

Anti-inflammatory effects of DhHP-6 in LPS-induced RAW264.7 macrophages and carrageenan-induced air pouch model in rat

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Research Article

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Abstract

DhHP-6 (Deuterohemin-Ala-His-Thr-Val-Glu-Lys) is a novel peptide mimic of peroxidases that was previously designed in our laboratory. Here, we explored the anti-inflammatory potential of DhHP-6 against lipopolysaccharide(LPS)stimulated inflammatory response in RAW264.7 cells and carrageenan-induced air pouch model rats. DhHP-6 treatment dramatically attenuated the production of nitric oxide (NO), IL-6, andTNF- α in LPS induced RAW264.7 cells. Also, it blocked phosphorylation and degradation of κ B α and suppressed the nuclear translocation of p65. DhHP-6 (0.2, 0.6, and 2.0 mg/kg) significantly reduced the levels of total proteins and WBC counts in the exudates of the air pouch model rats. Moreover, MDA contents in the plasma of rats were reduced and SOD activities were enhanced in the DhHP-6-treatment group. Our results strongly show the effectiveness of DhHP-6 as an anti-inflammatory agent. The mechanism could be related to the reduction of Reactive oxygen species(ROS), inhibition of NF- κ B nuclear translocation, and reduction of pro-inflammatory cytokines.

1. Introduction

Inflammation, characterized by redness, pain, and swelling, is considered a defensive immune response against external stimuli, such as toxins and pathogens [16]. However, uncontrolled inflammation may cause potential damage to the host. Chronic inflammation can develop into several inflammation-related diseases such as diabetes mellitus, ischemic heart disease, atherosclerosis, stroke, cancer, autoimmune diseases like rheumatoid arthritis, neurodegenerative conditions like Alzheimer's disease, and so on [17, 18, 25, 19, 28]. Therefore, uncontrolled inflammation needs to be timely controlled to avoid damages.

ROS are oxygen compounds produced from exogenous oxidants or intracellular metabolism. These free radical compounds with unpaired electrons exhibit strong chemical reactivity. During unbalanced production and elimination, excessive accumulation of ROS can damage intracellular molecules, such as DNA, proteins, and lipids inducing oxidative stress [6]. Several studies have linked oxidative stress to inflammation [10, 20]. ROS/RNS (Reactive nitrogen species) initiate the inflammatory processes by stimulating the synthesis/secretion of pro-inflammatory cytokines [14].

Deuterohemin-Ala-His-Thr-Val-Glu-Lys (DhHP-6), designed from microperoxidase-11 (MP-11), is a novel peroxidase mimetic peptide. Its active center is composed of Fe³⁺-containing heme and histidine. Notably, the peroxidase activity of DhHP-6 is ~93% ($3.9 \times 10^3 \text{ U} \cdot \mu\text{mol}^{-1}$) of that of natural peroxidase MP-11. Moreover, it is relatively more aqueous soluble and stable, and therefore, it can be easily synthesized and purified [32, 31].

Previously, we showed that DhHP-6 could effectively eliminate ROS exhibiting protective effects in many oxidative stress-related diseases such as Type 2 diabetes [32, 31], Alzheimer's disease [35, 36], and cerebral ischemia-reperfusion [11]. Importantly, the pathological changes in these diseases have been linked to oxidative stress-induced inflammation. We showed that DhHP-6 administration reduced the levels of ROS, amyloid- β plaques in the cerebral cortex and hippocampus, and pro-inflammatory

cytokines in APP^{swe}/PSEN1^{dE9}, a transgenic mouse model of Alzheimer's disease [36]. Although DhHP-6 was shown great therapeutic potential, its mechanism of alleviating inflammation remains elusive. Accordingly, here, we systematically explored the ROS scavenging bioactivity of DhHP-6 and its protective effect in alleviating inflammatory diseases.

LPS induced inflammation in RAW264.7 cells was used to study the anti-inflammatory activity and molecular mechanism of DhHP-6. Furthermore, we used carrageenan as an inducer to set up a rat subcutaneous air pouch model to examine the anti-inflammatory effect of DhHP-6 *in vivo*.

2. Materials And Methods

2.1 Chemicals and reagents

DhHP-6 was purchased from ShengnuoBiopharm Co., Ltd, Chengdu China. Dexamethasone sodium phosphate injection (equivalent to dexamethasone 5 mg/mL) was purchased from Jiangsu Lianshui Pharmaceutical Co., Ltd. LPS (L4391) from *Escherichia coli* O111:B4, and carrageenan (C1013) were purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) (11885084), fetal bovine serum (FBS) (10099-141), and trypsin (15050065) were purchased from Life Technologies/Gibco Laboratories (Grand Island, NY, USA). Penicillin-streptomycin solution (P1400), nucleoprotein extraction kit (R0050), bicinchoninic acid (BCA) protein assay kit (PC0020), D-Hank's solution (H1045), red blood cell lysate buffer (R1010), DAPI staining solution (C0065), and phosphate-buffered saline (PBS) (P1020) were purchased from Solarbio Life Sciences (Peking, China). Polyvinylidene fluoride (PVDF) membranes (ISEQ00010) were purchased from Millipore (Billerica, MA, USA). Enhanced chemiluminescence (ECL) reagent (RPN2232) was purchased from GE (Marlborough, MA, USA). WST-1 cell proliferation and cytotoxicity detection kit (C0035), and reactive oxygen species assay kit (S0033S) were purchased from Beyotime Biotechnology (Shanghai, China). Kits to assay superoxide dismutase (SOD)(A001-3-2), malondialdehyde(MDA)(A003-1-2), and NO (S0021M) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Mouse IL-6 Quantikine® ELISA Kit (D6050) and Mouse TNF- α Quantikine® ELISA Kit (MTA00B) were purchased from R&D Corporation (Minneapolis, MN, USA). Antibodies against NF- κ B/p65 (#8242T), I κ B α (#4814), p-I κ B α (#2859), β -Actin (#4970), Histone H3(#4499), induced nitric oxide synthase (iNOS) (#13120), and Alexa Fluor® 488 conjugate anti-rabbit IgG (H+L) (4412) were purchased from Cell Signaling (Beverly, MA, USA). Goat anti-rabbit IgG/HRP antibody (bs-0295G-HRP), and goat anti-mouse IgG/HRP antibody (bs-0296G-HRP) were purchased from Immunoway Biotechnology Company (Beijing, China).

2.2 Instruments

Mini protean electrophoresis and transblot system Instrument (Bio-Rad, Hercules, CA, USA); Microplate reader (BioTek Instruments, Inc., Winooski, VT, USA); Flow cytometer (Beckman, Indianapolis, IN, USA); Automatic chemiluminescence image analysis system (Tanon, Shanghai, China); Fluorescence microscope system (Leica, Wetzlar, Germany); Centrifuge (Eppendorf, Hamburg, Germany); Sysmex Microcell Counter (Sysmex, Kobe, Hyogo, Japan).

2.3 Cell line and cell culture

RAW264.7 (murine macrophage) cell line (P4), purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), was cultured in DMEM with 10% FBS, 100U/mL penicillin, and 100µg/ mL streptomycin at 37 °C with 5% CO₂ and 95% humidity. The medium was changed every day. Cells were passaged at 70-80% confluency.

2.4 Cell viability assay

Cell viability was measured using the WST-1 cell proliferation and cytotoxicity detection kit. Briefly, the cells (5×10^3 cells/well) were seeded into 96-well plates (six replicates) and cultured overnight. Next day, these were treated with DhHP-6 (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, or 64 µM in DMEM) for 24 h. Then, WST-1 solution (10µL/well) was added to each well and incubated for 3 h. A microplate reader was used to measure the optical densities at 450 nm.

2.5 Estimation of ROS

RAW264.7 cells (5×10^5 cells/well) were seeded into 6-well plates and incubated overnight. The cells were pretreated with various concentrations of DhHP-6 (0.25, 0.5, and 1 µM) for 2 h and then incubated with or without LPS (1 µg/mL in PBS) for another 12 h. The cellular ROS levels were estimated by ROS assay kit and a flow cytometer.

2.6 Assessment of NO levels and cytokines release

RAW264.7 cells (5×10^3 cells/well) were seeded into 96-well plates and incubated for 24 h. The cells were divided into five groups, namely the control group, model group, and three treatment groups of low (0.25 µM), middle (0.5 µM), and high (1 µM) doses. The control group was not treated with LPS or DhHP-6. The model group was treated with LPS (1 µg/mL) for 24 h. The cells of the treatment groups were pretreated with different concentrations of DhHP-6 (0.25, 0.5, and 1 µM) for 2 h, and then treated with LPS (1 µg/mL) for 24 h. Culture media was assayed for NO and inflammatory cytokines (IL-6 and TNF-α) using NO detection and ELISA kits, respectively.

2.7 Western blotting

RAW264.7 cells (5×10^5 cells/well) were seeded into 6-well plates and incubated overnight. These were pretreated with different concentrations of DhHP-6 (0.25, 0.5, and 1 µM) for 2 h. After LPS (1 µg/mL) stimulation of the RAW264.7 cells for 12 h, cytoplasmic proteins and nucleoprotein were extracted using a nucleoprotein extraction kit. Sample protein concentration was determined by the BCA kit. Protein samples (50 µg, 20 µL each) were separated by 10% or 12% SDS-PAGE gels and transferred onto PVDF membranes. After blocking the PVDF membrane with 5% nonfat milk for 1 h, the membrane was incubated with primary antibodies at 4 °C overnight. Then, another incubation was performed with a

secondary antibody for 1 h at room temperature (RT), and the bands were detected by ECL reagent. Protein blot images were captured using an automatic chemiluminescence image analysis system.

2.8 Immunofluorescence assay

RAW264.7 cells (1×10^5 cells/mL) were seeded into 24-well plates and incubated overnight. Then, these were pretreated with DhHP-6 (1 μ M) for 2 h, followed by LPS-stimulation (1 μ g/mL) for 12 h. After discarding the culture medium, cells were fixed in 4% paraformaldehyde for 0.5h, permeabilized with 0.1% Triton X-100 for 15 min, and blocked with 5% bovine serum albumin (BSA) for 1 h. Thereafter, the cells were washed with PBS, followed by incubation with NF- κ B p65 subunit antibody (1:100) for 1 h at 37 °C. Next, the cells were incubated with ALEXA FLUOR 488 labeled goat anti-rabbit antibody(1:1000)for 1 h. Cell nuclei were stained with DAPI. Fluorescence images were acquired with a fluorescence microscope system.

2.9 Animals

Male Sprague-Dawley (SD) rats (6-8 weeks old) were obtained from the Liaoning Changsheng biotechnology corporation (Certificate of Conformity: SCXK (Liao) 2020-0001). All animal protocols were performed following the guidelines of Guide for the Care and Use of Laboratory Animals of the Jilin University (License No.: 20210417) and were approved by the Animal Care Committee of Jilin University.

2.10 Carrageenan-induced air pouch model in rats and experimental design

Forty-eight SD rats were randomly divided into six groups: control group, model group, dexamethasone treatment group, and three DhHP-6 treatment (low, medium, and high dose) groups. Solid DhHP-6 was dissolved in normal saline to prepare DhHP-6 solution. The rats were correspondingly injected intraperitoneally (IP) with normal saline (10 mL/kg, control and model groups), dexamethasone (1 mg/kg), and DhHP-6 (0.2, 0.6, and 2 mg/kg), once a day during the seven days of the challenge period.

The air pouch model was established as described previously[5]. Briefly, on day 1, an airbag was formed by subcutaneous injection of 20 mL sterile air into the scapular region on the rat's back. Thereafter, the drugs were administered (IP) for seven consecutive days. On the third day, an additional 10 mL of sterile air was injected into the bag to prevent the cyst's closure. Subsequently, on the seventh day, 0.5 h after administration of the respective drugs, carrageenan (2 mL, 0.25% solution in 0.9% w/v saline) was injected into the bag to induce inflammation in all the groups except the control group. The control group received the same volume of saline (0.9% w/v).

2.11 Assessment of WBC count and proteins in the exudate

Six hours after the administration of carrageenan or saline, all the rats were anesthetized with sodium pentobarbital (3 mg/mL, 0.1 mL/kg, IP). The airbag was washed with 4 mL of D-Hank's solution, and the entire volume of lavage solution was collected. From each group, 0.1 mL of lavage solution was diluted with 1 mL of red blood cell lysate buffer. The WBCs count in the mixture was estimated using Sysmex

Microcell Counter. The lavage solutions were centrifuged for 10 min (1000g, 4 °C) to separate the supernatants. Protein concentrations in the supernatant were measured using the BCAkit.

2.12 Assessment of SOD activity and MDA content in the plasma

Blood samples were obtained from the abdominal aorta of anesthetized rats. These were centrifuged for 10 min(3000 g, 4 °C). From each group, 1 mL of the plasma was collected to analyze SOD activity and MDA content following the instructions of the kits.

2.13 Statistical analysis

All data were statistically evaluated using statistical software Origin 6.0, and the significance of the data was analyzed by single-factor analysis (one-way ANOVA). All the results are expressed as mean value \pm standard deviation (SD). *P* values < 0.05 were considered statistically significant. All the charts were drawn using Origin 6.0.

3. Results

3.1 Effect of DhHP-6 on cell viability

The cytotoxicity of DhHP-6 in RAW264.7 cells was determined by WST-1 assay (Fig. 1). We found that compared to the control group (untreated), low-dose DhHP-6 (0.125-1 μ M) treatment displayed no apparent toxicity in RAW264.7 cells. However, when the DhHP-6 concentration exceeded 4 μ M, cell viability reduced significantly. In comparison to the control group (100% viability), cell viability decreased to 69.13% after treatment with 32 μ M DhHP-6. Based on these observations, 0.25, 0.5, and 1 μ M DhHP-6 were selected as the three concentration levels for further experiments.

3.2 DhHP-6 inhibits LPS-induced ROS accumulation

ROS promote inflammatory disorder, meanwhile, inflammation can also enhance ROS production causing oxidative stress [27]. Next, we examined the effect of DhHP-6 on LPS induced ROS Levels. Notably, LPS enhanced ROS levels in RAW264.7 cells were significantly reduced if pretreated with DhHP-6 (Fig. 2).

3.3 DhHP-6 reduces LPS-induced pro-inflammatory cytokines

Pro-inflammatory cytokines produced by the infiltrated cells amplify acute inflammation [3, 12]. Therefore, we explored the impact of DhHP-6 on the production of pro-inflammatory cytokines (TNF- α and IL-6). The release of pro-inflammatory cytokines in the culture medium was determined by corresponding ELISA kits. As shown in Fig. 3, LPS treatment enhanced the production of TNF- α and IL-6 in RAW 264.7 cells. However, after DhHP-6 (0.25, 0.5, and 1 μ M) addition, the secretion of TNF- α and IL-6 significantly decreased compared to the LPS treated group. Moreover, it was a dose-dependent effect (Figs. 3A and B).

3.4 DhHP-6 reduces LPS-induced production of NO and expression of iNOS

Cellular production of NO is stimulated by nitric oxide synthase (NOS) which is namely divided into 3 types; endothelial (eNOS), neuronal (nNOS), and induced (iNOS) nitric oxide synthases. Under inflammatory conditions, the production of NO is regulated by iNOS while the expression of iNOS is influenced by endotoxins, cytokines, etc. [22]. We measured the iNOS levels in the RAW264.7 cells by Western blotting and found that the protein level of iNOS was markedly increased after LPS-stimulation. However, DhHP-6 pretreatment reduced the level of iNOS in a dose-dependent manner (Fig. 4B).

3.5 DhHP-6 inhibits LPS-stimulated nuclear translocation of NF- κ B

The expression of inflammatory cytokines and iNOS is regulated by Nuclear Factor Kappa B (NF- κ B) [15]. To understand the inflammation alleviating mechanism of DhHP-6, we examined its effect on the NF- κ B signaling pathway. As shown in Fig. 5A, RAW264.7 cells treated with LPS alone (for 12 h) showed a decreased level of I κ B- α and increased cytosolic level of phosphorylated I κ B- α . Meanwhile, the cytoplasmic levels of NF- κ B p65 also decreased in RAW264.7 cells, and it got accumulated in the nucleus. Interestingly, cellular pretreatment with DhHP-6 reversed these changes in a dose-dependent manner. Furthermore, immunofluorescence experiments were carried out to study the effect of DhHP-6 on the NF- κ B pathway. After LPS treatment, cytoplasmic p65 was translocated to the nucleus (Fig. 5B). However, the DhHP-6 treatment could inhibit the nuclear translocation of p65. These results suggest that DhHP-6 could attenuate inflammation by inhibiting the activation of the NF- κ B pathway.

3.6 DhHP-6 inhibits the exudation of WBCs and proteins in air pouch model rats

We found that compared to the sham group, the population of WBCs and protein contents in the exudates were significantly higher in the diseased model group ($p < 0.01$, Figs. 6A and B). At a high dose, DhHP-6 (2 mg/kg) significantly reduced the WBCs count and protein contents in the exudates (both having $p < 0.05$). A similar anti-inflammatory response was also observed in the dexamethasone-treated group ($p < 0.05$). In the low (0.2 mg/kg) and medium (0.6 mg/kg) dose DhHP-6 treated groups (0.2 and 0.6 mg/kg), the WBCs count and protein contents were not significantly different, compared to the model group (both $p > 0.05$). All these results indicate that high doses (2 mg/kg) of DhHP-6 may potentially inhibit the leucocytes and protein extravasation in response to inflammation.

3.7 DhHP-6 enhanced SOD activity and reduced MDA content in the plasma of air pouch model rats

The results (Figs. 7A and B) showed that compared to the sham group, SOD activities were significantly decreased, whereas MDA contents were increased in the rat's plasma of the diseased model group (both $p < 0.05$). However, compared to the model group, SOD activities increased significantly after treatment with a high (0.6 mg/kg) and medium (2.0 mg/kg) dose of DhHP-6 (both $p < 0.01$). However, low-dose DhHP-6 (0.2 mg/kg) showed no statistical difference ($p > 0.05$). Furthermore, compared with the model group, different doses of DhHP-6 (2.0, 0.6, and 0.2 mg/kg) significantly decreased the MDA contents in the plasma of air pouch model rats (compared with the model group, $p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively). All these results suggest a significant anti-oxidant effect of DhHP-6.

4. Discussion

The inflammatory response is a kind of host defense mechanism; however, an uncontrolled inflammatory response can lead to several chronic diseases [17, 18, 25, 19, 28]. Therefore, effective control of inflammation is essential to treat such diseases. Excessive accumulation of ROS can damage normal tissues further aggravating inflammatory reactions [10, 20, 14]. DhHP-6, a novel peroxidase mimetic peptide, can effectively scavenge ROS [32]. Previously, we showed the therapeutic effects of DhHP-6 in inflammation-related diseases; however, the mechanism remained unknown. Here, we examined the anti-inflammatory mechanism of DhHP-6.

Macrophages, one of the immune cells, produce major pro-inflammatory factors and play an indispensable role in inflammation. RAW264.7, a monocyte/macrophage-like cell line, is an established *in vitro* model for inflammation-related studies [8]. Endotoxins, such as LPS, are the key components of the cell wall of gram-negative bacteria [24]. LPS, from *Escherichia coli* O111:B4, is commonly used as an inducer to immune cells [4, 30], which binds to the toll-like receptors (TLRs) and initiates inflammation [34].

LPS stimulates the release of a variety of pro-inflammatory cytokines, such as TNF- α , IL-6, and NO [1]. We measured TNF- α , IL-6, and NO in the cell supernatant after LPS treatment with or without pretreatment with DhHP-6 and found that DhHP-6 reduced their levels. The production of NO is mainly regulated by iNOS [22], an inducible and Ca²⁺-independent isoform of NOS [29]. We tested the effect of DhHP-6 on iNOS expression in LPS-treated RAW264.7 cells and found that DhHP-6 could reduce iNOS levels.

LPS binds to the TLR4 receptor of macrophages, and thereby activates many intracellular inflammatory pathways. NF- κ B, an important nuclear transcription factor, promotes the transcription of inflammation-associated mediators, including iNOS, and pro-inflammatory factors such as TNF- α and IL-6; which contain NF- κ B binding motifs κ B DNA elements [33, 31, 9]. The prominent NF- κ B family members are p50, p52, Rel (p65), c-Rel, and RelB [9]. The p65-p50 heterodimer is the most common form of NF- κ B. Normally, the I κ B protein binds to the NF- κ B subunit to form a trimeric complex [13]. It keeps NF- κ B in the cytoplasm and inhibits its transport to the nucleus. However, under inflammation, I κ B kinase is activated to phosphorylate I κ B- α , triggering I κ B- α dissociation from NF- κ B. Furthermore, phosphorylation of I κ B- α leads to its ubiquitination at Lys 48 for proteasomal degradation. The inhibitory effect of I κ B on NF- κ B is relieved, and the cytoplasmic NF- κ B gets translocated to the nucleus to initiate the transcription of target genes [7]. NF- κ B signaling is modulated by posttranslational modifications, such as acetylation [26], phosphorylation [23], and methylation [2]. Oxidative stress is involved in the non-classical activation pathway of NF- κ B. ROS activate phosphatidylinositol 3-kinase (PI3K) which phosphorylates tyrosine 42 on I κ B [27]. Here, we showed that DhHP-6 pretreatment reduced the level of ROS and phosphorylation of I κ B, and promoted the level of I κ B in LPS stimulated RAW264.7 cells. Also, the nuclear transfer of NF- κ B and activation of NF- κ B signal pathway by LPS were inhibited.

To further verify the *in vivo* anti-inflammatory effect of DhHP-6, we established a murine inflammation model. Some animal inflammatory models are simulated by exogenous substances to activate immune cells. The subcutaneous air pouch is one of such models, which involves the subcutaneous injection of sterile air into the intra-scapular area of the back of the animal. This is then followed by an injection of irritants like carrageenan. Carrageenan, an acidic mucopolysaccharide, increases vascular permeability [5]. This inflammation model is convenient and the pathological changes include pouch lining of macrophage and fibroblast-like cells, increase in exudate. The cellular infiltration is extremely similar to human acute intra-articular synovitis. Therefore, we chose this model to explore the anti-inflammatory effects of DhHP-6. We showed that DhHP-6 exhibited anti-inflammatory activity, and inhibited the exudation of WBCs and protein in the air pouch mice model.

SOD is an important anti-oxidase that clears the superoxide anion ($O_2^{\cdot-}$) into hydrogen peroxide and molecular oxygen. The cellular level of MDA, the end product of lipid peroxidation, signifies *in vivo* oxidation status and is considered as an oxidative stress marker. Here, we showed that the peroxidase mimetic DhHP-6 (2.0 and 0.6 mg/kg) significantly increased the SOD activity ($p < 0.01$) while decreased the plasma content of MDA ($p < 0.01$). Overall, these results showed that DhHP-6 exerts a good antioxidant effect in the carrageenan-induced gasbag-synovitis model in rats.

In conclusion, we showed that DhHP-6 exhibited anti-inflammatory effects in both LPS induced RAW264.7 cells and the air pouch rat model. DhHP-6 may serve as a potential therapeutic agent for the treatment of acute and chronic inflammatory diseases.

Declarations

ETHICS APPROVAL

The study was approved by the Animal Care Committee of Jilin University and conformed to the Animal Ethical Standards and Use Committee at Jilin University.

CONSENT TO PARTICIPATE

Not Applicable

CONSENT FOR PUBLICATION

The participants have consented to the submission of this research to the journal.

AVAILABILITY OF DATA AND MATERIAL

We make sure that all data and materials support the published claims and comply with field standards. All data and materials are fully available.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. Material preparation, data collection, and analysis were performed by Fanwei Meng, Junfeng Ke, Jia Xu, and Jinze Li. The first draft of the manuscript was written by Fanwei Meng and Jia Xu. All authors helped with the revisions of the manuscript and approved the final manuscript.

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Figures

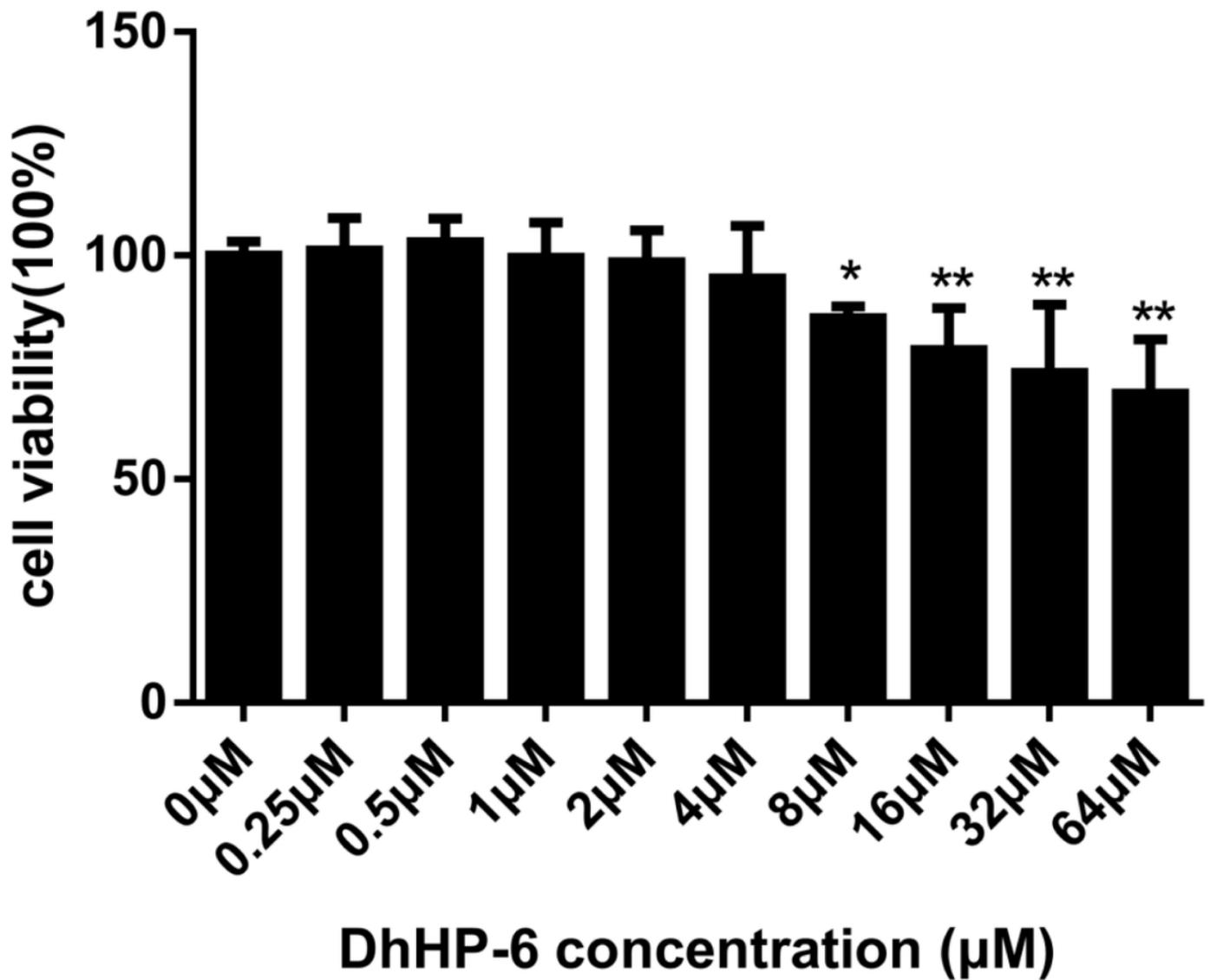


Figure 1

Effect of various concentrations of DhHP-6 on the viability of RAW264.7 cells. The cells were exposed to different concentrations of DhHP-6. The cytotoxicity of DhHP-6 was determined by WST1 assay after 24 h of treatment. P < 0.05 vs. NC group, **p < 0.01 vs. NC group.

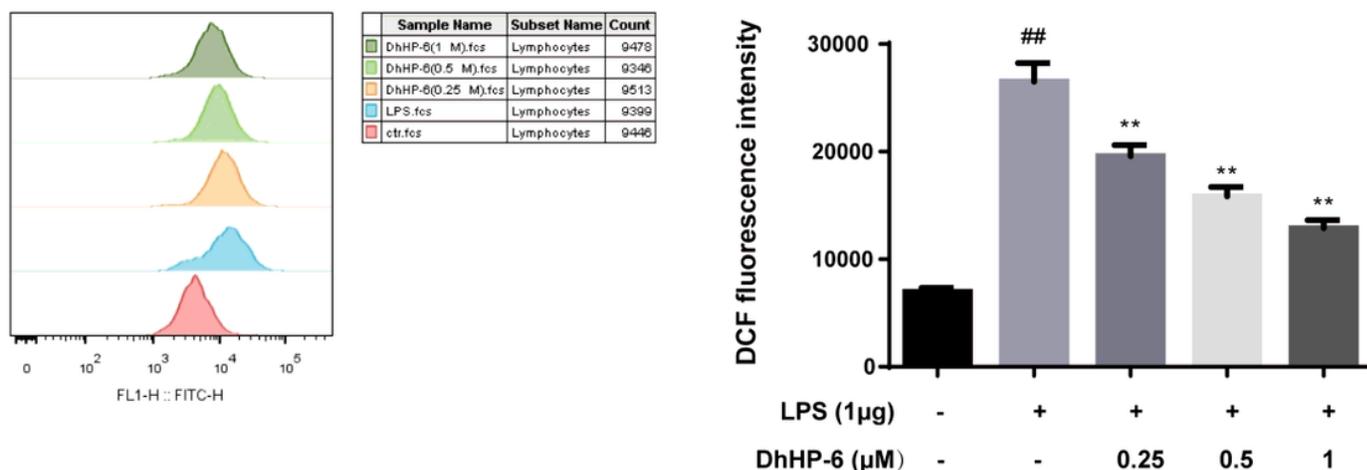


Figure 2

DhHP-6 inhibits LPS-induced ROS accumulation in RAW264.7 cells. The cells were treated with DhHP-6 (0.25, 0.5, and 1 μM) for 2 h before exposure to LPS (1 μg/mL) for 12 h. Then, the cellular ROS levels were measured by ROS assay kit. The mean fluorescence intensity of FITC in the different treated cell groups was measured by flow cytometry. ##p < 0.01 vs. Control group, *p < 0.05 vs. LPS group, **p < 0.01 vs. LPS group.

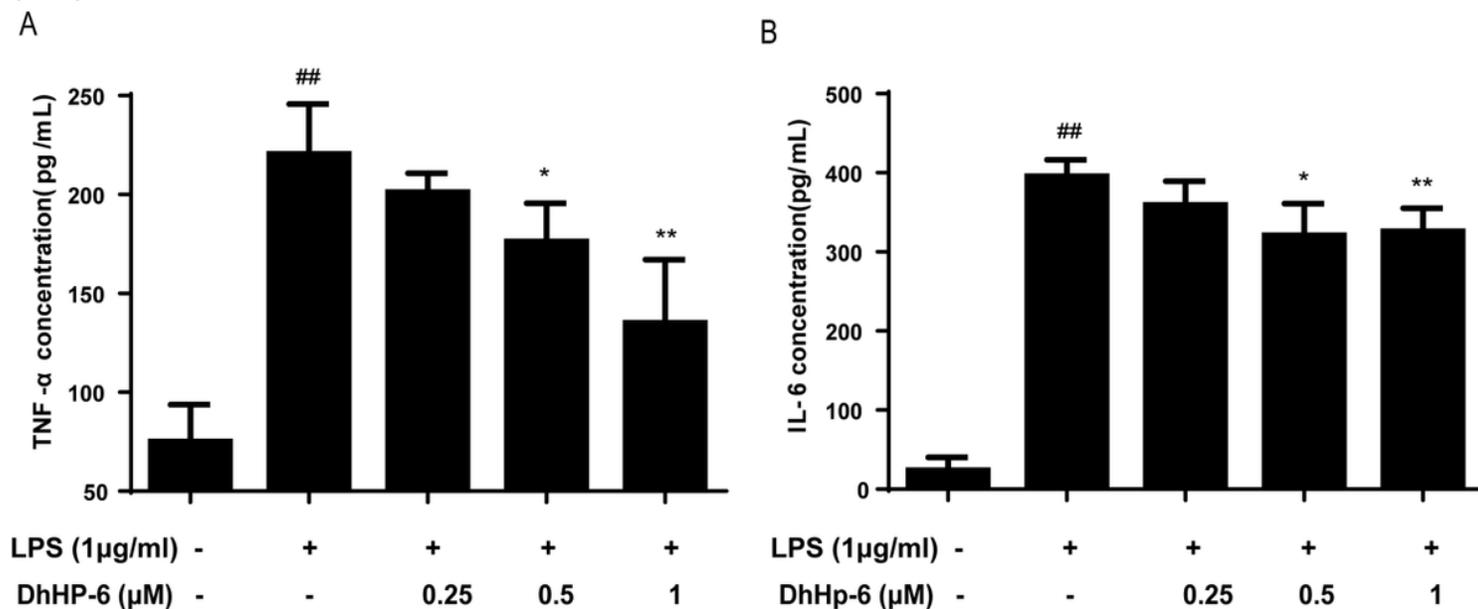
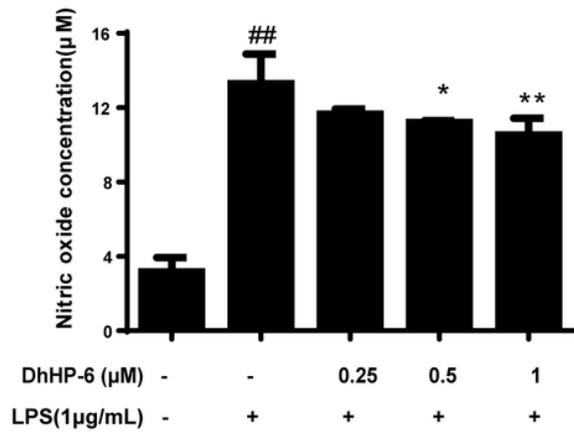


Figure 3

DhHP-6 reduces LPS-induced expression of pro-inflammatory cytokines in RAW264.7 cells. After different treatments, cell-secreted TNF-α, and IL-6 were measured by ELISA. TNF-α (A), and IL-6 (B) in the LPS group were significantly higher than in control groups; however, DhHP-6 could reduce them. All data are mean ± standard deviation (n=8). ##p < 0.01 vs. Control group, *p < 0.05 vs. LPS group, **p < 0.01 vs. LPS group.

A



B

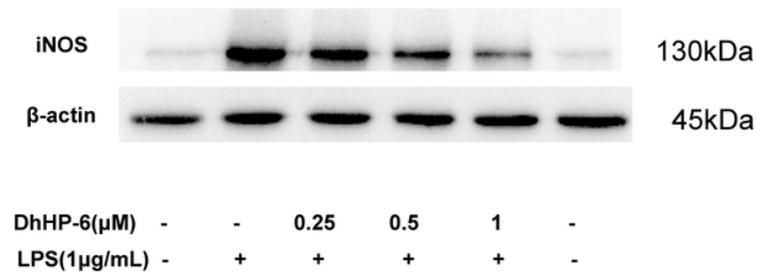
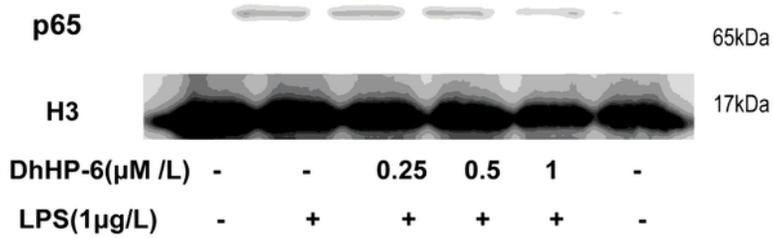
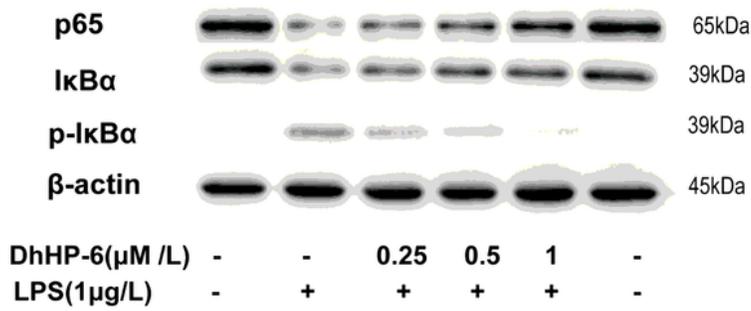


Figure 4

DhHP-6 reduces LPS-induced production of NO and expression of iNOS. (A) RAW264.7 cells were treated with DhHP-6 (0.25, 0.5, and 1 µM) for 2 h, followed by stimulation with LPS (1 µg/mL) for 24 h. The medium was collected to determine the NO level using the NO detection kit. (B) Likewise, iNOS expression was detected by Western blotting.

A



B

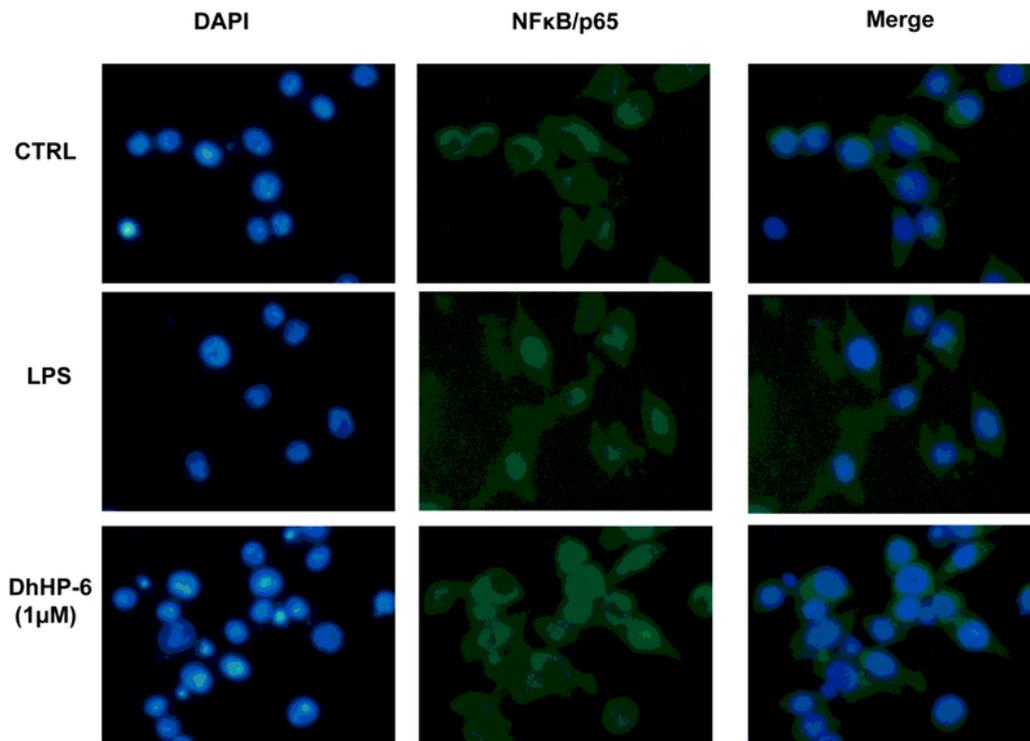
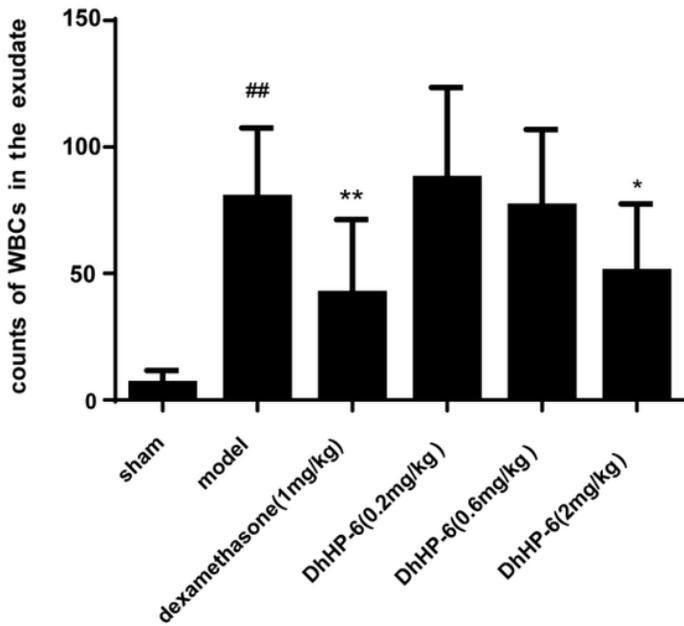


Figure 5

DhHP-6 inhibits LPS-stimulated nuclear translocation of NF-κB. (A) RAW264.7 cells were treated with DhHP-6 (0.25, 0.5, and 1 μM) for 2 h, followed by stimulation with LPS (1 μg/mL) for 12 h. The protein levels of P65, phosphorylated IκB in the cytoplasm, and p65 in the nucleus were detected by Western blotting. (B) p65 translocation was determined using immunofluorescence analysis (magnification, ×400).

A



B

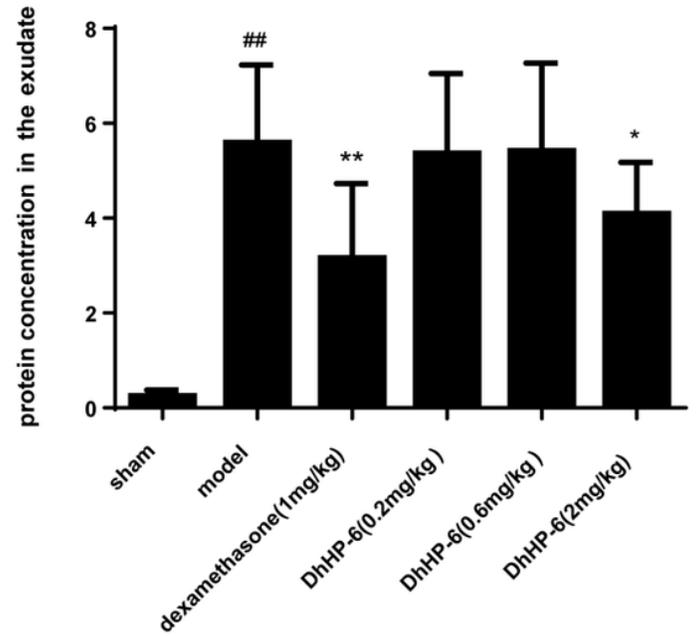
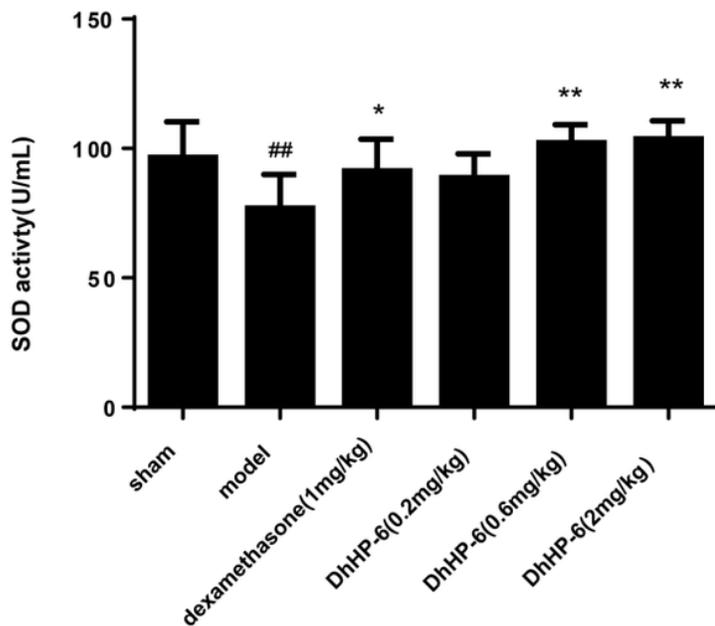


Figure 6

DhHP-6 inhibited the exudation of WBCs and protein in gasbag-synovitis-induced rats. The exudates were collected as described in the method section. WBCs count was measured in the lavage solutions (A) and total protein was measured by BCA protein kit (B). All data are mean \pm standard deviation (n=8). ##p < 0.01 vs. Control group, *p < 0.05 vs. LPS group, **p < 0.01 vs. LPS group.

A



B

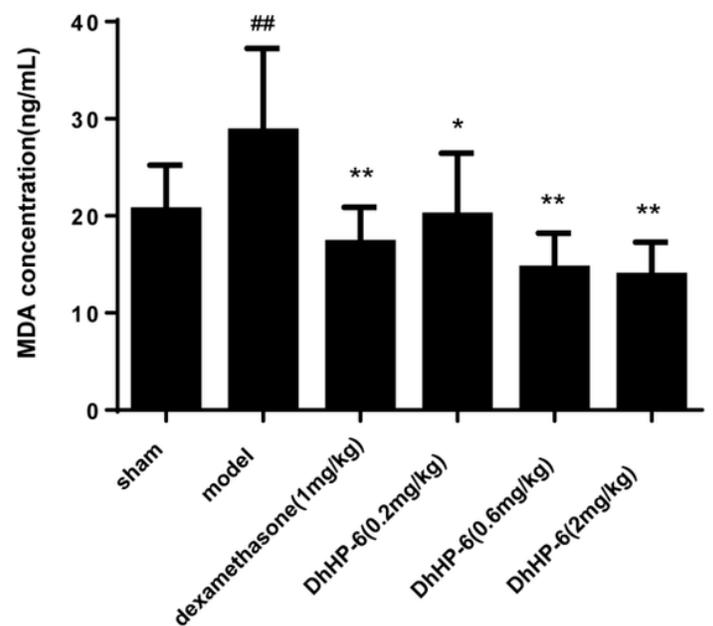


Figure 7

DhHP-6 enhanced SOD activities and reduced plasma MDA content in gasbag-synovitis-induced rats. The rat's plasma was collected to measure SOD activity (A) and MDA content. All data are mean \pm standard deviation (n=8). ##P < 0.01 vs. Control group, *P < group, **p < 0.01 vs. LPS group.