The guggulsterone derivative GG-52 inhibits NF-κB signaling in gastric epithelial cells and ameliorates ethanol-induced gastric mucosal lesions in mice

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Kim JM, Kim SH, Ko SH, Jung J, Chun J, Kim N, Jung HC, Kim JS. The guggulsterone derivative GG-52 inhibits NF-κB signaling in gastric epithelial cells and ameliorates ethanol-induced gastric mucosal lesions in mice. Am J Physiol Gastrointest Liver Physiol 304: G193–G202, 2013. First published November 1, 2012; doi:10.1152/ajpgi.00103.2012.—Gastric mucosal inflammation can develop after challenge with noxious stimuli such as alcohol. Specially, alcohol stimulates the release of inflammatory cytokines but does not increase gastric acid secretion, leading to gastric mucosal damage. The plant sterol guggulsterone and its novel derivative GG-52 have been reported to inhibit nuclear factor-κB (NF-κB) signaling in intestinal epithelial cells and experimental colitis. In the present study, we investigated the anti-inflammatory effects of GG-52 on gastric epithelial cells and on ethanol-induced gastric mucosal inflammation in mice. GG-52 inhibited the expression of interleukin-8 (IL-8) in gastric epithelial AGS and MKN-45 cell lines stimulated with tumor necrosis factor (TNF)-α in a dose-dependent manner. Pretreatment with GG-52 suppressed TNF-α-induced activation of IkB kinase (IKK) and NF-κB signaling in MKN-45 cells. In contrast, the inactive analog GG-46 did not produce significant changes in IL-8 expression or NF-κB activation. In a model of ethanol-induced murine gastritis, administration of GG-52 significantly reduced the severity of gastritis, as assessed by macroscopic and histological evaluation of gastric mucosal damage. In addition, the ethanol-induced upregulation of chemokine KC, a mouse homolog of IL-8, and phosphorylated p65 NF-κB signals were significantly inhibited in murine gastric mucosa pretreated with GG-52. These results indicate that GG-52 suppresses NF-κB activation in gastric epithelial cells and ameliorates ethanol-induced gastric mucosal lesions in mice, suggesting that GG-52 may be a potential gastroprotective agent.

gastric epithelial cells; nuclear factor-κB

GASTRIC MUCOSAL INJURIES SUCH as erosion or ulcers can result from an imbalance between host defense factors and exposures to harmful stimuli such as chemicals or toxic products. Among such stimuli, alcohol is one of the cardinal causes of gastric mucosal inflammation. Although the pathophysiological mechanisms of damage to the gastric mucosa by alcohol ingestion are not completely understood, alcohol directly impairs the gastric mucosal barrier in a dose-dependent manner, and both acidification mucosal cells and alcohol itself induce the release of inflammatory cytokines, leading to mucosal damage (7). Gastrin, released from antral G cells during meal ingestion, stimulates the parietal cell to secrete gastric acid (32). Ethanol itself does not increase plasma concentration of gastrin (33). Likewise, the substances found in certain alcoholic beverages such as beer or wine that are responsible for the increased gastric acid secretion include succinic acid and maleic acid, but these compounds do not affect gastrin level (35). The therapeutic principle behind gastric mucosal inflammation is to suppress gastric acid secretion. However, alcohol does not play a vital role in inducing gastric mucosal damage via gastric acid. Therefore, proton-pump inhibitors are not considered to be clinically useful in treating alcohol-induced gastric damage. In addition, long-term use of proton-pump inhibitors may be associated with increased risk of hip bone fracture or community-acquired pneumonia (38). Therefore, there is need for the development of new drugs capable of protecting gastric epithelial cells against alcohol-induced damage.

The gastric epithelial layer serves not only as a protective barrier against a variety of stimuli but also can provoke signals that induce mucosal inflammation. Mucosal inflammation is largely mediated by chemokines such as interleukin (IL)-8, which induces influx of neutrophils as a potent chemoattractant. Furthermore, activation of the proinflammatory gene transcriptional program in gastric epithelial cells after challenge by stimuli such as alcohol, Helicobacter pylori, or various inflammatory cytokines, including either tumor necrosis factor-α (TNF-α) and IL-1β, is associated with acute gastric inflammation (5). In such conditions, activation of transcriptional nuclear factor-κB (NF-κB) leads to upregulation of the expression of numerous proinflammatory genes (40). Therefore, modulation of NF-κB signaling in gastric epithelial cells may be a potential target for the treatment of gastritis (13, 37).

We have previously demonstrated that guggulsterone [4,17(20)-pregnadiene-3,16-dione] can block the NF-κB signaling pathway and attenuate acute murine colitis (4). Guggulsterone is an active compound extracted from the gum resin of the tree Commiphora mukul and has been used for thousands of years in the traditional Indian medicinal system to treat arthritis, obesity, lipid metabolism disorders, hypothyroidism, and inflammation (36). However, absorption of guggulsterone in the blood is potentially harmful; guggulsterone may cause unexpected suppression of NF-κB activity in other tissues, which may augment inappropriate apoptosis. To diminish intestinal absorption and prolong the retention period of guggulsterone in the gut lumen, we designed and biosynthesized several guggulsterone derivatives with increased lipophilicity and decreased solubility (12). Although the guggulsterone
derivative GG-52 exhibits anti-inflammatory activity in intestinal epithelial cells and is a potent inhibitor of NF-κB, little information is known about the GG-52-induced attenuation of gastritis. In this study, we determined whether GG-52 could affect the functions of human gastric epithelial cells in response to TNF-α stimulation. The guggulsterone derivative GG-52 was shown herein to inhibit NF-κB signaling, leading to downregulated expression of chemokine IL-8 in gastric epithelial cells stimulated with TNF-α. In addition, GG-52 attenuated ethanol-induced gastritis, suggesting the ability of GG-52 to modulate TNF-α-induced inflammation and protect gastric mucosa.

MATERIALS AND METHODS

Cell culture. Human gastric epithelial AGS (ATCC CRL 1739) and MKN-45 (JCRB) cell lines were cultured in RPMI-1640 (Sigma Chemical, St. Louis, MO) supplemented with 10% FBS (GIBCO-BRL, Gaithersburg, MD) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). Cells were seeded at 0.5–2 × 10^6 cells/well on six-well plates and allowed to attach overnight. After 12 h of serum starvation, cells were pretreated for 24 h with guggulsterone derivatives, after which cells were stimulated with TNF-α for the indicated period. In some experiments, curcumin (Sigma), PD-98059 (Calbiochem, La Jolla, CA), SB-203580 (Calbiochem), or the indicated period. In some experiments, curcumin (Sigma), PD-98059 (Calbiochem, La Jolla, CA), SB-203580 (Calbiochem), or derivatives were dissolved in dimethyl sulfoxide as a 50 mM stock solution and stored at 20°C. Toxicity testing of GG compounds was performed at ChemOn (Yongin, Korea) (12).

Real-time PCR and enzyme-linked immunosorbent assay. Total cellular RNA was extracted from cells using Trizol (GIBCO-BRL). Real-time PCR for IL-8 and human β-actin mRNA was performed as described previously (14). Briefly, 1 μg of extracted RNA was reverse transcribed and amplified using SYBR green PCR master mix and an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Primers designed using Primer Express version 2.0 were purchased from Applied Biosystems. Amplifications were performed in triplicate, and the data were normalized vs. β-actin for human IL-8. Levels of human IL-8 were measured using commercially available enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Real-time PCR for murine KC (CXCL1) and β-actin mRNA was performed as described above. The primers for murine KC were 5′-GGG CGC TTA TGC CCA AT-3′ and 5′-ACC TTC AAG CTC TGG ATG TTC TTG-3′ [NM_008176.3, Mus musculus chemokine (C-X-C motif) ligand 1 (CXCL1), mRNA]. The primers for murine β-actin were 5′-GTG ACG TTG ACA TCC GTA AAG A-3′ and 5′-GCC GGA CTC ATG TTC ATC C-3′ [NM_007393.3, β-actin, mRNA].

Electrophoretic mobility shift assay. Cells were harvested, and nuclear extracts were prepared as described previously (15). The total protein concentrations of extracts were determined using a Bradford assay (Bio-Rad, Hercules, CA). Electrophoretic mobility shift assay (EMSA) was performed using an assay kit (Promega, Madison, WI) as described previously (11, 15). In brief, 5 μg of nuclear extract were incubated for 30 min at room temperature with a double-stranded γ-32P-labeled oligonucleotide probe [sense strand, 5′-AGT TGA GGG GAC TTC TTT CCC AGG C-3′ for the NF-κB-binding site; sense strand, 5′-CGC TTG ATG ACT CAG GGA-3′ for the activator protein (AP)-binding site]. After incubation, bound and free DNA were resolved on 5% polyacrylamide gels. Supershift assay was used to identify the specific members of the activated NF-κB families. EMSA was performed as described above, except that rabbit antibodies (1 μg/reaction; Santa Cruz Biotechnology, Santa Cruz, CA) against NF-κB proteins p50, p52, p65, c-Rel, or Rel B were added during the binding reaction period, as described previously (14).

Transfection and reporter assays. Reporter plasmids, including pIL8-luciferase, p2X NF-κB-luciferase, and β-actin- and pRSV-β-galactosidase-luciferase transcriptional reporters, were provided by Dr. M. F. Kagnoff of the University of California, San Diego (6, 15). Cells in six-well dishes were transfected with 1.5 μg of plasmid DNA using Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA) as described previously (30). Briefly, transfected cells were incubated for 24 h at 37°C in a 5% CO2 incubator. Cells were then harvested, and whole cell lysates were prepared as described previously (30). Luciferase activity was determined in accordance with the manufacturer’s instructions (Tropix, Bedford, MA), and luminescence was quantitated for 10 s using a luminometer (MicroLumat Plus; Berthold, Bad Wildbad, Germany). Luciferase activity was determined and normalized relative to β-galactosidase expression (15). The wild-type (WT) plasmids for mitogen/extracellular signal-regulated kinase (MEK) 1, which is an upstream effector extracellular signal-regulated kinase (ERK), and for p38 (WT-p38) were purchased from Biomyx Technologies (San Diego, CA) (1). The recombinant adenovirus containing wild-type NF-κB-inducing kinase plasmid (Ad-WT-NIK) and control adenovirus were provided by Dr. Christian Jobin (University of North Carolina at Chapel Hill, Chapel Hill, NC) (16).

Immunoblot analysis. Immunoblot analyses were performed as described previously (12). Briefly, cells were washed with ice-cold PBS and lysed in 0.5 ml/well lysis buffer [150 mM NaCl, 20 mM Tris at pH 7.5, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml aprotinin]. Fifteen to fifty micrograms of protein per lane were size-fractionated on a 6% polyacrylamide minigel and electrophoretically transferred to a nitrocellulose membrane (0.1-μm pore size). Specific proteins were detected using phospho-IκB kinase (IKK)-α/β, pan-ERK1/2 (p44/p42), phospho-

Fig. 1. Chemical structures of the guggulsterone derivative GG-52 and its inactive analog GG-46. MW, mol wt.
ERK1/2, pan-c-Jun NH2-terminal kinase (JNK, p54/p46), phospho-JNK, pan-p38, phospho-p38, and actin (Cell Signaling Technology, Beverly, MA) as primary antibodies and peroxidase-conjugated antirabbit or anti-rabbit IgG as a secondary antibody. Specific binding of antibodies was detected by enhanced chemiluminescence (ECL system; Amersham Life Science, Buckinghamshire, UK) and exposure to X-ray film.

**IKK.** IKK activity on IκBα phosphorylation was determined using a commercially available HTScan IKK-β kinase assay kit (Cell Signaling Technology) according to the manufacturer’s instructions (19, 31). Briefly, cells treated with guggulsterone derivatives were washed with ice-cold PBS. Whole cell extracts were prepared using lysis buffer (150 mM NaCl, 20 mM Tris at pH 7.5, 0.1% Triton X-100, 1 mM PMSF, and 10 μg/ml aprotonin), after which lysates were cleared by centrifugation at 14,000 rpm for 10 min at 4°C. Levels of IKK activity were measured by colorimetric ELISA according to the manufacturer’s instructions.

We used ELISA kits for evaluating the roles of AP-1 and mitogen-activated protein kinases (MAPKs) in the GG-52 pretreatment condition. To measure the concentration of c-fos in the nuclear fraction of cells, a Trans-AP-1 family kit (Active Motif, Carlsbad, CA) was used. The activity of JNK, p38, and ERK1/2 was evaluated by FACE ELISA kits (Active Motif) in accordance with the protocols provided with the kits. The assay was performed according to the manufacturer’s instruction (20, 31).

**Ethanol-induced acute murine gastritis model.** Specific pathogen-free mice (C57BL/6NcrjBgi, mice, 20–25 g, 7–8 wk) were purchased from Orient Experimental Animal (Seoungnam, Korea). Animals were maintained on a 12:12-h light-dark cycle under specific pathogen-free conditions. Mice had ad libitum access to a standard diet and water until reaching the desired age (8–9 wk) and/or weight (18–21 g) (12). All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

In vivo studies were performed as described previously with some modification (10, 21, 24). Briefly, mice were divided into the following four groups: 1) negative control (n = 4), 2) an ethanol-treated group (n = 6), 3) an ethanol-treated group pretreated with GG-52 (50 mg/kg) (n = 6), and 4) an ethanol-treated group pretreated with GG-52 (200 mg/kg) (n = 6). Mice were administered 0.9% normal saline (negative control) or absolute ethanol (5 mg/kg) by oral gavage. The guggulsterone derivative GG-52 was suspended in 0.5% methylcellulose solution (vehicle) and administered once by oral gavage 1 h before ethanol exposure. As a negative control, 0.9% normal saline-treated mice received the vehicle in a comparable volume by the same route.

**Macroscopic assessment and histological evaluation of gastric damage.** Mice were killed 1 h after ethanol administration. Gross morphological index and histological index were scored as described previously (10, 21). Briefly, isolated stomachs were cut open along the greater curvature and washed in ice-cold saline. To investigate the degree of gross mucosal damage, the mucosal sides of the stomachs were photographed using a digital camera, and part of the mucosa was immediately fixed with a 10% formalin solution. The lesion severity was scored on a scale of 0 to 5: 0, normal; 0.5, light local reddening; 1, general reddening or small hemorrhage (<1 mm); 2, large hemorrhage (>1 mm); 3, small ulcer (<2 mm); 4, large ulcer (>2 mm); and 5, perforated ulcer. A single score was assigned to each lesion. A researcher who was blinded to the treatment conditions scored all gastric mucosal injuries.

For microscopic assessment of gastric lesions, stomachs fixed with formalin were cut longitudinally with 5-mm width from the cardia to the pylorus. Tissue specimens were dehydrated and embedded in paraffin. The longest portion was sectioned by 6 μm, followed by staining with hematoxylin and eosin. Histological quantification was performed in a blinded fashion using the scoring system. The severity of histological lesions was scored from 0 to 3: 0, normal; 1, slight damage of surface gastric mucosa or damage of just two or three glandular cells in the upper mucosal layer; 2, damage greater than that of score 1 and involving <50% of the thickness of the gastric mucosa; and 3, damage involving >50% of the thickness of the gastric mucosa.

**Immunohistochemical analysis.** Immunohistochemical analysis of the phosphorylated p65 subunit of NF-κB was performed as described previously (12, 31). Briefly, for the microwave antigen retrieval procedure, slides were immersed in 10 mM citrate buffer (pH 6.0) in a clean polyethylene chamber in a full-powered microwave for 10 min and then cooled for 10–20 min. After being washed with PBS, the sections were stained with rabbit polyclonal anti-phospho-p65 NF-κB antibody (Cell Signaling Technology) in an automated staining system (Tech Mate 1000 TM; Ventana, Tucson, AZ) according to the manufacturer’s recommendations.

**Statistical analysis.** The in vitro data such as real-time PCR, luciferase assay, and ELISA were statistically analyzed using Wilcoxon’s rank sum test. Differences between groups in a murine model were compared using Kruskal-Wallis test with Mann-Whitney U-test for post hoc analysis. P values <0.05 were considered statistically significant.

**RESULTS**

The guggulsterone derivative GG-52 inhibits the expression of IL-8 in gastric epithelial cells. Pretreatment with the guggulsterone derivative GG-52 significantly downregulated IL-8 mRNA level in AGS cells stimulated with TNF-α, as determined by real-time RT-PCR. In contrast, the inactive guggulsterone derivative GG-46 did not significantly alter the expression of IL-8. Thus the promoter activity in TNF-α-stimulated AGS cells stimulated with GG-52 was significantly decreased by about 10.22±0.33. IL-8 secretion, we measured the amount of IL-8 protein in culture supernatants. As shown in Fig. 2, IL-8 mRNA levels were accompanied by decreased protein secretion, we measured the amount of IL-8 protein in culture supernatants. As shown in Fig. 2, IL-8 mRNA levels were normalized to GAPDH mRNA level in AGS cells stimulated with TNF-α. The amount of GG-52 decreases the TNF-α-induced NF-κB DNA-binding activity in gastric epithelial cells. As shown in Fig. 4, pretreatment of MKN-45 cells with GG-52 reduced the promoter activity of NF-κB in TNF-α-stimulated cells. Inhibition of NF-κB activity was dependent on the concentration of GG-52. To confirm these results, NF-κB DNA-binding activity was determined by EMSA. As shown in Fig. 4, pretreatment of cells with GG-52 decreased TNF-α-induced NF-κB activity, whereas pretreatment with GG-46 did not influence TNF-α-induced NF-κB activation. To identify which specific NF-κB subunits comprise the suppressed NF-κB signal in TNF-α-
To evaluate whether overexpression of constitutively active NF-κB signals could affect GG-52’s effects on IL-8 secretion, MKN-45 cells were transfected with Ad-WT-NIK, which can overexpress NF-κB signals. Under the transfection condition, Ad-WT-NIK significantly recovered the GG-52-induced suppression of IL-8 secretion in TNF-α-stimulated cells (Fig. 4D). In contrast, combined treatment with GG-52 and TNF-α significantly decreased IL-8 secretion compared with TNF-α alone in the control virus-transfected cells. These results suggest that the GG-52-induced inhibition of NF-κB signal is predominantly composed of heterodimers of p65/p50.

GG-52 inhibits NF-κB signaling in MKN-45 cells by blocking IKK. TNF-α strongly induced a phosphorylation of IKK. We investi-
gated the possibility that GG-52 may exert an influence on the NF-κB signaling pathway by blocking IKK activity. GG-52 does not inhibit AP-1 signaling in TNF-α-stimulated MKN-45 cells. Because the promoter for IL-8 gene induction contains binding sites for both AP-1 and NF-κB, we investiga-
ted whether GG-52 may affect AP-1 activity. As shown in Fig. 5B, stimulation of MKN-45 cells with TNF-α resulted in an increase of IKK activity. Under these experimental conditions, pretreatment of cells with GG-52 significantly reduced TNF-α-induced IKK activity. These results indicate that the guggulsterone derivative GG-52 can inhibit the NF-κB signaling pathway by blocking IKK activity.

stimulated MKN-45 cells, we performed supershift assay. Spec-
cific antibodies to p50, p52, p65, c-Rel, and Rel B were used for these experiments. The supershift studies demonstrated that antibody to p65 shifted the entire signal and that antibody to p50 also caused a significant shift. However, anti-p52, anti-c-Rel, or anti-Rel B antibodies did not shift the NF-κB signals (Fig. 4C). To evaluate whether overexpression of constitutively

![Fig. 2. Effects of the guggulsterone derivative GG-52 on interleukin (IL)-8 expression in gastric epithelial cells stimulated with tumor necrosis factor (TNF)-α.](http://ajpgi.physiology.org/)

**Fig. 2.** Effects of the guggulsterone derivative GG-52 on interleukin (IL)-8 expression in gastric epithelial cells stimulated with tumor necrosis factor (TNF)-α. A and B: AGS and MKN-45 cells were pretreated with 50 µM of GG-52 or the inactive analog GG-46 for 24 h and then stimulated with TNF-α (10 ng/ml) for 6 h. mRNA expression of IL-8 was measured by real-time RT-PCR, in which levels were normalized to β-actin. Data are expressed as the fold change in mRNA transcript level relative to that of the unstimulated control (mean ± SE, n = 5). C: MKN-45 cells were pretreated with GG-52 or GG-46 (50 µM) for 24 h and then stimulated with TNF-α (10 ng/ml) for 24 h. Secretion of IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) (mean ± SE, n = 5). *P < 0.05 compared with TNF-α alone.

![Fig. 3. The guggulsterone derivative GG-52 inhibits IL-8 reporter gene activation in MKN-45 cells stimulated with TNF-α.](http://ajpgi.physiology.org/)

**Fig. 3.** The guggulsterone derivative GG-52 inhibits IL-8 reporter gene activation in MKN-45 cells stimulated with TNF-α. MKN-45 cells were transfected with pIL8-luciferase transcriptional reporter for 24 h. Transfected cells were pretreated with the indicated concentration of GG-52 or the inactive analog GG-46 for 24 h and then combined with TNF-α (10 ng/ml) for 6 h. Data are expressed as mean fold induction ± SE of luciferase activity relative to unstimulated controls (n = 5). The mean fold induction of β-actin reporter gene activity relative to the unstimulated controls remained relatively constant throughout each experiment. *P < 0.05 compared with TNF-α alone.
c-fos activity that was significantly reduced by pretreatment with the curcumin. However, pretreatment with GG-52 did not significantly reduce the levels of c-fos activity compared with TNF-α alone (Fig. 6B). Consistent with this, pretreatment with GG-52 did not also significantly change the levels of AP-1 reporter gene activity in TNF-α-stimulated MKN-45 cells although c-Jun significantly decreased the TNF-α-induced AP-1 activity (Fig. 6C). These results suggest that GG-52 does not affect AP-1 signaling in TNF-α-stimulated MKN-45 cells.

Effects of GG-52 on MAPK activity in TNF-α-stimulated MKN-45 cells. Because MAPK activation is associated with IKK and NF-κB signaling (19, 31, 39), we asked whether the effects of GG-52 may be attributable to the inhibition of MAPK activity. As shown in Fig. 7A, stimulation of MKN-45 cells with TNF-α increased the phosphorylated MAPK signals such as ERK, p38, and JNK. In this experimental condition, pretreatment with GG-52 clearly attenuated the phosphorylated ERK and p38 induced by TNF-α stimulation in a dose-dependent manner. However, the phosphorylated JNK signals seem not to be changed in the GG-52-pretreated condition. For objective measurement of MAPK activity, we used MAPK ELISA kits. In this experimental study, the following kinase inhibitors as positive controls were used: PD-98059, an inhibitor of MEK1/2, an MAPK that phosphorylates ERK1/2; pyridinyl imidazole SB-203580, which specifically inhibits p38; and SP-600125, which inhibits JNK. As shown in Fig. 7B, pretreatment with GG-52 significantly suppressed the phosphorylated activities of ERK and p38 under TNF-α-stimulated conditions. In contrast, pretreatment with GG-52 did not significantly reduce the phosphorylated JNK activity although SP-600125 significantly decreased the TNF-α-induced JNK.
activity. In these experimental conditions, pretreatment with GG-46 did not significantly reduce the level of each MAPK activity compared with TNF-α alone. Furthermore, transfection with MEK1- and p38-overexpressing plasmids significantly attenuated the GG-52-induced suppression of IL-8 secretion in TNF-α-stimulated cells (Fig. 7C). These results suggest that GG-52 may inhibit TNF-α-induced ERK and p38 kinase activation in gastric epithelial cells.

GG-52 attenuates ethanol-induced acute gastritis in mice. To test the in vivo ability of the guggulsterone derivative GG-52 to suppress inflammatory reactions, we used a murine model of ethanol-induced acute gastritis, as shown in Fig. 8, A and B. In this model, administration of GG-52 (50 mg/kg) did not significantly reduce the severity of ethanol-induced gastritis, although scores of both gross and histological damages were improved. In contrast, GG-52 (200 mg/kg) appeared to reduce both gross and histological findings (Fig. 6, A and B). In addition, scores of both gross and histological damages in the GG-52 (200 mg/kg)-treated group significantly attenuated acute gastritis compared with ethanol alone (Fig. 8, C and D).

Because our data of in vitro study showed that GG-52 could exert its anti-inflammatory effects by blocking IKK activity in gastric epithelial cells, we investigated this signaling in our ethanol-gastritis model to verify its significance in vivo. As shown in Fig. 9A, the ethanol-induced expression of chemokine KC, a mouse homolog of IL-8, was significantly decreased in mice pretreated with GG-52. To determine NF-κB activity in the in vivo model of gastritis, immunohistochemis-

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Fig. 6. Effects of guggulsterone derivative GG-52 on activator protein (AP)-1 signaling in MKN-45 cells. A: MKN-45 cells were pretreated with the indicated concentration of GG-52 for 24 h or curcumin (20 μM) for 30 min and then stimulated with TNF-α (20 ng/ml) for another 1 h. AP-1 DNA-binding activity in the nuclear extracts was assessed by EMSA. The data are representative of three independent experiments. B: MKN-45 cells were pretreated with GG-52 (50 μM) for 24 h or curcumin (20 μM) for 30 min and then stimulated with TNF-α (20 ng/ml) for the indicated period of time. Nuclear proteins were extracted, and 10 μg of each sample were subjected to assay. Data are expressed as means ± SE (n = 5). *P < 0.05 compared with TNF-α alone. C: MKN-45 cells were transfected with pAP-1-luciferase transcripational reporter. After 24 h, transfected cells were pretreated with GG-52 (50 μM) for 24 h or curcumin (20 μM) for 30 min and then were stimulated with TNF-α (20 ng/ml) for 1 h. The mean fold induction of the β-actin reporter gene activity relative to the unstimulated controls remained relatively constant throughout each experiment. Data are expressed as the mean fold induction ± SE relative to untreated controls (n = 5).

Fig. 7. Effects of guggulsterone derivative GG-52 on mitogen-activated protein kinase activity in MKN-45 cells. A: MKN-45 cells were stimulated with the indicated concentration of GG-52 for 24 h and then stimulated with TNF-α (20 ng/ml) for 30 min. Extracellular signal-regulated kinase (ERK) 1/2, p38, and c-Jun NH2-terminal kinase (JNK) activities were measured by immunoblot analysis. Results are representative of 5 independent experiments. B: MKN-45 cells were pretreated with GG-52 (50 μM) and GG-46 (50 μM) for 24 h or each inhibitor (PD-98059 (20 μM), SB-203580 (20 μM), or SP-600125 (20 μM)) for 30 min. Phosphorylated activities of ERK1/2, p38, or JNK were measured by each ELISA kit. Data are expressed as the mean fold induction ± SE relative to untreated controls (n = 5). *P < 0.05 compared with TNF-α alone. C: MKN-45 cells were transfected for 48 h with wild-type plasmids for mitogen/extracellular signal-regulated kinase (WT-MEK1), wild-type p38 (WT-p38), or control plasmids. Cells were pretreated with GG-52 (50 μM) for 6 h and then stimulated with TNF-α (20 ng/ml) for 24 h. Secretion of IL-8 was measured by ELISA (mean ± SE, n = 3). *P < 0.05.
Fig. 8. GG-52 attenuates ethanol-induced acute gastritis in a murine model. Mice were pretreated with GG-52 (50 or 200 mg/kg) or vehicle (0.5% methylcellulose) for 1 h, after which absolute ethanol (5 mg/kg) was administrated by oral gavage. The mice were killed 1 h after ethanol administration. A: gross appearances of gastric mucosa. Hemorrhages are shown in each specimen (arrowhead). B: histological findings of stomachs. Images stained with hematoxylin and eosin (H&E) that represent >50% of microscopic fields/group are shown (magnification, ×200). C and D: comparison of the gross damage scores (C) and histological damage scores (D) with or without pretreatment of GG-52 before ethanol administration. Results are means ± SE of 6 mice/group except the control vehicle group with 4 mice. *P < 0.05 compared with ethanol alone. NS, nonsignificant.
The findings of the present study showed that TNF-α/H9251 of excess production of inflammatory cytokines and increased induced a phosphorylated IKK activity, NF-κB activity may allow for the recovery suppressed of NF-κB activation (9, 29, 34), suppression of NF-κB activation (19, 31, 39). Nevertheless, it is possible that an association between MAPKs and IKK activation may activate NF-κB signaling in gastric epithelial cells because MAPK signaling can induce NF-κB activation (19, 31, 39).

The MAPK cascades are serine/threonine-specific kinases that play a key role in regulating the production of proinflammatory cytokines leading to inflammation (22). In the present study, pretreatment with GG-52 attenuated the phosphorylated ERK and p38 in the TNF-α-stimulated cells. In contrast, the phosphorylated JNK did not change in the GG-52-pretreated condition although SP-600125 (the JNK inhibitor) significantly decreased the TNF-α-induced JNK activation. These results suggest that GG-52 may inhibit TNF-α-induced ERK and p38 kinase activation in gastric epithelial cells. Nevertheless, further studies are required to clarify other factors that may contribute to the GG-52-induced decrease of IL-8 expression in gastric epithelial cells.

To clarify whether GG-52 can protect against gastric injury in vivo, we used an experimental murine model of ethanol-induced gastritis. We found that GG-52 can attenuate ethanol-induced acute gastritis. Consistent with observation of ethanol-induced NF-κB activation in gastric mucosa (18, 25, 28), ethanol administration upregulated expression of chemokine KC, a mouse homolog of IL-8, and expression of phosphorylated p65 subunit of NF-κB, whereas pretreatment with GG-52 significantly reduced these activating signals in mice. These results suggest that the guggulsterone derivative GG-52 may have a protective anti-inflammatory effect in the stomach.

try was performed. As shown in Fig. 9B, ethanol-induced gastritis was accompanied by an increased expression of phospho-p65 NF-κB in the gastric mucosa and submucosa. In contrast, phosphorylated NF-κB signals were markedly reduced in the presence of GG-52. These results suggest that GG-52 effectively protected against acute gastritis.

DISCUSSION

In the present study, we found that the guggulsterone derivative GG-52 significantly suppressed NF-κB signaling in gastric epithelial cells, resulting in the downregulation of IL-8 expression in cells stimulated with TNF-α. Moreover, GG-52 attenuated ethanol-induced acute gastritis in a murine model. These findings suggest that oral administration of the guggulsterone derivative GG-52 has anti-inflammatory and protective effects in gastric mucosa.

Although gastritis is induced by a variety of causes, mucosal damage and inflammation are its most common manifestations. Considering that TNF-α-mediated increase of inflammatory cytokines and gastric damage requires NF-κB activation (9, 29, 34), suppression of NF-κB activity may allow for the recovery of excess production of inflammatory cytokines and increased damage of gastric mucosa. Consistent with these possibilities, the findings of the present study showed that TNF-α strongly induced a phosphorylated IKK activity, NF-κB DNA-binding activity, and IL-8 expression, all of which were downregulated upon treatment with GG-52.

Many natural products such as flavonoids and plant ingredients have protective effects on gastritis (2, 3, 8, 17). These natural products are consumed in significant amounts as part of the human diet; however, it is possible that absorption of these products into the blood may be potentially harmful. For example, excessive absorption of guggulsterone may cause unexpected suppression of NF-κB activity in undesirable tissues, which may augment inappropriate apoptosis. To diminish intestinal absorption and prolong the retention period within the lumen, the present study used the guggulsterone derivative GG-52, which exhibits increased lipophilicity and decreased solubility. To examine the clinical use of GG-52, acute toxicity testing of GG-52 was performed in mice. Because 200 mg/kg of GG-52 produced a preventive effect in a murine model, toxicity testing was performed with oral administration of GG-52 at a daily dose of 2,000 mg/kg for 2 wk in mice. The result did not show any significant toxicity in mice. Thus the value of no observed adverse effect level for GG-52 was >2,000 mg/kg, which is 10 times the maximum therapeutic dose (12).

In the present study, GG-52 inhibited the expression of IL-8 by blocking NF-κB signaling in gastric epithelial cells, suggesting a possible mechanism for its anti-inflammatory actions in the stomach. However, pretreatment with GG-52 did not completely inhibit the activation of NF-κB signals in TNF-α-stimulated cells. Because the expression of IL-8 is regulated by transcription factors NF-κB and AP-1, the suppressive effects of GG-52 may have been due to AP-1 signaling in gastric epithelial cells. However, the present study showed that GG-52 did not influence AP-1 signaling in TNF-α-stimulated cells. Nevertheless, it is possible that an association between MAPKs and IKK activation may activate NF-κB signaling in TNF-α-stimulated cells because MAPK signaling can induce NF-κB activation (19, 31, 39).

The MAPK cascades are serine/threonine-specific kinases that play a key role in regulating the production of proinflammatory cytokines leading to inflammation (22). In the present study, pretreatment with GG-52 attenuated the phosphorylated ERK and p38 in the TNF-α-stimulated cells. In contrast, the phosphorylated JNK did not change in the GG-52-pretreated condition although SP-600125 (the JNK inhibitor) significantly decreased the TNF-α-induced JNK activation. These results suggest that GG-52 may inhibit TNF-α-induced ERK and p38 kinase activation in gastric epithelial cells. Nevertheless, further studies are required to clarify other factors that may contribute to the GG-52-induced decrease of IL-8 expression in gastric epithelial cells.

To clarify whether GG-52 can protect against gastric injury in vivo, we used an experimental murine model of ethanol-induced gastritis. We found that GG-52 can attenuate ethanol-induced acute gastritis. Consistent with observation of ethanol-induced NF-κB activation in gastric mucosa (18, 25, 28), ethanol administration upregulated expression of chemokine KC, a mouse homolog of IL-8, and expression of phosphorylated p65 subunit of NF-κB, whereas pretreatment with GG-52 significantly reduced these activating signals in mice. These results suggest that the guggulsterone derivative GG-52 may have a protective anti-inflammatory effect in the stomach.
Ethanol is a well-established irritant that induces inflammation in gastric mucosa and can induce NF-κB in gastric mucosal cells in experimental animals such as mice and rats (18, 25, 28). In addition, it is possible that plasma membrane damage incurred by ethanol may be due to an indirect effect on intracellular signaling, including ethanol-induced calcium signaling of NF-κB in gastric epithelial cells (26, 27). These reports support the in vivo physiological relevance of guggulsterone derivative GG-52-mediated suppression of inflammatory reactions according to a murine model of ethanol-induced gastritis. However, our findings should be further validated by studies using different gastritis models such as H. pylori-infected mice.

In conclusion, the guggulsterone derivative GG-52 was found to suppress NF-κB signaling by targeting MAPKs, IKK, and IL-8 expression in gastric epithelial cells. Furthermore, GG-52 attenuated gastritis in a murine model of ethanol-induced acute gastritis. These results suggest a role for GG-52 as a potential protective agent for the prevention of gastritis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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