Beyond proteinuria: VDR activation reduces renal inflammation in experimental diabetic nephropathy

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Diabetic nephropathy (DN) is the leading cause of end-stage renal disease in the Western world. Diabetes is associated with an initial increase in glomerular filtration rate (GFR). DN progression is characterized by the development of microalbuminuria in patients with normal renal function, followed by a progressive increase in urinary albumin excretion (UAE) and eventual decrease in GFR. Damage to highly differentiated glomerular podocytes is thought to be an early event in DN (35). In recent years, a direct association between inflammatory parameters and clinical markers of glomerular as well as tubulointerstitial damage has been demonstrated, suggesting that inflammation may be a pathogenic factor for the development and progression of DN (22). Hyperglycemia, angiotensin II, transforming growth factor (TGF)-β1, and proteinuria itself all play roles in stimulating renal inflammation and/or fibrosis and contribute to progression of DN.

Multiple factors have been implicated in the progression of DN, but inflammatory cytokines may be critical in the development of diabetic complications and nephropathy. Inflammatory markers such as IL-6, IL-18, monocyte chemoattractant protein-1 (MCP-1), and TNF-α are increased in the serum and urine of patients with diabetes and DN (22). Furthermore, urinary TNF-α is directly related to UAE, and strategies modulating TNF-α activity are associated with antialbuminuric effects. This increase in inflammatory parameters occurs early in the disease, correlates with the degree of albuminuria, and even precedes the increase in UAE (18, 37). Resident cells, such as mesangial, tubular epithelial, and podocytes, can produce cytokines and can express molecules that are part of the costimulatory pathway (30). Experimental animal models have recently provided evidence that inflammatory molecules, including TNF-α and MCP-1, may have a causative role in the development of DN (22).

The vitamin D receptor (VDR) is a ligand-activated transcription factor which, after activation, recruits cofactor molecules and binds to specific DNA binding sites to modify the expression of target genes (34). There is evidence that VDR is a modulator of glomerular injury. Calcitriol, the endogenous VDR ligand, decreases the glomerulosclerosis index and UAE in rats with subtotal nephrectomy (13, 31), and mice lacking the VDR are more susceptible to hyperglycemia-induced renal injury (39). The combination of a VDR activator and an ACE inhibitor protected mice from developing DN (41). In the clinical setting, chronic kidney disease (CKD) patients treated with paricalcitol (a selective VDR activator) showed a significant reduction of proteinuria (measured by a semiquantitative dipstick method) after 23 wk of therapy, independently of GFR, blood pressure, or ACE inhibition (1). However, in clinical trials the proteinuria response to VDR activators has not been uniform, raising the question of whether patients without reduced UAE might still be benefiting from VDR activator therapy. Thus, in Fishbane’s trial which included 50%
diabetic subjects, paricalcitol decreased UAE >10% in only 58% of patients (vs. 30% in the placebo group) (11). In the VITAL trial of DN patients, differences in the primary outcome (% change in urinary albumin/creatinine ratio in the combined paricalcitol group vs. placebo) just fell short of statistical significance (8). However, even in patients without reduced proteinuria, VDR activators might have beneficial effects. In this regard, another potential beneficial effect of VDR activation is an anti-inflammatory action. In animal models of primary glomerular disease, VDR activators reduces glomerular infiltration of inflammatory cells (23). Consistently, higher serum vitamin D levels in CKD patients are associated with decreased systemic inflammation (33). However, despite podocytes being key cells in proteinuria and DN, potential direct VDR modulation of high glucose-induced inflammation in cultured podocytes had not been studied.

The aim of our study was to investigate the regulation of renal inflammatory parameters in experimental DN by subantiproteinuric doses of VDR activators and the modulation by VDR activators of the inflammatory response elicited in cultured murine podocytes by high glucose.

METHODS

Animal study. Thirty male Sprague-Dawley rats were rendered diabetic by intravenous administration of streptozotocin (65 mg/kg) (7). Experimental methods used with laboratory animals comply with Law 5/1995 of June 21 by “Generalitat de Catalunya” of protection of animals used for experimentation and other scientific finalities and the Royal Decree 1201/2005 of October 10 on the protection of animals used for experimentation and other scientific finalities. Moreover, the present protocol was approved by the Ethic Animal Experimentation Committee of the University of Lleida.

Table 1. Weight and biochemical parameters in diabetic nephropathy animal model at the end of the 4-mo follow-up

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Diabetes</th>
<th>Diabetes + Calcitriol</th>
<th>Diabetes + Paricalcitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>393 ± 10</td>
<td>345 ± 10*</td>
<td>343 ± 7*</td>
<td>343 ± 13*</td>
</tr>
<tr>
<td>Serum calcium, mg/dl</td>
<td>10.5 ± 0.1</td>
<td>10.3 ± 0.2</td>
<td>10.6 ± 0.3</td>
<td>10.5 ± 0.2</td>
</tr>
<tr>
<td>Serum phosphate, mg/dl</td>
<td>7.4 ± 0.4</td>
<td>7.1 ± 0.5</td>
<td>6.3 ± 0.5</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.42 ± 0.01</td>
<td>0.43 ± 0.02</td>
<td>0.45 ± 0.04</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>Serum glucose, mg/dl</td>
<td>91.4 ± 2.08</td>
<td>490 ± 5*</td>
<td>472 ± 10*</td>
<td>505 ± 9*</td>
</tr>
<tr>
<td>Urine volume, ml/day</td>
<td>9.7 ± 1</td>
<td>152 ± 14*</td>
<td>148 ± 19*</td>
<td>132 ± 15*</td>
</tr>
<tr>
<td>Urinary albumin/creatinine ratio, mg/mg</td>
<td>0.073 ± 0.019</td>
<td>8.3 ± 1.7*</td>
<td>6.7 ± 1.6*</td>
<td>7.1 ± 1.2*</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>2.0 ± 0.14</td>
<td>2.0 ± 0.2</td>
<td>1.9 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. Sham.

Fig. 1. Vitamin D receptor (VDR) activation with paricalcitol or calcitriol downregulates total kidney expression of inflammatory mediator mRNA in experimental diabetic nephropathy (DN), quantified by qRT-PCR. Values are means ± SE. MCP-1, monocyte chemoattractant protein-1. *P < 0.05 vs. control. #P < 0.01 vs. diabetic vehicle. +P < 0.05 vs. diabetic vehicle.

However, despite podocytes being key cells in proteinuria and DN, potential direct VDR modulation of high glucose-induced inflammation in cultured podocytes had not been studied.

Thus the aim of our study was to investigate the regulation of renal inflammatory parameters in experimental DN by subantiproteinuric doses of VDR activators and the modulation by VDR activators of the inflammatory response elicited in cultured murine podocytes by high glucose.
After 48 h, the presence of hyperglycemia was confirmed and animals were randomized to different treatment groups: 10 animals were maintained as diabetic controls and received 1 IU/day insulin subcutaneously and intraperitoneal (ip) injections of vehicle; 10 animals received insulin and 250 ng/kg calcitriol three times a week ip; and 10 animals received insulin and 750 ng/kg paricalcitol three times a week ip for 4 mo. Ten vehicle-injected animals were used as nondiabetic controls. At the end of the treatment period, animals were placed in metabolic cages and a 24-h urine sample was collected. Then, rats were euthanized after isoflurane anesthesia and a terminal blood sample was collected.

Kidneys were perfused with sterile isotonic saline and divided into two parts. One part was fixed in 4% formaldehyde and embedded in paraffin, and the second part was kept in RNAlater and used to extract RNA.

Fig. 2. VDR activation with paricalcitol or calcitriol downregulates the glomerular expression of IL-6 protein in experimental DN. A: representative images by immunohistochemistry. Arrows point to stained glomerular cells with podocyte morphology. Original magnification ×200. B: quantification of glomerular IL-6 expression by immunohistochemistry. Values are means ± SE. *P < 0.0001 vs. control. #P < 0.0001 vs. diabetic vehicle. C: colocalization of IL-6 and WT-1 staining identifies podocytes as sources of glomerular IL-6 (arrows).
Analytic determinations. Serum and urine biochemistries were analyzed using a multichannel autoanalyzer (Roche/Hitachi Modular Analytics). The method used for calcium analysis was the ocresolphthalein complexone method, for phosphate the ammonium molybdate method, for creatinine the Jaffé reaction, and for microalbuminuria a turbidometric method. Blood glucose levels were tested with an Accu Check meter (Roche Diagnostics).

Cells and reagents. Conditionally immortalized mouse podocytes were a kind gift by Peter Mundel (19). Podocytes were propagated on type I collagen (Biochrom, Berlin, Germany) at 33°C in the presence of 10 U/ml mouse recombinant IFN-γ (permissive conditions, Immugenex, Los Angeles, CA) to enhance expression of a thermosensitive T antigen. Once cells had reached 70–80% confluence, differentiation and a quiescent phenotype were induced by culturing under nonper-

Fig. 3. VDR activation with paricalcitol or calcitriol downregulates the glomerular expression of MCP-1 protein in experimental DN. A: representative images by immunohistochemistry. Original magnification ×200. B: quantification of glomerular MCP-1 expression by immunohistochemistry. Values are means ± SE. *P < 0.0001 vs. control diabetic vehicle. #P < 0.0001 vs. diabetic vehicle. C: colocalization of MCP-1 and WT-1 staining identifies podocytes as sources of glomerular MCP-1 (arrows).
missive conditions at 37°C without INF-γ for >12 days, resulting in disappearance of the T antigen. Culture medium was DMEM containing 5 mM glucose (GIBCO, Grand Island, NY), 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured in serum-free media 24 h before the addition of the stimuli and throughout the experiment. Paricalcitol (Abbott) or calcitriol (10⁻¹⁰ M, Sigma, St. Louis, MO) was added 1 h before high glucose (DMEM containing 25 mM glucose) and murine TNF-α (30 ng/ml, Immugenex). This concentration of paricalcitol and calcitriol is close to the range of concentrations reached with therapeutic administration of paricalcitol or physiological levels of calcitriol in humans (8).

**ELISA.** Cells were stimulated with high glucose, paricalcitol, or calcitriol for 6 h. The concentration of murine MCP-1 in the cell culture supernatants was determined by ELISA (BD Pharmingen, San Diego, CA).

**RNA extraction and real-time RT-PCR.** Total RNA was extracted from kidneys and cells by the TRizol method (Invitrogen, Paisley, UK). One microgram RNA was reverse transcribed with a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) for 15 min at 25°C and 2 h at 37°C. Real-time PCR reactions were performed with the ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to the manufacturer’s protocol using the ΔΔCt method. Expression levels are given as ratios to GAPDH.

**Fig. 4. VDR activation with paricalcitol or calcitriol reduces the number of glomerular infiltrating CD43-positive leukocytes in experimental DN.** A: representative images by immunohistochemistry. Arrows point to glomerular CD43-positive cells. Original magnification ×200. B: quantification of glomerular CD43 staining by immunohistochemistry. Values are means ± SE. *P < 0.0001 vs. control. #P < 0.0001 vs. diabetic vehicle.
Predeveloped primer and probe assays (PDAR) were obtained for GAPDH, MCP-1, IL-18, IL-6, renin, fibronectin, and TNF-α from Applied Biosystems. The relative RNA amount was calculated by standard formulas (24).

**Immunohistochemistry.** Immunohistochemistry was carried out in paraffin-embedded tissue sections 5 μm thick (27). Primary antibodies were rabbit polyclonal anti-RelA, goat polyclonal anti-MCP-1, anti-IL6 or anti-WT1 (1:75, Santa Cruz Biotechnology) and mouse monoclonal anti-CD43 (1/100, Pharmingen). In brief, for costaining with anti-MCP-1 or anti-IL-6 antibodies and anti-WT1, slides were first incubated with anti-IL-6 or anti-MCP-1 and then with the respective biotin-conjugated secondary antibody. After developing of the reaction with ABCComplex, sections were incubated with anti-WT1 and then with a FITC secondary antibody. Sections were counterstained with Carazzi’s hematoxylin. Negative controls included incubation with a nonspecific immunoglobulin of the same isotype as the primary antibody. MCP-1 and IL-6 staining was evaluated by a quantitative scoring system using Image-Pro Plus software (Media Cybernetics, Bethesda, MD) in 20 randomly selected glomeruli/kidney. Anti-CD43 stains leukocytes (12). The total number of anti-CD43-stained cells was counted in 20 randomly glomeruli using Image-Pro Plus software. Samples from each animal were examined in a blinded manner.

Apoptotic cells were stained using terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL; In Situ Cell Death Detection Kit; Promega) according to the manufacturer’s instructions and as previously described (27).

Glomerular fibrosis was stained with Sirius red. Tissue sections were deparaffinized and stained for 30 min in direct Red 80 (Sigma) dissolved in Picro-sirius acid and then dehydrated.

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**Fig. 5. VDR activator effects on kidney NF-κB activation and renin expression in experimental DN.**

**A:** VDR activation with paricalcitol or calcitriol reduces the number of glomerular cells with nuclear p65/RelA staining, an indication of NF-κB activation. Representative image is shown of NF-κB p65/RelA immunohistochemistry in diabetic rats where nuclear RelA staining is observed in a glomerulus from a control diabetic rat. Original magnification ×200. **B:** VDR activation with paricalcitol or calcitriol tended to decrease kidney renin mRNA expression quantified by qRT-PCR. Values are means ± SE.
fibrotic area, expressed as a percentage of glomerular area, was assessed by ImageProPlus software (Media Cybernetics), analyzing 20 glomeruli/kidney (2, 25).

Statistics. Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as means ± SE. Significance at the P < 0.05 level was assessed by Student’s t-test for two groups of data and ANOVA for three of more groups, followed by a Scheffé post hoc test.

RESULTS

Subantiproteinuric doses of calcitriol or paricalcitol decrease glomerular inflammation in experimental DN. Clinical trials of paricalcitol in human DN have shown that paricalcitol has a modest and variable effect on proteinuria at doses that do not promote hypercalcemia or hyperphosphatemia (8). For our studies, we chose doses of paricalcitol and calcitriol that did not influence serum levels of calcium or phosphate (Table 1). In addition, VDR activators did not modify glycemia or creatinine clearance. Finally, there were no significant differences in UAE between animals receiving VDR activators and diabetic controls. Thus we have developed an animal model that allows exploring renal effects of VDR activators in DN in the absence of significant changes in proteinurin. DN was associated with increased whole kidney mRNA expression of several cytokines. Despite the nonsignificant effect on UAE, both calcitriol and paricalcitol reduced whole kidney expression of inflammatory cytokine mRNAs in DN (Fig. 1).

At the protein level, increased expression of IL-6 and MCP-1 protein was noted in DN glomeruli by immunohistochemistry (Figs. 2 and 3). Podocytes were among glomerular cells expressing inflammatory cytokines as evidenced by costaining with the podocyte marker WT-1 (Figs. 2C and 3C). Despite the nonsignificant effect on UAE, calcitriol and paricalcitol reduced glomerular IL-6 and MCP-1 protein expression (Figs. 2 and 3).

DN was associated with increased glomerular infiltration by CD43-positive leukocytes (Fig. 4). Consistent with their effect on chemotactic factors, calcitriol and paricalcitol also reduced glomerular infiltration by CD43-positive leukocytes in DN (Fig. 4). VDR activation may decrease the activation of NF-κB (4) and may also decrease renin expression (14), and both effects may result in decreased inflammation. Decreased renal glomerular inflammation was associated with decreased nuclear RelA, a key component of the canonical NF-κB activation pathway that promotes chemokine gene transcription (28, 29), in glomerular cells in rats treated with either calcitriol or paricalcitol (Fig. 5A). In addition, a trend toward decreased kidney renin mRNA expression was observed in VDR activator-treated DN rats (Fig. 5B).

Beyond inhibiting features of glomerular inflammation, some key histological parameters of glomerular injury were improved by VDR activator in diabetic rats. Thus the presence of glomerular apoptotic cells, as assessed by TUNEL staining, was only elevated in nontreated DN rats (Fig. 6). An increased extracellular matrix deposition is a key feature of DN. Indeed, control DN rats displayed increased glomerular extracellular matrix deposition as assessed by Sirius red staining (Fig. 7). This was improved by both VDR activators (Fig. 7).

Calcitriol and paricalcitol reduce expression of inflammatory mediators fibronectin and renin in cultured podocytes. A high glucose concentration is the main initiator of DN. A high glucose milieu increased the expression of IL-6 and MCP-1
mRNA in cultured podocytes (Fig. 8A). Podocytes are key cells in DN and in the genesis of proteinuria, and they express the VDR in culture and in vivo (15, 26). Despite this, the effect of VDR activation on the podocyte response to a diabetic milieu had not been studied at the cell culture level. Both paricalcitol and calcitriol at concentrations relevant for the clinic prevented the increase in IL-6 and MCP-1 mRNA expression in podocytes exposed to high glucose. Furthermore, both paricalcitol and calcitriol prevented the increase in MCP-1 protein released to the supernatant by podocytes exposed to high glucose (Fig. 8B). Thus both VDR activators, at the same concentration, had a protective effect on podocytes, preventing the inflammatory response to metabolic (high glucose) stress.

VDR activators also prevented the increase in fibronectin mRNA expression induced by high glucose (Fig. 8C), suggesting that they may also exert a direct effect on extracellular matrix production by podocytes as has been reported in the context of Fabry disease (26). In addition, VDR activators also prevented the increase in renin expression induced by high glucose in cultured podocytes (Fig. 8D).

DISCUSSION

The main observation of this study is that both calcitriol and paricalcitol, at doses that do not significantly modify calcemia, phosphatemia, or urinary albumin excretion, have an anti-
inflammatory action on DN glomeruli and in cultured podocytes exposed to high glucose.

Both calcitriol and paricalcitol have been previously studied in the context of experimental kidney disease. In 1993 calcitriol was reported to lower proteinuria in Heymann’s active nephritis, at the expense of promoting hypercalcemia (3). More recently, both paricalcitol and calcitriol were shown to lower proteinuria in the subtotal nephrectomy model of rat proteinuric kidney disease (17). The involvement of VDR activation in DN has also been demonstrated. Diabetic VDR knockout mice developed more severe albuminuria and glomerulosclerosis with podocyte effacement than their wild-type counterparts (39). Paricalcitol reduced albuminuria when associated with losartan in mice with streptozotocin-induced diabetes, but the effect of paricalcitol alone was moderate, not indicated to be statistically significant, and kidney inflammation or calcitriol was not explored (41). Paricalcitol inhibited the induction of kidney MCP-1, glomerulosclerosis, and glomerular macrophage infiltration in streptozotocin-diabetic mice (40). However, proteinuria was not evaluated and the in vivo effects of calcitriol were not assessed. More recently, paricalcitol reduced albuminuria and kidney inflammation in db/db diabetic mice (6). Thus none of the previous studies had addressed whether VDR activation by either paricalcitol or calcitriol might decrease renal inflammation even when proteinuria was not significantly reduced. In the present study, we used doses of calcitriol or paricalcitol that did not significantly decrease UAE in diabetic rats. The present animal model allowed us to study additional effects of VDR activation, beyond proteinuria. The anti-inflammatory action observed beyond proteinuria may be clinically significant for two reasons. First, in human DN, the urinary presence of inflammatory cytokines differentiated microalbuminuric patients with progressive vs. nonprogressive renal injury, suggesting both the importance of renal inflammation for kidney injury and the clinical dissociation between UAE and inflammation in some patients (36). This is in line with recent clinical trial results suggesting dissociation between an antialbuminuric effect and renal outcomes, at least in certain patients (16). Second, in clinical trials >40% of patients did not benefit from paricalcitol regarding proteinuria (11). Indeed, high paricalcitol doses (those more likely to cause adverse effects on serum calcium and phosphate) were needed to decrease albuminuria in human DN. Recently reported results from the VITAL randomized controlled trial of paricalcitol vs. placebo in proteinuric DN showed an antiproteinuric effect that was statistically significant only for the higher paricalcitol dose used (8).

Both bloodborne cells (mainly monocytes and macrophages) and intrinsic renal cells synthesize inflammatory cytokines in a diabetic context (10). The renal cortical mRNA expression of IL-6 and TNF is increased in rat DN (20, 21). A high glucose milieu, albuminuria, and inflammatory factors released in response to albuminuria or high glucose may all contribute to further inflammation by promoting the secretion of chemokines (22). Glomerular cells, including podocytes, have VDRs (15, 26). Calcitriol inhibited high glucose-induced fibronectin production in cultured mesangial cells and increased nephrin expression in cultured podocytes (39). Calcitriol also suppressed high glucose-induced activation of the renin-angiotensin system (RAS) and TGF-β in mesangial and juxtaglomerular cells (39). Furthermore, in cultured mesangial cells, calcitriol stabilized IκBα, leading to an inhibition of p65 translocation to the nucleus and subsequent reduction of NF-κB DNA binding (40). In tubular cells, paricalcitol prevented RelA binding to DNA, but not nuclear translocation (32). However, a direct anti-inflammatory action of VDR activation on podocytes in the context of a diabetic milieu had not been previously observed. We now show that VDR activation protects cultured podocytes from the direct proinflammatory and profibrotic effects of hyperglycemia. In the present model, an anti-inflammatory and antifibrotic effect of VDR activation was observed in vivo despite the lack of significant...
reduction in albuminuria, and, based on cell culture results, we hypothesize that it can be ascribed to a lower inflammatory response of podocytes to high glucose or inflammatory cytokines. Protection from high glucose-, cytokine-, or even albuminuria-induced inflammation might result in inhibition of inflammation-induced progression of DN by VDR activators, although this should be tested in clinical trials.

NF-κB (4) and renin (14) are key targets of VDR activators that may underlie the present observations. High glucose activates a local RAS in podocytes by promoting transcription of the renin gene and increasing angiotensin II production in an aliskiren-sensitive fashion (9). VDR activation may suppress the RAS, renin and angiotensinogen being the main targets. The activated VDR negatively regulates renin biosynthesis by directly transrepressing renin gene transcription in different cell types, including mesangial cells (38). Furthermore, VDR activation may decrease the expression of other RAS pathway genes through inhibition of NF-κB activation, as recently shown for angiotensinogen in podocytes (5). As mentioned above, VDR activation may inhibit NF-κB activation through different mechanisms. An effect of VDR activation on NF-κB activation is supported by the negative regulatory effect of VDR activators on the expression of the canonical NF-κB transcriptional targets MCP-1 and RANTES (29) in cultured podocytes and the reduced evidence for p65/RelA-containing complexes migrating to the nuclei of glomerular cells in vivo. Furthermore, expression of renin in podocytes was also down-regulated by VDR activation, pointing to a possible dual effect reducing inflammation by decreasing both NF-κB activation and renin expression.

A shortcoming of the study is that this model did not allow the evaluation of the effects of VDR activators on preservation of renal function, since renal function was not decreased in control DN rats. Our design, using daily administration of insulin to keep glucose levels under control, is closer to the clinical settings, but does not allow the investigation of renal function deterioration beyond proteinuria. However, it did provide a series of insights into actions on molecular mechanisms that are known to contribute to renal function deterioration.

In summary, our study provides insights into additional targets (i.e., decreased inflammation) that might be expected from VDR activator treatment even in the absence of decreased albuminuria. These insights might help design new primary outcome measures in future trials involving assessment of renal inflammation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS