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Title Page

Title : **Diterpenoid geranylgeraniol supplementation suppresses LPS-induced inflammation in rat**

Running title : GGOH inhibits NF- κ B by suppressing IRAK1 and TRAF6

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Abstract

Background

Geranylgeraniol (GGOH) is a natural diterpenoid occurring virtually in all dietary sources which possess the mevalonate pathway, including rice. Rich sources of GGOH include *Bixa orellana* commonly known for its annatto food colorant which has been used abundantly in many forms of food for daily consumption. One functional aspect of GGOH is its reported anti-inflammatory effect. In this study, we examined the mechanism of GGOH in suppressing inflammation induced by lipopolysaccharide.

Materials and methods

Eight weeks old male Wistar rats were supplemented with or without GGOH at incremental doses (48.3 – 4830 mg/kg diet). After ten days of feeding, the rats were ip. injected with 0.5 mg/kg bw LPS or vehicle then fasted for 18 h before sacrifice. Levels of inflammatory cytokines (IL-1 β , IL-6 and TNF α) were measured.

Results

Rats supplemented with GGOH at dosages 483 and 4830 mg/kg diet showed substantial suppression in plasma concentrations of IL-1 β , IL-6 and TNF α . The liver of these rats were also protected from damage as indicated by lower plasma ALT and AST activity. Livers of GGOH treated rats showed down-regulation of inflammatory and NF- κ B signal transducer genes, specifically *Irak1*, *Traf6* and *Tak1*. This suppression, including inhibition of NF- κ B, was observed up to protein level as indicated by western blot assay. It appears that GGOH involves the degradation of IRAK1 to effectively inhibit activation of NF- κ B after LPS stimulation.

Conclusions

We observed that GGOH treatment *in vivo* effectively inhibited NF- κ B activation after LPS stimulation. We obtained results that indicate GGOH suppresses NF- κ B signal transduction molecules and maintaining them at low levels to inhibit excessive inflammation.

Keywords: isoprenoid; geranylgeraniol; anti-inflammation; lipopolysaccharide; IRAK1 suppression; NF- κ B inhibition; THP-1 cell; rat liver.

1 Introduction

Natural bioactive compounds in dietary plants such as fruits, vegetables, grains and legumes have been epidemiologically shown to inhibit and prevent degenerative diseases. It has become increasingly essential knowledge that the bioactive substances in these products, or quite often referred to as chemo preventive agents, have an important role in the control and maintenance of inflammation [1, 2]. Results from epidemiological studies and animal experiments may elucidate some aspect of the mechanisms involved, and investigations on more comprehensive and detailed mechanism are still ongoing and our understanding will continue to evolve. Of this vast chemo protective agents gaining recent interest are isoprenoids or terpenoids, a class of secondary metabolite from the mevalonate pathway consisting of over 22,000 constituents showing very potent inflammatory gene modulation [1-3].

It has been postulated that the action of isoprenoids in inflammatory suppression lies in the modification of mevalonate pathway which may also be involved in the biosynthesis of these compounds. Indeed, several observations have reported increased expression of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and down-regulation of squalene synthase that result in cholesterol production after inflammatory stimulation with LPS, IL-1 β and TNF- α [4-6]. Paradoxically, it has also been reported that dysregulation to the cellular mevalonate pathway, which results in the decrease of both cholesterol and isoprenoids synthesis, account for inflammation in auto-inflammatory diseases such as the case for mevalonate kinase deficiency or hyperimmunoglobulin-D syndrome [3,4,7,]. Thus it was recommended that treatment with exogenous isoprenoids may help in the alleviation of these and possibly other auto-inflammatory diseases. Furthermore, many emerging evidence point to other potentially direct interaction between isoprenoids with the NF- κ B activation cascade [2,3,8], an essential mediator and transcription factor for inflammation [8-11]. Thus isoprenoids may provide immune-modulatory effect.

Geranylgeraniol (GGOH) has been demonstrated to exhibit anti-inflammatory actions in human peripheral blood mononuclear cells (PBMCs) [12,13] and a mouse model of alendronate induced inflammation [7]. It has been observed that the geranylgeranyl pyrophosphate (GGPP) precursor, GGOH, and other hydrophobic isoprenoid derivatives, e.g. farnesol (FOH) are able to permeate the cell easily as the former pyrophosphate groups are prevented from entering living cells [12,14]. In the cell, GGOH and FOH are subsequently converted into their respective pyrophosphate moieties (GGPP and farnesyl pyrophosphate) by two successive monophosphorylations [12,15].

There are several proposed mechanisms as to how GGOH may inhibit inflammation. As an intermediate of the mevalonate pathway, treatment with exogenous GGOH was able to replete the low levels of *de novo* synthesized isoprenoids commonly observed in inflammatory conditions and/or mevalonate dysregulation such as ubiquinones [7,12,14,16] with little explanation on how it mediates this effect. Additionally, GGOH may be used for the synthesis of GGPP that is required for post-translational attachment to other proteins or isoprenylation, proper translocation of various signaling proteins, and is key to secretion of IL-1 β in stimulated BPMCs [13,16]. Furthermore, it has been demonstrated that GGOH inhibits NF- κ B activation, along with other isoprenoid such as ursolic acid, in a manner independent of redox change; however, the clear mechanism of this action remains elusive [17,18]. Thus the objectives of this study was to investigate and clarify possible interactions between GGOH and signal transducers which

result in the inhibition of NF- κ B activation induced by LPS stimulation. In this study, we observed that GGOH shows a more direct role in suppressing signal transducers particularly IRAK1 and TRAF6 to substantially inhibit NF- κ B activation.

2 Methods

2.1 Materials

GGOH was provided by Tama Biochemical Co. Ltd, (Tokyo, Japan) and stored at -20°C. LPS [*E.coli* O111:B4 (Cat. #L2630)] used throughout this study was purchased from Sigma (St. Louis, MO, USA) and dissolved in sterilized saline as stock solution (1 mg·mL⁻¹). Phorbol 12-myristate 13-acetate (PMA) purchased from Sigma was dissolved in ethanol to promote differentiation of THP-1 cells. We used a vitamin K deficient diet for base diet (TD97053) purchased from Harlan Teklad (Madison, WI, USA) re-constituted with 0.75 mg·kg⁻¹ phyloquinone.

2.2 Cell culture

Human monocytic THP-1 cells were obtained from RIKEN BioResource Center (Tsukuba, Japan) and cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 U·mL⁻¹ penicillin, and 100 μ g·mL⁻¹ streptomycin at 37 °C and 5% CO₂ atmosphere. The THP-1 cells were differentiated for 48 h with the addition of 10 ng·mL⁻¹ PMA before further treatments. Upon differentiation, the medium was exchanged with fresh medium and treated with GGOH (10 μ M) for 24 h incubation. Cells were then treated with LPS (1 μ g·mL⁻¹) and 3 h incubation followed by mRNA isolation.

2.3 Animal experiment

Eight weeks old male Std:Wistar rats, weighing 130-150g, were purchased from SLC Japan (Shizuoka, Japan) and maintained in 12 h light dark cycle (08:00 – 20:00 light), 23 \pm 1°C, and 50 \pm 5% RH. For acclimatization, standard pellet feed (F2, Funabashi Farm, Chiba, Japan) was given for three days with distilled water. The rats were then divided into four groups; two control groups fed base diet with or without LPS challenge (0.5 mg·kg⁻¹ body weight, Con- and Con+); and two groups fed base diet supplemented with 483 mg·kg⁻¹ diet GGOH with or without LPS challenge (GG- and GG+). Experimental feed was given freely for ten days, followed by intraperitoneal injection of LPS and 18 h fasting prior to sacrifice. Euthanasia was by abdominal aorta exsanguination under diethyl ether anesthesia, from which plasma was obtained. Livers were promptly excised; snap frozen in liquid nitrogen and stored under -65°C until further analysis.

All procedures involving animals were conducted humanely in accordance to the approval of the Animal Research-Animal Care Committee at the Graduate School of Agricultural Sciences, Tohoku University.

2.4 Blood and liver biochemical markers assay

Blood taken by exsanguination were centrifuged in Na₂EDTA prepared tubes (final concentration 1.5 mg·mL⁻¹ blood) at 1,870 \times g for 15 min at 4°C; and the resulting plasma was divided into aliquots and stored at -30°C. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, total cholesterol and triglyceride were determined by enzymatic colorimetric methods (Wako Pure

Chemical Industries, Osaka, Japan) according to the manufacturer's instruction. Plasma concentrations of inflammatory cytokines were analyzed by Quantikine ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

2.5 RNA preparation and quantitative RT-PCR

Total RNA was isolated from excised liver, previously stored in RNAlater (Ambion, Tokyo, Japan), by tissue disruption in guanidine isothiocyanate-based reagent (Isogen, Nippon Gene, Tokyo, Japan) with bead type homogenizer Micro Smash MS-100 (Tomy Seiko, Tokyo, Japan) according to the manufacturer's instructions. For cell culture, cells were homogenized by repeated pipetting in Isogen. Isolated RNA was analyzed qualitatively by agarose gel electrophoresis, whereas absorbance ratio of 260 to 280 nm was used to determine quantitatively. cDNA was synthesized from 5 µg of total RNA, denatured with oligo-dT/random primers, 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 of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 20 units of RNaseOUT RNase inhibitor (Invitrogen); at 25°C for 5 min, then 50°C for 60 min and finally 70°C for 15 min in TaKaRa PCR Thermal cycler MP (Takara biomedical, Shiga, Japan). Aliquots of the synthesized cDNA were used as template for quantitative PCR by ABI 7300 (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. Measurement of expressed mRNA was first normalized to its eukaryotic elongation factor 1 α -1 (EF1 α 1) then compared with the expression of control to yield relative expression [19]. The sequences of primers used for each gene expression assay are shown in Table 1.

2.6 Western blot

Frozen liver samples were homogenized with polytron homogenizer (Polytron, Basel, Switzerland) in ice cold extraction buffer [20] 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 homogenate was then centrifuged at 15000 $\times g$ for 20 min at 4°C and the supernatant was collected. For cell samples, after 24 h GGOH incubation (10 µM), differentiated THP-1 cells were treated with 1 µg·mL⁻¹ LPS for the determined duration then lysed at 4 °C for 30 min with the above-mentioned extraction buffer containing inhibitors for proteinase and phosphatase. The cell lysate was centrifuged at 15,000 $\times g$ for 20 min and the supernatant was collected. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Tokyo, Japan). Twenty micrograms of protein was mixed with SDS gel loading buffer and resolved on a 10–20% SDS–polyacrylamide gel electrophoresis (Wako Pure Chemical Industries); subsequently, the proteins were transferred on to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was subjected to blocking for 1 h with TBS-T (10 mM Tris-HCl at pH 7.4, 150 mM NaCl and 0.1% Tween 20) containing 5% bovine serum albumin (Sigma), and then incubated with antibodies against MyD88 (Cell Signaling Technology, Danvers, MA; USA), IRAK1 (Cell Signaling Technology), phosphorylated IRAK1 (Thr209; Abcam, Tokyo, Japan), TAK1 (Cell Signaling Technology), phosphorylated TAK1 (Thr184/187; Cell Signaling Technology), phosphorylated IKK α /β (Ser176/180; Cell Signaling Technology), TRAF6 (Cell Signaling Technology), NF-κB p65 (Cell Signaling Technology) or phosphorylated NF-κB p65 (Ser536; Cell Signaling Technology), and detected with the Immobilon Western

Detection Reagent (Millipore) using 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 by its antibody (Sigma).

2.7 Statistical analysis

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

3 Results

3.1 GGOH suppresses inflammatory cytokines in rat independent of cholesterol alteration

Ten days of experimental feeding supplemented with or without GGOH (483 mg·kg⁻¹ diet) did not induce change in normalized organ weight among similarly treated rats (with or without LPS stimulation). We also observed no significant change in lung and testis weight amongst all groups. It was observed, however, that livers of LPS challenged rats show increase in weight when compared to its respective negative controls, indicating acute liver inflammation (Table 2). We obtained significant suppression in the concentration of plasma inflammatory cytokines IL-1 β , TNF- α and IL-6 of rats supplemented with 483 mg·kg⁻¹ GGOH (Figs. 1A-C). Additionally, we observed striking hepatic protection from acute LPS inflammation in GGOH supplemented rats as indicated by the attenuation of plasma ALT and AST activities (Figs. 1D&E). The results we observed are in agreement with those observed in other study involving liver damage induced by diethylnitrosamine with 2-acetylaminofluorene treatment [17].

LPS challenge was observed to increase plasma cholesterol concentrations significantly in both Con+ and GG+ when compared to non challenged groups (although not significant between challenged groups) (Fig. 1F). This result confirms observations that LPS treatment may increase plasma cholesterol in animal models [4,6,17]. However, due to the short duration of feeding with GGOH, no cholesterol change or improvement was observed among GGOH supplemented group. Similar result was observed in plasma triglyceride concentration (Fig. 1G). Despite no improvement in plasma cholesterol of GG+ rats, substantial inflammatory suppression indicates GGOH inhibits NF- κ B activation by other mechanism.

3.2 GGOH modulates inflammatory genes in rat liver

The striking suppression of acute inflammation-induced liver damage observed in GGOH supplemented rats intrigued us to further investigate changes in inflammatory gene expression modulated by GGOH. Messenger RNA isolated from livers of rats with or without GGOH supplementation was quantified by RT-PCR. We observed that liver of GGOH supplemented rats showed down-regulation of inflammatory genes (Figs. 2A-C), strongly correlated with the decrease in plasma inflammatory cytokines (Figure 1A-C). We presumed GGOH treatment interacts and affects suppression of molecules closely regulating NF- κ B activation and observed further down-regulation of signal transducer genes upstream of IKK complex, notably Irak1 and Traf6, in the liver of GGOH supplemented groups (Figs. 2D&E). This suppression was further observed in western blots of GGOH supplemented rat livers thus inhibiting of NF- κ B activation (Fig. 2F). Furthermore, gene suppression of Irak1 and Traf6 were observed in livers of GGOH

supplemented rats without LPS challenge thus indicating possible modulation of these gene expressions by GGOH. Suppression of TRAF6 and IRAK1 proteins was also observed in human HepG2 cells incubated with 10 μ M GGOH for 24 hours (Supplementary Figure 1).

3.3 Pre-incubation with GGOH suppresses inflammatory genes in human THP-1 cells

In an attempt to elucidate the molecular mechanism of GGOH in suppressing TLR4-NF- κ B inflammatory response to LPS stimulation, we turn to an established *in vitro* model using human differentiated monocytic THP-1 cells. One rationale is that differentiated THP-1 cells behave like native monocyte-derived macrophages more than other human myeloid cell lines, such as HL-60, U937, KG-1, or HEL cell lines; thus providing a valuable model for studying the mechanisms involved in regulation of macrophage-specific genes as they relate to physiological functions such as inflammatory responses [21]. The other reason is that hepatic tissue contains resident macrophagic cells, or Kupffer cells, and is subjected to further macrophagic infiltration during the onset of inflammatory challenge.

Differentiated THP-1 cells pre-incubated with GGOH for 24 h (1 μ M or 10 μ M) then treated with 1 μ g·mL⁻¹ LPS showed significant suppression of inflammatory cytokine mRNA expressions, primarily IL-1 β , TNF- α and IL-6 (Figs. 3A-C). The suppressions of these genes were observed clearly substantial ($p < 0.05$) in the cells pre-incubated with higher GGOH concentration. However, time course pre-incubation of GGOH did not show strong correlation of its inflammatory suppression effects (data not shown). Quantification of other NF- κ B target genes involved in inflammatory response, such as CCL2 and COX2, also showed significant down-regulation (Figs. 3D&E). To further validate our observation that GGOH effectively abolished NF- κ B activation after LPS stimulation, results from western blot of phosphorylated NF- κ B p65 (activated form) and total I κ B α indicate clear inhibition by 10 μ M GGOH 24 h pre-incubation (Fig. 3F).

3.4 GGOH suppresses IRAK1 expression and subsequent phosphorylation of NF- κ B signaling molecules

We observed that GGOH was able to suppress gene and protein expressions of Irak1 and Traf6 in rat liver and HepG2 cells. This observation was also confirmed in differentiated THP-1 pre-incubated with 10 μ M GGOH for 24 h. GGOH significantly suppressed mRNA expressions of IRAK1, TRAF6, and TAK1 in LPS stimulated cells (Fig. 4A). This was further demonstrated in the western blot assay that protein expressions of IRAK1 and TRAF6 were decreased after GGOH 24 h pre-incubation (0 min); and up to 30 min after LPS stimulation in TRAF6 (Fig. 4B). To clarify whether GGOH suppresses expressions of IRAK1 and TRAF6 prior to LPS stimulation, we incubated THP-1 cells with GGOH for transiently and observed mRNA expression change only for IRAK1. However, we acquired substantial protein suppression in western blot of IRAK1 and TRAF6 thus indicating a possible increase of degradation promoted by GGOH treatment (Supplementary Figure 2).

We further investigated whether GGOH treatment may affect the phosphorylation of these signaling proteins. We treated differentiated THP-1 cells with 10 μ M GGOH for 24 h followed by LPS stimulation then harvested at indicated times and immediately lysed for western blot analysis. The results obtained show effectively significant suppression of TAK1 and IKK α / β phosphorylation (Fig. 4C). Thus it

was apparent that GGOH treatment promotes the suppression of IRAK1 and TRAF6 resulting in the decrease of subsequent phosphorylation of TAK1 and IKK α/β ; and inhibition of NF- κ B activation.

4 Discussion and conclusions

In this study, we have demonstrated the inhibitory effect of GGOH on NF- κ B signaling cascade stimulated by LPS. The anti-inflammatory effect of GGOH treatment have been previously demonstrated in human PBMCs [12,13], however, to our knowledge this study is the first to report an insight to a more detailed molecular effect of GGOH on NF- κ B inhibition. Supplementation of exogenous isoprenoid has been numerously cited to affect cholesterol metabolism by post-translational inhibition of HMG-CoA reductase activity [3,16,17] and has been postulated as a possible anti-inflammatory effect. Although, it has been reported that 7 weeks of GGOH administration was shown to decrease both plasma cholesterol and NF- κ B activation in rats [17], our ten days feeding results indicate no improvement in plasma cholesterol and triglyceride concentrations (Figs. 1F&G). LPS challenge, however, was observed to elevate plasma cholesterol in groups treated with or without GGOH in the acute phase period. This elevation was also reported in previous studies involving, but not limited to, Syrian hamster and has been attributed to the observation that inflammatory responses greatly decrease hepatic squalene synthase activity [5,6,22]. However, we observed significant anti-inflammatory action with little improvement to plasma cholesterol concentration in GGOH treated rats. This observation may indicate a more direct effect by GGOH in suppressing molecules regulating NF- κ B activation. Thus we were able to obtain significant down-regulation of signal transduction genes, in particular IRAK1 and TRAF6, in livers of GGOH supplemented rats with or without LPS challenge (Fig. 2).

NF- κ B is an essential mediator of transcription in the immunological response of mammalian cells [9,23,25]. As a response towards pathogenic agents, such as LPS attaching to TLR4, adaptor proteins activate signaling cascade of ubiquitination and phosphorylation in signal transducers starting from recruitments of MyD88 and IRAKs and ultimately leading to degradation of I κ B and the nuclear translocation of both NF- κ B subunits [8,9,23]. The binding of NF- κ B to its responsive element in target genes then initiates transcription of hundreds of genes for a variety of functional cellular programs, including those for inflammatory response [23,24,26]. We have chosen to preliminary determine gene expressions of key inflammatory proteins IL-1 β , TNF- α and IL-6 based on the facts that these are rapid response target genes of NF- κ B activation from TLR4 signaling. It has also been reported that LPS induced secretion of IL-1 β may be alleviated by GGOH treatment [16].

We obtained results showing dose dependent suppression activity of GGOH on inflammatory gene expressions (Figs. 3A-C) in agreement with previous report, also showing effective activities at higher dosage [17]. Furthermore, in order to verify the inhibition of NF- κ B activation, it was demonstrated that other target genes including CCL2, COX2, are highly suppressed by GGOH treatment at higher concentrations (Figs. 3D&E). This observation was also observed in our experiments in which liver mRNA expressions of these NF- κ B target genes were down-regulated in GGOH supplemented rats (Figs 2A-C, and unpublished data of down-regulation of chemokines and cell adhesion molecules). The transcriptional suppression of these genes was further reinforced by significant decrease in plasma levels of inflammatory

cytokines. It is apparent that NF- κ B was inhibited from nuclear translocation as shown by markedly decreased NF- κ B p65 in nucleus [17] and our western blots of decreased phosphorylated p65, abundance of I κ B α (Fig. 2F&3F) and luciferase reporter assay (data not shown).

One mechanism that has been shown to inhibit NF- κ B signaling is inhibition of oxidative stress [27] which would be unlikely in this case as GGOH does not show anti-oxidative properties [28,29]. Another possible mechanism put forward is the interference in the processing of *ras*, one important activator of NF- κ B [24] or a direct inhibition of I κ B phosphorylation, and subsequent ubiquitination, with an apparent inhibition of NF- κ B p-65 phosphorylation as demonstrated with other (cyclic) isoprenoid in the form of ursolic acid [17,18]. Several small geranyl-geranylated proteins of Ras family, in particular Rho subfamily, are demonstrated to participate in the signaling pathways leading to the activation of NF- κ B and subsequent induction of cytokines and chemokines [30,31]. Treatment with statin may inhibit isoprenylation of these proteins which results in decrease of membrane translocation and increase of activated GTPase [31]; which negatively affects geranylgeranylation by GGOH treatment. Although interference to these small G-proteins cannot be ruled out, it would appear that GGOH treatment involves a different mechanism to inhibit NF- κ B activation.

We demonstrated that treatment with GGOH induce suppression of IRAK1 and TRAF6 protein expression as early as 60 min incubation (Supp. Fig. 2) and more prominently after 24 h incubation. GGOH was also observed to suppress the transcription of these genes under LPS stimulation (Fig. 4A). Similar observation of TRAF6 gene suppression and NF- κ B inhibition was reported in RAW264.7 cells by sesquiterpene lactone parthenolide [32]. GGOH may promote degradation of IRAK1 and TRAF6 as observed from substantial protein decrease in as early as 60 min after treatment (Supp. Fig. 2). Proteosomal degradation of TRAF6 may be attained by polyubiquitination of Lys-48, as opposed to Lys-63 for signal transduction, and this degradation has been reported to be promoted by the sesquiterpene lactone eupatolide [33]. However, degradation of IRAK1 has been reported to be initiated by rapid autophosphorylation of IRAK1 as a result of TLR/IL-1R stimulation [34], which correlates with our finding (data not shown). IRAK1 would then undergo ubiquitination to which its specific E3 ligase has yet to be elucidated [34,35]. This degradation has been shown to occur in tolerant THP-1 [36] and dendritic cells [37]. However, as GGOH is not a ligand to TLR or IL1R, this degradation effect is perhaps due to dysregulation of membrane bound adaptor proteins as GGOH enters cellular space. Nevertheless, as detailed mechanism of this effect remains unclear and requires further investigation we may conclude that GGOH suppresses protein expressions of IRAK1 and TRAF6 (Fig. 4d).

Another possible mechanism to inhibit NF- κ B activation is interference and suppression of ubiquitination or phosphorylation. It was observed that GGOH treatment affects substantial inhibition of TAK1 and IKK α / β phosphorylation. This may be partly due to GGOH suppressing gene and protein expression of TRAF6 after LPS stimulation thus inhibiting further signal transduction from TLR4. Another plausible explanation is the inhibition of IKK, NIK and possibly further upstream phosphorylations by diterpenoid derivatives of GGPP with similar four units of isoprene as mentioned [25]. This may explain the fact that GGOH shows effective phosphorylation inhibition after 24 h of preincubation in which conversions to these derivatives may have occurred abundantly, and that very short incubation with GGOH

does not show inhibited phosphorylation of IRAK1 and TAK1 (data not shown). A recent report showed menaquinone-4 (MK-4), one form of vitamin K2, exhibiting similar NF- κ B inhibition effect in differentiated THP-1 cells [38] by suppressing IKK α / β phosphorylation. Apart from its naphthoquinone ring, MK-4 and GGOH show strikingly similar chemical structures. Other studies have also reported that γ -tocotrienol with its multiple unsaturated isoprene units, but not γ -tocopherol, effectively inhibits NF- κ B activation by inhibiting TAK1 and all other downstream signaling protein in a TNF stimulated inflammation without affecting its DNA binding ability [8](Ahn *et al.*, 2007). Similar inhibition of TAK1 was also observed in celastrol (a novel isoprenoid) treated human embryonic kidney A293 cells stimulated with TNF- α to activate NF- κ B [39]. We view that more investigations on the details of how GGOH promotes degradation and inhibition of phosphorylations in NF- κ B signaling cascade is required. Recent studies have shown and will continue to reveal immunomodulatory effect by various isoprenoids and terpenoids, including GGOH, in the treatment of various diseases that provides alternative use of natural compounds to target signal transducers.

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6 Conflict of interest

None.

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Table 1. Sequences of primers used to detect and quantitated gene expressions from cDNA synthesized from isolated rat liver and differentiated THP-1 cell RNA.

Gene	Forward primer	Reverse primer
<i>Eef1a1</i>	GATGGCCCCAAATTCTTGAAG	GGACCATGTCAACAATTGCAG
<i>Il-6</i> **	AGAGGAGACTTCACAGAGGATACC	AATCAGAATTGCCATTGCACAAC
<i>Il-1β</i> **	GCTGACAGACCCCAAAAGATT	ATCTGGACAGCCCAAGTCAA
<i>Tnf-α</i> **	TAATGCTGATTTGGTGACCAGG	GTAGGGCGATTACAGTCACGG
<i>Myd88</i> **	ACTTGTTCTCTCTACCGTTGGTC	TCCAAGTACTCGAAACCCATC
<i>Irak1</i> **	CCAGAGAATCAAGTTTGAGGAG	GCCAGCTTTTGTACCATCTTC
<i>Traf6</i> **	ACCAATATCTGGGGCAATTC	ACAAATTGATGAGCGTCTGG
<i>IL-1β</i>	CTGATGGCCCTAAACAGATGAAGT	GCCTGAAGCCCTTGCTGTAGT
<i>TNF-α</i>	TGTTGTAGCAAACCCTCAAGCTG	AGGACCTGGGAGTAGATGAGGTACA
<i>IL-6</i>	ATGAGGAGACTTGCCTGGTGAA	ACTCTCAAATCTGTTCTGGAGGTACTC
<i>CCL2</i>	CAAGCAGAAGTGGGTTTCAGGAT	AAGTCTTCGGAGTTTGGGTTTG
<i>COX2</i>	TGAGCATCTACGGTTTGCTG	AACTGCTCATCACCCCATTC

** Sequence specific to detect and quantify rodent gene (*Rattus norvegicus*)

Table 2. Weight of selected organs normalized to 100g body weight after ten days of experimental feeding, ip. injection and 18 h fasting.

Group	Liver				Lung			Testis		
<i>Con -</i>	2.57	±	0.04	^a	0.40	±	0.01	1.14	±	0.01
<i>Con +</i>	3.12	±	0.07	^b	0.49	±	0.01	1.11	±	0.03
<i>GG -</i>	2.71	±	0.05	^a	0.38	±	0.02	1.13	±	0.01
<i>GG +</i>	3.19	±	0.05	^b	0.44	±	0.01	1.13	±	0.02

All values are mean ± SEM; n=4-8; different letters indicate significant difference as determined by Tukey post-hoc.

Figure legends.

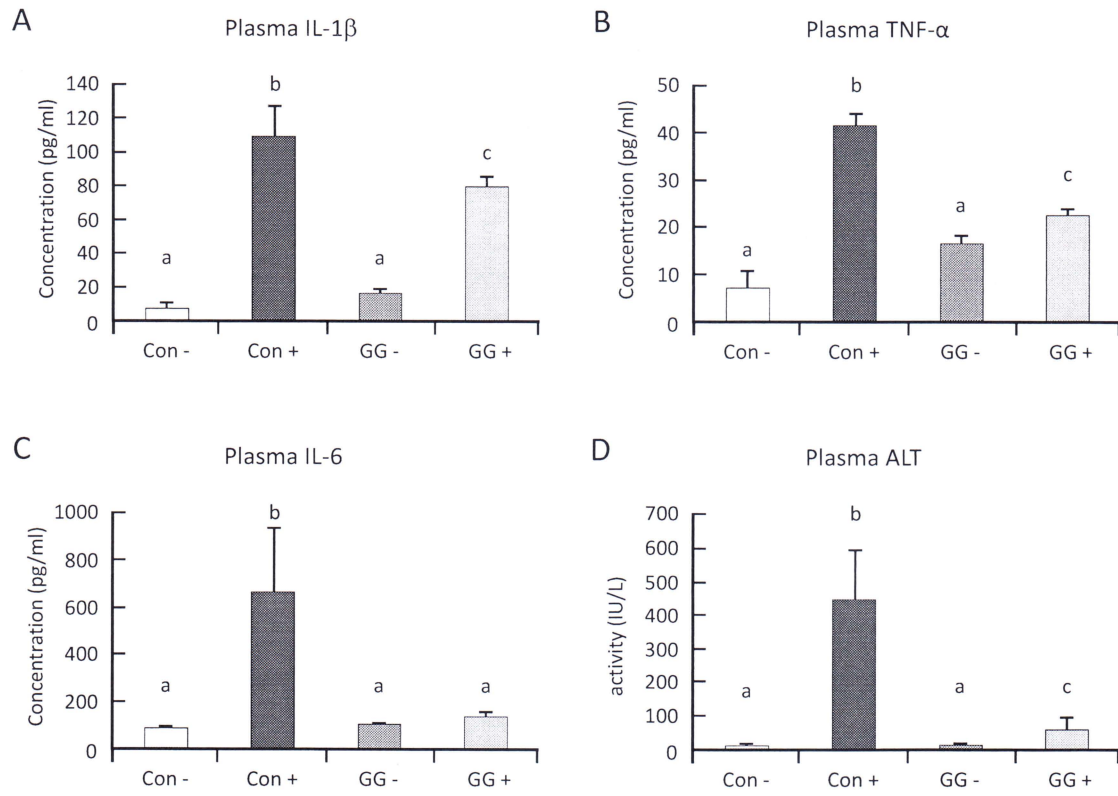
Figure 1. GGOH suppresses LPS induced inflammation and liver damage independent of cholesterol alteration *in vivo*. (A-C) Plasma inflammatory cytokines after LPS challenge were substantially decreased after ten days of 483 mg·kg⁻¹ diet GGOH supplementation (GG +). (D, E) Inflammation caused liver damage was similarly suppressed by GGOH supplementation as marked reduction in plasma concentrations of ALT and AST. (F) Plasma concentration of cholesterol was markedly increased in LPS challenged rats, however no alleviation was observed in GGOH supplemented group. (G) Plasma triglyceride concentration shows no alteration. All values are mean ± SEM; n=4-8. The values with different letters (a, b, and c) are significantly different at $p<0.05$.

Figure 2. GGOH effectively dampens inflammatory genes and NF-κB in the liver. (A-C) Inflammatory gene expressions were significantly suppressed in livers of rats supplemented with GGOH 483 mg·kg⁻¹ diet, correlating with decreased plasma cytokine concentrations. (D, E) Gene expressions of Irak1 and Traf6 were suppressed by GGOH supplementation in both LPS challenged and unchallenged rats. (F) Western blot of GGOH supplemented rat livers show suppressed Irak1 and Traf6 protein levels. All values are mean ± SEM; n=5-8. The values with different letters (a, b, and c) are significantly different at $p<0.05$. Photographs are representative of 5 rats.

Figure 3. GGOH preincubation prevents over-activation of NF-κB in LPS stimulated THP-1 cells. THP-1 cells were differentiated with PMA then pre-incubated with GGOH or not for 24 h before LPS stimulation. After 3 h stimulation, cells were harvested for RNA isolation described in Methods. (A-C) Pre-incubation with high GGOH concentration effectively inhibited over-expression of inflammatory cytokine genes. (D, E) Over-expressions of other NF-κB target genes involved in further inflammatory enhancement were also suppressed by GGOH preincubation. All values are mean ± SEM; n=3. The values with different letters (a, b, and c) are significantly different at $p<0.05$. (F) Representative photograph of suppressed NF-κB p65 phosphorylation and IκB degradation in GGOH pre-incubated cells. Cells were harvested after indicated time of LPS stimulation. Western blot results are from 3 independent experiments. *The values are significantly different compared from those of control at $p<0.05$.

Figure 4. GGOH preincubation modulates NF-κB signaling molecules. Differentiated THP-1 cells were incubated with or without 10μM GGOH for 24 h, then stimulated with LPS for indicated times. (A) After 3 h of LPS stimulation, THP-1 cells were harvested followed by RNA isolation and cDNA synthesis. Signal transduction gene expressions were determined by quantitative RT-PCR. Values are mean ± SEM; n=3. The values with different letters (a, b, and c) are significantly different at $p<0.05$. Western blot of total signal transduction proteins (B) and their phosphorylated state (C) were markedly dampened in cells pre-incubated with GGOH for 24 h. Photographs are representative of three independent experiments. *The values are significantly different compared from those of control at $p<0.05$.

Figure 1



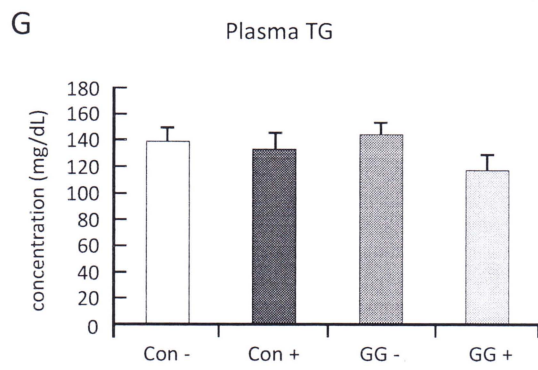
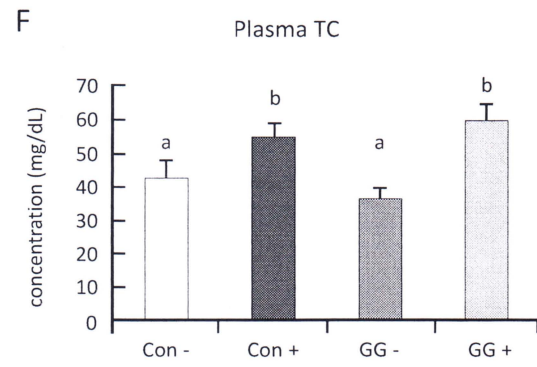
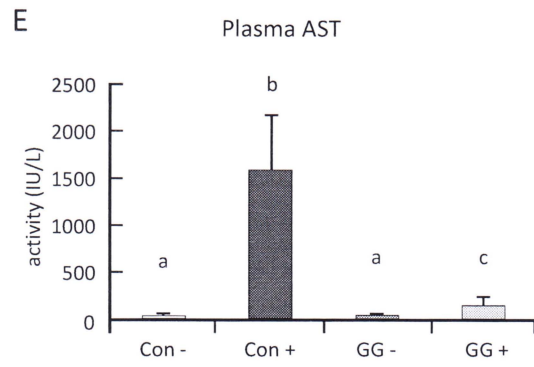
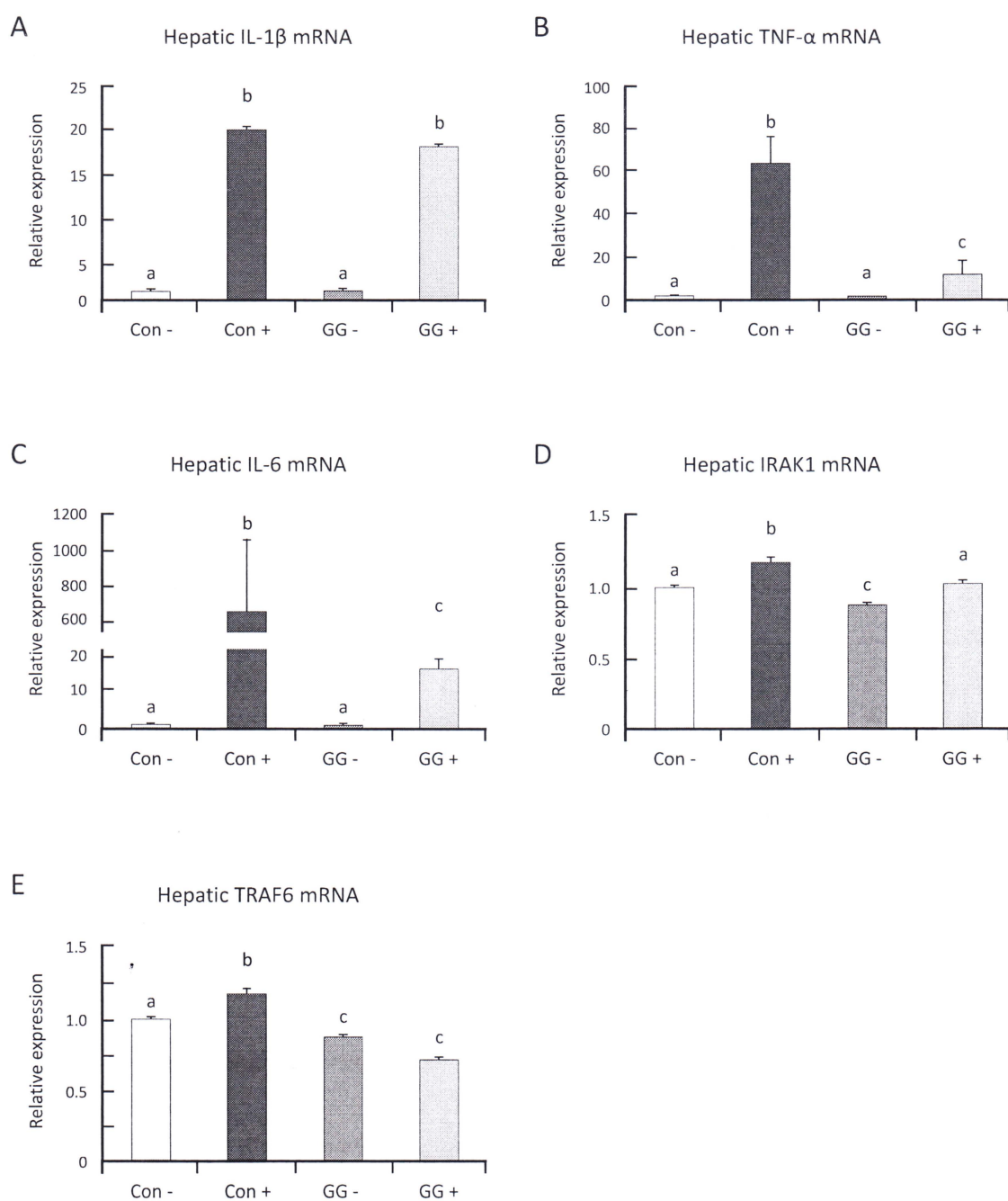


Figure 2



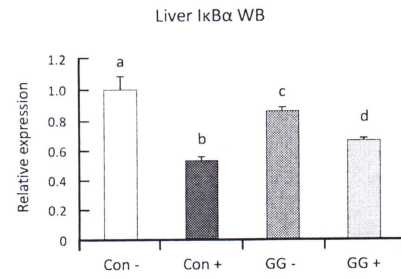
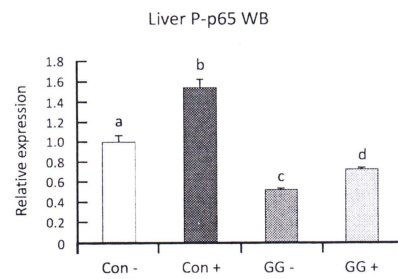
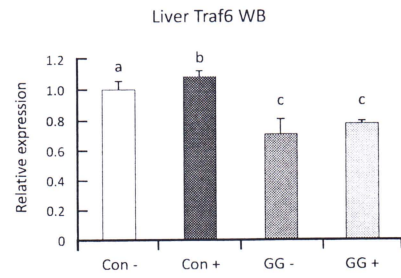
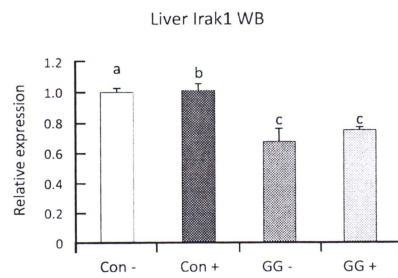
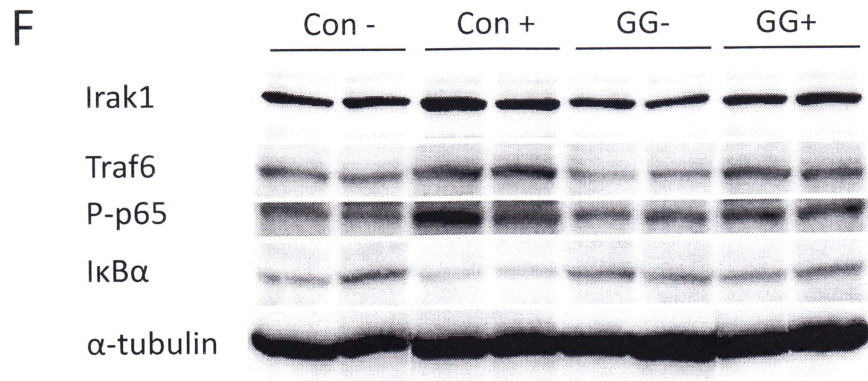
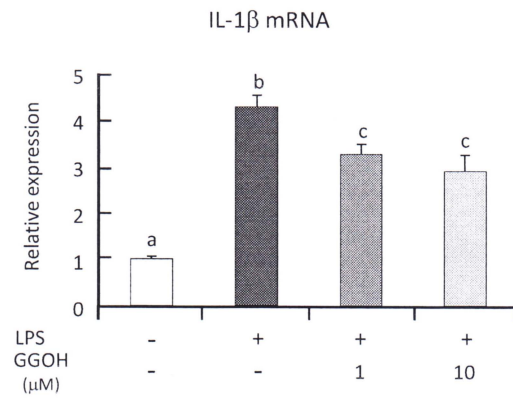
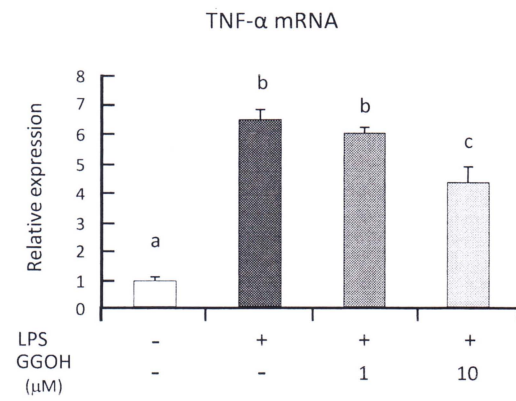


Figure 3

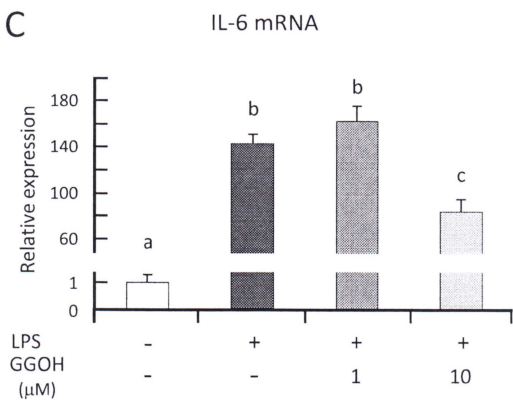
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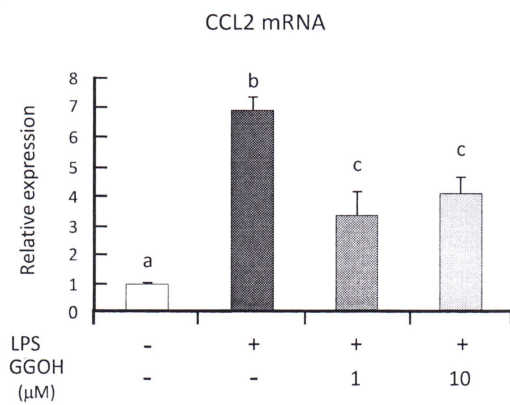
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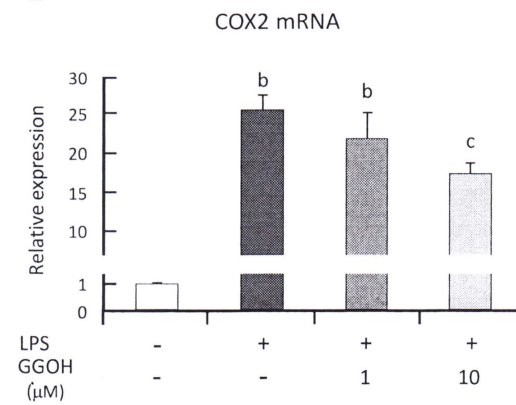
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D



E



F

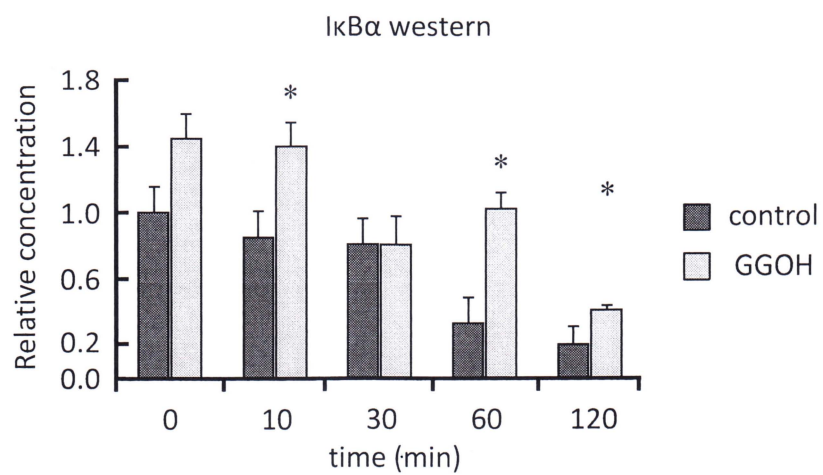
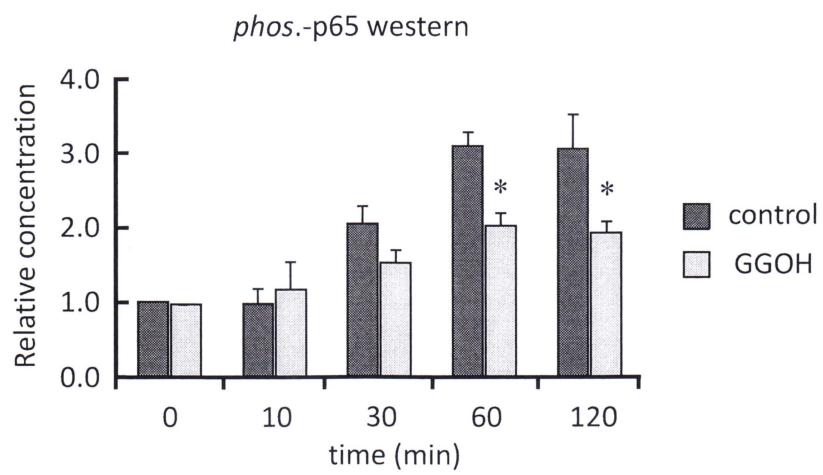
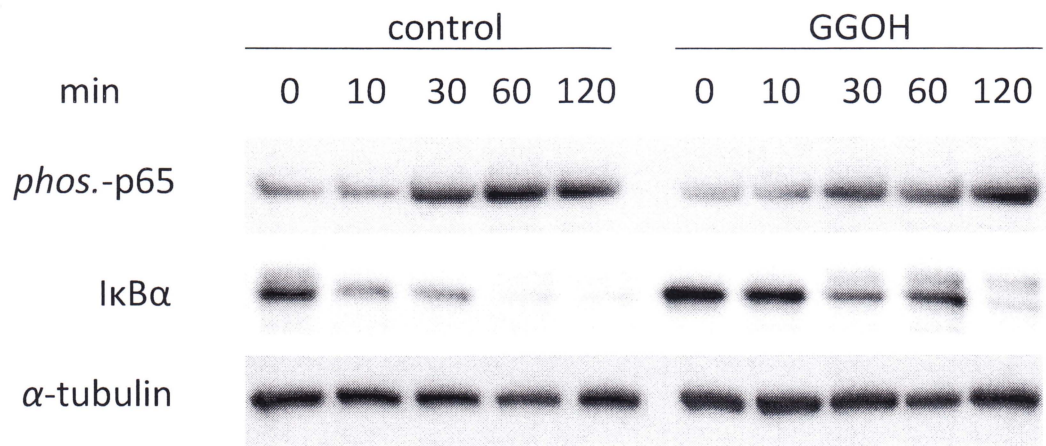
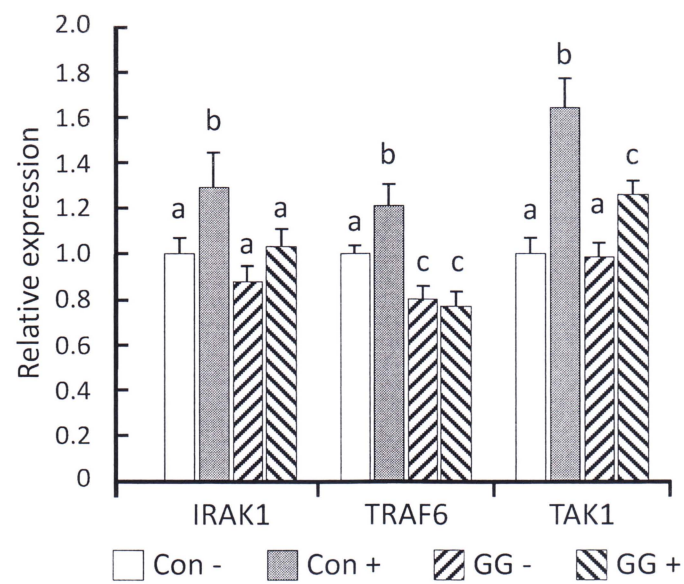
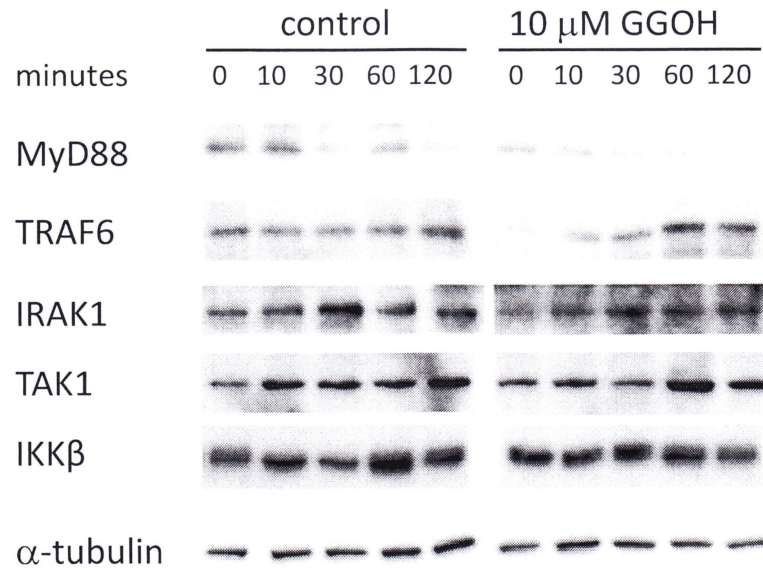


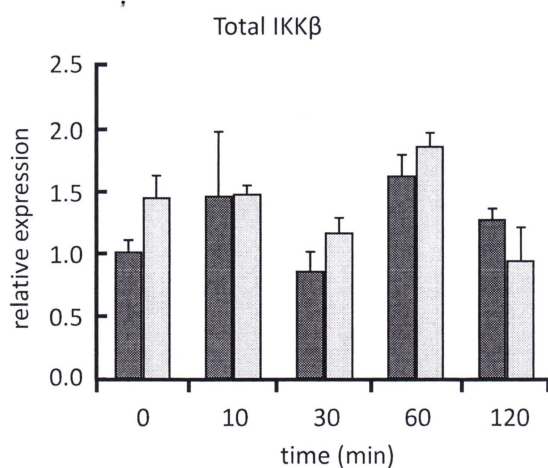
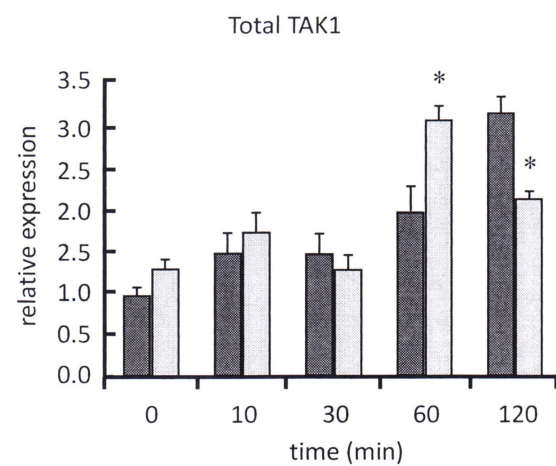
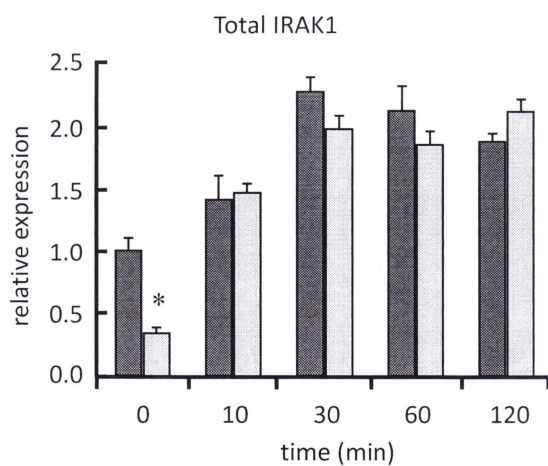
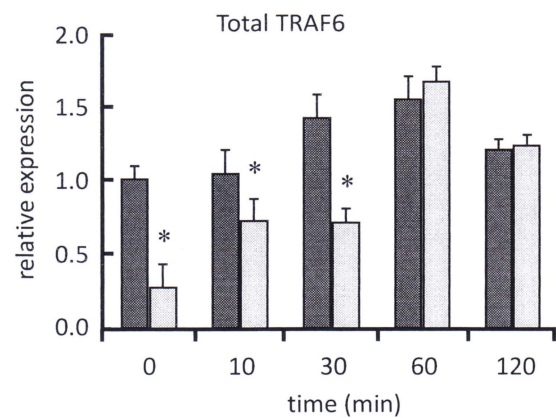
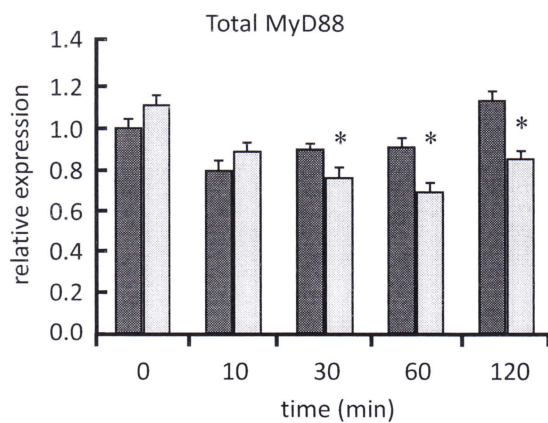
Figure 4

A



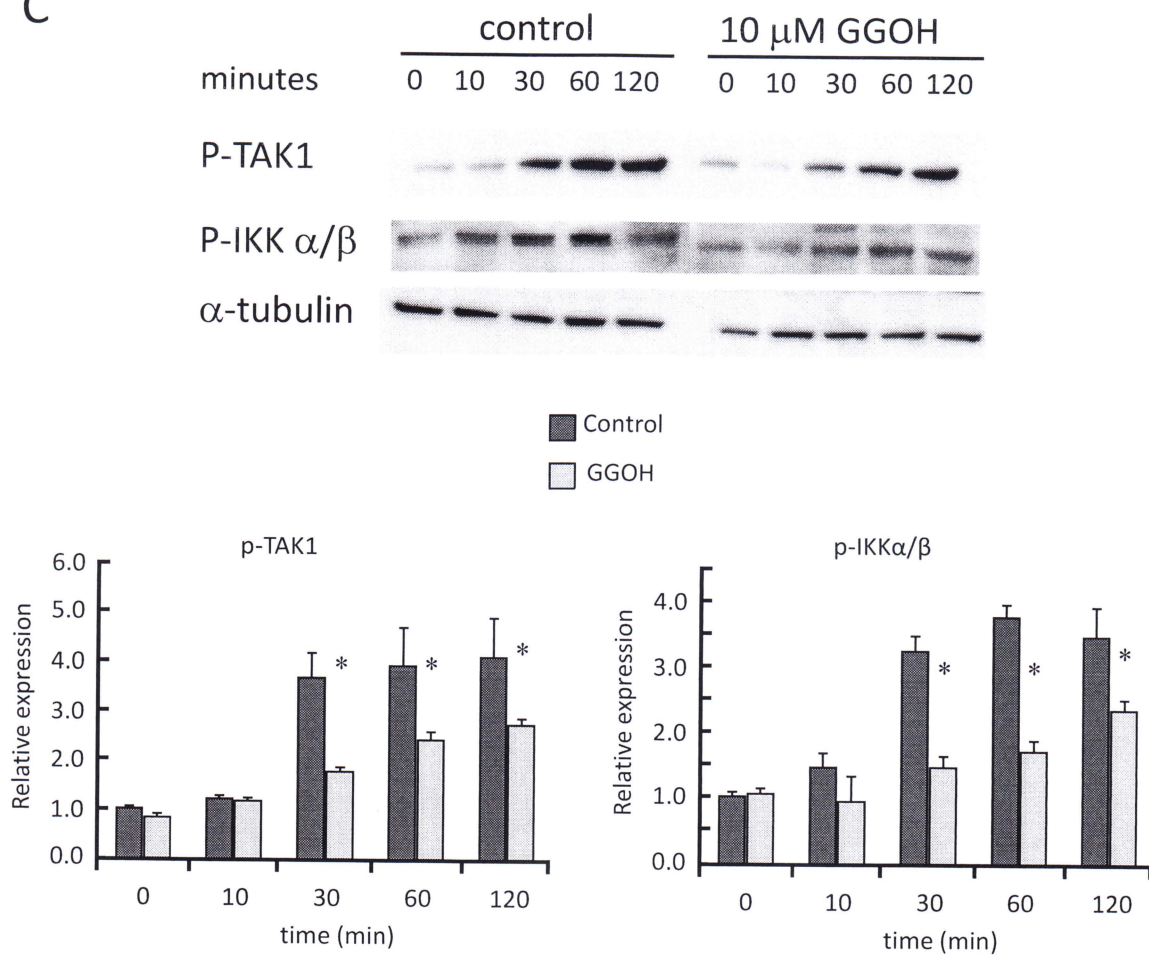
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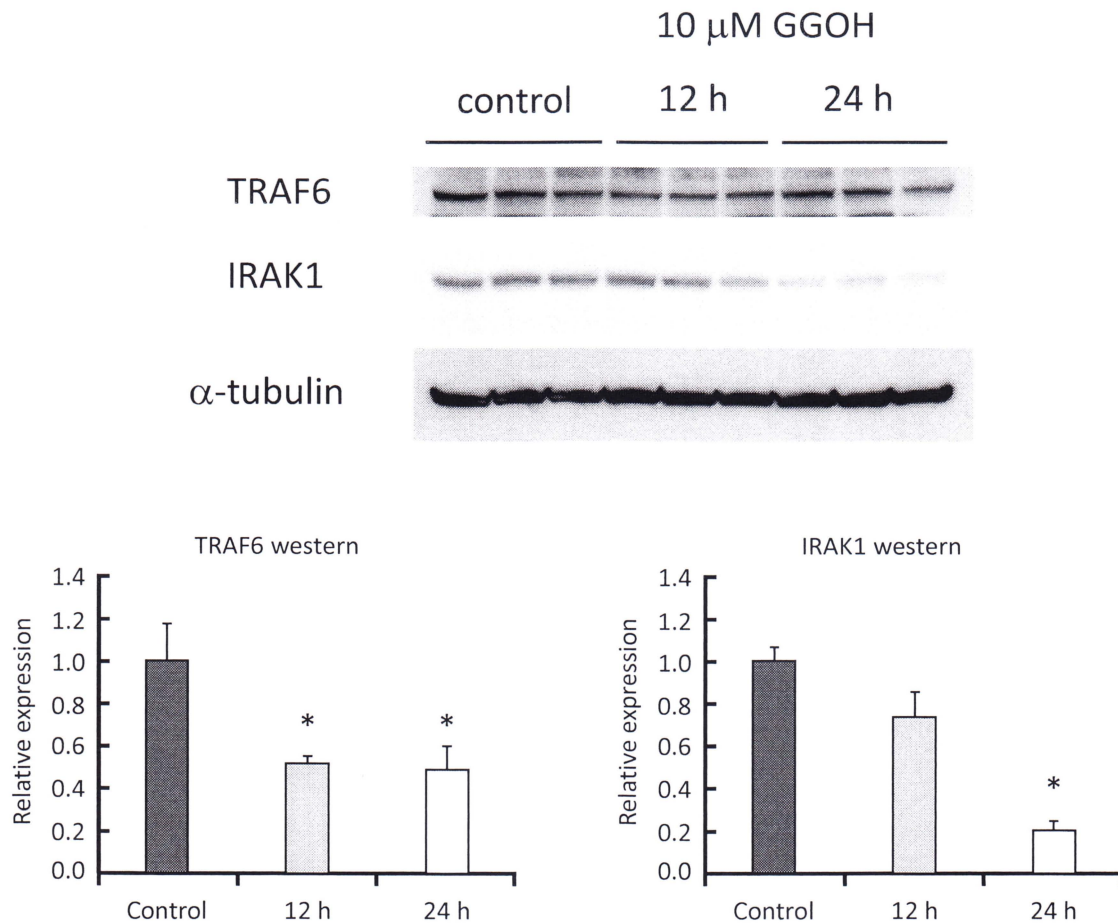


control
GGOH

C

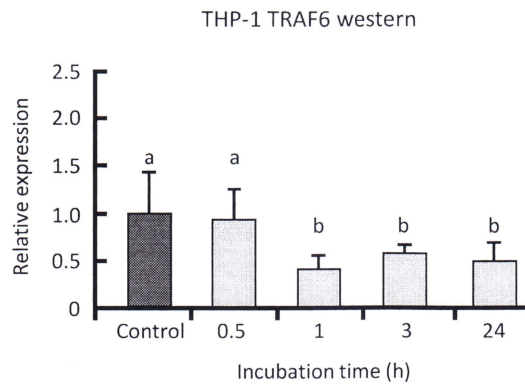
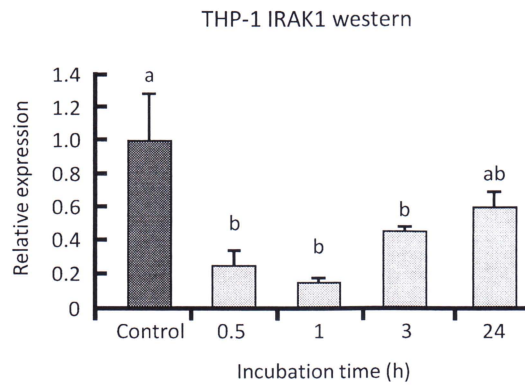
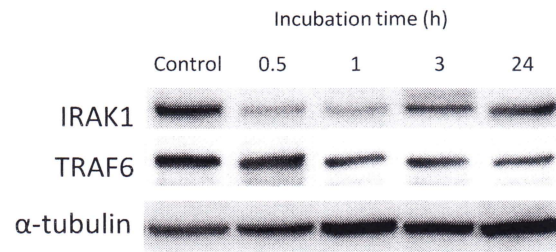


Supplementary Figure 1.



Supplementary Figure 1. Incubation with GGOH suppresses TRAF6 and IRAK1 expression in HepG2 cells. HepG2 cells were incubated with GGOH for 0, 12 or 24 h in DMEM with 10% FCS at 1.0×10^6 cells \cdot mL $^{-1}$. After incubation period, cells were rinsed and lysed and immunoblotted with corresponding antibodies. Values are mean \pm SEM; $n=3$; $p<0.05$.

Supplementary Figure 2.



Supplementary Figure 2. Short incubation with GGOH promotes degradation and suppression of IRAK1 and TRAF6 expression in THP-1 cells. THP-1 cells were incubated with GGOH for 0 (control) or indicated times in RPMI 1640 with 10% FCS at 1.0×10^6 cells·mL⁻¹. After incubation period, cells were rinsed and lysed and immunoblotted with corresponding antibodies. Values are mean \pm SEM; $n=3$; $p<0.05$. Photographs are representative of three independent experiments.