Targeted vitamin D receptor expression in juxtaglomerular cells suppresses renin expression independent of parathyroid hormone and calcium

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Previously, we showed that vitamin D receptor gene knockout leads to hyperreninemia independent of calcium metabolism; however, the contribution of parathyroid hormone to renin upregulation remained unclear. Here we separated the role of vitamin D and parathyroid hormone in the regulation of renin expression *in vivo* by generating transgenic mice that overexpressed the human vitamin D receptor in renin-producing cells using the 4.1 kb Ren-1c gene promoter. Targeting of human vitamin D receptor to the juxtaglomerular cells of the mice was confirmed by immunohistochemistry. Renal renin mRNA levels and plasma renin activity were decreased in these transgenic mice by about 50% and 30%, respectively, with no significant change in blood pressure, calcium, or parathyroid hormone levels. Moreover using vitamin D receptor knockout mice, we found that expression of the human receptor in their juxtaglomerular cells reduced renin expression in these mice without affecting calcium or parathyroid hormone status. Our study shows that suppression of renin expression by 1,25-dihydroxyvitamin D *in vivo* is independent of parathyroid hormone and calcium.

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The renin–angiotensin system is important in the regulation of blood pressure and intravascular volume and electrolyte homeostasis. Renin is the rate-limiting enzyme that converts angiotensinogen to angiotensin (Ang) I in the renin–Ang cascade. The circulating renin is mainly generated in the juxtaglomerular (JG) cells in the kidney, and the production and release of renin from the JG cells are tightly regulated by various physiological stimuli, including changes in renal perfusion pressure, tubular sodium chloride concentrations, and sympathetic nerve activity.[1] At the molecular level, renin gene expression is regulated by a network of transcription factors targeting the renin gene promoter.[2]

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active metabolite of vitamin D, is a multifunctional hormone that acts by activating the vitamin D receptor (VDR).[3] Our previous studies have demonstrated that $1,25(OH)_2D_3$ suppresses the renin–Ang system by negatively regulating renin gene expression,[4] and genetically mutant mice lacking VDR develop hyperreninemia, leading to renal and cardiovascular abnormalities.[5,6] As VDR knockout (KO) mice also develop hypocalcemia and secondary hyperparathyroidism,[7] it is important to clarify the relationship between these phenotypes. It is clear from our previous studies that renin upregulation is not due to the hypocalcemia associated with VDR inactivation, because normalization of serum calcium levels by dietary means failed to normalize renin expression in VDRKO mice;[5] however, it remains to be determined whether the high serum parathyroid hormone (PTH) concentration contributes to the increase in renin biosynthesis. In VDRKO mice, the serum PTH level is very high due to the secondary hyperparathyroidism, and because of the loss of VDR-mediated suppression by $1,25(OH)_2D_3$ the PTH level cannot be completely normalized even when the serum-ionized calcium level has been normalized by dietary means.[5] Therefore, the contribution of PTH to hyperreninemia cannot be completely excluded in VDRKO mice.

The data reported in the literature are inconclusive and somewhat conflicting regarding the role of PTH in the regulation of renin and blood pressure. For example, in one study PTH was thought to be vasodilative in hypertensive patients,[8] whereas in another PTH infusion was shown to

cause hypertension in normal subjects.[9] PTH was reported to directly stimulate renin production,[10] or increase renin production by modulation of tubular transport of sodium[11,12] or calcium.[13,14] These inconsistent and mostly observational data call for a further clarification of PTH effect on renin regulation.

To address the effect of PTH on renin upregulation and confirm the repressive role of VDR in renin regulation *in vivo*, in the present study we generated transgenic (Tg) mice that overexpress hVDR in the renin-producing cells. We demonstrated that the transgene regulates renin biosynthesis in mice independently of calcium and PTH.

RESULTS

We used the 4.1 kb mouse *Ren-1^C* gene promoter to drive hVDR expression in Tg mice because this 4.1 kb 5′-flanking region has been shown to contain all the components required for tissue- and cell-specific expression of renin gene,[15] and this promoter has been successfully used to target transforming growth factor-β1 and green fluorescent protein expression in renin-producing cells in Tg studies.[16,17] Thus, this promoter was ideal for our experimental purpose. Microinjection of the 6.3 kb R1C-hVDR construct (Figure 1a) resulted in 34 pups born, of which 7 founders were carrying the *hVDR* transgene, as identified by PCR and verified by Southern blotting (Figure 1b).

The expression of the *hVDR* transgene in Tg mouse lines was measured by northern (Figure 1c) and western blot analyses (Figure 1d) in the kidney of Tg line 35 (high copy number) and line 25 (low copy number). To see the maximal effect, we focused on Tg-35 in the following investigations. Immunostaining of kidney sections of Tg-35 mice with anti-VDR antibody localized *hVDR* transgene expression in the JG apparatus (Figure 1e). As reported previously,[16] a very low level of hVDR mRNA was detectable by reverse transcriptase–PCR in the submandibular gland of Tg-35 mice (data not shown).

We first determined the effect of the *hVDR* transgene on serum-ionized calcium and PTH levels. As shown in Figure 2, the values of serum-ionized calcium (Figure 2a) and intact PTH (iPTH) (Figure 2b) in Tg mice were within the normal range compared to wild-type (Wt) mice (Ca^{++} (mM): Tg ($n=4$), 1.36 ± 0.06 vs Wt ($n=7$), 1.34 ± 0.04; iPTH (pg/ml), Tg, 18.7 ± 3.2 versus Wt, 17.9 ± 7.2). These data indicate that the *hVDR* transgene had no effects on the PTH and calcium status in Tg mice.

We then determined the effect of the *hVDR* transgene on renin biosynthesis and blood pressure. Northern blot analysis showed that renin mRNA expression in the kidney was clearly reduced in Tg mice by more than 50% compared to the Wt counterparts (Figure 2c and d). The plasma renin activity (PRA) was reduced by about 30% in Tg mice compared to Wt mice (Figure 2e). These data are consistent with the notion that VDR downregulates renin expression. However, the mean blood pressure was not different between Tg and

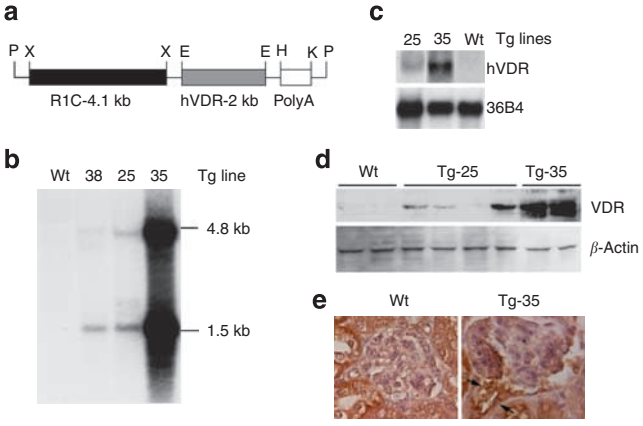

Figure 1 | Generation of hVDR transgenic mice. (**a**) Schematic map of the R1C4.1-hVDR DNA construct used for microinjection in Tg mouse production. P, *Pme*I; X, *Xba*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I. (**b**) Identification hVDR-Tg lines by Southern blot analysis. Genomic DNA (10 μg per lane) isolated from tail samples of Tg lines were digested with *Bgl*II, separated with 0.8% agarose gel, and probed with ^{32}P-labeled hVDR cDNA. Wt, wild-type control. (**c**) Northern blot showing expression of the *hVDR* transgene in Tg lines 25 and 35. (**d**) Western blot analysis of kidney lysates from Wt and Tg lines 25 and 35 with anti-VDR antibody. (**e**) Immunostaining of kidney sections of Wt and Tg-35 mice with anti-VDR antibody. Arrows indicate a strong staining in the JG region of the glomerulus.

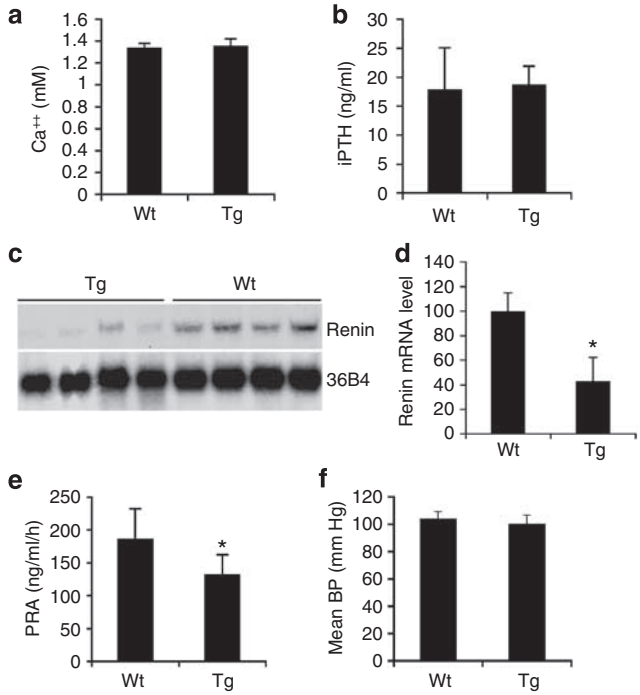

Figure 2 | Effect of the *hVDR* transgene on serum calcium and PTH status and renin biosynthesis. (**a**) Serum-ionized calcium levels; and (**b**) serum-intact PTH levels in Wt and Tg mice. (**c**) Northern blot showing renin mRNA levels in the kidney of Wt and Tg mice. (**d**) Quantification of renin mRNA in Wt and Tg mice based on the northern blot data. (**e**) Plasma renin activity (PRA) in Wt and Tg mice. (**f**) Mean blood pressure (BP) in Wt and Tg mice. Wt, wild-type mice, Tg, transgenic mice; *$P<0.05$ vs Wt, $n \geqslant 4$.

Wt mice (Figure 2f), indicating that the effect of *hVDR* transgene was not enough to change the blood pressure.

The next question is whether the *hVDR* transgene is able to normalize renin expression when it is reconstituted into the JG cells in VDRKO mice. The JG cells are highly specialized smooth muscle cells located in the afferent arteriole of the nephron. The main function of these cells is to produce and secret renin, and they are not involved in the regulation of calcium metabolism or PTH secretion. So reconstitution of VDR in these cells is not expected to change calcium and PTH status. To this end, we generated VDRKO mice that expressed the *hVDR* transgene in the JG cells (Tg-KO). Like VDRKO mice, Tg-KO mice also developed hypocalcemia and secondary hyperparathyroidism, and serum-ionized calcium and iPTH levels were not different between VDRKO and Tg-KO mice (Ca^{++} (mM): Tg-KO, 0.89 ± 0.03; VDRKO, 0.91 ± 0.02 vs Wt, 1.36 ± 0.05, and iPTH (ng/ml): Tg-KO, 1504 ± 236; VDRKO, 1693 ± 174 vs Wt, 39 ± 5) (Figure 3a and b). Therefore, we confirmed that the transgene had no effects on the calcium and PTH status in the VDRKO background. As shown previously,[5] renin expression was upregulated in VDRKO mice compared to Wt mice. Interestingly, renin mRNA levels in Tg-KO mice were markedly lower than those seen in VDRKO mice (Figure 3c and d), indicating that the *hVDR* transgene was able to rescue the renin upregulation seen in VDRKO mice. This confirms that VDR-mediated action of $1,25(OH)_2D_3$ suppresses renin expression in a calcium- and PTH-independent manner.

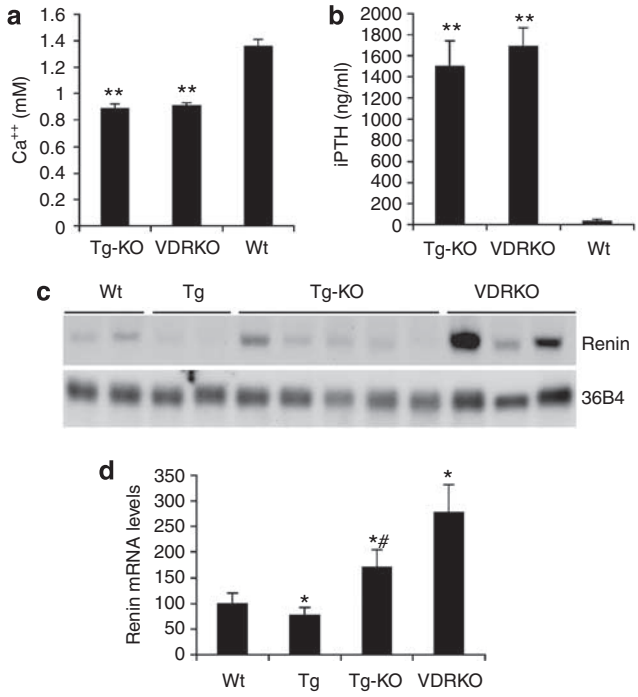

Figure 3 | Effect of the *hVDR* transgene on calcium and PTH status and renin expression in the VDRKO background. (**a**) Serum-ionized calcium levels; and (**b**) intact PTH levels in Wt, VDRKO, and Tg-KO mice. $^{**}P < 0.001$ vs Wt ($n = 4$–6). (**c**) Total RNAs were isolated from kidneys of Wt, Tg, Tg-KO, and VDRKO mice and renin mRNA expression was determined by northern blotting. (**d**) Quantitative data of renin mRNA based on the northern blot analyses. $^{*}P < 0.05$ vs Wt; $^{\#}P < 0.05$ vs VDRKO ($n = 4$–5).

DISCUSSION

Our previous studies have shown that VDR inactivation leads to hyperreninemia in VDRKO mice.[5] As VDRKO mice develop hypocalcemia and secondary hyperparathyroidism, the elevation of renin production may be caused by VDR inactivation *per se*, low serum calcium, high PTH, or a combination of these factors. We have demonstrated that renin upregulation is not due to hypocalcemia[5] and $1,25(OH)_2D_3$ directly suppresses renin gene transcription in a VDR-dependent manner in JG cells.[4] Other recent studies have further demonstrated that $1,25(OH)_2D_3$ and activated vitamin D analogs can suppress renin expression in the heart[18] and mesangial cells.[19] Zhou *et al.*[20] have confirmed that $1,25(OH)_2D_3$ downregulates renin expression in a calcium-independent manner in 25-hydroxyvitamin D 1α-hydroxylase knockout mice. However, the *in vivo* effect of PTH on renin expression remains unclear. Although no effect of PTH on renin expression was detected in As4.1 cell culture,[5] it is possible that, because the cyclic AMP–protein kinase A signaling pathway is constitutively active in these cells,[21] PTH is not able to transduce the signal in these cells. Therefore, the *in vitro* effect of PTH on renin expression is not clear either. Given that PTH is a major endocrine hormone, it is important to clarify its role in the renin regulation. One argument is to assess the PTH effect by treating animals with PTH, but the problem is that PTH will alter calcium and vitamin D status in the animals, which complicates the data interpretation.

In the present study we generated Tg mice that overexpress hVDR only in the renin-producing cells, and, as expected, the *hVDR* transgene did not alter the status of calcium and PTH in both wild-type and VDR-null backgrounds. Like Wt mice, Tg mice had normal calcium and PTH status, thus allowing for assessment of the role of VDR in renin regulation without the interference caused by abnormal calcium and PTH levels; on the other hand, Tg-KO mice developed hypocalcemia and secondary hyperparathyroidism as seen in VDRKO mice, and these mice allowed for assessment of VDR's role in renin regulation in the same pathophysiological environment as in VDRKO mice. Our data showed that hVDR overexpression suppressed renin expression under normal calcium and PTH conditions in Tg mice, and reconstitution of the JG cells with the *hVDR* transgene mostly normalized renin expression in Tg-KO mice. These data provide compelling evidence that VDR-mediated regulation of renin by $1,25(OH)_2D_3$ is independent of calcium and PTH, and PTH plays a very minor role, if any, in the upregulation of renin in VDRKO mice. It is of note that, despite the reduction in renin production by the *hVDR* transgene, the blood pressure of Tg mice was not changed. This is not surprising because alteration of renin, in this case 30% reduction in PRA, usually does not lead to alteration in

blood pressure under normal physiological conditions. For example, in normotensive healthy human subjects receiving renin inhibitor aliskiren treatment, blood pressure was unchanged even plasma Ang II levels was reduced by 75–89%.[22]

In sum, the main purpose of this Tg study was to address the relationship among calcium, PTH and $1,25(OH)_2D_3$ in renin regulation, and together the data unequivocally confirm that $1,25(OH)_2D_3$ negatively regulates renin gene expression *in vivo* through a VDR-dependent, and calcium- and PTH-independent mechanism.

METHODS

Generation of hVDR transgenic mice

The schematic map of the DNA construct (R1C4.1-hVDR) used to generate Tg mice is illustrated in Figure 1a. This construct was generated as follows: the 4.1 kb (from −4100 to +6) mouse *Ren-1^C* gene promoter was released from pR1C-4.1CAT[15] by *Xba*I digestion, purified and cloned into the *Xba*I site 5′ upstream to the hVDR coding sequence in pcDNA-hVDR. Then the 130 bp SV40 polyadenylation signal flanked by 5′ *Hin*dIII and 3′ *Kpn*I sites was cloned into the unique *Hin*dIII and *Kpn*I sites 3′ downstream to the hVDR coding sequence. The 6.3 kb R1C4.1-hVDR-PolyA fragment was cleaved by *Pme*I, purified and microinjected into the nuclei of single-cell-stage embryos of C57BL6 according to standard procedures[23] by the Transgenic Mouse Facility at the University of Chicago. Founders carrying the *hVDR* transgene were identified by PCR analysis of tail genomic DNA, using hVDR-specific primers 5′CGTGTGAATGATGGTGGAGGGAGCC3′ (forward), and 5′GTCT TGGTTGCCACAGGTCCAGGAC3′ (reverse). Integration of the transgene into the mouse genome was verified by Southern blot using ^{32}P-labeled hVDR cDNA as the probe. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Generation of transgenic VDR-null (Tg-KO) mice

VDRKO mice carrying the *hVDR* transgene were generated by a two-step breeding strategy. First step was Tg(+/−) × VDR(+/−) breeding to generate Tg(+/−)VDR(+/−) mice; second step was Tg(+/−)VDR(+/−) × Tg(+/−)VDR(+/−) breeding to obtain Tg(+/−)VDR(−/−) and Tg(+/+)VDR(−/−) mice. These mice were referred as Tg-KO mice.

Northern blot

Northern blot analysis was performed as described previously.[24] Briefly, total RNAs were extracted from the kidney using the TriZol reagent (Invitrogen, Carlsbad, CA, USA). Total RNAs were separated on an 1% agarose gel containing 0.6 M formaldehyde, transferred onto a Nylon membrane (MSI, Westborough, MA, USA) and crosslinked in an UV crosslinker (Bio-Rad, Hercules, CA, USA). The membranes were hybridized with ^{32}P-labeled cDNA probes according to the method of Church and Gilbert.[25] Variations in RNA loading were normalized with 36B4 probe.

Western blot

Kidney samples were placed in the Laemmli sample buffer,[26] boiled for 5 min, and dispersed by sonication. Protein concentrations were determined as described.[24] Western blots were carried out as described previously.[24] Briefly, the kidney lysates were separated by SDS–polyacrylamide gel electrophoresis, and then transferred onto Immobolin-P membranes. The membranes were incubated sequentially with primary antibody and peroxidase-conjugated second antibody, and the antigen was visualized using an enhanced chemiluminescent kit (Amersham, Piscataway, NJ, USA).

Plasma renin activity

PRA was determined using a commercial kit from DiaSorin (Stillwater, MN, USA). Briefly, first the renin activity was determined by measuring the conversion of angiotensinogen to Ang I, using plasma from bilaterally nephrectomized rats as the source for angiotensinogen substrate as described previously.[27] The nephrectomized rat plasma provides an excess of renin substrate (angiotensinogen) for the assay. The amount of Ang I generated was determined by radioimmunoassays. PRA is expressed as ng(AngI)/ml(mouse plasma)/h.

Immunohistochemical staining

Kidneys were fixed in 4% formalin made in phosphate-buffered saline (pH 7.2) overnight, processed, embedded in paraffin, and cut to 5 μm sections with a microtome. The paraffin sections were boiled in 10 mM Na citrate solution for 10 min to retrieve the antigens, and incubated with an anti-VDR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 3 h. After being stained with horseradish peroxidase-conjugated second antibody the antigen was visualized with a peroxidase substrate diaminobenzidine kit (Vector Laboratories, Burlingame, CA, USA) as described previously.[28]

Measurement of blood pressure

Mouse blood pressure was determined using an invasive left carotid artery cannulation method described previously.[5]

Serum-ionized calcium and intact PTH

Serum-ionized calcium levels were determined using a 634 Ca^{++}/pH analyzer. Serum iPTH levels were determined using a commercial enzyme-linked immunosorbent assay kit as described previously.[5]

Statistical analysis

Data were presented as mean ± s.e.m. Statistical comparisons were made using Student's *t*-test, with $P<0.05$ being considered significant.

DISCLOSURE

All the authors declared no competing interests.

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