Int J Clin Exp Med 2015;8(4):5065-5074
www.ijcem.com /ISSN:1940-5901/IJCEM0006594

Original Article

Cardioprotective role of vitamin D receptor in circulating endothelial cells of ApoE-deficient mice

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Received February 2, 2015; Accepted April 3, 2015; Epub April 15, 2015; Published April 30, 2015

Abstract: Atherosclerosis is the key course of coronary heart disease. In this study, we investigated the effect of vitamin D receptor on serum 1,25-$(OH)_2$D levels, lipid profiles, nitric oxide expression, apoptosis-related gene Bcl-2, fas protein levels, in ApoE-deficient mice. The proliferation activity of VDR-RNAi transfected endothelial cells was decreased, the ability of apoptosis was increased, nitric oxide concentration was decreased and eNOS protein level was significantly reduced. VDR-RNAi induced lipid metabolism abnormality, reduced eNOS and ApoE levels, promoted lipid peroxidation, damaged the endothelial function and accelerated the process of atheroscleros. Together, our data presented a novel role for VDR in the pathogenesis process of atheroscleros by up regulating eNOS protein expression which could lay a solid foundation of VDR-specific activator treatment for coronary artery disease.

Keywords: Vitamin D, vitamin D receptor, apolipoprotein E, atherosclerosis, endothelial cells

Introduction

Atherosclerosis and the subsequent cardiovascular complications, such as myocardial infarction, stroke, and ischemic heartfailure, is a major cause of death in the Western world [1]. Thus, identifying the underlying molecular mechanisms that may attenuate atherosclerotic plaque progression and ultimate rupture is necessary for developing effective preventive and therapeutic strategies [2]. Increasing evidence indicates that vitamin D receptors (VDR) have been found in all the major cardiovascular cell types including cardiomyocytes, arterial wall cells, and immune cells [3]. Experimental studies have established a role for vitamin D metabolites in pathways that are integral to cardiovascular function and disease, including inflammation, thrombosis, and the renin-angiotensin system [4]. The vitamin D receptor (VDR) is an intracellular receptor which binds 1,25-dihydroxyvitamin D, the active form of vitamin D [5]. The VDR has been identified in most human tissues (including the heart, brain, kidney, and muscle [6-8] as well as in sheep arteries (endothelium, intima, and media [9]. Vitamin D functions through vitamin D receptor, a transcription factor, and directly or indirectly controls more than 200 heterogeneous genes including the genes for the regulation of cellular differentiation, proliferation, and angiogenesis [10]. Vitamin D receptors are distributed in a variety of tissues including cardiomyocytes, vascular smooth muscle cells (VSMCs), endothelium, and cells of immune system [6].

In this study, we investigated the role of VDR in ApoE-deficient mice by examining the expression of serum 1,25-$(OH)_2$D levels, lipid profiles, nitric oxide expression, apoptosis-related gene Bcl-2, fas protein levels. However, the role of vitamin D receptor in the management of cardiovascular disease remains to be established. This study studies showing associations between vitamin D receptor and cardiovascular disease and the experimental studies that explore the mechanistic basis for these associations.

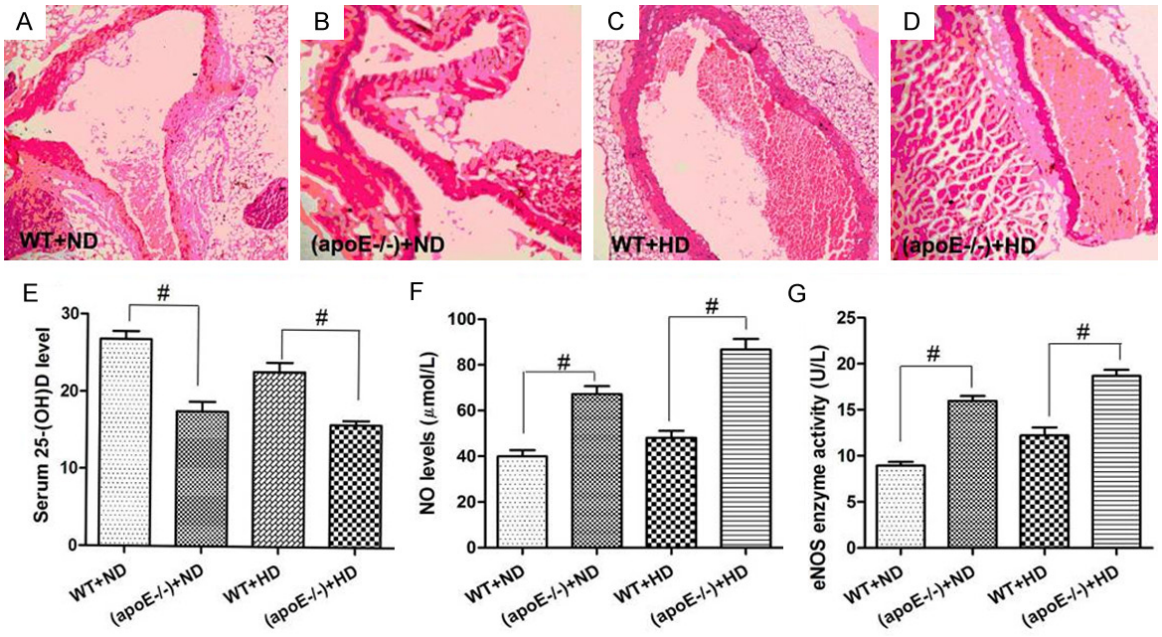

Figure 1. Morphology of aorta and serum 1,25-$(OH)_2$D levels changes in ApoE-deficient mice after high-fat diet treatment. Histology of transversal sections of aortas from wild type with normal diet (A. WT+ND); ApoE-/- mice with normal diet (B. ApoE-/-+ND); wild type with high-fat diet (C. WT+HD) or ApoE-/- mice with high-fat diet (D. ApoE-/-+HD). 20 × magnification, H&E, hematoxilin-eosin. Representative images of three aortas for each group. (E) The serum 1,25-$(OH)_2$D level of ApoE-/- mice was lower than the wild type mice; and it became even lower after high-fat treated. (F, G) ApoE-deficient mice showed increased NO concentration and eNOS enzyme activity compared with wild type mice with or without high fat diet.

Materials and methods

Animals

Eight-week-old male C57BL/6J mice (wild-type mice, WT) and homozygous apoE-/- mice were purchased from the Animal Center of Beijing University, Beijing, China. Animals were fed a high-fat diet (15% fat plus 1.25% cholesterol) for 4 weeks. A total of 32 male homozygous apoE-/- mice and 32 male C57BL/6J mice (wild type mice) were used in this experiment. The animals were housed individually in the specific pathogen free barrier facility at constant temperature (20-22°C) and humidity (45-55%) with a 12 hour light-dark cycle. The animal experiments were approved by the Committee on Animal Care of Hainan University and the procedures were performed in accordance with the guidelines of the institutional Animal Care and Use Committee; in conform to the NIH Guide for the Care and Use of Laboratory Animals.

Aorta dissection

Mice were anesthetized with an intraperitoneal injection of ketamine 10% (Ketostop; Drogas Pharma-Invetec, Santiago-Chile), xylazine 2% (Xylavet; Alfasan International BV, Holland), acepromazine 1% (Drag Pharma) and pre-medicated with 100 UI i.p of heparin. After deep anesthesia, a midline incision was made and the aorta obtained in block, and immersed in Krebs modified NaCl 115 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, EDTA 0.01 mM and glucose 11.1 mM. The heart was removed and immersed in 0.9% NaCl.

Western blotting

Cardiac tissue was homogenized in 1 ml of lysis buffer (Tris 50 mM, SDS 0.1%, NaCl 30 mM, EDTA 2 mM) supplemented with 10 μl of proteases inhibitors cocktail (MP Biomedicals, Solon, OH, EE.UU.) using an ultraturrax (3 cycles of 15 s). Then the homogenate was centrifuged at 4000 rpm for 10 min at 3°C. The supernatant was removed and mixed with loading buffer. Total protein concentration from hearts was determined using the BCA method, with the BCA Protein Assay de Thermo Scientifc, Pierce Biotechnology (Rockford, IL, EE.UU.). Protein samples of each fraction (100 μg) were separated by 7% SDS-PAGE and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA).

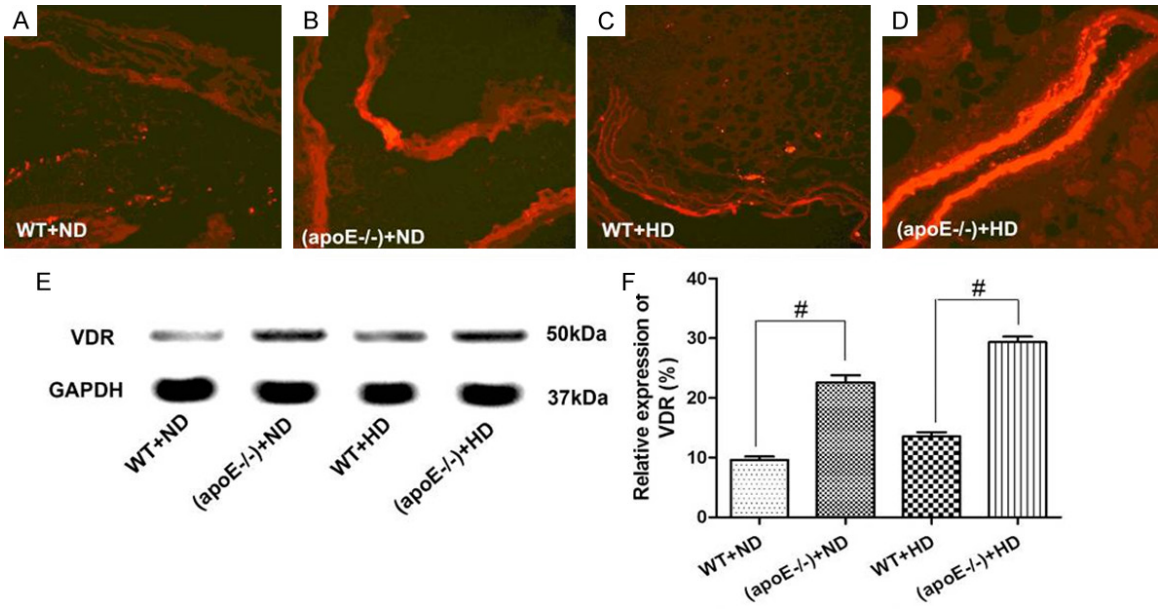

Figure 2. Vitamin D receptor epression in ApoE-deficient mice. Representative images of immunofluorescence analysis was used to assess the vitamin D receptor expression in aortas of four groups (A. WT+ND; B. ApoE-/-+ND; C. WT+HD D. ApoE-/-+HD). 20 × magnification. (E) Western blot analysis of VDR protein expression levels. (F) Statistical analysis revealed that VDR expression levels were higher in ApoE-deficient mice comparing to the wild type mice with or without high fat diet treatment (n=3; $^{*}P<0.01$).

The membrane was incubated with a primary antibody overnight at 4°C and then with an appropriate secondary antibody (2 hours at room temperature). The protein bands were visualized with standard methods, using Western LightingTM Chemiluminescence reagent (Perkin Elmer, Boston, MA), SuperSignal® West Femto (Pierce, Rockford, IL). Western blots were scanned and evaluated by densitometry analysis using Image J software.

Histology

Aortas were fixed in 10% formalin at pH 7.4. Once the samples were fixed and included in paraffin, sections of 5 μm were obtained using a rotating Microm HM325 microtome, then, were mounted on xylanized slides. Sections of hydrated and de-paraffnized tissues were stained with haematoxylin-eosin (HE).

Statistical analyses

Each experiment was performed in triplicate. Statistical analyses were carried out using SPSS statistical software, version19.0 (SPSS, Inc. Chicago, IL, USA) for Windows. Data were expressed as mean ± SD. Analysis of variance experiments (one-way ANOVA, factorial ANOVA and repeated-measures ANOVA), followed by the Student-Newman-Keuls test were performed, with $P<0.05$ considered statistically significant

Results

High-fat diet enhanced the atherosclerosis of ApoE-deficient mice by decreasing serum 1,25-$(OH)_2$D levels and increasing NO levels and eNOS enzyme activity

The histology of the aortas were analyzed by hematoxilin-eosin, we found a normal morphology in wild mice with normal diet group, with absence of atherosclerosis (**Figure 1A**), the wall of the aorta in ApoE-deficient mice with normal diet group was diffuse thickening, luminal stenosis was more apparent, with foam cells and lipid streaks, partly appeared fibrous plaque (**Figure 1B**). After high fat diet treated, atherosclerosis condition was aggravated in both wild type mice and ApoE-deficient mice groups (**Figure 1C**, **1D**). These results suggested that high fat diet strengthen atherosclerosis of ApoE-deficient mice.

Figure 1E showed the serum 1,25-$(OH)_2$D levels in these four groups, the serum 1,25-$(OH)_2$D

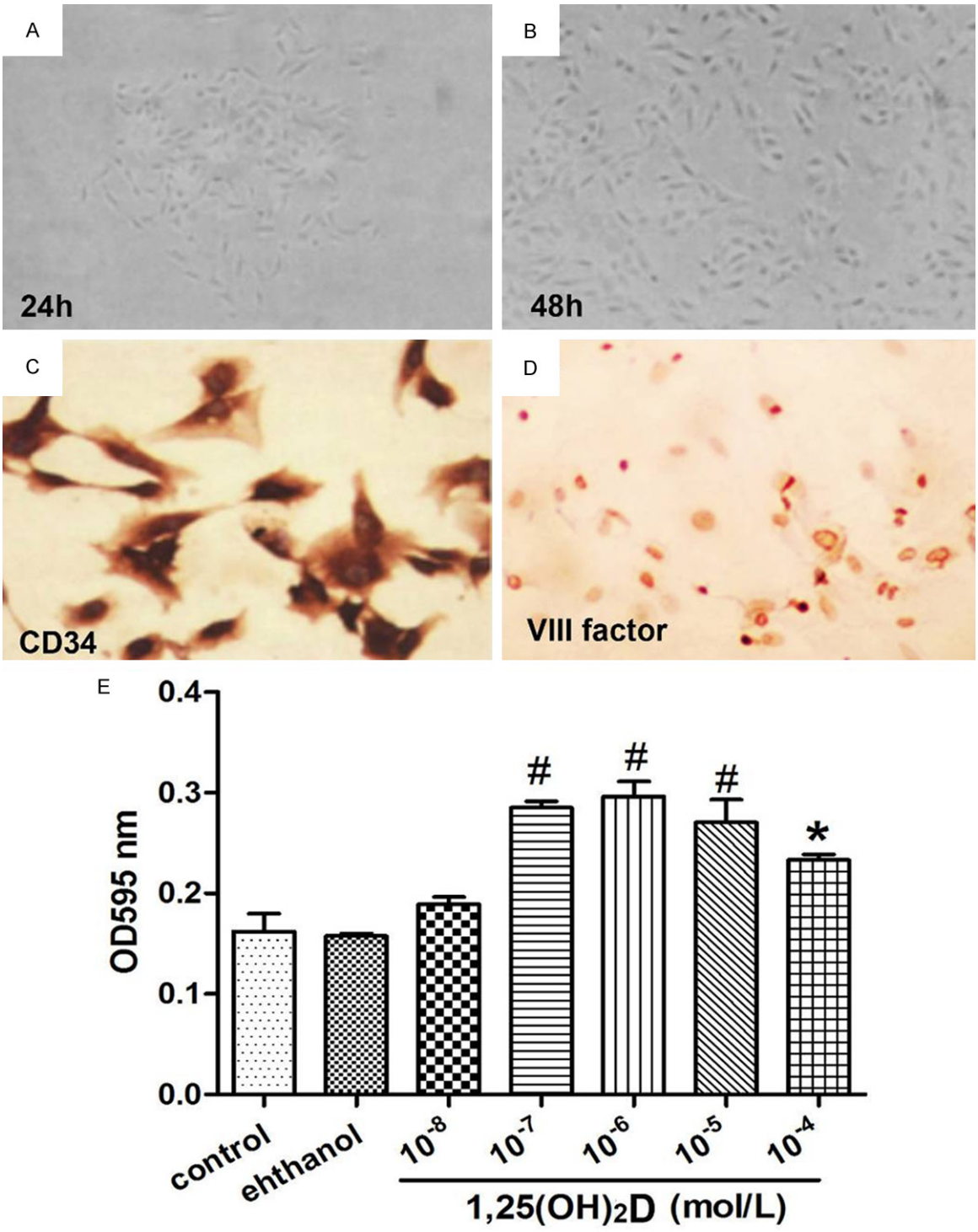

Figure 3. Effect of 1,25-$(OH)_2D$ on endothelial progenitor cells proliferation. A, B. Light microscope images of endothelial progenitor cells. C, D. The endothelial progenitor cells were identified by CD34 and VIII antibodies. E. The cell proliferation activity after 1,25-$(OH)_2D$ treatment (n=3; $^{*}P<0.01$, $^{*}P<0.05$).

levels were higher in wild type mice comparing to the ApoE-deficient mice with or without high fat diet.

Next the NO levels and eNOS enzyme activity were measured in these four groups (**Figure 1F**, **1G**). ApoE-deficient mice showed increased NO

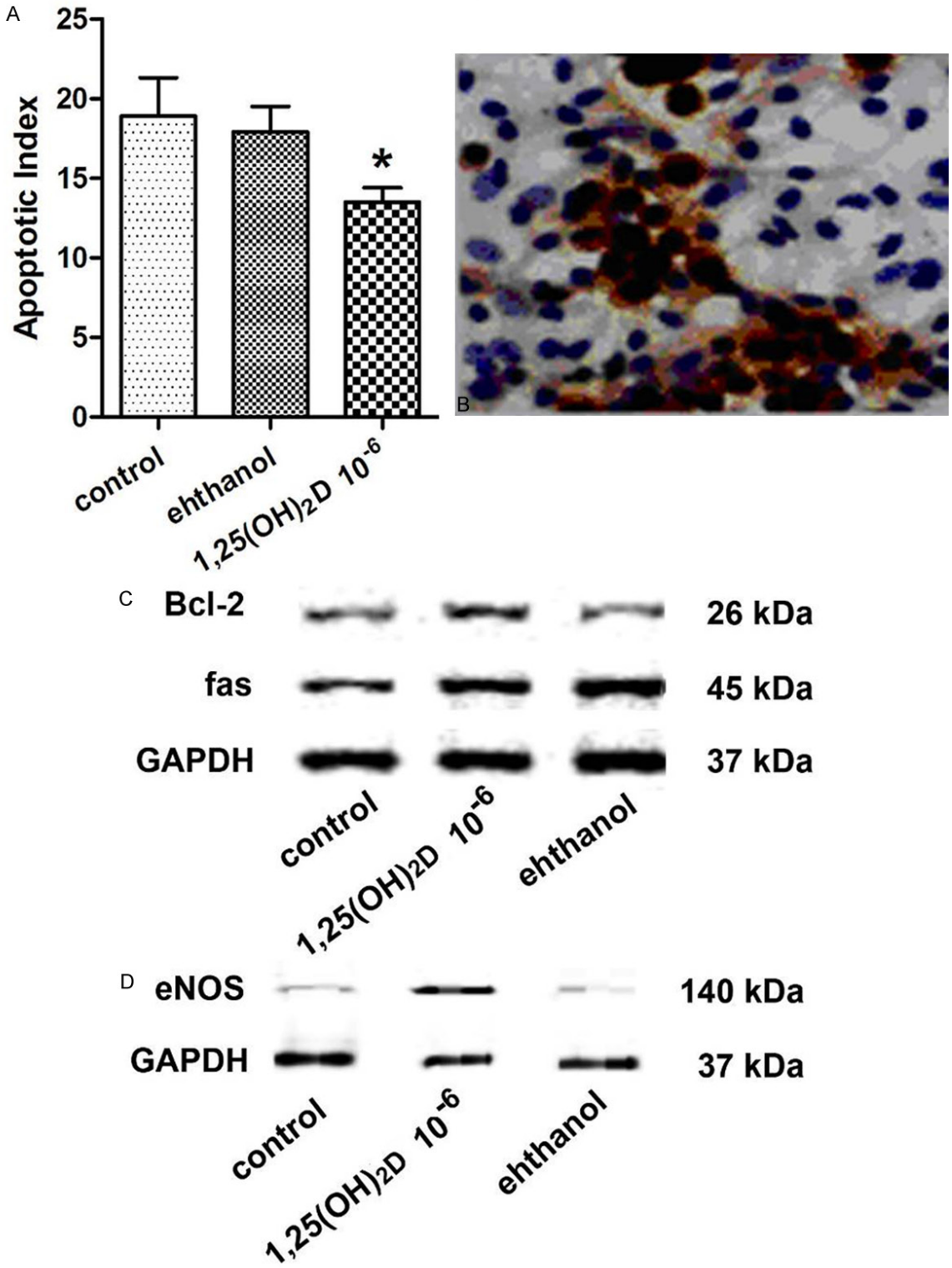

Figure 4. Effect of 1,25-$(OH)_2$D on endothelial progenitor cells Apoptosis. A. The apoptotic index of endothelial progenitor cells after1,25-$(OH)_2$D treatment. B. The representative TURNEL staining image of EC cells. C. The apoptotic marker proteins Bcl-2 and fas protein levels were measured by western blot. D. The eNOS protein levels of 1,25-$(OH)_2$D treated EC cells group was dramatically increased comparing to the ethanol or control treated groups. There was no statistical difference between control and ethanol treated groups.

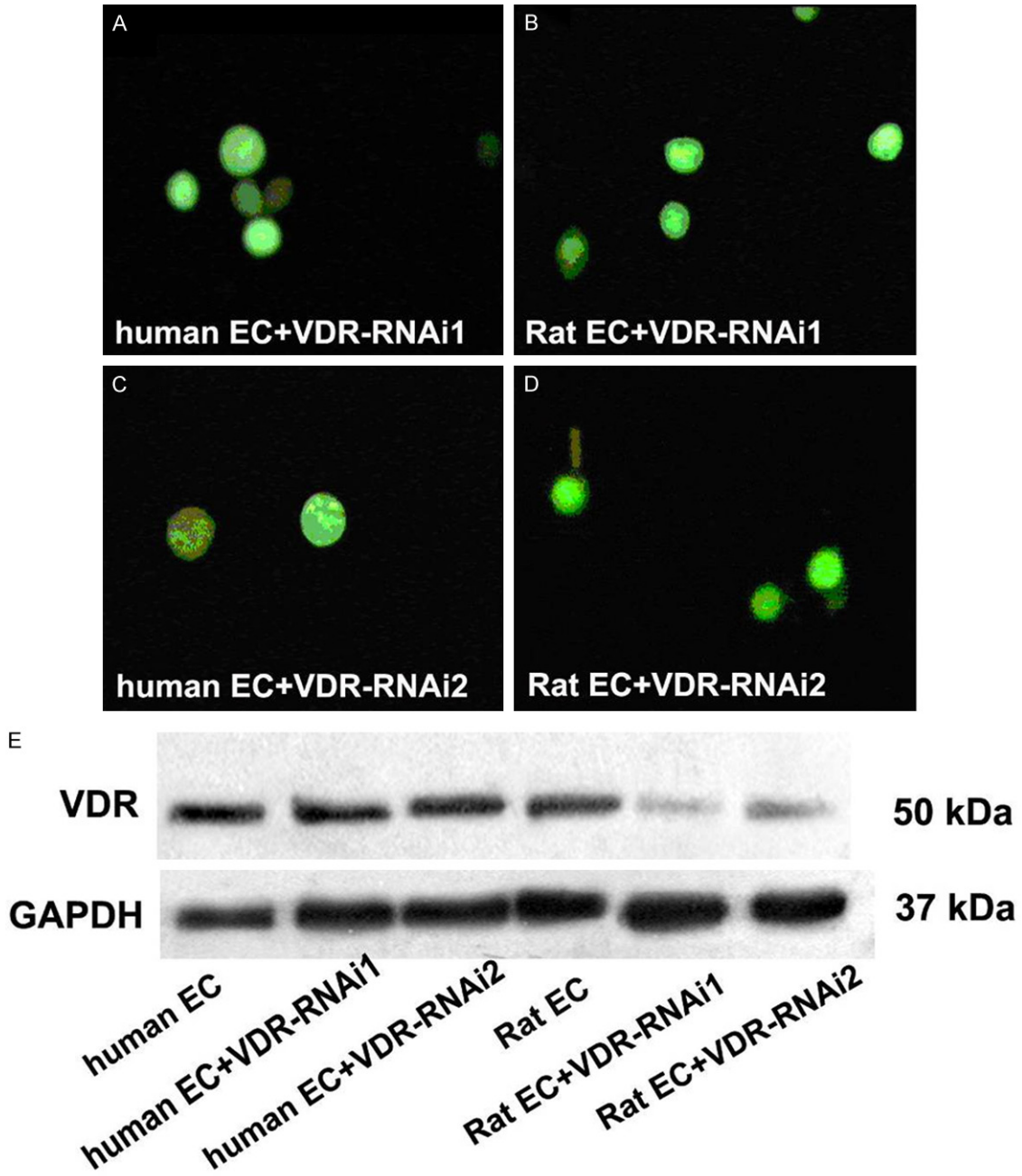

Figure 5. Construction of human and rat VDR RNA interference EC cells. Two VDR RNAi plasmids were constructed from rat and human race respectively. A-D. Representative images of rat or human VDR RNAi plasmids transfected EC cells. E. Western blot analysis was used to identify the effect of VDR RNAi plasmids transfection.

concentration and eNOS enzyme activity compared with wild type mice with or without high fat diet. These results demonstrated that high-fat diet enhanced the atheroscleros of ApoE-deficient mice by decreasing serum 1,25-$(OH)_2D$ levels and increasing NO levels and eNOS enzyme activity ($^*P<0.01$).

Upregulated vitamin D receptor epression in ApoE-deficient mice

Immunofluorescence analysis was used to assess the vitamin D receptor epression in aortas of four groups. Representative images showed that the vitamin D receptor epression

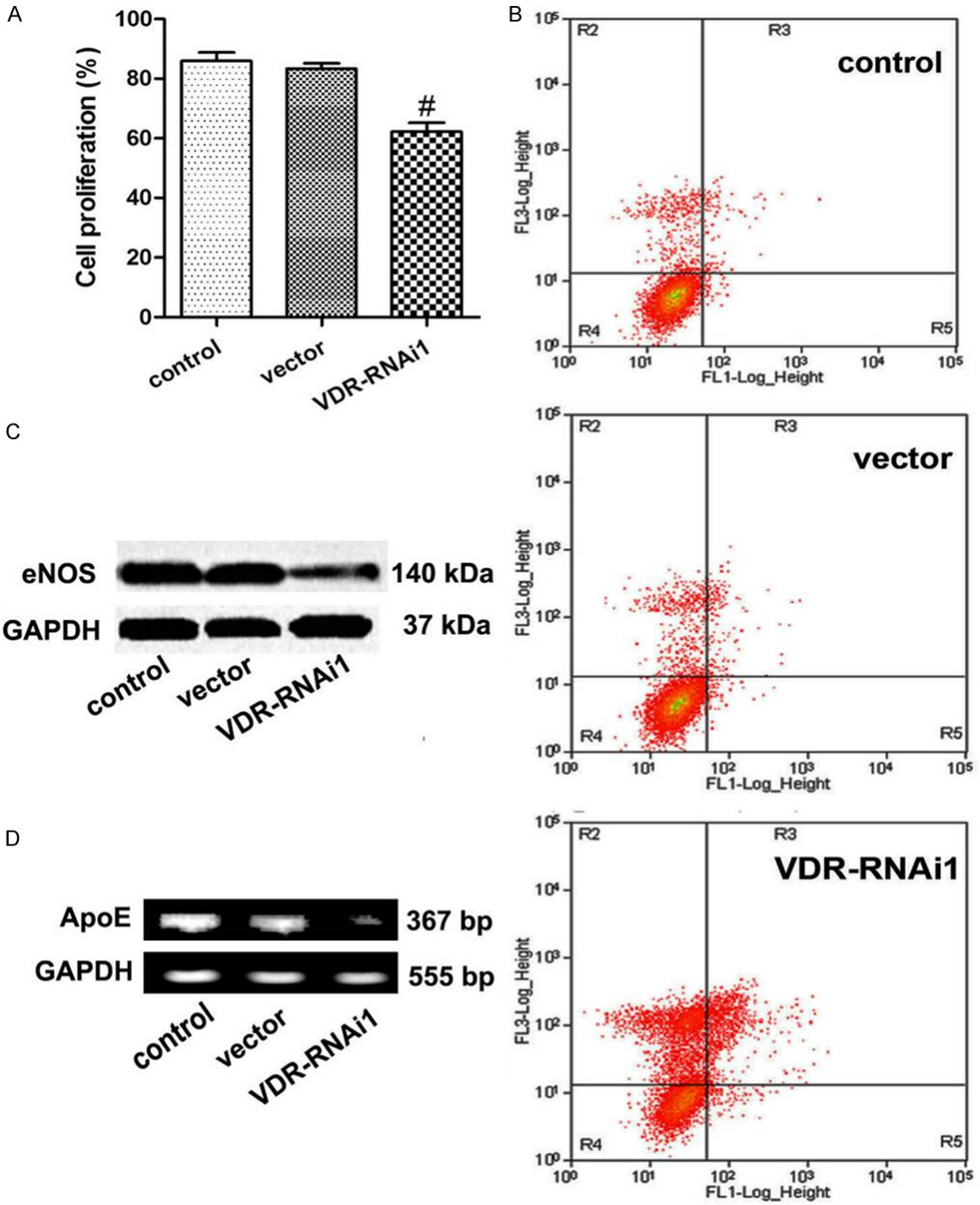

Figure 6. Effect of VDR-RNA1 on rat EC cells. A. Rat EC cells proliferation was measured in VDR-RNA1 transfected group, vector transfected and control group. VDR-RNA1 transfected EC cells proliferation was inhibited comparing to the control and vector transfected groups (n=3, $^{*}P<0.01$). B. Representative images of TURNEL staining of EC cells. The apoptosis index of VDR-RNA1 transfected EC cells was much higher than the control and vector transfected groups. C-D. VDR-RNA1 transfection also inhibit eNOS protein expression level and ApoE mRNA expression level.

level was upregulated in ApoE-deficient mice; the vitamin D receptor epression level was even higher after high fat diet treatment (**Figure 2A-D**). Western blot assay with VDR antibody confirmed this result (**Figure 2E**). Statistical analysis revealed that VDR expression levels

were higher in ApoE-deficient mice comparing to the wild type mice with or without high fat diet treatment (*P<0.01). This result was opposite to serum 1,25-$(OH)_2$D levels, which might including negative feedback regulation need further investigation.

Effect of 1,25-$(OH)_2$D on endothelial progenitor cells proliferation

The endothelial progenitor cells were susscessfully isolated (**Figure 3A**, **3B**) and identified by CD34 (**Figure 3C**) and VIII (**Figure 3D**) antibodies. Several concentrations of 1,25-$(OH)_2$D were selected to add into the culture of endothelial progenitor cells. **Figure 3E** showed the cell proliferation activity after 1,25-$(OH)_2$D treatment, endothelial progenitor cell proliferation activity was improved by 1,25-$(OH)_2$D. The 10^{-6} mol/L 1,25-$(OH)_2$D was used to the next experiment (*P<0.01, *P<0.05).

Effect of 1,25-$(OH)_2$D on endothelial progenitor cells apoptosis

Next, we evaluated endothelial progenitor cells apoptosis after 1,25-$(OH)_2$D treatment. **Figure 4A** showed apoptotic index of 1,25-$(OH)_2$D treated EC cells were lower comparing to the enthanol or control treated groups. **Figure 4B** was a representative image of TURNEL staining of EC cells. The apoptotic marker proteins Bcl-2 and fas protein levels were measured by western blot (**Figure 4C**). Bcl-2 protein expression level of 1,25-$(OH)_2$D treated EC cells group was dramatically increased comparing to the ethanol or control treated groups. However, fas protein level was dramatically decreased. The eNOS protein levels of 1,25-$(OH)_2$D treated EC cells group was dramatically increased comparing to the ethanol or control treated groups (**Figure 4D**). There was no statistical difference between control and ethanol treated groups. 1,25-$(OH)_2$D is the active form of vitamin D which could markbly supress EC cells apoptosis and promote Bcl-2 protein expression while downregulating fas protein levels, these results together implied vitamin D involving in EC cells apoptosis process.

Construction of human and rat VDR RNA interference EC cells

Two VDR RNAi plasmids were constructed from rat and human race respectively. Representative images of rat or human VDR RNAi plasmids transfected EC cells were shown in **Figure 5A-D.** Western blot analysis was used to identify the effect of VDR RNAi plasmids transfection, VDR-RNAi1 of rat EC had the highest inhibition efficiency **Figure 5E**.

Effect of VDR-RNA1 on rat EC cells

Rat EC cells proliferation was measured i*n* VDR-RNA1 transfected group, vector transfected and control group. VDR-RNA1 transfected EC cells proliferation was inhibited comparing to the control and vector transfected groups (**Figure 6A**, *P<0.01). **Figure 6B** showed representative images of TURNEL staining of EC cells. The apoptosis index of VDR-RNA1 transfected EC cells was much higher than the control and vector transfected groups. VDR-RNA1 transfection also inhibit eNOS protein expression level (**Figure 6C**) and ApoE mRNA expression level (**Figure 6D**).

Discussion

In the present study, we address a cardioprotective novel role for vitamin D receptor in circulating endothelial cells of ApoE-deficient mice. VDR deficiency not only promoted the development of atherosclerosis but also decreased the stability of atherosclerotic plaques.

Atherosclerosis is a pathological state of the vasculature that progress as dyslipidemia produces a state of endothelial dysfunction that ultimately leads to the accumulation of lipids Vitamin D plays a classical hormonal role in skeletal health by regulating calcium and phosphorus metabolism [11-13]. Vitamin D metabolites also have physiological functions in non-skeletal tissues, where local synthesis influences regulatory pathways via paracrine and autocrine mechanisms. The active metabolite of vitamin D, 1,25-dihydroxy vitamin D, binds to the vitamin D receptor that regulates numerous genes involved in fundamental processes of potential relevance to cardiovascular disease, including cell proliferation and differentiation, apoptosis, oxidative stress, membrane transport, matrix homeostasis, and cell adhesion [14, 15].

Our results demonstrated that high-fat diet enhanced the atherosclerosis of ApoE-deficient mice by decreasing serum 1,25-$(OH)_2$D levels

and increasing NO levels and eNOS enzyme activity. Treatment of normal aortas with exogenous peroxynitrite dramatically increased vascular O(2) (*-) production, seemingly from eNOS, because this effect was absent in vessels lacking endothelium, was blocked by NOS inhibition, and did not occur in vessels from mice lacking eNOS [16]. VDR expression levels were higher in ApoE-deficient mice comparing to the wild type mice with or without high fat diet treatment. This result was opposite to serum 1,25-$(OH)_2$D levels, which might including negative feedback regulation need further investigation.

1,25-$(OH)_2$D is the active form of vitamin D which could markbly supress EC cells apoptosis and promote Bcl-2 protein expression while downregulating fas protein levels, these results together implied vitamin D involving in EC cells apoptosis proces VDR-RNA1 transfected EC cells proliferation was inhibited comparing to the control and vector transfected groups. In conclusion, we provide evidence that VDR ablation associated with endothelial cells accelerates high-fat diet–induced atherogenesis and plaque instability by contributing to endothelial dysfunction and enhanced vascular inflammation. Given that acute clinical cardiovascular events occurred at sites of atherosclerosis due to a lack of effective ways of preventing the rupture of atherosclerotic plaques, our work highlights the important role of VDR as a therapeutic target in this clinical situation. Further clinical investigation is needed to determine whether increased VDR levels constitute an effective treatment for underlying atherosclerosis in patients or whether VDR is a potential target for the prevention of atherogenesis and the stabilization of atherosclerotic plaques.

Acknowledgements

This study was supported by Hainan Natural Science Foundation (No. 814314 and No. 309079).

Disclosure of conflict of interest

None

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