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Original article

1, 25-Dihydroxyvitamin D3 activates Apelin/APJ system and inhibits the production of adhesion molecules and inflammatory mediators in LPS-activated RAW264.7 cells

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ABSTRACT

Background: 1, 25-Dihydroxyvitamin D3 (1, 25$(OH)_2D_3$), an active form of vitamin D3, plays a crucial role in the mitigation of inflammation damage. Recent studies have revealed that apelin and its receptor (apelin/APJ system) could significantly ameliorate LPS-induced inflammation-response. This investigation aimed to appraise the effects of 1, 25$(OH)_2D_3$ on the apelin/APJ system and production of adhesion molecules and inflammatory mediators in LPS–activated RAW264.7 macrophage cells.
Methods: Murine RAW264.7 cells were pretreated with 1, 25$(OH)_2D_3$, followed stimulation with LPS (1 μg/mL) for 24 h. The effect of 1, 25$(OH)_2D_3$ on LPS-induced cell injury was determined by MTT assay, whereas, enzyme-linked immunosorbent assay (ELISA), qPCR and western blotting were used to evaluate cytokine production and apelin/APJ system expression. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) protein expression were measured by flow cytometry.
Results: The levels of IL-1β, IL-6, and TNF-α cytokines were significantly increased by incubation with LPS. LPS also increased the protein expression of adhesion molecules, including VCAM-1 and ICAM-1. However, pretreatment with 1, 25$(OH)_2D_3$ markedly inhibited LPS-induced production of inflammatory cytokines and adhesion molecules. Moreover, we found that 1, 25$(OH)_2D_3$ could induced the apelin/APJ system expression. Further experiments demonstrated the significant increase of apelin/APJ system expression at both the protein and mRNA levels in LPS-activated cells when pretreated with 1, 25$(OH)_2D_3$.
Conclusion: Taken together, our results indicated that 1, 25$(OH)_2D_3$ confers an anti-inflammatory effect through a likely mechanism involving a reduction in pro-inflammatory mediators and adhesion molecules *via* up-regulation of the apelin/APJ system in RAW264.7 cells.

Introduction

The inflammatory is a local response of the immune system defense against cell injury. The excessive inflammatory response to injury is known as a major problem for the progression of diseases such as atherosclerosis, inflammatory bowel disease, rheumatoid arthritis, and sepsis [1]. The production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and nitric oxide by macrophage have been proposed in development of Lipopolysaccharide (LPS)-induced cell injury. However, macrophage activation modulates the amount of pro-

Abbreviations: APJ, apelin receptor; 1, 25$(OH)_2D_3$, 1, 25-Dihydroxyvitamin D3; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; ICAM-1(CD54), intercellular adhesion molecule-1; IL, interleukin; LPS, lipopolysaccharide; MTT, 3-(4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromidefor; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1(CD106).

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inflammatory mediators synthesize in immune response, thereby amplify the host response to the cell injury. Conversely, excessive macrophage activation by an increase in cytokine production could have detrimental effects [2,3]. In addition, adhesion molecules such as induced vascular cell adhesion molecule-1 (VCAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are essential for the mediation of macrophages binding to epithelial cell in inflammatory conditions. Studies have revealed that activation of TNF-α by nuclear factor-κB (NF-κB) induced VCAM-1 and ICAM-1 expression to indicate the severity of inflammation in RAW264.7 cells [4,5]. Reports have confirmed that LPS, from the cell wall of Gram-negative bacteria, causes cytokines and reactive oxygen species (ROS) production in macrophage cells and associated with multiorgan dysfunction such as myocardial dysfunction in septic shock [6,7]. TNF-α production by LPS synergically enhanced large amounts of cytokine production in macrophage cells. Therefore, prevention of macrophages activation and inflammatory mediators may ameliorate the severity of inflammatory diseases [8,9].

Apelin, identified as an endogenous ligand of the orphan G protein-coupled receptor APJ, is known to have many important physiological functions. The main sources of apelin in serum are adipocytes cells and also found in a wide variety of human tissues. Apelin is initially synthesized as a 77-amino acid pre-propeptide that finally is cleaved to mature shorter peptides of 36, 17, 13 and 12 amino acids in length [10]. Many factors such as hypoxia, oxidative stress, inflammation are regulated by apelin expression [11]. A number of studies indicated that apelin has the main role in the inhibition of cell apoptosis, inflammation and macrophage activation [12,13]. The anti-inflammatory effect of apelin by down-regulation of the inflammatory cytokines in cultured cells has been shown *in vitro* and *in vivo* studies. It has been reported that apelin exhibited anti-inflammatory effects by a reduction in TNF-α and IL-6 in rat peritoneal macrophages. In fact, apelin may have anti-inflammatory properties that attenuate the functions of activated macrophages [14–16].

1, 25-Dihydroxyvitamin D3 (1, $25(OH)_2D_3$) is conceived as a key mediator in response to inflammation. 1, $25(OH)_2D_3$ has been demonstrated that displays extraskeletal, anti-inflammatory and immunomodulatory functions. Therefore, its deficiency may trigger various inflammatory diseases like atherosclerosis [17]. Investigations represent that 1, $25(OH)_2D_3$ can elevate the differentiation rate of monocyte toward macrophages so barricades the extrication of inflammatory cytokines and eventually, diminishes the potency of antigens representing the process to lymphocytes [18]. 1, $25(OH)_2D_3$ has been involved in up-regulation of anti-inflammatory cytokines like interleukin (IL)-10, provided that it implicates in down-regulation of pro-inflammatory cytokines production such as IL-6, IL-12, and TNF-α. It has been suggested that 1, $25(OH)_2D_3$ supplement can be used in chronic inflammatory disease to moderate excessive cytokine activity [19,20]. However, little is known from macrophage models about the relationship between apelin/APJ system and 1, $25(OH)_2D_3$. Considering the ability of 1, $25(OH)_2D_3$ against inflammation, we aimed to underline 1, $25(OH)_2D_3$ influence on the production of pro-inflammatory mediators in LPS-activated RAW264.7 cells and its direct effect on apelin expression in activated macrophage cells.

Material and methods

Cell culture and 1, 25(OH)$_2$D$_3$ treatments

Murine macrophage cell line (RAW264.7) was obtained from Iran National Cell Bank (NCBI code: C639, Pasteur Institute, Tehran). RAW264.7 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin in 5% CO2 at 37 °C in humidified atmosphere. The 125 $(OH)_2D_3$ (Cayman Chemicals, Ann Arbor, USA) was dissolved in ethanol for *in vitro* experiments and stored at −80°C until use. Cells with 70–80% confluence were detached from culture plates by using 0.25% Trypsin-EDTA solution (Gibco). After 3 to 4 passages, cells were used for different analyses. Prior to the treatment, cells were plated at a density of 5×10^4 cells/ml in 96-well plates for further cell viability measurement. To run this assay, the medium was replaced with fresh DMEM medium (0.5% FBS) containing diluted 1, $25(OH)_2D_3$ (25, 50, and 100 nM) for 4 h, followed stimulation with 1 μg/mL of LPS (E. coli serotype 0127, B8, Sigma) for an additional 24 h to make the cell injury model. Meanwhile, the control cells were set in DMEM medium (1, $25(OH)_2D_3$ untreated; 0.5% ethanol only). The viability of cells was measured by the MTT assay, after interventions, 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ((MTT) solution of 5 mg/mL in PBS) were added to the each wells (0.5 mg/ml per well) for another 4 h. Finally, the culture medium was carefully removed, and 50 μl of dimethyl sulfoxide (DMSO) was added into each well. The optical density at 570 nm was measured using the microplate reader (Bio-Tek, ELX 800, USA).

Flow cytometry determination of ICAM-1 (CD54) and VCAM-1 (CD106)

For flow cytometry, after incubation, cells were washed and incubated with fluorochrome-conjugated monoclonal antibodies (mAbs) for 45 min at 4 °C. Phycoerythrin (PE) labeled anti-VCAM-1 (CD106) and PE-anti-ICAM-1 (CD54) was purchased from BD Biosciences Inc. Background fluorescence was determined by measuring the fluorescent signal from cells stained with fluorochrome-labeled isotype-matched. Flow cytometry was performed using a FACSCalibur (BD Biosciences) flow cytometer, and data were analyzed using FlowJo software (Tree Star, Ashland, OR). In all the experiments, cells were gated on forward and side scatter to eliminate dead cells and debris.

Determination of IL-1β, IL-6, and TNF-α

RAW264.7 cells were seeded onto 6-well plates at a density of 1×10^6 cells/ml and incubated for overnight. Then, treated with 100 nM of 1, $25(OH)_2D_3$ for 4 h, followed stimulation with LPS (1 μg/ml) and incubation for an additional 24 h. The concentration of cytokines TNF-α, IL-1β, and IL-6 were quantified using mouse-specific ELISA kit (ExCell Biotech (Taicang) Co., Ltd., China) according to the manufacturer's instructions. The optical density (OD) was measured at 450 nm using a microplate reader (stat fax 2100, USA).

Real-time quantitative PCR

RT-qPCR analysis was performed following standard protocols as previously described [21]. Total RNA from Raw264.7 cells was isolated by RNAX-Plus solution kit (Cat: YT9065; YTA Co) according to the manufacturer instructions and 1 μg RNA was reverse transcripted into cDNA (Cat no. YT4500, Yekta Tajhiz Azma). TNF-α, IL-1β, IL-6, apelin, and APJ were quantified by using SYBR Green PCR Master Mix (Yekta Tajhiz, Tehran, Iran) on a LightCycler® 96 real-time PCR detection system (Roche Molecular Systems, Inc). β-actin was included as internal reference and data were analyzed by $2^{-\Delta\Delta Ct}$ method. The sequences of primers used for the PCR were exhibited in Table1.

Western blotting

The protein expression was measured by Western blotting analysis. All of the primary and secondary antibodies were purchased from Santa Cruz Biotechnology. Briefly, cells were

Table 1
Primer sequences used in quantitative real-time polymerase chain reaction (qPCR) analysis.

Gene name	Forward/Reverse Primer Sequence (5-3)	Accession number	Annealing (°C)
Apelin	F: CCTCCAGATGGAACAGGACTA R: GCGAAATTTCCTCCTGCCTCC	NM_013912.4	59
Apelin receptor (APJ)	F: TGGCTGACTTGACCTTTGTG R: GCAAAAGACACTGGCGTACA	NM_011784.3	58
IL-1β	F: GTTGACGGACCCCAAAAGAT R: CCTCATCCTGGAAGGTCCAC	NM_008361.4	59
IL-6	F: ACAAAGCCAGAGTCCTTCAGA R: TCCTTAGCCACTCCTTCTGT	NM_031168.2	58
TNF-α	F: ACTGAACTTCGGGGTGATCG R: TCTTTGAGATCCATGCCGTTG	NM_013693.3	59
β-actin	F: AGAGGGAAATCGTGCGTGAC R: CAATAGTGATGACCTGGCCGT	NM_007393.5	60

washed with PBS and lysed in ice-cold RIPA lysis buffer containing protease inhibitors (sc-24948). The supernatant was separated from cell debris using centrifugation at 12,000 *g* for 10 min at 4 °C, and protein concentration was determined *via* the Bradford assay (Bio-Rad, Hercules, California). An equal amount of 40 μg of protein from each sample was resolved in 12% Tris-glycine SDS polyacrylamide gel (SDS-PAGE). Then, protein bands were transferred in Tris-glycine buffer at 300 mA for 1 h on to polyvinylidene fluoride membranes (PVDF, Millipore Corporation, Bedford, MA, USA). After blocking for 2 h in 5% (w/v) skimmed dry milk in Tris-buffered saline containing Tween-20 (TBST) at room temperature, the membrane was washed and incubated with appropriate primary antibodies; Apelin (46 kDa);(1: 200 Santa Cruz, sc-293441), APJ (42 kDa);(1: 200 Santa Cruz, sc-33823) and β-actin (1: 4000 Santa Cruz, sc-69879) for 24 h at 4 °C. Afterward, the membrane was washed with TBST and incubated with secondary antibody (sc-2020) at room temperature for 2 h. Peroxidase labeling bands were visualized by using Western blotting luminol Reagent (Santa Cruz Technology, Santa Cruz, CA, USA). Semiquantitatively analyses on developed bands were performed by ImageJ software (version 1.41). The relative protein levels of apelin and APJ were normalized to β-actin. Blots were stripped and probed for β-actin.

Statistical analysis

All statistical calculations were performed using SPSS 19.0 software and GraphPad Prism 6.0 software. The normality distributions of results were analyzed by Kolmogorov–Smirnov test. Also, the Levene test was applied for the equality of variances. Differences between the groups were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) multiple range test. All date are expressed as mean ± SEM. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of 1, 25(OH)$_2$D$_3$ on the cell viability in LPS-activated RAW264.7 macrophage cells

To evaluate the effects of 1, 25(OH)$_2$D$_3$ and LPS on the cell viabilities of RAW264.7 cells, MTT assay was performed. The cells were pretreated with various concentrations of 1, 25(OH)$_2$D$_3$ (25, 50, and 100 nM), followed stimulation with LPS for 24 h. An MTT assay revealed that survival rate in LPS group was significantly decreased as compared with the control group ($p < 0.01$; Fig. 1), while in LPS treatment group, the significant increase in cell viability was observed when pretreated with 50 nM and 100 nM of 1, 25(OH)$_2$D$_3$ as compared with LPS group ($p < 0.05$, $p < 0.01$, respectively; Fig. 1). As shown in Fig. 1, up to 100 nM of 1, 25 (OH)$_2$D$_3$ treatment no cellular toxicity effects were observed in Raw264.7 cells. In addition, 1, 25(OH)$_2$D$_3$ attenuated LPS induced cell injury in macrophage cells in a concentration-dependent manner. Moreover, The average levels of 25(OH)D3, an indicator of optimal levels of vitamin D3 are thought to be about 70–100 nM in serum [22]. So, was used 100 nM of 1, 25(OH)$_2$D$_3$ in the treated group for subsequent experiments. Collectively, these results indicated that 1, 25(OH)$_2$D$_3$ significantly protect macrophage cells against LPS induced cytotoxicity in Raw264.7 cells.

1, 25(OH)$_2$D$_3$ inhibits the cytokines levels of TNF-α, IL-6, and IL-1β in LPS-activated RAW264.7 macrophage cells

A number of studies have confirmed that overproduction of proinflammatory mediators such as TNF-α, IL-6, and IL-1β, are essential for the induction of chronic inflammation. To determine the effect of 1, 25(OH)$_2$D$_3$ on the production of pro-inflammatory mediator, we used ELISA in our study. As shown in Fig. 2, treatment with LPS enhanced the activation of inflammatory cytokine both at the mRNA and protein levels in RAW264.7 cells ($p < 0.0001$; Fig. 2A, B). Meanwhile, mRNA and protein expression of IL-1β, IL-6, and TNF-α were significantly blocked by 1, 25(OH)$_2$D$_3$ (100 nM) in LPS treatment group ($p < 0.001$; Fig. 2A, B).

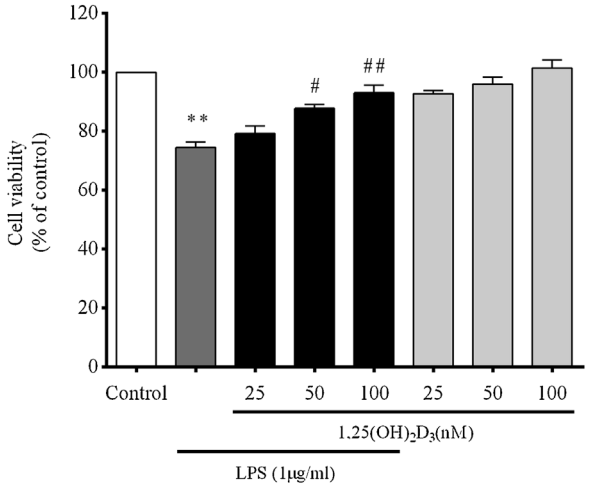

Fig. 1. Protective effect of 1, 25(OH)$_2$D$_3$ against LPS-activated RAW264.7 cells cytotoxicity. 1, 25(OH)$_2$D$_3$ was no-toxic to RAW264.7 cells. Cells were pretreated with 1, 25(OH)$_2$D$_3$ (25, 50, and 100 nM) for 4 h, and then stimulated with lipopolysaccharide (LPS; 1 μg/ml) for 24 h. MTT assay was performed to assess the cell viability of RAW264.7 cells. The values are expressed as the means ± SEM. $^{**}p < 0.01$ *vs.* control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* LPS group (n = 3 in each group).

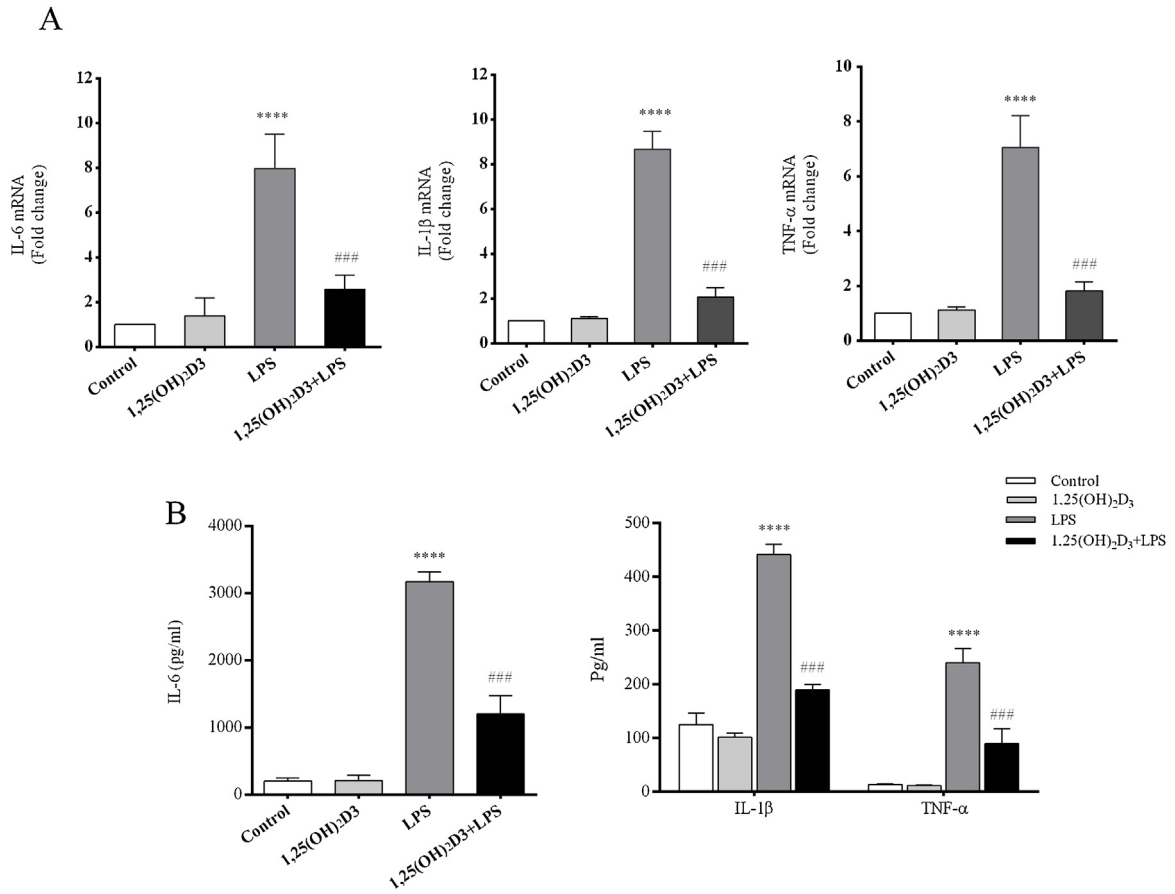

Fig. 2. 1, 25$(OH)_2D_3$ inhibits the inflammation in LPS-activated RAW264.7 cells. Cells were pretreated with 1, 25$(OH)_2D_3$ (100 nM) for 4 h, and then stimulated with lipopolysaccharide (LPS; 1 μg/ml) for 24 h. (A) mRNA expression profile for IL-6, IL-1β and TNF-α. (B) Protein levels of IL-6, IL-1β and TNF-α in RAW264.7 cells. The values are expressed as the means ± SEM. $^{****}p < 0.0001$ *vs.* control group, $^{###}p < 0.001$ *vs.* LPS group (n = 3 in each group).

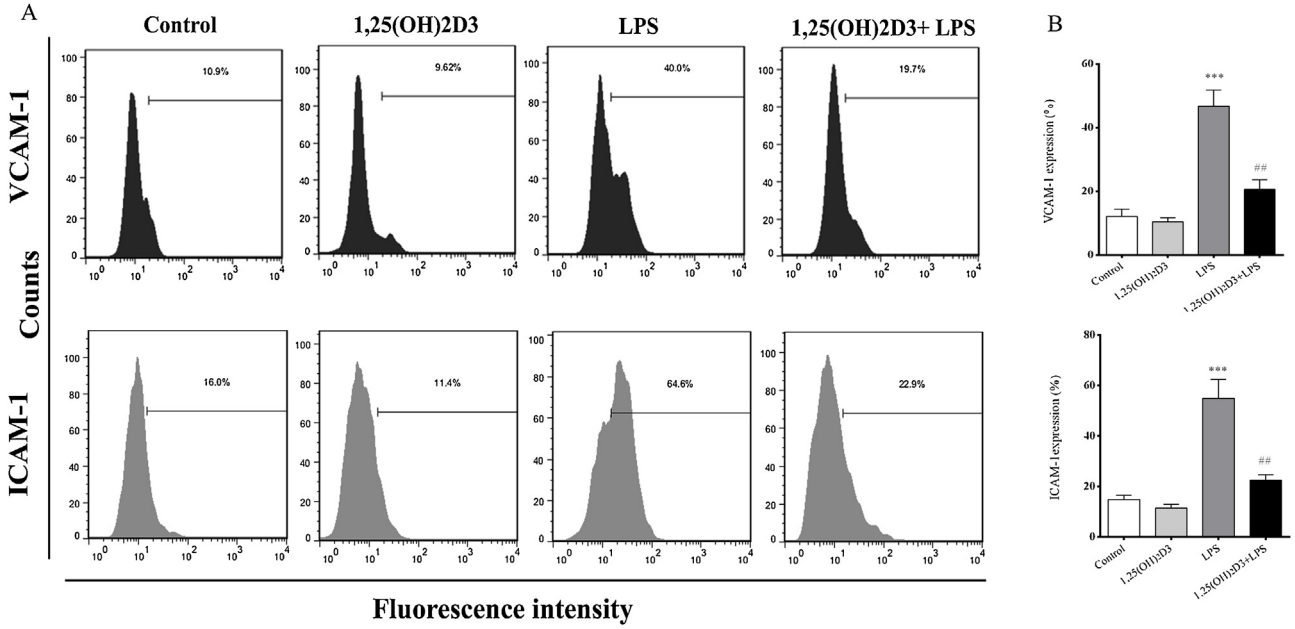

Fig. 3. 1, 25$(OH)_2D_3$ mitigates the surface expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in LPS-activated RAW264.7 cells. Cells were pretreated with 1, 25$(OH)_2D_3$ (100 nM) for 4 h, and then stimulated with lipopolysaccharide (LPS; 1 μg/ml) for 24 h. The surface protein expression was measured by Flow cytometry. The values are expressed as the means ± SEM. $^{***}p < 0.001$ *vs.* control group; $^{##}p < 0.01$ *vs.* LPS (n = 3 in each group).

1, 25(OH)$_2$D$_3$ suppresses the cell surface expression of ICAM-1 and VCAM-l in LPS-activated RAW264.7 macrophage cells

Expression of ICAM-1 (CD54) and VCAM-l (CD106) are important in the development of atherosclerosis plaque through activation of macrophage inflammatory cytokines. To investigate whether 1, 25(OH)$_2$D$_3$ could inhibit ICAM-1 (CD54) and VCAM-l expression we examined the protein expression of ICAM-1 (CD54) and VCAM-l by Flow cytometry in LPS-activated RAW264.7 macrophage cells. As shown in Fig. 3, There was no obvious difference between the control and 1, 25(OH)$_2$D$_3$ groups to the VCAM-1 and ICAM-1 expression. In LPS group, LPS increased significantly VCAM-1 and ICAM-1 protein expression on the surface of RAW264.7 cells as compared with control and 1, 25 (OH)$_2$D$_3$ group, respectively ($p < 0.001$; Fig. 3B). However, pretreated with 1, 25(OH)$_2$D$_3$ reduced protein expression of VCAM-1 and ICAM-1 in LPS treatment group ($p < 0.01$).

1, 25(OH)$_2$D$_3$ augments apelin/APJ system in LPS-activated RAW264.7 macrophage cells

Studies have demonstrated that apelin/APJ system has an important role in suppression of inflammation. We next determined whether 1, 25(OH)$_2$D$_3$ induced modulation of the apelin/APJ system expression in RAW264.7 cells. Thus, qPCR and western blotting were performed to analyze the mRNA and protein expression of apelin/APJ. As shown in Fig. 4, 1, 25(OH)$_2$D$_3$ significantly increased the expression of both apelin and APJ mRNA ($p < 0.05$; Fig. 4A). Interestingly, 1, 25(OH)$_2$D$_3$ showed a synergistic effect on apelin and APJ mRNA expression level in LPS treatment cells, although in the LPS group the difference did not reach statistical significance. However, apelin and APJ protein expression in RAW264.7 cells were consistent with its mRNA expression patterns. Similarly, immunoblotting analysis showed that both apelin and APJ protein expression was increased significantly under 1, 25(OH)$_2$D$_3$ administration ($p < 0.05$; Fig. 4B, C). These results showed that 1, 25(OH)$_2$D$_3$ significantly increased apelin and APJ expression specifically under LPS treatment.

Discussion

The activation of macrophage has been shown to play a crucial role in the enhanced expression of several pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α. The mechanisms underlying the macrophage activation have been the subject of intense investigation, especially its regulation and function in inflammation [23]. In this current study, we evaluated*in vitro* the effects of 1, 25(OH)$_2$D$_3$ on proinflammatory cytokines and adhesion molecules production with apelin/APJ system regulation in LPS-activated RAW264.7 macrophage cells. We indicated that 1, 25(OH)$_2$D$_3$ treatment associated with a significant reduction in TNF-α, IL-6, and IL-1β expression at mRNA and protein levels in LPS-activated RAW264.7 cells. However, this findings were in agreement with the previous studies that revealed the inhibitory effects of 1, 25(OH)$_2$D$_3$ against inflammatory cytokines production

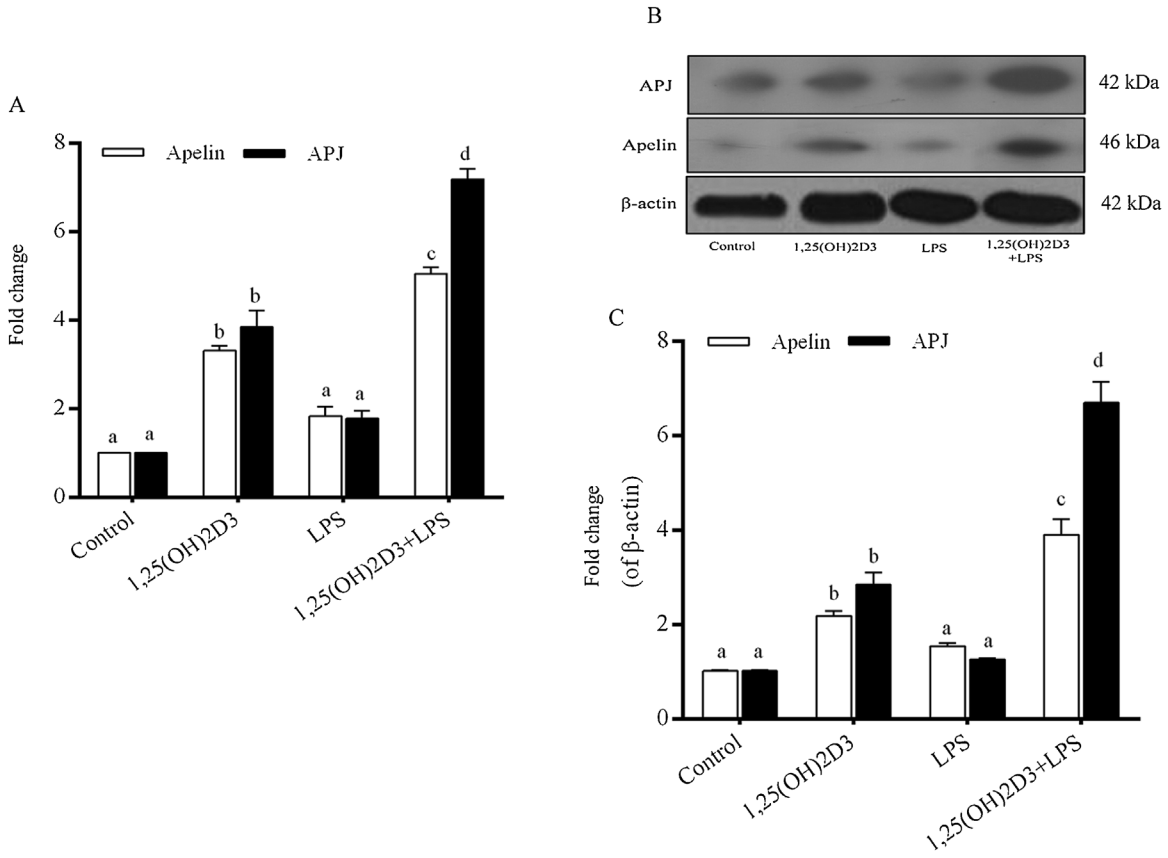

Fig. 4. 1, 25(OH)$_2$D$_3$ upregulates the protein and mRNA expression of apelin, and apelin receptor (APJ) in LPS-activated RAW264.7 cells. Cells were pretreated with 1, 25 (OH)$_2$D$_3$ (100 nM) for 4 h, and then stimulated lipopolysaccharide (LPS; 1 μg/ml) for 24 h. Relative expression profile for apelin and APJ in both protein and mRNA levels were measured using qPCR and western blot. (A) mRNA expression profile for apelin and APJ. (B) Western blot analysis of apelin and APJ in RAW264.7 cells. (C) Quantification of the density of expression level of apelin and APJ. The values are expressed as the means ± SEM. Western blot results are normalized to β-actin. Small letters compare every relative protein and mRNA expression values among groups; means followed by the same letter are not different ($p > 0.05$), while means followed by different letters show significant differences ($p < 0.05$), (n=3 in each group).

in activated murine and human macrophages [24,25]. Further experiments were carried out to investigate the effects of 1, 25 $(OH)_2D_3$ on VCAM-1 and ICAM-1 expression. The results clearly showed that VCAM-1 and ICAM-1 protein expression were completely abolished by 1, 25$(OH)_2D_3$ treatment in LPS-activated macrophages. A number of studies have shown that 1, 25$(OH)_2D_3$ has potent anti-inflammatory effects and therefore has been assumed for adjunctive therapy for numerous chronic diseases including asthma, rheumatoid arthritis, and atherosclerosis [26,27]. Studies have also revealed that 1, 25$(OH)_2D_3$ interferes with NF-kB as a master regulator of inflammatory mediator by the increased $I_kB\alpha$ expression, consequently interfering with the nuclear translocation of the activated NF-kB subunits [28]. Dong Ma et al. identified that 1, 25$(OH)_2D_3$ regulates cell proliferation and inflammation in macrophage cells by attenuating of NF-kB subunit interaction [29].

The peptide apelin and its G-protein coupled receptor, APJ, have been shown to be associated with the pathophysiology of cardiovascular diseases [11]. However, several studies have demonstrated the anti-inflammatory effects of apelin. Leeper et al. showed that the apelin medicated attenuating of pro-inflammatory cytokines in abdominal aortic aneurysm by inhibiting of macrophage [12]. Furthermore, Izgut-Uysal et al. confirmed that apelin inhibited the release of inflammatory cytokines in LPS-activated peritoneal macrophages [14]. Zhang et al. reported the protective effects of apelin administration against LPS-activated acute lung injury in vivo and in vitro experiment [30]. Moreover, Liu et al. indicated the anti-atherogenic effects of apelin by inhibition of foam cell formation in THP-1 macrophage cells [31]. In vitro evidence has shown that apelin repression by miR-497 facilitates foam cell formation in human THP-1 cell line [15]. Nevertheless, studies have attested the inhibitory effect of 1, 25$(OH)_2D_3$ against foam cell formation in macrophages from diabetics patients [32]. However, an actual relationship between miR-497 and 1, 25$(OH)_2D_3$ with regarding the apelin regulation is not completely elucidated. Yang et al. reported that apelin and APJ up-regulated in macrophage cells under hypoxia. They also indicated that apelin treatment could protect RAW264.7 macrophage from apoptosis and inflammation process by down-regulating chemokines and angiogenic factors [33]. Since apelin plays a crucial role in atherosclerosis, we encourage to determine that 1, 25$(OH)_2D_3$ could regulate apelin expression in LPS-stimulated RAW264.7 cells. The direct effect of 1, 25 $(OH)_2D_3$ on apelin expression in macrophage cells physiology has never been investigated to date. In this present *in vitro* study, we explored that pretreatment of stimulated RAW264.7 cells with 1, 25$(OH)_2D_3$ augments significantly apelin/APJ and improves LPS-activated cytotoxicity leading to the formation of inflammatory cytokines in RAW264.7 cells.

These reports generate clues for the effect of 1, 25$(OH)_2D_3$ on the apelin/APJ system activation in LPS-stimulated RAW264.7 macrophage cell. Recent work supports the protective effects of apelin by pleiotropic mechanisms against injury induced by LPS [13]. It is possible that some signaling pathway have been implicated in 1, 25$(OH)_2D_3$ treatment. However, the limitations of this investigation is the lack of study about the knockdown or overexpression of apelin/APJ system effects on production of inflammatory mediator for better understanding of the interplay between 1, 25$(OH)_2D_3$ and apelin/APJ system in inflammation. Taken together, our results demonstrated the anti-inflammatory effects of 1, 25$(OH)_2D_3$ in LPS-activated RAW264.7 cells by the prevention of inflammatory cytokines and adhesion molecules production. Of particular importance, our results indicated that 1, 25$(OH)_2D_3$, as native form of vitamin D3, may regulate inflammatory response in LPS-induced injury in macrophages through a likely activation of apelin/APJ system and provided the new insight into macrophage function.

Conflict of interest

The authors declare no conflict of interest.

Authors' contributions

Yousef Faridvand, Maohammad Nouri and Sammad Ghaffari designed experiments and analyzed results. Yousef Faridvand, Nazanin Bagherpour-Hassanlouei, Samira Nozari, Nasrin Nasiri and Hadi Rajabi performed experiments, wrote the manuscript, and helped the manuscript revising and language editing.

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