Dihydroarteannuin ameliorates lupus symptom of BXSB mice by inhibiting production of TNF-alpha and blocking the signaling pathway NF-kappa B translocation

Wei-dong Li a,⁎, Yan-jun Dong a,1, You-you Tu b,2, Zhi-bin Lin a,3

a Department of Pharmacology, Health Science Center, Peking University, 38 Xue yuan Road, Beijing, 100083, PR China
b Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine, Beijing, 100083, PR China

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Abstract

The aim of this study was to investigate the mechanisms of action of Dihydroarteannuin (DHA), a semi-synthesized agent from the starting material artemisinin extracted from the Chinese Traditional Herbs Artemisia annua, on ameliorating the symptoms of lupus on BXSB mice. The concentration of TNF-alpha in the culture supernatant of the peritoneal macrophages and in the sera of BXSB mice was determined by the ELISA method. NF-κB protein expression and translocation were assayed by the EMSA method and laser confocal scanning microscopy method, respectively. IκB-α and NF-κB p65 protein expression were determined by the Western blot method. Renal tissue of the BXSB mice was prepared for assaying inhibitory activity of DHA on NF-κB, p65 and IκB-alpha protein expression in vivo. The peritoneal macrophages were prepared for analysis inhibitory effects of DHA on translocation of NF-κB into nuclear in vitro. We found that DHA strongly reduced the production of TNF-alpha in the culture supernatant of the peritoneal macrophages and in the sera of BXSB mice in vitro or in vivo. The results demonstrated that DHA decreased the expression of NF-κB subunit p65 protein and the activation of NF-κB in the renal tissue of BXSB mice in vivo. DHA effectively inhibited the nuclear translocation of NF-κB in peritoneal macrophages of BXSB mice in vitro. Furthermore, it was demonstrated that the degradation of IκB-α protein was significantly inhibited by DHA. These observations suggested that the inhibitory effects of DHA on TNF-alpha production may result from the block in the NF-κB signaling pathway upstream of IκB degradation.

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1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of indeterminate etiology, characterized by episodic flares that are often associated with relentless disease progression, substantial morbidity, and increased mortality [1]. Murine models of lupus, including the BXSB mouse, have been proved invaluable in the analyses of the
pathogenesis of SLE. BXSB mice spontaneously develop a human lupus-like autoimmune disease and die from immune complex-mediated glomerulonephritis that is somewhat different in distribution and manifestations from renal diseases characteristic of mice of other autoimmune-prone strains [2,3].

For systemic lupus erythematosus’s patients, compared with normal people, TNF-alpha secreted is at a higher level in sera and as well as soluble receptors. SLE sera contain considerable biologically active TNF-alpha and peripheral blood lymphocytes of SLE patients contain functional TNF-alpha receptors in high expression. TNF-alpha is an agent capable to induce cell surface expression of these antigens in human keratinocytes and thus being proposed to be a potential inducer of an autoimmune response [4].

Nuclear factor-κB (NF-κB), an inducible eukaryotic transcription factor of the rel family, normally exists in an inactive cytoplasmic complex. Its predominant form is a heterodimer composed of p50 and p65 (Rel A) subunits, bound to inhibitory proteins of the IκB family, which is activated in response to primary (viruses, bacteria, UV) or secondary (inflammatory cytokines) pathogenic stimuli [5,6]. Stimulation triggers the release of NF-κB from IκB, resulting in NF-κB translocation to the nucleus, where it binds to the DNA at specific κB sites, rapidly inducing a variety of genes encoding signaling proteins. NF-κB, involved in many pathological events including the progression of SLE [7–10], is considered as an immediate early mediator of immune and inflammatory responses.

Dihydroarteannuin (DHA, MW, 284.35, molecular formation is C15H24O5, chemical structure see Fig. 1) represents one of the largest groups of sesquiterpene lactones that are generally accepted as biologically effective substrat of traditional herbal medicine and used as an anti-malarial agent in clinical treatment. In our previous reports, we found that the DHA exhibited significantly immunosuppressive activities on the LPS-induced IL-1 production on lupus-like BXSB mice. It inhibited the production of anti-dsDNA antibodies and improved the pathologic lesion of lupus nephritis in BXSB mice [11,12]. Following the study that DHA exerted an anti-lupus activity in BXSB mice, we expected to investigate the effect of DHA on the production of pro-inflammatory mediator, TNF-alpha, and the effect of DHA on signal conduction pathway. The aim of the present work was to prove the molecular mechanisms of DHA on occurring and the progress of lupus.

2. Materials and methods

2.1. Animals

BXSB male mice were obtained from the department of immunology, Peking University. All animals were exposed to 12 h light/dark cycles, were given food and water ad libitum, and were allowed to acclimate for at least 5 days prior to the experimental manipulation. Mice were given DHA (at different dosages described as below) in PBS solution once each day for 10 days by intragastrical (i.g.). At the end of the course of giving DHA, the mice were sacrificed and collected the blood. The blood was collected and pooled into 2 ml tubes. The sera were stored at −20 °C until measurement of the plasma concentration of the TNF-alpha.

2.2. Agents

DHA (purity >99%) was a generous gift from Professor You-you TU, Institute of Chinese Materia Medica, China

![Fig. 1. Chemical structure of Dihydroarteannuin.](image-url)

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![Fig. 2. Effect of DHA on the content of TNF-alpha in the cultured supernatant of peritoneal macrophage from BXSB mice in vivo.](image-url)

Fig. 2. Effect of DHA on the content of TNF-alpha in the cultured supernatant of peritoneal macrophage from BXSB mice in vivo. Mice of normal and model control groups were treated with NS for ten days. Testing group mice were treated with DHA once each day for ten days. Mice peritoneal macrophage were prepared and co-cultured with LPS 10 μg/ml for 12 h, then the TNF-alpha production were determined. Values are the mean±SEM of 3 separate experiments, each performed in triplicate: ΔΔP<0.01 vs the normal control group; **P<0.01 vs the model control group.
Academy of Traditional Chinese Medicine. Lipopolysaccharides (LPS, *E. coli* 0111:B4), and RPMI-1640 medium were from Sigma Co. Fetal calf sera (FCS) were purchased from Gibco-BRL Co.

2.3. Peritoneal macrophages preparation and culturing conditions

Mice were killed by carbon dioxide or diethyl ether inhalation. Peritoneal macrophages were prepared by peritoneal lavage with 10 ml of sterile PBS. The cells were spun down, washed once with sterile PBS, resuspended in Dulbecco’s minimal essential medium (DMEM) containing penicillin/streptomycin and 10% fetal calf sera and plated to 6-well culture dishes at a concentration of about 1×10⁶ cells/well. The macrophages were allowed to adhere to the dishes (2 to 3 h), the non-adhesive cells were removed, and the cells were kept in culture for up to 96 h. The culture medium was changed after 24 and 76 h. Then the cells were washed once with PBS and resuspended in 1 ml of PBS.

2.4. TNF-alpha assay

Macrophages from conditioned and sham-operated mice were collected and around 1 million cells were plated in 12 well dishes at a concentration of about 1×10⁶ cells/well. The macrophages were allowed to adhere to the dishes (2 to 3 h), the non-adhesive cells were removed, and the cells were kept in culture for up to 96 h. The culture medium was changed after 24 and 76 h. Then the cells were washed once with PBS and resuspended in 1 ml of PBS.

Fig. 3. Effect of DHA on the content of TNF-alpha in the supernatant from BXSB mice peritoneal macrophage cultured in vitro. Mice peritoneal macrophage were prepared and co-cultured with DHA for 2 h, lately with LPS 10 μg/ml for 12 h, then the TNF-alpha production were determined. Values are the mean±SEM of 3 separate experiments, each performed in triplicate. ∆∆P<0.01 vs the normal control group; **P<0.01 vs the LPS control group.

Fig. 4. Effect of DHA on the level of TNF-alpha in the sera from BXSB mice in vivo. Mice of normal and model control groups were treated with NS for ten days. Testing group mice were treated with DHA once time each day for ten days. Values are the mean±SEM of 3 separate experiments, each performed in triplicate. ∆∆P<0.01 vs the normal control group; *P<0.05, **P<0.01 vs the model control group.

Fig. 5. Effect of DHA on the renal tissue NF-κB activation in the BXSB mice in vivo. Mice of normal and BXSB model control groups were treated with NS for ten days. Testing group mice were treated with DHA once time each day for ten days. Crude nuclear extracts were prepared from the renal tissue and the effect of DHA on the NF-κB activation was determined using an electrophoretic mobility shift assay (EMSA). Lane 1: normal control; Lane 2: model control; Lane 3: DHA 125 mg/kg; Lane 4: DHA 25 mg/kg; Lane 5: DHA 5 mg/kg. Results are the representative of two separate experiments.

Fig. 6. Effect of DHA on the expression of NF-κB p65 in renal tissue of BXSB mice in vivo. Mice of normal and model control groups were treated with NS for ten days. Testing group mice were treated with DHA for ten days. The expression of NF-κB p65 was detected by the Western blot method. Lane 1: normal control; Lane 2: model control; Lane 3: DHA 125 mg/kg; Lane 4: DHA 25 mg/kg; Lane 5: DHA 5 mg/kg.
2.5. Preparation of cytosol and nuclear protein extracts

The cytosol and nuclear protein extracts were prepared from mice renal tissue. The extracts were obtained using a modification of the method described in detail before [13]. Mice of normal and BXSB model control groups were treated with NS for ten days. Testing group mice were treated with DHA for ten days. The expression of NF-κB p65 was detected by the Western blot method. Values are the mean±SEM of 3 separate experiments, each performed in triplicate. **P<0.01 vs the normal control group; ***P<0.01 vs the model control group.

2.6. Measurements of the level of active NF-κB by Electrophoretic Mobility Shift Assays (EMSA)

The DNA sequences of the double-stranded oligonucleotide specific for NF-κB were 5′-AGTTGAGGGGACTTCCCAGGCC-3′ and 5′-TCAACTCCCCCTGAAAGGTTCCG-3′ (Shanghai Sangon Biotechnology Co.). The level of active NF-κB in the nuclear extracts of control and treated cells was determined by its DNA binding activity by EMSA as described [14] elsewhere. Complementary strands were annealed, and double-stranded oligonucleotides were labeled with γ-32p-ATP (γ32P adenosine triphosphate) [γ32P] ATP using the Klenow fragment of DNA polymerase (Life Technologies). Nuclear extracts (5 μg of protein) were incubated for 10 min at 4 °C in a total volume of 20 μl containing 2 μg of poly(dl-dC) and 2 μl of 10×gel shift binding buffer (20 mM Tris–HCl, pH 7.9, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 20% glycerol). The extracts were then incubated for 30 min at 4 °C with 10000 cpm of 32P-labeled NF-κB probes. The samples were loaded on a 5% native polyacrylamide gel, and run in 0.5×TBE buffer. The gel was dried and subjected to autoradiography.

NF-κB-specific bands were confirmed by competition with a 100-fold excess of an unlabeled NF-κB probe, which resulted in a no shifted band. Double-stranded oligonucleotides were labeled with γ-32p-ATP (Beijing Free Biotechnology Co.) and T4 polynucleotide kinase (Promega Co.), then incubated with samples of 10 μg nuclear protein extract in binding buffer (10 mM Tris–HCl pH 7.8, 50 mM NaCl, 1 mM EDTA pH 8.0, 1 mM DTT, 1 mM MgCl2, glycerol 14%, 1 μg/μl poly dl-dC (Roche Co.) for 30 min at room temperature. The mixture was then electrophoresed on an 8% non-denaturing polyacrylamide gel in 1×TBE buffer. NF-κB-specific bands were confirmed by competition with a 100-fold excess of an unlabeled NF-κB probe. The gel was dried and subjected to autoradiography for 48 h at −70 °C.

2.7. Western immunoblot analysis

Nuclear extract proteins (20 μg) were separated with 10% SDS-PAGE, then electrotransferred onto nitrocellulose membranes in a buffer containing 25 mM Tris/192 mM glycine/20% methanol for 3 h at 80 V and 4 °C. The membranes were pre-incubated for 1 h at room temperature in Tris-buffered saline (TBS), containing 0.05% Tween-20 and 3% bovine serum albumin (pH 7.6). The nitrocellulose membranes were incubated with p65 or IκBα-specific antibodies purchased from Santa Cruz Biotechnology. Immunoreactive bands were then determined by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents [15]. The blots were washed in TBST and were incubated with secondary antibody (Santa Cruz Biotechnology). The DNA sequences of the double-stranded oligonucleotide specific for NF-κB were 5′-AGTTGAGGGGACTTCCCAGGCC-3′ and 5′-TCAACTCCCCCTGAAAGGTTCCG-3′ (Shanghai Sangon Biotechnology Co.). The level of active NF-κB in the nuclear extracts of control and treated cells was determined by its DNA binding activity by EMSA as described [14] elsewhere. Complementary strands were annealed, and double-stranded oligonucleotides were labeled with γ-32p-ATP (γ32P adenosine triphosphate) [γ32P] ATP using the Klenow fragment of DNA polymerase (Life Technologies). Nuclear extracts (5 μg of protein) were incubated for 10 min at 4 °C in a total volume of 20 μl containing 2 μg of poly(dl-dC) and 2 μl of 10×gel shift binding buffer (20 mM Tris–HCl, pH 7.9, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 20% glycerol). The extracts were then incubated for 30 min at 4 °C with 10000 cpm of 32P-labeled NF-κB probes. The samples were loaded on a 5% native polyacrylamide gel, and run in 0.5×TBE buffer. The gel was dried and subjected to autoradiography.

NF-κB-specific bands were confirmed by competition with a 100-fold excess of an unlabeled NF-κB probe. The gel was dried and subjected to autoradiography for 48 h at −70 °C.

Nuclear extract proteins (20 μg) were separated with 10% SDS-PAGE, then electrotransferred onto nitrocellulose membranes in a buffer containing 25 mM Tris/192 mM glycine/20% methanol for 3 h at 80 V and 4 °C. The membranes were pre-incubated for 1 h at room temperature in Tris-buffered saline (TBS), containing 0.05% Tween-20 and 3% bovine serum albumin (pH 7.6). The nitrocellulose membranes were incubated with p65 or IκBα-specific antibodies purchased from Santa Cruz Biotechnology. Immunoreactive bands were then determined by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents [15]. The blots were washed in TBST and were incubated with secondary antibody (Santa Cruz Biotechnology).
Fig. 9. Effect of DHA on the NF-κB p65 nuclear translocation in peritoneal macrophage from the BXSB mice in vitro. Mice peritoneal macrophages were prepared and co-cultured with DHA for 2 h, lately co-cultured with LPS 10 μg/ml for 2 h, then the p65 protein localization in peritoneal macrophages was determined with anti-p65 antibody and FITC-labelling anti-rabbit IgG antibody and laser confocal scanning microscopy. Staining with propidium iodide (PI) verified the location and integrity of nuclei. Negative control without the anti-p65 antibody. (A) Negative control; (B) BXSB mice + LPS group; (C) BXSB mice model group; (D) DHA 250 μM on BXSB mice + LPS group; (E) DHA 25 μM on BXSB mice + LPS group; (F) DHA 2.5 μM on BXSB mice + LPS group. The red color expressed the nuclear that dye by PI. The green color expressed the quantity of translocation of NF-κB p65 protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Biotechnology), and proteins were visualized by chemiluminescence (Amersham).

2.8. Confocal microscopy

Mice peritoneal macrophages were prepared from BXSB male mice, then the NF-κB p65 protein localization in peritoneal macrophages was determined with anti-p65 antibody and FITC-conjugated anti-rabbit IgG antibody and laser confocal scanning microscopy. Microscopic images were acquired with a TCS SP2 microscope workstation (Leica Inc. Germany) equipped with a laser-scanning confocal unit (Leica Inc. Germany). FITC was excited using the 488 nm argon laser emission line. PI was excited using the 543 nm argon laser emission line.

2.9. Statistics

Values expressed are means ± SEM. Data were analyzed by the Student t-test. Significance was defined as a P value of less than 0.05.

3. Results

3.1. Effect of DHA on TNF-alpha production in the cultured supernatant of peritoneal macrophage in BXSB mice in vivo and in vitro

To investigate further the role of TNF-alpha in disease pathogenesis and the potential of DHA as therapeutic agents in SLE, the effect of DHA on TNF-alpha cytokine production of peritoneal macrophages in BXSB mice in vitro or in vivo, were assayed respectively. The effect of DHA on TNF-alpha cytokine production in sera of BXSB mice in vivo was assayed. The effect of DHA on the production of TNF-alpha was studied in BXSB mice peritoneal macrophages in culture. A maximal production of TNF-alpha was measured at 4 h in lipopolysaccharide (LPS)-stimulated macrophages, and addition of DHA (0.25–250 μM) anticipated this increase by 1 h. We subsequently treated BXSB mice with DHA to determine the in vivo effects of DHA on SLE disease progression. It was found that there was a significant amelioration of clinical signs of disease, particularly treated in BXSB mice with DHA compared with controls and DHA significantly down-regulated overproduction of TNF-alpha LPS-stimulated with BXSB mice in vitro and in vivo. (see Figs. 2 and 3). DHA also inhibited TNF-alpha production in sera of BXSB mice. Compared with BXSB control mice, there was a significantly difference between them (see Fig. 4).

3.2. Effect of DHA on the activation of NF-κB in the renal tissue of BXSB mice in vivo

The promoter region in TNF-alpha genes contains binding motifs for NF-κB/Rel, et al. These appear to be important in mediating LPS induction the produce of TNF-alpha. To further investigate the role of NF-κB on TNF-alpha production, the effect of DHA on NF-κB activation was assessed with Electrophoretic Mobility Shift Assays. DHA (125, 25, 5 mg/kg) administrated by intragastrical way with BXSB mice for ten days. The peritoneal macrophages were collected and stimulated by LPS at 10 μg/ml final concentration. It had been found that DHA at 125 and 25 mg/kg dosage significantly inhibited the activation of NF-κB expression induced by LPS. In this report, the inhibitory mechanism of DHA on the LPS-induced NF-κB activation was characterized (see Fig. 5).

3.3. Expression of NF-κB p65 protein in renal tissue of BXSB mice

Western Blot Analysis of cytosolic protein for p65. Using specific antibody binding predominantly to the activated form of p65, a distinct staining pattern for these proteins in BXSB mice renal tissue (Figs. 6 and 7) was detected. The level of p65 protein expression was inhibited by DHA at the dosage of 125, 25 and 5 mg/kg, respectively.

3.4. Effect of DHA on the expression of IκB in renal tissue of BXSB mice in vivo

To further investigate whether the inhibition of transcription factors by DHA was mediated through the influence on the degradation of inhibitor of NF-κB, the effects of DHA on the expression of IκB protein were assessed by the Western blot method. The results demonstrated that the degradation of IκB-alpha protein was significantly inhibited by DHA at the dosage of 125 and 25 mg/kg, respectively (Fig. 8).

3.5. Effect of DHA on the NF-κB p65 nuclear translocation in peritoneal macrophage from the BXSB mice in vitro

In order to clearly understand the influence of DHA on the NF-κB p65 nuclear translocation, the NF-κB p65 nuclear shift situation in peritoneal macrophage from the BXSB mice was detected by the Confocal microscopy method. The results showed that DHA at the concentration of 250, 25 and 2.5 μM inhibited the translocation of NF-κB p65 (showed as Fig. 9).

4. Discussion

The murine models of autoimmune diseases, such as that of BXSB mice, are invaluable in assessing the efficacy of various therapeutic modalities because they exhibit histologic and serologic similarities to human autoimmune diseases. Male BXSB mice spontaneously develop autoimmune disease with features similar to systemic lupus erythematosus. The present work demonstrated that TNF-alpha appeared over production in peritoneal macrophages and in sera of BXSB mice.

In our previous work, anti-dsDNA antibody and TNF-alpha in serum of the BXSB mice were detected by
enzyme linked immunosorbent assay. Renal tissue was stained by HE and Masson method. The results showed that compared with the model group, the levels of anti-dsDNA antibody in serum and TNF-alpha level in serum were significantly lower ($P<0.0105$) at the DHA dosage of 125 and 25 mg/kg. We also found that DHA may reverse renal pathological changes in BXSB mice. The kidney organization freezes slice direct immunity fluorescence examination result demonstrated that immunity globulin (IgG, IgA, IgM) and complements (C3, C1q) in BXSB mice group displayed positive results, whereas, kidney organization freezes slice of the mice given DHA at 125 and 25 mg/kg dosages showed negative immunity fluorescence [12,13]. It clues to us that DHA has the certain protective function to the kidney of BXSB mouse.

It has been known that the activation of NF-κB must enter the nucleus. In the cytosol, NF-κB is bound to an inhibitory protein, IκB that prevents the translocation of NF-κB into the nucleus. Many inflammatory stimuli, such as tumor necrosis factor-alpha (TNF-alpha) or oxygen radicals, trigger NF-κB activation in immune cells by causing the phosphorylation of NF-κB, which in turn leads to rapid IκB ubiquitination and degradation by the proteasome. Once NF-κB is released from IκB, it translocates into the nucleus, where it stimulates expression of several genes, including key components of the inflammatory response (such as TNF-alpha, and interleukin-1), and growth factors (such as interleukin-2) required to mount an immune fluorescence cascade. In addition to the inhibition of inflammatory mechanisms that DHA acts to block the inflammatory cascade. In addition to the inhibition of inflammatory mediators known as TNF-alpha, it can influence the activation of NF-κB and translocation to the nucleus. This effect may be brought by inhibiting one of the proteins involved in the destruction of IκB. The result is that NF-κB remains sequestered outside the cell’s nucleus thereby preventing the NF-κB-induced inflammatory response. The effects of DHA on cytokine production and on the signal way of NF-κB can explain anti-lupus effects of DHA.

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References


