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Enhancement of ligand-dependent Vitamin D receptor transactivation by the cardiotonic steroid bufalin

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Abstract

Bufalin, a bufadienolide type cardiotonic steroid that is one of the major components of the toad venom-prepared traditional Chinese medicine called *Ch'an Su* or *Senso*, exhibits a cardiotonic action by inhibiting the membranous Na^+,K^+-ATPase. Bufalin also induces differentiation of leukemia cells alone or in combination with other differentiation inducers including 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. In this study, we performed a transient cotransfection assay using a Vitamin D receptor (VDR) expression vector and a luciferase reporter and found that although bufalin did not transactivate the VDR, it effectively enhanced VDR activity induced by 1,25$(OH)_2D_3$. Bufalin also augmented VDR activation by bile acid ligands, such as lithocholic acid and 3-ketocholanic acid. Other cardiotonic steroids including ouabain, digitoxigenin and cinobufagin did not enhance VDR activation. Bufalin did not bind directly to VDR but did modulate the interaction of VDR and cofactors, such as steroid receptor coactivator-1 and nuclear receptor corepressor. Bufalin treatment significantly increased the expression of an endogenous VDR target gene, CYP24, in kidney- and monocyte-derived cell lines treated with 1,25$(OH)_2D_3$. The data indicate that bufalin-mediated cellular mechanisms such as interaction with Na^+,K^+-ATPase may affect VDR transcriptional activity. Bufalin may be a useful tool in the investigation of VDR regulation by membrane-originating cellular signals and of pathophysiological mechanisms linking VDR to cardiovascular dysfunction.

Keywords: Vitamin D receptor; Bufalin; Cardiotonic steroid; Transcription; Nuclear receptor; Active Vitamin D

1. Introduction

The toad venom preparation *Ch'an Su* or *Senso* has been used as a cardiotonic and local anesthetic agent in China and Japan for centuries [1]. Bufalin is one of the major active components of this traditional Chinese medicine and has been shown to be a potent inducer of human leukemia cell differentiation [2]. Like other cardiotonic steroids including ouabain and digoxin, bufalin inhibits Na^+,K^+-ATPase activity and promotes the differentiation of leukemia cells [3,4]. The Na^+,K^+-ATPase is a membrane-bound enzyme consisting of α and β subunits that uses energy from ATP hydrolysis to pump Na^+ and K^+ ions across the cell membrane, establishing the membrane potential essential for a variety of cellular and physiological functions [5]. Inhibition of the Na^+,K^+-ATPase by cardiotonic steroids increases intracellular Na^+ ions and results in intracellular Ca^{2+} accumulation by modulating Na^+/Ca^{2+} exchanger activity, leading to increased force of cardiomyocyte contraction [5]. Although the role of the Na^+,K^+-ATPase in leukemia differentiation remains unclear, treatment with low dose bufalin dramatically enhanced the differentiation of myeloid leukemia cells

Abbreviations: 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; VDR, Vitamin D receptor; AF-2, activation function 2; RXR, retinoid X receptor; DR, direct repeat; ER, everted repeat; SRC-1, steroid receptor coactivator-1; N-CoR, nuclear receptor corepressor; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase

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induced by tumor necrosis factor-α and 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] [6]. These findings suggest that bufalin regulation of the Na^+,K^+-ATPase, or other cellular targets, enhances differentiation signals induced by $1,25(OH)_2D_3$.

$1,25(OH)_2D_3$, the active form of Vitamin D_3, regulates calcium and bone homeostasis, immunity, and cellular growth and differentiation through direct binding to the Vitamin D receptor (VDR; NR1I1) [7,8]. VDR is a member of the nuclear receptor superfamily of ligand-inducible transcription factors that regulate many physiological processes including cell growth and differentiation, embryonic development, and metabolic homeostasis [9,10]. Nuclear receptor transcriptional activity is modulated by ligands such as steroids, retinoids, and other lipid-soluble compounds. Upon ligand binding, nuclear receptors undergo a conformational change in the cofactor-binding site and activation function 2 (AF-2) helix that results in the dissociation of corepressors and recruitment of coactivators [11]. These cofactors form complexes with associated factors that induce chromatin remolding or recruitment of the basal transcriptional machinery and allow nuclear receptors to modulate the transcription of specific target genes.

VDR forms a heterodimer with the retinoid X receptor (RXR; NR2B) and binds preferentially to a DNA response element that consists of a direct repeat of a two hexanucleotide (AGGTCA or related sequence) motif separated by three nucleotides (DR3), or an everted repeat motif with a six nucleotides spacer (ER6) [7,12]. VDR is highly expressed in target organs that mediate calcium homeostasis, such as intestine, bone, kidney, and parathyroid glands. Several *VDR* gene mutations have been reported in hereditary Vitamin D-resistant rickets [13], and mice that lack VDR expression exhibits a phenotype similar to the human disease [14]. In addition to bone and mineral defects, VDR-null mice were also reported to have cardiovascular abnormalities [15,16]. $1,25(OH)_2D_3$ represses renin transcription in the kidney and heart through a VDR-mediated mechanism, and VDR-null mice exhibit cardiohypertrophy due to dysregulation of the renin–angiotensin system in both the systemic circulation and the myocardium [16]. Although the importance of VDR in cardiovascular biology is emerging, the effect of cardiotonic steroids on VDR function has not been investigated.

Previously, we and others reported that the differentiation-inducing effect of $1,25(OH)_2D_3$ on myeloid leukemia cells is enhanced in combination with other drugs including retinoids, hydroxyurea, ethacrynic acid and antioxidants, as well as bufalin [17–20]. In this study, we report that bufalin enhances VDR transactivation and VDR-mediated endogenous gene expression. Bufalin does not bind directly to the VDR ligand-binding pocket, but can modulate ligand-dependent cofactor interactions. Bufalin-regulated cellular mechanisms have the potential to modify VDR responsive gene expression.

2. Materials and methods

2.1. Compounds

Bufalin, ouabain, digitoxigenin, cinobufagin, and lithocholic acid were purchased from Sigma-Aldrich (St. Louis, MO). $1,25(OH)_2D_3$ was purchased from Calbiochem (San Diego, CA), and 3-ketocholanic acid was obtained from Steraroids (Newport, RI).

2.2. Plasmids

A fragment of human VDR (GenBank accession no. NM_000376) was inserted into the pCMX vector to make pCMX-VDR and the ligand-binding domain of VDR was inserted into the pCMX-GAL4 vector to make pCMX-GAL4-VDR [21]. The VDR amino acid fragment 90–415 was inserted into the pCMX-GAL4 vector to make pCMX-GAL4-VDR-dAF-2. A full-length VDR cDNA was inserted into the pCMX-VP16 vector to make pCMX-VP16-VDR [21]. The nuclear receptor-interacting domains of steroid receptor coactivator-1 (SRC-1) (amino acids 595–771; GenBank accession no. U90661) and nuclear receptor corepressor (N-CoR) (amino acids 1990–2416; GenBank accession no. U35312) were inserted into the pCMX-GAL4 vector to make pCMX-GAL4-SRC-1 and pCMX-GAL4-N-CoR, respectively [22]. VDR-responsive hCYP3A4-ER6x3-tk-LUC and GAL4-responsive MH100(UAS)x4-tk-LUC were utilized in luciferase reporter assay [23].

2.3. Cell culture and cotransfection assay

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and antibiotic–antimycotic (Nacalai, Kyoto) at 37 °C in a humidified atmosphere containing 5% CO_2. THP-1 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 unit/ml penicillin, and 100 μg/ml streptomycin.

Transfections were performed by the calcium phosphate coprecipitation assay as described previously [21]. Eight hours after transfection, test compounds were added. Cells were harvested after 16–24 h and were assayed for luciferase and β-galactosidase activities using a luminometer and a microplate reader (Molecular Devices, Sunnyvale, CA). Cotransfection experiments used 50 ng of reporter plasmid, 20 ng of pCMX-β-galactosidase, 15 ng of each receptor expression plasmid, and pGEM carrier DNA to give 150 ng of DNA per well of a 96-well plate. Luciferase data were normalized to an internal β-galactosidase control and represent the mean ± S.D. of triplicate assays.

2.4. Competitive ligand-binding assay

Human VDR protein was generated using the TNT Quick Coupled Transcription/Translation System (Pro-

mega, Madison, WI). The protein was diluted five-fold in ice-cold TEGWD buffer (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20 mM sodium tungstate, 10% glycerol). The diluted lysate was incubated with [26,27-methyl-^{3}H] 1,25(OH)$_2$D$_3$ (1 nM) for 16 h at 4 °C in the presence or absence of nonradioactive competitor compounds [21,24]. Bound and labeled 1,25(OH)$_2$D$_3$ was quantitated using scintillation counting.

2.5. Quantitative real-time RT-PCR analysis

Total RNAs from samples were prepared with the RNAgents kit (Promega) and cDNAs were synthesized using the ImProm-II Reverse (Promega). Real-time PCR was performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq (Takara Bio, Otsu, Japan) according to the instructions provided by the manufacturer [21]. Primers were as follows: CYP24A, 5′-TGA ACG TTG GCT TCA GGA GAA-3′ and 5′-AGG GTG CCT GAG TGT AGC ATC T-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.

3. Results

3.1. Bufalin enhances the transcriptional activity of VDR

To examine the effect of bufalin on VDR function, we transiently transfected HEK293 cells with a VDR expression vector and a luciferase reporter containing a VDR-responsive ER6 element derived from the CYP3A4 promoter [12]. Cells were treated with a range of concentrations of 1,25(OH)$_2$D$_3$ in the absence or presence of bufalin, and luciferase activity was analyzed. Due to endogenous VDR expression in kidney-derived HEK293 cells, 1,25(OH)$_2$D$_3$ increased hCYP3A4-ER6x3-tk-LUC reporter activity slightly in the absence of VDR cotransfection [22]. In the presence of transfected VDR, 1,25(OH)$_2$D$_3$ activated transcription in a concentration-dependent manner (Fig. 1A). Although bufalin treatment alone did not induce luciferase activity, combined treatment markedly enhanced the effect of 1,25(OH)$_2$D$_3$ on transactivation of both endogenous and transfected VDR. The addition of 3 nM bufalin increased the reporter activity two-fold when added to 3 nM 1,25(OH)$_2$D$_3$ (Fig. 1A). Next, GAL4-chimeric VDR was utilized. In order to control for the potentially confounding effects of endogenous receptors, a GAL4-chimeric VDR assay was utilized. 1,25(OH)$_2$D$_3$, with or without bufalin, did not induce GAL4 reporter activity in the absence of GAL4-VDR transfection (data not shown). While bufalin alone did not induce GAL4-VDR transactivation, this

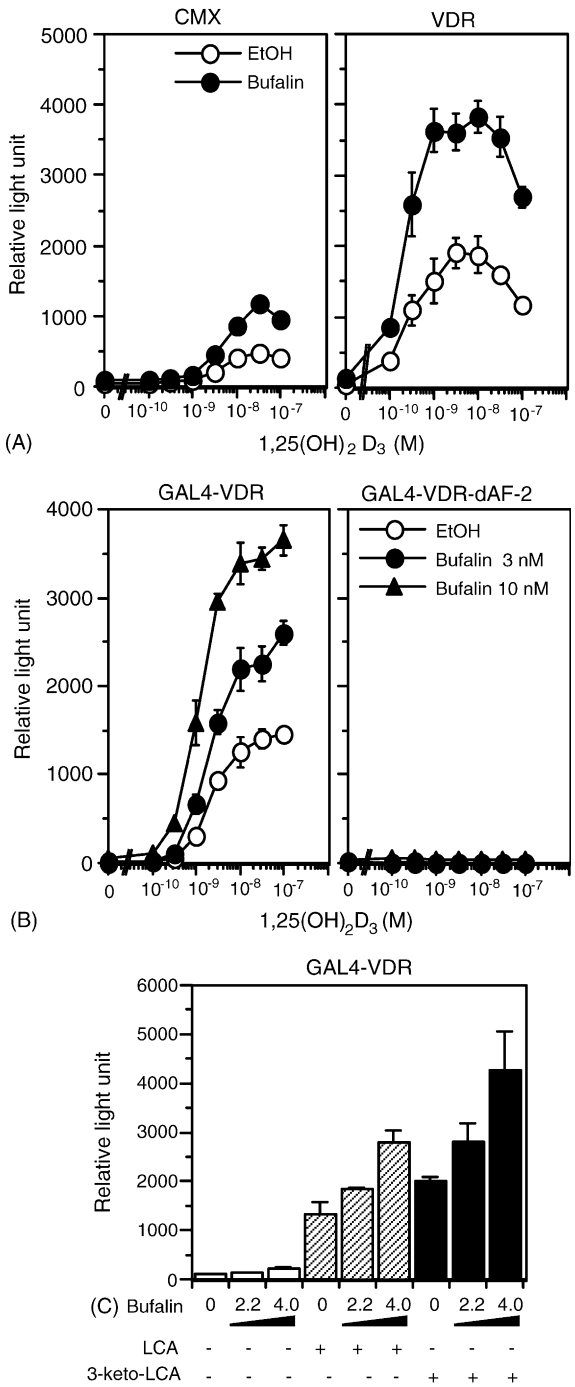

Fig. 1. Potentiation of ligand-dependent VDR transactivation activity by bufalin. (A) Bufalin enhances full-length VDR transactivation activity. HEK293 cells were cotransfected with a control CMX vector or CMX-VDR and the hCYP3A4-ER6x3-tk-LUC reporter, and were treated with 1,25(OH)$_2$D$_3$ at a range of concentrations, ethanol (EtOH), or 3 nM bufalin. (B) Bufalin enhances the transactivation activity of GAL4-VDR in an AF-2 domain-dependent manner. HEK293 cells were cotransfected with CMX-GAL4-VDR or CMX-GAL4-VDR-dAF-2 and the MH100(UAS)x4-tk-LUC reporter, and were treated with 1,25(OH)$_2$D$_3$ at a range of concentrations in combination with ethanol (EtOH) or bufalin (3 nM or 10 nM). (C) Bufalin enhances bile acid-induced VDR transactivation. HEK293 cells were cotransfected with CMX-GAL4-VDR and the MH100(UAS)x4-tk-LUC reporter, and were treated with 10 μM lithocholic acid (LCA) or 3-ketocholanic acid (3-keto-LCA) in combination with ethanol or bufalin (2.2 nM or 4.0 nM). The values represent mean ± S.D.

compound enhanced $1,25(OH)_2D_3$ activation of the chimeric receptor in a concentration-dependent manner (Fig. 1B). Upon ligand binding, nuclear receptors undergo a conformational change that induces C-terminal AF-2-dependent recruitment of coactivators such as SRC-1. We examined the effect of $1,25(OH)_2D_3$ and bufalin on a VDR AF-2 deletion mutant. The effect of $1,25(OH)_2D_3$ and bufalin on reporter transcription was completely abolished by the GAL4-VDR-dAF-2 mutant (Fig. 1B). Recently, we found that VDR functions as a receptor for bile acids such as lithocholic acid and 3-ketocholanic acid [12]. The effect of bufalin on bile acid-stimulated VDR activity was examined. The addition of bufalin augmented the VDR activity induced by lithocholic acid and 3-ketocholanic acid (Fig. 1C). Taken together, these data indicate that bufalin enhances ligand-dependent VDR activation.

The effect of other cardiotonic steroids on VDR activation was examined. Ouabain is an arrow poison derived from the African *Ouabaio* tree and *Strophanthus gratus* plants, and is a well-known inhibitor of the sodium pump [25]. Digitoxigenin is a cardiac glycoside derived from the leaves of the common foxglove plant *Digitalis purpurea* [26]. Cinobufagin is another component of the toad venom preparation *Ch'an Su* or *Senso* [1]. Although bufalin at 3 nM and 10 nM increased VDR transactivation, ouabain, digitoxigenin and cinobufagin at these concentrations did not enhance reporter activity (Fig. 2). These compounds did exert a nonspecific effect on the luciferase reporter assay at high concentrations (data not shown).

3.2. Bufalin modulates VDR-cofactor interaction

We examined the possibility that bufalin might directly bind VDR using a competitive binding assay. Isotopically

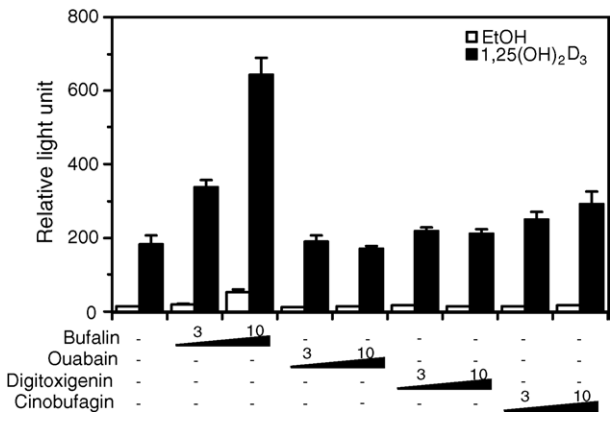

Fig. 2. Ouabain, digitoxigenin, and cinobufagin do not enhance $1,25(OH)_2D_3$-induced VDR transactivation. HEK293 cells were cotransfected with CMX-GAL4-VDR and the MH100(UAS)x4-tk-LUC reporter, and were treated with ethanol (EtOH) or $1,25(OH)_2D_3$ (3 nM) in combination with bufalin, ouabain, digitoxigenin, or cinobufagin (3 nM or 10 nM). The values represent mean ± S.D.

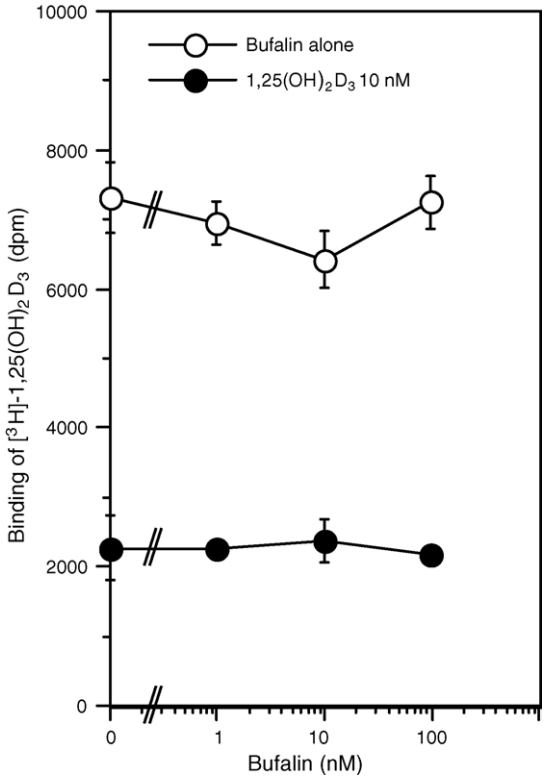

Fig. 3. Effect of bufalin on direct binding of $1,25(OH)_2D_3$ to VDR. In vitro translated VDR proteins were incubated with [^{3}H] $1,25(OH)_2D_3$ (1 nM) in the presence or absence of nonradioactive $1,25(OH)_2D_3$ (10 nM) in combination with 0 nM, 1 nM, 10 nM, or 100 nM bufalin. The values represent mean ± S.D.

labeled $1,25(OH)_2D_3$ was incubated with in vitro translated VDR protein in the absence or presence of test compounds. The addition of bufalin up to a concentration of 100 nM did not inhibit the binding of labeled $1,25(OH)_2D_3$ to VDR (Fig. 3). Unlabeled $1,25(OH)_2D_3$ competed with the labeled ligand as expected, and addition of bufalin did not affect the binding efficiency. Thus, bufalin does not activate VDR through a direct interaction.

The AF-2 domain of VDR, which forms a ligand-dependent interface for cofactor interaction, is required for bufalin action on the receptor as shown in Fig. 1B. The effect of bufalin on ligand-inducible cofactor recruitment was examined in the mammalian two-hybrid assay using a GAL4-SRC-1 receptor-interacting domain that contains the three LXXLL motifs and VP16-VDR chimeric expression vectors [22]. In this assay, the association of cofactor and receptor can be detected by the luciferase induction that results from recruiting the herpesvirus VP16 transactivation domain to the GAL4 response element. $1,25(OH)_2D_3$ alone induced the interaction of VDR and SRC-1 in a concentration-dependent manner as previously reported [22]. Interestingly, addition of 10 nM bufalin strongly enhanced the association of VDR and SRC-1 (Fig. 4A). While 1 nM $1,25(OH)_2D_3$ induced SRC-1 interaction at a suboptimal level,

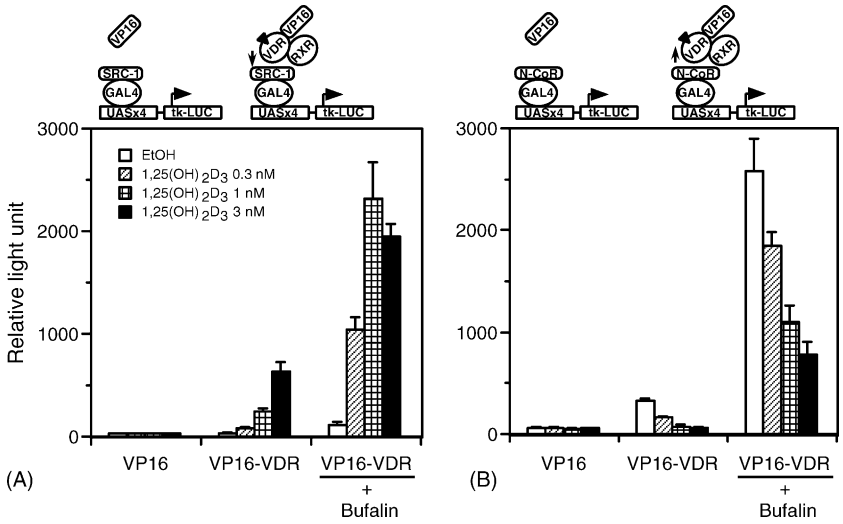

Fig. 4. Bufalin enhances the ligand-dependent interaction of VDR with SRC-1 and N-CoR. HEK293 cells were cotransfected with GAL4-chimeric vectors for SRC-1 or N-CoR in combination with the CMX-VP16 control or CMX-VP16-VDR vector and the MH100(UAS)x4-tk-LUC reporter, and were treated with ethanol (EtOH), 0.3 nM, 1 nM, or 3 nM $1,25(OH)_2D_3$ in the absence or presence of 10 nM bufalin. The values represent mean ± S.D.

full association was seen in combined treatment with bufalin. The mammalian two-hybrid assay using the GAL4-N-CoR chimeric corepressor measures the ligand-dependent dissociation of N-CoR from VDR. Unexpectedly, bufalin alone increased the interaction of VDR and N-CoR in the absence of $1,25(OH)_2D_3$, and the effect of $1,25(OH)_2D_3$ on dissociation of N-CoR from VDR was diminished in the presence of bufalin (Fig. 4B). These data suggest that bufalin modulates a ligand-inducible VDR conformation or an additional determinant of cofactor interaction.

3.3. Endogenous gene expression

We investigated the ability of bufalin to enhance the ability of $1,25(OH)_2D_3$ to induce an endogenous VDR target gene. VDR is expressed in kidney-derived HEK293 cells and monocyte-derived THP-1 cells (data not shown). As shown in Fig. 5, $1,25(OH)_2D_3$ (100 nM), and not bufalin (10 nM), induced expression of the VDR target gene CYP24. Addition of bufalin significantly increased CYP24 expression in both HEK293 cells and THP-1 cells. Therefore, bufalin enhances $1,25(OH)_2D_3$-stimualted VDR transcriptional activity on an endogenous target gene.

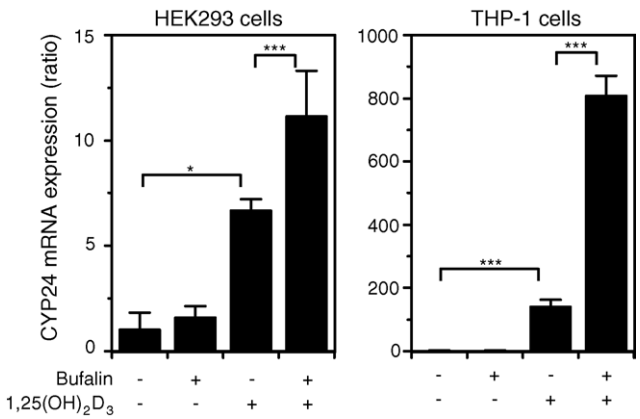

Fig. 5. Bufalin potentiates endogenous VDR target gene expression in HEK293 cells (A) and THP-1 cells (B). Cells were treated with bufalin (10 nM) and/or $1,25(OH)_2D_3$ (100 nM) for 24 h. Quantitative real-time PCR from mRNA for CYP24 was performed. The values represent mean ± S.D. $^{*}P < 0.05$; $^{***}P < 0.001$.

4. Discussion

Cardenolides, including ouabain and digitalis, and bufadienolides, including bufalin and cinobufagin, are cardiotonic steroids that inhibit the Na^+,K^+-ATPase and exert a positive inotropic effect on the heart [5]. In the transient transfection assay to HEK293 cells, bufalin, but not ouabain, digitoxigenin or cinobufagin, enhanced $1,25(OH)_2D_3$-dependent VDR transactivation (Fig. 2). Previous reports indicate that bufalin was the most potent of these compounds in inducing differentiation of human leukemia K562 cells and that this activity was correlated with Na^+,K^+-ATPase inhibition [2,6]. While bufalin at 10 nM effectively induced a differentiation marker in K562 cells, other cardiotonic steroids at this concentration showed a weak or marginal effect. Ouabain inhibited the Na^+,K^+-ATPase activity of HEK293 cells with an EC_{50} value of 100 nM [27]. We could not evaluate the effect of ouabain, digitoxigenin, and cinobufagin at high concentrations on VDR transactivation, because these compounds exerted a nonspecific effect on the luciferase reporter assay. Several isoforms of Na^+,K^+-ATPase have been identified for both α and β subunits [5]. However, the affinities of the isoforms for cardiotonic steroids including bufalin have not been elucidated. The physiological and pharmacological

relevance of Na^+,K^+-ATPase should be further investigated. Bufalin induced apoptosis in human leukemia U937 cells through activation of the Ras-Raf1-MEK-ERK and Rac-MEKK-SEK-JNK mitogen-activated protein kinase (MAPK) pathways [28,29]. Because the plasma membrane is thought to be impermeable to bufalin [3], activation of MAPK pathways may be indirect effects of Na^+,K^+-ATPase inhibition, and a precise mechanism remains unknown. Our data show that bufalin does not interact directly with VDR (Fig. 3) and suggest that bufalin affects intracellular signaling through interaction with a membrane-associated receptor. MAPK activation inhibits $1,25(OH)_2D_3$-dependent VDR transactivation activity through phosphorylation of the heterodimer partner RXRα [30]. Bufalin activation of MAPK pathways does not seem to account for potentiation of VDR activity because this effect is ligand-dependent (Fig. 1) and the MAPK inhibitor PD989059 did not repress VDR transactivation induced by $1,25(OH)_2D_3$ and bufalin (data not shown).

VDR activity is regulated by multiple cellular mechanisms. Ligand binding induces the dynamic association of multiprotein complexes, such as coregulatory complexes with histone acetylase or histone deacetylase activities, nonhistone acetylase DRIP/TRAP/SMCC coactivator complexes, and ATP-dependent chromatin-remodeling complexes [31]. Phosphoproteins that tansduce membrane receptor signals, such as SMAD3, also act as coregulators of VDR [32]. The second messenger cAMP, which mediates the biological effects of G protein-coupled receptors, such as the parathyroid hormone receptor, by activating protein kinase A, was reported to repress the VDR transactivation through an unknown mechanism [33]. These findings suggest that VDR activity is modulated by signals from the plasma membrane that modify the activity or expression of cofactor complexes. Bufalin modified the interaction of VDR with cofactors as shown in Fig. 4. Bufalin enhanced the ligand-dependent interaction between VDR and SRC-1, but also increased the association of VDR and the corepressor N-CoR. This apparent paradox may be explained by the finding that N-CoR binds to a SRC-1 family coactivator and enhances ligand-induced thyroid hormone receptor β activity [34]. Although the effect of bufalin on the interaction of VDR with additional cofactor complexes requires further investigation, the data suggest that bufalin may alter VDR activity by modulating cofactor interactions. Nonsteroidal bis-phenyl derivatives were reported to activate VDR [35] and to regulate coactivator recruitment to the VDR-RXR heterodimer without direct binding [36]. There is a possibility that small amounts of bufalin diffuse into cytosol and interact with VDR at a site other than the ligand-binding pocket. Elucidation of molecular mechanisms of bufalin enhancement of VDR transactivation may assist in the development of novel VDR-modulating drugs. VDR was recently reported to be localized in caveole-enriched regions of the plasma membrane and to modulate rapid calcium ion channel responses in osteoblasts [37,38]. A functional interaction between membranous and cytosolic VDR and the Na^+,K^+-ATPase might be involved in the regulation of intracellular Ca^{2+} homeostasis. The calmodulin antagonist calmidazolium and the calmodulin kinase inhibitor KN-93 did not affect VDR transactivation induced by $1,25(OH)_2D_3$ and bufalin (data not shown). Further investigation of the molecular mechanism of VDR activation by $1,25(OH)_2D_3$ and bufalin should prove useful in understanding the links between membrane signals and nuclear receptor function.

Recently, VDR was reported to be involved in cardiac function. Because $1,25(OH)_2D_3$ suppresses renin transcription in kidney and heart through VDR, VDR-null mice develop high blood pressure and cardiac hypertrophy [15,16]. The renin–angiotensin system plays a central role in the regulation of blood pressure, intravascular volume, and electrolyte homeostasis. In addition to the systemic renin–angiotensin system, local autocrine or paracrine renin–angiotensin mechanisms play a significant role in regulating cardiovascular function [39]. In the failing heart a vicious circle develops in the systemic and cardiac renin–angiotensin systems that induce vasoconstriction and result in increased afterload, decreased myocardial contractility, and worsened cardiac output. Several clinical trials reveal that angiotensin-converting enzyme inhibitors and angiotensin receptor blockers reduce morbidity and mortality in patients with chronic heart failure [40]. Another large randomized double-blind clinical trial indicated that the cardiotonic steroid digoxin did not reduce overall mortality in patients with chronic heart failure but did reduce hospitalization rates, defining a beneficial role of digoxin in the management of chronic heart failure [41]. Although pharmacological manipulation of the cardiac renin–angiotensin system by digitalis is not well known, VDR may play a role in this regulatory cascade. Bufalin effectively enhanced VDR transactivation induced by $1,25(OH)_2D_3$, but other cardiotonic steroids, including ouabain, digitoxigenin and cinobufagin, were ineffective (Fig. 2). This finding could be due to the observation that bufalin is the most potent inhibitor of the Na^+,K^+-ATPase in this chemical group [6]. Although there is a possibility that the bufalin effect is mediated by a Na^+,K^+-ATPase-independent mechanism, other cardiotonic steroids may exhibit similar activities at concentrations that exceed the sensitivity of the transfection assay. Several endogenous cardiac glycosides including ouabain have been reported to regulate Na^+ metabolism and the cardiovascular system [25]. Although a biosynthetic pathway has not been elucidated, endogenous compounds could potentially modulate VDR function. In conclusion, we found that bufalin enhances ligand-dependent VDR transactivation by modulating cofactor interaction. Bufalin should be a useful tool in

the investigation of VDR regulation by membrane-originating signals and of pathophysiological roles of VDR in diseases including heart failure.

Acknowledgments

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