1α,25-Dihydroxyvitamin D3 inhibits vascular cellular adhesion molecule-1 expression and interleukin-8 production in human coronary arterial endothelial cells

ヒト冠動脈血管内皮細胞における活性型ビタミンDの血管細胞接着分子-1発現とインターロイキン8産生の抑制効昊

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【研究の背景】

川崎病は，遷延する発熱，眼球結膜の充血，口唇の発赤・いちご舌，手足の硬性浮腫，不定形発疹，非化膿性頸部リンパ節腫脹を特徴とする，乳幼児に好発する全身性血管炎である．川崎病の病理学的特徴は，血管内皮細胞壊死を伴う汎血管炎で，中小血管への単核細胞浸潤が特徴である．川崎病の最も重要な合併症は冠動脈病変で，虚血性心疾患の原因となりうる冠動脈狭窄を形成することがある．約 10%の川崎病患者が，標準的な高用量ガンマグロブリン静注療法 (IVIG) に抵抗性であり，新しい治療介入案が望まれている．当研究室はこれまでに，炎症性サイトカインを制御する細胞内転写因子である nuclear transcription factor-κB (NF-κB) 活性化および tumor necrosis factor-α (TNF-α) などのサイトカインの産生上昇が，川崎病の炎症病態において重要な役割を果たしていることを明らかにしてきた．また川崎病急性期では，動脈内皮細胞が活性化し末梢血中で様々な可溶性接着分子濃度が上昇していることが報告されている．活性型ビタミン D ($1\alpha,25\text{-}(OH)_2D_3$) は電解質や骨格構造の維持に関与することは以前より報告されているが，近年抗炎症作用や免疫調節作用についての報告が散見される．活性型ビタミン D は，マクロファージにおける TNF-α 産生や NF-κB 活性を制御し，ヒト臍静脈内皮細胞で TNF-α に誘導された細胞間接着分子 (ICAM-1) および血管細胞接着分子 (VCAM-1) 発現を抑制すると報告されている．これらの報告より，活性型ビタミン D は急性期の川崎病血管炎に効果がある可能性が考えられた．

【目的】

ヒト冠動脈血管内皮細胞 (HCAECs) における接着分子発現と炎症性サイトカイン産生は，転写因子により制御されている．近年，活性型ビタミン D は抗炎症作用を有することが明らかになっている．当研究室ではこれまでに，HCAECs において活性型ビタミン D が TNF-α 刺激による NF-κB 活性化および E-selectin 発現を抑制することを報告してきた．本研究では，HCAECs における活性型ビタミン D の TNF-α 刺激による接着分子 (ICAM-1 と VCAM-1) 発現と，サイトカイン（インターロイキン-6 (IL-6)および IL-8）産生の抑制効果について検討した．

【方法】

HCAECs において，TNF-α 刺激による ICAM-1，VCAM-1 発現に対する活性型ビタミン D の抑制効果を，フローサイトメトリー法で測定した．同じく IL-6，IL-8 産生に対する活性型ビタミン D の抑制効果を ELISA 法で，さらに VCAM-1 および IL-8 mRNA 発現に対する活性型ビタミン D の抑制効果を real-time PCR 法で測定した．

【結果】

活性型ビタミンDで前処置したHCAECsにおいて，TNF-α刺激によるVCAM-1発現増加は10^{-7}M，10^{-8}Mの活性型ビタミンD下で有意に抑制された．10^{-8}M活性型ビタミンD下でVCAM-1発現は，同刺激4時間後に有意に抑制された．またIL-8産生増加は，10^{-8}Mの活性型ビタミンD下で有意に抑制された．10^{-8}M活性型ビタミンD下でIL-8産生は，同刺激4，8，12および24時間後に有意に抑制された．一方，ICAM-1発現とIL-6産生増加に活性型ビタミンDの前処置で抑制効果は認められなかった．VCAM-1 mRNA発現増加は，10^{-7}M，10^{-8}Mの活性型ビタミンD下で同刺激2，4時間後に有意に抑制され，IL-8 mRNA発現増加は，10^{-8}Mの活性型ビタミンD下で同刺激2，4および8時間後に有意に抑制された．

【考察】

本研究では，HCAECsにおいて活性型ビタミンDは，TNF-α刺激によるVCAM-1発現およびIL-8産生増加を有意に抑制したが，ICAM-1発現とIL-6産生増加に抑制効果を認めなかった．HCAECsにおけるTNF-α刺激による活性型ビタミンDのICAM-1とVCAM-1発現およびIL-6とIL-8産生抑制効果の違いについて，それらの遺伝子の複雑な転写調整の結果であると推測している．

本研究で使用した活性型ビタミンD濃度は，常用量投与での健常人の血漿中濃度と整合している．近年活性型ビタミンDはベーチェット病，炎症性腸疾患，多発性硬化症，敗血症，および炎症性多発関節炎などの炎症性疾患に対する補助療法としての可能性が報告されている．川崎病患者におけるIVIG療法は，冠動脈病変の発生率を低下させるが，少なくとも10%は不応症例である．本研究では，活性型ビタミンDにTNF-α刺激後4時間以降で抗炎症効果が認められた．従って本研究の結果から，活性型ビタミンDが，川崎病急性期において抗炎症効果を示す可能性が示唆された．しかも活性型ビタミンDは小児で広く使用され，かつ副作用がほとんどなく安全性が高いため，臨床応用へ導きやすいと考えられる．

【結語】

川崎病血管炎in vitroモデルにおいて，活性型ビタミンDは抗炎症効果を示し，川崎病の補助治療薬になる可能性が示唆された．

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Original Article

1α,25-dihydroxyvitamin D_3 inhibits vascular cellular adhesion molecule-1 expression and interleukin-8 production in human coronary arterial endothelial cells

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Abbreviations:

1α,25-$(OH)_2D_3$—1α,25-dihydroxyvitamin D_3

AP-1—Activator protein-1

ELISA—Enzyme-linked immunosorbent assay

HCAECs—Human coronary arterial endothelial cells

ICAM-1—Intercellular adhesion molecule-1

IL—Interleukin

IVIG—Intravenous immunoglobulin

KD—Kawasaki disease

NF-κB—Nuclear transcription factor-κB

RANTES—Regulated on activation normal T cell expressed and secreted

SD—Standard deviation

TNF-α—Tumor necrosis factor-α

VCAM-1—Vascular cellular adhesion molecule-1

PCR—polymerase chain reaction

Abstract

Kawasaki disease is an acute febrile vasculitis of childhood that is associated with elevated production of inflammatory cytokines, causing damage to the coronary arteries. The production of proinflammatory cytokines and expression of adhesion molecules in human coronary arterial endothelial cells (HCAECs) is regulated by nuclear transcription factor-κB (NF-κB) activation. We have previously reported that the active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), inhibits tumor necrosis factor-α (TNF-α)-induced NF-κB activation. In this study, we examined the anti-inflammatory effects of 1α,25-$(OH)_2D_3$ on TNF-α–induced adhesion molecule expression (vascular cellular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1)) and cytokine production (interleukin-6 (IL-6) and IL-8) in HCAECs. Pretreatment with 1α,25-$(OH)_2D_3$ significantly inhibited TNF-α–induced VCAM-1 expression and IL-8 production in HCAECs. Our results suggest that adjunctive 1α,25-$(OH)_2D_3$ therapy may modulate the inflammatory response during Kawasaki disease vasculitis.

Key words: Kawasaki disease; Coronary arterial endothelial cells; IL-8; VCAM-1; 1α,25-dihydroxyvitamin D_3

1. Introduction

Kawasaki disease (KD) is an acute illness of early childhood that is characterized by prolonged fever, diffuse mucosal inflammation, persistent edema of the extremities, polymorphous skin rash, and non-suppurative lymphadenopathy [1]. Histopathology results show that patients display panvasculitis with endothelial necrosis, as well as infiltration of mononuclear cells into small and medium-sized blood vessels [2]. The primary and most significant complication of KD is coronary arterial lesions, which may cause significant coronary stenosis, resulting in ischemic heart disease [3]. In earlier studies, we showed that activation of nuclear transcription factor-κB (NF-κB), a transcription factor regulating the expression of proinflammatory cytokines, and tumor necrosis factor-α (TNF-α) activity play important roles in the pathogenesis of KD [4–8]. In addition, arterial endothelial cells are known to be activated in acute KD, because the levels of soluble forms of various adhesion molecules are elevated in the peripheral blood [9,10].

The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) or calcitriol is associated with calcium and phosphorus homeostasis and maintenance of skeletal architecture [11]. Recently, several studies have reported that 1α,25-$(OH)_2D_3$ exhibits anti-inflammatory and immunomodulatory effects [11–13]. TNF-α production and NF-κB activation in macrophages, and TNF-α–induced expression of intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cellular adhesion molecule-1 (VCAM-1, CD106) in human umbilical vein endothelial cells are inhibited by 1α,25-$(OH)_2D_3$ [13–18]. These findings put forward the possibility that 1α,25-$(OH)_2D_3$ could be effective in treating endothelial cell inflammation in acute KD. In addition, approximately 10% of KD patients are resistant to the standard high-dose intravenous immunoglobulin (IVIG) therapy, highlighting the need for new approaches [19,20]. In this study, we determined whether 1α,25-$(OH)_2D_3$ inhibits adhesion molecule expression (ICAM-1 and VCAM-1) and cytokine production (interleukin-6 (IL-6) and IL-8) induced by TNF-α in human coronary arterial endothelial cells (HCAECs).

2. Materials and methods

2. 1. Cell culture and stimulation conditions

HCAECs were obtained from Lonza (Walkersville, MD, USA) and maintained at 37°C under humidified 5% CO_2 in a stationary culture. HCAECs were grown using the EGM-2 BulletKit (Lonza). The cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; GIBCO), 100 units/mL penicillin (GIBCO), and 100 μg/mL streptomycin (GIBCO).

HCAECs were exposed to 2 ng/mL TNF-α (R&D Systems, Minneapolis, MN, USA) with or without pretreatment with 1α,25-$(OH)_2D_3$ (Wako Junyaku Co., Osaka, Japan) for 30 min. The cells and cell culture supernatants were collected 4, 8, 12, and 24 h after the addition of TNF-α for subsequent analysis.

2. 2. Determination of ICAM-1 (CD54) and VCAM-1 (CD106) expression on HCAECs

The expression of ICAM-1 and VCAM-1 was determined by flow cytometric analysis. The cells were then labeled with phycoerythrin-conjugated anti-ICAM-1 and anti-VCAM-1 antibodies (Becton-Dickinson Biosciences, San Diego, CA, USA). Immunofluorescence staining was analyzed with a FACSCalibur flow cytometer equipped with CellQuest software (Becton-Dickinson Biosciences). Five thousand cells were analyzed in the flow cytometric studies.

2. 3. Determination of IL-6 and IL-8 production from HCAECs

The concentrations of IL-6 and IL-8 in cell culture supernatants were determined with a sandwich-type enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). The lower detection limits for IL-6 and IL-8 were 3.12 pg/mL and 31.2 pg/mL, respectively.

2. 4. Real-time polymerase chain reaction (PCR) of VCAM-1 and IL-8 in HCAECs

Real-time PCR was performed to determine the mRNA levels of IL-8 and VCAM-1 in HCAECs. Total cellular RNA was isolated using a QIAamp RNA Blood Mini Kit (Qiagen K.K., Tokyo, Japan), according to the manufacturer's instructions. A critical RNA purification procedure was

performed with deoxyribonuclease (DNase) I (Invitrogen, Leek, The Netherlands) to eliminate DNA. Reverse transcription (RT) was carried out with 1 μg of total RNA and oligo dT primers (Invitrogen) in a reaction volume of 20 μL, using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's instructions. Real-time PCR was carried out using TaqMan Gene Expression Assays and the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). IL-8 and VCAM-1 probes were purchased from Applied Biosystems. The PCRs were recorded and analyzed using the accompanying StepOne™ software (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems) served as the housekeeping gene for the reactions and the expression of the IL-8 and VCAM-1 genes was normalized to GAPDH.

2. 4. Statistical analysis

The data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using unpaired Student's *t*-tests, with $p < 0.05$ being considered as significant. Analyses and calculations were performed using SPSS-12.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3. 1. Inhibitory effects of 1α,25-$(OH)_2D_3$ on TNF-α–induced VCAM-1 expression

Flow cytometric analysis revealed that TNF-α significantly increased VCAM-1 expression in HCAECs ($p < 0.001$; Figure 1). Pretreatment with 10^{-7} and 10^{-8} M 1α,25-$(OH)_2D_3$ significantly inhibited TNF-α–induced expression of VCAM-1 ($p < 0.05$), but pretreatment with 10^{-9} M had no effect. The kinetics of VCAM-1 expression in HCAECs pretreated with 1α,25-$(OH)_2D_3$ prior to stimulation with TNF-α is shown in Figure 2. VCAM-1 expression was significantly inhibited 4 h after TNF-α stimulation ($p < 0.05$). Conversely, TNF-α treatment significantly increased ICAM-1 expression in HCAECs ($p < 0.001$; Figure 3), whilst pretreatment with 1α,25-$(OH)_2D_3$ had no effect 4h (Figure 3) or 6h (data not shown) after stimulation.

3. 2. Inhibitory effects of 1α,25-$(OH)_2D_3$ on TNF-α–induced IL-8 production

Analysis of cell culture supernatants by ELISA demonstrated that TNF-α significantly increased IL-8 production in HCAECs ($p < 0.001$; Figure 4). Pretreatment with 10^{-8} M 1α,25-$(OH)_2D_3$ inhibited TNF-α–induced production of IL-8, but pretreatment with 10^{-7} and 10^{-9} M 1α,25-$(OH)_2D_3$ had no effect (Figure 4). The kinetics of IL-8 production in HCAECs pretreated with 10^{-8} M 1α,25-$(OH)_2D_3$ prior to stimulation with TNF-α is shown in Figure 5. IL-8 production was significantly inhibited 4, 8, 12, and 24 h after TNF-α stimulation ($p < 0.05$, $p < 0.05$, $p < 0.01$, and $p < 0.05$, respectively).

Although TNF-α treatment significantly increased IL-6 production in HCAECs ($p < 0.001$; Figure 6), pretreatment with 1α,25-$(OH)_2D_3$ did not inhibit the release of IL-6 (Figure 6).

3. 3. Inhibitory effects of 1α,25-$(OH)_2D_3$ on TNF-α–induced VCAM-1 and IL-8 mRNA expression

Real-time PCR demonstrated that pretreatment with 10^{-7} M and 10^{-8} M 1α,25-$(OH)_2D_3$ inhibited TNF-α–induced VCAM-1 mRNA expression 2 h and 4 h after TNF-α stimulation ($p < 0.05$, $p < 0.05$, $p < 0.05$ and $p < 0.05$, respectively; Figure 7), but pretreatment with 10^{-9} M 1α,25-$(OH)_2D_3$ had no effect (Figure 7). In addition, 10^{-7} M, 10^{-8} M and 10^{-9} M 1α,25-$(OH)_2D_3$ had no inhibitory effects at 8 h after stimulation. Pretreatment with 10^{-8} M 1α,25-$(OH)_2D_3$ inhibited TNF-α–induced

IL-8 mRNA expression at 2h, 4h and 8h after stimulation ($p < 0.05$, $p < 0.05$ and $p < 0.05$, respectively; Figure 8), but pretreatment with 10^{-7} and 10^{-9} M 1α,25-$(OH)_2D_3$ had no effect (Figure 8).

4. Discussion

We previously demonstrated that NF-κB was markedly activated in peripheral blood monocytes/macrophages and T cells from children with acute KD [7]. Serum TNF-α levels are elevated in KD; moreover, KD patients with high levels of soluble TNF receptor in the serum appear susceptible to coronary artery lesions [4–6,9]. NF-κB is an essential transcription factor regulating genes that encode proinflammatory cytokines, chemokines, and adhesion molecules that mediate inflammation. The levels of VCAM-1 and ICAM-1, members of the immunoglobulin superfamily, and E-selectin are increased on endothelial cells after exposure to inflammatory cytokines such as TNF-α and IL-1β [18,21]. It is likely that TNF-α, induced primarily by NF-κB activation, causes systemic vasculitis associated with high levels of ICAM-1 and VCAM-1 expression in vascular endothelial cells during the acute phase of KD.

It has been reported that 1α,25-$(OH)_2D_3$ exhibits anti-inflammatory and immunomodulatory effects [11–13]. 1α,25-$(OH)_2D_3$ inhibited the production of interferon-γ and IL-12 in T cells; differentiation, maturation, activation, and survival in dendritic cells; and TNF-α production and NF-κB activation in macrophages [14–17]. 1α,25-$(OH)_2D_3$ inhibits the activation of NF-κB and the production of IL-6, IL-8, and "regulated on activation normal T cell expressed and secreted" (RANTES) in human microvascular endothelial cells. Furthermore, it has been shown to inhibit the expression of ICAM-1 and VCAM-1 in human umbilical vein endothelial cells [13,18]. We have previously shown that both mRNA and protein levels of the vitamin D receptor are expressed in HCAECs, and that 1α,25-$(OH)_2D_3$ inhibits TNF-α–induced NF-κB activation and E-selectin expression in HCAECs [22]. In the present study, pretreatment with 1α,25-$(OH)_2D_3$ significantly inhibited TNF-α–induced expression of VCAM-1 and production of IL-8 in HCAECs, but had no effect on the expression of ICAM-1 or production of IL-6. The concentrations of 1α,25-$(OH)_2D_3$

(10^{-9} to 10^{-7} M) used in our study are consistent with levels obtained in healthy human plasma after administration of a normal dose [23,24].

Our results also showed that 1α,25-$(OH)_2D_3$ inhibited TNF-α–induced mRNA expression of VCAM-1 and IL-8 as shown in Figures 7 and 8. 1α,25-$(OH)_2D_3$ is known to reduce the basal and TNF-α–induced binding activity of activator protein-1 (AP-1) and NF-κB to their response elements, and to consequently inhibit AP-1– and NF-κB–dependent tissue factor expression in monocytic cells [25]. Docosahexaenoic acid inhibits the expression of both ICAM-1 and VCAM-1, but had a more pronounced effect on VCAM-1 expression [26]. Furthermore, it has been reported that the *cis* elements located at the promoter region of the ICAM-1 and VCAM-1 genes are different [26–28]. The human ICAM-1 promoter contains binding sites for a number of transcription factors, including signal transducer and activator of transcription (STAT), NF-κB, AP-1, and antioxidant response elements [27]. On the other hand, the human VCAM-1 promoter includes binding sites for NF-κB, AP-1, GATA, and thyroid response elements [28]. IL-6 release has been found to be associated with NF-κB activity, while IL-8 release more closely correlated with AP-1 activity [29]. We speculate that the differences in the inhibitory effect of 1α,25-$(OH)_2D_3$ on VCAM-1 and ICAM-1 expression, and IL-8 and IL-6 production in TNF-α–induced HCAECs, result from the complicated transcriptional regulation of these genes.

Recently, it has been reported that 1α,25-$(OH)_2D_3$ may be used as a therapy against inflammatory diseases, including Behçet's disease, inflammatory bowel disease, multiple sclerosis, experimental sepsis and inflammatory polyarthritis [30–38]. IVIG therapy has been reported to be effective for reducing the incidence of coronary artery lesions in KD patients [39–41]. However, at least 10% of KD patients fail to exhibit defervescence with IVIG therapy [19,20]. Some reports have recommended alternative anti-inflammatory therapies with steroids, and recently, with ulinastatin and infliximab, for the treatment of KD [42–46]. In this study 1α,25-$(OH)_2D_3$ exhibits anti-inflammatory effects after TNF-α stimulation (from 4 h onwards). In addition, the inhibitory effects of 1α,25-$(OH)_2D_3$ in VCAM-1 expression were seen 4h after TNF-α stimulation. We therefore believe that 1α,25-$(OH)_2D_3$ may inhibit HCAECs activation by TNF-α in the acute phase of KD temporarily. Our present results suggest that adjunctive 1α,25-$(OH)_2D_3$ therapy may modulate

the inflammatory response during KD vasculitis, particularly during the acute phase. Importantly, 1α,25-$(OH)_2D_3$ has been widely used in children and has a high safety index and few side effects.

5. Conclusion

The present study showed that 1α,25-$(OH)_2D_3$ inhibited TNF-α–induced surface expression of VCAM-1 and production of IL-8 in HCAECs. The findings suggesting that adjunctive 1α,25-$(OH)_2D_3$ therapy may modulate the inflammatory response during KD vasculitis.

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6. References

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7. Figure Legends

Figure 1. 1α,25-$(OH)_2D_3$ inhibits VCAM-1 expression in TNF-α–treated HCAECs.

The expression of VCAM-1 in HCAECs was measured by flow cytometry. HCAECs were pretreated with 10^{-7}, 10^{-8} and 10^{-9} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 4 h. *$p < 0.05$, compared to cells treated with TNF-α. Data are presented as the mean ± SD.

Figure 2. The kinetics of VCAM-1 expression on HCAECs pretreated with 1α,25-$(OH)_2D_3$.

HCAECs were pretreated with 10^{-8} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 0, 4, 8, 12, and 24 h. White bars indicate cells stimulated by TNF-α only and black bars indicate cells pretreated with 10^{-8} M 1α,25-$(OH)_2D_3$ prior to stimulation with TNF-α. Data are presented as the mean ± SD with *$p < 0.05$, compared to cells treated with TNF-α.

Figure 3. 1α,25-$(OH)_2D_3$ inhibition of ICAM-1 expression in TNF-α–treated HCAECs.

The surface expression of ICAM-1 in HCAECs was measured by flow cytometry. HCAECs were pretreated with 10^{-7}, 10^{-8}, and 10^{-9} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 4 h. Data are presented as the mean ± SD.

Figure 4. Inhibitory effect of 1α,25-$(OH)_2D_3$ on IL-8 production in TNF-α–treated HCAECs.

IL-8 production in HCAECs was measured by ELISA. HCAECs were pretreated with 10^{-7}, 10^{-8}, and 10^{-9} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 24h. The data are expressed as the ratio of stimulated cells to cells cultured in medium alone. Data are presented as the mean ± SD with *$p < 0.05$, compared to cells treated with TNF-α.

Figure 5. Kinetics of IL-8 production on HCAECs pretreated with 1α,25-$(OH)_2D_3$.

HCAECs were pretreated with 10^{-8} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 0, 4, 8, 12, and 24 h. White bars indicate cells stimulated by TNF-α only, and black bars indicate cells pretreated with 10^{-8} M 1α,25-$(OH)_2D_3$ stimulated by TNF-α. Data are presented as the mean ± SD with **$p < 0.01$ and * $p < 0.05$, compared to cells treated with TNF-α only.

Figure 6. Inhibitory effect of 1α,25-$(OH)_2D_3$ on IL-6 production in TNF-α–treated HCAECs.
IL-6 production in HCAECs was measured by ELISA. HCAECs were pretreated with 10^{-7}, 10^{-8}, and 10^{-9} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 24h. Data are presented as the mean ± SD.

Figure 7. 1α,25-$(OH)_2D_3$ inhibits VCAM-1 mRNA expression in TNF-α–treated HCAECs.
The mRNA expression of VCAM-1 in HCAECs was measured by real-time PCR. HCAECs were pretreated with 10^{-7}, 10^{-8} and 10^{-9} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 2h (white bar), 4 h (gray bar) and 8h (black bar). The data are expressed as the ratio of stimulated cells to cells cultured in medium alone. *$p < 0.05$, compared to cells treated with TNF-α. Data are presented as the mean ± SD.

Figure 8. Inhibitory effect of 1α,25-$(OH)_2D_3$ on IL-8 mRNA expression in TNF-α–treated HCAECs.
IL-8 mRNA expression in HCAECs was measured by real-time PCR. HCAECs were pretreated with 10^{-7}, 10^{-8}, and 10^{-9} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 2h (white bar), 4 h (gray bar) and 8h (black bar). The data are expressed as the ratio of stimulated cells to cells cultured in medium alone. Data are presented as the mean ± SD with *$p < 0.05$, compared to cells treated with TNF-α.