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Anti-inflammatory effects of silkworm hemolymph on lipopolysaccharide-stimulated macrophages

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Abstract−Macrophages participate in several inflammatory pathologies such as sepsis and arthritis. We investigated the effect of silkworm hemolymph (SH) on the LPS-induced pro-inflammatory macrophages. SH inhibits LPS-induced nitric oxide (NO) production in RAW 264.7 cells and murine peritoneal macrophages. The decreased NO was reflected as a decreased amount of inducible nitric oxide synthase (iNOS) mRNA and protein. It was also found that SH inhibited pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α production. To elucidate the mechanism by which SH inhibits NO production and iNOS expression, we investigated that SH suppressed IκB phosphorylation, which leads to the activation of NF-κB followed by degradation of IκB. This observation suggests that SH is a potential therapeutic modulator for inflammation-associated disorders.

Key words: Macrophages, Silkworm Hemolymph, Nitric Oxide (NO), Inducible Nitric Oxide Synthase (iNOS), Pro-inflammatory Cytokines

INTRODUCTION

The innate immune response is the first line of defense against microbial infection and lies behind most inflammatory responses, which are triggered in the first instance by macrophages, polymorphonuclear leukocytes, and mast cells through their innate immune receptors [1]. Lipopolysaccharide (LPS) is an outer membrane glycolipid of gram-negative bacteria and a well-known inducer of the innate immune response [2]. Macrophages respond to LPS early and play a crucial role in the innate immunity. LPS-activated macrophages produce large amounts of inflammatory agents such as IL-1β, IL-6, and TNF-α, which serve as endogenous mediators of inflammation. LPS-activated macrophages also release nitric oxide (NO), which is produced by inducible nitric oxide synthase (iNOS). Engagement of LPS on the host cell initiates strong pro-inflammatory responses that stimulate host defenses but can lead to a pathological condition, septic syndrome, if the inflammatory responses are amplified and uncontrolled [3,4].

Animal models of septic shock showed that IFN-γ, TNF-α, and IL-10 are related to the regulation of LPS-induced NO release [5]. Analyses of cytokine mRNAs and proteins revealed that many pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and GM-CSF are induced in rheumatoid arthritis tissue [6]. Moreover, the expression of iNOS is regulated by pathways that involve MAPKs and NF-κB in macrophages [7]. NF-κB is a transcription factor that modulates the expression of a variety of genes involved in immune and inflammatory responses, including iNOS and TNF-α [8]. NF-κB is an important intermediary of LPS-induced signal transduction in macrophages.

Silkworm hemolymph (SH), which was used as a substitute for fetal bovine serum (FBS) [9], increased the expression of recombinant protein and the longevity of host cells [10,11], and the anti-apoptotic effect of SH was also reported in insect, mammalian, and human cell systems [12-14]. 30 K proteins are the anti-apoptotic protein in SH [15], and the potential applications of the 30 K proteins and their genes were also investigated to increase the productivity of recombinant proteins in mammalian cell culture system by inhibiting host cell apoptosis [16-21]. In addition to anti-apoptotic activity, SH recently improved the glycosylation pattern in recombinant N-linked glycoprotein and secreted human placental alkaline phosphatase (SEAP) [22,23]. In this study, we investigated the anti-inflammatory effect of SH in LPS-stimulated macrophages which participate in several inflammatory pathologies such as sepsis and arthritis. We herein report evidence that SH inhibits the iNOS and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) gene expressions in RAW 264.7 cells and peritoneal macrophages after LPS treatment.

MATERIALS AND METHODS

1. Cells and Reagents

Cells of the murine monocyte/macrophage cell line, Raw 264.7,

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were maintained in DMEM (Gibco-BRL; Gaithersburg, MD) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 1 mM sodium pyruvate, 2 mM glutamine, and 5 mM HEPES (complete DMEM). Peritoneal macrophages were harvested from 6- to 8-week-old female BALB/c mice (Samtako, Osan, Korea) which were injected with 10 mL of pre-warmed complete DMEM. After centrifugation and washing, cells were resuspended in complete DMEM, and aliquots of 5×10^5 cells in 2 mL of medium were cultured in 6-well plates (NUNC, Roskile, Denmark). LPS from *Escherichia coli* 0127:B8 was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in PBS. N^G-monomethyl-L-arginine monoacetate salt (NMMA) was purchased from Calbiochem (San Diego, CA).

2. Collection of Silkworm Hemolymph

Silkworms (*Bombyx mori*) were a kind gift from the Sericulture & Insect Research Institute (Suwon, Korea). The silkworm hemolymph was collected from fifth instar larvae by clipping the side of an abdominal leg. The collected hemolymph was heat-treated at 60 °C for 30 min, then chilled and centrifuged. The supernatant was filtered with a 0.2 μm membrane filter and used for supplementing the medium. To assess the effect of SH, 5% SH (v/v medium) was added to 5% FBS containing medium based on our previous reports [9, 10]. 10% FBS containing medium was used as control. 5% SH was replaced with 5% FBS to both maximize the effect of SH on inflammation and minimize the side effects such as growth inhibition caused by serum shortage.

3. Nitrite Assay

RAW 264.7 cells in six well plates were stimulated with LPS and supernatants were collected for analysis of nitrite using the Griess reagent. Briefly, 100 μL of 1 : 1 mixture of 0.1% naphthylethylenediamine in water and 1% sulfanilamide in 5% phosphoric acid was added to 100 μL medium and incubated for 10 min at room temperature. Absorbance at 540 nm was determined with a microplate reader (Bio-Rad, Hercules, CA) using a standard curve of nitrite solutions in culture medium.

4. Semiquantitative Reverse Transcription-polymerase Chain Reaction (RT-PCR)

The RT reaction was performed using the reverse transcription system (Promega, Madison,WI). The RT reaction was carried out at 42 °C for 1 h, followed by 5 min at 95 °C. The PCR reactions were performed by adding 10 μL of cDNA to 10 μL of the reaction mixture containing $MgCl_2$ 3.125 mM, Tris-HCl 12.5 mM, PH 8.3, 1.563 units of *Taq* DNA polymerase, and iNOS or beta-Actin-primers 10 pmol. The specific primers were: 5′-ACG GAG AAG CTT AGA TCT GGA GCA GAA GTG-3′ and 5′-CTG CAG GTT GGA CCA CTG GAT CCT GCC GAT-3′ for iNOS; 5′-TCC TTC GTT GCC GGT CCA CA-3′ and 5′-CGT CTC CGG AGT CCA TCA CA-3′ for beta-Actin. The conditions for PCR were: 94 °C for 2 min, 30 cycles of 94 °C for 1 min (denaturing), 55 °C for 2 min (annealing), and 72 °C for 1 min (extension) for amplifying 654-bp (iNOS) and 508-bp (beta-Actin) products. Finally, the extension of products was separated on 1.0% agarose gel, visualized using ethidium bromide, and then photographed with a molecular imager Gel Doc SR System (Bio-Rad, Hercules, CA).

5. Western Blotting

Whole cell extracts were prepared by incubation with RIPA solution (50 mM Tris, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.5% SDS, 1 mM PMSF, and 1× protease inhibitor cocktail from Sigma-Aldrich) on ice for 20 min. Protein was quantified by the Bradford method. Equal amounts of protein were subjected to electrophoresis on a 10% SDS PAGE gel and blotted onto a PVDF membrane (Millipore; Billerica, MA) using a semi-dry transferring apparatus (Bio-Rad; Hercules, CA). Transferred blots were blocked with 5% bovine serum albumin fraction V (Amresco; Solon, OH) in TTBS (10 mM Tris, 100 mM NaCl, and 0.05% Tween 20) overnight and incubated with indicated primary Ab at 4 °C for 6 h. After incubation with HRP-conjugated secondary Ab at 4 °C for 3 h, protein bands were visualized with an ECL chemiluminescence detection kit (West-Pico, Pierce; Rockford, IL). Immunoblots of tubulin were used as loading controls.

6. Determination of IL-1β, IL-6, and TNF-α in Culture Supernatant

Cells were stimulated with LPS for 24 h in the presence and absence of silkworm hemolymph, and concentrations of IL-1β, IL-6, and TNF-α were measured in culture supernatants by high-sensitivity enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

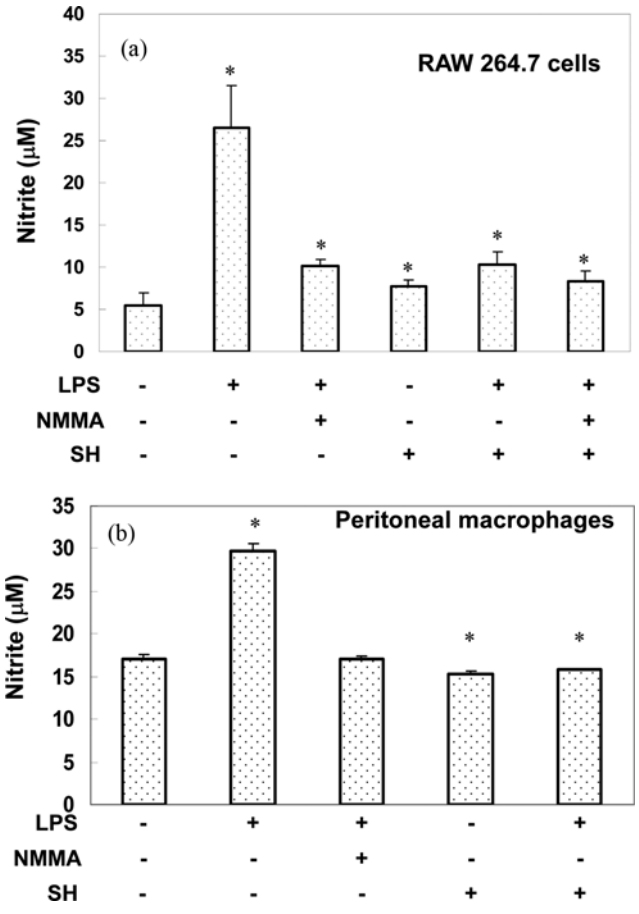

Fig. 1. Decrease of NO production by SH in LPS-stimulated RAW 264.7 cells and peritoneal macrophages. RAW 264.7 cells (a) and peritoneal adherent cells (b) were pre-incubated with the 5% SH for 1 h before being treated with LPS. Cells (5×10^5 cells/mL) were treated with LPS (0.1 μg/mL), NMMA (0.1 mM), or both, for 24 h. The culture supernatant was collected and analyzed for NO production. Each column shows the means±S.D. (*, P<0.05, n=3).

RESULTS

1. SH Decreases NO Production in LPS-stimulated Macrophages

To investigate the anti-inflammatory effect of SH, we first examined the production of NO in LPS-activated RAW 264.7 macrophage cells in the presence and absence of SH. Cells were harvested at 24 h after LPS treatment, and the accumulation of nitrite, the stable metabolite of NO, in the culture medium was measured by the Griess method. As shown in Fig. 1(a), NO production was increased by LPS treatment at the concentration of 0.1 μg/mL; however, it was markedly inhibited in the cells pre-incubated with 5% (v/v medium) SH compared with each control group (unstimulated macrophages, stimulated macrophages with NMMA, and unstimulated macrophages with SH). The NMMA is an iNOS inhibitor. We also found that the production of NO by SH was negligible, which means SH does not elicit the immune responses when used as a medium supplement.

We isolated the peritoneal macrophages from female BALB/c mice to examine the effect of SH on NO production in primary macrophages. Cells were pre-incubated with 5% (v/v medium) SH and then stimulated with 0.1 μg/mL of LPS. NO was not observed for the first thre days after LPS stimulation in peritoneal macrophages (Data not shown). NO productions at seven days after LPS treatment were compared in the cells incubated with and without SH (Fig. 1(b)). When the cells were pre-incubated with SH, the amount of nitrite was maintained at a background level similar to that in the unstimulated control confirming SH's inhibitory effect on NO production upon LPS treatment.

2. SH Inhibits the Expression of iNOS and Production of Pro-inflammatory Cytokines

NO production in LPS-treated macrophages rapidly increased until 24 h. NO was produced by inducible nitric oxide synthase (iNOS) when macrophages were stimulated with LPS. The expression levels of iNOS mRNA and protein were compared at 24 h after LPS (0.1 μg/mL) stimulation in the presence and absence of SH. Western blot (left panel) and RT-PCR (right panel) analysis indicated that iNOS protein as well as mRNA expression were markedly inhibited by SH.

We next investigated whether SH blocks the production of pro-inflammatory cytokines IL-1β, IL-6, and TNF-α to modulate the progression of inflammation induced by LPS. Cells were harvested at 24 h after LPS stimulation, and the released pro-inflammatory cytokines from the media were assayed by using sandwich enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 2(b), pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α burst into secretion from macrophages upon the stimulation with LPS, but their secretion was dramatically inhibited by SH.

3. Inhibitory Effect of SH are Mediated by NF-κB Suppression

NF-κB is a transcription factor that modulates the expression of variety of genes involved in immune and inflammatory responses, and it is an important intermediary of LPS-induced signal transduction in macrophages. The activation of NF-κB occurs only after the inhibitory IκB protein is phosphorylated and finally degraded in cytosol. In this experiment, a Western blot was conducted to investigate whether SH blocks the LPS-induced activation of NF-κB by inhibiting the phosphorylation of IκB. Cells were treated with LPS in the presence and absence of SH for 10 and 15 min. While

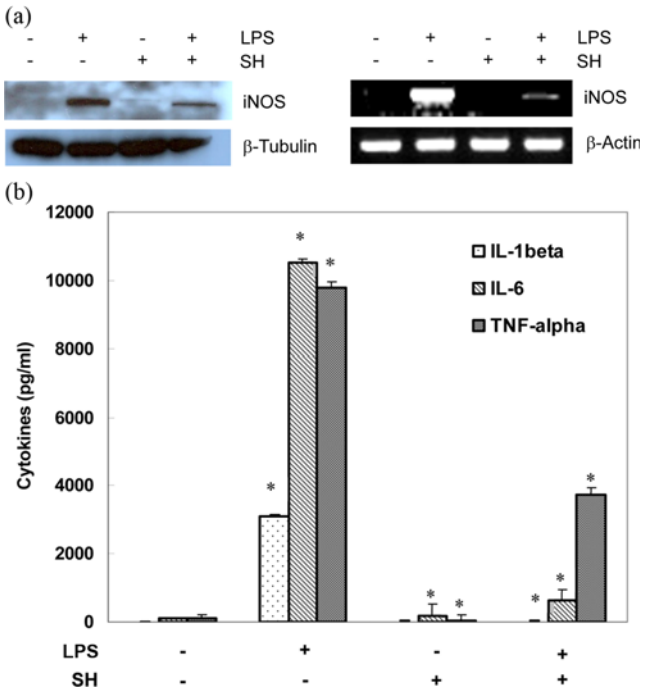

Fig. 2. Inhibitory effect of SH on the expression of iNOS and pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. Cells were pre-incubated with 5% SH before being treated with LPS (0.1 μg/mL) for 24 h. Cell lysates were then prepared and subjected to Western immunoblotting (left panel) and RT-PCR (right panel) for murine iNOS (a). The releases of pro-inflammatory cytokines from the media were analyzed by ELISA (b). Data are presented as means±S.D. (*, P<0.05, n=3).

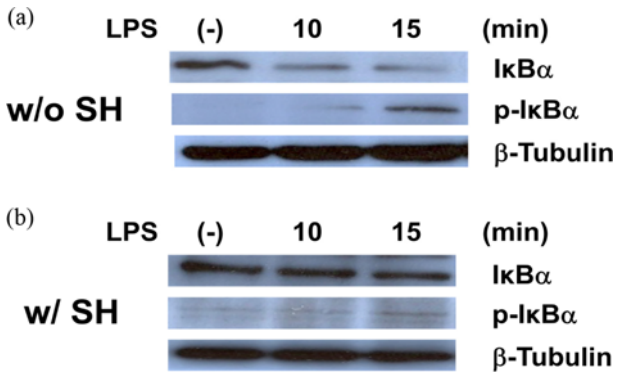

Fig. 3. Inhibitory effect of SH on the phosphorylation of IκB. RAW 264.7 cells were treated with LPS (0.1 μg/mL) for 10 and 15 min (a) and pre-incubated with 5% SH before being treated with LPS (b). 10 μg of whole cell protein extract was loaded onto a 10% SDS-PAGE gel and transferred to PVDF membranes. Western blotting was performed using a specific antibody for phosphorylated IκB (middle panel) and total IκB (top panel). As a control for equal loading of total protein, samples were immunoblotted with an antibody for β-tubulin (lower panel).

IκB was phosphorylated and degraded in the absence of SH after LPS treatment (Fig. 3(a)), it was not phosphorylated in the presence of SH, and thus the level of IκB remained the same regardless of LPS treatment (Fig. 3(b)). From these results, we suggest that SH inhibits the phosphorylation of IκB in LPS-stimulated macrophages. And this blocks the translocation of NF-κB to nucleus and exhibits anti-inflammatory effects as a result.

4. Deproteinized Extract of SH Decreases the Production of NO and Pro-inflammatory Cytokines

As a first step to figure out the potential anti-inflammatory components and see if they are non-protein fractions such as small molecules or peptides in SH, it is necessary to separate non-protein fractions from SH. Therefore, we investigated which one showed anti-inflammatory activity between the protein fraction and deproteinized extract of SH, since the protein fraction (mainly SP1, SP2, and 30 K proteins) of SH exhibits an anti-apoptotic activity in various systems [16-19,24].

Deproteinized extract of SH, which is called SHEX (silkworm hemolymph extract), was prepared by heat-treatment at 100 °C for 30 min and centrifugation, as most of effective proteins in SH are denatured and removed at this condition [25]. After the centrifugation, the supernatant was used as SHEX. Cells were pre-incubated with SHEX (5% v/v medium) and then NO production was measured after LPS treatment as described above. Interestingly, SHEX decreased NO production and also inhibited the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α as shown in Fig. 4(a) and (b), respectively.

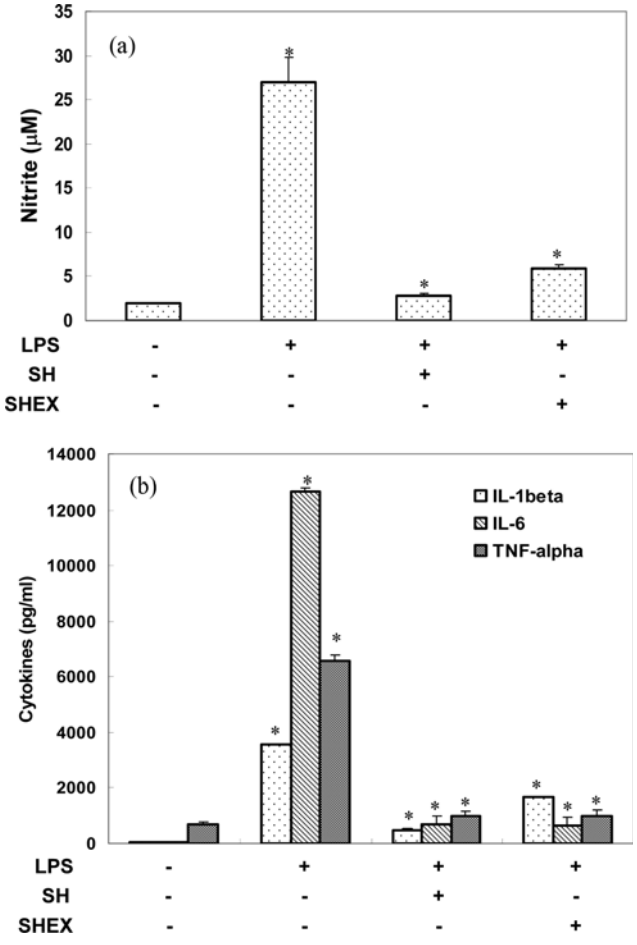

Fig. 4. Effect of SHEX on NO production and the expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells. Cells were pre-incubated with the 5% SH or 5% SHEX for 1 h before being treated with 0.1 μg/mL of LPS for 24 h. The culture supernatants were subsequently isolated and used for the analysis of NO production (a) and expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α (b). Data are presented as means±S.D. (*, P<0.05, n=3).

DISCUSSION

We have reported that SH exhibits the inhibiting activity of apoptosis induced by various inducers such as actinomycin D, camptothecin, sodium butyrate, staurosporine, and viruses in insect, mammalian, and human cell systems. Among these inducers, staurosporine involves multiple cellular and molecular events containing the production of reactive oxygen species (ROS). SH efficiently inhibits staurosporine-induced apoptosis by maintaining the mitochondrial transmembrane potential, which results in less oxidative stress according to the previous results [19]. The generation of ROS in phagocytic leukocytes such as monocytes and macrophages is also one of the most important hallmarks of the inflammatory process [26]. Based on this, we have demonstrated the role of SH in the inflammation process induced by LPS in this article.

First, NO which is a member of ROS was measured as an inflammation mediator in LPS-stimulated macrophages. NO production remarkably decreased by SH pre-incubation in RAW 264.7 cells and mouse primary macrophages (Fig. 1). This finding gives a first clue that SH shows the anti-inflammatory effect in progression of inflammation induced by LPS. As we expected, decreased NO production was due to the iNOS down-regulation as shown in RT-PCR and Western blotting results (Fig. 2(a)). In addition to NO, pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α produced by macrophages are major mediators of inflammatory responses and also play a prominent role in the development of sepsis [27]. Excessive production of these mediators is observed in many pathological conditions such as sclerosis, rheumatoid arthritis, and atherosclerosis [28,29]. We examined the production of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in LPS-stimulated macrophages, and it was drastically inhibited when cells were pre-incubated with SH (Fig. 2(b)). These results suggest that SH modulates the progression of inflammation by the inhibition of iNOS and pro-inflammatory cytokines production. Thus the inhibition of these mediators by SH can be an effective therapeutic strategy for preventing inflammatory reaction and diseases.

NF-κB is a dimeric transcription factor that is involved in the regulation of a large number of genes that control various aspects of the immune and inflammatory response. In the resting state, NF-κB is present in the cytosol and is bound to the inhibitory protein, IκB. In response to pro-inflammatory stimuli, IκB is first phosphorylated in its N-terminal domain by a large multikinase complex, then poly-ubiquitinylated, and finally degraded by the proteosome [30]. The released NF-κB is transported into the nucleus, where it initiates gene transcription upon binding its cognate DNA motifs in regulatory segments of genes. As described above, we have investigated the effect of SH on NF-κB activation in LPS-stimulated mac-

rophages. LPS-induced phosphorylation of IκB in cytosol were chased at time-scale. When cells were stimulated by LPS, IκB began to be phosphorylated within 10 min. In contrast, SH pre-incubation inhibited the phosphorylation and degradation of IκB, which further prevented the subsequent activation of NF-κB. These serial effects resulted in the down-regulation of pro-inflammatory cytokine genes. This indicates that the inhibition of LPS-induced inflammatory response by SH is mediated through the blocking of NF-κB activation.

LPS is known as a potent inducer of apoptosis as well as inflammation. Therefore, the production of NO and cytokines can be affected by anti-apoptotic effect of SH. However, we doubt this possibility as SH only inhibits cell surface death receptor-independent apoptosis from our recent experiments. LPS can induce apoptosis mainly by the autocrine production of TNF-α by macrophages [31]. As TNF-α mediated apoptosis directly transmit apoptosis signal to caspase without Bax translocation, SH cannot inhibit apoptosis in LPS-treated macrophages. Moreover, deproteinized extract of SH which does not have 30 K protein and SP still showed the anti-inflammation effect in this system; this is also the proof that anti-apoptotic effect of SH is not involved in anti-inflammation. In addition, although cytotoxicity of the SH has not been observed in any cells that we have experienced, long-term toxicity of SH and its effective components will be carefully tested for further applications.

Deproteinized extract of SH decreased the production of NO as well as pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α as shown in Fig. 4(a) and (b). This suggests that the components responsible for anti-inflammatory effect are contained in SHEX, deproteinized fraction of SH. There are some reports showing the presence of LPS-binding proteins in SH [32,33]. However, the SH used is heat-inactivated and it is likely that these proteins are denatured and removed upon centrifugation. Moreover, deproteinized extract of SH showed the anti-inflammation effect. There are some reports of bioactive peptides with immune-modulating activities in silkworm [34,35]. The silkworm paralytic peptide (PP) exerts multiple biological activities involved in defense reaction and growth regulation. These are small peptides that can be included in the SHEX and so they can one. Further study is required to elucidate the components and molecular mechanism involved in the anti-inflammatory activity of SH. To generalize and develop the detailed anti-inflammatory mechanism of SH and its components, we are planning to use and combine various activators and inhibitors of inflammation in macrophages supplemented with SH or its components. In conclusion, this article reports for the first time that SH acts as an inhibitor of NO production in murine macrophages and peritoneal macrophages activated by LPS and it is through inhibition of iNOS expression. Besides, SH down-regulates the pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α as well as iNOS by blocking NF-κB activation. This finding provides a new therapeutic approach for various inflammation disorders such as septic syndrome and arthritis.

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