Vitamin D Deficiency Accelerates Coronary Artery Disease Progression in Swine

Songcang Chen, Vicki J. Swier, Chandra S. Boosani, Mohamed M. Radwan, Devendra K. Agrawal

Objective—The role of vitamin D deficiency in coronary artery disease (CAD) progression is uncertain. Chronic inflammation in epicardial adipose tissue (EAT) has been implicated in the pathogenesis of CAD. However, the molecular mechanism underlying vitamin D deficiency–enhanced inflammation in the EAT of diseased coronary arteries remains unknown. We examined a mechanistic link between 1,25-dihydroxyvitamin D–mediated suppression of nuclear factor-κB (NF-κB) transporter, karyopherin α4 (KPNA4) expression and NF-κB activation in preadipocytes. Furthermore, we determined whether vitamin D deficiency accelerates CAD progression by increasing KPNA4 and nuclear NF-κB levels in EAT.

Approach and Results—Nuclear protein levels were detected by immunofluorescence and Western blot. Exogenous KPNA4 was transported into cells by a transfection approach and constituted lentiviral vector. Swine were administered vitamin D–deficient or vitamin D–sufficient hypercholesterolemic diet. After 1 year, the histopathology of coronary arteries and nuclear protein expression of EAT were assessed. 1,25-dihydroxyvitamin D inhibited NF-κB activation and reduced KPNA4 levels through increased vitamin D receptor expression. Exogenous KPNA4 rescued 1,25-dihydroxyvitamin D–dependent suppression of NF-κB nuclear translocation and activation. Vitamin D deficiency caused extensive CAD progression and advanced atherosclerotic plaques, which are linked to increased KPNA4 and nuclear NF-κB levels in the EAT.

Conclusions—1,25-dihydroxyvitamin D attenuates NF-κB activation by targeting KPNA4. Vitamin D deficiency accelerates CAD progression at least, in part, through enhanced chronic inflammation of EAT by upregulation of KPNA4, which enhances NF-κB activation. These novel findings provide mechanistic evidence that vitamin D supplementation could be beneficial for the prevention and treatment of CAD. (Arterioscler Thromb Vasc Biol. 2016;36:1651-1659. DOI: 10.1161/ATVBAHA.116.307586.)

Key Words: 1,25-dihydroxyvitamin D ■ atherosclerosis ■ coronary artery disease ■ KPNA4 ■ vitamin D

Coronary artery disease (CAD) and its complication, myocardial infarction arising from atherosclerosis, are the most common life-threatening cardiovascular disorders. Vascular endothelial dysfunction, increased inflammatory cytokine release, immune cell infiltrates, and subsequent stimulation of vascular smooth muscle cell proliferation are the pathophysiological basis of atherosclerosis. The inflammatory signals are classically considered to originate from blood-borne immune cells. However, accumulated data from the past decade indicate that pathogenic inflammatory adipokines from epicardial adipose tissue (EAT) contribute to the development and progression of CAD. The majority of clinical data have demonstrated that EAT volume is a strong predictor of coronary atherosclerosis and directly associated with the severity of atherosclerotic lesions. EAT is located between the outer wall of the myocardium and the visceral layer of pericardium. Almost all coronary atherosclerotic lesions occur in the segments of coronary arteries that are encased in EAT. In contrast, the arterial segments associated with the myocardial bridge, which is absent of EAT, have limited or no atherosclerotic plaques. Furthermore, the excision of dysfunctional EAT slows the progression of porcine coronary atherosclerosis and transplantation of inflammatory perivascular adipose tissue accelerates the progression of atherosclerosis. These studies provide sufficient evidence that the inflammation-induced dysregulation of EAT plays an important role in promoting the progression of CAD. Compared with other adipose deposits, EAT displays a unique phenotype and is able to produce local inflammatory cytokines to influence the progression of CAD. These cytokines predominate consist of nuclear factor kappa B (NF-κB) transcription factor and the products of its target genes, including monocyte chemoattractant protein-1, interleukin (IL)-6, IL-8, IL-1β, and tumor necrosis factor (TNF)-α. Therefore, it is important to distinguish EAT from the pericardial fat, which consists of EAT and paracardial fat. The latter is located between the parietal and visceral pericardium.

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Activation of NF-κB has been implicated in physiological immunity and pathological inflammation in many different tissues and cells.\(^\text{16-18}\) The NF-κB family consists of 5 members: RelA (p65), NF-κB1 (p105/p50), NF-κB2 (p100), RelB, and c-Rel, which share \(\approx\)300 amino acid–long N-terminal Rel homology domain. In the resting state, the inhibitor of κB protein (IκB), IκBα, binds to NF-κB dimer through a robust nuclear export sequence. This binding masks the nuclear localization signals of p65 to keep the dimer in the cytoplasm. On activation either by canonical or noncanonical pathways, IκBα is phosphorylated, which leads to rapid, signal-induced polyubiquitination and degradation by the proteasome. The liberating NF-κB complex with unmasked nuclear localization signals binds to karyopherin alpha 4 (KPNA4; also known importin α3), a nuclear membrane protein that functions as a shuttle of protein nuclear translocation.19 KPNA4–importin α complex recognizes and binds to nuclear localization signals in the NF-κB dimer, p65/p50. The reaction docks KPNA4–importin β–NF-κB complex to the cytoplasmic side of the nuclear pore complex, leading to the translocation of NF-κB dimer into the nucleus. It has been shown that the activation of the NF-κB complex is facilitated predominantly by KPNA4-mediated nuclear translocation.20 The nuclear NF-κB dimer acts as a transcription factor that binds to both promoter and enhancer elements in its target genes to regulate both transcriptional activities and chromatin remodeling.

Vitamin D exerts anti-inflammatory activity and has a cardiovascular protective function,\(^\text{21-23}\) in addition to its regulation of calcium and phosphorus metabolism. Vitamin D is synthesized in the skin by ultraviolet light exposure or from diet and hydroxylated in the liver to generate 25-hydroxyvitamin D, the plasma vitamin D marker. The latter is hydroxylated by 1α-hydroxylase in the proximal renal tubule or local tissues to generate 1,25-dihydroxyvitamin D (VD3), a biologically active form of vitamin D. VD3 as a nuclear hormone regulates gene transcription and exerts its biological effects through binding to the vitamin D receptor (VDR). Recent human studies have shown that vitamin D deficiency is an independent risk factor associated with CAD.\(^\text{24-26}\) However, whether vitamin D deficiency directly causes the progression of CAD or vice versa remains unclear. It is possible that patients with progressive CAD reduce outdoor activity or vitamin D intake caused by low appetite, leading to vitamin D deficiency. It has been shown that vitamin D signaling inhibits NF-κB activation in many kinds of cells including adipocytes.\(^\text{27-31}\) However, the exact molecular mechanism remains to be elucidated. Vitamin D deficiency increases the expression of the NF-κB target gene, monocyte chemoattractant protein-1, TNF-α, and IL-6 in swine EAT,\(^\text{32}\) and CAD patients with vitamin D deficiency have elevated inflammatory cytokines in the EAT.\(^\text{33}\) These results indicate that vitamin D signaling negatively regulates NF-κB activation in EAT. However, the molecular mechanism underlying vitamin D deficiency–enhanced NF-κB activation in EAT is largely unknown. Here, we examined VD3-mediated suppression of KPNA4 expression and reduction of NF-κB activation, and a mechanistic link between the 2 proteins in swine epicardial preadipocytes. Furthermore, we determined whether vitamin D deficiency accelerates CAD progression by increasing nuclear NF-κB and KPNA4 levels in the EAT of swine fed an atherogenic diet.

### Materials and Methods

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

### Results

**VD3 Inhibits NF-κB Activation**

To determine the molecular mechanism by which VD3 inhibits NF-κB activation, we established a primary culture of preadipocytes from adult swine EAT. After the treatment with 10\(^{-8}\) mol/L VD3 (the same concentration used in all experiments in this study) for 24 hours and TNF-α (20 ng/mL), an optimum dose was used in this study; Figure IA in the online-only Data Supplement) for 25 minutes, the cells were fixed for immunofluorescent assay. As shown in Figure 1A, VD3 alone had no effect on the intracellular location of p65, a major NF-κB unit, compared with control. TNF-α stimulated p65 nuclear translocation from cytoplasm, showing that almost all p65 proteins were located in the nuclei. Treatment with VD3 prevented TNF-α stimulated p65 translocation from cytoplasm to nuclei. To confirm and quantify VD3-dependent suppression of NF-κB activation, the preadipocytes were treated as described above and the nuclear extract was prepared for Western blot analysis. As shown in Figure 1B, treatment with TNF-α resulted in a 4- to 7-fold increase in the levels of nuclear p65. Preincubation of VD3 had no effect on basal nuclear p65 levels, but significantly reduced TNF-α induced nuclear p65 by 65%. The nuclear translocation of p65 is essential and the critical step for NF-κB activation. These results provide convincing evidence that TNF-α stimulates NF-κB activation through cytoplasmic to nuclear translocation in the preadipocytes, which is blocked by VD3. In addition to its suppressive effect on TNF-α–stimulated activation of NF-κB, VD3 also inhibited another proinflammatory stimulus, IL-1β–induced NF-κB nuclear translocation (Figure IB in the online-only Data Supplement).

To determine whether IκB plays a potential role in VD3-mediated inhibition of NF-κB activation, we treated the preadipocytes with VD3 or TNF-α for different time periods. As shown in Figure 1C, treatment with TNF-α led to 4-fold increase in IκBα phosphorylation. However, VD3 had no significant effect on TNF-α–stimulated IκBα phosphorylation, suggesting that the exact molecular mechanisms remain unclear, and another potential target may be responsible for the VD3 effect.
VD3 Suppresses KPNA4 Expression Through Increased VDR Expression

To explore the molecular mechanism on the suppressive role of VD3 in NF-κB activation, we focused on the nuclear membrane transporter KPNA4, a major shuttle responsible for the transportation of activated NF-κB from cytoplasm to nucleus. As demonstrated in Figure 2A, VD3 inhibited KPNA4 mRNA expression starting at 3-hour treatment through 24 hours of VD3 exposure. The maximum inhibitory effect of VD3 was ≈70% after 6-hour incubation. The reduction of mRNA levels was accompanied with decreased KPNA4 protein expression. Treating cells with VD3 for 12 and 24 hours led to a 50% and 75% reduction of KPNA4 translation, respectively (Figure 2B). These results suggest that VD3 inhibits NF-κB activation possibly through the suppression of KPNA4 transcription and translation.

To examine whether VD3 amplifies VDR expression, we incubated the preadipocytes with VD3 at different time periods. As shown in Figure 2C, exposure to VD3 for 5 hours resulted in a 4-fold increase in VDR expression. VDR levels decreased with time, but maintained significantly high level compared with control throughout 24-hour treatment. To determine whether the increased VDR levels contribute to the effect of VD3 on the suppression of KPNA4 expression, we knocked down VDR expression by the specific siRNA. As expected in Figure 2D, VDR siRNA effectively blocked VDR expression and significantly reduced VD3-induced VDR levels compared with those in control siRNA. Knockdown of VDR disrupted the suppressive role of VD3 in KPNA4 expression (Figure 2E), suggesting that VD3-mediated inhibition of KPNA4 expression is VDR dependent. VD3 can be synthesized and degraded locally in many different cells by 1-α-hydroxylase and 24-hydroxylase, respectively. To determine whether these 2 enzymes express in the preadipocytes, which are regulated by VD3, the cellular extracts prepared as above were detected with antibodies against these 2 enzymes. The expression of both enzymes was significantly lower than that of VDR. VD3 had no significant effect on 1-α-hydroxylase and 24-hydroxylase expression (Figure IIA and IIB in the online-only Data Supplement). To explore whether prohibitin plays a potential role in the VD3-mediated anti-inflammatory process, we treated preadipocytes with VD3 or TNF-α for different time periods. Neither VD3 nor TNF-α had a significant effect on prohibitin mRNA or protein levels (Figure IIC and IID in the online-only Data Supplement). These results ruled out the participation of prohibitin in the anti-inflammatory role of VD3 in the preadipocytes. To detect whether TNF-α exerts any effect on KPNA4 expression, we incubated the cells with TNF-α in presence or absence of VD3. TNF-α had no significant effect on VDR expression (Figure IIIB in the online-only Data Supplement). As shown in Figure 2F, VD3 alone significantly decreased KPNA4 expression, and the effect was not markedly changed by the combined treatment of VD3 and TNF-α. These results indicate that TNF-α-dependent activation of NF-κB is not implicated in KPNA4 expression, but VD3 prevents the translocation of activated NF-κB from cytoplasm to nuclei, which is likely because of suppression of KPNA4 expression.
VD3 Reduces NF-κB Activation Through Targeting KPNA4

To determine a mechanistic link between vitamin D–dependent reduction of KPNA4 and suppression of NF-κB activation, we used 2 independent gain-of-function approaches to elucidate this question. First, we made KPNA4-lentivirus-GFP expression vector to generate the viruses, and then used the viruses to infect the preadipocytes. KPNA4-positive cells were selected by puromycin, and exogenous KPNA4 expression was confirmed by the detection of GFP expression (Figure IV in the online-only Data Supplement). We then cultured KNPA4-positive preadipocytes and pretreated them with VD3 for 24 hours and TNF-α for 25 minutes. Cells were then fixed for immunofluorescent assay. As shown in Figure 3, VD3 alone had no effect on the cellular distribution of p65, and TNF-α promoted p65 nuclear accumulation in KPNA4-positive cells, which were similar in wild-type cells. However, in KPNA4-positive cells, VD3 failed to block TNF-α–induced p65 nuclear translocation, suggesting that rescuing vitamin D–mediated suppression of KPNA4 levels by increased KPNA4 expression restores the ability of TNF-α–mediated NF-κB nuclear translocation in the presence of VD3. Second, to examine whether increased KPNA4 expression participates in the effect of VD3 on NF-κB function, we transfected CMV6 (cytomegalovirus 6) promoter-driven KPNA4 expression vector with NF-κB-luciferase/renilla-luciferase into preadipocytes, and then treated cells with VD3 for 24 hours and TNF-α for the final 8 hours. As shown in Figure 4, treatment with TNF-α led to a 3-fold increase in NF-κB luciferase activity normalized to renilla-luciferase activity (P<0.01). This was significantly reduced by ≈50% with VD3 treatment (P<0.01), indicating that VD3 is able to block TNF-α–mediated NF-κB activation. Cotransfection with 0.1 μg of KPNA4 expression vector modestly decreased VD3-dependent inhibition of NF-κB activation by ≈25% (P<0.05). Increased KPNA4 expression by cotransfection with 0.3 μg of the vector led to complete recovery of vitamin D–mediated inhibition, suggesting that KPNA4 dose dependently rescues VD3-mediated suppression of NF-κB activity. Of note, transfection of 0.3 μg of KPNA4 significantly increased TNF-α–induced NF-κB luciferase activity compared with that of control vector (P<0.05), suggesting that increased KPNA4 levels would promote available NF-κB from cytoplasm to nuclei. Taken together, these results provide a mechanistic link between VD3-mediated suppression of KPNA4 expression and NF-κB activation and suggest that KPNA4 is a major target responsible for vitamin D–mediated suppression of NF-κB activation in the preadipocytes.

Vitamin D Deficiency Exacerbates the Progression of Swine CAD Linked to Increased KPNA4 and Nuclear p65 Levels in the EAT

To determine a causal relationship between vitamin D deficiency and CAD progression, we fed swine with vitamin D–deficient or vitamin D–sufficient hypercholesterolemic...
diets for 1 year. In one group of animals, vitamin D–deficient hypercholesterolemic diet (Diet-1) contained ≈500 IU/d of vitamin D, and the sufficient control diet (C-1) contained 1500 IU/d of vitamin D. In another group, vitamin D–deficient hypercholesterolemic diet (Diet-2) contained undetectable vitamin D levels, and the control diet (C-2) contained 1300 IU/d of vitamin D. Just before euthanization, the plasma 25-hydroxyvitamin D levels were 17±1.8 ng/mL in the vitamin D–deficient Diet-1 group versus 28±4.0 ng/mL in the vitamin D–sufficient Diet-1 group versus 7.2±0.7 ng/mL in the Diet-2 group versus 23±5.3 ng/mL in the C-2 group. Their age, body weight, and plasma cholesterol levels at the time of euthanization were similar between the vitamin D–deficient and control groups (Table I in the online-only Data Supplement). As shown in Figure 5, administration of the vitamin D–sufficient hypercholesterolemic diets for 1 year led to the development of atherosclerotic plaques, which were similar between C-1 and C-2 groups (≈15% versus ≈12% stenosis). The novel results here are that vitamin D deficiency accelerated CAD progression on either Diet-1 or Diet-2 versus the control diet (≈35% versus ≈15% stenosis on Diet-1, \( P<0.01 \); ≈50% versus ≈12% stenosis on Diet-2, \( P<0.01 \)), and that as shown in Figure 5E, the severity of vitamin D deficiency seems to be causally related to the extent of CAD progression.
(≈35% stenosis on Diet-1 in Figure 5C with 25-hydroxyvitamin D levels of ≈17 ng/mL versus ≈50% stenosis on Diet-2 in Figure 5D with 25-hydroxyvitamin D levels at ≈7 ng/mL). Furthermore, the atherosclerotic plaques were more advanced in swine fed Diet-2 (Figure 5C) compared with the plaques in swine fed Diet-1 (Figure 5A).

To examine whether vitamin D deficiency–dependent exacerbation of CAD progression is linked to an increase in KPNA4 and nuclear NF-κB levels in EAT, the EAT of the right coronary arteries as shown in Figure V in the online-only Data Supplement from the swine on Diet-2 or C-2 diet was isolated and embedded for quantitative immunofluorescent assay. The administration of vitamin D–deficient Diet-2 led to a >2-fold increase in KPNA4 expression (P<0.01) and nuclear NF-κBp65 levels (P<0.01) in the EAT compared with the levels in swine fed Diet-1 (Figure 5A).

To examine whether vitamin D deficiency–dependent exacerbation of CAD progression is linked to an increase in KPNA4 and nuclear NF-κB levels in EAT, the EAT of the right coronary arteries as shown in Figure V in the online-only Data Supplement from the swine on Diet-2 or C-2 diet was isolated and embedded for quantitative immunofluorescent assay. The administration of vitamin D–deficient Diet-2 led to a >2-fold increase in KPNA4 expression (P<0.01) and nuclear NF-κBp65 levels (P<0.01) in the EAT compared with the levels in the EAT of vitamin D–sufficient swine (Figure 6). These results suggest that vitamin D deficiency–induced increase in KPNA4 expression promotes the transport of more activated NF-κB complexes into the nuclei, leading to NF-κB activation and an inflammatory reaction in EAT, contributing to the progression of CAD.

**Discussion**

The major findings in this study are as follows: (1) VD3 inhibits NF-κB activation and reduces KPNA4 levels through increased VDR expression in preadipocytes from swine EAT; (2) exogenous KPNA4 rescues VD3-dependent suppression of NF-κB activation; and (3) vitamin D deficiency exacerbates the progression of swine CAD linked to increased KPNA4 and nuclear p65 levels in the EAT.

VD3 has been shown to exert anti-inflammatory activities in adipose tissue. Activation of NF-κB signaling plays a central role in an inflammatory reaction. Several studies have demonstrated that VD3 inhibits inflammation and cytokine release through suppression of NF-κB activation. However, the precise molecular mechanism remains largely unknown. In this study, we established the primary preadipocyte culture from adult swine EAT to elucidate the inhibitory effect of VD3 on TNF-α–stimulated inflammatory reaction. It has been shown that human preadipocytes produce significantly higher inflammatory adipokines than mature adipocytes. We found that TNF-α rapidly stimulated NF-κB translocation from cytoplasm to nuclei and drove the transcription of the target gene containing κB-response elements, and the effects were significantly blocked by VD3. Furthermore, VD3 also inhibited IL-1β–induced NF-κB activation. These findings provide strong evidence that VD3 is able to prevent the activation of the NF-κB signaling pathway in preadipocytes. Some, but not one, studies have shown that VD3 reduces NF-κB activation via targeting IκBα expression or phosphorylation, a key step in NF-κB activation that releases the NF-κB complex, leading to its nuclear translocation. In our culture system, we confirmed that TNF-α stimulated phosphorylation of IκBα. However, we did not see a significant effect of VD3 on the either expression or TNF-α–stimulated phosphorylation of IκBα. Although the inconsistent results might be because of different cell types or
different cultured conditions, our results rule out that IκBα is a critical target responsible for VD3-dependent suppression of NF-κB activation in the preadipocytes.

Previous studies have shown that activated NF-κB is transported into nuclei predominantly by KPNA4, 18, 20 and VD3 is able to inhibit KPNA4 expression in bronchial smooth muscle cells. 36 However, it remains to be elucidated whether VD3-mediated inhibition of KPNA4 is responsible for its reduction of NF-κB activity. We demonstrated that VD3 exerted its maximum inhibitory effect on KPNA4 transcription and translation after 6- and 24-hour incubation, respectively. VD3 induced VDR expression with a maximal effect at 5 hours, and the induction was still significant, but gradually decreased with the time of treatment. The effect is temporally orchestrated with its inhibitory role in KPNA4 transcription. Furthermore, the suppressive role of VD3 in KPNA4 expression disappeared by the knockdown of VDR, suggesting that VD3 inhibits KPNA4 expression through amplification of VDR levels. Preadipocytes also expressed low levels of 1α-hydroxylase and 24-hydroxylase. However, VD3 had no significant effect on their expression. These results indicate that VD3 gradually decreases its inhibitory effect on KPNA4 mRNA levels predominately associated with attenuated VDR induction, rather than increase in its degradation or decrease in local VD3 synthesis.

Because KPNA4 is responsible for NF-κB transportation from cytoplasm to nuclei, a critical step for NF-κB activation, VD3-mediated suppression of KPNA4 transcription leading to impaired KPNA4 protein levels may be associated with attenuated NF-κB activation in the preadipocytes. To determine a causal link between VD3-dependent inhibition of KPNA4 expression and NF-κB activation, we performed 2 independent gain-of-function experiments. Our key finding in the present study is that increases in KPNA4 levels by 2 different exogenous expression vectors provide multiple KPNA4 shuttles for activated NF-κB transportation from cytoplasm to nuclei. This process rescues VD3-dependent suppression of TNF-α–induced NF-κB nuclear translocation and transcriptional activity. The novel finding provides a mechanistic explanation that VD3 inhibits NF-κB activation through suppression of KPNA4 expression, reducing the reserved nuclear transporter number and thus leading to the retention of accumulated free NF-κB dimer in the cytoplasm.

It remains unclear whether VD3 directly or indirectly inhibits KPNA4 transcription. Prohibitin has been shown to inhibit KPNA4 expression in intestinal epithelial cells, 37 and VD3 increases prohibitin levels in bronchial smooth muscle cells. 38 These studies suggest that vitamin D may suppress KPNA4 expression through the upregulation of prohibitin expression in the preadipocytes. However, our data revealed no effect of VD3 on inhibitin mRNA and protein levels, suggesting that prohibitin does not mediate VD3-dependent suppression of KPNA4 expression in the swine preadipocytes. Whether VD3 negatively controls NF-κB activity through a direct binding to KPNA4 promoter needs further investigation.

Figure 6. Quantitative immunofluorescence (IF) showing increased nuclear p65 and KPNA4 levels in the epicardial adipose tissue (EAT) of swine fed on vitamin D–deficient hypercholesterolemic Diet-2 vs sufficient hypercholesterolemic diet (C-2). The experiments were performed using the sections from right coronary arteries and the EAT of 3 different swine from each group. Karyopherin α4 (KPNA4; A) and nuclear p65 (B) levels were determined by the overlap of the staining using the specific antibodies and DAPI. The representative immunofluorescent images are shown and the graphs display the intensity of immunofluorescence measured in relative units (RU) in ≈150 nuclei in 5 to 6 random fields from 3 animals per group. **P<0.01 vs individual control group. DAPI indicates 4′,6-diamidino-2-phenylindole.
Human observational study has shown that vitamin D deficiency is associated with the extent of CAD. However, data from the observational studies cannot answer the critical question whether vitamin D deficiency leads to CAD progression or vice versa. In addition, there are many confounders that affect the conclusion from observational studies, including participants with different ages, sexes, races, outdoor activity, vitamin D intake, obesity, histories of renal failure, stroke, hypertension, and other chronic diseases. In contrast, the anatomy and physiological function of coronary arteries in swine are similar to human, and the confounding factors mentioned above can be eliminated using the swine model. We administered 2 groups of swine with hypercholesterolemic diets containing deficient or sufficient vitamin D for 1 year. All other conditions were kept the same for each swine. Our results provide a direct causal relationship between vitamin D deficiency and CAD progression. One year later, we repeated the above experiment using a severely vitamin D-deficient diet. Combining the data from the different vitamin D diets, the findings in this study show that the severity of vitamin D deficiency seemed to be causally related to CAD progression and advanced stage atherosclerotic plaques in swine fed hypercholesterolemic diets.

Although a mechanistic link between dysfunction of EAT and CAD has not been completely established, increased EAT volume resulting in the dysregulation of inflammatory adipokine release contributes to the development and progression of CAD. Studies have shown that the administration of inflammatory mediators outside the arterial adventitia leads to immune cell invasion, increases in intimal thickness, and arterial remodeling, and increased coronary vasa vaso-neovascularization precedes endothelial dysfunction in swine fed a high-cholesterol diet. These data suggest that local adipokines from EAT could diffuse into interstitial fluid across the adventitia into the vascular wall by paracrine factors or be directly transported into downstream coronary arteries by vasoconstrictive factors. This hypothesis supports a notion of outside to inside signaling of atherosclerosis, in which the inflammatory milieu in EAT promotes the development and progression of CAD. Recently, in atherosclerotic swine, McKenney et al. clearly showed that EAT excision attenuated the progression of CAD, but their observation did not have a clear mechanistic link. Here, we provide critical data on EAT signaling mechanisms underlying CAD. Vitamin D deficiency increases nuclear translocation of freely available cytoplasmic NF-κB complexes generated by an atherogenic diet through increased KPNA4 expression, leading to enhanced NF-κB activation in EAT, which contributes to CAD progression.

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Disclosures
None.

References
Highlights

- 1,25-dihydroxyvitamin D inhibits nuclear factor-κB activation and reduces karyopherin α4 levels in preadipocytes of swine epicardial adipose tissue.
- 1,25-dihydroxyvitamin D exerts its effect through increased expression of vitamin D receptor in preadipocytes.
- Exogenous karyopherin α4 rescues 1,25-dihydroxyvitamin D-dependent suppression of nuclear translocation and action of nuclear factor-κB.
- Vitamin D deficiency exacerbates the progression of swine coronary artery disease linked to increased karyopherin α4 and nuclear p65 levels in the epicardial adipose tissue.
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Materials and Methods

Primary preadipocyte culture

The Institutional Animal Care and Use Committee of Creighton University approved the animal research protocol. The EAT samples around coronary arteries of adult Yucatan microswine were pooled, and minced with scissors into small pieces (2-3 mm³) under an aseptic condition. The minced tissue samples were washed twice using PBS containing penicillin/streptomycin, placed in digestion solution (0.25% type 1 collagenase in DMEM/F12 medium) and incubated in a shaker (250 rpm) at 37°C for 60-80 minutes. The undigested tissue was separated from collagenase-digested samples by filtration through a 100-μm mesh fabric. Matured fat cells were separated from the pellets containing preadipocytes by centrifugation at 400 g at 4°C for 10 minutes. The preadipocytes were subsequently washed two times, and then resuspended in the growth medium (DMEM/F12 medium containing 10% FBS). The primary cultured preadipocytes (70–80 % confluent cells) were arrested by replacing 10% FBS medium with 0.2% FBS DMEM/F12. After 24 hours, cells were incubated with 10 nmol/L VD3 (calcitriol) (D1530 Sigma-Aldrich, St. Louis, MO) or 25 ng/ml recombinant TNF-α or IL-1β (PeproTech, Inc. NJ) at a different time period. The control group was treated with 1/1000 medium volume of ethanol or water. Cells were used for immunofluorescent analysis, or harvested for preparation of total RNA, total protein, or nuclear extract to do real-time PCR or Western blot analysis, respectively. Some cells were used for transfection experiments.

Animal study

Swine were housed and cared for according to NIH and USDA guidelines in the Animal Resource Facility of Creighton University under IACUC-approved protocol number 0831. Adult Yucatan™ miniature swine (30-40 lbs) were purchased from Sinclair Bio-resources, Windham, MA, and fed with 1–1.5 lb/swine/day of atherogenic vitamin D-sufficient or atherogenic vitamin D-deficient diets for one year. Swine were fed with two different vitamin D-deficient high-cholesterol diets. The first group of animals was randomly divided into two subgroups. The control subgroup was fed with a mixture of Teklad swine Diets 8753 and 9487 (Envigo, Madison, Wisconsin USA) and 8% chocolate. This is an atherogenic vitamin D-sufficient control (C-1) diet that provides 1,500 IU of vitamin D3 per day. The vitamin D-deficient subgroup was fed a mixture of TD8753, 10125, 0.55% sodium cholate and 9% chocolate an atherogenic diet (Diet-1), which provides about 500 IU of vitamin D3 per day. The second group of animals was similarly divided into two subgroups. The control subgroup (C-2) was fed with mixed TD150250 (Envigo, Madison, Wisconsin USA), an atherogenic diet that provides 1,300 IU of vitamin D3 per day. The vitamin D-deficient subgroup was fed with TD150038 (Diet-2), which is completely devoid of vitamin D. Serum calcium and parathyroid hormone (PTH) levels were measured every 4-8 weeks in the vitamin D-deficient animals. The PTH levels started to rise above the normal level after 5 months and the total serum calcium levels started to decrease below the normal level after 6 months of feeding with the vitamin D-deficient diet. Once the PTH rose above the normal level (>65 pg/ml), the pigs were supplemented with oral calcium in the form of calcium citrate tablets (Puritan’s Pride, USA) 0.5 to 1 g per day according to their PTH and calcium levels, to maintain normal or near-normal PTH and calcium levels. After one year of the diets, the animals were euthanized and venous blood was collected from each animal for 25(OH) D measurement and complete metabolic profile.
VDR protein knockdown, whole cell and nuclear protein preparation and Western blot

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Lentiviral particles expressing KPNA4 were developed using the Lenti-X expression system from Clontech. Lenti-X 293T cells were cultured in DMEM medium containing 10% tetracycline-reduced serum in the presence of penicillin and streptomycin antibiotics. To generate lentivirus particles, about 7 μg of the plasmid DNA pLVX-EF1α-KPNA4-AcGFP1 was diluted with 557 μl of Xfect Reaction buffer along with 36 μl of Lenti-X HTX Packaging plasmids. The DNA mix was then vortexed briefly and allowed to incubate at room temperature for 2 minutes. In a separate tube, 7.5 μl of Xfect Polymer was diluted in 592.5 μl of Xfect Reaction Buffer, briefly vortexed and added to the tube containing the pLVX-EF1α-KPNA4-AcGFP1 and Lenti-X Packaging plasmids. The mixture was then incubated at room temperature for 10 minutes and directly added to Lenti-X 293T cells at a density of 70% confluence with 10 ml of culture medium. The cells were allowed to grow overnight and the supernatant was discarded in 10% bleach solution. After adding fresh culture medium, the cells were allowed to grow for additional 24 hours and the supernatant containing lentiviral particles was collected and stored at -80 °C. About 20 μl of the supernatant was used to quantify the titer of the virus using Lenti-X GoStick, which shows two distinct bands on the GoStick if the titer is above 5 x 10^5 IFU/ml.

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Based on the intensity of the band that appears on the GoStick, about 100 to 500 μl of the viral supernatant was used to transduce cells grown in a 10 cm plate. Virus supernatant was mixed with Lenti-X accelerator (10:1) for 30 minutes, and the mixture was added directly to the cell culture medium to transduce overnight. The next day, medium supernatant was discarded in 10% bleach solution and fresh medium was added to the transduced cells. The cells were allowed to grow for one to two more days and the lentiviral transduction efficiency was evaluated by observing GFP expression. After visualizing the GFP, the cells were digested and subjected to puromycin selection (2 μg/ml) for 4 days prior to the experiments.

Histology

Whole heart was removed after the swine was euthanized. Right coronary arteries from the aorta to the ventricular branch with EAT were excised (some samples from left anterior descending arteries and left circumflex arteries). The arteries were fixed in 10% buffered formalin for 24 hours. The arteries were sectioned into 4 to 5 mm portions and placed into tissue cassettes numbered 1 to 5 (1 being the larger portion of the right coronary, the origin at the aorta, and 5 being the smaller distal region at the branch with the acute marginal artery). The sections in the tissue cassettes were processed with ethanol and xylene and embedded into paraffin blocks. The paraffin blocks (1 through 5) were sectioned and stained with H&E. The percent stenosis was determined for each section (1 through 5) and the mean of these values was obtained to reflect the mean percent stenosis of the right coronary artery for each swine. The luminal and internal elastic lamina area was measured by NIH ImageJ software.
Briefly, percent area stenosis was calculated as \[1-(\text{luminal area/\text{internal elastic lamina area}})\times100\]. Some slides were used for immunofluorescent assay.

**Immunofluorescent assay**

After fixing the cultured cells with 4\% paraformaldehyde or after deparaffinization and rehydration of the slides containing swine EAT, antigen retrieval was performed prior to immunostaining. Fixed cells on the slides were incubated for 2 hours in block/permeabilizing solutions containing PBS, 0.25 \% Triton X-100, and 5\% (v/v) goat serum at room temperature. The slides were subsequently incubated with primary antibodies including rabbit anti-p65, rabbit anti-KPNA4, at 4°C overnight. After washing with PBS four times for 5 minutes each, a secondary antibody (affinity purified goat anti-rabbit cyanine 3 (cy3) antibody, 1:300) (Jackson ImmunoResearch, Westgrove, PA) was applied to the sections for 2 hours in dark. Negative controls were run in parallel with normal host IgG including ChromPure rabbit IgG or complete omission of primary antibody. Sections were washed with PBS for 5 minutes four times. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). A single layer of nail polish was placed around the edge of slide to prevent escape of mounting media from the coverslip. Pictures were taken within 1 to 24 hours of mounting using an Olympus DP71 camera. All parameters during image acquisition were the same between the groups. To measure the fluorescent intensities of KPNA4 and nuclear NF-κB in EAT, a region was drawn around each nuclei which were confirmed by DAPI co-staining for the assessment, and the identical region near it without a fluorescent object was drawn and measured for the background subtraction. The nuclear net-integrated intensities = integrated density – (area of selected nucleus \times mean fluorescence of background readings), were calculated using NIH Image J software. The nuclear net-integrated intensities from 150 cells of EAT each group were quantified (n= 3 per group).

**Statistical Analysis**

The data were analyzed using the GraphPad Prism 5.0 (GraphPad Software, Inc). The values are presented as mean ± SD. ANOVA with Bonferroni’s multiple comparison test or two-tailed unpaired student t-test were used to analyze statistically significant differences between the groups.

**Supplementary Figures and Table:**

**Suppl. Figure I. Pro-inflammatory cytokines stimulated activation of NF-κB p65. (A)**
Effect of different doses of TNF-α on p65 nuclear translocation: the optimum dose of TNF-α was 20 ng/ml in mediating p65 nuclear translocation. The experiments were repeated twice. The representative images and pooled data in the graph are shown. **(B)** VD3 prevented IL-1β-induced p65 nuclear translocation. The cells were fixed for immunofluorescent assay after being treated with 10⁻⁸ mol/L VD3 for 24 hours and 20 ng/ml IL-1β for 25 minutes. The experiments were repeated three times and the representative immunofluorescent images are shown.
Suppl. Figure II. Effect of VD3 on vitamin D signaling molecules or prohibitin. (A and B) VD3 had no effect on the expression of 1-α-hydroxylase and 24-hydroxylase in the preadipocytes. The experiments were repeated twice. (C and D) VD3 had no significant effect on prohibitin mRNA and protein levels in the preadipocytes. The experiments were repeated three times using different samples.

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A

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nuclear P65/Lamin B (Fold Induction)

0 20 30 50

B

C

VD3

IL-1β

VD3+IL-1β

Suppl. Figure I

DAPI Merge
**Suppl. Figure II**

**A**

- **1-α-hydroxylase/GAPDH**
  - (Fold Induction)
  - (Hours)
  - VD3 (10⁻⁸ mol/L)
  - 0 5 10 24

**B**

- **24-hydroxylase/GAPDH**
  - (Fold Induction)
  - (Hours)
  - VD3 (10⁻⁸ mol/L)
  - 0 5 10 24

**C**

- **Prohibitin/GAPDH mRNA**
  - (% Control)
  - (Hours)
  - VD3 (10⁻⁸ mol/L)
  - 0 3 6 12 24

**D**

- **Prohibitin/GAPDH**
  - (% Control)
  - (Hours)
  - VD3 (10⁻⁸ mol/L)
  - 0 6 12 24
**A**

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<th>(Hours)</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>24</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
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Graph showing KPNA4 and GAPDH expression over time with bars indicating fold induction.

**B**

<table>
<thead>
<tr>
<th></th>
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<td>GAPDH</td>
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Graph showing VDR and GAPDH expression with bars indicating fold induction.
Suppl. Figure IV
Materials and Methods

Primary preadipocyte culture

The Institutional Animal Care and Use Committee of Creighton University approved the animal research protocol. The EAT samples around coronary arteries of adult Yucatan microswine were pooled, and minced with scissors into small pieces (2-3 mm3) under an aseptic condition. The minced tissue samples were washed twice using PBS containing penicillin/streptomycin, placed in digestion solution (0.25% type 1 collagenase in DMEM/F12 medium) and incubated in a shaker (250 rpm) at 37°C for 60-80 minutes. The undigested tissue was separated from collagenase-digested samples by filtration through a 100-μm mesh fabric. Matured fat cells were separated from the pellets containing preadipocytes by centrifugation at 400 g at 4°C for 10 minutes. The preadipocytes were subsequently washed two times, and then resuspended in the growth medium (DMEM/F12 medium containing 10% FBS). The primary cultured preadipocytes (70–80 % confluent cells) were arrested by replacing 10% FBS medium with 0.2% FBS DMEM/F12. After 24 hours, cells were incubated with 10 nmol/L VD3 (calcitriol) (D1530 Sigma-Aldrich, St. Louis, MO) or 25 ng/ml recombinant TNF-α or IL-1β (PeproTech, Inc. NJ) at a different time period. The control group was treated with 1/1000 medium volume of ethanol or water. Cells were used for immunofluorescent analysis, or harvested for preparation of total RNA, total protein, or nuclear extract to do real-time PCR or Western blot analysis, respectively. Some cells were used for transfection experiments.

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High cholesterol diet or pro-atherogenic cytokines (e.g. TNF-\(\alpha\))

\(\text{NF-\(\kappa\)B}
\]

Vitamin D Deficiency

VD3/VDR

An adipocyte in EAT

I\(\kappa\)B\(\alpha\) → NF-\(\kappa\)B → Target gene (e.g. IL-6, IL-1\(\beta\) and MCP-1) expression and the protein secretion

Nuclear membrane

Swine coronary artery with advanced atherosclerotic plaque

EAT attached to coronary artery

EAT