Disruption of Nuclear Vitamin D Receptor Gene Causes Enhanced Thrombogenicity in Mice

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Running Title: VDR Knockout Mice and Thrombosis
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Summary (243 words)

Vitamin D metabolites influence the expression of various genes involved in calcium homeostasis, cell differentiation and regulation of the immune system. Expression of these genes is mediated by the activation of the nuclear vitamin D receptor (VDR). Previous studies have shown that a hormonally active form of vitamin D, 1\(\alpha\),25-dihydroxyvitamin D\(_3\), exerts anticoagulant effects in cultured monocytic cells. To clarify whether activation of VDR plays any anti-thrombotic actions in vivo, hemostatic/thrombogenic systems were examined in normocalcemic VDR knockout (KO) mice on a high calcium diet and compared with wild type and hypocalcemic VDRKO mice that were fed a regular diet. Platelet aggregation was significantly enhanced in normocalcemic VDRKO mice compared to wild type and hypocalcemic VDRKO mice. Aortic endothelial nitric oxide (NO) synthase expression and urinary NOx excretions were reduced in hypocalcemic VDRKO mice, but not in normocalcemic VDRKO mice. Northern blot and RT-PCR analyses revealed that the gene expression of antithrombin in the liver as well as that of thrombomodulin in the aorta, liver and kidney was down-regulated in hypo- and normocalcemic VDRKO mice. Whereas tissue factor mRNA expression in the liver and kidney was up-regulated in VDRKO mice regardless of plasma calcium level. Furthermore, VDRKO mice manifested an exacerbated multi-organ thrombus formation after exogenous lipopolysaccharide injection regardless of the calcemic conditions. These results demonstrate that activation of nuclear VDR elicits anti-thrombotic effects in vivo, and suggest that the VDR system may play a physiological role in the maintenance of anti-
thrombotic homeostasis.
Introduction

In many target organs, vitamin D exerts a variety of biological functions such as calcium homeostasis, cell proliferation, and cell differentiation. Most of these actions are exerted through the transcriptional control of target genes by the activation of the nuclear vitamin D receptor (VDR). VDR is a ligand-inducible transcription factor that belongs to the nuclear receptor superfamily (1). VDR forms a heterodimer with the retinoid X receptor and binds to specific vitamin D responsive elements on target genes (2,3), which initiate sequences of events that lead to the activation or repression of target gene transcription by recruiting transcriptional cofactor complexes. Previous studies demonstrated that VDR knockout (KO) mice manifest a variety of phenotypic abnormalities, including hypocalcemia, osteopenia, growth retardation, alopecia, (4,5), impaired immunity (6), hypertension with cardiac hypertrophy (7) and abnormal skeletal muscle development (8), and that most, but not all of these phenotypic abnormalities can be rescued by normalization of serum mineral levels (6,9).

In addition to its classical target tissues, VDR is also expressed in monocytic cells (10) and vascular endothelial cells (11), suggesting potential roles of vitamin D in anti-thrombotic functions. Koyama and colleagues have found that a hormonally active form of vitamin D, 1\(^\text{[\text{.[25-dihydroxyvitamin D3, as well as retinoic acid, exert anticoagulant effects by up-regulating the expression of the anticoagulant glycoprotein, thrombomodulin (TM), and by down-regulating the expression of a critical coagulation factor, tissue factor (TF), in cultured monocytic cells and human peripheral monocytes (10,12). However, it remains unclear whether activation of nuclear VDR can elicit}}

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anti-thrombotic actions in vivo. The present study was undertaken to clarify the physiological role of VDR-mediated actions in the hemostatic/thrombogenic system in vivo.
Experimental Procedures

Animal preparation

VDR homozygous mutants (4) and wild type (WT) control littermates were maintained as hybrids with C57BL/6J and CBA genetic backgrounds in a specific pathogen free facility with a 12-hour light and dark cycle. As previously reported (4,9), a lactose-rich high calcium (Ca) diet can normalize hypocalcemia in VDRKO mice, while a high calcium diet causes considerable hypercalcemia in WT mice. Therefore, after weaning (at 3 weeks of age) we divided the mice into 3 groups as follows: group 1 are WT mice fed a regular diet (containing 1.2% Ca and 1.0% phosphorus, Japan Clea, Tokyo, Japan), group 2 are VDRKO mice fed a regular diet (hypocalcemic), and group 3 are VDRKO mice fed a lactose-rich high Ca diet (normocalcemic). Experiments were conducted in 12 weeks old mice that had been weaned then fed a specified diet for 9 weeks.

Blood collection

Blood was collected from the inferior vena cava of mice under ether anesthesia. The blood was drawn with 21-G needles into plastic syringes containing 0.1 volume of 3.8% trisodium citrate solution. Some whole blood samples were immediately used for platelet aggregation analysis and other whole blood samples were centrifuged at 2000×g for 10 minutes to obtain plasma. The plasma was stored at −80°C until assayed.
**Measurement of plasma calcium and parathyroid hormone**

Plasma levels of calcium and parathyroid hormone (PTH) were determined using the OCPC method and a rat PTH IRMA Kit (Immunotopics Inc., San Clemente, California, USA), respectively.

**Comparison of platelet aggregation with a screen filtration pressure method**

Measurements of platelet aggregation with a screen filtration pressure (SFP) aggregometer (WBA analyzer from SSR Engineering Co., Ltd. Yokohama Japan) were performed according to the previously described method (13,14). Reaction tubes containing 200 µl aliquots of whole blood were placed in an incubation chamber at 37°C for 30s, followed by addition of 22.2 µl of a serial concentration (1, 2, 4, 8 or 16 µM) of ADP (Sigma St. Louis, MO, USA). Two minutes after the addition of ADP, the blood samples were filtered through microsieve screens connected to a pressure sensor. A negative pressure of -130mmHg was established as 100%. The 0% pressure baseline was established as -6mmHg, rather than 0mmHg, due to the viscosity of whole blood. The platelet aggregation pressure of each reaction tube was determined as the pressure rate (%). For the SFP aggregometer study, the pressure rate was standardized using a grading curve produced by plotting four or five concentrations of ADP on the x-axis and pressure rate (%) on the y-axis. The concentration of ADP causing a 50% increase in pressure rate was calculated and applied as the platelet aggregatory threshold index (P ATI).
Measurement of daily urinary excretion of nitric oxide metabolites

To evaluate urinary excretion of nitric oxide (NO) metabolites (NOx), mice were individually housed in metabolic cages that provided free access to tap water and food for 24 hours. NOx levels, from the murine urine, were determined by HPLC (15).

Estimation of plasma prothrombin time and activated partial thromboplastin time

Plasma prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined by a scattered light detection method (16).

Measurement of plasma antithrombin activity

Plasma antithrombin (AT) activity was measured using N-test ATIII-S (Nittobo, Tokyo, Japan), which determines anticoagulant activity using a chromogenic substrate, according to the manufacture’s instructions.

Western blot analysis

Aortic tissue samples were homogenized in an ice-cold tissue protein extraction reagent (T-PER™, PIERCE, Rockford, ILL. USA) containing protease inhibitors. Equal amounts of protein (50µg) were loaded onto 10% acrylamide gels and then transferred to nitrocellulose membranes using a wet blot apparatus. Membranes were blocked overnight at 4°C in blocking buffer (10mM Tris-HCl, 10mM NaCl, 0.1% polyoxyethylenesorbitan monolaurate) with 5% skim milk. The first antibody, mouse anti-human endothelial nitric oxide synthase (eNOS) monoclonal antibody (1:1000
dilution, BD Transduction Laboratories, San Jose, CA), was applied for 1 hour at room temperature. After a 30-min wash with the blocking buffer, membranes were probed with the second antibody (1:5000 dilution anti-mouse IgG HRP-linked whole antibody, Amersham Biosciences, Buckinghamshire, UK) for 1 hour at room temperature. Membranes were washed again for 30 min. The membranes were then incubated with chemiluminescent reagents (ECL Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, UK) for 5 min, and then exposed to X-ray film. Band intensities were quantified using a NIH image system.

**RNA isolation**

Aortas, livers and kidneys were rinsed with physiological saline. Total RNA was isolated by an acid guanidinium thiocyanate-phenol-chloroform extraction using TRIzol R Reagent (Invitrogen life technologies, Carlsbad CA, USA). RNA concentrations were measured spectrophotometrically at 260 nm and then samples were stored in diethyl dicarbonate-treated water at −80°C.

**Northern blot analysis**

Approximately 20 μg total RNA of the liver was fractionated on 1% formaldehyde-agarose gels and transferred to Hybond nylon membranes (Amersham Pharmacia Biotech, Little Chalfont, England) by capillary action in a high salt solution (20 × SSC). Blots were prehybridized in a hybridization solution for 1 hour at 42°C, followed by overnight hybridization with a digoxigenin-labeled specific oligonucleotide probe (DIG
Northern Starter Kit, Roche Diagnostics, Indianapolis, IND, USA). Blots were washed twice in 2×SSC/0.1% SDS at room temperature for 5 min and then washed twice in 0.2×SSC/0.1% SDS at 68°C for 15 min before exposure to X-ray film. The forward and reverse sequences of the AT oligonucleotide probe were: 5’-ATGATGTACCAGAA-GGCAA-3’ and 5’-GGAATGCCTCGGAGACATAG-3’, respectively. AT mRNA was estimated after correcting for loading differences by measuring the amount of 28S rRNA.

**RT-PCR analysis**

Expression of tissue factor (TF), thrombomodulin (TM) and G3PDH mRNA in aorta, liver and kidney tissue were quantified by a RT-PCR method (8). Primers used were as follows: TF; forward 5’-CGGGTGCAGGCATTCCAGAG-3’ and reverse 5’-CTCCGTGGGA-CAGAGAGGAC -3’, TM; forward 5’-CAGGCTACCAGTGGCTG-CAG-3’ and reverse 5’-AGAGTTAGGGTCACAGTCTGC -3’, G3PDH; forward 5’-ACCACAGTCCATGCCATCAC-3’ and reverse 5’-TCCACCACCCCTGTGCTGTA -3’. PCR products were electrophoresed on 2.0% agarose gels, stained with ethidium bromide, visualized by ultraviolet transillumination and photographed. Expression levels of TF and TM were expressed relative to the G3PDH signal.

**Lipopolysaccharide-induced thrombus formation experiments and Immunohistochemical analysis**

Mice were injected intraperitoneally with 5 mg/kg of lipopolysaccharide (LPS)
(Escherichia coli serotype 0111: B4; Sigma, St Louis, MO). Six hours later, mice were sacrificed and lung, aorta, liver and kidney tissue were removed. The tissue samples were immediately placed in 20% neutrally buffered formalin and stored overnight. After fixation, samples were embedded in paraffin and 3 μm thick sections were produced. These tissue sections were deparaffinized, hydrated and treated with a protease cocktail (P-8038, Sigma-Aldrich Co., St. Louis, MO, USA) for 10 minutes at room temperature. After washing with deionized water, endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes and then endogenous biotin was blocked with the DAKO Biotin Blocking System (DAKO Cytomation, Glostrup, Denmark). After washing with deionized water and blocking non specific staining by incubation with 10% porcine serum (GEMINI Bio-Products, Woodland, CA, USA) in PBS for 10 minutes at room temperature, the slides were incubated with rabbit anti-human fibrin-fibrinogen antibody (DAKO, Cytomation, Glostrup, Denmark, 1:200 dilution) overnight at 4°C. The slides were then rinsed with PBS and incubated with (1:500) biotinylated F(ab’)2 fragments of swine anti-rabbit immunoglobulins (DAKO, Cytomation, Glostrup, Denmark) for 30 minutes at room temperature. After rinsing with PBS, the slides were incubated with diluted (1:500) peroxidase-conjugated streptavidin (DAKO, Cytomation, Glostrup, Denmark) for 30 minutes at room temperature. Following incubation in 100ml PBS containing 20mg “3,3’-Diaminobenzidine, tetrahydrochloride”, 30% hydrogen peroxide of 20ul and 65mg sodium azide for 5 minutes at room temperature, the slides were counterstained with hematoxylin for 1 minute. Finally, the sections were mounted after washing with
deionized water and dehydration. The percentage of the glomeruli with fibrin deposition (%GFD) were calculated in all areas of each histological specimen of the kidney. Partially stained glomeruli were categorized as positive.

**Statistical analysis**

Values for each parameter within a group were expressed as mean ± SEM. For comparisons between genotypes, statistical significance was assessed using a one way ANOVA. Statistical significance was considered at P < 0.05.
Results

Plasma Ca and PTH levels in WT and VDRKO mice

The plasma Ca levels of VDRKO mice fed a regular diet were significantly low compared to WT mice (Fig. 1A). When VDRKO mice were fed a high Ca diet, plasma Ca levels were restored to levels similar to those in WT mice (Fig. 1A). As expected, marked elevation in plasma PTH levels were detected in VDRKO mice compared to WT mice. Consistent with the normalization of hypocalcemia from a high Ca diet (Fig. 1B), elevated plasma PTH levels decreased to similar levels observed in WT mice.

Aberrant platelet aggregation in VDRKO mice

To examine the effect of VDR activation in platelet function, we first evaluated ADP-induced whole blood aggregation using an SFP method in 12-week old mice (Fig. 2A). PATI values in hypocalcemic VDRKO mice were markedly increased compared to those in WT mice (P<0.01) (Fig. 2B). In contrast, PATI values in normocalcemic VDRKO mice were significantly lower than those in WT mice (P<0.05) (Fig. 2B). These results demonstrate that hypocalcemia is responsible for the suppression of platelet aggregation in VDRKO mice fed a regular diet, and that VDR itself has a suppressive effect on platelet aggregability.

Decreased urinary level of nitric oxide metabolites in hypocalcemic VDRKO mice was restored by a high Ca diet
Because platelet aggregation is affected by the bioavailability of NO, we then analyzed urinary excretion of NOx (Fig. 3A). Daily urinary excretion of NOx in VDRKO mice was significantly decreased compared to WT mice. Feeding a high Ca diet (2.0% Ca) restored the urinary level of NOx in VDRKO mice to a level similar to that in WT mice. Thus, it is unlikely that the enhanced platelet aggregation in normocalcemic VDRKO mice is mediated by a change in NO level.

*Impaired aortic eNOS expression in hypocalcemic VDRKO mice was normalized by high Ca diet*

Western blot analysis demonstrated that eNOS protein levels in hypocalcemic VDRKO mice decreased to 45 ± 20% of WT mice. This may be a cause of decreased levels of urinary NOx excretion in hypocalcemic VDRKO mice (Fig. 3B). By feeding a high Ca diet, eNOS protein levels in VDRKO mice returned to levels similar to those in WT mice (Fig. 3B). These results indicate that reduced eNOS protein levels in VDRKO mice are mediated by hypocalcemia.

*VDRKO mice manifested normal prothrombin time and activated partial thromboplastin time*

Although PT and APTT in WT, hypo- and normocalcemic VDRKO mice were measured, no significant differences were noted among these mice in either PT (10.6 ± 0.4, 10.6 ± 0.2, 10.8 ± 0.6 sec in WT, hypo- and normocalcemic VDRKO mice, respectively) or APTT (58.0 ± 3.8, 54.5 ± 4.4, 56.4 ± 6.0 sec in WT, hypo- and
normocalcemic VDRKO mice, respectively). These results demonstrate that the loss of VDR function does not lead to impaired blood coagulation, and is consistent with the fact that VDRKO mice do not show bleeding tendency.

**Reduced plasma activity and gene expression of antithrombin in VDRKO mice**

We next examined the plasma activity of AT in these mice. Mean activities of plasma AT showed reductions in VDRKO mice regardless of their plasma Ca levels compared to those in WT mice (Fig. 4A). In addition, gene expression levels of AT in the liver were reduced by approximately 20% in both hypo- and normocalcemic VDRKO mice compared to those in WT mice (Fig. 4B). These results demonstrate that activated VDR positively regulates AT gene expression in the liver.

**Aberrant gene expression of TF and TM in VDRKO mice**

As shown in Fig. 5, TF mRNA expression levels in the liver and kidney were enhanced in both hypo- and normocalcemic VDRKO mice compared to those in WT mice, and tended to be higher in the aorta of normocalcemic VDRKO mice. In contrast, TM mRNA levels in the aorta, liver and kidney were all reduced in VDRKO mice compared to those in WT mice (Fig. 6). Normalization of plasma Ca levels failed to correct the aberrant expression patterns of TF and TM genes in VDRKO mice (Fig. 5 and 6). These results indicate that activation of VDR elicits down-regulation of TF and up-regulation of TM gene expression, *in vivo.*
Lipopolysaccharide-induced thrombus formation was exacerbated in VDRKO mice

In order to test the possibility that the VDR system counteracts against thrombotic stimuli, 5 mg/kg of LPS was injected intraperitoneally to WT and VDRKO mice. All mice survived the treatment until sacrifice. Immunohistochemical analysis revealed that hypo- and normocalcemic VDRKO mice exhibited increased fibrin deposition in the glomeruli and peritubular capillaries of the kidney compared to WT mice (Fig. 7). Exacerbated fibrin deposition was also noted in the hepatic sinusoids of VDRKO mice regardless of plasma Ca levels (Fig. 7). No fibrin deposition was observed in the aorta or lung tissues from any group of mice. (data not shown). To compare the degree of fibrin deposition quantitatively in the kidney, we compared the %GFD among three groups of mice. As shown in Fig. 8, the %GFD was significantly higher in both hypo- and normocalcemic VDRKO mice than that in WT mice. No fibrin deposition in the liver and kidney was observed when a vehicle was injected in both types of VDRKO mice (data not shown).
Discussion

The present study demonstrates that ADP-induced platelet aggregation was significantly enhanced in normocalcemic VDRKO mice. Platelets are fragments of megakaryocytes that contribute to thrombus formation (17). Both normal hemostasis and abnormal thrombosis depend on various regulatory factors within platelets (18). Physiological plasma Ca concentration is among the most important factors for normal platelet aggregation. Coordination of calcium flux through platelet-platelet contact serves to propagate calcium signaling throughout the developing thrombus to maintain thrombus growth (19). Thus, it is plausible to assume that the impaired platelet aggregation in hypocalcemic VDRKO mice was due to the hypocalcemia in these mice. In contrast, molecular mechanisms underlying the accelerated platelet aggregation in normocalcemic VDRKO mice are unknown, and further investigations are required to understand VDR function in megakaryocytes and platelets.

There have been in vitro studies that demonstrate that a hormonally active form of vitamin D up-regulates TM gene expression and down-regulates TF gene expression in monocytic cells (10,12). However, in vivo effects of the vitamin D/VDR system on these factors have never been tested. In the present study, the gene expression of AT in the liver as well as that of TM in the aorta, liver and kidney in VDRKO mice was down-regulated, whereas TF mRNA expression in the liver and kidney was up-regulated in VDRKO mice regardless of plasma calcium levels. Thus, the vitamin D/VDR system is shown to enhance the expression of anti-thrombotic factors, while inhibiting the expression of a thrombogenic factor, TF.
AT is a plasma glycoprotein synthesized by hepatocytes, and is one of the crucial inhibitors of blood coagulation through thrombin inactivation. Recently, AT deficient mice were generated by gene targeting, and homozygous AT null mice were shown to be prenatally lethal due to extensive thrombosis in the myocardium and liver sinusoids along with generally massive bleeding (20). In addition, heterozygous AT deficient mice showed a tendency toward thrombus formation in the kidney after LPS injection (21). The present observations suggest that the vitamin D/VDR system may affect AT activity through transcriptional control of AT gene expression. Although Niessen et al. reported ligand-dependent enhancement of human AT gene expression by retinoid X receptor [] and thyroid hormone receptor [] (22), their study revealed no effect of 1,25-dihydroxyvitamin D3 on AT production. The reason why they were unable to observe the effect of the vitamin D/VDR system on AT gene transcription may be the use of a shortened AT gene promoter in their study (22).

Because all-trans retinoic acid up-regulates TM expression in both monocytic and vascular endothelial cells via the retinoic acid responsive element (RARE)-mediated transcriptional activation of the TM gene (10), and because RARE on the TM gene promoter is very similar to the vitamin D-responsive element (VDRE), there is a possibility that the effect of the vitamin D/VDR system on the up-regulation of TM gene expression is mediated via the binding of liganded VDR to the RARE of the TM gene (10,12). In contrast, there is no evidence for transcriptional regulation of TF gene expression by the vitamin D/VDR system through VDRE or RARE. The TF gene promoter contains two activator protein (AP)-1-binding sites and a nuclear factor (NF)-
kB site, and functional interactions between these two factors are required for maximal induction of TF gene transcription by TNF-α in vascular endothelial cells and by LPS in monocyctic cells (23). Because interleukin-12 production from activated monocyctic THP-1 cells is suppressed by 1,25-dihydroxyvitamin D3 through the inhibition of NF-kB activation (24), the vitamin D/VDR system may suppress TF gene expression via modulation of NF-kB activation.

The present study also demonstrates that VDRKO mice manifest an exacerbated multi-organ thrombus after exogenous LPS injection regardless of the calcemic conditions. In agreement with the present results, Asakura et al. demonstrated a beneficial effect of the active form of vitamin D3 against thrombosis, using a LPS-induced disseminated intravascular coagulation rat model (25). Enhanced platelet aggregation, down-regulated expression of AT and TM along with up-regulation of TF expression in both hypo- and normocalcemic VDRKO mice, can all contribute to the enhanced thrombogenicity of VDRKO mice. In addition, deficiency of NO is associated with arterial thrombosis and thrombus formation in the renal vasculature of animal models and patients with endothelial dysfunction (26,27). However, because reduced eNOS levels and urinary NOx excretion in hypocalcemic VDRKO mice were reversed in normocalcemic VDRKO mice, the exacerbated thrombus formation due to LPS injection in normocalcemic VDRKO mice cannot be explained by a change in NO production. Although further investigation is needed to clarify the anti-thrombogenic effects of vitamin D3, the present results are consistent with the notion that the vitamin D/VDR system plays an important role in maintaining normal anti-thrombotic
homeostasis in vivo.
References


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Acknowledgements

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Footnotes

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The abbreviations used are: VDR, vitamin D receptor; WT, wild type; KO, knockout; Ca, calcium; PTH, parathyroid hormone; SFP, screen filtration pressure; PATI, platelet aggregatory threshold index; NO, nitric oxide; AT, antithrombin; TF, tissue factor; TM, thrombomodulin; LPS, lipopolysaccharide; GFD, glomeruli with fibrin deposition
Figure Legends

Figure 1.
Plasma levels of calcium (A) and PTH (B) in WT mice and VDRKO mice at 12 weeks of age. The number of mice examined in (A) and (B) were: WT (1.2% Ca) = 18, KO (1.2% Ca) = 14, and KO (2.0% Ca) = 14.
Values are expressed as mean ± SEM. **P < 0.01

Figure 2.
(A) Representative results of platelet aggregation analyzed by SFP method in WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age. Four or five different concentrations of agonist (ADP) were plotted along the horizontal axis and their individual pressure rates (%) were plotted along the vertical axis.
(B) The mean concentrations of agonist inducing a 50% pressure rate were calculated as PATI. The number of mice examined were: WT (1.2% Ca) = 14, KO (1.2% Ca) = 10, and KO (2.0% Ca) = 14.
Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01

Figure 3.
(A) Daily urinary excretion levels of NOx in WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age. The number of mice examined were: WT (1.2% Ca) = 18, KO (1.2% Ca) = 14, KO (2.0% Ca) = 14.
Values are expressed as mean ± SEM. *P < 0.05

(B) Western blot analysis of eNOS protein levels in the aorta. Protein levels of eNOS in aortic tissues of WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age were determined by Western blot analysis and quantified densitometrically. Each lane contains 50 μg protein. Six aortic tissues were examined in each group.

Figure 4.

(A) Plasma AT activity in WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age. The number of mice examined were: WT (1.2% Ca) = 18, KO (1.2% Ca) = 18 and KO (2.0% Ca) = 16. Values are expressed as mean ± SEM. *P < 0.05

(B) Northern blot analysis of hepatic AT gene expression in WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age. Six individual hepatic tissue samples were examined in each group. Values are expressed as mean ± SEM.

Figure 5.

Quantitative analysis of TF mRNA levels in the aorta, liver and kidney were performed by RT-PCR as described in the Methods section. Total RNA was isolated from each organ of WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age. Six individual tissue samples were examined in each group. Values are expressed as mean ± SEM.
Figure 6.

TM mRNA levels in the aorta, liver and kidney were analyzed by RT-PCR as described in the Methods section. Total RNA was isolated from each organ of WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age. Six individual aorta, liver and kidney tissue samples were examined in each group. Values are expressed as mean ± SEM.

Figure 7.

Immunohistochemical stainings with anti-fibrin-fibrinogen antibody of the liver (upper panels) and kidney (lower panels) after LPS injection, in WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age. Larger numbers of fibrin depositions were detected in both VDRKO mice compared to WT mice. Magnification, ×200.

Figure 8.

Percentage of glomeruli with fibrin deposition after LPS injection, in WT mice (1.2% Ca) and VDRKO mice (1.2% Ca, 2.0% Ca) at 12 weeks of age. LPS was intraperitoneally injected into mice as described in the Methods section. After 6 hours, renal specimens were subjected to immunohistochemical analysis. The percentage of glomeruli with fibrin deposition was significantly higher in VDRKO mice than WT mice. Six individual renal tissue samples were examined in each group. Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01
Figure 1

A

Plasma Calcium (mg/dl)

**  **

WT  KO  KO

Dietary Ca  1.2%  2.0%

B

Plasma PTH (pg/ml)

**  **

WT  KO  KO

Dietary Ca  1.2%  2.0%
Figure 2

A

Pressure Rate (%)

0 1 2 4 8

ADP (μM)

WT (1.2% Ca diet)

KO (1.2% Ca diet)

KO (2.0% Ca diet)

B

ADP-PATI (μM)

0 2 4 6 8 10 12

WT KO KO

Dietary Ca 1.2% 2.0%

* * *
Figure 3

A

Dietary Ca

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Daily Urinary NOx

(nmol/10g body weight)

B

Aorta

eNOS 140kDa

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eNOS Protein Relative Intensity
Figure 4

A

Plasma AT Activity (%)

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B

Liver

AT

28S

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Figure 5

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Aorta

Liver

Kidney

Dietary Ca

1.2%  2.0%

WT  KO  KO

Tissue Factor mRNA Relative Intensity

3.0

2.0

1.0

0

3.0

2.0

1.0

0

3.0

2.0

1.0

0
Figure 6

Aorta

Liver

Kidney

Dietary Ca 1.2% 2.0%

Thrombomodulin mRNA Relative Intensity

WT KO KO

WT KO KO

WT KO KO

WT KO KO
Figure 7

Liver

Kidney

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Figure 8

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[Bar graph showing fibrin deposition in glomeruli with different dietary calcium levels (1.2% and 2.0%) for WT and KO genotypes.]
Disruption of nuclear vitamin D receptor gene causes enhanced thrombogenicity in mice


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