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Vitamin D receptor agonists increase klotho and osteopontin while decreasing aortic calcification in mice with chronic kidney disease fed a high phosphate diet

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Vascular calcification is common in chronic kidney disease, where cardiovascular mortality remains the leading cause of death. Patients with kidney disease are often prescribed vitamin D receptor agonists (VDRAs) that confer a survival benefit, but the underlying mechanisms remain unclear. Here we tested two VDRAs in a mouse chronic kidney disease model where dietary phosphate loading induced aortic medial calcification. Mice were given intraperitoneal calcitriol or paricalcitol three times per week for 3 weeks. These treatments were associated with half of the aortic calcification compared to no therapy, and there was no difference between the two agents. In the setting of a high-phosphate diet, serum parathyroid hormone and calcium levels were not significantly altered by treatment. VDRA therapy was associated with increased serum and urine klotho levels, increased phosphaturia, correction of hyperphosphatemia, and lowering of serum fibroblast growth factor-23. There was no effect on elastin remodeling or inflammation; however, the expression of the anticalcification factor, osteopontin, in aortic medial cells was increased. Paricalcitol upregulated osteopontin secretion from mouse vascular smooth muscle cells in culture. Thus, klotho and osteopontin were upregulated by VDRA therapy in chronic kidney disease, independent of changes in serum parathyroid hormone and calcium.

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There is a heavy burden of cardiovascular morbidity and mortality in patients with chronic kidney disease (CKD).[1] Disordered mineral metabolism occurs early in CKD and is characterized by secondary hyperparathyroidism, elevated fibroblast growth factor-23 (FGF23) levels, and klotho and 1,25-dihydroxyvitamin D deficiency. Higher 25-hydroxyvitamin D levels are associated with lower mortality in CKD patients,[2] and observational studies have noted a survival advantage when dialysis patients are treated with vitamin D receptor agonists (VDRAs).[3,4] VDRAs are currently approved for the treatment of secondary hyperparathyroidism, but their association with improved survival is independent of parathyroid hormone (PTH) levels,[5,6] suggesting that other pleiotropic effects of VDRAs are involved. In contrast to its beneficial effects, high-dose calcitriol has also been associated with hypercalcemia, and VDRAs were associated with vascular calcification (VC) in some animal models.[7,8] *In vitro* data are also conflicting; calcitriol has been shown to increase vascular smooth muscle cell (VSMC) calcification in some studies[9,10] but not others.[11,12] Paricalcitol (19-nor-1α,25$(OH)_2D_2$) is an analog of calcitriol that causes less hypercalcemia[13] and may have a survival benefit over calcitriol.[14] Data from rodent studies are mixed, but suggest a beneficial effect of VDRAs, especially paricalcitol, on VC.[7,8,12,15,16] Despite human and experimental data suggesting benefits with VDRA therapy, the underlying mechanisms remain to be clarified.

Many mechanisms contribute to uremic VC, including systemic calcium/phosphate imbalances, decreased expression of calcification inhibitors, VSMC osteogenic differentiation, and elastin remodeling.[17] The VSMC phenotype change is particularly striking and can be triggered by elevated extracellular phosphate.[18–20] Large observational studies have correlated elevated serum phosphate with increased cardiovascular mortality in end-stage kidney disease,[21] CKD,[22] and

the general population.[23] It is noteworthy that phosphate loading occurs early in CKD stage 3, as evidenced by increased serum levels of FGF23, which precedes overt hyperphosphatemia.[24]

The outcome of VDRA therapy is difficult to predict because of the myriad of vasculotropic effects (both anticalcific and procalcific) downstream of vitamin D receptor activation.[25] This complexity emphasizes the need for *in vivo* studies to assess the overall consequence of VDRA therapy on VC. In the present study, we evaluated calcitriol and paricalcitol in DBA/2J mice that develop marked arterial medial calcification (AMC) when subjected to CKD and high-phosphate diet.[26,27] We demonstrate that both VDRAs decreased the extent of VC independently of serum calcium and PTH and identify underlying beneficial mechanisms that include (1) increased serum klotho and (2) upregulation of VSMC osteopontin.

RESULTS

VDRA therapy was associated with ~50% less AMC and normalized serum phosphate

CKD was surgically induced using partial renal ablation; non-CKD (NC) controls were not surgically manipulated. Mice were randomized to receive VDRA therapy intraperitoneal for 3 weeks (see Figure 1 for experimental timeline). The doses tested were 30 ng/kg calcitriol (C30), 100 ng/kg paricalcitol (P100), and 300 ng/kg paricalcitol (P300). C30 and P100 reflect doses used in current clinical practice, and we also tested a higher dose of paricalcitol to look for dosage effect. Diets used were normal (0.5%) phosphate (NP) and high (1.5%) phosphate (HP) diets.

The extent of VC was assessed via aortic arch calcium content in all mice. Aortic calcium content in CKD mice on high (1.5%) phosphate diet (CKD + HP) mice was 8.5-fold higher than that in NC control mice on normal (0.5%) phosphate diet (NC + NP) mice. Consistent with previous reports,[26,27] CKD mice on normal (0.5%) phosphate diet (CKD + NP) mice did not develop aortic calcification. CKD + HP mice on calcitriol and paricalcitol developed significantly less AMC, and there was no statistical difference between the two VDRAs (Figure 2a). Alizarin Red-S staining of thoracic aorta sections confirmed that calcification was restricted to the medial layer (Figure 2b). H&E staining showed straightening of elastic fibers and no atherosclerotic lesions at areas of calcification; BM8 staining for macrophages confirmed lack of inflammation (data not shown).

Serum parameters for the 11 treatment groups are summarized in Table 1. At the time of randomization, average BUN in CKD mice was 35 ± 4.7 mg/dl as compared with 28 ± 4.6 mg/dl in NC mice; at termination, the average BUN in CKD mice (38.7 ± 6.4 mg/dl) remained significantly higher than that in control mice (26.9 ± 5.3 mg/dl; $P < 0.05$). CKD + HP mice developed significant hyperphosphatemia and markedly elevated FGF23 levels. Serum phosphate was corrected to normal levels and FGF23 was significantly lowered with VDRA therapy. In CKD + NP mice, calcitriol significantly raised serum calcium levels, whereas paricalcitol did not. There was a nonsignificant trend for higher serum calcium in the CKD + HP mice treated with VDRAs. PTH levels were not significantly affected by VDRA therapy in CKD mice, but VDRA therapy significantly raised PTH levels in the NC control mice on high (1.5%) phosphate diet (NC + HP) group. We noted less mortality in the VDRA-treated CKD + HP groups (19/20 mice per group survived until termination of the study, vs. 16/20 mice in the CKD + HP group); however, our study was not designed for survival analysis.

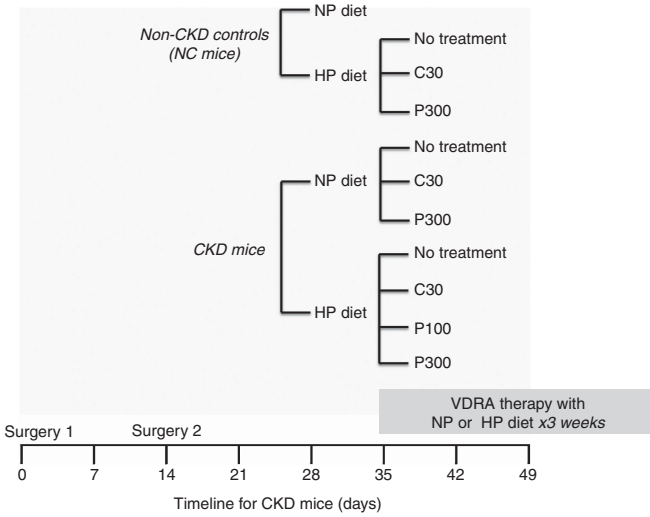

Figure 1 | Experimental design. Chronic kidney disease (CKD) was induced by partial renal ablation: the right kidney was exposed, decapsulated, and electrocauterized (surgery 1), followed by left total nephrectomy 2 weeks later (surgery 2). Non-CKD control (NC) or CKD mice were placed on normal (0.5%) phosphate diet (NP) or high (1.5%) phosphate diet (HP) for 3 weeks. Concurrently, mice were either given no treatment, 30 ng/kg calcitriol (C30), 100 ng/kg paricalcitol (P100), or 300 ng/kg paricalcitol (P300). The vitamin D receptor agonists (VDRAs) were administered via intraperitoneal (i.p.) injections three times a week for 3 weeks.

VDRA effects on AMC were independent of serum PTH levels

To determine whether the development of tertiary hyperparathyroidism might explain the lack of serum PTH lowering by VDRA therapy, a separate cohort of CKD mice ($n = 8$) were placed on the HP diet for 18.5 days and then switched to the NP diet for 3 weeks. The PTH levels in these mice decreased from 1349 ± 612 to 406 ± 346 pg/ml and were equivalent to levels in the original CKD + NP group, thus ruling out tertiary hyperparathyroidism. Alternatively, we considered that the very high (1.5%) phosphate diet used in the present studies might have blunted the ability of VDRA to reduce PTH levels. The influence of dietary phosphate content was examined using a lower (but still above normal) 0.9% phosphate diet that was previously shown to induce VC in CKD mice.[26] CKD mice fed the 0.9% phosphate diet and treated with the P300 dose showed lowering of serum PTH to 184 ± 211 pg/ml ($n = 5$) compared with 608 ± 285 pg/ml in the untreated group ($n = 3$) ($P = 0.05$). Taken together,

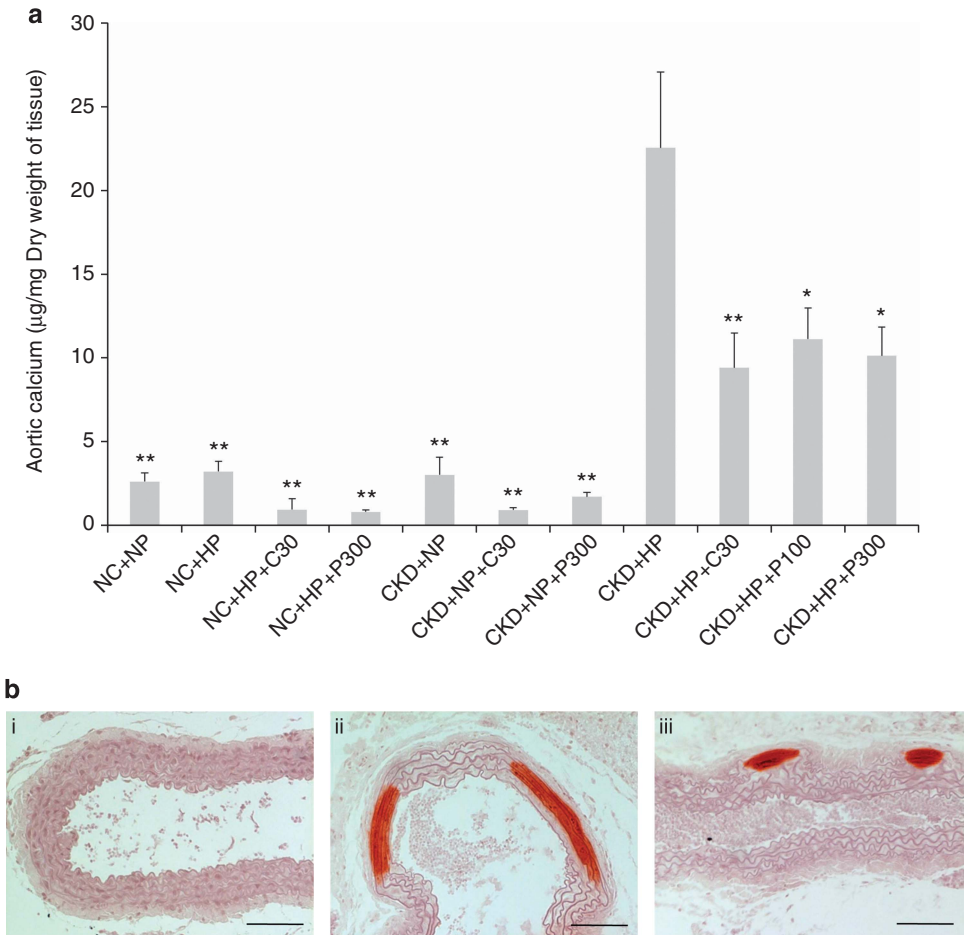

Figure 2 | Chronic kidney disease (CKD) mice on high (1.5%) phosphate diet (CKD + HP) developed vascular calcification that was significantly decreased by vitamin D receptor agonist (VDRA) therapy. (**a**) Aortic arch calcium content expressed as μg calcium normalized to mg dry weight (mean ± s.e.m.). *$P<0.01$ and **$P<0.001$ compared with the CKD + HP group. Calcification was not different between the NC + NP (non-CKD control mice on normal (0.5%) phosphate diet), NC + HP (non-CKD control mice on high (1.5%) phosphate diet), and CKD + NP (CKD mice on normal (0.5%) phosphate diet) subgroups ($P=1$ for all *post-hoc* Tukey analyses). Aortic calcium content was analyzed from all mice in the study (refer to Table 1 for *n* per group). (**b**) Thoracic aorta with Alizarin Red-S stain showing marked medial calcification in the CKD + HP animal (ii) compared with the NC + NP animal (i), and significantly less calcification in an animal treated with paricalcitol (CKD + HP + P300, iii). Bar = 150 μm; original magnification × 10. C30, calcitriol 30 ng/kg; P100, 100 ng/kg paricalcitol; P300, 300 ng/kg paricalcitol.

these data suggest that the 1.5% phosphate diet drove PTH secretion even in the presence of pharmacological VDRA doses, allowing us to uncover PTH-independent VDRA inhibitory effects against VC.

Serum and urine klotho, and tubular phosphate excretion, were increased in CKD + HP mice treated with VDRA

Fractional excretion of phosphate (FE_{phos}) was significantly higher in VDRA-treated CKD + HP mice, compared with NC + NP and CKD + HP mice (Figure 3a). Furthermore, 24-h phosphate excretion mirrored the changes in FE_{phos} (Figure 3b). As PTH levels were not significantly changed by VDRA therapy and could not account for the increased tubular phosphate excretion and correction of hyperphosphatemia, the FGF23/klotho axis was examined. FGF23 levels in CKD + HP mice were ~5-fold higher than that in NC + NP mice, and were significantly lowered by VDRA therapy (Table 1). This FGF23 drop was likely in response to normalization of serum phosphate levels, as FGF23 levels were highly correlated with serum phosphate levels in this study ($R=0.58$; $P<0.001$).

Serum levels of klotho, a cofactor for FGF23, were then examined using immunoprecipitation–immunoblot. Figure 3c and d demonstrates that serum klotho levels were depressed in CKD. In striking contrast to serum FGF23 levels, calcitriol and paricalcitol both markedly increased serum klotho in CKD + HP mice (130 kDa band), and these levels were significantly higher even when compared with NC controls. Interestingly, coexistence of CKD and phosphate loading was required for VDRA upregulation of serum klotho; VDRA therapy *per se* did not significantly increase klotho levels in NC + HP or CKD + NP mice (Figure 3d).

Table 1 | Serum parameters in the various treatment groups

Treatment group	*n*	BUN (mg/dl)	Ca (mg/dl)	P (mg/dl)	PTH (pg/ml)	FGF23 (pg/ml)	OPN (ng/ml)
Non-CKD groups							
NC+NP	9	22 ± 6[a]	9.9 ± 0.9	9.5 ± 2.2	249 ± 24	203 ± 98	173 ± 41
NC+HP	10	29 ± 5[a]	9.9 ± 0.6	8.3 ± 0.8	681 ± 173[b]	473 ± 147[c]	495 ± 93
NC+HP+C30	10	26 ± 3[a]	8.9 ± 0.6	10.3 ± 1.3	1699 ± 154[b]	ND	435 ± 119
NC+HP+P300	9	30 ± 4[a]	9.3 ± 0.6	9.6 ± 1.6	1253 ± 214[b]	ND	441 ± 120
CKD groups							
CKD+NP	10	40 ± 8	10 ± 1.5	10.3 ± 1.1	451 ± 81	229 ± 108	185 ± 84
CKD+NP+C30	10	34 ± 5	11.9 ± 1.4[d]	9.8 ± 0.8	726 ± 188	ND	367 ± 79
CKD+NP+P300	10	45 ± 5	10.1 ± 0.6	10.6 ± 1	457 ± 145	ND	219 ± 64
CKD+HP	16	41 ± 6	9.1 ± 1.6	12.2 ± 1.4	1822 ± 168[e]	1176 ± 368	746 ± 196
CKD+HP+C30	19	36 ± 5	10.4 ± 0.6	9.1 ± 0.7[f]	1728 ± 127[e]	386 ± 168[g]	610 ± 108
CKD+HP+P100	19	34 ± 3	10.2 ± 0.8	8.3 ± 1.2[f]	1684 ± 262[e]	ND	359 ± 67[h]
CKD+HP+P300	19	40 ± 5	9.8 ± 0.8	9 ± 0.7[f]	1701 ± 113[e]	336 ± 124[g]	436 ± 93[h]

Abbreviations: BUN, blood urea nitrogen; Ca, calcium; C30, calcitriol 30 ng/kg; CKD, chronic kidney disease; FGF23, fibroblast growth factor 23; HP, high (1.5%) phosphate; *n*, number of mice in each treatment group; ND, not determined due to insufficient serum; NP, normal (0.5%) phosphate; OPN, osteopontin; P, phosphate; P100, paricalcitol 100 ng/kg; P300, paricalcitol 300 ng/kg; PTH, parathyroid hormone.

Non-CKD control (NC) or CKD mice were placed on normal or high phosphate diet for 3 weeks, and given no treatment, C30, P100 or P300. Each drug was given i.p. three times a week. All parameters were measured at time of termination except for PTH, which was measured within 24 h following the penultimate VDRA injection. For BUN and OPN, n=10 per group except for NC+NP and NC+HP+P300 groups where n=9. For serum Ca, n=8 per group except for NC+NP, NC+HP, NC+HP+C30, CKD+NP+C30 and CKD+NP+P300 groups where n=5. For serum P, n=8 per group; for intact PTH, n=6 per group. For FGF23, n=9 except for NC+NP group where n=8. Data are mean ± s.d.

[a]The non-CKD control groups had significantly lower BUN compared with the CKD+HP group ($P<0.001$).

[b]PTH levels were significantly higher when control mice were placed on the high phosphate diet (P=0.001 compared with NC+NP group); there was a further significant rise in PTH with C30 and P300 treatment ($P<0.001$ compared with NC+HP group).

[c]FGF23 levels were significantly increased in high phosphate-fed control mice ($P<0.05$ compared with NC+NP group).

[d]Calcitriol significantly raised serum calcium in normal phosphate-fed CKD mice ($P<0.05$ vs. CKD+NP group). Although there was a trend for increased serum calcium in the high phosphate-fed CKD mice treated with calcitriol and paricalcitol, the differences were not statistically significant compared with the CKD+HP group.

[e]PTH levels in high phosphate-fed CKD mice were not significantly affected by VDRA therapy (P-value was not significant compared with CKD+HP group). Similarly, PTH levels were not significantly different among the CKD+NP groups.

[f]Calcitriol and paricalcitol lowered serum phosphate in CKD, high phosphate-fed mice ($P<0.001$ vs. CKD+HP group).

[g]FGF23 levels were decreased with VDRA therapy ($P<0.001$ compared with CKD+HP group).

[h]OPN levels were significantly lower in paricalcitol-treated groups ($P<0.001$ vs. CKD+HP group). OPN was also decreased in the CKD+HP+C30 group but P-value was not significant.

In addition, immunoblot detected a trend for increased urine klotho in VDRA-treated CKD + HP mice compared with untreated CKD + HP animals (Supplementary Figure S1 online). Urinary klotho concentrations did not correlate with proteinuria; moreover, average proteinuria was the same in the control and CKD groups (4.4 mg/day).

Elevated serum and urine klotho were not explained by increased renal or parathyroid gland klotho protein levels following VDRA treatment

In an attempt to identify the source of klotho upregulation by VDRAs, we first examined the kidney (the major site of klotho synthesis under healthy conditions[28,29]). Immunoblot of kidney lysates is shown in Figure 4a, with the corresponding densitometric analysis graphed in Figure 4b. Klotho protein levels were depressed with high-phosphate feeding alone (NC + HP mice), consistent with a previous report whereby high-phosphate feeding was associated with decreased klotho expression and ectopic calcifications in the kidneys of healthy mice.[30] Klotho protein levels were low in CKD mice (decreased by 30–45 × compared with NC + NP mice) and remained low following VDRA therapy, which was also evident on kidney immunostaining (Figure 4c). Quantitative RT-PCR showed an ~50% decrease in kidney klotho mRNA levels in CKD mice regardless of VDRA treatment (data not shown).

Klotho expression in parathyroid glands was next examined by immunostaining of neck tissues that had been dissected out *en bloc* (Supplementary Figure S2A online). Analysis of the fractional area with positive staining showed equivalent parathyroid gland klotho expression in VDRA-treated versus untreated CKD + HP mice (Supplementary Figure S2B online). To determine whether the degree of parathyroid gland hyperplasia might contribute to increased serum klotho, serial sectioning and three-dimensional reconstruction were performed.[31] We found no difference in parathyroid gland volumes following VDRA treatment (Supplementary Figure S2C and D online). Thus, elevated serum klotho levels could not be explained by parathyroid hyperplasia in response to VDRA treatment.

Similarly, there was no klotho upregulation in other tissues surveyed. Klotho expression in human aortas was recently reported,[32] but klotho was not detected in aortas from healthy or CKD mice by RNA or protein analyses (Supplementary Figure S3A and B online). Finally, we examined archival tissue from CKD mice that had been placed on 0.9% phosphate diet and treated with VDRA. No upregulation of klotho expression was observed by immunoblot of brain, heart, lung, and liver lysates (Supplementary Figure S3C online), suggesting that increased steady-state expression in these tissues does not contribute to elevated serum klotho in VDRA-treated mice. The current data

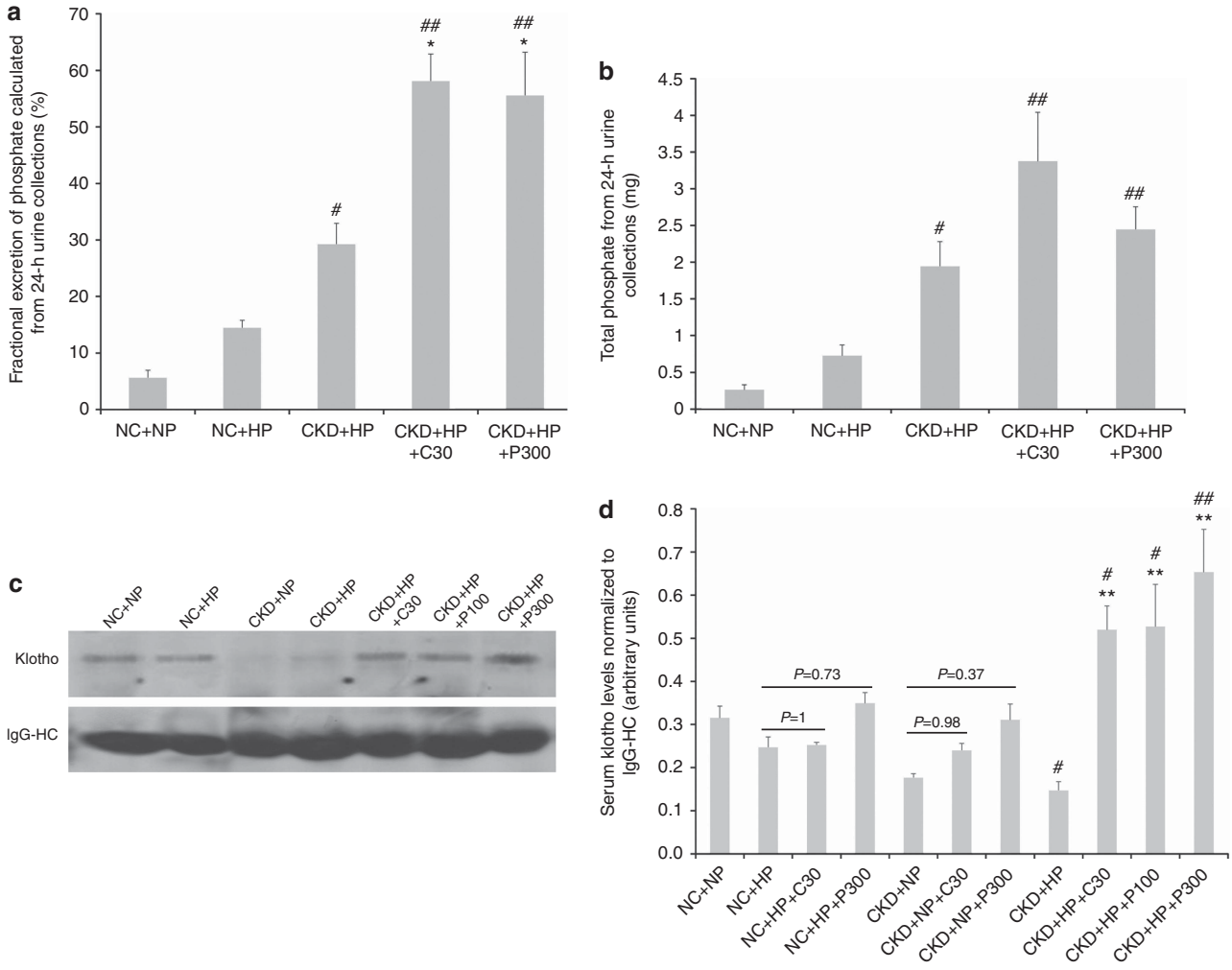

Figure 3 | Renal phosphate excretion and serum klotho were increased by vitamin D receptor agonist (VDRA) treated. (**a**) Correction of hyperphosphatemia correlated with increased fractional excretion of phosphate in VDRA-treated CKD + HP animals (fractional excretion of phosphate (FE_{phos}) calculated from 24-h urine collections expressed as mean percentage ± s.e.m., $n = 6$ for the NC + NP and NC + HP groups, $n = 4$ for the CKD + HP group, $n = 5$ for the CKD + HP + C30 and CKD + HP + P300 groups. (**b**) Total urinary phosphate from 24-h urine collections was significantly increased in CKD + HP animals compared with the NC + NP group. There was a trend for increased total phosphate excretion in VDRA-treated CKD + HP mice ($P = 0.06$ and $P = 0.9$ for the CKD + HP + C30 and CKD + HP + P300 groups, respectively, compared with CKD + HP animals). (**c**) Representative blot of serum klotho protein in individual mice from select groups (upper panel, 130 kDa band). The same blot was stripped and reprobed for immunoglobulin G heavy chain (IgG-HC) as loading control (lower panel). (**d**) Serum klotho levels were decreased in CKD, and were increased by VDRA therapy in high phosphate–fed CKD mice to levels that were significantly higher than that in control mice. VDRAs did not significantly raise serum klotho in the NC + HP and CKD + NP groups (*post-hoc* Tukey *P*-values shown on chart). Levels expressed as arbitrary units normalized to IgG-HC using densitometric analyses (mean ± s.e.m.); $n = 5$ for the NC + NP group, $n = 6$ for the NC + HP, CKD + NP and CKD + HP groups, $n = 3$ in the remaining groups. $^{*}P < 0.01$ and $^{**}P < 0.001$ compared with the CKD + HP group, and $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ compared with the NC + NP group.

cannot rule out simultaneous increased expression and shedding as a mechanism of increasing circulating klotho.

VDRA therapy increased VSMC osteopontin (OPN) expression *in vivo* and *in vitro*

In contrast to serum OPN, which decreased in parallel with decreased VC (Table 1), aorta immunostaining showed increased VSMC cytoplasmic expression of OPN in the VDRA-treated CKD + HP mice (Figure 5a and b). Consistent with previous findings,[26] no OPN staining was observed in aortas from NC mice (data not shown). As serum klotho was elevated in VDRA-treated CKD + HP mice, the question was raised whether OPN upregulation was mediated by VDRA or by klotho. This was tested *in vitro* by treating mouse VSMCs with 2 ng/ml klotho (with/without FGF23) or 50 nmol/l paricalcitol. Paricalcitol significantly increased OPN levels in the conditioned media after 48 h, whereas klotho with/without FGF23 had no significant effect (Figure 5c). OPN mRNA levels were unchanged (data not shown). We further evaluated other major regulators of VSMC calcification

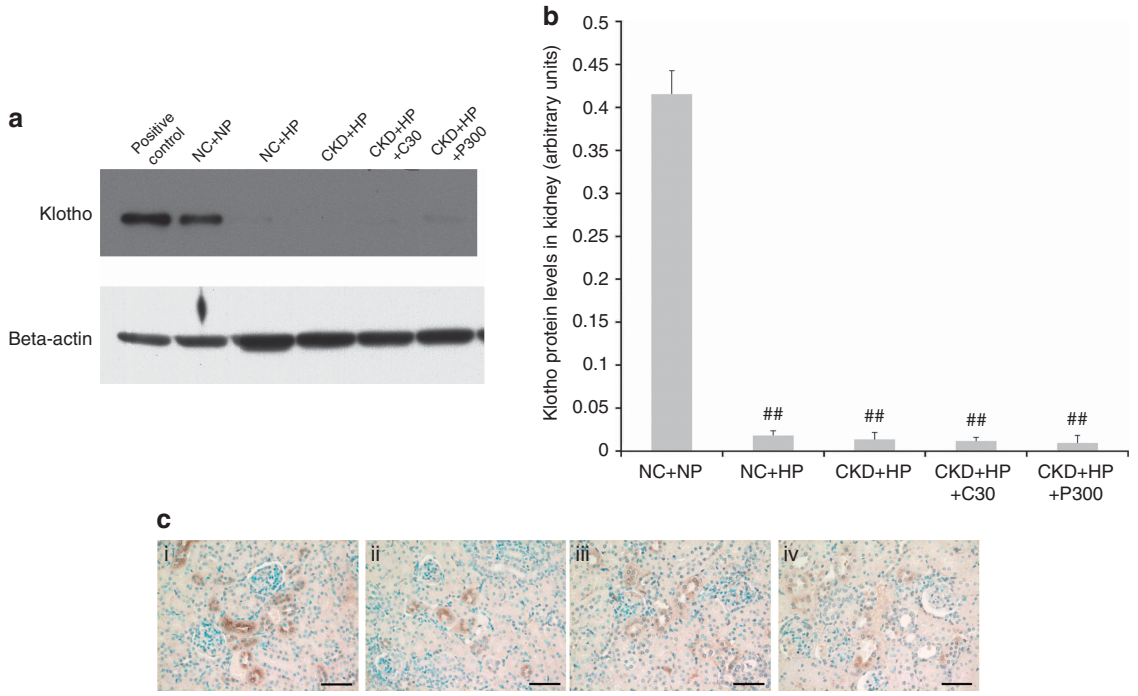

Figure 4 | Vitamin D receptor agonist (VDRA) treatment did not increase kidney klotho levels. (**a**) Representative western blot showing klotho protein (130-kDa band) from whole-kidney lysates (upper panel). The same blot was stripped and reprobed for beta-actin as loading control (lower panel). (**b**) Klotho protein in the kidney was significantly decreased by high-phosphate diet (in both control and CKD mice, $^{\#\#}P<0.001$ compared with the NC + NP non-CKD group). VDRA treatment did not increase the expression of kidney klotho in CKD mice. Levels expressed as arbitrary units normalized to beta-actin using densitometric analyses (mean ± s.e.m., $n=3$ for all groups). (**c**) Decreased klotho immunostaining in kidneys from NC + HP and CKD mice; (i) NC + NP animal, (ii) NC + HP animal, (iii) CKD + HP animal, and (iv) CKD + HP + P300 animal. Bar = 50 μm; original magnification × 20.

including matrix gla protein and the sodium-phosphate cotransporters PiT-1 and PiT-2, and VDRA therapy did not change the expression of any of these genes (Supplementary Figure S4 online).

VDRA therapy did not affect elastin remodeling in CKD mice

Elastin degradation is prominent in our mouse CKD model, irrespective of dietary phosphate, and precedes overt AMC.[27] In end-stage kidney disease, elastin breakdown has been reported alongside the upregulation of matrix metalloproteinase-2,[33] and vitamin D deficiency has been associated with higher circulating concentrations of matrix metalloproteinase-9.[34] Examination of elastin integrity by eosin fluorescence showed prominent elastin degradation in the aortas from all CKD groups (30–50 lamellae breaks per cross-sectional area vs. four lamellae breaks in the NC + NP group). VDRA treatment did not decrease the extent of elastin breaks. There was also no significant difference between CKD groups in terms of aortic arch desmosine content (data not shown), indicating no difference in the amount of functional elastin. Finally, immunostaining showed equivalent levels of elastolytic matrix metalloproteinase-2 across CKD groups (data not shown). Taken together, these data suggest that the improvement in AMC following VDRA treatment was not due to changes in elastin remodeling.

DISCUSSION

We describe a mouse CKD model that, when challenged with a high-phosphate diet, develops robust AMC in conjunction with metabolic derangements that include hyperphosphatemia, elevated serum PTH, FGF23, and OPN, and klotho deficiency. VDRA therapy for 3 weeks with either calcitriol or paricalcitol resulted in significantly less aortic calcification, and this effect was independent of changes in serum PTH and calcium levels. The lower extent of AMC was associated with elevated serum klotho levels (significantly higher than that in NC controls), increased phosphaturia, and normalized serum phosphate and FGF23 levels. In addition, OPN expression in aortic VSMCs was increased by VDRA treatment *in vivo* and *in vitro*, in contrast to circulating OPN levels that decreased in conjunction with reduced VC.

High doses of VDRAs stimulate VC, often in association with hypercalcemia.[12,15,16,35] As in our current study, Mathew *et al.*[15] noted a protective effect of both calcitriol and paricalcitol against VC when they used lower (more physiological) dosages. Other groups have reported differential stimulation of VC by calcitriol but not by paricalcitol in CKD rats,[12,16] but their experimental protocols differed in two major aspects: (1) the CKD animals did not develop VC, and therefore the studies were not capable of detecting beneficial anticalcification effects, and (2) the degree of experimental CKD was more severe.

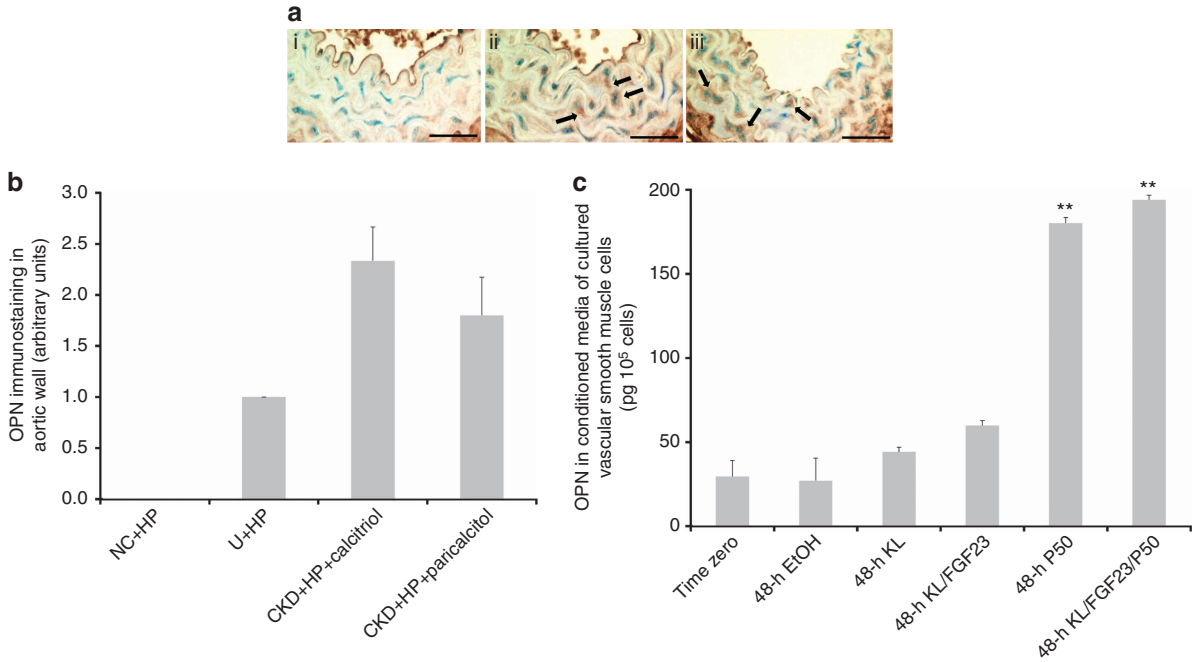

Figure 5 | (**a**) Arterial medial osteopontin (OPN) levels were increased by vitamin D receptor agonist (VDRA) treatment. (i) OPN expression was low but detectable in the aortic media of chronic kidney disease (CKD) mice fed a high (1.5%) phosphate diet (CKD + HP). VDRA treatment increased smooth muscle cell expression of OPN in the aortic media in the (ii) CKD + HP + C30, and (iii) the CKD + HP + P300 animal. Arrows point to aortic medial cells expressing OPN. Bar = 30 μm; original magnification × 40. (**b**) Quantification of OPN immunostaining showed no OPN expression in aortas from non-CKD controls, weak staining in high phosphate–fed CKD mice, and increased levels in VDRA-treated CKD mice (mean ± s.e.m., $n = 3$ for all except CKD + HP + paricalcitol, $n = 5$, where P100 and P300 samples were grouped). (**c**) Treatment of cultured vascular smooth muscle cells (VSMCs) with 50 nmol/l paricalcitol increased OPN levels in the media (significantly higher levels by enzyme-linked immunosorbent assay (ELISA) at 48 h compared with time zero, $^{**}P < 0.001$). Klotho (KL, 2 ng/ml) with/without fibroblast growth factor-23 (FGF23; 2 ng/ml) did not upregulate OPN secretion. Three wells were sampled per time point, per treatment group; data are mean ± s.e.m. EtOH, ethanol control; P50, paricalcitol 50 nmol/l.

Although VDRA therapy was associated with a slight lowering of PTH and a trend for increased serum calcium, these changes were not significant (Table 1). Our data suggest that the high dietary phosphate content (1.5%) used in the present study to achieve more rapid onset of AMC in mice with mild CKD, compared with the 0.9% phosphate[12,26,36] and 1.2% phosphate[8,16,37] diets used for longer time periods in previous studies, drove PTH secretion despite VDRA supplementation. Indeed, CKD mice on a 0.9% phosphate diet showed the expected lowering of PTH levels when treated with VDRA. Dietary phosphate influence was also evident in the NC + HP group, in which VDRA therapy was associated with increased PTH levels. A possible explanation is that VDRAs increased intestinal phosphate uptake, leading to the induction of PTH secretion as a mechanism to maintain phosphate homeostasis. The increased 24-h total urinary phosphate in VDRA-treated CKD + HP mice (Figure 3b) is consistent with increased intestinal phosphate uptake, especially with calcitriol therapy ($P = 0.06$ between CKD + HP + C30 and CKD + HP groups). Overall, our study provided a unique opportunity to examine the beneficial vascular effects of VDRAs independent of changes in PTH and calcium.

CKD is a state of klotho deficiency,[38,39] and restoration of circulating klotho is an attractive therapeutic target. There has been accumulating evidence that soluble klotho can mediate phosphaturia independent of FGF23. Soluble klotho in the absence of FGF23 inhibits NaPi cotransporters in cultured OK cells and in cell-free membrane vesicles.[40] In addition, i.v. administration of klotho leads to decreased renal expression of NaPi-2a and hypophosphatemia, even in FGF23-null mice.[40] On the other hand, transmembrane klotho is the coreceptor for renal FGF23 signaling, which results in phosphaturia via downregulation of NaPi-2a and -2c in the proximal tubule.[41] The ectodomain of klotho has been shown to bind to exogenously expressed FGF receptors (FGFRs), suggesting that soluble klotho may be able to mediate the formation of the FGF23–FGFR–klotho complex.[42,43] The potential role of soluble klotho in FGF23 signaling *in vivo* remains unknown at this time, but appears to be a less plausible mechanism for phosphaturia, as *in vitro* assays have shown that the affinity of FGFRs for the klotho ectodomain is log-fold lower than their affinity for full-length transmembrane klotho.[42]

Hyperphosphatemia can perpetuate VC via several pathways,[44] and correction of this metabolic derangement has been shown to impede the development of VC in clinical trials.[45,46] Indeed, transgenic mice that overexpress klotho, when subjected to CKD, showed increased phosphaturia and

less VC.[39] Klotho was also recently shown to have direct anticalcification effects at the vascular wall, via inhibition of sodium-dependent phosphate uptake and VSMC osteoblastic transformation.[39]

The VDRA-associated increased serum klotho was modulated by phosphate excess (Figure 3d). A striking and significant increase in serum klotho was evident only in the setting of *both* CKD and dietary phosphate loading. Interestingly, a phosphate modulatory effect was previously described with respect to vitamin D effects on gene expression in cultured human VSMCs.[47]

Serum klotho is thought to arise from shedding of membrane-bound klotho from tissues in which it is normally expressed, and the major site of klotho synthesis is the kidney.[28,29] Calcitriol upregulates klotho in the kidneys of healthy mice,[48] and functional vitamin D–responsive elements have been located upstream of both the human and mouse klotho genes.[49] VDRAs have also been shown to upregulate klotho in cultured human and mouse kidney–derived cell lines.[50] Thus, it was surprising that our study found persistently low klotho mRNA and protein levels from the remnant kidneys of VDRA-treated CKD mice (Figure 4), suggesting that the kidney was not the source of serum klotho. Furthermore, although klotho was easily detected in parathyroid glands by immunostaining, no difference was detected in CKD + HP mice following VDRA treatment (Supplementary Figure S2 online). We have not ruled out inhibition of klotho degradation, nor the possibility that accelerated shedding of klotho into serum and urine accounted for the increased levels. Recent studies have identified the proteases involved in klotho shedding,[51,52] although the *in vivo* significance of the various shed/secreted isoforms remains unknown. Additional studies are needed to determine the mechanisms underlying increased klotho in the setting of CKD and VDRA therapy.

Serum FGF23 levels decreased significantly in the VDRA-treated CKD + HP mice, and it is likely that production of FGF23 was downregulated in parallel with correction of hyperphosphatemia. FGF23 strongly correlated with serum phosphate levels in the current studies ($R = 0.58$; $P < 0.001$). Although VDRAs have been shown to markedly increase FGF23 expression from bone in healthy[53] and CKD mice,[54] studies in vitamin D receptor–null ($VDR^{-/-}$) mice demonstrated that phosphate can upregulate FGF23 expression independent of vitamin D.[55] Lowering of FGF23 may confer cardiovascular benefits, as FGF23 has been shown to induce left ventricular hypertrophy independent of klotho.[56] Interestingly, heterogeneity in the response of FGF23 levels to calcitriol treatment was recently reported in a cohort of CKD patients; in addition, the study noted a significant correlation between change in serum phosphate levels and change in FGF23.[57]

Upregulation of OPN in aortic VSMCs was the other major finding of this study. OPN is a potent local inhibitor of VC[58] and has been detected in calcified medial wall deposits from CKD patients.[59] Wu-Wong *et al.*[47] previously reported that OPN mRNA levels in human coronary VSMCs were upregulated after treatment with 100 nmol/l paricalcitol for 6 days. We noted upregulation of secreted OPN protein from mouse VSMCs after 48 h of culture with 50 nmol/l paricalcitol. Klotho with/without FGF23 did not upregulate OPN expression *in vitro*, suggesting that the increased OPN expression observed in aortic VSMCs was stimulated by VDRA treatment and not by klotho. Recently, increased aortic wall expression of OPN was reported in CKD rats treated with a supra-therapeutic dose of calcitriol.[60] Whether this upregulation of OPN signifies an overall osteogenic transformation of VSMCs that, while deterring mineral deposition, may be maladaptive in terms of vascular wall contractility remains unclear.

In summary, calcitriol and paricalcitol decreased phosphate-induced AMC in CKD mice. We describe novel mechanisms, including increased serum klotho levels and upregulation of vascular wall OPN, that could contribute to the beneficial anticalcification effects of these VDRAs. Further studies are needed to determine whether these findings are clinically relevant in terms of cardiovascular end points.

MATERIALS AND METHODS

Animal studies

Female DBA/2J mice aged 8–10 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME). Diets included a normal phosphate (NP) diet containing 0.5% phosphate or a high-phosphate (HP) diet containing 1.5% phosphate (Dyets, Bethlehem, PA). The mice underwent partial renal ablation as previously described,[26] whereas NC control mice were not surgically manipulated. HP diet and VDRA treatment (intraperitoneal injections 3 × / week for 3 weeks) were started 2 weeks after renal ablation. Calcitriol (Sigma–Aldrich, St. Louis, MO) and paricalcitol from Abbott (Abbott Park, IL) were dissolved in 100% ethanol and diluted in 5% ethanol to desired concentrations. Further details are available in Supplementary Information online.

Serum chemistries

Blood was drawn from the saphenous vein 4–7 days after surgery 2 to measure blood urea nitrogen (BUN). Interim blood draw was performed within 24 h after a penultimate VDRA dose to assess PTH levels. Terminal blood was collected via cardiac puncture following a 2–4-h fast. The following assays were used: the QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA) for BUN; the *o*-cresolphthalein complexone kit from Teco Diagnostics (Anaheim, CA) for calcium; the standard bioanalyzer at Phoenix Central Laboratory (Everett, WA) for phosphate; the mouse FGF23 C-terminus ELISA kit (Immutopics, San Clemente, CA); the DuoSet mouse osteopontin ELISA kit (R&D Systems, Minneapolis, MN); and the mouse intact PTH enzyme-linked immunosorbent assay kits (ALPCO Diagnostics, Salem, NH and Immutopics). Reference ranges based on levels in NC + NP mice were as follows: PTH 213.9–274.8 pg/ml and FGF23 76.3–423.5 pg/ml.

Klotho immunoblot

Rat anti-human klotho monoclonal antibody (KM2076) was used for klotho western blot in urine and immunoprecipitation-enriched

serum, as well as in tissue lysates. Details are available in Supplementary Information online.

Quantification of aortic calcium and desmosine

Aortic arch segments were lyophilized and decalcified with 0.6 N HCl at 37 °C for 24 h. The calcium content of the supernatant was determined with the *o*-cresolphthalein complexone kit (Teco Diagnostics). Aortic calcium content was normalized to the dry weight of the tissue (μg Ca/mg dry weight). Decalcified aortic arch segments were hydrolyzed in 6 N HCl at 100 °C for 24 h and the supernatant was analyzed for desmosine content as previously described.[61]

Metabolic cage studies

The mice underwent a 24-h urine collection in individual metabolic cages (Tecniplast, Exton, PA). Urine collections coincided with blood collections so as to have corresponding serum data. Serum creatinine was measured using the Quantichrom Creatinine assay kit (BioAssay Systems). Serum phosphate, urine phosphate, and urine creatinine levels were measured using an autoanalyzer (Phoenix Central Laboratory). Fractional excretion of phosphate was calculated using the following formula: FE_{phos} = serum creatinine × urine phosphate/urine creatinine × serum phosphate. Urinary protein was determined using the Pierce Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL).

Histology and immunohistochemistry

The following primary antibodies were used for immunostaining: klotho (R&D Systems, AF1819), osteopontin (R&D Systems, AF808), matrix metalloproteinase-2 (R&D Systems, AF1488), and BM8 (eBioscience, San Diego, CA, 14-4321). Details are available in Supplementary Information online.

Quantitative RT-PCR

Details are available in Supplementary Information online.

VSMC osteopontin expression

VSMCs from C57BL/6 mice (passage 7) were a gift from Mei Speer. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing antibiotics/antimycotic and 10% FBS. At passage 9, VSMCs were seeded at a density of 2×10^4 cells/well in six-well plates. At confluence, serum concentration was lowered to 1% FBS. After an initial 24-h incubation with 1% FBS (time zero), five treatment groups were initiated: (1) vehicle (EtOH) control because paricalcitol is dissolved in 5% EtOH, (2) klotho 2 ng/ml, (3) klotho and FGF23 each at 2 ng/ml, (4) paricalcitol 50 nmol/l, and (5) klotho + FGF23 + paricalcitol. Recombinant mouse FGF23 (2629-FG/CF) and klotho (1819-KL) were purchased from R&D Systems; paricalcitol was provided by Abbott. OPN in the conditioned media was detected using the DuoSet mouse osteopontin ELISA kit (R&D Systems) and normalized to no. of cells/well (averaged from three wells; Beckman Coulter Z1 Particle Counter, Brea, CA). RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) for quantitative RT-PCR analysis.

Statistical analysis

SPSS software v16.0 (SPSS, Chicago, IL) was used to compare group means using one-way ANOVA with Tukey's *post-hoc* analysis. Simple linear regression was used to obtain correlation coefficients between two parameters. Significance for all tests was set at $P < 0.05$.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Average urine klotho levels were higher in the VDRA-treated groups, and the increase was statistically significant in the CKDH + HP + C30 group ($P < 0.05$ compared to CKD + HP group).
Figure S2. VDRA treatment did not increase parathyroid klotho or gland volume.
Figure S3. VDRA treatment did not increase klotho protein in aorta, brain, heart, lung and liver.
Figure S4. Aorta MGP, PiT-1 and PiT-2 mRNA levels were not affected by VDRA treatment.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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