Vitamin D, or cholecalciferol, is a lipophilic, secosteroid hormone. Vitamin D levels are maintained either through dietary consumption or through de novo synthesis in the basal layers of the epidermis following exposure to ultraviolet light. Vitamin D is a prohormone and must undergo sequential hydroxylations by (1) vitamin D 25-hydroxylase to generate 25-hydroxyvitamin D (25(OH)D) and (2) 25-hydroxyvitamin D 1α-hydroxylase1 to generate the most polar and bioactive vitamin D metabolite, 1,25 dihydroxyvitamin D (1,25(OH)2D). Because of the short half-life of 1,25(OH)2D in plasma, the major circulating form of vitamin D, 25(OH)D, is currently considered the best indicator of vitamin D status in the body.2

1,25(OH)2D exerts its biological activity by binding to and activating the vitamin D receptor (VDR). The liganded VDR heterodimerizes with the retinoid X receptor, or occasionally the retinoic acid receptor, to form the regulatory complex that binds to the vitamin D response element to modulate gene expression.3

A billion people affected worldwide.1 Epidemiological data indicate that vitamin D deficiency (circulating 25(OH)D levels <20 ng/dL) is common in patients with cardiovascular disease.1 Reduced plasma 25(OH)D levels are associated with increased risk of hypertension,4,5 ischemic heart disease, myocardial infarction, and early mortality.6 In selected interventional studies, investigators have shown that vitamin D treatment improves endothelial function,7–9 lowers blood pressure,10 and reverses cardiac hypertrophy11,12 in humans. These clinical data support the notion that vitamin D deficiency is a risk factor for the development of cardiovascular disease and that restoration of vitamin D levels may mitigate this risk.

Until recently, studies of 1,25(OH)2D action were largely confined to the intestinal mucosa and bone where its calcitropic properties have been shown to be critical in supporting skeletal mineralization. More recently, it has become apparent that 1,25(OH)2D targets multiple organ systems, modulating functions ranging from epidermal development to immune response to cardiovascular function. VDR and the synthetic machinery for production of 1,25(OH)2D

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Endothelial Function

Elimination of Vitamin D Receptor in Vascular Endothelial Cells Alters Vascular Function

Wei Ni, Stephanie W. Watts, Michael Ng, Songcang Chen, Denis J. Glenn, David G. Gardner

See Editorial Commentary, pp 1187-1188

Abstract—Vitamin D deficiency has been associated with cardiovascular dysfunction. We evaluated the role of the vitamin D receptor (VDR) in vascular endothelial function, a marker of cardiovascular health, at baseline and in the presence of angiotensin II, using an endothelial-specific knockout of the murine VDR gene. In the absence of endothelial VDR, acetylcholine-induced aortic relaxation was significantly impaired (maximal relaxation, endothelial-specific VDR knockout=58% versus control=73%; P<0.05). This was accompanied by a reduction in endothelial NO synthase expression and phospho–vasodilator-stimulated phosphoprotein levels in aortae from the endothelial-specific VDR knockout versus control mice. Although blood pressure levels at baseline were comparable at 12 and 24 weeks of age, the endothelial VDR knockout mice demonstrated increased sensitivity to the hypertensive effects of angiotensin II compared with control mice (after 1-week infusion: knockout=155±15 mm Hg versus control=133±7 mm Hg; P<0.01; after 2-week infusion: knockout=164±9 mm Hg versus control=152±13 mm Hg; P<0.05). By the end of 2 weeks, angiotensin II infusion–induced, hypertrophy-sensitive myocardial gene expression was higher in endothelial-specific VDR knockout mice (fold change compared with saline-infused control mice, type-A natriuretic peptide: knockout mice=3.12 versus control=1.7; P<0.05; type-B natriuretic peptide: knockout mice=4.72 versus control=2.68; P<0.05). These results suggest that endothelial VDR plays an important role in endothelial cell function and blood pressure control and imply a potential role for VDR agonists in the management of cardiovascular disease associated with endothelial dysfunction. (Hypertension. 2014;64:1290-1298.) • Online Data Supplement

Key Words: gene targeting ■ hypertension ■ receptors, calcitriol ■ vitamin D
are present in multiple cell types throughout the heart and vasculature.\textsuperscript{13–15} We reported previously that the ligand VDR possesses antihypertrophic activity in the heart.\textsuperscript{9,11} The expression of the VDR in cardiac myocytes and fibroblasts is upregulated following exposure to hypertrophic stimuli in vitro and in hypertrophied hearts in vivo.\textsuperscript{15} In the vasculature, vitamin D has been shown to control vascular smooth muscle and endothelial cell proliferation.\textsuperscript{15,16} Several, although not all,\textsuperscript{17} animal studies suggest that vitamin D promotes blood vessel relaxation and reduces contractile responses to vasoconstrictors in animals with cardiovascular dysfunction.\textsuperscript{18,19} The identity of the vascular cells targeted by vitamin D in these studies remains undefined. Endothelial dysfunction is seen in a variety of conditions that adversely impact the cardiovascular system, including hypertension, diabetes mellitus, hypercholesterolemia, and chronic renal failure,\textsuperscript{20} conditions that are also known to be associated with vitamin D deficiency. These associations suggested the possibility of a link between endothelial dysfunction and impaired signaling through the liganded VDR.

In an effort to address this issue in a directed fashion, we have generated a mouse with selective deletion of the VDR gene in endothelial cells. We show that these mice demonstrate impaired endothelium-dependent vasorelaxation and an augmented blood pressure response to the administration of angiotensin II (AngII). Collectively, the data suggest that VDR ligands exert an endothelium-dependent, palliative effect on the vascular tree that is likely to prove important in the maintenance of cardiovascular homeostasis.

**Methods**

A full description of Materials and Methods is available in the online-only Data Supplement.

**Endothelial Cell–Specific VDR Knockout Mice**

Generation and characterization of a mouse with a targeted (loxP-bordered) VDR gene allele (VDR\textsuperscript{L/L}) has been reported previously.\textsuperscript{21} We reported previously that the liganded VDR gene in endothelial cells isolated from hearts of control and VDR\textsuperscript{L/L} mice.*** We used the Tie-2 promoter-driven Cre transgene\textsuperscript{22} to promote

\begin{center}
\textbf{Figure 1.} Validation of expression of vitamin D receptor (VDR) in endothelial cells and generation of an endothelial cell–specific VDR knockout mouse. A, VDR expression in human umbilical endothelial cells (HUVECs). Parallel Western blots were incubated with antibody (Ab) alone or Ab with a 5X competing peptide (CP). B, VDR protein was visualized by immunofluorescence (red, arrows) with anti-VDR in HUVECs. Nuclei (blue) are stained with 4',6-diamidino-2-phenylindole (DAPI). Cell borders (green) were defined with anti-CD31. C, Schematic representation of the generation of endothelial cell–specific VDR gene knockout (VDR\textsuperscript{CreKO}) mouse. D, Mouse with homozygous LoxP-bordered VDR allele was crossed with a Tie-2 Cre transgenic mouse to generate VDR\textsuperscript{CreKO} mouse. Each mouse with Cre transgene showed a minor band corresponding to VDR gene deletion. VDR genotyping was performed by polymerase chain reaction using primers indicated with arrows (C). E, VDR mRNA expression in endothelial cells and nonendothelial cells isolated from hearts of control and VDR\textsuperscript{CreKO} mice. ***P <0.001 (n=4). F, VDR protein expression in endothelial cells isolated from hearts of control and VDR\textsuperscript{CreKO} mice. L indicates loxP; and VDR\textsuperscript{L/L}, VDR floxed mouse, served as control.
\end{center}
VDR gene deletion in endothelial cells. Figure 1D lanes 1 to 5 show genotyping of offspring resulting from mating a VDR<sup>lox/lox</sup> female mouse with a VDR<sup>lox/lox</sup>Tie-2 Cre<sup>+</sup> (endothelial cell–specific VDR gene knockout [VDR<sup>ECKO</sup>]) male mouse, a breeding strategy we used to generate all mice for the experiments presented below.

We isolated and cultured endothelial cells and nonendothelial cells (consisting of a mixture of cardiac myocytes, fibroblasts, and vascular smooth muscle cells) from the hearts of control (VDR<sup>lox/lox</sup>) and VDR<sup>ECKO</sup> mice. VDR mRNA levels in endothelial cells were reduced by 87% in VDR<sup>ECKO</sup> mice, whereas VDR expression levels in the nonendothelial cell population were unchanged (Figure 1E). Compared with the endothelial cells isolated from control mice, VDR protein expression in endothelial cells from VDR<sup>ECKO</sup> mice was significantly reduced (Figure 1F). As predicted, deletion of VDR in endothelial cells did not affect plasma calcium or phosphorus levels (Figure S1 in the online-only Data Supplement), indicating that there was no perturbation of systemic mineral metabolism.

Impaired Vasodilation and Reduced Endothelial NO Synthase Expression in Aorta From VDR<sup>ECKO</sup> Mouse

Previous reports have linked vitamin D deficiency to impaired endothelial function. We investigated the role of endothelial VDR in supporting endothelial function by examining endothelium-dependent vasorelaxation in an ex vivo model. We compared the vasorelaxant response to acetylcholine in isolated aortae taken from control versus VDR<sup>ECKO</sup> mice. Figure 2A shows the real-time tracing of aortic tension from control versus VDR<sup>ECKO</sup> mice. Aortae from both groups were challenged with phenylephrine to generate half-maximal, phenylephrine-induced contraction. Increasing concentrations of acetylcholine were then added to the tissue bath to induce endothelium-dependent relaxation. VDR<sup>ECKO</sup> mouse aortae demonstrated impaired vasorelaxation compared with control aortae (Figure 2B). The relaxation to a maximal concentration of sodium nitroprusside (NO donor, 10<sup>-5</sup> mol/L), an endothelium-independent vasodilator, was not affected by VDR deletion in endothelial cells (Figure 2A). Similarly, there was no difference in the aortic contraction to phenylephrine in VDR<sup>ECKO</sup> versus control mice (Figure 2C). Collectively, these results demonstrate that endothelial cell–specific deletion of the VDR gene interferes selectively with endothelium-dependent vasorelaxation in mouse aorta.

Acetylcholine-induced relaxations were completely blocked by the endothelial NO synthase (eNOS) inhibitor (N<sup>ω</sup>-nitro-L-arginine) in both groups (Figure 2B), confirming the fact that NO is the principal mediator of acetylcholine-induced relaxation in mouse aorta. By inference, this suggests that the different vasorelaxant responses that we observed between control and VDR<sup>ECKO</sup> mice could reflect differences in eNOS expression and activity. As shown in Figure 3A, we found that eNOS mRNA levels were reduced to 62% of those found in control aortae. We found a similar reduction in eNOS protein levels (±37%) in aortae from VDR<sup>ECKO</sup> versus control mice (Figure 3B). The levels of phospho–vasodilator-stimulated phosphoprotein, a cGMP-sensitive marker of NO activity (Figure 3C), were also reduced. These results support the notion that VDR deletion in endothelial cells results in impaired NO signaling. Endothelin-1, a peptide produced predominantly in endothelial cells, is a powerful vasoconstrictor and mitogen that stimulates fibrogenesis. A vitamin D analog has been shown to repress high matrix stiffness-induced endothelin-1 gene transcription. However, we found no change in preproendothelin-1 mRNA expression in aortae from VDR<sup>ECKO</sup> versus control mice (data not shown).

Figure 2. Impaired blood vessel relaxation in aorta from endothelial cell–specific VDR gene knockout (VDR<sup>ECKO</sup>) mice. A, Representative tracings of acetylcholine (ACh)-induced, concentration-dependent aortic relaxation. NO donor sodium nitroprusside (SNP) relaxed aorta to baseline. B, Aortae from VDR<sup>ECKO</sup> mice exhibit impaired ACh-induced relaxation. NO synthase inhibitor N<sup>ω</sup>-nitro-L-arginine (LNNA) completely abolished ACh-induced aortic relaxation in aorta from control and VDR<sup>ECKO</sup> mice. VDR floxed mouse, served as control (VDR<sup>L/L</sup>)+LNNA (n=3); VDR<sup>ECKO</sup> (n=5). C, Comparable contractile response to phenylephrine (PE) in aorta from control and VDR<sup>ECKO</sup> mice. *P<0.05 (n=5 for each group).
Because reactive oxygen species, such as superoxide, have been shown to impair endothelial function in several systems, we assessed superoxide levels by dihydroethidium staining on frozen cross sections of aortae from control and VDR ECKO mice at 12 weeks of age. We found a significantly higher level of superoxide in the endothelium from VDR ECKO mice compared with that in the control mice, and this difference extended to the entire vascular wall (Figure S2).

It is important to note that Tie-2 Cre expression has also been reported in myeloid cells. Because macrophages from the myeloid lineage are known to contain VDR and might contribute to the phenotypes we are seeing, we sought to determine whether macrophage number was altered in the VDR ECKO aortae at baseline. To address this, we analyzed aortic cell suspensions for the presence of CD11b+ and F4:80+ cells (CD11b is a marker for the myeloid lineage and F4:80+ cells for macrophage) by fluorescence-activated cell sorter analysis. Isolated aortic cells were stained with CD45 antibody to identify leukocytes in the preparation, and there was no signifcant difference in CD45+ cell number in the control versus VDR L/L versus VDR ECKO aortic suspensions. If anything there appeared to be a slight, albeit nonsignificant, trend toward a smaller number of macrophages in the VDR ECKO aortae. Macrophage number was consistent with what has previously been reported in aortic cell suspensions. Our data suggest that the impaired vasorelaxant activity seen in VDR ECKO mice results from VDR deletion in endothelial cells.

Blood Pressure and Cardiac Response to AngII Infusion Is Increased in Mice Deficient in Endothelial VDR

At 24 weeks of age, VDR ECKO mice had similar blood pressure and heart weight/body weight ratio compared with control mice (Figure S4). Cross-sectional hematoxylin and eosin staining of aorta and carotid artery showed normal blood vessel morphology in the VDR ECKO mouse (Figure S4). Collectively, these data suggest that, at baseline, the cardiovascular phenotype of the VDR ECKO mice is not appreciably different from the controls.

To evaluate the phenotype of the VDR ECKO mouse under the challenge of cardiovascular stress, we subjected 12- to 14-week-old VDR ECKO and control mice to 2 weeks of AngII infusion. We used a low dose of AngII (500 ng/kg/min) to induce a mild increase in blood pressure with minimal cardiac hypertrophy. Blood pressures were measured using the tail cuff method before, 1 week, and 2 weeks after initiation of vehicle or AngII infusion. Blood pressures, before osmotic minipump implantation, were comparable at 12 to 14 weeks of age in control versus VDR ECKO mice. After 1 week, mice receiving AngII had minimal-to-moderate increases in blood pressure, as expected (Figure 4). However, the level of systolic blood pressure induction was significantly higher in VDR ECKO mice versus controls (155.8±14.9 versus 132.9±7.8 mm Hg, P<0.01). Blood pressure levels continued to rise by the end of 2 weeks of AngII infusion, and the difference between VDR ECKO and control mice (Figure 4A–4C) was maintained.

AngII infusion caused thickening of the aortic wall (fold change compared with saline-infused VDR L/L control mice, AngII-infused VDR L/L mice=1.38±0.02, P<0.05; saline-infused VDR ECKO mice=1.03±0.01 versus AngII-infused VDR ECKO mice=1.25±0.1, P<0.05), as demonstrated by hematoxylin and eosin staining (Figure 5). In addition, AngII treatment resulted in increased vascular fibrosis, as demonstrated by Masson trichrome staining of aortic cross sections (Figure 5). However, there were no differences between AngII-treated VDR ECKO controls and VDR ECKO mice in either vessel wall thickness or fibrosis.

The effects of AngII on the heart were evaluated after the 2 weeks of infusion. Figure 6A shows hematoxylin and eosin staining of cardiac sections from the 4 groups of animals. Gravimetric analysis showed similar increases in heart weight/body weight ratio in control and VDR ECKO mice with AngII infusion (Figure 6B). The size of cardiac myocytes was
AngII infusion induced a mild degree of cardiac fibrosis in both control and VDR<sup>ECKO</sup> mice as demonstrated by Masson trichrome staining of cardiac sections (Figure 7A). Gene expression analysis showed that collagen 1a1 and collagen 3a1 mRNA increased with AngII infusion, as expected<sup>35</sup> (Figure 7B). Additionally, as noted with the hypertrophic marker genes above, collagen 3a1 was induced to a greater extent in hearts from VDR<sup>ECKO</sup> mice.

**Discussion**

The present study provides the first evidence of a direct protective role for the endothelial VDR in regulating vascular tone. Our results show that the deletion of VDR in endothelial cells leads to endothelial dysfunction (demonstrated by impaired blood vessel relaxation) and sensitization of mice to the hypertensive effects of AngII infusion. Given the acknowledged importance of endothelial function to cardiovascular homeostasis, the protective activity of VDR in vascular endothelium may account for at least some of the reported beneficial effects attributed to vitamin D in preventing or reversing cardiovascular disease.<sup>36,37</sup>

Several epidemiological studies have demonstrated that individuals with low plasma concentrations of 25(OH)D, the best measure of vitamin D status in humans, are at higher risk for all-cause as well as cardiovascular mortality,<sup>38,39</sup> myocardial infarction,<sup>40</sup> and hypertension.<sup>4,5</sup> However, these relationships remain controversial,<sup>41</sup> and interventional studies have, by and large, failed to demonstrate major effects of vitamin D on reversing cardiovascular disease outcomes,<sup>42</sup> suggesting that this is an area worthy of further investigation.

One potential target for vitamin D in the cardiovascular system is the endothelial cell. Endothelial cells have been shown to express VDR as well as the ligand-generating 1α-hydroxylase,<sup>14</sup> implying that they possess the capacity for orchestrating local vitamin D-dependent regulatory activity that is confined to the vascular wall. Experimental animal studies support a role for vitamin D in regulating endothelial function. Expression of eNOS was shown to be reduced by calcemia in the VDR<sup>−/−</sup> mouse. In another study, VDR<sup>ECKO</sup> mice were normocalcemic (Figure S1). In another study, advanced
glycation end products reduced eNOS gene expression and enzymatic activity. These were restored after treatment with 1,25(OH)2D. VDR activators (eg, paricalcitol) have also been shown to mitigate endothelial dysfunction in animals with renal insufficiency. In rats with 5/6 nephrectomy, VDR gene expression was found to be reduced in the endothelium but not in the smooth muscle layer of the vascular wall. This endothelial-specific VDR deficiency was restored after vitamin D analog treatment. Two weeks of paricalcitol treatment dose dependently improved acetylcholine-induced (endothelium-dependent) relaxation in blood vessels from 5/6 nephrectomized rats. In addition, the improved relaxation was abolished by an NO synthase inhibitor suggesting that paricalcitol affected endothelial function by increasing NO production, a finding that complements those reported here.

Several clinical studies have demonstrated an inverse correlation between plasma 25(OH)D levels and endothelial function, assessed by flow-mediated vasodilation, in humans. Tarcin et al showed that 25(OH)D deficiency was associated with endothelial dysfunction, and vitamin D replacement was found to be effective in reversing this dysfunction. Jablonski et al found that endothelium-dependent brachial artery flow-mediated dilation was lower in vitamin D–insufficient and vitamin D–deficient versus vitamin D–sufficient individuals, whereas endothelium-independent brachial artery dilation did not differ between the groups. Of note, they found an increase in inflammation-linked markers in vitamin D–deficient versus –sufficient subjects and a reduction in both VDR and 1α-hydroxylase expression. They speculated that reduced VDR and local 1,25(OH)2D synthesis might account for the link between vitamin D insufficiency and endothelial dysfunction. Harris et al showed that 16 weeks of vitamin D supplementation (60,000 IU monthly) led to significant improvement in flow-mediated dilation in overweight black adults. In view of the well-established link between endothelial dysfunction and adverse cardiovascular events, one could speculate that the increased risk of cardiovascular events and mortality noted above is, at least in part, a reflection of the impaired endothelial function that accompanies inadequate vitamin D activity.

Natriuretic peptides, especially type-B natriuretic peptide, have been considered as diagnostic markers for asymptomatic and symptomatic cardiac dysfunction as well as prognostic markers in patients with heart failure. A recent study showed that even modest elevations in type-B natriuretic peptide in asymptomatic hypertensive patients can be linked to subclinical cardiac remodeling, inflammation, and extracellular matrix changes. Increases in both type-A natriuretic peptide and type-B natriuretic peptide gene expression were amplified in the hearts from VDRECKO mice after AngII infusion, implying a direct result of dysfunctional cardiac endothelial cells, which support cardiac myocyte and fibroblast function, or whether this is an indirect phenomenon resulting from the modestly elevated blood pressures seen in VDRECKO mice.
Vitamin D deficiency has been linked to increased oxidative stress in previous studies, whereas vitamin D supplementation has been shown to reverse this process in a variety of tissues, including the vasculature. The antioxidant effect of vitamin D was shown to be mediated by the mitogen-activated protein kinase/extracellular signal-regulated kinases/sirtuin-1 axis in endothelial cells. Chronic treatment of the spontaneously hypertensive rat with calcitriol normalized elevations in reactive oxygen species and expression of angiotensin II receptor type 1 and nicotinamide adenine dinucleotide phosphate-oxidase subunits in the vasculature. More recently, calcitriol treatment of renal arteries from hypertensive patients, in vitro, has been shown to improve endothelial function and reduce oxidative stress by reducing angiotensin II receptor type 1 and nicotinamide adenine dinucleotide phosphate-oxidase subunits and increasing superoxide dismutase 1 and superoxide dismutase 2 expression. Our data are consistent with these previous findings and demonstrate the important role of VDR in supporting endothelial function more directly in an in vivo model.

As noted above, Tie2-Cre has been shown to be expressed in cells of the myeloid lineage (eg, macrophage), raising the possibility that deletion of the VDR in these cells could contribute to the phenotype observed here. We have examined both aortic sections (immunocytochemistry) and dispersed aortic cells (fluorescence-activated cell sorter) for the presence of immune cells, including cells of the myeloid lineage, and found no difference between the VDRECKO and control mice. This suggests that the observed effects on vasorelaxant activity and NOS gene expression and activity result from deletion of the VDR gene in the endothelial cell. Although it would seem likely that this would have an effect on blood pressure as well, given the complexity of blood pressure regulation in vivo and the known proinflammatory effects of AngII in the cardiovascular system, it remains possible that some portion of the effects of Tie2-Cre–generated VDR deletion could result from coincident reduction of liganded VDR activity in myeloid cells.

**Perspectives**

The fact that VDR is expressed in many, if not most, cells of the cardiovascular system makes it difficult to disentangle the mechanism(s) of vitamin D's cardiovascular effects using subjects with vitamin D deficiency or whole animal VDR deletion, because many confounding factors (ie, changes in parathyroid hormone levels or calcium homeostasis) could potentially contribute to the phenotype. Using a mouse model with selective elimination of VDR in endothelial cells, we have provided direct evidence that vitamin D regulates endothelium-dependent blood vessel relaxation and arterial blood pressure, possibly by modulating eNOS gene expression. Our data add another piece of evidence supporting the hypothesis that vitamin D exerts palliative effects in the cardiovascular system and imply that vitamin D or its less-calcemic analogs may be useful in the prevention and treatment of cardiovascular and metabolic diseases that are associated with endothelial dysfunction.

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**Disclosures**

None.

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Novelty and Significance

What Is New?
- We showed a direct protective role of the endothelial vitamin D receptor in regulating vascular tone in vivo.
- Blood pressure and reactive oxygen species generation were higher in the endothelial cell–specific VDR gene knockout mouse versus controls.

What Is Relevant?
- Endothelial dysfunction contributes to the development of hypertension and other cardiovascular diseases.
- Vitamin D action in endothelial cells plays a role in protecting cardiovascular homeostasis, supporting the notion of using vitamin D agonists in preventing and treating cardiovascular diseases.

Summary
These findings indicate that vitamin D receptor is an important regulator of endothelium-dependent vasorelaxation. Our data provide additional support for the hypothesis that vitamin D exerts palliative effects in the cardiovascular system and implies that vitamin D or its less-calcemic analogs may be useful in the prevention and treatment of cardiovascular and metabolic diseases that are associated with endothelial dysfunction.
Elimination of Vitamin D Receptor in Vascular Endothelial Cells Alters Vascular Function
Wei Ni, Stephanie W. Watts, Michael Ng, Songcang Chen, Denis J. Glenn and David G. Gardner

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DATA SUPPLEMENT
ELIMINATION OF VITAMIN D RECEPTOR IN VASCULAR ENDOTHELIAL CELLS ALTERS VASCULAR FUNCTION

Short title: Endothelial cell-specific VDR knockout

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Methods

AngII Infusion and Blood Pressure Measurement

Mice were anesthetized with 2.5% isoflurane. Alzet micro-osmotic pumps (Model 1002, DURECT Corporation, Cupertino, CA) with a release rate of 0.25ul/hour were implanted subcutaneously between the scapulae of the mouse to deliver saline or AngII (500 ng kg\(^{-1}\) min\(^{-1}\)) continuously for 14 days. Mouse blood pressures were measured using a conventional tail-cuff method. Briefly, mice were trained on the Hatteras Instruments SC1000 Blood Pressure Analysis System for 4 days before pump implantation. Ten consecutive measurements on the day before, day 7 and the last day of infusion were averaged to calculate systolic and diastolic blood pressure. Mean arterial blood pressure was calculated as 2/3 diastolic pressure plus 1/3 systolic blood pressure.

Mouse Endothelial Cell Isolation and Human Umbilical Vein Endothelial Cell (HUVEC) Culture

Vascular endothelial cells were isolated from hearts of 12 week old mice. Left ventricle was removed, cut transversely in two halves and rinsed with cold Hank’s Balanced Salt Solution. Single cell suspensions were obtained utilizing a combination of enzymatic dissociation with collagenase II (600 U/ml) and mechanical force with the GentleMACS\(^{\text{TM}}\) Dissociator (Miltenyi Biotec Inc.) according to the manufacturer’s protocol. To select endothelial cells, cell suspensions were incubated with rat anti-mouse CD31 (protein expressed on the surface of endothelial cells) antibody (BD Pharmingen, 2.5 ug/ml) in 1% BSA-PBS at 4\(^\circ\)C for 30 min. Cells were washed with 0.5% BSA-PBS to remove unbound primary antibody. Endothelial cells were then magnetically labeled with goat anti-rat IgG MicroBeads (Miltenyi Biotec Inc.). The cell suspension was loaded on to a MS MACS column which was placed in the magnetic field of a MACS\(^{\circ}\) separator. Unbound cells were collected and cultured in 5% FBS-DMEM H21. The magnetically retained cells in the column were eluted after removing the column from the magnetic field, plated on collagen I-coated tissue culture plate (BD BioCoat\(^{\text{TM}}\) Collagen I Cellware, BD Biosciences) and cultured in Endothelial Cell Medium-complete (ScienCell Research Laboratories).

HUVECs were purchased at passage 3 from ScienCell Research Laboratories. HUVECs were cultured and expanded in Endothelial Cell Medium-complete. At passages 4-7, HUVECs were collected and used to measure VDR protein expression by Western blot analysis and immunocytochemistry.

Isometric Contractions/Relaxations of Mouse Aorta

Mouse aorta strips were mounted in tissue baths for isometric tension recordings using Grass transducers and a PowerLab Data Acquisitions system (Colorado Springs, CO, USA). Aortic strips were placed under optimal resting tension (250 mg) and equilibrated in a tissue bath for 1 hour in aerated (95% O\(_2\)/5% CO\(_2\)) physiological salt solution \(^{1}\) at 37\(^\circ\)C before exposure to pharmacological reagents. Administration of an initial concentration of the \(\alpha_1\) adrenergic agonist
phenylephrine (PE, $10^{-5}$ M) was used to assess blood vessel viability. To compare contractile function of mouse aorta, concentration-response curves for PE were generated in a cumulative manner. Tissues were then washed repeatedly until tone returned to baseline. To examine the status of the arterial endothelium, tissues were contracted with a half-maximal concentration of PE (determined previously; $10^{-8}$–$10^{-7}$ M). Once the contraction plateaued, cumulative concentrations of the muscarinic agonist acetylcholine (ACh; $10^{-9}$M–$10^{-5}$ M) were administered, followed by the NO donor sodium nitroprusside (SNP, $10^{-5}$ M) to determine the ability of vessels to relax to an NO stimulus. In some experiments, vehicle or the nitric oxide synthase inhibitor N(G)-nitro-L-arginine (L-NNA) (100 µM) was added prior to the addition of PE to contract tissues so that agonist-induced relaxation could be measured.

**Quantitative PCR**

RNA from primary cultures of mouse endothelial cells was obtained using QIAshredder (QIAGEN) and RNeasy Mini Kit (QIAGEN) according to manufacturer’s instructions. Mouse aortae were cleaned of fat, snap-frozen in liquid nitrogen and powdered. Trizol reagent (600 µl per sample) was added and the individual samples were homogenized. After a 5-minute incubation at room temperature, 120 µl chloroform was added to the mixture and shaken vigorously for 20 seconds, followed by 3 minutes incubation at room temperature. Aqueous and organic phases were separated by centrifugation at 10,000g for 15 minutes at 4 °C. Aqueous phase was collected and mixed with an equal volume of 100% RNA-free ethanol and loaded on to RNeasy column for RNA extraction and DNase I treatment. RNA concentration was measured by NanoDrop (Thermo Scientific).

Equal amounts of RNA were used to reverse transcribe cDNA with SuperScript® III Reverse Transcriptase (Life Technologies). Real-time quantitative PCR was performed using Applied Biosystems 7900 HT (Applied Biosystems, Foster City, CA). Expression levels of genes of interest were quantified and normalized to GAPDH as an internal control. Sense and anti-sense primers for SYBR®-Green based detection were as follow: mouse VDR (sense: AGGACAACCGGCGACACT and antisense: CTTACGCTGCACCTCCTCAT), MMP2 (sense: TCCCCGGCCGATGTCG and antisense: GCAGCCATAGAAGTGTTCAAGGT). Other gene products of interest were analyzed using Taqman-based detection with primer sets from Applied Biosystems: mouse ANP (Mm01255748_g1), BNP (Mm00435304_g1), collagen 1a1 (Mm00801666_g1) and collagen 3a1 (Mm01254476_m1) and GAPDH (4352932E).

**Western Blotting**

Mouse aortas were cleaned of fat, pulverized in liquid nitrogen and solubilized in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 10% glycerol, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 1 mmol/L glycophosphate) containing protease inhibitors (1 Complete tablet/50 ml, Roche Diagnostics, Indianapolis, IN). HUVECs were rinsed in PBS and harvested in lysis buffer. Homogenates were centrifuged at 11,000 rpm for 10 min at 4°C. Supernatant protein was collected and measured by Bradford assay (Pierce Coomassie Assay Reagent). Equal amounts of total protein were loaded and separated on Criterion Tris-HCl gradient gel (4%-15%) and transferred to polyvinylidene difluoride (PVDF) membrane. VDR, eNOS, p-VASP and VASP expression were detected using
a rabbit antibody against VDR (C-20, Cat # sc-1008, Santa Cruz Biotechnology), a mouse antibody against eNOS (Cat # 610297, BD biosciences), P-VASP (Cell Signaling Technologies, Cat # 3132S) or VASP (Cell Signaling Technologies Cat # 3114S) respectively. In some experiments, blots were incubated with 5-fold (mass/mass) excess of competing VDR peptide (Cat # sc-1008p, Santa Cruz Biotechnology) pre-mixed with anti-VDR antibody. Blots were then incubated with the appropriate horseradish peroxidase-linked secondary antibody. ECL® reagent (Amersham Life Sciences, Arlington Heights, IL, U.S.A.) was used to visualize bands. GAPDH, assessed using anti-GAPDH antibody (Santa Cruz Biotechnology), served as an internal control.

**Immunocytochemistry**

HUVECs were cultured in Endothelial Cell Medium-complete until confluent, fixed with Z-fix (Anatech Ltd, Battle Creek, MI) and rinsed with PBS. After incubation with blocking solution (5% BSA in PBS containing 0.1% Tween-20), cells were incubated with rabbit anti-VDR (1:200, Santa Cruz Biotechnology) and mouse anti-CD31 (1:200, Abcam, ab24590) overnight, followed by incubation with Alexa 555 goat-anti rabbit (1:200, Life technologies) and Alexa 488 donkey anti-mouse antibody (1:200, Life technologies) for 1 hour. Coverslips were mounted with VECTASHIELD Mounting Medium containing DAPI for nuclear counterstain.

**Histological Analysis**

Histological analyses of blood vessels and hearts were performed on tissue fixed overnight in Z-fix (Anatech Ltd, Battle Creek, MI). Tissue was embedded in paraffin blocks for sectioning at 5 μm thickness. Sections were stained with hematoxylin/eosin (tissue morphology), Masson’s trichrome (interstitial collagen) or wheat germ agglutinin-TRITC conjugate (50 μg/ml) (myocyte cross-sectional area). For the cell size analyses, we used an ImageJ macro developed by Dr. Kees Straatman at University of Leicester for automated batch processing in ImageJ. The mean cardiomyocyte area was evaluated by measurement of over 1300 cells from at least 6 hearts per genotype.

**Superoxide analysis in aorta section**

Dihydroethidium (DHE) fluorescent analysis was performed on frozen mouse aorta sections (5 μm) incubated with DHE (Sigma, 10 μM) for 10 minutes at 37 °C, and washed with phosphate-buffered saline (PBS). Images were acquired using a confocal laser scanning microscope (Leica TCS SP5) with same exposure time for all aortic sections. Confocal images were analyzed by Image J. DHE fluorescence density was normalized to auto-fluorescence acquired with the default setting for Alexa 488 on Leica TCS SP5 from the same image.

**Flow Cytometric Analysis of Aorta and Spleen Cell Suspensions**

Aortic cell suspensions were prepared and analyzed according to the procedure of Butcher et al. Briefly, the vasculature was cleared with PBS containing 2% heparin to remove the blood from the aorta. The aorta was then excised and the adherent adipose tissue and para-aortic lymph nodes were dissected away leaving the aorta and adventia intact. The aorta was digested in PBS containing 125 U/ml Collagenase type XI, 60 U/ml Hyaluronidase type 1-s, 60 U/ml DNase I,
and 450 U/ml Collagenase type I for 1 hour at 37 °C (all enzymes from Sigma Aldrich, St Louis, MO). A single cell suspension was obtained by passing the tissue through a 70 µm mesh filter. The spleen was removed and placed in DMEM + 2% fetal calf serum. A single cell suspension was obtained after passing through subsequent 70 µm and 40 µm mesh filters. The suspension was treated with ACK buffer (Lonza, Walkerville, MD) to remove red blood cells. The aorta and spleen cells were re-suspended in PBS containing 1% BSA. Cells (aorta: 7.5 x 10⁴ and spleen 5x10⁵) were treated with Fc block and stained with the following conjugated antibodies: CD11b-phycocerythrin (PE) (eBioscience cat # 12-0112), F4:80- fluorescein isothiocyanate (FITC) (eBioscience cat # 11-4801) and CD45-allophycocyanin (APC) (eBioscience cat # 9017-9459). Dead cells were analyzed by staining with LIVE/DEAD Fixable Violet kit (Invitrogen) according to the manufacturer’s instructions. Acquisition was performed on a BD FACS Calibur flow cytometer, and the data analyzed using FlowJo software.

Statistical Analysis

Data were analyzed using ANOVA, with Newman-Keuls posthoc test, or Students t-test, where appropriate. Results are presented as mean ± standard error of the mean (SEM).

Figure Legend (Supplement Figures)

Supplement Figure 1. Plasma calcium and phosphorus levels are not affected by VDR deletion in endothelial cells. VDRL/L= VDR floxed mouse, served as control; VDRECKO= endothelial cell specific VDR gene knockout mouse. VDRL/L (n=7); VDRECKO (n=5). No statistically significant difference was noted; p = 0.2 (calcium) and p = 0.26 with Student t-test.

Supplement Figure 2. Superoxide levels are increased in aortic wall with VDR deletion in endothelial cells. A. Representative images show superoxide detected by DHE staining. B. Quantification of superoxide signal in endothelial layer and whole blood vessel wall. Superoxide signal was normalized to green auto-fluorescence for each image. N= 4 each group. ** p<0.01, *** p<0.001.

Supplemental Figure 3. Flow cytometric analysis of splenic and aortic cell suspensions in VDRL/L and VDRECKO. Spleen or aortic cells suspensions were stained with CD45, CD11b and F4:80 antibodies. (A) Live cells were determined using LIVE/DEAD Fixable Violet staining (x-axis) and the CD45 positive cell (y-axis) population was identified. Representative plots are shown for (A) spleen and (B) aorta. The CD45 positive population was further analyzed for the presence of CD11b (y-axis) and F4:80 positive cells (x-axis). Representative plots are shown for (C) spleen and (D) aorta. The results of 3-4 independent experiments are quantified for number of CD45 positive cells present in the VDRL/L (N=3) and VDRECKO (N=4) aortic suspensions (E), CD11b and F4:80 positive cells expressed as a percentage of live cells (F) and as a percentage of CD45 positive cells (G). No significant (N.S.) differences were noted.

Supplement Figure 4. VDRECKO mice showed normal heart and blood vessel morphology and blood pressure at 6 months of age. A. H&E staining of aortae and carotid arteries from 24-week old VDRL/L or VDRECKO mice. B. Tail cuff blood pressure measurements of 24-week old VDRL/L
or VDR\textsuperscript{ECKO} mice, VDR\textsuperscript{L/L} (n = 8); VDR\textsuperscript{ECKO} (n = 11). C. H&E staining of hearts from 24-week old VDR\textsuperscript{L/L} or VDR\textsuperscript{ECKO} mice. D. Left-ventricle weight to body weight ratio (LVW/BW) from 24-week VDR\textsuperscript{L/L} or VDR\textsuperscript{ECKO} mice, n = 15 each group. VDR\textsuperscript{L/L} = VDR floxed mouse, served as control; VDR\textsuperscript{ECKO} = endothelial cell specific VDR gene knockout mouse.

References

Supplement Figure S1

Plasma analyses

![Graph showing plasma analyses for Calcium and Phosphorus with two groups: VDR<sup>UL</sup> and VDR<sup>DECKO</sup>.](image)

- **Calcium**: The bars for both groups show similar values, with VDR<sup>UL</sup> slightly lower than VDR<sup>DECKO</sup>.

- **Phosphorus**: VDR<sup>DECKO</sup> has a significantly higher value compared to VDR<sup>UL</sup>.

The y-axis represents mg/dl, with a scale from 0 to 15.
Supplement Figure S2

A

B

**Endothelial Layer**

**Whole Vessel Wall**

Arbitrary Unit

Arbitrary Unit

VDR^{L/L}  VDR^{ECKO}

VDR^{L/L}  VDR^{ECKO}
Supplement Figure S3

A. SPLEEN
   VDR\textsuperscript{L/L}  \hspace{1cm}  VDR\textsuperscript{ECKO}

B. AORTA
   VDR\textsuperscript{L/L}  \hspace{1cm}  VDR\textsuperscript{ECKO}

C. CD45 / CD11b
   F4:80

D. CD45 / CD11b
   F4:80

E. CD45 / live cells (%)
   VDR\textsuperscript{L/L}  \hspace{1cm}  VDR\textsuperscript{ECKO}
   N.S.

F. F4:80 + CD11b / live cells (%)
   VDR\textsuperscript{L/L}  \hspace{1cm}  VDR\textsuperscript{ECKO}
   N.S.

G. F4:80 + CD11b / CD45 (%)
   VDR\textsuperscript{L/L}  \hspace{1cm}  VDR\textsuperscript{ECKO}
   N.S.
Supplement Figure S4

A

VDR<sub>L/L</sub>  VDR<sub>ECKO</sub>

Aorta

Carotid Artery

C

VDR<sub>L/L</sub>  VDR<sub>ECKO</sub>


B

![BP (mmHg) Bar Graph](image)

SBP  DBP  MBP

C

VDR<sub>L/L</sub>  VDR<sub>ECKO</sub>

LVW (mg)/BW (g)