Vitamin D receptor activation, left ventricular hypertrophy and myocardial fibrosis

Sara Panizo¹, Sara Barrio-Vázquez¹, Manuel Naves-Díaz¹, Natalia Carrillo-López¹, Isabel Rodríguez¹, Amalia Fernández-Vázquez², Jose M. Valdivielso³, Ravi Thadhani⁴ and Jorge B. Cannata-Andía¹

¹Servicio de Metabolismo Óseo y Mineral. Instituto Reina Sofía de Investigación. REDinREN del ISCIII. Universidad de Oviedo. Hospital Universitario Central de Asturias, Oviedo, España, ²Servicio de Anatomía Patológica Centro Médico de Asturias, Oviedo, España, ³Laboratorio de Nefrología Experimental, IRB Lleida, Hospital Universitario Arnau de Vilanova, Lleida, España and ⁴Division of Nephrology, Massachusetts General Hospital, Boston, MA, USA

Correspondence and offprint requests to: Jorge B. Cannata-Andía; E-mail: cannata@hca.es

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ABSTRACT

Background. Left ventricular hypertrophy (LVH), a common complication in chronic kidney disease (CKD), is associated with high cardiovascular mortality. The aim of this experimental study was to analyze the effect of different vitamin D receptor activators (VDRAs) on both LVH and myocardial fibrosis in chronic renal failure (CRF).

Methods. Male Wistar rats with CRF, carried out by 7/8 nephrectomy, were treated intraperitoneally with equivalent doses of VDRAs (calcitriol, paricalcitol and alfacalcidol, 5 days per week) during 4 weeks. A placebo group (CRF + vehicle) and a Sham group with normal renal function served as controls. Biochemical, morphological, functional and molecular parameters associated with LVH were evaluated, as well as cardiomocyte size, LV wall and septum thickness and heart–body weight ratio similar to those observed in the Sham group. At molecular levels, all VDRAs treatment prevented LVH, with values of cardiomyocyte size, LV wall and septum thickness and heart–body weight ratio similar to those observed in the Sham group. Biochemical, morphological, functional and molecular parameters associated with LVH were evaluated, as well as cardiac fibrosis, collagen I, transforming growth factor β1 (TGFβ1) and matrix metalloproteinase-1 (MMP1) expression.

Results. All VDRAs treatment prevented LVH, with values of cardiomyocyte size, LV wall and septum thickness and heart–body weight ratio similar to those observed in the Sham group. At molecular levels, all VDRAs attenuated atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) expression compared with CRF + vehicle. The phosphorylation of ERK1/2, a signal for activating growth, was stimulated in the CRF + vehicle group; VDRAs use prevented this activation. Paricalcitol was the only VDRA used that maintained in the normal range all parameters associated with myocardial fibrosis (total collagen, collagen I, TGFβ1 and MMP1).

Conclusions. Our findings demonstrated that the three VDRAs used induced similar changes in bone metabolic parameters and LVH. In addition, paricalcitol was the only VDRA which showed a relevant beneficial effect in the reduction of myocardial fibrosis, a key factor in the myocardial dysfunction in CKD patients.

INTRODUCTION

Cardiovascular disease is the main cause of death in patients with chronic kidney disease (CKD) [1]. Abnormalities of left ventricular structure and function are frequent among CKD patients; >70% of them have left ventricular hypertrophy (LVH) by the time dialysis is initiated [2]. Although LVH is a well-recognized cardiovascular risk factor in the general population, CKD patients develop early LVH in the course of renal disease [3, 4]. For the same level of blood pressure, CKD
patients have a greater left ventricular mass than non-CKD patients, suggesting that other specific CKD-associated factors may contribute to its development [5, 6].

LVH is primarily a remodeling process against pressure or volume overload that involves an increase of cardiac work [7]. In CKD patients, cardiac remodeling is clinically manifested by changes in cardiac size, shape and function in response to cardiac injury or increased cardiac load [8]. Cardiomyocytes and fibroblasts are commonly involved in the remodeling process. Cardiomyocytes increase their size, meanwhile fibroblasts increase collagen synthesis that leads to fibrosis. These changes progressively lead to apoptosis or necrosis of cardiomyocytes which are replaced by fibroblasts and extracellular collagen [9].

In CKD-MBDs (CKD-Mineral Bone Disorders), it has been current practice to use vitamin D receptor activators (VDRAs) like calcitriol, paricalcitol and alfalcaldol to reduce parathyroid hormone (PTH) levels [10]. In addition, its use has been associated with reductions in cardiovascular events [11–13], left atrial volume [14], vascular calcification [15], and cardiovascular and overall mortality [16–18]. Experimental results suggested that VDRAs may reduce LVH likely through a direct effect on the myocardium [19]. In agreement, VDR knockout mice exhibited cardiac hypertrophy [20] and cardiomyocytes in culture, showing that calcitriol effectively reduces cardiomyocyte size and proliferation [21, 22].

To further explore this topic, we investigated the effects of different VDRAs on the cardiomyocytic and fibrotic changes observed in the LVH secondary to chronic renal failure (CRF).

MATERIALS AND METHODS

Experimental animals

CRF was carried out in male Wistar rats (350–400 g) by 7/8 nephrectomy as previously detailed [23]. The animals were fed a standard rodent chow containing 0.6% calcium, 0.6% phosphorus, 1000 IU vitamin D3/kg (Panlab, Barcelona, Spain), and were housed in wire cages. Water and food administration was ad libitum. One week after surgery, a total of 29 animals were divided into four groups. Group 1 (CRF + calcitriol, n = 8) received calcitriol (10 ng/kg body weight/day); Group 2 (CRF + paricalcitol, n = 7) received paricalcitol (30 ng/kg body weight/day); Group 3 (CRF + alfalcaldol, n = 7) received alfacaldol (20 ng/kg body weight/day) and Group 4, (placebo group, CRF + vehicle, n = 7) received vehicle (corn oil 0.8 mL/kg body weight/day). Calcitriol [23], paricalcitol and alfacaldol doses were established as a function of the ratios currently used in clinical practice and in our laboratory to achieve the same degree of PTH suppression (unpublished observations). Pilot preliminary studies carried out in normal and CRF rats showed that in 4 weeks, significant differences in LVH were observed. All compounds were dissolved in ethanol and diluted with corn oil to a final volume of 0.8 mL corn oil/kg body weight/injection and administered intraperitoneally 5 days/week over a period of 4 weeks. A group of rats (Sham + vehicle, n = 7) with normal renal function was also included in the study as the reference group.

After 4 weeks of treatment, the rats were introduced in metabolic cages, and 24-h urine was collected; afterwards, they were sacrificed using CO₂ anesthesia. At sacrifice, serum samples were drawn for analyses, the aortas were collected, the hearts were removed, washed twice with saline solution, blotted dry and weighed. The left ventricle (LV) was frozen at −80°C until analysis; it was then divided into three pieces: two of them were used for RNA and protein extraction, and the third fragment was fixed, embedded in paraffin and sliced for the histological studies.

The protocol was approved by the Laboratory Animal Ethics Committee of Oviedo University.

Blood pressure measurement

During the fourth week, systolic (SBP) and diastolic (DBP) blood pressures were recorded using an automated, non-invasive tail-cuff method (LSI Letica, Barcelona, Spain). To minimize the procedure-induced stress, the animals were accustomed to the instrument for four consecutive days prior to the definitive measurements, which consisted of a set of a minimum of three repetitive measurements in each animal. Only stable, reproducible values were considered.

Biochemical markers

Serum creatinine, calcium, and phosphorus and creatinine clearance were measured using a multichannel auto analyzer (Hitachi 717; Boehringer Mannheim, Berlin, Germany). Serum iPTH was measured by IRMA (Rat PTH kit Immutomics, San Juan Capistrano, CA), and serum intact FGF23 was assessed using a Sandwich ELISA kit (Kainos Laboratories, Tokyo, Japan), following the manufacturer’s protocols.

Total calcium content in the aorta

The aortas were washed with a saline solution and homogenized in HCl (0.6 N) for 24-h to extract calcium. After centrifugation, the pellet was transferred to a lysis buffer (125 mM Tris, 2% SDS, pH 6.8), for protein extraction. The calcium content was determined in the HCl solution by the O-cresolphthalein complexone method [24] (Sigma, Saint Louis, MO) and the total protein determined by the Lowry method (Bio Rad, Hercules, CA).

Morphological and histological changes

Cardiomyocyte diameter and LV wall and septum thickness were measured using an optical microscope (model DMRXA2, Leica Microsystems, Wetzlar, Germany) coupled to a digital video camera (model Dc-100, Leica Microsystems) in deparaffined sections of the LV and stained with hematoxylin–eosin. Captured images were analyzed using an image analysis system (Leica Q500IW, Leica Microsystems) and specific software (Leica QWIN standard version 2.3, Leica Microsystems). The mean myocyte diameter was determined by measurement of transnuclear widths of random, longitudinally oriented 20 myocytes. The LV wall and septum thickness were measured using a pre-design software which pooled and analyzed a set of at least 50 blinded radius measurements from the center of the LV to its outer edge.
The myocardial total collagen content was determined by using Masson’s trichrome and Sirius red staining [19]. The stained collagen area was measured using a semiautomatic image analysis software (Leica QWIN standard version 2.3, Leica Microsystems). The measurements were blinded, and the results were expressed as percentages of the total myocardial area. The collagen fiber–muscular tissue ratio was then calculated.

**Immunohistochemistry**

Collagen I localization was assessed by immunohistochemistry in 5 μm sections incubated overnight at 4°C in 1:2000 anti-collagen (Merck Millipore, Darmstadt, Germany), and then washed and incubated with a biotinylated secondary antibody following the manufacturer’s instructions (Dako REAL EnVision, Denmark). Along with Masson’s trichrome staining, semiautomatic image analysis software (Leica QWIN standard version 2.3, Leica Microsystems) was used. A negative control without primary antibody was used to set the level of the lowest detectable staining intensity. Briefly, the image of each heart was converted to grayscale; then, using the optical density function of the software, pixels that fell within a designed threshold were counted, thus obtaining a mean value of grey color density. Collagen I stained was expressed as the average optical density.

**RNA extraction, cDNA synthesis and quantitative RT-PCR**

Total RNA was extracted from the LV by the TRizol method (Sigma, Saint Louis, MO). Total RNA concentration and purity were quantified by UV-Vis spectrophotometry (NanoDrop Technologies, Wilmington, DE) measuring its absorbance at 260 and 280 nm. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) following the manufacturer’s instructions. Gene expression was measured in LV by qRT–PCR using an ABI Prism 7000 Sequence Detection System (Applied Biosystems.). TaqMan Real-time PCR amplification was performed with a gene-specific primer (Gene Expression Assays from Applied Biosystems) for a brain natriuretic peptide (BNP), atrial natriuretic peptide (ANP), renin and vitamin D receptor (VDR). Rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a housekeeping gene. The relative quantitative evaluation of the target gene was performed by comparing threshold cycles using the ΔΔCT method [25].

**Western blot analysis**

Western blot analysis in LV tissue was performed as previously described [26]. After blotting, the membrane was incubated overnight with an anti-pERK 1/2 antibody (dilution 1:1000; Cell Signaling, Danvers, MA), anti-ERK 1/2 antibody (dilution 1:1000; Cell Signaling), anti-Matrix metalloproteinase-1 (MMP1) antibody (dilution 1:1000; Acris antibodies, San Diego, CA), anti-transforming growth factor β1 (TGFβ1) antibody (dilution 1:1000; Santa Cruz Biotechnology) and anti-GAPDH (dilution 1:30 000 Santa Cruz biotechnologies). Secondary antibody binding was detected using the ECL western blotting detection kit (Amersham Biosciences, Buckinghamshire, England) and the ChemiDoc gel imaging system model XRS + (Bio-Rad, Hercules, CA).

**Statistical analysis**

Statistical comparisons between groups were carried out by using one-way analysis of variance and Student’s *t*-test. Bivariate correlation analyses were performed. The results were expressed as mean ± SD. Differences were considered significant when *P* < 0.05. All statistical analyses were performed using SPSS 17.0 for Windows (SPSS, Chicago, IL).

**RESULTS**

**Effect of uremia and VDRA use on biochemical markers, calcium content of the aorta, SBP and DBP**

Table 1 shows values of the above-mentioned parameters in the different groups after 4 weeks of treatment. No significant differences in the degree of renal failure were observed among the four CRF groups. As expected, VDRA treatment significantly increased serum FGF23 and calcium levels while decreasing serum PTH levels in all groups. In the alfacalcidol group, a significant increase in serum phosphorus was observed. No significant differences in the calcium content of the aorta, SBP and DBP were observed between groups (Table 1).

**Effect of uremia and VDRA use on LVH**

LVH was evaluated by measuring morphological and molecular parameters (Table 2). Morphological parameters: cardiomyocyte diameter showed a significant increase in the CRF + vehicle group, and the use of VDRAs showed a non-significant trend toward its decrease. The LV wall and septum thickness (the latter not shown) were significantly greater in the CRF + vehicle group compared with the Sham group. All VDRAs prevented the increase in LV wall and septum thickness, showing significant differences with respect to the CRF + vehicle group. The heart–body weight ratio was significantly higher in the CRF + vehicle group when compared with the Sham group. A slight but non-significant decrease in the heart–body weight ratio was observed with the use of VDRAs (Table 2).

Molecular parameters: BNP, ANP and renin heart gene expression significantly increased in the CRF + vehicle group. They decreased with the use of VDRAs, but only BNP and renin expression showed statistically significant differences. Regarding VDR expression, a slight but not significant decrease was observed in the CRF + vehicle group. The use of calcitriol and paricalcitol almost doubled VDR expression; however, the changes were not statistically significant (Table 2).

The (pERK 1/2)/(ERK 1/2) ratio showed a significant increase in the CRF + vehicle group; the use of VDRAs significantly reduced the (pERK 1/2)/(ERK 1/2) ratio (Figure 1). The relationship between the (pERK 1/2)/(ERK 1/2) ratio and the LV wall thickness and the heart–body weight ratio with the use of placebo or VDRAs is shown in Figures 2A and 2B. A positive and significant correlation between the (pERK 1/2)/(ERK 1/2) ratio and the LV wall thickness (*r* = 0.72) and heart–body weight ratio (*r* = 0.86) was observed in the CRF + vehicle rats (Figure 2A). The relationship disappeared with the use of VDRAs (Figure 2B).
Effect of uremia and VDRAs use on fibrosis parameters

Morphological parameters: CRF + vehicle rats showed a significant increase in collagen fibers stained by Masson’s tri-chrome (Figure 3A), and similar results were observed using Sirius red staining (data not shown). The use of VDRAs decreased the percentage of collagen fibers but it only reached statistical significance in the paricalcitol-treated rats, in which the percentage of collagen fibers observed after 4 weeks of treatment was similar to that observed in the Sham + vehicle group (Figure 3A). The proportion of collagen–muscular

Table 1. Biochemical parameters, calcium content of the aorta and blood pressure

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>CRF + vehicle</th>
<th>CRF + calcitriol</th>
<th>CRF + paricalcitol</th>
<th>CRF + alfacalcidol</th>
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</thead>
<tbody>
<tr>
<td>Creatinine clearance (mL/min)</td>
<td>2.01 ± 0.32</td>
<td>0.71 ± 0.30\textsuperscript{a}</td>
<td>0.57 ± 0.10\textsuperscript{a}</td>
<td>0.64 ± 0.22\textsuperscript{a}</td>
<td>0.51 ± 0.19\textsuperscript{a}</td>
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<tr>
<td>FGF23 (pg/mL)</td>
<td>368.14 ± 55.54</td>
<td>792.00 ± 214.44\textsuperscript{a}</td>
<td>1256.25 ± 14.25\textsuperscript{a,b}</td>
<td>1197.00 ± 25.14\textsuperscript{a,b}</td>
<td>1220.43 ± 32.99\textsuperscript{a,b}</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>10.93 ± 0.30</td>
<td>11.79 ± 0.49</td>
<td>13.53 ± 2.04\textsuperscript{a}</td>
<td>14.17 ± 0.87\textsuperscript{a}</td>
<td>14.04 ± 0.64\textsuperscript{a}</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>5.00 ± 2.64</td>
<td>20.66 ± 33.20\textsuperscript{a}</td>
<td>2.94 ± 6.08\textsuperscript{b}</td>
<td>0.83 ± 0.57\textsuperscript{b}</td>
<td>0.50 ± 0.34\textsuperscript{b}</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>6.90 ± 1.05</td>
<td>5.61 ± 2.16</td>
<td>6.56 ± 1.36</td>
<td>7.05 ± 0.89</td>
<td>8.19 ± 1.39\textsuperscript{b}</td>
</tr>
<tr>
<td>Calcium content of aorta (µg calcium/mg protein)</td>
<td>128.95 ± 70.93</td>
<td>183.38 ± 71.26</td>
<td>211.36 ± 155.54</td>
<td>173.18 ± 85.52</td>
<td>167.13 ± 92.56</td>
</tr>
<tr>
<td>SBP (mm/Hg)</td>
<td>131.71 ± 7.63</td>
<td>143.75 ± 12.34</td>
<td>146.31 ± 6.77</td>
<td>136.29 ± 2.94</td>
<td>135.76 ± 6.43</td>
</tr>
<tr>
<td>DBP (mm/Hg)</td>
<td>93.50 ± 10.59</td>
<td>106.84 ± 19.01</td>
<td>103.97 ± 11.67</td>
<td>95.16 ± 23.5</td>
<td>100.11 ± 17.22</td>
</tr>
</tbody>
</table>

Data represent mean ± standard deviation.
SBP, systolic blood pressure; DBP, diastolic blood pressure.
\textsuperscript{a}P < 0.005 versus Sham.
\textsuperscript{b}P < 0.005 versus CRF + vehicle.

Table 2. LVH morphological and molecular parameters

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>CRF + vehicle</th>
<th>CRF + calcitriol</th>
<th>CRF + paricalcitol</th>
<th>CRF + alfacalcidol</th>
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<tbody>
<tr>
<td>Morphological parameters</td>
<td></td>
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<tr>
<td>Cardiomyocytes diameter (µm)</td>
<td>9.52 ± 1.07</td>
<td>10.67 ± 1.16\textsuperscript{a}</td>
<td>9.74 ± 0.50</td>
<td>9.78 ± 0.048</td>
<td>9.87 ± 0.37</td>
</tr>
<tr>
<td>LV wall thickness (mm)</td>
<td>1.98 ± 0.23</td>
<td>2.29 ± 0.28\textsuperscript{a}</td>
<td>1.97 ± 0.20\textsuperscript{b}</td>
<td>1.88 ± 0.18\textsuperscript{b}</td>
<td>2.03 ± 0.26\textsuperscript{b}</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>2.56 ± 0.14</td>
<td>2.90 ± 0.38\textsuperscript{a}</td>
<td>2.76 ± 0.46</td>
<td>2.72 ± 0.39</td>
<td>2.77 ± 0.28</td>
</tr>
<tr>
<td>Molecular parameters</td>
<td></td>
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<tr>
<td>mRNA BNP/GAPDH (RU)</td>
<td>1.00 ± 0.95</td>
<td>5.64 ± 2.45\textsuperscript{a}</td>
<td>1.79 ± 1.25\textsuperscript{b}</td>
<td>0.94 ± 0.75\textsuperscript{b}</td>
<td>0.65 ± 0.30\textsuperscript{b}</td>
</tr>
<tr>
<td>mRNA ANP/GAPDH (RU)</td>
<td>1.00 ± 1.12</td>
<td>14.27 ± 13.70\textsuperscript{a}</td>
<td>4.43 ± 2.60</td>
<td>3.11 ± 2.90</td>
<td>3.22 ± 3.82</td>
</tr>
<tr>
<td>mRNA renin/GAPDH (RU)</td>
<td>1.00 ± 0.69</td>
<td>6.06 ± 2.87\textsuperscript{a}</td>
<td>1.61 ± 1.51\textsuperscript{b}</td>
<td>0.74 ± 0.49\textsuperscript{b}</td>
<td>0.60 ± 0.46\textsuperscript{b}</td>
</tr>
<tr>
<td>mRNA VDR/GAPDH (RU)</td>
<td>1.00 ± 0.69</td>
<td>0.81 ± 0.39</td>
<td>1.50 ± 0.57</td>
<td>1.46 ± 1.00</td>
<td>1.18 ± 0.42</td>
</tr>
</tbody>
</table>

Data represent mean ± standard deviation.
\textsuperscript{a}P < 0.005 versus Sham.
\textsuperscript{b}P < 0.005 versus CRF + vehicle.
tissues reached 2.9% in the Sham group, and it increased to 11.7% in the CRF + vehicle group. After 4 weeks of VDRA use, it decreased in all groups achieving statistical significance only in the paricalcitol group.

Molecular parameters: similar results in the ratio between collagen and muscular tissues were observed using immunohistochemistry. The calcitriol and alfacalcidol groups showed a slight decrease in the ratio, whereas a significant reduction was observed in the paricalcitol group (Figure 3B).

Figure 4 demonstrates that the TGFβ1 values followed the same pattern observed in collagen quantification. CRF + vehicle rats showed a significant increase of TGFβ1, and the use of VDRAs reduced the increases of TGFβ1 achieving statistical significance only in the CRF + paricalcitol-treated group (Figure 4A). The results of MMP1, involved in collagen degradation, almost mirrored those of TGFβ1 and collagen, and the paricalcitol group showed the highest level of MMP1 expression (Figure 4B).

**DISCUSSION**

This is the first experimental *in vivo* study comparing the effects of three VDRAs on LVH and myocardial structure by analyzing the proportion of fibrotic and muscular tissues. All VDRAs showed a clear trend toward a reduction of LVH development, as evaluated through different parameters: cardiomyocyte diameter, LV wall and septum thickness, and heart–body weight ratio (Table 2). Paricalcitol was the most effective VDRA in the prevention of cardiac fibrosis, which is considered a critical component of the myocardial dysfunction in CKD patients (Figure 3).

This study confirms previous findings, proving that the three VDRAs used were efficient in suppressing PTH secretion in rats with CRF. In addition, the three VDRAs significantly increased serum calcium levels, like in other experimental studies we did not observe less hypercalcemic and...
hyperphosphatemic effects of paricalcitol [15, 24, 27–30]. The reasons for these differences are not clear but at least it can be partly explained by the different ratios and dose of the VDRAs used in the compared studies.

Some previous studies suggested a dose ratio of 1.5/1–2/1 for alfacalcidol/calcitriol and 3/1–4/1 for paricalcitol/calcitriol [24, 31, 32]. In our study, 2/1 and 3/1 ratios for alfacalcidol and paricalcitol were respectively chosen based on the previous literature [24, 31, 32], but also on a pilot study carried out in our laboratory, in which the dose chosen was based on a similar magnitude of PTH suppression. As expected, because of the non-calcific dose of VDRAs used and due to the limited time of administration (4 weeks), no significant increases in the calcium content of the aortas were observed in the four groups of rats with CRF receiving VDRAs compared with the sham group.

Like other authors [19, 33], we did not observe any significant influence of VDRAs on SBP or DBP, a finding that allows us to speculate that, at current doses, the known capability of VDRAs to reduce blood pressure (BP) [34, 35] cannot be perceived due to the low dose, but also because the VDRAs effect on BP can be overcome by other factors. In contrast to the lack of positive results in BP, the morphological and molecular changes observed in the heart on LVH and myocardial fibrosis were positive, and they merit a detailed discussion.

VDRAs at equivalent doses did lead to beneficial changes in LVH despite the increases in serum FGF23 levels, which has recently been shown to be able to induce LVH [36, 37], but with FGF 23 increases far higher to the 50% observed in our study. In addition, any possible negative effect due to the moderate increases in FGF23 might have been overcome by the beneficial effects of the VDRAs on the heart.

LVH in CKD is the result of several mechanisms that take place to readapt the cardiac function to tissue needs. Several factors act as main triggers of these mechanisms, including vessel stiffness—mainly in the aorta but also in medium-size caliber arteries—anemia, the elevation of PTH levels [6, 38] and the

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**FIGURE 3**: Effect of VDRA treatment on morphological parameters of fibrosis: (A) Masson’s trichrome staining quantification in all rats. Data represent mean ± standard deviation. *P < 0.005 versus Sham, # P < 0.005 versus CRF + vehicle. The table collects the values of fibrotic–muscular tissue for all groups. (B) Quantification of average optical density of collagen I by immunohistochemistry (RU). Data represent mean ± standard deviation. *P < 0.005 versus Sham, # P < 0.005 versus CRF + vehicle.
in CKD patients. The increase of may mimic those occurring during the development of LVH reasonable to postulate that the progressive increases in the observed after 1 year of treatment with paricalcitol. In fact, we found a remarkable decrease in the muscular tissue ratio observed in our experimental study (Figure 3A and B). The results observed in the molecular parameters. In fact, in the CRF + vehicle group, a positive association between increases in the (pERK1/2)/(ERK1/2) ratio and wall thickness and the heart/body weight was observed, demonstrating the important role played by the activation of the ERK pathway in the development of LVH (Figure 2A). The direct positive effect of VDRA use on LV mass is shown in Figure 2B, which demonstrates that the positive association between the two mentioned parameters disappears with the use of VDRAs. In fact, VDRA use reduces ERK activation but also the wall thickness and the heart–body weight ratio (Table 2 and Figure 2).

In myocardial hypertrophy, mitogen-activated protein kinases (MAPKs) play an important role [40, 41]. This pathway represents a complex system involved in hypertrophic growth. Activation of MAPKs has also been documented in different heart diseases, including dilated and ischemic cardiomyopathies. MAPKs include extracellular signal-regulated kinases ERK1/2, a pathway found to be mainly responsible for growth signaling stimulation [42]. Uncontrolled activation of ERK signaling by uremia could trigger a hypertrophic cardiomyopathy. In fact, an endogenous inhibitor of the ERK pathway, Sprouty-1, has been reported to be induced in human hearts during hypertrophy regression after implantation of a left ventricular assist device [43].

Another important finding of our study is related with the reduction of fibrotic heart tissue observed after VDRA use, particularly paricalcitol. The remodeling process that occurs in the LV involves not only increases in cardiomyocytes size but also in fibrotic heart tissue, which implies that increases in the heart size are partly due to the elevated proportion of fibrotic tissue at the expense of a proportional reduction in muscular tissue, a fact that should have a negative impact on cardiac function undetectable by measuring LV mass. In fact, the fibrotic–muscular tissue ratio in the Sham group was 2.9%, showing a 4-fold increase to 11.7% in the CRF + vehicle rats at the expense of reducing muscular tissue (Figure 3A and B).

The beneficial effect of VDRAs reducing fibrosis and consequently the fibrotic–muscular tissue ratio has been clearly shown in our study with all VDRAs used, but especially with paricalcitol. In fact, we found a remarkable decrease in the fibrotic–muscular tissue ratio in the heart in the paricalcitol group (Figure 3A) assessed by three methods (Masson’s trichrome staining, Sirius red staining and immunohistochemistry of collagen I), achieving a fibrotic–muscular tissue ratio equal to that of the control non-uremic group after 4 weeks of treatment (Figure 3A and B).

In the paricalcitol group, the differential beneficial effect in the reduction of fibrosis may be explained by the observed extremely frequent high BP observed in CKD patients. The progressive LVH has two main components: muscular tissue hypertrophy evidenced by the increase of cardiomyocyte size and the undesirable growth of fibrosis triggered and favored by the hypertrophied cardiomyocytes which lead to increases in fibroblast and collagen synthesis. Thus, the natural evolution of these changes progressively leads to the partial replacement of cardiac muscular tissue with fibrotic tissue. In a normal heart, the fibrotic–muscular tissue ratio is low, as it has been recently shown in other studies [19] and confirmed by our own work (2.9% in the non-uremic control rats). Unfortunately, the fibrotic–muscular tissue ratio present whenever LV mass increases cannot be detected by the use of conventional diagnostic methods which measure LV mass, such as nuclear magnetic resonance and echocardiography. These two methods were used in the recently published PRIMO clinical trial, in which no changes in LV mass were observed after 1 year of treatment with paricalcitol.

According to our morphological and molecular results, it is reasonable to postulate that the progressive increases in the fibrotic–muscular ratio observed in our experimental study may mimic those occurring during the development of LVH in CKD patients. The increase of fibrotic tissue at the expense of the reduction of muscular tissue may be one important factor responsible for the impairment in the atrial volume and diastolic function, which are key players in the myocardial dysfunction observed in CKD patients.

Several recent epidemiological studies have shown an encouraging, positive association between the use of VDRAs and survival, in part due to a lower cardiovascular mortality [18, 38]. However, the mechanisms involved in these possible advantages are still poorly understood. According to our results and in agreement with a recent experimental study [39], the use of VDRAs prevents the growth of the LV mass. The results observed in the morphological parameters were in agreement with those observed in the molecular parameters. In fact, in the CRF + vehicle group, a positive association between increases in the (pERK1/2)/(ERK1/2) ratio and wall thickness and the heart/body weight was observed, demonstrating the important role played by the activation of the ERK pathway in the development of LVH (Figure 2A). The direct positive effect of VDRA use on LV mass is shown in Figure 2B, which demonstrates that the positive association between the two mentioned parameters disappears with the use of VDRAs. In fact, VDRA use reduces ERK activation but also the wall thickness and the heart–body weight ratio (Table 2 and Figure 2).

In myocardial hypertrophy, mitogen-activated protein kinases (MAPKs) play an important role [40, 41]. This pathway represents a complex system involved in hypertrophic growth. Activation of MAPKs has also been documented in different heart diseases, including dilated and ischemic cardiomyopathies. MAPKs include extracellular signal-regulated kinases ERK1/2, a pathway found to be mainly responsible for growth signaling stimulation [42]. Uncontrolled activation of ERK signaling by uremia could trigger a hypertrophic cardiomyopathy. In fact, an endogenous inhibitor of the ERK pathway, Sprouty-1, has been reported to be induced in human hearts during hypertrophy regression after implantation of a left ventricular assist device [43].

Another important finding of our study is related with the reduction of fibrotic heart tissue observed after VDRA use, particularly paricalcitol. The remodeling process that occurs in the LV involves not only increases in cardiomyocytes size but also in fibrotic heart tissue, which implies that increases in the heart size are partly due to the elevated proportion of fibrotic tissue at the expense of a proportional reduction in muscular tissue, a fact that should have a negative impact on cardiac function undetectable by measuring LV mass. In fact, the fibrotic–muscular tissue ratio in the Sham group was 2.9%, showing a 4-fold increase to 11.7% in the CRF + vehicle rats at the expense of reducing muscular tissue (Figure 3A and B).

The beneficial effect of VDRAs reducing fibrosis and consequently the fibrotic–muscular tissue ratio has been clearly shown in our study with all VDRAs used, but especially with paricalcitol. In fact, we found a remarkable decrease in the fibrotic–muscular tissue ratio in the heart in the paricalcitol group (Figure 3A) assessed by three methods (Masson’s trichrome staining, Sirius red staining and immunohistochemistry of collagen I), achieving a fibrotic–muscular tissue ratio equal to that of the control non-uremic group after 4 weeks of treatment (Figure 3A and B).

In the paricalcitol group, the differential beneficial effect in the reduction of fibrosis may be explained by the observed
increase of MMP1 (Figure 4B), an interstitial collagenase that degrades type I, II and III structural collagens. The significant increase of MMP1 could be responsible for a greater collagen degradation reducing its accumulation, and thus, helping to preserve the proportion of muscular tissue. Previous studies have demonstrated the relevant role played by MMP1; in fact, it has been shown that inactivation of MMP1 synthesis in the infarcted myocardium leads to collagen accumulation, and the infusion of MMP1 in the heart increases collagen degradation reducing myocardial fibrosis [44]. The results obtained in our study in the paricalcitol-treated rats (less collagen and higher MMP1) are reinforced by the results obtained in TGFβ1 expression. Paricalcitol was the only VDRA used that showed a significant reduction in TGFβ1. It is well known that TGFβ1 is involved in the differentiation of myofibroblasts into fibroblasts, and that it causes an excess of collagen production [45–47].

The reduction of TGFβ1 observed with all VDRAs, which was more marked and significant only in the paricalcitol group, gives additional support to the antiﬁbrillar proﬁle of all VDRAs, but particularly to paricalcitol. This effect seems to be associated with a higher capacity of paricalcitol in the inhibition of genes involved in the production (TGFβ1) and degradation (MMP1) of collagen in the heart, respectively. The beneﬁcial effects of paricalcitol reducing ﬁbrosis in heart could be comparable with the protective effect observed in the kidney, where it has been described that paricalcitol suppresses the expression of ﬁbrogenic signals and ameliorates kidney injury [48], inhibiting the renal expression of TGFβ1, collagen 1 and other ﬁbrogenic genes, similar to what we observed in the heart.

Overall, all results obtained in our model of CKD with the use of paricalcitol are in partial agreement with the recent experimental data published in a murine model of pressure overloaded [49] and also with clinical ﬁndings described as post hoc analyses of the PRIMO study carried out in CKD 3–4 patients [14, 50, 51]. In the former, in non-uremic hypertensive mice, paricalcitol reduced myocardial ﬁbrosis and preserved diastolic LV function [49]. Similarly in the PRIMO trial, 1 year of treatment with paricalcitol failed to reduce LV mass, but attenuation in the rises of BNP plasma levels [52], reduction in cardiovascular hospitalizations (mainly from congestive heart failure) and, more importantly, reduction in the left atrial volume were observed pointing out a manifest improvement in the diastolic function and myocardial stretch. The CRF model used in our study yields a reduction of renal function equivalent to stage CKD 3. Unfortunately, we did not perform any functional tests; nevertheless, the BNP results, the remarkable reduction of ﬁbrosis and the normalization of the ﬁbrotic–muscular ratio observed in our study with the use of paricalcitol allow us to speculate that it is possible that part of the beneﬁcial effect on the atrial volume and diastolic function observed in the PRIMO trial carried out in CKD patients could be at least partly due to the important reduction in the myocardial ﬁbrosis induced by paricalcitol.

In summary, the administration of different VDRAs to uremic rats induced similar changes in the calcium–phosphorus–PTH axis, attenuated the growth of cardiomyocytes, the LV wall thickness, and reduced the heart–body weight ratio. However, paricalcitol was the most effective VDRA in the prevention of myocardial ﬁbrosis, reducing collagen I and TGFβ1 expression and increasing MMP1 synthesis, evidencing a high level of collagen degradation. All these ﬁndings suggest a beneﬁcial class effect of paricalcitol on the reduction of the myocardial ﬁbrosis and preservation of the muscular tissue of the heart.

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CONFLICT OF INTEREST STATEMENT

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REFERENCES

Nephronophthisis (NPH) is a rare recessive disease caused by several different gene mutations. Most gene products localize to the cilium, and thus, the various NPH manifestations including kidney cysts and situs inversus have been linked to ciliary defects. Result. Here, we describe that targeted knockdown of NPHP2 significantly reduced the number of cilia on polarized MDCK cells. As one of the underlying molecular mechanisms, we identified a direct interaction between NPHP2 and Aurora A kinase.