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1, 25$(OH)_2D_3$-induced interaction of vitamin D receptor with p50 subunit of NF-κB suppresses the interaction between KLF5 and p50, contributing to inhibition of LPS-induced macrophage proliferation

Dong Ma [a, b], Ruo-nan Zhang [a], Ya Wen [c], Wei-na Yin [d], Disi Bai [b], Guo-ying Zheng [b], Jin-shui Li [a], Bin Zheng [a], Jin-kun Wen [a, *]

[a] Department of Biochemistry and Molecular Biology, Key Laboratory of Neural and Vascular Biology, Ministry of Education, Hebei Medical University, Shijiazhuang, 050017, PR China
[b] School of Public Health, North China University of Science and Technology, Tangshan, Hebei, 063000, PR China
[c] Department of Neurology, Second Hospital of Hebei Medical University, Shijiazhuang, 050017, PR China
[d] Department of Pediatrics, Handan First Hospital, 056000, PR China

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ABSTRACT

KLF5 and nuclear factor κB (NF-κB) regulate cell proliferation and inflammation. Vitamin D signaling through vitamin D receptor (VDR) exerts anti-proliferative and anti-inflammatory actions. However, an actual relationship between KLF5, NF-κB and VDR in the inflammation and proliferation of macrophages is still unclear. Here, we showed that LPS and proinflammatory cytokines stimulate KLF5 gene expression in macrophages, and that 1, 25$(OH)_2D_3$ suppresses LPS-induced KLF5 expression and cell proliferation via upregulation of VDR expression. Mechanistic studies suggested that KLF5 interacts with p50 subunit of NF-κB to cooperatively induce the expressions of positive cell cycle regulators cyclin B1 and Cdk1/Cdc2 in LPS-treated macrophages. Further studies revealed that 1, 25$(OH)_2D_3$-induced interaction of VDR with p50 decreases LPS-induced interaction of KLF5 with p50. Collectively, we identify a novel regulatory pathway in which 1, 25$(OH)_2D_3$ induces VDR expression and promotes VDR interaction with p50 subunit of NF-κB, which in turn attenuates the association of KLF5 with p50 subunit of NF-κB and thus exerts anti-inflammatory and anti-proliferative effects on macrophages.

1. Introduction

Inflammation is a fundamental adaptation to the loss of cellular and tissue homeostasis with many important physiological roles, including host defence, tissue remodeling and repair, and the regulation of metabolism [1]. Macrophages are crucial mediators of the inflammatory response and play an important role in a broad spectrum of acute (e.g., pathogen infection, sepsis) and chronic inflammatory responses (e.g., insulin resistance, atherosclerosis, chronic wounds, and tumorigenesis) [2]. Accumulating evidence shows that the inflammatory response requires the activation of a complex transcriptional program that is both cell-type- and stimulus-specific and involves the dynamic regulation of hundreds of genes [3]. Zinc finger transcription factor KLF5 and nuclear factor κB (NF-κB) family of transcription factors, particularly p65 and p50, are well known to regulate cell proliferation, inflammation, immune responses, and tumor development [4,5]. For example, recent evidence shows that collecting duct expression of Klf5 is essential for inflammatory responses to the unilateral ureteral obstruction (UUO) [6]. NF-κB plays a central role in upregulating the expression of adhesion molecules and other pro-inflammatory genes [7]. However, the role of NF-κB and KLF5 in linking inflammation and cell proliferation had not yet been shown.

Although it has been known for many years that the biologically active vitamin D metabolite — 1,25-dihydroxyvitamin D3 (1,25$(OH)_2D_3$) is associated with systemic calcium homeostasis, there are now increasing evidences showing that 1, 25$(OH)_2D_3$ regulates cell proliferation, differentiation, apoptosis and immune responses [8]. Previous studies have shown that the biological effects of 1,25$(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), which is present in many cells, including macrophages, cardiomyocytes, vascular smooth muscle cells, and endothelial cells

* Corresponding author.
E-mail address: wjk@hebmu.edu.cn (J.-k. Wen).

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[9], and that vitamin D signaling through VDR exerts anti-proliferative, anti-inflammatory, pro-apoptotic, and pro-differentiating actions [10]. Mechanistic study shows that VDR is directly involved in the regulation of nuclear factor-κB (NF-κB) activation [11]. Although NF-κB and VDR are well studied with respect to their role in macrophages, the actual relationships between KLF5, NF-κB, and VDR in the determination of macrophage fate are not yet completely elucidated.

In this study, we tested KLF5 expression and cell proliferation in LPS-induced macrophages and further dissected the molecular mechanism underlying the regulation of KLF5 expression and cell proliferation by 1, $25(OH)_2D_3$. We show for the first time that 1, $25(OH)_2D_3$ promotes competition of VDR with KLF5 for binding to p50 subunit of NF-κB, contributing to inhibition of LPS-induced macrophage proliferation.

2. Materials and methods

2.1. Reagents

Ultrapure *E. coli* LPS was purchased from Invitrogen (San Diego, USA), and $1,25(OH)_2D_3$ (VitD), cycloheximide (CHX), actinomycin D (ActD) and DAPI were purchased from Sigma (St. Louis, MO). RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Hyclone Laboratories, Inc. TRIzol RNA extraction reagent was obtained from Molecular Research Center, Inc. The reverse transcription system was obtained from Promega.

2.2. Cell culture and treatment

Murine macrophage cells (RAW264.7) were routinely cultured in RPMI 1640 medium containing 10% FBS at 37 °C in a 5% CO_2 incubator. Cells were treated for different times or concentrations with LPS (1 μg/mL) in 2% FBS-supplemented medium. Macrophages were also stimulated with TNF-α (10 ng/mL) and IFN-γ (400 IU/mL) in the presence or absence of $1,25(OH)_2D_3$ (10–100 nM).

2.3. Transient transfection of macrophages

Ad-KLF5 and Ad-GFP were made as described previously [12]. Macrophages were infected with Ad-KLF5 or Ad-GFP as described previously [13]. Small interfering RNAs (siRNAs) specific for mouse VDR and KLF5 were synthesized by Gene Pharma (Shanghai, China). Non-specific siRNA (NS siRNA) was purchased from Santa Cruz Biotechnology. The siRNA sequences used in these studies were as follows: VDR siRNA: 5′-CCCUUCAAUGGAGAUUGCCGCAUCA-3′ and 5′-UGAUGCGGCAAUCUCCAUUGAAGGG-3′; KLF5 siRNA: 5′- GUUCCACAGACGUCAAUGATT-3′ and 5′-UCAUUGACGUCUGUGGAACTT-3′; NS-siRNA: 5′-UUCUCCGAACGUGUCACGUTT-3′ and 5′-ACGUGACACGUUCGGAGAATT-3′. Transfection was performed using Lipofectamine™ reagent (Invitrogen) according to the manufacturer's instructions. Twenty-hours after transfection, macrophages were treated with LPS (1 μg/mL). Then cells were harvested and lysed for Western blotting or co-immunoprecipitation assays.

2.4. Cell counting

The cell number was determined using a Countess™ automated cell counter (Invitrogen) after treatment with LPS, $1,25(OH)_2D_3$ or LPS plus $1,25(OH)_2D_3$ for the different periods. Untreated cells were used to obtain baseline count. Each sample was counted three times, and the average value from triplicate experiments was measured.

2.5. MTS assay

After appropriate treatment, viability of the macrophages cultured in 96-well plates was measured using the MTS assay, as previously described [14]. In brief, the medium of cultured macrophages was replaced with 100 μL serum-free 1640 medium containing 10 μL of Cell Titer 96 Aqueous one solution (Promega, G3582) and incubated at 37 °C for 4 h. Then, 60 μL of medium from each well was transferred to a new 96-well plate, and the absorbance at 490 nm was measured using a Multiskan spectrum (Thermo).

2.6. Cell cycle analysis

Cells were treated with the indicated conditions and harvested by trypsinization, and then cells were washed with ice-cold PBS, fixed with ethanol, incubated for 5 min with 0.5% Triton X-100 and stained with propidium iodide in PBS containing 25 mg/mL RNase. Stained cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA) [15].

2.7. RNA isolation and RT-PCR

Total RNA was extracted from macrophages using the TRizol according to the manufacturer's instructions. 1 μg of RNA was subjected to reverse transcription using first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. qRT-PCR of mRNAs was performed using Platinum SYBR Green qPCR Super Mix UDG Kit (Invitrogen), and real-time PCR experiments were carried on a ABI 7500 FAST system (Life Technologies). Relative amount of transcripts was normalized with GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ formula as previously described [16]. All PCRs were performed in triplicate. The primer sequences were as follows: mouse GAPDH, 5′-CGTCCCGTAGACAAAATGGT-3′ (forward) and 5′-GAGGTCAATGAAGGGGTCG-3′ (reverse); mouse KLF5, 5′-ACCAGACGGCAGTAATGGACAC-3′ (forward) and 5′-ATTGTAGCGGCATAGGACGGAG-3′ (reverse); mouse VDR, 5′-CACAAGACCTACGACCCCAC-3′ (forward) and 5′-CATCATGTCCAGTGAGGGGG-3′ (reverse); mouse PCNA, 5′-AGAAGAGGAGGCGGTAACCA-3′ (forward) and 5′- GGAGACAGTGGAGTGGCTTT-3′ (reverse).

2.8. Western blot analysis

The cell lysates were separated by SDS-PAGE, and transferred onto PVDF membrane (Millipore). Membranes were blocked with 5% milk in TTBS for 2 h at 37 °C and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-KLF5 (1:1000, GTX103289, GeneTex), mouse anti-VDR (1:1000, ab-89626, Abcam), rabbit anti-p50 (1:500, ab-38338, Abcam), mouse anti-PCNA (1:1000, ab-18197, Abcam), rabbit anti-Cdc2 (1:1000, 10762-1-AP, Proteintech), rabbit anti-cyclin B1 (1:1000, sc-70898, Santa Cruz), rabbit anti-TLR4 (1:1000, 19811-1-AP, Proteintech), rabbit anti-p-STAT5 (1:1000, sc-11761, Santa Cruz), rabbit anti-STAT5 (1:1000, 12071-1-AP, Proteintech) and rabbit anti-β-actin (1:5,000, Proteintech). Membranes were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Protein bands were detected by ECL plus (Thermo Scientific) and β-actin was served as an internal control for protein loading.

2.9. Co-immunoprecipitation assay

Co-immunoprecipitation was performed as described previously [17]. The cell lysates were immunoprecipitated with anti-p50 antibodies for 1 h at 4 °C, followed by incubation with protein

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