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IRAK4 as a Molecular Target in the Amelioration of Innate Immunity–Related Endotoxic Shock and Acute Liver Injury by Chlorogenic Acid

Sun Hong Park,* Seung-Il Baek,* Jieun Yun,* Seungmin Lee,* Da Young Yoon,* Jae-Kyung Jung,* Sang-Hun Jung,*† Bang Yeon Hwang,* Jin Tae Hong,* Sang-Bae Han,* and Younsoo Kim*

Mice lacking the IL-1R–associated kinase 4 (IRAK4) are completely resistant to LPS-induced endotoxic disorder or the TLR9 agonist CpG DNA plusα-galactosamine–induced acute liver injury (ALI), whereas wild-type strains succumb. However, translational drugs against sepsis or ALI remain elusive. Lonicerae flos extract is undergoing the clinical trial phase I in LPS-injected healthy human volunteers for sepsis treatment. In the current study, chlorogenic acid (CGA), a major anti-inflammatory constituent of lonicerae flos extract, rescued endotoxic mortality of LPS-intoxicated C57BL/6 mice, as well as ameliorated ALI of healthy human volunteers for sepsis treatment. In the current study, chlorogenic acid (CGA), a major anti-inflammatory constituent of lonicerae flos extract, rescued endotoxic mortality of LPS-intoxicated C57BL/6 mice, as well as ameliorated ALI of C3H/HeJ mice via directly affecting the kinase activity of IRAK4, a proximal signal transducer in the MyD88-mediated innate immunity that enhances transcriptional activity of NF-κB or AP-1. CGA consequently attenuated protein or mRNA levels of NF-κB/AP-1 target genes encoding TNF-α, IL-1α, IL-6, and high-mobility group box-1 in vivo under endotoxiaemia or ALI. Finally, this study suggests IRAK4 as a molecular target of CGA in the treatment of innate immunity–related shock and organ dysfunction following insult of various TLR pathogens from bacteria and viruses. The Journal of Immunology, 2015, 194: 000–000.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ALI, acute liver injury; CGA, chlorogenic acid; CLP, cecal ligation and puncture; GaN,α-galactosamine; HMG-B1, high-mobility group box-1; iNOS, inducible NO synthase; IP-10, IFN-γ–induced protein 10; irak4–/–, IL-1R–associated kinase 4 knockout; IRAK4, IL-1R–associated kinase 4; IRF3, IFN regulatory factor 3; Kr, Michaelis–Menten constant; MBP, myeloid basic protein; MD-2, myeloid differentiation protein 2; MNB, N-(2-morpholinoethyl)-2-(3-nitrobenzoyl)benzimidazole; rh, recombinant human; TAK1, TGF-β–activated kinase 1; TAK242, ethyl (6R)–[N-(2-chloro-4-fluorophenyl)sulfonyl]cyclohex-1-ene-1-carboxylate (TAK-242) are recently considered as the drug candidates completing clinical trials, phase II and III, in the treatment of sepsis patients (13, 14). Eritoran is a synthetic analog of lipid A from nonpathogenic Rhodobacter sphaeroides and antagonizes endotoxic LPS binding to MD-2 (13, 15). TAK-242 specifically interacts with the Cys747 residue on the intracellular domain of TLR4, thus blocking the recruitment of adaptor molecules for innate immunity (14, 16). Therapeutic index of MD-2 antagonist (eritoran) or TLR4 inhibitor (TAK-242) is limiting to the patients infected with Gram-negative bacteria. Unfortunately, eritoran or TAK-242 has failed to...
improve survival rates of patients with severe sepsis (13, 14). However, the failure of sepsis study suggests a need for trial design rethinking focused on more homogeneous populations or specific biomarkers to monitor patients under treatment (17). In particular, sepsis patients enrolled in the clinical trials are not selected on the basis of the presence of LPS in the circulation (13, 14), suggesting that there may be mixed with redundant systemic inflammatory response syndrome pathways other than the MD-2/TLR4. Therefore, novel drug targets responding to various TLR pathogens from Gram-positive bacteria and viruses are requisite in the combination treatment of septic disorders.

The cecal ligation and puncture (CLP) or bacterial LPS-intoxicated rodent models mimic many of the pathophysiological features of innate immunity-imbalanced septic disorders (18, 19).

Chlorogenic acid (CGA; Fig. 1A) protects against CLP-induced septic mortality and multiorgan injury in mice (20). We recently reported that CGA is a major anti-inflammatory constituent of lonicerae flos extract, which improves Escherichia coli LPS-induced endotoxicemia in mice (21). Thereby, the lonicerae flos extract is further developed as a drug candidate currently undergoing a phase I trial (Korea Food and Drug Administration, Identifier HSP Injection_101 Version 2.00) for sepsis treatment. Moreover, CGA ameliorates LPS-induced acute hepatotoxicity in α-galactosamine (GalN)–sensitized mice (22). This liver injury is also known as an innate immunity–related disorder, but differs from LPS-induced systemic endotoxic shock in that it involves a sudden hepatocellular apoptosis with minimal necrosis (23). In the current study, we focused on molecular mechanism of CGA in the amelioration of LPS-induced endotoxic shock and LPS plus GalN (LPS/GalN)–induced acute liver injury (ALI) in mice, suggesting IRAK4 as a therapeutic target against innate immunity–related shock or organ dysfunction following insult of various TLR pathogens from bacteria and viruses.

Materials and Methods

Materials

CGA-JK001 (Fig. 1B) with >97% purity was prepared from CGA by reacting with palladium on carbon under H2 gas infusion. Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or Cell Signaling Technology (Danvers, MA). Recombinant human (rh)MD-2 and ELISA kits to determine protein levels of TNF-α, IL-1α, IL-6, HMGB-1, IFN-β, or IP-10.

Cell culture

Mouse peritoneal macrophages were cultured in DMEM supplemented with 10% FBS, benzylenicillin (143 U/ml), and streptomycin (100 μg/ml) under an atmosphere of 37°C and 5% CO2.

Animal experiments

C57BL/6 or C57/B16 mice were obtained from Korea Research Institute of Bioscience & Biotechnology (Ochang, Korea). Mice were challenged with LPS (40 mg/kg, i.p.) or LPS (10 mg/kg, i.p.)/GalN (500 mg/kg, i.p.) and then treated with CGA (i.v.) for in vivo experiments. Mice were treated with CGA (i.p.) for 24 h. Peritoneal macrophages were isolated from the mice and then stimulated with LPS (30 ng/ml) or other agonist for ex vivo experiments. Mouse peritoneal macrophages were stimulated with LPS (30 ng/ml) in the presence of CGA for in vitro experiments. All animals received humane care in compliance with the protocols approved by the Animal Experimentation Ethics Committee of the Chungbuk National University institute.

Cytokine ELISA

Sera from mice or supernatants from macrophage cultures were loaded onto ELISA kits to determine protein levels of TNF-α, IL-1α, IL-6, HMGB-1, IFN-β, or IP-10.

Histological examination

Hepatic lobules were removed from mice and fixed in 10% formalin. Serial sections (3-μm thick) of the specimens were stained with H&E.

Western blot analysis

Cell extracts were resolved on SDS-acrylamide gels by electrophoresis and transferred to a polyvinylidene difluoride membrane. Either 5% nonfat milk in PBS containing Tween 20 or 5% BSA in TBS containing Tween 20 was used as the blocking buffer. The blots were incubated with primary Ab at 4°C overnight and then reacted with appropriate HRP-labeled secondary Ab at room temperature for 3–5 h. The immune complex was then reacted with an ECL reagent (GE Healthcare, Chalfont St. Giles, U.K.).

RT-PCR analysis

Total RNAs were subjected to RT-PCR with an RNA PCR kit (Bioneer, Daejeon, Korea) for determining mRNA levels of TNF-α, IL-1α, HMGB-1, IFN-β, IP-10, or TNF. Nucleotide sequences of the PCR primers were previously described (21). 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. The RT-PCR products were resolved on agarose gels by electrophoresis and stained with 1% ethidium bromide.

Measurement of LPS binding to MD-2

LPS (15 μg/ml) was immobilized to microplates and then incubated with rhMD-2 (100 nM) for 2 h. After rinsing, microplates were added with anti-MD-2 Ab followed by HRP-labeled secondary Ab. The immune complex was reacted with o-phenylenediamine (2 mg/ml) containing 0.2% H2O2 for 20 min and stopped with 1 N H2SO4. Absorbance values were then measured at 490 nm.

Confocal microscopy

Mouse peritoneal macrophages were fixed in 4% formalin, permeabilized with 0.5% Triton X-100, and then blocked with 1% BSA. For immunostaining, these cells were reacted with anti-p-IRAK4 or anti-NF-κB p65 Ab followed by Alexa Fluor 568-labeled secondary Ab. After nuclei staining with DAPI (3 μM), the cells were analyzed under a confocal fluorescence microscope.

In vitro kinase assay

rhIRAK4 or other protein kinase was reacted with myeloid basic protein (MBP; 0.33 mg/ml) or casein (2 mg/ml) as the exogenous substrate and [γ-32P]ATP (5 μCi) as the probe at 30°C for 1 h. Aliquots of the reaction mixture were spotted onto P81 phosphocellulose papers, washed three times with 0.75% H3PO4 followed by one wash with 100% acetone, and then measured the radioactivity as counts per minute. The Michaelis–Menten constant (Km) and maximal velocity (Vmax) of rhIRAK4-catalyzed MBP phosphorylation were determined by Lineweaver-Burk plot.

Chromatin immunoprecipitation assay

Chromatin fragments were precipitated with anti-NF-κB p65 Ab (2 μg) or anti-κ-Fos Ab (2 μg). The precipitated chromatin was subjected to quantitative real-time PCR. Nucleotide sequences of primer pairs are as follows: NF-κB p65 (−586/−468, TNF-α promoter), sense 5′-ATGCA-CACCTCCCAACCATCGAA-3′ and antisense 5′-CTTCTGGAAGCCTGGTCATAAAG-3′; NF-κB p50 (−208/−89, TNF-α promoter), sense 5′-TTCCTTGATGCTCCTGGTGTCC-3′ and antisense 5′-CCACCGAGATTCTGCGCAATG-3′; and internal control 18S RNA, sense 5′-GGGAGGCTGAGAAACCGG-3′ and antisense 5′-GGTGTGAGAGATTTGTTAT-3′.

NO quantification

Culture supernatants of mouse peritoneal macrophages were reacted with 0.1% sulfanilamide and 0.1% N-(1-naphthyl)ethylendiamine in 5% H3PO4 and then measured absorbance values at 540 nm with NaNO2 as a standard.

Statistical analysis

Results are expressed as mean ± SEM from at least three independent experiments. Data were statistically evaluated using the ANOVA procedure followed by the Dunnett test. The p values < 0.05 were considered as significantly different.
Results

**CGA rescued endotoxic LPS-challenged mice**

C57BL/6 mice were intoxicated with *E. coli* LPS at a lethal dose (40 mg/kg, i.p.) and then treated with CGA or other sample (i.v.). LPS alone-injected mice were sacrificed due to endotoxic shock, and <10% of them survived past 72 h (Supplemental Fig. 1A). Treatment with CGA at the same time as LPS challenge increased survival rates of septic mice up to ~70% at the dosage 3 mg/kg, as did TAK-242, whereas CGA-JK001 was not effective (Fig. 1C). CGA-JK001 is a dihydrogenated derivative of CGA (Fig. 1A, 1B), thus suggesting an important role of \( \alpha,\beta \)-unsaturated carbonyl moiety in the therapeutic index. Moreover, posttreatment with CGA or TAK-242 at 2–8 h after LPS challenge rescued endotoxic mice in a time-dependent manner (Fig. 1D). Therefore, CGA can improve LPS-induced endotoxemia with similar levels of effectiveness to TAK-242 in the comparison of dose responses and time-dependent kinetics.

We then examined cytokine levels in the blood of endotoxic mice. C57BL/6 mice were intoxicated with *E. coli* LPS (i.p.) and immediately treated with vehicle or CGA (i.v.). LPS alone-injected mice had markedly increased TNF-\( \alpha \) levels at 2 h after LPS challenge, IL-1\( \alpha \) or IL-6 levels at 5 h, and HMGB-1 levels at 12 h in the blood over the vehicle group. Treatment with CGA attenuated LPS-induced TNF-\( \alpha \), IL-1\( \alpha \), IL-6, or HMGB-1 levels in the blood (Table I). In another experiment, C57BL/6 mice were inoculated with whole *E. coli* (i.p.) and immediately treated with vehicle or CGA (i.v.). Treatment with CGA also decreased bacterial infection-induced TNF-\( \alpha \) release in the blood (Supplemental Fig. 1B), suggesting its anti-inflammatory action protecting from the cytokine storm in endotoxemia.

**CGA ameliorates ALI of LPS/GalN-challenged mice**

C57BL/6 mice were intoxicated with whole *E. coli* inoculated with whole *E. coli* in the blood (Table I). In another experiment, C57BL/6 mice were intoxicated with *E. coli* (i.p.) and immediately treated with vehicle or CGA (i.v.). LPS alone–injected mice were sacrificed due to endotoxic shock, and <10% of them survived past 72 h (Supplemental Fig. 1A). Treatment with CGA at the same time as LPS challenge increased survival rates of LPS/GalN-intoxicated mice, such that 50–80% of mice were alive at the dosages 3–10 mg/kg, in which silymarin was also effective (Fig. 1E). Moreover, treatment with CGA or silymarin ameliorated LPS/GalN-induced ALI, especially congestion in the hepatic lobules (Fig. 2A). We next assessed whether CGA could affect specific phosphorylation of NF-\( \kappa \)B p65 at Ser\(^{320}\) residue, c-Jun at Ser\(^{63}\) residue, or IRF3 at Ser\(^{396}\) residue as molecular markers enhancing each transcriptional activity (24, 25). Treatment with CGA inhibited LPS/GalN-induced phosphorylation of NF-\( \kappa \)B p65 or c-Jun but did not affect p-IRF3 levels in the liver lobules (Fig. 2B). Accordingly, treatment with CGA attenuated mRNA levels of NF-\( \kappa \)B/AP-1 target genes encoding TNF-\( \alpha \) and IL-1\( \alpha \) in the tissues (Fig. 2C).

**CGA interrupts MyD88-dependent early signal cascade**

CGA itself did not affect LPS binding to the receptor component rhMD-2 in cell-free reactions, whereas the antagonist lipid IVa inhibited (Fig. 3A), thus excluding a direct effect of CGA on LPS scavenging or antagonism. To elucidate a mechanism, we investigated whether CGA could affect proximal signaling events after agonist binding to its receptor. C57BL/6 mice were treated with vehicle or CGA (i.p.) for 24 h, and peritoneal macrophages derived from the mice were then stimulated with LPS. Upon exposure to vehicle followed by LPS alone, peritoneal macrophages from C57BL/6 mice markedly increased the autophosphorylation of IRAK4 at Thr\(^{345}\) and Ser\(^{346}\) residues (Fig. 3B), indicating that MyD88-mediated innate immunity was stimulated. Treatment with CGA inhibited LPS-stimulated autophosphorylation of IRAK4, as did N-(2-morpholinylethyl)-2-(3-nitrobenzoylamido)benzimidazole (MNB; dual inhibitor of IRAK4 and IRAK1), whereas CGA-JK001 was not effective (Fig. 3B). In another ex vivo experiment, treated with vehicle, CGA, or the hepatoprotective silymarin (i.v.). LPS/GalN alone–injected mice were sacrificed due to ALI, and <5% of them survived past 24 h (Supplemental Fig. 1A). Treatment with CGA increased survival rates of LPS/GalN-intoxicated mice, such that 50–80% of mice were alive at the dosages 3–10 mg/kg, in which silymarin was also effective (Fig. 1E). Moreover, treatment with CGA or silymarin ameliorated LPS/GalN-induced ALI, especially congestion in the hepatic lobules (Fig. 2A). We next assessed whether CGA could affect specific phosphorylation of NF-\( \kappa \)B p65 at Ser\(^{320}\) residue, c-Jun at Ser\(^{63}\) residue, or IRF3 at Ser\(^{396}\) residue as molecular markers enhancing each transcriptional activity (24, 25). Treatment with CGA inhibited LPS/GalN-induced phosphorylation of NF-\( \kappa \)B p65 or c-Jun but did not affect p-IRF3 levels in the liver lobules (Fig. 2B). Accordingly, treatment with CGA attenuated mRNA levels of NF-\( \kappa \)B/AP-1 target genes encoding TNF-\( \alpha \) and IL-1\( \alpha \) in the tissues (Fig. 2C).

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CGA also inhibited Pam3CSK4 (TLR1/2 agonist mimicking the tricatylated lipoprotein from Gram-positive bacteria), MALP-2 (TLR2/6 agonist as a diacylated lipoprotein from Mycoplasma fermentans), IL-1α-, or HMGB-1-stimulated autophosphorylation of IRAK4 in peritoneal macrophages from C3H/HeJ mice (Fig. 3C, 3D). However, LPS alone, TNF-α alone, or treatment with CGA did not induce or affect IRAK4 autophosphorylation in the cells (Fig. 3C, 3D). This insensitivity is attributable to a missense mutation disrupting TLR4 function in C3H/HeJ mice (26, 27) and suggests a bypass of IRAK4 in TNF-α-stimulated signal cascades.

For in vitro experiments with intact cells, peritoneal macrophages were isolated from C57BL/6 mice and then stimulated with LPS, ssRNA (TLR7 agonist mimicking the viral RNAs), or IL-1α in the presence of CGA. Treatment with CGA decreased LPS-induced IRAK4 autophosphorylation in the cells (Supplemental Fig. 2A, 2B, 2C). Therefore, CGA can resolve MyD88/IRAK4-dependent innate immunity in macrophages stimulated with various TLR agonists from bacteria and viruses or with even endogenous cytokines.

The inhibition of IRAK4 autophosphorylation should affect TAK1 activation in the MyD88-dependent early signal cascades for innate immunity, because IRAK4 is located upstream of TAK1.

### Table I. Effect of CGA on cytokine levels in the blood of LPS-challenged mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNF-α at 2 h (pg/ml)</th>
<th>IL-1α at 5 h (pg/ml)</th>
<th>IL-6 at 5 h (pg/ml)</th>
<th>HMGB-1 at 12 h (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle alone</td>
<td>136 ± 55</td>
<td>19 ± 43</td>
<td>104 ± 76</td>
<td>175 ± 198</td>
</tr>
<tr>
<td>LPS alone</td>
<td>8170 ± 473**</td>
<td>1820 ± 245**</td>
<td>7167 ± 316**</td>
<td>9542 ± 388*</td>
</tr>
<tr>
<td>LPS + CGA (0.3 mg/kg)</td>
<td>6280 ± 255**</td>
<td>1754 ± 146</td>
<td>6417 ± 592</td>
<td>8350 ± 414**</td>
</tr>
<tr>
<td>LPS + CGA (1 mg/kg)</td>
<td>4237 ± 342**</td>
<td>1004 ± 250**</td>
<td>4710 ± 267**</td>
<td>2580 ± 298**</td>
</tr>
<tr>
<td>LPS + CGA (3 mg/kg)</td>
<td>1527 ± 227**</td>
<td>726 ± 162**</td>
<td>2805 ± 208**</td>
<td>1071 ± 183**</td>
</tr>
<tr>
<td>LPS + CGA (10 mg/kg)</td>
<td>821 ± 149**</td>
<td>161 ± 134**</td>
<td>893 ± 165**</td>
<td>634 ± 396**</td>
</tr>
</tbody>
</table>

C57BL/6 mice were challenged with *E. coli* LPS (40 mg/kg, i.p.) and immediately treated with vehicle or CGA (i.v.). Blood was collected at 2, 5, or 12 h after LPS injection. Data are mean ± SEM from three independent experiments.

* p < 0.05 versus vehicle alone–injected group, ** p < 0.05 versus LPS alone–challenged group.

CGA directly inhibits the kinase activity of IRAK4 or IRAK1

To understand whether CGA could directly affect the catalytic activity of IRAK4 or IRAK1, we carried out in vitro kinase assays. Catalytically active rhIRAK4 or rhIRAK1 was treated with CGA in cell-free reactions and then reacted with MBP as an exogenous substrate.

**FIGURE 2.** Effect of CGA on ALI of LPS/GalN-challenged mice. C57BL/6 mice were intoxicated with *E. coli* LPS (10 μg/kg)/GalN (500 mg/kg) (i.p.) and immediately treated with vehicle, CGA, or silymarin (i.v.). (A) Hepatic lobules were removed from the mice, serially sectioned, and then stained with H&E. Original magnification ×100. (B) Cell extracts from the liver tissues were subjected to Western blot (WB) analysis with paired Abs against p-NF-κB p65 and total NF-κB p65, p-c-Jun and total c-Jun, or p-IRF3 and total IRF3. (C) Total RNAs from the liver tissues were subjected to RT-PCR to determine mRNA levels of TNF-α or IL-1α with the internal control β-actin. Data are mean ± SEM from three independent experiments. * p < 0.05 versus LPS/GalN alone–injected group.
substrate and [γ-32P]-ATP as the probe. Treatment with CGA inhibited the kinase activity of IRAK4 more efficiently than that of IRAK1, whereas CGA-JK001 was not effective (Fig. 5A, 5B). In a kinetic study, rhIRAK4 alone exhibited a $K_m$ value of 0.35 μM and a $V_{max}$ value of 24,560 Δcpm/min (Fig. 5C). Treatment with CGA decreased the $V_{max}$ value of rhIRAK4-catalyzed kinase activity but did not change the $K_m$ value (Fig. 5C), suggesting a noncompetitive mechanism with respect to cofactor ATP. However, CGA did not significantly affect the kinase activity of rhTAK1-TAB, rhJNK, or rhTBK1 in cell-free reactions, in which LLZ 1640-2, the JNK inhibitor SP 600125, or the TBK1 inhibitor BX-795 was effective (Fig. 5D–F).

**CGA consequently suppresses NF-κB or AP-1 activation**

The inhibition of IRAK4 or IRAK1 should affect MyD88-dependent activating pathways of NF-κB and AP-1 in LPS-stimulated macrophages, because IRAKs located upstream from the branching point between the two pathways (3, 8). C57BL/6 mice were treated with CGA (i.p.) for 24 h, and peritoneal macrophages derived from the mice were then stimulated with LPS for ex vivo experiments. Treatment with CGA sequentially inhibited LPS-induced phosphorylation of IkBα at Ser32 and Ser36 residues (Fig. 6A), IkBα degradation (Fig. 6B), nuclear import of NF-κB (Fig. 6C), and DNA-binding ability of NF-κB in the cells (Fig. 6D), which are requisite to NF-κB activation downstream from IRAKs (3, 28). In another experiment, peritoneal macrophages were isolated from C57BL/6 mice and stimulated with LPS or IL-1α in the presence of CGA for in vivo experiments. Treatment with CGA inhibited IkB kinase α/β phosphorylation as well as IkBα degradation in the IL-1α- or LPS-activated cells, as did MNB (Supplemental Fig. 3A, 3B), suggesting the involvement of IRAK4-catalyzed kinase activity for NF-κB activation (28).

To investigate the effect of CGA on the AP-1–activating pathway, C57BL/6 mice were treated with CGA (i.p.) for 24 h, and peritoneal macrophages derived from the mice were then stimulated with LPS for ex vivo experiments. Treatment with CGA inhibited LPS-induced phosphorylation of JNK1/2 at the Thr183 residue or p38 at Thr 180 residue (Fig. 7A), that of c-Jun at Ser63 residue (Fig. 7B), and DNA-binding ability of AP-1 in the cells (Fig. 7C).

**CGA downregulates expression of inflammatory genes**

We next examined whether CGA could affect LPS-induced expression of NF-κB/AP-1 target genes such as TNF-α, IL-1α, HMGB-1, and iNOS (29). C57BL/6 mice were treated with CGA (i.p.) for 24 h, and peritoneal macrophages derived from the mice were then stimulated with LPS. Treatment with CGA decreased E. coli LPS–induced protein or mRNA levels of TNF-α, IL-1α, and HMGB-1 in the cells (Fig. 8A–C). However, treatment with CGA did not affect E. coli LPS–induced expression of the IRF3 target genes encoding IFN-β or IP-10 (Fig. 8D–F). Moreover, treatment with CGA inhibited E. coli LPS–induced NO production in a dose-dependent manner (Fig. 9A) as well as decreased Pseudomonas aeruginosa LPS–, Salmonella typhimurium LPS–, or Serratia marcescens LPS–induced NO production (Fig. 9B–D). Accordingly, treatment with CGA attenuated E. coli LPS–induced protein and mRNA levels of iNOS (Fig. 9E, 9F). Taken together, CGA can downregulate LPS-induced expression of NF-κB/AP-1 target genes but not those under the control of IRF3.

**Discussion**

In the current study, CGA rescued endotoxic mortality of LPS-intoxicated mice and also ameliorated LPS/GalN-induced ALI in mice. As a molecular mechanism, CGA directly inhibited the kinase activity of IRAK4 or IRAK1 in cell-free reactions and thus interrupted MyD88-dependent activating pathways of NF-κB and AP-1 in macrophages. CGA consequently suppressed expression of NF-κB/AP-1 target genes encoding TNF-α, IL-1α, IL-6, or HMGB-1 in vivo under endotoxemia or ALI. However, CGA did...
not affect LPS binding to the receptor component MD-2, kinase activities of TAK1, JNK, and TBK1, or TRIF-dependent expression of IRF3 target genes encoding IFN-β and IP-10.

The pathophysiologic importance of IRAK4 or IRAK1 in response to TLR-mediated innate immunity has been revealed in gene knockout or -in mice. IRAK4 knockout (irak4\(^{-/-}\)) mice are

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**FIGURE 4.** Effect of CGA on TAK1 autophosphorylation and cell viability. C57BL/6 mice were treated with vehicle, CGA, or other sample (i.p.) for 24 h. Peritoneal macrophages were isolated from the mice and then stimulated with LPS (A), IL-1α (B), or TNF-α (C) for 10–20 min. Cell extracts were subjected to Western blot (WB) analysis with anti-p-TAK1 or anti-TAK1 Ab. (D) C57BL/6 mice were treated with vehicle or CGA (i.p.) for 24 h. Peritoneal macrophages derived from the mice were then incubated for 20 h in the absence or presence of LPS. Cell viability was analyzed by MTT method and is represented as absorbance values at 540 nm (A\(_{540}\)). Data are mean ± SEM from three independent experiments. *p < 0.05 versus LPS alone– or each cytokine alone–stimulated group.

**FIGURE 5.** Effect of CGA on the kinase activity of IRAK4 or other protein kinase. Catalytically active rhIRAK4 (A), rhIRAK1 (B), rhTAK1-TAB (D), rhJNK (E), or rhTBK1 (F) was treated with CGA for 10 min in cell-free reactions. In vitro kinase activity was then monitored by the incorporation of [\(^{32}\)P] from the probe [\(^{32}\)P]-ATP onto MBP or casein as an exogenous substrate. Data are mean ± SEM from three to five independent experiments. (C) Kinetic data of rhIRAK4-catalyzed MBP phosphorylation are represented as mean 1/V, an inverse of the initial increase of cpm values per min (Δcpm/min), from three independent experiments with variable concentrations of ATP. *p < 0.05 versus each protein kinase alone–containing group.
completely resistant to LPS-induced endotoxic shock or CpG ODN (TLR9 agonist mimicking the viral or bacterial unmethylated CpG DNA) plus GalN (CpG ODN/GalN)–induced ALI, whereas wild-type mice succumb and die (30, 31). The irak4<sup>−/−</sup> mice have virtually no production of TNF-α, IL-1, or IL-6 in the blood following intoxication with LPS or CpG ODN/GalN (30). Moreover, inactive IRAK4 knockin mice (irak4<sup>KN/KN</sup>, a missense mutation in the loss of kinase activity) elude LPS- or CpG ODN/GalN-induced shock (31). In another proof of concept, irak1<sup>−/−</sup> mice are partially resistant to LPS-induced endotoxic shock (32). The irak1<sup>−/−</sup> mice show significantly but not completely decreased TNF-α or IL-6 levels in the blood following intoxication with LPS or IL-1β as compared with those in wild-type mice (33).

Anti-inflammatory properties of CGA have previously reported in numerous rodent models. CGA rescues CLP-induced septic mortality and multiorgan injury in mice, in which it suppresses TNF-α, IL-1β, IL-6, or HMGB-1 levels in the blood but enhances bacteria clearance (20). CGA ameliorates LPS-induced hepatic injury in mice with decreased TLR4 or TNF-α production in liver tissues (22), as well as LPS-induced pulmonary injury with attenuated leukocyte infiltration into the airway alveolar fluid (34). CGA reduces CCl4-induced hepatic inflammation and fibrosis in rats, in which it inhibits NF-κB–activating pathways such as IκBα phosphorylation, IκBα degradation and nuclear import of NF-κB downregulates expression of TNF-α, IL-1β, IL-6, or iNOS in liver tissues (35). CGA also protects against adjuvant-induced arthritis in rats with decreased TNF-α or IL-1β production in knee joints (36).

**FIGURE 6.** Effect of CGA on NF-κB–activating pathway. C57BL/6 mice were treated with vehicle or CGA (i.p.) for 24 h. Peritoneal macrophages were isolated from the mice and then stimulated with LPS for 10–20 min (A), 30–40 min (B), or 1 h (C and D). Cell extracts were subjected to Western blot (WB) analysis with anti-p-IκBα or anti-IκBα Ab (A) and anti-IκBα or anti-GAPDH Ab (B). (C) The cells were subjected to confocal fluorescence microscopy, displaying the NF-κB p65 stained with Alexa Fluor 568–labeled Ab as a red color and the nuclei stained with DAPI as a blue color. Original magnification ×400. (D) Chromatin fragments were precipitated with anti–NF-κB p65 Ab followed by quantitative real-time PCR with specific primers targeting to the TNF-α promoter region (−586/−468). Data are mean ± SEM from three independent experiments. *p < 0.05 versus LPS alone–stimulated group. ChIP, chromatin immunoprecipitation.

**FIGURE 7.** Effect of CGA on AP-1–activating pathway. C57BL/6 mice were treated with vehicle or CGA (i.p.) for 24 h. Peritoneal macrophages were isolated from the mice and then stimulated with LPS for 30–40 min (A and B) or 1 h (C). Cell extracts were subjected to Western blot (WB) analysis with paired Abs against p-JNK1/2 and total JNK1/2 or p-p38 and total p38 (A) and those against p–c-Jun and total c-Jun (B). (C) Chromatin fragments were precipitated with anti–c-Fos Ab followed by quantitative real-time PCR with specific primers targeting to the TNF-α promoter region (−2208/−89). Data are mean ± SEM from three independent experiments. *p < 0.05 versus LPS alone–stimulated treatment group. ChIP, chromatin immunoprecipitation.
However, none of the previous findings have indicated primary target molecule of CGA in the treatment of diverse inflammatory disorders. In the current study, CGA directly inhibited the kinase activity of IRAK4 in a noncompetitive mechanism with respect to cofactor ATP. Moreover, CGA attenuated Gram-negative bacteria LPS–, Gram-positive bacteria TLR agonist–, viral TLR agonist–, IL-1α–, or HMGB-1–stimulated IRAK4 autophosphorylation as well as downregulated expression of NF-κB/AP-1 target genes, thus protecting from the cytokine storm under endotoxemia or ALI. Therefore, CGA can resolve MyD88/IRAK4-dependent innate immunity in response to various TLR pathogens, whereas the MD-2/TLR4 inhibitor eritoran or TAK-242 is confined to the sepsis patients infected with Gram-negative bacteria. Finally, this study suggests a pharmacological importance of IRAK4 in the treatment of innate immunity–related shock, organ dysfunction, or inflammatory disorders following insult of various TLR pathogens from not only Gram-negative and Gram-positive bacteria, but also viruses, which is a translational strategy of drug discovery from the proof of concept in gene knockout or -in mice.

**FIGURE 8.** Effect of CGA on LPS-induced cytokine expression. C57BL/6 mice were treated with vehicle or CGA (i.p.) for 24 h. Peritoneal macrophages were isolated from the mice and then stimulated with LPS for 4–6 h (C and F) or 20 h (A, B, D, and E). Aliquots of the culture supernatants were loaded onto ELISA kits to determine protein levels of TNF-α (A), IL-1α (B), IFN-β (D), or IP-10 (E). Total RNAs were subjected to RT-PCR to determine mRNA levels of NF-κB/AP-1 target genes (C) or IRF3 target genes (F) with the internal control β-actin. Data are mean ± SEM from three independent experiments. *p < 0.05 versus LPS alone–stimulated group.

**FIGURE 9.** Effect of CGA on LPS-induced NO production or iNOS expression. C57BL/6 mice were treated with vehicle or CGA (i.p.) for 24 h. Peritoneal macrophages were isolated from the mice and then stimulated with various LPS stocks from *E. coli* (A), *P. aeruginosa* (B), *S. typhimurium* (C), or *S. marcescens* (D) for 20 h. Aliquots of the culture supernatants were used to determine NO levels with NaNO₂ as a standard. C57BL/6 mice were treated with vehicle or CGA (i.p.) for 24 h. Peritoneal macrophages were isolated from the mice and then stimulated with *E. coli* LPS for 6 h (F) or 20 h (E). (E) Cell extracts were subjected to Western blot (WB) analysis with anti-iNOS or anti-GAPDH Ab. (F) Total RNAs were subjected to RT-PCR to determine mRNA levels of iNOS with the internal control β-actin. Data are mean ± SEM from three independent experiments. *p < 0.05 versus each LPS alone–stimulated group.
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Disclosures
The authors have no financial conflicts of interest.

References
SUPPLEMENTAL FIGURE 1. Time-dependent survival rates of mice under endotoxemia or ALI, and effect of CGA on bacterial infection-induced TNF-α release. (A) C57BL/6 mice were challenged with LPS (i.p.) for endotoxic shock or LPS/GalN (i.p.) for ALI, and their survival rates were scored in time-dependent manners. (B) C57BL/6 mice were injected with E. coli (3-5 x 10^6 cells/mouse, i.p.) and immediately treated with CGA (i.v.). Sera were then loaded onto ELISA kits to determine protein levels of TNF-α. Data are mean ± SEM from three independent experiments. *P < 0.05 vs. whole E. coli alone-injected group.
SUPPLEMENTAL FIGURE 2. Effect of CGA on TLR agonist- or IL-1α-induced IRAK4 autophosphorylation in intact cells. Peritoneal macrophages-derived from C57BL/6 mice were pretreated with CGA or the IRAK1/4 inhibitor MNB for 2 h and stimulated with LPS (30 ng/ml), ssRNA or IL-1α for 5-10 min in the presence of CGA or MNB. (A) The cells were subjected to confocal fluorescence microscopy, displaying the p-IRAK4-stained with Alexa Fluor 568-labelled antibody as a red color and the nuclei-stained with DAPI as a blue color. (B, C) Cell extracts were subjected to Western blot analysis (WB) with anti-p-IRAK4 or anti-IRAK4 antibody.
SUPPLEMENTAL FIGURE 3. Effects of CGA on IL-1α- or LPS-induced IKKα/β phosphorylation and IκBα degradation. Peritoneal macrophages-derived from C57BL/6 mice were pretreated with CGA or the IRAK1/4 inhibitor MNB for 2 h and stimulated with IL-1α or LPS for 30-40 min in the presence of CGA or MNB. (A) Cell extracts were subjected to Western blot analysis (WB) with anti-p-IKKα/β or anti-IKKα/β antibody. (B) Cell extracts were subjected to WB with anti-IκBα or anti-GAPDH antibody.