Anti-inflammatory effect of hispidin on LPS induced macrophage inflammation through MAPK and JAK1/STAT3 signaling pathways

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Abstract
Severe inflammatory reactions caused by macrophage activation can trigger a systemic immune response. In the present study, we observed the anti-inflammatory properties of hispidin on LPS induced RAW264.7 macrophage cells. Our results showed that hispidin treatment significantly reduced the production of cellular NO, IL-6 and reactive oxygen species (ROS) while has not inhibitory effect on TNF-α productions. Excitingly, hispidin treatment retains the phagocytosis ability of macrophages which enabling them to perform the function of removing foreign invaders. Signaling studies showed, hispidin treatment dramatic suppressed the LPS induced mitogen activated protein kinases (MAPK) and JAK/STAT activations. In conclusion, our findings suggest that hispidin may be a new therapeutic target for clinical treatment of macrophages-mediated inflammatory responses.

Keywords: Hispidin, LPS, Macrophage, ROS, Signal transduction

Introduction
Inflammation is an important component of the innate immune response and can be caused by a variety of irritants, including invasive pathogenic microorganisms, harmful mechanical and chemical agents, cell fragmentation, and autoimmune responses [1]. On the other hand, the inflammatory response can increase microvascular caliber and vascular permeability, recruit white blood cells and release inflammatory mediators [2]. Although the inflammatory response is initially occurs to protect the bodies from the damages, but the extensive studies have indicated that long-term and excessive inflammation is associated with various clinical manifestations such as arthritis [3], cardiovascular complications [4] and tumor progression [5]. Though the non-steroidal anti-inflammatory drugs (NSAIDs) are widely used as anti-inflammatory drugs [6], but long-term treatments can cause many adverse reactions in the body. Studies have shown that gastrointestinal bleeding and other complications, including peptic ulcers, are associated with long-term use of NSAIDs [7, 8].

Lipopolysaccharide (LPS), as a component of cell wall of Gram-negative bacteria, is one of the strongest inflammatory factors found in current research and is widely used in the induction of inflammation in animal and cell experiments [9–11]. Macrophages activated by LPS mainly involve three signaling pathways including...
Shao et al. has also demonstrated that hispidin can inhibit cemic regulatory functions [27–29]. Recently, Hong Jun Gymnopilus spectabilis showed anti-the fruiting body of Fomitiporia ellipsoidea [24–26]. Hispidin isolated from sp., fermentation products and the fruiting bodies of syndrome (SIRS). Inflammatory cytokines and mediators is an important efficient inhibits the synthesis or release of these pro-inflammatory mediators results in increasing the tissue and cellular damages which can promote severe inflammatory responses in vivo and in vitro [16, 17]. Therefore, efficiently inhibits the synthesis or release of these pro-inflammatory cytokines and mediators is an important strategy for the treatment of systemic immune response syndrome (SIRS).

The “Sanghuang” are well-known traditional medicinal fungi which are commonly used in East Asian countries [18]. It was reported that “Sanghuang” or its extracts have anti-oxidant, anti-bacterial, anti-tumor, regulate blood sugar, improve immune response and blood lipid functions in clinical treatments [19–21]. A paper published in Nature in 2019 showed that during the optimization of the “Sanghuang” strain, the obtained A67 strain had stronger antioxidant activity and inhibited tumor cell proliferation than the SH1 strain before optimization. Exploring the reasons for this, the author found that A67 contains more polyphenols than SH1, among which hispidin is the most abundant [22]. Hispidin, a polyphenolic substance, identified as 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyranone by Edwards in 1961, was first isolated from the fruiting body of I. hispidus by Zopf (1889) [23]. Subsequently, the researchers isolated hispidin and its derivatives from the ethanol extract of the fruiting bodies of Inonotus sp., I. xeronicus, P. linteus and Phellinus sp., fermentation products and the fruiting bodies of Fomitiporia ellipsoidea [24–26]. Hispidin isolated from the fruiting body of Gymnopilus spectabilis showed antioxidant activities, such as scavenging superoxide radicals. It's antioxidant activity was about 3–5 times that of water-soluble vitamin E, which is equivalent to that of BHA and caffeic acid [24]. It has also been reported that hispidin has anti-cancer, anti-bacterial and hypoglycemic regulatory functions [27–29]. Recently, Hong Jun Shao et al. has also demonstrated that hispidin can inhibit the macrophage mediated inflammatory response by down-regulating the NF-κB activations [30], however, the effect of hispidin on LPS induced macrophage ROS levels, phagocytosis as well as cytokines productions and its molecular signaling mechanisms has remained unclear.

In the present study, we investigated the inhibitory effect of hispidin on the release of inflammatory mediators, cellular ROS level and phagocytic function in RAW264.7 macrophages stimulated by LPS and explore the possible intracellular signaling regulated by hispidin on LPS induced macrophages.

Materials and methods
Reagents and chemical
Lipopoly saccharides (LPS) and hispidin were both purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). The fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (General Electric Healthcare Life Sciences, Mississauga, Canada), penicillin streptomycin (P/S) was purchased from solarbio (Solarbio life sciences, Beijing, P. R. China).

Cell culture
The RAW264.7 macrophage cells (Shanghai BOGO Industrial Co., Ltd., Shanghai, China), was cultivated in DMEM supplemented with 10% FBS and 1% P/S (100 U/ml and 100 mg/ml, respectively). In the logarithmic growth phase, RAW264.7 macrophage cells maintained in DMEM at 37 °C and 5% CO2. After pretreatment with various concentrations of hispidin they were treated with 1 μg/ml LPS for the indicated time.

Cell viability assay
RAW264.7 macrophage cells were seeded into 96 well plates at a concentration of 4 × 103 cells per well, with different concentrations (0 μg/ml to 20 μg/ml) of hispidin treated 24 h. Subsequently, a solution of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich,) was added to each well and then incubated in an incubator (37 °C, 5% CO2) for 4 h. After incubating 4 h, remove the supernatant and add DMSO to dissolve formazan. Finally, use a UV MAX kinetic microplate reader (Molecular Devices, LLC) to measure the absorbance at 490 nm.

Griess method detect the production of NO
The production of NO is reflected by the color rendering degree of nitrite generated after the reaction of culture medium with Griess reagent. Collect the culture supernatants and mix it with an equal volume of Griess reagent [0.1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 2.5% H3PO4]. Use a UV MAX kinetic microplate reader (Molecular Devices, LLC) to measure the absorbance at 540 nm.

Western blotting
Harvested cells were lysed with protein lysis buffers (20 mM HEPES-OH, pH 7.0; 50 mM NaCl; 10% glycerol and 0.5% Triton X 100) and extracted the total proteins. Then, the total proteins were denatured for 5 min and using 12% sodium dodecyl sulfate-polyacrylamide gel
electrophoresis to separate it and transferred into nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Using 5% skimmed milk to block the membranes for 30 min, at room temperature. They were then incubated with polyclonal rabbit anti-iNOS, anti IκB-α, anti β-actin; mouse monoclonal anti-p-P38, anti p-JNK, anti p-ERK, anti P38, anti JNK, anti ERK primary antibodies (dilution, 1:1000) at 4 °C overnight. The membranes were washed five times with TBST (Tris buffered saline (TBS) containing Tween [150 mM NaCl, 10 mM Tris HCl (pH 7.5) and 0.2% Tween-20]) and were subsequently incubated with horseradish peroxidase conjugated goat anti mouse IgG or anti rabbit IgG for 1 h at room temperature. After removing the excess antibodies by washing with TBST, specific conjugates were detected using a chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK) according to the manufacturer's protocol.

Cytokine assays
The concentration of IL-6 and TNF-α in the cell culture supernatants were measured by using enzyme-linked immunosorbet assay (ELISA) kits for IL-6 and TNF-α. RAW264.7 macrophage cells (2 × 10⁵ cells) were plated into a 48-well plates and incubated with different concentrations of hispidin (0 μg/ml to 20 μg/ml) and 1 μg/ml LPS for 24 h. In order to determine the concentration IL-6 of and TNF-α that had been released from the cells, the culture supernatants were collected and measured according to the manufacturer’s protocol.

Flow cytometry measure the phagocytosis and ROS
Analysis the phagocytosis of macrophages by flow cytometry according to previously reported methods [31]. Alexa 488 conjugated BioParticles (Invitrogen; Thermo Fisher Scientific) were added to serum-free medium, sonicated, and the cells were incubated at 37 °C for 15 min. Subsequently, the cells were washed three times with phosphate-buffered saline (PBS) and resuspended in 500 μl PBS. The internalized fluorescence from 10,000 cells was immediately detected by using a FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). In order to detect ROS levels, cells were incubated with 10 mM DCFH-DA (Invitrogen; Thermo Fisher Scientific, Waltham, USA), a fluorescent indicator of ROS, which was incubated at 37 °C for 15 min, and DCF-DA fluorescence intensity from 10,000 cells was measured using FACScan (BD Biosciences). The results were analyzed using WinMDI (Version 2.9, BD Biosciences) software.

Statistical analysis
The data are presented as the mean standard error of the mean. Differences between experimental groups were analyzed by one-way analysis of variance and a Tukey test. GraphPad Prism software version 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze results. P < 0.05 was considered to indicate a statistically significant difference.

Results
Hispdin inhibits the production of NO in LPS-treated RAW264.7 macrophage cells
The chemical structure of hispidin is presented in Fig. 1a. To ensure the effects of hispidin on macrophage cell viability, RAW264.7 macrophage cells were treated with hispidin at various concentrations (0, 1, 5 10 and 20 μg/ml) for 24 h. As demonstrated in Fig. 1b, hispidin has no effect on macrophage cell viability. To clarify whether hispidin has anti-inflammatory properties, the NO levels in RAW264.7 macrophage cell culture supernatants were examined. The results showed that treatment with hispidin could dose- and time-dependently decrease the production of NO in LPS-treated RAW264.7 macrophage cells (Fig. 1c, d). Furthermore, the expression level of iNOS (an enzyme involved in NO production) protein was also observed with western blotting. As shown in Fig. 1e–h, hispidin treatment significantly down-regulated the LPS induced iNOS protein expression in a dose- and time-dependent manner.

Effect of hispidin on LPS induced production of IL-6 and TNF-α in RAW264.7 macrophage cells
It was well known that TNF-α and IL-6 are the major cytokines which produced by macrophage cells response to LPS stimulations. To verify whether the hispidin could influence the secretion of pro-inflammatory cytokines in LPS-treated RAW264.7 macrophage cells, the content of cytokines which produced by macrophage cells response to LPS stimulations. To verify whether the hispidin could influence the secretion of pro-inflammatory cytokines in LPS-treated RAW264.7 macrophage cells was examined. The results showed that treatment with hispidin had no significant effect on LPS induced TNF-α production, though there has a slight inhibitory effect at 6 h (Fig. 2c, d).

Effect of hispidin on LPS induced cellular ROS level and phagocytosis in RAW264.7 macrophage cells
ROS has been proved to be an important signaling factor in LPS-treated macrophages. LPS-treated macrophages produce a large amount of ROS, and activate various signaling pathways to induce macrophage over-activation [32, 33]. To examine whether hispidin affects the ROS levels in LPS-treated macrophages, RAW264.7 macrophage cells were pre-treated with hispidin (20 μg/ml) for 30 min, followed by treatment with LPS (1 μg/
ml) for 24 h. Cellular ROS levels were examined by flow cytometry and fluorescence microscopy. The results showed that LPS treatment upregulated the level of ROS in RAW264.7 macrophage cells, which were significantly down-regulated by pre-treatment with hispidin (Fig. 3a–c). Phagocytosis, an important marker of macrophage activation, was also detected by hispidin treatment. The results showed that LPS treatment significantly increased the phagocytosis of RAW264.7 macrophage cells, but unfortunately, hispidin treatment could not affect the LPS induced macrophage phagocytosis (Fig. 3d–f).

**Hispidin exerts anti-inflammatory effects by suppresses LPS-induced MAPK and JAK1-STAT3 signaling pathways in RAW264.7 macrophage cells**

The NF-κB, MAPK and JAK1/STAT3 signaling pathways are crucial mediators of pro-inflammatory cytokines production in LPS-treated macrophages [14, 34–36]. To better define the underlying mechanism of anti-inflammatory effect of hispidin in LPS-treated RAW264.7 macrophage cell, the above three signaling pathways were detected. The results revealed that phosphorylation levels of ERK, JNK, and p38 were dramatic up-regulated by LPS treatments which were significantly inhibited by hispidin treatment in RAW264.7 macrophage cells (Fig. 4a–d). Furthermore, the effect of hispidin on JAK/STAT signaling pathways were also examined in LPS induced RAW264.7 macrophage cells. The results revealed that hispidin treatment significantly down-regulated LPS induced JAK1 and STAT3 phosphorylation levels in RAW264.7 cells (Fig. 4e–g). These results comprehensively illustrates that hispidin inhibits LPS-mediated macrophage inflammatory response by extensively inhibiting intracellular signaling pathways.

**Discussion**

Inflammatory response plays a key role in the occurrence and development of various diseases, while macrophage is a target cell for regulating inflammatory related diseases. When stimulated by LPS, macrophage can activate...
related signal pathways NF-κB, MAPKs, JAK/STAT and then secrete a mass of NO, prostaglandin 2, and pro-inflammatory cytokines, such as IL-6, TNF-α, IL-1β [15].

Nitric oxide (NO) is a highly active diatomic molecule, which is catalyzed by NO synthase to produce L-arginine in mammalian cells. As the second messenger of intracellular signal transduction, nitric oxide has a wide range of pathophysiological functions in the regulation of cardiovascular, digestive, immune and respiratory systems. Nitric oxide synthase includes structural constitutive nitric oxides synthase (cNOS) and inducible nitric oxide synthase (iNOS). iNOS is almost non-expressed in normal cells, but only in inflammation, hypoxia, tumor and other pathological states, followed by a large number of catalytic induced NO generations in a short period of time [37]. When macrophages are stimulated by substances such as LPS, the expression of iNOS is increased which in turn induces the secretion of large amounts of NO and promote the development of diseases such as inflammation [38]. In the process of inflammation, the pro-inflammatory cytokines, such as IL-6 and TNF-α plays an important role in the pathogenesis of many diseases [17]. Therefore, effectively suppressing the secretion of NO and pro-inflammatory cytokines is one of the important strategies to control the inflammatory response. In the present study, treatment with hispidin could significantly decreased the production of NO and IL-6 as well as iNOS protein expression level in LPS-induced RAW264.7 macrophages cells, but not TNF-α (Figs. 1, 2). Our results strongly suggests that hispidin could play an essential roles in inhibiting the inflammatory response processed upon LPS stimulation. The different effects of compounds on inhibitory functions on TNF-α and IL-6 productions have been reported in many researches. Our previous studies have also reported that 16α, 17α-epoxypregnenolone-20-oxime could inhibit the LPS induced NO and IL-6 productions but not TNF-α in BV2 microglia and RAW264.7 macrophage cells [39, 40].

Fig. 2 Effect of Hispidin on LPS induced TNF-α and IL-6 production in RAW264.7 macrophage cells. Cells were pre-treated with 20 µg/ml Hispidin for 30 min, followed by treatment with LPS (1 µg/ml) for the indicated durations. a, b IL-6 and c, d TNF-α productions were measured in the culture media by enzyme-linked immunosorbent assay (ELISA). Data are presented as the mean ± standard error of the mean for three different samples. *P < 0.05, and ***P < 0.001.
Furthermore, it was also reported that knockdown of Peroxiredoxin I could efficiently affect the expression of IL-10 but not IL-1β and TNF-α in LPS induced RAW264.7 macrophage cells [41]. These findings suggest that there have different regulatory mechanisms on cytokines production upon LPS stimulation in macrophage cells, but possible mechanisms should be further studied.

Furthermore, the inhibitory effect of hispidin on cellular ROS level in LPS treated macrophages was also showed in our results (Fig. 3). It is well known that macrophages are activated with the increase of ROS and causes oxidative stress and amplify the inflammatory response [42]. It has been reported that exposure to ozone could up-regulate the expression of inflammatory mediator, cause lung inflammation; increase MDA level and GSH-PX activity; induce oxidative stress in the lung tissue; results in leading to worsening of asthma [43]. Our results about the inhibitory effect hispidin on ROS productions deeply demonstrates that the hispidin can be used as a potential anti-inflammatory drug candidate for treatment to LPS induced immune responses. When exogenous microbial pathogens invade the body, in order to eliminate them, macrophages will greatly improve their phagocytic ability after being activated. However, we examined the effect of hispidin on the phagocytic capacity of macrophages induced by LPS and found that hispidin had no effect on the phagocytic capacity of macrophages (Fig. 3d–f). The phenomenon that hispidin only inhibits the inflammatory response induced by LPS without affecting the phagocytosis capacity has aroused our interest, even though we have not studied the phenomenon in this study. After consulting the paper, we learned that when studying the reason that the Gram-positive bacteria Listeria monocytogenes and macrophages can coexist, it was found that when the bacteria infects J774 cells, the PKC family in the cells can translocate, resulting in less bacteria entering into the macrophages and escaping from the early phagocytes of macrophages. Hispidin, a PKC inhibitor [44], can inhibit the transfer of PKC β, allowing macrophages to engulf more bacteria and reduce the escape of bacteria from early phagosomes [45]. This suggests that whether hispidin does not affect the phagocytosis of LPS induced macrophages is also due...
to its ability to inhibit PKC in macrophages, which will be proved in subsequent experiments.

Sum up, our results also revealed that hispidin has the characteristics of high efficiency and low cytotoxicity in macrophage cells (Figs. 1, 2, 3, 4), therefore, we strongly believe that hispidin can be used as a candidate drug to inhibit inflammatory response and it can be widely used in future researches.

The major signaling pathways involved in macrophage activation are NF-κB, MAPKs and JAK/STAT. In many studies of anti-inflammatory drugs, many of them can exert their anti-inflammatory effects by inhibiting the activation of the these signaling pathways [14, 34–36, 46]. Recently, Hong Jun Shao et al. reported that hispidin could inhibit the activation of NF-κB in LPS-induced macrophages which results in suppressed the production of cytokines productions [30] suggests the anti-inflammatory properties of hispidin on LPS stimulated macrophage cells, but the molecular mechanisms remains unknown.

MAPKs are serine/threonine protein kinases, which mainly include c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase (ERK), and p38 MAPK [47]. ERK regulates cell proliferation and differentiation, JNK is involved in stress response, p38 is closely related to inflammation, apoptosis and other processes. MAPKs, which are relatively independent but interconnected between different signals, transmit signals from the outside of the cell to the inside through a three-stage kinase cascade reaction activated by MAPK kinase (MKKK), MAPK kinase (MKK), and MAPK [48]. MAPKs play an important regulatory roles in the expression and secretion of various inflammatory mediator, especially in the macrophages activated by LPS, which can produce ROS dependence phosphorylation [49–51]. In the present study, Hispidin can significantly inhibit the phosphorylation of P38, ERK, and JNK (Fig. 4a–d) suggest that the inhibitory effect of Hispidin on LPS induced macrophage activations were relayed on suppressing the MAPK signaling pathway which were known as ROS-dependent activated signaling cascades.

Cytokines signal transduction is mainly through mediating the JAK/STAT signaling pathway [52]. Cytokines bind to receptors on cell membrane, causing JAK to be phosphorylated which in turn phosphorylates downstream of STAT, eventually inducing a transcription response and increasing gene expression of related inflammatory mediators [53]. In the present study, we detected the phosphorylation of JAK1 and STAT3, and found that hispidin can slightly inhibit the phosphorylation of JAK1 and STAT3. Our results indicates that hispidin treatment can also reduce the production of inflammatory mediators by inhibiting the activation of JAK1/STAT3 (Fig. 4).
Taken together, our studies suggest that hispidin exerts anti-inflammatory properties by attenuating the production of NO, IL-6 and intracellular ROS levels in LPS-induced macrophages by inhibiting the activation of MAPKs and JAK1/STAT3 signaling pathways. Excitingly, hispidin treatment retains the phagocytosis ability of macrophages, enabling them to perform the function of removing foreign invaders. Our findings may provide a new sight of hispidin as a therapeutic target for clinical treatment of macrophages-mediated inflammatory responses.

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Authors’ contributions
Data curation: D-QC, Y-HJ, W-LL, Y-XG, YYM and D-PX. Formal analysis: Y-HH, M-HJ, G-NS, D-SL, S-JK and J-SK. Funding acquisition: JL, L-YY and TK. Supervision: Y-HH, Y-DC and H-NS. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

Competing interests
The authors declare that they have no competing interests.

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