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Gomisin A Ameliorates Endoplasmic Reticulum Stress-induced Hepatic Steatosis

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Previously, we have shown that *Schisandra chinensis* (Turcz.) Baill. (*S. chinensis*) has a protective effect against endoplasmic reticulum (ER) stress-induced hepatic steatosis. Gomisin A is a bioactive phytoestrogen derived from *S. chinensis*. In the present study, the *in vitro* and *in vivo* effects of gomisin A on ER stress and hepatic steatosis were investigated. We quantified the expression of markers of ER stress, including glucose regulated protein 78 (GRP78), C/EBP homolog protein (CHOP), and X-box-binding protein-1 (XBP-1), in HepG2 cells treated with tunicamycin or palmitate. Tunicamycin treatment in HepG2 cells induced the expression of markers of ER stress, including GRP78, CHOP, and XBP-1c. However, treatment with gomisin A reduced the expression of markers of ER stress. These inhibitory effects were also observed in palmitate-incubated HepG2 cells. The *in vivo* inhibitory effects of gomisin A were assessed in mice injected with tunicamycin or fed with a high fat diet (HFD). Gomisin A reduced the expression of markers of ER stress and decreased triglyceride levels in the livers of mice after tunicamycin injection or HFD feeding. Furthermore, gomisin A decreased the expression of inflammatory genes in palmitate-incubated HepG2 cells and the liver of HFD-fed obese mice. These results suggest that gomisin A inhibits ER stress and ameliorates hepatic steatosis induced by ER stress.

Key words : Endoplasmic reticulum (ER) stress, gomisin A, hepatic steatosis, high fat diet, inflammation

Introduction

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+} [22]. 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) [22]. However, prolonged ER stress can lead to cell death and cause several diseases including ischemia/reperfusion injury, heart disease, and diabetes [1, 3, 23, 26]. Recent studies show that hepatic ER stress is observed in metabolic diseases such as obesity and diabetes [1, 3, 23, 26]. ER stress contributes to development of insulin resistance and hepatic steatosis in non-alcoholic fatty liver disease (NAFLD) [2, 17, 18, 20].

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[7]. 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 [2, 17, 20]. Thus, an agent that can attenuate ER stress may be a good therapeutic option for the treatment of NAFLD.

The fruit of *S. 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 [4-6, 15, 19]. In addition, hepatoprotective activities of *S. chinensis* have also been reported [8, 9, 14, 25]. *S. chinensis* contains various bioactive constituents, including lignans, triterpenoids, polysaccharides, and sterols [10]. Lignans such as deoxyschizandrin, gomisin A, and gomisin N are the main functional constituents of *S. chinensis*. Gomisin A was reported to possess hepatoprotective, anti-

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oxidative, and anti-inflammatory effects [11, 13, 16, 24].

In the present study, we investigated the *in vitro* and *in vivo* inhibitory effect of gomisin A 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 gomisin A were investigated in tunicamycin-injected mice or high fat diet (HFD) obese mice.

Materials and Methods

Reagents and cell culture

Gomisin A was purchased from ChemFaces (Wuhan, China). Tunicamycin and palmitate were purchased from Sigma-Aldrich (St. Louis, MO, USA). The human hepatocellular carcinoma cell line HepG2 was obtained from the American Type Culture Collection (Manassas, VA, USA). HepG2 cells were cultured in Dulbecco's Minimum Eagle's Essential Medium (DMEM, Hyclone, South Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 U/ml penicillin, and 20 μg/ml streptomycin.

Quantitative PCR (qPCR)

Total RNA was isolated from the liver of the experimental mice and HepG2 cells with TRIzol™ (Invitrogen, Darmstadt, Germany). cDNA was synthesized using the GoScript Reverse Transcription System (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. The primers (Cosmo Genetech, Seoul, Korea) used in this study are listed in Table 1.

Animal study

C57BL/6 mice (8 weeks of age) were purchased form 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 A (5 mg/kg body weight), and treatment with tunicamycin and a high dose of gomisin A (20 mg/kg body weight). Gomisin A was administered orally for 4 days. On Day 4, tunicamycin (1 mg/kg body weight) was administered intraperitoneally for 24 hr via injection. On Day 5, gomisin A was again administered for 24 hr. To see the protective effects of gomisin A against HFD-induced hepatic steatosis, C57BL/6 mice were fed a normal diet (ND) or an HFD for six weeks. Then, the HFD-fed mice were randomly divided into the following three groups (n=6 per group): an HFD (distilled water-treated) group, HFD+low-dose gomisin A (5 mg/kg of body weight) group, and HFD+high-dose gomisin A (20 mg/kg of body weight) group. The experimental diets were the AIN93G-based on the High-fat diet containing 60% kcal fat and the control diet containing 10% kcal fat. 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

Table 1. List of primers for q-PCR

1. Human

Gene	Forward primer	Reverse primer
hGRP78	ATGATGCTGAGAAGTTTGCTGA	GGAAAGTTTACCTCCCAGCTTT
hCHOP	AGGGAGAACCAG GAAACG	TCC TGC TTG AGC CGT TCA TTC
hXBP-1	TGCTGAGTCCGCAGCAGGTG	GCTGGCAGGCTCTGGGGAAG
hTNF-α	TGCTTGTTCCTCAGCCTCTT	ATGGGCTACAGGCTTGTCACT
hIL-6	ACTCACCTCTTCAGAACGAAT	CCATCTTTGGAAGGTTCAGGTTG
hMCP-1	CCCCAGTCACCTGCTGTTAT	TGGAATCCTGAACCCACTTC

2. Mouse

Gene	Forward primer	Reverse primer
mGRP78	GAAAGGATGGTTAATGATGCTGAG	GTCTTCAATGTCCGCATCCTG
mCHOP	CAGTCATGGCAGCTGAGTCC	TAGGTGCCCCCAATTTCATC
mXBP-1	GAG TCC GCA GCA GGT G	GTG TCA GAG TCC ATG GGA
mTNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
mIL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
mMCP-1	GCATCCACGTGTTGGCTCA	CTCCAGCCTACTCATTGGGATCA

and use of laboratory animals (ED-PNU2015-0010).

Oil Red O (ORO) staining

Liver specimens were sectioned in blocks and fixed in 10% formalin. After fixation, tissues were dehydrated with a graded series of ethanol and xylene, embedded in paraffin, cut into 3 μm sections. Liver slides were washed with 60% isopropanol for 5 min and stained with Oil-red O working solution (1.5 mg/ml Oil-red O/60% isopropanol) for 15 min at RT. The slides were washed with distilled water and photographed under a light microscope (Carl Zeiss, DE/Axio Imager A1, Germany).

Biochemical analysis

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) 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 hr at room temperature and centrifuged (3,000 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.

Statistical analysis

Data were expressed as the mean ± SEM. 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 A inhibits ER stress in HepG2 cells

An MTT assay revealed that gomisin A was not cytotoxic to HepG2 cells at a concentration of 100 μM, (data not shown). Then, we investigated the inhibitory effect of gomisin A on ER stress in HepG2 cells treated with tunicamycin. As shown in Fig. 1A, tunicamycin alone increased transcription of markers of ER stress, including GRP78, CHOP, and XBP-1. In contrast, gomisin A suppressed this induced transcription in a dose-dependent manner. We then repeated our investigation of the inhibitory effects of gomisin A against ER stress in HepG2 cells with palmitate, because tunicamycin is not a physiological inducer of ER stress. As shown in Fig. 1B, palmitate incubation increased transcription of markers of ER stress such as GRP78, CHOP, and

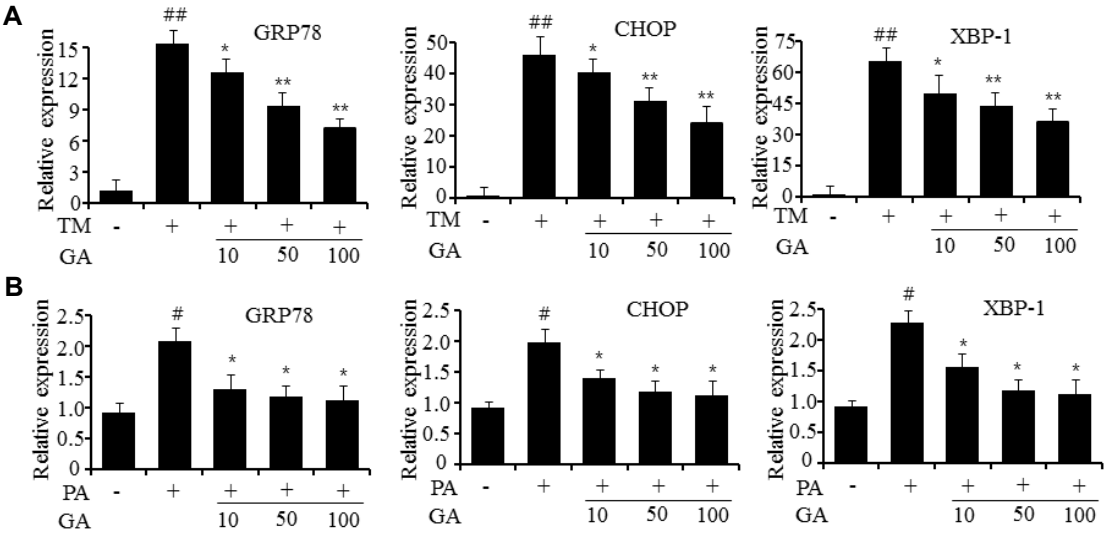

Fig. 1. Gomisin A inhibits ER stress in HepG2 cells. (A) q-PCR analysis of markers of ER stress in tunicamycin-treated HepG2 cells. HepG2 cells were pre-incubated in the absence or presence of gomisin A (10, 50, or 100 μM) for 16 hr prior to addition of tunicamycin (2 μg/ml) for 6 hr. TM; tunicamycin, GA; gomisin A. (B) q-PCR analysis of markers of ER stress in palmitate-incubated HepG2 cells. HepG2 cells were pre-incubated in the absence or presence of gomisin A (10, 50, or 100 μM) for 16 h prior to adding palmitate (400 μM) for 24 hr. PA; palmitate, GA; gomisin A. Values are expressed as the mean ± SEM (n=3) independent experiments). $^{\#}p$ <0.05, $^{\#\#}p$<0.01 vs. untreated control. *p <0.05, **p<0.01 vs. tunicamycin or palmitate-treated control.

XBP-1. Gomisin A suppressed palmitate-induced markers of ER stress. Taken together, these results demonstrated that gomisin A inhibits ER stress in HepG2 cells treated with tunicamycin or palmitate.

Gomisin A downregulates the expression of inflammatory genes in HepG2 cells

It has been previously reported that ER stress correlates with inflammation[21]. Tunicamycin increased pro-inflammatory cytokine production in an animal model. Therefore, we further determined the role of gomisin A 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 including tumor necrosis factor(TNF)-α, interleukin(IL)-6 and monocyte chemoattractant protein (MCP)-1 increased by treatment with tunicamycin (Fig. 2A) or palmitate (Fig. 2B). However, gomisin A was found to block the increased expression of these genes in tunicamycin (Fig. 2A) or palmitate (Fig. 2B)-treated HepG2 cells. These results suggest that gomisin A inhibits ER stress-related inflammation.

Gomisin A alleviates tunicamycin-induced hepatic ER stress and TG accumulation in mice

We next investigated the inhibitory effects of gomisin A on hepatic ER stress and TG accumulation in mice injected with tunicamycin intraperitoneally. The protective effect of gomisin A against hepatic steatosis was investigated by ORO staining and measurement of hepatic TG levels. Tunicamycin injection increased ORO staining in the liver, but gomisin A significantly decreased the staining (Fig. 3A). Hepatic TG levels were markedly increased after tunicamycin injection. However, gomisin A prevented this increase in TG levels (Fig. 3B). These results indicate that gomisin A improves hepatic steatosis that is induced by ER stress. We next examined whether gomisin A ameliorates tunicamycin-induced hepatic toxicity by measuring serum AST and ALT levels. As shown in Fig. 3C, tunicamycin injection increased the levels of AST and ALT, whereas gomisin A 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 A is associated with a reduction in hepatic ER stress. As shown in Fig. 3D, tunicamycin injection increased the expression of markers of ER stress, whereas gomisin A administration repressed this effect. Taken together, these findings indicate that gomisin A can prevent ER stress-induced hepatic steatosis and improve hepatic injury in mice.

Gomisin A inhibits hepatic ER stress and hepatic steatosis in HFD obese mice

Then, we verified the *in vivo* inhibitory effects of gomisin A on hepatic steatosis in HFD obese mice. ORO staining showed that HFD feeding elevated TG in mouse livers, but

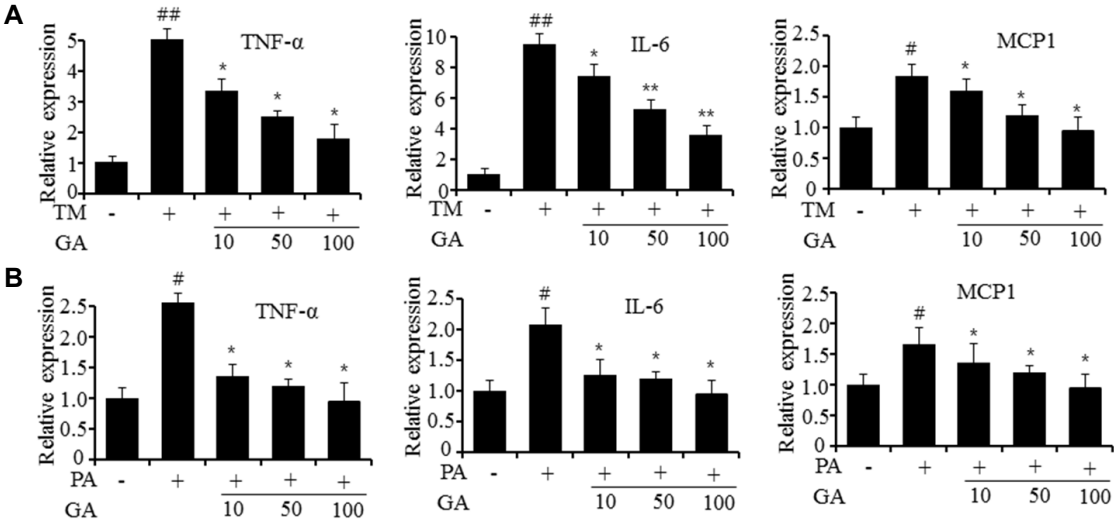

Fig. 2. Gomisin A represses the expression of inflammatory genes in HepG2 cell. (A) qPCR of TNF-α, IL-6 and MCP-1 in tunicamycin-treated HepG2 cells. TM; tunicamycin, GA; gomisin A. (B) qPCR of TNF-α, IL-6 and MCP-1 in palmitate-treated HepG2 cells. PA; palmitate, GA; gomosin A. Values are expressed as the mean ± SEM (n=3 independent experiments). $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. untreated control. $^{*}p<0.05$, $^{**}p<0.01$ vs. tunicamycin or palmitate-treated control.

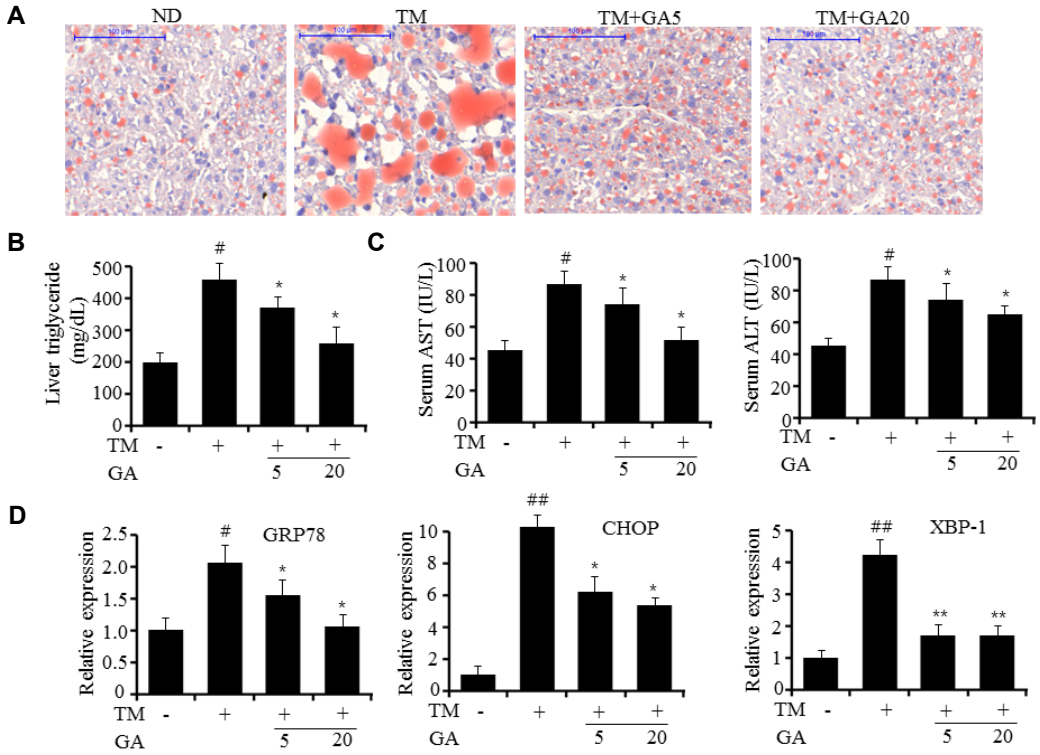

Fig. 3. Gomisin A inhibits ER stress and decreases TG accumulation in the livers of tunicamycin-injected mice. C57BL/6 mice were orally administrated gomisin A (5 or 20 mg/kg body weight) for 4 days and tunicamycin (1 mg/kg body weight) was intraperitoneally injected on Day 5. (A) Oil red O staining. (B) Measurement of hepatic TG levels. (C) Measurement of serum AST and ALT levels. (D) q-PCR analysis of markers of ER stress. TM; tunicamycin, GA; gomisin A. Values are expressed as the mean ± SEM (n=6 mice per group). $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. untreated control. $^{*}p<0.05$, $^{**}p<0.01$ vs. tunicamycin-treated control.

gomisin A treatment blocked the elevation of hepatic TG (Fig. 4A). Consistent with this, gomisin A significantly reduced HFD-induced hepatic TG levels (Fig. 4B). These results suggest that gomisin A inhibits hepatic steatosis in HFD obese mice. We next examined whether gomisin A ameliorates HFD-induced hepatic toxicity by measuring serum GOT and GPT levels. As shown in Fig. 4C, HFD increased the levels of GOT and GPT, whereas gomisin A efficiently reduced the levels of both.

We then examined the expression of markers of ER stress in livers of HFD obese mice. As shown in Fig. 4D, HFD increased the expression of markers of ER stress, whereas gomisin A administration repressed this effect. These results suggest that gomisin A inhibits ER stress and ameliorates hepatic steatosis in HFD-induced obese mice.

Gomisin A downregulates the expression of inflammatory genes in the liver of HFD obese mice

Finally, we examined inflammation by measuring the expression of inflammatory genes in the livers of HFD obese mice. As shown in Fig 5, the mRNA levels of the inflammatory genes such as TNF-α, IL-6 and MCP-1 were markedly increased in the livers of HFD obese mice compared to lean control mice. However, gomisin A significantly inhibited the this increase of inflammatory genes in the liver of HFD obese mice. These results indicate that gomisin A inhibits inflammation in the liver of HFD obese mice.

Discussion

Previously, we found that *S. chinensis* extract inhibited ER stress and prevented the development of hepatic steatosis [12]. 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. Our study demonstrated that gomisin A, a bioactive phytoestrogen derived from *S. chinensis*, 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

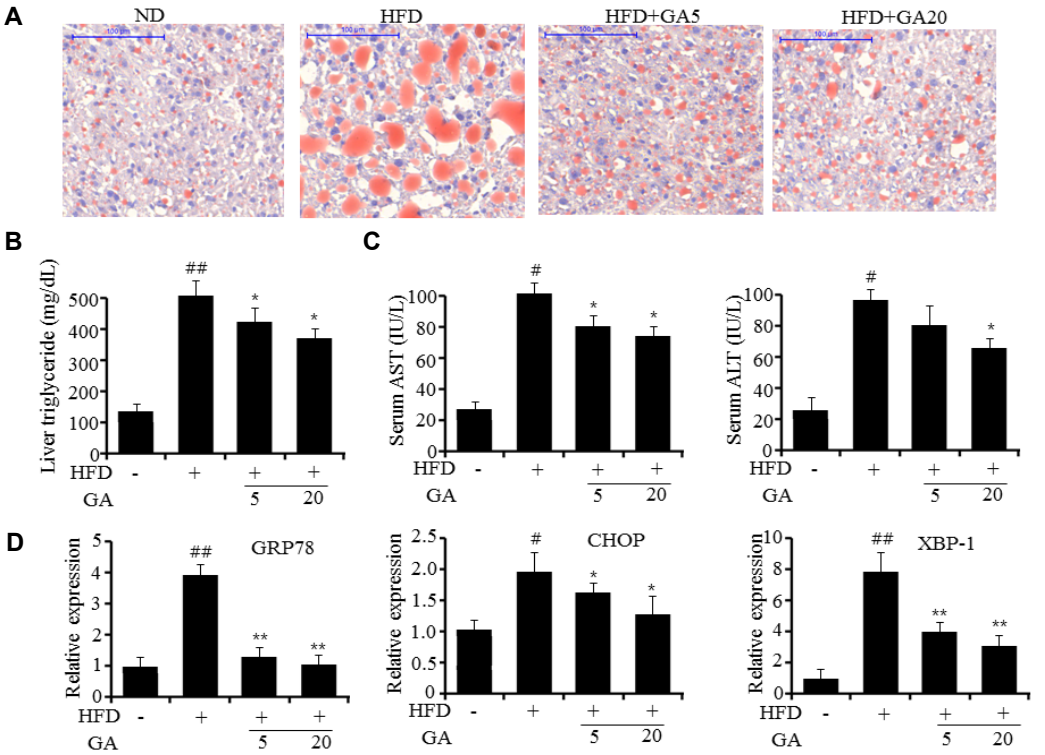

Fig. 4. Gomisin A ameliorates hepatic steatosis in high-fat diet (HFD)-induced obese mice. C57BL/6 mice were fed a ND or HFD for six weeks, and gomisin A was administered to the HFD-fed mice for an additional eight weeks (n=6 mice per group). (A) Oil red O staining. (B) Measurement of hepatic TG levels. (C) Measurement of serum GOT and GPT levels. (D) q-PCR analysis of markers of ER stress. Values are expressed as mean ± SEM (n=6). $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. ND group. $^{*}p<0.05$, $^{**}p<0.01$ vs. HFD group.

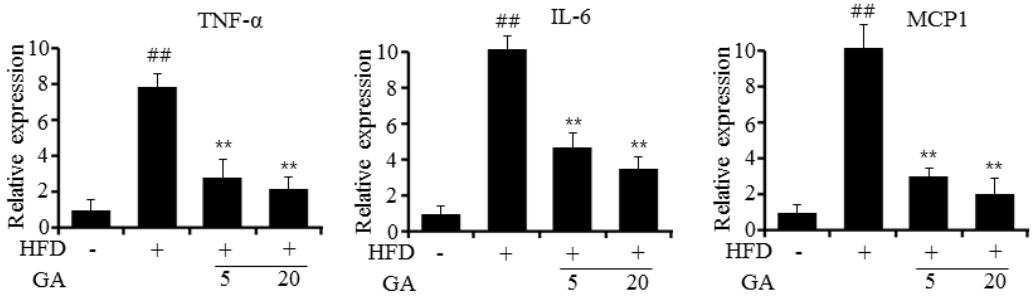

Fig. 5. Gomisin A inhibits the expression of inflammatory genes in the liver of HFD obese mice. The expression of TNF-α, IL-6 and MCP-1 was measured by qPCR. GA; gomosin A. Values are expressed as the mean ± SEM (n=6). $^{\#\#}p<0.01$ vs. ND group. $^{**}p<0.01$ vs. HFD group.

stress inducer. We demonstrated the protective effects of gomisin A against ER stress in HepG2 cells treated with both pharmaceutical (tunicamycin) and physiological (e.g. palmitate) inducers. mRNA levels of GRP78, CHOP, and XBP-1 were used as markers of ER stress. Our data showed that gomisin A repressed mRNA levels of GRP78, CHOP, and XBP-1 in palmitate-treated HepG2 cells as well as in tunicamycin-treated HepG2 cells. This indicates that gomisin A has a protective effect against pharmaceutical and physiological inducers of ER in liver cells. An adaptive response known as the UPR is induced under pathological conditions [22]. 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-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 A 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 A has an inhibitory effect on all three branches.

To confirm the protective effect of gomisin A against ER stress-induced hepatic steatosis *in vivo*, a low dose or high dose of gomisin A 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 A efficiently blocked increases in hepatic markers of ER stress and TG accumulation. In addition, gomisin A reduced tunicamycin-mediated elevation in levels of the biomarkers of liver injury, AST and ALT. Taken together, these results suggest that gomisin A attenuates ER stress and ameliorates ER stress-induced NAFLD and liver injury *in vivo*. The protective effects of gomisin A against hepatic steatosis were further confirmed in HFD-induced obese mice. HFD induced hepatic steatosis in mice, which was characterized by increased hepatic lipid accumulation; however, gomisin A treatment efficiently blocked hepatic lipid accumulation.

Furthermore, we investigated the effect of gomisin A on inflammation in ER stress-induced HepG2 cells. It has been previously reported that ER stress correlates with inflammation[21]. The expressions of TNF-α, IL-6 and MCP-1 were measured in HepG2 cells treated with tunicamycin or palmitate in the absence or presence of gomisin A. Our data showed that gomisin A reduced the levels of TNF-α, IL-6 and MCP-1 that were induced by incubation with tunicamycin or palmitate. The anti-inflammatory effects were also confirmed in the liver of HFD obese mice. These results indicated that gomisin A inhibits inflammation by attenuation of ER stress.

In conclusion, gomisin A efficiently attenuated ER stress and thus halted the development of NAFLD. Thus, gomisin A is a promising lead compound for future drug development.

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초록 : Gomisin A의 비알코올성 지방간 보호효과

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본 연구는 소포체스트레스(endoplasmic reticulum stress)에 의해 유발되는 지방간(hepatic steatosis)에 대한 오미자추출물(*Schisandra chinensis*)의 주요성분인 gomisin A의 지방간 보호 효능에 대하여 연구하였다. 이를 위해 HepG2 세포에 소포체스트레스 유도물질인 tunicamycin 또는 palmitate을 처리하여 세포에서의 지방간 모델을 만들어 실험을 진행 하였으며, 소포체스트레스 표지자(marker)인 GRP78, CHOP, XBP-1의 발현을 측정하였다. Tunicamycin 처리한 세포에서는 GRP78, CHOP, XBP-1의 발현이 증가되었으나, gomisin A를 처리 한 세포에서는 이들의 발현 증가가 억제됨을 확인하였다. 이는 palmitate를 처리한 HepG2 세포에서도 palmitate에 의해 증가하는 소포체스트레스 표지자들이 gomisin A을 처리한 세포에서 발현이 감소함을 확인하였다. 이러한 결과에 의해, gomisin A는 소포체스트레스를 억제함을 알 수 있었다. 다음으로 gomisin A가 *in vivo*에서 소포체스트레스 및 지방간에 대한 보호효과가 있는지 확인하기 위해, tunicamycin과 고지방(high fat diet)으로 식이 한 쥐에서 소포체스트레스와 지방간의 보호효능에 대해 실험을 진행하였다. Tunicamycin과 고지방식이을 한 쥐의 간에서 중성지방이 증가하였으나, gomisin A를 처리한 쥐의 간에서 중성지방의 수준이 유의적으로 감소함을 확인하였다. 소포체스트레스 표지자들 역시 tunicamycin이나 고지방식이을 한 쥐에서 증가되나 gomisin A를 처리한 쥐에서 감소됨을 확인하였다. Gomisin A의 염증 반응에서의 조절기능을 확인하기 위하여 TNF-α, IL-6 그리고 MCP1과 같은 염증관련 유전자들의 발현을 분석한 결과, tunicamycin이나 고지방식이을 한 쥐에서 염증유전자들의 발현이 증가하였으나 gomisin A를 처리한 쥐에서는 유의적으로 감소하였다. 종합적으로 본 연구 결과에 의하면, gomsin A는 소포체스트레스를 억제하여 지방간의 생성을 저해함을 알 수 있었다.