Cardiac hypertrophy in vitamin D receptor knockout mice: role of the systemic and cardiac renin-angiotensin systems

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Xiang, Wei, Juan Kong, Songcang Chen, Li-Ping Cao, Guilin Qiao, Wei Zheng, Wenhua Liu, Xinmin Li, David G. Gardner, and Yan Chun Li. Cardiac hypertrophy in vitamin D receptor knockout mice: role of the systemic and cardiac renin-angiotensin systems. Am J Physiol Endocrinol Metab 288: E125–E132, 2005. First published September 14, 2004; doi:10.1152/ajpendo.00224.2004.—Our recent studies suggest that 1,25-dihydroxyvitamin D3 functions as an endocrine suppressor of renin biosynthesis. Genetic disruption of the vitamin D receptor (VDR) results in overstimulation of the renin-angiotensin system (RAS), leading to high blood pressure and cardiac hypertrophy. Consistent with the higher heart-to-body weight ratio, the size of left ventricular cardiomyocytes in VDR knockout (KO) mice was markedly increased compared with wild-type (WT) mice. As expected, levels of atrial natriuretic peptide (ANP) mRNA and circulating ANP were also increased in VDRKO mice. Treatment of VDRKO mice with captopril reduced cardiac hypertrophy and normalized ANP expression. To investigate the role of the cardiac RAS in the development of cardiac hypertrophy, the expression of renin, angiotensinogen, and AT-1a receptor in the heart was examined by real-time RT-PCR and immunostaining. In VDRKO mice, the cardiac renin mRNA level was significantly increased, and this increase was further amplified by captopril treatment. Consistently, intense immunostaining was detected in the left ventricle of captopril-treated WT and VDRKO mice by use of an anti-renin antibody. Levels of cardiac angiotensinogen and AT-1a receptor mRNAs were unchanged in the mutant mice. These data suggest that the cardiac hypertrophy seen in VDRKO mice is a consequence of activation of both the systemic and cardiac RAS and support the notion that 1,25-dihydroxyvitamin D3 regulates cardiac functions, at least in part, through the RAS.

Cardiac hypertrophy, usually characterized by enlarged left ventricular cardiomyocytes, is a common and often lethal complication of arterial hypertension. At the molecular level, cardiac hypertrophy is often accompanied by activation of the so-called fetal gene program in the left ventricle. This program includes the genes encoding atrial natriuretic peptide (ANP), α-skeletal actin, and β-myosin heavy chain (6, 27). These genes are normally expressed in late fetal and early neonatal heart tissues and are extinguished in adult ventricular myocardiun. The increase of ANP is regarded as a cardioprotective response because of the associated natriuretic, anti-hypertrophic, anti-fibrotic, and anti-hypertensive activities (18).

The renin-angiotensin system (RAS) plays a central role in the regulation of blood pressure, intravascular volume, and electrolyte homeostasis. It is well known that renin is produced mainly in the juxtaglomerular cells in the kidney and released into the blood stream, where it converts the liver-derived angiotensinogen (AGT) to angiotensin (ANG) I, which is converted to ANG II, the central effector of the RAS, by ANG-converting enzyme (ACE). Apart from its endocrine action, the RAS has also been found inside many organs, including the heart, kidney, and brain, suggesting that the RAS may also function in a paracrine fashion (17). ANG II is known to possess potent vasoconstrictive activity, which increases peripheral resistance to blood flow and raises blood pressure (3, 14). Although the RAS is involved in the development of pressure overload cardiac hypertrophy (2), ANG II can also directly induce cardiomyocyte hypertrophy independently of its blood pressure-elevating activity (1, 6, 8, 23). Recently, it has been reported that expression of some components of the cardiac RAS is increased in hypertensive rat models (13, 24, 30, 34), suggesting that the RAS also plays an autocrine-paracrine role in the development of cardiac hypertrophy.

We have shown recently that vitamin D receptor (VDR)-deficient [VDR(-/-)] mice develop high blood pressure accompanied by an increase in heart weight-to-body weight ratio. This reflects, at least in part, effects from activation of the systemic RAS (15, 19). Because vitamin D has been shown previously to directly regulate cardiac functions (39), it is unclear whether the cardiac hypertrophy is due to a direct effect of VDR deficiency or is secondary to activation of the RAS in this model. To address this question in the present study, we sought to determine whether the cardiac hypertrophy in VDR(-/-) mice is associated with activation of the systemic and cardiac RAS, and whether inhibition of RAS activation can reverse the hypertrophic process. Our results suggest that activation of both systemic and cardiac RAS plays an important role in the development of cardiac hypertrophy in VDR(-/-) mice.

MATERIALS AND METHODS

Animals and treatment. Wild-type [WT; or VDR(+/+)] and VDR(-/-) mice were produced from VDR(+/-) breeding, as described previously (20). The body weight of VDR(-/-) mice is 10–20% less than that of WT mice (20). The blood pressure of VDR(-/-) mice is ~20 mmHg higher than that of WT mice, but blood electrolyte levels of the mutant mice are normal (19). Mice were housed in a barrier facility with a 12:12-h light-dark cycle and fed autoclaved normal rodent chow and tap water. Age- and sex-matched 2- to 3-mo-old WT and VDR(-/-) mice were used in this study. In

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some experiments, WT and VDR(−/−) mice were treated with captopril (dissolved in drinking water at 100 mg·kg body wt⁻¹·day⁻¹), as reported previously (15, 19), for 2 wk before death. Mice were killed by exsanguination under anesthesia. The use of mice in this study was approved by the Institutional Animal Care and Use Committee of the University of Chicago.

RNA isolation and Northern blot. Total RNAs were extracted from freshly dissected tissues from WT and VDR(−/−) mice. The tissues were then processed, embedded in paraffin, and hybridized with 32P-labeled cDNA probes as described previously (19). The sections were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). RNA loading was normalized using 36B4 cDNA probe, the acidic ribosomal phosphoprotein PO (16).

ANP measurement. ANP concentrations in the plasma were determined with a commercial radioimmunoassay RIA kit (Phoenix Pharmaceuticals, Mountain View, CA) according to the manufacturer’s instructions.

Histological and immunohistochemical analyses. Freshly dissected hearts were fixed immediately in 4% formaldehyde-PBS (pH 7.4) solution overnight. The tissues were then processed, embedded in paraffin, and cut into 6-μm sections by use of a Leica 2030 microtome. The sections of left ventricles were stained with hematoxylin-eosin and examined under a regular microscope or stained with FITC-labeled wheat germ agglutinin (Sigma, 1:5 dilution) for 2 h at room temperature and then examined under a fluorescence microscope to visualize the myocyte membrane. Heart sections were also stained with an anti-renin antibody as reported previously (19), and the signals were visualized with a DAB peroxidase substrate kit (Vector Labs, Burlingame, CA). The relative size of the cardiomyocytes was quantified by measuring the diameter of the myocytes, which was the distance between the two plasma membranes of a cell in longitudinal section. The measurement was done using ImageJ software. Data were obtained from >30 cells randomly selected from 5–10 microscopic fields of left ventricle slides.

Real-time RT-PCR. The mRNA levels of renin, AGT, and type 1A ANG II receptor (AT-1aR) in the heart were quantified by real-time RT-PCR. Briefly, first-strand cDNAs were synthesized from 5 μg of total heart RNAs in a 50-μl reaction using Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies) and oligo(dT)₁₂–₁₈ as the primer. The cDNAs were then used as the template (5 μl reaction) for real-time PCR amplification. Real-time PCR was carried out using a Cepheid Smart Cycler (Cepheid, Sunnyvale, CA) and a SYBR green PCR reagent kit (Applied Biosystems, Foster City, CA). The PCR primers for mouse renin, AGT, AT-1aR, and GAPDH genes were designed on the basis of cDNA sequences deposited in the GenBank database and are listed in Table 1. GAPDH was used as the internal control for each reaction. All primers were tested for their specificity by conventional PCR before being used for the real-time PCR quantitative studies. The critical threshold (Cₜ) value for each gene was obtained from the real-time PCR reactions, and the starting amount of each target mRNA was calculated on the basis of a calibration curve and the Cₜ value. The relative amount of mRNA was normalized to GAPDH mRNA.

RESULTS

Cardiac hypertrophy in VDR null mice. We (15, 19) previously showed that VDR(−/−) mice had a dramatic increase in renal renin expression, plasma ANG II, and aldosterone levels and developed higher blood pressure. As expected, the heart weight-to-body weight ratio of VDR(−/−) mice was significantly higher (by 20%) than that of WT mice (Fig. 1A), indicating the presence of cardiac hypertrophy in the mutant mice. Histological analyses confirmed that the size of left ventricular myocytes was markedly increased (by ~22% in diameter) in VDR(−/−) mice compared with WT mice (Fig. 1B and C). Consistent with these findings, the mRNA levels of ANP, an important marker of myocardial hypertrophy, were significantly increased in the heart of VDR(−/−) mice (Fig. 2A). Quantitative data showed that the increase in ANP mRNA levels in VDR(−/−) mice ranged from 50 to 100% compared with WT mice (Fig. 2B). More, the concentration of plasma ANP was also significantly increased (by 84%) in the mutant mice (Fig. 2C). Interestingly, the increase in ANP mRNA levels in the atria (60%) (Fig. 2D) was larger than that seen in the ventricles (31%) in VDR(−/−) mice (Fig. 2E). Ventricular α-actin expression was also increased by 24%, but the value did not reach statistical significance (P = 0.164, n = 4; data not shown). No significant changes in brain natriuretic peptide expression were detected in the heart of VDR(−/−) mice (data not shown).

Role of the RAS in cardiac hypertrophy. To address whether the myocardial hypertrophy seen in VDR(−/−) mice is a consequence of stimulation of the RAS caused by VDR inactivation, the mice were treated with captopril, an ACE inhibitor that blocks ANG II production. As shown in Fig. 3, 2 wk of captopril treatment drastically increased the expression of renin in the kidney of both WT and VDR(−/−) mice (Fig. 3A and B), confirming that the treatment was effective. The reason for the increase in renin gene expression is the disruption of the ANG II feedback loop by captopril, which is an important regulatory mechanism in renin biosynthesis. It is worth pointing out that, after 5 days of captopril treatment (as showed previously by our laboratory, Ref. 15), the approximately threefold difference in renal renin expression remained evident between treated WT and VDR(−/−) mice, whereas the 2 wk of treatment here appeared to have maximized the stimulation of renin expression in both WT and VDR(−/−) mice; therefore, the difference between them became minimal (Fig. 3B).

The captopril treatment had little effect on the heart-to-body weight ratio in WT mice but significantly reduced the heart-to-body weight ratio in VDR(−/−) mice (Fig. 4A). Similarly, the treatment also reduced ANP mRNA expression in VDR(−/−) mice to the levels observed in WT mice (Fig. 4B). Consistently, captopril treatment diminished the left ventricular cardiomyocyte hypertrophy in VDR(−/−) mice (Fig. 5). These results suggest that the cardiac hypertrophy and in-

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### Table 1. Primers for real-time RT-PCR analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>PCR Primers</th>
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<tbody>
<tr>
<td>Renin</td>
<td>forward 5′-GAGGCGCTGTGTTGACGAAATG-3′</td>
</tr>
<tr>
<td></td>
<td>reverse 5′-TGTCAGATCCAGAAGCTGGA-3′</td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>forward 5′-CACCCCTGCATGCAGTCCTGG-3′</td>
</tr>
<tr>
<td></td>
<td>reverse 5′-GTCGAGCTGACGCCCCTCCACG-3′</td>
</tr>
<tr>
<td>AT-1a receptor</td>
<td>forward 5′-GGAACAGCAGTGGTGAGTGATC-3′</td>
</tr>
<tr>
<td></td>
<td>reverse 5′-CTTGGACACAGTGGAGCAGAAG-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward 5′-CAACTTTTGTCTTTGGTGAACG-3′</td>
</tr>
<tr>
<td></td>
<td>reverse 5′-ACGACTTGGGGGTTAGGAACAC-3′</td>
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AT-1a receptor, ANG II type 1a receptor.
creased ANP gene expression in VDR(−/−) mice are largely a consequence of elevated ANG II production resulting from RAS activation (19).

Role of the cardiac RAS. Previous studies suggested that the cardiac RAS may be involved in the development of hypertension and cardiac hypertrophy in hypertensive animal models (13, 24, 34). We therefore measured renin, AGT, and AT-1aR mRNA levels within the hearts of VDR(−/−) mice by real-time RT-PCR. As shown in Fig. 6, the mRNA level of cardiac renin was increased 2.5-fold in VDR(−/−) mice compared with WT mice (Fig. 6A). Interestingly, captopril treatment dramatically induced cardiac renin mRNA expression in both WT and VDR(−/−) mice, and renin mRNA levels in the treated VDR(−/−) mice remained much higher than those seen in the treated WT mice (Fig. 6A). This result was supported by immunostaining of heart sections with a renin-specific antibody. Although the heart sections from WT mice were hardly stained by the antibody (Fig. 7A), positive staining was detected in the left ventricle (particularly the distal portion of the left ventricle) in VDR(−/−) mice (Fig. 7B), reflecting the higher renin mRNA levels. More intense staining was seen in approximately the same area in the left ventricle of captopril-treated WT and VDR(−/−) mice (Fig. 7, C and D). This pattern of renin expression reflects renin mRNA levels detected in the heart (Fig. 6A). A similar pattern of renin induction was observed previously in the kidneys of captopril-treated WT and VDR(−/−) mice (15), suggesting the existence of a similar feedback inhibition loop for renin regulation in the heart. In contrast, compared with WT mice, the expression of cardiac AGT and AT-1aR mRNAs in VDR(−/−) mice was not significantly different with or without captopril treatment (Fig. 6, B and C).
The upregulation of ANP, which can be blocked or attenuated by captopril treatment. Thus VDR(-/-) mice develop ANG II-dependent cardiac hypertrophy. Histologically, it represents a mild concentric ventricular hypertrophy with an increased left ventricular wall. The present data are consistent with our early observations that blocking the RAS diminishes the increase in blood pressure and over-drinking behavior in VDR(-/-) mice (15, 19). We also show that the cardiac hypertrophy in VDR(-/-) mice is associated with a significant increase in renin levels within the heart. These observations suggest that cardiac hypertrophy in VDR(-/-) mice may be a consequence of stimulation of the RAS both in the systemic circulation and within the myocardium, leading to hypertension and activation of the ANG II-mediated hypertrophic program (29). Because ACE is also involved in the inactivation of bradykinin, inhibition of ACE by captopril may also affect the kallikrein-kinin system by preventing degradation of bradykinin (36). Therefore, the question of whether the kallikrein-kinin system also contributes to the development of the cardiovascular abnormalities in VDR(-/-) mice needs further investigation.

Tissue RAS has been reported to play an important role in regulating blood pressure and cardiovascular functions (17). In particular, increased cardiac RAS has been linked to hypertension and cardiac hypertrophy. In genetically hypertensive rat models such as spontaneously hypertensive rats and hyperterglyceridemic rats, which develop hypertension and cardiac hypertrophy with increasing age, components of the cardiac RAS, including renin, AGT, ACE, and/or AT-1aR, were found to be significantly elevated compared with normal rats (13, 22, 24, 30, 34). Autocrine release of ANG II has been shown to mediate stretch-induced hypertrophy of neonatal cardiomyocytes in culture (29). Thus activation of the cardiac RAS likely contributes to the development of cardiac hypertrophy. Local cardiac RAS may act independently of the systemic RAS in
inducing hypertrophy (12, 23), which may partially explain the in vivo cardioprotective effect of ACE and AT receptor blockade in preventing or attenuating ventricular hypertrophy, even in the absence of high circulating renin levels (7, 25). The present study shows that cardiac renin, but not AGT and AT-1aR, is significantly elevated in VDR(−/−) mice. This result closely parallels our early finding that vitamin D specifically suppresses renin gene expression in mouse kidney (19). The induction of renin within the heart may increase intracardiac ANG II production and thus contribute to the development of the hypertrophic state in VDR(−/−) mice. It is possible that the intracardiac renin comes from uptake of circulating (pro)renin protein, as suggested by other studies (28); however, the dramatic increase in cardiac renin mRNA levels in captopril-treated mice argues against this possibility, suggesting that at least part of the intracardiac renin is from de novo synthesis due to renin gene activation in the heart. Regulation of the RAS is complex and involves multiple pathways. One interesting question is whether vitamin D, in addition to regulating renin, is also involved in the regulation of other components of the RAS. Because both hepatic and cardiac AGT and AT-1aR are not affected by VDR inactivation, as shown previously (19) and in the present study, we speculate that vitamin D is unlikely to directly regulate AGT and AT-1aR expression in mice.

Because VDR(−/−) mice have a dramatic increase in renin production, it is interesting to compare these mice with other hypertensive rodent models. The endothelial nitric oxide synthase (eNOS) knockout mice have a level of hypertension comparable to that of VDR(−/−) mice, with a twofold increase in plasma renin levels (31, 43). These mice also develop cardiac hypertrophy (4, 43). Ventricular ANP expression is markedly increased, which was thought to compensate for the loss of the eNOS contribution to left ventricular relaxation (11). The 2K1C renal artery stenosis rats, on the other hand, develop much more severe high-renin hypertension and ventricular hypertrophy (10). ANP expression is drastically induced in the ventricles and much less so in the atria, and an increase in both ANG II and blood pressure is necessary for cardiac ANP induction in 2K1C rats (40). In VDR(−/−) mice, the increase in ventricular ANP expression appears to be less dramatic compared with the eNOS knockout and 2K1C models, which may reflect the relatively moderate level of ventricular hypertrophy seen in VDR(−/−) mice.

Under normal conditions, ANP is mainly produced in the atrium, and little is produced in the ventricle. Atrial ANP production increases in responses to volume overload and atrial distention, whereas ventricular ANP production increases when ventricular hypertrophy develops in response to chronic increase in pressure overload (3). Compared with WT mice, VDR(−/−) mice have much higher water intake and urine excretion (19). Constant increased water intake causes a tran-
sient volume overload and atrial stretch, leading to upregulation of ANP in the atrium. The increase in atrial ANP production, in turn, stimulates diuresis and helps to maintain the steady-state volume balance. Therefore, the more pronounced increase in atrial ANP may well reflect a compensatory physiological response to the increased water intake, whereas the upregulation of ventricular ANP expression is a pathophysiological consequence of ventricular hypertrophy resulting from ANG II-induced vasoconstriction and the prohypertrophic action of ANG II. Lack of direct suppression of ANP gene expression by liganded VDR may also contribute to the greater elevation in atrial ANP levels (41). Therefore, VDR(−/−) mice appear to develop concentric cardiac hypertrophy mostly due to pressure overload caused by activation of the RAS.

Because VDR is expressed in cardiac myocytes (9, 33), the heart is believed to be a vitamin D target organ. However, regulation of cardiovascular functions by vitamin D appears to be complicated. In vitamin D-deficient rats, blood pressure and the ratio of heart weight to body weight, as well as cardiac and vascular muscle contractile responses, are increased (37, 39). This may involve both direct and indirect actions of vitamin D (38). Interestingly, the vitamin D-deficient rats also show a marked increase in plasma renin activity in both hypocalcemic and normocalcemic states (38), suggesting that the activation of the RAS also contributes to the aberrant cardiovascular function in this setting. In vitro studies have suggested that vitamin D directly regulates cardiomyocyte function. In primary cultures of neonatal rat cardiomyocytes, 1,25-dihydroxyvitamin D3 inhibits ventricular myocyte proliferation (26) and antagonizes endothelin-induced myocyte hypertrophy (42). Genes associated with myocyte proliferation and hypertrophy, including c-myc, PCNA, and ANP, are suppressed by 1,25-dihydroxyvitamin D3 (5, 26, 41, 42). The present study suggests that vitamin D regulates cardiovascular functions through activation of the RAS, and it does not exclude other more direct effects on the cardiac myocytes. Taken together, the available data suggest that vitamin D regulates cardiovascular functions by multiple mechanisms involving both direct effects on the cardiomyocytes and indirect effects that are dependent on suppression of the systemic and cardiac RAS.
Cardiovascular disease is a major cause of death among patients with chronic kidney disease, and vitamin D deficiency is a common problem among these patients. Interestingly, recent studies have shown that vitamin D and vitamin D analogs given primarily to treat secondary hyperparathyroidism and the associated calcium and phosphate metabolic abnormalities of chronic kidney disease are associated with a significant reduction in the risk of death and of cardiovascular death among patients on hemodialysis, suggesting that the protective effect of vitamin D is beyond calcium-phosphorus metabolism (32, 35). The cardiovascular protection of vitamin D is consistent with its role as a negative endocrine regulator of the RAS and its anti-proliferative effect on myocardial cell hypertrophy and proliferation. As a major pathogenic factor to hypertrophy and proliferation, the angiotensin II-mediated cardiovascular and renal diseases. Pharmacol Rev 52: 11–34, 2000.


