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 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 hypercalcemia.

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 attenuates 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 transcalcithia 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

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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 VDR-mediated transcription. The effective dose of 1,25(OH)2D3 on phosphorylation levels and nuclear translocation of SMAD3 binding was lower in 1,25(OH)2D3-treated versus untreated control kidneys. 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 cyp24a1 expression (Figure 2A). The TGF-β–dependent elevation of cyp24a1 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 cyp24a1 expression (Figure 2B and Supplemental Figure 1B). TGF-β–dependent elevation of cyp24a1 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)_{2}D_{3} 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)_{2}D_{3} 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)_{2}D_{3} 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)_{2}D_{3} 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)_{2}D_{3}. 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)_{2}D_{3} 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 and 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).
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 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-ΔH12 compared with full-length VDR. Similar results were obtained by deleting H12 from VDR-LBD (VDR-LBD-ΔH12). Consistent with the results of transcription assays (Figure 4B), in vitro pulldown experiments showed that VDR-LBD-ΔH12 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. 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-\(\beta\)–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-\(\beta\)–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-\(\beta\)–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 593–780) or SMAD3-MH1 (aa 1–132). Unlike 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 formation of H12, leading to a nonagonistic state in the VDR.

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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 Serpine1 promoter was inhibited by these compounds (Supplemental Figure 3C).

**DLAM derivatives suppress renal fibrosis without inducing hypercalcemia.** To examine whether the suppression of renal fibrosis by 1,25(OH)2D3 is due to the inhibition of SMAD signaling by VDR, we tested the effect of DLAM-iPr and DLAM-4P on renal fibrosis caused by UUO. In mouse kidney, DLAM-iPr and DLAM-4P did not increase the level of Cyp24a1 mRNA, whereas 1,25(OH)2D3 did (Figure 8A). In contrast, administration of DLAM-iPr and DLAM-4P significantly reduced Serpine1, Acta2, and Col1a1 mRNA levels in UUO kidneys (Figure 8A). Consistent with these results, UUO-induced PAI-1 and α-SMA protein expression was also inhibited by DLAM-iPr and DLAM-4P (Figure 8B and Supplemental Figure 11). While the level of pSMAD3 was not affected by the VDR ligands in UUO mice (Figure 8B and Supplemental Figure 11), ChIP experiments revealed suppressed binding of SMAD3 to the Serpine1 promoter in response to DLAM-iPr and DLAM-4P treatment (Figure 8C), confirming the inhibition of TGF-β–SMAD signal transduction. Consistent with these results, both DLAM-iPr and DLAM-4P led to a decrease in the size of the area stained with aniline blue, anti–FSP-1 antibody, or anti–α-SMA antibody in UUO kidneys (Figure 8, D and E). There was no appreciable increase in serum calcium in mice treated with DLAM-iPr or DLAM-4P (Figure 8F), in concordance with the inability of these ligands to induce VDR-mediated transcription.

Several studies have shown that vitamin D analogs are renoprotective in experimental animal models with renal diseases. One of these analogs, paricalcitol, is a VDR agonist and is used as a drug for prevention and treatment of secondary hyperparathyroidism associated with chronic renal failure. Preclinical studies have shown that paricalcitol induces less hypercalcemia than 1,25(OH)2D3 (55). We therefore compared paricalcitol and DLAM compounds in terms of their VDR-agonistic, fibrotic, and hypercalcemic effects in UUO kidneys. Unlike DLAMs, mRNA expression of Cyp24a1 was elevated by paricalcitol (Figure 8A and Supplemental Figure 7). In contrast, similar to DLAMs, paricalcitol decreased Acta2 and Col1a1 mRNA levels (Supplemental Figure 7) as well as the size of the area stained with aniline blue, anti–FSP-1 antibody, or anti–α-SMA antibody (Supplemental Figure 8). Paricalcitol did not decrease the typical level of the TGF-β target gene Serpine1 in UUO kidneys (Supplemental Figure 7). More importantly, unlike DLAMs, paricalcitol significantly elevated serum calcium levels compared with vehicle in our experimental condition (Supplemental Figure 9). Next, we tested DLAM-iPr and DLAM-4P using an alternative renal fibrosis model, i.e., folic acid–induced nephropathy (56). Mice were intraperitoneally injected with folic acid after administration of vehicle or DLAM derivatives and analyzed after 14 and 129 days (short- and long-term analysis, respectively). In the short-term analysis, mRNA levels of Tgfb1, Serpine1, Acta2, and Col1a1 increased in mice injected with folic acid (Supplemental Figure 10A). In agreement with the results of the UUO model, mRNA levels of Serpine1, Acta2, and Col1a1 decreased in response to 1,25(OH)2D3, DLAM-iPr, and DLAM-4P, whereas Tgfb1 mRNA was not affected (Supplemental Figure 10A). Similar results were observed in Western blot analyses, in which PAI-1 and α-SMA protein levels significantly decreased in response to 1,25(OH)2D3, DLAM-iPr, and DLAM-4P, whereas SMAD3 and pSMAD3 levels were not affected (Supplemental Figure 10B). In the long-term analysis, Serpine1, Acta2, and Col1a1 expression decreased in response to treatment with DLAM-iPr or DLAM-4P (Figure 9A). Masson’s trichrome and immunohistochemical staining of kidneys as well as measurement of serum creatinine showed significant inhibition of progressive fibrosis and renal failure in DLAM-iPr– and DLAM-4P–treated mice (Figure 9, B–D). Unlike 1,25(OH)2D3, DLAM-iPr and DLAM-4P did not increase serum calcium levels in the short-term analysis. Moreover, even in the long-term analysis, DLAMs only slightly increased the serum calcium level (Figure 9E).

Taken together, these results show that DLAM-iPr and DLAM-4P reduce renal fibrosis by inhibiting TGF-β–SMAD signal transduction, but do not induce hypercalcemia via VDR transactivation. Therefore, these synthetic ligands may be good candidates for use in treating renal fibrosis.

**Discussion**

TGF-β plays a crucial role in the maintenance of tissue homeostasis. 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.
Figure 9

DLAM-iPr and DLAM-4P ameliorate folic acid–induced nephropathy. (A) DLAM-iPr and DLAM-4P suppress the expression of proinflammatory genes 129 days after folic acid injection. We prepared RNA from kidneys of mice treated with folic acid and the indicated ligands, and Cyp24a1, Serpine1, Acta2, and Col1a1 mRNA levels were examined by qPCR. (B) Representative photomicrographs of Masson’s trichrome staining (top row, whole kidney; second row, magnified view of boxed region, enlarged x3.24), FSP-1– (third row), and α-SMA–specific immunofluorescence staining (bottom row) of kidney sections from mice that received folic acid (vehicle). 3 days after injection, mice were treated with vehicle, 0.6 μg/kg/d 1,25(OH)2D3, 60 μg/kg/d DLAM-iPr, or 60 μg/kg/d DLAM-4P by continuous infusion from osmotic pumps. Mice were analyzed 129 days after folic acid injection. Scale bars: 3.0 mm (top row); 500 μm (second row); 20 μm (bottom 2 rows). (C) Fibrotic area (μ) (n = 4 per group), FSP-1–positive cells in stained sections (μ) (n = 10 per group), and α-SMA–positive area (μ) (n = 10 per group). (D) and (E) Serum creatinine (D) and calcium (E) concentrations. Short-term, 14 days; Long-term, 129 days. *P < 0.05; **P < 0.01; ***P < 0.001.

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 also had an inhibitory effect on TGF-β–SMAD signal transduction. From these observations, it appears that 1,25-lactone is not merely a waste metabolite of 1,25(OH)2D3, but is a natural compound with specific biological functions. Because 1,25(OH)2D3 fully activated VDR-mediated transcription and 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 effects 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-kB–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-kB–mediated inflammatory networks results in downregulation of Tgfbi 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 Tgfbi 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. Intestinal myofibroblasts have been widely regarded as the major source of intestinal 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 FSP1–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, RXRα, 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′-GGTTCAGGAGTTCA-3′) and 9-tandem CAGA sequence (62) were subcloned into a pGL3-Basic reporter plasmid, resulting in VDRE-Luc and CAGA-Luc, 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; 100 mM KCl; 0.2 mM EDTA; 1 mg/ml 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 (NacalaiTesque) 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.

UUO model. Cs7BL/6 mice were purchased from CLEA Japan Inc. We induced kidney injury by UUO. Under 2.5% avertin (NacalaiTesque) 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 UUO 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 administered 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) every day after UUO 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.

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 α-SMA (Sigma-Aldrich). We used Alexa Fluor 594–conjugated secondary antibody (Invitrogen).

Statistics. 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.


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