KINSENOSIDE ISOLATED FROM ANOECTOCHILUS FORMOSANUS SUPPRESSES LIPOPOLYSACCHARIDE-STIMULATED INFLAMMATORY REACTIONS IN MACROPHAGES AND ENDOTOXIN SHOCK IN MICE

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Running head: Kinsenoside suppresses LPS-stimulated inflammation

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ABSTRACT—In the present study, we reported that kinsenoside, a major component of *Anoectochilus formosanus*, inhibited inflammatory reactions in mouse peritoneal lavage macrophages (MPLMs) and protects mice from endotoxin shock. In lipopolysaccharide (LPS)-stimulated MPLMs, kinsenoside inhibited the inflammatory mediators, such as nitric oxide, tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1) and macrophage migration inhibitory factor (MIF) production. Furthermore, kinsenoside decreased the formation of a nuclear factor-κB (NF-κB)–DNA complex and nuclear p65 and p50 protein levels. Kinsenoside inhibited NF-κB translocation through both IκBα-dependent and –independent pathway. In contrast, it stimulated anti-inflammatory cytokine IL-10 generation, and enhanced the mRNA expression of IL-10 and suppressor of cytokine signaling-3 in the same cells induced by LPS. In an animal model, both pre- and post-treatment of kinsenoside increased the survival rate of ICR mice challenged by LPS (80 mg/kg, i.p.). Pretreatment with kinsenoside decreased serum levels of TNF-α, IL-1β, IL-10, MCP-1 and MIF at 1 h after sublethal dose of LPS (40 mg/kg, i.p.) in mice. In contrast, kinsenoside enhanced serum IL-10 level at 24 h after LPS injection in mice. In conclusion, kinsenoside inhibited the production of inflammatory mediators and enhanced anti-inflammatory cytokin generation. Therefore, kinsenoside can alleviate acute inflammatory hazards.

KEYWORDS—cytokine, inflammation, kinsenoside, lipopolysaccharide.

INTRODUCTION

During the inflammatory process, macrophages play a central role in mediating various immunopathological responses, including the production of inflammatory mediators and anti-inflammatory cytokines (1). Macrophages are activated by various mediators, including lipopolysaccharide (LPS) which is a constituent of the cell wall of gram-negative bacteria (1). There are many regulators involved in the inflammation process, and the nuclear factor-κB (NF-κB) signaling pathway appears to have a central function in the regulation of the inflammation (2). In unstimulated cells, NF-κB is constitutively localized in the cytosol. Following activation, including LPS stimuli, the NF-κB is translocated to the nucleus, where it activates the transcription of target genes, including genes encoding for pro-inflammatory cytokines, chemokines, inducible nitric oxide synthase, and anti-inflammatory cytokines (2).

Kinsenoside [3-(*R*)-3-β-D-glucopyranosyloxybutanolide; Fig. 1] is a significant and active compound of the *Anoectochilus formosanus* (Orchidaceae), an important ethnomedicinal plant of Taiwan. *A. formosnus* is used as health food for liver disease in Taiwan and other Asian countries (3). Currently, we have reported that a standardized aqueous extract of *A. formosanus* significantly reduces thioaetamide-induced hepatic fibrosis in mice, probably through inhibition of hepatic Kupffer cell activation (4). *In vitro* study also showed that kinsenoside inhibited the tumor necrosis factor α (TNF-α) secretion from Kupffer cells, hepatic macrophages, to LPS stimulation (4). These results suggest that kinsenoside may protect the liver from thioacetamide-induced injury through inhibiting the activation of Kupffer cells, by suppressing inflammation. Therefore in this study, we examined the anti-inflammatory properties of kinsenoside both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Preparation of kinsenoside

The identity and purity of kinsenoside (> 85 %) was analyzed by HPLC according to the previous report (5).

Animal

Male ICR mice were obtained from BioLASCO Co., Ltd. (Taipei, Taiwan), and fed with a standard laboratory diet and tap water *ad libitum*. Experimental animals were housed in an air-conditioned room at 22-25°C with a 12-h light/dark cycle. All animals received humane care, and the study protocols were in compliance with institutional guidelines of China Medical University for the use of laboratory animals.

Isolation of mouse peritoneal lavage macrophages (MPLMs)

Mouse macrophages were isolated from the peritoneal cavity of 7-week-old mice, 3 days after the injection of 1 ml endotoxin-free 5% thioglycollate medium (BD Pharmingen, San Jose, USA). Peritoneal fluid from mice were harvested from peritoneal cavities by infusing 5 ml ice-cold sterile Hank’s buffered salt solution. After centrifugation at 300 *g* for 5 min, the cell pellets were suspended in DMEM (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum, penicillin 100 U/ml, and streptomycin 100 U/ml. The cells were plated onto 24-well (1×10^6 cells) or 6 cm culture dish (1×10^7 cells) for cytokine detection or RNA, protein extract respectively. Non-adherent cells were removed after 4 h culture.

For the experiments, cells cultured for 4 h were washed and cultured in fresh medium with various concentrations of kinsenoside (10, 25 and 50 μM) 30 min before

LPS (1 μg/ml) treatment. For NO and cytokines detection, the supernatant was collected 24 h after LPS treatment. For RNA extract, cells were collected 24 h after LPS treatment. RT-PCR analyses for interleukin 10 (IL-10) and suppressor of cytokine signaling-3 (SOCS-3) were performed as described a little later in the text (section of RT-PCR analysis).

Nitrite and cytokines determinations

The production of nitric oxide (NO) was determined by measuring the accumulation of nitrite in culture medium based on the Griess reaction. Nitrite in the culture medium was determined by Griess reagent (Sigma St. Louis, MO). Concentrations of cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-10, monocyte chemoattractant protein-1 (MCP-1) (eBioscience, San Diego, CA) and macrophage migration inhibitory factor (MIF, R&D Systems, Minneapolis, MN) in the supernatants of cell culture were determined using an enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Preparation of cytoplasmic and nuclear extracts and Western blot analysis

The cytoplasmic and nuclear protein extracts were described previously (6). The MPLMs were incubated with or without LPS in the presence or absence of kinsenoside. The cells (1.0×10^7) were washed with ice-cold phosphate-buffered saline and suspended in 0.2 ml hypotonic lyses buffer [10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM dithiothreitol (DTT), 1 mM phenylmethysulfonyl fluoride (PMSF), 1 mM sodium fluoride (NaF), 1 mM orthovanadate (Na3VO4), 10 μM EGTA, 10 μM EDTA] containing 0.5 % Nonidet P-40 and microcentrifuged at 12000 × g for 1 min. The homogenate was centrifuged, and supernatant containing the cytoplasmic extracts

was removed and stored frozen at -80°C. The nuclear pellet was lysed in 20 μl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 mM NaF, 1 mM Na3VO4, 10 μM EGTA, 10 μM EDTA) for 1 hour of intermittent mixing, the extract was centrifuged. The lysates were centrifuged at 12,000 ×*g* for 15 min at 4 °C, and supernatants containing nuclear extracts were secured. The protein concentration was determined using the Bradford protein assay reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacture's instruction.

The MPLMs cytoplasmic and nuclear protein extracts (40 and 20 μg, respectively) were separated by 12% SDSPAGE and transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk, and then incubated overnight at 4^0C with various primary antibodies in TBS containing 0.1% Tween-20. The primary antibodies were obtained from the following sources: anti-p65, anti-phospho-p65 (anti-pp65), anti-phospho-IκBα, anti-IκBα from Cell signaling (Danvers, USA); and anti-proliferating cell nuclear antigen (PCNA), anti-α-tubulin, anti-p50 from Santa Cruz (CA, USA). Thereafter, the blot was washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h, and then developed by enhanced chemiluminescence (Thermo, Rockford, USA). PCNA and α-tubulin were used as an internal control in nuclear and cyoplasmic experiments, respectively.

Electrophoretic mobility shift assay (EMSA)

To determine NF-κB activation, the sequence of the NF-κB–binding oligonucleotide used as a fluorescence DNA probe was cy5.5-5'-AGCTTGGGGACTTTCCGA-3' (Bio-protech, Taipei, Taiwan). The DNA binding reaction was performed at room temperature in a volume of 20 μl, which contained the binding buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM DTT), 1

μg of poly (dI-dC), 50 nM cy5.5 labeled probe, 0.5% Tween-20 and 15 μg of nuclear extracts. After incubation for 30 minutes, the samples were electrophoresised on native 5% acrylamide gels prepared in 0.5× TBE buffer (Amresco, Solon, Ohio). Supershift assay using anti-p65 and anti-p50 antibody were also conducted to confirm the specificity of NF-κB DNA-binding activity. "Cold" represents nuclear extract preincubated with an excess of unlabelled oligonucleotide. Subsequently the gel was transferred to and imaged on a LI-COR Odyssey infrared imaging system (LI COR Biosciences, Nebraska) at 700 nm channels and 169 μm resolution. The density of fluorescence in each band was measured in triplicate with the use of the LI-COR imaging software.

RT-PCR analysis

Total RNA was isolated from the MPLMs cultured by the acid guanidinium thiocyanate-phenol-chloroform extract method, as described by Chomzynski and Sacchi (7). A 3-μg sample of total RNA was subjected to reverse transcription (RT) with moloney murine leukemia virus reverse transcriptase in a 50-μl reaction volume. Aliquots of the RT mix were used in amplification by polymerase chain reaction of fragments of IL-10 and SOCS-3 with the mice primer pairs listed in Table 1. The expression levels of all the transcripts were normalized to that of *GAPDH* mRNA in the same tissue sample. PCR product identities were confirmed by sequence analysis. The PCR products were separated on a 2% agarose gel and recorded on Polaroid film; the bands were quantified with a densitometer.

Survival rate after LPS-induced endotoxic shock

When the mice attained a weight of 24-26 g, they were used in the experiments.

The mice were divided randomly into three groups, such as model, and two kinsenoside treatment groups, according to their body weight. In the prophylatic experiment, LPS (80 mg/kg) was administered by intraperitoneal injection 1 h after kinsenoside (100, 300 mg/kg; i.p.) or saline treatment. The number of surviving mice was observed for 72 h. In the therapeutic experiment, kinsenoside (100, 300 mg/kg; i.p.) and saline were intraperitoneal injection 1 h and 6 h after LPS (80 mg/kg, i.p.) administration. The number of surviving mice was observed for 72 h.

LPS-induced inflammatory model in mice

To examine the effect of kinsenoside on serum cytokines profile in acute inflammation, the sub-lethal dose of 40 mg/kg of LPS was used in this experiment. When the mice attained a weight of 24-26 g, forty mice were randomly divided into 4 group (containing 10-12 mice per group), such as control, model, and two kinsenoside (100, 300 mg/kg; i.p.) treatment groups. Except control group, LPS was administered by intraperitoneal injection 1 h after kinsenoside (100, 300 mg/kg; i.p.) treatment. The control group was given equal volume of saline to substitute LPS.

After 1 h of LPS treatment, blood was collected from the mice *via* the retro-orbital sinus. At the end of the experiment (24 h after LPS injection), mice were sacrificed under CO_2 anesthesia and blood was withdrawn from the abdominal vein.

The concentrations of the sera TNF-α, IL-6, IL-10, MCP-1 and MIF were measured by ELISA using commercially available kits (eBioscience, San Diego, CA) according to the manufacturer’s instructions.

Statistical analysis.

Results are expressed as the mean ± SD. All experimental data were analyzed by

one-way analysis of variance following the Dunnet test. The statistical significance of differences between groups of animals in survival experiments was analyzed by the Kaplan-Meier test. Difference with $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Kinsenoside on inflammatory mediators production in LPS-stimulated MPLMs

The culture treatment of MPLMs with 1 μg/ml LPS for 24 h caused a dramatic increase in IL-1β, IL-10, TNF-α, NO, MCP-1 and MIF production (Fig. 2). LPS-induced the production of IL-1β, TNF-α, NO, MCP-1 and MIF were inhibited by kinsenoside (10 - 50 μM) pretreatment in a concentration-dependent manner. In contrast, the release of IL-10 was increased by kinsenoside (50 μM) pretreatment. MPLMs did not undergo any change in viability after exposure to LPS + kinsenoside (data unpublished).

Effect of kinsenoside on protein expression of IκBα, pIκBα, pp65, p65 and p50 in LPS-stimulated MPLMs

MPLMs were incubated with LPS in the presence or absence of kinsenoside for 30 min. Treatment with LPS for 30 min increased cytoplasmic protein expression levels of pIκBα, pp65, but not IκBα, by Western blot analysis (Fig. 3A). The expression levels of pIκBα and pp65 in the LPS group were 145% and 123% respectively, all of them greater than those in control group (Fig. 3). Kinsenoside treatment did not affect the expression of IκBα. Pretreatment of kinsenoside led to decrease of 45% (25 μM)

and 63% (50 μM) of pIκBα expression and also 43% (25 μM) and 55% (50 μM) of pp65 expression (Fig. 3A).

MPLMs were incubated with LPS in the presence or absence of kinsenoside for 30 min. Treatment with LPS for 30 min caused translocations of p65 and p50 into nucleus by Western blotting. The expression levels of p65 and p50 in the LPS group were 126% and 151% respectively, all of them greater than those in control group (Fig. 3B). Pretreatment of kinsenoside led to decrease of 25% (25 μM) and 31% (50 μM) of p65 expression and also 18% (25 μM) and 37% (50 μM) of p50 expression (Fig. 3B).

Effect of kinsenoside on LPS-induced NF-κB activation

The MPLMs were pretreated with kinsenoside for 30 min, and then treated with LPS (1 μg/ml) for 30 min. LPS treatment caused a significant increase in the DNA-binding activity of NF-κB, as determined by EMSA (Fig. 4A). Pretreatment with kinsenoside (25 and 50 μM) significantly suppressed the induced DNA-binding activity of NF-κB by LPS. Supershift assay of NF-κB-DNA probe binding showed that LPS-activated NF-κB consisted of p50 and 65 subunits (Fig. 4B).

Kinsenoside inhibited the mRNA expression of IL-10 and SOCS-3 in LPS-stimulated MPLMs

Fragments of *IL-10* and *SOCS-3* genes were amplified by RT-PCR (Fig. 5A). The *IL-10/GAPDH* and *SOCS-3/GAPDH* ratios in the LPS group were 118% and 120% respectively, all of them greater than those in control group (Fig. 5B). Pretreatment with kinsenoside (25, 50 μM) led to 18.9% and 24.5%, 22.2% and 31.5% increase in *IL-10/GAPDH* and *SOCS-3/GAPDH* ratios, respectively.

Kinsenoside increased the survival rate after LPS-induced endotoxic shock

Mice received an i.p. injection of LPS (80 mg/kg). Six hours after LPS, mice clearly displayed the symptoms of endotoxic shock, such as decreased motor activities, ruffled fur, diarrhea, and ocular exudates. Fig. 6A showed that 48 h after LPS, 90% of mice treated with saline died. In 48 h, 50% and 40% of mice treated with kinsenoside 100, 300 mg/kg died, respectively.

Since the studies described above suggested that kinsenoside prevents endotoxin shock, we next examined the therapeutic effects of kinsenoside. In order to investigate the therapeutic effects, kinsenoside were treated at 1 h and 6 h after LPS injection. As shown in Fig. 6B, 83.3% of the mice died within 24 h in the saline treatment. In 36 h, 70% and 50% of mice treated with kinsenoside 100, 300 mg/kg died, respectively.

Effect of kinsenoside on serum cytokine levels in LPS-induced endotoxic shock

To determine the effects of kinsenoside on inflammatory cytokines involved in the endotoxaemia of LPS, serum levels of IL-1β, IL-10, TNF-α, MCP-1 and MIF were determined by ELISA. Kinsenoside was administered to mice 1 h prior to LPS treatment and each cytokine level was determined at 1 and 24 h after LPS injection. One hour after LPS injection, serum concentrations of IL-1β, IL-10, TNF-α, MCP-1 and MIF increased 2.7-, 27-, 7.9-, 3.1- and 9.3-fold in LPS-treated mice as compared with the control group (Fig. 7A-C and Fig. 8A-B). Administration of kinsenoside (100, 300 mg/kg, i.p.) significantly inhibited the increase of serum concentrations of IL-1β, IL-10, TNF-α, MCP-1 and MIF.

There were no significant differences in serous IL-1β, TNF-α and MCP-1 levels at 24 h between LPS-treated group and control group. The kinsenoside treatment did not

affect the serum levels of IL-1β, TNF-α and MCP-1 (Fig. 7D, F and Fig. 8C). Twenty four hours after LPS injection, serum concentration of IL-10 and MIF increased 4.8- and 7.6-fold in LPS-treated mice as compared with the control group (Fig. 7E). Kinsenoside (300 mg/kg, i.p.) inhibited the increase of serum concentrations of MCP-1. In contrast, administration of kinsenoside (100, 300 mg/kg, i.p.) led to 45% and 59% increase in serum concentration of IL-10.

DISCUSSION

For the first time, this study clearly demonstrated that mice treated with kinsenoside exhibited improved survival to endotoxic shock. The protective mechanisms may involve decreased production of inflammatory cytokines and increased secretion of anti-inflammatory mediators.

Bacterial LPS/endotoxin, a cell wall component of Gram-negative bacteria, is a potent activator of mammalian cells, such as macrophages (1). It has been demonstrated that LPS, after binding to receptors on macrophages, could induce this inflammatory cells to release free radical NO (8), various cytokines, including TNF-α and IL-1β and IL-10 (9-11). In this study, MPLMs were pretreated with kinsenoside for 30 min, and then the cells were stimulated by LPS for 24 h. We found that kinsenoside significantly inhibited LPS-induced NO, TNF-α and IL-1β secretions in MPLMs.

Downstream to LPS activation, the ubiquitous transcription factor NF-κB activates transcription of a wide variety of genes, including those coding for iNOS, TNF-α and IL-1β (8, 12). The result of EMSA study showed that kinsenoside inhibited the LPS-induced DNA binding activity of p65 and p50. Western blot analysis of nuclear protein also showed that kinsenoside suppressed the nuclear

translocation of p65 and p50 proteins. We clearly demonstrated that anti-inflammatory effects of kinsenoside may be exerted partly via inhibition of NF-κB signaling.

In unstimulated cells, NF-κB is present in the cytosol as a homodimer or heterdimer, and in particular is linked to the inhibitory protein IκB. NF-κB signaling is generally considered to occur through either the classical or alternative pathway. In the classical pathway, NF-κB activation results from the phosphorylation, ubiquitination, and proteasome-mediated degradation of inhibitory IκB protein, and then followed by the nuclear translocation and DNA binding of NF-κB (2). In the alternative IκB-independent pathway, direct phosphorylation of NF-κB subunit p65 also modulates NF-κB transcription activity (13). In this study, our results showed that the expression of cytoplasmic pIκBα and pp65 were increased under LPS-stimulation. Kinsenoside significantly inhibited the expression of pIκBα and pp65, which indicated that kinsenosed inhibited NF-κB translocation through both the IκBα-dependent and –indepdent pathways.

IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of NO and pro-inflammatory cytokines in MPLMs (14). In this study, the production and mRNA expression of IL-10 in macrophages stimulated by LPS were enhanced by kinsenoside. These results suggest that some of the anti-inflammatory effects of kinsenoside may be partly mediated by the release of IL-10, which, in turn inhibited the release of NO and pro-inflammatory cytokines.

It has been reported that LPS-induced transcriptional activation of IL-10 is mediated by NFκB pathway in mouse macrophage (15). In this study, kinsenoside inhibited NFκB activation in MPLMs, but enhanced IL-10 release. This result indicated that kinsenoside could enhance IL-10 release but not through NFκB

pathway. Dong et al. showed that enhanced SOCS-3 gene expression could promote IL-10 production in placental trophoblast cells (16). In the present study, LPS-induced mRNA expression of IL-10 and SOCS-3 in MPLMs were enhanced by kinsenoside treatment. Kinsenoside induces SOCS-3 expression might contribute to the production of IL-10. In addition, SOCS protein are a family of intracellular proteins that control cytokine signaling by suppressing cytokine transduction process (17). It has been shown that SOCS-3 mediates the inhibitory effects of IL-10 on LPS-induced macrophage activation (18). Kinsenoside induces SOCS-3 expression might contribute to the production and anti-inflammatory actions of IL-10.

Bacterial LPS in the bloodstream induces overexpression of various inflammatory mediators, and large amounts of inflammatory mediators produced in the body are thought to contribute to the LPS-induced symptoms of endotoxic shock and molarity (19). Thus, survival rate in murine endotoxic shock has been used as an indicator of anti-inflammatory effect. In an animal model with lethal endotoxemia (LPS 80 mg/kg, i.p.), the pre- or post-treatments of kinsenoside increased the LPS-induced survival rate. These observation strongly supports the hypothesis that kinsenoside has anti-inflammatory activity, not only *in vitro* but also *in vivo*.

The current study utilized a sub-lethal dose of 40 mg/kg of LPS, which allows for examining the profile of cytokines not only at the early but also at the late stage of LPS-induced acute inflammation. In this study, kinsenoside treatment decreased serum TNF-α and IL-1β levels at 1 h after LPS challenge in mice. Since the serum levels of TNF-α and IL-1β at 24 h after LPS injection is very low, no effect of kinsenoside is observed. Kinsenoside suppressed TNF-α release *in vivo* was stronger than that *in vitro*, which indicated that kinsenoside was involved in other mechanisms *in vivo* to inhibit pro-inflammatory cytokines secretion. Currently, Borovikova et al.

showed that the efferent vagus nerve inhibited the release of TNF-α and other proinflammatory cytokines and attenuates the development of endotoxin-induced shock in rodents (20). The possibility of how kinsenoside activates vagally-mediated neural anti-inflammation *in vivo* is required further to investigate.

Kinsenoside treatment decreased the serum IL-10 level at 1 h after LPS challenge in mice. In contrast, kinsenoside enhanced the serum IL-10 level at 24 h after LPS challenge. It is known that host response to injury is finely regulated by the formation of both pro-inflammatory and anti-inflammatory cytokines. While pro-inflammatory cytokines, such as TNF-α and IL-1β, stimulate the body's defense mechanism by promoting inflammatory reactions in order to fight inflammatory stimuli and restore homeostasis; anti-inflammatory cytokines such as IL-10, lower inflammatory responses and prevent defense mechanisms from causing damage by overshooting (21).

In the early phase, the serum levels of TNF-α and IL-1β were lower in the kinsenoside treated group, therefore, the serum level of IL-10 of kinsenoside treated group was lower than the LPS alone group. In the late stage, kinsenoside enhanced the serum IL-10 level. *In vitro* study also clearly demonstrated that kinsenoside stimulated IL-10 release from MPLMs. These results suggested that the increased survival rate of kinsenoside-treated mice with endotoxemia may partly mediate the increase of anti-inflammatory cytokines.

Both MIF and MCP-1 were produced in macrophages induced by LPS (22, 23). In MPLMs cell culture system, kinsenoside inhibited MIF and MCP-1 secretion. In vivo study, we found that both serum concentrations of MIF and MCP-1 were increased by LPS treatment. It has been shown that anti-MIF antibody and blocker of MCP-1 synthesis could increase survival rates in LPS-induced endotoxemia (24, 25).

Kinsenoside decreased the LPS-induced MIF and MCP-1, indicating that it protected mice from LPS-induced endotoxin shock, partly through inhibiting the production of MIF and MCP-1.

In conclusion, our results demonstrated the beneficial effects of kinsenoside on endotoxic shock in mice. Multiple mechanisms were involved in the anti-inflammatory action of kensenoside. Firstly, kinsenoside inhibited proinflammatory cytokins release via NF-κB pathway. Secondary, kinsenoside enhanced the anti-inflammatory cytokine release might be mediated through activation of SOCS-3. Finally, kinsenoside reduced the MIF and MCP-1 productions.

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Figure legends

FIG. 1. **Structure of kinsenoside.**

FIG. 2. **Effects of kinsenoside on LPS-induced IL-1β, IL-10, TNF-α, NO, MCP-1 and MIF productions in MPLMs.** MPLMs were pre-incubated for 30 min with indicated concentrations of kinsenoside and then activated for 24 h with 1 μg/ml LPS. The cell culture media were collected for measuring IL-1β (A), IL-10 (B), TNF-α (C), NO (D), MCP-1 (E) and MIF (F) productions by ELISA as described in materials and methods. Values were expressed as means ± SD (n = 3). $^{\#\#}P < 0.01$ as compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the LPS + Vehicle group.
Con: control

FIG. 3. **Effects of kinsenoside on LPS-induced protein expression of pIkBα, IkBα, pp65, p65 and p50 in MPLMs.** MPLMs were pre-incubated for 30 min with indicated concentrations of kinsenoside and then activated for 30 min with 1 μg/ml LPS. A, Cytoplasmic fractions were obtained for the detection of IKKα, pIκBα, IκBα, pp65 levels. B, Nuclear fractions were obtained for the detection of p65 and p50 levels. Values were expressed as means ± SD (n = 3). $^{\#}P < 0.05$ as compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the LPS + Vehicle group.
Con: control; kin: kinsenoside

FIG. 4. **Kinsenoside inhibited LPS-induced NF-κB activation.** A. MPLMs were incubated either alone or with the presence of kinsenoside for 30 min, then treated with LPS (1 μg/ml) for 30 min for testing nuclear NF-κB levels by EMSA as

described in materials and methods. B. EMSA results showed a supershift of complex formed in the presence of anti p50 and anti p65 antibodies. The p50and p65 subunits cause a specific binding of NF-κB to consensus DNA sequence. ‘‘Cold’’ represent that the nuclear extract was preincubated with an excess of unlabelled oligonucleotide. Values were expressed as means ± SD (n = 3). $^{\#}P < 0.05$ as compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the LPS + Vehicle group.

Con: control

FIG. 5. **Effects of kinsenoside on *IL-10* and *SOCS-3* mRNA expression in LPS-stimulated MPLMs.** MPLMs were pre-incubated 30 min with indicated concentrations of kinsenoside and then activated for 24 h with 1 μg/ml LPS. A, *IL-10* and *SOCS-3* mRNAs were amplified by RT-PCR. B, their expression levels were measured and quantified by densitometric scanning. Values were expressed as means ± SD (n = 3). $^{\#}P < 0.05$ as compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the LPS + Vehicle group.

Con: Control; kin: kinsenoside

FIG. 6. **Effects of kinsenoside on the survival of mice challenged with LPS.** All mice were injected i.p. with LPS (80 mg/kg). A, Single kinsenoside treatment was administered at 1 h before LPS challenge (n = 10/group). B, Kinsenoside was administered at 1 h and 6 h after LPS challenge (n =12/group). $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the LPS + Vehicle group.

Kin: kinsenoside

FIG. 7. **Effects of kinsenoside on serum levels of IL-1β, IL-10 and TNF-α after LPS-challenge.** A-C, Mice were administered with kinsenoside 1 h before LPS (40 mg/kg, i.p.) challenge, and serum were collected 1 h after LPS challenge for cytokine expression. D-F, Mice were administered with kinsenoside 1 h before LPS (40 mg/kg, i.p.) challenge, and serum were collected 24 h after LPS challenge for cytokine expression. Values were expressed as means ± SD (n = 10). $^{\#\#}P < 0.01$ as compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the LPS + Vehicle group.

Con: control

FIG. 8. **Effects of kinsenoside on serum levels of MCP and MIF after LPS-challenge.** A-B, Mice were administered with kinsenoside 1 h before LPS (40 mg/kg, i.p.) challenge, and serum were collected 1 h after LPS challenge. C-D, Mice were administered with kinsenoside 1 h before LPS (40 mg/kg, i.p.) challenge, and serum were collected 24 h after LPS challenge. Values were expressed as means ± SD (n = 10). $^{\#\#}P < 0.01$ as compared with the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the LPS + Vehicle group.

Con: control