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Vitamin D suppresses lipopolysaccharide-induced inflammatory response in vascular smooth muscle cells *via* inhibition of the p38 MAPK signaling pathway

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Inflammation and vascular smooth muscle cells (VSMCs) play key roles in the development of many cardiovascular diseases (CVDs). Although vitamin D decreases the risks of inflammation related diseases including CVDs, the links between vitamin D, VSMCs and vascular inflammation remained unclear. In this study, we investigated the anti-inflammatory effect and signaling pathways of vitamin D in lipopolysaccharide (LPS)-induced VSMCs. $1,25(OH)_2D_3$ treatment inhibited the significant upregulation of COX-2, PGE_2, TNF-α and IL-6 and p38 phosphorylation induced by LPS in A10 cells. Blocking p38 signaling attenuated the inhibitory effect of $1,25(OH)_2D_3$ on the upregulation of COX-2 and phosphorylation of p38. These results indicate $1,25(OH)_2D_3$ suppresses inflammatory response in LPS-induced VSMCs through p38 MAPK signaling pathway.

1. Introduction

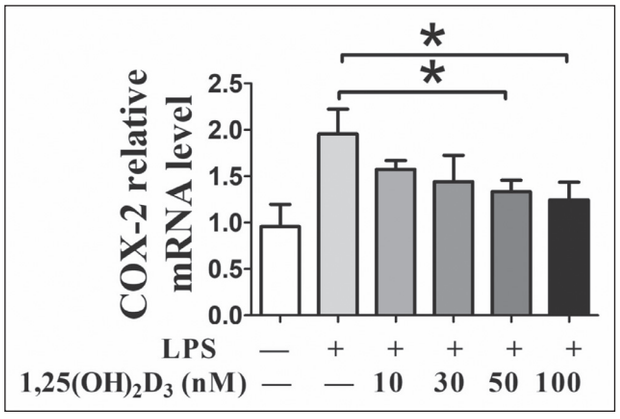
Inflammation is essential in the pathogenesis of cardiovascular diseases (CVD) (Ferrucci and Fabbri 2018). Vascular smooth muscle cells (VSMCs) are deeply involved in vascular inflammatory response to injury or infection through releasing inflammatory mediators, producing cytokines and growth factors, undergoing excessive proliferation and migration, and depositing extracellular matrix (ECM), altogether finally contributing to vascular remodeling and adverse outcomes (Wadey et al. 2018; Wu et al. 2014; Doran et al. 2008).

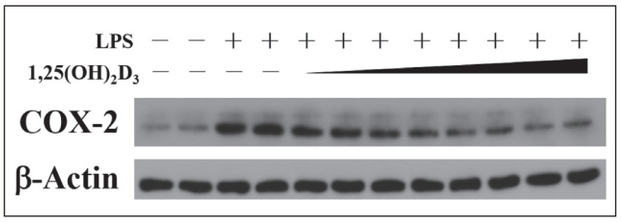
Prostanoids, which are synthesized from arachidonic acids (AA) by two isoforms of cyclooxygenases (COX-1 and COX-2), are important inflammatory mediators which are significantly increased in inflammatory response (Vane 1976; Ricciotti and FitzGerald 2011; Tilley et al. 2001). Among the two isoforms of COX, COX-1 is constitutively distributed in most tissues and mainly responsible for producing basal levels of prostaglandins to serve homeostatic functions and protect the gastrointestinal mucosa (Ornelas et al. 2017), whereas COX-2 is inducible and contributive to releasing prostaglandins under internal or external stimuli such as an infection, an injury or carcinogenesis (Ricciotti and FitzGerald 2011). Inhibition of COXs by nonsteroidal anti-inflammatory drugs (NSAIDs) leads to prostanoids inhibition and therefore anti-inflammatory, analgesic, antipyretic actions, which highlights the pro-inflammatory role of prostanoids (Vane 1976; Ricciotti and FitzGerald 2011). Moreover, inhibition of COX-2, but not COX-1, was reported to reduce vascular neointimal hyperplasia after mechanical injury (Yang et al. 2004) and angiotensin II induced abdominal aortic aneurysm (King et al. 2006).

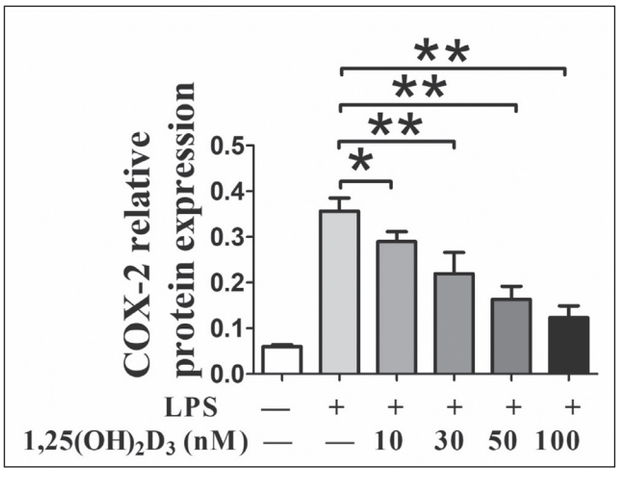
COX-2-derived PGE_2 is the main prostanoid released by inflammatory cells and vascular cells including VSMCs that participate in all processes of acute inflammation (pain, swelling, redness, fever) (Funk 2001). In vascular inflammation, PGE_2 promotes vascular remodeling through binding to one or more of its four cognate receptors (EP1-EP4) under various pathological situations such as hypertension (Avendaño et al. 2016), atherosclerosis (Wang et al. 2006), aneurysm (Wang et al. 2008; Camacho et al. 2013) and neointimal hyperplasia (Wang et al. 2011; Zhang et al. 2013). Deletion or inhibition of COX-2 or microsomal prostaglandin E synthase-1(mPGES-1), the dominant enzyme that catalyzes the isomerization of PGH_2 into PGE_2 can attenuate those pathological changes (Avendaño et al. 2016; Zhang et al. 2013). Together, these results suggest that the COX-2/PGE_2 pathway plays important pro-inflammatory roles in inflammatory response of VSMCs.

Vitamin D is a fat-soluble hormone which plays a vital role in multiple physiological processes, such as mineral homeostasis, cellular proliferation and differentiation, immune responses and inflammation through genomic responses via vitamin D receptors (VDRs) (Clark and Mach 2016; Wei et al. 2018; Ding et al. 2013; Christakos et al. 2016) or rapid non-genomic actions (Rebsamen et al. 2002; Hii and Ferrante 2016). Vitamin D, which is obtained either from photosynthesis in the skin or from dietary intake, is hydroxylated to 25-hydroxycholecalciferol (25(OH)D) or calcidiol in the liver, and then in the kidney to produce the biologically active compound 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) or calcitriol. Due to low sunlight exposure and insufficient dietary sources, vitamin D deficiency is common worldwide (Holick 2007).

Previous observational studies reported that vitamin D deficiency was linked to increasing risk of various pathological changes and illnesses including inflammation and cardiovascular diseases (Al Mheid et al. 2013; Holick 2007; Wimalawansa 2018; Christakos et al. 2016; Guillot et al. 2010). *In vitro* and *in vivo* studies suggested that vitamin D could suppress pro-inflammatory mediators as well as increase anti-inflammatory cytokines, *via* inhibition of NF-κB and mitogen-activated protein kinases (MAPKs) p38 pathways in endothelial cells (Paˊl et al. 2018; Talmor et al. 2008; Kudo et al. 2012). Our previous study also demonstrated that $1,25(OH)_2D_3$ inhibited secretion of pro-inflammatory mediators TNF-α and IL-6 through suppression of COX-2 pathway in macrophages (Wang Q et al. 2014). However, the anti-inflammatory effect and mechanism of vitamin D on LPS-stimulated VSMCs is poorly understood. To prove the hypothesis that vitamin D might suppress LPS-induced inflammatory response in VSMCs via COX-2 and MAPKs pathways, we investigated the anti-inflammatory effects of vitamin D and its intermediary pathways in rat VSMCs.







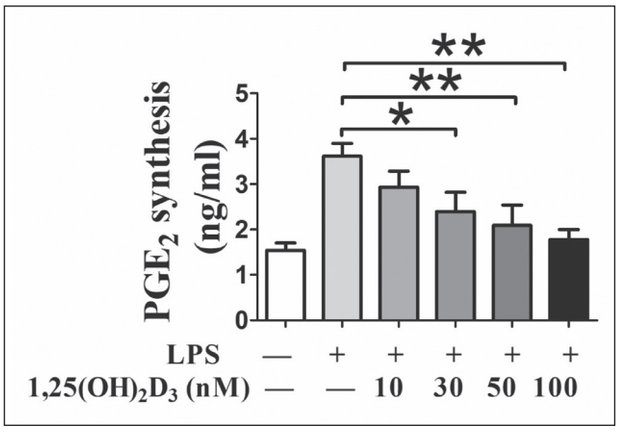


Fig. 1: $1,25(OH)_2D_3$ inhibits COX-2 expression and PGE_2 production in VSMCs. A10 cells were treated with different dosages of $1,25(OH)_2D_3$ (10nM, 30nM, 50nM and 100nM) in the presence of LPS. The mRNA (A) and protein expressions (B) of COX-2 were analyzed by quantitative RT-PCR and Western blotting. Densitometric quantitation of COX-2 expression by comparison to β-actin as shown in (C). Effect of $1,25(OH)_2D_3$ treatment on PGE_2 as shown in (D). *P <0.05, **P < 0.01; n=3.

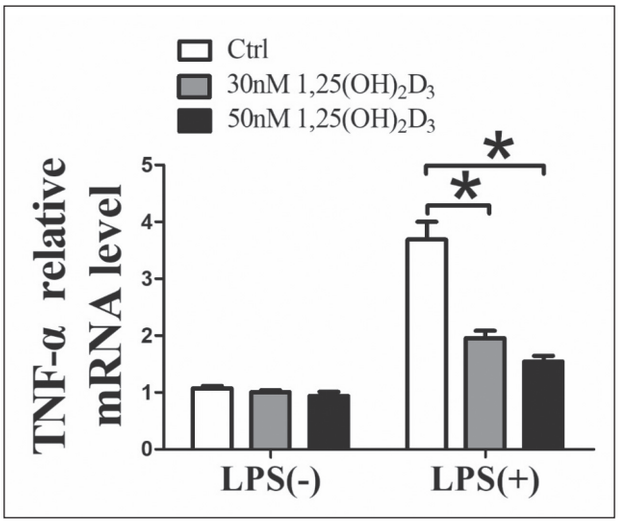
2. Investigations and results

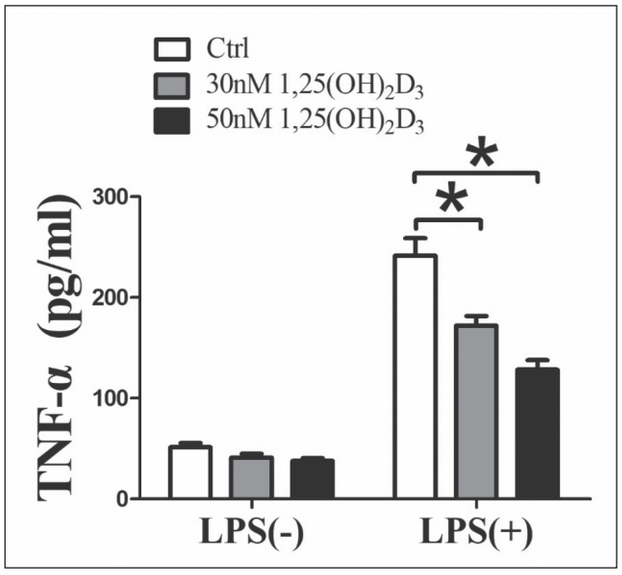
2.1. $1,25(OH)_2D_3$ inhibits COX-2 and PGE_2 production in A10 VSMCs

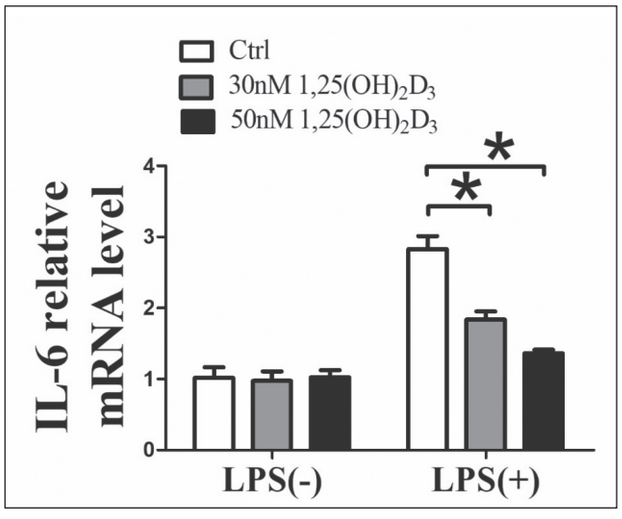
To determine whether $1,25(OH)_2D_3$ affects the expression of COX-2 and PGE_2, A10 cells were pre-treated with different doses of $1,25(OH)_2D_3$ in the presence of LPS. Then the cells were harvested for COX-2 measurement and the supernatant was used for PGE_2 measurement. Both COX-2 expression (Fig. 1A-C) and PGE_2 (Fig. 1D) production were remarkably upregulated in LPS-stimulated cells compared to the control group without LPS stimulation. $1,25(OH)_2D_3$ dose-dependently suppressed LPS-induced COX-2 mRNA (Fig. 1A) and protein expression (Fig.1B, C). Although 10 nM $1,25(OH)_2D_3$ inhibited COX-2 protein expression ($P< 0.05$), higher concentrations (50 or 100 nM) of $1,25(OH)_2D_3$ had more significant effect on COX-2 mRNA levels ($P< 0.05$) (Fig. 1A) and protein levels ($P< 0.01$) (Fig. 1B, C). $1,25(OH)_2D_3$ also exhibited concentration-dependent suppression on LPS-induced PGE_2 production, with higher concentrations (50 or 100 nM) having more notable influence ($P< 0.01$) (Fig. 1D). These results indicate that $1,25(OH)_2D_3$ reduced LPS-induced COX-2 and PGE_2 production.

2.2. $1,25(OH)_2D_3$ downregulates secretions of TNF-α and IL-6 in A10 VSMCs

To determine whether $1,25(OH)_2D_3$ can attenuate the expression of pro-inflammatory cytokines, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and enzyme linked immunosorbent assay (ELISA) were used to examine the mRNA and protein level of TNF-α and IL-6 in A10 VSMCs. Both mRNA level and secretion of TNF-α (Fig. 2A, B) and IL-6 (Fig. 2C, D) were upregulated remarkably in stimulated by LPS ($P< 0.05$), while $1,25(OH)_2D_3$ did not significantly influence the basal level of TNF-α and IL-6 without LPS stimulation (Fig. 2A-D). $1,25(OH)_2D_3$ did not significantly influence the basal level of TNF-α and IL-6 in A10 VSMCs without LPS stimulation. Both mRNA level and secretion of TNF-α (Fig. 2A, B) and IL-6 (Fig. 2C, D) were upregulated remarkably in A10 VSMCs stimulated by LPS ($P< 0.05$). $1,25(OH)_2D_3$ treatment (30 nM or 50 nM) suppressed mRNA transcripts of both TNF-α and IL-6 (Fig. 2A,C, $P< 0.05$), although the difference between two different doses of $1,25(OH)_2D_3$ treatment was statistically insignificant. Consistently, $1,25(OH)_2D_3$ also attenuated secretions of both TNF-α and IL-6 proteins (Fig.2B, D, $P< 0.05$). $1,25(OH)_2D_3$ treatment (50 nM) were more effective in inhibiting IL-6 secretion than that for 30nM (Fig. 2D, $P< 0.05$), whereas these two concentrations presented no statistical difference in inhibiting TNF-α secretion (Fig. 2B).







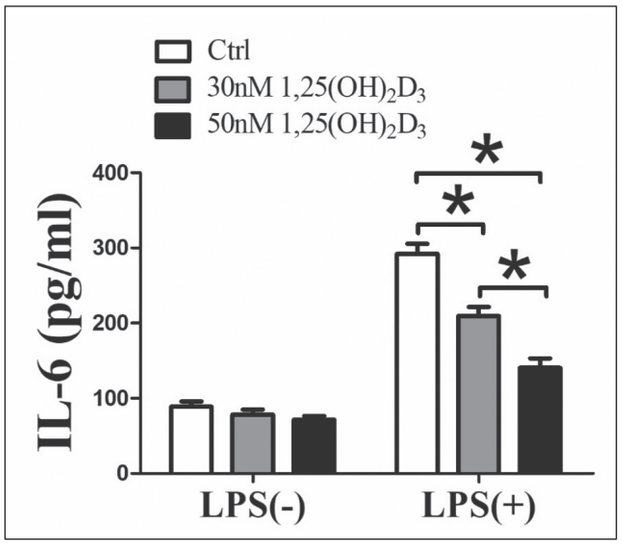
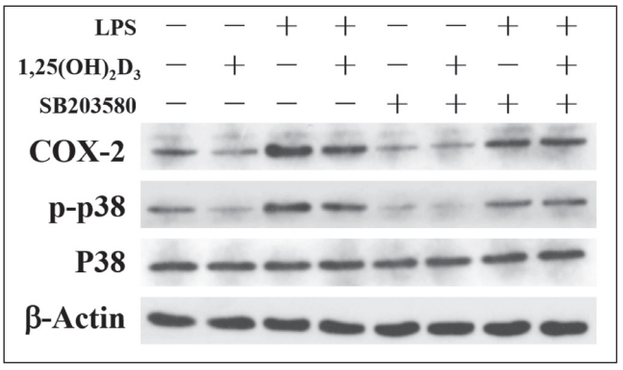
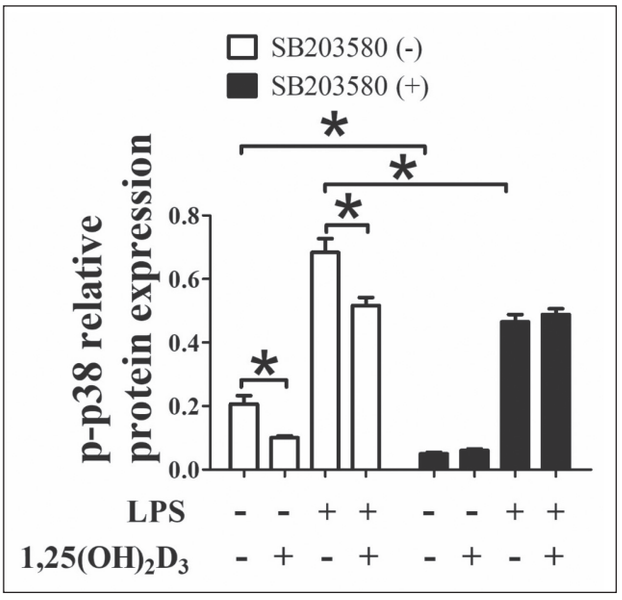


Fig. 2: $1,25(OH)_2D_3$ inhibits the expression and secretion of pro-inflammatory cytokines in VSMCs. (A, B) Effect of $1,25(OH)_2D_3$ on mRNA expression and secretion of TNF-α in A10 cells in presence and absence of LPS stimulation. (C, D) Effect of $1,25(OH)_2D_3$ on mRNA expression and secretion of IL-6 in A10 cells in absence and presence of LPS stimulation. *P <0.05, **P < 0.01; n=3.

2.3. $1,25(OH)_2D_3$ inhibits p38 MAPK signaling in A10 VSMCs

MAPKs can be activated by stress and inflammatory stimuli including LPS and promote the expression of pro-inflammatory





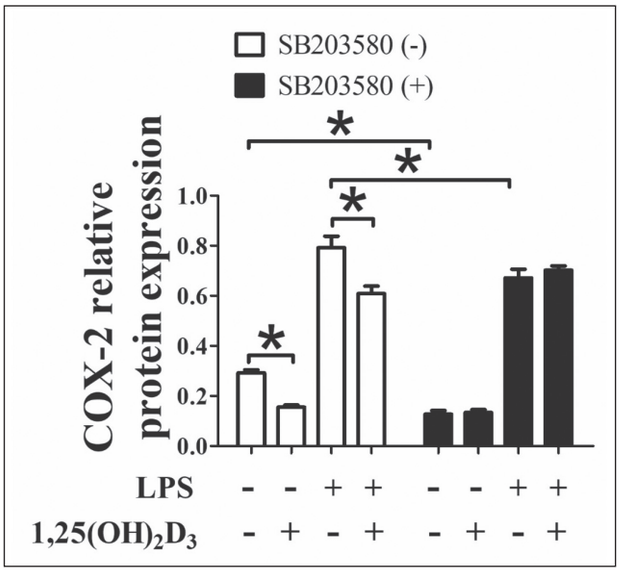
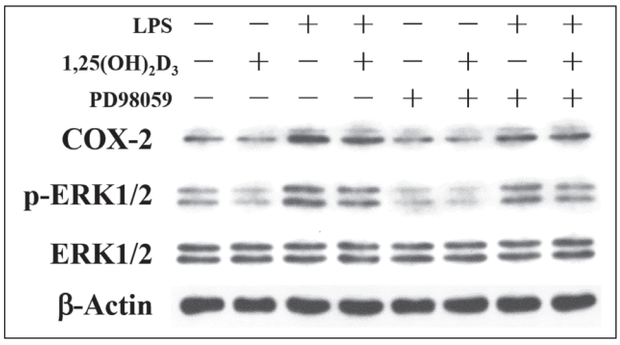
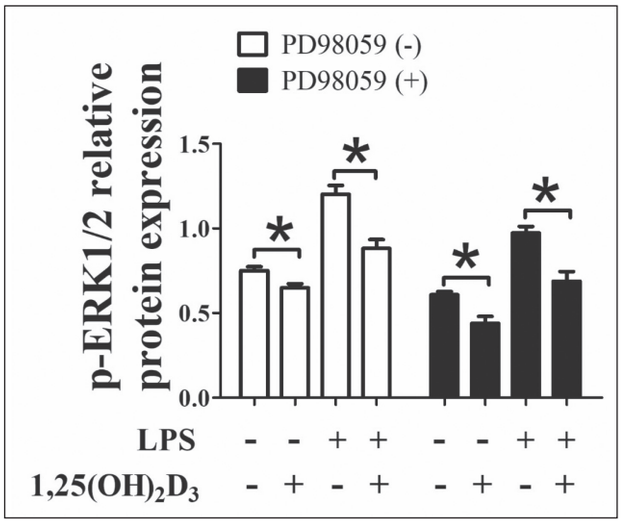


Fig. 3: Effect of $1,25(OH)_2D_3$ on P38 pathway in VSMCs. (A) Effect of P38 MAPK inhibitor-SB203580 (5μM) on phosphorylation of p38 (p-p38) and COX-2 expression in A10 cells. Densitometric quantitation of expression of p-p38 and COX-2 by comparison to β-actin as shown in (B, C). *P <0.05, **P < 0.01; n=3.

cytokines (Kyriakis and Avruch 2012). The best investigated MAPKs are p38 MAPK, extracellular signal-regulated kinases (ERKs), and c-Jun N-terminal kinases (JNKs) (Hazzalin and Mahadevan 2002). To explore the anti-inflammatory mechanism of $1,25(OH)_2D_3$, we measured the phosphorylation situation of p38 MAPK. LPS treatment induced a notable increase of phosphorylation of p38 MAPK (Fig. 3A, B) and COX-2 expression (Fig. 3A, C), which were both suppressed by $1,25(OH)_2D_3$ (50 nM). Interestingly, p38 MAPK inhibitor-SB203580 (5μM) blunted the suppression effect of $1,25(OH)_2D_3$ on LPS-induced p38 phosphorylation





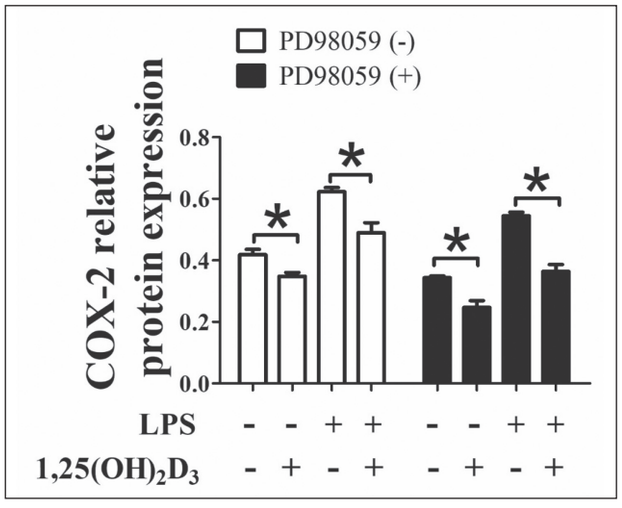
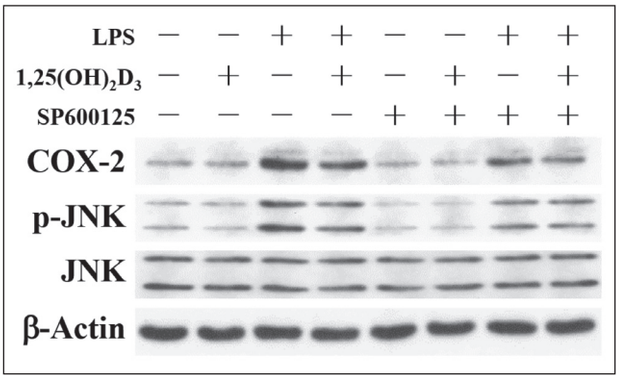
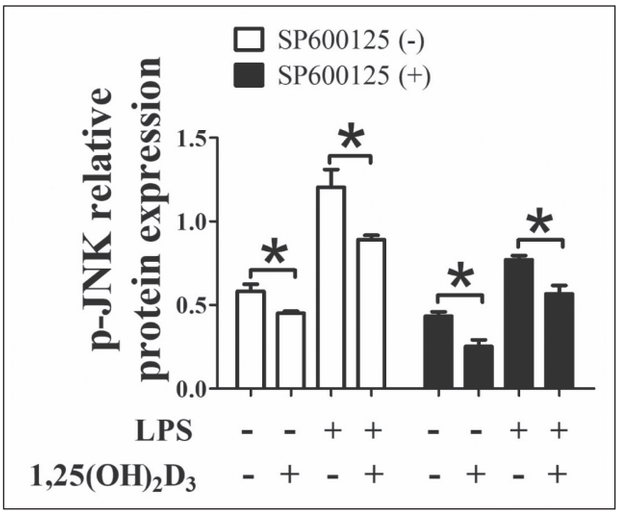


Fig. 4: Effect of $1,25(OH)_2D_3$ on ERK1/2 MAPK pathway in VSMCs. (A) Effect of ERK1/2 MAPK inhibitor-PD98059 (200 μM) on phosphorylation of ERK1/2 (p-ERK1/2) and COX-2 expression in A10 cells. Densitometric quantitation of expression of p-ERK1/2 and COX-2 by comparison to β-actin as shown in (B, C). *P <0.05, **P < 0.01; n=3.





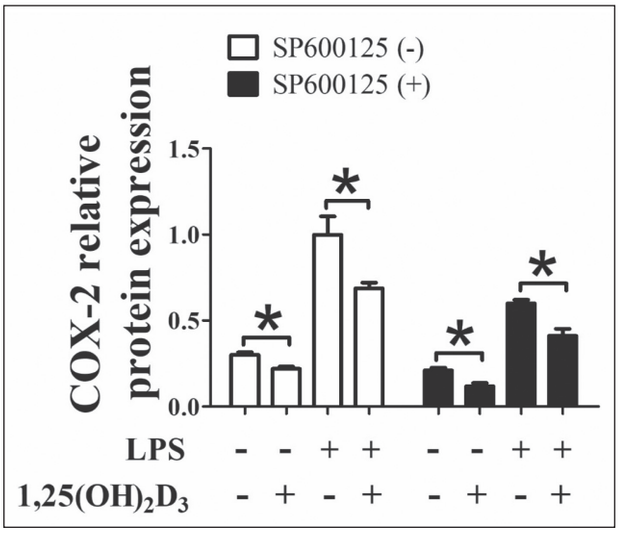


Fig. 5: Effect of $1,25(OH)_2D_3$ on JNK pathway in VSMCs. (A) Effect of JNK MAPK inhibitor-SP600125 (5μM) on phosphorylation of JNK (p-JNK) and COX-2 expression in A10 cells. Densitometric quantitation of expression of p-JNK and COX-2 by comparison to β-actin as shown in (B, C). *P <0.05, **P < 0.01; n=3.

(Fig.3A, B, P< 0.05) and subsequently restored COX-2 expression (Fig. 3A, C, P< 0.05).

2.4. Effect of $1,25(OH)_2D_3$ on ERK MAPK signaling in A10 VSMCs

LPS treatment induced a notable increase of phosphorylation of ERK MAPK (Fig. 4A, B) and COX-2 expression (Fig. 4A, C) in

A10 VSMCs, which were both suppressed by $1,25(OH)_2D_3$ (50 nM). However, ERK MAPK inhibitor- PD98059 (200 μM) did not show significant changes of $1,25(OH)_2D_3$ on LPS-induced ERK1/2 phosphorylation (Fig. 4A, B, $P< 0.05$) and COX-2 expression (Fig. 4A, C, $P< 0.05$).

2.5. Effect of 1,25(OH)$_2$D$_3$ on JNK MAPK signaling in A10 VSMCs

LPS treatment induced a remarkable increase of phosphorylation of JNK MAPK (Fig. 5A, B) and COX-2 expression (Fig. 5A, C) in A10 VSMCs, which were both suppressed by $1,25(OH)_2D_3$ (50nM). However, we did not observe the visible influences of $1,25(OH)_2D_3$ on LPS-induced JNK phosphorylation (Fig. 5A, B, $P< 0.05$) and COX-2 expression (Fig. 5A, C, $P< 0.05$) by adding JNK MAPK inhibitor- SP600125 (5 μM) .

3. Discussion

Vitamin D plays an important role in inflammatory response in vasculatures through regulating inflammatory mediators and modulating the functions of inflammatory cells and vascular cells (Christakos et al. 2016; Guillot et al. 2010). Vitamin D deficiency was associated with increased risk of several cardiovascular changes such as arterial stiffness (Al Mheid et al. 2011), vascular remodeling (Pa´l et al. 2018), and atherosclerosis (Faridi et al. 2017). VSMC is a major cell type in vascular wall and responsible for producing extracellular matrix. However, it was suggested that VSMCs also plays an important role in inflammatory response in blood vessels. Recent studies reported that, although VSMCs exist mainly in tunica media, a large number of VSMCs exist in the intima as well, particularly in older patients (Doran et al. 2008). VSMCs can produce multiple inflammatory molecules that contribute to the initiation and advancement of intimal thickening and atherosclerosis (Wadey et al. 2018; Doran et al. 2008). *In vitro*, vitamin D was reported to modulate tissue factor (TF) expression (Martinez-Moreno et al. 2016), decreased bone morphogenetic protein 2 (BMP-2)(Guerrero et al. 2012), trigger the shedding of TNF receptor 1 (TNFR1) and decreases responsiveness to TNF-α (Yang et al. 2015) in VSMCs under stimulation by TNF-α. However the genomic effect of vitamin D on inflammatory response in VSMCs stimulated by LPS remains unknown. LPS, as a risk factor for CVDs, could activate inflammatory responses in VSMCs (Yang et al. 2015; Jiang et al. 2014). The A10 cell line, derived from the thoracic aorta of rat embryo, was commonly used as a model of non-differentiated VSMC that bear great resemblance to neointimal cells (Rao et al. 1997). In this study, we used A10 cells to investigate the anti-inflammatory effect of vitamin D on VSMCs. We found here that LPS remarkably upregulated the production of pro-inflammatory mediators (COX-2, PGE_2, TNF-α and IL-6) in rat VSMCs, and the expression of these mediators was significantly inhibited by treatment of $1,25(OH)_2D_3$ for 24 h in the presence of LPS. Our present study indicates that vitamin D can suppress the expression of pro-inflammatory molecules produced by LPS-induced VSMCs that might contribute to reducing risk of CVDs.

COX-2 and COX-2 derived PGE_2 are important pro-inflammatory mediators in vasculatures (Vane 1976; Ricciotti and FitzGerald 2011; Tilley et al. 2001; Funk 2001). Consistent with these reports, we found COX-2 expression as well as PGE_2 synthesis to be very low in VSMCs in the absence of LPS and was induced markedly in inflammation, as shown in Figs. 1, 3, 4, and 5. In addition, constitutive levels of pro-inflammatory cytokines (TNF-α, IL-6) were low in VSMCs, but the expressions of both cytokines were considerably induced by LPS, as shown in Fig. 2. TNF-α plays an essential role in the induction of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1, thus enhancing leukocyte recruitment , promoting VSMCs proliferation and migration, and blocking endothelial regrowth after injury (Raines and Ferri 2005; Hopkins 2013). IL-6 induces expression of monocyte chemotactic protein-1 (MCP-1) (Hosaka et al. 2017), recruits monocytes, increases vascular calcification (Callegari et al. 2014), and is a strong independent marker to predict mortality (Lindmark et al. 2001). Hence, our current study confirms VSMCs play a vital role in vascular inflammation through production of multiple pro-inflammatory mediators.

COX-2 derived PGE_2 can increase the production of inflammatory cytokines such as IL-1β, IL-6 and MCP-1(Babaev et al. 2008; Bayston et al. 2003). Therefore, anti-PGE_2 treatment using antibodies yielded an inhibition of carrageenan-induced paw inflammation and IL-6 production (Portanova et al. 1996). Moreover, our previous studies found that downregulation of COX-2 attenuates the secretion of TNF-α and IL-6 in macrophages (Wang et al. 2014). On the other hand, PGE_2 is also induced by cytokines such as IL-1β, and TNF-α (Soler et al. 2000; Warner and Libby 1989). To our knowledge, we first demonstrated that vitamin D dose-dependently inhibited the expression of COX-2 and PGE_2 in VSMCs stimulated by LPS *in vitro*. Notably, we also found that $1,25(OH)_2D_3$ suppressed the basal level of COX-2 in A10 cells in the absence of LPS. In addition, vitamin D attenuated the production of TNF-α and IL-6 in a dosage-dependent manner. Taken together, these studies strongly indicate that COX-2/PGE_2 forms a regulatory loop with pro-inflammatory cytokines such as TNF-α and IL-6 in collaboration to create an overlapping pro-inflammatory effect in vasculatures, and vitamin D might decrease the production of pro-inflammatory cytokines through COX-2/PGE_2 pathway to inhibit inflammation.

COX-2 expression has been reported to be regulated by MAPKs in a variety of cell types (Scotece et al. 2018; Guolan etal. 2018; Chen et al. 2003). MAPKs are activated by simultaneous phosphorylation of both tyrosine (Tyr) and threonine (Thr) in a conservative Thr-X-Tyr motif in the activation loop of subdomain VIII (Kyriakis and Avruch 2012). MAPK pathways can be activated by many different stimuli including hormones, growth factors, cytokines, danger-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs) such as LPS (Kyriakis and Avruch 2012). Once activated, MAPKs participate in functions including gene expression, apoptosis, proliferation, and differentiation (Kyriakis and Avruch 2012). The most investigated mammalian MAPKs include p38, ERK, and JNKs (Kyriakis and Avruch 2012; Hopkins 2013). P38 MAPK post-transcriptionally stabilize COX-2 mRNA (Chun and Surh 2004), whereas JNKs and ERKs activate COX-2 transcription by binding the activator protein-1 (AP-1) site of its promoter (Kyriakis and Avruch 2012). The P38 MAPK inhibitor SB203580 is a pyridinyl imidazole which binds to the ATP binding site to inhibit specifically the enzyme activity of p38α and p38β (Davies et al. 2000; Gum et al. 1998). The ERK1/2 MAPK inhibitor PD98059 targets two ERK-specific MAP2Ks (MKK1 and MKK2), and exhibits a strong interaction with the inactive unphosphorylated kinases than phosphorylated ones and, thus, prevents the phosphorylation of MKK1, and/or the conformational change (Bain et al. 2007; Alessi et al. 1995). The JNK MAPK inhibitor SP600125 forms a hydrogen-bond with ATP binding site of JNKs and has similar potency for JNK-1, -2, and -3 isoforms (Bennett et al. 2001). Consistent with previous studies, we found that LPS induced a notable increase of phosphorylation of p38, ERK1/2 and JNK; p38 MAPK inhibitor (SB203580), ERK1/2 MAPK inhibitor (PD98059) and JNK MAPK inhibitor (SP600125) all downregulated COX-2 in the presence and absence of LPS. Generally, COX-2 expression was mediated by activation of MAPKs. LPS might initiate MAPKs signaling pathway through TLR4 receptor to promote cytokine secretion (Son et al. 2008), proliferation (Jiang et al. 2014; Yang G et al. 2015) and migration of VSMCs (Yang et al. 2015). Moreover, $1,25(OH)_2D_3$ was demonstrated to interfere with MAPKs signaling in different types of cells (Zhang et al. 2012; Hosokawa et al. 2015), but the effect of $1,25(OH)_2D_3$ on MAPKs pathway in VSMCs was not clear. In this study, we found that $1,25(OH)_2D_3$ treatment downregulated LPS-induced COX-2 expression and p38 phosphorylation, and p38 MAPK inhibitor blunted the suppression effect of $1,25(OH)_2D_3$ on both p38 phosphorylation and COX-2 expression stimulated by LPS, while ERK MAPK inhibitor and JNK MAPK inhibitor had no notable influence on the effect of $1,25(OH)_2D_3$ on phosphorylation of ERK1/2 and JNK, respectively, and COX-2 inhibition.

Taken together, our findings indicates that $1,25(OH)_2D_3$ suppresses COX-2 induction in LPS-induced VSMCs through inhibition of p38 signaling pathway, but not ERK1/2 and JNK pathway.
In conclusion, $1,25(OH)_2D_3$ inhibits COX-2 expression in VSMCs via p38 MAPK signaling pathway. Therefore, $1,25(OH)_2D_3$ might be used as an adjuvant therapy to reduce inflammation for CVDs.

4. Experimental

4.1. Cell culture and treatment

The Rat VSMC cell line A10 was purchased from the American Type Culture Collection Shanghai Cell Institute Country Cell Bank (Shanghai, China). Cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, California, USA) which were supplemented with 10 % fetal bovine serum (FBS)(Gibco, California, USA), penicillin (100 U/ml, Gibco-Invitrogen, Paisley, UK) and streptomycin (100 U/ml, Gibco-Invitrogen, Paisley, UK), and were cultivated at 37 ˚C in a humidified atmosphere of 5 % CO_2. A10 cells at 70 % confluence were serum-starved for 24 h and pretreated with different concentration of 1,25-dihydroxy-vitamin D_3 (Sigma-Aldrich, St. Louis, USA) for 24 h. The supernatant was removed, and cells were cultured in serum-free medium, with or without treatment of LPS (5 μg/mL, Sigma-Aldrich, St. Louis, USA). Then the supernatant was collected and stored at -80 ˚C for ELISA and enzyme immunoassay. The cells were washed with PBS and lysed with Trizol reagent (Invitrogen, California, USA) before stored at -80˚C for qRT-PCR and western blotting assay.

4.2. Quantitative real time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, California, USA) and then reverse transcribed to cDNA by use of the Moloney murine leukemia virus reverse transcriptase (TaKaRa, Japan). The mRNA expression levels of COX-2, TNF-α and IL-6 were evaluated by quantitative RT-PCR (Applied Biosystems, USA). The primers were as followes: the COX-2 primer sequences were forward primer 5'- AGAGCAGAGAGATGAAATACC -3' and reverse primer 5'- AGGAGAACAGATGGGATTAC -3'; the IL-6 primer sequences were forward primer
5'- GTTGCCTTCTTGGGACTGATG -3' and reverse primer 5'-ACTGGTCTGTTGTGGGTGGT -3'; the TNF-α primer sequences were forward primer 5'-GAAACAGTCTGCGAGGTGTG -3' and reverse primer 5'- TTCTTCTTGCAGCCACACAC -3'.

4.3. Western blotting

The treated A10 cells were washed once in ice-cold PBS and were placed into lysis buffer. After centrifugation, total protein concentration was collected and quantified using a Bio-Rad protein assay. The proteins were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were blocked using TBST (0.1 % Tween-20, 150 mM NaCl, 50 mM Tris, pH 7.4) containing 5 % non-fat milk and then incubated with primary antibodies against COX-2 (Abcam, MA, USA), phospho-ERK1/2 MAPK (Cell Signaling Technology, MA, USA), ERK1/2 MAPK (Cell Signaling Technology, MA, USA), phospho-p38 MAPK (Cell Signaling Technology, MA, USA), p38 MAPK (Cell Signaling Technology, MA, USA), phospho-JNK MAPK (Cell Signaling Technology, MA, USA), JNK MAPK (Cell Signaling Technology, MA, USA), and β-actin(Sigma-Aldrich, St. Louis, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Cell Signaling Technology, MA, USA) were used as secondary antibodies. Signals were detected using enhanced chemiluminescence (ECL, Pierce).

4.4. Analysis of PGE_2 activity by enzyme immunoassay

For the PGE_2 assay, A10 cells were cultured in 6-well plates and treated with different concentrations of $1,25(OH)_2D_3$ (10 nM, 30 nM, 50 nM and 100 nM) for 24 h. Then the cells were cultured with fresh DMEM containing 5 % FBS before stimulated with LPS (5μg/ml) for 12 h. The supernatant was collected immediately for PGE_2 assay or stored at -80 °C for measurement. PGE_2 was measured using an enzyme immunoassay kit (R&D systems, MN, USA) according to the manufacturer's instructions.

4.5. Analysis of TNF-α, IL-6 by enzyme linked immunosorbent assay (ELISA)

The supernatant of cultured A10 cells were collected and the levels of TNF-α and IL-6 were assayed using ELISA kits from R&D Systems (MN, USA) according to the manufacturer's instructions.

4.6. Statistical analysis

All experiments were carried out at least three times, and the data were presented as mean±standard deviation (SD). Values were analyzed using one-way analysis of variance (ANOVA) and Student's t-test with SPSS 13.0 software (SPSS, Chicago, USA). $P < 0.05$ was considered statistically significant.

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