1α,25-Dihydroxyvitamin D3 targeting of NF-κB suppresses TNF-α induced adhesion molecules expression in human endothelial cells

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Abstract

Background Vitamin D and its analogues have been documented to be associated with endothelial dysfunction in various diseases. However, the underlying mechanism remains unknown. Here, we conducted an in vitro study to evaluate the effect of 1α,25-dihydroxyvitamin D3, the active form of vitamin D, on adhesion molecules expression in human endothelial cells. The possible mechanism involved in this process was also explored.

Methods Human umbilical vein cells (HUVECs) were cultured and treated according to the experiment requirement. Western Blot and RT-PCR were used to evaluate the expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin. ChIP assay, immunofluorescence, Western Blot and co-immunoprecipitation were used to assess the effect of 1α,25-dihydroxyvitamin D3 on NF-κB signaling.

Results 1α,25-dihydroxyvitamin D3 inhibited VCAM-1 and E-selectin mRNA and protein expression after TNF-α stimulation. ChIP assay showed that TNF-α increased the p65 binding to the promoter of VCAM-1 and E-selectin, which was suppressed by 1α,25-dihydroxyvitamin D3. 1α,25-dihydroxyvitamin D3 affected TNF-α induced IκBα phosphorylation and p65 NF-κB activation, leading to an inhibition of p65 nuclear translocation. These effects were reversed by a specific vitamin D receptor siRNA (VDR-siRNA). Co-immunoprecipitation revealed that 1α,25-dihydroxyvitamin D3 induced an increased binding of VDR to p65, which inhibited the ability of p65 binding to target gene promoters.

Conclusions 1,25-dihydroxyvitamin D3 suppresses TNF-α induced adhesion molecules expression in human endothelial cells by blocking the NF-κB pathway, and this was VDR dependent.

Background

Cardiovascular (CV) diseases remain the leading cause of death in modern societies with endothelial dysfunction being the common pathway of various risk factors. As an important non-traditional risk factor for CV events, vitamin D and its analogues have been documented to be associated with endothelial dysfunction in a diverse group of people, including healthy humans [1, 2], patients with chronic kidney disease (CKD) [3–7] [8], diabetes [9], lupus [10], stroke [11], obesity [12], and rheumatoid arthritis [13]. Although the physiological mechanisms linking vitamin D to endothelial dysfunction in these settings have not been established, it may involve vascular inflammation. During inflammatory process, the phenotype of endothelial cells turns to be activated [14]. And endothelial activation induces an upregulated expression of adhesion molecules, such as E-selectin and vascular cell adhesion molecule–1 (VCAM–1), which play a pivotal role in leukocyte-endothelium interactions, eventually leading to atherosclerosis and CV diseases.

Tumor necrosis factor-α (TNF-α) is one of the primary mediators of endothelial activation, which contributes to the inflammatory endothelial cells response and is initiated through activation of the classical NF-κB pathway [15]. Vitamin D has been documented to have anti-inflammatory effect in various diseases. It is reported that in healthy women, serum 25(OH)D concentrations are negatively
correlated with TNF-α concentrations [16]. Supplementation of paricalcitol, a vitamin D analogue, was associated with a reduction of serum levels of TNF-α in CKD patients [17]. The relationship between vitamin D and NF-κB signaling has also been reported. Suzuki et al. documented in their study that in human coronary arterial endothelial cells, vitamin D inhibits activation of NF-κB signaling pathway as well as the expression of its downstream target E-selectin [18]. However, it is still unknown which stage of NF-κB pathway is affected by vitamin D in endothelial cells. Here, we conducted an in vitro study to evaluate the effect of 1α,25-dihydroxyvitamin D3, the active form of vitamin D, on TNF-α induced adhesion molecules expression in human endothelial cells. We also explored the effect of 1α,25-dihydroxyvitamin D3 on various stages of NF-κB pathway, including early activation, nuclear translocation and its binding to VCAM–1 and E-selectin promoters, to provide additional insight into the molecular mechanisms linking vitamin D to endothelial function.

Results

**Treatment of HUVECs with 1α,25-(OH)₂D₃ inhibits TNF-α-induced VCAM–1 and E-selectin release**

As shown in Figure 1, when HUVECs were stimulated with TNF-α 40ng/ml for 24 h, the expression of VCAM–1 and E-selectin were significantly increased. Pretreatment of HUVECs with various concentrations of 1α,25-(OH)₂D₃ for 30min markedly reduced VCAM–1 and E-selectin mRNA and protein levels, with the maximum reduction observed in the middle concentration (10⁻⁸ M 1α,25-(OH)₂D₃) state. Therefore, vitamin D is able to suppress the induction of adhesion molecules by TNF-α in HUVECs. After transfected with a specific VDR-siRNA, the inhibitory effect of 1α,25-(OH)₂D₃ on expression of adhesion molecules was reversed, demonstrating that the inhibition is VDR-dependent. The degree of VDR knockdown by VDR-siRNA was assessed in these cells at both mRNA and protein level. As shown in Figure 2, a strong reduction of VDR transcripts and protein expression was observed by RT-PCR and western blots.

*NF-κB signaling pathway is critical for mediating HUVECs activation*

It is reported that NF-κB signaling pathway plays an important role in the process of endothelial activation. To confirm this, HUVECs were incubated with TNF-α 40ng/ml in the absence or presence of a specific NF-κB inhibitor (NF-κB SN50). As shown in Figure 3, inhibition of NF-κB signaling reduced TNF-α induced VCAM–1 and E-selectin expression in these cells, indicating that an intact NF-κB signaling is required for the activation of HUVECs.

**Effect of 1α,25-(OH)₂D₃ on NF-κB signaling pathway**

Having confirmed that NF-κB pathway is important in mediating VCAM–1 and E-selectin expression in HUVECs, we hypothesized that 1α,25-(OH)₂D₃ might inhibit VCAM–1 and E-selectin expression by...
modulating NF-κB pathway. To test this hypothesis, we examined the effect of 1α,25-(OH)2D3 on IkBα and p65 NF-κB phosphorylation, p65 NF-κB nuclear translocation, and p65 NF-κB binding to VCAM–1 and E-selectin promoters.

We first investigated whether 1α,25-(OH)2D3 can modulate the signaling events after p65 NF-κB nuclear translocation, in other words, p65 NF-κB binding to VCAM–1 and E-selectin promoters. As shown in Figure 4, ChIP assay demonstrated that 1α,25-(OH)2D3 abrogated the binding of p65 NF-κB to its cognate cis-acting element in VCAM–1 and E-selectin promoters in HUVECs. After transfected with a specific VDR-siRNA, the inhibitory effect of 1α,25-(OH)2D3 on p65 DNA binding was reversed. This result indicates that 1α,25(OH)2D3 blunts TNF-α induced adhesion molecules expression by blocking the NF-κB binding activity.

We further investigated the p65 NF-κB nuclear translocation after various treatments. As shown in Figure 5, immunofluorescence staining HUVECs with anti-p65 antibody demonstrated that upon stimulation with TNF-α, p65 NF-κB rapidly translocated into the nuclei in HUVECs, and 1α,25-(OH)2D3 can inhibit the nuclear translocation. However, 1α,25-(OH)2D3 did not affect p65 NF-κB nuclear translocation in HUVECs transfected with a specific VDR-siRNA.

Since IkBα is the major inhibitor of NF-κB that binds to p65 NF-κB and blocks its activation and subsequent nuclear translocation, we explored further the effect of 1α,25(OH)2D3 on IkBα phosphorylation, as well as p65 NF-κB phosphorylation and activation. As shown in Figure 6, when HUVECs were incubated with TNF-α 40 ng/ml, IkBα and p65 NF-κB were rapidly phosphorylated. Pretreatment of HUVECs with 10^{-8} M 1α,25-(OH)2D3 significantly inhibited IkBα and p65 NF-κB phosphorylation. In HUVECs transfected with a specific VDR-siRNA, the inhibitory effect of 1α,25-(OH)2D3 on IkBα and p65 NF-κB phosphorylation was reversed. Therefore, 1α,25(OH)2D3 appeared to suppress NF-κB signaling, at least in part, by reducing IkBα and p65 NF-κB phosphorylation in HUVECs.

**Effect of 1α,25-(OH)2D3 on the interaction between VDR and p65 NF-κB**

It is reported that 1α,25-(OH)2D3 can upregulate the expression of VDR. Meanwhile, in human proximal tubular cells, vitamin D abolishes p65 NF-κB binding to RANTES promoter by facilitating VDR/p65 interaction. Our aforementioned results that effect of 1α,25-(OH)2D3 can be reversed by a specific VDR-siRNA suggests that vitamin D exerts its beneficial effect at least partly through VDR. Thus, we further explored the effect of 1α,25-(OH)2D3 on VDR expression and the interaction between VDR and p65 NF-κB in HUVECs. As presented in Figure 7, 1α,25-(OH)2D3 upregulated the expression of VDR in HUVECs. Meanwhile, in HUVECs that overexpressed p65 NF-κB, increased p65 was detected in the cell lysates precipitated by anti-VDR antibody after stimulation by TNF-α and/or 1α,25-(OH)2D3 (Figure 8). Hence, it is possible that an increased formation of VDR/p65 complex after 1α,25-(OH)2D3 treatment reduced the
level of free p65, thereby affected its binding to promoters of targeting genes, resulting in a inhibition of p65-mediated gene transcription.

**Discussion**

This study demonstrated that 1α,25-(OH)2D3, the active form of vitamin D, suppresses TNF-α induced upregulation of VCAM–1 and E-selectin expression in HUVECs. Mechanically, 1α,25-(OH)2D3 targets NF-κB, a principal signaling pathway involved in the regulation of inflammatory reaction in various circumstances, in a VDR-dependent manner. 1α,25-(OH)2D3 can also upregulate the expression of VDR, promote the binding of VDR to p65, and thus inhibit the ability of p65 binding to VCAM–1 and E-selectin gene promoters. Since endothelial activation is a critical process that contributes to the pathogenesis of atherosclerosis and CV diseases, inhibition of endothelial activation may be an important mechanism by which vitamin D exerts its beneficial activity in ameliorating CV events.

As a fat-soluble secosteroid, vitamin D is responsible for calcium and phosphorus metabolism, and multiple other biological effects, including regulation of immune system and endothelial function. We have shown in our previous study that in CKD patients, hypovitaminosis D was associated with decreased brachial artery flow-mediated dilation (FMD) and increased VCAM–1 and E-selectin [7], and vitamin D supplementation can improve endothelial function [8]. The present study revealed that 1α,25-(OH)2D3 suppresses TNF-α induced high expression of VCAM–1 and E-selectin in HUVECs, which provides additional evidence for the endothelial protective effect of vitamin D through in vitro experiment. In agreement with our findings, Martinesi et al [19] demonstrated that 1α,25(OH)2D3 was able to reduce VCAM–1 level previously enhanced by TNF-α, though in their study this effect was not seen on E-selectin expression, which might be explained by the experimental conditions. In fact, in their experiments, HUVECs were incubated with the association of TNF-α and 1α,25(OH)2D3, adding the two compounds at the same time.

Another question worth to mention is the intervention concentration of 1α,25(OH)2D3. In this study we pretreated HUVECs with various concentrations of 1α,25-(OH)2D3 (10−9 to 10−7 M) and found that 1α,25(OH)2D3 at 10−8 M had the strongest inhibitory effect on TNF-α induced adhesion molecules expression. Likewise, Kudo et al [20], showed in their study that, in human coronary arterial endothelial cells (HCAECs), 1α,25(OH)2D3 at a concentration of 10−8 M also had a slightly stronger suppressive effect on VCAM–1 expression after stimulation with TNF-α in contrast to 1α,25(OH)2D3 concentrations of 10−7 M and 10−9 M. Martinesi et al [19]. reported that in the absence of growth factors, 1α,25(OH)2D3 at 10−8 M had a mildly stronger inhibitory effect on the expression of adhesion molecules in HUVECs when compare to 1α,25(OH)2D3 at 10−7 M. Since the hormone alone cannot alter the expression of adhesion molecules [19], it’s possible that a higher concentration (10−7 M) may have severer inhibitory effects on HUVECs proliferation (as reported by Zehnder et al. [21]), and thus leads to a decreased expression of these molecules. Of note, there is also a study showing that 1α,25(OH)2D3 at 10−7 M had more obvious inhibitory effect on E-selectin expression as well as NF-κB activation when compared to
10^{-8} \text{ M} \text{ and } 10^{-9} \text{ M} \text{ in HCAECs} \text{ [18]. Therefore, it's reasonable to use concentrations of } 1\alpha,25-(\text{OH})2\text{D3 consistent with levels obtained in healthy human plasma after administration of a normal dose, that is } 10^{-9} \text{ to } 10^{-7} \text{ M as reported by literatures [22, 23].}

Inhibition of NF-\kappa B signaling with NF-\kappa B SN50 reduced TNF-\alpha stimulated VCAM–1 and E-selectin expression in HUVECs, indicating that an intact NF-\kappa B signaling is required for inflammatory cytokine induced endothelial activation. Previous investigations have reported that expression of NF-\kappa B was greater in vascular endothelial cells of subjects with vitamin D deficiency [24]. Hence, it's possible that vitamin D exerts its inhibitory effect on endothelial activation by inhibiting NF-\kappa B signaling pathway. It is well known that the activity of NF-\kappa B includes I\kappa B\alpha and p65 NF-\kappa B phosphorylation, p65 NF-\kappa B nuclear translocation, and p65 NF-\kappa B binding to target gene promoters. Thus, the activity of NF-\kappa B can be regulated at multiple sites. The present study demonstrated that 1\alpha,25-(\text{OH})2\text{D3 can affect p65 NF-\kappa B binding to VCAM–1 and E-selectin promoters. Although the exact mechanism by which vitamin D disrupts this interaction remains unclear, our data suggest that, in HUVECs, at least part of the mechanism stems from an increase or stabilization of I\kappa B\alpha, thus abolishing p65 nuclear translocation. In agreement with our findings, several studies reported that in mesangial cells, pancreatic islet cells and mouse embryonic fibroblasts, vitamin D can also inhibit NF-\kappa B signaling through increasing I\kappa B\alpha and reducing p65 NF-\kappa B nuclear translocation, and eventually abolishing its binding to gene promoters [25–27]. Based on this point, more studies are needed to elucidate how vitamin D regulates I\kappa B\alpha. However, it should be noted that in human proximal tubular cells, paricalcitol, a vitamin D analogue, influences neither I\kappa B\alpha nor p65 nuclear translocation, but only abolishes the binding of p65 to target gene promoter [28]. Although the exact reason behind this discrepancy is still unknown, it could be related to different cell types.

As expected, in HUVECs transfected with a specific VDR-siRNA, the inhibitory effect of 1\alpha,25(\text{OH})2\text{D3 on VCAM–1 and E-selectin expression as well as NF-\kappa B signaling pathway was abolished, indicating that VDR is required to mediate the repressive action of 1\alpha,25(\text{OH})2\text{D3. However, 1\alpha,25-(\text{OH})2\text{D3 can upregulate the expression of VDR in HUVECs, which physically interacts with p65 and potentially blocks p65 binding to DNA. This increased VDR expression and VDR–p65 physical association may serve as another mechanism by which vitamin D destroys the binding of p65 to gene promoters. In fact, it was also reported in fibroblasts, human proximal tubular cells and osteoblasts [26, 28, 29], but not in mesangial cells [27], which might be explained by the cell-type specificity.}

Though our data strongly indicate that 1\alpha,25(\text{OH})2\text{D3 inhibits endothelial adhesion molecules expression primarily by triggering a VDR-mediated sequestration of NF-\kappa B pathway, it remains to be elucidated whether this mechanism is applicable in vivo. Besides, 1\alpha,25(\text{OH})2\text{D3 may regulate endothelial activation by other routes as well, given that vitamin D has pleiotropic effects.}

**Conclusions**
In conclusion, we have shown in this study that 1α,25-(OH)2D3 can inhibit proinflammatory cytokine induced endothelial activation, and this potentially endothelial protective role seems to be mediated by its ability to induce the VDR-mediated sequestration of NF-κB signaling. In view of the importance of endothelial cell activation in the occurrence and development of CV events, the endothelial protective effect of 1α,25-(OH)2D3 may become a novel target for the prevention and treatment of such diseases.

Methods

Cell culture

Normal cryopreserved HUVECs (obtained from KeyGEN BioTECH, Nanjing, China) were thawed rapidly in 37–40 degrees water bath and grown in F12K medium (KeyGEN BioTECH, Nanjing, China) at 37 °C in a 5% CO2 humid incubator. When the cells reached 80% confluence, they were passaged using 0.25% Trypsin (KeyGEN BioTECH, Nanjing, China). Cells at passages 3 to 4 were used for experiments.

Cytokine treatment

HUVECs were incubated with various concentrations of 1α,25-(OH)2D3 (Sigma-Aldrich, USA) for 30min and then exposed to 40 ng/ml TNF-α (PeproTech Inc. USA) for 24 h, unless otherwise indicated. For blocking NF-κB signaling, HUVECs were pretreated with a specific NF-κB inhibitor NF-κB SN50 (MCE, USA) for 1 h and then incubated with TNF-α 40ng/ml for 24h. For assessing NF-κB early activation, HUVECs were pretreated with 1α,25-(OH)2D3 for 30min, followed by incubation with TNF-α for various periods as indicated.

Transfection with VDR-siRNA

HUVECs were seeded into 24-well plates (Corning Incorporated, USA) at a density of 5.0 × 10⁵ cells per well in 500μl of antibiotic-free medium and incubated for 24 hours prior to transfection. Cells were then incubated with a mixture of siRNA (200 nM final concentration), medium and lipofectamine 2000 buffer (Invitrogen, Carlsbad, CA, USA) for 4 h, washed and incubated for further 48 h at 37°C. The nucleotide sequence of VDR-siRNA is 5'-UCCGUGCCUCTGGCTTTCUCTTCUUTT–3', directed against a sequence of the human VDR gene started at 842. siRNA-mediated knock-down efficiency was checked by quantitative Western-Blot and RT–PCR.

RNA isolation and quantitative RT–PCR analysis

HUVECs were treated with various reagents as indicated. Total RNA was extracted from HUVECs using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNAs synthesized from total RNAs were used as templates for PCR amplification with gene-specific primer pairs. The sequences
of the primer sets were as follows: E-selectin, 5’-CCGAGCGAGGCTACATGAAT–3’ (forward), and 5’-GAGACTCAGCTGGACCC–3’ (reverse); VCAM–1, 5’-TCGTGATCCTTGGAGCGCTCA–3’ (forward), and 5’-AGGAAAAGAGCTCTGCTGC–3’ (reverse); and VDR, 5’-GCTTGTCAAAAGGCGGCAG–3’ (forward), and 5’-CCAAAGGTCTTGTCGG–3’ (reverse). The internal control for the PCR reaction was GAPDH. PCR application was performed in an ABI Step one plus Real time-PCR system.

**Western Blot Analysis**

The preparation of whole-cell lysates and Western blot analysis of protein expression were carried out using routine procedures. Briefly, HUVECs after various treatments as indicated were washed with ice-cold PBS and lysed in the presence of Protease Inhibitor Cocktail (Sigma-Aldrich, USA) for 15 min in ice-cold lysis buffer. Cell extracts were centrifuged at 14,000 x g for 15 min in a 4°C pre-cooled centrifuge and the supernatant was stored at −70°C. For Western blot analysis, protein lysates were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membrane and immunoblotted using standard protocols. Proteins were visualized using horseradish peroxidase detection reagents according to manufacturer's instructions (New-SUPER ECL, KeyGEN BioTECH, Nanjing, China), and exposed to autoradiographic film (G:BOX chemiXR5). Gel-Pro32 software was used to grayscale the results. The primary antibodies: Anti-VCAM–1 (ab134047), anti-E-selectin (ab18981), anti-p65 NF-κB (ab32536), anti–phospho-p65 NF-κB (phospho S529) (ab109458), anti-IκBα (ab32518), anti–phospho-IκBα (phospho S36) (ab133462) and anti-VDR (ab109234), were all obtained from Abcam (Cambridge, UK).

**ChIP assays**

NF-κB binding to VCAM–1 and E-selectin gene promoters in HUVECs was determined by ChIP assays using a commercially available ChIP assay kit (Lake Placid, NY, USA). Briefly, HUVECs after various treatments as indicated were treated with 1% formaldehyde to cross-link histones to DNA, and then treated with stop buffer for 5 minutes to stop the cross-linking. The chromatin was extracted and fragmented by sonication. The sonicated chromatin were incubated with anti-p65 antibody overnight at 4°C, followed by incubation with protein A–agarose for 2 h. The precipitates were washed, and chromatin complexes were eluted. After reversal of the cross-linking, the DNA was purified, and used as templates for PCR using the primers flanking the NF-κB binding sites in VCAM–1 and E-selectin gene promoters. The sequences of primers used for ChIP assay were as follows: VCAM–1, 5’- GAGGAGCAGGTAGGACTT–3’ (forward), and 5’- CTGAGGTCTGGAATCTATAACT–3’ (reverse); and E-selectin, 5’-GCCTTCACCTCAGCCTTGAG–3’ (forward), and 5’-ACATTGTGGCAACATCAGTATCCT–3’ (reverse). The PCR products were run on 1.5% agarose gel and stained with ethidium bromide.

**Co-Immunoprecipitation**
Co-Immunoprecipitation was carried out using an established method. Briefly, HUVECs were transfected with NF-κB expression vector (pNF-κB/p65) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for 48h, and then incubated with or without TNF-α 40 ng/ml and/or 1α,25-(OH)2D3 (10⁻⁸) for 1h, before subjected to co-immunoprecipitation. Cells were lysed in 1 ml of non-denaturing lysis buffer that contained 10μl phosphatase inhibitors, 1μl protease inhibitor, and 5μl 100mM PMSF. Cell lysates were incubated with 2μg of anti-VDR overnight at 4°C, followed by precipitation with 20 μl of Protein G Agarose for 3h at 4°C. The precipitated complexes were separated on 10% SDS-PAGE gels and immunoblotted with anti-p65 NF-κB antibody.

**Immunostaining**

HUVECs were pretreated with or without 10⁻⁸ M 1α,25(OH)2D3 for 24 h and then exposed to TNF-α 40ng/ml for 1h. The cells were fixed with 4% paraformaldehyde for 30 min, and incubate with normal goat serum for 20 min to block unspecific binding of the antibodies. After that, cells were stained with anti-p65 antibody for 2h in a humidified chamber, followed by staining with fluorescein isothiocyanate (FITC)–conjugated secondary antibody (Abcam, Cambridge, UK) for 1h in the dark. Each slide was stained with 4-diamidino–2-phenylindole (DAPI) for 5min to visualize the nucleus. Slides were viewed with an Olympus IX51 microscope equipped with a digital camera (Japan).

**Statistical Analyses**

Analyses were performed by IBM SPSS statistics 22. Results are presented as mean ± standard deviation (SD). Differences between groups were assessed by t test. Statistical significance was assumed at a two-tailed value of \( P < 0.05 \).

**Abbreviations**

HUVECs: Human umbilical vein cells; NF-κB: Nuclear factor-κB; VCAM–1: Vascular cell adhesion molecule–1; ChIP: Chromatin immunoprecipitation; VDR: Vitamin D receptor; VDR-siRNA: Vitamin D receptor-small interfering RNA; CV: Cardiovascular; CKD: Chronic kidney disease; TNF-α: Tumor necrosis factor-α; FMD: Flow-mediated dilation; HCAECs: Human coronary arterial endothelial cells; RT-PCR: Real-time polymerase chain reaction.

**Declarations**

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Authors’ contributions

Z-QY and J-CM designed the study. Z-QY, Z-YS, and FY performed the experiments. LJ and JB analyzed the data. Z-QY ZW, SC and C-DW made the figures. Z-QY drafted and revised the manuscript. J-CM, S-QY and T-TF revised the manuscript. ZM supervised the experimental work. All authors read and approved the final version of the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**

**Figure 1**

1α,25-(OH)2D3 inhibits TNF-α induced VCAM-1 and E-selectin expression in HUVECs in vitro. HUVECs transfected with or without a specific VDR-siRNA were incubated with 40 ng/ml TNF-α in the presence or absence of various concentrations of 1,25-(OH)2D3 as indicated. Western blot (A, B and C) and RT-PCR (D and E) showed that 1α,25-(OH)2D3 inhibited VCAM-1 and E-selectin expression induced by TNF-α in HUVECs, and the inhibitory effect can be reversed by VDR-siRNA. Data are mean ± SD of three experiments. *, P< 0.05 versus control; †, P< 0.05 versus TNF-α treated alone; ‡, P< 0.05 versus the groups with same concentrations of 1α,25-(OH)2D3.
**Figure 2**

Western blot (A and B) and RT-PCR (C) for VDR in lysates from cells transfected with VDR-siRNA and control. Data are mean ± SD of three experiments. *, P< 0.05 versus control.

**Figure 3**

NF-κB signaling is critical for mediating VCAM-1 and E-selectin expression in HUVECs. HUVECs were treated with 40 ng/ml TNF-α in the absence or presence of NF-κB inhibitor. (A,B and C) Western blot and (D and E) RT-PCR showed that Inhibition of NF-κB signaling using NF-κB SN50 abrogated TNF-α induced VCAM-1 and E-selectin expression in HUVECs. Data are mean ± SD of three experiments. *, P< 0.05 versus TNF-α treated alone.
Figure 4

1α,25-(OH)2D3 inhibits p65 NF-κB binding to VCAM-1 and E-selectin promoters in HUVECs. HUVECs transfected with or without a specific VDR-siRNA were treated with TNF-α in the absence or presence of 10-8 M 1α,25-(OH)2D3 for 24h.
Figure 5

1α,25-(OH)2D3 affect TNF-α induced p65 NF-κB nuclear translocation in HUVECs. HUVECs transfected with or without a specific VDR-siRNA were pre-treated with or without 10-8 M 1α,25(OH)2D3 for 24 h. The cells were then exposed to TNF-α 40ng/ml for 1h, fixed and stained with anti-p65 antibody or 4-diamidino-2-phenylindole (DAPI) to visualize the nucleus. The cells were observed under a fluorescent microscope.

Figure 6
1α,25-(OH)2D3 can inhibit TNF-α induced NF-κB early activation. HUVECs transfected with or without a specific VDR-siRNA were pre-treated with or without 10-8 M of 1α,25(OH)2D3 for 30min, followed by incubation with TNF-α 40 ng/ml for various periods as indicated. Whole cell lysates were immunoblotted with indicated antibodies. A: Western blot demonstrated that 1α,25(OH)2D3 significantly inhibited TNF-α induced IκBα phosphorylation. B: Densitometric quantification of p-IκBα protein bands normalized to IκBα. C: 1α,25(OH)2D3 significantly altered TNF-α induced p65 NF-κB phosphorylation. D: Densitometric quantification of p-p65 protein bands normalized to p65.

Figure 7

Effect of 1α,25-(OH)2D3 on the expression of VDR in HUVECs. HUVECs were treated with 40 ng/ml TNF-α or 10-8 M 1α,25-(OH)2D3 for 24h. Western blot (A) and RT-PCR (B) for VDR in lysates from cells. Data are mean ± SD of three experiments. *, P< 0.05 versus control.

Figure 8

Effect of 1α,25-(OH)2D3 on the interaction between VDR and p65 NF-κB in HUVECs. Co-immunoprecipitation showed that 1α,25-(OH)2D3 increased the binding of VDR to p65 NF-κB. HUVECs transfected with p65 NF-κB expression vector were treated with TNF-α, 1α,25-(OH)2D3, or both. Cell lysates were then immunoprecipitated with anti-VDR antibody, followed by immunoblotting with anti-p65.
Whole cell lysates were also immunoblotted with anti-p65 to normalize p65 abundance. Data from three independent experiments are shown. *, P <0.05 versus control, #, P <0.05 versus TNF-α or 1α,25-(OH)2D3 treated alone.