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# Heart Remodeling and Ischemia–Reperfusion Arrhythmias Linked to Myocardial Vitamin D Receptors Deficiency in Obstructive Nephropathy Are Reversed by Paricalcitol

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## Abstract

Cardiovascular disease is often associated with chronic kidney disease and vice versa; myocardial vitamin D receptors (VDRs) are among the probable links between the 2 disorders. The vitamin D receptor activator paricalcitol protects against some renal and cardiovascular complications. However, the structural and electrophysiological effects of myocardial vitamin D receptor modification and its impact on the response to ischemia–reperfusion are currently unknown. This work attempted to determine whether obstructive nephropathy induced myocardial changes (in rats) linked to vitamin D receptor deficiency and to ventricular arrhythmias in Langendorff-perfused hearts. Unilateral ureteral-obstructed and Sham-operated rats were treated with either paricalcitol (30 ng/kg/d intraperitoneal) or vehicle for 15 days. In 5 hearts from each group, we found that obstructed rats showed a reduction in VDRs and an increase in angiotensin II type I receptor expression (messenger RNA and protein), suffered fibrosis (determined by Masson trichrome stain) and myofibril reduction with an increase in mitochondrial size, and had dilated crests (determined by electron microscopy). These changes were reversed by paricalcitol. In 8 additional hearts per group, we found that obstructed rats showed a higher incidence of ventricular fibrillation during reperfusion (after 10 minutes of regional ischemia) than did those treated with paricalcitol. The action potential duration was prolonged throughout the experiment in paricalcitol-treated rats. We conclude that the reduction in myocardial vitamin D receptor expression in obstructed rats might be related to myocardial remodeling associated with an increase in arrhythmogenesis and that paricalcitol protects against these changes by restoring myocardial vitamin D receptor levels and prolonging action potentials.

## Keywords

arrhythmias, paricalcitol, reperfusion injury, ureteral obstruction, ventricular remodeling, vitamin D receptor

## Introduction

Cardiovascular events occur with increased frequency in patients with chronic kidney disease (CKD).<sup>1,2</sup> Ventricular arrhythmias are particularly prevalent among patients with CKD, even when those patients do not have any kind of electrolyte imbalance.<sup>3–5</sup> Mortality risk is also increased in patients with CKD who have acute coronary syndrome.<sup>6</sup>

The vitamin D receptors (VDRs) are a potential link between these conditions. Vitamin D receptors are expressed in the cardiovascular system, and their activation counteracts myocardial hypertrophy and hypertension by modulating the renin–angiotensin system (RAS), inflammation, and fibrosis.<sup>2</sup> In clinical terms, low levels of serum 25-hydroxyvitamin D are associated with an increase in mortality, in particular with

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sudden cardiac death and coronary disease.<sup>7-9</sup> Vitamin D receptor activation by paricalcitol (19-nor-1,25-dihydroxyvitamin D<sub>2</sub>) increased survival in patients with CKD, independent of their parathyroid hormone and calcium levels, but the mechanism of action for this effect remains unclear.<sup>10</sup>

We postulate that CKD, even when kidney function is not significantly impaired, induces myocardial remodeling caused by a reduction in the VDR pathways and that these changes are proarrhythmic, independent of hydroelectrolytic status. To test this hypothesis, we used an animal model of unilateral kidney disease that shows decreases in renal VDR expression and in which paricalcitol treatment restores the VDR pathways.<sup>11</sup> In this study, we evaluated whether reduction in VDR expression occurs also at the myocardial level and whether this modification might be linked to structural changes and/or modifies the electrophysiological response to the ischemia–reperfusion protocol.

## Methods and Materials

All experimental procedures were previously approved by the Ethics Committee on Laboratory Animal Research from the Medical Sciences College, National University of Cuyo, Mendoza (IACUL A5780-01), Argentina, in accordance with the guidelines of the Committee of Ethical Animal Experimentation of Argentina. The animals used in the present study corresponded with those previously described by our group; this prior study analyzed changes at the renal cortical level.<sup>11</sup>

### *Surgical Procedure and Pharmacological Treatment*

The urinary tracts (left kidney) of female Wistar-Kyoto rats, each animal weighing from 180 to 200 g, were surgically obstructed. Animals were anesthetized with 60 mg/kg of ketamine and 0.1 mg/kg of acepromazine by intraperitoneal injection. Using an aseptic technique, a midline abdominal incision was performed, as previously described.<sup>11</sup> After surgical closure of the abdominal incision, the rats were given free access to water and food. Finally, 15 days after the operation, the hearts and kidneys were harvested from both the obstructed and the Sham-operated animals. Rats were considered to have a successful ureteral obstruction when ureteral dilation was >2 mm (as determined using a millimetric eyepiece). Both groups (Sham and obstructed) were treated either with vehicle (100 µL of propylene glycol/d) or with paricalcitol (30 ng/kg/d in 100 µL of propylene glycol; provided by Abbott Laboratories, Abbott Park, Illinois), both being injected intraperitoneally for 15 days (from surgery until killing). Each of the 4 groups included 5 animals to be used for the molecular and structural studies and 8 animals assigned to the ischemia–reperfusion experiment.

### *Systolic Blood Pressure Measurement*

After weighing the animals, the systolic blood pressure (SBP) was monitored indirectly in conscious, prewarmed, slightly restrained rats. Monitoring was accomplished using the tail-

cuff method, and each animal's SBP was recorded using a CODA device (Kent Scientific Corporation, Torrington, Connecticut) the day before the isolated heart experiments. The rats were trained on the apparatus several times before measurement.

### *Histological Studies*

Myocardial tissue samples from 5 hearts from each group were fixed in 10% phosphate-buffered formalin (pH = 7.1) for 24 to 48 hours before being embedded in paraffin and serially sectioned (5 µm) on a microtome (Leica Microsystems, Deerfield, Illinois). The paraffin sections were subjected to staining with Masson trichrome stain, as previously described. For this experiment, ventricular sections were treated sequentially with Weigert iron hematoxylin solution for 10 minutes, Biebrich scarlet–acid fuchsin for 2 minutes, phosphotungstic acid/phosphomolybdic acid for 10 minutes, and aniline blue for 5 minutes. Tissue was destained in 1% acetic acid for 2 minutes, dehydrated through graded ethanol to xylene, and mounted for examination by light microscopy. For all morphologic evaluations, the observer was blinded to the origin of the histological sections. A standard point-counting method was used to quantify the fibrosis of the myocardial samples. Ten consecutive fields were randomly selected in the ventricular tissue and were evaluated at ×400 magnification on a 10 × 10 grid-imprinted reticule. Blue-stained interstitial fibrotic areas were assessed by using an image analyzer (ImageJ 1.43; Wayne Rasband, National Institutes of Health, Bethesda, Maryland). All points not counted within cardiomyocytes and vascular space were considered interstitial. The number of grid points containing blue collagen staining in the interstitium was divided by the total number of points in the fields (1000) to obtain the percentage of the fractional area of interstitial collagen deposition. Results were expressed as a percentage of the measured area.

### *Immunohistochemical Studies*

Heart paraffin sections (5–6 µm thick) were dewaxed in xylol, rehydrated, and incubated with 3% H<sub>2</sub>O<sub>2</sub> for 30 minutes to quench endogenous peroxidase activity. After washing in Tris-buffered saline (0.05 mol/L Tris-HCl, 0.15 mol/L NaCl), pH 7.6, and non-specific blocking with 10% bovine serum albumin for 30 minutes at room temperature, the sections were immunostained to reveal VDR and angiotensin II type 1 receptor (AT<sub>1</sub>R). Antibodies applied were mouse monoclonal antibody against VDR (D-6) and rabbit polyclonal antibody against AT<sub>1</sub>R (306; Santa Cruz Biotechnology, Inc, Santa Cruz, California), diluted at 1:500. A commercial immunoperoxidase kit was used (Dako EnVision; Dako Corporation, Carpinteria, California). Positive reactions were evaluated considering the specific location of immunostaining (heart structure and cell compartment: nucleus, cytoplasm, and membrane) and the intensity of the immunoreaction. The negative controls included tissues unexposed to primary antibodies and tissues exposed to control immunoglobulin G. The positive controls were human breast cancer biopsy samples. We used a

**Table 1.** Sets of Primers for the RT-PCR Procedure.<sup>a</sup>

Primer	Sequence	Annealing, °C	Predicted Product Size, bp
<b>VDR</b>			
Sense	5'-GAC TTT GAC CGG AAC GT GCG -3'	62	227
Antisense	5'-CAT CAT GCC GAT GT CCA CAC -3'		
<b>AT<sub>1</sub>R</b>			
Sense	5'-GCACACTGGCAATGTAATGC-3'	55	385
Antisense	5'-GTTGAACAGAACAAGTGACC-3'		
<b>TGF<sub>β</sub></b>			
Sense	5'-GGACTACTACGCCAAAGAAG-3'	54	294
Antisense	5'-TCAA AAG ACAG CCA CT CAG G -3'		
<b>β-Actin</b>			
Sense	5'-TGGAGAAGAGCTATGAGCTGCCTG-3'	65	201
Antisense	5'-GTGCCACCAGACAGCACTGTGTTG-3'		

Abbreviations: VDR, vitamin D receptors; AT<sub>1</sub>R, angiotensin II type I receptor; TGF<sub>β</sub>, transforming growth factor β; RT-PCR, reverse transcription-polymerase chain reaction.

<sup>a</sup> Sense and antisense sequences, annealing temperatures, and predicted product size in bp are shown.

scoring system slightly modified from one reported previously.<sup>12</sup> Briefly, we used the following intensity scores: 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining.

### Electron Microscopy

Immediately upon being separated from their respective organs, tissue samples were fixed by immersion in a fixative solution (1:10). Fixative solution was obtained by diluting 1 phosphate-buffered saline tablet, following the manufacturer's instructions, in 200 mL of double-distilled water and 2% glutaraldehyde (v/v), 2% of fresh p-formaldehyde (v/v), and 2% of picric acid as saturated solution. After 2 hours at room temperature, the samples were reduced and placed in an osmium tetroxide (OsO<sub>4</sub>) solution overnight at 4°C. The next day, the samples were dehydrated in alcohol-acetone, grading up to 100%, and embedded in Epon 812 (Sigma, St Louis, Missouri). Ultrathin sections were obtained with an Ultracut microtome (Leitz, Folly Beach, South Carolina) and stained with lead citrate and uranyl, using conventional staining methods. Observations were made and micrographs were created using a Zeiss EM 900 microscope (Zeiss, Oberkochen).

### Reverse Transcription-Polymerase Chain Reaction and Semiquantification of Messenger RNA

Total ribonucleic acid from ventricular myocardial tissue was obtained using Trizol reagent (Life Technologies Inc Gibco/Brl Division, Grand Island, New York). Ribonucleic acid of 1 μg was denatured in the presence of 0.5 μg/50 μL oligo (dT) 15 primer and 40 U recombinant ribonuclease inhibitor (Promega, Madison, WI). Reverse transcription was performed in the presence of the mixture, using 200 U of reverse transcriptase in reaction buffer and 0.5 mmol/L of deoxyribonucleotide triphosphate (each), and incubated for 60 minutes at 42°C. The complementary DNA (10 μL) was amplified by polymerase chain reaction under standard conditions. Each sample was analyzed to determine levels of VDR, transforming growth factor β (TGF<sub>β</sub>),

AT<sub>1</sub>R, and β-actin (primers in Table 1, Integrated DNA Technologies, Inc, Coralville, Iowa). The VDR, AT<sub>1</sub>R, and TGF<sub>β</sub> signals were standardized against the β-actin signal for each sample, and the results were expressed as a ratio.

### Langendorff-Perfused Rat Hearts

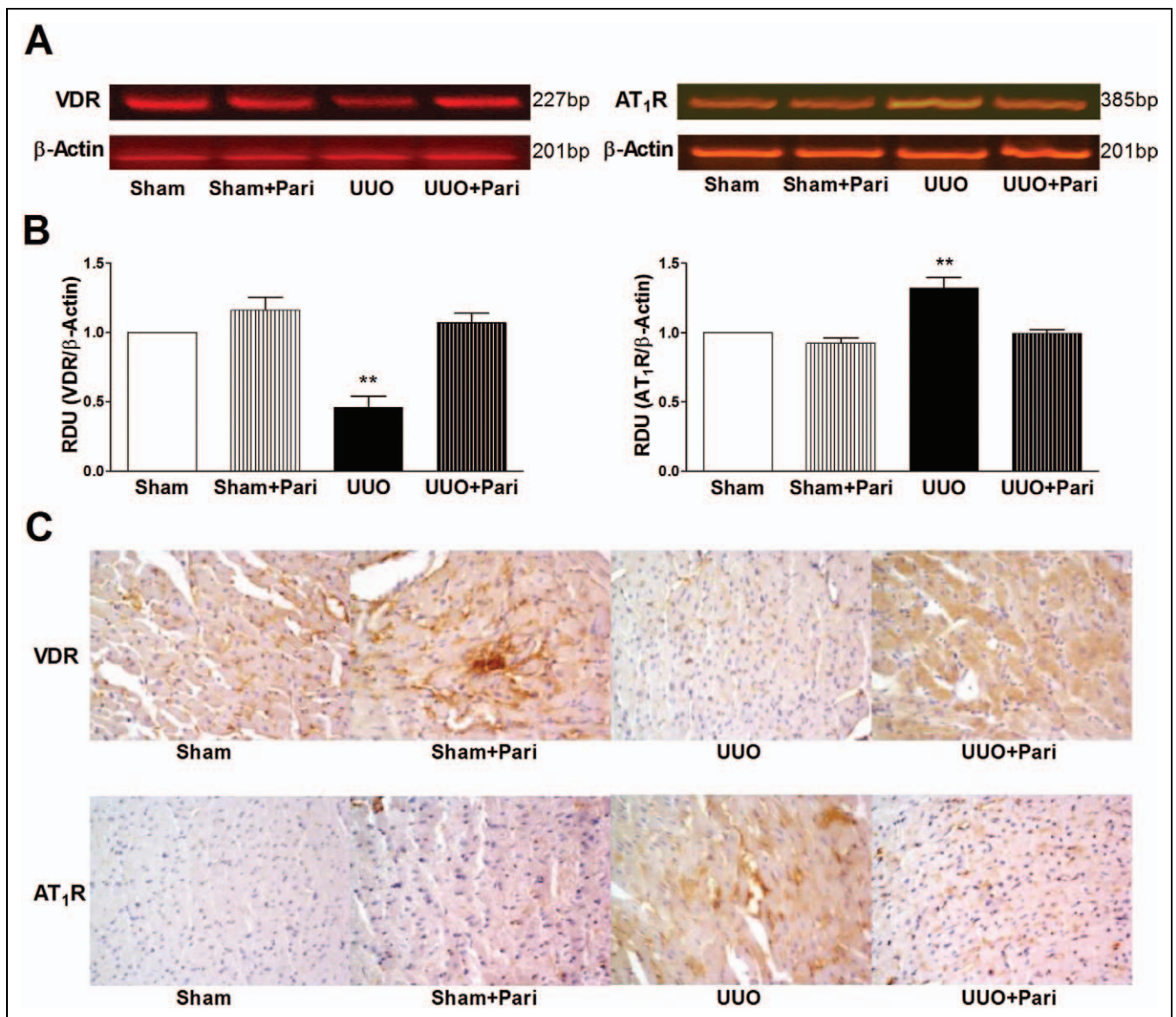
After killing, 8 hearts from each group were rapidly excised and kept at 4°C until being connected to a perfusion system (which always took less than 3 minutes). The hearts were perfused at constant pressure of 80 cm H<sub>2</sub>O with a modified Krebs-Henseleit solution containing (in mmol/L) 121 NaCl, 25 NaHCO<sub>3</sub>, 1.2 Na<sub>2</sub>HPO<sub>4</sub>, 5 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, and glucose. When equilibrated with 5% CO<sub>2</sub> in O<sub>2</sub> at 36.5°C ± 0.5°C, the pH was 7.4 ± 0.02.

The coronary flow was measured throughout the experiment. It was used as an index of adequate perfusion and as a criterion to assess the efficiency of coronary ligation. A reduction of at least 25% during occlusion was considered satisfactory. In order to validate reproducibility, we reoccluded the artery at minute 10 of reperfusion, perfused Evans blue, weighed the hearts, and after 1 hour of cooling at -20°C, sliced the ventricles transversely from apex to base into 2 mm slices. The zones colored other than blue were considered the areas at risk. The slices were photographed for planimetric analysis (ImageJ 1.43; Wayne Rasband, National Institutes of Health, Bethesda, Maryland) and weighed for adjusted expression of the area at risk. We included only those hearts with an ischemic area greater than 40% of the ventricles in order to guarantee the reproducibility of the reperfusion arrhythmia incidence.<sup>13</sup>

### Reperfusion Arrhythmias and Action Potentials

After 20 minutes of stabilization, we continuously obtained the surface electrogram equivalent of lead II and epicardial transmembrane potential using a Hewlett-Packard 1500A (Hewlett-Packard Company USA, Palo Alto, California) and a custom-made microelectrode amplifier, respectively. Both





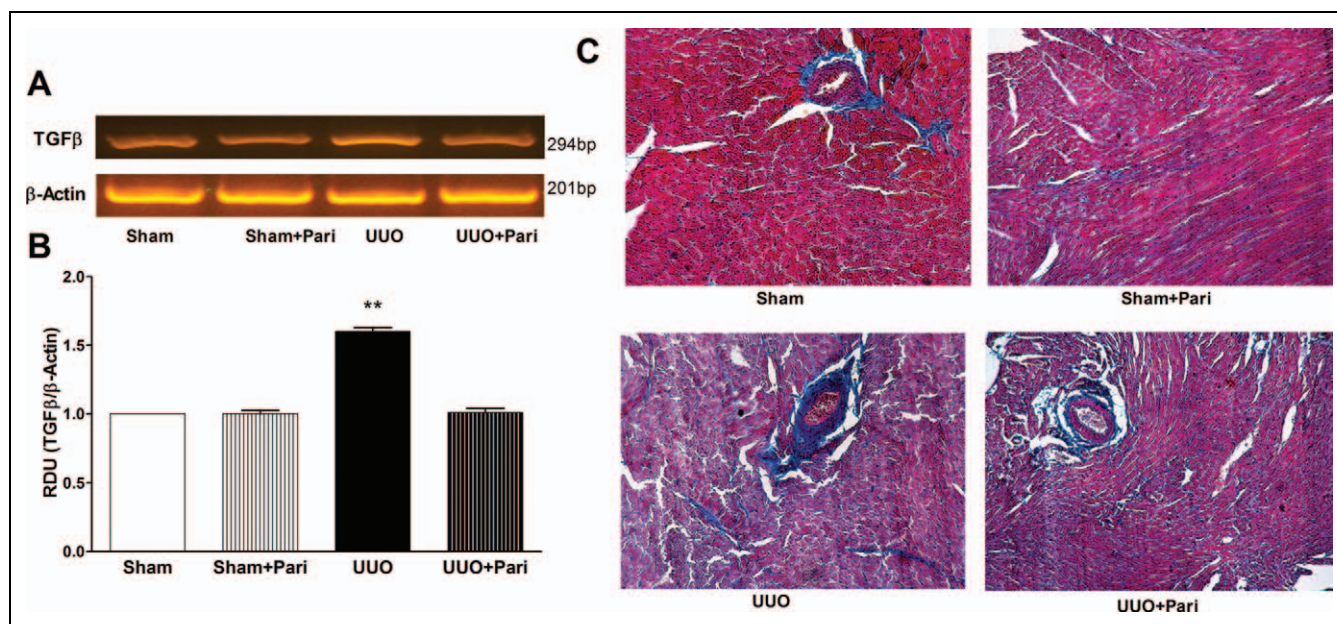
**Figure 1.** Vitamin D receptor and AT<sub>1</sub>R expression. A, Representative gels of VDR (227 bp) and AT<sub>1</sub>R (385 bp) mRNA in myocardial samples obtained from Sham-operated rats treated with vehicle (Sham), from obstructed rats (UUO), and from rats treated with paricalcitol (Sham + Pari and UUO + Pari, respectively). The corresponding housekeeping β-actin (201 bp) is included subsequently. B, Vitamin D receptor–β-actin mRNA ratio shows lower expression in myocardial samples from UUO versus Sham-operated (Sham) rats as well as a higher AT<sub>1</sub>R–β-actin mRNA ratio (\*\**P* < .01 for both). Paricalcitol treatment maintained VDR and AT<sub>1</sub>R mRNA expression. Results are mean ± standard error of the mean (SEM); *n* = 5. C, Immunohistochemical staining of VDR and AT<sub>1</sub>R in myocardial samples. Images were taken at ×400. AT<sub>1</sub>R indicates angiotensin II type I receptor; mRNA, messenger RNA; RDU, relative densitometry units; UUO, unilateral ureteral obstruction; VDR, vitamin D receptor.

signals were digitized with an analog-to-digital converter (NI PCI-6221; National Instruments, Austin, Texas) and recorded using LabView Signal Express 2.5 (National Instruments, Austin, Texas). After a period of 10 minutes, preischemia, the hearts underwent 10 minutes of regional ischemia by ligation of the left anterior descending coronary artery followed by 10 minutes of reperfusion. Ventricular arrhythmias were classified according to the Lambeth convention.<sup>14</sup> We evaluated the incidence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) and also the arrhythmia severity each minute using the following scores: 0 = sinus rhythm; 1

= premature ventricular beats or bigeminy; 2 = Salvos; 3 = nonsustained VT (<30 seconds); and 4 = sustained VT (>30 seconds) or VF.<sup>15</sup> We analyzed the following parameters of epicardial transmembrane potentials: action potential amplitude, resting potential, and action potential duration at 90% of repolarization.

### Statistical Analysis

Data were expressed as mean ± standard error of the mean, and statistical analysis was performed using analysis of



**Figure 2.** Myocardial TGF $\beta$  expression and Masson trichrome staining. A, Representative gel of TGF $\beta$  (294 bp) mRNA. The corresponding housekeeping  $\beta$ -actin is included subsequently. B, Graphical representation of TGF $\beta$ - $\beta$ -actin mRNA ratio shows higher mRNA expression in obstructed rat hearts. \*\* $P < .01$  Sham versus unilateral ureteral obstruction (UUO). Results are mean  $\pm$  standard error of the mean (SEM);  $n = 5$ . C, Masson trichrome-stained sections of myocardial samples. Magnification:  $\times 400$ . mRNA indicates messenger RNA; RDU, relative densitometry units; TGF $\beta$ , transforming growth factor  $\beta$ .

variance (ANOVA) or 2-way repeated-measures ANOVA followed by a Bonferroni posttest and Fisher exact test, as appropriate. Variables not normally distributed were analyzed using the Kruskal-Wallis test followed by Dunn posttest. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, California).

## Results

### Vitamin D Receptor and AT $_1$ R Myocardial Expression

The VDR expression in the hearts of the obstructed rats was lower than that found in the hearts of the Sham-operated group (the intensity score for Sham was 2.1; for Sham + Pari, the score was 2.3; for unilateral ureteral obstruction [UUO], the score was 1.0\*; and for UUO + Pari, the score was 2.6;  $P < .05$ , Sham vs UUO); conversely, AT $_1$ R expression was higher (the intensity score for Sham was 1.1; the score for Sham + Pari was 1.2; the score for UUO was 2.5\*; the score for UUO + Pari was 1.6;  $P < .05$ , Sham vs UUO). Paricalcitol restored VDR and AT $_1$ R expression in samples obtained from the UUO + Pari group (Figure 1).

### Myocardial Structural Remodeling

We did not see any changes in SBP (Sham,  $110 \pm 5$ ; Sham + Pari,  $112 \pm 7$ ; UUO,  $118 \pm 5$ ; UUO + Pari,  $116 \pm 7$ ;  $P =$  not significant [ns]) nor did we see myocardial hypertrophy (relative heart weight in mg/g: Sham,  $3.8 \pm 0.1$ ; Sham + Pari,  $3.7 \pm 0.3$ ; UUO,  $3.9 \pm 0.2$ ; UUO + Pari,  $3.7 \pm 0.2$ ;  $P =$  ns) associated

with UUO, but the microscopic structure revealed evidence of increased fibrosis (Sham,  $34.7 \pm 5.4\%$ ; Sham + Pari,  $29.5 \pm 6.2\%$ ; UUO,  $68.9 \pm 7.4\%*$ ; UUO + Pari,  $34.1 \pm 6.9\%$ ;  $*P < .05$ , Sham vs UUO) as determined by Masson trichrome staining, the presence of which was consistent with the increase in TGF $\beta$  expression at the messenger RNA level (Figure 2). Paricalcitol-treated hearts did not appear to be any different from Sham vehicle-treated ones. Hearts from obstructed rats showed myofibrillar density reduction and mitochondrial edema (Figure 3). However, paricalcitol prevented the loss of myofibrillar content and prevented as well mitochondrial edema.

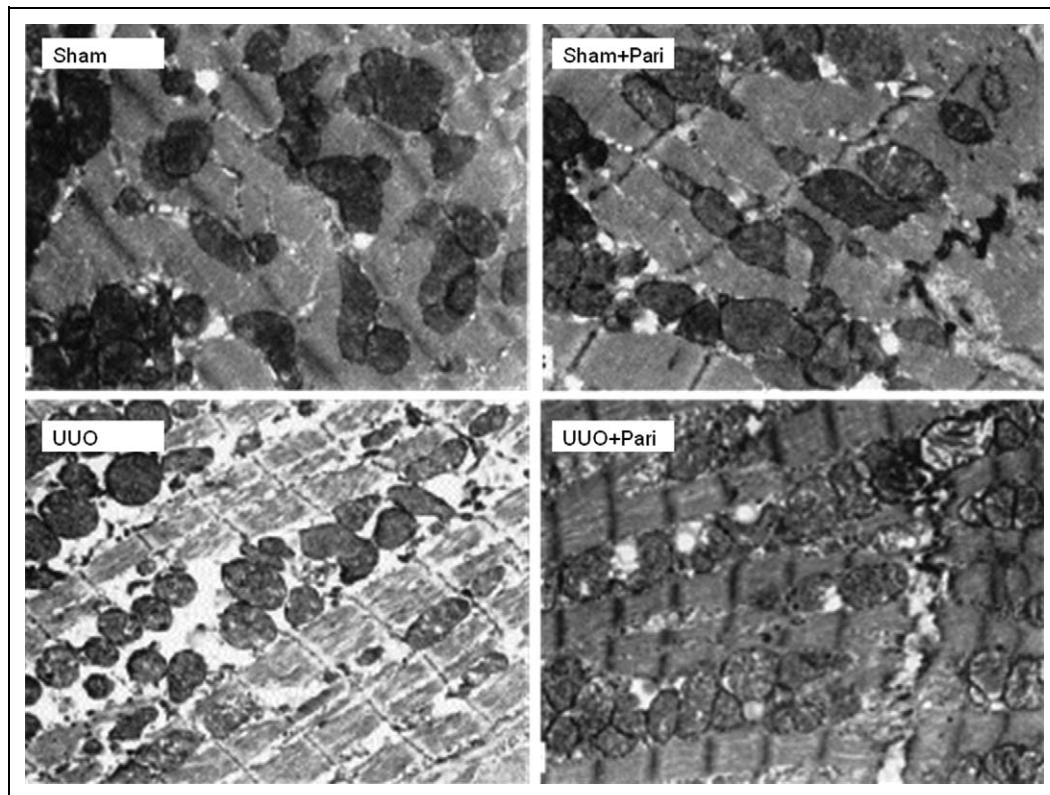
### Coronary Flow and Area at Risk

Coronary flow presented similar values in all of the groups, and the degree of reduction was stable after coronary occlusion. The ischemic area measured at the end of the experiment did not differ between groups (both results are shown in Table 2).

### Ventricular Arrhythmias

We did not find any differences in basal heart rate between the groups, and all had a reduction of around 20 to 40 beats/min during ischemia (Table 2). Ventricular arrhythmias occurred at the onset of reperfusion in the UUO + vehicle-treated hearts (Figure 4). The administration of paricalcitol reduced the incidence and duration of VF but did not modify VT (Figure 4 and Table 3). Similar durations of VT were followed by a predominant sinus rhythm during reperfusion in all groups other than the UUO + vehicle group, in which VT was followed by





**Figure 3.** Electron microscopy: myocardial effect of unilateral obstruction and paricalcitol treatment. The upper row corresponds to electron microscopy photograph ( $\times 60\,000$ ) from myocardial samples obtained from Sham-operated rats treated with vehicle (Sham) and with paricalcitol (Sham + Pari). The lower row shows data from myocardial samples obtained from rats after 15 days of unilateral obstruction and after having been treated with vehicle (UUO) or paricalcitol (UUO + Pari). UUO indicates unilateral ureteral obstruction.

**Table 2.** Coronary Flow and Heart Rate During the Experimental Protocol and the Size of Ischemic Area.<sup>a</sup>

	Coronary Flow, mL/g			Heart Rate, beats/min			Ischemic Area, %
	Preischemia	Prereperfusion	Minute 10 of Reperfusion	Preischemia	Prereperfusion	Minute 10 of Reperfusion	
Sham	6.8 $\pm$ 0.3	4.0 $\pm$ 0.4 <sup>b</sup>	5.5 $\pm$ 0.4	292 $\pm$ 13	261 $\pm$ 17	278 $\pm$ 16 (7)	46 $\pm$ 5
Sham + Pari	6.9 $\pm$ 0.4	3.9 $\pm$ 0.3 <sup>b</sup>	5.4 $\pm$ 0.5	297 $\pm$ 14	262 $\pm$ 15	272 $\pm$ 13 (8)	47 $\pm$ 6
UUO	6.8 $\pm$ 0.5	4.1 $\pm$ 0.5 <sup>b</sup>	5.4 $\pm$ 0.3	293 $\pm$ 18	261 $\pm$ 17	277 $\pm$ 21 (4)	46 $\pm$ 6
UUO + Pari	6.9 $\pm$ 0.4	4.1 $\pm$ 0.4 <sup>b</sup>	5.6 $\pm$ 0.5	300 $\pm$ 17	265 $\pm$ 17	279 $\pm$ 12 (8)	45 $\pm$ 5

Abbreviations: SEM, standard error of the mean; UUO, unilateral ureteral obstruction.

<sup>a</sup> All values represent mean  $\pm$  SEM of  $n = 8$ . The heart rate during reperfusion corresponds to the number of hearts in sinus rhythm indicated in the parentheses.

<sup>b</sup>  $P < .01$  versus preischemia.

sustained VF. These results are supported by a reduction in the severity score (Figure 4), which also indicates that the antiarrhythmic effect was sustained, since vehicle-treated hearts maintained a high level of severity.

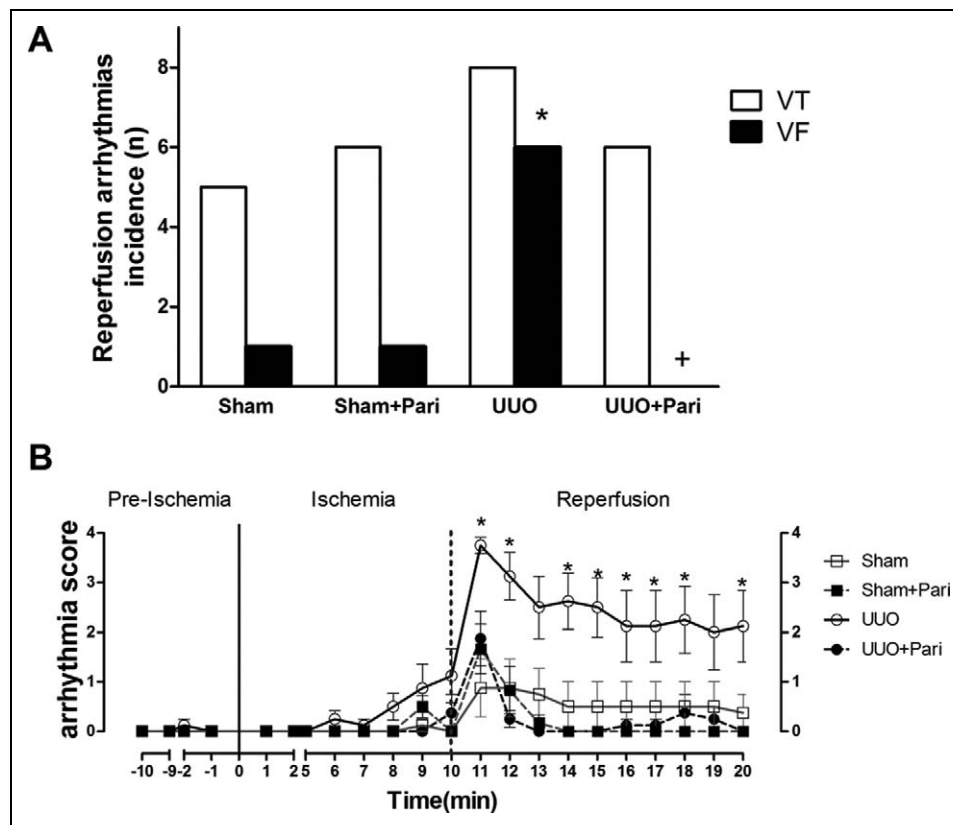
### Transmembrane Potential

Analysis of the transmembrane potential of epicardial cardiomyocytes determined that paricalcitol lengthened the action potential duration at 90% repolarization in both treated groups. This effect was found during preischemia and previous to

reperfusion. The UUO hearts maintained short action potential duration during reperfusion. The amplitude and the resting potential were very similar in all groups during the experiment (Figure 5).

### Discussion

The present study relates myocardial VDR reduction in UUO rats with arrhythmogenic remodeling reversed by paricalcitol. This is the first report of a reduction in VDR occurring at the myocardial level, in which the reduction is associated with



**Figure 4.** Ventricular arrhythmias during reperfusion. A, Incidence of ventricular arrhythmias during reperfusion. White bars indicate the number of hearts that developed ventricular tachycardia (VT) and black bars indicate those who had ventricular fibrillation (VF) for each group. \* $P = .0406$ , Sham versus UUO; and  $^+P = .007$ , UUO versus UUO + Pari, both by Fisher exact test;  $n = 8$  each. B, Arrhythmia severity score throughout the experimental periods. Continuous connecting lines indicate vehicle-treated rats and dashed connecting lines indicate paricalcitol-treated rats. Sham groups are indicated by squares and UUO by circles. \* $P < .05$  Sham versus UUO by 2-way repeated measures analysis of variance (ANOVA). UUO indicates unilateral ureteral obstruction.

**Table 3.** Reperfusion Arrhythmias Duration.<sup>a</sup>

	Ventricular Tachycardia	Ventricular Fibrillation
Sham	23.0 (0-41)	0.0 (0-45)
Sham + Pari	21.5 (8-65)	0.0 (0-28)
UUO	40.5 (12-72)	366.0 (198-548) <sup>b</sup>
UUO + Pari	29.5 (16-66)	0.0 (0-41) <sup>c</sup>

Abbreviation: UUO, unilateral ureteral obstruction.

<sup>a</sup> All values correspond to the median (first quartile to third quartile) expressed in seconds.

<sup>b</sup>  $P < .01$  Sham versus UUO analyzed by Kruskal-Wallis test followed by Dunn posttest.

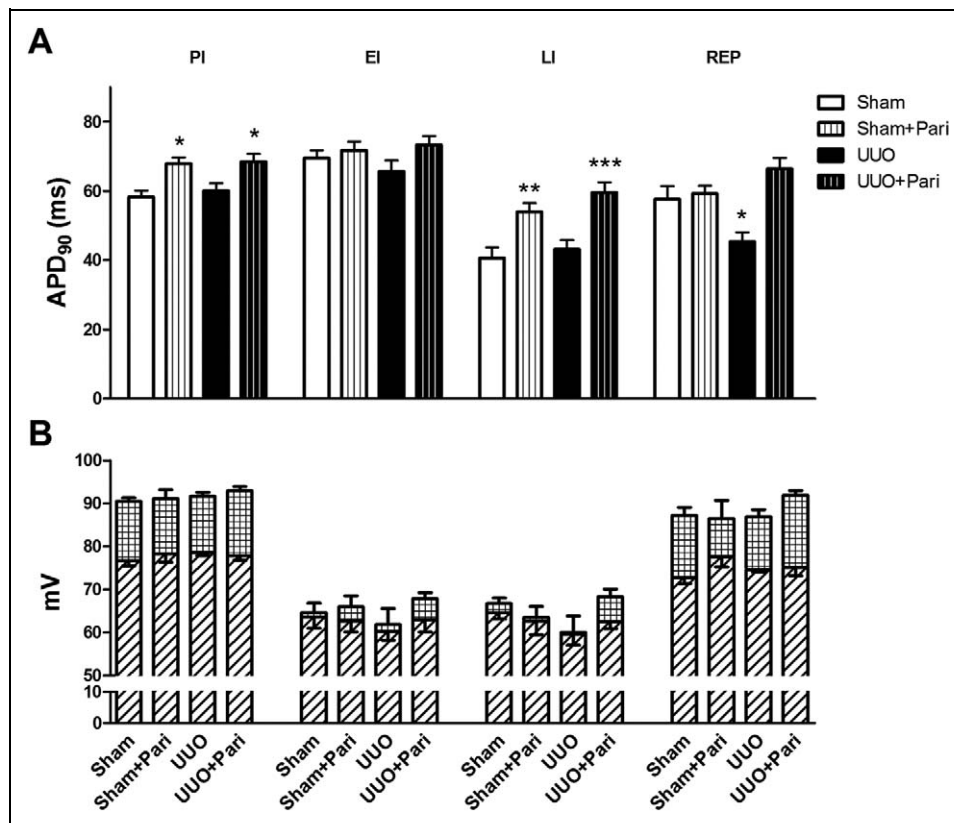
<sup>c</sup>  $P < .01$  UUO versus UUO + Pari analyzed by Kruskal-Wallis test followed by Dunn posttest.

kidney disease but without any other hemodynamic stimulus, which has been shown to be a VDR expression modifier. Previous reports using the subtotal nephrectomy model of CKD did not find any differences in myocardial VDR expression, but the animals developed myocardial hypertrophy.<sup>16</sup> Others reported that salt-associated hypertrophy induced VDR expression as a counterbalance.<sup>17</sup> Furthermore, a deletion of the receptor induced myocardial hypertrophy.<sup>18</sup> So, the differences here described in respect to previous reports could be attributed

to the fact that our kidney disease model partially reduced VDR without any hemodynamic stimulus and/or myocardial hypertrophy. Since we did not find any changes in calcium homeostasis, we speculate that the obstructed kidney might generate inflammatory components that affect VDR expression, but this hypothesis has been confirmed only at the renal level.<sup>19</sup> The specific mechanism involved in myocardial VDR reduction in UUO is not clear and deserves further evaluation.

Our finding helps clarify the role of myocardial VDR expression in the pathophysiological mechanisms linking CKD to cardiovascular disease, which is itself caused by the exacerbation of myocardial angiotensin effects. A long time ago, an original work suggested a possible link between vitamin D and the RAS.<sup>20</sup> Inappropriate stimulation of the RAS resulting from vitamin D deficiency has been associated with hypertension, heart attack, stroke, and hypertrophy of the left ventricle and vascular smooth muscle cells.<sup>21</sup> More recently, experimental and clinical evidence indicates that vitamin D deficiency and tissular angiotensin II (ANG II) upregulation play a pivotal role in the progression of renal disease associated with obstructive nephropathy,<sup>22</sup> and that VDR attenuates obstructive renal injury at least in part by suppressing the RAS.<sup>23</sup> The pleiotropic actions of vitamin D and its analogs are mediated by a specific





**Figure 5.** Effects of the UO and paricalcitol treatment on the membrane potential response to ischemia–reperfusion. **A**, Changes in action potential duration at 90% repolarization ( $APD_{90}$ ) with ischemia and reperfusion for each group. Data were obtained before ischemia, preischemia (PI), at early ischemia (EI; first 5 minutes) and late ischemia (LI; last 5 minutes), and during reperfusion (REP). The bars represent means  $\pm$  standard error of the mean (SEM),  $n = 8$ . \* $P < .05$ ; \*\* $P < .01$ ; and \*\*\* $P < .001$  for comparisons versus Sham group for the same period. **B**, The diagonally striped bars represent the resting potential, and the total height of each bar corresponds to the action potential amplitude. The overshoot is indicated by the checkered pattern. UO indicates unilateral ureteral obstruction.

VDR, a ligand-dependent transcription factor that belongs to the steroid/nuclear receptor gene family.<sup>24,25</sup> In agreement, we find significantly higher  $AT_1R$  expression and lower VDR expression resulting from UO. Although our findings are not causative, the remodeling here described is in agreement with the postulated counterbalance between VDR and the RAS at the myocardial level.<sup>18,26,27</sup> Indeed, in a recent report, we showed that  $AT_1R$  modulated by VDR is involved in the mechanism by which paricalcitol provides renal protection in spontaneously hypertensive rats.<sup>28</sup>

Angiotensin II, via the  $AT_1R$ , is involved in the modulation of ventricular structure and function in CKD. Angiotensin II causes significant proinflammatory activity that induces the expression of cytokines, chemokines, adhesion molecules, growth factors, and reactive oxygen species.<sup>29</sup> Previous reports that associate  $AT_1R$  and mitochondrial malfunction are in agreement with the ultrastructural changes described here.<sup>30</sup> In addition, the myofibrillar reduction could be attributed to the regulatory effect of vitamin D on the expression of myocardial myosin.<sup>31,32</sup> We attribute these changes to the myocardial VDR pathway because they were prevented in paricalcitol-treated rats. In addition,  $AT_1R$  blockade interferes with ANG II-mediated  $TGF\beta$  expression.<sup>33</sup> Vitamin D

receptors are negative regulators of  $TGF\beta$ /Smad signaling. Impaired VDR signaling with reduced expression of VDR and decreased levels of its ligand may thus contribute to hyperactive  $TGF\beta$  signaling.<sup>34</sup> Important to our understanding, paricalcitol showed a relevant beneficial effect in the reduction of myocardial fibrosis, a key factor in the myocardial dysfunction suffered by patients with CKD.<sup>35</sup>

In regard to arrhythmogenesis, it is well known that myocardial remodeling generates electrical instability.<sup>36</sup> The influence of a structural kidney alteration at the myocardial level may not be evident under basal conditions but modifies the response to stressful situations such as ischemia and reperfusion. Unilateral ureteral obstruction rats showed remodeling and an increase in VF during reperfusion. Both the increase in  $AT_1R$  and  $TGF\beta$  could underlie the arrhythmogenesis that is observed in UO rat hearts. The level of  $AT_1R$  expression plays an important role in ischemia–reperfusion arrhythmogenesis. The  $AT_1R$  knockout mice do not display ventricular arrhythmias after ischemia–reperfusion.<sup>37</sup> The  $AT_1R$  is unregulated after ischemia–reperfusion injury.<sup>38</sup> Losartan, a specific  $AT_1R$  blocker, effectively reduces ischemia–reperfusion arrhythmias in transgenic rats with cardiac  $AT_1R$  overexpression.<sup>39</sup> The pathway that relates  $AT_1R$  and  $TGF\beta$  signaling is involved in a number

of human diseases, including heart failure and myocardial arrhythmias.<sup>40</sup> Transforming growth factor  $\beta$  increases sarcoplasmic reticulum  $\text{Ca}^{2+}$  content and the frequency of spontaneous intracellular  $\text{Ca}^{2+}$  oscillations.<sup>40</sup> Paricalcitol prevented the changes in the pattern of expression, the structural changes, and also the lethal arrhythmias. However, lengthening of the action potential duration of paricalcitol could also explain the antiarrhythmic effect found, as it is known that this variable is a determinant of the myocardial refractory period and the generation of reentry circuits. In fact, it could explain the antifibrillatory effect, due to the interference of microreentrant circuits, without affecting the brief self-limited episodes of VT observed even in Sham-operated hearts, probably because the latter are usually the expression of macroreentrant circuits.<sup>41</sup>

We support our electrophysiological findings in a recent publication that describes an action potential lengthening in 1,25-dihydroxyvitamin D-treated, isolated rabbit left atrium that exerts an antiarrhythmic effect.<sup>42</sup> However, we did not identify the mechanisms by which the drug induces action potential prolongation. Since isolated hearts were not exposed to the drug during the experiment, it might be possible to attribute this effect to changes in ionic current expression. We did not find any reports of electrocardiogram changes in patients receiving paricalcitol. The electrophysiological effects of paricalcitol should be further investigated.

We conclude that myocardial VDR pathways are a link between UUO kidney disease and cardiac remodeling and that their presence in a host predisposes that host to lethal arrhythmias during reperfusion. This interesting new model will enable researchers to evaluate the complex interaction of the kidneys and the heart.

## Summary

The reduction in myocardial vitamin D receptor expression in unilateral ureteral obstructed rats induced myocardial remodeling associated with an increase in arrhythmogenesis; paricalcitol protects against these changes by restoring myocardial vitamin D receptor levels and prolonging action potentials.

## Perspectives

Despite overwhelming data that link sudden cardiac death with CKD, this is a relatively unexplored field in the basic research area, mainly because of the difficulty of finding an adequate experimental model. Furthermore, other cardiovascular effects of VDR expression reduction due to kidney disease could be evaluated using this animal model of UUO. In addition, electrophysiological properties of paricalcitol deserve further investigation in order to clarify whether or not these effects might be responsible for the improved prognosis that has been described in treated patients.

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## Author Contributions

All authors contributed to conception and design of the study, with substantial contribution to data acquisition, analysis and interpretation of the data, drafting of the article, and critical revision of the article for intellectual content.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## References

- Collins AJ, Li S, Gilbertson DT, Liu J, Chen SC, Herzog CA. Chronic kidney disease and cardiovascular disease in the Medicare population. *Kidney Int Suppl.* 2003;87(S24-S31).
- Wang L, Manson JE, Song Y, Sesso HD. Systematic review: vitamin D and calcium supplementation in prevention of cardiovascular events. *Ann Intern Med.* 2010;152(5):315-323.
- Bleyer AJ, Hartman J, Brannon PC, Reeves-Daniel A, Satko SG, Russell G. Characteristics of sudden death in hemodialysis patients. *Kidney Int.* 2006;69(12):2268-2273.
- Karnik JA, Young BS, Lew NL, et al. Cardiac arrest and sudden death in dialysis units. *Kidney Int.* 2001;60(1):350-357.
- Pilz S, März W, Wellnitz B, et al. Association of vitamin D deficiency with heart failure and sudden cardiac death in a large cross-sectional study of patients referred for coronary angiography. *J Clin Endocrinol Metab.* 2008;93(10):3927-3935.
- Pun PH, Smarz TR, Honeycutt EF, Shaw LK, Al-Khatib SM, Middleton JP. Chronic kidney disease is associated with increased risk of sudden cardiac death among patients with coronary artery disease. *Kidney Int.* 2009;76(6):652-658.
- Drechsler C, Pilz S, Obermayer-Pietsch B, et al. Vitamin D deficiency is associated with sudden cardiac death, combined cardiovascular events, and mortality in haemodialysis patients. *Eur Heart J.* 2010;31(18):2253-2261.
- Pilz S, Tomaschitz A, Drechsler C, Dekker JM, März W. Vitamin D deficiency and myocardial diseases. *Mol Nutr Food Res.* 2010;54(8):1103-1113.
- Campbell NG, Varaganam M, Sawhney V, et al. Mild chronic kidney disease is an independent predictor of long-term mortality after emergency angiography and primary percutaneous intervention in patients with ST-elevation myocardial infarction. *Heart.* 2012;98(1):42-47.

10. Teng M, Wolf M, Lowrie E, Ofsthun N, Lazarus JM, Thadhani R. Survival of patients undergoing hemodialysis with paricalcitol or calcitriol therapy. *N Engl J Med*. 2003;349(5):446-456.
11. Garcia IM, Altamirano L, Mazzei L, et al. Role of mitochondria in paricalcitol-mediated cytoprotection during obstructive nephropathy. *Am J Physiol Renal Physiol*. 2012;302(12):F1595-F1605.
12. Gago FE, Tello OM, Diblasi AM, Ciocca DR. Integration of estrogen and progesterone receptors with pathological and molecular prognostic factors in breast cancer patients. *J Steroid Biochem Mol Biol*. 1998;67(5-6):431-437.
13. Curtis MJ, Hearse DJ. Reperfusion-induced arrhythmias are critically dependent upon occluded zone size: relevance to the mechanism of arrhythmogenesis. *J Mol Cell Cardiol*. 1989;21(6):625-637.
14. Walker MJ, Curtis MJ, Hearse DJ, et al. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. *Cardiovasc Res*. 1988;22(7):447-455.
15. Curtis MJ, Walker MJ. Quantification of arrhythmias using scoring systems: an examination of seven scores in an in vivo model of regional myocardial ischaemia. *Cardiovasc Res*. 1988;22(9):656-665.
16. Wu-Wong JR, Noonan W, Nakane M, et al. Vitamin d receptor activation mitigates the impact of uremia on endothelial function in the 5/6 nephrectomized rats. *Int J Endocrinol*. 2010;2010:625852.
17. Bodyak N, Ayus JC, Achinger S, et al. Activated vitamin D attenuates left ventricular abnormalities induced by dietary sodium in Dahl salt-sensitive animals. *Proc Natl Acad Sci USA*. 2007;104(43):16810-16815.
18. Bae S, Singh SS, Yu H, Lee JY, Cho BR, Kang PM. Vitamin D signaling pathway plays an important role in the development of heart failure after myocardial infarction. *J Appl Physiol*. 2013;114(8):979-987.
19. Xiong M, Gong J, Liu Y, Xiang R, Tan X. Loss of vitamin D receptor in chronic kidney disease: a potential mechanism linking inflammation to epithelial-to-mesenchymal transition. *Am J Physiol Renal Physiol*. 2012;303(7):F1107-F1115.
20. Jensen LP, Ras G, Boes EG. Hypercalcaemia in pregnancy: a case report. *S Afr Med J*. 1980;57(17):712-713.
21. Zittermann A. Vitamin D and disease prevention with special reference to cardiovascular disease. *Prog Biophys Mol Biol*. 2006;92(1):39-48.
22. Klahr S. Urinary tract obstruction. *Semin Nephrol*. 2001;21(2):133-145.
23. Zhang Y, Kong J, Deb DK, Chang A, Li YC. Vitamin D receptor attenuates renal fibrosis by suppressing the renin-angiotensin system. *J Am Soc Nephrol*. 2010;21(6):966-973.
24. Dusso AS, Brown AJ. Mechanism of vitamin D action and its regulation. *Am J Kidney Dis*. 1998;32(suppl 2):S13-S24.
25. Rachez C, Freedman LP. Mechanisms of gene regulation by vitamin D(3) receptor. A network of coactivator interactions. *Gene*. 2000;246(1-2):9-21.
26. Ferder M, Inserra F, Manucha W, Ferder L. The world pandemic of vitamin D deficiency could possibly be explained by cellular inflammatory response activity induced by the renin-angiotensin system. *Am J Physiol Cell Physiol*. 2013;304(11):C1027-C1039.
27. Freundlich M, Quiroz Y, Zhang Z, et al. Suppression of renin-angiotensin gene expression in the kidney by paricalcitol. *Kidney Int*. 2008;74(11):1394-1402.
28. García IM, Altamirano L, Mazzei L, et al. Vitamin D receptor-modulated Hsp70/AT1 expression may protect the kidneys of SHR at the structural and functional levels [published online November 13, 2013]. *Cell Stress Chaperones*. 2013.
29. Manucha W, Vallés PG. Apoptosis modulated by oxidative stress and inflammation during obstructive nephropathy. *Inflamm Allergy Drug Targets*. 2012;11(4):303-312.
30. Vazquez-Medina JP, Popovich I, Thorwald MA, et al. Angiotensin receptor mediated oxidative stress is associated with impaired cardiac redox signaling and mitochondrial function in insulin resistant rats. *Am J Physiol Heart Circ Physiol*. 2013;305(4):H599-H607.
31. O'Connell TD, Weishaar RE, Simpson RU. Regulation of myosin isozyme expression by vitamin D3 deficiency and 1,25-dihydroxyvitamin D3 in the rat heart. *Endocrinology*. 1994;134(2):899-905.
32. Wang GF, Nikovits W Jr, Bao ZZ, Stockdale FE. Irx4 forms an inhibitory complex with the vitamin D and retinoic X receptors to regulate cardiac chamber-specific slow MyHC3 expression. *J Biol Chem*. 2001;276(31):28835-28841.
33. Wolf G. Renal injury due to renin-angiotensin-aldosterone system activation of the transforming growth factor-beta pathway. *Kidney Int*. 2006;70(11):1914-1919.
34. Zerr P, Vollath S, Palumbo-Zerr K, et al. Vitamin D receptor regulates TGF- $\beta$  signalling in systemic sclerosis [published online January 21, 2014]. *Ann Rheum Dis*. 2014. doi:10.1136/annrheumdis-2013-204378.
35. Panizo SI, Barrio-Vázquez S, Naves-Díaz M, et al. Vitamin D receptor activation, left ventricular hypertrophy and myocardial fibrosis. *Nephrol Dial Transplant*. 2013;28(11):2735-2744.
36. Tomaselli GF, Zipes DP. What causes sudden death in heart failure? *Circ Res*. 2004;95(8):754-763.
37. Harada K, Komuro I, Hayashi D, Sugaya T, Murakami K, Yazaki Y. Angiotensin II type 1a receptor is involved in the occurrence of reperfusion arrhythmias. *Circulation*. 1998;97(4):315-317.
38. Yang BC, Phillips MI, Ambuehl PE, Shen LP, Mehta P, Mehta JL. Increase in angiotensin II type 1 receptor expression immediately after ischemia-reperfusion in isolated rat hearts. *Circulation*. 1997;96(3):922-926.
39. de Boer RA, van Geel PP, Pinto YM, et al. Efficacy of angiotensin II type 1 receptor blockade on reperfusion-induced arrhythmias and mortality early after myocardial infarction is increased in transgenic rats with cardiac angiotensin II type 1 overexpression. *J Cardiovasc Pharmacol*. 2002;39(4):610-619.
40. Ramos-Mondragón R, Galindo CA, Avila G. Role of TGF-beta on cardiac structural and electrical remodeling. *Vasc Health Risk Manag*. 2008;4(6):1289-1300.
41. Ng GA, Brack KE, Patel VH, Coote JH. Autonomic modulation of electrical restitution, alternans and ventricular fibrillation initiation in the isolated heart. *Cardiovasc Res*. 2007;73(4):750-760.
42. Hanafy DA, Chang SL, Lu YY, et al. Electromechanical effects of 1,25-dihydroxyvitamin D with antiatrial fibrillation activities. *J Cardiovasc Electrophysiol*. 2014;25(3):317-323.