Vitamin D Protects Against Atherosclerosis via Regulation of Cholesterol Efflux and Macrophage Polarization in Hypercholesterolemic Swine

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Objective—Prevalence of vitamin D (VD) deficiency and its association with the risk of cardiovascular disease prompted us to evaluate the effect of VD status on lipid metabolism and atherosclerosis in hypercholesterolemic microswine.

Approach and Results—Yucatan microswine were fed with VD-deficient (0 IU/d), VD-sufficient (1000 IU/d), or VD-supplemented (3000 IU/d) high-cholesterol diet for 48 weeks. Serum lipids and 25(OH)-cholecalciferol levels were measured biweekly. Histology and biochemical parameters of liver and arteries were analyzed. Effect of 1,25(OH)2D3 on cholesterol metabolism was examined in human hepatocyte carcinoma cell line (HepG2) and human monocyte cell line (THP-1) macrophage-derived foam cells. VD deficiency decreased plasma high-density lipoprotein levels, expression of liver X receptors, ATP-binding membrane cassette transporter A1, and ATP-binding membrane cassette transporter G1 and promoted cholesterol accumulation and atherosclerosis in hypercholesterolemic microswine. VD promoted nascent high-density lipoprotein formation in HepG2 cells via ATP-binding membrane cassette transporter A1–mediated cholesterol efflux. Cytochrome P450 (CYP)27B1 and VD receptor were predominantly present in the CD206+ M2 macrophage foam cell–accumulated cores in coronary artery plaques. 1,25(OH)2D3 increased the expression of liver X receptors, ATP-binding membrane cassette transporter A1, and ATP-binding membrane cassette transporter G1 and promoted cholesterol efflux in THP-1 macrophage–derived foam cells. 1,25(OH)2D3 decreased intracellular free cholesterol and polarized macrophages to M2 phenotype with decreased expression of tumor necrosis factor-α, interleukin-1β, interleukin-6 under lipopolysaccharide stimulation. 1,25(OH)2D3 markedly induced CYP27A1 expression via a VD receptor–dependent c-Jun N-terminal kinase (JNK) 1/2 signaling pathway and increased 27-hydroxycholesterol levels, which induced liver X receptors, ATP-binding membrane cassette transporter A1, and ATP-binding membrane cassette transporter G1 expression and stimulated cholesterol efflux that was inhibited by VD receptor antagonist and JNK1/2 signaling inhibitor in THP-1 macrophage–derived foam cell.

Conclusions—VD protects against atherosclerosis in hypercholesterolemic swine via controlling cholesterol efflux and macrophage polarization via increased CYP27A1 activation. (Arterioscler Thromb Vasc Biol. 2015;35:2432-2442. DOI: 10.1161/ATVBAHA.115.306132.)

Key Words: atherosclerosis cardiovascular disease cholesterol efflux hydroxycholesterols macrophage polarization vitamin D

Atherosclerosis is a chronic inflammatory disease characterized by accumulation of macrophages in the arterial intima, which along with an associated inflammatory response in blood vessel walls initiates the formation and progress of atherosclerotic lesions.1 In atherosclerotic plaques, macrophage-derived foam cell formation is a hallmark of the progression of atherosclerosis that incites inflammatory rupture of plaques and leads to life threatening cardiovascular complications.2 It is well established that an atherogenic lipid profile, including increased serum low-density lipoprotein (LDL), promotes the massive accumulation of cholesterol in macrophages, whereas high-density lipoprotein (HDL) inhibits macrophages-derived foam cell formation via promoting cholesterol efflux and transporting it to the liver for excretion, a process termed reverse cholesterol transport.3 In atherosclerotic lesions, macrophages are submitted to microenvironmental factors, including cytokines and lipid signals, which might differentiate these cells into morphologically and functionally distinct states.

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functionally distinct phenotypes that are usually characterized by the classical proinflammatory M1 macrophages and alternatively activated anti-inflammatory M2 macrophages. Several ligand-activated nuclear hormone receptors, such as liver X receptor-α (LXR-α) and peroxisome proliferator-activated receptor-γ, are important lipid sensors that not only regulate expression of genes involved in lipid metabolism but also regulate macrophage polarization.

Vitamin D (VD) deficiency has been rising in the general population. Approximately 50% of the population worldwide has low levels of plasma 25-hydroxyvitamin D (25(OH)D3 <20 ng/mL), a stable marker of VD status. Beyond its well-defined role in calcium homeostasis, VD has recently been identified as an important factor in cardiovascular health. Clinical studies have indicated that plasma 25(OH)D3 level below 20 ng/mL is associated with increased risk for coronary heart disease. Several mechanisms, including protection of endothelial function, modulation of immune response, inhibition of vascular smooth muscle cell growth, have been proposed to account for the antiatherosclerotic effect of VD. Recently, several large retrospective studies demonstrated that VD deficiency is associated with atherogenic lipid profile, including increased serum LDL and decreased HDL levels. High 25(OH)D3 concentrations in the elderly are associated with low prevalence of metabolic syndrome with more beneficial HDL cholesterol (HDL-C) levels, the biologically active form of VD, suppresses foam cell formation by reducing oxidized LDL uptake in diabetic subjects. However, repletion of 25-hydroxyvitamin D levels in the short term has been reported not to improve the lipid profile in human. Thus, further studies are warranted to define the exact role of VD on the lipid metabolism and atherosclerosis.

In the present study, we found that VD deficiency decreased plasma HDL levels and promoted the progression of atherosclerosis in hypercholesterolemic swine via impaired LXRs/ABCA1/ABCG1 pathway, a crucial regulator in the formation and function of HDL. Our experiments revealed that 1,25(OH)2D3 promotes nascent HDL formation in human hepatocyte carcinoma cell line (HepG2) cells via ABCA1-mediated cholesterol efflux. In human monocytic cell line (THP-1) macrophages–derived foam cell, 1,25(OH)2D3 increased the expression of LXRs, ABCA1, and ATP-binding membrane cassette transporter G1 (ABCG1) and promoted cholesterol efflux and polarized macrophages to an M2 phenotype with decreased expression of inflammatory cytokines under lipopolysaccharide stimulation. 1,25(OH)2D3 causes upregulation of cytochromes P450 (CYP)27A1 via a VD receptor (VDR)–dependent c-Jun N-terminal kinase (JNK) 1/2 signaling, which plays a crucial role in the activation of LXRs/ABCA1/ABCG1 pathway.

Results

VD Deficiency Decreases Plasma HDL Level and Promotes Atherosclerosis in Hypercholesterolemic Swine

To investigate the effect of VD on the lipid metabolism and the progression of atherosclerosis, microswine were fed on VD-deficient (VD Def), VD-sufficient (VD Suf), and VD-supplemented (VD Sup) high-cholesterol diets. As shown in Figure 1A, VD Def diet produced significant VD deficiency, whereas VD Sup diet increased the serum level of 25(OH)D3, a stable marker of VD status. Lipid levels of animals fed with high-cholesterol diets were increased with time. Compared with VD Suf group, a significant decrease in HDL-C was observed as early as the end of the third month in VD Def group (VD Def group, 68.4±7.9 mg/dL; VD Suf group, 87.8±6.2 mg/dL; VD Sup group, 98.5±10.3 mg/dL; P<0.01). Furthermore, the VD Sup group had significantly higher level of HDL-C at month 12 compared with the VD Suf group and VD Def group; VD Sup group, 117.3±12.8 mg/dL; VD Suf group, 98.3±9.6 mg/dL; P<0.05; Figure 1B). Compared with VD Suf group, VD deficiency decreased while VD supplementation decreased the levels of LDL cholesterol. Nevertheless, there was no statistically significant difference among these groups (Figure 1B).

To investigate the effect of VD status on the atheroma progression in hypercholesterolemic swine, the histological analyses of fatty streak in thoracic aorta from different treatment groups were compared. As shown in Figure 1C, relative aortic fatty streak lesion area was 49.35±6.17% for the VD Def group, 34.11±2.56% for the VD Suf group, and 24.28±3.05% for the VD Sup group. Compared with VD Suf group, the progression of aortic fatty lesion formation under high-cholesterol diet. To further evaluate the extent of atherosclerosis, common carotid arteries from 3 dietary groups were analyzed for total cholesterol and cholesteryl ester deposition (Figure 1D). Total cholesterol contents of the arteries were significantly higher in the swine from VD Def group than in swine from the VD Suf or VD Sup group (P<0.01, 58.9±9.81 versus 36.8±5.28 µmol/g artery or 58.9±9.81 versus 35.5±4.44 µmol/g artery). Free cholesterol content in the common carotid artery was significantly increased by 73.2% or 93.2% in the VD Def group compared with VD Suf or VD Sup groups, respectively, further suggesting that VD deficiency exacerbates the lipid accumulation and atherosclerosis in hypercholesterolemic microswine.

Materials and Methods

Materials and methods are available in the online-only Data Supplement.
VD Deficiency Decreases 27-Hydroxycholesterol Level in Liver of Hypercholesterolemic Swine

To examine the molecular signaling targets that could explain the difference in HDL levels response to VD status, we first performed microarray analysis to examine differentially expressed genes in liver, which is the major organ for the synthesis of HDL. A total of 24,124 genes were examined using the porcine GeneChip for liver of pigs (n=4 for each treatment groups). A total of 342 mRNAs were identified as being up- or downregulated >2-fold in the VD Def group compared with VD Suf and VD Sup groups. Of these genes, 38 genes were regulated in a VD dose-dependent manner. Analysis of these genes using Gene Ontology Biological Process revealed that differential expression of genes primarily regulates the following biological functions: oxidative stress, lipid metabolism and inflammatory response, and cell signaling and interactions (Table I in the online-only Data Supplement). These results support the concept that in addition to its established role in regulating calcium homeostasis, VD seems to play an important role in the modulation of metabolic inflammatory diseases, including atherosclerosis. Prominent effects of VD on the genes in the lipid metabolism were noted. Interestingly, the transcription of genes encoding oxysterol receptor LXR pathway, ligand-activated transcription factors that raise HDL-C levels, was significantly decreased in the VD Def group (Table I in the online-only Data Supplement and Figure 2A). After further validation by Western blot, the key proteins in the LXR pathway, including LXR-α and LXR-β, its target gene ABCA1, and steroid catabolic gene CYP27A1 were decreased in VD Def group compared with VD Suf and VD Sup groups (Figure 2B). CYP27A1 plays a crucial role in the biogenesis of oxysterols, crucial ligands for the activation of LXRs. To clarify whether the regulated effect of VD on the HDL metabolism is related to the oxysterol metabolism, the levels of steroid catabolic intermediates, including 27-hydroxycholesterol (HOC), desmosterol, and 24,25-epoxycholesterol, in the liver were measured in the 3 groups. Compared with VD Suf and VD Sup groups, VD deficiency decreased the levels of 27-HOC. However, there was no significant difference in the levels of desmosterol and 24,25-epoxycholesterol in the 3 groups (Figure 2C). In the cultured HepG2 cell, 1,25(OH)2D3 (10 nM) treatment substantially upregulated the expression of CYP27A1 in a time-dependent manner (Figure 2D) and increased 27-HOC concentrations in oxidized LDL-loaded HepG2 cells (Figure 2E).

The activation of LXRs by oxysterol ligands induces the expression of ABCA1, which interacts with apolipoprotein A-I (apoA-I) and promotes the cholesterol efflux that plays a crucial role in the biogenesis of nascent HDL. Because 1,25(OH)2D3 increases 27-HOC production in HepG2 cells, we investigated whether 1,25(OH)2D3 plays a role in nascent HDL formation. After labeling overnight with [1,2-3H(N)]cholesterol, HepG2 cells were treated with LXR agonist T0901317 (5 μM),
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27-HOC (1 μM), and 1,25(OH)2D3 (10 nM), respectively, for 18 h and then incubated with lipid-free apoA-I (25 μg/mL) for 12 h. The medium was collected and filtered through a 0.45 μm polyvinylidene fluoride membrane to separate non-HDL and HDL. The radioactivity in [3H]-HDL fraction was determined by liquid scintillation counter. We found that the 1,25(OH)2D3 and the LXR agonist T0901317 and 27-HOC significantly increased cholesterol efflux to apoA-I (Figure 2F). After the incubation, ABCA1 protein expression in HepG2 cells was markedly increased by T0901317, 27-HOC, and 1,25(OH)2D3 (Figure 2G). Overall, these data suggest that 1,25(OH)2D3 promotes the nascent HDL formation in HepG2 cells by upregulating CYP27A1 activity and ABCA1 expression.

**VD Increases Cholesterol Efflux and M2 Polarization in Coronary Artery of Hypercholesterolemic Swine**

To determine the exact mechanism of VD on the inhibition of atherosclerotic progress, we stained coronary plaques from hypercholesterolemic swine. As shown in Figure 3A, VD metabolism, signal-related proteins, including CYP27B1, and VDR were predominantly present in the macrophage foam cell–accumulated cores of plaques in coronary artery. Vitamin D3 and its biologically active form, 1,25(OH)2D3, have been confirmed to have crucial effects on macrophage function via binding to its receptor VDR. Therefore, this data suggest that VD-mediated inhibition of atherosclerosis may be the result from the direct effect of VD on arterial wall, besides its effect on HDL levels. Macrophages, with extreme polarization phenotypes M1 and M2 macrophages, are the major cell components in atherosclerotic lesion that contribute to the foam cell formation and secretion of inflammatory factors. To clarify the exact role of VD in macrophage function, we compared the staining intensity between M1 marker CCR7 and M2 marker mannose receptor (CD206) in the regions of CYP27B1-positive and VDR-positive cores in plaques. We found significantly greater staining of M2 macrophage marker, CD206, in VDR-positive area, whereas proinflammatory M1...
macrophage marker CCR7 showed minimal immunoreactivity in this area (Figure 3A), suggesting that VD may play a role in the regulation of macrophage differentiation and inflammatory response in atherosclerotic lesion. To verify this hypothesis, we examined the expression of proinflammatory cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-1β) by ELISA. B, Proteins from the common carotid arteries of different treatment groups of swine were isolated and analyzed to determine the levels of inflammatory cytokines (Figure 3B, left) by Movat stain; right, Immunohistochemistry. C, ATP-binding membrane cassette transporter A1 (ABCA1)/ATP-binding membrane cassette transporter G1 (ABCG1) are coexpressed with VDR at sites of M2 macrophage foam cells in coronary atherosclerotic lesions of hypercholesterolemic swine. D, Western blot analysis of ABCA1, ABCG1, liver X receptors (LXR) α, and LXRβ expression in the common carotid arteries of different hypercholesterolemic swine. Values are means±SEM for n=3 animals per group. Statistical differences between groups were detected by 1-way ANOVA (*P<0.05, vitamin D–deficient [VD Def] compared with vitamin D–sufficient [VD Suf] or vitamin D–supplemented [VD Sup]; #P<0.05, VD Sup or VD Suf, n=3).

**Figure 3.** Vitamin D increases cholesterol efflux and M2 macrophage polarization in artery of hypercholesterolemic swine. A, Vitamin D signal-related CYP27B1 and vitamin D receptor (VDR) are expressed at sites of M2 macrophage (CD206 positive) foam cells in coronary atherosclerotic lesions of hypercholesterolemic swine (left, Movat stain; right, Immunohistochemistry). B, Proteins from the common carotid arteries of different treatment groups of swine were isolated and analyzed to determine the levels of inflammatory cytokines (Figure 3B, left) by Movat stain; right, Immunohistochemistry. C, ATP-binding membrane cassette transporter A1 (ABCA1)/ATP-binding membrane cassette transporter G1 (ABCG1) are coexpressed with VDR at sites of M2 macrophage foam cells in coronary atherosclerotic lesions of hypercholesterolemic swine. D, Western blot analysis of ABCA1, ABCG1, liver X receptors (LXR) α, and LXRβ expression in the common carotid arteries of different hypercholesterolemic swine. Values are means±SEM for n=3 animals per group. Statistical differences between groups were detected by 1-way ANOVA (*P<0.05, vitamin D–deficient [VD Def] compared with vitamin D–sufficient [VD Suf] or vitamin D–supplemented [VD Sup]; #P<0.05, VD Sup or VD Suf, n=3).

1,25(OH)2D3 Promotes Cholesterol Efflux and M2 Polarization in Macrophage-Derived Foam Cells via Upregulating 27-HOC

To further determine whether 1,25(OH)2D3 altered cholesterol efflux in macrophages, we analyzed the effect of 1,25(OH)2D3 on ABCA1-mediated cholesterol efflux in THP-1 macrophage–derived foam cells. As shown in Figure 4A, oxidized LDL-induced macrophage-derived foam cells cultured in 1,25(OH)2D3–deficient media exhibited a significantly greater staining in oil red after treatment.
with apoA-I compared with macrophage-derived foam cells cultured in 1,25(OH)\(_2\)D\(_3\)-supplemented media. Next, we examined the effect of 1,25(OH)\(_2\)D\(_3\) on cholesterol content and cholesterol efflux in THP-1 macrophage–derived foam cells. As shown in Figure 4B and 4C, the ability of apoA-I to decrease cholesterol content and to promote cholesterol efflux in THP-1 macrophage–derived foam cell was significantly increased in 1,25(OH)\(_2\)D\(_3\)-supplemented media compared with 1,25(OH)\(_2\)D\(_3\)-deficient media. To determine whether the increased ability of apoA-I to promote cholesterol efflux in 1,25(OH)\(_2\)D\(_3\)-supplemented media is related to cholesterol transporters, we analyzed the expression of ABCA1 and ABCG1 in these cells. Analysis of protein levels by Western blotting demonstrated a 1,25(OH)\(_2\)D\(_3\)-induced upregulation in the levels of ABCA1 and ABCG1 expression in THP-1 macrophage–derived foam cells (Figure 4D).

Macrophages-derived foam cells from atherosclerotic subjects have been shown to exhibit classic (M1) proinflammatory phenotype.\(^5\)\(^\text{,}^7\) LXR-\(\alpha\) activation and increasing ABCA1 levels have been found to inhibit inflammation and induce expression of IL-10, a marker of the M2 macrophage phenotype.\(^1\)\(^8\)\(^\text{,}^19\) To investigate whether the increased activation of LXR-\(\alpha\) induced by 1,25(OH)\(_2\)D\(_3\) results in the macrophage phenotypic polarization, we compared the expression of M1 or M2 marker in basal, IL-4, or lipopolysaccharide condition. Compared with 1,25(OH)\(_2\)D\(_3\)-deficient media, the macrophages from 1,25(OH)\(_2\)D\(_3\)-supplemented media have a significant increase in IL-4–stimulated mRNA expression of CD206 and MGL-1 (Figure 4E). Cells were analyzed by flow cytometry using anti-mannose receptor CD206 antibody. As shown in Figure 4F, 1,25(OH)\(_2\)D\(_3\) robustly increased IL-4–induced expression of CD206, suggesting 1,25(OH)\(_2\)D\(_3\)
promotes the M2 polarization of macrophage-derived foam cells. In vitro stimulation with lipopolysaccharide demonstrated that macrophages from 1,25(OH)2D3-supplemented media produced significantly less proinflammatory cytokine, tumor necrosis factor-α, compared with macrophages from 1,25(OH)2D3-deficient media (Figure 4G). These results support the concept that 1,25(OH)2D3 in macrophage is antiatherogenic via promotion of cholesterol efflux and anti-inflammatory macrophage polarization by upregulating LXR-α pathway.

To clarify whether the regulated effect of 1,25(OH)2D3 on macrophage is dependent on 27-HOC, we investigated the effect of 1,25(OH)2D3 on CYP27A1 expression. 1,25(OH)2D3 treatment substantially increased the expression of CYP27A1 in THP-1 macrophage-derived foam cells (Figure 4H). We also examined the effect of 27-HOC on the expression of ABCA1 and ABCG1 and found that 27-HOC induced ABCA1 and ABCG1 expression in a dose-dependent manner in THP-1 macrophage–derived foam cells (Figure I in the online-only Data Supplement). These results suggest that the 27-HOC/LXR-α pathway plays a crucial role in the regulation of macrophage cholesterol efflux and anti-inflammatory effect of 1,25(OH)2D3.

1,25(OH)2D3 Induces 27-Hydroxycholesterol in a VDR-Dependent JNK Signal Manner

CYP27A1 expression is controlled by activated JNK signal. In multiple cell types, the JNK pathway has been demonstrated to be activated by 1,25(OH)2D3. After treatment with 1,25(OH)2D3 for 30 minutes, the phosphorylation of JNK1/2 was detected, and it was further enhanced at 3 h (Figure 5A). We further used JNK signal inhibitor SP600125 to determine whether 1,25(OH)2D3 is exerting its CYP27A1 induction effects through activated JNK signal. THP-1 macrophage–derived foam cells were exposed to SP600125 or 1,25(OH)2D3. SP600125 almost completely blocked 1,25(OH)2D3-induced expression of phosphorylation of JNK1/2 in THP-1 macrophages (Figure 5B). The ability of 1,25(OH)2D3 to induce CYP27A1, ABCA1, and ABCG1 mRNA expression in THP-1 macrophage–derived foam cells was significantly impaired by SP600125.
of vitamin D3 supplementation in modifying lipid profile and increased the level of HDL-C, suggesting the beneficial effect significantly prevented the development of atherosclerosis and decreased atherosclerosis and decreased HDL-C level.32 Mutations of ABCA1 in human underlie the expression of ABCA1, and LXR-α expression as well as the production of the endogenous liver X receptors (LXR) ligand, 27-hydroxycholesterol (27-HOC), which stimulated ABCA1 expression in hepatocytes almost totally reduces the circulating ABCA1, and LXR-α expression in VDR siRNA cells was significantly abolished compared with control siRNA-untreated cells (Figure 5F), indicating a VDR-dependent effects of 1,25(OH)2D3 on LXR-α and its target genes. We further examined the role of VDR in the 1,25(OH)2D3-induced cholesterol efflux using liquid scintillation counter. The ability of 1,25(OH)2D3-induced cholesterol efflux in VDR siRNA cells was markedly decreased compared with that in control siRNA-treated cells (Figure 5G).

Discussion

Although clinical studies suggest that VD deficiency is related to a higher risk for cardiovascular disease, the exact role of VD in the progression of cardiovascular diseases has not been well defined.23 Because a higher plasma VD concentration is usually associated with a healthier lifestyle, it is unclear whether VD status is causally related to disease or is merely a marker of health.8,20 In this study, we fed hypercholesterolemic swine with different VD diet, resulting in different plasma 25(OH)D levels that provide the direct evidence for the effect of VD status on the progression of the disease. We found that VD deficiency promoted the progress of atherosclerosis and decreased plasma HDL-C level, a crucial cardiovascular protective factor, under hypercholesterolemic condition. Supplementation of vitamin D3 in the diet (3000 IU per day×24 weeks) significantly prevented the development of atherosclerosis and increased the level of HDL-C, suggesting the beneficial effect of vitamin D3 supplementation in modifying lipid profile and decreasing cardiovascular disease. In a randomized clinical study, including 57 postmenopausal VD Def women, Catalano et al found that the supplementation with 25(OH)D3 (calcifediol) for 24 weeks significantly increased HDL-C level even after adjustment for age, baseline body mass index, 25(OH)D3, and lipid levels.24 In another randomized double-blind placebo-controlled clinical trial, including 70 participants with type 2 diabetes mellitus, it was found that the supplementation of calcitriol (1,25(OH)2D3, 0.5 μg per day) for 12 weeks increased the HDL-C level compared with the placebo group.25 However, Ponda et al have reported no improvement in the lipid profile after short-term supplementation of vitamin D3 (50000 IU of vitamin D3 weekly for 8 Weeks) in VD Def adults with elevated risk for cardiovascular disease, suggesting that the dose and duration of treatment determine the outcomes, and this remains to be elucidated. Oxyesters are the endogenous ligands for the LXRs, ligand-activated transcription factors that can regulate a crucial checkpoint in cholesterol homeostasis.26 The sterol 27-hydroxylase (CYP27A1) is a mitochondrial cytochrome P450 and is involved in the synthesis of oxyesters, HOC, 3β-hydroxy-5-cholestenolic acid, and 25(OH)D3, which play a crucial role in cholesterol, bile acids, and VD metabolism.27 It was reported previously that vitamin D, and its biologically active form, 1,25(OH)2D3, can regulate expression of various CYP.28–30 Using a microarray method, we found that VD deficiency induces a response for the expression of the mRNA of genes associated with oxidative stress, lipid metabolism, and inflammation pathways in the liver. VD supplementation increased liver mRNA levels of oxidation–reduction and oxyester-related CYP genes, including CYP1A1, CYP1A2, CYP2J34, and CYP27A1. In addition, we found that VD upregulates the expression of LXRs and ABCA1 in liver in a dose-dependent manner.

ABCA1 is a lipid transport protein transcriptionally regulated by nuclear receptors LXR-α, which facilitates cellular cholesterol efflux to lipid-poor apoA-I and plays a key role in the formation and function of HDL.31 In mice, specific lack of ABCA1 in hepatocytes almost totally reduces the circulating HDL-C level.32 Mutations of ABCA1 in human underlie the
HDL deficiency syndrome, Tangier disease, suggesting that primarily hepatic ABCA1 determines the generation of nascent HDL particles.\(^{31,32}\) To clarify whether the regulated effect of VD on the HDL metabolism is related to the ABCA1 expression, we investigated the effect of 1,25\((\text{OH})_2\)D\(_3\) on the expression of ABCA1 and nascent HDL formation in hepatocytes and found that 1,25\((\text{OH})_2\)D\(_3\) promoted cellular cholesterol efflux to lipid-poor apoA-I, a major component of nascent HDL via upregulating ABCA1 expression. In several cholesterol-loaded cells, including hepatocytes and macrophages, LXR-\(\alpha\)/ABCA1 pathway has been found to be upregulated to promote the cholesterol efflux via a 27-HOC–dependent manner.\(^{33,34}\) In this study, we have shown for the first time that 1,25\((\text{OH})_2\)D\(_3\) facilitates the upregulation of LXR-\(\alpha\)/ABCA1 in cholesterol-loaded hepatocytes and macrophages via promoting the expression of CYP27A1. This effect could be supported by a recent clinical data, showing a significant positive correlation between the levels of 27-OHC and 25(OH)D\(_3\) under hypercholesterolemic condition.\(^{35}\) Interestingly, the LXR target genes, such as ABCG5/8, SREBP-1c, and SR-B1, were not found to be regulated by 1,25\((\text{OH})_2\)D\(_3\) in HepG2 cells (Figure II in the online-only Data Supplement). The conserved consensus cis-acting element DR4-dependent transcription is necessary for LXR-\(\alpha\)–induced ABCA1 expression in HepG2 cells.\(^{27}\) The activation of VDR by 1,25\((\text{OH})_2\)D\(_3\) has been found to recruit coactivators at DR4-type response element that leads to the expression of target genes.\(^{37,38}\) These results suggest that the recruitment of coactivators at the response element induced by 1,25\((\text{OH})_2\)D\(_3\) may be playing a critical role in supporting the transactivation of LXR-\(\alpha\)/ABCA1 pathway, though the exact mechanisms remain to be further examined.

Several studies have shown that 1,25\((\text{OH})_2\)D\(_3\) can regulate the differentiation, maturation, and function of macrophages,\(^{2,39,40}\) which prompted us to investigate whether the antiatherogenic effects of VD results from the direct effect of VD on the arterial wall. In this study, we found that VDR and CYP27B1, a key enzyme to control the synthesis of 1,25\((\text{OH})_2\)D\(_3\), are mainly expressed at the M2 macrophage foam cell–accumulated cores of plaques in the coronary artery. VD regulates the macrophage M1/M2 phenotype in diabetic nephropathy rats.\(^{36}\) In vitro, 1,25\((\text{OH})_2\)D\(_3\) has also been found to switch high glucose–induced M1/M2 polarization to suppress cholesteryl ester formation and to enhance cholesterol efflux in M2 macrophages.\(^{41,42}\) In atherosclerotic lesion, we found that VDR is primarily coexpressed with ABCA1/ABCG1 at M2 macrophage–derived foam cell. In vitro, 1,25\((\text{OH})_2\)D\(_3\) has been found to promote the cholesterol efflux and polarize macrophages to proinflammatory M2 phenotype. In macrophages, ABCA1 and ABCG1, another ABC family of transporter, play critical role in mobilizing cholesterol out of macrophages and onto extracellular HDL.\(^{43}\) In addition to its lipid transport function, ABCA1 and ABCG1 have been found to play a crucial role in the regulation of cytokine-triggered macrophage polarization programs and inflammatory response.\(^{44,45}\) Several studies have shown that the cholesterol export activity of ABCA1/ABCG1 could account for its potent anti-inflammatory properties.\(^{46,47}\) Knockdown of ABCA1 in macrophage results in an increase of cell membrane cholesterol and lipid raft content, which facilitate toll-like receptor 4-MyD88-mediated proinflammatory response.\(^{48}\) In this study, we found that 1,25\((\text{OH})_2\)D\(_3\) markedly induced the expression of ABCA1 and ABCG1 in macrophages, suggesting that enhanced cholesterol efflux may play a crucial role in anti-inflammatory effect of VD.

Previously, JNK/c-jun pathway has been reported to upregulate the expression of CYP27A1 in hepatocytes.\(^{20}\) 1,25\((\text{OH})_2\)D\(_3\) activates the JNK/c-jun pathway in various cell lines.\(^{21,22,49}\) Consistently, our study demonstrated that 1,25\((\text{OH})_2\)D\(_3\) upregulates the phosphorylation of JNK in macrophages. Furthermore, inhibitor of JNK activation blocks 1,25\((\text{OH})_2\)D\(_3\)-induced upregulation of CYP27A1 and 27-HOC content, suggesting that JNK/c-Jun activation plays an important role in antiatherogenic activity of VD. Both VDR and LXR-\(\alpha\) are crucial lipid-activated nuclear receptors that shape macrophage function.\(^{30}\) Earlier studies have shown that VDR is regulated by LXR-\(\alpha\) through its ability to modulate sterol regulatory element–binding protein present in the promoter region of VDR gene.\(^{31}\) In our study, we found that LXR-\(\alpha\) pathway is also regulated by VDR through its ability to modulate CYP27A1 and 27-HOC (Figure 6), suggesting the existence of crosstalk between LXR and VDR that stimulates the intracellular pathway to exert the antiatherogenic effect.

In conclusion, the findings in this study revealed a novel mechanistic link between VD deficiency and cardiovascular risk. VD supplementation significantly increased the levels of plasma HDL and the expression of 27-HOC/LXR-\(\alpha\) pathway, which contributes to alleviate cholesterol toxicity and macrophage proinflammatory polarization, resulting in protection against the development of atherosclerosis.

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Disclosures
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References
Vitamin D Regulates Cholesterol Eflux


### Significance

Epidemiological studies suggest that vitamin D deficiency is related to a proatherogenic lipid profile and inflammation. However, the exact role of vitamin D in the progress of atherosclerosis has not been fully elucidated. Here, we found that vitamin D deficiency accelerated the development of atherosclerosis in hypercholesterolemic swine, which is similar to the lipid metabolism and the progress of atherosclerosis in human. Vitamin D deficiency results in impaired cholesterol efflux in the liver and artery that leads to decreased high-density lipoprotein level, cholesterol accumulation, and M1 macrophage polarization in vascular wall. The 1,25(OH)2D3 markedly induced CYP27A1 expression and increased 27-hydroxycholesterol levels, which induced cholesterol efflux. These results suggest that vitamin D supplementation might serve as a novel therapeutic modality to increase high-density lipoprotein level and decrease severity of atherosclerosis in patients with cardiovascular disease risk.
Vitamin D Protects Against Atherosclerosis via Regulation of Cholesterol Efflux and Macrophage Polarization in Hypercholesterolemic Swine
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**Supplementary Table I:** Microarray results showing that the 38 genes were regulated in a Vitamin D dose-dependent manner in the liver of hypercholesterolemic swine. The total RNA from liver tissue of swine was extracted to carry out the microarray.

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<th>Gene</th>
<th>Gene Full Name</th>
<th>Probe ID</th>
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<th>Fold Change (Suf vs Def)</th>
<th>Process, GO Terms</th>
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Supplementary Figure I. THP-1 macrophage-derived foam cells were cultured on six-well plates. Cells were treated with 27-hydroxycholesterol (27HOC) (10nM, 100nM, 1 μM and 10 μM) for 24h. The protein was isolated for western-blot analysis of ABCA1 and ABCG1 expression. Statistical differences between groups were detected by one-way ANOVA and Student’s t test (**p < 0.05, compared to control, n=3).

Supplementary Figure II. HepG2 cells were treated with 1,25(OH)₂D₃ (10nM) for 24h, the relative mRNA expression of ABCG5, ABCG8, SREBP-1c, and SR-B1 were determined by quantitative real-time PCR (P > 0.05, control vs 1,25(OH)₂D₃ group).
### Supple Table II: Primer sets for real-time RT-PCR analysis of gene expression

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<tr>
<th>Gene Name</th>
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<td>ABCA1</td>
<td>AGTAGTGGATGGTGTCTTTC</td>
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<td>ABCG1</td>
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<td>LXR α</td>
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<td>CD206</td>
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<td>GAPDH</td>
<td>GGTGTGAACCATGAGAAGATAGA</td>
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MATERIALS AND METHODS

Animals and Diets - The Institutional Animal Care and Use Committee of Creighton University approved the experimental animal research protocol in the present study. Yucatan miniswines purchased from Lonestar Laboratories (Sioux Center, IA) were randomized into three groups with similar body weight (30-40 lbs). Group 1 swine (n=6) were fed with vitamin D-deficient high cholesterol diet (Harlan, USA) with the following major ingredients: 19% casein “vitamin free”, 23.5% sucrose, 23.9% corn starch, 13% maltodextrin, 4% soybean oil, 4% cholesterol, and 10% cellulose. Group 2 swine (n=4) were fed with vitamin D sufficient (1000IU/d) high cholesterol diet. Group 3 swine were fed with vitamin D supplement (3000IU/d) high cholesterol diet. Animals were housed in the Creighton Animal Facility under controlled conditions, 12:12 light-dark cycle at 20-24°C, without exposure to sunlight and fed a controlled diet to avoid any variation in the 25(OH)D levels due to season or diet. Venous blood (10 ml) from the ear vein was drawn every 2 weeks to examine serum 25(OH)D levels and complete lipid profile.

Antibodies and Reagents – apoA1, ABCA1, ABCG1, LXR-α, LXR- β, VDR, CYP27B1, CYP27A1, p-JNK, CD206, and CCR7 antibodies were obtained from Abcam (Abcam, UK). OxLDL were purchased from Kalen Biomedical, LLC. 1,25(OH)₂D₃ and recombinant human apoA-I were purchased from Peprotech (Rocky Hill, NJ, USA). Cholesterol and Desmosterol were purchased from Sigma. 27-hydroxycholesterol (27-HOC) and synthetic LXR agonist T0901317 were purchased from Santa Cruz. 24,25- epoxycholesterol (EPO) was purchased from ABCam (Abcam, UK).

Morphological Examination of Atherosclerotic Lesions- Atherosclerotic lesions were analyzed by a method described previously [1]. The aorta was dissected from the aortic valve to the aortic hiatus, and as much adventitia as possible was removed to prevent errors during Oil red staining of the vessel. After overnight fixation in 10% formalin, the aorta was opened longitudinally and rinsed in 70% ethanol for 10 min and then stained with Oil-red stain solution for 60 min. Destaining was carried out for 30 min in 70% ethanol. Lipid deposition in the aorta was determined by morphological assessment of the percentage of lesion-covered aorta as visualized by oil-red staining of the region. The atherosclerotic lesions were analyzed using image analysis software (NIH Image). The aortic lesion area was calculated as a percentage as follows: atherosclerotic lesion area ÷ area of the aorta ×100.

Movat’s Pentachrome Stain- Sections were stained with 5% sodium thiosulfate for 5 min, washed for 5 min under cold running water, stained in 1% alcian blue for 20 min and washed again. The slides were placed in preheated alkaline alcohol (60°C) for 10 min, washed under running water than stained with hematoxylin according to Weigert (10 min), washed with water, then placed in Movat’s solution for 60 min and washed again. Samples were stained with acid fuchsin for 1 min, differentiated in 1% acetic acid and incubated with 5% phosphotungstic acid for 5 min (Movat pentachrome stain Kit, Diapath S.p.A). The slides were transferred directly into 1% acetic acid for 5 min and washed finally.

Immunohistochemistry (IHC) and Immunofluorescence (IFC) – Paraffin embedded samples, after deparaffinization and rehydration, were treated by steam heating for antigen retrieval (20-30 min). Immersing the slides in a 3%
hydrogen peroxide solution for 20 min inhibited endogenous peroxidase. After pre-incubation of the tissue sections with 10% serum for 1 h, the sections were incubated with primary antibodies overnight at 4°C. After consecutively incubated with biotinylated secondary antibody or a secondary antibody (affinity purified goat anti-mouse and goat anti-rabbit cyanine 3 (cy3) antibody, 1:200) (Jackson ImmunoResearch, Westgrove, PA) for 1 h, slides were incubated with ABC solution (VECTASTAIN Elite ABC system, Vector Laboratories, Burlingame, CA) for IHC or counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for IFC. Sections incubated without the primary antibody served as negative controls. Slides were examined using an inverted microscope (Olympus).

RNA Isolation and microarray Analysis- Total RNA from liver tissues were extracted by using TRIzol reagent (Invitrogen) in accordance with the manufacturer’s instructions. The mRNA expression profiles of liver tissue samples from mini-swine were carried out utilizing the porcine Affymetrix GeneChip (AFF-900624). Differentially expressed genes were considered significant at 2-fold.

Relative Quantitative Real-Time PCR- Relative quantitative real-time PCR (RT-PCR), using SYBR Green detection chemistry, was performed on 7500 real time PCR system (Applied Biosystems). Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the \( \Delta \Delta C_t \) method and expression of GAPDH was used as the internal control. The porcine primers used for the RT-PCR are shown in On-line supplementary Table II.

Western Blot Analysis- Proteins (15-20µg of extracts) were loaded on 5-10% SDS-polyacrylamide electrophoresis gel and electrophoresed for 2 h at 100V in buffer containing 25mM Tris base, 250 mM glycline and 0.1% SDS. After electrophoresis, the proteins were electrically transferred to the immobilon-P transfer membrane in buffer containing 25 mM Tris, 192 mM glycline, 20% methanol. After transfer, the membrane was blocked in TBST (20mM Tris base pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% skimmed milk for 4 h at room temperature. The membrane was then incubated with antibodies in the blocking solution at 4°C overnight. Thereafter the membrane was washed three times with TBST for 30 min, incubated with secondary antibody in the blocking solution for 50 min at room temperature, and washed three times with TBST for 30 min. The proteins were visualized using a chemiluminescence method (Bioimaging System, UVP, Upland, CA). Densitometric analysis was done to relatively quantify the amount of protein.

High Performance Liquid Chromatography for oxysterols – liver tissue oxysterols were determined as described by a method described previously with minor modification [2]. Briefly, the liver total lipid of pig (100mg) was extracted by adding 4 mL of ice-cold chloroform/methanol (3:1, vol/vol), containing 0.005% (vol/vol) butylated hydroxytoluene (BHT), and 20α-hydroxycholesterol (20nM) as an internal standard for HPLC. A cholesterol-rich fraction was isolated from the total lipid by solid-phase extraction. The residue was dissolved in methanol and stored at 4°C until analysis. Oxysterols were determined by HPLC using a column (Silica 5 μm, 4.6×250mm). Mobile phase: 96.5% hexane, 2.5% isopropanol, 1% glacial acetic acid. All oxysterols were detected by UV spectrophotometer at 240 nm. The concentration of the compound in tissue and cells was calculated from a
calibration curve of oxysterols standard.

**Cells** - HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC® HB-8065™) containing 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO2. THP-1 cells were cultured in RPMI1640 medium (Sigma) containing 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO2. After 3 to 4 days, cells were treated with PMA (100nM) for 3 days, and then the medium was replaced by serum-free medium containing oxLDL (50 µg/ml) for 48 h to become fully differentiated macrophages foam cells before their use in experiments.

**Oil-Red Staining** - The lipid accumulation in THP-1 macrophages was evaluated by Oil Red O staining. Briefly, 10% formalin-fixed cells were stained with Oil Red O for 20 minutes, and then the samples were counterstained with hematoxylin for 2 minutes. Results were examined by light microscopy.

**Cholesterol/Cholesterol Ester Measurements** - Total cholesterol/cholesterol ester in arterial samples and cells were measured using a commercial assay according to the manufacturer instructions (ab65359; Abcam, Cambridge, UK). Briefly, tissues were washed in ice-cold saline, blotted dry between sheets of filter paper, and weighed. Cholesterol and cholesteryl ester contents in the tissues and cells were measured using cholesterol assay buffer after they were extracted with chloroform: Isopropanol: NP-40 (7:11:0.1) in a micro-homogenizer. The cholesterol standard was diluted to 25 ng/µl by adding 10µl of the cholesterol standard to 790µl of cholesterol assay buffer, and mixed well. The absorbance was measured at Ex/Em = 535/590 nm using the fluorescence assay.

**Cellular Cholesterol Efflux Experiments** - Cells were cultured as indicated above. Then they were labeled with 0.2 µCi/ml [³H]cholesterol. After 72 h, cells were subsequently washed with phosphate-buffered saline (PBS) and incubated overnight in RPMI 1640 medium containing 0.1% (w/v) bovine serum albumin (BSA) to allow equilibration of [³H]cholesterol in all cellular pools. Equilibrated [³H]cholesterol-labeled cells were washed with PBS and incubated in 2 ml of efflux medium containing RPMI 1640 medium and 0.1% BSA with 25 µg/ml human plasma apoA-I. A 150 µl sample of efflux medium was obtained at the times designated and passed through a 0.45-µm filter to remove any floating cells. Monolayers were washed twice in PBS, and cellular lipids were extracted with isopropanol. Medium and cell-associated [³H] cholesterol was then measured by liquid scintillation counter. Percent efflux was calculated by the following equation: [total media counts/(total cellular counts + total media counts)]×100%.

**Flow cytometry** - After pelleted down, the cells were fixed in 3.7% formaldehyde for 10 min and permeabilization with 0.1% PBS-Tween for 20 min. The cells were then incubated in 1x PBS / 10% normal goat serum to block non-specific protein-protein interactions followed by the CD206 antibody (2µg/1x10⁶ cells, Abcam, UK) at RT. The secondary antibody used was DyLight® 488 goat anti-mouse IgG at 1/500 dilution for 30 min at 22°C. Isotype control antibody was mouse IgG1 used under the same conditions. The cells were then analyzed using a BD FACS Aria (BD Biosciences).

**Transfection of siRNA** - The siRNA against VDR, and an irrelevant nonfunctional 21-nucleotide siRNA duplex, which was used as a control, was purchased from Santa Cruz Biotech using FuGENE6 transfection reagent
(Roche Applied Science) according to manufacturer’s instructions. The respective sequences were as follows: sense: GCUGUUUAUUUGACAGAGATT; antisense: UCUCUGUCAAAUAAAACAGCAA. Scrambled siRNA sense, UUGGGAUGACUGAGGUACCUGA, and antisense, UCAGGUACUCAGUCAUCACA.

**Statistical Analysis**  Data are expressed as means ± S.E.M. Results were analyzed by one-way ANOVA or two-way ANOVA and Student’s t test, using SPSS 13.0 software. Statistical significance was obtained when P values were less than 0.05.

**REFERENCES**
