Vitamin D receptor deficiency increases systolic blood pressure by upregulating the renin-angiotensin system and autophagy

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Abstract. The vitamin D receptor (VDR) may regulate blood pressure via multiple pathways. The present study investigated the underlying mechanism by which VDR deficiency increases blood pressure. A total of 16 8-week-old male littermate mice were randomly divided into the VDR knockout and wild-type groups (VDR−/− and VDR+/+, respectively). Blood pressure was measured using a four-channel PowerLab data acquisition and ADI software analysis system. After euthanasia, vascular smooth muscle cells (VSMCs) were isolated from the VDR−/− and VDR+/+ mice. Oxidative stress, renin-angiotensin system (RAS) activation and autophagy markers were measured in the isolated VSMCs using reverse transcription-quantitative PCR (RT-qPCR), western blotting and transmission electron microscopy (TEM) assays. Mean systolic pressure was significantly higher in the VDR−/− mice compared with the VDR+/+ mice. RT-qPCR and western blotting analyses indicated that RAS markers (angiotensin II and type 1 receptor) were significantly upregulated, oxidative stress was increased (evidenced by reduced superoxide dismutase and peroxiredoxin-4) and autophagy was activated (upregulation of autophagy related protein 7, Beclin 1 and microtubule-associated proteins 1A/1B light chain 3A) in the VDR−/− VSMCs compared with the VDR+/+ VSMCs. TEM demonstrated that there were more autophagy bodies in the VDR−/− VSMCs compared with the VDR+/+ VSMCs. In conclusion, VDR deficiency was associated with high blood pressure. The mechanism underlying the increase in blood pressure caused by VDR deficiency may involve activation of the RAS, as well as increased oxidative stress and autophagy of VSMCs.

Introduction

Vitamin D signaling plays a role in regulating blood pressure through influencing vascular endothelial function, oxidative stress and activation of the renin-angiotensin-system (RAS), as well as increasing insulin resistance. The widespread effect of vitamin D relies on the extensive presence of the vitamin D receptor (VDR), which is expressed in every human tissue and nearly all nucleated cells, although at varying levels. It is currently hypothesized that almost all biological actions of vitamin D are mediated by its active form, 1,25(OH)2D, signaling mainly through the intracellular VDR (1). Vitamin D signaling has been associated with elevated plasma renin and angiotensin (Ang) II levels (2). Animal experiments have indicated that 1,25(OH)2D3 can inhibit renin gene transcription (3), and Zhou et al (4) revealed that blood pressure is higher in la(OH)ase−/− mice compared with wild-type mice, which is accompanied by elevated mRNA expression levels of renin, plasma aldosterone and Ang II. These studies indicate that 1,25(OH)2D3 may influence blood pressure by regulating the central and peripheral RAS through an anti-oxidative stress mechanism. Another in vivo experiment (5) suggested that vitamin D deficiency increases Ang II and oxygen anion levels in local vascular smooth muscle cells (VSMCs). Several studies have also demonstrated that 1,25(OH)2D3 deficiency can increase blood pressure by inducing oxidative stress pathways and over-activating the central RAS (6-8).

When vitamin D levels are adequate, a number of the intracellular oxidative-stress-related activities are downregulated. Suboptimal concentrations of serum 25(OH)D fail to subdue oxidative stress conditions, augment intracellular oxidative damage and decrease the rate of apoptosis. Superoxide dismutase (SOD) belongs to a group of antioxidant enzymes that play a significant role in regulating oxidative stress in cells (9). Peroxiredoxin 4(Prdx4), a typical endoplasmic reticulum-resident 2-Cys antioxidant of peroxiredoxins, can fine-tune hydrogen peroxide catabolism, which affects cell survival by affecting reox balance, oxidative protein folding and hydrogen peroxide signaling (10). Vitamin D can regulate autophagy at different levels, including induction, nucleation and elongation to maturation and degradation, which affects the occurrence and development of diseases (11).

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We previously investigated the association between VDR gene polymorphisms and hypertension. We revealed polymorphisms in VDRrs11574129, rs2228570 and rs739837 in 2,409 patients with hypertension and 3,063 controls, and that the rs2228570 polymorphism is significantly correlated with risk of hypertension (12). However, the mechanism by which vitamin D signaling regulates blood pressure remains unclear. Therefore, the present study established a VDR deficiency animal model using VDR knockout mice to investigate how VDR regulates blood pressure.

Materials and methods

Animals. VDR<sup>+/-</sup> mice were derived by homologous recombination in embryonal stem cells as described previously (gifted from Dr Marie Demay; Massachusetts General Hospital, MA, USA) (13). VDR wild-type (VDR<sup>++</sup>) and knockout (VDR<sup>−/-</sup>) mice were identified using western blotting analysis of tail blood samples (Fig. S1). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. For sample preparation, 100% carbon dioxide was used to euthanize the animals (14).

Experimental mice were raised in the SPF Animal Center of Nanjing Medical University, where the room temperature was maintained at 20-24°C and ~60% humidity with a 12 h light/dark schedule. A total of 168-week-old male littersmates were randomly divided into the VDR<sup>++</sup> and VDR<sup>−/-</sup> groups (n=8 mice per group). After weaning, the mice were fed a regular diet or a 'rescue diet' (Harlan Teklad; Envigo) containing 20% lactose, 1.25% phosphorus and 2% calcium for 8 weeks.

Blood pressure measurements. The ML125 non-invasive blood pressure (NIBP) system (AD Instruments) was used to measure systolic blood pressure in conscious animals. A pneumatic pulse sensor cuff was placed on the tails. After habituation to this setting for 7 days, systolic blood pressure was recorded. To obtain accurate blood pressure recordings, the mice were kept in a motionless and undisturbed state during the measurement. Conditioning was achieved once the mice were processed gently without forcing restraint. Systolic blood pressure was recorded consecutively for 3 days in chambers (Sigma‑Aldrich) containing proteinase inhibitors. Systolic blood pressure (NIBP) system (AD Instruments) was used for the PCR amplification using the SYBR® Green PCR reagent kit (Toyobo Life Science) in a PCR cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH expression was applied as a loading reference. The target mRNA was amplified in the following thermocycling conditions: initial denaturation for 10 min at 95°C, 40 cycles of denaturation for 15 sec at 95°C, annealing for 40 sec at 55°C, extension for 30 sec at 72°C and final extension for 7 min at 72°C. The following PCR primers were used for corresponding gene detection: Ang-II (NC_000023.11) forward primer: 5'-CCT GAG GGC AAG GGA GGA G‑3' and reverse primer: 5'-GAG GGC AGG GGT AAA AGA GAG‑3'; AT1R (NC_000003.12) forward primer: 5'-ATG TTT TTT TGT GGT GCT TTG‑3' and reverse primer: 5'-CCT GAG AGG GTG CAG GAG AA‑3'; SOD1 (NC_000021.9) forward primer: 5'-AAC CAT CCA CTT GGT GGC TTG‑3' and reverse primer: 5'-CAG GGC ACA GAG GCA AGG‑3'; SOD2 (NC_000022.4) forward primer: 5'-CCT GAG AGG GTG CAG GAG AA‑3'; SOD3 (NC_000021.9) forward primer: 5'-AAC CAT CCA GAG GCA‑3' and reverse primer: 5'-GGT CTC CCA ACAT GCC TC TCT‑3'; GAPDH forward primer: 5'-GAC GCG GAG A TG CTC‑3' and reverse primer: 5'-GCC GTG TCT CAC CAC TT‑3'. Relative expression level of the respective target gene was calculated according to the 2<sup>−ΔΔCq</sup> method (16).

Western blotting. VSMCs were lysed in RIPA buffer (Sigma‑Aldrich; Merck KGaA) containing proteinase inhibitors (Sigma‑Aldrich). The protein concentration of the lysates was determined using a BCA kit (Merck KGaA). Aliquots (20 µg) of the extracted protein sample were boiled for 5 min, loaded on an 10% SDS‑PAGE gel, separated by electrophoresis and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% milk at room temperature in phosphate-buffered saline/0.05% Tween 20 (PBST) for 3 h and then incubated with a monoclonal antibody against renin (1:1,000; cat. no. 70R‑1584; Fitzgerald) overnight at 4°C. After three washes with PBST, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000; cat. no. A0208; Beyotime Institute of Biotechnology) at room temperature for 1 h. Finally, the probed bands were visualized using an Enhanced Chemiluminescence reagent (PerkinElmer) and analyzed using ImageJ (version 1; National Institutes of Health) (15). Other used antibodies were as
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Transmission electron microscopy (TEM). For cellular TEM observation, VSMCs were cultured for 120 min and then fixed with 2.5% glutaraldehyde and post-fixed with 3% osmium tetroxide for 2 h at room temperature. The specimen was dehydrated in a graded series of ethanol, embedded with Epon812, and stained by uranium acetate and aluminum citrate. Epon resin and then observed with a Hitachi-600 TEM (Hitachi, Ltd.) to evaluate the formation of autophagosomes in the cells (17).

Statistical analysis. All data were statistically analyzed using SPSS software (version 13.0; SPSS, Inc.). In the present study, all data are presented as the mean ± standard deviation. Average data between the groups was compared using the unpaired Student’s t-test. The Levene test was applied for distribution analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Systolic blood pressure is elevated in VDR−/− mice. Systolic blood pressure was measured using the NIBP system in all mice. The systolic blood pressure of the VDR−/− mice was significantly higher compared with the VDR+/+ littermate control mice (Fig. 1).

The RAS is upregulated in VDR−/− mice. To understand how VDR deficiency affects hypertension and the RAS, the expression levels of RAS factors Ang II and AT1R were measured using western blotting and RT-qPCR assays in VSMCs isolated from both VDR−/− and VDR+/+ mice. Protein expression of AT1R was significantly increased in the VDR−/− VSMCs compared with the VDR+/+ VSMCs (Fig. 2A). The mRNA levels of Ang II and AT1R were significantly upregulated in the VDR−/− VSMCs compared with the VDR+/+ VSMCs (Fig. 2B). These results suggested that deletion of VDR upregulated the RAS in mice.

Oxidative stress is elevated in VDR−/− mice. The association between hypertension in the VDR−/− mice and oxidative stress levels were determined in the VSMCs. mRNA levels of SOD were measured using RT-qPCR and protein expression of Prdx4 were measured using western blotting analysis in the VSMCs isolated from both VDR−/− and VDR+/+ mice. The results demonstrated that the mRNA levels of SOD were significantly downregulated in the VDR−/− VSMCs compared with the VDR+/+ VSMCs (Fig. 3A). The protein expression of Prdx4 was significantly decreased in the VDR−/− VSMCs compared with the VDR+/+ VSMCs (Fig. 3B). These data indicated that VDR deficiency upregulated oxidative stress in mice.

Discussion

Oxidative stress can injure blood vessels and serve as a pathogenic factor in hypertension. Numerous studies have demonstrated that there is an imbalance between the anti-oxidative defense system and the production of oxygen free radicals, causing a high level of oxidative stress in patients with hypertension (18-20). Dysfunction of vascular endothelial cells caused by oxidative stress is considered to be the main cause of hypertension (21). Oxidative stress is closely associated with endothelial cell inflammation, hypertrophy, apoptosis, migration, fibrosis and vascular remodeling in hypertension (19,22).

Since the VDR is widely distributed in vascular endothelial cells, VSMCs and cardiomyocytes, the role of VDR in hypertension has received extensive attention. In a previous observational study, activation of the VDR is associated with lower cardiovascular risk and improved survival (23). VDR deficiency can elevate intracellular oxidative stress (23), and VDR agonists have been demonstrated to synergistically alleviate diabetic atherosclerosis.
by inhibiting oxidative stress (24). Consistent with previous findings, the present study revealed that SOD mRNA levels and Prdx4 protein expression were significantly downregulated in VDRΔ mice compared with the VDR Δ mice. These data suggested that VDR deficiency could increase oxidative stress.
Oxidative stress reflects an imbalance between the reactive oxygen species and a biological ability to detoxify or repair the resulting damage. SOD is an enzyme that downregulates O2‑byscavenging potentially damage-free radical moieties. It acts as a major anti-oxidative enzyme in almost all organisms. Therefore, SOD level reflects the anti-oxidative capacity. The higher the level of SOD, the higher capacity of anti-oxidation, which results the positive balance of anti-oxidation and pro-oxidation. Oppositely, the lower level of the SOD, the lower capacity of anti-oxidation, which causes the negative balance of anti-oxidation and pro-oxidation or oxidative damage (23).

The results of the present study revealed that SOD level was decreased in the VDR‑/‑ mice, which indirectly reflects the upregulated oxidative stress. Indeed, this pattern is consistent with a number of previous findings. For example, in primary angle closure glaucoma, oxidative stress is increased accompanied with a decrease of SOD level (25). Under normal circumstances, production and clearance of reactive oxygen species (ROS) are in equilibrium. However, once ROS production exceeds clearance, a large number of oxygen free radicals will be generated in the body. In patients with hypertension, increased production of ROS results in decreased levels of SOD, destruction of unsaturated fatty acids and increased lipid peroxidation, causing increased production of malondialdehyde.

Vitamin D signaling plays an important role in the inhibition of renin secretion and synthesis. Disruption of VDR signaling transduction leads to RAS activation, cardiac hypertrophy and hypertension (26). It has been demonstrated that VDR+ mice, a model of vitamin D signal disruption, develop hypertension (26). Vitamin D inhibits the renin-angiotensin-aldosterone system by blocking renin gene expression (3). Plasma renin and Ang II levels are negatively correlated with 1,25(OH)2D3 (27). Knockout of VDR and cytochrome P450 27B1 in mice results...

Figure 4. Protein expression of ATG7, Beclin1, LC3A and p62 in VSMCs. Protein expression of ATG7, Beclin1, LC3A and p62 in VSMCs isolated from VDRKO and VDRWT mice was detected using western blotting analysis. (A) Representative images of the corresponding western blot images. The average intensities of (B) ATG7, (C) p62, (D) Beclin1 and (E) LC3A are summarized in the corresponding bar graphs from three independent assays. WT levels were set at 1.0 for data normalization. KO, knockout/VDR‑/‑; WT, wild-type/VDR+/+. VSMCs, vascular smooth muscle cells; ATG7, autophagy-related protein 7; p62, nucleoporin p62; LC3A, microtubule-associated proteins 1A/1B light chain 3A; VDR, vitamin D receptor.
in elevated serum renin and RAS activity and increased blood pressure (5). Xiang et al (28) reported an increase in renin and Ang II mRNA levels in the hearts of 1α(OH)ase and VDR knockout mice. The present study demonstrated that AT1R and Ang II levels increased significantly in VDR−/− VSMCs, consistent with the previous findings. Vitamin D can inhibit renin gene expression by activating the cAMP response elements at the promoter region of the renin gene (4). Overexpression of VDR can inhibit renin production in renal paracyclic cells (29). Based on the result of the present study and the literature, we hypothesize that VDR deficiency induces overexpression of renin, thus activating the RAS in mouse VSMCs. However, further research is needed to confirm this hypothesis.

Autophagy plays an important role in human health. Numerous disorders are associated with autophagy imbalances, such as hypertension and cardiac disease (30). Vitamin D has been reported to regulate autophagy through multiple pathways, including gene induction, nucleation and elongation of protein maturation and degradation (31). However, the mechanism by which the VDR regulates autophagy has not been fully determined. An improved understanding of this mechanism could be useful for clinical diagnosis and treatment of relative diseases.

To the best of our knowledge, thus far, studies on vitamin D-mediated regulation of autophagy have mainly focused on the phosphatidylinositol 3 kinase/Beclin-1 pathway, Ca2+ levels, toll-like receptor signaling pathway, antimicrobial peptides and lysosomes, autophagy related gene expression and inflammatory factors (31). Ang II, a vasoactive peptide, plays a notable role in numerous vascular disorders. An imbalance in vascular autophagy, excessive VSMC proliferation and vascular remodeling can lead to increased vascular resistance and lumen stenosis, resulting in increased blood pressure (32,33). Hypertensive rats demonstrated endothelial dysfunction in aortic and mesenteric arteries, with decreased phosphorylated (p)-Akt, p-mTOR and autophagy marker protein p62, and increased LC3 II/I levels (34). Ang II and glomerular podocyte autophagic activity increased significantly in hypertensive rat kidneys; therefore, high blood pressure caused by kidney injury may be associated with Ang II-induced glomerular podocyte autophagy. Excessive autophagy causes endothelial dysfunction in rats, but it has been revealed that endothelial function can be improved and blood pressure can be reduced through regulating autophagy (35). Ang II induces autophagy through the IAT1R/Rhoda/Rho kinase pathway, causing hypertrophy of VSMCs (36). The interaction between autophagy, oxidative stress and the RAS plays a notable role in vascular remodeling and vascular damage caused by hypertension (37,38).

The objective of the present study was to explore the possible mechanism of VDR deficiency on the RAS and cellular autophagy in a mouse model of vitamin D deficiency. The present study data demonstrated that VDR deficiency increased oxidative stress via downregulating SOD levels and Prdx4 expression, and activating autophagy via upregulation of ATG7, Beclin1 and LC3A expression levels in VSMCs. We hypothesize that the increased autophagy level induced by VDR deficiency may be associated with activation of the RAS and signaling pathways downstream of oxidative stress.

In conclusion, the present study suggested that VDR deficiency increased blood pressure by elevating oxidative stress factors and RAS activity, in addition to causing excessive autophagy of VSMCs. The present study offered novel insight into the mechanism by which VDR regulates blood pressure and provided theoretical evidence to guide clinicians on administering 1,25(OH)2D3 for the prevention and treatment of hypertension.

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Authors' contributions
YZ performed the research conception and design. XT, JL, YZ and XY performed the experiments. JJ and ZT analyzed and checked the data, and drafted the manuscript. JJ, ZT and XY prepared figures. JJ and YZ edited and revised manuscript. YZ was primarily responsible for final content. JJ and YY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (approval no. IACUC‑1910005). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


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