Conclusions — VDR activation reduces dissecting AAA formation induced by Ang-II in apoE−/− mice and may constitute a novel therapeutic strategy to prevent AAA progression. (Arterioscler Thromb Vasc Biol. 2016;36:1587-1597. DOI: 10.1161/ATVBAHA.116.307530.)

Key Words: abdominal aortic aneurysm ☐ calcitriol ☐ chemokines ☐ endothelial cells inflammation ☐ dilatation

Abdominal aortic aneurysm (AAA) is a localized dilatation of the abdominal aorta and occurs most commonly in ≤6% of adults >65 years of age. The clinical approach to AAA is currently limited to surgical repair and is not indicated in patients with small AAA or who are asymptomatic. Pathological features of AAA include chronic vascular inflammation of the aortic wall, progressive extracellular matrix degradation, and increased neovascularization. Because of the high mortality rate associated with AAA, new effective therapeutic strategies are needed to prevent its progression.

Clinical and experimental data support a role for the renin–angiotensin system in AAA pathogenesis. Angiotensin-II (Ang-II), the major effector peptide of renin–angiotensin system, is implicated in both vascular inflammation and pathological vascular growth. Indeed, chronic subcutaneous infusion of Ang-II in apoE−/− mice is a model of AAA formation that shares some characteristic features of human disease, including leukocyte infiltration, chemokine secretion, matrix metalloproteinases (MMPs) release, enhanced new capillary formation, and degradation of the elastic layer of the aortic wall. Although a valuable preclinical model of AAA, increasing evidence indicates that Ang-II–induced lesions in mice should be considered as pseudoaneurysms or dissecting AAA as relevant differences are observed in human AAA, including aneurysm localization and evolution.

Epidemiological evidence has established a relationship between low plasma levels of vitamin D₃ and increased

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risk of cardiovascular disease.10–12 More recently, whereas vitamin D₃ deficiency seems to be an independent risk factor associated with thoracic aortic dilatation,13 an inverse relationship between vitamin D₃ levels and AAA development in humans has been established.14 The underlying mechanisms explaining these associations, however, are not clearly understood.14

Calcitriol (1,25-dihydroxyvitamin D₃) is the active form of vitamin D₃, a lipid-soluble vitamin that stimulates absorption of calcium. Beyond its critical function in bone and calcium homeostasis, it also acts on the immune and cardiovascular systems.15 The binding of calcitriol to the vitamin D receptor (VDR) activates VDR to bind to vitamin D response elements in target genes that are involved in processes of potential relevance to cardiovascular disease.16 Indeed, VDR activation exerts anti-inflammatory, antiproliferative, and antioxidative properties, which may additionally play important roles in vitamin D₃–mediated cardiovascular protection.17–19 Furthermore, increasing evidence suggests that vitamin D₃ is an important endogenous regulator of the renin–angiotensin system.20–22 Accordingly, VDR-deficient mice display high renin and Ang-II levels and develop hypertension.20 In addition to regulating the components involved in synthesizing Ang-II, calcitriol also alters the expression of Ang-II AT1 receptors in several target tissues.23,24 Therefore, vitamin D₃ seems to affect on a range of cellular and molecular mechanisms of importance to AAA pathogenesis.25 It is uncertain, however, whether VDR activation can affect dissecting AAA promoted by Ang-II, and the vascular inflammatory and angiogenic response associated with this disease.

In an attempt to find more effective therapeutic strategies to halt the progression of AAA, this study aimed to examine the effect of calcitriol on Ang-II–induced dissecting AAA development in apoE–/– mice. We demonstrate for the first time that calcitriol treatment significantly attenuates dissecting AAA formation. Calcitriol-cotreated mice exhibit reduced macrophage infiltration, and MMP and chemokine expression in the suprarenal aortic walls, which seem to be mediated through activation of VDR–retinoid X receptor alpha (RXRα) interactions. We also show that VDR activation inhibits Ang-II–induced mononuclear leukocyte–endothelial cell interactions, morphogenesis, and endothelial release of proangiogenic chemokines.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Calcitriol Treatment Inhibits Ang-II–Induced Dissecting Aneurysm Formation
ApoE–/– mice were infused subcutaneously with Ang-II (n=36) or saline (n=10) for 28 days. Ang-II–infused mice received orally vehicle (n=12) or calcitriol administered every other day at a dose of 0.1 μg/kg (n=12) or 1 μg/kg (n=12; Figure 1). Within the initial 14 days of treatments, 2 (17%) Ang-II–infused apoE–/– mice and 2 (17%) Ang-II plus low-dose calcitriol–treated mice died as a result of rupture of the aorta in the suprarenal abdominal region, whereas no deaths occurred in mice treated with calcitriol at the higher dose (Figure 1B). As expected, no dissecting aneurysms were present in saline-infused control mice at day 28 (Figure 1C and 1D). Ang-II–infused apoE–/– mice showed a larger maximal external diameter of the suprarenal aorta than saline-infused mice, which was significantly reduced in mice treated with 1-μg/kg calcitriol (Figure 1C, P<0.05). On the basis of the classification system of Daugherty et al.,28 the dissecting aneurysms induced by Ang-II were more severe than those cotreated with calcitriol (1 μg/kg; Figure 1D, P<0.05). Furthermore, the increased diameter and area of ascending aortas induced by Ang-II infusion was significantly reduced in calcitriol-cotreated (1 μg/kg) mice (Figure IA and IB in the online-only Data Supplement, P<0.05). Calcitriol administration did not provoke changes in the systolic blood pressure or the lipid profile in apoE–/– mice chronically infused with Ang-II (Table I in the online-only Data Supplement).

Calcitriol Reduces Leukocyte Infiltration and Neovascularization in Dissecting AAA Induced by Ang-II
Inflammation and angiogenesis are prominent features of AAA.17,27 Immunohistochemical analysis of suprarenal aortic sections showed that Ang-II infusion for 28 days promoted leukocyte recruitment of CD68+ cells in the adventitia and media (Figure 2A), which was significantly reduced in calcitriol-cotreated (1 μg/kg; Figure 1C, P<0.05). When compared with the control group, Ang-II–infused animals presented a significant increase in CD31+ capillary vessels (Figure 2F and 2G) and also expression of vascular endothelial growth factor (VEGF) mRNA (Figure 2H) in aortic tissue. Mice cotreated with calcitriol had less capillary formation and VEGF mRNA expression in the suprarenal aortas than Ang-II–infused animals (Figure 2G and 2H, P<0.05).

Calcitriol Modulates Ang-II–Induced Extracellular Matrix Degradation in Dissecting AAA
Histological examination of Ang-II–induced dissecting AAA revealed a structural deformity within the adventitia of the
abdominal aorta together with a loss of vascular smooth muscle cells and also focal elastin destruction (Verhoeff–van Gieson staining) in the medial layer (Figure II in the online-only Data Supplement), and this was ameliorated in calcitriol-cotreated mice (Figure IIA through IID in the online-only Data Supplement).

Because MMPs are intimately involved in extracellular matrix destruction and aortic wall remodeling in AAA,28 we questioned the effect of calcitriol on the balance between MMP-2 and MMP-9 and their endogenous inhibitor tissue inhibitor of metalloproteinases (TIMP-1). Immunohistochemistry revealed that Ang-II–infused mice had higher expression of MMP-2 and MMP-9 and also TIMP-1 protein than vehicle-treated mice in the suprarenal aorta (Figure 3A), and this correlated with a significant increase in mRNA expression (Figure 3B through 3D, \( P < 0.05 \)). By contrast, MMP-2 (Figure 3A, \( P < 0.05 \)) and MMP-9 expression (Figure 3C, \( P < 0.05 \)) was lower in calcitriol-cotreated mice, whereas TIMP-1 expression was higher (Figure 3D, \( P < 0.05 \)) in suprarenal aortas.

Calcitriol attenuates extracellular signal–regulated kinases 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK), and nuclear factor-κB (NF-κB) phosphorylation in Ang-II–induced dissecting AAA.29,30 We therefore evaluated the impact of calcitriol cotreatment on these pathways in suprarenal aneurysmal tissue. Western blot analysis of the suprarenal aorta revealed significantly higher levels of ERK1/2 (Figure 4A), p38 MAPK (Figure 4B), and p65 (Figure 4C) phosphorylation in apoE−/− mice subjected to Ang-II infusion for 28 days than in vehicle-treated mice. Phosphorylation of ERK1/2, p38 MAPK, and NF-κB was significantly reduced in the suprarenal aortas of calcitriol-cotreated mice (Figure 4, \( P < 0.05 \)).

**Effects of Calcitriol Treatment on Ang-II–Induced Vascular Responses in the Early Phase of Dissecting AAA Formation**

Additional studies were performed to investigate whether calcitriol treatment modulated the early phase of Ang-II–induced vascular dysfunction. CD68-positive cells or neutrophils (myeloperosidase) were not detected in the suprarenal aortas at 5 days after Ang-II infusion. (Figure IIIA and IIIB in the online-only Data Supplement). However, mRNA expression of CCL2, CCL5, and CXCL1 was increased in the suprarenal aortas of Ang-II–infused apoE−/− mice, but not in calcitriol-cotreated mice (Figure IIIC through IIIE in the online-only Data Supplement). Moreover, VEGF mRNA expression remained unchanged after Ang-II infusion or calcitriol cotreatment (Figure IIIF in the online-only Data Supplement, \( P > 0.05 \)).

Elastin fibers and smooth muscle cells in aorta segments of Ang-II–infused animals seemed intact in all treatment
Ang-II treatment resulted in a modest increase in MMP-2 expression after 5 days (Figure IVB and IVC in the online-only Data Supplement, \( P > 0.05 \)). MMP-9 expression increased significantly in suprarenal aortas after 5 days of Ang-II infusion, and this was prevented by calcitriol cotreatment (Figure IVB and IVD in the online-only Data Supplement, \( P < 0.05 \)). In addition, calcitriol cotreatment led to significantly higher levels of TIMP-1 expression than Ang-II infusion alone (Figure IVB and IVE in the online-only Data Supplement, \( P < 0.05 \)).

A significant increase in ERK1/2, p38 MAPK, and p65 phosphorylation was detected in the suprarenal aortas of apoE\(^{-/-}\) mice infused with Ang-II for 5 days (Figure VA in the online-only Data Supplement, \( P > 0.05 \)). MMP-9 expression increased significantly in suprarenal aortas after 5 days of Ang-II infusion, and this was prevented by calcitriol cotreatment (Figure IVB and IVD in the online-only Data Supplement, \( P < 0.05 \)). In addition, calcitriol cotreatment led to significantly higher levels of TIMP-1 expression than Ang-II infusion alone (Figure IVB and IVE in the online-only Data Supplement, \( P < 0.05 \)).

A significant increase in ERK1/2, p38 MAPK, and p65 phosphorylation was detected in the suprarenal aortas of apoE\(^{-/-}\) mice infused with Ang-II for 5 days (Figure VA in the online-only Data Supplement, \( P > 0.05 \)). MMP-9 expression increased significantly in suprarenal aortas after 5 days of Ang-II infusion, and this was prevented by calcitriol cotreatment (Figure IVB and IVD in the online-only Data Supplement, \( P < 0.05 \)). In addition, calcitriol cotreatment led to significantly higher levels of TIMP-1 expression than Ang-II infusion alone (Figure IVB and IVE in the online-only Data Supplement, \( P < 0.05 \)).

Reverse-transcription polymerase chain reaction analysis of VDR expression in the suprarenal and ascending aorta of mice infused for 5 days with Ang-II revealed that although a modest increase was detected in calcitriol-treated animals, none of the treatments significantly altered VDR expression (Figure VE in the online-only Data Supplement, \( P > 0.05 \)). Because activation of VDR by its ligand can result in heterodimerization with other nuclear receptors, such as RXR\(\alpha\),\(^{31}\) we performed immunoprecipitation experiments to investigate the potential involvement of VDR–RXR\(\alpha\) heterodimer complexes in the responses elicited by calcitriol. Immunoprecipitation was performed in mouse aortas using an anti-RXR\(\alpha\) antibody for immunoprecipitation and an anti-VDR antibody for Western blotting. An augmented VDR–RXR\(\alpha\) interaction was observed in both suprarenal and ascending aortas of calcitriol-treated mice (Figure VF in the online-only Data Supplement).
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Calcitriol Decreases Mononuclear Leukocyte–Endothelial Cell Interactions and Morphogenesis Induced by Ang-II

To connect our observations in mice to humans, we investigated the effect of calcitriol on Ang-II–induced vascular inflammation and angiogenesis in primary human umbilical vein endothelial cell (HUVEC) cultures. We first evaluated mononuclear leukocyte–endothelial cell interactions in vitro using a dynamic flow chamber assay.32 Freshly isolated human mononuclear cells were perfused across HUVEC monolayers stimulated or not with Ang-II (1 μmol/L) for 24 hours and adhesion was measured. Mononuclear leukocyte adherence to endothelial cells was significantly higher in cells stimulated with Ang-II than in cells treated with vehicle (0.01% DMSO [dimethyl sulfoxide]; Figure VI in the online-only Data Supplement, P<0.05). To determine the effects of calcitriol on Ang-II–induced mononuclear cell recruitment, HUVEC were pretreated with calcitriol (1–100 nmol/L) 24 hours before Ang-II stimulation. Significant reductions in Ang-II–mediated mononuclear adherence were achieved with calcitriol, and this occurred in a concentration-dependent manner (Figure VIB in the online-only Data Supplement, P<0.05).

Calcitriol Decreases Ang-II–Induced Endothelial Production of Chemokines and VEGF

Ang-II signaling through AT1 receptor interactions induces the production and release of different CC and CXC chemokines34–36 and VEGF.34 To determine whether calcitriol affected the levels of these factors, we collected conditioned media from treated cells and performed ELISA. A significant increase was detected in the levels of CCL2, CCL5, CXCL1, and VEGF in culture medium of HUVEC subjected to Ang-II stimulation for 24 hours (Figure VIC through VIF, in the online-only Data Supplement, P<0.05). Calcitriol pretreatment (100 nmol/L) reduced the Ang-II–induced increase in CCL2, CCL5, CXCL1, and VEGF in culture medium of HUVEC subjected to Ang-II stimulation for 24 hours (Figure VIC through VIF, in the online-only Data Supplement, P<0.05).

VDR and RXRα Are Involved in the Anti-Inflammatory and Antiangiogenic Activity Exerted by Calcitriol in Human Endothelial Cells

To explore the underlying mechanisms for the observed effects of calcitriol, we investigated the potential involvement of the VDR in these responses. We used RNA interference to specifically downregulate VDR expression. HUVEC transfected
with an siRNA targeting VDR demonstrated a >75% reduction in VDR protein levels after 48 hours relative to control siRNA-transfected cells (Figure 5A, P <0.05). Of note, VDR silencing abolished the inhibitory effects of calcitriol (100 nmol/L) on mononuclear leukocyte arrest (Figure 5B), tubulogenesis (Figure 5C), and cytokine release (Figure 5D through 5G) induced by Ang-II in HUVEC.

We used an identical approach to silence RXRα expression (Figure 6A). Consistent with the results for VDR silencing, RXRα silencing blocked the inhibitory effects elicited by the VDR ligand (100 nmol/L) on leukocyte arrest (Figure 6B, P <0.05) and tubulogenesis (Figure 6C, P <0.05) in HUVEC stimulated with Ang-II.

To extend these findings, we performed immunocytochemistry using antibodies to VDR and RXRα. Although significant amounts of unliganded VDR and RXRα were found in the cytoplasm of endothelial cells, calcitriol exposure resulted in the translocation of VDR and RXRα into the nucleus (Figure 6D). As expected, immunoprecipitation assays revealed an enhanced VDR–RXRα interaction in the presence of calcitriol (Figure 6E).

**Calcitriol Inhibits the Activation of Ang-II–Induced MAPK Signaling Pathways in Human Endothelial Cells**

Finally, as ERK1/2, p38 MAPK, and NF-κB signaling pathways seem to play a role in Ang-II–induced dissecting AAA formation, we examined their potential involvement in the inflammatory and angiogenic response provoked by Ang-II in human endothelial cells. A 15-minute challenge with 1-μmol/L Ang-II triggered a marked phosphorylation of ERK1/2, p38 MAPK, and p65 NF-κB in HUVEC (Figure VII in the online-only Data Supplement, P <0.05). Preincubation of the cells with calcitriol (100 nmol/L, 24 hours) decreased the Ang-II–induced phosphorylation of these signaling pathways (Figure VIIA through VIIC in the online-only Data Supplement, P <0.05).

**Discussion**

Vitamin D₃ deficiency is emerging as a new cardiovascular risk factor associated with the development of AAA. Indeed, clinical studies suggest that low levels of vitamin D₃ are associated with inflammation and the consequent endothelial dysfunction, and correlate with AAA development and enhanced aortic diameters. By contrast, VDR activation seems to exert anti-inflammatory properties, and it is effective at inhibiting atherosclerosis lesion formation in animal models. Despite these findings, the direct effects of vitamin D₃ on AAA development are largely unknown. This study was designed to evaluate the effect of orally administered calcitriol on dissecting AAA development in the Ang-II–infused apoE−/− mouse model.

An imbalance of renin–angiotensin system is associated with AAA pathogenesis. In this study, we provide evidence that oral calcitriol treatment for 28 days inhibits dissecting AAA formation induced by Ang-II and also reduces macrophage infiltration. It is well recognized that macrophages play critical roles in the pathophysiology of AAA by secreting inflammatory chemokines/cytokines and MMPs, which lead to aortic structural disruption. We found that suprarenal aortic expression of the inflammatory markers CCL2, CCL5, and CXCL1 were decreased in calcitriol-cotreated mice. Although significant amounts of unliganded VDR and RXRα were found in the cytoplasm of endothelial cells, calcitriol exposure resulted in the translocation of VDR and RXRα into the nucleus (Figure 6D). As expected, immunoprecipitation assays revealed an enhanced VDR–RXRα interaction in the presence of calcitriol (Figure 6E).
of these chemokines and their receptors have been detected in the microenvironment of the AAA wall in humans, and in experimental models. Indeed, CCR2 deficiency or dual blockade of CCR1/CCR5 receptors drastically reduces the recruitment of monocytes into the aortic lesion, inhibits MMPs and neovascularization, and preserves aortic wall structure, resulting in attenuated aneurysm development.

A growing body of evidence suggests the potential interplay between angiogenesis and inflammation, and new microvessel formation in aortic aneurysmal disease is related to the risk of rupture and complications. Because newly formed blood microvessels facilitate the continuous recruitment of immune cells that release a variety of key mediators, such as proinflammatory and proangiogenic chemokines and growth factors, it is plausible to suggest that exacerbation of inflammation may be directly involved in the amplification of the angiogenic process. Of interest, suprarenal aortic neovascularization and VEGF levels were unchanged during the acute phase of dissecting AAA formation; however, a marked increase in angiogenesis and VEGF levels were detected during the chronic phase of Ang-II–induced dissecting AAA. It is well established that Ang-II stimulation of vascular endothelial cells results in the production of an array of leukocyte-recruiting chemokines and VEGF, CCL2 and CCL5 together with the ELR+ CXC chemokine CXCL1/growth-related oncogene-α are involved in neovascularization, and previous data by our group revealed that CCL2, CCL5, and VEGF are likely key molecules in the angiogenic activity elicited by Ang-II. Our present study suggests that the marked increased of VEGF and neovascularization in dissecting AAA could be attributable to the cellular proliferation and intense infiltration of the suprarenal aortic walls by inflammatory cells. Previous reports demonstrated that calcitriol can suppress VEGF–induced endothelial cell sprouting and angiogenesis in vivo and in vitro. We found that the suprarenal aortas of chronic calcitriol–treated mice presented a marked reduction in neovascularization and VEGF levels. These changes might be because of the reduction in leukocyte infiltration provoked by calcitriol because inflammatory cells including monocytes/macrophages recruited during aortic aneurysm development are an important source of VEGF.

Dysregulation of MMP production and activity represents an additional major mechanism in the initiation and the progression of AAA. MMPs are released by endothelial cells and smooth muscle cells, but also by infiltrating inflammatory cells, predominantly macrophages, and contribute to medial

![Figure 5](http://atvb.ahajournals.org/)

Figure 5. Knockdown of vitamin D3 receptor (VDR) by siRNA abrogates the inhibitory effect of calcitriol on angiotensin-II (Ang-II)–induced mononuclear cell adhesion, tube formation, and cytokine release. Human umbilical vein endothelial cells (HUVEC) were transfected with control or VDR-specific siRNA. At 48 h post transfection, cells were treated with calcitriol (D3, 100 nmol/L) for 24 h and then with Ang-II (1 μmol/L) for 24 h. At 48 h post transfection VDR protein expression was determined by Western blot. Protein quantification was performed by densitometry, and protein levels were normalized to β-actin. Results are the means±SD (n=5 independent experiments). A, VDR protein expression relative to β-actin. Results are the means±SD (n=5 independent experiments). B, Human mononuclear cells (10⁶ cells/ml) were perfused across the endothelial monolayers for 5 min at 0.5 dyn/cm², and leukocyte adhesion was quantified. Data are the means±SD of the number of tube-like structures in 5 low-magnification (×100) fields (n=5 independent experiments performed in triplicate). C, Endothelial differentiation was performed on Matrigel. Data represent mean±SD of the number of tube-like structures in 5 low-magnification (×100) fields (n=5 independent experiments performed in triplicate). D, (C-C motif) ligand 2 (CCL2), (E) (C-C motif) ligand 5 (CCL5), (F) (C-X-C motif) ligand 1 (CXCL1), and (G) vascular endothelial growth factor (VEGF) release were determined by ELISA in the cell-free supernatants of transfected HUVEC. Results are expressed as pM concentration and presented as means±SD (n=5 independent experiments performed in triplicate). *P<0.05 vs vehicle; †P<0.01 vs Ang-II.)
smooth muscle cell apoptosis, degradation of elastin and subsequently AAA formation.\(^2,4^1\) MMP activity is modulated by TIMPs,\(^4^1\) and the MMP/TIMP ratio is critical for controlling tissue damage.\(^5^3\) In agreement with previous reports,\(^5^4\) we found an increase in MMP-9 in suprarenal aortas before dissecting AAA formation and 28 days after Ang-II infusion; however, MMP-2 expression was prominent only at the later stages of dissecting AAA development. Interestingly, calcitriol-cotreated mice had reduced medial elastin destruction together with a reduction in MMP-2 and MMP-9 and an increase in TIMP-1 levels in suprarenal aortas. Consistent with our data, activation of VDR can downregulate MMP-2 and MMP-9 and upregulate TIMP-1 in several vascular cells and tissues.\(^5^5-5^7\) Therefore, our current findings suggest that VDR activation may play an important role in the preservation of vessel wall integrity.

AT1 receptors are expressed in endothelial cells.\(^5^8\) Under dynamic flow conditions or during endothelial differentiation in vitro, pretreatment of endothelial cells with calcitriol resulted in significant reductions in leukocyte arrest and tubulogenesis induced by Ang-II. Proinflammatory and proangiogenic effects of Ang-II through its interaction with AT1 receptors have been described both in vitro and in animal models.\(^3^,3^4,4^8\) However, although previous studies have demonstrated that whole-body AT1a receptor deficiency reduces aneurysms,\(^5^9\) depletion of AT1a receptors in endothelial or smooth muscle cells did not affect Ang-II–induced dissecting AAA in mice.\(^6^0\) Therefore, the main cell type stimulated by Ang-II to induce dissecting AAA remains to be determined.

On interaction with calcitriol, VDR acts as a transcription factor through its binding to distinct responsive elements, probably through the formation of heterodimers with RXR receptors.\(^3^1,6^1\) Our data in apoE\(^-/-\) mice show that VDR expression in the suprarenal or ascending aorta remained unchanged after Ang-II infusion or calcitriol cotreatment. However, VDR–RXR interaction was markedly increased in calcitriol-treated mice. Similarly, knockdown of endothelial

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Figure 6. Knockdown of retinoid X receptor alpha (RXRα) by siRNA blocks the suppressive effect of calcitriol on angiotensin-II (Ang-II)–induced mononuclear cell adhesion and tube formation. Human umbilical vein endothelial cells were transfected with control or RXRα-specific siRNA. At 48 h post transfection, cells were pretreated with calcitriol (D3, 100 nmol/L) for 24 h and then with Ang-II (1 μmol/L) for 24 h. A, Protein expression was determined by Western blot. Protein quantification was performed by densitometry, and protein levels were normalized to β-actin. Data represent the mean±SD (n=5 independent experiments). \(†P<0.05\) vs values in the siRNA control group. A representative gel is shown. B, Human mononuclear cells (10\(^6\) cells/mL) were perfused across the endothelial monolayers, and leukocyte adhesion was quantified. Data represent the mean±SD (n=5 independent experiments). C, Endothelial differentiation was performed on Matrigel. Data represent the mean±SD of the number of tube-like structures in 5 low-magnification (×100) fields (n=5 independent experiments performed in triplicate). \(*P<0.05\) vs vehicle; \(†P<0.05\) vs Ang-II. D, Immunofluorescence analysis showing localization of nuclear receptors, VDR and RXR, in endothelial cells with the different treatments. Immunoreactivity was visualized using Alexa Fluor 633 (VDR, red) and Alexa Fluor 488 (RXR, green) secondary antibodies. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; blue). E, VDR–RXR α interaction was assessed by immunoprecipitation of RXRα and subsequent Western blotting for VDR. A representative blot is shown (n=5 independent experiments).
RXRα reversed the inhibitory effects mediated by calcitriol on Ang-II–induced mononuclear leukocyte adhesion and morphogenesis, and immunoprecipitation studies revealed a clear interaction between VDR and RXR in human endothelial cells treated with calcitriol. Given that RXRα activation can inhibit mononuclear cell adhesion and early angiogenic properties,\(^6\) it seems probable that calcitriol functions, in part, via VDR–RXRα heterodimerization.

Activation of ERK1/2, p38 MAPK, and NF-κB were detected in the acute and the chronic phase of Ang-II infusion in apoE\(^{−/−}\) mice. ERK1/2 and p38 MAPK are upstream regulators of NF-κB, and the latter can regulate the expressions of numerous genes including those related to proinflammatory and proangiogenic responses in AAA development.\(^{6,65}\) Along this line, activation of ERK1/2 and p38 is associated with MMP upregulation and subsequent AAA formation.\(^{6,66,67}\)

Recently, endothelial selective blockade of NF-κB signaling in transgenic mice reduced Ang-II–induced dissecting AAA in apoE\(^{−/−}\) mice. ERK1/2 and p38 MAPK are upstream regulators of NF-κB, and the latter can regulate the expressions of numerous genes including those related to proinflammatory and proangiogenic responses in AAA development.\(^{6,65}\) Along this line, activation of ERK1/2 and p38 is associated with MMP upregulation and subsequent AAA formation.\(^{6,66,67}\)

Together, phosphorylation of ERK1/2 and p38 MAPK, and NF-κB signaling pathways are modulated by VDR ligands.\(^{6,66,67}\) Overall, the vascular protective effects displayed by VDR activation indicates that the impaired activation of the NF-κB pathway limits the release of inflammatory/proangiogenic molecules, resulting in blunted endothelial activation.

Finally, it is important to note that the doses and regimen of calcitriol used in this study were based on previous reports, with doses ranging from 0.1 to 10 μg/kg every other day in mice with no toxicity effects or hypercalcemia.\(^{3,8,70,71}\) In humans, doses of calcitriol 0.5 μg/kg or 38 μg/d showed minimal toxicity when used as intermittent therapy in clinical cancer trials.\(^{72}\) To minimize the calcemic side effects, a large number of vitamin D analogs with tissue-specific effects and low calcemic side effects have been developed in recent years.\(^{73}\) However, whether calcitriol at the doses used in our study in mice can offer a safe and effective approach for AAA in humans remains an open question and further clinical research with selective nonhypercalcemic VDR ligands is needed.

In conclusion, this study provides the first evidence that chronic treatment with calcitriol reduces Ang-II–induced dissecting AAA formation in mice. These effects are associated with a reduced inflammatory response and regulation of extracellular matrix homeostasis through VDR–RXRα heterodimerization. In light of these results, activation of VDR may represent a promising therapeutic target for AAA treatment.

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**Disclosures**

None.

**References**

Calcitriol decreases mononuclear leukocyte–endothelial cell interactions, morphogenesis, and chemokine release induced by Ang-II in human endothelial cells.

The effects of calcitriol are mediated via activation of vitamin D3 receptor–RXRα heterodimerization.

Vitamin D3 receptor activation might be a promising therapeutic target for pharmacological intervention of aortic aneurysm formation.

Highlights
Vitamin D Receptor Activation Reduces Angiotensin-II–Induced Dissecting Abdominal Aortic Aneurysm in Apolipoprotein E–Knockout Mice
Sara Martorell, Luisa Hueso, Herminia Gonzalez-Navarro, Aida Collado, Maria-Jesus Sanz and Laura Piqueras

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Calcitriol

Ang-II

ERK1/2, p38 MAPK and NF-κB

MMP-9, MMP2
CCL2, CCL5, CXCL1 and VEGF

Macrophage infiltration
Neovessel formation

Abdominal aortic aneurysm formation

Calcitriol + VDR RXR

+ Ang-II

+ ERK1/2, p38 MAPK and NF-κB

+ MMP-9, MMP2
CCL2, CCL5, CXCL1 and VEGF

+ Macrophage infiltration
Neovessel formation

+ Abdominal aortic aneurysm formation
Vitamin D Receptor Activation Reduces Angiotensin-II-Induced Dissecting Abdominal Aortic Aneurysm in Apolipoprotein E Knockout Mice

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Animal studies
Protocols followed the European Union guidelines for animal care and protection and were approved by the ethics review board of the School of Medicine, University of Valencia. All efforts were made to minimize the number of animals used and their suffering. ApoE\(^{-/-}\) C57BL/6 male mice were supplied by Charles River Laboratories (Chatillon-sur-Chalaronne, France). Mice were housed under specific pathogen-free conditions with free access to a normal chow diet and water, at a constant temperature of 22±2°C and humidity 60%-65% with a 12 h dark/light cycle (lights on at 0800h).

Experimental protocol
Eight-week-old male apoE\(^{-/-}\) mice were used in these studies. An osmotic minipump (Alzet, Model 2004, Charles River) implanted subcutaneously in the dorsum of the neck was used to infuse Ang-II (CAS 4474-91-3, Calbiochem) at a rate of 500 ng kg\(^{-1}\) min\(^{-1}\) for 28 days and mice were fed a Western-type diet (0.15% cholesterol with 42% fat calories; cat\#: E15721, ssniff Spezialdiäten GmbH, Soest, Germany). ApoE\(^{-/-}\) mice were randomly allocated to the following treatments: i) treatment with calcitriol at a dose of 0.1 µg.kg\(^{-1}\) (n=12); ii) treatment with calcitriol at a dose of 1 µg.kg\(^{-1}\) (n=12); and iii) treatment with vehicle (n=12). Calcitriol or vehicle (carboxymethylcellulose) was administered by oral gavage every other day for 28 days. Control saline-infused apoE\(^{-/-}\) mice (n=10) were fed with a normal rodent laboratory diet (cat# E15720, ssniff Spezialdiäten GmbH). In another set of experiments, apoE\(^{-/-}\) mice were infused with Ang-II and treated with oral calcitriol (1 µg.kg\(^{-1}\)) or vehicle for 5 days (n=10 per group). The doses of calcitriol used in this study were based on the published efficacy and pharmacokinetic data from murine models.\(^{1-3}\)

A pilot study was initially carried out in eight-week-old male apoE\(^{-/-}\) mice (n=14) infused with Ang-II at a rate of 1000 ng/kg/min for 28 days. In that study, 4 of 14 animals (28%) died as a result of aortic rupture during the first week of Ang-II infusion (data not shown). The mortality rate was higher but within the limits observed in a recent meta-analysis published by Trachet and colleagues;\(^{4}\) however following the recommendations of the ethics review board of our institution, we performed the present study with a lower dose of 500 ng/kg/min of Ang-II in combination with a Western diet.

Aneurysm quantification
At the end of the experimental protocols, mice were anesthetized and cut open ventrally. Left cardiac ventricles were perfused with phosphate-buffered saline (10 mL) with an exit through the severed right atrium. The aorta was exposed under a dissecting microscope, the periadventitial tissue was removed and the aorta was photographed. Suprarenal regions of the abdominal aorta were identified between the last pair of intercostal arteries and the right renal branch. Maximal outer diameter of the suprarenal aorta was measured \textit{ex vivo} using ImageJ software (NIH). Aneurysm severity (stage I to stage IV) was evaluated based on previous studies:\(^{5}\) Type I, dilated lumen in the suprarenal region of the aorta without thrombus or with a little thrombus; Type II, dilated lumen in the suprarenal region of the aorta with a pronounced bulbous form that contains thrombus; Type III, dilated lumen in the suprarenal region of the aorta with multiple aneurysms; Type IV, attributed to ruptured aneurysms. Necropsies were performed on all mice that died during the experimental treatment. Aortic rupture was defined as the observation of blood clots in either the retroperitoneal cavity (abdominal aortic rupture) or thoracic cavity (thoracic aortic rupture). Considering tissue degradation, these animals were excluded from the analysis, but were used for mortality and rupture rate data.

The \textit{ex vivo} diameter of the ascending aorta and the intimal area of the thoracic aorta were measured as previously described.\(^{6}\) Scoring of ascending and abdominal aneurysms was determined by an investigator blinded to treatments. On determination
of the diameter, area, and classification, a second investigator matched the scored aneurysms to the different treatments of the mice.

**Measurement of blood pressure and lipid profile**

Systolic blood pressure was measured in conscious mice using a non-invasive tail-cuff system (Model LE5002 Pressure Meter, PANLAB, Barcelona, Spain) using methods similar to those described previously. Conscious mice were restrained on a warming chamber (Model LE5610, PANLAB). Mice were acclimatized to the instrument for at least one week before implantation of the osmotic pumps. To avoid variations in blood pressure due to day cycle, all measurements were carried out between 9 and 11 a.m. Individual mice received five initial pressure readings to acclimate them to the procedure, and then additional cycles were measured to obtain the mean systolic pressure. The criterion for acceptance of data were acquisition of at least 10 of 20 measurements and a standard deviation of <30 mm Hg for each session.

Plasma lipid levels were measured in mice fasted overnight. Total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) were determined using enzymatic procedures (WAKO, St Louis, MI).

**Histological and immunohistochemical analysis**

The suprarenal aorta samples were divided into two sequential regions (A and B) cutting at the level of maximal dilatation under a Leica DMD108 Digital microscope (Leica Microsystems). Region A was kept at −80 °C for molecular analysis. Region B was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and mounted on double gelatin-coated glass slides. Tissue cross sections from region B were obtained starting at the level of maximal dilatation. At least 8 slides, containing ≈5 tissue cross sections (5 µm thick) from each animal were examined (mean number of aortic sections per mouse = 42 ± 5). Hematoxylin and eosin (H&E) or Verhoeff-van Gieson stains (elastic fibers) (Sigma Aldrich, MI) were performed. Elastin degradation per field was evaluated under 40× magnification in suprarenal aortic sections from the different groups of mice. Qualitative evaluation of elastin integrity was performed by a blinded observer on digital images by semiquantitative grading as described. Elastin preservation was graded as follows: grade 1, non-elastin degradation, well organized elastin laminae; grade 2, elastic laminae with some interruptions and breaks; grade 3, elastic laminae with multiple interruptions and breaks; grade 4, severe elastin fragmentation or loss.

Inflammatory infiltration and microvessel formation in aortas were measured as described. After peroxidase inactivation (H2O2 0.3%) and blockade with horse serum, aorta cross-sections were incubated overnight (4°C) with the following primary antibodies: a rat monoclonal anti-mouse CD68 (1:50 dilution, cat# MCA1957GA, Serotec) and a rabbit polyclonal antibody to myeloperoxidase (1:25 dilution, cat# ab9535, Abcam, Cambridge, UK) were used for quantification of inflammatory cells. Specific labeling was detected with an Alexa Fluor 633 goat anti-rat secondary antibody (1:500 dilution, cat# A21094, Molecular Probes) or Alexa Fluor 488 goat anti-rabbit secondary antibody (1:500 dilution, cat# A11034, Molecular Probes). SMC staining was carried out using a monoclonal anti-alpha smooth muscle actin antibody conjugated to Cy3 (1:100 dilution, cat# C6198, Sigma-Aldrich). CD31 staining was performed with a rabbit polyclonal anti-mouse-CD31 (1:50 dilution, cat# ab28364, Abcam). For MMP analysis, a rabbit polyclonal anti-mouse MMP-2 (dilution 1:100, cat# ab38898, Abcam) antibody was used. For TIMP-1 detection, a rabbit polyclonal anti-mouse TIMP-1 (1:100 dilution, cat# ab38978, Abcam) was used. Specific labeling was detected with a biotin-conjugated goat anti-rabbit secondary antibody (1:400 dilution, cat# 4E0432, Dako). In order to confirm specificity of antibodies, isotype controls (cat# A2143; cat# A11126, both from Abcam) or secondary antibodies only were used as negative controls (Supplemental Figure VIII). Fields from each suprarenal aortic section were captured, digitized, and analyzed (Axio Observer A1, Carl Zeiss, NY). Scoring was performed blinded on coded slides.
PCR
Total RNA was extracted from aortas by homogenization and converted to cDNA by standard methods. PCR was performed with the Luminaris Color HiGreen HigRox qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA) using the ABI Cycler 7900 Fast System thermal cycler. The following murine primer sequences were used for amplification (Forward: Fw; Reverse: Rv): GAPDH Fw 5' TGACCACAGTCCATGCATC-3' and Rv 5'-GACGGACACATTGGGGTGAG-3'; MCP1/CCL2 Fw 5'-GCCACACGACCACGACCAG-3' and Rv 5'-GGCATACAGTCCAGTC-3'; RANTES/CCL5 Fw:5'-CTCGGTCCCTGGGAAATGGA-3' and Rv:5'-TGCTGATTCTTTGGGTTTCT-3'; KC/CXCL1 Fw 5'-GAAGCTCTGGGTGGATGATG-3' and Rv:5'-GAGCTCTGGTTCAGAAA-3'; VEGF Fw 5'-TCTCACCGGAAAGACCGATT-3' and Rv 5'-CTGTAACCGTGACGCATGATG-3'. mRNA levels were normalized to the levels of GAPDH as an endogenous control. We also used specific primers pre-designed by Applied Biosystems for analysis of MMP-2 (Mm00439498_m1); MMP-9 (Mm00442991_m1), TIMP-1 (Mm00441818_m1) and VDR (Mm00437297_m1)

Western blotting
Protein concentration was determined by the Bradford method.11 Samples were denatured, subjected to SDS-PAGE using a 10% running gel, and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with 3% BSA in TBS solution and membranes were incubated overnight with primary rabbit polyclonal antibodies. Primary antibodies used were as follows: p44/p42 MAPK/ERK1/2 (cat# 4695), phosphorylated p44/p42 MAPK/ERK1/2 (Thr202/Tyr204, cat# 4377), p-38 MAPK (cat# 9212), phosphorylated-p38 MAPK (Thr180/Tyr182, cat#9211) and NF-κB p65 (cat# 4764) and phosphorylated-p65 (Ser536, cat# 3033), all at a dilution of 1:500 (Cell Signaling Technology, Danvers, MA) and a rat monoclonal antibody against VDR (1:100 dilution, cat# ab8756, Abcam). Membranes were subsequently washed, incubated for an additional hour with the secondary HRP-linked anti-rabbit antibody (1:2000 dilution, cat# 0448, Dako) or HRP-linked anti-rat antibody (1:2000 dilution, cat# 0450, Dako) and developed using an ECL procedure (GE Healthcare, Madrid, Spain). Signals were recorded using a luminescent analyzer (FujiFilm Image Reader LAS 4000, Fuji, Tokyo, Japan) and analyzed with ImageJ software (NIH).

Human studies
All research with human samples complied with the principles outlined in the Declaration of Helsinki and was approved by the institutional ethics committee of the University Clinic Hospital Valencia (Valencia, Spain). Written, informed consent was obtained from all volunteers.

Cell culture
Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase treatment12 and maintained in human endothelial cell specific medium (EBM-2) supplemented with endothelial growth media (EGM-2) and 10% FCS. All experiments and transfection assays were performed in HUVEC at passage 1. In post-transfection studies cells were at passage 2.

Leukocyte-HUVEC interactions under flow conditions.
HUVEC at passage 1 were grown to confluence and stimulated with 1 µM Ang-II for 24 h. Cells were incubated with calcitriol (1-100 nM) 24 h prior to stimulation with Ang-II. Human mononuclear cells were obtained fromuffy coats of healthy donors by Ficoll-Hypaque density gradient centrifugation. The GlycoTech flow chamber was assembled and placed onto an inverted microscope stage and freshly isolated human mononuclear cells (1×10⁵ ml⁻¹) were then perfused across the endothelial monolayers. In all experiments, leukocyte adhesion was determined after 5 min at 0.5 dynes cm⁻². Cells interacting on the surface of the endothelium were visualized and recorded (×20 objective, ×10 eyepiece) using phase-contrast microscopy (Axio Observer A1).

Endothelial Cell Differentiation Assay
HUVEC were seeded on growth factor-depleted Matrigel (BD Biosciences) as described. Phase contrast micrographs were recorded and the number of tubes in 5 low-power (×100) random fields were counted.

**Transfection with small interfering RNA**

Confluent HUVEC at passage 1 were transfected with control siRNA, VDRα-specific siRNA or RXR-specific siRNA (Dharmacon, Lafayette, CO) for 48 h using Lipofectamine RNAiMAX (Invitrogen, Calsbad, CA) as described. Forty-eight hours post-transfection, VDR and RXRα expression was determined by western blotting using a rabbit monoclonal antibody against human RXRα (1:500, cat# ab125001, Abcam) and a rat monoclonal antibody against human VDR (dilution 1:100, cat# ab8756, Abcam). Membranes were subsequently washed, incubated for an additional hour with a polyclonal HRP-linked goat anti-rabbit secondary antibody (1:2000 dilution, cat# 0448, Dako) or a polyclonal HRP-linked rabbit anti-rat secondary antibody (1:2000 dilution, cat# 0450, Dako) and developed using an ECL procedure (GE Healthcare).

**Chemokines and VEGF detection**

The human chemokines CCL2, CCL5, CXCL1, and VEGF were measured in HUVEC culture supernatants using antibody pairs from R&D Systems (ELISA Duoset kits, Abingdon, UK). After coating the plates overnight with the primary antibody, non-specific binding sites were blocked with 3% BSA for 1 h. Supernatants and standards were added to PBS/0.5%/BSA/0.05% sodium azide for 2 h. Biotinylated detector antibodies were added for 2 h, followed by neutravidin-HRP for 1 h. All plate washes were carried out in four cycles with freshly prepared PBS/0.2% Tween20. Enhanced K-Blue tetramethylbenzidine substrate was added for 30 min and the enzyme reaction was stopped by addition of 0.19 M sulfuric acid. Absorbance was read at 450 nm. Experiments were performed in triplicate. Results are expressed as pM chemokine in the supernatant.

**Immunofluorescence**

After treatments, HUVEC were washed with PBS, fixed with methanol/acetone (1:1), and blocked in PBS/1% BSA solution. VDR and RXR distribution in HUVEC monolayers was visualized by indirect immunofluorescence (Axio Observer A1) using a rabbit monoclonal antibody against human RXRα (1:100 dilution, cat# ab125001, Abcam) and a rat monoclonal antibody against human VDR (1:100 dilution, cat# ab8756, Abcam). Immunofluorescence signals were detected using an Alexa Fluor 633 goat anti-rat secondary antibody (1:500 dilution, cat# A21094, Molecular Probes) or an Alexa Fluor 488 goat anti-rabbit secondary antibody (1:500 dilution, cat# A11034, Molecular Probes). To confirm specificity of antibodies, isotype controls (cat# 172730; cat# ab18451 both from Abcam) or secondary antibodies only were used as negative controls. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI).

**Immunoprecipitation**

Aortic walls or endothelial cell extracts were prepared in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, and protease (1 mM PMSF, 40 µg.ml⁻¹ aprotinin, and 40 µg.ml⁻¹ leupeptin) and phosphatase (1 mM sodium orthovanadate and 1 mM NaF) inhibitors. Protein (200 µg) was incubated with 5 µg of the rabbit monoclonal antibody against RXRα (cat# ab125001, Abcam). Immunocomplexes were precipitated using anti-rabbit IgG beads (cat# 8800, eBioscience, San Diego, CA) and suspended in sample buffer containing freshly added 50 mM dithiothreitol (DTT). Western blotting was performed with a rat monoclonal antibody against VDR (dilution 1:500, cat# ab8756, Abcam). Membranes were incubated with the secondary HRP-linked anti-rabbit antibody (1:2000 dilution, cat# 0448, Dako) and chemiluminescent signals were developed with ECL (GE Healthcare).
Additional Materials
Calcitriol was from Tocris Bioscience (Bristol, UK). Unless stated, all other reagents were from Sigma-Aldrich.

Statistical analysis.
Values are expressed as individual data points, percentage or means±SD when appropriate. For comparisons of two groups, Student’s t test was used in data that passed both normality (Kolmogorov-Smirnov test) and equal variance (Levene test); otherwise, a non-parametric Mann Whitney U test was performed. For comparisons among multiple groups, one way analysis of variance (ANOVA) followed by post hoc analysis (Bonferroni test) was used in data that passed both normality and equal variance; otherwise, a non-parametric Kruskal–Wallis test followed by Dunn’s post hoc analysis was used. To assess statistical significance of aneurysm subtype variables, chi-square analysis was performed. Survival curves were analyzed using the Logrank test. Data were considered statistically significant at P<0.05.

References


SUPPLEMENTAL MATERIAL

Vitamin D Receptor Activation Reduces Angiotensin-II-Induced Dissecting Abdominal Aortic Aneurysm in Apolipoprotein E Knockout Mice

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Supplemental Figures

Supplemental Figure I

**Supplemental Figure I.** Effects of calcitriol (D$_3$, 1 µg.kg$^{-1}$) on Ang-II-induced ascending aortic aneurysms. (A) Ascending aorta diameter (mm). (B) Intimal area of the thoracic aorta (mm$^2$). Data are expressed as the mean ± SD and also as individual data points. *$P<0.05$ vs. respective control mice. †$P<0.05$ vs. Ang-II-infused animals.
Supplemental Table I

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<td>Ang-II</td>
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**Supplemental Table I.** Calcitriol (D₃) treatment does not alter systolic blood pressure (SBP), plasma cholesterol (TC), HDL-cholesterol (HDL-C) or triglycerides in apoE⁻/⁻ mice. Data represent the mean ± SD. *P<0.05 vs. respective control mice.
Supplemental Figure II

Effects of calcitriol (D₃, 1 µg.kg⁻¹) on the elastic fibers and vascular smooth muscle cell lamina in the suprarenal aortas of Ang-II infused apoE⁻/⁻ for 28 days. (A) Representative photomicrographs of H&E staining. Bars-500 µm. (B) Representative images of immunohistochemical stains for elastin fiber (Van Gieson); arrows show the sites of elastin destruction. Higher magnifications of vessel areas are indicated in the right panels. Bars-100 µm. (C) Representative immunostaining for smooth muscle α-actin (red). Nuclei were stained with DAPI (blue). Arrows show the sites of loss of medial smooth muscle cells. Bars-100 µm. (D) Severity of elastin degradation was semiquantified as: grade 1, no degradation; grade 2, mild degradation; grade 3, severe degradation; and grade 4, presence of aortic rupture. Data represent the mean ± SD (n= 5 animals/group). *P<0.05 vs. vehicle-infused mice; †P<0.05 vs. Ang-II-infused animals.
**Supplemental Figure III.** Effects of calcitriol (D$_3$, 1 µg.kg$^{-1}$) on suprarenal aortas of mice infused with Ang-II for 5 days. Representative photomicrographs of (A) CD68+ cells and (B) myeloperoxidase (MPO) staining. Bars-100 µm. Gene expression of (C) CCL2, (D) CCL5 and (E) CXCL1. Data represent the mean ± SD of the ratio between each gene and gapdh expression. (n=4-5 animals/group). *P<0.05 vs. vehicle-infused mice; †P<0.05 vs. Ang-II-infused animals. (F) Immunohistochemical staining of CD31. Bars-50 µm. (G) VEGF expression by real-time RT-PCR.
Supplemental Figure IV. Effects of calcitriol (D₃, 1 µg.kg⁻¹) on suprarenal aortas of mice infused with Ang-II for 5 days. (A) Representative photomicrographs of H&E staining, elastin fiber and smooth muscle α-actin. B) Immunohistochemical staining of MMP2, MMP9 and TIMP-1 in the suprarenal aorta of apoE⁻/⁻ mice. Bars-50 µm. Gene expression of (C) MMP2, (D) MMP9 and (E) TIMP-1 was analyzed by real-time RT-PCR. Data represent the mean ± SD of the ratio between each gene and gapdh expression (n=5 animals/group) *P<0.05 vs. vehicle-infused mice; †P<0.05 vs. Ang-II-infused animals.
Supplemental Figure V. Effects of calcitriol (D₃, 1 µg.kg⁻¹) on ERK1/2, p38 MAPK and NFκB phosphorylation and VDR expression in aortas of apoE⁻⁻ mice infused with Ang-II for 5 days. (A) Representative western blots and (B-D) densitometric analysis of phospho ERK1/2/total ERK1/2, phospho p38/total p38 and phospho p65/total p65 in the suprarenal aortic tissues. Data represent the mean±SD (n=4 animals/group) of protein densitometry. *P<0.05 vs. vehicle-infused mice; †P<0.05 vs. Ang-II-infused animals. (E) Gene expression of VDR was analyzed by real-time RT-PCR in the suprarenal and ascending aortas of apoE⁻⁻ mice. Data represent the mean ± SD of the ratio between VDR and gapdh expression (n=4 animals/group). (F) VDR/RXRα interaction was assessed by immunoprecipitation of RXRα and subsequent western blotting for VDR in the suprarenal and ascending aorta of apoE⁻⁻ mice treated with calcitriol for 5 days.
Supplemental Figure VI. Calcitriol (D₃) decreases Ang-II-induced mononuclear endothelial cell interactions, tubulogenesis and chemokine release in human venous endothelial cells. HUVEC were incubated with vehicle (0.01 % DMSO) or calcitriol (0.1-100 nM) 24 h before Ang-II stimulation (1 µM, 24 h). (A) Freshly isolated human mononuclear cells (10⁶ cells ml⁻¹) were perfused across the endothelial monolayers for 5 min at 0.5 dyn/cm² and leukocyte adhesion was quantified. Results are the mean ± SD (n=5 independent experiments); *P<0.05 relative to vehicle group, †P<0.05 relative to Ang-II stimulated cells. (B) Endothelial differentiation assay was performed on Matrigel. Graph represent mean ± SD of the number of tube-like structures in 5 low-magnification (×100) fields (n=5 independent experiments performed in triplicate). *P<0.01 vs. vehicle; †P<0.05 vs. Ang-II. Representative photomicrographs are shown. Calcitriol decreases (C) CCL2, (D) CCL5 and (E) CXCL1 and (F) VEGF release in Ang-II-stimulated endothelial cells. Chemokines and VEGF release were determined by ELISA in cell-free supernatants. Results are expressed as pM concentration and are presented as mean ± SD (n=5 independent experiments performed in triplicate). *P<0.05 vs. vehicle; †P<0.01 vs. Ang-II.
Supplemental Figure VII. Phosphorylation of ERK1/2, p38 MAPK and p65 induced by Ang-II was reduced by pretreatment of HUVEC with calcitriol. Cells were pretreated with calcitriol (D$_3$, 100 nM) for 24 h. After treatments, HUVEC were stimulated with Ang-II (1 µM, 15 min). Representative western blots of (A) phospho ERK1/2/total ERK1/2, (B) phospho p38 MAPK/total p38MAPK and (C) phospho p65/total p65. Data represent the mean ± SD (n=5 independent experiments). *P<0.05 vs. vehicle; †P<0.05 vs. Ang-II.
Supplemental Figure VIII

A  Suprarenal aortic tissue

B  HUVEC

Supplemental Figure VIII. No signals were detected when respective isotype control antibodies were used instead of the primary antibodies or when secondary antibodies were used alone as negative controls. Representative images of immunohistochemical and immunofluorescence analysis: (A) suprarenal aortic tissue and (B) HUVEC.