

Anti-inflammatory effect of 1,25-dihydroxyvitamin D₃ is associated with crosstalk between signal transducer and activator of transcription 5 and the vitamin D receptor in human monocytes

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Received June 16, 2014; Accepted January 26, 2015

DOI: 10.3892/etm.2015.2321

Abstract. 1,25-Dihydroxyvitamin D₃ (1,25-(OH)₂D₃) has an anti-inflammatory effect on human monocytes incubated with sera from patients with type 2 diabetes/diabetic nephropathy; however, the detailed mechanism behind the effect remains to be explored. The current study further validated the effects of 1,25-(OH)₂D₃ and lipopolysaccharide (LPS) + human recombinant interleukin (IL)-15 on the expression of the vitamin D receptor (VDR) and phosphorylated signal transducer and activator of transcription 5 (p-STAT5) in human monocytes and explored the possible interaction between VDR and p-STAT5. Synchronized THP-1 cells were divided into pre-intervened groups, namely the control, LPS + IL-15 and 1,25-(OH)₂D₃ groups, according to their differing treatments. The expression of STAT5 and p-STAT5 was evaluated by western blot analysis; the concentration of IL-6 in the supernatant was determined using an enzyme-linked immunosorbent assay; the expression of cytoskeletal proteins was observed using immunofluorescence and laser confocal microscopy; and the possible intranuclear interaction between VDR and p-STAT5 was investigated using immunofluorescence, immuno-coprecipitation and western blot analysis. LPS + IL-15 upregulated p-STAT5 expression and the IL-6 level (P<0.05), with cytoskeletal rearrangement. These effects were partially prevented through pretreatment with 1,25-(OH)₂D₃. The LPS + IL-15 group and the 1,25-(OH)₂D₃ group exhibited an interaction between p-STAT5 and VDR in the nucleus, with the latter group showing a significant increase compared with the former (P<0.05). The immuno-coprecipitation

results provided evidence of the interaction between VDR and p-STAT5, which suggests the existence of STAT5-VDR crosstalk in THP-1 monocytes. Cytoskeletal rearrangement, VDR and p-STAT5 potentially have interactions in THP-1 monocytes. The anti-inflammatory effect of 1,25-(OH)₂D₃ may be associated with crosstalk between STAT5 and VDR, which further induces cytoskeletal rearrangement.

Introduction

Inflammation and inappropriate immune activity may be closely associated with the development of type 2 diabetes mellitus (T2DM) and diabetic nephropathy (DN) (1-4). In T2DM, monocytes demonstrate pro-inflammatory characteristics and an increase in the expression of inflammatory factors (3-7).

Vitamin D can prevent the development of numerous chronic diseases, such as diabetes (8,9), infectious diseases (10,11) and autoimmune diseases (12,13). Vitamin D may have a preventative effect on T2DM, since it is known that the concentration of a key metabolite, 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), is independently associated with insulin sensitivity and β-cell function among individuals at risk of T2DM (9). 1,25-(OH)₂D₃ suppresses the expression of toll-like receptor (TLR) 2 and TLR4 proteins and mRNA in human monocytes in a time- and dose-dependent manner, and reduces the effectiveness of the monocyte response to bacterial cell wall components in line with a vitamin D receptor (VDR)-dependent mechanism, presumably due to the reductions in the levels of TLR2 and TLR4 (11). 1,25-(OH)₂D₃ downregulates the expression of TLR by monocytes and triggers hyporesponsiveness to pathogen-associated molecular patterns (12). Due to these factors, vitamin D has attracted attention from researchers.

1,25-(OH)₂D₃ is an active metabolite of vitamin D. Its interaction with the VDR in target cells regulates calcium phosphate metabolism, exerts an anti-inflammatory effect, controls cell proliferation, induces cell differentiation, affects immunoregulation and enhances glucose metabolism (14-16). In a pilot study, it was found that 1,25-(OH)₂D₃ had an anti-inflammatory effect on human monocytes incubated with sera from patients with T2DM and DN with uremia, and that

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Key words: 1,25-dihydroxyvitamin D₃, monocyte, lipopolysaccharide, interleukin-15, vitamin D receptor, signal transducer and transcription activator

it may exert an anti-inflammatory effect by regulating the signal transduction pathways that control VDR and signal transducer and activator of transcription 5 (STAT5) expression (1). Considering that these findings were obtained from the preliminary research phase, however, as well as that reports on the anti-inflammatory effect of 1,25-(OH)₂D₃ are rare, this anti-inflammatory effect requires further validation. Furthermore, the mechanism underlying the effect remains to be explored.

Based on the aforementioned results, the present study aimed to further validate the effect of 1,25-(OH)₂D₃ on the expression of VDR and phosphorylated STAT5 (p-STAT5) in human monocytes, as well as cytoskeletal rearrangement, and to explore the possible interaction between VDR and p-STAT5. The results of this study may shed new light on the multiple functions of vitamin D and lay a theoretical foundation for further exploration in related fields.

Materials and methods

Materials and reagents. Rabbit polyclonal VDR antibody (cat. no. ab3508) was purchased from Abcam (Cambridge, UK) and rabbit polyclonal p-STAT5 antibody (cat. no. 9351) was obtained from Cell Signaling Technology, Inc. (Denver, MA, USA). Mouse monoclonal STAT5 (cat. no. sc-377069) and p-STAT5 (cat. no. sc-81524) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). p-STAT5 was fluorescein isothiocyanate (FITC)-labeled and VDR was tetramethylrhodamine (TRITC)-labeled. In addition, horseradish peroxidase-labeled goat anti-mouse immunoglobulin (Ig)G (cat. no. ZB-2305); horseradish peroxidase-labeled goat anti-rabbit IgG (cat. no. ZB-2301); FITC-labeled goat anti-mouse IgG (cat. no. ZF-0312); TRITC-labeled goat anti-rabbit IgG (cat. no. ZF-0316) were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China); and mouse monoclonal anti-β-actin (cat. no., ICM001-100) was purchased from Beijing 4A Biotech Co., Ltd. (Beijing, China). 1,25-(OH)₂D₃, lipopolysaccharide (LPS) and F-actin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human interleukin (IL)-15 was manufactured by Peprotech (Rocky Hill, NJ, USA).

THP-1 cells have been widely used in investigations of human monocytes and macrophages *in vitro* (17,18). The THP-1 cell line (TcHu 57) used in this study was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

Cell culture and grouping. The THP-1 cells were re-suspended in RPMI-1640 culture medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies), penicillin and streptomycin (HyClone Laboratories, Inc., Logan, UT, USA) both at 100 U/ml, and flask-cultured in 5% (v/v) CO₂ at 37°C. Prior to each experiment, the cells were allowed to grow in serum-free medium for 24 h to ensure that all cells were synchronized at the G0 phase.

The synchronized cells were divided into the control, LPS + IL-15 and 1,25-(OH)₂D₃ groups according to their differing treatment. The control group was treated with phosphate-buffered saline (PBS) only. In the LPS + IL-15 group, LPS at 1 μg/ml and IL-15 at 100 ng/ml were added for 4 h of

incubation. In the 1,25-(OH)₂D₃ group, the cells were pre-treated with 1,25-(OH)₂D₃ at 1x10⁻⁷ mol/l for 48 h, followed by 4 h of incubation with 1 μg/ml LPS and 100 ng/ml IL-15.

Western blot analysis. The protein expression of STAT5 and p-STAT5 was observed using western blot analysis. Following treatment according to the grouping method, ice-cold protein extraction buffer (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China), supplemented with 1% protease inhibitor and 1% phosphorylation inhibitor (Nanjing KeyGen Biotech Co. Ltd.), was added for protein extraction. Protein concentrations were determined according to the instructions indicated in the bicinchoninic acid (BCA) protein concentration detection kit (Nanjing KeyGen Biotech Co. Ltd.). The protein sample was mixed with sample-loading buffer (Beyotime Institute of Biotechnology, Shanghai, China) and heated to 100°C for 5 min of loading. Proteins were separated in 6 or 10% Tris-glycine polyacrylamide gradient gels (Beyotime Institute of Biotechnology). The obtained proteins were transferred onto a nitrocellulose membrane (Invitrogen Life Technologies, Carlsbad, CA, USA) and then blocked with Tris-buffered saline-Tween® containing 5% bovine serum albumin (Cell Signaling Technology, Inc.) for 1 h. The membrane was incubated overnight at 4°C with the primary rabbit polyclonal anti-STAT and anti-p-STAT5 antibodies (1:500 dilution) or β-actin (1:5,000 dilution). Following washing, the membrane was incubated at room temperature for 2 h with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (ZSGB-BIO, Beijing, China) (1:3,000 dilution with blocking buffer, Abcam). Protein expression was then detected according to the instructions provided in the chemiluminescent staining reagent kit (Beyotime Institute of Biotechnology). The average pixel density was analyzed with UN-SCAN-IT gel analysis software (Silk Scientific Inc., Orem, UT, USA).

Cell slide preparation. Following treatment, the THP-1 cells were applied onto adhesive polylysine-coated glass slides (Abcam) and fixed in 4% paraformaldehyde at ambient temperature for 20–30 min for immunofluorescence and laser confocal experiments.

Laser confocal microscopy. Monocytic cytoskeletons were characterized using laser confocal microscopy. The cells were washed in PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 for 10 min. Following PBS washing, they were incubated with 1:20 rhodamine-labeled phalloidin (Sigma-Aldrich). Specific conjugation to F-actin was allowed at 25°C (room temperature) for 40 min. The cells were washed with PBS and then mounted in an anti-photobleaching mounting medium (Santa Cruz Biotechnology, Inc.). Photomicrographs were captured with an Axio LSM 710 laser confocal microscope (Carl Zeiss GmbH, Jena, Germany) at a magnification of x2,000.

Fluorescence microscopy. VDR and p-STAT5 proteins were localized using fluorescence microscopy. The slides were washed thrice with PBS. Triton™ X-100 (0.3%) was added for 10 min of membrane permeabilization. Following another three washes with PBS (5–10 min per wash), they were blocked with bovine serum for 30 min.

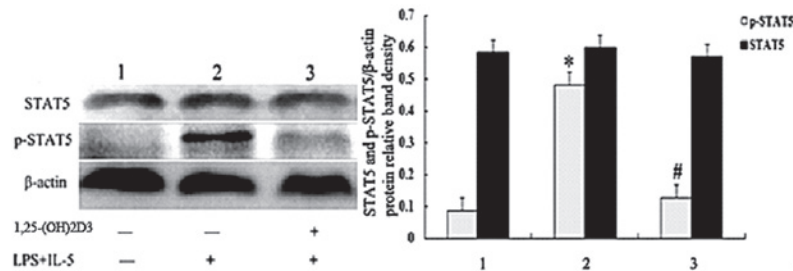


Figure 1. STAT5 and p-STAT5 protein expression determined by western blot analysis. Data are expressed as mean \pm standard error of the mean. Lane/bar 1, the control group; 2, the LPS + IL-15 group; 3, the 1,25-(OH)₂D₃ group. * $P < 0.05$ vs. control; # $P < 0.05$ vs. the LPS + IL-15 group. STAT5, signal transducer and activator of transcription 5; p, phosphorylated; LPS, lipopolysaccharide; IL, interleukin; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃.

Primary antibodies (rabbit polyclonal anti-VDR and mouse monoclonal anti-p-STAT5 antibodies, 1:200 dilution) were applied for incubation at 4°C overnight. Following three washes with PBS, the slides were dried in air. The samples were incubated with secondary antibodies (goat anti-mouse secondary antibody and goat anti-rabbit secondary antibody, 1:50 dilution) at room temperature away from light for 1-2 h. Following washing, the samples were incubated with 4',6-diamidino-2-phenylindole (DAPI) solution at room temperature for 5-10 min. The slides were washed thrice and then covered with coverslips. Observation was performed under an Axio Observer A1 fluorescence microscope (magnification, $\times 10-40$; Carl Zeiss AG, Oberkochen, Germany) and images were captured.

Enzyme-linked immunosorbent assay (ELISA). The IL-6 secretion in the cell culture supernatant was detected using an ELISA kit (PeproTech). The procedures were conducted in accordance with the manufacturer's instructions. The samples were measured in duplicate.

Co-immunoprecipitation. The interaction between VDR and p-STAT5 was validated using the co-immunoprecipitation technique.

The cells in the different groups were collected following treatment. After washing twice with pre-cooled PBS, the cells were dissociated in 1 ml pre-cooled nuclear protein extraction solution (Nanjing KeyGen Biotech Co. Ltd.) and then centrifuged at 700 \times g for 15 min. The supernatant was collected. Approximately 5 μ g TRITC-labeled rabbit anti-VDR polyclonal antibody (1:100 dilution; cat. no. ab3508; Abcam) was added and agitation was performed at 4°C for 4 h. Protein A-Sepharose beads (Pierce Biotechnology, Inc., Rockford, IL, USA) were added, followed by agitation at 4°C for 30 min. The sample was then centrifuged at 700 \times g for 15 min. The beads bearing antigen-antibody complexes were collected. Following thrice washing with pre-cooled lysis buffer (Sangon Biotech Co., Ltd., Shanghai, China), the beads were transferred into a fresh electrophoresis tube and loading buffer was added. The suspension was boiled for 4 min, and then SDS-PAGE gradient gel electrophoresis was performed. The proteins were transferred to polyvinylidene fluoride membranes (Sigma-Aldrich) and incubated with the FITC-labeled mouse monoclonal anti-p-STAT5 antibody (1:50 dilution; cat. no. sc-81524; Santa Cruz Biotechnology,

Inc.) overnight at 4°C. Following washing, the secondary antibody (goat anti-rabbit and anti-mouse IgG; 1:50 dilutions) was added and incubation proceeded at room temperature for 1 h. The membranes were washed and chromogenic developmental reagent (Beyotime Institute of Biotechnology) was added in the absence of light. Each experiment was repeated thrice.

Statistical analysis. Measurement data were presented as mean \pm standard error of the mean. Statistical analysis was carried out using SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA) using a t-test for comparisons between groups and one-factor analysis of variance for comparisons among groups. Differences of $P < 0.05$ were considered to be statistically significant.

Results

STAT5 and p-STAT5 protein expression. To assess the response of 1,25-(OH)₂D₃ pretreated monocytes to LPS + IL-15, the protein expression of STAT5 and p-STAT5 was observed using western blot analysis. The results are shown in Fig. 1.

In the LPS + IL-15 group, the cells exhibited a significantly higher level of p-STAT5 expression compared with that in the control group (0.481 ± 0.16 vs. 0.086 ± 0.024 ; $P = 0.016$). In the 1,25-(OH)₂D₃ group, however, p-STAT5 expression did not show a significant difference compared with that in the control group (0.092 ± 0.028 vs. 0.086 ± 0.024 ; $P > 0.05$). No significant differences in the level of STAT5 expression were observed among the three groups (the expression levels in the control, LPS + IL-15 and 1,25-(OH)₂D₃ groups were 0.580 ± 0.098 , 0.594 ± 0.086 and 0.568 ± 0.105 , respectively; $P > 0.05$).

Monocytic cytoskeletons. Monocytic cytoskeletons in the different groups were characterized using laser confocal microscopy. The results are shown in Fig. 2. In the control group, THP-1 cytoskeletons were primarily distributed at the periphery of the cytoplasm (Fig. 2A). In the LPS + IL-15 group, the cell morphology altered, the actins were remodeled, the actin bands at the cytoplasmic periphery disappeared and the actin masses noticeably shrank. In addition, the lysis of microfilaments was observed in certain cells (Fig. 2B). In the 1,25-(OH)₂D₃ group, the pretreatment with 1,25-(OH)₂D₃ partially prevented the effects caused by LPS + IL-15 (Fig. 2C).

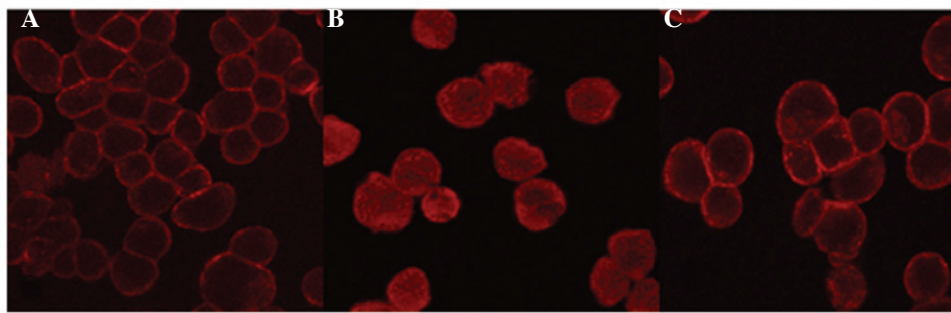


Figure 2. THP-1 cells viewed by laser confocal microscopy (rhodamine-labeled phalloidin staining; magnification, x2,000). (A) Control group; (B) LPS + IL-15 group; (C) 1,25-(OH)₂D₃ group. LPS, lipopolysaccharide; IL, interleukin; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃.

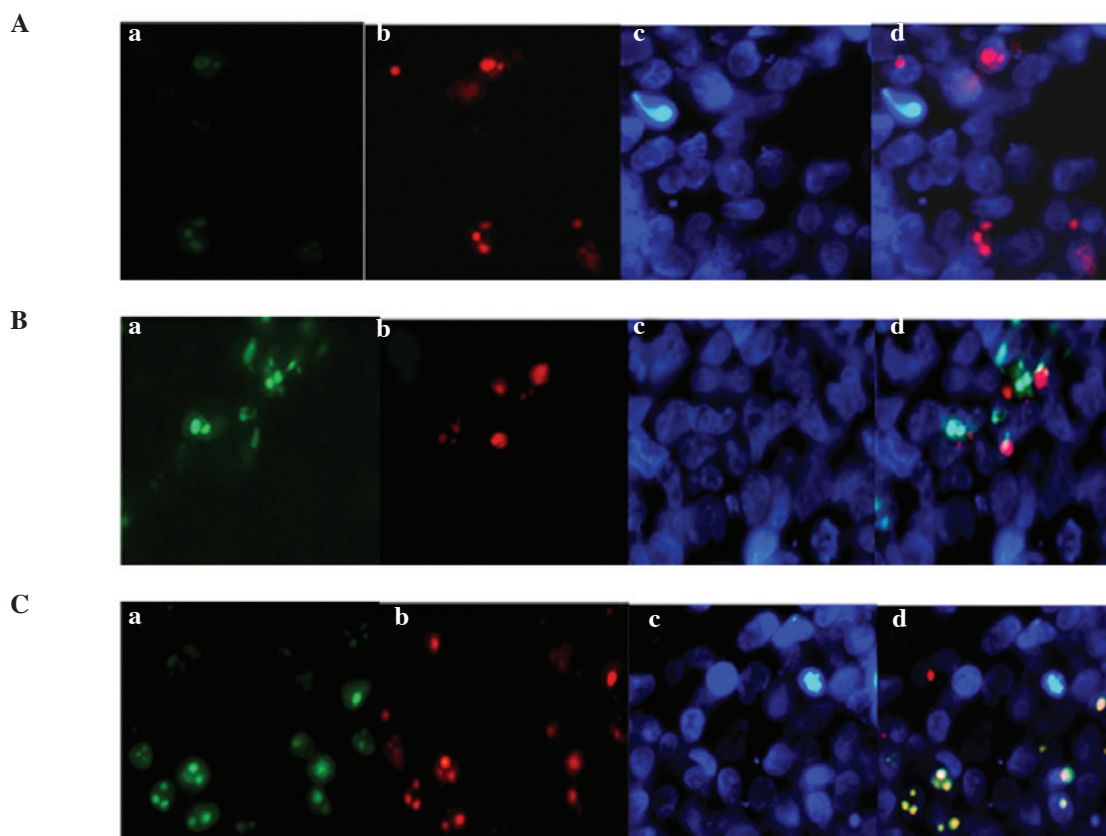


Figure 3. Co-localization of VDR and p-STAT5 using laser confocal microscopy and immunofluorescence microscopy (magnification, x400). (A) Control group; (B) LPS + IL-15 group; (C) 1,25-(OH)₂D₃ group. Staining for (a) p-STAT5 (fluorescein isothiocyanate) and (b) VDR (tetramethylrhodamine); (c) 4',6-diamidino-2-phenylindole staining and (d) merged image. VDR, vitamin D receptor; p-STAT5, phosphorylated signal transducer and activator of transcription.

Co-localization of VDR and p-STAT5. To detect the interaction between VDR and p-STAT5, co-localization was performed using immunofluorescence. The cells were grouped and treated according to the method described previously. Prior to stimulation with LPS + IL-15, STAT5 was expressed in the cytoplasm. Subsequent to LPS + IL-15 stimulation, STAT5 was phosphorylated and expressed in the nucleus. In all three groups, VDR was mostly expressed in the nucleus with a small amount expressed in the membrane. p-STAT5 was almost undetectable in the control group (Fig. 3A). Compared with the control group, the LPS + IL-15 group exhibited a greater amount of nuclear p-STAT5 and some co-expressed VDR + p-STAT5 complexes (Fig. 3B;

indicated in yellow). The pretreatment with 1,25-(OH)₂D₃ markedly enhanced the nuclear expression levels of VDR and p-STAT5; their co-localization was more noticeable than that in the LPS + IL-15 group (stained in yellow in Fig. 3C).

IL-6 level. As shown in Fig. 4, the IL-6 level in the LPS + IL-15 group was significantly higher compared with that in the control and 1,25-(OH)₂D₃ groups (53.122 ± 17.756 vs. 0.063 ± 0.006 and 13.472 ± 5.056 pg/ml, respectively; both $P < 0.01$).

Co-immunoprecipitation. The possible interaction between VDR and p-STAT5 was further tested using the co-immunoprecipitation method. VDR and proteins interacting with p-STAT5

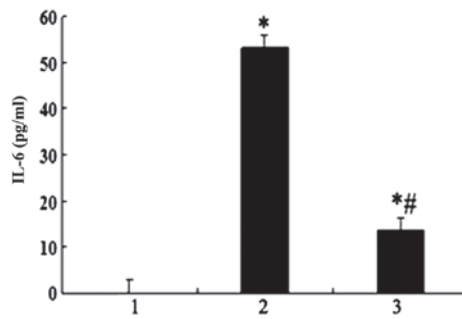


Figure 4. IL-6 level in the THP-1 cell supernatant. Bar 1, the control group; 2, the LPS + IL-15 group; 3, the 1,25-(OH)₂D₃ group. *P<0.01 vs. the control group; #P<0.01 vs. the LPS + IL-15 group. IL, interleukin; LPS, lipopolysaccharide; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃.

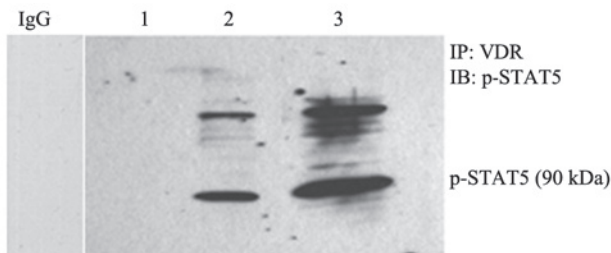


Figure 5. Possible intranuclear interaction of VDR with p-STAT5 as indicated by immunofluorescence. Lane 1, the control group; 2, the LPS + IL-15 group; and 3, the 1,25-(OH)₂D₃-pretreated group. VDR, vitamin D receptor; p-STAT, phosphorylated signal transducer and activator of transcription. LPS, lipopolysaccharide; IL, interleukin; IB, immunoblotting; IP, immunoprecipitation; IgG, immunoglobulin G.

were precipitated from the cell extracts, and western blot analysis was performed (Fig. 5). In the control group, when LPS and IL-15 were absent, p-STAT5 was not observed. In the LPS + IL-15 group, p-STAT5 became noticeable in VDR-containing protein complexes. In the 1,25-(OH)₂D₃ group, the association between VDR and p-STAT5 became more evident compared with that in the LPS + IL-15 group. These data, as well as the immunofluorescence results, provided evidence of the interaction between VDR and p-STAT5. Each experiment was repeated three times and yielded a consistent result.

Discussion

1,25-(OH)₂D₃ is an active metabolite of vitamin D that has multiple activities (14-16). It exerts action via the VDR; therefore, the effect of vitamin D is dependent on the VDR level. Our previous study (5) demonstrated that 1,25-(OH)₂D₃ and LPS + IL-15 influenced the expression of VDR and STAT5 in serum-incubated monocytes from patients with T2DM and uremia caused by DN: LPS + IL-15 upregulated the expression of p-STAT5, whereas pretreatment with 1,25-(OH)₂D₃ significantly inhibited this effect. Considering the wide potential clinical application of vitamin D, however, a further step was taken in the present study to validate the anti-inflammatory effect of 1,25-(OH)₂D₃ and to explore the mechanism underlying this effect.

The results of the present study showed that THP-1 cells exhibited a distorted morphology following stimulation with

LPS + IL-15; the cytoskeletons became depolymerized and remodeled, actin bands disappeared at the periphery, and actin masses emerged. Furthermore, LPS + IL-15 significantly strengthened the DNA binding activity of nuclear p-STAT5 and increased the level of IL-6 in the supernatant. These changes were significantly inhibited, however, through 1,25-(OH)₂D₃ pretreatment. These results were consistent with those found previously (5), strengthening the evidence of the effects of 1,25-(OH)₂D₃ on the expression of VDR and p-STAT5 as well as cytoskeletal rearrangement in human monocytes.

According to the literature (19), 1,25-(OH)₂D₃ promotes the formation of STAT1-VDR complexes in THP-1 monocytes; it significantly weakens the transcriptional activity of VDR but enhances STAT1 transcription. The signaling pathways controlled by VDR and STAT may, therefore, be interconnected and the anti-inflammatory effect of 1,25-(OH)₂D₃ may be associated with the effects of vitamin D on these pathways.

The Janus kinase (JAK)/STAT signaling pathway is one of the essential signal transduction channels involved in multiple cell behaviors, such as growth, development, division, differentiation, apoptosis and functional synchronization (20). IL-15 is a JAK/STAT signaling-mediated soluble cytokine that is able to promote inflammation (21). During chronic micro-inflammation in patients with T2DM and uremia caused by DN, LPS affects the production of IL-6, IL-15, IL-18 and IL-10 by its action on intracellular TLR4 or TLR2 (22-26). It promotes the secretion of pro-inflammatory cytokines, such as IL-6 and IL-15. These cytokines bind to cell-borne receptors to activate the tyrosine kinase, JAK (22,27,28), which, in turn, activates STAT5 to form p-STAT5 via phosphorylation. p-STAT5 takes the form of homo- or hetero-dimers or oligomers. It enters the nucleus, where it binds to promoter VDR DNA to regulate VDR transcription. This process can be partially prevented, however, through 1,25-(OH)₂D₃ pretreatment. Following the binding of 1,25-(OH)₂D₃ to the VDR, 1,25-(OH)₂D₃/VDR/retinoid X receptor complexes are formed and VDR DNA-binding sites are exposed. These sites are bound by p-STAT5 and VDR-STAT5 complexes are formed. The formed complexes induce the production of anti-inflammatory cytokines and inhibit the secretion of pro-inflammatory cytokines, thereby preventing the occurrence of inflammation to a certain degree. Such an effect of 1,25-(OH)₂D₃ on monocytes may reflect the interaction between 1,25-(OH)₂D₃ with the VDR and the JAK/STAT signaling pathway. To test whether a crosstalk between VDR and STAT5 occurs, monocytes were incubated with 1,25-(OH)₂D₃ prior to their treatment with LPS and IL-15 in this study. The results obtained from the immunofluorescence and co-immunoprecipitation experiments suggest that crosstalk between these two proteins does exist in THP-1 cells; it is this crosstalk that further induces cytoskeletal rearrangement.

This study has a limitation: The results would be more convincing if an animal or human *in vivo* study or a study on monocytes directly isolated from patients with diabetes was conducted.

In conclusion, 1,25-(OH)₂D₃ may exert an anti-inflammatory action by influencing the crosstalk between STAT5 and

VDR to a certain extent. The present study sheds new light on the mechanism behind the effect of vitamin D in its wide application and provides a new therapeutic target in the treatment of diseases, such as T2DM and uremia caused by DN.

Acknowledgements

This study was supported by the Science and Technology Department of Guizhou Province [Guizhou Branch of Social Research Programs SY word (2012) No. 3116], the Science and Technology Department of Guizhou, Zunyi Medical College, Zunyi Municipal Science and Technology Bureau of Science and Technology Joint Fund [Guizhou Branch of J word (2013) No. 53], and the Zunyi Medical College Foundation for Doctors (Project No. F-588).

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