Paricalcitol Inhibits Renal Inflammation by Promoting Vitamin D Receptor–Mediated Sequestration of NF-κB Signaling

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ABSTRACT

Inflammation is a pathologic feature of a variety of chronic kidney diseases. Several lines of evidence suggest a potential anti-inflammatory role for vitamin D in chronic kidney disease, but the underlying mechanism remains unknown. Here, the effect of the synthetic vitamin D analogue paricalcitol on renal inflammation was investigated in a mouse model of obstructive nephropathy. Paricalcitol reduced infiltration of T cells and macrophages in the obstructed kidney. This inhibition of inflammatory cell infiltration was accompanied by a decreased expression of RANTES and TNF-α. Induction of RANTES was localized primarily to the tubular epithelium, underscoring a role for tubular cells in renal inflammation. In a human proximal tubular cell line (HKC-8), paricalcitol inhibited RANTES mRNA and protein expression and abolished the ability of tubular cells to recruit lymphocytes and monocytes after TNF-α stimulation. Although RANTES induction depended on NF-κB signaling, paricalcitol affected neither TNF-α–mediated IκBα phosphorylation and degradation nor p65 NF-κB activation and nuclear translocation. Instead, chromatin immunoprecipitation assay showed that paricalcitol abolished the binding of p65 to its cognate cis-acting element in the RANTES promoter. The vitamin D receptor (VDR) and p65 formed a complex in tubular cells after paricalcitol treatment, which inhibited the ability of p65 to trans-activate gene transcription. In vivo, paricalcitol did not block NF-κB nuclear translocation after obstructive injury but did increase the expression and nuclear distribution of VDR. These results suggest that paricalcitol inhibits renal inflammatory infiltration and RANTES expression by promoting VDR-mediated sequestration of NF-κB signaling.


Deficiency in vitamin D and its active metabolites is a common pathologic feature that occurs early in the pathogenesis of chronic kidney disease (CKD). Numerous clinical studies have shown a high prevalence of calcitriol deficiency in CKD, even in patients with reasonable GFR.1,2 In humans, active vitamin D reduces proteinuria and all-cause mortality in CKD, which is independent of serum parathyroid hormone, phosphorus, and calcium levels.3–5 Evidence is also mounting that vitamin D analogue is renoprotective in different experimental nephropathies.6 Although early studies largely focused on primary glomerular diseases,7–9 active vitamin D was shown to display beneficial effects in obstructive nephropathy,10 a model characterized by inflammatory infiltration, tubular atrophy, and interstitial fibrosis. The therapeutic effects of active vitamin D in obstructive nephropathy are thought to be mediated by its ability to preserve tubular epithelial integrity via inhibiting epithelial-to-mesenchymal transition (EMT)10; however, in view of its
pleiotropic property, it is conceivable that vitamin D could elicit its renoprotection by a multitude of actions. One of the potential mechanisms could be related to its ability to modulate renal inflammation after injury.

Renal inflammation, characterized by the infiltration of inflammatory cells including T cells and macrophages to kidney parenchyma, is an imperative pathologic process in the evolution of CKD. Inflammatory infiltration contributes to the initiation and progression of CKD in several ways. On the one hand, inflammatory cells release proinflammatory, chemotactic cytokines (chemokines), thereby leading to the formation of a vicious self-accumulation circle. On the other hand, production and secretion of profibrotic cytokines by inflammatory cells such as monocytes/macrophages and T cells create a fibrogenic microenvironment, leading to generation of the matrix-producing effector cells through fibroblast activation and tubular EMT. Not surprising, decline of renal function in patients with CKD often correlates closely to the extent of in-

Figure 1. Paricalcitol reduces renal inflammatory T cell infiltration. (A through E) Immunohistochemical staining revealed an increased infiltration of CD3+ T cells in the obstructed kidney at 7 d (B) and 14 d (D), respectively, after UUO, compared with sham control (A). Paricalcitol treatment at 0.3 \( \mu \)g/kg body wt effectively inhibited the infiltration of T cells in the obstructed kidneys at 7 d (C) and 14 d (E) after UUO, respectively, as indicated. Bar = 20 \( \mu \)m. (F) Graphic presentation of quantitative data. Data are means ± SEM of five animals per group. *\( P < 0.01 \) versus sham control; †\( P < 0.01 \) versus vehicle.

Figure 2. Paricalcitol inhibits renal inflammatory macrophage infiltration. (A through E) Immunohistochemical staining showed an increased infiltration of F4/80+ myeloid cells including macrophages and renal dendritic cells in the obstructed kidney at 7 d (B) and 14 d (D), respectively, after UUO, compared with sham control (A). Paricalcitol at 0.3 \( \mu \)g/kg body wt inhibited the infiltration of the F4/80+ cells in the obstructed kidneys at 7 d (C) and 14 d (E), respectively, after UUO as indicated. Bar = 20 \( \mu \)m. (F) Graphic presentation of quantitative data. Data are means ± SEM of five animals per group. *\( P < 0.01 \) versus sham control; †\( P < 0.01 \) versus vehicle.
Inhibition of renal inflammation by different maneuvers is therapeutically effective, resulting in an amelioration of renal fibrotic lesions in various experimental animal models. Several lines of evidence have suggested a potential anti-inflammatory activity of vitamin D in CKD. In animal models of primary glomerular diseases, administration of vitamin D reduces glomerular infiltration of inflammatory cells. Consistently, a decreased infiltration is associated with higher serum vitamin D level in patients with CKD. Vitamin D may exert its immunomodulatory action through regulating the activity of many types of immune cells such as macrophages, dendritic cells, and T cells. Furthermore, evidence also points to an inhibitory effect of vitamin D on the signaling of NF-κB, a key transcription factor that is thought to mediate acute and chronic inflammation by regulating the gene expression of cytokines, chemokines, and adhesion molecules; however, it remains ambiguous as to the molecular mechanism.
by which vitamin D inhibits inflammation in the setting of CKD.

In this study, we demonstrated that paricalcitol, an active vitamin D analogue, reduces inflammatory cell infiltration and RANTES, also known as CC-chemokine ligand 5 (CCL5), expression \textit{in vivo}. We further showed that paricalcitol induced a physical interaction of vitamin D receptor (VDR) and p65 NFκB, resulting in sequestration of the ability of p65 to bind to cis-acting element, thereby causing the repression of NFκB-mediated gene transcription. Our findings provide significant mechanistic insights into the understanding of the molecular details underlying vitamin D inhibition of renal inflammation.

RESULTS

Paricalcitol Inhibits Renal Inflammatory Infiltration

To assess the potential effect of paricalcitol on renal inflammation, we first examined renal infiltration of the CD3+ T cells in the obstructed kidney after unilateral ureteral obstruction (UUO). As shown in Figure 1, compared with sham controls, obstructive injury caused significant T cell infiltration in the kidney at 7 d after UUO, as illustrated by immunohistochemical staining for CD3 antigen. Prolonged, continuous obstruction for 14 d markedly increased renal infiltration of the CD3+ T cells in the obstructed kidney, when compared with 7 d after UUO (Figure 1, D \textit{versus} B); however, paricalcitol treatment effectively inhibited the infiltration of T cells in the obstructed kidney at different time points (Figure 1, C and E). A computer-aided quantitative analysis also demonstrated a dramatic suppression of inflammatory T cell infiltration by paricalcitol at 7 and 14 d after UUO, respectively, in obstructive nephropathy (Figure 1F).

We also examined the effects of paricalcitol on monocyte/macrophage infiltration in the obstructed kidney. As shown in Figure 2, UUO instigated significant infiltration of the F4/80 antigen–positive myeloid cells, including macrophages and dendritic cells in renal interstitium. Similarly, administration of paricalcitol substantially reduced F4/80-positive cell infiltration at different time points after UUO (Figure 2, C, E, and F).

Paricalcitol Inhibits Renal mRNA Expression of RANTES and TNF-α

We next examined the expression of RANTES, an important proinflammatory chemokine that plays a crucial role in inciting infiltration of T cells and other inflammatory cells such as monocytes, in the obstructed kidney. As shown in Figure 3, reverse transcriptase–PCR (RT-PCR) results revealed that approximately seven- and 11-fold induction of RANTES/CCL5 mRNA was observed in the obstructed kidney at 7 and 14 d, respectively, when compared with sham controls. Interestingly, we found that paricalcitol markedly inhibited RANTES mRNA expression in different time points (Figure 3, A and B); however, paricalcitol seemed to have little effect on monocyte chemoattractant protein-1 (MCP-1), also known as CCL2, mRNA expression in the obstructed kidney (Figure 3).

We further investigated the expression of TNF-α, a key inflammatory cytokine that is produced primarily by the infiltrated cells, in the obstructed kidney after UUO. As presented in Figure 3, UUO dramatically induced renal TNF-α mRNA expression at 7 and 14 d after UUO, respectively. Similarly, paricalcitol inhibited TNF-α expression in the obstructed kidney in a dosage-dependent manner.

RANTES Protein Is Induced Specifically in Renal Tubules after Obstructive Injury

We also examined RANTES protein expression and its localization in the obstructed kidney by immunohistochemical staining. As shown in Figure 4A, no or little RANTES protein was observed in normal, sham-operated kidney; however, RANTES protein expression was markedly induced in the obstructed kidney at 7 and 14 d, respectively. RANTES protein was localized predominantly in renal tubular epithelia (Figure 4, B and D). Consistent with the mRNA data (Figure 3), paricalcitol treatment significantly reduced RANTES protein expression in the obstructed kidney at different time points (Figure 4, C and E).

Paricalcitol Inhibits Tubular RANTES Induction In Vitro

Given that tubular epithelial cells are the primary sites of RANTES production \textit{in vivo} in obstructive nephropathy, we next examined the regulation of renal RANTES expression by paricalcitol using an \textit{in vitro} cell culture system. To this end, human proximal tubular epithelial cells (HKC-8) were incubated with TNF-α in the presence or absence of paricalcitol, and RANTES expression was examined. As shown in Figure 5, A and B, Western blot analysis
Paricalcitol Reduces the Recruitment of Inflammatory Cells In Vitro

To evaluate the significance of tubular expression of RANTES in recruiting inflammatory cells, we examined the chemotactic ability of the supernatants of tubular cells by using chemotaxis assay. To this end, HKC-8 cells were treated without or with TNF-α (2 ng/ml) in the absence or presence of various concentrations of paricalcitol for 24 h. The supernatants of these cultures were used in a chemotaxis assay to assess their ability to attract splenocytes (A and B) or THP-1 cell (C and D) migration across the filter of Transwell chambers. Freshly isolated mouse splenocytes (A and B) and cultured THP-1 cells (C and D) were used. (A and C) Paricalcitol treatment reduced the ability of HKC-8 cell supernatants to attract both splenocyte (A) and THP-1 cell (C) migration. (B and D) Neutralization of RANTES in HKC-8 cell supernatants with specific anti-RANTES antibody (2 μg/ml) reduced splenocyte (B) and THP-1 cell (D) migration. The same amount of normal IgG was used as controls. Data are expressed as the percentages of migrated cells in total cells added and presented as means ± SEM of three experiments. *P < 0.05 versus control; †P < 0.05 versus TNF-α treated alone. (E) Representative pictures show the migrated THP-1 cells in the bottom chambers of the Transwell plates in various groups as indicated.

Paricalcitol Does not Affect NF-κB Early Activation and Its Nuclear Translocation

Having established that NF-κB signaling is important in mediating RANTES induction (Figure 7), we reasoned whether paricalcitol inhibits RANTES expression and renal inflammation by modulating NF-κB pathway. To test this hypothesis, we first examined the effect of paricalcitol on IκBα phosphorylation and its subsequent degradation, as well as p65 NF-κB induced by TNF-α in tubular cells, indicating that an intact NF-κB signaling is required for RANTES induction. In reciprocal experiments, ectopic expression of p65 NF-κB sensitized tubular epithelial cells to express RANTES in response to a low level of TNF-α (0.2 ng/ml) stimulation (Figure 7B, lane 2 versus lane 3). Interestingly, paricalcitol (10⁻⁷ M) also inhibited RANTES expression in the tubular cells overexpressing p65 NF-κB (Figure 7B, lane 3 versus lane 4).
phosphorylation and activation. When HKC-8 cells were incubated with proinflammatory TNF-α, IκBα and p65 NF-κB were rapidly phosphorylated starting at the time point as early as 5 min (Figure 8, A and B); however, treatment of HKC-8 cells with paricalcitol did not significantly affect the kinetics and magnitude of IκBα and p65 NF-κB phosphorylation. Likewise, paricalcitol also did not influence IκBα degradation triggered by TNF-α in HKC-8 cells (Figure 8B). We next assessed the p65 NF-κB nuclear translocation after various treatments. Immunofluorescence staining demonstrated that upon stimulation with TNF-α, p65 NF-κB rapidly translocated into the nuclei in HKC-8 cells, and paricalcitol did not affect the nuclear translocation of p65 NF-κB (Figure 8C). These results suggest that paricalcitol inhibits tubular RANTES expression by a mechanism independent of NF-κB early activation and its nuclear translocation.

Paricalcitol Abolishes NF-κB Binding to RANTES Promoter by Facilitating VDR/p65 Interaction

The inability of paricalcitol to affect NF-κB activation prompted us to examine whether it modulates the signaling events after p65 nuclear translocation. To test this, we first investigated the possibility that paricalcitol might negatively influence the in vivo binding of p65 NF-κB to RANTES promoter region. As shown in Figure 9, A and B, TNF-α induced p65 binding to its cognate cis-acting NF-κB element in human RANTES promoter, as revealed by chromatin immunoprecipitation (ChIP) assay; however, treatment of HKC-8 cells with paricalcitol abolished the binding of p65 to RANTES promoter induced by TNF-α. To assess the consequence of this paricalcitol-mediated inhibition of p65/DNA binding, we examined the effect of paricalcitol on NF-κB–mediated gene expression by using a luciferase reporter assay. As demonstrated in Figure 9C, paricalcitol effectively repressed...
the NF-κB-mediated reporter gene expression after TNF-α stimulation. These results indicate that paricalcitol can specifically target a key event in NF-κB signaling.

We next investigated the potential mechanism underlying the paricalcitol inhibition of p65 binding to RANTES promoter. By co-immunoprecipitation, we found that paricalcitol facilitated the interaction between VDR and p65 NF-κB in HKC-8 cells that overexpressed p65. As shown in Figure 9, D and E, after stimulation by TNF-α and paricalcitol, increased p65 was detectable in the immunocomplexes precipitated by anti-VDR antibody. Similarly, in HKC-8 cells without ectopic expression of exogenous p65, paricalcitol induced a physical interaction between VDR and endogenous p65 NF-κB (Figure 9F). This suggests that an increased VDR/p65 complex formation after paricalcitol treatment would lead to a reduced availability of free p65, thereby sequestering its ability to bind to cis-acting element, causing a repression of p65-mediated gene transcription.

**Paricalcitol Does not Affect NF-κB Nuclear Translocation but Restores VDR Expression In Vivo**

We further investigated how paricalcitol inhibits NF-κB signaling and renal inflammation in obstructive nephropathy in vivo. As showed in Figure 10A, UUO markedly induced p65 phosphorylation and activation, as revealed by Western blot analysis of whole-kidney lysates using phospho-specific anti-p65 antibody; however, paricalcitol did not inhibit p65 phosphorylation in the obstructed kidney at 7 d after UUO (Figure 10A). Immunofluorescence staining demonstrated that p65 NF-κB was localized largely in the cytoplasm of the tubular cells in normal kidney (Figure 10C); however, obstructive injury apparently induced its nuclear translocation. Similar to an in vitro situation, administration of paricalcitol did not affect the activation and nuclear translocation of p65 in obstructed kidney (Figure 10C).

We also examined the expression and localization of VDR after different treatments. Consistent with a previous report,10 obstructive injury caused a suppression of VDR, and paricalcitol largely restored VDR expression after UUO (Figure 10B). Interestingly, immunostaining exhibited that a large proportion of VDR was localized in the nuclei in normal kidney (Figure 10C), suggesting an active role of the vitamin D system in the maintenance of normal tubular integrity. After UUO, the overall level of VDR was reduced and little nuclear VDR was observed; however, paricalcitol not only restored VDR protein level but also induced VDR to redistribute completely into the nuclei. It is plausible, therefore, that the nuclear VDR after paricalcitol treatment would bind to the activated p65 in the nuclei and sequester its gene-transactivating ability.

**DISCUSSION**

The results presented in this study demonstrate that paricalcitol, an active vitamin D analogue, displays a potent anti-inflammatory effect through repression of NF-κB-mediated gene transcription in obstructive nephropathy. These findings suggest that paricalcitol could be an effective treatment for renal inflammation in obstructive nephropathy.
flammatory activity by effectively inhibiting T lymphocyte and macrophage infiltration and proinflammatory cytokines RANTES and TNF-α expression in a mouse model of obstructive nephropathy. Mechanistically, paricalcitol specifically target NF-κB, a principal signaling transducer that is involved in mediating proinflammatory responses in virtually all circumstances. Paricalcitol induces VDR binding to the p65 subunit of NF-κB and prevents it from interacting with cis-acting DNA element, thereby sequestering its ability to transactivate the transcription of its targeted genes. Because chronic inflammation is a critical process that contributes to the pathogenesis of CKD in many ways, inhibition of inflammation could be an important mechanism by which vitamin D exerts its beneficial activity in ameliorating renal interstitial fibrosis after obstructive injury; therefore, our findings shed new light on the understanding of the therapeutic effects of active vitamin D in CKD.

Although an anti-inflammatory effect of vitamin D is well documented, previous studies largely emphasized its role in modulating immune cell activity; however, paricalcitol inhibits RANTES expression that is localized almost exclusively in renal tubules after injury, suggesting that tubular epithelial cells are likely the primary target of vitamin D in eliciting its anti-inflammatory action. Tubular epithelial cells, the largest cell population in kidney parenchyma, are not a bystander in the development of renal inflammation. Instead, increasing evidence indicates that they contribute directly and actively to renal inflammatory response after injury. Tubular production of RANTES inevitably builds up the chemokine gradient, which serve as chemotactic signals to attract the migrating leukocytes to move along the gradient and eventually reach the tubulointerstitial site of inflammation. In this regard, tubular expression and secretion of RANTES is a critical step that sets in motion toward the peritubular infiltration of inflammatory cells. As shown by chemotaxis assay, RANTES is a major chemotactic component in the supernatants of tubular cells after TNF-α stimulation, which plays an important role in attracting both splenocyte (rich in lymphocytes) and THP-1 cell (monocytic cell line) migration (Figure 6); therefore, by inhibiting RANTES expression in tubular cells, paricalcitol directly targets a key event in the circuit of inflammatory responses in obstructive nephropathy.

The influx of inflammatory cells from the peripheral circulation to the injured sites is a complex process in which chemokines play a fundamental role. Chemokines not only act as the directional signals to sort and guide effector leukocyte mi-

Figure 10. Paricalcitol does not block NF-κB activation and its nuclear translocation but restores VDR expression and enhances its nuclear translocation in the obstructed kidney after UUO in vivo. (A) Western blot demonstrated that UUO induced p65 NF-κB phosphorylation and paricalcitol treatment did not block p65 NF-κB activation in vivo. Kidney lysates were immunoblotted with anti-phospho-specific p65 NF-κB antibody. (B) Western blot showed that VDR expression was restored significantly in the obstructed kidney and paricalcitol treatment largely restored VDR expression in vivo. Numbers (1, 2, and 3) denote each individual animal in a given group. (C) Immunofluorescence staining also demonstrated that paricalcitol restored VDR expression but did not block p65 NF-κB nuclear translocation in the obstructed kidney in vivo. Kidney sections were immunostained for total p65 (red) and the nuclei (green; top). (Bottom) Staining for VDR (red). Arrowheads indicate the nuclear staining of VDR and p65 NF-κB. Bar = 20 μm.
calcitcal on NF-κB signaling is operated at the postnuclear translocation stage. Of note, several reports suggested that vitamin D inhibits NF-κB signaling by increasing IκBα and reducing p65 NF-κB nuclear translocation in mesangial cells, mouse embryonic fibroblasts, and pancreatic islet cells.33–35 The reason behind this discrepancy remains unknown, but it could be related to the cell-type specificity. In this study, we demonstrated that paricalcitol induced a complex formation between VDR and p65 NF-κB, consistent with previous reports in fibroblasts and osteoblastic cells.33,34 It should be noted that in the p65-overexpressing tubular cells, addition of TNF-α (or paricalcitol) alone slightly induced the p65/VDR interactions (Figure 9). This could be attributable to the presence of a trivial amount of vitamin D in the cultured conditions, which promoted the p65/VDR interaction when the cells overproduce p65. Neither TNF-α nor paricalcitol alone induced endogenous p65/VDR interaction (Figure 9F), while these cells did not overexpress p65. The physical interaction between VDR and p65 evidently prevents activated NF-κB from binding to the DNA element in the promoter of RANTES gene (Figure 9B). These findings underscore that paricalcitol targets specifically and directly a key nuclear event in NF-κB signal circuit, resulting in an effective sequestration of this proinflammatory signaling.

The data presented here strongly suggest that paricalcitol inhibits renal inflammation primarily by triggering a direct, VDR-mediated sequestration of NF-κB signaling; however, although the in vitro data are clear, it remains to be determined whether this mechanism is operative in vivo and whether the VDR ligation is necessary for the paricalcitol effect. In addition, we cannot exclude the possibility that paricalcitol may regulate RANTES and TNF-α expression by a NF-κB–independent mechanism. Furthermore, paricalcitol may exert its anti-inflammatory actions by other indirect routes as well, given that vitamin D has pleiotropic effects.37,38 Along this line, Li et al.39 reported that vitamin D is a negative regulator of renin gene expression, and, therefore, vitamin D analogue could inhibit renin expression, resulting in a reduced activation of the renin-angiotensin system in diseased kidney. Because angiotensin II is a known proinflammatory stimulus, it is conceivable that paricalcitol could inhibit renal inflammation by targeting the renin-angiotensin system in CKD. In addition, vitamin D is shown to induce the expression of hepatocyte growth factor,40 a cytokine that has potent anti-inflammatory and antifibrotic properties.41–43

In summary, we have shown in this study that paricalcitol attenuates renal infiltration of inflammatory cells and inhibits tubular RANTES expression in obstructive nephropathy. This anti-inflammatory effect of paricalcitol seems to be mediated by its ability to induce the VDR-mediated sequestration of NF-κB signaling; therefore, in addition to preserving tubular epithelial integrity by blocking EMT, as previously reported,10 active vitamin D also exerts its beneficial activities in CKD by inhibiting renal inflammation through directly disrupting NF-κB signaling.
CONCISE METHODS

Animals
Male CD-1 mice that weighed approximately 18 to 22 g were purchased from Harlan Sprague-Dawley (Indianapolis, IN). UUO was performed using an approved protocol by the Institutional Animal Care and Use Committee at the University of Pittsburgh, as described previously.6,44 Sham-operated mice were used as normal controls. Paricalcitol (provided by Abbott laboratories, Abbott Park, IL) was administered by daily subcutaneous injection at the dosages of 0.1 and 0.3 μg/kg body wt, respectively. Mice that underwent UUO and received an injection of the same volume of vehicle (ethanol) were used as vehicle controls. Groups of mice (n = 5) were killed at 7 and 14 d after UUO, respectively, and the kidneys were removed for various analyses.

Cell Culture and Cytokine Treatment
Human proximal tubular epithelial cells (HKC, clone-8) were provided by Dr. L. Racusen (Johns Hopkins University, Baltimore, MD). Cell culture and cytokine treatments were carried out according to the procedures described previously.44 Briefly, HKC-8 cells were seeded in complete medium that contained 10% FBS at approximately 70% confluence. After an overnight incubation, cells were serum-starved in serum-free medium for 24 h before addition of cytokines. Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN). HKC-8 cells were incubated with various concentrations of paricalcitol in the absence or presence of TNF-α (2 ng/ml) for 24 h, unless otherwise indicated. For some experiments, such as assessing NF-κB activation, HKC-8 cells were pretreated with paricalcitol for 0.5 h, followed by incubation with TNF-α for various periods as indicated. For blocking NF-κB signaling, HKC-8 cells were pretreated with a cell-permeable inhibitor peptide NF-κB-SN50 (Calbiochem, La Jolla, CA; 20 μM) for 1 h and then incubated with TNF-α. In some experiments, HKC-8 cells were transfected with either p65 NF-κB expression vector (pNF-κB/p65; provided by Dr. J.A. Schmid, Medical University Vienna, Vienna, Austria) or empty pcDNA3 control vectors for 48 h and then treated with low concentration of TNF-α (0.2 ng/ml). Whole-cell lysates or conditioned media were prepared and then subjected to various analyses.

Western Blot Analysis
The preparation of whole-cell lysates and kidney tissue homogenates and Western blot analysis of protein expression were carried out by using routine procedures as described previously.10 The primary antibodies were obtained from following sources: Anti-RANTES (sc-1410) and anti-VDR (sc-1008; Santa Cruz Biotechnology, Santa Cruz, CA); anti-p65 NF-κB, anti–phospho-p65 NF-κB (Ser536), anti-IκBα, and anti–phospho-IκBα (Ser32; Cell Signaling Technology, Danvers, MA); anti–glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX).

Immunohistochemical and Immunofluorescence Staining
Immunohistochemical staining of kidney sections was performed by an established protocol.10 In brief, paraffin-embedded sections were stained with anti-CD3 (sc-20047; Santa Cruz Biotechnology), anti-F4/80 (14-4801-82; ebioscience, San Diego, CA), and anti-RANTES (500-P118; PeproTech, Rocky Hill, NJ) antibodies using the Vector M.O.M. immunodetection kit, according to the protocol specified by the manufacturer (Vector Laboratories, Burlingame, CA). Indirect immunofluorescence staining was carried out according to the procedures described previously.46 Briefly, cells or kidney cysotyes were incubated with the specific primary anti-p65 and anti-VDR antibodies, followed by staining with cyanine Cy3–conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Some slides were double stained with sytox green (Invitrogen, Carlsbad, CA) to visualize the nuclei. Slides were viewed with a Nikon Eclipse E600 microscope equipped with a digital camera (Melville, NY) or Leica TCS-CL confocal microscope (Microsystems, Heidelberg, Germany). Nonimmune normal control IgG was used to replace the primary antibody as negative control, and no staining occurred. CD3, F4/80, and RANTES staining were semiquantified by a computer-aided morphometric analysis (MetaMorph; Universal Imaging Co., Downingtown, PA). Briefly, a grid containing 117 (13 × 9) sampling points was superimposed on images of cortical high-power field (×400). The number of grid points overlying positive area (except tubular lumen and glomeruli) was counted and expressed as a percentage of all sampling points, as described previously.10 For each kidney, 10 randomly selected, nonoverlapping fields were analyzed in a blinded manner.

RT-PCR
For determination of RANTES and TNF-α mRNA expression, a semiquantitative RT-PCR was used. Total RNA was prepared from kidney tissue and cultured HKC-8 cells. After reverse transcription of the RNA, cDNA was used as a template in PCR reactions using gene-specific primer pairs. Approximately 20 to 25 cycles for amplification in the linear range were used. After quantification of band intensities by use of densitometry, the relative steady-state level of mRNA was calculated after normalization to β-actin. The sequences of the primer sets were as follows: RANTES (human), 5’-ACC CTG CTG CTG TGC CTA C (sense), and 5’-GTT TCA CGC CAT TCT CCT G (antisense); RANTES (mouse), 5’-GTG CCC ACD TCA AGG ATG AT (sense) and 5’-GGG AAG CGT ATA CAG GGT CA (antisense); TNF-α, 5’-CTG GCA CAG TGA CCT GGA CAG GAA CCT GGA AGA TCC TG-3’ (antisense); MCP-1, 5’-CCC ACT CAT CTG CTA CTA C-3’ (sense) and 5’-TTC TGT GGA TCA GCA CAG A-3’ (antisense). The sequences of β-actin primer set were described previously.46

Chemotaxis Assay
Cell chemotaxis assay was performed using a 24-well Transwell plate as described previously.29 Human monocyte cell line (THP-1; American Type Culture Collection, Manassas, VA) was maintained in RPMI-1640 medium supplemented with 10% FCS. Splenocytes, which contain lymphocytes, as well as macrophages, natural killer cells, and dendritic cells, were freshly prepared from mouse spleen using an established procedure as described previously.47 Briefly, spleens were removed from CD1 mice and a single-cell suspension in Hank’s buffer prepared using 40-μm cell strainers. Red blood cells
were lysed using standard lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA [pH 7.4]). Splenocytes were then washed and used in chemotaxis assays. Cultured THP-1 cells and mouse splenocytes (5 × 10⁶ in 100 μl) were added onto the upper chamber of the Transwell insert (5-μm polycarbonate filter; Corning, Corning, NY). HKC-8 cells–conditioned media (0.5 ml) were added in the lower chamber of the Transwell. For the neutralizing experiments, anti-VDR antibody (5 μg/ml; Santa Cruz Biotechnology) or control goat IgG was added to the conditioned media in the lower chamber. After incubation at 37°C for 4 h, the number of cells migrated to the lower chamber of the Transwell was counted. Data are expressed as percentage of the migrated cells in total number of input cells.

**ChIP Assay**

ChIP assay was performed to analyze in vivo interactions of NF-κB and its cognate cis-acting element in RANTES promoter. This assay was carried out essentially according to the protocols specified by the manufacturer (ChIP assay kit; Upstate, Charlottesville, VA). Briefly, HKC-8 cells after various treatments as indicated were cross-linked in vivo. HKC-8 cells after various treatments as indicated were cross-linked and the supernatant was diluted 10-fold. An aliquot of total diluted lysate was used for total genomic DNA as input DNA control. The ChIP samples were used as a template for PCR using the primer sets containing protease inhibitors. The chromatin solution was sonicated, and the supernatant was diluted 10-fold. An aliquot of total diluted lysate was used for total genomic DNA as input DNA control. The anti-p65 NF-κB antibody was added and incubated at 4°C overnight, followed by incubation with protein A–agarose for 1 h. The precipitates were washed, and chromatin complexes were eluted. After reversal of the cross-linking at 65°C for 4 h, the DNA was purified, and ChIP samples were used as a template for PCR using the primer sets for human RANTES promoter regions (from −208 to −10) containing NF-κB response element. The sequences of primers used for ChIP assay were as follows: Forward, 5′-TTGGTGCTTGGTCAAA- and reverse, 5′-CCCTTATAGGGCCAGTTGA-3′.

**Transient Transfection and Luciferase Reporter Assays**

The effect of paricalcitol on NF-κB–mediated gene transcription was assessed by using the PathDetect NF-κB-Luc cis-reporter system (Stratagene, La Jolla, CA), in which NF-κB response element was linked to the firefly luciferase gene. HKC-8 cells were co-transfected by using Lipofectamine 2000 reagent (Invitrogen) with pNF-κB-Luc plasmid (1 μg). An internal control reporter plasmid (0.05 μg) Renilla reniformis luciferase driven under thymidine kinase (TK) promoter (pRL-TK; Promega, Madison, WI) was also co-transfected for normalizing the transfection efficiency. The transfected cells were incubated in serum-free medium without or with TNF-α (2 ng/ml) in the absence or presence of paricalcitol (10⁻⁷ M) as indicated. Luciferase assay was performed using the Dual Luciferase Assay System kit according to the manufacturer’s protocols (Promega). Relative luciferase activity (arbitrary unit) was reported as fold induction over the controls after normalization for transfection efficiency.

**Immunoprecipitation**

Immunoprecipitation was carried out by using an established method. Briefly, HKC-8 cells were transfected with p65 NF-κB expression vector (pNF-κB/p65) by using lipofectamine 2000 reagent (Invitrogen) and then incubated with or without 10⁻⁷ M paricalcitol in the absence or presence of 2 ng/ml TNF-α for 1 h. In the experiments for assessing endogenous p65/VDR interaction, HKC-8 cells were directly treated with paricalcitol, TNF-α, or both and then subjected to co-immunoprecipitation. Cells were lysed on ice in 1 ml of non-denaturing lysis buffer that contained 1% Triton X-100, 0.01 M Tris-HCl (pH 8.0), 0.14 M NaCl, 0.025% NaN₃, 1% protease inhibitors cocktail, and 1% phosphatase inhibitors cocktails I and II (Sigma). After preclearing with normal IgG, cell lysates (0.5 mg of protein) were incubated overnight at 4°C with 4 μg of anti-VDR (Santa Cruz Biotechnology), followed by precipitation with 30 μl of protein A/G Plus-Agarose for 1 h at 4°C. The precipitated complexes were separated on SDS–polyacrylamide gels and immunoblotted with anti-p65 NF-κB antibody.

**Statistical Analysis**

Statistical analysis of the data was carried out using SigmaStat software (Jandel Scientific, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by Student-Newman-Keuls test. P < 0.05 was considered significant.

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**DISCLOSURES**

None.

**REFERENCES**


