Effect of 1,25-(OH)$_2$D$_3$ and lipopolysaccharide on mononuclear cell inflammation in type 2 diabetes mellitus and diabetic nephropathy uremia

E.L. Wu and H.X. Cui

Hemopurification Center, Binzhou People’s Hospital, Binzhou, China

Corresponding author: E.L. Wu
E-mail: wuelwu@163.com

ABSTRACT. The prevention and treatment of type-2 diabetes mellitus (T2DM) and diabetic nephropathy (DN), which are disorders with high incidence rates, is of primary importance. In this study, we analyzed the effect of 1,25-(OH)$_2$D$_3$ and lipopolysaccharide (LPS) in combination with interleukin (IL)-15 on the inflammatory immune response and expression of vitamin D receptor (VDR) in mononuclear cells of T2DM and DN uremia (DNU) patients. The human acute monocytic leukemia cell line THP-1 was treated with peripheral blood serum isolated from 30 healthy controls and T2DM and DNU patients each, cultured in the presence or absence of 1,25-(OH)$_2$D$_3$, and subsequently treated with LPS and IL-15. The VDR mRNA and protein expression in THP-1 cells was detected by real-time polymerase chain reaction and western blot (and immunofluorescence assay), respectively, and IL-6 and IL-10 concentrations in the culture supernatant were detected by enzyme-
linked immunosorbent assay. LPS treatment induced a significant decrease in VDR mRNA expression in T2DM and DNU serum-treated THP-1 cells compared to the control cells (P < 0.05). The VDR protein expression in DNU serum-treated THP-1 cells was also significantly down-regulated (P < 0.05). LPS treatment induced IL-6 secretion in serum-treated THP-1 cells (P < 0.05), while 1,25-(OH)\(_2\)D\(_3\) treatment inhibited IL-6 secretion to some extent. These findings suggested that LPS down-regulates the expression of VDR in mononuclear cells of T2DM and DNU patients and induces an imbalance in the pro-inflammatory and anti-inflammatory cytokine response, while 1,25-(OH)\(_2\)D\(_3\) partially reversed the effect of LPS and protected patients with T2DM and DNU.

**Key words:** Type-2 diabetes mellitus; Diabetic nephropathy; 1,25-(OH)\(_2\)D\(_3\); Lipopolysaccharide

**INTRODUCTION**

Type-2 diabetes mellitus (T2DM) and diabetic nephropathy (DN) are serious disorders with high rates of incidence, especially over the past few years (Mukhopadhyaya et al., 2010). DN has a complex pathogenesis; the occurrence and development of DN cannot always be controlled despite the patients exhibiting normal blood glucose, blood pressure, and blood lipid levels (Afkarian et al., 2013). Therefore, the pathogenesis of DN may be attributed to inflammation, in addition to known factors such as the renin-angiotensin system, oxidative stress, and glucose and lipid metabolism disorders.

A recent study (Xu et al., 2014) suggests that vitamin D deficiency may be correlated with low-grade chronic inflammation, as well as the occurrence and development of T2DM and its complications. Additionally, studies focusing on DN (Matilainen et al., 2010; Luis-Rodriguez et al., 2012) have reported that all stages of the pathological process of DN involve inflammatory factors. T2DM mononuclear cells are known to overexpress inflammatory factors (Ke and Yu, 2012). Patients with T1DM or T2DM present an increase in the inflammatory marker concentration. The infiltration of monocyte/macrophage may be a central step in the occurrence and development of DN; therefore, the immune inflammation theory considers DN as a chronic low-grade inflammatory disease with natural immune activation characteristics (Pervin et al., 2013). Vitamin D or 1,25-(OH)\(_2\)D\(_3\) has a proven immune regulatory effect (Cani et al., 2007; Ko et al., 2008). Vitamin D and its analogues can upregulate the expression of vitamin D receptors (VDR) in mononuclear cells. However, the effect of 1,25-(OH)\(_2\)D\(_3\)-induced inflammatory response (via mononuclear cells) in patients with T2DM and DN uremia (DNU) remains to be elucidated.

VDR, which belongs to a superfamily of steroid hormones and thyroid receptors, transforms into 1,25-(OH)\(_2\)D\(_3\) by combining with its ligand, vitamin D3. The anti-inflammatory and immunoregulatory effects of 1,25-(OH)\(_2\)D\(_3\) have attracted a considerable amount of attentions over the past few years (Gu et al., 2011). Interleukin (IL)-6 and IL-12 are cytokines generated by mononuclear macrophages. Low concentrations of IL-6 and IL-12 have been previously shown to regulate the inflammatory reaction and promote tissue recovery; at higher concentrations, chain and amplification reactions can
be induced, leading to impaired organ structure and function (Devaraj et al., 2011). IL-10, a pleiotropic cytokine, presents anti-inflammatory and immunoregulatory functions (Sadeghi et al., 2006). The aim of this study was to elucidate the regulatory effect of lipopolysaccharide (LPS) and active vitamin D3 on mononuclear cells of patients with T2DM and DNU by simulating the peripheral blood microenvironment of T2DM and DNU using serum from patients with these disorders, as well as by stimulating in vitro cultured THP-1 cells using LPS and I; -15.

MATERIAL AND METHODS

Study subjects

Thirty healthy people (16 males and 14 females) with no previous history of endocrine and metabolic diseases such as diabetes, hypertension, coronary heart disease, and nephropathy, were recruited to this study between May 2010 and May 2013. During the same time period, 30 patients with T2DM (17 males and 13 females) and DNU (16 males and 14 females), admitted to the Nephrology or Endocrinology Department of the Binzhou People’s Hospital, Shandong, China, were recruited to this study. Diabetes was diagnosed according to the Diagnostic and Classification Standards of the WHO (1999). All T2DM patients conformed to the following criteria: serum glutamic acid decarboxylase antibody (GADA) level < 1.0, fasting blood glucose level <7.0 mM, and hemoglobin A1c (HbA1c) <6.50%. The diagnosis of DN was in conformance with the criteria of chronic renal failure (uremia period): serum creatinine level ≥707 µM or endogenous creatinine clearance rate <10 mL/min. Patients with T1DM, secondary diabetes, acute infectious disease, poor cardiac and liver function, connective tissue disease, autoimmune disease, tumor, cardiocerebral events, or asthma, those who developed diabetes-associated acute complications or infections over the past three months, patients who were medicated with drugs that could influence the study results, or pregnant or breastfeeding females were excluded from this study. The subjects were of Han Chinese ethnicity with no genetic relationship. The study design was approved by the Ethics Committee of Binzhou People’s Hospital, Shandong, China; signed informed consent forms were obtained from all study subjects.

Materials and reagents

RPMI1640 and fetal calf serum (FCS) were purchased from Gibco (Waltham, MA, USA). Active vitamin D or 1,25-(OH)2D3 and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal VDR antibody was purchased from Abcam (Cambridge, UK) and the goat anti-rabbit secondary antibody was obtained from Zhongshan Jinqiao (Beijing, China). The ELISA kit used to detect IL-6 and IL-10 was obtained from MyBioSource (San Diego, CA, USA). The GADA detection kit was produced by BioMerica (Irvine, CA, USA) and HbA1c levels were detected by ELISA using a Diastar HbA1c analyzer (Axis-Shield Company, Norway), based on the principles of chemiluminescence. Blood indices were detected using a XE-2100 automatic analyzer (Sysmex, Kobe, Japan). Renal function and fasting blood glucose and blood lipid levels were detected by testing venous blood from the patients with a fully automated biochemical analyzer (Olympus, Tokyo, Japan). Urinary albumin was detected using a radioimmunoassay.
Methods

Preparation and inactivation of serum

Fasting venous blood (6 mL) was collected from each subject. Three milliliters of each blood sample was sent to the clinical laboratory at our hospital to test for blood indices, blood lipid level, and liver and renal function. The remaining 3 mL was centrifuged at 1006 g for 15 min after a 30-min incubation period (at room temperature). The serum (top layer) was collected, filtered with a 0.22-μm Millipore filter (Merck-Millipore, Darmstadt, Germany), sterilized, and stored in a refrigerator at -20°C.

Grouping

Culture solution was removed from THP-1 cells at the logarithmic phase of growth. The cells were centrifuged at 3000 g for 10 min, resuspended with serum free medium, and counted. The cells (2 mL solution per well) were then inoculated on a 6-well culture plate at a concentration of 5 x 10⁵ cells/mL and cultured at 37°C in 5% CO₂ incubator for 24 h. Subsequently, the cells were divided into three groups based on the type of added serum, as follows: healthy control group (N = 30; 200 μL 5.0% serum from healthy subjects); T2DM group (N = 30; 200 μL 5.0% serum from T2DM patients); and the DNU group (200 μL 5.0% serum from DNU patients). Each serum type was added to two wells. Each group was divided into the VD3 + LPS and LPS subgroups: the samples were cultured in culture solution supplemented with (or not) 1,25-(OH)₂D₃ (10⁻⁷ M) for 48 h and subsequently intervened with LPS at a final concentration of 1 μg/mL for 24 h. The mononuclear cells and culture solution were collected, and the exact serum, LPS, and 1,25-(OH)₂D₃ concentrations were confirmed by preliminary analyses.

Cultivation of mononuclear cells

The THP-1 cell line was obtained from the cell bank of the Chinese Academy of Sciences and cultured as described in the previous subsection.

Detection of VDR mRNA expression with RT-PCR

Total RNA was extracted from the cells using the TRIzol reagent (Guangzhou Xiangbo Biotech. Co., Ltd., Guangzhou, China), purified, and reverse transcribed into complementary DNA. The primer sequences and PCR amplification conditions were designed as follows: internal reference β-actin - upstream primer 5'-GACCCAGATCATGTTTGAGACC-3', downstream primer 5'-ATCTCCTTCTGCATCCTGTCG-3', product length 595 bp, annealing temperature 60°C, 39 cycles; VDR - upstream primer 5'-AGCCCTTGGGTCTGAGATGTCG-3', downstream primer 5'-GTCGGGTGGTTCCTGGATGATGC-3', product length 302 bp, annealing temperature 66°C, 39 cycles. The PCR products were electrophoresed on a 1.5% agarose gel and the gel images were generated by an automatic gel imager (Beijing Kechuang Ruixin Company, Beijing, China). The relative content of VDR was expressed as the ratio of gray values of the VDR and β-actin bands.
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Detection of VDR protein expression with western blot

The cells were disrupted on ice and subsequently centrifuged at 12,000 g at 4°C for 5 min. The protein concentration in the supernatant was determined. Individual proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel; the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked with TBST containing 5% skim milk powder for 90 min. The membrane was then incubated overnight with rabbit anti-human VDR antibody (1:500) (4A Biotech Co., Ltd., Beijing, China) and rabbit anti-mouse β-actin antibody (1:5000) (4A Biotech Co., Ltd.) at 4°C. The membrane was washed and subsequently incubated with horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (IgG) (1:1500; Beijing Zhongshan Jinqiao Company; Beijing, China) for 2 h at 25°C; the membrane was washed again, developed, and fixed. Images of the membrane were then obtained and the images were analyzed (Cani et al., 2008).

Detection of cytokines

The IL-6 and IL-10 concentrations in the cell supernatant was detected by enzyme-linked immunosorbent assay (ELISA) using a commercial ELISA kit (MyBioSource, USA). We also detected the IL-6 and IL-10 concentrations in the supernatant of mononuclear cells treated with serum, 1,25-(OH)_2D_3, and LPS. The optical density of each well was measured at 450 nm, and the IL-6 and IL-10 content was expressed as pg/mL.

Detecting the VDR protein expression in mononuclear cells with immunofluorescent assay

Serum-, 1,25-(OH)_2D_3-, and LPS-treated cells were washed with phosphate buffer solution, fixed with 4% paraformaldehyde, blocked with FCS, and finally processed with 0.5% Triton X-100 permeable membrane for 10 min. The cells were then incubated overnight with the primary antibody (1:200) at 4°C and subsequently with the fluorescent secondary antibody (1:100) for 1 h. The cells were observed under a 10 x 20 fluorescence microscope (five fields were randomly selected from every image). Then optical density was calculated and the average value was taken as the final result.

Statistical analysis

All experimental data are reported as means ± standard deviation (one independent experiment). The data was statistically analyzed using SPSS v.19.0 (IBM, Armonk, NY, USA). Pairwise comparison was performed using the t-test. The data was compared between groups with one-way analysis of variance (ANOVA). The differences were considered to be statistically significant when P < 0.05. P < 0.01 indicated a very significant difference.

RESULTS

Effects of LPS on VDR mRNA and protein expression in mononuclear cells

We observed a statistically significant downregulation in the expression of VDR
mRNA in the T2DM and DNU groups compared to the normal control group after treating with LPS for 24 h (P < 0.05); however, the difference between the T2DM and DNU groups had no statistical significance (Figure 1).

Similarly, the VDR protein expression was significantly down-regulated in the mononuclear cells of patients with DNU compared to the normal controls 24 h after LPS intervention (P < 0.05) (Figure 2).

**Effect of 1,25-(OH)\(_2\)D\(_3\) on VDR mRNA and protein expression in mononuclear cells stimulated by LPS**

The cells were treated with LPS (1 \(\mu\)g/mL) for 24 h after 48-h culturing in medium containing 1,25-(OH)\(_2\)D\(_3\) (10\(^{-7}\) M). The expression of VDR mRNA was up-regulated in T2DM and DNU mononuclear cells treated with both VD3 and LPS compared to T2DM and DNU cells treated with LPS alone. However, the VDR mRNA expression did not differ significantly among the mononuclear cells from the three groups when treated with 1,25-(OH)\(_2\)D\(_3\) (P > 0.05) (Figure 3).

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Similarly, the VDR protein expression was upregulated in T2DM and DNU mononuclear cells treated with both VD3 and LPS compared to T2DM and DNU cells treated with LPS alone. However, VDR expression did not differ significantly among mononuclear cells from the three groups when treated with 1,25-(OH)₂D₃ (P > 0.05) (Figure 4).

Effect of 1,25-(OH)₂D₃ on IL-6 and IL-10 levels in supernatant of THP-1 culture solution treated with LPS

We observed a significantly higher level of IL-6 in the supernatant of the THP-1 cultures belonging to the T2DM and DNU groups compared to the normal control group after 24-h intervention with LPS (P < 0.01). However, IL-10 levels were significantly lower in these two groups compared to the normal control group (P < 0.01). IL-6 expression was significantly down-regulated and IL-10 was significantly up-regulated in mononuclear cells treated with VD3 + LPS, compared to those treated with LPS alone. However, although the IL-6 and IL-10 expressions in the T2DM and DNU groups differed from those in the normal control group, this difference was not statistically significant (P > 0.05) (Table 1).
Results of cellular immunofluorescence

VDR was mainly distributed in the cell nucleus and was also mildly expressed on the membrane. VD3 intervention resulted in strong VDR expression in the cell nucleus and on the membrane of mononuclear cells; however, the cells treated with LPS showed low VDR expression (P < 0.05) (Figure 5).

<table>
<thead>
<tr>
<th>Groups</th>
<th>IL-6 (pg/mL, N = 30, mean ± standard deviation)</th>
<th>IL-10 (pg/mL, N = 30, mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control</td>
<td>15.14 ± 1.62</td>
<td>14.87 ± 1.73</td>
</tr>
<tr>
<td>T2DM</td>
<td>24.08 ± 2.95*</td>
<td>9.26 ± 2.37*</td>
</tr>
<tr>
<td>DNU</td>
<td>70.76 ± 4.44*</td>
<td>9.02 ± 1.85*</td>
</tr>
<tr>
<td>T, 25-(OH),D3 + LPS group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control</td>
<td>10.33 ± 0.85</td>
<td>22.44 ± 4.85</td>
</tr>
<tr>
<td>T2DM</td>
<td>11.21 ± 1.20</td>
<td>20.23 ± 4.24</td>
</tr>
<tr>
<td>DNU</td>
<td>10.85 ± 1.42</td>
<td>19.41 ± 3.88</td>
</tr>
</tbody>
</table>

*P < 0.01 compared to the normal control group

LPS, lipopolysaccharide; T2DM, type 2 diabetes mellitus; DNU, diabetic nephropathy uremia.

DISCUSSION

The anti-inflammatory and immunoregulatory effects of 1,25-(OH)2D3, an active metabolic product of vitamin D, have been the focus of a number of studies conducted in recent years (Liu et al., 2011; Patrick et al., 2012). In addition to the calcium-phosphorus metabolism, it has also been shown to regulate the proliferation and differentiation of multiple cell types (Pittas et al., 2012). Panichi et al. (2001) reported that active VD3 inhibits the effects of transmitters, inflammatory factors such as IL-1, IL-6, and tumor necrosis factor-α, relieve the pathological changes occurring in the glomerulus, protect renal function, and play an important role in the prevention of diabetes and its complications. VDR, a member of the nuclear receptor superfamily of proteins, is expressed in most cells (including monocyte/macrophage) of the immune system. A recent report has suggested that deficiencies in VD
and VDR are correlated with the development of chronic inflammatory diseases such as obesity, cardiovascular disease, and diabetes, and their complications (Zhang et al., 2012). 1,25-(OH)\(_2\)D\(_3\) mediates VDR in a dose-dependent manner. However, few studies have analyzed the regulatory effect of 1,25-(OH)\(_2\)D\(_3\) on VDRs expressed on mononuclear cells in patients with T2DM and DNU.

In this study, THP-1 cells were cultured with serum extracted from patients with T2DM and DNU who expressed insufficient 25-(OH)VD, and the expression of VDR was observed in these cells under stimulation by 1,25-(OH)\(_2\)D\(_3\) and LPS. The results of this study suggested that the VDR mRNA and protein expression was down-regulated in LPS-stimulated mononuclear cells obtained from patients with T2DM and DNU compared to that in the normal control group; the down-regulation was more significant in the DNU group (P < 0.01). These results indicated an increase in the endotoxin levels in patients with T2DM and DNU, which in turn resulted in the development of dysfunctional mononuclear cells (because of the endotoxin-rich microenvironment). LPS has been reported to promote the release of inflammatory factors such as IL-6 by mononuclear cells in T2DM and DNU patients, in order to accelerate and amplify the inflammatory and immune reaction. This may be an important inflammatory and immune mechanism mediated by mononuclear cells in patients with T2DM and DNU (Pickup, 2004). 1,25-(OH)\(_2\)D\(_3\) can partially reverse the effect of LPS, induce the upregulation of VDR mRNA and protein in mononuclear cells of patients with T2DM and DNU, and induce the up- and down-regulation of IL-10 and IL-6, respectively. The development of uremia results in a decrease in the density and binding force of VDR; therefore, increasing the expression of 1,25-(OH)\(_2\)D\(_3\) can upregulate VDR expression, and vice-versa. The extreme sensitivity of mononuclear cells to external stimuli under the induction of serum from T2DM and DNU patients is a protection mechanism (Adams et al., 2009).

In summary, we verified the anti-inflammatory mechanism of 1,25-(OH)\(_2\)D\(_3\) in the inflammatory immune response of T2DM and DNU. 1,25-(OH)\(_2\)D\(_3\) exerts a protective effect on patients with T2DM and DN. When induced with serum from patients with T2DM and DN, mononuclear cells were abnormally sensitive to external stimulus, which is a cellular protective mechanism. Therefore, timely supplementation of active vitamin D3 to patients lacking in vitamin D and likely to develop T2DM and DNU could be useful in the prevention and treatment of T2DM and DNU.

**Study limitations**

LPS may activate nuclear transcription factor NF-κB via the toll-like receptors to induce the proliferation and differentiation of cells, regulate the transcription of cytokines, and further amplify the immune and inflammatory reaction. However, the mechanism with which LPS induces VDR down-regulation, the relevant intracellular signal transduction pathways, and the effect of 1,25-(OH)\(_2\)D\(_3\) on the inflammatory and immune reactions in patients with T2DM and DNU remains to be elucidated. Currently, the study of the relationship between mononuclear cells and DNU and its mechanism is still at the initial stage. The occurrence and development of DN is extremely complex, especially in the human body, as it involves multiple risk factors and genetic backgrounds. This limitation cannot be avoided because this is an *in vitro* study involving a single factor, with inflammatory immunity as the research perspective.
Conflicts of interest

The authors declare no conflict of interest.

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