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Inhibition of IRAK-4 activity for rescuing endotoxin LPS-induced septic mortality in mice by lonicerae flos extract

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ABSTRACT

Lonicerae flos extract (HS-23) is a clinical candidate currently undergoing Phase I trial in lipopolysaccharide (LPS)-injected healthy human volunteers, but its molecular basis remains to be defined. Here, we investigated protective effects of HS-23 or its major constituents on *Escherichia coli* LPS-induced septic mortality in mice. Intravenous treatment with HS-23 rescued LPS-intoxicated C57BL/6J mice under septic conditions, and decreased the levels of cytokines such as tumor necrosis factor α (TNF-α), interleukin (IL)-1β and high-mobility group box-1 (HMGB-1) in the blood. Chlorogenic acid (CGA) and its isomers were assigned as major constituents of HS-23 in the protection against endotoxemia. As a molecular mechanism, HS-23 or CGA isomers inhibited endotoxin LPS-induced autophosphorylation of the IL-1 receptor-associated kinase 4 (IRAK-4) in mouse peritoneal macrophages as well as the kinase activity of IRAK-4 in cell-free reactions. HS-23 consequently suppressed downstream pathways critical for LPS-induced activation of nuclear factor (NF)-κB or activating protein 1 (AP-1) in the peritoneal macrophages. HS-23 also inhibited various toll-like receptor agonists-induced nitric oxide (NO) production, and down-regulated LPS-induced expression of NF-κB/AP-1-target inflammatory genes in the cells. Taken together, HS-23 or CGA isomers exhibited anti-inflammatory therapy against LPS-induced septic mortality in mice, at least in part, mediated through the inhibition of IRAK-4.

1. Introduction

Sepsis-related disorders are currently hypothesized to involve a characteristic pathophysiology of overwhelming inflammatory reaction accompanied by compensatory immune depression, which can lead to multiple organ injury, shock and even death [1,2]. However, therapeutic strategy against septic disorders remains elusive. An insult of LPS, a glycolipid in the outer membrane of Gram-negative bacteria, induces endotoxemia in rodent models that mimic many of the initial clinical features of human sepsis increasing inflammatory cytokines and also causing progressive multi-organ dysfunction [3,4]. Mammalian hosts recognize endotoxin LPS through a receptor complex named as myeloid differentiation protein 2 (MD-2)/toll-like receptor 4 (TLR4) [5–7]. The intracellular toll/IL-1 receptor (TIR) domain of TLR4 recruits adaptor molecules such as myeloid differentiation factor 88 (MyD88) and TIR-containing adaptor inducing interferon (IFN)-β (TRIF) [8,9]. TLR4 signaling is thereby divided into MyD88- and TRIF-dependent pathways. MyD88 is also an essential part of other TLR pathways except that of TLR3, but TRIF is confined to those of TLR3 and TLR4 [10].

The MyD88-dependent signal cascade activates the transcription factors of NF-κB and AP-1, which up-regulate inducible NO synthase (iNOS) and inflammatory cytokines including TNF-α, IL-1β, IL-6 and HMGB-1 [8–10]. Upon LPS ligation, TLR4 recruits MyD88 to activate IRAK-4 and IRAK-1 [11]. Phosphorylation of the IRAKs results in the subsequent activation of TNF receptor-associated factor 6 (TRAF6) and transforming growth factor-β-activated kinase 1 (TAK-1) [12]. TAK-1 then activates the inhibitory κB (IκB) kinase (IKK) complex, which phosphorylates cytoplasmic IκB proteins, leading to proteasome-mediated degradation of IκBs and thus allowing a free NF-κB dimer to translocate into the nucleus for transcriptional activity [13,14]. TAK-1 also stimulates mitogen-activated protein kinases (MAPKs) such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinases 1 and 2 (ERK-1/2), leading to AP-1 activation followed by transcriptional

Abbreviations: AP-1, activating protein 1; CGA, chlorogenic acid; HS-23, lonicerae flos extract; IRAK-4, IL-1 receptor-associated kinase 4; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; TBK-1, TNF receptor-associated factor (TRAF) family member-associated NF-κB activator (TANK)-binding kinase 1; TLR, toll-like receptor; TRIF, toll/IL-1 receptor (TIR)-containing adaptor inducing IFN-β.

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control of target genes [8,9,13]. The TRIF-dependent pathway activates another transcription factor IRF3 via TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IKK*i*, which triggers gene expression of type I IFNs [8,9].

Lonicerae flos, a flower bud of *Lonicera japonica* (Caprifoliaceae), is a traditional Korean or Chinese medicine used in the treatment of inflammatory and infectious diseases. Lonicerae flos extract (HS-23, Fig. S1A) increases survival rates of cecal ligation and puncture (CLP)-induced septic rats, appearing in a patent KR 10-0841408, but its molecular basis remains to be defined. HS-23 is further developed as a clinical candidate currently undergoing Phase I trial (KFDA, Identifier, HSP Injection_101 Version 2.00) in LPS-injected healthy human volunteers. In the current study, we investigated protective effects of HS-23 or its major constituents (CGA isomers) on *Escherichia coli* LPS-induced septic mortality in mice, and suggested the IRAK-4-catalyzed kinase activity as a molecular target.

2. Materials and methods

2.1. Materials

HS-23 was prepared from the water extracts of lonicerae flos (Fig. S1A). CGA, cryptochlorogenic acid (crypto-CGA), neochlorogenic acid (neo-CGA), loganin and vogeloside were isolated as major constituents from HS-23, and their chemical structures are shown in Fig. S1B. Primary and secondary antibodies used in this study were purchased from Santa Cruz Biotech (Santa Cruz, CA) or Cell Signaling Tech (Danvers, MA). Recombinant human (rh)IRAK-4 or rhTBK1 polypeptide was purchased from SignalChem (Parkwood Way Richmond, Canada). MD-2 polypeptide and ELISA kits were purchased from R&D Systems (Minneapolis, MN). All other chemicals including LPS from *E. coli* 0111:B4 were otherwise purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Experimental animals and treatment regimens

C57BL/6J mice were obtained from KRIBB (Ochang, Korea), and maintained in accordance with the conditions recommended in Guide for the Care and Use of Laboratory Animals. *E. coli* LPS was dissolved in PBS, and HS-23 or its major constituent in a vehicle (10% *N*,*N*-dimethylacetamide and 10% cremophor in PBS). Mice were intoxicated with an intraperitoneal injection (ip) of LPS (40 mg/kg), and then treated with an intravenous injection (iv) of HS-23 or its major constituent for *in vivo* experiments. Mice were also treated with HS-23 or its major constituent (ip) for 24 h, their macrophages were isolated from peritoneal cavity and then stimulated with LPS (100 ng/ml) for *ex vivo* experiments. Animal experiments were carried out following the protocols approved by Animal Experimentation Ethics Committee in CBNU institute.

2.3. Cell culture

Peritoneal macrophages from C57BL/6J mice were cultured in Dulbecco's modified Eagle's media containing 10% FBS, benzylpenicillin potassium (143 U/ml) and streptomycin sulfate (100 μg/ml) under 37 °C and 5% CO_2 atmosphere.

2.4. Measurement of inflammatory cytokines

Sera from LPS-injected mice (*in vivo*) or culture supernatants from LPS-activated mouse peritoneal macrophages (*ex vivo*) were loaded onto ELISA kits to determine TNF-α, IL-1β, HMGB-1 or IFN-β levels.

2.5. Measurement of LPS binding to MD-2

LPS (15 μg/ml) was immobilized to microplates and then incubated with MD-2 polypeptide (100 nM) for 2 h in cell-free reactions. After rinsing, microplates were added with anti-MD-2 antibody followed by horseradish peroxidase-labeled secondary antibody. The immune complex was reacted with *o*-phenylenediamine (2 mg/ml) containing 0.2% H_2O_2 for 20 min, and stopped with 1 N H_2SO_4. Absorbance values were then measured at 490 nm.

2.6. Immunoblotting analysis

Cell extracts were resolved on SDS–acrylamide gels by electrophoresis and transferred to a polyvinylidene difluoride membrane. Either 5% non-fat milk in PBS containing Tween 20 or 5% BSA in Tris-buffered saline containing Tween 20 was used as the blocking buffer. The blots were incubated at 4 °C overnight with primary antibody, and then reacted with appropriate horseradish peroxidase-labeled secondary antibody at room temperature for 3–5 h. The immune complex was visualized by reacting with an enhanced chemiluminescence reagent (GE Healthcare, Chalfont St. Giles, United Kingdom).

2.7. In vitro kinase assay

rhIRAK-4 (50 ng) or rhTBK1 polypeptide (120 ng) was reacted at 30 °C for 1 h with myeloid basic protein (MBP, 2 μg) as an exogenous substrate and [γ-^{32}P]ATP (5 μCi) as a probe. Aliquots of the reaction mixture were spotted onto P81 phosphocellulose papers, and washed three times with 0.75% phosphoric acid followed by one wash with 100% acetone. Radioactivity was then measured as count per min (cpm) using a liquid scintillation counter. The Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) of rhIRAK-4-catalyzed kinase activity were also determined by Lineweaver–Burk plot.

2.8. NO quantification

Peritoneal macrophages from C57BL/6J mice were stimulated with LPS (100 ng/ml), Pam3CSK4 (0.3 μg/ml) or CpG oligodeoxynucleotide (CpG ODN, 5 μM) for 20 h. Aliquots of the culture supernatant were reacted with 0.1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 5% phosphoric acid, and then measured absorbance values at 540 nm with $NaNO_2$ as a standard.

2.9. RT-PCR analysis

Total RNAs were subjected to RT-PCR using an RNA PCR kit (Bioneer, Daejeon, Korea). In brief, total RNAs were reversely transcribed at 42 °C for 1 h and then subjected to 25–30 cycles of PCR consisting of 30-s denaturation at 94 °C, 60-s annealing at 50–60 °C and 90-s extension at 72 °C. Nucleotide sequences of RT-PCR primers and the sizes of PCR products are described in Table S1. RT-PCR products were resolved on agarose gels by electrophoresis and visualized after staining with ethidium bromide.

2.10. Statistical analysis

Results are expressed as means ± standard deviation (SD), and these data were statistically evaluated using the ANOVA procedure followed by the Dunnett's test. Statistical values of $P < 0.05$ were considered significantly different.

3. Results

3.1. HS-23 or CGA isomers rescued septic mortality of LPS-challenged mice

C57BL/6J mice were intoxicated with LPS (ip) at a lethal dose (40 mg/kg), and then treated with HS-23 (iv). Upon exposure to LPS alone, mice were sacrificed by septic shock beginning at 12–16 h after LPS challenge, and none of them survived past 72 h (Fig. 1A and B). Co-treatment with HS-23 and LPS increased survival rates of septic mice, such that 70–80% of mice were alive at 72 h after LPS challenge in the HS-23 (3–10 mg/kg)-treated groups (Fig. 1A). Moreover, post-treatment with HS-23 (5 mg/kg, iv) at 1–3 h after LPS challenge prolonged survival rates of septic mice in a time-dependent manner (Fig. 1B). Major constituents of HS-23 were identified as CGA, crypto-CGA, neo-CGA, loganin and vogeloside (Fig. S1B). C57BL/6J mice were intoxicated with LPS (40 mg/kg, ip), immediately treated with each of the constituents (10 mg/kg, iv), and their survival rates were then observed at 72 h after LPS challenge. Treatment with CGA, crypto-CGA or neo-CGA increased survival rates of LPS-intoxicated septic mice, while those with loganin or vogeloside did not affect significantly (Fig. 1C).

To understand anti-inflammatory activity *in vivo*, we assessed whether HS-23 could affect cytokine levels in the blood of LPS-intoxicated mice. Upon exposure to LPS alone (ip), C57BL/6J mice dramatically increased TNF-α levels at 1 h after LPS challenge, IL-1β levels at 5 h, and HMGB-1 levels at 12 h as compared with the vehicle group (Fig. 1D–F). Co-treatment with HS-23 (iv) and LPS (ip) differentially attenuated TNF-α, IL-1β or HMGB-1 levels in the blood as compared with the LPS alone-challenged group (Fig. 1D–F).

3.2. HS-23 or CGA isomers directly inhibited IRAK-4 activity

HS-23 itself did not inhibit LPS binding to MD-2 polypeptide in cell-free reactions, in which the MD-2 antagonist lipid IVa was effective (Fig. 2A), excluding a direct effect of HS-23 on the receptor binding or LPS scavenging. To elucidate a molecular mechanism, we examined whether HS-23 could affect proximal signaling events in the cytoplasm after LPS binding to its receptor. C57BL/6 mice were treated with vehicle or HS-23 (ip) for 24 h, and peritoneal macrophages-isolated from the mice were then stimulated with LPS for *ex vivo* experiments. Upon exposure to vehicle followed by LPS alone, peritoneal macrophages markedly increased the autophosphorylation of IRAK-4 at Thr-345 and Ser-346 residues (Fig. 2B), which can be used as an active index in the stimulation of MyD88-dependent signal cascade [9,11]. However, we could not detect significant levels of the phosphor (p)-IRAK-4 in peritoneal macrophages elicited by vehicle or HS-23 itself without LPS stimulation (Fig. 2B), excluding possible contamination with endotoxin or other stimuli of TLR signaling in the vehicle or HS-23. Treatment with HS-23 inhibited LPS-induced autophosphorylation of IRAK-4 in the cells, whereas no significant change was observed in total levels of IRAK-4 (Fig. 2B).

To understand whether HS-23 could directly affect the catalytic activity of IRAK-4, we carried out the *in vitro* kinase assays. rhIRAK-

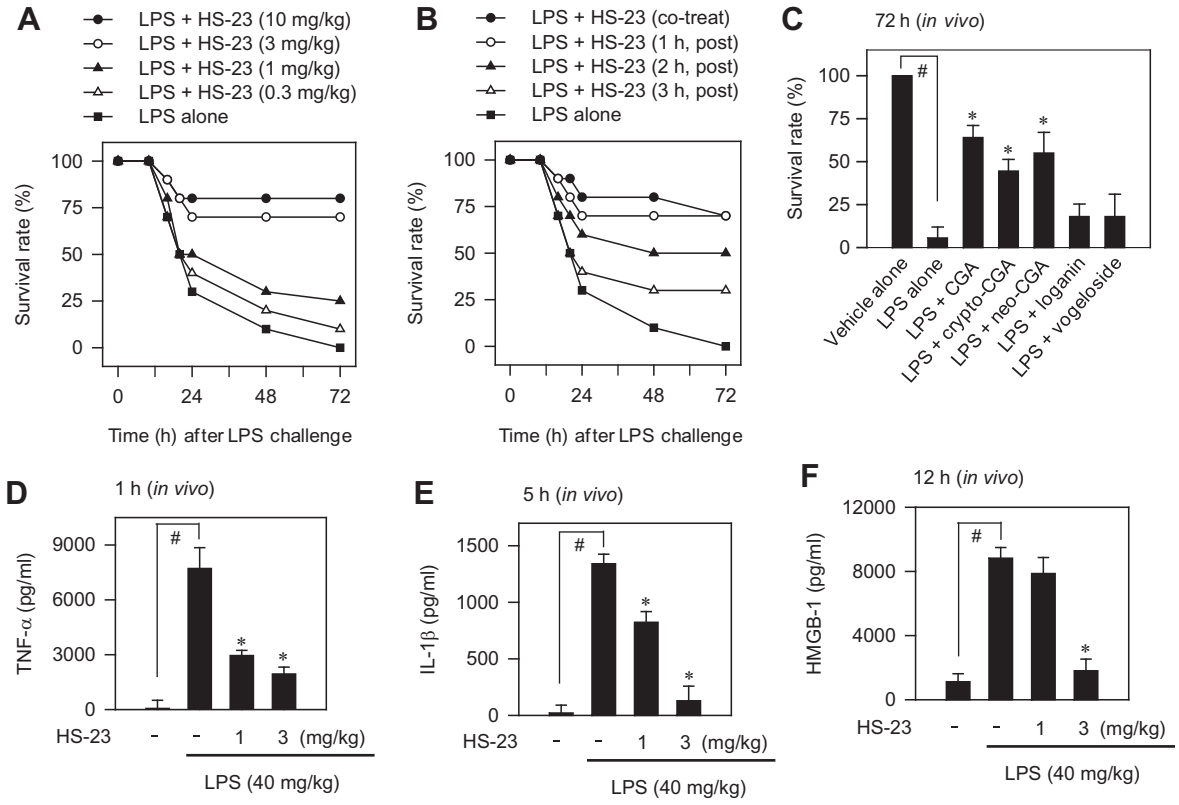

Fig. 1. Effects of HS-23 or its major constituents on survival rate of LPS-intoxicated mice. (A) C57BL/6J mice (each group, $n = 10$) were intoxicated with LPS (40 mg/kg, ip), and immediately treated with vehicle or HS-23 (iv). (B) C57BL/6J mice (each group, $n = 10$) were intoxicated with LPS (40 mg/kg, ip), and then treated with HS-23 (5 mg/kg, iv) at 1–3 h after LPS challenge. Survival rates of the mice were examined until 72 h after LPS challenge. Data are mean values from three independent experiments. (C) C57BL/6J mice (each group, $n = 10$) were intoxicated with LPS (40 mg/kg, ip), immediately treated with vehicle or each of the major constituents of HS-23 (10 mg/kg, iv), and their survival rates were then examined at 72 h after LPS challenge. Data are represented as means ± SD from three independent experiments. $^{\#}P < 0.05$ vs. vehicle alone-treated group. $^{*}P < 0.05$ vs. LPS alone-challenged group. C57BL/6J mice (each group, $n = 3$–5) were intoxicated with LPS (ip), immediately treated with vehicle or HS-23 (iv), and their bloods were collected at 1 h after LPS challenge (D), at 5 h (E), or at 12 h (F). Sera were then loaded onto ELISA kits to determine TNF-α, IL-1β or HMGB-1 levels.

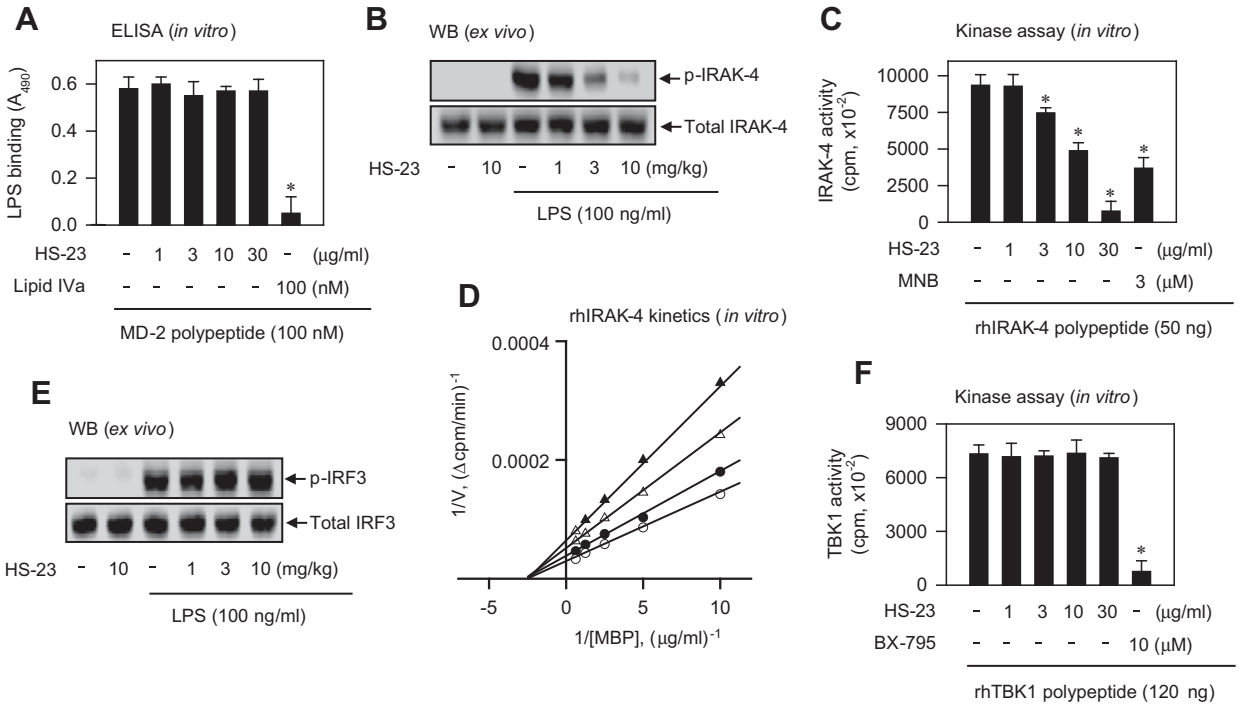

Fig. 2. Effect of HS-23 on LPS-induced activation of IRAK-4 or IRF3. (A) LPS (15 μg/ml) was immobilized to microplates, and then incubated with cell-free MD-2 polypeptide for 2 h in the presence of HS-23. LPS binding to the receptor component MD-2 was determined by ELISA. Data are represented as means ± SD from three independent experiments. *$P < 0.05$ vs. MD-2 alone-containing group. C57BL/6J mice were treated with vehicle or HS-23 (ip) for 24 h. Peritoneal macrophages were isolated from the mice, and then stimulated with LPS for 5–10 min (B) or 30–40 min (E). Cell extracts were subjected to Western blot analysis (WB) with anti-p-IRAK-4 or anti-IRAK-4 antibody (B) or with anti-p-IRF3 or anti-IRF3 antibody (E). rhIRAK-4 (C and D) or rhTBK1 (F) was treated with HS-23 for 10 min in cell-free reactions. *In vitro* kinase activity of rhIRAK-4 or rhTBK1 was then measured by monitoring the incorporation of [^{32}P] from probe [γ-^{32}P]ATP onto the exogenous substrate MBP. Data are represented as means ± SD from three independent experiments. *$P < 0.05$ vs. rhIRAK-4 or rhTBK1 alone-containing group. (D) Kinetic data of rhIRAK-4-catalyzed kinase activity are represented as mean values of 1/V, an inverse of the initial increase of cpm values per min (Δcpm/min), from three independent experiments with various concentrations of MBP. Symbols are in the absence (open circle) or presence of HS-23 at 1 μg/ml (solid circle), 3 μg/ml (open triangle) or 10 μg/ml (solid triangle).

4 was treated with HS-23 in cell-free reactions, and then reacted with MBP as an exogenous substrate and [γ-^{32}P]ATP as a probe. Treatment with HS-23 dose-dependently inhibited the rhIRAK-4-catalyzed kinase activity, in which the IRAK-4 inhibitor *N*-(2-morpholinylethyl)-2-(3-nitrobenzoylamido)-benzimidazole (MNB) was also effective (Fig. 2C). In a kinetic study, rhIRAK-4 itself exhibited the K_m value of 0.4 μg/ml and the V_{max} value of 30,260 Δcpm/min (Fig. 2D). Treatment with HS-23 dose-dependently decreased the V_{max} value of rhIRAK-4-catalyzed kinase activity but did not change the K_m value (Fig. 2D), suggesting that HS-23 exerted as a noncompetitive inhibitor with respect to the substrate MBP. Moreover, treatment with CGA, crypto-CGA or neo-CGA significantly inhibited LPS-induced autophosphorylation of IRAK-4 in mouse peritoneal macrophages as well as the kinase activity of rhIRAK-4 in cell-free reactions (Fig. S2A and B).

Endotoxin LPS also stimulates TRIF-dependent phosphorylation of IRF3 at Ser-396 residue via TBK1 [8,9]. Treatment with HS-23 affected neither LPS-induced phosphorylation of IRF3 in peritoneal macrophages nor the kinase activity of rhTBK1 in cell-free reactions, in which the TBK1 inhibitor *N*-(3-((5-iodo-4-((3-(2-thienylcarbonyl)amino)propyl)amino)-2-pyrimidinyl)amino)phenyl)-1-pyrrolidinecarboxamide (BX-795) was effective as expected (Fig. 2E and F).

3.3. HS-23 consequently inhibited LPS-induced NF-κB or AP-1 activating pathways

The inhibition of IRAK-4 activity should affect NF-κB activation and also AP-1 activation in LPS-stimulated macrophages, since IRAK-4 is located upstream from the branching point between the two pathways [8,9,11]. To prove a cross-relationship between IRAK-4 inhibition and the resultant cellular responses, C57BL/6J mice were treated with vehicle or HS-23 (ip) for 24 h, and peritoneal macrophages-isolated from the mice were then stimulated with LPS for *ex vivo* experiments. Upon exposure to vehicle followed by LPS alone, peritoneal macrophages markedly increased the phosphorylation of IκBα at Ser-32 and Ser-36 residues or NF-κB p65 at Ser-536 residue within 5–10 min, and then degraded cytoplasmic IκBα within 30–40 min (Fig. S3A–C), which are essentially requisite to NF-κB activation. Treatment with HS-23 inhibited LPS-induced phosphorylation of IκBα or NF-κB p65, as well as LPS-induced degradation of IκBα in peritoneal macrophages (Fig. S3A–C). To investigate the effect of HS-23 on the AP-1 activating pathway, we assessed LPS-induced phosphorylation of MAPKs or c-Jun as signal transducers downstream from IRAK-4 activation. Treatment with HS-23 also inhibited LPS-induced phosphorylation of ERK-1/2 at Thr-202 and Tyr-204 residues, p38 at Thr-180 and Tyr-182 residues or c-Jun at Ser-63 residue in peritoneal macrophages (Fig. S3D and E).

3.4. HS-23 down-regulated LPS-induced expression of inflammatory genes

We asked further whether HS-23 could affect LPS-induced expression of NF-κB- and AP-1-target genes. C57BL/6J mice were treated with vehicle or HS-23 (ip) for 24 h, and peritoneal macrophages-isolated from the mice were then stimulated with various TLR agonists such as LPS, Pam3CSK4 or CpG ODN for *ex vivo* experiments. Pam3CSK4 is a TLR1/2 agonist and CpG ODN as a TLR9 agonist, also promoting the activation of NF-κB or AP-1

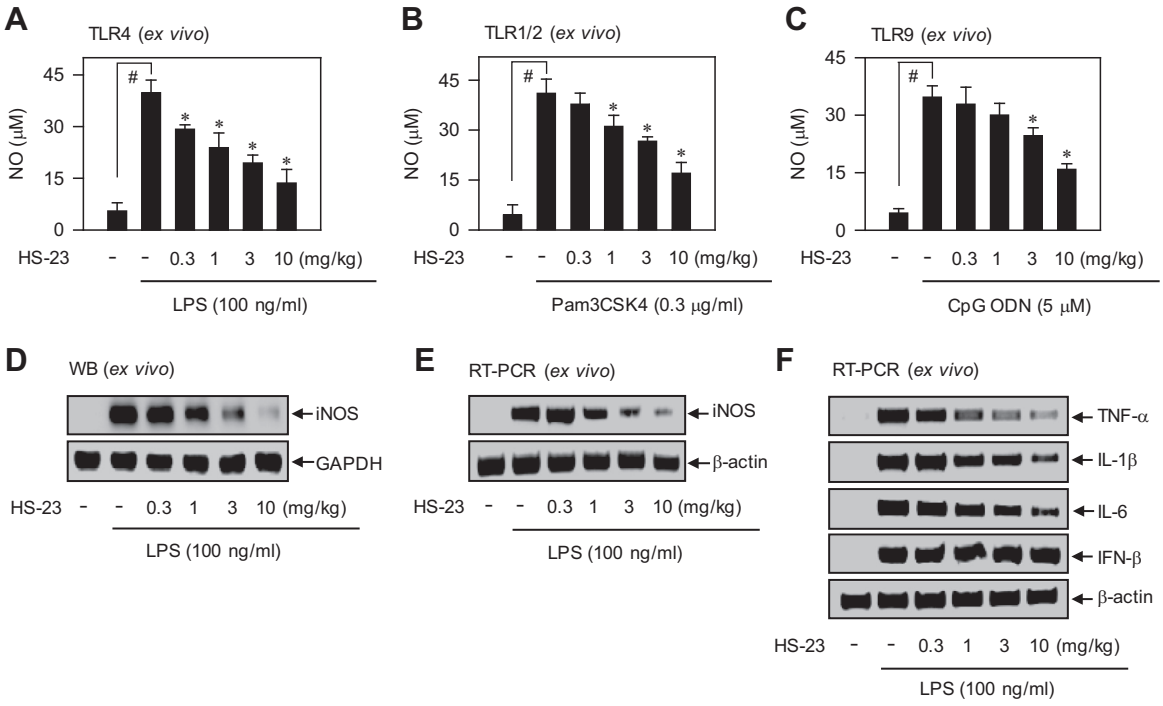

Fig. 3. Effects of HS-23 on TLR agonist-induced NO production and LPS-induced expression of inflammatory genes. C57BL/6J mice were treated with vehicle or HS-23 (ip) for 24 h. Peritoneal macrophages were isolated from the mice, and then stimulated with LPS (A), Pam3CSK4 (B) or CpG ODN (C) for 20 h. Aliquots of the culture supernatant were used to determine NO levels with $NaNO_2$ as a standard. Data are represented as means ± SD from three independent experiments. $^{\#}P < 0.05$ vs. vehicle plus media alone-treated group. $^{*}P < 0.05$ vs. vehicle plus each TLR agonist alone-treated group. C57BL/6J mice were treated with vehicle or HS-23 (ip) for 24 h. Peritoneal macrophages were isolated from the mice, and then stimulated with LPS for 4–6 h (E and F) or 20 h (D). Cell extracts were subjected to Western blot analysis (WB) with anti-iNOS or anti-GAPDH antibody (D). Total RNAs were subjected to RT-PCR analysis of iNOS (E) or cytokines (F) with β-actin as an internal control.

through MyD88 and IRAK-4 [10]. Treatment with HS-23 inhibited LPS-induced NO production, as well as Pam3CSK4- or CpG ODN-induced NO production in peritoneal macrophages (Fig. 3A–C). Protein levels of iNOS were barely detectable in resting peritoneal macrophages treated with vehicle alone, as determined by Western blot analysis, but markedly increased upon exposure to vehicle followed by LPS alone (Fig. 3D). Treatment with HS-23 decreased LPS-inducible protein levels of iNOS in the cells (Fig. 3D). Accordingly, HS-23 attenuated LPS-induced mRNA levels of iNOS, as determined by RT-PCR (Fig. 3E). Moreover, treatment with HS-23 differentially inhibited LPS-inducible protein levels of TNF-α or IL-1β in peritoneal macrophages (Table S2), as well as attenuated LPS-induced mRNA levels of TNF-α, IL-1β or IL-6 (Fig. 3F). However, HS-23 did not affect LPS-induced protein and mRNA levels of the IRF3-target gene, IFN-β (Table S2 and Fig. 3F). These results indicate that HS-23 can down-regulate LPS-induced expression of NF-κB/AP-1-target inflammatory genes, but not those under the control of IRF3.

4. Discussion

In the current study, lonicerae flos extract HS-23 rescued septic mortality of *E. coli* LPS-intoxicated mice. As proposed in Fig. S4, molecular target of HS-23 appeared to be IRAK-4 as a proximal signal transducer in the MyD88-dependent activating pathways of NF-κB or AP-1. HS-23 consequently down-regulated LPS-induced expression of NF-κB/AP-1-target genes encoding TNF-α, IL-1β or HMGB-1 *in vivo* under septic conditions and those such as TNF-α, IL-1β, IL-6 or iNOS in *ex vivo* experiments with peritoneal macrophages. Moreover, CGA, crypto-CGA and neo-CGA were assigned as major constituents of HS-23 in the protection against endotoxemia.

HS-23 is further developed as a clinical candidate currently undergoing Phase I trial in LPS-injected healthy human volunteers. It will be interesting to ascertain the clinical efficacy of HS-23 as an IRAK-4 inhibitor, translating from preclinical outcomes to sepsis patients. Physiological importance of IRAK-4 in response to endotoxin LPS has been revealed in the gene knock-out or -in mice. IRAK-4-null (IRAK-$4^{-/-}$) mice are completely resistant to LPS-induced septic shock, while wild-type mice succumb to shock and die [15,16]. IRAK-$4^{-/-}$ mice or IRAK-4 kinase inactive knock-in mice (IRAK-4 KI mice with a positional mutation leading to a loss of the kinase activity of IRAK-4) also protect against CpG ODN/galactosamine-induced shock [16]. Moreover, LPS-challenged IRAK-$4^{-/-}$ mice or IRAK-4 KI mice exhibit dramatically reduced levels of inflammatory cytokines (TNF-α, IL-1, IL-6) in the blood as compared with wild-type mice [15–17].

In the current study, major constituents of HS-23 were identified as CGA, crypto-CGA, neo-CGA, loganin and vogeloside. Treatment with CGA, crypto-CGA or neo-CGA protected against LPS-induced septic shock in mice, while those with loganin or vogeloside were not effective. Numerous anti-inflammatory properties of CGA had been previously reported but not those of crypto-CGA or neo-CGA yet. CGA ameliorates CLP-induced septic mortality and organ injury in mice, CCl_4-induced hepatic inflammation and fibrosis in rats, adjuvant-induced arthritis in rats, and carrageenan-induced paw edema in rats [18–21]. However, none of them suggest primary target molecule of CGA in the anti-inflammatory therapy. In the current study, CGA, crypto-CGA or neo-CGA directly inhibited the catalytic activity of IRAK-4 as did HS-23, suggesting that IRAK-4 can be considerable as a potential therapeutic target of endotoxemia or other inflammatory disorders.

Taken together, lonicerae flos extract HS-23 or its major constituents (CGA isomers) protected against *E. coli* LPS-induced systemic

inflammatory response evoking septic mortality in mice. The IRAK-4-catalyzed kinase activity is a molecular target of HS-23 or CGA isomers in the suppression of endotoxin LPS-induced expression of NF-κB/AP-1-target inflammatory genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.045.

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