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Caffeoylglycolic acid methyl ester, a major constituent of sorghum, exhibits anti-inflammatory activity *via* the Nrf2/heme oxygenase-1 pathway†

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Sorghum contains diverse pharmacologically active phytochemicals including tannins, phenolic acids and anthocyanins. In the present study, we show that caffeoylglycolic acid methyl ester (CGME), a major constituent of the grains of *Sorghum bicolor*, exerted anti-inflammatory effects by inducing HO-1 expression. Treatment of RAW264.7 cells with CGME induced HO-1 protein and mRNA expression. CGME increased nuclear translocation of nuclear factor-E2-related factor 2 (Nrf2) and knockdown of Nrf2 by siRNA blocked CGME-mediated HO-1 induction. SP600125 (a JNK inhibitor) or LY294002 (a PI3K inhibitor) blocked CGME-induced HO-1 expression and nuclear translocation of Nrf2, suggesting that CGME induces HO-1 expression *via* activating Nrf2 through a PI3K and JNK pathway. Consistent with the notion that HO-1 has anti-inflammatory properties, CGME inhibited the production of nitric oxide (NO), prostaglandin E₂ (PGE₂) and interleukin-6 (IL-6) as well as the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and IL-6 in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. CGME also protected C57BL/6 mice from LPS-induced mortality. However, inhibition of HO-1 abrogated the inhibitory effects of CGME on the production of NO, COX-2 and IL-6 in LPS-stimulated RAW264.7 cells. Taken together, these findings suggest that CGME exerts an anti-inflammatory effect through the Nrf2/HO-1 pathway, and may be a potential HO-1 inducer for preventing or treating inflammatory diseases.

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Introduction

Inflammation is a central feature of many pathophysiological conditions in response to tissue injury and as a host defense against invading microbes. Macrophages are one type of critical immune cells in the regulation of inflammatory responses. Activated macrophages secrete a number of different inflammatory mediators, including tumor necrosis factor- α , interleukin-6 (IL-6), prostaglandin E₂ (PGE₂), nitric oxide (NO) and reactive oxygen species.¹ Excessive or unregulated production of these mediators has been implicated in mediating or exacerbating a number of diseases including rheumatoid arthritis, osteoarthritis, sepsis, and also carcinogenesis.²

Heme oxygenases (HOs) catalyze the oxidation of heme to the products carbon monoxide, biliverdin, and ferrous iron. Two distinct variants of HOs have been described in humans

and rodents, each encoded by a different gene: HO-2, which is constitutively expressed, and HO-1, which is potently induced in many cell types by heme, inflammatory cytokines, and oxidative stress-related factors.³ HO-1 expression is regulated mainly at the transcriptional level through the signaling pathways involving phosphoinositide 3-kinase (PI3K)/AKT and mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase-1/2 (ERK1/2) and p38 kinase.^{4,5} The transcription factor nuclear factor-E2-related factor 2 (Nrf2) plays a predominant role in HO-1 expression.^{5,6} HO-1 participates in maintaining cellular homeostasis and plays an important protective role in tissues to reduce oxidative injury and attenuate the inflammatory response.^{5,6} The protective actions of HO-1 are thought to be mediated by the by-products of its enzymatic activity, bilirubin and carbon monoxide, which have antioxidant, anti-inflammatory, anti-apoptotic, and anti-mitogenic potential.^{7,8} HO-1 expression or carbon monoxide treatment inhibits the production of inflammatory cytokines and chemokines induced by inflammatory mediators in activated macrophages.^{9–12} An increasing number of therapeutic agents have been reported to exert their anti-inflammatory effects by inducing HO-1.^{13–16} Therefore, targeting HO-1 with natural phytochemicals may be

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a valuable strategy for preventing or treating inflammatory diseases.¹⁷

The grains of *Sorghum bicolor* (L.) Moench are rich sources of caffeoylglycerols such as 1-*O*-caffeoylglycerol (1-*O*-CG) and caffeoylglycolic acid methyl ester (CGME).¹⁸ CGME is reported to possess antioxidant and human immunodeficiency virus integrase inhibitory activity, and also exhibit inhibitory activity against LPS-induced NO production in RAW264.7 cells.^{18–20} However, the anti-inflammatory effects of CGME and its underlying mechanisms are not elucidated well. In the present study, we identified CGME and 1-*O*-CG as potent inducers HO-1 expression from *S. bicolor*. We here show that CGME inhibits LPS-induced inflammatory responses *in vitro* and *in vivo* by inducing HO-1 *via* activating Nrf2 pathway.

Materials and methods

Cell culture

RAW264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's Modified Essential Medium supplemented with penicillin (100 units per ml)–streptomycin (100 µg ml⁻¹) and 10% heat-inactivated fetal bovine serum (Cambrex, Charles City, IA, USA). The cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C.

Reagents

SB203580, SP600125, U0126, and LY294002 were purchased from Calbiochem (San Diego, CA, USA). Tin protoporphyrin IX (SnPP) and copper protoporphyrin IX (CuPP) were obtained from Porphyrin Products Inc. (Logan, UT, USA). Cyclohexamide, actinomycin D, *N*-acetyl-L-cysteine (NAC) and anti- α -tubulin antibodies were purchased from Sigma (St. Louis, MO, USA). Antibodies to HO-1, iNOS, Nrf2, poly ADP-ribose polymerase (PARP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa 546-conjugated goat anti-mouse and DAPI (4',6'-diamidino-2-phenylindole) from Invitrogen (Molecular Probes, Eugene, OR, USA). PGE₂ ELISA kit was from R&D systems (Minneapolis, MN, USA). ELISA kit for IL-6 was from BioLegend (San Diego, CA, USA).

Isolation of caffeoylglycolic acid methyl ester and 1-*O*-caffeoylglycerol

CGME and 1-*O*-CG was isolated from the grains of *Sorghum bicolor* (L.) Moench var. *hwanggeumchal* as previously described.¹⁸ CGME and 1-*O*-CG were obtained as white amorphous powders, and showed [M]⁺ peaks at *m/z* 252 and 254, respectively, by electron ionization-mass spectrometry. Their structures are shown in Fig. 1. The purity of CGME and 1-*O*-CG was checked by ¹H and ¹³C nuclear magnetic resonance spectra, and their spectra showed highly pure signals without any other impurities (ESI 1†). CGME and 1-*O*-CG were solubilized in 100% dimethyl sulfoxide and used at a final concentration of less than 0.05% dimethyl sulfoxide.

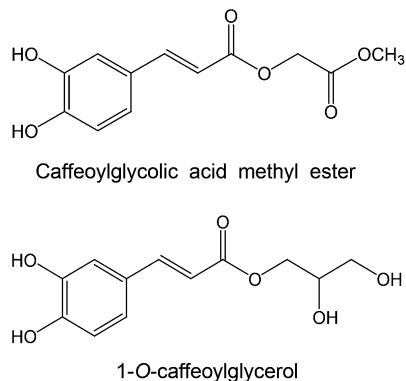


Fig. 1 Chemical structures of caffeoylglycolic acid methyl ester and 1-*O*-caffeoylglycerol.

Measurement of NO, PGE₂, and IL-6, and the cell viability assay

RAW264.7 cells were seeded in 24-well plates at 5×10^5 cells per well. The plates were pretreated with various concentrations of the test compounds for 30 min and then incubated for another 24 h with or without 1 µg ml⁻¹ of LPS. In some experiments, SnPP or CuPP was added to the plates together with the test compounds. Nitrite concentration in the culture supernatant was measured by the Griess reaction. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite. The amounts of IL-6 and PGE₂ in the culture supernatant were measured by immunoassay kits according to the manufacturer's protocol. Cell viability was measured with a MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide]-based colorimetric assay.

Measurement of bilirubin

RAW264.7 cells were seeded in 6-well plates at 10^6 cells per well. The next day, the medium of the wells was changed and the cells were incubated with various concentrations of CGME for 24 h. The amounts of bilirubin in the culture supernatant were measured by QuantiChrom™ Bilirubin Assay Kit according to the manufacturer's protocol (BioAssay Systems, CA, USA).

Measurement of intracellular reactive oxygen species (ROS) level

The intracellular ROS level was monitored utilizing 2',7'-dichlorofluorescein diacetate (DCF-DA) according to a protocol provided by Invitrogen. Raw264.7 cells were pre-loaded for 30 min with PBS containing 25 µM DCF-DA in the dark. Subsequently, the dye was removed and RAW264.7 cells were incubated with various concentrations of CGME at 37 °C for 30 min in the dark. Fluorescence was analyzed at an excitation wavelength of 480 nm and an emission wavelength of 535 nm by a Synergy MX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). The ROS level was expressed as DCF-DA fluorescence.

Western blot analysis

To prepare whole cell lysates, cells were lysed with a buffer [50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g ml}^{-1}$ pepstatin A, 10 $\mu\text{g ml}^{-1}$ aprotinin, 2 mM benzamidine, 50 mM NaF, 5 mM sodium orthovanadate, and 150 mM NaCl]. Nuclear and cytoplasmic fractions were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Fisher Scientific, Rockford, IL, USA) following the manufacturer's instruction. Equal amounts of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-P membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were blocked with 5% skim milk at room temperature for 1 h, and then incubated for 2 h with primary antibodies (1 : 1000 dilution). After washing, membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (1 : 2000 dilution). The signal was detected using an enhanced chemiluminescence system (Intron, Seongnam, Korea).

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR)

The cells were harvested and total RNA was isolated using RNeasy Mini Kits according to the manufacturer's instructions (Qiagen, Santa Clarita, CA, USA). One μg of total RNA was used to synthesize first strand cDNA using a RT-PCR kit (Invitrogen, Carlsbad, CA, USA). The following primers were used. HO-1, 5'-CGC AAC AAG CAG AAC CCA-3' (sense) and 5'-TGA CGC CAT CTG TGA GGG-3' (antisense). The cDNA for β -actin was also amplified as a control in a similar way using the following primers: 5'-GTG GGG CGC CCC AGG CAC CA-3' (sense) and 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3' (antisense). The following conditions were used for PCR amplification: 94 °C for 5 min for 1 cycle, and then 94 °C for 1 min, 56 °C for 30 s and 72 °C for 1 min for 27 cycles. Real-time qPCR amplification was carried out using TOPreal qPCR 2 \times PreMIX (SYBR Green, Enzynomics, Daejeon, Korea) and Rotor-Gene Q real-time PCR cycler (Qiagen). The following primers were used. iNOS, 5'-GGC AAA CCC AAG GTC TAC GTT-3' (sense) and 5'-TCG CTC AAG TTC AGC TTG GT-3' (antisense); COX-2, 5'-TGA GTA CCG CAA ACG CTT CT-3' (sense) and 5'-CTC CCC AAA GAT AGC ATC TGG-3' (antisense); IL-6, 5'-TCC ATC CAG TTG CCT TCT TGG-3' (sense) and 5'-CCA CGA TTT CCC AGA GAA CAT G-3' (antisense); β -actin, 5'-GGG AAA TCG TGC GTG ACA TCA AAG-3' (sense) and 5'-AAC CGC TCG TTG CCA ATA GT-3' (antisense). Optimized real-time PCR conditions were 95 °C for 10 min followed by 40 cycles at 95 °C for 10 s and 60 °C for 15 s and 72 °C for 20 s. All reactions were performed in triplicate and β -actin was used as an internal control. Quantification of relative gene expression was analyzed using the 2- $\Delta\Delta\text{Ct}$ method.

Small interfering RNA and transfection

Scrambled small interfering RNA (siRNA) and Nrf2 siRNA were purchased from OriGene Technologies (Rockville, MD, USA). RAW264.7 cells were seeded in 60 mm plates. After 12 h

incubation, the cells were transfected with Nrf2 siRNA or scrambled siRNA using Fugene HD according to the manufacturer's instructions (Promega, Madison, WI, USA). After transfection for 48 h, the cells were used for experiments.

Immunofluorescence and confocal microscopy

Cells were rinsed once in PBS, fixed in fresh 4% paraformaldehyde for 5 min at room temperature, and permeabilized in 0.5% TritonX-100. Nonspecific sites were blocked by incubation with PBS containing 1% goat serum before incubating the cells with an antibody against Nrf2 (1 : 200 dilution). After four washes in PBS, cells were incubated with goat anti-rabbit secondary Alexa Fluor 546 (1 : 250 dilution) for 3 h at room temperature, washed, stained with DAPI, and mounted. Confocal images were acquired using an OLYMPUS FV1000 inverted laser scanning confocal microscope equipped with an external argon, HeNe laser Green, and HeNe laser Red. Using a UPLSAPO 60 \times NA1.35 oil immersion objective (OLYMPUS), images were captured at the colony midsection.

Effect of CGME on LPS-induced mortality in C57BL/6 mice

The investigation is approved by the Animal Research Committee of Kangwon National University. Male C57BL/6 mice (20–22 g) were housed in plastic cages and maintained at 22 \pm 2 °C and 50–60% relative humidity with 12 h light-dark cycles throughout the experiment. The animals were maintained in these facilities for at least 1 week before the experiment. Mice (8 mice per group) were injected intraperitoneally with CGMC (50 mg kg⁻¹ and 20 mg kg⁻¹) dissolved in dimethyl sulfoxide : chremophor-EL : PBS (1 : 1 : 8 by volume) or control vehicle 1 h before LPS (*Escherichia coli* 0111:B4; Sigma-Aldrich, 40 mg kg⁻¹ body weight) injection. Mice survival was monitored for 5 days after injecting LPS, after which no further loss of mice occurred.

Statistical analysis

Data are expressed as the mean \pm standard error of mean (SEM). Statistical comparisons were made with one-way analysis of variance (ANOVA) and the difference between the experimental groups was further compared by the Fisher least significant difference test.

Results

CGME induces HO-1 expression in RAW264.7 cells at the transcriptional level

To investigate the anti-inflammatory effect of CGME, we first determined whether CGMC induces HO-1 expression in RAW264.7 cells (Fig. 2). RAW264.7 cells were treated with increasing concentrations of CGME for 6 h and expression of HO-1 was determined by Western blot analysis (Fig. 2A). CGME increased the expression level of HO-1 protein in a concentration-dependent manner. A time course experiment of HO-1 induction with 30 μM CGME revealed that HO-1 protein was increased 3 h after treatment and that its level continued to increase steadily even at 12 h (Fig. 2B). In addition, we found

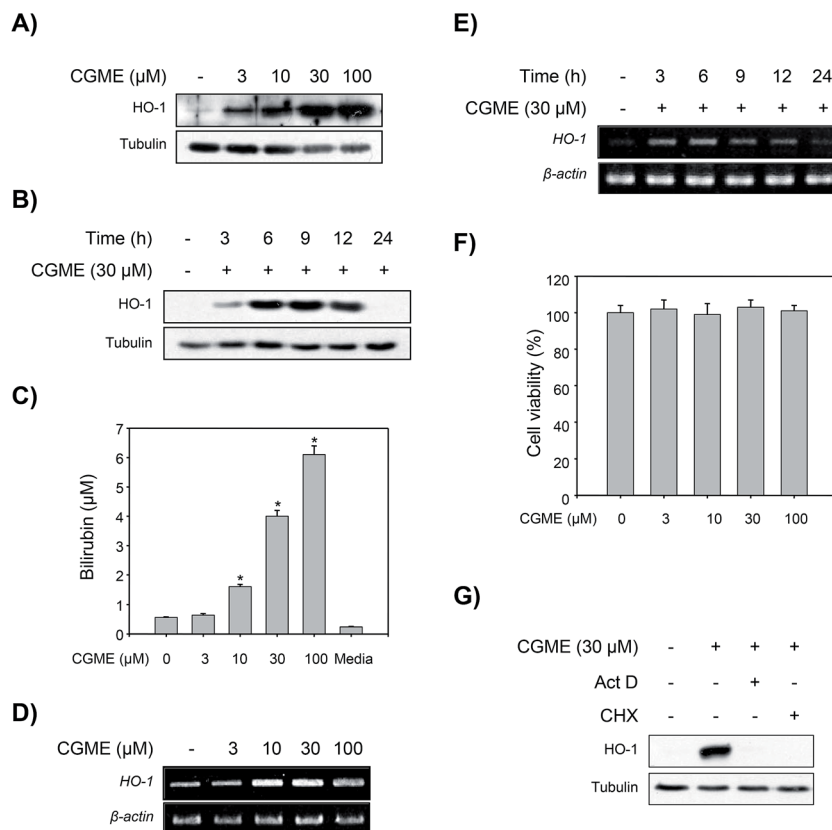


Fig. 2 CGME up-regulates HO-1 expression at the transcriptional level. (A) RAW264.7 cells were treated for 6 h with the indicated concentrations of CGME. Subsequently, total cell lysates were prepared and HO-1 expression levels were determined by Western blot analysis. (B) RAW264.7 cells were treated with 30 μM CGME for the indicated periods of time. Total lysates were prepared and HO-1 expression levels were determined by Western blot analysis. (C) CGME induces bilirubin production. RAW264.7 cells were treated with the indicated concentrations of CGME for 24 h. Subsequently, the amounts of bilirubin in the culture supernatant were determined. As a negative control, the amount of bilirubin in the culture media was also determined (media). Data are presented as mean \pm SEM (*, $p < 0.01$ compared with vehicle treated control, $n = 6$). (D) RAW264.7 cells were treated with indicated concentrations of CGME for 3 h. Subsequently, total RNAs were prepared and HO-1 mRNA expression levels were determined by RT-PCR. (E) RAW264.7 cells were treated with 30 μM of CGME for the indicated periods of time. Total RNAs were prepared and HO-1 mRNA expression levels were determined by RT-PCR analysis. (F) RAW264.7 cells were treated with the indicated concentrations of CGME for 24 h and cell viability was evaluated using a MTT assay. Data are presented as mean \pm SEM ($n = 6$). (G) RAW264.7 cells were treated with 30 μM CGME for 6 h in the presence of actinomycin D (Act. D, 50 ng ml^{-1}) or cycloheximide (CHX, 10 $\mu\text{g ml}^{-1}$). Subsequently, total lysates were prepared to determine HO-1 expression levels by Western blot analysis.

that treatment of RAW264.7 cells with CGME increased bilirubin production in culture medium in a concentration-dependent manner (Fig. 2C).

We next investigated whether CGME induced the expression of HO-1 at the transcriptional level. Treatment of RAW264.7 cells with increasing concentrations of CGME for 3 h increased the mRNA expression level of HO-1 in a concentration-dependent manner, as assessed by RT-PCR analysis (Fig. 2D). A time course experiment of HO-1 induction at 30 μM CGME revealed that the expression level of HO-1 mRNA was increased by 3 h and reached a peak at 6 h, and then this induction returned to the basal levels 24 h after treatment (Fig. 2E). On the other hand, CGME did not affect cell viability as assessed by the MTT assay at concentrations that induced HO-1 expression (Fig. 2F), indicating that CGME induces HO-1 expression without affecting cell viability.

We then attempted to determine the effect of actinomycin D and cycloheximide on CGME-mediated HO-1 expression

(Fig. 2G). Cotreatment of RAW264.7 cells with CGME and actinomycin D, a transcriptional inhibitor, significantly suppressed CGME-mediated HO-1 expression, confirming that CGME may induce HO-1 expression at the transcriptional level. Cycloheximide, a translational inhibitor, also blocked HO-1 expression induced by CGME. Taken together, these results suggested that CGME induced the expression of HO-1 at the transcriptional level.

CGME induces activation of Nrf2 via the JNK and AKT pathway

Since it is known that HO-1 expression is regulated mainly at the transcriptional level through the signaling pathways involving AKT and MAPKs such as c-Jun NH₂-terminal kinase (JNK), extracellular signal regulated kinase-1/2 (Erk1/2) and p38 kinase,^{5,6} we determined whether inhibition of AKT or MAPKs affected CGME-mediated HO-1 mRNA induction (Fig. 3A).

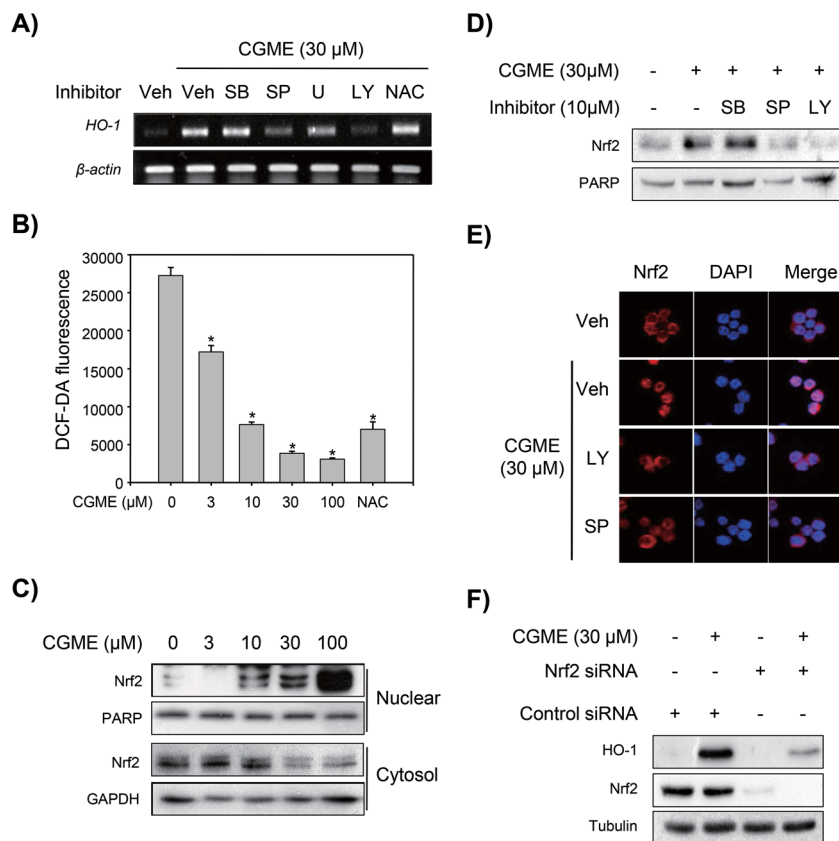


Fig. 3 CGME induces HO-1 expression via Nrf2 activation. (A) RAW264.7 cells were treated with CGME alone or in the presence of SB203580 (SB, 10 μM), SP600125 (SP, 10 μM), U0126 (U, 10 μM), LY294002 (LY, 10 μM), or *N*-acetylcysteine (NAC, 100 μM). The mRNA expression levels of HO-1 were determined by RT-PCR analysis. Veh: vehicle. (B) CGME decreases intracellular ROS level. RAW264.7 cells were treated with the indicated concentrations of CGME or NAC (100 μM) for 30 min. DCF-DA was used to determine the generation of intracellular ROS. Data are presented as mean ± SEM (*, $p < 0.01$ compared with vehicle treated control, $n = 6$). (C) RAW264.7 cells were treated for 2 h with indicated concentrations of CGME. Nuclear and cytosol extracts were subjected to Western blot analysis to determine the level of Nrf2. PARP was used as the nuclear marker and GAPDH as the cytosol protein marker. (D) RAW264.7 cells were treated for 2 h with CGME alone or in the presence of SB203580 (SB, 10 μM), SP600125 (SP, 10 μM), or LY294002 (LY, 10 μM). Nuclear extracts were subjected to Western blot analysis to determine the level of Nrf2. PARP was used as the nuclear marker. (E) RAW264.7 cells were treated for 2 h with CGME (30 μM) alone or in the presence of SP600125 (SP, 10 μM) or LY294002 (LY, 10 μM). Cells were immunostained with Nrf2 antibody. DAPI stains nuclei of cells. Veh: vehicle. (F) RAW264.7 cells transfected with the control siRNA or Nrf2-targeted siRNA were treated with CGME for 6 h. Whole cell lysates were subjected to Western blot analysis to determine the expression levels of HO-1.

Cotreatment of SB203580 (a p38 inhibitor) or U0126 (a MEK inhibitor) failed to modulate both CGME-induced mRNA of HO-1. In contrast, SP600125 (a JNK inhibitor) or LY294002 (a PI3K inhibitor) significantly blocked CGME-induced mRNA expression of HO-1, suggesting that the PI3K/AKT and JNK pathway could play a critical role in CGME-mediated HO-1 induction. We also determined whether NAC, an antioxidant, modulates CGME-induced HO-1 expression. Cotreatment of RAW264.7 cells with CGME and NAC did not change HO-1 mRNA expression induced by CGME (Fig. 3A). Moreover, treatment of RAW264.7 cells with CGME decreased the level of intracellular ROS in a concentration-dependent manner, as NAC did (Fig. 3B), suggesting that oxidative stress is not involved in the induction of HO-1 by CGME.

Nrf2 has been reported to play a central role in the induction of HO-1.²¹ Therefore, we examined whether CGME can activate Nrf2 in RAW264.7 cells. Treatment of RAW264.7 cells with

CGME increased the nuclear translocation of Nrf2 in a concentration-dependent manner (Fig. 3C), whereas SP600125 or LY294002 blocked this translocation (Fig. 3D). We confirmed this result with immunofluorescence, where CGME again induced nuclear translocation of Nrf2 and SP600125 or LY294002 blocked CGME-induced nuclear translocation of Nrf2 (Fig. 3E). Moreover, knockdown of Nrf2 by siRNA blocked CGME-mediated HO-1 expression (Fig. 3F), suggesting that CGME induces HO-1 expression through the activation of Nrf2 via the PI3K/AKT and JNK pathway.

CGME suppresses production of NO, PGE₂ and IL-6 in LPS-stimulated RAW264.7 cells, and protects LPS-induced mortality in C57BL/6 mice

To investigate the anti-inflammatory effect of CGME, we determined whether CGME inhibits LPS-induced production of NO, PGE₂ and IL-6. RAW264.7 cells were stimulated with 1 μg ml⁻¹

of LPS for 24 h in the presence of increasing concentrations of CGME, and the amount of NO, PGE₂ and IL-6 and in the culture supernatant was measured (Fig. 4A–C). The stimulation of RAW264.7 cells with LPS led to a significant increase in the levels of NO, PGE₂ and IL-6 in the culture supernatant. However, pretreatment of RAW264.7 cells with CGME inhibited the LPS-induced production of NO, PGE₂ and IL-6 in a concentration-dependent manner with IC₅₀ values of 26.6 ± 4.7, 29.5 ± 4.3 and 27.3 ± 3.8 μM, respectively.

We next investigated whether CGME protected C57BL/6 mice from LPS-induced lethality (Fig. 4D). Mice were injected with either CGME or vehicle, and 1 h later, they were challenged with 40 mg kg⁻¹ LPS intraperitoneally. Intraperitoneal injection of LPS into control mice resulted in death of 75% mice (*n* = 8) within 5 days. In contrast, all mice were rescued from death by administration of 50 mg kg⁻¹ CGME.

CGME downregulates the expression levels of iNOS, COX-2 and IL-6

Next, we determined whether CGME suppresses LPS-induced iNOS, COX-2 and IL-6 mRNA expression. RAW264.7 cells were stimulated for 8 h with 1 μg ml⁻¹ of LPS in the presence of increasing concentrations of CGME, and then real-time qPCR analysis was performed (Fig. 5A–C). CGME treatment concentration-dependently decreased mRNA levels of iNOS, COX-2 and IL-6, suggesting that this compound could suppress

LPS-induced iNOS, COX-2 and IL-6 expression at the transcriptional level. Western blot analysis also revealed that CGME treatment concentration-dependently decreased protein levels of iNOS and COX-2 (Fig. 5D).

Inhibition of HO-1 blocks the anti-inflammatory activity of CGME and 1-O-CG

We examined whether CGME-mediated HO-1 induction could be responsible for the anti-inflammatory effects of CGME. Thus, we utilized a specific HO-1 inhibitor, SnPP, and an inactive compound, CuPP. SnPP dramatically reversed CGME-mediated suppression of NO and PGE₂ production in LPS-stimulated RAW264.7 cells (Fig. 6A and B). In contrast, CuPP showed no effect. Moreover, knockdown of Nrf2 by siRNA significantly reversed CGME-mediated suppression of iNOS expression in LPS-stimulated RAW264.7 cells (Fig. 6C). Taken together, these observations suggest that inducing HO-1 mediates the inhibitory effects of CGME on LPS-induced inflammatory responses in macrophages.

1-O-CG is also a major constituent of *S. bicolor*.¹⁸ We determined whether 1-O-CG could exert anti-inflammatory effects *via* inducing HO-1. 1-O-CG also induced HO-1 expression in a concentration-dependent manner (Fig. 6D) and SnPP dramatically reversed 1-O-CG-mediated NO in LPS-stimulated RAW264.7 cells (Fig. 6E). Taken together, these results suggest

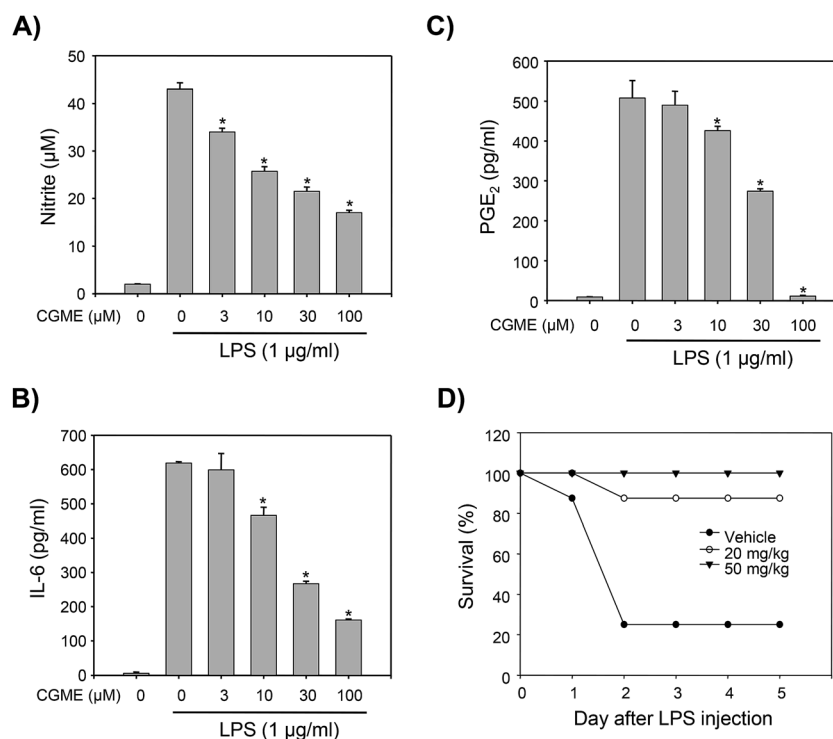


Fig. 4 CGME inhibits LPS-induced inflammatory responses *in vitro* and *in vivo*. (A, B and C) RAW264.7 cells were pretreated with the indicated concentrations of CGME for 30 min, followed by stimulation with LPS (1 μg ml⁻¹). After 24 h incubation, the amounts of NO (A), PGE₂ (B) and IL-6 (C) in the culture supernatants were determined. Data are presented as mean ± SEM (*, *p* < 0.01 compared with LPS-only treated control, *n* = 6). (D) Effect of CGME on LPS-induced mortality in C57BL/6 mice. C57BL/6 mice (8 mice per group) were challenged with LPS after the mice were injected with CGME or control vehicle. Survival was determined during 5 days after LPS injection.

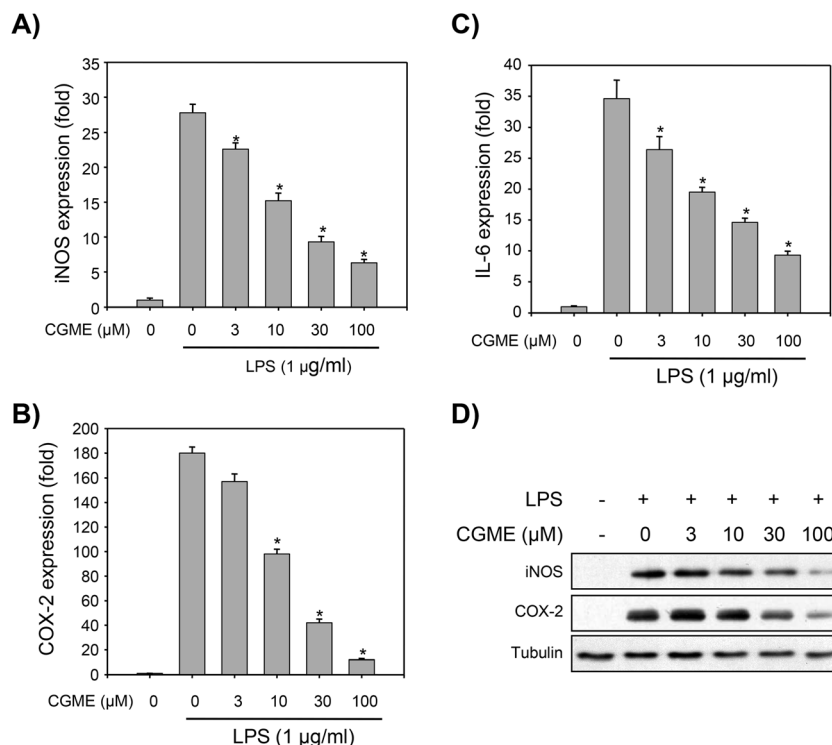


Fig. 5 CGME inhibits the expression of iNOS, COX-2 and IL-6. (A, B and C) RAW264.7 cells were pretreated for 30 min with the indicated concentrations of CGME, followed by stimulation with LPS ($1 \mu\text{g ml}^{-1}$) for 8 h. Subsequently, total RNAs were prepared and the mRNA expression levels of iNOS, COX-2 and IL-6 were determined by real-time qPCR. Data are presented as mean \pm SEM (*, $p < 0.01$ compared with LPS-only treated control, $n = 5$). (D) RAW264.7 cells were pretreated for 30 min with the indicated concentrations of CGME, followed by stimulation with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h. Subsequently, total lysates were prepared and the expression levels of iNOS and COX-2 were determined by Western blot analysis.

that the caffeoylglycerols such as CGME and 1-*O*-CG could exert the anti-inflammatory effects *via* induction of HO-1 expression.

Discussion

In the present study, we demonstrated the potential involvement of HO-1 induction in the anti-inflammatory activity of CGME, a major constituent of the grains of *S. bicolor*. We found that CGME induced HO-1 expression at the transcriptional level by activating of Nrf2 in RAW264.7 cells, and that this induction was correlated with suppression of NO, PGE₂ and IL-6 production in LPS-stimulated RAW264.7 cells. This is the first report showing that CGME, a caffeoylglycerol derivative, suppressed LPS-induced inflammatory responses *in vitro* *via* induction of HO-1 expression and protected C57BL/6 mice from LPS-induced lethality.

Sorghum is a rich source of various phytochemicals including tannins, phenolic acids, anthocyanins, phytosterols and policosanols.^{22,23} These phytochemicals have potential to significantly impact human health.²² The extracts of leaf sheaths and bran of *S. bicolor* have been demonstrated to have anti-inflammatory activity in LPS-induced RAW264.7 cells *in vitro* and *in vivo*, although the chemical components which are reckoned to be responsible for activity of the extracts were not identified.^{24,25} Recently, we showed that the caffeoylglycerols

such as CGME and 1-*O*-CG are the major constituents of the grains of *S. bicolor* and inhibit LPS-induced iNOS expression and NO production in RAW264.7 cells.¹⁸ In the present study, we showed that CGME inhibits LPS-induced inflammatory responses in RAW264.7 cells by inducing HO-1 *via* activating the Nrf2 pathway. 1-*O*-CG also induced HO-1 and inhibition of HO-1 abolished the anti-inflammatory effect of 1-*O*-CG. Thus, induction of HO-1 by caffeoylglycerols such as CGME and 1-*O*-CG may be important in the understanding of a novel mechanism for the anti-inflammatory activity of *S. bicolor*.

The induction of HO-1 is widely recognized as an effective cellular strategy to counteract a variety of cellular damage and inflammation.¹⁷ A growing body of evidence has shown that HO-1 efficiently represses inflammatory responses by inhibiting the production of various inflammatory cytokines. Several plant-derived components, including curcumin, resveratrol, malabaricone C and 2'-hydroxychalcone, induce HO-1 and exert anti-inflammatory activities in different types of cells.^{26–32} Similar to these findings, CGME induced HO-1 expression *in vitro*, and suppressed LPS-induced inflammatory responses *in vitro* and *in vivo*.

Our results show that inhibiting HO-1 activity by treatment with SnPP, a HO-1 inhibitor, abrogated the inhibitory effects of CGME on the production of NO and PGE₂ in LPS-stimulated RAW264.7 cells. However, CuPP, an inactive compound did

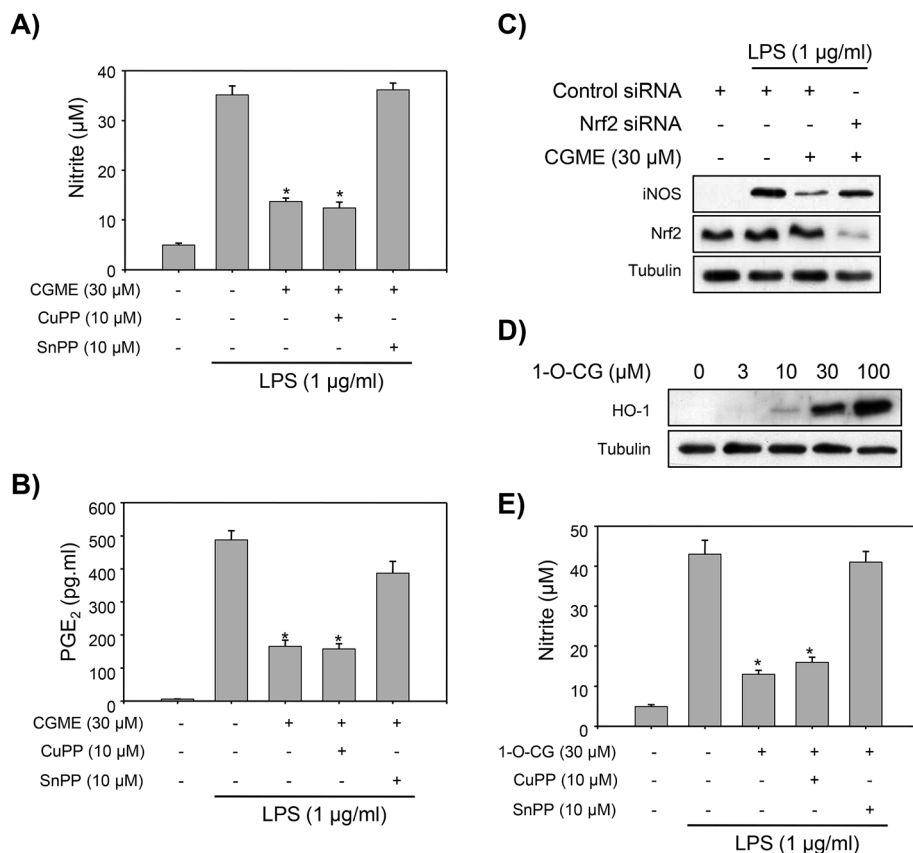


Fig. 6 Inhibition of HO-1 activity reverses the anti-inflammatory effects of CGME and 1-O-CG in RAW264.7 cells. (A and B) RAW264.7 cells were pretreated with CGME in the presence of SnPP or CuPP for 30 min and then stimulated with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h. The amounts of NO (A) and PGE₂ (B) in culture supernatants were determined. Data are presented as mean \pm SEM (*, $p < 0.01$ compared with LPS-only treated control, $n = 3$). (C) RAW264.7 cells transfected with the control siRNA or Nrf2-targeted siRNA were pretreated for 30 min with vehicle or CGME, followed by stimulation with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h. Subsequently, total lysates were prepared and the expression level of iNOS was determined by Western blot analysis. (D) RAW264.7 cells were treated for 6 h with the indicated concentrations of 1-O-CG. Subsequently, total cell lysates were prepared and HO-1 expression levels were determined by Western blot analysis. (E) RAW264.7 cells were pretreated with 1-O-CG in the presence of SnPP or CuPP for 30 min and then stimulated with LPS ($1 \mu\text{g ml}^{-1}$) for 24 h. The amounts of NO in the culture supernatants were determined. Data are presented as mean \pm SEM (*, $p < 0.01$ compared with LPS-only treated control, $n = 3$).

not. These data suggest that CGME-induced HO-1 expression is at least partially responsible for the anti-inflammatory effects of CGME. The anti-inflammatory actions of HO-1 are attributed to several factors, including degradation of the pro-oxidant heme, formation of the antioxidant biliverdin/bilirubin, as well as release of anti-inflammatory/anti-apoptotic carbon monoxide.^{4,8} Although the exact mechanisms involved in the anti-inflammatory effects of HO-1 have not been fully elucidated, one or more enzymatic by-products of HO-1 have been evaluated as possible factors capable of inhibiting macrophage-mediated inflammation.^{4,8} As CGME induced the expression of the anti-inflammatory HO-1 in RAW264.7 cells, the observed anti-inflammatory effects of CGME might be mediated, at least in part, by one or more HO-1 by-products.

HO-1 expression is regulated mainly at the transcriptional level.^{4,6,21} The most crucial transcription factor during HO-1 expression appears to be Nrf2.²¹ Nrf2 is a member of the basic-leucine zipper transcription factor family that controls the expression of antioxidant response element-regulated antioxidant and cytoprotective genes such as NAD(P)H: quinone

oxidoreductase 1 and HO-1.³³ Under basal conditions, the Kelch-like ECH-associated protein (Keap1) binds to Nrf2 and sequesters it in the cytoplasm, which results in a lower accumulation of Nrf2 in the nucleus and reduced transcription of the HO-1 gene.^{34,35} Most of HO-1 inducers derived from plants activate Nrf2 through signaling pathways involving protein kinase C, MAPKs, and PI3K.^{15,26–30,36} Oxidation of redox-sensitive cysteines within Keap1 releases Nrf2, and Nrf2 then translocates from the cytosol to the nucleus and binds to the HO-1 gene. In the present study, we showed that NAC, a potent antioxidant, did not block CGME-mediated induction of HO-1 and CGME decreased the intracellular ROS level in RAW264.7 cells, suggesting that reactive oxygen species are not involved in this induction. The JNK and AKT pathway also serves as the important signaling pathway in the induction of HO-1. Several lines of evidence have shown that HO-1 expression is regulated by the JNK- and PI3K/AKT-Nrf2-dependent pathway. For example, carnosol and fluvastatin induce HO-1 expression through the activation of Nrf2 *via* the PI3K/AKT pathway.^{37,38} Acrolein induces Nrf2 nuclear translocation and HO-1

expression *via* the activation of JNK.³⁹ In this study, we also showed that inhibition of AKT or JNK completely blocked CGME-induced both HO-1 expression and Nrf2 activation, suggesting that CGME induces HO-1 expression *via* activating the JNK- and PI3K/AKT-Nrf2 pathway.

Activation of the Nrf2 has been shown to protect against neurodegenerative diseases, aging, diabetes, cardiovascular disease, inflammation, pulmonary fibrosis, acute pulmonary injury, and cancer.^{40–43} Epidemiological studies indicate that sorghum consumption significantly lowers the risk of cardiovascular disease or cancer.^{44–46} Numerous studies have demonstrated the health benefits of polyphenols, and special attention has been paid to their beneficial effects against cardiovascular disease and cancer.⁴⁷ Growing evidence indicates that increased oxidative stress under pathophysiological conditions plays a key role in the development of cardiovascular disease and cancer. The beneficial effects of polyphenols on both pathological processes may be the consequence of direct and indirect antioxidant activity.⁴⁷ For example, polyphenols may affect the cellular redox state by inducing anti-oxidant defense enzymes such as glutamate-cysteine ligase and HO-1 *via* Nrf2 activation.^{47,48} Resveratrol confers endothelial protective effects which are mediated by the activation of Nrf2 in the vascular endothelium.⁴⁹ In the present study, we showed that CGME, one of the polyphenols, activates Nrf2 and decreases the level of intracellular ROS. In this regard, our findings that CGME activated Nrf2 imply that CGME or CGME-enriched extract from *S. bicolor* could be valuable for the prevention or treatment of Nrf2-dependent pathological conditions such as cardiovascular disease and cancer. However, the effects of CGME on cardiovascular disease and cancer remain to be elucidated.

In summary, we demonstrated that CGME, a major constituent of the grains of *S. bicolor*, induces HO-1 expression by activating the Nrf2 pathway and CGME-induced HO-1 expression is considerably associated with the anti-inflammatory effects of CGME. Furthermore, our data extend our understanding of the molecular mechanisms underlying the biological activities of caffeoylglycerols such as CGME and 1-O-CG, and the grains of sorghum. Our results also suggest that CGME-and/or 1-O-CG-enriched extracts from the grains of sorghum may be applied as supplemental and/or functional foods having a beneficial effect against inflammatory diseases.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

CGME	Caffeoylglycolic acid methyl ester
1-O-CG	1-O-Caffeoylglycerol
COX-2	Cyclooxygenase-2
CuPP	Copper protoporphyrin IX
DAPI	4',6-Diamidino-2-phenylindole
DCF-DA	2',7'-Dichlorofluorescein diacetate
ERK1/2	Extracellular signal regulated kinase-1/2

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HO	Heme oxygenase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
NAC	N-Acetyl-L-cysteine
NO	Nitric oxide
Nrf2	Nuclear factor-E2-related factor 2
PARP	Poly ADP-ribose polymerase
PGE ₂	Prostaglandin E ₂
PI3K	Phosphoinositide 3-kinase
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
siRNA	Small interfering RNA
SnPP	Tin protoporphyrin IX
qPCR	Quantitative PCR

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