Inflamm. Res. (2014) 63:81–90
DOI 10.1007/s00011-013-0674-4

Inflammation Research

ORIGINAL RESEARCH PAPER

# Anti-inflammatory effects of chlorogenic acid in lipopolysaccharide-stimulated RAW 264.7 cells

**Su Jung Hwang · Yong-Wan Kim · Yohan Park · Hyo-Jong Lee · Kyu-Won Kim**

Received: 27 May 2013 / Revised: 30 September 2013 / Accepted: 3 October 2013 / Published online: 15 October 2013
© Springer Basel 2013

**Abstract**
*Objectives and design* Chlorogenic acid, which belongs to the polyphenols, is an anti-oxidant and anti-obesity agent. In this study, we investigated the role of chlorogenic acid in inflammation.
*Materials and methods* Anti-inflammatory effects of chlorogenic acid were examined in lipopolysaccharide (LPS)-stimulated murine RAW 264.7 macrophages and BV2 microglial cells. We observed the level of various inflammation markers such as nitric oxide (NO), inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and chemokine (C-X-C motif) ligand 1 (CXCL1) under LPS treatment with or without chlorogenic acid. To clarify the specific effect of chlorogenic acid, we evaluated the adhesion activity of macrophages and ninjurin1 (Ninj1) expression level in macrophages. Finally, we confirmed the activation of the nuclear factor-κB (NF-κB) signaling pathway, which is one of the most important transcription factors in the inflammatory process.
*Results* Chlorogenic acid significantly inhibited not only NO production but also the expression of COX-2 and iNOS, without any cytotoxicity. Chlorogenic acid also attenuated pro-inflammatory cytokines (including IL-1β and TNF-α) and other inflammation-related markers such as IL-6 in a dose-dependent manner. Additionally, endotoxin-induced adhesion of macrophages and the expression level of ninjurin1 (Ninj1) were decreased by chlorogenic acid. Finally, chlorogenic acid inhibited the nuclear translocation of NF-κB.
*Conclusions* Chlorogenic acid may be beneficial for the prevention and treatment of anti-inflammatory diseases.

**Keywords** Chlorogenic acid · Nitric oxide · Cytokines · Ninjurin1 · Inflammation

Responsible Editor: Graham R. Wallace.

S. J. Hwang · Y.-W. Kim · Y. Park · H.-J. Lee (✉)
College of Pharmacy, Inje University, 607 Obang-dong, Gimhae, Gyungnam 621-749, Republic of Korea
e-mail: hjlee@inje.ac.kr

K.-W. Kim (✉)
SNU-Harvard NeuroVascular Protection Research Center, College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Republic of Korea
e-mail: qwonkim@snu.ac.kr

K.-W. Kim
Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul 151-742, Republic of Korea

## Introduction

Inflammation can be divided principally into acute and chronic inflammation. Acute inflammation is the body's normal protective response to an injury, irritation, or surgery [1]. However, chronic inflammation induces various chronic diseases including cancer, cardiovascular diseases, Alzheimer's disease, type II diabetes, arthritis, autoimmune diseases, neurological diseases, and pulmonary diseases [2, 3]. During the inflammatory process, interleukins or growth factors induce the proliferation and activation of leukocytes [4]. Activated leukocytes then mediate inflammation and programmed cell death [5, 6]. Understanding these mechanisms could therefore provide therapeutic clues to modulate the activity of leukocytes during acute or chronic inflammation.

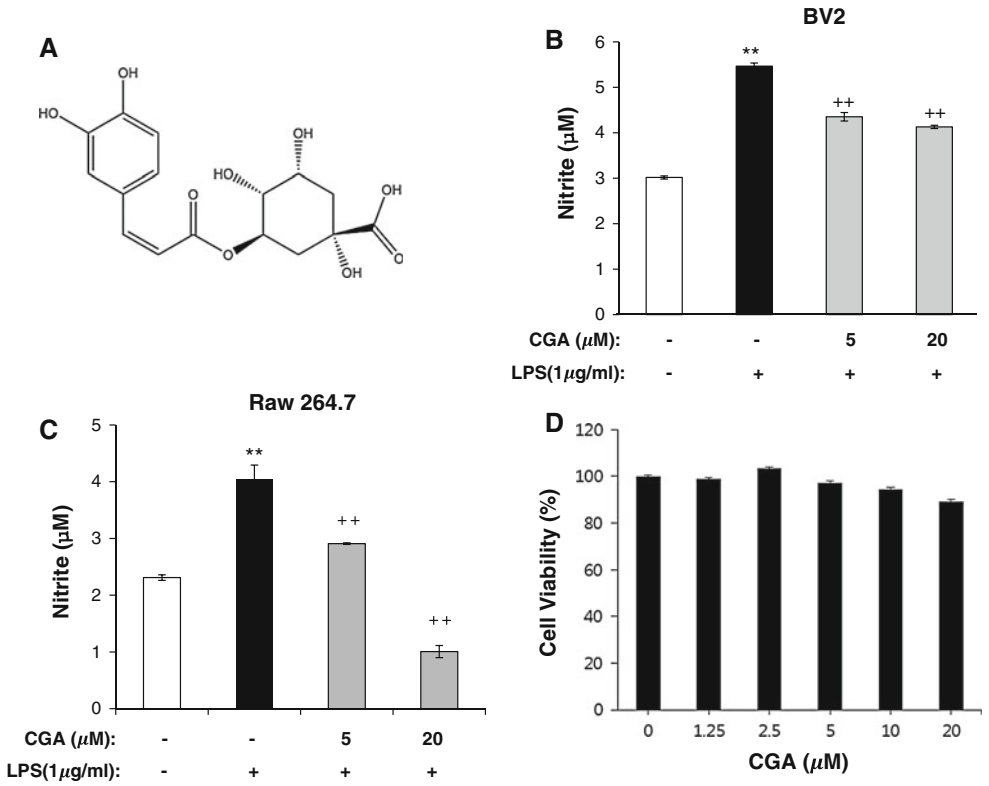


**Fig. 1** Effects of chlorogenic acid on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated BV2 microglia and RAW 264.7 macrophages. **a** Chemical structure of chlorogenic acid. **b**, **c** NO assay using the Griess reagent. Cells were treated with LPS (1 μg/ml) in the absence or presence of chlorogenic acid at different concentrations (0, 5, and 20 μM, respectively) for 24 h. Amounts of NO were determined using the Griess reagent and a standard curve created using $NaNO_2$ in BV2 (**b**) and RAW 264.7 cells (**c**); $^{**}P < 0.01$, vehicle vs. LPS-treated group; $^{++}P < 0.01$, LPS vs. LPS plus chlorogenic acid-treated group. **d** Cell viability assays using the MTT assay were then performed for 24 h following treatment with chlorogenic acid (0, 1.25, 2.5, 5, 10, and 20 μM). Values are expressed relative to that of vehicle-treated cells, normalized to 100 %. Values shown in the *graphs* are mean ± standard deviation (SD) and were obtained from three independent experiments

During the inflammatory process, various inflammatory mediators, including nitric oxide (NO), prostaglandin $E_2$ ($PGE_2$), pro-inflammatory cytokines, and adhesion molecules are closely associated with the classical symptoms of inflammation such as pain, heat, redness, swelling, and loss of function. NO is generated from amino acid L-arginine by the enzymatic action of inducible NO synthase (iNOS) which is stimulated during inflammation by bacterial endotoxins (e.g., lipopolysaccharide) and cytokines [7, 8]. The expression of pro-inflammatory cytokine and adhesion molecules is regulated by nuclear factor-κB (NF-κB), which is composed of p50 and p65 subunits [9, 10]. Under normal conditions, it is sequestered in the cytosol where it is bound to the inhibitor IκBα. When inflammation is induced by injury, irritation, or surgery, IκB undergoes ubiquitin-mediated degradation by proteasomes. The removal of IκB unmasks the nuclear-localization signals in both subunits of NF-κB, allowing their translocation to the nucleus. In the nucleus, NF-κB activates transcription of numerous target genes such as pro-inflammatory cytokine and adhesion molecules [11, 12]. Thus, inhibitors acting on the NF-κB pathway can be candidates for therapeutic agents against acute or chronic inflammation.

Chlorogenic acid (Fig. 1a) is an ester formed from cinnamic acids and quinic acid and is also known as 5-*O*-caffeoylquinic acid (5-CQA) (IUPAC nomenclature) or 3-CQA (pre-IUPAC nomenclature) [13]. It is synthesized in the process of aerobic respiration and is abundant in coffee beans, potatoes, and apples [14]. It is reported that chlorogenic acid has various functions such as anti-oxidant and hypotensive activity [15, 16]. Furthermore, chlorogenic acid prevents cardiovascular disease by increasing high-density lipoprotein [17]. Most natural products containing chlorogenic acid showed anti-inflammatory effects, suggesting that chlorogenic acid may be a potential anti-inflammatory agent [9, 18]. To date, however, it remains largely unknown, and so we need to study the anti-inflammatory effects of chlorogenic acid.

Here, we investigated the anti-inflammatory effects of chlorogenic acid and how it induces an anti-inflammatory effect in lipopolysaccharide (LPS)-inflamed murine RAW 264.7 macrophage cells. Our findings suggest that

chlorogenic acid may be a candidate for use in the treatment of a variety of inflammatory diseases.

## Materials and methods

### Reagents and cells

RAW 264.7 and BV2, widely used murine microglia/macrophage cell lines, were obtained from the Korean Cell Line Bank (KCLB) and grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL) supplemented with 10 % fetal bovine serum (FBS; Gibco BRL), penicillin (100 U/ml)/streptomycin (100 μg/ml) (Gibco BRL). LPS, chlorogenic acid, and Griess reagent were purchased from Sigma-Aldrich.

### Nitric oxide assay

Nitric oxide assay was performed as described previously [19]. RAW 264.7 and BV2 cells at $5 \times 10^4$ cells/well were cultured in flat-bottom 96-well plates in triplicate for 24 h and then incubated with LPS (1 μg/ml) with or without pretreatment with chlorogenic acid for 1 h. After 24 h of culture, the culture supernatant was collected; this was used as a measure of NO production. The culture supernatant (50 μl) was mixed with an equal volume of Griess reagent and the absorbance was measured at 550 nm. Finally, the concentration of nitrite was calculated from a standard curve drawn with known concentrations of sodium nitrite dissolved in DMEM. The relative cell viability of each parallel experimental group ($n = 3$) was expressed in percentage (%) based on a no-additions (NA) control, while untreated controls were considered as 100 % viable.

### Reverse transcriptase–polymerase chain reaction (RT-PCR) and real-time PCR

RT-PCR was performed as described previously [20]. RAW 264.7 cells were cultured in the presence of each sample alone or in combination with LPS in a 6-well plate ($1 \times 10^6$ cells/ml) for 6 h. Total cellular RNA was isolated using the TRIzol (Invitrogen) following the manufacturer's instructions. Total RNA (3 μg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega). The PCR primers used in this study are listed below and were purchased from Bioneer: forward strand iNOS 5′-cagctgggctgtacaaacctt-3′, reverse strand iNOS 5′-cattg gaagtgaagcgtttcg-3′; forward strand IL-1β 5′-aagggct gcttccaaacctttgac-3′, reverse strand IL-1β 5′-tgcctgaag ctcttgttgatgtgc-3′; forward strand TNF-α 5′-catcttctca aaattcgagtgacaa-3′, reverse strand TNF-α 5′-tgggagtaga caaggtacaaccc-3′; forward strand COX-2 5′-ttcaaaaga agtgctggaaaaggt-3′, reverse strand COX-2 5′-gatcatctctacc tgagtgtcttt-3′; forward strand IL-6 5′-gaggataccactcccaaca gacc-3′, reverse strand IL-6 5′-aagtgcatcatcgttgttcataca-3′; forward strand CXCL1 5′-tggggacacctttagcatc-3′, reverse strand CXCL1 5′-cttgaaggtgttgccctc-3′; forward strand β-actin 5′-agagggaaatcgtgcgtgac-3′, reverse strand β-actin 5′-g gccgtcaggcagctcatag-3′. A variable number of cycles were used to ensure that amplification occurred in the linear phase. PCR amplification employed β-actin as the internal control, and PCR products were separated on a 2.0 % agarose gel and visualized by RedSafe nucleic acid staining (Intron, Korea), and UV irradiation. Real-time PCR was done with the same primers of RT-PCR and performed on a Rotor-Gene Q real-time PCR cycler (Qiagen) using Rotor-Gene SYBR Green RT-PCR kit (Qiagen) according to the manufacturer's instructions.

### Western blot analysis

RAW 264.7 cells were cultured in the presence of LPS or in combination with each sample in a 6-well plate ($1 \times 10^6$ cells/well). After removal of the supernatants, extracts of RAW 264.7 cells were directly prepared in lysis buffer (0.5 % Triton, 50 mM β-glycerophosphate pH 7.2, 0.1 mM sodium vanadate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl urea, 2 μg/ml of leupeptin, and 4 μg/ml of aprotinin). The lysates were resolved by 10 % SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline (10 mM Tris–Cl pH 7.4) containing 0.5 % Tween 20 and 5 % nonfat dry milk, incubated with the first specific antibody in blocking solution for 5 h at room temperature, washed, and incubated with the second antibody for 1 h at room temperature. The protein bands were detected by chemiluminescence (FUSION-SL4, Vilber). Monoclonal antibodies against iNOS, β-actin, and peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology. In a parallel experiment, cytosolic protein was prepared using buffer A [20 mM HEPES (pH 8.0), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mm benzamidine, and 0.2 mm sodium orthovanadate]. The cell lysates were collected in homogenization tubes and were cleared by centrifugation twice at 2,500 rpm for 5 min at 4 °C. The cell pellets were discarded, and the supernatant was subjected to another centrifugation at 13,000 rpm for 30 min at 4 °C. At this stage, the supernatant was further centrifuged at 40,000 rpm for 1 h at 4 °C. The resulting supernatant was labeled the cytosolic fraction and used. Polyclonal

antibodies against IκB and GAPDH were purchased from Santa Cruz Biotechnology.

Cell viability assay

RAW 264.7 cells were plated into 96-well plates at $5 \times 10^4$ cells/well and allowed to attach for 24 h. Prior to chlorogenic acid treatment, media was removed and replaced with 0.1 ml of fresh media. Confluent cells were stimulated with different concentrations of chlorogenic acid. After 24 h, the media was removed and replaced with 0.1 ml fresh media. Next, 20 µl of CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) was added to each well, incubated at 37 °C for 2 h and then read at 490 nm.

Cell adhesion assays

In the cell–matrix adhesion assay, RAW 264.7 cells were added to each well coated with EMCs, which are fibronectin (Invitrogen), type I collagen (BD), laminin (BD), and gelatin (Sigma). After incubation for 15 min, RAW 264.7 cells were washed twice with PBS. Attached cells were stained with Crystal violet and washed twice. After lysis with 0.2 % NP-40, absorbance of lysates was analysed with ELISA at 590 nm.

Induction of endotoxin shock

ICR mice were injected three times intraperitoneally (i.p.) with a dose of 6.6 µg of LPS (330 µg/kg of body weight)

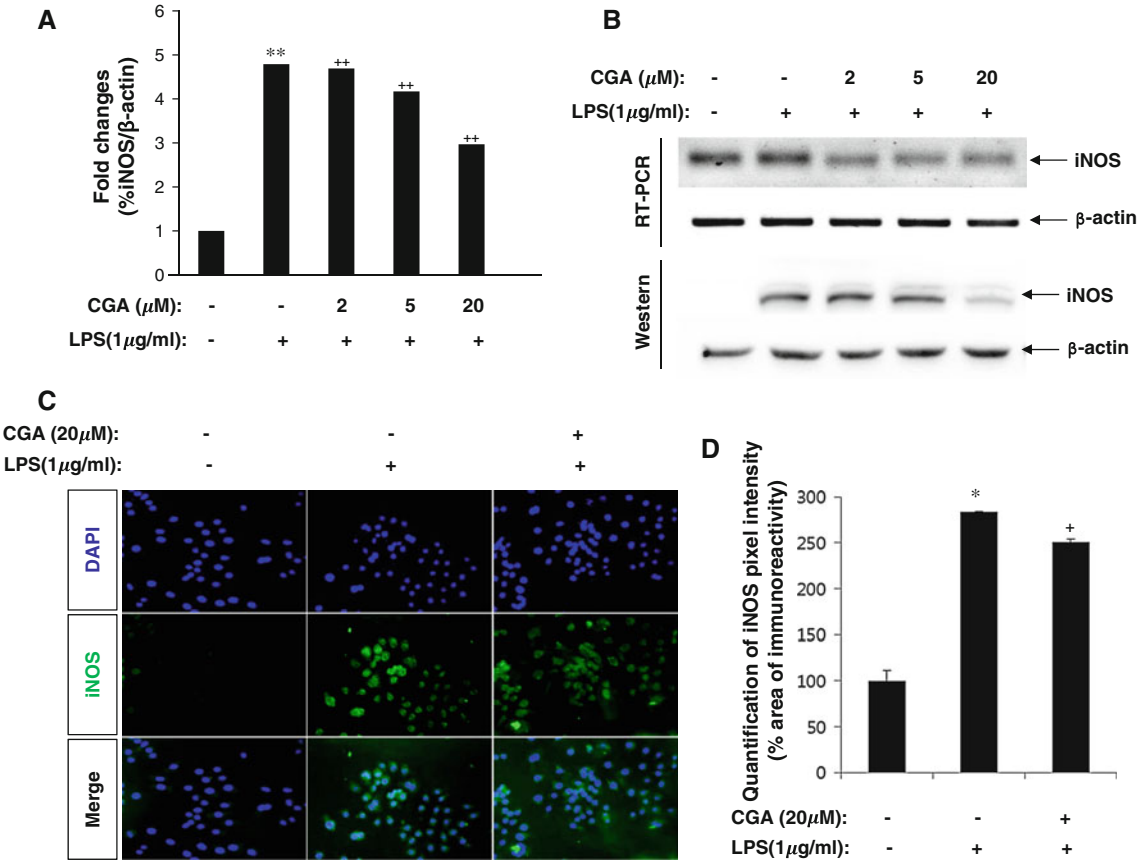


**Fig. 2** Effects of chlorogenic acid on inducible NO synthase (iNOS) expression in LPS-stimulated RAW 264.7 cells. **a**, **b** Cells were treated with LPS (1 µg/mL) in the absence or presence of chlorogenic acid at different concentrations (0, 2, 5, and 20 µM, respectively) for 24 h. The iNOS mRNA level at 24 h was analyzed using real-time PCR (**a**) and conventional RT-PCR (**b**, *upper*) and then the iNOS protein level at 24 h (**b**, *lower*) was determined using Western blotting after LPS stimulation; $^{**}P < 0.01$, vehicle vs. LPS-treated group; $^{++}P < 0.01$, LPS vs. LPS plus chlorogenic acid-treated group. **c** After LPS (1 µg/ml) stimulation either with or without chlorogenic acid treatment for 24 h, cells were fixed and immunostained using an iNOS antibody and a goat anti-rabbit IgG conjugated with Alexa Fluor 488 (*green*). Cells were also stained with DAPI (4′,6-diamidino-2-phenylindole) to visualize nuclei (*blue*). **d** Immunostaining of iNOS was quantified using ImageJ (NIH Image). Values were normalized to 100 % for the immunoreactivity of the untreated control group. Error values are mean ± SD for each group ($n = 1$); $^{*}P < 0.05$, vehicle vs. LPS-treated group; $^{+}P < 0.05$, LPS vs. LPS plus chlorogenic acid-treated group. Images are 40× magnification (color figure online)

and/or with a dose of 0.1 mg of chlorogenic acid (5 mg/kg body weight) for 3 days. Animals were killed at 24 h after LPS administration.

### Immunofluorescent microscopy and quantification

RAW 264.7 or BV2 cells were plated at $1 \times 10^5$ cells/well in 4-well chamber slides (Falcon). For immunostaining, cells were washed twice with cold phosphate-buffered saline, fixed in 4 % paraformaldehyde for 10 min, permeabilized with 0.1 % Triton X-100 for 15 min, and blocked with 10 % normal goat serum for 30 min. Slides were incubated for 18 h at 4 °C with primary antibody at a 1:500 dilution, washed, and then incubated for 50 min with Alexa546-conjugated IgG (Molecular Probes) at a 1:1,000 dilution as secondary antibodies. Antibodies used for immunostaining were: ninjurin1 (Ninj1; 1:500, a kind gift from Dr. J. Milbrandt), iNOS (1:500, BD Biosciences), and p65 (1:200, Cell Signaling). Nuclei were stained using 4′-6-diamidino-2-phenylindole (DAPI, Invitrogen). Images were obtained with an Axiovert M200 microscope (Zeiss). Pixel intensities of iNOS, Ninj1, and DAPI were measured as percent area of immunoreactivity, using ImageJ, then recorded and compared statistically. All pixel intensities were measured and compared using images at ×40 magnification.

### Data analysis and statistics

Quantification of band intensity was analyzed using ImageJ (http://rsb.info.nih.gov/ij/) and normalized to the density of the GAPDH, tubulin, or Ponceau S staining band. All data are presented as mean ± SD and changed into relative percentage. Statistical comparisons between groups were done using Student's $t$ test. $P < 0.05$ was considered statistically significant.

## Results

### Chlorogenic acid inhibits NO production in LPS-stimulated murine microglia/macrophages without cell toxicity

First, we investigated the effects of chlorogenic acid on LPS-induced NO production in mouse BV2 microglia and RAW 264.7 macrophages. Chlorogenic acid significantly inhibited LPS-induced NO production in a dose-dependent manner (Fig. 1b, c). To examine whether the inhibitory effect of chlorogenic acid on NO production resulted from cellular toxicity, cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The data showed that chlorogenic acid had no effect on cell viability up to a concentration of 20 μM (Fig. 1d). These results suggest that chlorogenic acid has an inhibitory effect on NO production without cell toxicity in microglial cells.

### Chlorogenic acid inhibits iNOS expression in LPS-stimulated RAW 264.7 cells

Next, we examined the effects of chlorogenic acid on LPS-induced *iNOS* gene expression in RAW 264.7 cells. LPS-induced *iNOS* mRNA (Fig. 2a, b; upper) and protein (Fig. 2b; lower) expression were also significantly attenuated in a dose-dependent manner by chlorogenic acid. Moreover, immunofluorescence staining and quantification of iNOS pixel intensity (% area of immunoreactivity) showed that LPS-induced iNOS up-regulation is abolished by chlorogenic acid treatment (Fig. 2c, d). These results indicate that chlorogenic acid

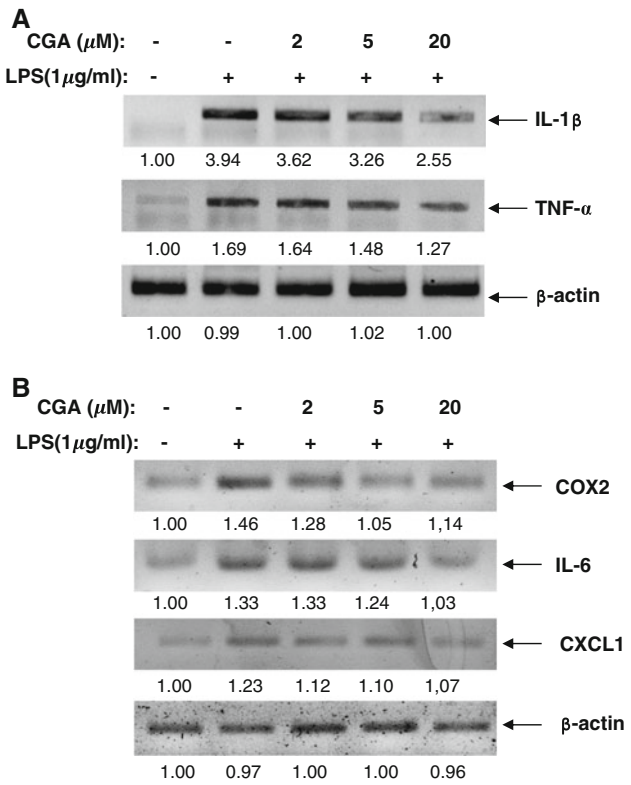


**Fig. 3** Effects of chlorogenic acid on the mRNA expression of pro-inflammatory cytokines and other inflammatory-related genes in LPS-stimulated RAW 264.7 cells. **a**, **b** Cells were treated with LPS (1 μg/mL) in the absence or presence of chlorogenic acid at different concentrations (0, 2, 5, and 20 μM, respectively) for 24 h. The mRNA expression levels of pro-inflammatory cytokine genes (*interleukin-1β* and *tumor necrosis factor-α*) (**a**) and other inflammation-related genes (*cyclooxygenase-2*, *chemokine (C-X-C motif) ligand 1 (CXCL1)*, and *interleukin-6*) at 24 h (**b**) were then determined after LPS (1 μg/ml) stimulation. Expression levels of mRNA were represented as relative to that of control and normalized to 1

has an inhibitory effect on iNOS induction in RAW 264.7 cells.

## Chlorogenic acid inhibits expression of pro-inflammatory cytokines in LPS-stimulated RAW 264.7 cells

We examined the effects of chlorogenic acid on the expression of pro-inflammatory cytokines such as IL-1β and TNF-α. LPS-induced expressions of *IL-1β* and *TNF-α* were significantly inhibited by chlorogenic acid (20 μM) at 24 h (Fig. 3a). To confirm the anti-inflammatory effect of chlorogenic acid, we further investigated the effect of chlorogenic acid on the expression of other inflammation-related genes. As shown in Fig. 3b, LPS caused mRNA induction of *COX-2*, *IL-6*, and *CXCL1* and chlorogenic acid significantly attenuated LPS-induced *COX-2*, *IL-6*, and *CXCL1* mRNA levels. Taken together, these results suggest that chlorogenic acid may reduce the expression of pro-inflammatory cytokines and chemokines.

## Chlorogenic acid inhibits Ninj1 expression and cell-to-matrix adhesion in LPS-stimulated RAW 264.7 cells

Adhesion molecules are important for a recognition system between leukocytes and other cells or cellular matrix proteins during inflammation. Among them, ninjurin1 (Ninj1) is known to increase movement to the site of the inflammation and the activity of leukocytes in both developmental processes and inflammatory responses [21]. Thus, we further investigated the effect of chlorogenic acid on the expression of Ninj1 both in vitro and in vivo and the

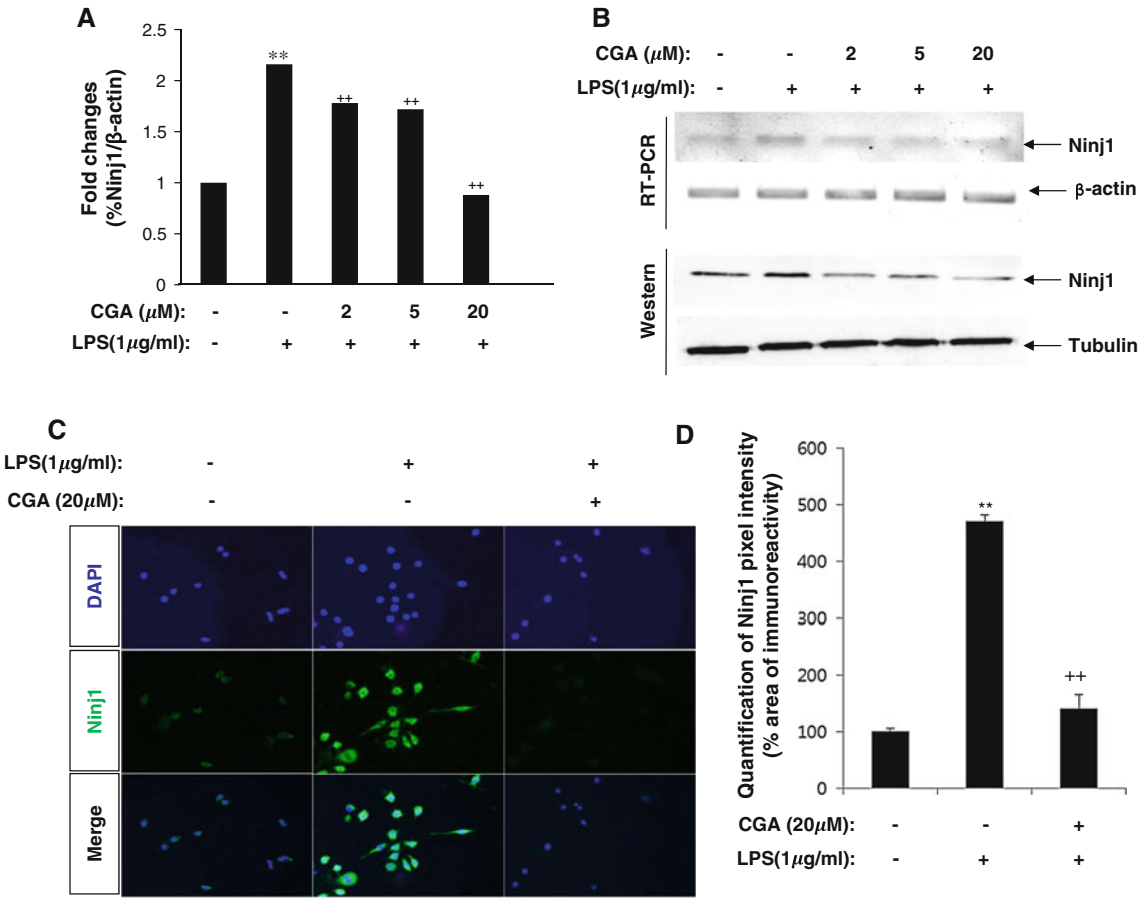


**Fig. 4** Effects of chlorogenic acid on Ninj1 expression in LPS-stimulated RAW 264.7 cells. **a**, **b** Cells were treated with LPS (1 μg/mL) in the absence or presence of chlorogenic acid at different concentrations (0, 2, 5, and 20 μM, respectively) for 24 h. Ninj1 mRNA level at 24 h was analyzed using real-time PCR (**a**) and conventional RT-PCR (**b**, *upper*) and then the iNOS protein level at 24 h (**b**, *lower*) was determined using Western blotting after LPS stimulation; $^{**}P < 0.01$, vehicle vs. LPS-treated group; $^{++}P < 0.01$, LPS vs. LPS plus chlorogenic acid-treated group. **c** Cells were treated with LPS (1 μg/ml) in the absence or presence of chlorogenic acid at 20 μM concentration. Immunofluorescent staining of Ninj1 (*green*). Nuclei were counterstained using DAPI (*blue*). **d** Immunostaining of Ninj1 was quantified using ImageJ (NIH Image). Values were normalized to 100 % for the immunoreactivity of the untreated control group. Error values are mean ± SD for each group ($n = 1$); $^{**}P < 0.01$, vehicle vs. LPS-treated group; $^{++}P < 0.01$, LPS vs. LPS plus chlorogenic acid-treated group. Images are 40× magnification (color figure online)

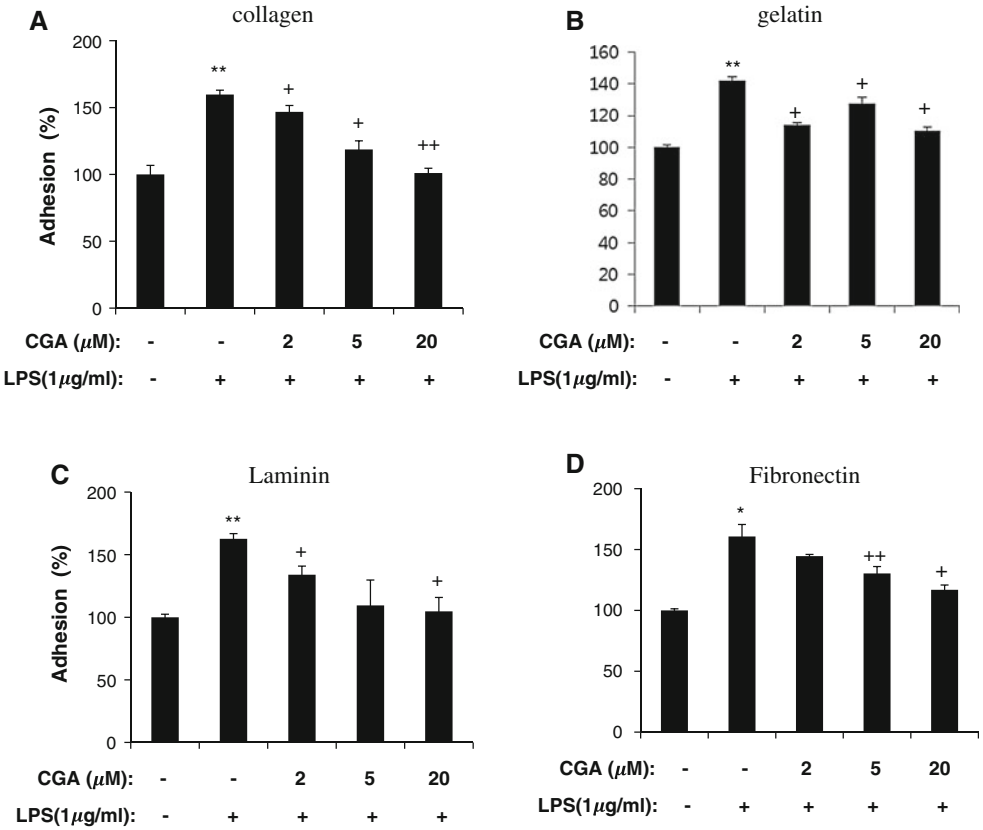


**Fig. 5** Effects of chlorogenic acid on the cell-to-matrix adhesion activity in LPS-stimulated RAW 264.7 cells. **a**, **b** RAW 264.7 cells suspended in serum-free media were added to each well coated with extracellular matrix such as gelatin, type I collagen, laminin, and fibronectin. Non-adherent cells were removed by washing and the remaining adherent cells were quantified as described in "Materials and methods". Cell-to-matrix adhesion assays used RAW 264.7 cells with either LPS (1 μg/ml) alone or chlorogenic acid. Adhesion values are expressed relative to the adhesion of vehicle-treated cells, normalized to 100 %. Values shown in the *graphs* are mean ± SD and were obtained from three independent experiments; $^{*}P < 0.05$, vehicle vs. LPS-treated group; $^{+}P<0.05$, LPS vs. LPS plus chlorogenic acid-treated group

adhesive activity of microglia/macrophages. LPS-induced expression of Ninj1 mRNA and protein was also significantly attenuated in a dose-dependent manner by chlorogenic acid (Fig. 4a, b). Moreover, immunofluorescence staining and quantification analysis of Ninj1 pixel intensity (% area of immunoreactivity) showed that chlorogenic acid abolishes Ninj1 up-regulation mediated by LPS treatment (Fig. 4c, d). Next, we examined the effect of chlorogenic acid on cell-to-matrix adhesion of RAW 264.7 cells. Under LPS-induced inflammatory conditions, the adhesion of RAW 264.7 cells on gelatin, type I collagen, laminin, and fibronectin matrix was increased compared with the control (Fig. 5). However, chlorogenic acid abolished the LPS-induced adhesion activity of RAW 264.7 cells in a dose-dependent manner (Fig. 5). Thus, these results showed that chlorogenic acid down-regulates Ninj1 expression and has an inhibitory effect on the adhesion activity of RAW 264.7 cells.

## Inhibitory effects of chlorogenic acid are mediated by NF-κB suppression in LPS-stimulated murine microglia/macrophages

Since the activation of NF-κB by LPS can induce the expression of pro-inflammatory mediators, we checked the effect of chlorogenic acid on the NF-κB signaling pathway under LPS-induced inflammation. To evaluate whether chlorogenic acid could influence the turnover and subcellular distribution of IκB proteins, and therefore NF-κB activation, the amounts of IκBα in the cytosol were determined by immunoblot analysis. As Fig. 6a shows, the marked decrease of IκB proteins elicited by LPS was markedly impaired in the presence of chlorogenic acid. Moreover, an important nuclear accumulation of p65 (NF-κB) was observed in cells treated with LPS, and chlorogenic acid treatment significantly attenuated the observed nuclear translocation (Fig. 6b, c). Taken together, these results indicated that chlorogenic acid's inhibition of the NF-κB signaling pathway may be the mechanism responsible for the suppression of NO and pro-inflammatory cytokines in LPS-stimulated murine microglia/macrophages.

## Chlorogenic acid inhibits the entry of Ninj1-expressing macrophages into mouse retina under endotoxin treatment

To investigate the effect of chlorogenic acid on the activation of macrophages in vivo, we used the LPS-inflamed mouse model and examined the entry of activating Ninj1-expressing macrophages into the retina. In untreated mice, Ninj1-expressing macrophages were not seen around

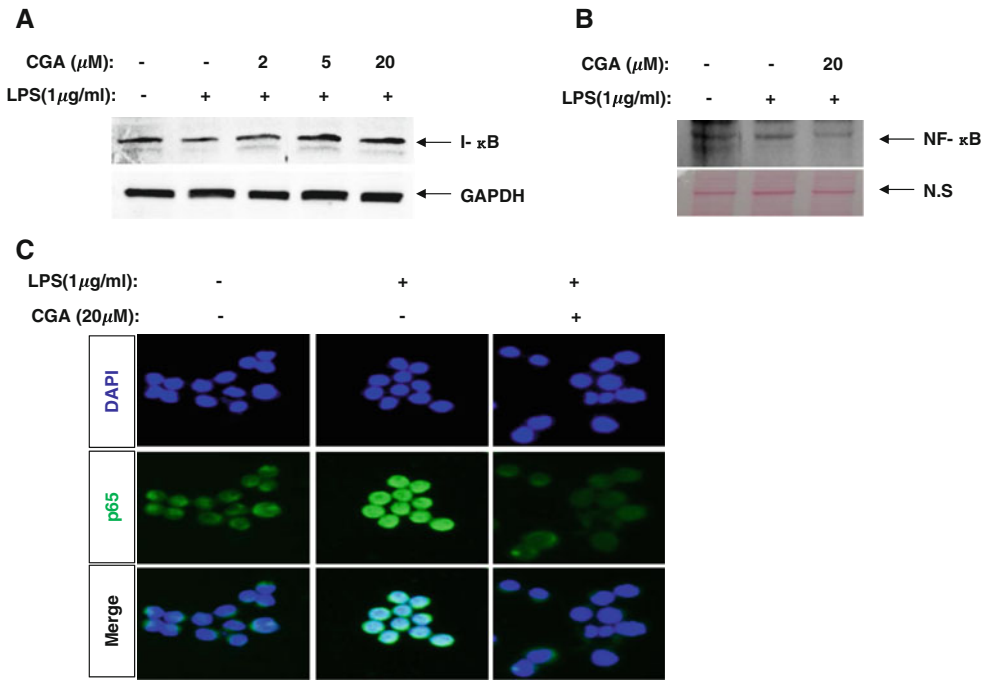


**Fig. 6** Effects of chlorogenic acid on NF-κB signaling pathway in LPS-stimulated RAW 264.7 cells. **a** Cells were treated with LPS (1 μg/ml) in the absence or presence of chlorogenic acid at different concentrations (0, 2, 5, and 20 μM, respectively) for 24 h. Total cytosolic protein was subjected to 15 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting using anti-IκBα. **b** Cells were treated with LPS (1 μg/ml) in the absence or presence of chlorogenic acid at 20 μM for 24 h. Total nuclear protein was subjected to 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Western blotting using anti-NF-κB. *NS* indicates a non-specific immunoreactive band. Ponceau staining was used as a standard. **c** Cells were treated with LPS (1 μg/ml) in the absence or presence of chlorogenic acid at 20 μM concentration. Immunofluorescent staining of NF-κB (*green*); nuclei were counterstained using DAPI (*blue*). Images are 40× magnification (color figure online)

retinal vessels (Fig. 7a). When the mice were treated with LPS, the number of Ninj1-expressing macrophages was increased and this was blocked by chlorogenic acid treatment. We confirmed this with Western blot analysis. LPS treatment enhanced the expression level of Ninj1 in retina lysates (Fig. 7b) and chlorogenic acid treatment led to the reduction of Ninj1 expression when compared with the LPS-treated group. These results suggest that chlorogenic acid may inhibit the migration and activation of macrophages during retinal inflammation.

## Discussion

In this study, we demonstrated the inhibitory activities of chlorogenic acid in RAW 264.7 cells and mouse retina under LPS-induced inflammation, at least in part owing to its regulation of NF-kB and Ninj1. Those results include: (1) decreased NO production mediated by down-regulation of iNOS; (2) suppression of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6, as well as the chemokine CXCL1 through down-regulation of NF-κB; (3) inhibition of Ninj1, which is important for leukocyte infiltration. This was consistent with previous findings, in which chlorogenic acid reduces inflammation and fibrosis through inhibition of the TLR4 signaling pathway in carbon tetrachloride ($CCl_4$)-induced liver fibrosis [22] and LPS-inflamed keratinocytes [23]. Although further studies are needed for the role of chlorogenic acid in inflammation, our present study provides an important proof-of-principle for the development of chlorogenic acid as an anti-inflammatory agent.

LPS is a cell wall component of Gram-negative bacteria, and plays a central role in the pathogenesis of septic shock [24]. When macrophages are exposed to bacterial products such as LPS, the LPS binds to Toll-like receptor 4 (TLR4) which activates two major signaling pathways, myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adaptor inducing IFN-β (TRIF), which result in activation of NF-κB. When the NF-κB signaling pathway is activated, macrophages secrete NO and pro-inflammatory cytokines, and express adhesion molecules [25–27]. Therefore, putative agents that can regulate the NF-κB activation and adhesion molecules have the potential to improve many inflammation-related symptoms in patients. In particular, the activation, adhesion, and homing of leukocytes are regarded as a mostly effective therapeutic target for inflammation-related diseases [21, 27]. Ninj1 is

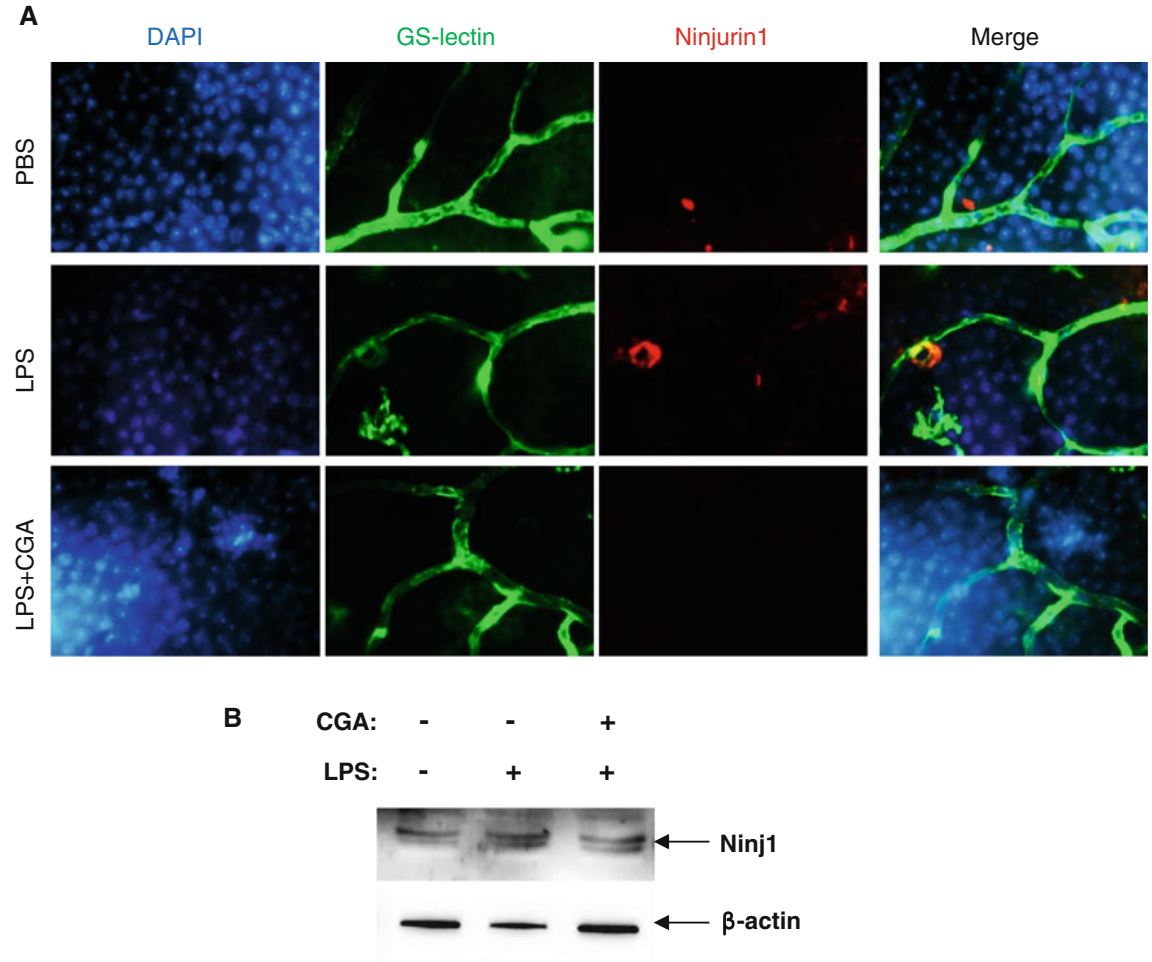


**Fig. 7** Effects of chlorogenic acid on Ninj1 expression in LPS-induced retinal inflammation model. **a** Immunostaining of adult whole-mounted retinas with antibodies for Ninj1 (*red*) and GS-lectin (*green*). Ninj1-positive cells appeared only after intraperitoneal injection of LPS in rat adult retina (7 weeks). Nuclei were counter-stained using DAPI (*blue*). **b** Ninj1 expression in eye lysates after 0–330 μg/kg of LPS injection in adult rat (7 weeks) in vivo. Mice were injected three times intraperitoneally (IP) with a dose of 6.6 μg of LPS (330 μg/kg of body weight) and/or with a dose of 0.1 mg of chlorogenic acid (5 mg/kg body weight) over 3 days. Animals were killed at 24 h after last LPS administration (color figure online)

an important adhesion molecule whose functions include immune surveillance, cell interaction, cell differentiation, and trafficking of leukocytes [28, 29]. Furthermore, Ninj1 is associated with multiple sclerosis, and regulates the migration of myeloid cells [21, 28]. In the present study, we observed that chlorogenic acid attenuates Ninj1 expression and decreases the cell-to-matrix adhesion ability of RAW 264.7 cells. Thus, chlorogenic acid will be a putative anti-inflammatory drug for regulating the adhesion and trafficking of leukocytes in leukocyte-mediated inflammatory diseases.

Various natural products including polyphenols such as resveratrol, flavonoids, and chlorogenic acid have various advantages for health [30, 31]. It has been widely accepted that chlorogenic acid has many health benefits as an anti-aging, anticancer, and anti-hypertension agent [15, 17, 18]. However, there is some controversy over whether chlorogenic acid prevents diabetes and allergy. For example, it is reported that chlorogenic acid reduced insulin responses and early fasting glucose [32]. In contrast, other studies show that chlorogenic acid enhances glucose uptake in skeletal muscle cells via AMPK activation, contributing a beneficial effect on type 2 diabetes mellitus [16, 33, 34]. Therefore, considering the controversial effect of chlorogenic acid in some diseases, extensive efforts are required to develop chlorogenic acid as a therapeutic agent.

In this study, we showed the inhibitory effects of chlorogenic acid on expression of NO, pro-inflammatory cytokines, and an important adhesion molecule Ninj1 regulated by the NF-κB pathway in LPS-stimulates RAW 264.7 cells. In conclusion, chlorogenic acid is a potential therapeutic drug for treating inflammatory diseases such as sepsis.

**Acknowledgments** This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Ministry of Education, Science and Technology (MEST) through the Global Research Laboratory Program (2011-0021874), the World Class University Program (R31-2008-000-10103-0), and the Global Core Research Center (GCRC) Program (2012-0001187).

## References

1. Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clin Exp Immunol. 2007;147:227–35.
2. Boyce JA. Eicosanoids in asthma, allergic inflammation, and host defense. Curr Mol Med. 2008;8:335–49.
3. Lee IT, Yang CM. Inflammatory signalings involved in airway and pulmonary diseases. Mediators Inflamm. 2013;2013:791231. doi:10.1155/2013/791231.
4. Barin JG, Rose NR, Cihakova D. Macrophage diversity in cardiac inflammation: a review. Immunobiology. 2011;217:468–75.
5. Papageorgiou AP, Heymans S. Interactions between the extracellular matrix and inflammation during viral myocarditis. Immunobiology. 2011;217:503–10.
6. Chen YW, Pat B, Gladden JD, Zheng J, Powell P, Wei CC, et al. Dynamic molecular and histopathological changes in the extracellular matrix and inflammation in the transition to heart failure in isolated volume overload. Am J Physiol Heart Circ Physiol. 2011;300:H2251–60.
7. Korhonen R, Lahti A, Kankaanranta H, Moilanen E. Nitric oxide production and signaling in inflammation. Curr Drug Targets Inflamm Allergy. 2005;4:471–9.
8. Montiel-Duarte C, Ansorena E, Lopez-Zabalza MJ, Cenarruzabeitia E, Iraburu MJ. Role of reactive oxygen species, glutathione and NF-kappaB in apoptosis induced by 3,4-methylenedioxymethamphetamine ("Ecstasy") on hepatic stellate cells. Biochem Pharmacol. 2004;67:1025–33.
9. Francisco V, Costa G, Figueirinha A, Marques C, Pereira P, Miguel Neves B, et al. Anti-inflammatory activity of Cymbopogon citratus leaves infusion via proteasome and nuclear factor-kappaB pathway inhibition: contribution of chlorogenic acid. J Ethnopharmacol. 2013;148:126–34.
10. Wu Y, Liu Y, Huang H, Zhu Y, Zhang Y, Lu F, et al. Dexmedetomidine inhibits inflammatory reaction in lung tissues of septic rats by suppressing TLR4/NF-kappa B pathway. Mediators Inflamm. 2013;2013:562154. doi:10.1155/2013/562154.
11. Nam NH. Naturally occurring NF-κB inhibitors. Mini Rev Med Chem. 2006;6:945–51.
12. Li X, Li Z, Zheng Z, Liu Y, Ma X. Unfractionated heparin ameliorates lipopolysaccharide-induced lung inflammation by downregulating nuclear factor-kappaB signaling pathway. Inflammation. 2013; doi: 10.1007/s10753-013-9656-5.
13. Gonthier MP, Verny MA, Besson C, Remesy C, Scalbert A. Chlorogenic acid bioavailability largely depends on its metabolism by the gut microflora in rats. J Nutr. 2003;133:1853–9.
14. Hulme AC. The isolation of chlorogenic acid from the apple fruit. Biochem J. 1953;53:337–40.
15. Zang LY, Cosma G, Gardner H, Castranova V, Vallyathan V. Effect of chlorogenic acid on hydroxyl radical. Mol Cell Biochem. 2003;247:205–10.
16. Ong KW, Hsu A, Tan BK. Anti-diabetic and anti-lipidemic effects of chlorogenic acid are mediated by AMPK activation. Biochem Pharmacol. 2013;85:1341–51.
17. Suzuki A, Yamamoto N, Jokura H, Yamamoto M, Fujii A, Tokimitsu I, et al. Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats. J Hypertens. 2006;24:1065–73.
18. Jung HA, Park JC, Chung HY, Kim J, Choi JS. Antioxidant flavonoids and chlorogenic acid from the leaves of Eriobotrya japonica. Arch Pharm Res. 1999;22:213–8.
19. Han HE, Kim TK, Son HJ, Park W, Han PL. Activation of autophagy pathway suppresses the expression of iNOS, IL6 and cell death of LPS-stimulated microglia cells. Biomol Ther. 2013;21:21–8.
20. Kang TJ, Moon JS, Lee SY, Yim DS. Polyacetylene compound from *Cirsium japonicum* var. *ussuriense* inhibits the LPS-induced inflammatory reaction via suppression of NF-κB activity in Raw 264.7 cells. Biomol Ther 2011; 19:97–101.
21. Lee HJ, Ahn BJ, Shin MW, Choi JH, Kim KW. Ninjurin1: a potential adhesion molecule and its role in inflammation and tissue remodeling. Mol Cells. 2010;29:223–7.
22. Shi H, Dong L, Jiang J, Zhao J, Zhao G, Dang X, et al. Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway. Toxicology. 2013;303:107–14.
23. Lee SA, Jung EB, Lee SH, Kim YJ, Bang H, Seo SJ, et al. 3,4,5-Tricaffeoylquinic acid inhibits the lipopolysaccharide-stimulated production of inflammatory mediators in keratinocytes. Pharmacology. 2012;90:183–92.
24. Sachithanandan N, Graham KL, Galic S, Honeyman JE, Fynch SL, Hewitt KA, et al. Macrophage deletion of SOCS1 increases sensitivity to LPS and palmitic acid and results in systemic inflammation and hepatic insulin resistance. Diabetes. 2011;60:2023–31.
25. Lee H, Bae S, Choi BW, Yoon Y. WNT/beta-catenin pathway is modulated in asthma patients and LPS-stimulated RAW264.7 macrophage cell line. Immunopharmacol Immunotoxicol. 2011;34:56–65.
26. Karpurapu M, Wang X, Deng J, Park H, Xiao L, Sadikot RT, et al. Functional PU.1 in macrophages has a pivotal role in NF-{kappa}B activation and neutrophilic lung inflammation during endotoxemia. Blood. 2011;118:5255–66.
27. Lee HJ, Kim KW. Anti-inflammatory effects of arbutin in lipopolysaccharide-stimulated BV2 microglial cells. Inflamm Res. 2012;61:817–25.
28. Ahn BJ, Lee HJ, Shin MW, Choi JH, Jeong JW, Kim KW. Ninjurin1 is expressed in myeloid cells and mediates endothelium adhesion in the brains of EAE rats. Biochem Biophys Res Commun. 2009;387:321–5.
29. Lee HJ, Ahn BJ, Shin MW, Jeong JW, Kim JH, Kim KW. Ninjurin1 mediates macrophage-induced programmed cell death during early ocular development. Cell Death Differ. 2009;16:1395–407.
30. Tucsek Z, Radnai B, Racz B, Debreceni B, Priber JK, Dolowschiak T, et al. Suppressing LPS-induced early signal transduction in macrophages by a polyphenol degradation product: a critical role of MKP-1. J Leukoc Biol. 2011;89:105–11.
31. Baowen Q, Yulin Z, Xin W, Wenjing X, Hao Z, Zhizhi C, et al. A further investigation concerning correlation between anti-fibrotic effect of liposomal quercetin and inflammatory cytokines in pulmonary fibrosis. Eur J Pharmacol. 2010;642:134–9.
32. van Dijk AE, Olthof MR, Meeuse JC, Seebus E, Heine RJ, van Dam RM. Acute effects of decaffeinated coffee and the major coffee components chlorogenic acid and trigonelline on glucose tolerance. Diabetes Care. 2009;32:1023–5.
33. Prabhakar PK, Doble M. Synergistic effect of phytochemicals in combination with hypoglycemic drugs on glucose uptake in myotubes. Phytomedicine. 2009;16:1119–26.
34. Ong KW, Hsu A, Tan BK. Chlorogenic acid stimulates glucose transport in skeletal muscle via AMPK activation: a contributor to the beneficial effects of coffee on diabetes. PLoS One. 2012;7:e32718.