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ORIGINAL CONTRIBUTION

Dietary supplementation with geranylgeraniol suppresses lipopolysaccharide-induced inflammation via inhibition of nuclear factor-κB activation in rats

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Abstract
Purpose The isoprenoid geranylgeraniol (GGOH) inhibits its nuclear factor-kappa B (NF-κB) activation in the liver, yet the mechanism remains unclear. We investigated the modulation and inhibition of lipopolysaccharide (LPS)-induced NF-κB signaling in the liver of rats fed a GGOH-supplemented diet.
Methods Rats were fed a diet supplemented with or without GGOH for 10 days. Rats were then intraperitoneally injected with 0.5 mg/kg LPS or vehicle (sterilized saline) and fasted for 18 h. Plasma levels of the inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, and the liver damage indicators alanine and aspartate aminotransferases (ALT and AST) were assessed. Liver mRNA and proteins were assayed for changes in NF-κB target genes and signal transduction genes.
Results Rats fed a high-dose, GGOH-supplemented diet showed significantly lower levels of plasma inflammatory cytokines and ALT and AST activities. In the liver, GGOH significantly suppressed NF-κB activation and mRNA expression of its pro-inflammatory target genes. Furthermore, GGOH supplementation substantially suppressed mRNA expression of signal transducer genes upstream of the IκB kinase complex. Western blotting of liver extracts further demonstrated the substantial decrease in total IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), leading to lower signal transduction and inhibition of NF-κB after LPS.
Conclusion A 10-day, high-dose, GGOH-supplemented diet was sufficient to inhibit LPS-induced inflammation and activation of NF-κB in rat livers. GGOH significantly modulated NF-κB signaling molecules, inhibiting its signal transduction and activation in the liver, thus protecting against liver damage.

Keywords Liver inflammation · Geranylgeraniol · *Irak1* · *Traf6* · NF-κB inhibition

Abbreviations

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
FOH	Farnesol
GOH	Geraniol
GGOH	Geranylgeraniol
GGPP	Geranylgeranyl pyrophosphate
Irak1	Interleukin-1 receptor-associated kinase 1
Tak1	TGF-beta activated kinase 1
TLR	Toll-like receptor
Traf6	TNF receptor-associated factor 6

Introduction

Natural bioactive components in dietary plants, such as fruits, vegetables, grains, and legumes, have epidemiologically been shown to inhibit and prevent degenerative diseases. The bioactive substances in their products, often called chemo-preventive phytochemicals, have important roles in the inhibition and control of inflammation [1, 2].

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Among the vast number of chemo-protective agents gaining attention are isoprenoids and terpenoids, a class of secondary metabolites from the mevalonate and 2-C-methyl-D-erythritol 4-phosphate pathways with over 22,000 constituents that show very potent inflammatory gene modulation [1–3].

Geranylgeraniol (GGOH; 3,7,11,15-tetramethylhexadeca-2E,6E,10E,14-tetraen-1-ol) is a C20 isoprenoid that is found in fruits, vegetables, and grains, including rice [2, 4]. GGOH may also be obtained from the hydrolyzation of geranylgeranyl pyrophosphate (GGPP) by intestinal alkaline phosphatase (one form of GGPPase) [4]. GGOH may be extracted from annatto food colorant, found in many kinds of food and categorized as GRAS (generally recognized as safe). Thus, the significance of chronic consumption of GGOH is an important line of study. GGOH and other hydrophobic isoprenoids, such as farnesol (FOH) and geraniol (GOH), may easily permeate enterocytes [4–6]. In the cell, both GGOH and FOH are thought to be subsequently converted into their respective pyrophosphate moieties (GGPP and farnesyl pyrophosphate—FPP) by two successive monophosphorylation events [5, 7].

GGOH exhibits anti-inflammatory actions in lipopolysaccharide (LPS)-simulated macrophage-like human THP-1 cells [8], monocytes from mevalonate kinase-deficient patients, and murine macrophage-like RAW 264.7 cells [9]. Several mechanisms have been proposed to explain how GGOH may inhibit inflammation. As a product of the mevalonate pathway, treatment with exogenous GGOH was shown to restore the low levels of de novo-synthesized isoprenoids, such as ubiquinone, commonly observed in inflammatory conditions and mevalonate dysregulation [5, 6, 10]. However, the mechanism remains unclear. GGOH may be used for the synthesis of GGPP as required for post-translational attachment to some proteins (isoprenylation) and proper translocation of various signaling proteins [10], which may be closely related to the inflammatory response. Furthermore, GGOH inhibits nuclear factor-κB (NF-κB) activation, along with other isoprenoids, such as ursolic acid, in a manner independent of redox change; however, the clear mechanism of this action remains elusive [11, 12].

Thus, the objective of this study was to investigate and clarify the mechanism of the anti-inflammatory effect of dietary GGOH supplementation in LPS-challenged rats. We observed a novel finding in the liver of these rats, namely that GGOH suppressed the basal expression and maintained a low concentration of signal transducers, particularly interleukin (IL)-1 receptor-associated kinase 1 (Irak1) and tumor necrosis factor (TNF) receptor-associated factor 6 (Traf6), thus substantially inhibiting LPS-induced NF-κB activation. Our observations indicate that GGOH may play a direct role in modulating the innate inflammatory response.

Materials and methods

Materials

GGOH was provided by Tama Biochemical Co. Ltd. (Tokyo, Japan) and stored at −20 °C. LPS (*Escherichia coli* O111:B4) was purchased from Sigma (St. Louis, MO) and dissolved in sterilized saline as a stock solution (1 mg/mL). We used a vitamin K-deficient diet for the base diet (TD97053), which was purchased from Harlan Teklad (Madison, WI) and reconstituted with 0.75 mg/kg phylloquinone, as was previously reported [13].

Animal experiments

Eight-week-old male Wistar rats, weighing 130–150 g, were purchased from SLC Japan (Shizuoka, Japan) and maintained on a 12/12-h light–dark cycle (08:00–20:00 light) at 23 ± 2 °C and 50 % ± 5 % relative humidity. For acclimatization, standard pellet feed (F2, Funabashi Farm, Chiba, Japan) was given for 3 days with distilled water. The rats were then divided into eight groups: two control groups fed the base diet with or without intraperitoneal LPS challenge (0.5 mg/kg body weight, Con+ and Con−); two groups fed the base diet supplemented with 48.3 mg/kg GGOH with or without LPS challenge (Lo GG+ and Lo GG−); two groups fed the base diet supplemented with 483.0 mg/kg GGOH with or without LPS challenge (Mid GG+ and Mid GG−); and two groups fed the base diet supplemented with 4,830 mg/kg GGOH with or without LPS challenge (Hi GG+ and Hi GG−). Experimental feed was given freely for 10 days, followed by intraperitoneal injection of LPS or sterilized saline then 18-h fasting prior to sacrifice. Fasting was observed to be essential in sensitizing rats to LPS challenge [14]. Euthanasia was performed by abdominal aorta exsanguinations, from which plasma was obtained, under diethyl ether anesthesia. Livers were promptly excised, placed in RNAlater (Ambion, Tokyo, Japan) or snap-frozen in liquid nitrogen, and stored at −65 °C until further analysis.

Ethical guidelines

The experimental plan for the present study was approved by the Animal Research Animal Care Committee of Tohoku University (No. 20-dounou-21). All experiments were carried out in accordance with the guidelines issued by this committee and Japanese governmental legislation (2005). The same committee supervised the care and use of the rats used in the study.

Assay for biochemical markers in plasma

Blood taken by exsanguination was centrifuged in Na_2EDTA-prepared tubes (final EDTA concentration 1.5 mg/mL blood) at 1,870×*g* for 15 min at 4 °C. The resulting plasma was divided into aliquots and stored at −30 °C. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, total cholesterol, and triglyceride levels were determined by enzymatic, colorimetric methods (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. Plasma concentrations of inflammatory cytokines were analyzed by Quantikine ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

RNA preparation and quantitative reverse transcriptase mediated polymerase chain reaction (RT-PCR)

Total RNA was isolated from excised liver by tissue disruption in guanidine isothiocyanate-based reagent (Isogen, Nippon Gene, Tokyo, Japan) using the bead-type homogenizer Micro Smash MS-100 (Tomy Seiko, Tokyo, Japan) according to the manufacturer's instructions. Isolated RNA was analyzed qualitatively by agarose gel electrophoresis, and an absorbance ratio of 260–280 nm was used to determine quantitative amounts of RNA. cDNA was synthesized from 5 μg total RNA and denatured with oligo-dT/random primers and 10 mM dNTP at 65 °C. The denatured RNA was then incubated in 50 mM Tris–HCl buffer (pH 8.3), 0.1 mM DTT, 50 units Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), and 20 units RNaseOUT RNase inhibitor (Invitrogen) at 25 °C for 5 min, then at 50 °C for 60 min, and finally at 70 °C for 15 min in a TaKaRa PCR Thermal cycler MP (Takara Biomedicals, Shiga, Japan). Aliquots of the synthesized cDNA were used as a template for quantitative PCR in an ABI 7300 (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. Measurement of expressed mRNA was first normalized to eukaryotic elongation factor 1α-1 (EF1α1) then compared with the expression of control mRNA to yield relative expression levels [13]. The sequences of primers used for each mRNA expression assay are shown in Table 1.

Western blot analysis

Frozen liver samples were homogenized in ice-cold extraction buffer [15] containing inhibitors for proteinase (Complete Mini proteinase inhibitor cocktail tablet, Roche Applied Science, Mannheim, Germany) and phosphatase (PhosSTOP phosphatase inhibitor cocktail tablet, Roche Applied Science). Tissue homogenates were centrifuged at 15,000×*g* for 20 min at 4 °C, and the supernatant was collected. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). Twenty micrograms of protein were mixed with SDS gel loading buffer and resolved by 10–20 % SDS-polyacrylamide gel electrophoresis (Wako Pure Chemical Industries). The proteins were then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were subjected to blocking for 1 h with TBS-T (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1 % Tween-20) containing 5 % bovine serum albumin (Sigma), incubated with antibodies against IRAK1 (Cell Signaling Technology, Danvers, MA), phosphorylated IκB kinase (IKK) α/β (Ser176/180; Cell Signaling Technology), TRAF6 (Cell Signaling Technology), transforming growth factor-beta activated kinase 1 (TAK1; Cell Signaling Technology), phosphorylated NF-κB p65 (Ser536; Cell Signaling Technology), or IκBα (Cell Signaling Technology), and detected by Immobilon Western Detection Reagent (Millipore) using a luminescent image analyzer LAS-4000mini (Fujifilm, Tokyo, Japan). The relative expression level of each protein was normalized according to the amount of α-tubulin detected in each sample (Sigma).

Statistical analysis

Values are represented as the mean value ± SEM. One-way analysis of variance followed by the Fisher's least significant difference test was used to evaluate the differences between groups unless otherwise stated. SPSS version 11.0 (SPSS Inc., Chicago, IL) was used for all data analysis. Statistical significance was determined at $p < 0.05$.

Results

GGOH supplementation suppresses LPS-induced inflammatory cytokines in rats

Ten days of experimental feeding supplemented with or without GGOH at any dose did not induce significant changes in final body weight, body weight gain, and average daily feed consumed (Table 2). After experimental feeding, LPS was administered to rats intraperitoneally. We observed significant suppression ($p < 0.027$) of plasma levels of inflammatory cytokines (IL-1β, TNF-α, and IL-6) in rats in the Mid GG+ and Hi GG+ groups (Fig. 1A–C). Additionally, we observed a significant decrease ($p < 0.031$) in liver damage, as measured by plasma AST and ALT activity, in rats fed the diet supplemented with higher concentrations of GGOH (Mid GG+ and Hi GG+) (Fig. 1D, E). These results indicate that dietary supplementation with higher concentrations of GGOH for 10 days suppressed LPS-induced

Table 1 Nucleotide sequences of primers used in quantitative RT-PCR

Gene	Forward primer	Reverse primer
Eef1a1	GATGGCCCCAAATTCTTGAAG	GGACCATGTCAACAATTGCAG
Il-6	AGAGGAGACTTCACAGAGGATACC	AATCAGAATTGCCATTGCACAAC
Il-1β	GCTGACAGACCCCAAAAGATT	ATCTGGACAGCCCAAGTCAA
Tnf-α	TAATGCTGATTTGGTGACCAGG	GTAGGGCGATTACAGTCACGG
Ccl2	AAGAAGCTGTAGTATTTGTCAC	ATCTCACTTGGTTCTGGTC
Vcam1	ATGGGAAGGTGAAGACAG	TAGGGAATGAGTAGATGTCCA
Irak1	CCAGAGAATCAAGTTTGAGGAG	GCCAGCTTTTGTACCATCTTC
Traf6	ACCAATATCTGGGGCAATTC	ACAAATTGATGAGCGTCTGG
Tak1	GAAAAACCAGGCAAAGCAAC	CACGATCCTCGCTTCTATTTC

Table 2 Growth and plasma parameters of rats after experimental feeding

	Con−	Lo GG−	Mid GG−	Hi GG−	Con+	Lo GG+	Mid GG+	Hi GG+
Body weight (g)	224.4 ± 3.9	235.1 ± 1.3	210 ± 2.0	208.6 ± 1.9	225.6 ± 3.3	222.3 ± 2.9	225.1 ± 2.5	227.5 ± 2.0
Weight gain (g)	47.6 ± 4.7	44.4 ± 4.2	45.4 ± 2.1	41.2 ± 2.2	33.5 ± 3.7	30.2 ± 3.1	33.7 ± 2.8	36.4 ± 3.8
Average feed consumed (g/day)	16.3 ± 0.5	17.7 ± 0.2	17.2 ± 0.6	16.6 ± 0.6	16.4 ± 0.6	15.7 ± 0.6	16.6 ± 0.8	16.2 ± 0.6
Plasma glucose (mg/dL)	159.6 ± 9.5	174.4 ± 6.5	140.1 ± 12.3	135.2 ± 9.4	117.4 ± 16.5	124.0 ± 9.2	121.5 ± 9.1	140.0 ± 9.6
Plasma TG (mg/dL)	139.5 ± 10.6	145.2 ± 9.1	121.7 ± 14.0	138.0 ± 8.2	133.8 ± 12.4	126.4 ± 12.1	118.3 ± 12.2	124.1 ± 9.3
Plasma TC (mg/dL)	42.9 ± 5.5[a]	36.7 ± 3.0[a]	40.8 ± 1.6[a]	40.9 ± 2.4[a]	55.1 ± 4.2[b]	64.8 ± 3.1[b]	59.8 ± 5.4[b]	59.2 ± 4.9[b]

All values represent the mean ± SEM; $n = 4$–8. Values with different letters (a and b) are significantly different at $p < 0.05$

inflammation and subsequent liver injury. Our observations are in agreement with those observed in another study involving liver damage induced by diethylnitrosamine with 2-acetylaminofluorene treatment [11].

LPS challenge increased plasma cholesterol levels significantly ($p < 0.023$) in the Con+ and all GGOH-supplemented groups when compared with non-challenged groups (Table 2). This result corresponds to earlier observations that LPS treatment may increase plasma cholesterol levels in animal models of inflammation [11, 16, 17]. However, there was no difference in plasma cholesterol levels among the LPS-challenged groups. Thus, the mevalonate pathway, including cholesterol synthesis, could not be altered by GGOH supplementation, and the anti-inflammatory effect of GGOH supplementation could not be attributed to the modulation of mevalonate pathway in this experiment.

GGOH suppresses mRNA expression of LPS-induced inflammatory genes in rat liver

One of the early signs of LPS challenge is injury to the liver, because LPS may concentrate rapidly in hepatic tissue, and up to 80 % of injected LPS may accumulate within a few hours [18], initiating inflammation. LPS clearance and liver injury are marked by elevated plasma ALT and AST [14]. The striking suppression of LPS-induced liver damage observed in the Mid GG+ and Hi GG+ groups (Fig. 1D, E) encouraged us to further investigate changes in inflammatory gene expression modulated by a higher concentration of GGOH. mRNA isolated from the livers of rats with or without GGOH supplementation was quantified by RT-PCR. We observed that the liver of Mid GG+ and Hi GG+ rats showed down-regulation of *Il-6, Il-1β*, and *Tnf-α* (Fig. 2A–C), which strongly correlated with the decrease in plasma inflammatory cytokine levels (Fig. 1A–C). Other inflammatory genes as targets of NF-κB transactivation, *Ccl2* and *Vcam1*, were also suppressed in rats fed the diet supplemented with a higher GGOH concentration (Fig. 2D, E). Next, we measured protein levels of LPS-induced phosphorylated NF-κB p65 (nuclear activated form) and its upstream signaling molecules in rat liver by western blot analysis. Amounts of phosphorylated p65 and IKKα/β, an upstream kinase of NF-κB activation, were significantly increased in the LPS-challenged groups. However, GGOH supplementation significantly suppressed ($p < 0.039$) these phosphorylated protein levels (Fig. 2F). The amounts of IκBα in LPS-challenged groups were significantly decreased compared with non-challenged groups. In the Mid GG+ and

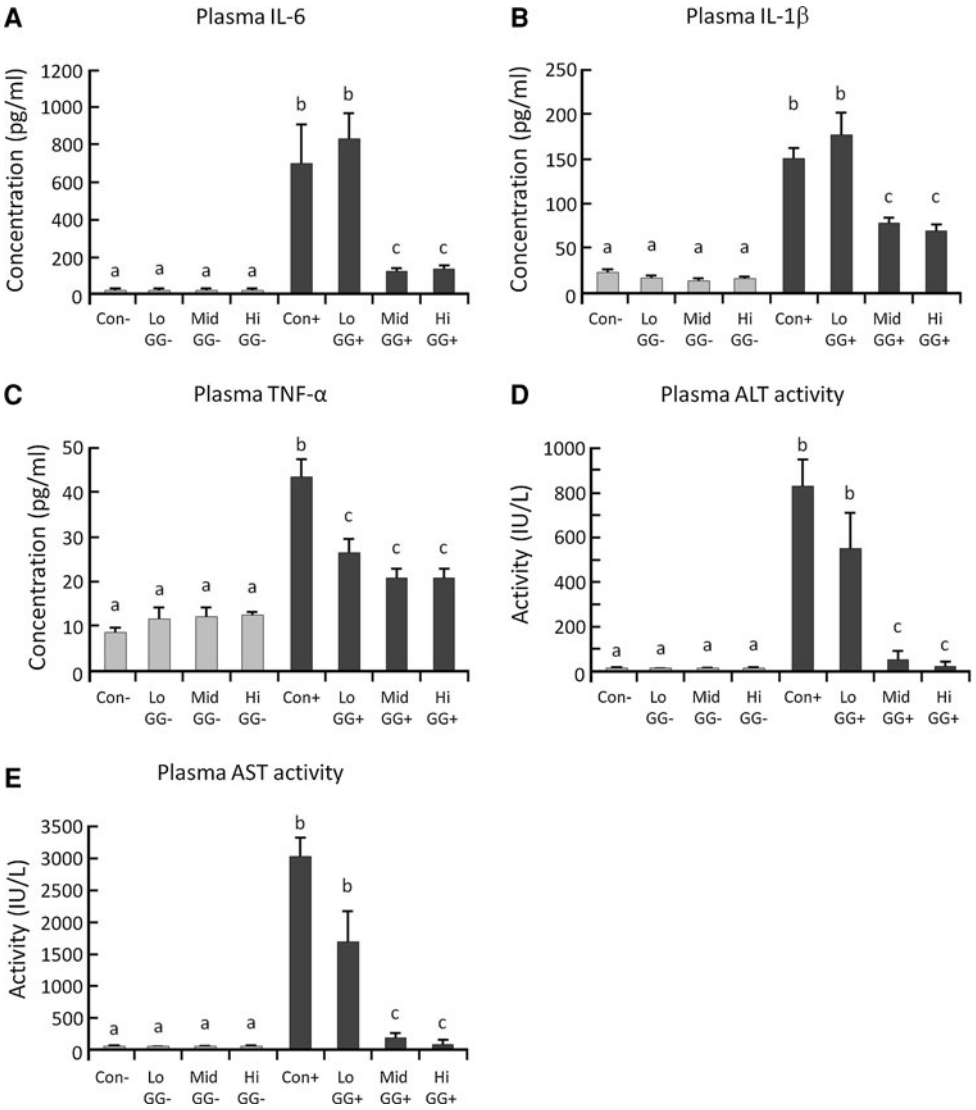

Fig. 1 GGOH supplementation suppressed LPS-induced inflammation and organ damage. **A–C** Plasma inflammatory cytokine levels after LPS challenge were substantially decreased after 10 days of GGOH supplementation (Mid GG+ and Hi GG+). **D**, **E** Organ damage caused by LPS challenge was suppressed by GGOH supplementation, as marked by a reduction in plasma activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). All values represent the mean ± SEM; $n = 4–8$. Values with *different letters* (*a*, *b*, and *c*) are significantly different at $p < 0.05$

Hi GG+ groups, IκBα levels were significantly increased compared with the Con+ group ($p < 0.022$). These results indicate that the suppression of mRNA expression of NF-κB target genes was caused by NF-κB inhibition in the liver of Mid GG+ and Hi GG+ rats.

GGOH supplementation suppresses protein levels of IRAK1, TRAF6, and TAK1, originating from transcriptional down-regulation

We hypothesized that GGOH treatment would affect the suppression of molecules that closely regulate NF-κB activation. Western blot analysis showed a significant decrease ($p < 0.037$) in signal transducer proteins in the liver of Mid GG+ and Hi GG+ rats. Specifically, the expression of IRAK1, TRAF6, and TAK1 (Fig. 3A) may be one cause of suppressed phosphorylated IKKβ/α, thus inhibiting NF-κB activation. It was apparent that basal expression (without LPS challenge) of IRAK1 and TRAF6 was significantly lower in the Mid GG- and Hi GG- groups, compared to controls, thus preventing the normally rapid response to LPS stimulation. Quantitative RT-PCR analysis showed no changes in the mRNA levels of Toll-like receptor 4 (*Tlr4*) or LPS-binding protein (data not shown); however, we observed a correlating down-regulation ($p < 0.031$) of signal transducer genes upstream of the IKK complex, starting from *Irak1*, *Traf6*, and *Tak1* in the livers of Mid GG+ and Hi GG+ rats (Fig. 3A–D). Furthermore, suppression of mRNA expression levels of these transducer genes (with the exception of *Irak1*) was observed in the livers of GGOH-supplemented rats without LPS challenge, indicating possible transcriptional modulation by GGOH.

Discussion

In this study, we demonstrated an inhibitory effect of GGOH on the LPS-induced NF-κB signaling cascade. The anti-inflammatory effect of GGOH treatment has been

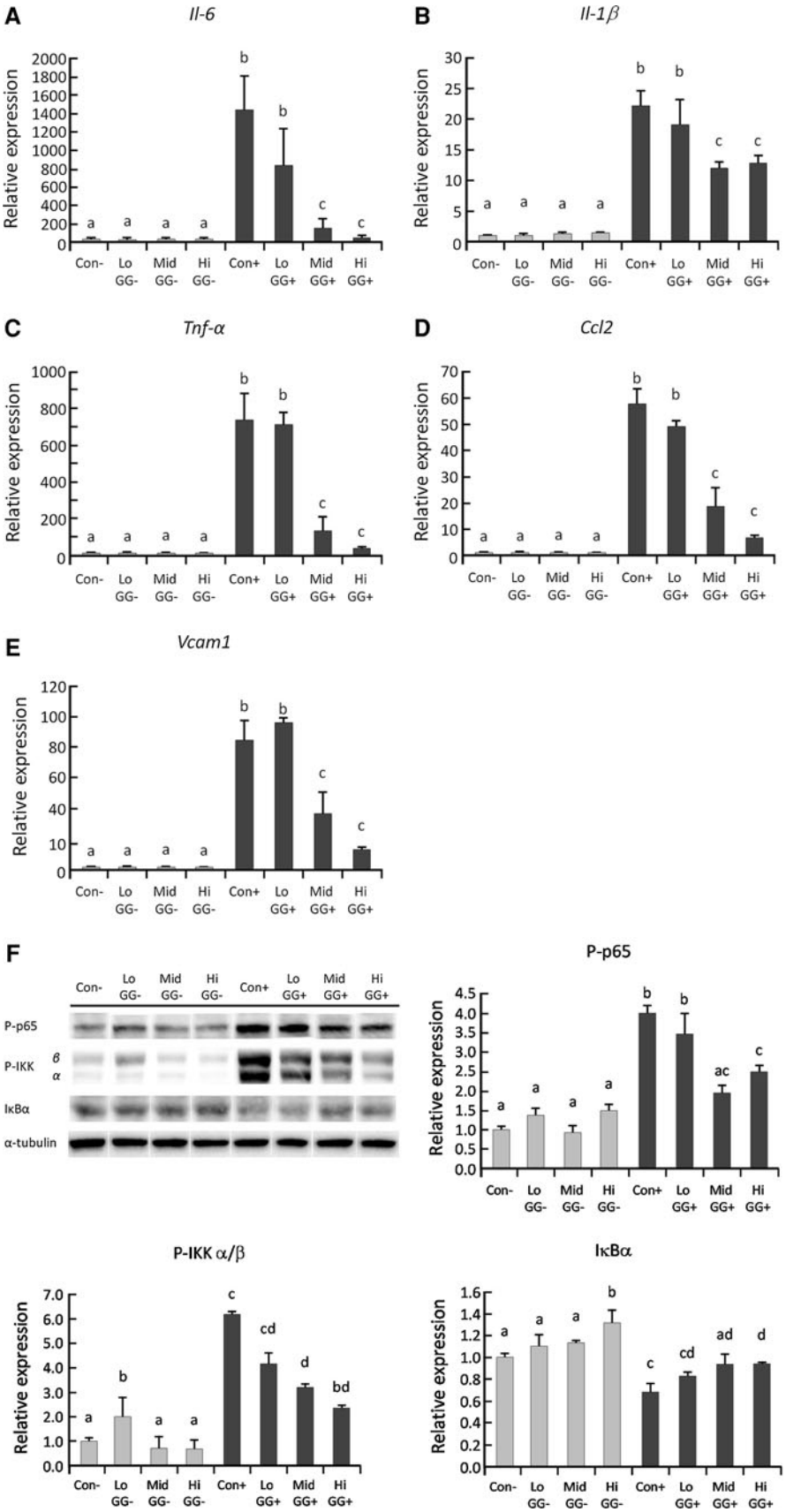

Fig. 2 GGOH suppressed LPS-induced inflammatory gene expression and NF-κB activation in the liver. **A**–**C** Messenger RNA levels of proinflammatory cytokines induced by LPS challenge were significantly suppressed in livers of rats in the Mid GG and Hi GG groups. mRNA levels of Ccl2 (**D**) and Vcam1 (**E**), additional target genes of NF-κB, were suppressed by higher doses of GGOH supplementation. **F** Western blot analysis of higher GGOH-supplemented rat livers indicated decreased phosphorylated NF-κB p65 stemming from suppressed phosphorylation of IKKα/β. All values represent the mean $\pm$ SEM; $n = 5$–8. Values with *different letters* are significantly different at $p < 0.05$. Photographs are representative of five rats

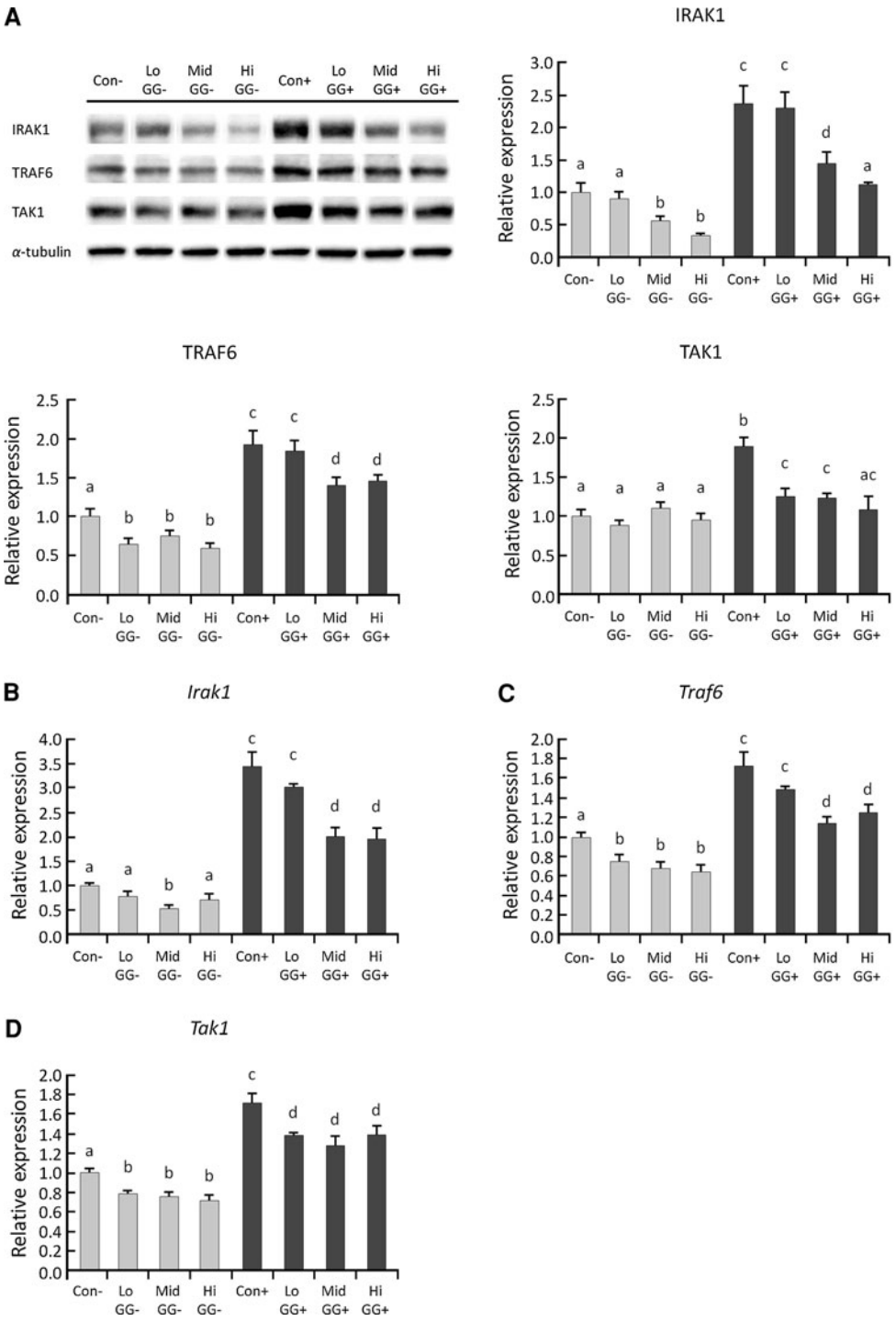

Fig. 3 GGOH supplementation suppressed protein and transcriptional levels of *Irak1*, *Traf6*, and *Tak1* in rat liver. **A** *IRAK1*, *TRAF6*, and *TAK1* protein levels were suppressed in livers of GGOH-treated rats as quantified by immunoblotting assays. Transcript levels of the signal transducers **B** *Irak1*, **C** *Traf6*, and **D** *Tak1* were suppressed by GGOH supplementation in non-challenged and challenged rats. All values represent the mean ± SEM; $n = 5$–8. Values with *different letters* are significantly different at $p < 0.05$. Photographs are representative of five rats

previously shown in LPS-stimulated human monocytic cells [8, 9]; however, to our knowledge, this study is the first to report a more detailed molecular effect of GGOH on NF-κB inhibition in an LPS-challenged rat model. Supplementation with exogenous isoprenoids, such as beta-ionone, limonene, GOH, and GGOH, has been cited on numerous occasions to affect cholesterol metabolism via the post-translational inhibition of HMG-CoA reductase activity [11], which has been observed as one anti-inflammatory effect. It was reported that 7 weeks of GGOH administration decreased NF-κB activation in rats challenged with diethylnitrosamine, which was attributed to a decrease in plasma cholesterol [11]. Although our 10-day feeding schedule was considered transient, our results indicated that there were no changes in either plasma cholesterol or triglyceride levels (Table 2).

NF-κB is an essential mediator of transcription during mammalian immunological responses [19–21]. During the response to pathogenic agents, such as LPS attachment to TLR4, adaptor proteins activate signaling cascades of ubiquitination and phosphorylation in signal transducers, beginning with the recruitment of Myd88 and IRAKs, and ultimately leading to the degradation of IκB via phosphorylation of the IKK complex and the nuclear translocation of NF-κB [19, 20, 22]. The binding of NF-κB to its response element in the promoter region of target genes then initiates the transcription of hundreds of genes in a variety of functional cellular programs, including those for inflammatory responses [20, 21, 23]. We chose to determine the mRNA expression levels of key inflammatory genes (*Il-1β*, *Tnf-α*, and *Il-6*) because of their rapid response in NF-κB activation downstream of TLR4 signaling. It has been reported that the LPS-induced secretion of IL-6 [8] and IL-1β may be alleviated by GGOH treatment [9, 16]. Our results show that dietary supplementation with higher doses of GGOH suppresses mRNA expression of inflammatory cytokines in the liver (Fig. 2A–C), which is in agreement with a previous report showing that NF-κB activation was inhibited by gavage-administered GGOH at a higher dose (16 mg/100 g body weight in rats) [11]. Furthermore, to verify the inhibition of NF-κB activation, we demonstrated that other target genes, including *Ccl2* and *Vcam1*, are highly suppressed by GGOH treatment at higher concentrations (Fig. 2D, E). The transcriptional suppression of these genes was further reinforced by a significant decrease in the plasma levels of inflammatory cytokines. It is apparent that the nuclear translocation of NF-κB was inhibited, as shown by a marked decrease in NF-κB p65 in the nucleus [11], a decrease in phosphorylated p65 (nuclear form of p65), and abundant IκBα, as shown by western blot analyses (Fig. 2F). We have observed similar western blot results (abundance of IκB and decrease of phosphorylated p65) in human HepG2 cells and macrophage-like THP-1 cells, preincubated with GGOH for 24 h before LPS stimulation (*unpublished data*). As the liver is a complex tissue comprising of hepatocytes and residential macrophages (Kupffer cells), in addition to other cells, our unpublished in vitro results correlates similar condition occurring in rat liver. As to the effect of GGOH on other cells in the liver such as liver sinusoidal endothelial cells (LSEC), stellate cells and others, further detailed investigation is required.

Activation and translocation of NF-κB relies on the phosphorylation and subsequent degradation of its inhibitor, IκB. In the event of an inflammatory response, such as TLR4 activation, IKKβ is the major kinase responsible for the phosphorylation of IκBα. Activation of IKKβ, in turn, requires signal transduction, beginning with the recruitment of IRAK1, followed by TRAF6, and forming a complex with TAB 2/3-TAK1 to complete the signaling cascade.

Dietary supplementation with high doses of GGOH suppressed the abundance of signal transducer proteins, namely IRAK1, TRAF6, and TAK1, in the liver of rats. It was apparent that the suppression of these proteins originated from the significantly lower transcriptional levels of their respective genes, as observed in the livers of non-LPS-challenged rats. This effect would precipitate in a lower inflammatory response to LPS, as the abundance of these signaling proteins is significantly limited. Similar gene suppression of *Traf6* and NF-κB inhibition by terpenoids was observed in sesquiterpene lactone parthenolide-treated murine RAW264.7 cells [24]. Other terpenoids, such as γ-tocotrienol [22] and celastrol [25], have been observed to inhibit the activity TAK1 in suppressing NF-κB activation. What is certain is that high-dose GGOH dietary supplementation for 10 days caused basal transcriptional repression of the transducer genes *Irak1*, *Traf6*, and *Tak1*. Thus, GGOH-mediated transcriptional suppression appears to limit the abundance and activation rate of signal transducers and IKK activation after LPS-TLR4 stimulation. However, further investigation is required to elucidate the mechanism that GGOH utilizes to suppress transcriptional levels of these genes. It is noteworthy that similar IKK inhibition was reported in the treatment of differentiated THP-1 cells with menaquinone-4, which shares strikingly similar chemical structure apart from its naphthoquinone ring [8].

Treatment with a high concentration of GGOH was presumed to decrease the synthesis of farnesyl and GGPP, which is required for farnesylation and geranylgeranylation processes of certain proto-oncogenes [11], such as *ras*, which is involved in the activation of NF-κB [21]. In another study (Giriwono et al., unpublished data), it was determined by tandem mass spectrometry that the livers of GGOH-supplemented rats contained an increased abundance of GGOH in its original form, with little change to other moieties, such as GGPP or GOH. This would indicate that GGOH is an active form that enables the modulation of these genes and proteins to inhibit NF-κB activation, with little effect on the alteration of prenylation and subsequent anti-inflammatory action. This view is in agreement with other reports describing normal level of *ras* protein in fibroblasts of hyperimmunoglobulinemia D syndrome (HIDS) patients treated with GGOH [16].

In summary, dietary supplementation with the isoprenoid GGOH at high concentrations was effective in suppressing LPS-induced inflammation and liver damage. The short duration of feeding did not alter cholesterol levels significantly, but was substantial in decreasing transcription of the signal transducers *Irak1* and *Traf6*, which would result in their lower protein abundance. This effect

subsequently limits the normal, innate inflammatory response, thus inhibiting IKK phosphorylation and NF-κB activation. Additional investigations would be valuable to further understand how GGOH is able to modulate and suppress the transcription of these signaling molecules.

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Conflict of interest The authors declare no conflicts of interest.

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