60 cells treated with $1,25(OH)_2D_3$ and/or bufalin for 24 h. While CYP24A1 expression in untreated HL60 cells was marginal, treatment of HL60 cells with $1,25(OH)_2D_3$ at 10 nM increased CYP24A1 expression more than 10,000-fold compared to that in untreated cells (Fig. 1B). Effective concentrations of $1,25(OH)_2D_3$ (10–30 nM) for CYP24A1 induction in HL60 cells cultured in 10% serum-containing medium were comparable to those in the previous reports [19,20]. While the effect of bufalin alone was not significant, $1,25(OH)_2D_3$ in combination with bufalin induced CYP24A1 expression more effectively than $1,25(OH)_2D_3$ alone (Fig. 1B). $1,25(OH)_2D_3$ (100 nM) in combination with bufalin gave the most robust induction of CYP24A1

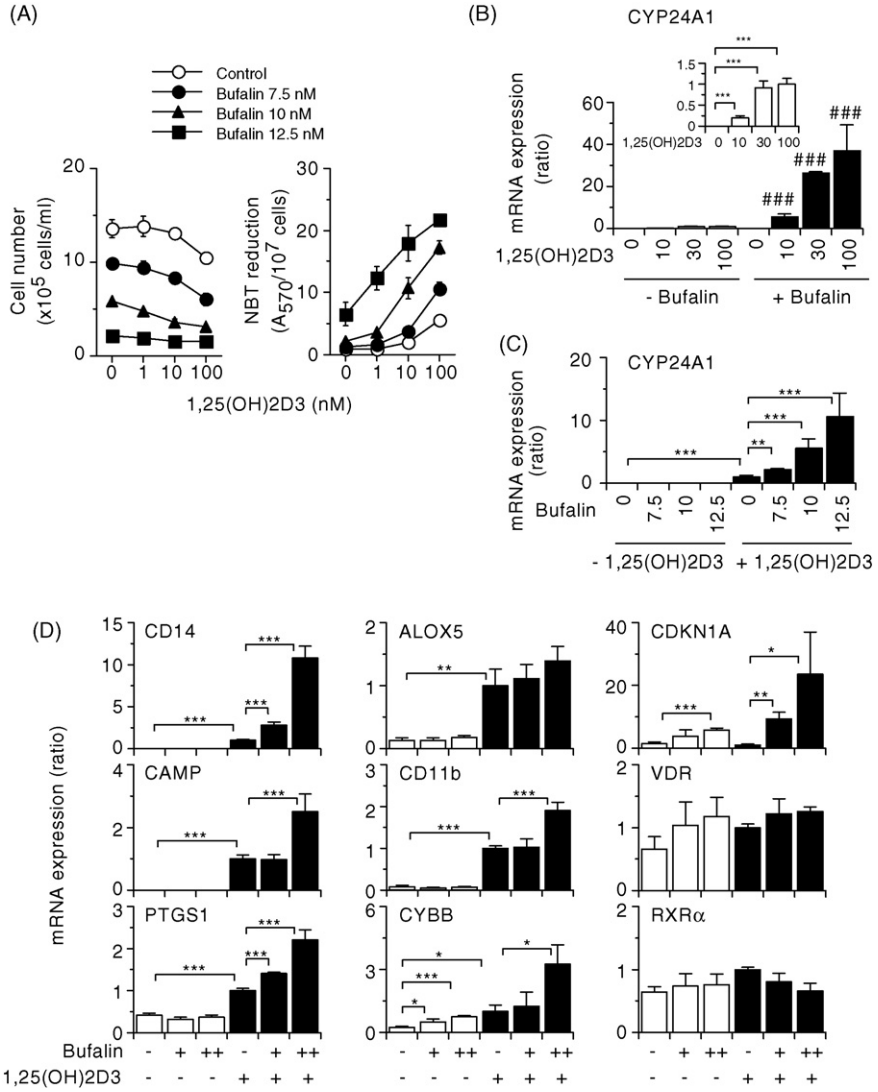

Fig. 1. Effects of $1,25(OH)_2D_3$ and bufalin on differentiation and gene expression in human leukemia HL60 cells. (A) Cell proliferation and NBT-reducing activity. Cells were cultured with $1,25(OH)_2D_3$ and bufalin at the indicated concentrations for 3 days. (B) Expression of CYP24A1 is induced by $1,25(OH)_2D_3$ in combination with bufalin. Cells were cultured with several concentrations of $1,25(OH)_2D_3$ in the absence or presence of 12.5 nM bufalin for 24 h. $^{###}P<0.001$ compared with values in the absence of bufalin. (C) Concentration-dependent effect of bufalin on expression of CYP24A1 induced by $1,25(OH)_2D_3$. Cells were cultured with several concentrations of bufalin in the absence or presence of 100 nM $1,25(OH)_2D_3$ for 24 h. (D) Expression of other VDR targets and differentiation-related genes. Cells were cultured with bufalin at 0 nM (−), 7.5 nM (+), or 10 nM (++) in the absence (−) or presence of 100 nM $1,25(OH)_2D_3$ (+) for 24 h. The values represent means ± SD of triplicate assays. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

in HL60 cells. These findings suggest that the enhancing effects of bufalin on the vitamin D signaling pathway is more apparent in combination with high concentrations of $1,25(OH)_2D_3$. Bufalin alone did not induce the expression of CYP24A1 in HL60 cells but enhanced CYP24A1 expression induced by 100 nM $1,25(OH)_2D_3$ in a concentration-dependent manner (Fig. 1C). Thus, bufalin enhances $1,25(OH)_2D_3$-induced CYP24A1 expression in HL60 cells.

Next, we examined the combined effects of bufalin (7.5 nM or 10 nM) and $1,25(OH)_2D_3$ (100 nM) on the expression of other VDR targets and differentiation-associated genes. VDR-binding elements have been reported in the CD14, CAMP and ALOX5 genes [21–23]. Expression of CD14, ALOX5, PTGS1 (also called cyclooxygenase 1), CD11b, and CYBB (also called gp91-phox) is associated with myeloid differentiation [12,24,25]. Bufalin has been reported to induce CDKN1A (also called p21), which is associated with cell cycle arrest, in endometriotic stromal cells [26]. Bufalin at 7.5 nM and 10 nM, concentrations that did not induce NBT-reducing activity (Fig. 1A), did not change CD14, CAMP, ALOX5, PTGS1, or CD11b expression (Fig. 1D). Like CYP24A1 expression, bufalin enhanced the expression of CD14, CAMP, PTGS1, and CD11b in combination with 100 nM $1,25(OH)_2D_3$, but did not induce ALOX5. Bufalin weakly induced the expression of CYBB and CDKN1A and further enhanced CYBB expression induced by $1,25(OH)_2D_3$. Although $1,25(OH)_2D_3$ (100 nM) was not effective in CDKN1A expression, bufalin plus $1,25(OH)_2D_3$ effectively activated its expression. Bufalin and $1,25(OH)_2D_3$ did not change the expression of VDR and RXRα, a heterodimer partner of VDR. Thus, bufalin and $1,25(OH)_2D_3$ synergistically induce differentiation markers and VDR target gene expression in HL60 cells.

3.2. Effects of mitogen-activated protein kinase inhibitors on gene expression

In addition to differentiation induction, bufalin induces apoptosis of myeloid leukemia cells in serum-depleted culture or at high concentration (1 μM) by activation of mitogen-activated protein (MAP) kinase pathways [27,28]. We examined the involvement of MAP kinase pathways in gene induction by bufalin (10 nM) and $1,25(OH)_2D_3$ (100 nM) using pharmacologic inhibitors. PD98059, an inhibitor of the Erk MAP kinase pathway, did not suppress

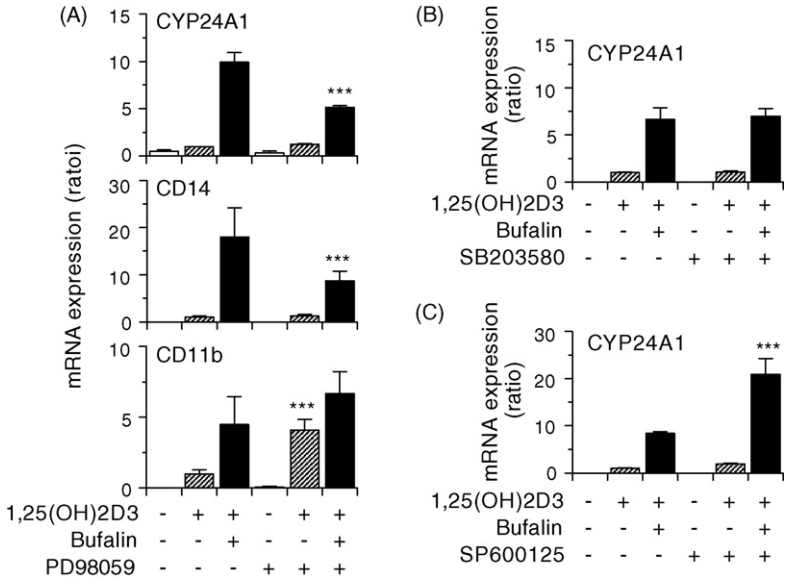

Fig. 2. Effects of MAP kinase pathway inhibitors on gene expression induced by $1,25(OH)_2D_3$ plus bufalin in HL60 cells. (A) The Erk pathway inhibitor PD98059 inhibits bufalin-enhanced expression of CYP24A1 and CD14, but not CD11b. (B) The p38 MAP kinase inhibitor SB203580 does not affect CYP24A1 expression induced by $1,25(OH)_2D_3$ plus bufalin. (C) The JNK MAP kinase inhibitor SP600125 enhances CYP24A1 expression induced by $1,25(OH)_2D_3$ plus bufalin. Cells were treated with 100 nM $1,25(OH)_2D_3$ and/or 10 nM bufalin in combination with 5 μM PD98059, 10 μM SB203580, or 5 μM SP600125 for 24 h. The values represent means ± SD of triplicate assays. $^{***}P < 0.001$ compared with values in the absence of an inhibitor.

expression of CYP24A1 and CD14 induced by $1,25(OH)_2D_3$, but decreased bufalin-enhanced gene expression (Fig. 2A). PD98059 enhanced CD11b expression induced by $1,25(OH)_2D_3$, and did not affect activation by bufalin. SB203580, a specific inhibitor of p38 MAP kinase, did not affect CYP24A1 expression induced by $1,25(OH)_2D_3$ alone or in combination with bufalin (Fig. 2B). Although a c-Jun N-terminal protein kinase (JNK) MAP kinase pathway has been reported to be involved in bufalin-induced apoptosis [29], the JNK MAP kinase inhibitor SP600125 increased CYP24A1 expression induced by $1,25(OH)_2D_3$ plus bufalin (Fig. 2C). This finding suggests that the effect of bufalin on CYP24A1 expression is mediated by a distinct mechanism from apoptosis induction. Inhibitor studies reveal that the effect of bufalin on gene expression in HL60 cells is partially mediated by the Erk MAP kinase pathway.

3.3. Combination of $1,25(OH)_2D_3$ with other cardiotonic steroids

In our previous study, we examined the effects of other cardiotonic steroids (cinobufagin, ouabain, and digitoxigenin) on VDR transactivation in a HEK293 cell transfection assay and found no effect of these steroids at 3 nM and 10 nM on ligand-activated VDR [11]. We examined the effects of these steroids on expression of the endogenous VDR target CYP24A1. Although they did not significantly increase CYP24A1 expression in the absence or presence of $1,25(OH)_2D_3$ at 10 nM, cinobufagin at 12.5 nM effectively enhanced $1,25(OH)_2D_3$-induced CYP24A1 expression (Fig. 3). Ouabain and digitoxigenin had no effect at 12.5 nM. Therefore, bufadienolides (bufalin and cinobufagin) and not cardenolides (ouabain and digitoxigenin) enhance CYP24A1 expression.

3.4. Time-dependent gene expression and nuclear expression of VDR protein

We examined the time course of expression of CYP24A1, CD14, CD11b and CDKN1A in HL60 cells treated with $1,25(OH)_2D_3$ (100 nM) and/or bufalin (10 nM). $1,25(OH)_2D_3$ increased the expression of CYP24A1, CD14 and CD11b in a time-dependent manner (Fig. 4A). While bufalin did not enhance gene expression in HL60 cells at 12 h treatment, it enhanced the time-dependent induction of CYP24A1, CD14 and CD11b by $1,25(OH)_2D_3$ from 24 to 72 h of treatment. $1,25(OH)_2D_3$ did not increase CDKN1A expression. Bufalin treatment alone increased CDKN1A expression slightly, an effect that was potentiated by $1,25(OH)_2D_3$ at 24 h (Fig. 1D). Interestingly, bufalin plus $1,25(OH)_2D_3$ synergistically induced CDNK1A

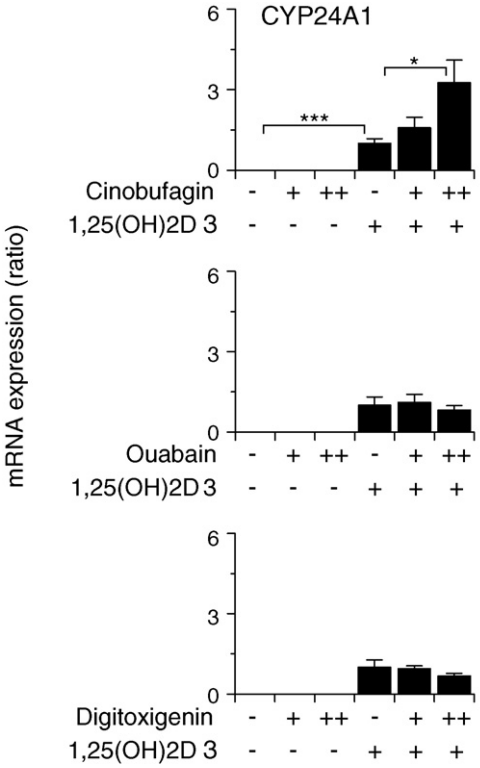

Fig. 3. Cinobufagin, but not ouabain or digitoxigenin, enhances CYP24A1 expression induced by $1,25(OH)_2D_3$ in HL60 cells. Cells were treated with cinobufagin, ouabain, or digitoxigenin at 0 nM (−), 10 nM (+), or 12.5 nM (++) in the absence (−) or presence of 100 nM $1,25(OH)_2D_3$ for 24 h. The values represent means ± SD of triplicate assays. $^{*}P < 0.05$; $^{***}P < 0.001$.

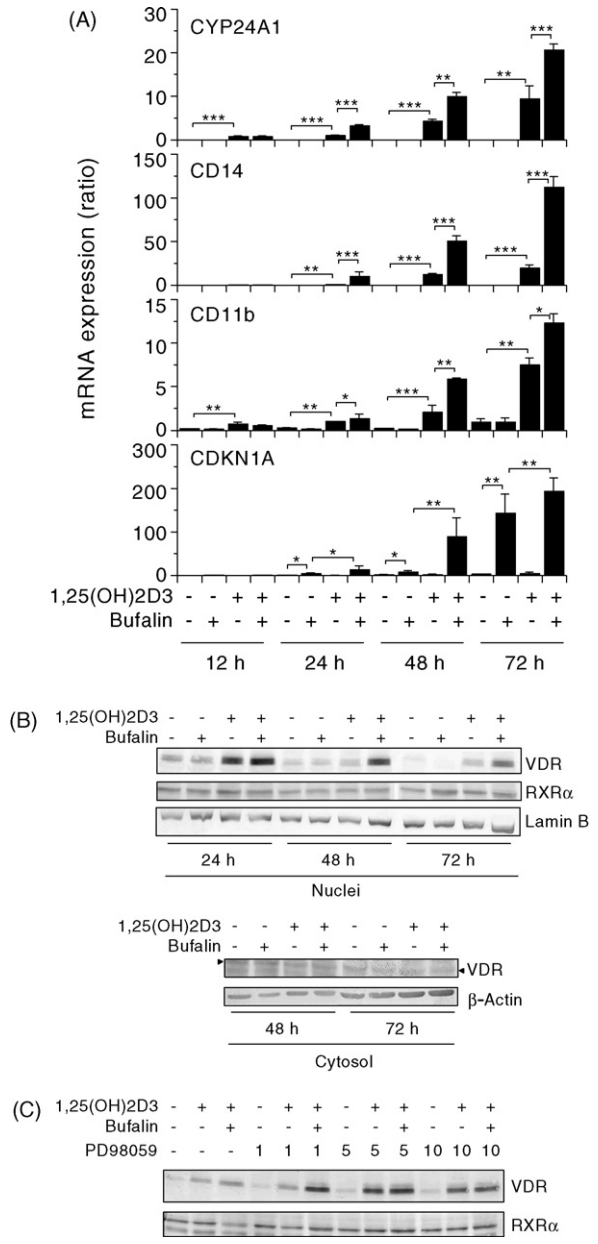

Fig. 4. Time course of gene expression (A) and nuclear VDR accumulation (B) induced by $1,25(OH)_2D_3$ and bufalin in HL60 cells. Cells were treated with 100 nM $1,25(OH)_2D_3$ and/or 10 nM bufalin for 12 h, 24 h, 48 h, and 72 h. The values represent means ± SD of triplicate assays. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Each lane was loaded with 5 μg and 1 μg of nuclear proteins for VDR/RXRα and lamin B, respectively, and 15 μg of cytosolic proteins for VDR and β-actin. (C) Effect of an Erk MAP kinase pathway inhibitor on nuclear VDR translocation. Cells were treated with 100 nM $1,25(OH)_2D_3$ and 10 nM bufalin in combination with PD98059 at 0 μM (−), 1 μM (1), 5 μM (5) or 10 μM (10) for 48 h. Western blotting was repeated twice with similar results.

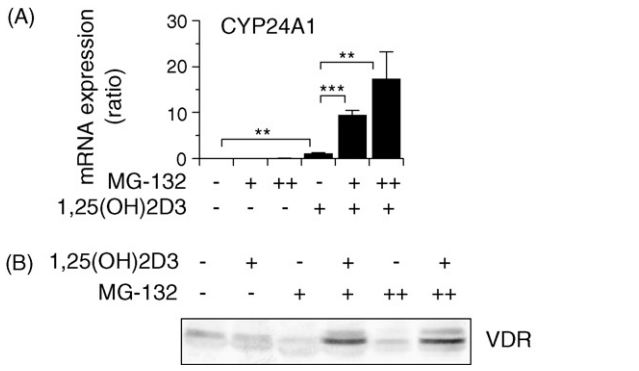

Fig. 5. Effects of a proteasome inhibitor on CYP24A1 expression (A) and nuclear VDR expression (B) induced by $1,25(OH)_2D_3$ in HL60 cells. Cell were treated with 0 nM (−), 10 nM (+), or 100 nM (++) MG-132 in the absence or presence of 100 nM $1,25(OH)_2D_3$ for 24 h (A) and 48 h (B). The values represent means ± SD of triplicate assays. $^{**}P < 0.01$; $^{***}P < 0.001$. Western blotting was repeated twice with similar results.

expression at 48 h, and at 72 h only bufalin had additional activity (Fig. 4A). With the finding that bufalin inhibited proliferation of HL60 cells (Fig. 1A), bufalin may induce CDNK1A via a VDR-independent mechanism.

VDR proteins fused with green fluorescent protein are localized in both the cytosol and nucleus and accumulates in the nucleus in response to $1,25(OH)_2D_3$ binding [30]. To examine localization of VDR, we examined protein expression of VDR in nuclear extracts and cytosol by immunoblotting. As reported previously [31], $1,25(OH)_2D_3$ treatment for 3 h increased nuclear expression of VDR in HL60 cells (data not shown). We examined the combined effects of $1,25(OH)_2D_3$ and bufalin on the time course of nuclear VDR expression. While $1,25(OH)_2D_3$ maintained nuclear VDR levels at 24 h, bufalin did not increase nuclear VDR expression alone or with $1,25(OH)_2D_3$ at 24 h (Fig. 4B). Nuclear VDR levels decreased after 48 h treatment with $1,25(OH)_2D_3$. Surprisingly, addition of bufalin maintained nuclear VDR levels induced by $1,25(OH)_2D_3$ at both 48 h and 72 h in HL60 cells. Neither $1,25(OH)_2D_3$ nor bufalin changed nuclear RXRα expression. Cytosolic VDR protein levels were relatively low and did not change after treatment with $1,25(OH)_2D_3$, as reported previously [31]. Since the MAP kinase inhibitor PD98059 inhibited bufalin-enhanced expression of CYP24A1 and CD14 (Fig. 2A), we next examined the effect of PD98059 on nuclear VDR expression. While treatment of HL60 cells with PD98059 alone did not change nuclear VDR levels, PD98059 enhanced nuclear VDR expression induced by $1,25(OH)_2D_3$ as effectively as bufalin, and did not further increase the VDR expression induced by $1,25(OH)_2D_3$ plus bufalin (Fig. 4C). The increased expression of nuclear VDR by $1,25(OH)_2D_3$ plus PD98059 was not correlated with mRNA expression of CYP24A1 and CD14 (Fig. 2A). PD98059 treatment did not change nuclear expression of RXRα. These findings indicate that nuclear expression of VDR by $1,25(OH)_2D_3$ plus bufalin is not mediated by activation of Erk MAP kinase pathway.

Inhibition of proteasome has been reported to block VDR degradation and enhance VDR-mediated gene expression in keratinocytes [32]. We examined the effects of a proteasome inhibitor MG-132 on gene expression and nuclear VDR expression in HL60 cells. MG-132 alone did not induce the expression of CYP24A1 in HL60 cells but enhanced CYP24A1 expression induced by 100 nM $1,25(OH)_2D_3$ (Fig. 5A). Like bufalin, MG-132 increased nuclear VDR levels induced by $1,25(OH)_2D_3$ at 48 h (Fig. 5B). Thus, increased nuclear VDR expression by proteasome inhibition is associated with enhanced expression of a VDR target gene in HL60 cells.

We examined the effects of $1,25(OH)_2D_3$ and bufalin on CYP24A1 mRNA expression and nuclear VDR expression in myeloid leukemia THP-1 and U937 cells. As reported previously [11], bufalin enhanced CYP24A1 expression induced by $1,25(OH)_2D_3$ in THP-1 cells at 24 h (Fig. 6A). However, a time-dependent increase of CYP24A1 expression was not observed in THP-1 cells treated with $1,25(OH)_2D_3$ plus bufalin. $1,25(OH)_2D_3$ treatment for 24 h increased nuclear VDR expression in THP-1 cells (Fig. 6B). Nuclear VDR proteins induced by $1,25(OH)_2D_3$ were still observed at 48 h and 72 h, and were not further increased by bufalin combination. Bufalin also enhanced CYP24A1 expression induced by $1,25(OH)_2D_3$ in U937 cells at 24 h (Fig. 6C). While CYP24A1 expression by $1,25(OH)_2D_3$ was decreased at 72 h, $1,25(OH)_2D_3$ plus bufalin slightly increased CYP24A1 expression at 48 h. Differently from HL60 and THP-1

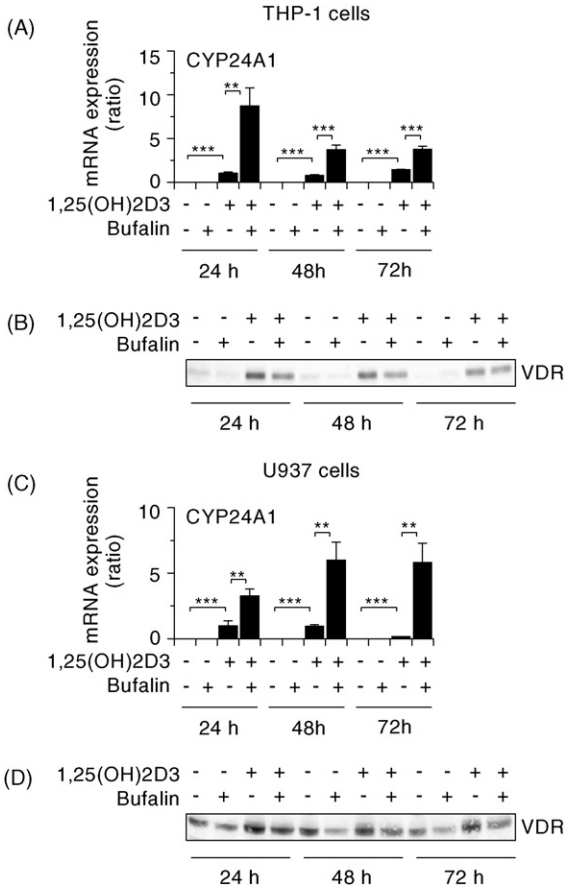

Fig. 6. Effects of $1,25(OH)_2D_3$ and bufalin on CYP24A1 mRNA expression and nuclear VDR accumulation in myeloid leukemia THP-1 and U937 cells. CYP24A1 expression (A, C) and nuclear VDR expression (B, D) in THP-1 (A, B) and U937 cells (C, D). Cells were treated with 100 nM $1,25(OH)_2D_3$ and/or 10 nM bufalin for 24 h, 48 h, and 72 h. The values represent means ± SD of triplicate assays. $^{**}P < 0.01$; $^{***}P < 0.001$. Each lane was loaded with 5 μg of nuclear proteins. Western blotting was repeated twice with similar results.

cells, VDR proteins were highly expressed in nuclei of untreated U937 cells, and were slightly increased by $1,25(OH)_2D_3$ treatment (Fig. 6D). Addition of bufalin did not further increase nuclear VDR expression. Thus, a time-dependent induction of VDR target genes is cell type-selective and is associated with bufalin-dependent nuclear VDR expression.

3.5. Histone acetylation and binding of VDR to the CYP24A1 promoter

To examine whether bufalin-enhanced gene expression is associated with binding of VDR to the CYP24A1 promoter, we performed ChIP assays in HL60 cells. A ligand-dependent conformational change of VDR recruits a coactivator protein complex with a histone acetyltransferase activity [33], and $1,25(OH)_2D_3$ induces histone 4 acetylation on the CYP24A1 promoter [34]. We examined histone acetylation on the CYP24A1 promoter in HL60 cells by ChIP using an anti-acetyl-lysine histone 4 antibody. As shown in Fig. 7, $1,25(OH)_2D_3$ weakly induced histone acetylation on the CYP24A1 promoter from 2 to 48 h of treatment. Addition of bufalin strongly enhanced histone acetylation on the CYP24A1 promoter. $1,25(OH)_2D_3$ induced VDR binding to the CYP24A1 promoter at 24 h and 48 h, an effect that was enhanced by bufalin (Fig. 7). Thus, bufalin-mediated enhancement of VDR activation is associated with histone acetylation and VDR recruitment to target gene promoters.

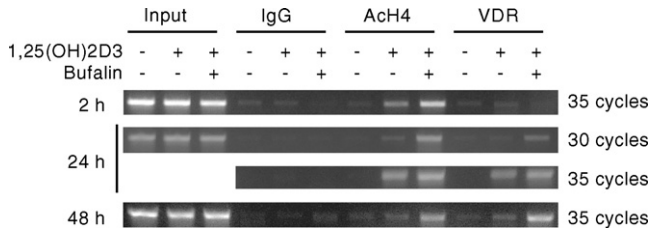

Fig. 7. Histone acetylation and VDR recruitment to the CYP24A1 promoter. Cells were treated with 100 nM $1,25(OH)_2D_3$ and 10 nM bufalin for 2 h, 24 h, and 48 h, and ChIP assay was performed using control IgG, anti-acetyl-lysine histone 4 antibody (AcH4), or anti-VDR antibody. The promoter sequence in the ChIP complex was detected by 30 or 35 PCR cycles. The experiments were repeated twice with similar results.

4. Discussion

We show here that bufalin stabilizes $1,25(OH)_2D_3$-dependent nuclear expression of VDR in HL60 cells. Nuclear expression of VDR is regulated by several mechanisms, including import, export, synthesis and degradation. Studies using fluorescent protein chimeras of VDR demonstrated that VDR is located in nuclei in the absence of ligand and that addition of ligand increases nuclear localization [30]. $1,25(OH)_2D_3$ induces a rapid increase in synthesis of VDR protein and its transport to the nucleus in HL60 cells [31]. Since $1,25(OH)_2D_3$-induced nuclear expression of VDR is not associated with decreased cytoplasmic VDR levels, nuclear appearance of VDR may be derived from *de novo* synthesis. We observed that $1,25(OH)_2D_3$ increased nuclear VDR levels at 24 h treatment but that this effect diminished after 48 h in HL60 cells. Addition of bufalin did not change nuclear VDR expression from 3 to 24 h, suggesting that bufalin does not affect nuclear import or *de novo* synthesis of VDR. Enhancement of ligand-dependent VDR transactivation by bufalin is associated with sustained nuclear expression of VDR after 48 h treatment with $1,25(OH)_2D_3$, an effect that requires $1,25(OH)_2D_3$. These findings suggest that bufalin suppresses ligand-dependent VDR nuclear export or degradation. $1,25(OH)_2D_3$ inhibits ubiquitination of VDR and proteasome inhibitors enhance ligand-dependent VDR target gene expression in keratinocytes [32]. We also observed that a proteasome inhibitor MG-132, like bufalin, enhanced $1,25(OH)_2D_3$-induced CYP24A1 expression and nuclear VDR expression in HL60 cells. Bufalin may increase nuclear VDR expression by inhibiting degradation. Unlike in HL60 cells, nuclear VDR proteins were expressed stably in THP-1 and U937 cells, and the effect of bufalin on nuclear VDR expression was not observed in these cells. Ligand-dependent nuclear localization of VDR is regulated in a cell type-dependent manner [14]. Further studies are required to elucidate the mechanism of nuclear localization and degradation of VDR.

Bufalin, and to a lesser extent cinobufagin, enhanced CYP24 expression induced by $1,25(OH)_2D_3$ in HL60 cells. Our previous study did not detect an effect of cinobufagin on VDR transactivation in transfected HEK293 cells [11]. Bufalin and cinobufagin are bufadienolide cardiotonic steroids that inhibit Na^+,K^+-ATPase activity [10], and bufalin is a more effective inhibitor than cinobufagin [9]. The differences in the effects of cinobufagin in our experiments may be concentration-related. Ouabain and digitoxigenin, unlike bufalin and cinobufagin, did not enhance $1,25(OH)_2D_3$-induced CYP24 expression, although ouabain inhibits Na^+,K^+-ATPase as effectively as cinobufagin [35]. The only established receptor for cardiotonic steroids is the α subunit of the Na^+,K^+-ATPase, a protein with four isoforms (α1, α2, α3 and α4) [36]. Interaction of the bufadienolides with a distinct α subunit may result in their enhancing effect on VDR transactivation. Bufalin has been reported to activate the Erk and JNK MAP kinase pathways [28,29]. These effects of bufalin are thought to be mediated by the Na^+,K^+-ATPase signalosome through

a Na^+ pump-independent mechanism [10]. PD98059, an Erk MAP kinase pathway inhibitor, inhibited the effect of bufalin on expression of CYP24A1 and CD14, but not that of CD11b. This finding suggests that Erk MAP kinase activation is not necessary for bufalin action but is involved downstream of VDR transactivation or in a gene-selective regulatory mechanism. PD98059 increased VDR nuclear expression induced by $1,25(OH)_2D_3$ and did not change bufalin-enhanced VDR nuclear expression. PD98059-dependent nuclear VDR accumulation did not result in increased CYP24A1 expression, indicating that increased nuclear VDR is not sufficient for target gene induction. The enhancing effect of bufalin on CYP24A1 expression may be mediated by both nuclear VDR expression and Erk MAP kinase activation. Bufalin has been shown to direct cytoplasmic membrane components adjacent to the nucleus by regulating endosomal pH and endocytosed membrane traffic in neuronal precursor cells most likely via a Na^+,K^+-ATPase-mediated mechanism [37]. Since the relation between endosomal function and degeneration of exported nuclear proteins remains unknown, further investigation is required to elucidate the role of the Na^+,K^+-ATPase in the regulation of nuclear proteins, including VDR.

Digoxin treatment reduces mortality and hospitalization in patients with ambulatory chronic systolic and diastolic heart failure, and several cardiotonic steroids, including ouabain, digoxin and marinobufagin, are endogenously produced in mammals [10]. Emerging evidence suggests that endogenous cardiotonic steroids act as hormones that regulate cardiovascular function. Vitamin D has been also shown to be involved in cardiovascular homeostasis. $1,25(OH)_2D_3$-activated VDR suppresses renin expression and VDR-null mice develop cardiovascular disease, such as hypertension and cardiac hypertrophy, due to dysregulation of the renin–angiotensin system [38,39]. Clinical studies have shown that vitamin D deficiency is associated with high risk of cardiovascular disease [40,41]. Thus, vitamin D regulates not only bone and mineral homeostasis but also cardiovascular function, suggesting a cooperative mechanism with cardiotonic steroids in the body. Recently, Imura et al. reported that α-Klotho binds to the α subunit of the Na^+,K^+-ATPase and is necessary for the activation and membrane accumulation of Na^+,K^+-ATPase induced by low extracellular Ca^{2+} [42]. Secretion of parathyroid hormone in response to low extracellular Ca^{2+}, which stimulates $1,25(OH)_2D_3$ production, is also dependent on α-Klotho and the Na^+,K^+-ATPase. These mechanisms stimulate Ca^{2+} absorption in the intestine and kidney. $1,25(OH)_2D_3$ induces α-Klotho gene expression in the kidney and α-Klotho, in combination with fibroblast growth factor 23, downregulates the production of $1,25(OH)_2D_3$ by repressing 25-hydroxyvitamin D_3 1α-hydroxylase gene expression [43,44]. Fibroblast growth factor 23 is also upregulated by $1,25(OH)_2D_3$ [45]. In addition, liganded VDR inhibits expression of parathyroid hormone and 25-hydroxyvitamin D_3 1α-hydroxylase genes by forming a negative transcription factor complex on their promoters [46,47]. Thus, vitamin D_3 and the Na^+,K^+-ATPase, as well as α-Klotho, parathyroid hormone and fibroblast growth factor 23, are involved in a regulatory network that mediates calcium homeostasis. The role of the Na^+,K^+-ATPase in leukemia cell differentiation and mechanisms of $1,25(OH)_2D_3$-induced differentiation remain unknown. Further studies on the effects of bufalin on VDR function may be helpful in the elucidation of cellular differentiation induction by $1,25(OH)_2D_3$ as well as the relationship between calcium homeostasis and cardiovascular function.

We observed that bufalin at 10–12.5 nM inhibited proliferation of HL60 cells and enhanced the differentiation of HL60 cells induced by $1,25(OH)_2D_3$ at 1–10 nM. Animal studies on Liu-Shen-Wan, a Chinese formula containing bufalin, indicate that these concentrations of bufalin are tolerated [48,49]. Phase I trials of high dose $1,25(OH)_2D_3$ have shown that peak plasma concentrations of 1.5–6.0 nM can be achieved with minimal toxicity [50,51]. These findings suggest that bufalin can enhance therapeutic effects of $1,25(OH)_2D_3$ *in vivo*. $1,25(OH)_2D_3$ and its synthetic analogs in combination with bufalin may be useful in the treatment of myeloid leukemia.

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