Original Article
Dihydroartemisinin modulation of toll-like receptors-4 signaling pathway in lipopolysaccharide-stimulated RAW264.7 murine macrophages

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Abstract: Objective: To investigate how dihydroartemisinin (DHA) modulate Toll-like receptors-4 signaling pathway in LPS-stimulated RAW264.7 murine. Methods: IRF3 was detected in RAW264.7 cells treated with lipopolysaccharide (LPS) (1 μg/ml), lipopolysaccharide and dihydroartemisinin (LPS/DHA) (10 μM) and equal volume of culture medium respectively by using immunocytochemistry staining. IFN-α/β mRNA was extracted from Raw264.7 cells in LPS, LPS/DHA and control group, and then was measured by real-time PCR. The concentration of IFN-β in the supernatants was detected by enzyme linked immunosorbent assay. Evaluation on TRAF6 and IRF3 protein expression was carried out by western blot. Results: LPS increased the expression level of IRF3 while DHA significantly inhibited the effect of LPS. Compared with the control group, the IFN-β mRNA level of LPS group dramatically increased. DHA significantly diminished LPS-induced IFN-β gene expression (**P < 0.01 vs. control group; ##P < 0.01 vs. LPS group). However, though LPS slightly upregulated IFN-α expression, no significant difference was observed among the three groups. The protein level of IFN-β in cells supernatants was increased in LPS group, while DHA decreased the release of IFN-β in LPS/DHA group (***P < 0.01 vs. control group; ###P < 0.01 vs. LPS group). LPS enhanced IRF3 expression and DHA inhibited LPS-stimulated effect in this study. Whereas no statistically significant difference of TRAF6 protein level was observed between LPS group and LPS/DHA group. Conclusion: DHA has potent immunosuppressive effect in vitro by inhibiting TRIF-IRF3 signaling pathway of TLR4 and IFN-β production.

Keywords: Systemic lupus erythematosus (SLE), dihydroartemisinin (DHA), toll-like receptors-4 (TLR4), IRF3, IFN-β

Introduction

Systemic lupus erythematosus (SLE) is a non-organ-specific multisystem autoimmune disease with excessive inflammatory responses and various immunological abnormalities, which ultimately lead to multi-organ failure [1]. Although the etiology of SLE remains unclear, previous studies have indicated that macrophages, a group of antigen-presenting cells, are commonly involved in inflammatory responses, and have played pivotal roles in the pathogenesis of SLE [2, 3].

Toll-like receptors-4 (TLR4) is one of the most important pattern recognition receptors (PRR), which is part of the type I transmembrane protein family and is situated at the cell surface. It plays a key role in pathogen recognition and activation of innate immunity [4]. The extracellular leucine-rich repeat (LRR) domain can recognize biomacromolecules [5]. TLR4 is well-known for recognizing lipopolysaccharide (LPS), a component of gram-negative bacteria. Its ligands also include several exogenous microbial ligands like viral proteins, polysaccharide and endogenous proteins such as low-density lipoprotein, beta-defensins, and heat shock protein, which released from damaged tissues and dying cells [6-8]. They recognize pathogen-associated molecular patterns (PAMPs), which are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. In addi-
tion, TRIF pathway of TLR4 ultimately leads to the activation of IRF3. A genetic polymorphism in the promoter region of IRF3 was found to be associated with higher IRF3 expression and with increasing risk of developing SLE [9].

Macrophages plays an important role in pathophysiology of SLE [10, 11] and expresses TLR4 abundantly [12]. Zhang H reported that anti-dsDNA Abs activated NLRP3 inflammasome in monocytes/macrophages from SLE patients by binding to TLR4 and inducing the production of mitochondrial ROS [3]. Ji J found that FC-99 possessed anti-inflammation activity and directly interacted with interleukin-1 receptor-associated kinase 4 (IRAK4), which was a pivotal molecule in TLR4 signaling [13]. TLR4 signaling might play a key role in immune disease. Our previous study showed that Dihydroartemisinin (DHA) was an immunosuppressant with beneficial effects in mouse spleen cell of immune diseases [1]. Inspired by these observations, we examined the immunosuppressive potential of combined treatment with DHA of murine macrophage cell line (RAW264.7).

DHA, a sesquiterpene lactone isolated from the Chinese herb Artemisia annua (A. annua), is a metabolite of artemisinin. A. Annua, as immunomodulator, has been widely used as a Chinese herbal medicine treatment for about 20 different disorders, including cancer and fever [14]. Artemisinin and its derivatives have been widely used as antimalarial drugs that function in killing multidrug-resistant strains of malarial parasites [15] with excellent safety profile [16]. Besides, DHA has been reported to possess preferential immune-regulatory effect [17-20]. The current investigation aims to examine whether DHA inhibits the activation of TLR4 signaling pathway involving type I IFNs in LPS-stimulated RAW264.7 murine macrophages.

Materials and methods

Reagents

DHA was purchased from Zhejiang Institute for Food and Drug Administration (Hangzhou, Zhejiang Province, China). Lipopolysaccharides from Escherichia coli 055:B5 were purchased from Sigma Chemicals (St. Louis, MO, USA). TRAF6 and IRF3 antibodies were purchased from Abcam (Cambridge, UK). Mouse IFN-β enzyme-linked immunosorbent assay (ELISA) kit was purchased from Cusabio (DE, USA).

Data collection and pathway enrichment analysis

We collected targets of dihydroartemisinin through STITCH (confidence range > 0.4) [21] and literature search, resulting in 26 targeting proteins. DAVID [22] was applied to conduct pathway enrichment analysis for the 26 protein products. Enriched pathways were defined as those with adjusted p-values < 0.05.

Cell culture and growth conditions

Mouse macrophage Raw264.7 cells were purchased from the Shanghai Cell Resource Center, Chinese Academy of Sciences (Shanghai, China). Macrophages were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂. Cells were stimulated with LPS (1 μg/ml) (LPS group), LPS and DHA (10 μM) (LPS/DHA group) and equal volume of culture medium (control group) respectively. DHA was dissolved in Dimethyl sulfoxide (DMSO) at the final concentration of less than 0.01%.

Immunocytochemistry staining

The Raw264.7 cells were grown in sterile dishes with LPS (1 μg/ml) (LPS group), LPS/DHA (10 μM) (LPS/DHA group) and equal volume of culture medium (control group) respectively for 2 hours, and then washed with phosphate-buffered saline (PBS). Cells were fixed in acetone for 10 minutes, then washed with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for additional 40 minutes. Proteins were immunodetected with IRF3 antibody (1:100, Abcam) for 12 hours at 4°C and with goat anti-rabbit IgG (1:100, Abcam) as secondary antibody for 1 hour at room temperature. Dishes were observed under the confocal laser scanning biological microscope (Olympus, Japan).

Western blot

Raw264.7 cells were incubated with LPS (1 μg/ml) (LPS group) and LPS/DHA (10 μM) (LPS/DHA group) respectively at 37°C for 24 hours. The cells added with the equal volume of culture medium were used as solvent control.

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Raw264.7 cells were collected and washed with PBS, and the protein was extracted by RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). The protein concentration was measured with BCA protein assay kit (Beyotime). Protein samples (50 μg) were separated by 10% Tris-glycine SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk in Tris buffered saline with Tween (TBST) at room temperature for 1 hour followed by TBST washed. After that, the membranes were incubated with primary antibodies for TRAF6 (1:1000, Abcam), IRF3 (1:1000, Abcam) and β-actin (1:1000, Beyotime) at 4°C for 12 hours. After TBST washed, the membranes were incubated with the secondary antibodies by IRDye 680 goat anti-mouse IgG (1:10000, LI-COR, Bioscience) or IRDye 800CW goat anti-rabbit IgG (1:10000, LI-COR, Bioscience) for 2 hours at room temperature. Protein bands were detected by Odyssey fluorescent scanner (LI-COR; Bioscience, Lincoln, NE, USA).

ELISA

Cells (2.0 × 10^6 cells/ml) were seeded in 6-well plates and were treated with LPS (1 μg/ml) (LPS group), LPS/DHA (10 μM) (LPS/DHA group) and equal volume of culture medium (control group), respectively for 24 hours. Supernatants were harvested and stored at -20°C. Afterward, concentrations of IFN-β in the supernatants were detected by using ELISA kit (Cusabio, DE, USA).

Real-time PCR

For RNA extraction, Raw264.7 cells (2.0 × 10^6 cells/ml) were cultured in 6-well plates and incubated with LPS (1 μg/ml) (LPS group), LPS/DHA (10 μM) (LPS/DHA group) and equal volume of culture medium (control group), respectively for 6 hours. Total RNA was isolated from Raw264.7 cells by using TRIzol reagent (Qiagen, Germany) and reverse transcribed into cDNA by using the SuperScript II reverse transcriptase (Takara Bio, Shiga, Japan) and oligo (dT) primers. To exam the mRNA levels of IFN-α and IFN-β, cDNA was amplified by real-time PCR with a SYBR Premix Ex Taq RT-PCR kit (Bio-Rad, Hercules, CA) and GAPDH was used as loading control. The relative expression levels of IFN-α and IFN-β mRNA were calculated by applying 2^(-ΔΔCt) method. The primers were used as follows: IFN-β (forward: 5'-CCC TAT GGA GAT GAC GGA GA-3', reverse: 5'-ACC CAG TGC TGG AGA AAT TG-3'), IFN-α (forward: 5'-AGT GAG CTG ACC CAG CAG AT-3', reverse: 5'-AGA CAG CCT TGC AGG TCA TT-3') and GAPDH (forward: 5'-TGC ACC ACC AAC TGC TTA G-3', reverse: 5'-GGA TGC AGG GAT GAT GTT C-3').

Table 1. The 23 KEGG pathways with p-values < 0.05 generated by DAVID

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<th>KEGG pathway</th>
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<th>Benjamini</th>
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<td>Drug metabolism-cytochrome P450</td>
<td>3.00E-32</td>
<td>1.10E-30</td>
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<tr>
<td>Steroid hormone biosynthesis</td>
<td>4.00E-31</td>
<td>1.00E-29</td>
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<td>Chemical carcinogenesis</td>
<td>8.10E-31</td>
<td>1.60E-29</td>
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<tr>
<td>Drug metabolism-other enzymes</td>
<td>1.50E-30</td>
<td>2.40E-29</td>
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<tr>
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<td>6.60E-29</td>
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Regulation of TLR4 expression by dihydroartemisinin

**Results**

*Pathway enrichment analysis*

We performed pathway enrichment analysis of the 26 targets of dihydroartemisinin and found that the targets were enriched in 23 pathways with p-values < 0.05, including apoptosis, metabolic pathways and Toll-like receptor signaling pathway (Table 1). Among them, Toll-like receptor signaling pathway plays a key role in activation of innate immunity.

*Immunocytochemistry staining*

As shown by immunocytochemistry staining, the fluorescence intensity displayed the expres-
Regulation of TLR4 expression by dihydroartemisinin

Expression of IRF3. Normal expression of IRF3 was detected in RAW264.7 cells in control group. The expression of IRF3 detected in RAW264.7 cells in LPS group was higher than control group (P < 0.01). The expression of IRF3 measured in RAW264.7 cells in LPS/DHA group was similar as control group (P > 0.01). LPS significantly increased the expression level of IRF3 while DHA markedly inhibited expression of LPS-stimulated IRF3 (Figure 1) (P < 0.01).

Western blot

As shown by western blot, the gray band represented the expression of IRF3 and TRAF6. β-actin was detected as loading control. Normal expression of TRAF6 was detected in RAW264.7 cells in control group. The expression of TRAF6 detected in RAW264.7 cells in LPS group was higher than control group (P < 0.01). The expression of TRAF6 measured in RAW264.7 cells in LPS/DHA group was lower than LPS group (P > 0.01). There are no significant differences of TRAF6 observed among the three groups (Figure 2A) (P > 0.01). Normal expression of IRF3 was detected in RAW264.7 cells in control group. The expression of IRF3 detected in RAW264.7 cells in LPS group was higher than control group (P < 0.01). The expression of IRF3 measured in RAW264.7 cells in LPS/DHA group was lower than LPS group (P < 0.01). LPS significantly increased the expression level of IRF3 while DHA markedly inhibited expression of LPS-stimulated IRF3 in the cell suspension (Figure 2B) (P < 0.01).

RT qPCR

Normal mRNA level of IFN-β was detected in RAW264.7 cells in control group. The mRNA level of IFN-β detected in RAW264.7 cells in LPS group was significantly higher than control group (P < 0.01). The mRNA level of IFN-β measured in RAW264.7 cells in LPS/DHA group was significantly lower than LPS group (P < 0.01). LPS significantly increased the mRNA level of

Figure 2. Effect of DHA on LPS-induced IRF3 and TRAF6 expression. Three separated experiments were performed and mean ± SEM were calculated. **P < 0.01 vs. control group; ##P < 0.01 vs. LPS group.
Regulation of TLR4 expression by dihydroartemisinin

IFN-β while DHA markedly inhibited expression of LPS-stimulated IFN-β in the cell suspension (P < 0.01) (Figure 3A). Normal mRNA level of IFN-α was detected in RAW264.7 cells in control group. The mRNA level of IFN-α detected in RAW264.7 cells in LPS group was higher than the control group (P > 0.01). The mRNA level of IFN-α measured in RAW264.7 cells in LPS/DHA group was lower than LPS group (P > 0.01). Though LPS slightly upregulated IFN-α mRNA levels, no statistically significant differences of IFN-α were observed among the three groups (P > 0.01) (Figure 3B).

**ELISA**

The concentration of IFN-β was detected in cell supernatant by using ELISA. Normal expression of IFN-β was detected in RAW264.7 cells in control group. The expression of IFN-β detected in RAW264.7 cells in LPS group was significantly higher than control group (P < 0.01). The expression of IFN-β measured in RAW264.7 cells in LPS/DHA group was significantly lower than LPS group (P < 0.01). LPS significantly increased the expression of IFN-β in cell supernatant while DHA markedly inhibited expression of LPS-stimulated IFN-β (P < 0.01) (Figure 4).

**Discussion**

This study indicated that DHA may suppress the activation of TLR4-IRF signaling pathway triggered by LPS in RAW264.7 murine macrophages. In general, TLR4 had 2 major downstream signaling pathways, the MyD88-dependent pathway and the TRIF-dependent pathway. TRIF played a key role in TRIF-
dependent pathway, activating non-canonical inhibitors of nuclear factor kappa B kinase (IKKs), and finally led to the activation of IRF3 [23], which resulted in the transcription of target genes, such as Type I interferon [24-26].

Immunocytochemistry staining and Western blot indicated that LPS increased the expression level of IRF3 while DHA significantly inhibited expression of LPS-stimulated IRF3. DHA had been reported to regulate cellular functions, angiogenesis, immunity and tumor cell growth [19]. Li B reported that artesunate, a derivative of dihydroartemisinin, forcefully inhibited the release of IL-6 and TNF-α and decreased the expressions of TLR9 and TLR4 mRNA induced by CpG oligodeoxynucleotide (CpG ODN), LPS or heat-killed E. coli [27].

Western blot showed that there was no difference among three groups in TRAF6 protein expression. TRAF6, which played an important role in MyD88-dependent pathway of TLR4, formed a complex with UBC13 and UEV1A, and activated TAK1. Thus, our finding showed that DHA took effect on TLR4 signaling pathway via TRIF-IRF3 pathway rather than MyD88-dependent pathway.

The detection of IFN-α/β showed that LPS dramatically increased gene expression of IFN-β and DHA obviously down-regulated LPS-stimulated IFN-β gene expression. However, there was no statistically significant difference in IFN-α gene expression. ELISA showed that DHA decreased the release of IFN-β induced by LPS. Previous research proved that Type I interferons was a link between innate and adaptive immune system, and one active interferon-mediated signaling; overexpression of genes regulated by type I interferon was commonly regarded as an important feature of SLE [28]. Moreover Type I interferons affected adaptive immunity through activating dendritic cells and binding to interferon receptors on B cells, T cells, neutrophils and natural killer cells [29].

In summary, our research results showed that DHA had potent immunosuppressive effects in vitro by inhibiting TRIF-IRF3 signaling pathway of TLR4 and IFN-β production in macrophage. Besides our study implies that DHA has the potential as a therapeutic treatment of SLE, but the details of DHA effected on SLE patient still need further studies.

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Disclosure of conflict of interest

None.

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