A Citrus Flavonoid Hesperidin Suppresses Infection-Induced Endotoxin Shock in Mice

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Administration of a citrus flavonoid hesperidin (HES) to mice before LPS challenge significantly reduced tumor necrosis factor (TNF-α) production in a dose-dependent manner. Treatment of HES 3 h before intraperitoneal (i.p.) infection with 10⁸ CFU Salmonella typhimurium aroA resulted in rescue from lethal shock as similar to LPS-nonresponder mice. Not only bacterial numbers in livers and spleens but also plasma LPS levels significantly decreased by pretreatment with HES. In addition, HES markedly suppressed plasma levels of TNF-α and high mobility group box chromosomal protein 1 (HMGB-1), decreased the number of apoptotic cells in livers and normalized the activated states of blood coagulation factors such as prothrombin time and platelet numbers caused by infection. Pretreatment of LPS with HES suppressed the chromogenic Limulus reaction.

Key words Salomonella-infection; endotoxin shock; flavonoid; hesperidin; blood coagulation factor; Limulus reaction

Septic shock caused by Gram-negative bacteria is initiated by lipopolysaccharide (LPS), the main constituent of the outer membrane, and triggered by TNF-α which is a main representative among various types of cytokines produced by LPS-stimulated macrophages.¹⁻³ Thus, screenings for substances capable of inhibiting endotoxin shock have mainly focused on the suppression of TNF-α production. An inhibitory substance(s) from cinnamon bark has recently been shown to reduce TNF-α release by directly binding to the lipid A moiety of LPS.⁴ In a previous study,⁵ we showed that naringin, the main flavonoid of grapefruit, suppressed LPS-induced TNF-α production. Since sensitization with α-D-galactosamine (GalN) renders mice hypersensitive to TNF-α released by LPS-stimulated macrophages,⁶⁷ the inhibitory effect of naringin on lethal shock could convincingly be demonstrated in GalN-sensitized mice.⁵

Hesperidin (HES), a common constituent of citrus fruits, possesses significant inhibitory activities on inflammation, hypotension and analgesia,⁸⁻¹⁰ colony stimulating factor-inducing activity,¹¹ nitric oxide synthase inhibition,¹² and anti-apoptotic efficacy.¹³ Recently we established a new endotoxin shock model based on intraperitoneal (i.p.) infection with high numbers (10⁸ CFU) of attenuated Salmonella typhimurium and compared the effect of the antibiotics fosfomycin and imipenem.¹⁴ We describe in this study that pretreatment with HES, capable of reducing both LPS-elicited and infection-induced TNF-α production, inhibited infection-induced lethal shock, which resembles clinical cases.

MATERIALS AND METHODS

Mice Female BALB/c and C57BL/6 (B6) mice were purchased from Clea Japan Inc. (Tokyo, Japan). Breeding pairs of BALB/lps d mice were obtained from the Max-Planck-Institute for Immunobiology (Freiburg in Breisgau, Germany). All mice were kept at the animal facility of the Kitasato University School of Science under specific pathogen free conditions and fed autoclaved food and water. For infections all mice were housed in plastic cages with a filter set in a clean-air streamed box of a P-2 level room and fed autoclaved food and water. The experiments described in this study were performed in adherence with the National Institutes of Health guidelines on the use of experimental animals. Approval of the Animal Use Committee of the Kitasato University School of Science was obtained prior to initiating the experiments.

Chemicals, Glassware and Plastics Heparin (1000 units/ml, Novo Nordisk A/S, Denmark), Endospecy® (Seikagaku Corp., Tokyo), an endotoxin-free alkaline reagent consisting of 0.1 N KOH, 0.1% Triton X-100, 10 mM CaCl₂, 30 mM N,N’-bis(2-hydroxyethyl)glycine (Bicine), 0.07% ethyleneimine polymer and 0.1% polybrene (Seikagaku Corp.), the reference endotoxin standard of USP (Lot F) and Escherichia coli 0111:B4 (Lot 735900, Difco Laboratories, Detroit, U.S.A.) were used. A highly purified LPS preparation from Salmonella abortus equi was obtained from Dr. Chris Galanos (Max-Planck-Institute for Immunobiology). The stock solution (1 mg/ml) of S. abortus equi LPS dissolved in distilled water was clear. Working solutions were made with pyrogen-free saline (Otsuka Pharmaceutical Co., Tokushima, Japan). All glassware used in measurement of endotoxin was heated at 250°C for 2 h to eliminate potentially contaminating environmental endotoxin. Endotoxin-free plastic tips and plastic pipettes were purchased from Seikagaku Corp. and Corning Inc. (Corning, NY, U.S.A.), respectively. Hesperidin (HES): Wako Pure Chemical Industries, Osaka, Japan) was dissolved in dimethyl sulfoxide (DMSO: Wako Pure Chemical Industries) for Limulus test and suspended in saline or distilled water (Otsuka Pharmaceutical Co.) for in vivo experiments.

Bacteria and Inoculation An attenuated strain of S. typhimurium SL7207 aroA, obtained from Dr. Bruce Stocker (Stanford University Medical School, Stanford, CA) via Dr. Stephan Kaufmann (Max-Planck-Institute for Infection Biology, Berlin, Germany), was used. Bacteria were grown in tryptic soy broth (Nissui Pharmaceutical Co. Ltd., Tokyo) for...
18 h at 37 °C. After washing with phosphate buffered saline (PBS), bacterial numbers were calculated from a standard curve made based on turbidity and colony numbers counted. Mice were injected i.p. with bacterial suspension (5×10^8 CFU/ml) in a volume of 0.2 ml. The inoculum size was checked by plating diluted aliquots on tryptic soy agar (Difco Laboratories, Detroit, MI, U.S.A.).

**Preparation of Blood Samples** Following deep anesthesia with diethyl ether, blood was taken from mice by cardiac puncture using a 1-ml syringe with a 26G needle. For measuring TNF titers, endotoxin units and fibrinogen concentration, blood was immediately mixed with 20 μl heparin and then centrifuged at 10000×g for 1 min at room temperature. Plasma samples were collected carefully and used immediately or kept at −80 °C until use. For counting platelet numbers, blood samples (0.5-ml aliquots) were immediately mixed with 0.1 ml of 3.8% sodium citrate solution (Nipro Inc., Tokyo).

**Determination of TNF Titer** Plasma TNF titers were estimated by a cytotoxicity test using TNF-sensitive L929 (CSF6) cells in the presence of actinomycin D-mannitol as described previously. Four hours before the termination of cultures, aliquots (10 μl) of WST-1 reagents (Dojindo, Kumamoto, Japan) were added to each well of 96-well microplates. The resultant formation of formazan by viable cells was estimated by measuring absorbance at 450 nm (reference at 690 nm). The TNF titers in plasma samples were determined by multiplying the sample dilution and the value of a recombinant murine TNF-α (Genzyme Inc., Boston, MA, U.S.A.) giving 50% cytotoxicity.

**Determination of Endotoxin in Plasma** To eliminate the influence of plasma components on the Limulus cascade reactions, aliquots (20 μl) of plasma samples in endotoxin-free test tubes were treated with aliquots (80 μl) of an alkaline solution (Seikagaku Corp.) at 37 °C for 10 min. The treated samples were diluted serially at 1:10 with pathogen-free water (Otsuka Pharmaceutical Co.) and kept at 4 °C until testing. Endotoxin in the pretreated and diluted samples was determined quantitatively by an automated kinetic chromogenic assay using Endospecy® and a microplate well reader SK601 (Seikagaku Corp.). Aliquots (25 μl) of the pretreated and diluted samples were distributed in duplicate into a 96-well microplate and then mixed with 100 μl of Endospecy®. The mixture was incubated at 37 °C for 30 min. Absorbance at 405 nm per minute was measured during incubation and the mean EU value was calculated automatically.

**Western Blotting of HMGB-1** The presence of HMGB-1 in plasma was detected by Western blotting as described previously. Briefly, plasma samples were mixed with the sample buffer, heated in boiling water for 2 min and analyzed using a 12.5% gel by SDS-PAGE. After blotting, HMGB-1 was visualized using an anti-mouse polyclonal rabbit anti-HMGB-1 antibody (PharMingen), and a VECTASTAIN ABC-PO kit (Vector Laboratories Inc., Burlingame, CA, U.S.A.).

**Measurement of Platelet Numbers, Prothrombin Time (PT), Fibrinogen (Fib) and Fibrin Degradation Product (FDP) Concentration** Platelet numbers were differentially counted by a SS-3000 autoanalyzer (Sysmex Co., Tokyo). The PT, Fib and FDP concentration in plasma was measured by a Coagulex 100 autoanalyzer (International Reagent Co., Kobe, Japan).

**Histology** Liver samples of BALB/c mice 24 h after infection were fixed with 10% formalin in 0.01 M phosphate buffer (pH 7.4) and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE).

**Detection of Apoptotic Cells in Liver** Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using an in situ apoptosis detection kit from Takara Shuzo Co., Ltd., Kyoto, Japan. Staining of liver sections was performed according to the manufacturer’s recommendation.

**Determination of Antimicrobial Activity in Vitro** Freshly prepared bacteria as described above were suspended in fresh tryptic soy broth at different concentrations of HES or fosfomycin (FOF) (Meiji Seika Ltd., Tokyo) as a positive control. The optical density was measured at different time points during incubation.

**Observation of Peritoneal Exudate Cells (PEC) after Administration of HES** BALB/c mice were injected i.p. with either 1 mg HES in saline or saline alone in a volume of 0.3 ml. After 3 h PEC were recovered with Hank’s buffer solution, smeared on a glass slide and stained with May-Grunwald Giemsa. For each specimen 100 PEC were enumerated microscopically for differential cell counting.

**Statistical Analysis** Statistical significance of the data was determined by the Scheffe of post-hoc and log-rank tests or Student’s t-test. A p value of less than 0.05 was taken as significant.

**RESULTS**

**Suppressive Effect of HES on LPS-Induced TNF Production in Vivo** To evaluate whether HES can suppress LPS-induced TNF production in vivo, B6 mice were injected i.p. with the indicated dose of HES 3 h before an intravenous (i.v.) injection with 0.1 μg S. abortus equi LPS. HES significantly suppressed serum TNF-α production in a dose-dependent manner (Fig. 1).

**Inhibition of Infection-Induced Lethal Shock by HES** When female BALB/c mice were infected i.p. with 10^7 CFU of S. typhimurium aroA, lethal shock was caused in all infection-control mice within 42 h (Fig. 2). In contrast, LPS-non-

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![Fig. 1. Dose Effects of HES on LPS-Induced TNF Release](image-url)
responder BALB/lpsd mice survived during this period, proving that mortality was due to endotoxin shock. Pretreatment with HES resulted in rescue from infection-induced lethal shock at all doses (0.3, 1, 3 mg) tested, similar to the LPS-nonresponder mice.

Since LPS is the initiating molecule of endotoxin shock, we measured whether plasma LPS levels released during infection were decreased by treatment with HES. Plasma samples were collected at different time points from infected BALB/c mice with or without i.p. treatment with 1 mg HES 3 h before infection and evaluated by a chromogenic Limulus assay. Significantly lower levels of plasma LPS were seen in HES-treated mice compared to those of infection alone (controls) (Table 1).

To determine effects on a late mediator of endotoxin shock, the plasma levels of HMGB-1, a non-histon DNA-binding protein, were monitored by immunoblotting. Detectable amounts of plasma HMGB-1 were found in infection control mice 24 h after infection. Treatment with HES resulted in suppression of HMGB-1 release to plasma (Fig. 5).

Protective Effect of HES on Infection-Induced Liver Damage To determine potential protective effects of HES on infection-induced histological liver damage, livers were removed from three groups, i.e., untreated control mice (A), infection controls (B), and HES-pretreated and infected mice (C). In livers of infection controls, fibrin precipitation in the blood vessels caused severe thrombosis (Fig. 6B) and ultimately interruption of the blood flow (Table 2), resulting in severe liver damage due to necrotic death of cells supported by the affected vessels. HES treatment decreased fibrin precipitation and prevented thrombosis-dependent hepatocyte death. Moreover, a suppressive effect of HES on apoptosis in decreases in bacterial numbers were observed in both organs of HES-treated mice compared to infection control mice, whereas bacterial numbers in both organs 30 min after infection were almost identical. Representative data of bacterial growth in livers and spleens are shown in Fig. 4.

Suppressive Effect of HES on Infection-Induced TNF-α and HMGB-1 Release in Vivo In view of the central role of TNF-α as early mediators of endotoxin shock, infection-induced plasma TNF levels, determined by a bioassay using L929 cells, peaked at 90 min after infection, similar to LPS injection (data not shown). Treatment of mice with 1 or 3 mg HES 3 h before infection significantly lowered TNF-α levels compared with those of infection alone (controls) (Table 1).

Table 1. Inhibitory Effect of HES on Infection-Induced TNF-α Production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNF-α titer (ng/ml)</th>
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<tbody>
<tr>
<td>Infection alone</td>
<td>10.76±0.38</td>
</tr>
<tr>
<td>Infection+1 mg HES</td>
<td>5.67±0.00**</td>
</tr>
<tr>
<td>Infection+3 mg HES</td>
<td>2.55±0.60***</td>
</tr>
</tbody>
</table>

Results represent arithmetic mean±S.D. of 3 mice per group. **p<0.01 and ***p<0.001 vs. controls (by the Scheffe of post-hoc test).
liver of infected mice was observed. Frequency of apoptotic cells was estimated by counting 1000 liver cells in each specimen (Table 3).

**HES Normalized Impaired Coagulation Caused by Infection** To independently determine whether HES affects blood coagulation parameters such as PT, Fib and FDP concentration and platelet numbers, blood samples were collected 14 h after infection from BALB/c mice treated with 1 mg HES 3 h before infection. The PT values of infected controls increased markedly (more than 60 s), but were significantly reduced by administration of HES (Table 2). Fibrinogen levels decreased during infection and conversely were enhanced in HES-treated mice. Likewise, administration of HES was able to significantly block the depletion of platelets, which occurred in infection control mice. These results confirmed that treatment with HES could counteract coagulation-associated changes initiated by infection.

**Antimicrobial Activity of HES and Phagocytic Peritoneal Exudate Cells (PEC) after i.p. Administration of HES** For evaluation of potential antimicrobial activity of HES in vitro, *S. typhimurium* aroA bacilli were incubated for 5.5 h in tryptic soy broth containing 1 mg/ml of HES. HES did not show antimicrobial activity under these conditions. We therefore monitored phagocytic PEC after i.p. administration of HES. HES depleted macrophages but at the same time strongly increased the influx of neutrophils. To independently confirm the identity of neutrophils, cells were smeared, stained with May–Gruenwald Giemsa and counted microscopically. The granulocyte population increased from 10.0/μl (3.6% in control mice to 75.3/μl (17.8% in mice treated with HES 3 h earlier, whereas a declining trend for total monocytes was noted (73.7/μl (3.1 and 11.0/μl (9.9%). Lymphocyte ratios remained unaffected (13.7/μl (6.1 and 13.7/μl (8.1%).

**DISCUSSION**

The present study showed that pretreatment with HES could suppress infection-induced endotoxin shock in mice. We measured LPS levels in plasma and bacterial numbers in...
livers and spleens. Compared with those of infection controls, pretreatment with HES resulted in significant reduction of bacterial numbers during infection (Fig. 4), followed by significantly lower levels of plasma LPS (Fig. 3). Lower levels of plasma LPS than those in infection controls resulted in significant suppression of plasma TNF-α levels, an early mediator of endotoxin shock (Table 1). HMGB-1 levels in plasma of HES-pretreated mice also decreased compared with those of controls (Fig. 5). Although Wang et al. showed that HMGB-1 would be a late mediator in endotoxin shock because of its TNF-inducing activity, Hasunuma et al. showed that upregulation of HMGB-1 was not due to secretion by TNF-α-stimulated macrophages but due to release at the terminal stage from hepatocytes with severe damage. A significant decrease of apoptotic hepatocytes was observed in HES-treated mice (Table 3). Thus, lower levels of plasma HMGB-1 seems due to decreases in the number of severely damaged hepatocytes caused by treatment with HES. The downstream effects of HES on the blood coagulation system also appear to significantly contribute to the protection from endotoxin shock.

We demonstrated the inhibitory activity of HES on LPS-elicited Limulus reaction (Table 4). Although we have not yet determined the mechanism by which HES inhibits the Limulus activity of LPS, it is possible that one answer to the question of why HES could counteract endotoxin shock is its direct action on LPS. Future studies will have to address in detail by what mechanisms HES exerts these effects.

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