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Calcium-independent and 1,25(OH)$_2$D$_3$-dependent regulation of the renin-angiotensin system in 1α-hydroxylase knockout mice

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To determine whether the cardiovascular effect of 1,25(OH)$_2$D is dependent on calcium and/or phosphorus, mice with targeted deletion of the 25(OH)D 1α-hydroxylase and their wild-type littermates were fed a normal diet or a diet to rescue the ambient serum calcium and phosphorus levels. Mice on the normal diet were treated daily with vehicle or 1,25(OH)$_2$D$_3$ while mice on the rescue diet received vehicle, captopril or losartan. After four weeks the vehicle-treated knockout mice developed hypertension, cardiac hypertrophy and impaired cardiac function along with an up-regulation of the renin-angiotensin system in both renal and cardiac tissues. Although the serum calcium and phosphorus levels were normalized in knockout mice on the rescue diet, abnormalities in blood pressure, cardiac structure-function and the renin-angiotensin system remained. In contrast, 1,25(OH)$_2$D$_3$ not only normalized serum calcium and phosphorus levels but also normalized blood pressure, cardiac structure-function and the renin-angiotensin system. Treatment of the knockout mice with either captopril or losartan normalized blood pressure and cardiac structure and function although renin expression remained elevated. This study shows that 1,25(OH)2D plays a protective role in the cardiovascular system by repressing the renin-angiotensin system independent of extracellular calcium or phosphorus.

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KEYWORDS: vitamin D; renin-angiotensin system; hypertension; cardiac hypertrophy; 1α-hydroxylase gene knockout

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The most well-known function of 1,25(OH)$_2$D is to maintain calcium and phosphorus homeostasis and to promote bone mineralization. However, apart from these traditional calcium-related actions, 1,25(OH)$_2$D$_3$ and its synthetic analogs are being increasingly recognized for their potent antiproliferative, pro-differentiation, and immunomodulatory activities. The actions of 1,25(OH)$_2$D are mediated mostly through the vitamin D receptor (VDR). Physiological and pharmacological actions of 1,25(OH)$_2$D$_3$ in various systems, along with detection of VDR in various target cells, suggest potential therapeutic applications of VDR ligands in osteoporosis, cancer, secondary hyperparathyroidism, and autoimmune diseases.[1] Furthermore, recent evidence indicates that the vitamin D endocrine system also plays a critical role in regulation of blood pressure, volume homeostasis, and cardiac function.[2–5]

Previous studies reported that the serum level of 1,25(OH)$_2$D$_3$ is inversely associated with blood pressure in normotensive and hypertensive subjects;[6–8] it is also inversely correlated with plasma renin activity in patients with essential hypertension.[9] A recent epidemiological study of a large normotensive population in the United States also found an inverse association between serum vitamin D levels and blood pressure.[10] In clinical trials, vitamin D treatment reduced blood pressure in hypertensive or elderly patients.[11,12] Administration of 1,25(OH)$_2$D$_3$ has also been reported to reduce blood pressure, plasma renin activity, angiotensin II (Ang II) levels, and myocardial hypertrophy.[5,13,14] The clinical and epidemiological evidence therefore suggests that vitamin D may regulate blood pressure via regulating the renin–angiotensin system (RAS).

Recently, Li *et al.* investigated the role of vitamin D in the cardiovascular system by employing a mouse model with targeted ablation of the *VDR* gene (VDR$^{-/-}$).[2,4,15] They found that both renin mRNA and protein levels in the kidney, as well as plasma Ang II production, were drastically increased in VDR$^{-/-}$ mice.[2] More recently they have found that 1,25(OH)$_2$D$_3$ suppresses *renin* gene transcription by blocking the activity of the cyclic AMP-response element in the *renin* gene promoter.[16] As a consequence of aberrant

RAS over-stimulation, $VDR^{-/-}$ mice developed hypertension, cardiac hypertrophy, and excess-drinking behavior. Plasma and urinary aldosterone levels were also increased in $VDR^{-/-}$ mice.[4,17] Their data suggest that $1,25(OH)_2D_3$ acts as a negative endocrine regulator of renin production *in vivo*. However, the effect of $1,25(OH)_2D_3$ deficiency on the cardiovascular system has not been fully explored in animals.

We[18] and others[19] have previously reported a mouse model deficient in $1,25(OH)_2D$ by targeted ablation of the *1α(OH)ase* gene ($1\alpha(OH)ase^{-/-}$). These mice developed hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, and skeletal abnormalities characteristic of rickets. These abnormalities are similar to those reported in $VDR^{-/-}$ mice,[15,20,21] except that $VDR^{-/-}$ mice also developed alopecia. In this study, we used $1\alpha(OH)ase^{-/-}$ mice to assess the effect of $1,25(OH)_2D$ deficiency on the RAS, blood pressure, and cardiac function. Our data demonstrate that hypertension induced by vitamin D deficiency can lead to detrimental effects on cardiac function, that treatment with $1,25(OH)_2D_3$ can reverse these findings, and that the effect of $1,25(OH)_2D_3$ on the cardiovascular system is calcium- and phosphorus-independent.

RESULTS

Effect of $1,25(OH)_2D_3$ deficiency on systolic blood pressure, cardiomyocytes, and cardiac systolic function

Both systolic blood pressure (Figure 1a) and heart weight-to-body weight ratio were increased significantly (Figure 1b) in $1\alpha(OH)ase^{-/-}$ mice compared with their wild-type littermates. The left ventricles and cardiomyocytes were markedly hypertrophic in $1\alpha(OH)ase^{-/-}$ mice, as demonstrated by cross sections of the ventricles (Figure 1c) and the cardiomyocyte morphology (Figure 1d). Quantitative data confirmed that the thickness of interventricular septum (Figure 1e) and cardiomyocyte diameters (Figure 1f) were increased significantly in $1\alpha(OH)ase^{-/-}$ mice. Echocardiographic analyses (Figure 2a) revealed that the interventricular septum thickness diastolic (IVSd; Figure 2b), the relative wall thickness (RWT; Figure 2c), and left-ventricular mass (LVM) relative to body weight (Figure 2d) were also increased in $1\alpha(OH)ase^{-/-}$ mice; however, indices of cardiac systolic function, including fractional shorting (FS; Figure 2e) and ejection fraction (EF; Figure 2f) were reduced in $1\alpha(OH)ase^{-/-}$ mice compared with wild-type mice. These results demonstrate that $1,25(OH)_2D_3$ deficiency leads to hypertension, cardiac hypertrophy, and impaired cardiac systolic function in mice.

Effect of $1,25(OH)_2D_3$ deficiency on the renal RAS

To determine whether hypertension and cardiac hypertrophy in $1\alpha(OH)ase^{-/-}$ mice were associated with the activation of the RAS, plasma renin, Ang II, and aldosterone levels were determined by radioimmunoassay, renal renin, and angiotensinogen (Aogen) mRNA levels were examined by real-time reverse transcription-polymerase chain reaction (RT-PCR) and renal renin protein levels were determined by western blotting. Plasma levels of renin (Figure 3a), Ang II (Figure 3b), and aldosterone (Figure 3c) were increased by more than 1.5-fold, and renal *renin* mRNA (Figure 3d) and protein levels (Figure 4a and b) and *Aogen* mRNA levels (Figure 3e) were markedly upregulated in $1\alpha(OH)ase^{-/-}$ mice compared with wild-type mice. These results demonstrate that $1,25(OH)_2D_3$ deficiency leads to upregulation of the RAS in mice.

Cardiac abnormalities and RAS overactivation cannot be rescued by normalization of ambient calcium and phosphorus levels in $1\alpha(OH)ase^{-/-}$ mice

To determine whether cardiac abnormalities and upregulation of RAS can be corrected by normalization of ambient calcium and phosphorus levels in $1\alpha(OH)ase^{-/-}$ mice, 6-week-old, sex-matched, wild-type and $1\alpha(OH)ase^{-/-}$ mice were fed a 'rescue diet' diet containing 2% calcium, 1.25% phosphorus, and 20% lactose for 4 weeks before analyses. The rescue diet normalized serum calcium and phosphorus levels in the mutant mice (Figure 4c and d), but failed to normalize the blood pressure (Figure 1a), cardiac hypertrophy (Figures 1b–f and 2a–d), cardiac systolic function (Figure 2e and f), and upregulation of the RAS (Figure 3 and 4a and b) seen in $1\alpha(OH)ase^{-/-}$ mice. These findings indicate that cardiac abnormalities and activation of the RAS associated with $1,25(OH)_2D_3$ deficiency are independent of the calcium and phosphorus status in mice.

Cardiac abnormalities and the RAS are normalized by administration of $1,25(OH)_2D_3$ in $1\alpha(OH)\ ase^{-/-}$ mice

To determine whether cardiac abnormalities and upregulation of RAS in $1\alpha(OH)ase^{-/-}$ mice can be normalized by administration of $1,25(OH)_2D_3$, 6-week-old, sex-matched, wild-type and $1\alpha(OH)ase^{-/-}$ mice on the normal diet were treated daily with vehicle or 62.5 ng $1,25(OH)_2D_3$/mouse for 4 weeks. As expected, $1,25(OH)_2D_3$ treatment normalized serum calcium and phosphorus levels in $1\alpha(OH)ase^{-/-}$ mice (Figure 4c and d). $1,25(OH)_2D_3$ also reduced blood pressure (Figure 1a), cardiac hypertrophy (Figures 1b–f and 2a), and normalized cardiac systolic function (Figure 2e and f) in mutant mice. Moreover, levels of plasma renin (Figure 3a), Ang II (Figure 3b), and aldosterone (Figure 3c), as well as that of *renin* mRNA (Figure 3d) and protein (Figure 4a and b) and *Aogen* mRNA (Figure 3e), were reduced significantly in $1\alpha(OH)ase^{-/-}$ mice by administration of $1,25(OH)_2D_3$ compared wit vehicle-treated $1\alpha(OH)ase^{-/-}$ mice. These findings demonstrate that although $1,25(OH)_2D$ deficiency leads to activation of the RAS and cardiovascular abnormalities, these abnormalities can be effectively treated with $1,25(OH)_2D_3$.

Cardiac abnormalities are rescued by captopril or losartan in $1\alpha(OH)ase^{-/-}$ mice

Given that Ang II is the central effector of the RAS, we assessed whether cardiac abnormalities can be corrected by administration of the angiotensin-converting enzyme inhibitor captopril or the Ang-α type 1 receptor antagonist

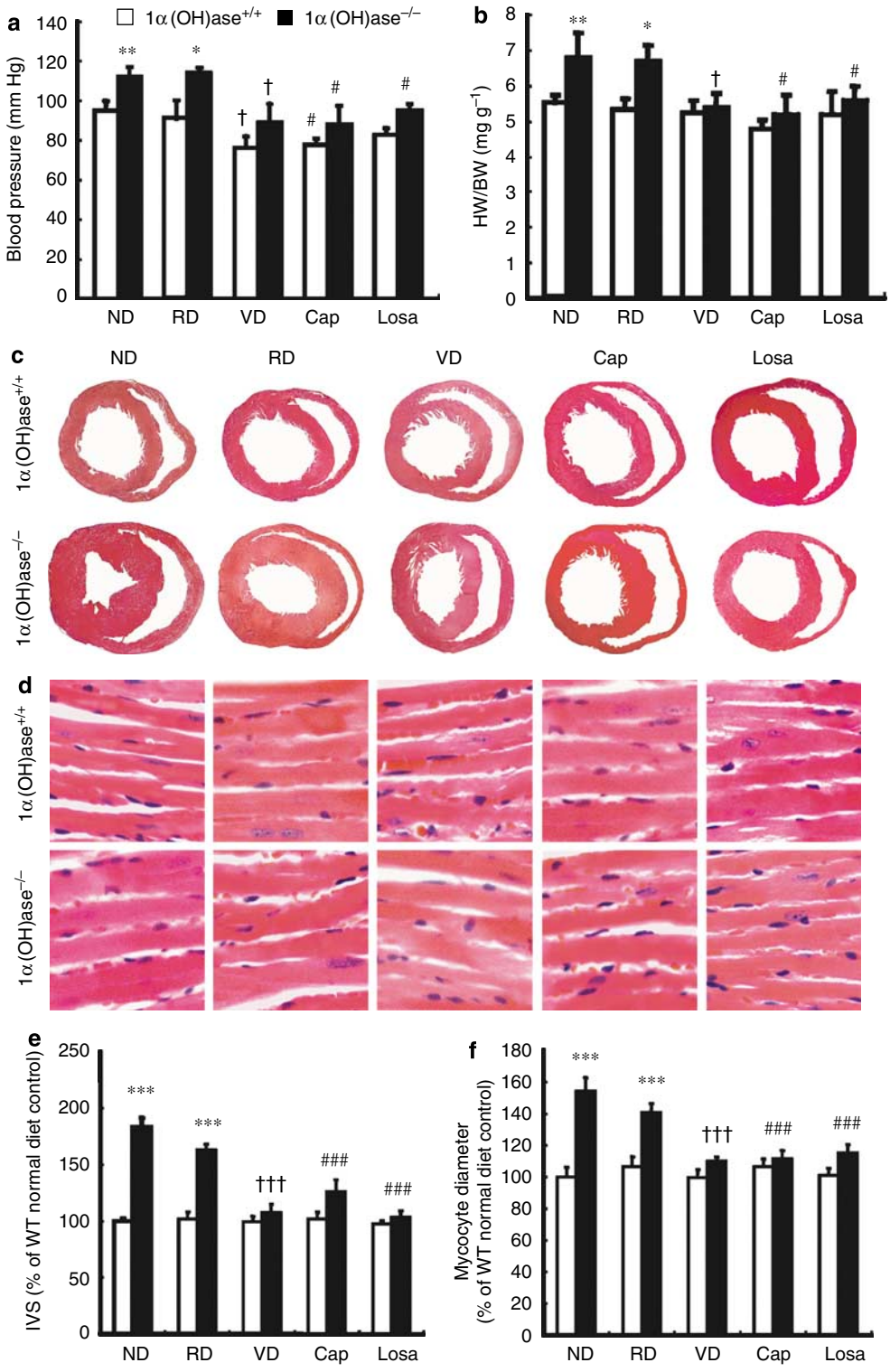

Figure 1 | Hypertension and cardiac hypertrophy in 1α(OH) ase$^{-/-}$ mice. After weaning sex-matched wild-type (1α(OH)ase$^{+/+}$) and 1α(OH)ase$^{-/-}$ mice were fed a normal diet or a 'rescue diet' diet. After 3 weeks on a normal diet, eight animals of each genotype received daily treatment of vehicle (ND), or 62.5 ng 1,25(OH)$_2$D$_3$, intraperitoneally (VD) for four subsequent weeks. After 3 weeks on the 'rescue diet', eight animals of each genotype received vehicle (RD) or 100 mg kg^{-1} captopril (Cap) or 30 mg kg^{-1} losartan (Losa). At the end of this time period, systolic blood pressure (**a**) and heart weight-to-body weight (HW/BW) ratio (**b**) were measured. Representative micrographs of the cross sections of the ventricles (**c**) and the longitudinal sections of cardiomyocytes (**d**) are shown. The thickness of interventricular septum (IVS) (**e**) and cardiomyocyte diameters (**f**) were measured and presented as percentage of wild-type values. Each value is the means ± s.e.m. of determinations in eight mice of each group. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with vehicle-treated wild-type mice. $^{\dagger}P<0.05$; $^{\dagger\dagger\dagger}P<0.001$ compared with vehicle-treated mice fed with the normal diet (ND). $^{\#}P<0.05$; $^{\#\#\#}P<0.001$ compared with vehicle-treated mice fed with a 'rescue diet' (RD).

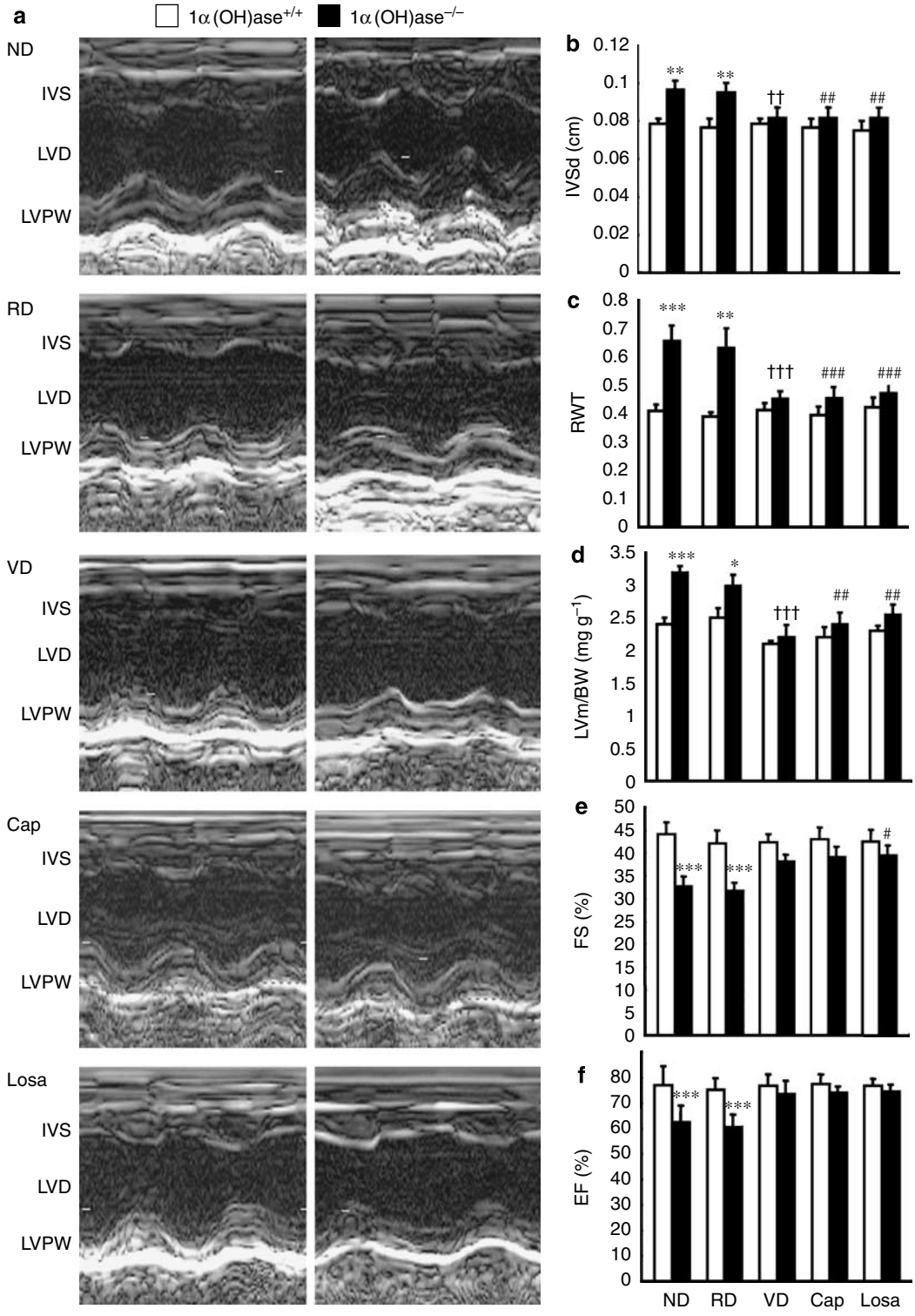

Figure 2 | Cardiac hypertrophy and impaired cardiac systolic function in 1α(OH)ase$^{-/-}$ mice. (**a**) Representative graphs of echocardiography from each group described in Figure 1. The parameters of echocardiography were measured and calculated as described under Materials and Methods. (**b**) IVSd. (**c**) RWT. (**d**) LVM relative to BW. (**e**) FS. (**f**) EF. Each value is the means ± s.e.m. of determinations in eight mice of each group. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with vehicle-treated wild-type mice. $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$ compared with vehicle-treated mice fed with the normal diet (ND). $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ compared with vehicle-treated mice fed with a 'rescue diet' (RD).

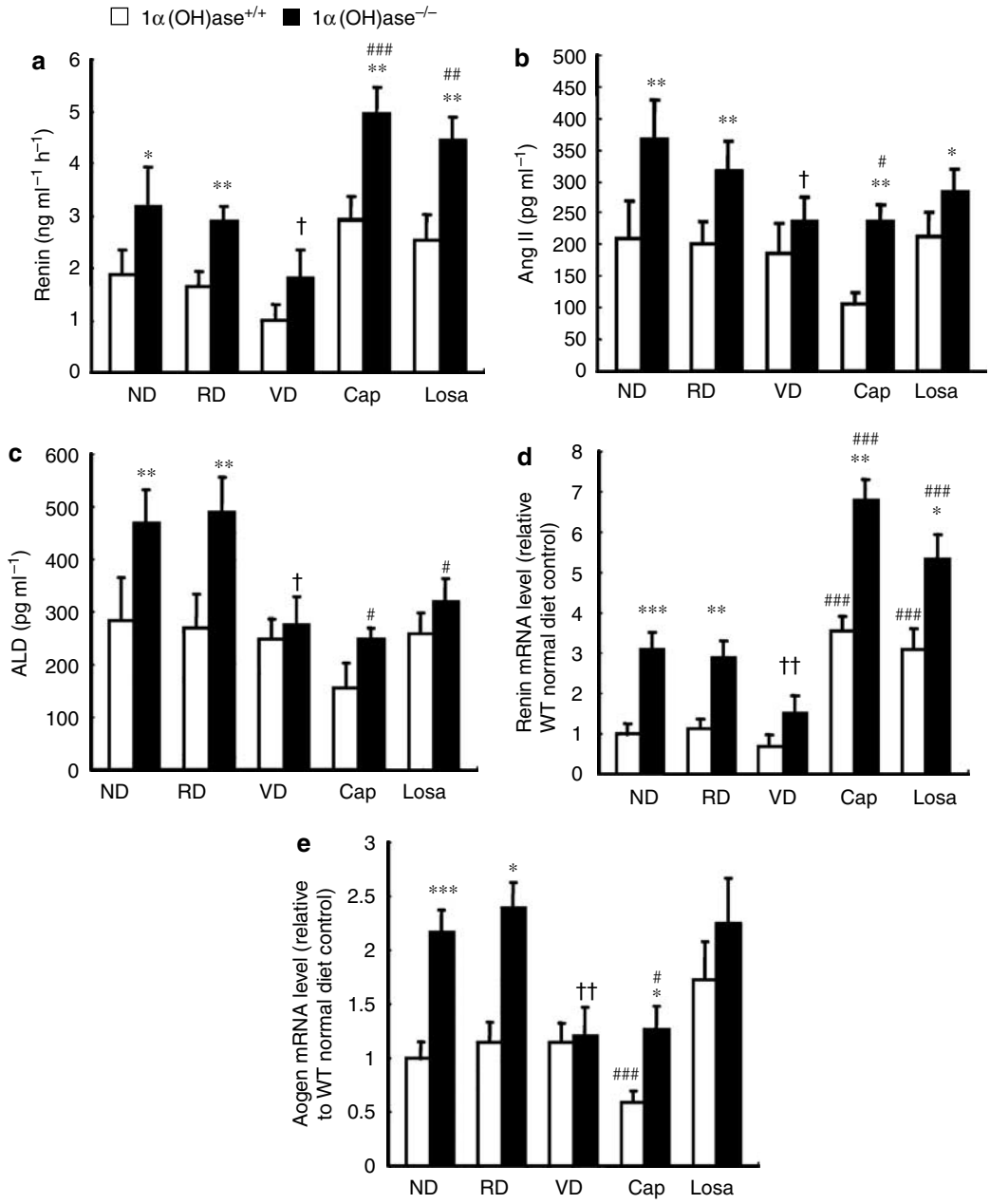

Figure 3 | Upregulation of the RAS in 1α(OH)ase$^{-/-}$ mice. Mice from each group were treated as described in Figure 1. The peripheral blood was harvested and plasma was separated. Plasma renin (**a**), Ang II (**b**), and aldosterone (**c**) were determined by radioimmunoassay. Renal renin (**d**) and Aogen (**e**) mRNA levels were examined by real-time RT-PCR. The renal renin and Aogen mRNA levels were calculated as a ratio to the GAPDH mRNA level and expressed relative to levels of vehicle-treated wild-type mice fed with the normal diet (ND) or a 'rescue diet' (RD). Each value is the means ± s.e.m. of determinations in eight mice of each group. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with vehicle-treated wild-type mice. $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$ compared with vehicle-treated mice fed with the normal diet (ND). $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ compared with vehicle-treated mice fed with a 'rescue diet' (RD).

losartan in 1α(OH)ase$^{-/-}$ mice. The 6-week-old, sex-matched, wild-type and 1α(OH)ase$^{-/-}$ mice on the 'rescue diet' were treated with the vehicle, 100 mg kg^{-1} captopril, or with 30 mg kg^{-1} losartan daily for 4 weeks; systolic blood pressure was measured and cardiac structure, systolic function, and the RAS parameters were examined. Results showed that blood pressure (Figure 1a), cardiac hypertrophy (Figure 1b–f, Figure 2a–d), cardiac systolic function (Figure 2e and f), and plasma aldosterone levels (Figure 3c) in 1α(OH)ase$^{-/-}$ mice were normalized by captopril or losartan. Compared with the rescue diet control, both captopril and losartan increased plasma renin (Figure 3a), renal *renin* mRNA (Figure 3d), and protein (Figure 4a and b) levels in wild-type mice because of their disruption of the feedback inhibition loop in renin synthesis; as a result, plasma renin, and *renin* mRNA and protein levels were even more elevated in these 1α(OH)ase$^{-/-}$

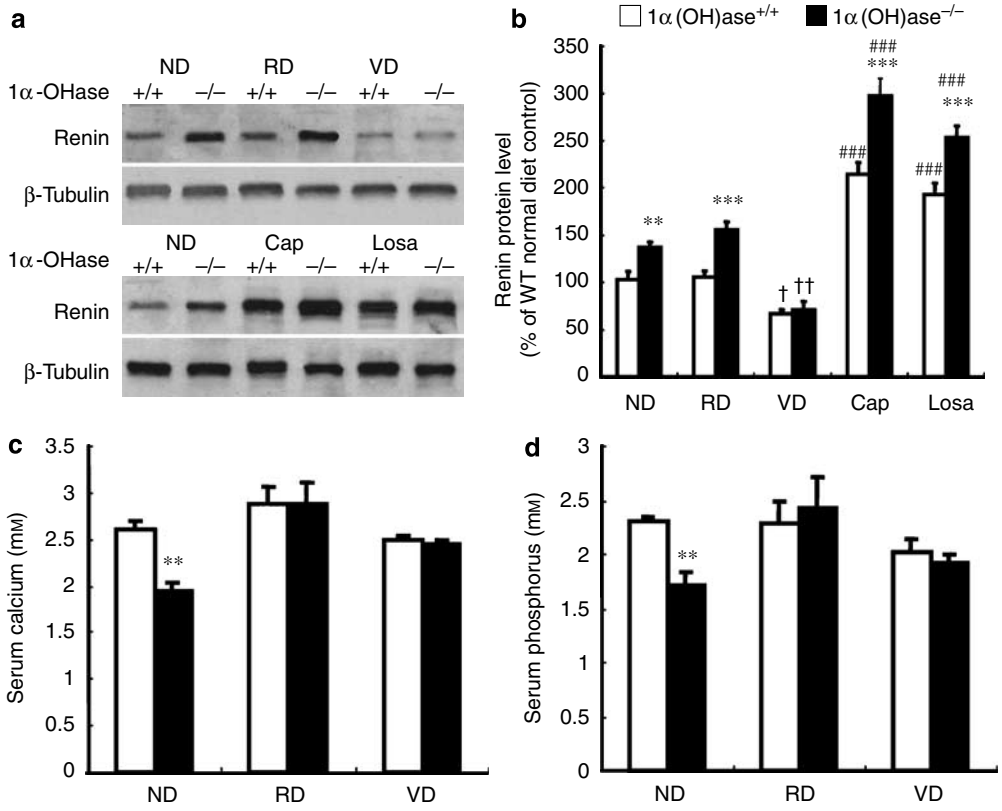

Figure 4 | Levels of renin protein and serum calcium and phosphorus. (**a**) Representative western blots of renal extracts to determine renin protein levels. β-Tubulin was used as loading control. (**b**) Renal renin protein levels relative to β-tubulin protein levels were assessed by densitometric analysis and expressed as percentage of the levels of vehicle-treated wild-type mice fed the normal diet (ND) or a 'rescue diet' (RD), respectively. (**c**) Serum calcium and (**d**) phosphorus were determined as described under Materials and Methods. Each value is the means ± s.e.m. of determinations in eight mice of each group. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; compared with vehicle-treated wild-type mice. $^{\dagger}P<0.05$; $^{\dagger\dagger}P<0.01$; $^{\dagger\dagger\dagger}P<0.001$ compared with vehicle-treated mice fed with the normal diet (ND). $^{\#\#\#}P<0.001$ compared with vehicle-treated mice fed with a 'rescue diet' (RD).

mice (Figures 3a, d and 4a, b). Plasma Ang II (Figure 3b) and *Aogen* mRNA levels (Figure 3e) were reduced significantly by captopril in wild-type mice, but were not altered by losartan, in both wild-type and 1α(OH)ase$^{-/-}$ mice. These findings confirm that the cardiac abnormalities seen in 1α(OH)ase$^{-/-}$ mice are mainly caused by over-activation of the RAS, and $1,25(OH)_2D_3$ protects the cardiovascular system through repression of the RAS cascade.

Effect of $1,25(OH)_2D_3$ deficiency on the expression of VDR and 1α(OH)ase and the RAS in cardiac tissue

We first determined whether the VDR and 1α(OH)ase were expressed in cardiac tissue. The results showed that *VDR* mRNA (Figure 5a) was detectable by real-time RT-PCR in both 1α(OH)ase$^{-/-}$ mice and wild-type mice, whereas *1α(OH)ase* mRNA was found only in wild-type mice (Figure 5b).

We next determined whether cardiac hypertrophy in 1α(OH)ase$^{-/-}$ mice was associated with activation of the cardiac RAS. Cardiac *renin* and *Aogen* mRNA levels were examined by real-time RT-PCR and cardiac renin protein levels were determined by western blotting. Results showed that cardiac *renin* mRNA (Figure 5c) and protein levels (Figure 5e and f) and *Aogen* mRNA levels (Figure 5d) were markedly upregulated in 1α(OH)ase$^{-/-}$ mice compared with wild-type mice. The rescue diet failed to normalize the upregulation of the cardiac RAS (Figure 5c–f) seen in 1α(OH)ase$^{-/-}$ mice. The level of cardiac *renin* mRNA (Figure 5c) and protein (Figure 5e and f) and *Aogen* mRNA (Figure 5d) were reduced significantly in 1α(OH)ase$^{-/-}$ mice by administration of $1,25(OH)_2D_3$ compared with the vehicle-treated 1α(OH)ase$^{-/-}$ mice. These results demonstrate that $1,25(OH)_2D_3$ not only regulates the renal RAS, but also regulates the cardiac RAS in mice in a calcium-independent and $1,25(OH)_2D_3$-dependent manner.

DISCUSSION

In this study, we employed a genetic approach to determine the effect of $1,25(OH)_2D_3$ deficiency on the cardiovascular system. Our data demonstrate that mice deficient in $1,25(OH)_2D_3$ synthesis with targeted ablation of the 1α(OH)ase gene developed hypertension, cardiac hypertrophy, and impaired cardiac systolic function, due to activation of the RAS. Administration $1,25(OH)_2D_3$ to 1α(OH)ase$^{-/-}$ mice not only normalized serum calcium and phosphorus

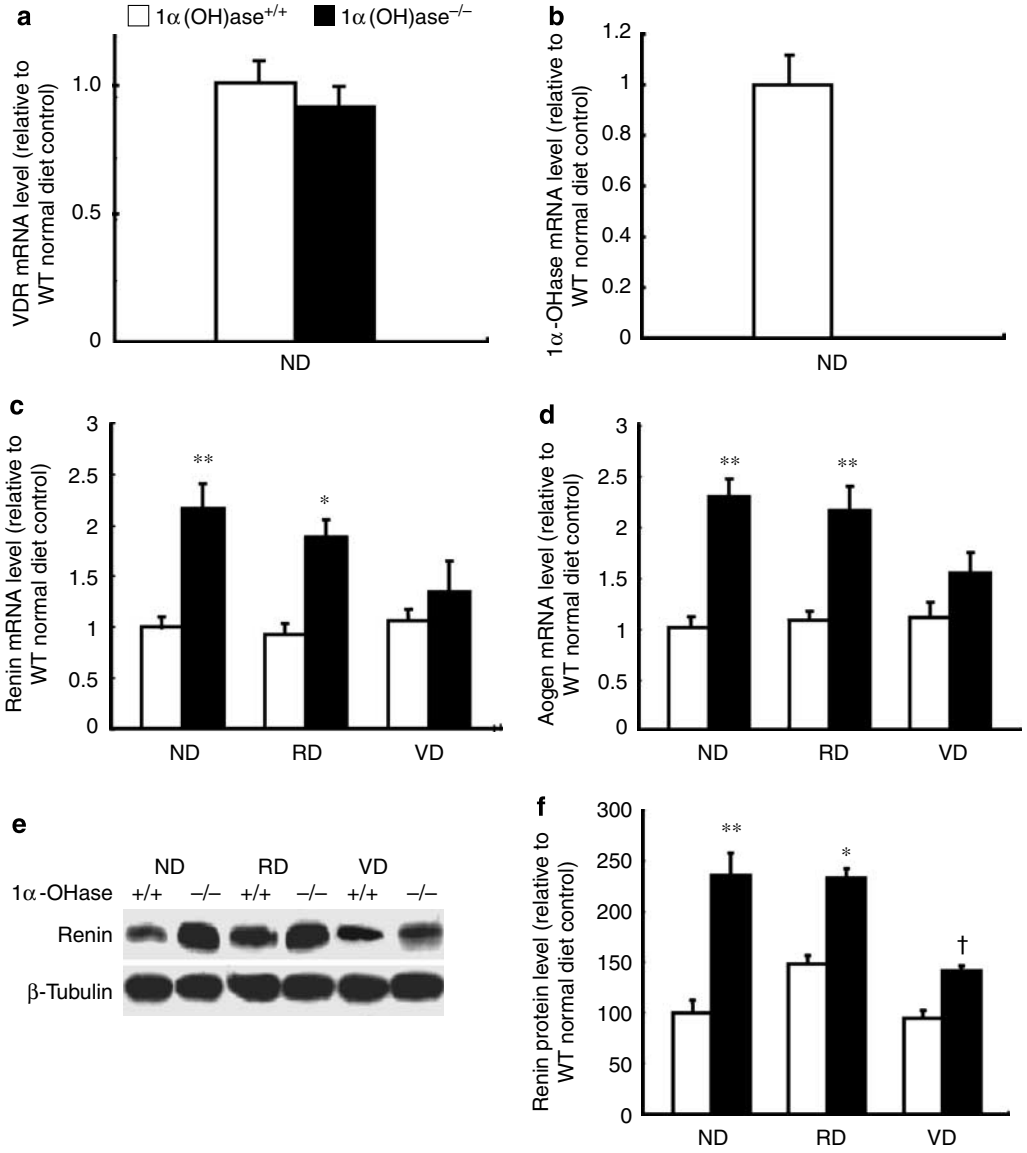

Figure 5 | Expression of 1α(OH)ase and VDR in cardiac tissue and the effect of 1,25(OH)$_2$D$_3$ deficiency on the cardiac RAS. The cardiac VDR (**a**) and 1α(OH)ase (**b**) mRNA levels were examined by real-time RT-PCR in vehicle-treated wild-type and 1α(OH)ase$^{-/-}$ mice fed a normal diet (ND). The cardiac VDR and 1α(OH)ase mRNA levels were calculated as a ratio relative to the GAPDH mRNA level. The cardiac renin (**c**) and Aogen (**d**) mRNA levels were examined by real-time RT-PCR. The cardiac renin and Aogen mRNA levels were calculated as a ratio relative to the GAPDH mRNA level and expressed relative to levels in vehicle-treated wild-type mice fed with the normal diet (ND). (**e**) Representative western blots of cardiac extracts to determine renin protein levels. β-Tubulin was used as a loading control. (**f**) Cardiac renin protein levels relative to β-tubulin protein levels were assessed by densitometric analysis and expressed as percentage of the levels of vehicle-treated wild-type mice fed the normal diet (ND). Each value is the means ± s.e.m. of determinations in eight mice of each group. *$P<0.05$; **$P<0.01$ compared with vehicle-treated wild-type mice. $^{\dagger}P<0.05$ compared with vehicle-treated mice fed with the normal diet (ND).

levels, but also normalized the RAS, blood pressure, cardiac hypertrophy, and cardiac functions. However, normalization of serum calcium and phosphorus in 1α(OH)ase$^{-/-}$ mice with dietary means failed to normalize the RAS and the cardiac abnormalities. These results indicate that 1,25(OH)$_2$D$_3$ regulates the RAS and cardiac function by a calcium- and phosphorus-independent mechanism.

The RAS plays a central role in regulation of blood pressure and water/salt balance under physiological conditions. Renin is the rate-limiting enzyme that converts Aogen to angiotensin I, which is then converted by angiotensin-converting enzyme to Ang II, the central effector of the RAS. Ang II increases salt retention and blood pressure by stimulating aldosterone secretion and by increasing vasoconstriction. A pivotal involvement of the RAS in the pathophysiology of cardiovascular disease is supported by a large body of basic and clinical evidence.[22–25] Over-stimulation of the RAS leads to hypertension, cardiac hypertrophy,

and impaired cardiac activity. Most of the pathological effect of Ang II is mediated via its binding to the AT1 receptor on the plasma membrane.[26] The key role of Ang II involved in the mechanism of diseases is strongly corroborated by a large number of interventional studies. In fact, pharmacologic interference of RAS activity, by both preventing Ang II formation with angiotensin-converting enzyme inhibitors or by blocking its binding to cell membrane receptors with selective antagonists, is associated with highly beneficial outcomes in major disease conditions, including hypertension, renal failure, heart failure, myocardial infarction, stroke, and others.[27] In this study, we showed that accompanying the hypertension, cardiac hypertrophy, and impaired cardiac systolic function in 1α(OH)ase$^{-/-}$ mice was upregulation of the RAS. Renin mRNA and protein expression, and plasma renin, Ang II, and aldosterone levels were all markedly elevated in the mutant mice relative to their wild-type littermates. Remarkably, blood pressure, cardiac hypertrophy, and cardiac function were normalized not only by the administration of captopril or losartan, but also by 1,25$(OH)_2D_3$. In 1α(OH)ase$^{-/-}$ mice; 1,25$(OH)_2D_3$ treatment suppressed renin expression, whereas treatment with captopril or losartan led to further increases in renin production, due to interruption of the feedback inhibition of renin production by these compounds. These data provide strong evidence that cardiac abnormalities in 1α(OH)ase$^{-/-}$ mice are a consequence of over-activation of the RAS, and that 1,25$(OH)_2D_3$ plays a protective role in the cardiovascular system by suppressing the RAS.

Our previous studies using 1α(OH)ase$^{-/-}$ mice showed that parathyroid gland size and development of the cartilaginous growth plate are each regulated by calcium and 1,25$(OH)_2D_3$, but independent of the VDR, whereas parathyroid hormone secretion and mineralization of bone were dependent on ambient calcium levels, but were independent of the 1,25$(OH)_2$D/VDR system.[28] Thus, although the phenotypes of the 1α(OH)ase$^{-/-}$ mice and VDR$^{-/-}$ mice in the skin, parathyroid glands, and cartilaginous growth plate are not completely identical,[28] the phenotypes in the cardiovascular system are similar in both mutant animal models. This is very strong evidence that 1,25$(OH)_2D_3$ regulates cardiovascular functions via the VDR.

Our data are consistent with previous reports that showed that mice with targeted ablation of the VDR gene also develop hypertension and cardiac hypertrophy due to upregulation of the RAS,[2,4] and our studies emphasize the detriment to cardiac function, which ensues in vitamin D-deficient animals. In clinical studies, it has previously been reported that administration of 1,25$(OH)_2D_3$ to hypertensive or elderly patients leads to reduction in blood pressure and myocardial hypertrophy.[11–14] In chronic kidney disease patients on hemodialysis, treatment with active vitamin D analogs significantly reduced the risk of cardiovascular death,[29] although the exact mechanism of action underlying the beneficial effect of the vitamin D has been unclear.

Previous studies have shown that cardiac RAS activation plays a major role underlying the structural and functional abnormalities associated with genetic sympathetic hyperactivity-induced heart failure in mice,[30] and the cardiac hypertrophy seen in VDR$^{-/-}$ mice is a consequence of activation not only of the systemic, but also of the cardiac RAS.[4] Our results demonstrated that cardiac *renin* mRNA and protein levels and *Aogen* mRNA levels were markedly upregulated in 1α(OH)ase$^{-/-}$ mice compared with wild-type mice, and were not normalized by dietary high calcium and phosphate, but were reduced significantly in 1α(OH)ase$^{-/-}$ mice by administration of 1,25$(OH)_2D_3$ compared with vehicle-treated 1α(OH)ase$^{-/-}$ mice. These results support the notion that 1,25$(OH)_2D_3$ not only regulated the renal RAS, but also regulated the cardiac RAS in mice in a calcium-independent and 1,25$(OH)_2D_3$-dependent manner, and that 1,25$(OH)_2D_3$ regulates cardiac functions, at least partially, through the cardiac RAS.

Recently VDR has been found in adult rat cardiac myocytes primarily localized to the t-tubule,[31] and we also demonstrated mRNA expression of the *VDR* in cardiac tissue. In addition, we demonstrated mRNA expression of the *1α(OH)ase* enzyme in cardiac tissue. This suggests that local conversion of 25 hydroxyvitamin D to 1,25$(OH)_2D_3$ may occur and that 1,25$(OH)_2D_3$ may then act in a paracrine, autocrine, or intracrine manner to modulate cardiac function such as the cardiac RAS. Nevertheless, in the model of global inactivation of the 1α(OH)ase we employed, the effects we observed on the rescue of both the cardiac and renal RAS were clearly due to circulating 1,25$(OH)_2D_3$ added exogenously. It remains to be determined what the relative contributions might be of circulating endogenous 1,25$(OH)_2D_3$ provided by renal synthesis, relative to local 1,25$(OH)_2D_3$ provided by cardiac conversion of 25hydroxyvitamin D. It also remains to be determined whether the activity of the cardiac 1α(OH)ase enzyme might be impaired in cardiac disease or might be impaired in renal failure. However, in the model of global inactivation of the 1α (OH)ase we used, both cardiac and renal enzymes were inactivated and exogenously administered circulating 1,25$(OH)_2D_3$ was clearly effective in rescuing the impaired RAS phenotype. These studies, and those by others,[2,4,5] demonstrating the efficacy of circulating 1,25$(OH)_2D_3$ on the cardiovascular system may now provide an explanation for the reduction in blood pressure and myocardial hypertrophy in hypertensive and elderly patients, and the decreased cardiovascular mortality of chronic kidney disease patients receiving systemic administration of active vitamin D forms.[29]

In summary, we demonstrate in this study that genetically mutant mice deficient in 1,25$(OH)_2D_3$ biosynthesis develop hypertension, cardiac hypertrophy, and impaired cardiac systolic function due to the over-stimulation of the renal and cardiac RAS. 1,25$(OH)_2D_3$ regulates renin biosynthesis and cardiovascular function by a calcium- and phosphorus-independent mechanism *in vivo*. Our data obtained from 1α(OH)ase$^{-/-}$ mice are consistent with, as well as confirm, the cardiovascular abnormalities of VDR$^{-/-}$ mice reported

previously, and provide new data on the detrimental effect of vitamin D deficiency on cardiac function, thus strengthening the notion that vitamin D plays a protective role in the cardiovascular system. These studies also provides hope for a useful and beneficial intervention in patients with potentially defective cardiac 1α (OH)ase and in those who have kidney failure.

MATERIALS AND METHODS

Animals and treatment

The generation and characterization of $1\alpha(OH)ase^{-/-}$ mice were previously described by Panda *et al.*[18] $1\alpha(OH)ase^{-/-}$ Mice were generated through breeding of heterozygous mice and identified by PCR with tail genomic DNA as the template, and wild-type littermates were used as controls in all the experiments. The use of animals in this study was approved by the Institutional Animal Care and Use Committee of the University.

Forty pairs of age- and gender-matched $1\alpha(OH)ase^{-/-}$ and wild-type littermates were randomly divided into five groups. After weaning they were fed a normal diet or a 'rescue diet' diet containing 2% calcium, 1.25% phosphorus, and 20% lactose (Harlan Teklad, Madison, WI, USA) for 3 weeks. For four subsequent weeks, animals on the normal diet received daily treatment of the vehicle or 62.5 ng of $1,25(OH)_2D_3$ intraperitoneally, and animals on the 'rescue diet' received the vehicle, 100 mg kg^{-1} captopril, an inhibitor of angiotensin-converting enzyme, or 30 mg kg^{-1} losartan, an Ang II type I receptor antagonist, by daily gavage.

Measurements of blood pressure

Systolic blood pressure was measured in conscious animals using a noninvasive computerized tail-cuff system (ML125 NIBP system; Powerlab, AD Instruments, Castle Hill, NSW, Australia). Mice were placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail. Mice were allowed to habituate to this procedure for 7 days before the measurement of systolic blood pressure. To obtain accurate blood pressure reading, mice remained at a still and unperturbed status throughout the measuring period. Conditioning occurred more readily when mice were handled gently and not forced to enter the restraint. The chamber was kept at 31–33 °C. Systolic blood pressure was measured during three consecutive days. Ten separate determinations of systolic blood pressure were made over a 10-min interval and the determinations recorded were averaged.

Echocardiographic analysis

Transthoracic echocardiography was performed using a 14.0-MHz imaging transducer (GE Ultrasound Machine Vivid 7). Mice from each group were anesthetized by peritoneal injection with 5 μl g^{-1} of 7.5% chloral hydrate. The left hemithorax of each mouse was shaved carefully and the heart was imaged in the two-dimensional mode in the parasternal long-axis view. From this view, an M-mode cursor was positioned perpendicular to the interventricular septum and posterior wall of the left ventricle at the level of the papillary muscles, and M-mode images were obtained for measurements of wall thickness and chamber dimensions. Left-ventricular end-diastolic dimension (LVDd), left-ventricular end-systolic dimension (LVDs), left-ventricular posterior wall thickness diastolic (LVPWd), IVSd, and heart rate were measured. All measurements were performed using the leading edge-to-leading edge convention adopted by the American Society of Echocardiography. EF was calculated by Teichholz formula. Percent FS, LVM, and RWT were calculated as follows:

$$FS = [(LVDd - LVDs)/LVDd] \times 100$$
$$LVM = 1.055 \times [(LVDd + IVSd + LVPWd)^3 - LVDd^3]$$
$$RWT = (IVSd + LVPWd)/LVDd$$

Measurement of blood parameters

Serum calcium and phosphate and $1,25(OH)_2D_3$ levels were measured as described previously.[28] Mouse plasma renin, angiotensin II (Ang II), and aldosterone concentrations were determined by radioimmunoassay, using commercial radioimmunoassay kits. The procedures were preformed according to the manufacturer's instructions.

Determination of heart-to-body weight ratio and histological analysis

The body weight and the weight of the freshly dissected hearts were measured and the ratio of heart weight to body weight was calculated. The whole heart was fixed in a 4% formaldehyde-phosphate-buffered saline (pH 7.4) solution overnight. The cardiac atria were removed by cutting through the coronary sulcus and the cardiac ventricles were embedded in wax and cut into 5-μm cross sections along the cutting face below the coronary sulcus using a rotary microtome. Serial sections of ventricles were collected every 50 μm, stained with hematoxylin—eosin, and examined under a regular microscope. The interventricular septum thickness and the diameter of the cardiomyocytes were measured using Northern Eclipse image analysis software.

Western blot analysis

The kidney and heart were dissected and immediately placed into radioimmunoprecipitation assay lysis buffer containing a cocktail for proteinase inhibitors for protein extraction. Protein extracts from the kidneys from each group were boiled for 5 min in the sample buffer and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membrane was blocked for 3 h at room temperature with 5% non-fat dry milk in phosphate-buffered saline/Tween 20. The blots were incubated overnight at 4 °C with monoclonal antibodies against renin (Fitzgerald, Hornby, Ontario, Canada), followed by incubation for 1 h with anti-mouse secondary antibody (horseradish peroxidase conjugated). Immunoreactive bands were visualized using enhanced chemiluminescence with ECL reagent treatment and exposure to hyperfilm-ECL. The intensity of the bands was measured using Image J version 1.29.

RNA isolation and real-time RT-PCR

RNA was isolated from mouse kidney and heart using Trizol reagent (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. The mRNA levels of *renin* and *Aogen* in the kidney and heart and the mRNA levels of *1α(OH)ase* and *VDR* were quantified by real-time RT-PCR.[32] Briefly, first-strand cDNAs were synthesized from 2 μg of total kidney RNAs in a 25-μl reaction using Moloney murine leukemia virus reverse transcriptase and oligo(dT) as the primer. The cDNAs were then used as the template (1 μl per reaction) for real-time PCR amplification. Real-time PCR was carried out using an Applied Biosystems Cycler and a SYBR Green PCR reagent kit (Toyobo Co., Osaka, Japan). The PCR primers for mouse renin, Aogen, 1α(OH)ase, VDR, and glyceraldehyde-3-

Table 1 | Real-time RT-PCR primers used with their name, orientation (S, sense; AS, antisense), sequence, annealing temperature (T_m), and length of amplicon (bp)

Name	S/AS	Sequence	T_m (°C)	bp
Renin	S	GAGGCCTTCCTTGACCAATC	64	188
	AS	TGTGAATCCCACAAGCAAGG		
Aogen	S	CACCCCTGCTACAGTCCATTG	64	221
	AS	GTCTGTACTGACCCCCTCCAG		
1α(OH)ase	S	GCAGAGGCTCCGAAGTCTTC	55	774
	AS	TGTCTGGGACACGGGAATTC		
VDR	S	GGCTTCCACTTCAACGCTATG	61	388
	AS	CTTACGTCTGCACGAATTGG		
GAPDH	S	CATTTCACTCAAGGTTGTCAGC	64	346
	AS	ATCATACTTGGCAGGTTTCTCC		

Aogen, angiotensinogen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; VDR, vitamin D receptor.

phosphate dehydrogenase (GAPDH) genes are shown in Table 1. GAPDH was used as the internal control for each reaction. All primers were tested for their specificity by conventional RT-PCR before being used for real-time RT-PCR quantitative studies. A melting curve was used to identify a temperature where only the amplicon, and not primer dimers, accounted for the SYBR green-bound fluorescence. Results were analyzed with the SDS 7500 software and the relative amount of mRNA was normalized to GAPDH mRNA.

Statistical analysis

Data are presented as means ± s.e.m. Statistical comparisons were made using a one-way analysis of variance, with $P<0.05$ being considered significant.

DISCLOSURE

The author(s) declare that they have no competing interests.

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