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Calcitriol prevents inflammatory gene expression in macrovascular endothelial cells

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ABSTRACT

Background: Calcitriol (vitamin D) supplementation has been proposed for therapeutical use in vascular diseases due to its immunomodulatory activity, preventing inflammation and promoting angiogenesis. In the present study, we hypothesised whether calcitriol downregulates pro-inflammatory gene expression without affecting angiogenesis and anti-inflammatory gene expression in LPS-induced endothelial cells.

Method: In order to evaluate the effect of calcitriol in suppressing inflammatory gene expression in the endothelium, endothelial cells were exposed to the physiological concentration of calcitriol followed by stimulation with lipopolysaccharide (LPS). Gene expression of interleukin (IL)-1β, Transforming Growth Factor (TGF)-β, Human β–defensin (HBD)-2, angiogenin (ANG) and cathelicidin (LL-37) were quantified by quantitative polymerase chain reaction.

Results: The results from six independent experiments conducted in duplicate, showed that calcitriol decreased IL-1β ($p < 0.01$) and HBD-2 expression ($p < 0.01$) when compared to non-treated cells. However, calcitriol treatment had no effect on TGF-β, ANG and LL-37 gene expression.

Conclusion: Calcitriol prevents inflammatory gene expression, but does not affect expression of angiogenic genes in endothelial cells, which suggest the potential use of calcitriol to prevent endothelial activation through the downregulation of IL-1β and HBD-2.

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Calcitriol; vitamin D; inflammation; septic shock; defensins; endothelial activation

Introduction

$1,25(OH)_2D_3$, or calcitriol, is the active metabolite of vitamin D, which is known to have multiple roles. The main functions of calcitriol are linked with calcium homeostasis, skin keratinocyte proliferation and endocrine system regulation.[1] In addition, calcitriol has immunomodulatory properties such as suppression of pro-inflammatory cytokines.[2] It has been reported that the risk of hypertension, ischemic heart disease and myocardial infarction is associated with vitamin D deficiency.[3] Similarly, in patients with diabetes, low vitamin D levels increase the risk of cardiovascular disease (CVD) and atherosclerosis,[5] which is probably mediated by correlated elevations in plasma of inflammatory markers. In contrast, administration of calcitriol suppresses foam cell formation by reducing oxidised low-density lipoprotein cholesterol uptake by macrophages from diabetics, so possibly reducing the risk of developing CVD and atherosclerosis.[6]

Mortality from CVD is significantly higher in individuals with type 2 diabetes mellitus (T2DM). Notably, the risk of vascular complications is associated with hyperglycaemia,[7] which is a systemic pathological state related to the overexpression of adhesion molecules, chemokine and cytokine release from endothelium, as well as reactive oxygen species production.[8] However, other molecules may be implicated in its pathogenesis, such as antimicrobial peptides (AMPs). Recent reports showed that AMPs are involved in the pathogenesis of various diseases.[9] Patients with chronically elevated levels of AMPs may develop chronic inflammation, as seen in psoriasis and rosacea,[10] active ulcerative colitis and could be implicated with chronic vasculitis.[11] AMPs have been reported to adhere to human endothelial cells.[12] Similarly, evidence suggests that defensins, an AMP from neutrophils, could be implicated in the accumulation and internalisation of LDL and lipoproteins at the tunica intima, which could be involved in early atherosclerotic formation process.[13] Also, it has been reported that the AMP cathelicidin (LL-37) is present in human atherosclerosis lesions.[14] Furthermore, stimulation of human coronary artery endothelial cells with defensin results in monocyte adhesion and induction of oxidative stress, which accelerates foam cell formation

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Table 1. Probe sequences.

Gene	Protein	F-Sequence	R-Sequence	SProbe Sequence
DEFB4	Human β-defensin-2 (HBD2)	5′-GAGGGAGCCCTTTTCTGAATC-3′;	3′-GTCTCCCTGGAACAAAATGC-5′	TGTGGCTG
CAMP	Cathelicidin (LL-37)	5′-GTCTGGGTCCCCATCCAT-3′	3′-TCGGATGCTAACCCTACG-5′	TCCAGGTC
ANG	Angiogenin	:5′-CATTGTCCTGCCCGTTTC-3′	3′-CAGCACGAAGACCAACAACA-5′	GCTGAGGA
IL-1B	Interleukin- 1β	5′-TCTTTGGGTAATTTTTGGGATCT-3′	3′-TACCTGTCCTGCGTGTTGAA-5′	AGCTGGAG
TGF-B	Transforming Growth Factor-β	5′-CAG CCGGTTGCTGAGGTA-3′	3′-TGACCTTGATTATTTTGCATACC-5	TGGAGCTG
HPRT1	Hypoxanthine phosphoribosyltransferase	5′-CGAGCAAGACGTTCAGTCCT-3′	3′-TGACCTTGATTATTTTGCATACC-5′	GAGGATTT

and activation of platelets.[15] In addition, patients with autoimmune diseases such as systemic lupus erythematosus who experienced a cardiovascular event, had higher human β-defensin (HBD)-2 levels.[16] Similarly, plasma angiogenin (ANG, angiogenesis-related peptide) levels were increased in acute coronary syndrome patients when compared to apparently healthy controls, which could be involved in its pathogenesis.[17]

Studies in mice and rat models with vascular inflammatory disease, showed that vitamin D supplementation has therapeutical effects, with anti-coagulant activity and downregulation of pro-inflammatory cytokines, and the inhibition of vascular endothelium LPS-induced activation.[18] These associations suggest the possibility of a link between endothelial dysfunction and AMPs in vitamin D-deficient patients. In the present study, we hypothesised that calcitriol downregulates pro-inflammatory gene expression without affecting angiogenesis and anti-inflammatory cytokines in LPS-induced endothelial cells.

Material and methods

Cell culture

The cell line EAhy926 (ATCC, CRL-2922, Manassas, VA, USA) obtained from umbilical cord endothelial cells, were seeded in cell culture flasks (Nunclon, Roskilde-Kamstrupyej, Denmark) and cultured with high glucose Dulbecco's Modified Eagle Medium (DMEM, Corning, Manassas VA, USA) supplemented with 10% Foetal Bovine Serum (FBS, Cellgro Corning, Manassas VA, USA) and Endothelial Cell Growth Supplement (ECGS, Sigma-Aldrich, Germany), 100 UI/ml of penicillin and 100 mg/ml of streptomycin (Gibco, Carlsbad, CA). Incubations were performed in humidified 5% CO_2 at 37°C. 4×10^4 cells were seeded in a 24 well plate and treated with 10^{-9} M $1,25(OH)_2D_3$ (Sigma-Aldrich, St. Louis, MO, USA) and 10 ng/ml or 100 ng/ml of LPS from *E. coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO, USA) for 18h. An equal amount of absolute ethanol (Sigma-Aldrich, St. Louis, USA) (0.1% v/v) in 1% FBS was used as a control. In additional experiments, cells were pre-treated and/or post-treated with 10^{-9}M (1nM) calcitriol after pre-stimulation or post-stimulation with LPS for 2 h. Subsequently, cells were collected and subjected to RNA extraction and cDNA synthesis.

RNA isolation, reverse transcription and gene expression analysis determined by real time polymerase chain reaction (PCR)

Total RNA from each cell culture was extracted with TRIzol (Gibco, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse mRNA transcription was performed using 5µg of RNA, 2µM Oligo $(dT)_{12-18}$ as template-primer (Invitrogen, Carslbard, CA, USA), 0.1M DTT (Invitrogen, Carslbard, CA), 10mM dNTPs (Qiagen, Duesseldorf, Germany) and 200 units of superscript™ II RT (Invitrogen, Carslbard, CA, USA) according to the manufacturer's instructions. Real-time qPCR was performed using a LightCycler 480 thermocycler (Roche Applied Science Inc, Indianapolis, IN, USA), using specific hydrolysis probes and primers, both were designed with universal probe library software (Roche Applied Science Inc., USA) (Table 1). Identical qPCR conditions were performed for all genes and in all cases normalised against hypoxanthine phosphoribosyl transferase (HPRT), used as housekeeping gene internal control. Estimation of the relative levels of gene expression was performed using the formula $2^{-\Delta\Delta Ct}$ described previously by Livak [19]. This method is based on the expression levels of a target gene vs. one reference gene (HPRT) comparing between control group and target group.

Statistical analysis

Normality of all data was determined using the D'Agostino and Pearson omnibus normality test for each data-set. For nonparametric data, the multiple comparison test Kruskal–Wallis followed by a Dunn's *post hoc* test was performed, whereas for the data that showed no normal distribution, differences were calculated by an ANOVA followed by a Tukey's multiple comparison test. Statistical analysis was performed using the GraphPad Prism Software (Graph Prism Software version 5.02, San Diego, CA).

Results

Calcitriol inhibits LPS-induced IL-1β and HBD2 gene expression

The results showed that calcitriol-LPS co-stimulation decreased IL-1β (Figure 1(A)) and HBD2 (Figure 1(B)) gene expression, respectively. In order to evaluate the

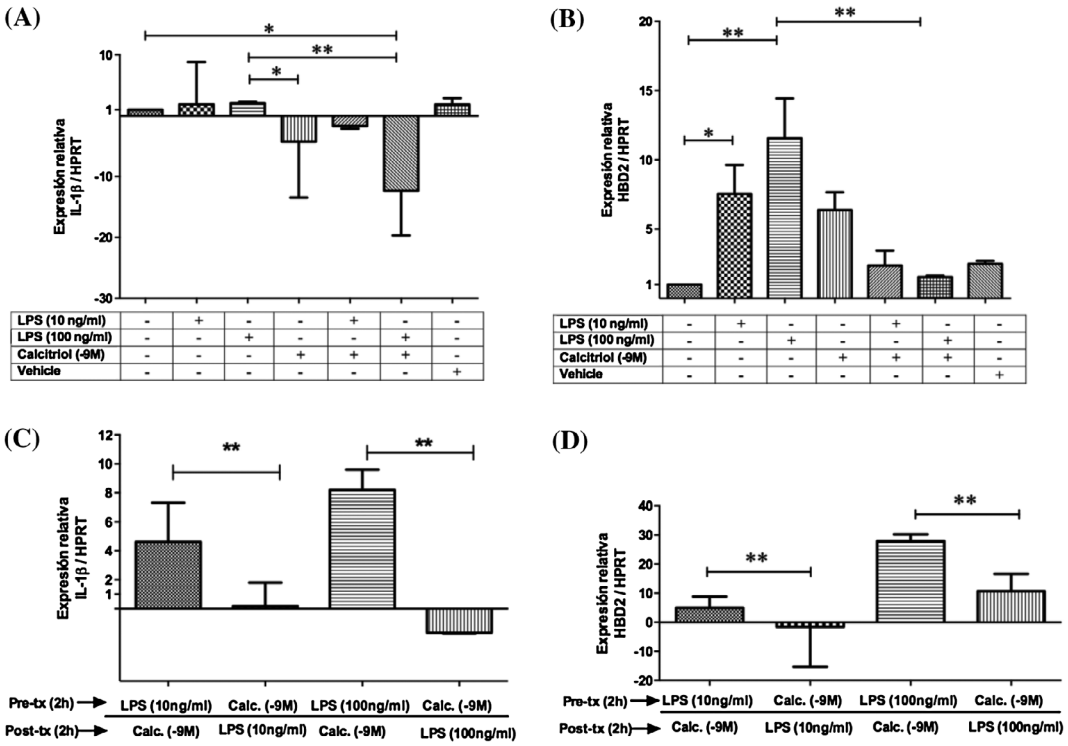

Figure 1. Pre-treatment with calcitriol decreases LPS-induced IL-1β and HBD2 mRNA expression in endothelial cells. Endothelial cells were treated with calcitriol (10^{-9}M) and LPS (10ng/ml or 100ng/ml, respectively) for 18 h and IL-1β (A) and HBD2 (B) gene expression were measured by qPCR. Additional experiment was performed, the cells were pre-treated or post-treated with 10^{-9}M of calcitriol 2h, after pre-stimulation or post-stimulation with LPS during 2 h. Finally, IL-1β (C) and HBD2 (D) gene expression was measured by qPCR. Data is expressed as median ± interquartile range to 1A, 1C, 1D. Statistics were calculated by Kruskal-Wallis and Dunn's *post hoc* tests were performed. To Figure 1B, data is expressed as mean ± SD of 6 independent experiments by triplicate each one. Statistics were calculated by ANOVA with Tukey's multiple comparison tests were performed. *$p < 0.05$;**$p < 0.01$.

efficiency of calcitriol in preventing pro-inflammatory gene expression, cells were pre-treated with calcitriol for 2h, then stimulated with LPS for 2h and vice versa. Calcitriol post-treatment did not suppress IL-1β or HBD2 gene expression. However, calcitriol pre-treatment decreased the LPS-induced IL-1β gene expression (Figure 1(C)). Similarly, calcitriol pre-treatment attenuated the LPS-induced HBD2 gene expression (Figure 1(D)).

Calcitriol-LPS co-stimulation did not change expression of angiogenesis-related genes

Calcitriol treatment is known to exert anti-angiogenic or pro-angiogenic effects depending on the tissue. Here, we evaluated the effect of calcitriol-LPS co-stimulation on induction TGF-β, ANG and LL-37 gene expression in endothelial cells. The results showed that there were no significant changes in pro-angiogenic gene expression when compared with control cells (Figure 2(A–C)).

Discussion

It has been previously reported that pro-inflammatory molecules such as cytokines and defensins are implicated in endothelial dysfunction.[20] There is evidence that calcitriol directly regulates AMPs gene expression in human keratinocytes because the promoter regions of LL-37 and HBD2 contain vitamin D response element consensus that mediate calcitriol-dependent gene expression.[2] In the present study, we demonstrate that calcitriol pre-treatment to macrovascular endothelial cells inhibited LPS-induced response, decreasing IL-1β and HBD2 gene expression and we speculate that the same behaviour can be seen at the protein level. The mechanism involved in HBD2 regulation by calcitriol has not been addressed in this model, previous studies have shown that calcitriol downregulates the expression of Toll-like receptors (TLR)-2 and TLR-4 in human monocytes [21] and myometrial smooth muscle.[22] Likewise, mRNA levels of TLR-2 and TLR-4 were suppressed when keratinocytes were treated with calcitriol prior to stimulation with LPS or UVB irradiation [2]. Therefore, we speculate that calcitriol downregulates TLR-4 or/and blocks LPS-induced p65 nuclear translocation through the inhibition of IκBα phosphorylation, resulting in blockade of the NF-κB activation pathway in macrophages [23] attenuating the LPS-induced HBD2 and IL-1β gene expression; however, further studies are needed to confirm this hypothesis. Similar studies using microvascular endothelial cells have shown that pretreatment with calcitriol inhibited the LPS-induced

Table 2. Summary.

What is known about this subject:
- Calcitriol has immunomodulatory properties such as suppression of pro-inflammatory cytokines in micro-endothelial cells.
- HBD-2 is a host defence peptide that induces significant pro-inflammatory responses.

What this paper adds:
- Calcitriol downregulates IL-1B and HBD-2 gene expression in LPS-stimulated endothelial cells.
- Calcitriol has no effect on TGF-β, ANG and LL-37 gene expression in LPS-stimulated endothelial cells.
- This suggests the potential use of calcitriol to reduce inflammation.

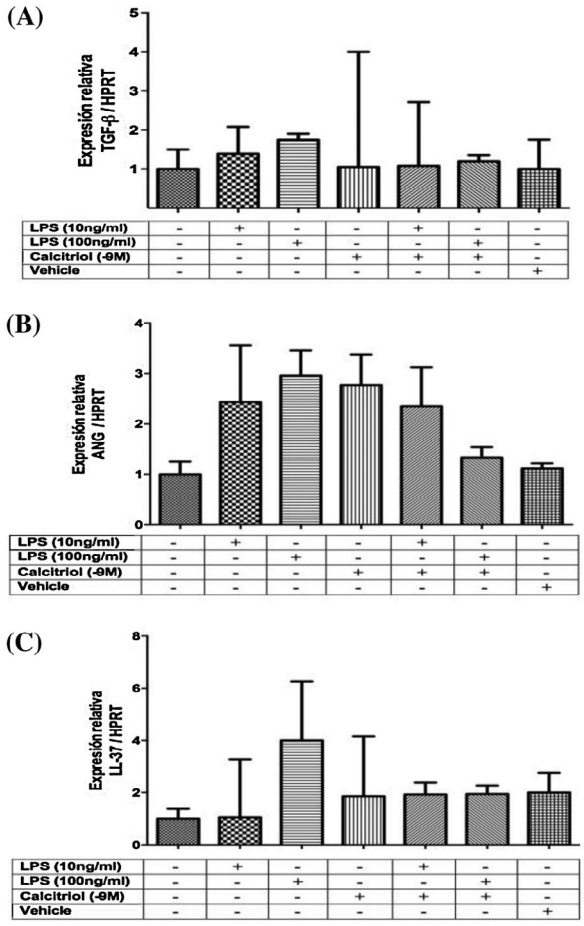

Figure 2. Calcitriol-LPS co-stimulation did not change expression of TGF-β, ANG5 and LL-37 genes expression in human endothelial cells. Endothelial cells were calcitriol-LPS co-stimulated for 18 h and TGF (A); ANG5 (B); LL-37 (C) gene expression were measured by qPCR. Data is expressed as median ± interquartile range of six independent experiments by triplicate each one. Statistics were calculated by Kruskal–Wallis and Dunn's *post hoc* tests were performed.

pro-inflammatory cytokines IL-6, IL-8 and chemokine RANTES.[24] Altogether, these results suggest that calcitriol promotes anti-inflammatory responses in macrovascular endothelia.

Manifestations of severe clinical conditions such as septic shock (such as oedema) are accentuated by an increased vascular permeability mediated by angiogenic genes.[25] It has been reported that TGF-β is able to induce vascular permeability by p38 MAPK activation, leading to the loss of cell-cell contacts causing intravascular leakage of fluid.[26] Similarly, ANG is a human plasma protein implicated in the induction of tissue plasminogen activator on endothelial cells to produce plasmin, which is a key enzyme to degrade fibrin clots. Interestingly, this protease has been related with the rupture of atherosclerotic plaque.[27] Likewise, a study showed that LL-37 is present in human atherosclerosis lesions and it was associated with advanced atherosclerotic lesions.[14] Therefore, we speculated that calcitriol-LPS co-stimulation was able to module angiogenic factors such as TGF-β, ANG and LL-37. The results showed that both stimuli do not affect the angiogenic molecules expression, suggesting that the anti-inflammatory activity of calcitriol *in vitro* is mediated by different mechanisms not necessarily related to the downregulation of angiogenic genes. In a clinical assay using a single dose of calcitriol (2 µg intravenously) therapeutically in patients with acute kidney failure and sepsis, no change in the expression of inflammatory biomarkers such as hCAP18, IL-6, TNF-α and IL-10 [28] was reported. This study suggests that although calcitriol inhibits pro-inflammatory molecules *in vitro*, therapeutic doses should be assessed for clinical trials Table 2.

In conclusion, calcitriol was found to suppress the LPS-mediated induction of IL-1β and HBD-2 gene expression in endothelial cells but did not affect TGF-β, ANG and LL-37 gene expression *in vitro*. This study will help to understand the pathogenesis of several pathologies, involving novel molecules such as HBD-2 and suggests other functions of calcitriol to protect the endothelium. This work represents an advance in biomedical science because it increases the knowledge of the use of calcitriol as immunomodulator in macro-vascular endothelial cells, and this knowledge can be used to further study inflammatory conditions such as septic shock.

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Disclosure statement

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