Regular Article

Protective Effect of Gomisins N against Endoplasmic Reticulum Stress-Induced Hepatic Steatosis

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Gomisin N is a physiological substance derived from *Schisandra chinensis*. In the present study, the *in vitro* and *in vivo* effects of gomisins N on endoplasmic reticulum (ER) stress and hepatic steatosis were investigated. We quantified the expression of markers of ER stress, including glucose regulated protein 78 (GRP78), CCAAT/enhancer binding protein (C/EBP) homolog protein (CHOP), and X-box-binding protein-1 (XBP-1), and triglyceride (TG) accumulation, in HepG2 cells treated with tunicamycin or palmitate. Tunicamycin treatment in HepG2 cells induced expression of markers of ER stress and increased TG levels; Gomisins N reversed these effects, reducing the expression of markers of ER stress and TG levels. Similar effects were seen following palmitate pretreatment of HepG2 cells. The inhibitory effects of gomisins N were further confirmed in mice injected with tunicamycin. Gomisins N reduced expression of markers of ER stress and decreased TG levels in mouse liver after tunicamycin injection. Furthermore, gomisins N decreased expression of inflammatory and lipogenic genes in palmitate-incubated HepG2 cells. These results suggest that gomisins N inhibits ER stress and ameliorates hepatic steatosis induced by ER stress.

Key words gomisins N; endoplasmic reticulum (ER) stress; hepatic steatosis; lipogenesis; inflammation

The endoplasmic reticulum (ER) plays critical roles in the synthesis of secreted and membrane proteins by mediating protein folding, production of lipids and sterols, and the storage of intracellular Ca^{2+} .¹⁾ However, pathological factors that disrupt ER homeostasis lead to the accumulation of unfolded protein in the ER lumen, provoking ER stress. Cells usually survive early stress by attenuating protein translation, removing unfolded proteins, and upregulating protein chaperons *via* the unfolded protein response (UPR).¹⁾ However, prolonged ER stress can lead to cell death and cause several diseases including ischemia/reperfusion injury, heart disease, and diabetes.^{2–5)} Recent studies show that hepatic ER stress is observed in metabolic diseases such as obesity and diabetes.^{2–5)} ER stress contributes to development of insulin resistance and hepatic steatosis in non-alcoholic fatty liver disease (NAFLD).^{6–9)}

NAFLD is a common hepatic disorder that is characterized by excessive lipid accumulation in the liver. The first stage consists of hepatic steatosis caused by triglyceride (TG) accumulation in hepatocytes. Symptoms range from simple hepatic steatosis to steatohepatitis, fibrosis, and hepatocarcinoma.¹⁰⁾ Because the prevalence of NAFLD is increasing, it is necessary to develop agents that can prevent hepatic lipid accumulation and treat NAFLD-associated hepatic disorders. It has been reported that ER stress is an important pathological factor in this pathological process.^{6–9)} Thus, an agent that can attenuate ER stress may be a good therapeutic option for the treatment of NAFLD.

The fruit of *Schisandra chinensis* has been used as a traditional herbal medicine in China, Korea, Japan, and Russia. Several studies have demonstrated the diverse pharmacological activities of *S. chinensis*, which include anti-oxidant, anti-tumor, anti-obesity, anti-inflammatory, and cardioprotective effects.^{11–15)} In addition, hepatoprotective activities of *S. chinensis* have also been reported.^{16–19)} A recent study of ours

showed that *S. chinensis* has a protective effect against ER stress-induced hepatic steatosis (unpublished data). *S. chinensis* contains various bioactive constituents, including lignans, triterpenoids, polysaccharides, and sterols.²⁰⁾ Lignans such as deoxyschizandrin, gomisins A, and gomisins N are the main functional constituents of *S. chinensis*. Gomisins N was reported to possess hepatoprotective,²¹⁾ anti-cancer,²²⁾ and anti-inflammatory effects.²³⁾

In the present study, we investigated the *in vitro* and *in vivo* inhibitory effect of gomisins N on ER stress and ER stress-induced hepatic steatosis. The *in vitro* inhibitory effects were examined in HepG2 cells treated with pharmaceutical (tunicamycin) or physiological (palmitate) stress inducers. The *in vivo* protective effects of gomisins N were investigated in tunicamycin-injected mice.

MATERIALS AND METHODS

Reagents Gomisins N was purchased from CoreSciences (Seoul, Korea). Tunicamycin and palmitate were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), while anti-glucose regulated protein 78 (GRP78) anti-CCAAT/enhancer binding protein (C/EBP) homolog protein (CHOP), and anti-X-box-binding protein-1 (XBP-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell Culture The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). HepG2 cells were cultured in Dulbecco's minimum Eagle's essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 U/mL penicillin, and 20 $\mu\text{g}/\text{mL}$ streptomycin.

Quantitative Polymerase Chain Reaction (qPCR) Total RNA was isolated from the liver of the experimental mice and HepG2 cells with TRIzolTM (Invitrogen, Darmstadt, Germany).

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Fig. 1. Gomisin N Inhibits ER Stress and Decreases TG Accumulation in HepG2 Cells

(A) qPCR analysis of markers of ER stress in tunicamycin-treated HepG2 cells. HepG2 cells were pre-incubated in the absence or presence of gomisin N (10, 50, or 100 μ M) for 16 h prior to addition of tunicamycin (2 μ g/mL) for 6 h. Tu; tunicamycin, GN; gomisin N. (B) Western blot analysis of GRP78, CHOP, and XBP-1 in tunicamycin-treated HepG2 cells. A representative image from three independent experiments is shown. (C) qPCR analysis of markers of ER stress in thapsigargin-treated HepG2 cells. HepG2 cells were pre-incubated in the absence or presence of gomisin N (100 μ M) for 16 h prior to addition of thapsigargin (1 μ M) for 6 h. Thap; thapsigargin, GN; gomisin N. (D) Measurement of intracellular TGs in tunicamycin-treated HepG2 cells. Values are expressed as the mean $\pm$ S.E.M. ($n=3$ independent experiments). [#] $p<0.05$ vs. untreated control. ^{*} $p<0.05$ vs. tunicamycin or thapsigargin-treated control.

cDNA was synthesized using the GoScript Reverse Transcription System (Promega, Madison, Wisconsin, U.S.A.) according to the manufacturer's protocol. The primers used in this study are listed in Supplementary material, Table S1.

Western Blot Proteins (40 μ g per well) from HepG2 cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel and transferred to polyvinylidene difluoride (PVDF) membranes. The blocked membranes were incubated with primary antibodies to a 78-kDa glucose-regulated protein (GRP78; BiP) and C/EBP homologous protein (CHOP) as markers of ER stress. Proteins were detected using an enhanced chemiluminescence (ECL) Western blot detection kit (Amersham, Uppsala, Sweden).

Animal Study C57BL/6 mice (8 weeks of age) were purchased from Central Lab. Animal Inc. (Seoul, Korea). They were randomly divided into 4 groups ($n=5$): no treatment group, treatment with tunicamycin alone, treatment with tunicamycin and a low dose of gomisin N (1 mg/kg body weight), and treatment with tunicamycin and a high dose of gomisin N (30 mg/kg body weight). Gomisin N was administered orally for 4 d. On Day 4, tunicamycin (1 mg/kg body

weight) was administered intraperitoneally for 24 h *via* injection. On Day 5, gomisin N was again administered for 24 h. Animal experiments were approved by the Pusan National University Animal Experiment Ethics Committee and were conducted in accordance with the institutional guidelines for the care and use of laboratory animals.

Biochemical Analysis Serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels were determined using a commercial kit (AM 101-K, Asan Pharmaceutical, Korea). Hepatic lipids were extracted from the liver according to the following procedure: Briefly, liver tissues were homogenized in a chloroform-methanol solution (2:1, v/v), and then incubated for 1 h at room temperature and centrifuged (3000 rpm, 10 min). The obtained bottom layer (organic phase) was dried overnight. After dissolving in ethanol, hepatic TG and total cholesterol (TC) were determined using a TG and TC kit (AM 157S-K and AM 202-K, Asan Pharmaceutical, Korea) and normalized to the protein concentration. HepG2 cells were homogenized in chloroform-methanol-H₂O solution (8:4:3, v/v/v). The TG content of HepG2 cells was determined using the same protocol and kit for hepatic TG

Fig. 2. Gomisin N Inhibits Palmitate-Mediated ER Stress and Decreases TG Accumulation in HepG2 Cells

HepG2 cells were pre-incubated in the absence or presence of gomisin N (10, 50, or 100 μ M) for 16 h prior to adding palmitate (400 μ M) for 24 h. (A) qPCR analysis of markers of ER stress. PA; palmitate, GN; gomisin N. (B) Western blot analysis of GRP78, CHOP, and XBP-1. A representative image from three independent experiments is shown. (C) ORO staining. A representative image from three independent experiments is shown. (D) Measurement of intracellular TGs. Values are expressed as the mean $\pm$ S.E.M. ($n=3$ independent experiments). [#] $p<0.05$ vs. untreated control. ^{*} $p<0.05$ vs. palmitate-treated control.

analysis.

Histological Analysis Liver sections were stained with hematoxylin and eosin (H&E) or oil red O (ORO) for histological analysis. For H&E staining, mouse liver was fixed in 10% formalin and dehydrated by serial incubations in ethanol and xylene. Dehydrated liver was embedded in paraffin, cut into 3- μ m sections, and stained with H&E. Mouse liver was sectioned while frozen prior to staining with ORO. Stained liver sections were observed at 200 $\times$ magnification by using a light microscope (Carl Zeiss, DE/Axio Imager A1, Germany).

Statistical Analysis Data were expressed as the mean $\pm$ standard error of the mean (S.E.M.) Statistically significant differences were determined by one-way ANOVA followed by Duncan's multiple-range tests. For all statistical analyses, p values below 0.05 were considered significant.

RESULTS

Gomisin N Inhibits ER Stress and Decreases TG Accumulation in HepG2 Cells The 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay revealed that gomisin N was not cytotoxic to HepG2 cells at a concentration of 100 μ M (data not shown). To investigate the inhibitory effect of gomisin N on ER stress, HepG2 cells were incu-

bated in the absence or presence of different concentrations of gomisin N (10, 50, or 100 μ M) for 16 h. Tunicamycin was added to the medium, and incubation was continued in the absence or presence of gomisin N for 6 h. As shown in Fig. 1A, tunicamycin alone increased transcription of markers of ER stress, including GRP78, CHOP, and XBP-1. In contrast, gomisin N suppressed this induced transcription in a dose-dependent manner. Western blots showed that GRP78, CHOP, and XBP-1 proteins were also decreased by gomisin N, which is consistent with their decreased mRNA levels (Fig. 1B). Treatment with thapsigargin, another pharmaceutical inducer of ER stress, increased markers of ER stress that were efficiently inhibited by gomisin N (Fig. 1C). We next examined whether gomisin N inhibits TG levels in tunicamycin-treated HepG2 cells. As shown in Fig. 1D, tunicamycin treatment increased intracellular TG levels in HepG2 cells. However, this effect was efficiently inhibited by gomisin N (Fig. 1D). Taken together, these results demonstrated that gomisin N inhibits ER stress and decreases intracellular TG levels in HepG2 cells.

Gomisin N Inhibits Palmitate-Mediated ER Stress and Decreases TG Accumulation in HepG2 Cells We then repeated our investigation of the inhibitory effects of gomisin N against ER stress in HepG2 cells with palmitate, because tunicamycin is not a physiological inducer of ER stress.

Fig. 3. Gomisin N Inhibits ER Stress and Decreases TG Accumulation in the Livers of Tunicamycin-Injected Mice

C57BL/6 mice were orally administrated Gomisin N (1 or 30 mg/kg body weight) for 4 d and tunicamycin (1 mg/kg body weight) was intraperitoneally injected on Day 5. (A) Liver morphology and measurement of TG & TC levels. Tu; tunicamycin, GN; gomisin N. (B) ORO staining and H & E staining (magnification $\times 200$). (C) Measurement of GOT and GPT levels. (D) qPCR analysis of markers of ER stress. Values are expressed as the mean $\pm$ S.E.M. ($n=5$ mice per group). # $p<0.05$ vs. untreated control. * $p<0.05$ vs. tunicamycin-treated control.

HepG2 cells were incubated in the absence or presence of various concentrations of gomisin N (10, 50, or 100 μ M) for 16 h. Palmitate was added to the culture medium, and incubation was continued in the absence or presence of gomisin N for 24 h. As shown in Fig. 2A, palmitate incubation increased transcription of markers of ER stress such as GRP78, CHOP, and XBP-1. Gomisin N suppressed palmitate-induced markers of ER stress. The protein levels of GRP78, CHOP, and XBP-1 were also decreased by gomisin N (Fig. 2B). TG levels were measured by ORO staining. Palmitate incubation increased intracellular TG levels in HepG2 cells, whereas gomisin N efficiently inhibited this increase in TG levels (Fig. 2C). These results suggest that gomisin N alleviates palmitate-induced ER stress and prevents TG accumulation in HepG2 cells.

Gomisin N Alleviates Tunicamycin-Induced Hepatic ER Stress and TG Accumulation in Mice We next investigated the ability of gomisin N to alleviate hepatic ER stress and TG accumulation *in vivo*. C57BL/6 mice were pre-administered gomisin N, and then administered injections with tunicamycin for 24 h. Morphological examination of the liver revealed that liver was yellow in color in tunicamycin-injected mice (Fig. 3A), indicating that tunicamycin injection induced severe

hepatic steatosis. However, pre-administration with gomisin N significantly inhibited this effect. The protective effect of gomisin N against hepatic steatosis was also confirmed by measurement of hepatic TG levels. Hepatic TG levels were markedly increased after tunicamycin injection. However, gomisin N prevented this increase in TG levels (Fig. 3A). Total cholesterol (TG) levels induced by tunicamycin were also decreased by gomisin N (Fig. 3A). ORO staining and H&E staining also demonstrated that tunicamycin-induced TG accumulation was significantly reduced by gomisin N (Fig. 3B). These results indicate that gomisin N improves hepatic steatosis that is induced by ER stress. We next examined whether gomisin N ameliorates tunicamycin-induced hepatic toxicity by measuring serum GOT and GPT levels. As shown in Fig. 3C, tunicamycin injection increased the levels of GOT and GPT, whereas gomisin N efficiently reduced the levels of both.

We examined the expression of markers of ER stress in livers to determine whether improvement of hepatic steatosis by gomisin N is associated with a reduction in hepatic ER stress. As shown in Fig. 3D, tunicamycin injection dramatically increased the expression of markers of ER stress, whereas

Fig. 4. Gomisin N Represses the Expression of Inflammatory Genes and Lipogenic Genes in HepG2 Cell

(A) qPCR of IL-6, TNF- α , and MCP-1 in tunicamycin or palmitate-treated HepG2 cells. Tu; tunicamycin, PA; palmitate, GN; gomisin N. (B) qPCR of FAS, ACC1, and GPAT in palmitate-treated HepG2 cells. PA; palmitate, GN; gomisin N. Values are expressed as the mean $\pm$ S.E.M. ($n=3$ independent experiments). # $p<0.05$ vs. untreated control. * $p<0.05$ vs. tunicamycin or palmitate-treated control.

gomisin N administration repressed this effect. Taken together, these findings indicate that gomisin N can prevent ER stress-induced hepatic steatosis and improve hepatic injury in mice.

Gomisin N Downregulates the Expression of Inflammatory and Lipogenic Genes in HepG2 Cells We further determined the role of gomisin N in ER stress-induced inflammation by measuring the expression of inflammatory genes in HepG2 cells treated with tunicamycin or palmitate. The expression of the inflammatory genes such as interleukin (IL)-6, tumor necrosis factor (TNF)- α , and monocyte chemoattractant protein (MCP)-1 increased by treatment with tunicamycin or palmitate. However, gomisin N was found to efficiently block the increased expression of these genes (Fig. 4A). It is well known that lipogenesis is the primary pathway by which ER stress induces hepatic steatosis.^{7,8)} To determine the mechanism involved in gomisin N-mediated improvement in hepatic steatosis, we measured the expression of the lipogenic genes such as fatty acid synthase (FAS), acetyl-coenzyme A carboxylase 1 (ACC1), and glycerol-3-phosphate acyltransferase (GPAT) in HepG2 cells treated with palmitate. As shown in Fig. 4B, the expression of the lipogenic genes FAS, ACC1, and GPAT increased in palmitate-incubated HepG2 cells. However, gomisin N significantly reduced the expression of these

lipogenic genes (Fig. 4B). These results indicate that gomisin N represses the expression of genes involved in hepatic lipogenesis and may therefore play a role in the inhibition of TG accumulation.

DISCUSSION

Previously, we found that *S. chinensis* extract inhibited ER stress and prevented the development of hepatic steatosis. This supports the hypothesis that *S. chinensis* extract can be used as a potential therapeutic agent for treatment of ER stress-induced diseases, including hepatic steatosis. Gomisin N is one of the active components in *Schisandra chinensis* and has been reported to have diverse pharmacological activities. These activities include a hepatoprotective activity against carbon tetrachloride (CCl₄)-induced liver injury, anti-cancer activity through the target gene Wnt/ β -catenin,^{21,22)} and anti-inflammatory effects.²³⁾ However, the potential for a protective effect against ER stress and associated hepatic steatosis remained unknown. Our study is the first to report that gomisin N inhibits ER stress and prevents the development of ER stress-induced NAFLD.

Tunicamycin and thapsigargin are pharmaceutical ER stress inducers, whereas palmitate is a physiological ER stress in-

ducer. We demonstrated the protective effects of gomisin N against ER stress in HepG2 cells treated with both pharmaceutical (e.g. tunicamycin or thapsigargin) and physiological (e.g. palmitate) inducers. Protein and mRNA levels of GRP78, CHOP, and XBP-1 were used as markers of ER stress. Our data showed that gomisin N repressed mRNA levels of GRP78, CHOP, and XBP-1 in palmitate-treated HepG2 cells as well as in tunicamycin or thapsigargin-treated HepG2 cells. This indicates that gomisin N has a protective effect against pharmaceutical and physiological inducers of ER in liver cells. It has been reported that gomisin N improves hepatic oxidative stress.²⁴ Therefore, gomisin N-mediated protective effect against ER stress may be due to antioxidant capacity.

An adaptive response known as the UPR is induced under pathological conditions.¹ UPR is characterized by the activation of three distinct signal transduction pathways mediated by three transmembrane proteins. They include protein kinase RNA-activated (PKR)-like ER kinase (PERK)-CHOP, inositol-requiring enzyme (IRE) 1-XBP-1 phosphorylated c-Jun N-terminal kinase (pJNK), and activating transcription factor (ATF)-6-GRP78. These three branches of the UPR attenuate protein synthesis, increase protein-folding capability, and degrade terminally unfolded and misfolded proteins within the ER. According to our current results, gomisin N inhibited the expression of GRP78, CHOP, and XBP-1. These proteins are representative of signaling mediators of the three branches of the UPR, suggesting that gomisin N has an inhibitory effect on all three branches.

We next investigated whether gomisin N prevents TG accumulation concomitant with inhibition of ER stress. In accordance with the attenuation of ER stress, gomisin N efficiently blocked tunicamycin-mediated increases in cellular TG levels in HepG2 cells. This inhibitory effect was revealed by ORO staining and measurement of TG levels. These results demonstrate that gomisin N exerts an inhibitory effect on ER stress-induced TG accumulation in HepG2 cells.

To confirm the protective effect of gomisin N against ER stress-induced hepatic steatosis *in vivo*, a low dose or high dose of gomisin N was pre-administered into C57BL6J mice, followed by injection with tunicamycin. Tunicamycin increased markers of ER stress and TG level in the mouse liver. However, pre-administration of both low and high doses of gomisin N efficiently blocked increases in hepatic markers of ER stress and TG accumulation. In addition, gomisin N decreased serum TG and TC. This is consistent with the reduction of hepatic TG content. Moreover, we found that gomisin N reduced tunicamycin-mediated elevation in levels of the biomarkers of liver injury, GOT and GPT. Taken together, these results suggest that gomisin N attenuates ER stress and ameliorates ER stress-induced NAFLD and liver injury *in vivo*.

Furthermore, we investigated the effect of gomisin N on inflammation in ER stress-induced HepG2 cells. It has been previously reported that ER stress correlates with inflammation.²⁵ Tunicamycin increased pro-inflammatory cytokine production in an animal model. The expressions of IL-6, TNF- α , and MCP-1 were measured in HepG2 cells treated with tunicamycin or palmitate in the absence or presence of gomisin N. Our data showed that gomisin N reduced the levels of IL-6, TNF- α , and MCP-1 that were induced by incubation with tunicamycin or palmitate, suggesting that gomisin N in-

hibits inflammation by attenuation of ER stress.

To determine the mechanism by which gomisin N improves ER stress-induced hepatic steatosis, we investigated the effect of gomisin N on lipogenesis. A previous study showed that tunicamycin decreased the expression of lipogenic genes such as FAS, ACC1, and GPAT in the livers of tunicamycin-injected mice despite the presence of hepatic steatosis.²⁶ It has been reported that fatty acids induce ER stress through induction of lipogenesis.²⁷ Therefore, we evaluated the anti-lipogenic effects of gomisin N in palmitate-treated HepG2 cells. Expression of lipogenic genes including FAS, ACC1, and GPAT were increased in HepG2 cells by incubation with palmitate alone, whereas gomisin N blocked an increase in lipogenic gene expression. These results suggest that gomisin N may exert inhibitory effects on fatty acid-induced hepatic steatosis through suppression of lipogenesis.

In conclusion, gomisin N efficiently attenuated ER stress and thus halted the development of NAFLD. Our experimental evidence supports the conclusion that gomisin N may be used as a potential therapeutic agent for the treatment of ER stress-induced disease. Thus, gomisin N is a promising lead compound for future drug development.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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