Paricalcitol modulates ACE2 shedding and renal ADAM17 in NOD mice beyond proteinuria

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Diabetic nephropathy is the leading cause of end-stage renal disease in the developed world (34). Previous studies have suggested that the renin-angiotensin system (RAS) is a major mediator of progressive renal injury in diabetic nephropathy. Drugs that target the RAS, including angiotensin-converting enzyme (ACE) inhibitors and ANG II type 1 receptor blockers, have been shown to reduce the progression of glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria (1, 4, 6, 21, 31). The systemic components of the RAS are altered in diabetes mellitus (32) and the intrarenal RAS is thought to play the major damaging role (25). The angiotensin-converting enzyme 2 (ACE2) has been identified in humans and differs from angiotensin-converting enzyme (ACE) in that it preferentially removes carboxy-terminal hydrophobic or basic amino acids (5, 12). Whereas ACE forms ANG II from ANG I, ACE2 is a major route of ANG II metabolism to ANG 1–7 (5, 39). Thus ACE2 activity may counterbalance the ANG II-promoting effects of ACE by preventing ANG II accumulation in tissues where ACE2 and ACE are both expressed (41, 47). We have previously demonstrated that circulating and renal ACE2 activity is increased early in non-obese diabetic (NOD) mice and that this increase persists as diabetic nephropathy progresses. In addition, this increase can be prevented by chronic administration of insulin (33). Although the source for circulating ACE2 is not currently known, different studies suggest that ACE2 may be actively shed from the vascular surface through metalloproteinases such as a disintegrin and metalloproteinase domain-10 and -17 (ADAM10 and ADAM17, respectively) (17). Recent studies have explored the relationship between ACE2 and ADAM17 in experimental models of diabetic nephropathy (8, 42). Those studies with Akita and db/db mice demonstrated a decrease in ADAM17 when blood glucose is controlled and, as a consequence, urinary ACE2 is decreased. Urinary ACE2 activity was increased in Akita mice and was associated with increased renal ACE2 and ADAM17 protein expression. In addition, renal proximal tubular cells treated with ADAM17 inhibitor showed reduced ACE2 shedding into the media, confirming ADAM17-mediated shedding of ACE2 (36). These results indicate that ACE2 may be actively shed from the vascular surface and proximal tubule luminal surfaces resulting in ACE2 in the urine.

The hormonal form of vitamin D, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], is a negative endocrine regulator of the RAS. The molecule 1,25(OH)2D3 suppresses renin biosynthesis (22, 51), and null mutant mice lacking the vitamin D receptor (VDR) gene develop hyperreninemia, high blood pressure, and cardiac hypertrophy (19, 22, 48). Recent studies showed that in the diabetic state, VDR-null mice developed more severe nephropathy than wild-type mice (52), suggesting that vitamin D plays a protective role against hyperglycemia-induced renal injury via its involvement in regulation of the RAS.

With these premises, the role of paricalcitol, the synthetic vitamin D analog, on modulating ACE2, remains unknown. In the present study, we examined the role of paricalcitol in modulating circulating and renal ACE2 in NOD mice. We also studied the renal content of ADAM17 as a responsible agent for ACE2 shedding.

Riera M, Anguiano L, Clotet S, Roca-Ho H, Rebull M, Pascual J, Soler MJ. Paricalcitol modulates ACE2 shedding and renal ADAM17 in NOD mice. Am J Physiol Renal Physiol 310: F534–F546, 2016. First published December 23, 2015; doi:10.1152/ajprenal.00082.2015.—Circulating and renal activity of angiotensin-converting enzyme 2 (ACE2) is increased in non-obese diabetic (NOD) mice. Because paricalcitol has been reported to protect against diabetic nephropathy, we investigated the role of paricalcitol in modulating ACE2 in these mice. In addition, renal ADAM17, a metalloprotease implied in ACE2 shedding, was assessed. NOD female and non-diabetic control mice were studied for 21 days after diabetes onset and divided into various treatment groups. Diabetic animals received either vehicle; 0.4 or 0.8 μg/kg paricalcitol, aliskiren, or a combination of paricalcitol and aliskiren. We then studied the effect of paricalcitol on ACE2 expression in proximal tubular epithelial cells. Paricalcitol alone or in combination with aliskiren resulted in significantly reduced circulating ACE2 activity in NOD mice but there were no changes in urinary albumin excretion. Serum renin activity was significantly decreased in mice that received aliskiren but no effect was found when paricalcitol was used alone. Renal content of ADAM17 was significantly decreased in animals that received a high dose of paricalcitol. Renal and circulating oxidative stress (quantified by plasma H2O2 levels and immunolocalization of nitrotyrosine) were reduced in high-dose paricalcitol-treated mice compared with non-treated diabetic mice. In culture, paricalcitol incubation resulted in a significant increase in ACE2 expression compared with nontreated cells. In NOD mice with type 1 diabetes, paricalcitol modulates ACE2 activity, ADAM17, and oxidative stress renal content independently from the glycemic profile and urinary albumin excretion. In tubular cells, paricalcitol may modulate ACE2 by blocking its shedding. In the early stage of diabetic nephropathy, paricalcitol treatment counterbalances the effect of diabetes on circulating ACE2 activity. Our results suggest that additional use of paricalcitol may be beneficial in treating patients with diabetes under standard therapeutic strategies.

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PARICALCITOL MODULATES ACE2/ADAM17 IN NOD MICE

Table 1. Animal characteristics at the end-point of the study

<table>
<thead>
<tr>
<th>Number</th>
<th>Age, wk</th>
<th>Blood glucose*, mM</th>
<th>Blood glucose†, mM</th>
<th>UAE, mg albumin/ mg creatine</th>
<th>Kidney-to-body weight ratio, %</th>
<th>Heart-to-body weight ratio, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>12</td>
<td>21.42 ± 1.01</td>
<td>7.50 ± 0.86</td>
<td>9.30 ± 0.32</td>
<td>0.90 ± 0.03</td>
<td>0.43 ± 0.02</td>
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<tr>
<td>C-PARI_H</td>
<td>6</td>
<td>22.02 ± 0.02</td>
<td>7.13 ± 0.71</td>
<td>8.82 ± 0.54</td>
<td>0.81 ± 0.06</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>NOD</td>
<td>20</td>
<td>19.78 ± 0.69</td>
<td>25.66 ± 1.66</td>
<td>34.92 ± 0.63</td>
<td>1.61 ± 0.05</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>NOD+PARI_L</td>
<td>21</td>
<td>19.90 ± 0.63</td>
<td>25.41 ± 1.47†</td>
<td>32.18 ± 1.47†</td>
<td>1.51 ± 0.04†</td>
<td>0.36 ± 0.01†</td>
</tr>
<tr>
<td>NOD+PARI_H</td>
<td>20</td>
<td>20.19 ± 0.58</td>
<td>20.41 ± 2.36†</td>
<td>32.10 ± 2.16†</td>
<td>1.58 ± 0.04†</td>
<td>0.37 ± 0.02†</td>
</tr>
<tr>
<td>NOD+ALSK</td>
<td>16</td>
<td>21.02 ± 0.59</td>
<td>24.32 ± 2.13†</td>
<td>32.89 ± 2.12†</td>
<td>1.51 ± 0.05†</td>
<td>0.39 ± 0.02†</td>
</tr>
<tr>
<td>NOD+ALSK+PARI_L</td>
<td>13</td>
<td>19.19 ± 0.77</td>
<td>25.65 ± 1.46†</td>
<td>32.59 ± 1.34†</td>
<td>1.62 ± 0.05†</td>
<td>0.40 ± 0.03†</td>
</tr>
</tbody>
</table>

CONT, control mice; C-PARI-H, control mice given 0.8 µg/kg paricalcitol; NOD+PARI_L, NOD mice given 0.4 µg/kg paricalcitol; NOD+PARI_H, NOD mice given 0.8 µg/kg paricalcitol; NOD+ALSK, NOD mice given aliskiren; NOD+ALSK+PARI_L, NOD mice given 0.4 µg/kg paricalcitol and aliskiren in combination. UAE, urinary albumin excretion. *Time = 0 days. †Time = 21 days. □ P < 0.05 vs. CONT; □ P < 0.05 vs. NOD.

MATERIALS AND METHODS

Animals. Female NOD/ShiLtJ and control female NOR/LJ mice (The Jackson Laboratory, Bar Harbor, ME) were used as a model of type 1 diabetes mellitus. The development of insulin-dependent diabetes in NOD mice is preceded by insulinitis at 3–4 wk of age. Virtually all female and male NOD mice have insulinitis by 3 mo of age, but only 20% of males and 80% of females develop diabetes between 16 and 24 wk of age (35).

The mice were housed in groups of three per cage with ad libitum access to mouse chow and water under a 12:12-h light:dark cycle. Animals were maintained under specific pathogen-free conditions in ventilated microisolators. The Institutional Animal Care and Use Committee of Hospital del Mar-Institut Hospital del Mar d’Investigacions Mèdiques approved all procedures, and all experiments adhered to the Spanish Guide for the Care and Use of Laboratory Animals.

Animals included in the study were chosen on the basis of blood glucose level measurements (ACCU-CHEKCompact; Roche, Germany) in NOD mice starting at 12 wk of age. Diabetes was considered established after two consecutive values of >13.87 mM.

Study groups. Diabetic mice were randomly assigned to one of five study groups and followed for 21 days: 1) NOD (n = 20), NOD mice that received vehicle; 2) NOD+PARI_L (n = 21), NOD mice that received 0.4 µg/kg (low-dose) of paricalcitol; 3) NOD+PARI_H (n = 20), NOD mice that received 0.8 µg/kg (high-dose) of paricalcitol; 4) NOD+ALSK (n = 16), NOD mice that received aliskiren (a specific renin inhibitor); and 5) NOD+ALSK+PARI_L (n = 13), NOD mice that received 0.4 µg/kg of paricalcitol and aliskiren.

NOR mice were used as nondiabetic controls. A control group (CONT) received vehicle (n = 12), another group received high-dose paricalcitol (C-PARI_H) (n = 6).

Therapeutic interventions. Paricalcitol was diluted in 90:10 propylene glycol:ethanol (Sigma-Aldrich, Spain) at a final concentration of 0.16 or 0.32 µg/ml depending on the treatment group. Animals received the assigned dose intraperitoneally thrice weekly.

Aliskiren was diluted in saline at 189 mg/ml. The solution was administered continuously by miniosmotic pumps (ALZET; Durect Corp, USA) at 0.11 µl/h. Hence animals received 0.5 mg of aliskiren per day. Miniosmotic pumps were implanted subcutaneously under ketamine/medetomidine anesthesia. After surgery, atipamezol was injected subcutaneously to reverse the effects of medetomidine.

Monitoring. Mice were monitored for body weight and blood glucose levels weekly and then sacrificed after 21 days of follow-up. Under sodium pentobarbital anesthesia blood was collected by intracardiac puncture and serum was maintained at −20°C until it was used. Afterward, mice were perfused with cold PBS by transcardiac puncture to flush out blood. Kidneys and hearts were then removed, weighed, and processed for several purposes. Half of one kidney was fixed in a 10% neutral-buffered formalin solution and processed for paraffin embedding according to standard procedures. The remaining tissue was snap-frozen and kept at −80°C until it was used.

Blood pressure. Noninvasive blood pressure was measured using the tail-cuff method (CODA; Kent Scientific, Torrington, CT). Values were obtained from conscious-trained mice on six consecutive morning sessions by the same research assistant. Mice were placed on a heating platform at 33°C in an acyclic restrainer, and a tail-cuff and pulse sensor was introduced along the tail. The tail cuff is connected to a cylinder of compressed air through an arrangement of inlet and outlet valves that permit inflation and deflation of the cuff at a constant rate. The animals need to become familiar with the procedure and remain calm within the restrainer. Results were calculated as the mean from the valid values of 30 measurements in each session and expressed in mmHg.

Albuminuria. Urinary albumin excretion was determined using the albumin-to-creatinine ratio (ACR) on morning spot urine collections. A commercial ELISA kit was used for albumin quantification (Albuwell M; Exocell, Philadelphia, PA) and a colorimetric assay kit (Creatinine Companion) was used to quantify creatinine levels (41).

ACE2 enzymatic activity. The ACE2 fluorescent enzymatic assay was performed as previously described using an ACE2-quenched fluorogenic substrate [Mca-Ala-Pro-Lys(Dnp)-OH; Enzo LifeSciences, Farmingdale, NY] (33). In addition, recombinant ACE2 (5 ng) and ascending doses of paricalcitol were incubated to discern a possible direct effect of paricalcitol on the enzymatic activity assay, as an internal control. After a 16-h incubation, paricalcitol did not substantially modify recombinant ACE2 activity (data not shown).

For serum and urine analysis, a 2-µl sample was incubated with ACE2 assay buffer [100 mM Tris-HCl, 600 mM NaCl, 10 µM ZnCl2, pH 7.5 in the presence of protease inhibitors 100 µM captopril, 5 µM amastatin, 5 µM bestatin, and 10 µM Z-Pro-prolin (from Sigma-Aldrich and Enzo LifeSciences)] and 10 µM fluorogenic substrate in a final volume of 100 µl at 37°C for 16 h. Serum ACE2 cleaves the substrate proportionally to the enzyme activity. The plates were read using a fluorescence plate reader (Tecan Infinite 200; Germany) at λex320 nm and λem400 nm. Results were expressed as relative fluorescent units (RFU) per microliter per hour (RFU·µl−1·h−1).

Table 2. Percentages of reduction in urinary albumin excretion

<table>
<thead>
<tr>
<th></th>
<th>NOD+PARI_L</th>
<th>NOD+PARI_H</th>
<th>NOD+ALSK</th>
<th>NOD+ALSK+PARI_L</th>
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<tbody>
<tr>
<td>Albumin reduction, %</td>
<td>17.1</td>
<td>38.4</td>
<td>53.6</td>
<td>14.3</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>44.091.0</td>
<td>9.076.0</td>
<td>27.088.0</td>
<td>−9.5/85.8</td>
</tr>
</tbody>
</table>
For tissues, 50 mg of kidney cortex was homogenized in buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.025 mM ZnCl₂ (Sigma-Aldrich)], 0.1 mM PefablocSC Plus and EDTA-free protease inhibitor cocktail tablet (Roche) and clarified at 14,000 g for 10 min at 4°C. After measuring protein concentration using the MicroBCA assay kit (Thermo Scientific, Waltham, MA), samples were diluted in the ACE2 assay buffer as used for serum and urine samples. To each well, 0.25 µl of protein in 50 µl of assay buffer was added with or without the specific ACE2 inhibitor MLN-4760 (final concentration 10 µM) and the reaction was initiated by addition of 50 µl of the substrate (final concentration 5 µM). Activity was determined after a 4-h incubation at 37°C. Experiments were carried out in duplicate for each data point. Results after subtraction of the inhibition value were expressed as RFU·µg⁻¹·h⁻¹.

ACE enzymatic activity. The ACE fluorescent enzymatic assay was performed as previously described with modifications (29, 38). The assay quantifies the fluorescent adduct of the enzyme-catalyzed product after incubation with mouse samples. For serum, 0.25 µl of sample was incubated with 75 µl of assay buffer containing 5 mM Hip-His-Leu in 0.4 M borate buffer and 0.9 M NaCl, pH 8.3, for 25 min at 37°C in a 96-well plate. The reaction was stopped by adding 0.18 ml of 0.28 N NaOH. The fluorescent product was formed when 20 mg/ml of o-phthaldialdehyde in methanol was added and, after 10 min, the solution was acidified with 3 N HCl (all from Sigma-Aldrich). Fluorescence was read using Tecan Infinite 200 at λex360 nm and λem485 nm. Results were expressed as RFU/µl.

For kidney tissue, the homogenate obtained for ACE2 enzymatic activity detection was used. ACE activity was determined in tubes by adapting the protocol for serum samples by adding 1 g of renal protein. After a 25-min incubation, samples were clarified at 800 g for 5 min and dispensed into a 96-well plate. Results were expressed as RFU/µg.

Fig. 1. Cardiovascular parameters. Blood pressure and heart rate were measured 1 wk before the end of the study. A: systolic blood pressure was increased in diabetic animals compared with controls. Aliskiren treatment resulted in lower systolic and diastolic blood pressures in diabetic animals. B: control animals showed higher heart rate values than NOD mice. No treatment effect was observed. *P < 0.05 vs. CONT; #P < 0.05 vs. NOD. CONT, control animals; CONT+PARI_H, control mice that received 0.8 g/kg of paricalcitol; NOD, NOD mice that received vehicle; NOD+PARI_L, NOD mice that received 0.4 µg/kg of paricalcitol; NOD+PARI_H, NOD mice that received 0.8 µg/kg of paricalcitol; NOD+ALSK, NOD mice that received aliskiren; NOD+ALSK+PARI_L, NOD mice that received aliskiren and 0.4 µg/kg of paricalcitol.
**Serum renin activity.** A commercial kit (SensoLyte 520 Mouse Renin Assay Kit; AnaSpec, Belgium) was used to detect renin activity in 5 μl of serum. Activity was detected after a 2-h incubation at 37°C with the specific fluorogenic substrate provided with the kit. In addition, the specificity of the assay was assessed by the use of aliskiren. Fluorescence was detected in the Tecan Infinite 200 at λex490 nm and λem520 nm. Results were expressed as RFU/5 μl.

**Quantification of ANG II and ANG 1–7.** Cortical content of ANG II and ANG 1–7 were determined using commercial ELISA kits (Peninsula Laboratories). For this purpose, 50 mg of renal cortex was homogenized in 1 ml of pure methanol and centrifuged and evaporated at 3,000 g at 37°C for 1 h. Samples were reconstituted with ELISA buffer. Protein content was determined by bicinchoninic acid assay (Pierce). ELISA was performed according to the manufacturer’s instructions. Results were expressed as picograms of peptide per milligram of total renal protein (pg/mg).

**Renal cortex ADAM17 quantification.** ADAM17 was determined in 5 μg of total protein extracts from renal cortex obtained for ACE2 activity detection. Renal ADAM17 levels were determined with a Human TACE/ADAM17 DuoSet (R&D Systems, UK) following the manufacturer’s instructions. Results were calculated using the standard curve provided by the kit and expressed as picograms of ADAM17 per micrograms of total renal protein (pg/μg).

**Western blot analysis.** Kidney cortex samples were homogenized and Western blot analysis was performed as previously described (33).

The following antibodies were used: ACE2 (1:2,000; Abcam, UK), ACE (1:1,000; Bioworld Technology, St. Louis Park, MN), and β-actin (1:4,000; Sigma Aldrich). Protein bands were detected by chemiluminescence (ECL Plus, GE Healthcare Life Science, Germany) on Curix RP2 film (Agfa Healthcare, Belgium). Band densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD) and results were expressed as ACE2 or ACE to β-actin ratio.

**Immunohistochemistry.** Paraffin blocks were cut into 3-μm sections. Samples were first boiled in 10 mM sodium citrate solution (pH 6.0) for antigen retrieval. The sections were incubated with anti-ACE2 (R&D Systems), anti-renin (E-17) (Santa Cruz Biotechnology, Dallas, TX), and antinitrotyrosine (Millipore, Billerica, MA) antibodies. Proteins were visualized with the EnVision System-HRP (Dako, Denmark). Sections were counterstained with hematoxylin and analyzed in a blinded fashion. The intensity of the staining was quantified with ImageJ software (33).

**Gene expression.** Total RNA was extracted from 50 mg of renal cortex using TriPure Isolation Reagent (Roche). RNA quantity and purity were analyzed with NanoDrop (ND-1000V3.3). First-strand cDNAs were synthesized from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) by incubating for 10 min at 25°C, 120 min at 37°C, and 5 min at 85°C. Real-time PCR was performed in the LightCycler 480 System (Roche) using SYBR Green I Master and the standard curve method for relative quantification. The β-actin gene served as the housekeeping gene. The sequences of the primers were as follows: Ace2_F, ACC CTT CCT ACA TCA GCC CCA CTG; Ace2_R, TGT CCA AAA TCT ACC CCA CAT AT; Ace_F, CGC CGC TAT GAG GAC AAA TA; Ace_R, ATG TCT CCC AGC AAA TGG GC; Ren_F, TAC GAG TCC CGG AAT TCA AC; Ren_R, AAG AAG CCC AGG ATG TTC TT; Actb_F, TCA CCA TCC ACT ACG GAT CA; and Actb_R, CAC AGT GAT TCC ACC CAC AG.

**Oxidative stress.** Oxidative stress is generally identified by indirect markers such as peroxynitrite production. For this reason, nitrotyrosine staining was used to evaluate superoxide and peroxynitrite levels in renal tissues as previously described (30). Sections (3 μm) of paraffin-embedded tissues were stained with rabbit antinitrotyrosine antibody and with EnVision+System-HRP (DAKO). Slides were counterstained with hematoxylin. Ten microphotographs at ×40 were taken for each sample, and brown-stained areas were quantified with ImageJ software. Data were expressed as arbitrary units of mean gray value (AU of MGV).

As a byproduct of the superoxide dismutase oxidative stress response, serum hydrogen peroxide content was also measured. Following the manufacturer’s instructions, H2O2 content in serum samples was detected using the Amplex Red Assay kit (Invitrogen, UK). The Amplex Red reagent, in combination with horseradish

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**Fig. 2. Angiotensin-converting enzyme-2 (ACE2) activity.** A: serum ACE2 enzyme activity was significantly increased in all NOD mice compared with CONT mice. Administration of paricalcitol in any combination resulted in significantly decreased serum ACE2 activity in NOD mice. B: in the renal cortex, ACE2 activity showed significant differences when NOD and CONT group mice were compared. C: ACE2 activity was measured in spot urine and expressed as milligrams of creatinine. No statistical differences were found among treated and nontreated diabetic animals. For all of these techniques, 10 animals from the CONT group, 6 animals from the CONT+PARI_H group, and 12 animals from each NOD group were analyzed. RFU, relative fluorescent units. *P < 0.05 vs. CONT; #P < 0.05 vs. NOD.
peroxidase, detects H2O2 concentration in 2.5 μl of serum. Results are expressed in micromole (μM).

**Cell culture studies.** A cortical tubule epithelial cell line, MTC (a kind gift from Dr. López Novoa), was grown in RPMI 1640 (Invitrogen) with 10% FBS and antibiotics in 5% CO2 at 37°C. Subconfluent cells serum-starved for 24 h were incubated with 0, 0.04, 0.4, and 4 ng/ml of paricalcitol for 24 h more. Media in these experiments contained high concentrations of glucose (25 mM). For immunofluorescent ACE2 detection, the same experiments were performed on sterilized glass coverslips in 24-well plates. After incubation, cells were washed in PBS and kept at −20°C in methanol. ACE2 primary antibody (R&D Systems) was detected with AlexaFluor 555 secondary antibody (Life Technologies). Coverslips were mounted on glass slides using Mowiol/DAABC+DAP solution (all from Sigma-Aldrich).

Cell staining was examined with a high-resolution spectral confocal Leica TCS SPE microscope. Intensity of staining was measured with ImageJ software and results were expressed as arbitrary units of fluorescence per cell. All analyses were performed in a blinded fashion.

**Statistical analyses.** Statistical analyses were performed using statistical software SPSS 16.0. Because the sample size was small, nonparametric tests were conducted. A Mann-Whitney U-test was used to compare the differences between the groups.

![Graph A](image)

Fig. 3. ACE2 gene and protein expression. A: using real-time quantitative PCR (RT-qPCR), ACE2 gene expression was quantified as that related to the β-actin gene. Gene expression was significantly increased in all treated diabetic groups compared with the NOD group. The increase was also observed in the paricalcitol-treated control group. Six animals from the control groups and 9 animals from the NOD groups were analyzed. B and C: the same number of animals in which ACE2 was detected in renal cortex were used for Western blot analysis. ACE2 protein expression was significantly greater in diabetic animals than in nondiabetic animals (*P < 0.05). Paricalcitol administration resulted in significantly higher ACE2 protein expression in diabetic and control animals.

D and E: immunohistochemistry localized ACE2 protein expression to the renal cortex, where high levels of it were detected in paricalcitol-treated groups. *P < 0.05 vs. CONT; #P < 0.05 vs. NOD.
used to compare between two groups. In addition, a Kruskal-Wallis test was performed for multiple comparisons in the study. Significance was defined as $P < 0.05$ and data are expressed as means ± SE.

RESULTS

Physiological parameters. NOD mice were used to determine whether administration of vitamin D analog modifies physiological parameters and albuminuria. Diabetic mice were treated with paricalcitol (high or low dose), aliskiren alone, or both in combination. NOR mice were used as nondiabetic controls. Glucose levels were similar in all diabetic groups at the time of inclusion (Table 1). At the end of the study, 21 days after diabetes diagnosis, the mean blood glucose levels in all NOD groups were maintained around 30 mM without statistical differences between them.

When percentages of kidney weight to body weight were compared between groups, NOD mice exhibited a significant increase compared with NOR mice, indicating the effect of early diabetes on renal hypertrophy. Only treatment with low-dose paricalcitol (NOD+PARI_L) or aliskiren (NOD+ALSK) modulated this effect and significantly decreased the percentages compared with the untreated diabetic group.

Heart/body weight ratio was not different between control and diabetic groups. However, paricalcitol-treated (at both doses) NOD mice showed significantly reduced heart/body weight ratio compared with the untreated NOD group (Table 1).

ACR in spot urine sample was determined to evaluate albuminuria at the end of the study. In this model of early diabetic nephropathy, NOD mice exhibit significantly in-

Fig. 4. ACE2 protein expression in MTC cells. To determine the effect of paricalcitol on tubular cells, MTC cells were incubated in high glucose (25 mM), and one of three different concentrations of paricalcitol was added: 0.04, 0.4, or 4 ng/ml. After 24 h of incubation, ACE2 was immunolocalized (A) and quantified using ImageJ software (B). ACE2 expression increased in a dose-dependent manner with paricalcitol incubation. *$P < 0.05$ vs. CONT.
creased albuminuria compared with control mice (Table 1).
Treatments resulted in slightly decreased levels of ACR in diabetic mice compared with untreated diabetic animals but without statistical significance (Table 2).

**Blood pressure and cardiac rate.** Systolic blood pressure (SBP) in conscious animals was significantly increased in NOD mice compared with control group mice (Fig. 1A). No effect of paricalcitol was observed. As expected, aliskiren treatment resulted in a significant decrease in SBP compared with untreated NOD mice. Diastolic blood pressure measurements followed the same profile (Fig. 1A). The tail-cuff method also allowed us to measure heart rates in all animals. Heart rates were significantly decreased in untreated diabetic mice compared with the nondiabetic group. No differences were found when nontreated and treated diabetic groups were compared (Fig. 1B).

**ACE2 activity and expression.** To study the effect of vitamin D analog on ACE2 we performed enzymatic activity and expression assays in serum and kidney cortex. As shown in our previous work, circulating ACE2 activity was significantly increased in the diabetic group compared with the nondiabetic control group (Fig. 2A). Paricalcitol administration resulted in significantly decreased ACE2 activity in all treated diabetic mice. Interestingly, aliskiren administration alone did not modify ACE2 activity in diabetic mice.

ACE2 enzyme activity was also tested in renal cortex homogenates. As shown in serum, ACE2 activity was significantly increased in renal cortex from diabetic mice compared with nondiabetic control mice (Fig. 2B). However, no differences were observed between treated and nontreated diabetic groups. In addition, a similar pattern was observed when ACE2 enzyme activity was assessed in urine (Fig. 2C).

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*Fig. 5. ACE expression and activity. A: serum ACE activity was elevated in all diabetic mice compared with CONT mice. B: ACE activity was decreased in diabetic animals compared with animals in the CONT group. High-dose paricalcitol led to a significant increase in ACE activity by returning values to basal levels. C: protein expression quantification by ImageJ software; D: representative image of a Western blot. E: gene expression from renal cortex. Protein and gene expressions displayed similar profiles without statistical differences. Of note, high-dose paricalcitol resulted in a significant increase in renal ACE gene expression compared with all other groups. For these analyses, 10 animals in each group were studied. *P < 0.05 vs. CONT; #P < 0.05 vs. NOD.*
ACE2 gene and protein expressions were analyzed in the renal cortex of all animals by real-time PCR and Western blot. Furthermore, immunohistochemistry studies in renal samples were also performed. Ace2 gene expression levels were similar in diabetic and nondiabetic mice. Interestingly, ace2 gene expression was significantly increased in all diabetic treated mice compared with diabetic untreated mice (Fig. 3A). As previously shown by other researchers (3, 20, 45), the lack of increase in ace2 gene expression in nontreated diabetic animals was not translated to the protein level (Fig. 3). Thus ACE2 protein expression in kidney cortex was significantly increased in diabetic mice compared with control mice. Paricalcitol administration alone resulted in significantly increased ACE2 protein and gene expressions in kidney cortex in both control and diabetic mice compared with vehicle-treated mice (Fig. 3). As shown in Fig. 3E, kidney ACE2 staining was observed mainly in the brush border of proximal tubules. Note that the intensity and distribution of the positive signal resembled the profile described by Western blot analysis.

ACE2-dependent paricalcitol expression in tubular epithelial cells. To test the direct effect of paricalcitol on renal tubular epithelial cells, cultured MTC cells in high-glucose medium were exposed to increasing doses of paricalcitol for 24 h. As shown in Fig. 4, paricalcitol at 0.4 and 4.0 ng/ml resulted in significantly greater ACE2 expression in renal tubular epithelial cells compared with control cells. Thus incubation with paricalcitol resulted in increased ACE2 expression in a dose-dependent manner.

ACE activity and expression. To elucidate the possible interplay of ACE2 with other RAS components we examined the modifications in ACE, not only its serum and cortical enzyme activity, but also its gene and protein expression. As shown in Fig. 5A, circulating ACE activity in diabetic mice was greater than it was in control mice. No differences were observed in the treated diabetic groups of mice.

As previously described, ACE activity was significantly decreased in the renal cortex of diabetic mice compared with controls (Fig. 5B). Administration of high-dose paricalcitol resulted in ACE activity being significantly restored. Thus ACE activity was significantly increased in paricalcitol-treated mice compared with untreated diabetic mice. The other treatments had no influence on cortical ACE activity. In addition, administration of high-dose paricalcitol resulted in significantly higher ACE gene expression in renal tissue (Fig. 5, C and D).

Renin activity and expression. Previous studies have shown the effect of paricalcitol in reducing cortical renin gene expression. For this reason, this study also explored this effect. Renin activity was tested by using recombinant renin and aliskiren. In addition, the direct effect of aliskiren on activity detection in the NOD group was also assessed (Fig. 6A). Treatment of diabetic mice exhibited a significant increase in serum renin activity compared with controls. Administration of aliskiren resulted in a significant decrease in renin activity in the treated diabetic group. Paricalcitol exerted minimal effects on serum renin activity compared with results from untreated diabetic mice (Fig. 6B). In concordance with previous studies, as shown in Fig. 6C, administration of high-dose paricalcitol resulted in significantly decreased renin gene expression in diabetic mice. In contrast, mice that received aliskiren exhibited a large increase in renin gene expression compared with untreated diabetic and control mice. Immunohistochemistry revealed a significant increase in protein expression in the renal cortex in aliskiren-treated mice compared with NOD mice (Fig. 7). High-dose paricalcitol induced different patterns of expression in the tubule than in the juxtaglomerular apparatus: although it
led to higher renin expression in the tubule, no changes were observed in the juxtaglomerular apparatus.

Renal ANG II and ANG 1–7 levels. Renal levels of ANG II and ANG 1–7 were measured in a small portion of renal cortex of diabetic mice. ANG II levels were significantly decreased in the group that received paricalcitol and aliskiren in combination (Fig. 8A). There were no changes in the other treated groups. When ANG 1–7 was analyzed, the aliskiren-treated group exhibited a significantly lower level than other groups (Fig. 8B). As the balance of two peptides, ANG 1–7 and ANG II ratio was significantly greater in the group that received paricalcitol and aliskiren in combination (Fig. 8C).

Oxidative stress. Because oxidative stress has also been implicated in the pathogenesis of renal injury mediated by RAS peptides, we performed two different assays; we measured H$_2$O$_2$ in serum and nitrotyrosine in paraffin-embedded kidney tissue from diabetic mice.

Administration of high-dose paricalcitol or paricalcitol and aliskiren in combination resulted in significantly lower levels of circulating H$_2$O$_2$ compared with untreated diabetic mice (Fig. 9A). In addition, administration of high-dose paricalcitol resulted in significantly decreased nitrotyrosine staining compared with untreated diabetic mice (Fig. 9, B and C).

Renal ADAM17 content. ADAM17 is one of the metalloproteinases implicated in ACE2 shedding. For this reason we determined ADAM17 content in renal cortex extracts from each group (Fig. 10). As expected, untreated diabetic mice showed higher levels of ADAM17 than nondiabetic control mice. Administration of high-dose paricalcitol resulted in significantly decreased ADAM17 levels compared with those in untreated diabetic mice.

![Fig. 7. Renal renin expression.](https://example.com/fig7)

![Graph A](https://example.com/graphA)

![Graph B](https://example.com/graphB)

![Graph C](https://example.com/graphC)

![Graph D](https://example.com/graphD)

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Fig. 7. Renal renin expression. In cortical tubules at $\times$20 magnification, immunohistochemistry showed an increase in renin expression in groups that received aliskiren (A and B) and juxtaglomerular JG apparatus at $\times$40 magnification (C and D) compared with nontreated NOD mice. AU of MGV, arbitrary units of mean gray value. *$P < 0.05$ vs. CONT; #P < 0.05 vs. NOD.
Paricalcitol administration did not lead to a modification of blood glucose levels or urinary albumin excretion in diabetic mice. ACE2 activity has been shown to be altered in diabetic kidney disease, hypertensive renal disease, and various models of kidney injury (3, 40, 49, 50). Our group has recently demonstrated that circulating and renal ACE2 is increased in NOD mice, and that enzyme expression was lowered by insulin treatment (33). Thus when blood glucose levels are normalized with insulin therapy, both circulating and renal ACE2 returns to basal levels (33). Interestingly, in the present work we showed that circulating ACE2 activity is downregulated by paricalcitol administration, but blood glucose levels and blood pressure do not change, suggesting a direct implication of paricalcitol in controlling circulating ACE2 levels independent of blood pressure and glycemic levels.

Previous studies in experimental animal models have investigated the renoprotective effect of paricalcitol administration in diabetic kidney disease. Zhang et al. (53) demonstrated a slight reduction in ACR levels in monotherapy with paricalcitol or losartan. However, combination therapy leads to a significant reduction in albuminuria in STZ-diabetic mice. In concordance, this therapy has resulted in a complete normalization of the structure of the glomerular filtration barrier, preventing glomerular basement membrane thickening and podocyte effacement (53). In that work the authors hypothesized that the beneficial effect of paricalcitol was that it blocked the compensatory renin rise, leading to more effective RAS inhibition. In contrast, in our study paricalcitol administration either as monotherapy or in combination with aliskiren was unable to prevent albuminuria in the NOD model. One limiting point would be the origin of the urine samples. Collecting urine from metabolic cages instead from the morning urinary spot would be more informative. However, in concordance with our results, a recently published work that explored the potential anti-inflammatory effects of VDR agonists at different doses demonstrated that paricalcitol did not modulate proteinuria in STZ-diabetic rats (37). The dose unable to modulate proteinuria was 0.75 μg/kg, which is very similar to the high-dose treatment used in our study (0.8 μg/kg). In agreement with these results, in a large randomized clinical trial, low-dose paricalcitol did not have an antiproteinuric effect in patients with diabetic nephropathy (11).

Another beneficial effect of paricalcitol is that it activates both antihypertrophic and antifibrotic activity in a murine model of cardiac hypertrophy and fibrosis generated by chronic infusion of ANG II (7, 15, 43). Accordingly, in our model, animals given paricalcitol showed significant reductions in heart/body weight ratios compared with other diabetic animals. Furthermore, the antihypertrophic activity of VDR agonists/agonists has been demonstrated by several studies in rodent models of cardiac hypertrophy. Thus paricalcitol and 1,25(OH)2D3 have been shown to reverse or prevent the hypertrophic phenotype in various models of experimental hypertension such as Dahl S rats (10), spontaneously hypertensive rats (18), and heart failure-prone spontaneously hypertensive rats (23). In addition, this protective effect was also observed in the 5/6 nephrectomy rodent, a model of chronic renal failure (26).

In our hands, NOD mice treated with high-dose paricalcitol showed similar SBP values compared with untreated diabetic animals. However, low-dose administration trended to de-

**DISCUSSION**

The role of vitamin D analogs in modifying circulating ACE2 in diabetes mellitus is unknown. In this work we demonstrated that administration of paricalcitol resulted in reduced circulating ACE2 activity in type 1 diabetic NOD mice. Interestingly, this modulation of ACE2 was associated with decreased ADAM17 in the kidneys of these mice. Of note, paricalcitol administration did not lead to a modification of blood glucose levels or urinary albumin excretion in diabetic mice.
crease SBP without statistical difference. To our knowledge, modulation of blood pressure by paricalcitol administration has not been shown (19, 46, 54). As expected, mice given aliskiren exhibited a significant decrease in SBP compared with non-treated diabetic mice. Thus modulation of paricalcitol in circulating or renal RAS components may be independent of blood pressure control. Plasma renin activity was significantly lower in aliskiren-treated NOD mice. As expected, aliskiren treatment resulted in increased renal mRNA and protein expression of renin. These results are in concordance with studies by Choi et al. (9) who used an experimental unilateral ureteral obstruction mouse model.

The increase in serum ACE2 activity in diabetes has been previously published (33, 44, 47, 49). However, our study describes the effects of paricalcitol on ACE2 activity. Interestingly, compared with nontreated diabetic animals, in diabetic animals, paricalcitol at both doses resulted in significantly decreased circulating ACE2 activity. Although the source for circulating ACE2 is not currently known, different studies suggest that ACE2 may be actively shed from the cell surface through metalloproteinase such as ADAM10 and ADAM17 (17). Recent studies have explored the relationship between ACE2 and ADAM17 in experimental models of diabetic nephropathy (8, 42). Those works with Akita and db/db mice have demonstrated a decrease in ADAM17 when blood glucose is controlled and, as a consequence, ACE2 is decreased. None of these works describe a pharmacological intervention against ADAM17. The direct effect of paricalcitol in inhibiting this sheddase in the context of renal osteodystrophy and parathyroid hyperplasia in renal disease has been previously explored (2, 13). Recently, Morgado-Pascual et al. (27) demonstrated the inhibitory and protective effects of paricalcitol on ADAM17 in cultured tubular epithelial cells.

Our study indicates a direct involvement of paricalcitol in the modulation of circulating ACE2 activity in animals in which blood glucose is pathologically elevated. At the renal level, ACE2 protein

Fig. 9. Oxidative stress. A: hydrogen peroxide concentration in plasma from diabetic mice was significantly decreased in mice that received high-dose paricalcitol. B and C: quantification of nitrotyrosine in renal cortex from NOD mice. A significant reduction was observed only in the group that received high-dose paricalcitol. For these analyses, n = 10 animals in each group. *P < 0.05 vs. CONT; #P < 0.05 vs. NOD.

Fig. 10. ADAM17 content in renal cortex was determined by ELISA. Diabetes resulted in a significant increase in ADAM17 renal content. Treatments reduced these levels. High-dose paricalcitol resulted in a significant decrease in these levels compared with animals in the nontreated diabetic group. For these analyses, n = 10 animals in each group. *P < 0.05 vs. CONT; #P < 0.05 vs. NOD.
increases in diabetes and further elevation was observed in paricalcitol-treated mice. This increase was coupled with higher levels of gene expression in treated diabetic mice. By contrast, renal ACE activity was decreased in diabetic mice and administration of high-dose paricalcitol significantly restored this decrease. In addition, renal ace gene expression was significantly increased after high-dose paricalcitol treatment. When ANG II and ANG (1–7) were measured, an increase in the ratio was observed in treated diabetic animals that reached statistical significance with paricalcitol and aliskiren in combination. The effect of diabetes on ACE2 and ACE expression within the kidney was contralregulated by vitamin D administration. One expects that paricalcitol administration will favor the ANG1-7/ANG II axis, however, the lack of this effect maybe related to the increase in ACE activity and gene expression in our model.

In diabetic animals, renal tubules depicted high levels of ACE2 enzymatic activity that may increase when paricalcitol is administered. Activity detection is unable to show differences between treated and nontreated animals. However, when using Western blots and real-time PCR, these differences become visible, showing that diabetic animals treated with paricalcitol express higher levels of protein and gene in renal cortex than nontreated diabetic animals. These results suggest that the increase in ACE2 protein expression within the kidney cortex may be related to the increase in ACE2 mRNA levels in paricalcitol-treated animals. To check the effect of paricalcitol within the renal tubules, in vitro assays with MTC cells were performed. Our studies in cultured cells demonstrated that tubular cells treated with paricalcitol showed an increase in ACE2 protein expression, again indicating this protective role of paricalcitol in the diabetic milieu. Of note, previous studies have shown that insulin administration to the podocyte also resulted in greater ACE2 expression. This effect was associated with a decrease in fibrosis markers and podocyte apoptosis (24, 25). Whether the beneficial effect of insulin administration in diabetic nephropathy is in part related to glycemic milieu control, our results indicate that paricalcitol exerts its renoprotective effect without changing the glycemic status.

Another contribution of our work has been to study the effect of paricalcitol in modulating renal oxidative stress in this diabetic mouse model. This analysis was performed by means of quantifying plasma H$_2$O$_2$ levels and the immunolocalization of nitrotyrosine as an indicator of nitric oxide-dependent oxidative stress. We demonstrated that administration of high-dose paricalcitol offered the most effective treatment for reducing circulating and renal oxidative stress. In accordance with our findings, a recent study that used rats with a phenotype that resembles type 2 diabetes describes a reduction in oxidative stress and renal lesions when animals were treated with the active vitamin D analog maxacalcitol (28). Furthermore, the antioxidant role of paricalcitol administration also has been described in other experimental models and clinical studies (14, 16).

In conclusion, in this model of type 1 diabetes, paricalcitol exerts a renoprotective effect in terms of modifying circulating and renal ACE2 and reducing oxidative stress. Interestingly, this effect is observed beyond lowering blood pressure and proteinuria. We postulate that administration of paricalcitol by keeping high levels of ACE2 in tubular cells and low levels of ACE2 within the circulation may help to slow the progression of diabetic nephropathy.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

M. Riera, J.P., and M.J.S. conception and design of research; M. Riera, L.A., S.C., H.R.-H., and M. Rebull performed experiments; M. Riera, L.A., S.C., and M.J.S. analyzed data; M. Riera and M.J.S. interpreted results of experiments; M. Riera prepared figures; M. Riera and M.J.S. drafted manuscript; M. Riera, J.P., and M.J.S. edited and revised manuscript; J.P. and M.J.S. approved final version of manuscript.

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