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A nonclassical vitamin D receptor pathway suppresses renal fibrosis

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The TGF-β superfamily comprises pleiotropic cytokines that regulate SMAD and non-SMAD signaling. TGF-β–SMAD signal transduction is known to be involved in tissue fibrosis, including renal fibrosis. Here, we found that 1,25-dihydroxyvitamin D3–bound [1,25(OH)2D3-bound] vitamin D receptor (VDR) specifically inhibits TGF-β–SMAD signal transduction through direct interaction with SMAD3. In mouse models of tissue fibrosis, 1,25(OH)2D3 treatment prevented renal fibrosis through the suppression of TGF-β–SMAD signal transduction. Based on the structure of the VDR-ligand complex, we generated 2 synthetic ligands. These ligands selectively inhibited TGF-β–SMAD signal transduction without activating VDR-mediated transcription and significantly attenuated renal fibrosis in mice. These results indicate that 1,25(OH)2D3-dependent suppression of TGF-β–SMAD signal transduction is independent of VDR-mediated transcriptional activity. In addition, these ligands did not cause hypercalcemia resulting from stimulation of the transcriptional activity of the VDR. Thus, our study provides a new strategy for generating chemical compounds that specifically inhibit TGF-β–SMAD signal transduction. Since TGF-β–SMAD signal transduction is reportedly involved in several disorders, our results will aid in the development of new drugs that do not cause detectable adverse effects, such as hypercalcaemia.

Introduction

The TGF-β superfamily is a large, evolutionarily conserved group of cytokines (1, 2). TGF-β regulates a wide range of cellular processes by signaling through high-affinity TGF-β receptors (3–6). Binding of TGF-β to its receptors triggers phosphorylation of SMAD transcription factors, which are central mediators of TGF-β signal transduction (7–9). SMAD2 and SMAD3 are receptor activated, whereas SMAD4 serves as a common partner for all receptor-activated SMAD proteins (10–12). Signaling pathways involving non-SMAD proteins such as MAPK or PI3K are activated directly by ligand-bound TGF-β receptors to reinforce, attenuate, or otherwise modulate downstream cellular responses (13).

Perturbations in TGF-β signal transduction play a role in numerous human diseases. For instance, upregulated TGF-β production has been linked to fibrotic disease (14–17) and cancer progression (18, 19). Furthermore, activation of SMAD signaling by TGF-β exacerbates tissue fibrosis (15, 20–23). Numerous studies show that SMAD3 deficiency in mice attenuated cutaneous (24), hepatic (25), renal (26–28), and pulmonary (29) fibrosis. Although TGF-β and TGF-β receptor blockers have been developed for clinical use, current understanding of the pathologic roles of SMAD proteins suggest that specifically targeting SMAD signaling may result in better therapeutic profiles.

The vitamin D receptor (VDR) is a member of the nuclear receptor superfamily and functions as a ligand-inducible transcription factor (30–32). Binding of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] to the receptor ligand-binding domain (LBD) of the VDR induces a conformational change in the receptor and dimerization with retinoid X receptors. The resulting heterodimers then bind DNA, vitamin D–responsive element (VDRE), to stimulate gene expression (classical genomic action). The VDR-LBD is crucial for ligand-dependent transcriptional activity (33). Crystal structure analyses indicate that the LBDs of the VDR and other nuclear receptors contain 12 conserved helices (34). Of particular note, the C-terminal helix 12 (H12) in LBD plays an important role in binding coactivators, including SRC-1, to the ligand-bound receptor (35). VDR modulates the transcription of vitamin D–regulated genes involved in intestinal calcium/phosphate absorption and remodeling of bone to maintain calcium homeostasis. Although direct regulation of gene expression by VDR depends on the presence of VDRE in the promoters of target genes, some 1,25(OH)2D3-regulated genes do not contain VDRE in their promoters and are thought to be regulated indirectly. Thus, VDR can not only affect gene expression by binding to VDRE, but can also regulate other gene expressions by associating with several transcription factors, such as Sp1 (36) and β-catenin/TCF (37, 38). In addition to the classical genomic action, 1,25(OH)2D3 has also been shown to initiate many biological responses via the rapid response pathway of VDR. The localization of VDR to the plasma membrane caveolae results in activation of signal transduction pathways that generate rapid responses, such as transcalcaltachia or insulin secretion, which are activated by signal via plasma membrane–localized VDR (nongenomic action) (39). Deficiencies in vitamin D and its active metabolites are known pathologic features of chronic kidney diseases (40). Previous studies have shown that vitamin D supplementation suppresses renal fibrosis (41–48). These studies have suggested that vitamin D...
Figure 1
1,25(OH)₂D₃ suppresses renal fibrosis by inhibiting TGF-β signal transduction in UUO mice. (A) Representative photomicrographs of Masson’s trichrome staining (top row, whole kidney; second row, magnified view of boxed region, enlarged ×4.57). (B) FSP-1–positive cells (n = 12 per group), or α-SMA–positive immunofluorescence staining (bottom row) of kidney sections from sham-operated control or UUO mice injected with vehicle or 1,25(OH)₂D₃. Scale bars: 1.0 mm (top row); 200 μm (second row); 20 μm (bottom 2 rows). (B) Fibrotic areas (n = 4 per group). serum calcium concentration. ∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.001.

metabolites and its synthetic analogs may play a role in therapeutic suppression of renal fibrosis through stimulation of VDR-mediated transcription, although there is little agreement concerning the underlying mechanism.

Here, we report that the VDR inhibits TGF-β-SMAD signal transduction. Our results indicate that the VDR decreases TGF-β-dependent SMAD transcriptional activity by inhibiting recruitment of SMAD3 to the promoter regions of TGF-β target genes in a 1,25(OH)₂D₃-dependent manner. Animal model experiments revealed that 1,25(OH)₂D₃ suppressed renal fibrosis by inhibiting TGF-β-SMAD signal transduction. Using computational structural analysis, we identified 2 synthetic VDR ligands — the 1α,25-dihydroxyvitamin D₃,26,23-lactam (DLAM) derivatives DLAM-iPr and DLAM-4P — that selectively inhibited TGF-β-SMAD signal transduction without activation of classical VDR-mediated transcription. The effective dose of 1,25(OH)₂D₃ cannot be used clinically because it results in significant hypercalcemia. In contrast, DLAM-iPr and DLAM-4P suppressed renal fibrosis in a mouse model without causing hypercalcemia. Thus, inhibition of TGF-β-SMAD signal transduction through VDR nonclassical pathway suppresses renal fibrosis. Our findings provide new insights relevant to the functions of the VDR and to the development of drugs to treat TGF-β–related diseases.

Results
VDR suppresses renal fibrosis in mice subjected to unilateral ureteral obstruction via inhibition of TGF-β–SMAD signal transduction. It has been reported that the VDR plays a role in therapeutic suppression of renal fibrosis (41–49), although there is little agreement concerning the underlying mechanism. Previous studies have suggested that activation of SMAD signaling by TGF-β exacerbates tissue fibrosis (15, 20–23). Therefore, we examined whether 1,25(OH)₂D₃ suppresses renal fibrosis by inhibiting TGF-β–SMAD signal transduction in mice subjected to unilateral ureteral obstruction (UUO), an established model of tubulointerstitial renal fibrosis. Masson’s trichrome staining of renal tissues in mice revealed that aniline blue–positive areas in UUO kidneys were significantly larger than those in sham-operated control kidneys (Figure 1, A and B).

In contrast, stained areas were significantly smaller in the kidneys of 1,25(OH)₂D₃-treated versus untreated UUO mice. Staining using fibroblast-specific protein–1 (FSP-1) and α-SMA as markers of renal fibrosis revealed the progression of fibrosis in the kidneys of UUO mice, whereas administration of 1,25(OH)₂D₃ produced dose-dependent decreases in FSP-1– and α-SMA–positive cells (Figure 1, A and B).

UUO kidneys contained 15-fold more Tgfb1 mRNA than did control kidneys (Figure 1C). Western blot analysis revealed that the level of phosphorylated SMAD3 (pSMAD3) was higher in UUO kidneys (Figure 1D and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI67804DS1), indicative of activation of TGF-β-SMAD signal transduction. We then examined the expression of 3 TGF-β target genes involved in fibrosis: Serpine1 (encoding plasminogen activator inhibitor 1 [PAI-1]), Acta2 (encoding α-SMA), and Col1a1 (encoding type I collagen) (50). Real-time quantitative RT-PCR (qPCR) analysis showed that Serpine1, Acta2, and Col1a1 mRNA levels increased in UUO versus control kidneys (Figure 1C). Interestingly, 1,25(OH)₂D₃ treatment did not affect the levels of Tgfb mRNA and pSMAD3 in UUO kidneys, whereas Serpine1, Acta2, and Col1a1 expression was dose-dependently decreased by 1,25(OH)₂D₃ (Figure 1, C and D, and Supplemental Figure 1A). In addition, ChIP assay revealed an increased level of SMAD3 binding to the Serpine1 promoter in UUO kidneys, which was decreased by 1,25(OH)₂D₃ treatment (Figure 1E).

Based on these data, it is reasonable to suggest that 1,25(OH)₂D₃ suppresses TGF-β-SMAD signal transduction in UUO kidneys. To test this possibility, we isolated mouse kidney fibroblasts (Supplemental Figure 2) and examined the effects of 1,25(OH)₂D₃ on expression of TGF-β target genes. As expected, TGF-β-stimulated Serpine1, Acta2, and Col1a1 mRNA expression, which was decreased by 1,25(OH)₂D₃ treatment (Figure 1F). These results suggest that 1,25(OH)₂D₃ ameliorates TGF-β-SMAD signal transduction, which may lead to suppression of renal fibrosis.

Meanwhile, 1,25(OH)₂D₃ also significantly increased the serum calcium level (Figure 1G), which reflects a dysregulation of calcium homeostasis resulting from stimulation of the transcriptional activity of the VDR. Thus, although 1,25(OH)₂D₃ may be therapeutic for renal fibrosis, the associated elevation in serum calcium may have serious adverse consequences that would prevent its clinical application.

1,25(OH)₂D₃ inhibits recruitment of SMAD3 to TGF-β target gene promoters. We next examined the effects of 1,25(OH)₂D₃ on expression of TGF-β target genes in the murine renal epithelial TCMK-1 cell line. qPCR analysis confirmed that 1,25(OH)₂D₃ induced expression of the target gene cyp24a1 (Figure 2A). Treatment of TCMK-1 cells with TGF-β increased Serpine1 and Acta2 transcription (Figure 2A). The TGF-β–dependent elevation of Serpine1 mRNA was reduced in a 1,25(OH)₂D₃-dependent manner (Figure 2A). Knocking down VDR expression in TCMK-1 cells abolished the effect of 1,25(OH)₂D₃ on Serpine1 expression (Figure 2B and Supplemental Figure 1B). TGF-β–dependent elevation of Acta2 mRNA levels was also reduced in a 1,25(OH)₂D₃-dependent manner (Figure 2A).

To explore the mechanism underlying VDR-dependent inhibition of TGF-β-SMAD signal transduction, we evaluated the effect of 1,25(OH)₂D₃ on phosphorylation levels and nuclear transloca-
Figure 2

1,25(OH)₂D₃ inhibits TGF-β-dependent transcription via VDR. (A) TCMK-1 cells were cultured in the presence or absence of 5 ng/ml TGF-β and 0.001, 0.1, or 10 nM (+) 1,25(OH)₂D₃ for 8 hours, and Cyp24a1, Serpine1, and Acta2 mRNA levels were determined using qPCR. (B) Knockdown of VDR expression eliminated the effects of 1,25(OH)₂D₃ on TGF-β-induced Serpine1 expression in TCMK-1 cells. Western blotting confirmed reduced expression of VDR protein in response to 2 VDR-specific siRNAs (siVDR-1 and siVDR-2). Cyp24a1 and Serpine1 mRNA levels in response to VDR knockdown and treatment with 5 ng/ml TGF-β and/or 10 nM 1,25(OH)₂D₃ were determined. (C) TCMK-1 cells were treated with or without 5 ng/ml TGF-β and 0.001, 0.1, or 10 nM 1,25(OH)₂D₃, SMAD2, pSMAD2, SMAD3, pSMAD3, SMAD4, PAI-1, and β-actin protein levels were determined by Western blotting. (D) TCMK-1 cells cultured in the presence or absence of 5 ng/ml TGF-β or 10 nM 1,25(OH)₂D₃ were stained with anti-SMAD2/3 or anti-VDR antibodies. Scale bars: 50 μm. (E) ChIP assay was performed with control IgG or anti-SMAD3 antibodies. Immunoprecipitated DNA was examined using qPCR with primers specific for the Serpine1 or Acta2 promoter. *P < 0.05; **P < 0.01; ***P < 0.001.
tion of SMAD2 and SMAD3. Western blot analysis revealed that TGF-β–induced pSMAD2 and pSMAD3 levels were not affected by 1,25(OH)2D3 (Figure 2C and Supplemental Figure 1C). In addition, PAI-1 and α-SMA levels were decreased (Figure 2C and Supplemental Figure 1C). Immunofluorescence staining revealed that TGF-β–induced nuclear accumulation of SMAD2 and SMAD3 was not affected by 1,25(OH)2D3 (Figure 2D). We next examined the recruitment of SMAD3 to the Serpine1 or Acta2 promoter regions, since SMAD3 has DNA-binding ability (51). ChIP assay demonstrated that the interaction between SMAD3 and the Serpine1 or Acta2 promoter was potentiated by TGF-β, but was suppressed in the presence of 1,25(OH)2D3 (Figure 2E). In addition, 1,25(OH)2D3–dependent suppression of Serpine1 expression and inhibition of SMAD3 binding to the Acta2 promoter were also observed in the human renal proximal tubular epithelial HK-2 cell line (Supplemental Figure 3). These results suggest that VDR suppresses TGF-β–SMAD signal transduction by reducing the recruitment of SMAD3 to TGF-β target genes in an 1,25(OH)2D3-dependent manner.

To determine the role of VDR in inhibiting SMAD3 recruitment to TGF-β target genes, we sought to identify the VDR regions responsible for inhibiting TGF-β–SMAD signal transduction. To address this issue, we performed a cell-based transcriptional reporter assay using reporter plasmids bearing VDRE (referred to herein as VDRE-Luc) or SMAD-binding elements (CAGA-Luc) as well as 2 VDR mutants, VDR-mC (D42G/G43S/G46V; ref. 52) and VDR-LBD, both of which lacked DNA-binding ability (Figure 3A and see Methods). Expression of VDR-mC or VDR-LBD in HEK293 cells did not induce the transcription of VDRE-Luc (Figure 3A). In contrast, 1,25(OH)2D3–dependent suppression of TGF-β–SMAD signal transduction was observed in cells that expressed VDR-mC or VDR-LBD as well as VDR (Figure 3A). These
results indicate that the DNA-binding ability and induction of transactivation of VDR are not necessary for the 1,25(OH)2D3-dependent inhibition of TGF-β–SMAD signal transduction.

Because VDR-mediated transcription was not necessary for 1,25(OH)2D3-dependent inhibition of TGF-β–SMAD signal transduction, we next tested the interaction between VDR and SMAD proteins. In vitro binding assays showed that SMAD3, but not SMAD2 or SMAD4, bound to the VDR-LBD in a 1,25(OH)2D3-dependent manner (Figure 3B and Supplemental Figure 1D). To identify the regions responsible for VDR binding in SMAD3, we generated mad homology 1 (MH1), MH1 plus linker (MH1L), and MH2 domains of SMAD3 (Figure 3C). In the binding assay, we identified the domain SMAD3-MH1 as the region that specifically bound to the VDR-LBD (Figure 3C and Supplemental Figure 1E).

Since SMAD3-MH1 possessed DNA binding ability, we investigated whether VDR and 1,25(OH)2D3 affect binding of SMAD3 to DNA. EMSA showed that SMAD3-MH1 formed a complex with a DNA probe containing a SMAD3-binding element, but formation of this complex was prevented in the presence of both VDR-LBD and 1,25(OH)2D3 (Figure 3D). These results indicated that the VDR binds to SMAD3-MH1, thereby inhibiting the ability of SMAD3 to bind to DNA.

The C-terminal H12 is necessary for VDR-mediated suppression of TGF-β–SMAD signal transduction. In classical genomic action, VDR binds to specific response elements (i.e., VDRE) and regulates transcription of target genes. In contrast, our results indicated that VDR suppresses TGF-β–SMAD signal transduction by binding to SMAD3. Therefore, the classical genomic action of VDR may not be involved in the suppression of renal fibrosis by 1,25(OH)2D3. To test this possibility, we attempted to generate VDR ligands able to inhibit TGF-β–SMAD signal transduction without activating VDR-mediated transcription.

We first examined the structural underpinnings of VDR-mediated inhibition of TGF-β–SMAD signal transduction. The structure of the 1,25(OH)2D3–VDR-LBD complex has previously been examined using X-ray crystallography (34). In this complex, 1,25(OH)2D3 associates with and stabilizes H12 of the VDR-LBD (Figure 4A) to facilitate interactions with coactivators, including SRC-1 (35). Because H12 has a critical role for VDR-mediated transcription, we examined the effect of H12 deletion on VDR-dependent suppression of TGF-β–SMAD signal transduction. We confirmed that the transcriptional activity of the VDR was abolished by deleting H12 (VDR-AH12; Figure 4B). H12 deletion also abolished the inhibition of TGF-β–dependent transcription, as shown using
VDR-DH12 compared with full-length VDR. Similar results were obtained by deleting H12 from VDR-LBD (VDR-LBD-DH12). Consistent with the results of transcription assays (Figure 4B), in vitro pulldown experiments showed that VDR-LBD-DH12 did not bind to SMAD3-MH1 (Figure 4C and Supplemental Figure 1F). These results demonstrated that the H12 region of the VDR-LBD is required for interaction with SMAD3.

An endogenous vitamin D metabolite induces weak stimulation of VDR transcription, but suppression of TGF-β–SMAD signal transduction. Because H12 is required to interact with both SMAD3 and coactivators, VDR ligands that specifically inhibit TGF-β–agonistic VDR, 1,25-lactone was docked in the ligand-binding pocket of the VDR-LBD in complex with 1,25(OH)2D3 (PDB: 1DB1) using MOE. 1,25-lactone and 1,25(OH)2D3 are shown with residues forming hydrogen bonds. H12 is shown in magenta. Residues between A231 and Y236 have been omitted for clarity. *P < 0.05; **P < 0.01; ***P < 0.001.
These data suggest that 1,25-lactone induces a conformational change in H12 that stimulates partial VDR-mediated transcription, yet also inhibits TGF-β–SMAD signal transduction. Based on the results of our molecular docking simulation study, we attempted to develop compounds that would specifically inhibit TGF-β–SMAD signal transduction via VDR without VDR-mediated transcription activation.

**DLAM derivatives suppress TGF-β–SMAD signal transduction without activating VDR-mediated transcription.** Based on the results of our molecular docking simulation study, we attempted to develop compounds that would specifically inhibit TGF-β–SMAD signal transduction via VDR without VDR-mediated transcription activation. Given the structure of 1,25-lactone and its effect on VDR activity, we hypothesized that further modification of 1,25-lactone to directly affect the conformation of H12 may decrease stimulation of VDR-mediated transcription, but still inhibit TGF-β–SMAD signal transduction. To validate our hypothesis, we focused on 1,25-lactone–mimicking VDR ligands, i.e., DLAM derivatives (54). Previously, we showed that DLAMs suppress the promyelocytic leukemia cell differentiation induced by 1,25(OH)2D3 (54). More interestingly, these derivatives possess a variety of substituents on the nitrogen
atom of the lactam skeleton at the position of 1,25(OH)_{2}D_{3} side chain. We expected the substituents in these compounds to have steric effects on the conformation of H12; therefore, we examined the effects of DLAM-iPr and DLAM-4P (Figure 6A).

Similar to the case of 1,25-lactone, molecular docking analyses of DLAM-iPr- and DLAM-4P–VDR-LBD complexes revealed that the vitamin D secosteroid backbones of these DLAM molecules interact with the VDR-LBD in a configuration similar to 1,25(OH)_{2}D_{3} (Figure 6B). In addition, the simulation study showed that VDR-LBD residues His305 and His397 do not form a hydrogen bond network, as seen with 1,25(OH)_{2}D_{3} (Supplemental Figure 5). Furthermore, we confirmed that the DLAM-iPr and DLAM-4P substituents are directed toward H12, and that the long substituent of DLAM-4P apparently interferes with the agonistic position of H12 in the VDR-LBD (Figure 6B).

In a transcription assay, although 1,25-lactone activated VDR-mediated transcription from VDRE-Luc, DLAM-iPr and DLAM-4P showed relatively weak stimulation of VDR-mediated transcription and marked inhibition of TGF-β–SMAD signal transduction (Figure 6C and Supplemental Figure 6). In particular, 1,25-lactone appeared to have a markedly high effect on VDR transcriptional activity (compare Figure 6C with Figure 5B), because of differences in the sensitivities of the VDR activity to ligands between the transcription reporter and qPCR assays. In TCMK-1 cells, DLAM-iPr and DLAM-4P abrogated mRNA expression of TGF-β–dependent Serpine1 and Acta2 without inducing Cyp24a1 (Figure 6D). Similar to TCMK-1 cells, DLAM compounds decreased Serpine1 expression in HK-2 cells (Supplemental Figure 3, A and B). In mouse fibroblasts, TGF-β–induced expression of Serpine1, Acta2, and Col1a1 mRNA was suppressed by DLAM-iPr and DLAM-4P without inducing Cyp24a1 expression (Figure 6E).

These data suggest that both the lactam skeleton and the substituents of DLAM-iPr and DLAM-4P modulate the conformation of H12, leading to a nonagonistic state in the VDR.

To confirm that the DLAM derivatives induce the specific conformation of VDR to interact with SMAD3, we performed in vitro pull-down assays with glutathione S-transferase–bound VDR-LBD (GST-VDR-LBD) and the transcriptional cofactor SRC-1 (aa 593–730). Unlike 1,25(OH)_{2}D_{3} (Figure 7A and Supplemental Figure 1H). In contrast, similar to 1,25(OH)_{2}D_{3}, DLAM-iPr and DLAM-4P did not induce formation of a complex between the VDR and SRC-1 (Figure 7A and Supplemental Figure 1H). In contrast, similar to 1,25(OH)_{2}D_{3}, the DLAM derivatives did induce formation of a complex between the VDR and SMAD3-MH1 (Figure 7A and Supplemental Figure 1H). These data suggest that both the lactam skeleton and the substituents of DLAM-iPr and DLAM-4P modulate the conformation of H12, leading to a nonagonistic state in the VDR.
that the binding of SMAD3 to the Serpine1 and Acta2 promoters was inhibited by both DLAM-iPr and DLAM-4P in TCMK-1 cells (Figure 7C). In HK-2 cells, SMAD3 binding to the promot-
Serpine1
ers was inhibited by both DLAM-iPr and DLAM-4P in TCMK-1 cells
promoters
that the binding of SMAD3 to the and Serpine1
and
Serpine1
promoter. Samples were normalized to input DNA level (n = 3 per group). (D) Representative photomicro-
graphs of Masson's trichrome staining (top row, whole kidney; sec-
ond row, magnified view of boxed region, enlarged x4.37), FSP-
1−
(third row), and α-SMA–specific immunofluorescence staining (bottom row) of kidney sections from sham-operated or UUO mice injected with vehicle, 1.25(OH)2D3, DLAM-iPr, or DLAM-4P. Scale bars: 1.0 mm (top row); 200 μm (second row); 20 μm (bottom 2 rows). (E) Fibrotic areas (n = 3 per group), FSP–1–positive cells (n = 12 per group), and α-SMA–positive areas (n = 8 per group) in stained kidney sections. (F) Serum calcium concentration. *P < 0.05; **P < 0.001.

Discussion
TGF-β plays a crucial role in the maintenance of tissue homeo-
ostasis. Disrupted TGF-β signal transduction has been implicated in many human diseases, including neoplastic, autoimmune, fibrotic, and cardiovascular conditions as well as tumor progression. In the present study, we demonstrated that the natural vitamin D metabolite 1,25(OH)2D3 suppressed TGF-β–SMAD signal transduction in fibrogenesis of the kidneys. In addition, we observed that 1,25(OH)2D3-bound VDR interacted directly with SMAD3-MH1, which contains a DNA-binding motif. This interaction decreased binding of SMAD3 to DNA and inhibited TGF-β–SMAD signal transduction. Using DLAM-iPr and DLAM-4P, which specifically inhibit TGF-β–SMAD signal transduction without activation of classical VDR-mediated transcription, we demonstrated that VDR suppresses renal fibrosis by inhibiting TGF-β–SMAD signal transduction; thus, VDR suppresses renal fibrosis via a nonclassical pathway.
Vitamin D undergoes a sequential 2-step metabolism in the liver and kidneys to form 1,25(OH)2D3, which is thought to be the most potent metabolite of vitamin D. 1,25(OH)2D3 regulation of VDR-mediated classical genomic actions in target organs such as intestine, bone, and parathyroid glands plays an important role in the in vivo maintenance of calcium homeostasis. Although other effects of 1,25(OH)2D3 have been reported, including inhibition of cell proliferation, induction of cell differentiation, modulation of immune responses, stimulation of insulin secretion, and various neurological effects, the detailed action mechanisms of these effects remain to be determined. In this study, we found that 1,25(OH)2D3-bound VDR directly interacted with SMAD3 and regulated SMAD-mediated signal transduction independent of its classical genomic action. Since 1,25(OH)2D3 has multiple effects beyond the maintenance of calcium homeostasis mentioned above, it may regulate these effects through nonclassical action of VDR.

Although vitamin D is metabolized into at least 30 known metabolites, 1,25(OH)2D3 alone has been shown to be capable of producing most of the biological responses attributed to vitamin D; however, our results suggest that another vitamin D metabolite, 1,25-lactone, is also biologically effective. 1,25-lactone has been isolated and identified as a metabolite of 1,25(OH)2D3 in sera from animals administered pharmacologic doses of 1,25(OH)2D3, as well as from humans under physiological conditions (57, 58). Although the precise metabolic half-life of 1,25-lactone has not been determined, it appears to be longer than that of 1,25(OH)2D3 (59). Our results revealed that 1,25(OH)2D3 attenuated renal fibrosis by inhibiting TGF-β–SMAD signal transduction and that 1,25-lactone showed minimal activity with an inhibitory effect on TGF-β–SMAD signal transduction, the metabolism of 1,25(OH)2D3 to form 1,25-lactone may involve shifting of VDR function from the classical genomic action to a nonclassical action.

Our results indicate that 1,25(OH)2D3 suppresses renal fibrosis through inhibition of TGF-β–SMAD signal transduction, whereas previous studies have examined the effect of VDR agonists on a number of signaling pathways as a means of inhibiting renal fibrosis. For example, VDR agonists have been reported to inhibit the renin-angiotensin system (41, 43, 45, 48), reduce NF-κB–dependent transcription (44, 46, 47), and increase HGF production (42), which antagonizes TGF-β binding to the cell surface receptors. Attenuation of renin-angiotensin signaling and NF-κB–mediated inflammatory networks results in downregulation of Tgfβ1 mRNA synthesis and leads to reduced phosphorylation of SMAD2 and SMAD3. In addition, increased HGF levels reduce phosphorylation of SMADs. In the UUO and folic acid–induced mouse models, we observed that Tgfβ1 mRNA levels and SMAD3 phosphorylation were not affected by 1,25(OH)2D3. In addition, similar effects were observed with DLAM-iPr and DLAM-4P, neither of which stimulated VDR-mediated transcription. Our observations indicate that 1,25(OH)2D3, DLAM-iPr, and DLAM-4P suppress renal fibrosis primarily via inhibition of TGF-β–SMAD signal transduction. However, we cannot exclude the possibility of multiple suppressive effects of VDR on renal fibrosis.

Given its almost universal appearance in chronic kidney disease, renal fibrosis is a promising treatment target. During the fibrotic process, a preferential target for therapy is the fundamental cell responsible for the exaggerated production and deposition of ECM. Interstitial myofibroblasts have been widely regarded as the major source of interstitial ECM. In addition, renal fibrosis is often associated with inflammatory infiltration of monocytes and macrophages, which are essential for the regulation of immune responses and the development of inflammation. Therefore, understanding the origin of these cells in kidney undergoing fibrosis is of great interest in chronic kidney disease; however, it is still controversial. In this study, we clearly showed that α-SMA–positive myofibroblasts and FSP-1–positive macrophages were significantly reduced by treatment with DLAM compounds and that DLAMs decreased expression levels of TGF-β–induced fibrogenic genes in mouse kidney fibroblasts. From these results, we concluded that renal fibroblasts and/or macrophages, as well as myofibroblasts, are the targets of DLAM-iPr and DLAM-4P. We believe that better understanding of these potent target cells for DLAM compounds might lead to more efficacious treatments and that new insights into the onset and progression mechanisms of the underlying diseases offer the promise of improved approaches to therapy for renal fibrosis.

It has been reported that 1,25(OH)2D3 and VDR regulate intestinal calcium/phosphate absorption and remodeling of bone to maintain calcium homeostasis via its genomic action. Furthermore, the ligand and receptor stimulate rapid responses (and corresponding nongenomic actions) in the intestine (rapid Ca2+/Cl− absorption), pancreatic β cells (insulin secretion), endothelial cells (cell migration), osteoblasts (exocytosis), and Sertoli cells (exocytosis or secretion) (39). Although 1,25(OH)2D3 affects cell proliferation and differentiation, it remains unclear how this ligand regulates these biological events via its receptor. Vitamin D deficiency is associated with skeletal disease, hyperparathyroidism, and chronic diseases (60), which affects exogenous compounds such as DLAMs; therefore, there is an increased risk of diseases related to vitamin D deficiency after inhibiting the binding between 1,25(OH)2D3 and VDR or pleiotropic VDR actions. However, our present study showed that DLAMs ameliorated renal fibrosis without altering serum calcium levels or inducing hypercalcemia. Furthermore, we found that DLAM-treated mice did not exhibit any abnormalities related to impaired vitamin D metabolism, such as reduced body weight or behavioral disorders, suggesting the absence of detectable adverse effects of DLAMs. Thus, we conclude that DLAM-iPr and
DLAM-4P have little effect on normal vitamin D signaling via VDR in vivo. However, we cannot exclude the possibility that DLAMs induce effects via rapid VDR response or other actions.

TGF-β-SMAD signal transduction is reported to be involved in several disorders, including cardiac, hepatic, skin, pulmonary, and renal fibrosis. The data from this study suggest that specific stimulation of nonclassical action of VDR by synthetic ligands can suppress tissue fibrosis in several organs. Recently, it has been reported that calcipotriol, an agonist for VDR, prevents carbon tetrachloride-induced liver fibrosis through inhibiting transcriptional activation by SMAD3 (61). In VDR-positive hepatocyte stellate cells, TGF-β-SMAD signal transduction causes redistribution of genome-wide VDR binding sites and facilitates VDR binding at SMAD3 profibrotic target genes via TGF-β-dependent chromatin remodeling. In the presence of calcipotriol, VDR binding to the coregulated genes reduces SMAD3 occupancy at these sites, inhibiting fibrosis. Although the ligand-dependent action mechanism of VDR on suppression is somewhat different between liver and kidney, our DLAM compounds may possibly suppress hepatic fibrosis as well as renal fibrosis. Also, our results may provide a new strategy for development of drugs that do not possess detectable adverse effects. To date, studies have extensively explored the relationship between activation or repression of nuclear receptor-mediated transcription and several human diseases. Since nuclear receptors have long been recognized as transcription factors that bind directly to the genome, drug design for the receptors has typically targeted classical transcriptional regulation. In the present study, we observed that the nonclassical action of VDR plays an important role in protection against renal fibrosis progression. Although it has been observed that some hormonal effects occur regardless of the classical mechanism of nuclear receptor action, the molecular target for the nonclassical effects of nuclear receptors has not been demonstrated conclusively. In addition, although we believe that this nonclassical VDR pathway is involved in protection against renal fibrosis, we suspect that the role of nonclassical pathways of other nuclear receptors in suppressing the progression of human diseases should be studied further. In the case of drug development for targeting the nonclassical pathway of nuclear receptors, a synthetic compound that can selectively stimulate the desired effect, but is dissociated from the classical genomic pathway of the receptor, is required for effective therapy. Our report is the first to demonstrate that it is possible to selectively stimulate a specific activity and to block disease without detectable adverse effects by modifying nuclear receptor ligands. Establishing a general method by which to develop synthetic ligands that stimulate specific nonclassical functions of nuclear receptors is the subject of future studies. Thus, our findings may provide a paradigm for the development of drugs to treat various human diseases by specifically stimulating certain activities of nuclear receptors.

Methods

Further information can be found in Supplemental Methods.

Cell culture and transfection. TCMK-1, HK-2, and HEK293 cells were maintained in DMEM supplemented with 10% FBS. Transfection was performed with PerFectin Transfection Reagent (Gene Therapy Systems) according to the manufacturer’s protocols.

Expression vectors. DNA fragments encoding VDRs, RXRβ, and ALK5 TD were amplified by PCR and cloned into pcDNA3 expression vectors containing sequences coding for FLAG. VDR-mc is a form of VDR bearing 3 amino acid substitutions in the DNA-binding domain (mutation of Asp42, Gly43, and Gly46 to Gly, Ser, and Val, respectively) (52). ALK5 TD is a constitutively active TGF-β type I receptor (mutation of Thr204 to Asp). 3-tandem VDRE sequence (5′-GTATGTCTCAGATGAA-3′) and 9-tandem CAGA sequence (62) were subcloned into a pGL3-Basic reporter plasmid, resulting in VDRE-TD and CAGA-TD, respectively.

Real-time RT-PCR. 24 hours after seeding cells in phenol red-free DMEM containing 4% charcoal-stripped FBS, we replaced the culture medium with fresh phenol red-free DMEM containing 0.2% charcoal-stripped FBS. After 24 hours of incubation, we added ligands and cultured for 8 hours. We performed qPCR analyses using the Thermal Cycler Dice Real Time System instrument and software (Takara). Primers for qPCR are listed in Supplemental Table 1.

In vitro GST pulldown assay. Immobilized GST fusion proteins were preincubated for 30 minutes at 4°C with 10 μM 1,25(OH)2D3 or 1 mM DLAMs in GST-binding buffer (20 mM Hepes-KOH, pH 7.9, 10 mM KCl; 0.2 mM EDTA; 1 mM Mg2+BSA; 20% glycerol). Immobilized proteins on beads were added to 4 volumes of GST-binding buffer containing 0.01% Tween 20, then incubated at 4°C for 4 hours with 0.5 μg purified His-tagged proteins in the absence or presence of 10 μM 1,25(OH)2D3 or 1 mM DLAMs. After 3 washes in 500 μl GST-binding buffer containing 0.02% Tween 20, elution was performed by incubation in GST-binding buffer containing 0.2% N-lauroylsarcosine (NacalaTesque) at room temperature for 30 minutes. Samples were resolved by SDS-PAGE and analyzed by Western blotting.

EMSA. DNA binding assays were performed essentially as described previously (63), with minor modifications. His-tagged VDR-LBD was preincubated for 30 minutes at 4°C with 10 μM 1,25(OH)2D3 or 1 mM DLAMs in PBS, and 3 volumes of EMSA buffer (20 mM Tris-HCl, pH 7.9; 180 mM KCl; 0.2 mM EDTA; 0.05% NP-40; 0.5 mM PMSF; 1 mM DTT) containing 1 mg/ml BSA, His-tagged SMAD3-MH1, and 1 μM biotinylated SBE oligonucleotide with 10 μM 1,25(OH)2D3 or 1 mM DLAMs were added. The SBE sequence used was 5′-GTATGTCTCAGATGAA-3′ (64). Gel shift assays were carried out at 4°C.

UO model. C57BL/6 mice were purchased from CLEA Japan Inc. We induced kidney injury by UO. Under 2.5% avertin (NacalaTesque) anesthesia, the left ureter was ligated at 2 points and cut between the ligatures in order to prevent retrograde urinary tract infection. Sham-operated mice had their ureters manipulated, but not ligated. We initiated subcutaneous injections with 1,25(OH)2D3 (0.3 or 0.6 μg/kg body weight), DLAM-iPr (60 μg/kg body weight), or DLAM-4P (60 μg/kg body weight) every day from the day after UO until day 7; the other mice received vehicle (propylene glycol). We initiated subcutaneous injections with 1,25(OH)2D3 (0.3 or 0.6 μg/kg body weight), DLAM-iPr (60 μg/kg body weight), or DLAM-4P (60 μg/kg body weight) every day from the day after UO until day 7; the other mice received vehicle (propylene glycol). We anesthetized at least 8 mice by intraperitoneal injection of avertin at 7 days. Kidneys were fixed in 4% formaldehyde and embedded in paraffin blocks. For RNA and protein analysis, kidney samples were fresh frozen. We prepared total RNA with Sepazool (NakaraiTesque) and performed qPCR. Serum calcium was measured with Fuji DRY-CHEM (FUJIFILM) using CA-P III slide.

Folic acid administration model. We induced kidney injury by a single intraperitoneal injection of folic acid (250 mg/kg body weight for the 14-day experiment; 300 mg/kg body weight in PBS for the 129-day experiment). In the 14-day experiment, we subcutaneously administered 1,25(OH)2D3 (0.6 μg/kg body weight), DLAM-iPr (60 μg/kg body weight), or DLAM-4P (60 μg/kg body weight) on the day after folic acid injection; other mice received vehicle (propylene glycol). In the 129-day experiment, mice were administrated vehicle (propylene glycol), DLAM-iPr (60 μg/kg body weight), or DLAM-4P (60 μg/kg body weight) via a subcutaneously implanted osmotic pump (Alzet 1004; Alzet Osmotic Pump) 3 days after folic acid injection. The pumps were replaced every month. Mice were analyzed at 129 days after folic acid injection. Serum calcium and creatinine levels were measured with Fuji Dry-Chem (Fujiﬁlm) using CA-P III and CRE-P I11 slides, respectively.

Histological analysis. For analysis of collagen deposition, paraffin-embedded kidneys were sectioned at 4 μm, and Masson’s trichrome stain was
performed by standard procedure at the Laboratory Animal Resource Center, University of Tsukuba. The amount of collagen deposition (blue area) was then digitally quantified using Image J software as described previously (65). For immunofluorescence, the paraffin-embedded kidney sections were deparaffinized, rehydrated, heat-inactivated using autoclave, permeabilized with NP-40, and incubated with primary antibody against FSP-1 (DAKO Japan) or e-SMA (Sigma-Aldrich). We used Alexa Fluor 594–conjugated secondary antibody (Invitrogen).

Statistical analysis

All values described in the text and figures are expressed as mean ± SD. Significance of differences was determined by 2-tailed Student’s t-test. A P value less than 0.05 was considered significant.

Study approval

All animal experiments were performed in accordance with institutional guidelines of Laboratory Animal Resource Center, University of Tsukuba.


