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Guggulsterone sensitizes hepatoma cells to TRAIL-induced apoptosis through the induction of CHOP-dependent DR5: Involvement of ROS-dependent ER-stress

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ABSTRACT

Guggulsterone (GGS) has anti-tumor and anti-angiogenesis potential by suppressing nuclear factor-κB and STAT3 activity. Although GGS has been suggested as a potential therapeutic agent for treating various cancers, the underlying molecular mechanisms are unknown. Therefore, we investigated whether GGS sensitizes hepatocellular carcinoma cells (HCC) to apoptosis mediated by tumor necrosis factor-related apoptosis inducing ligand (TRAIL). The apoptotic mechanism induced by treatment with a GGS/TRAIL combination involved the loss of mitochondrial transmembrane potential and consequent activation of caspases. GGS also induced upregulation of the death receptor DR5 for TRAIL. The effects seemed to be associated with eIF2α and CHOP activation, which are related to the endoplasmic reticulum (ER) stress response and apoptosis. This relationship was suggested by the observation that CHOP downregulation by specific siRNA attenuated both GGS-mediated DR5 upregulation and the cytotoxicity induced by GGS/TRAIL co-treatment. Moreover, salubrinal, a specific eIF-2α phosphorylation-inducing agent, enhanced the expression of CHOP and DR5 induced by GGS and sensitized cells to GGS/TRAIL-induced apoptosis. Thus, GGS-induced eIF2α phosphorylation seems to be important for CHOP and DR5 upregulation. Furthermore, these events were accompanied by an increase in the generation of reactive oxygen species. Pretreatment with *N*-acetyl-L-cysteine and glutathione inhibited GGS-induced ER-stress, and CHOP and DR5 upregulation and almost completely blocked GGS/TRAIL-induced apoptosis. These results collectively indicate that DR5 induction via eIF-2α and CHOP is crucial for the marked synergistic effects induced by TRAIL and GGS. Taken together, these results indicate that a GGS/TRAIL combination could represent a novel important tool for cancer therapy.

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, is a potent apoptosis-inducing cytokine. TRAIL appears to specifically kill a wide variety of cancer cells in culture and xenografted tumors but has a little or no effect on normal cells [1–3]. TRAIL-induced apoptosis is associated with the interaction of its ligands with two closely related membrane receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5). This interaction results in cooperation with the adaptor molecule FADD leading to the recruitment and cleavage of the initiator caspase-8 and the consequent activation of an effector caspase such as caspase-3 [4,5]. Nevertheless, cancer cells acquire TRAIL resistance through multiple unknown mechanisms. Thus, it is important to identify therapeutic agents capable of sensitizing resistant cancer cells to TRAIL-induced apoptosis.

Interestingly, many pharmaceutical products currently available originate from plant extracts, and various phytochemicals have anti-tumor properties both *in vivo* and *in vitro*. Guggulsterone (GGS), a plant polyphenol obtained from the gum resin of *Commiphora mukul*, has been used in ayurvedic medicine for centuries to treat obesity, diabetes, hyperlipidemia, atherosclerosis, and osteoarthritis [6,7]. Recently, GGS has been shown to have cholesterol-lowering activity as a farnesoid X receptor antagonist

Abbreviations: GGS, guggulsterone; HCC, hepatocellular carcinoma cells; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; ER, endoplasmic reticulum; CHOP, C/EBP homologous transcription factor; DR4, TRAIL-R1; DR5, TRAIL-R2; NF-κB, nuclear factor-κB; eIF2α, initiation factor-2α; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine; GSH, glutathione; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; HE, hydroethidine; DCFDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA.

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[8] and to possess anticancer potential in various human cancer cells by inhibiting nuclear factor (NF)-κB and STAT3 [9–13]. Despite these observations, the molecular mechanisms underlying the anticancer effects of GGS in TRAIL-induced apoptosis are poorly understood.

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver. Because only 10–20% of HCC can be completely removed by surgery to extend the lifespan of patients with HCC, sorafenib, a receptor tyrosine kinase inhibitor, has been used [14]. Although patients with HCC require chemotherapy (including TRAIL), many types of HCC cell lines examined in many studies show TRAIL resistance [15], despite a report that a variety of tumor cells respond to TRAIL. However, recent studies have shown that DR5 upregulation by chemotherapy agents could overcome TRAIL resistance [16,17]; therefore, DR5 upregulation may be a potential target to increase the tumor-specific effect of TRAIL in HCC.

DR5 expression can be regulated at the transcriptional level through C/EBP homologous transcription factor (CHOP), which is also known as growth arrest and DNA damage gene 153, via binding to the CHOP-binding site in the DR5 gene 5′-flanking region [18,19]. CHOP expression is primarily regulated at the transcriptional level and is one of most induced genes during endoplasmic reticulum (ER) stress [20]. Although the mechanism of ER stress-mediated CHOP expression is not fully understood, several mechanisms have been suggested. Under ER stress conditions, BiP binds to unfolded proteins and activates each transducer. Activated PERK, by release of BiP, phosphorylates Ser51 on eukaryotic initiation factor-2α (eIF2α), which then upregulates ATF4 and CHOP transcription [21,22]. Recent studies have demonstrated that induction of CHOP by ER stress is nearly attenuated in PERK null cells and eIF2α^{S51A} cells. These results indicate that the PERK/eIF2α signaling pathway plays a critical role in the induction of CHOP during ER stress [23,24]. It is well established that PERK/eIF2α signaling mediates CHOP expression; however, a potential role for PERK/eIF2α signaling in the regulation of DR5 expression by ER stress has not been fully studied. In particular, the effect of ER stress on DR5 expression in HCC remains unknown.

In this study, we showed that the combined treatment of TRAIL-resistant HCC with subtoxic doses of GSS and TRAIL dramatically induced HCC cell death. We also showed that GSS induced CHOP-dependent DR5 upregulation via generation of reactive oxygen species (ROS) and ER stress. Therefore, a combined treatment with GSS and TRAIL may synergistically stimulate and accelerate the death receptor-mediated apoptotic signaling pathway.

2. Materials and methods

2.1. Antibodies and reagents

GGS, *N*-acetyl-L-cysteine (NAC), glutathione (GSH) and 4′6′-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich (St. Louis, MO). Antibodies against Bid, caspase-9, DR5, IAP-1, IAP-2, XIAP, Bcl-2, Bad, eIF2α, PERK, phospho (p)-PERK, ATF4, IRE, BiP and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against PARP, JNK, (p)-JNK, p-eIF2α and p-IRE were purchased from Cell Signal (Beverly, MA). Antibodies for caspase-3, caspase-8, cytochrome *c*, CHOP and serine-threonine phosphatase PP2A were purchased from Upstate Biotechnology (Lake Placid, NY). Peroxidase-labeled donkey anti-rabbit, sheep anti-mouse

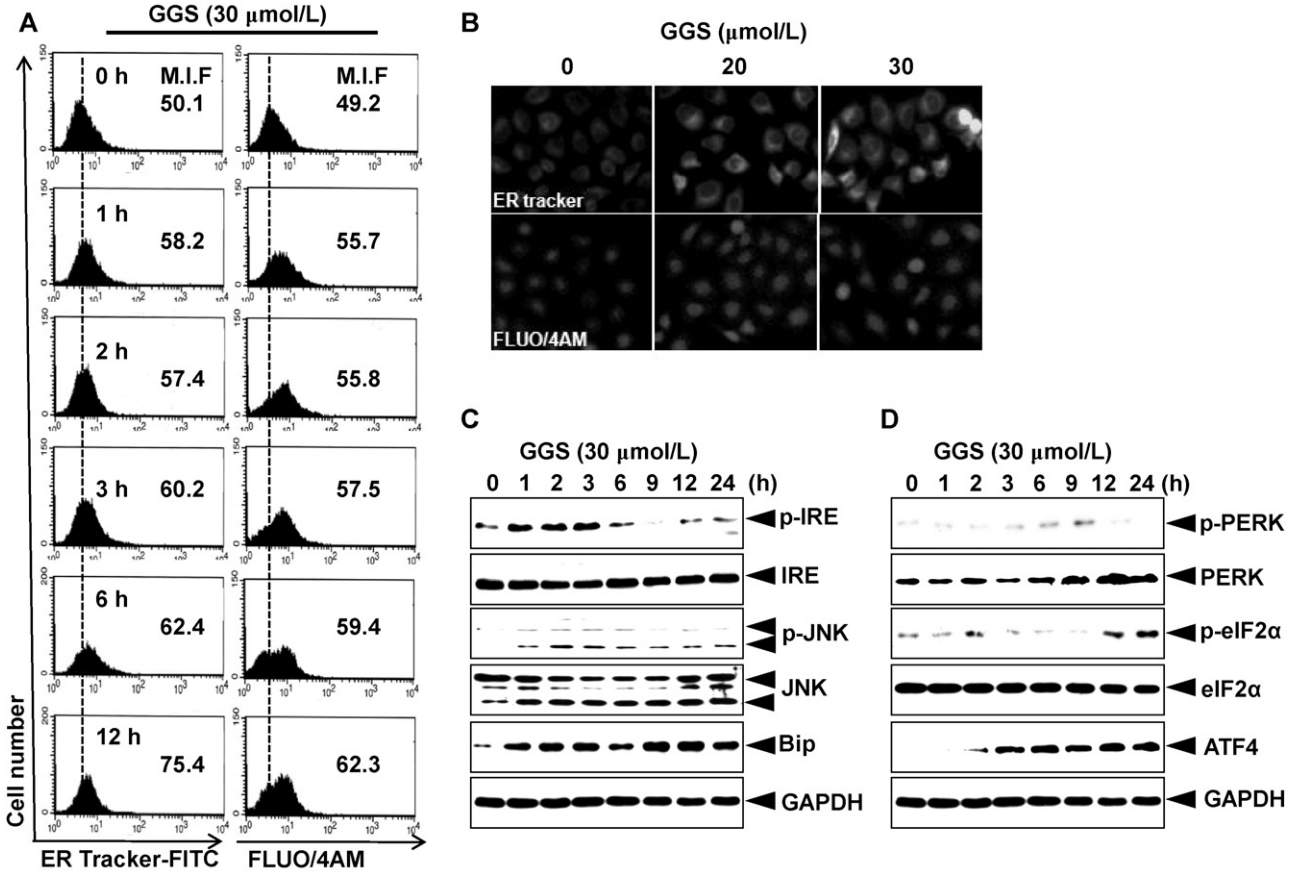

Fig. 1. GGS induces ER stress with high levels of ER stress marker proteins. (A and B) Hep3B cells were treated with 30 μM GGS at the indicated times and then stained with FITC-conjugated ER Tracker and Fluo-4AM. The fluorescence intensity of the ER Tracker and Fluo-4AM was analyzed by flow cytometry and fluorescence microscope at 12 h. (C and D) Hep3B cells were treated with 30 μM GGS for the indicated times. Cells were harvested and the indicated proteins were detected by Western blot analysis.

immunoglobulin and recombinant human TRAIL/Apo2 ligand (the nontagged 19-kDa protein, amino acids 114–281) were purchased from KOMA Biotechnology (Seoul, Republic of Korea). The blocking antibodies against DR4 and DR5 were purchased from R&D Systems (Minneapolis, MN). ER-Tracker-FITC, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$), hydroethidine (HE) and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) were purchased from Molecular Probes (Eugene, OR). z-VAD-fmk and salubrinal were purchased from Calbiochem (San Diego, CA). Calcium staining dye, Fluo-4AM, was purchased from Invitrogen (Carlsbad, CA).

2.2. Cell line and cell growth assay

Human hepatocellular carcinoma cell lines Hep3B and HepG2 were cultured in DMEM (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics (Sigma). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assays were used to determine cell viability.

2.3. Flow cytometric analysis

Cells were fixed with 1 U/ml of RNase A (DNase free, Sigma) and 10 μg/ml of propidium iodide (Sigma–Aldrich) overnight at room temperature in the dark. A FACSCalibur flow cytometer (Becton Dickenson, San Jose, CA) was used to analyze the level of apoptotic cells containing sub-G_1 DNA content. For annexin-V and $DiOC_6$ staining, live cells were incubated with annexin-V (R&D Systems) and $DiOC_6$.

2.4. DNA fragmentation assay

Cells were lysed in a buffer containing 10 mM of Tris (pH 7.4), 150 mM of NaCl, 5 mM of EDTA, and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10,000 × *g* for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1) and analyzed.

2.5. Western blotting analysis

Total cell extracts were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology, Sungnam, Republic of Korea). Total cell extracts were separated on polyacrylamide gels and then standard procedures were used to transfer them to the nitrocellulose membranes. The membranes were developed using an ECL reagent (Amersham, Arlington Heights, IL).

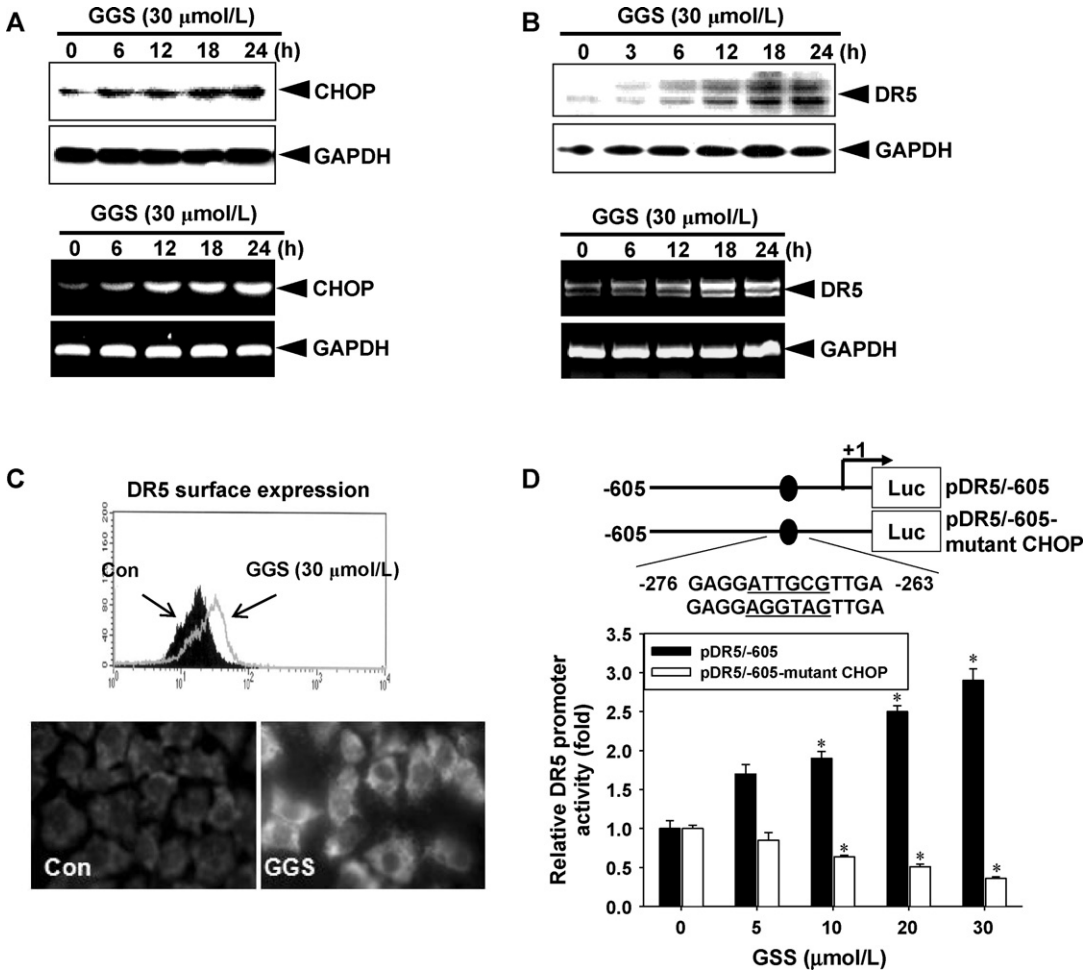

Fig. 2. GGS activates DR5 transcription through CHOP induction. (A and B) Hep3B cells were treated with up to 30 μM GGS for the indicated times, and RT-PCR and Western blot analysis of CHOP (A) and DE5 (B). GAPDH was used as an internal control. (C) Hep3B cells were incubated with or without 30 μM of GGS for 24 h, and a fluorescence microscope and flow cytometry were used to analyze DR5 surface expression. (D) Schematic structures (*upper*) of the DR5 promoter constructs were used to measure luciferase activity. Mutations were introduced into the CHOP consensus sites. Data are expressed as overall mean ± SE from three independent experiments. Statistical significance was determined by two-way ANOVA (*$P < 0.05$ vs. untreated control).

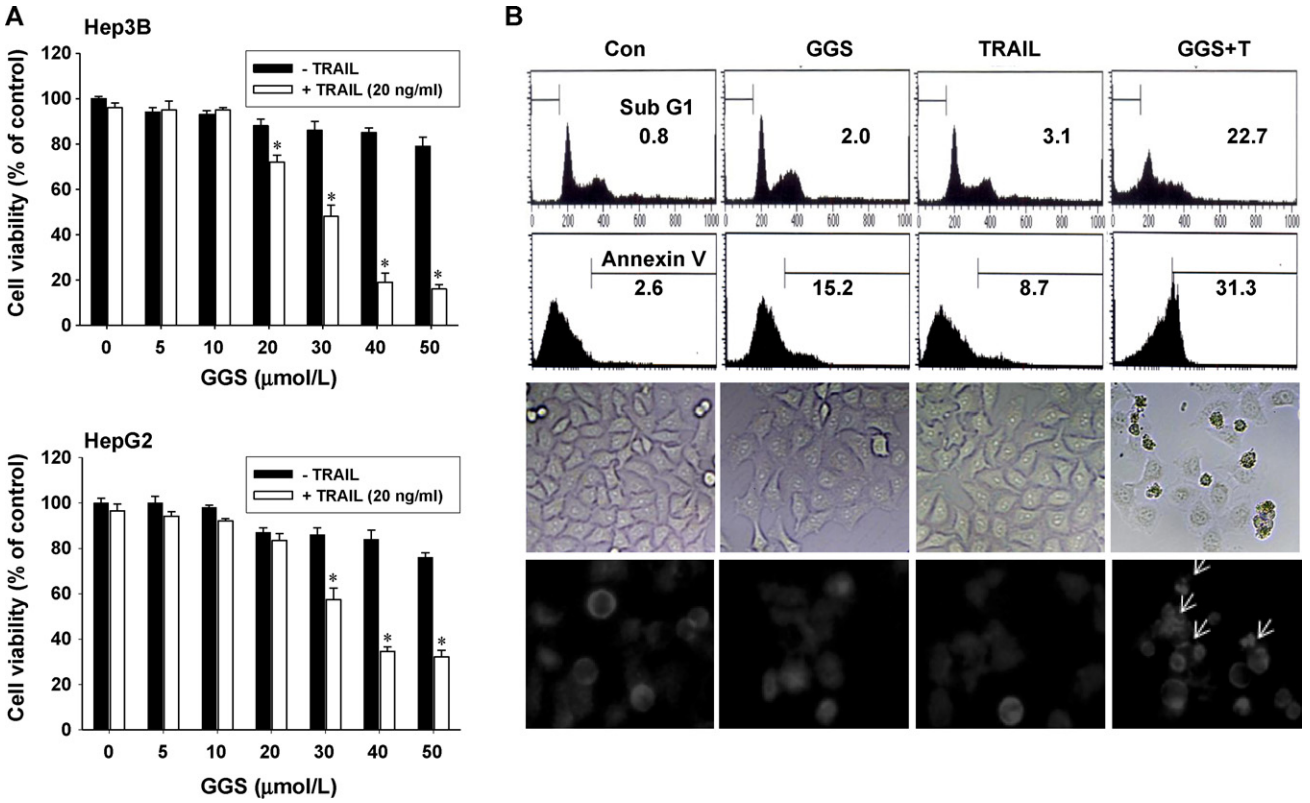

Fig. 3. GGS sensitizes TRAIL-induced cell death. Hep3B and HepG2 cells were treated with GGS at the indicated concentrations and with or without 20 ng/ml TRAIL for 24 h. (A) Cell viability was tested by the MTT assay. (B) Hep3B cells were treated with 20 ng/ml of TRAIL alone, 30 μM of GGS alone, or a combination of both for 24 h. Flow cytometry analysis of the DNA content and annexin-V$^+$ of the cells was performed. Cell morphology was examined under light microscopy (×400). Nuclear condensation and destruction were analyzed by DAPI staining. (C) Mitochondrial potential was measured using $DiOC_6$ dye. (D) The translocation of cytochrome *c* was analyzed by Western blot analysis. (E) Relative activity of caspase-3, -8 and -9 was determined according to the manufacturer's protocol. (F) Cells were harvested and the indicated proteins were detected by Western blot analysis. GAPDH was used as a loading control. Data are expressed as overall mean ± SE from three independent experiments. Statistical significance was determined by two-way ANOVA (*$P < 0.05$ vs. untreated control).

2.6. Measurement of ROS

Cells were plated at a density of 5×10^4, and allowed to attach for 24 h, and then exposed to 30 μM of GGS for indicated times. The cells were stained with 10 μM DCFDA and 5 μM HE for 15 min at 37 °C and flow cytometry was used to determine the fluorescence intensity of DCFDA and HE in the cells.

2.7. In vitro caspase activity assay

A caspase activation kit was used according to the manufacturer's instructions (R&D Systems) to measure the activity of caspase-like proteases.

2.8. RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and RT-PCR was conducted. The total RNAs were amplified by PCR using the following primers: DR5 sense 5′-AAG ACC CTT GTG CTC GTT GTC-3′, DR5 antisense 5′-GAC ACA TTC GAT GTC ACT CCA-3′, CHOP sense 5′-CAA CTG CAG AGA TGG CAG CTG A-3′, CHOP antisense 5′-CTG ATG CTC CCA ATT GTT CAT-3′, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5′-GTC TTC ACC ACC ATG GAG-3′ and GAPDH antisense 5′-CCA CCC TGT TGC TGT AGC-3′.

2.9. Fluorescent microscopy and staining

Indirect staining with primary rabbit anti-human DR5 followed by FITC-conjugated IgG was used to analyze cells for the surface expression of DR5. Briefly, 5×10^5 cells were stained with anti-DR5 antibody. After incubation, the cells were washed twice and reacted with FITC-conjugated rabbit polyclonal IgG for 30 min. For ER staining, FITC-conjugated ER-Tracker was diluted 1:1000 in the regular medium. Then, the pre-warmed probe-containing medium was added to the cells and incubated for 30 min. After the cells were washed with PBS, fluorescence microscope was used to analyze.

2.10. Plasmids, transfection and luciferase assays

The pDR5/*Sac*I plasmid [containing DR5 promoter sequence (−2500/+30)] and pDR5/−605 [containing DR5 promoter sequence (−605/+3)] were gifts from Dr. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan). To analyze the promoter regions responsible for GGS, the reporter constructs containing single (mCHOP) point mutations (from T.K. Kwon, School of Medicine, Keimyung University, Daegu, Republic of Korea) at putative CHOP-binding sites of the DR5 promoter were used. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega, Madison, WI). Luciferase activity was normalized by β-galactosidase activity in cell lysates and expressed as an average of three independent experiments.

2.11. Transfection of CHOP small interfering RNA (siRNA)

The siRNA of CHOP was purchased from Santa Cruz Biotechnology. The cells were transfected with siRNA using N-TER nanoparticle siRNA transfection system (Sigma–Aldrich) according to the manufacturer's recommendations.

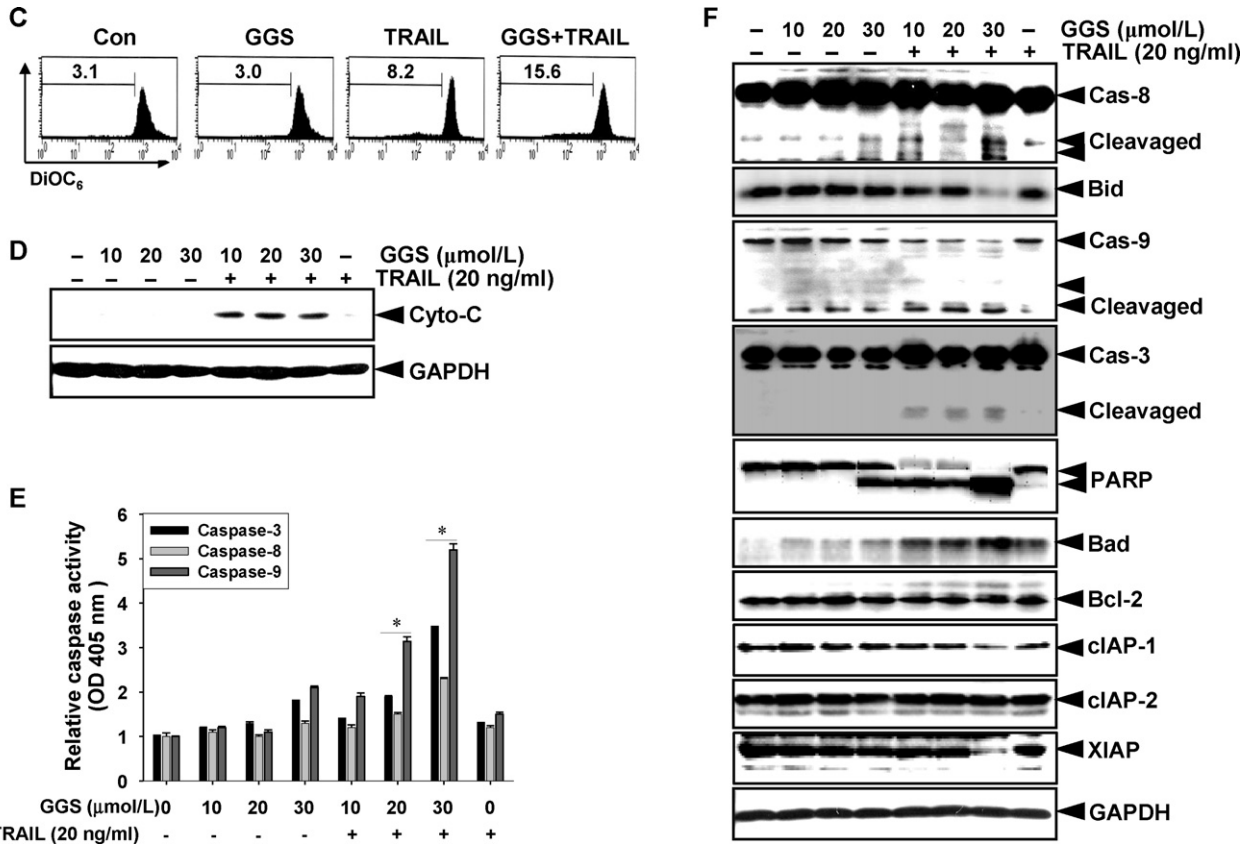

Fig. 3. (*Continued*).

2.12. Nuclear staining

After treatment with GGS with or without TRAIL for 24 h, cells were harvested and washed in ice-cold PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were stained with DAPI solution for 10 min at room temperature. Nuclear morphology of the cells was examined by fluorescence microscopy.

2.13. Electrophoretic mobility shift assay (EMSA)

DNA–protein binding assays were carried out with the nuclear extract. Synthetic complementary NF-κB- and Sp1-binding oligonucleotides (5′-AGT TGA **GGG GAC TTT CC**C AGG C-3′ and 5′-ATT CGA TC**G GGG CGG GGC** GAG C-3′, respectively; Santa Cruz Biotechnology) were 3′-biotinylated using a biotin 3′-end DNA labeling kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Assays were performed using a Lightshift EMSA Optimization kit (Pierce) according to the manufacturer's protocol.

2.14. Statistical analysis

Statistical analyses were conducted using SigmaPlot software (version 11.0). Values were presented as mean ± SE. Significant differences between the groups were determined using two-way ANOVA. Statistical significance was regarded at $P < 0.05$.

3. Results

3.1. GGS induces ER stress in HCC

The ER is the primary organelle for post-translational modification, assembly of newly synthesized proteins, and calcium storage [25]. Therefore, we first confirmed whether GGS induces ER stress. Treatment with GGS increased the intensity of ER-tracker-FITC dye in Hep3B cells in a time-dependent manner (left panel of Fig. 1A and upper panel of Fig. 1B). This dye specifically stains the ER in live cells, and the results showed that GGS-treated Hep3B cells exhibited significantly increased green staining compared to the control, suggesting that GGS induced ER stress. ER stress induced by GGS was also accompanied by Ca^{2+} release, which was detected using Fluo-4AM calcium indicators (right panel of Fig. 1A), and this was typified by increased green staining in Hep3B cells (lower panel of Fig. 1B). Next, we investigated the levels of several ER stress marker proteins (e.g. IRE, JNK, BiP, PERK, eIF2α, and ATF4), which usually increase during ER stress. GGS treatment rapidly increased the levels of BiP as early as 1 h. GGS treatment also elevated IRE1 and JNK phosphorylation levels (Fig. 1C). Therefore, we examined whether GGS-induced ER stress is associated with the PERK/eIF-2α/ATF4 signaling pathway. GGS treatment increased phosphorylated PERK and eIF-2α, as well as expression of ATF4, which is a transcriptional factor induced during ER stress responses (Fig. 1D). Taken together, these results indicate that GGS induces ER stress in Hep3B cells.

3.2. GGS induces CHOP-dependent DR5 expression

Given that CHOP is a prominent ER stress-marker protein involved in ER stress-mediated apoptosis and regulates DR5 expression [26,27], we next investigated whether GGS-induced ER stress is related to CHOP expression. By Western blot analysis and RT-PCR, we determined that GGS time-dependently increased CHOP expression in Hep3B cells (Fig. 2A). Our results also showed that the DR5 protein and RNA levels increased significantly by GGS treatment (Fig. 2B). A flow cytometric analysis and DR5 immune staining showed that DR5 cell surface expression significantly

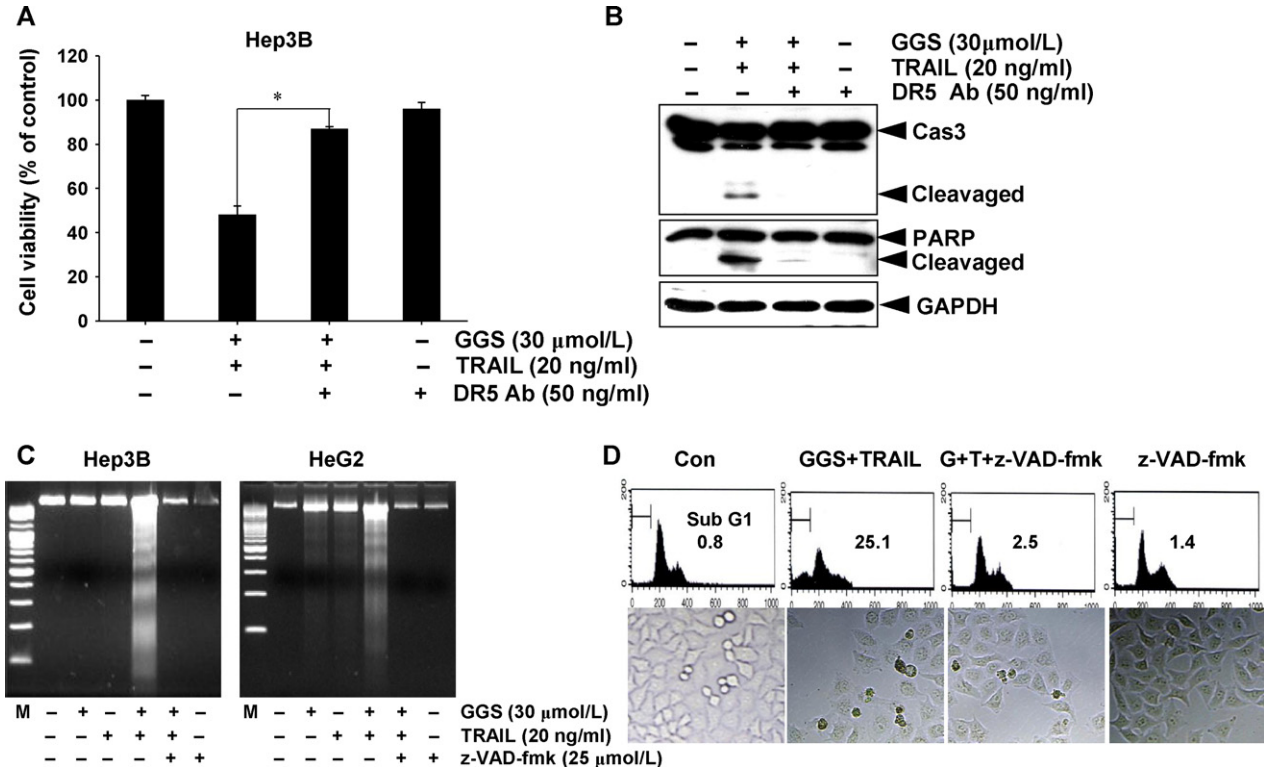

Fig. 4. DR5-dependent caspase activation was involved in the sensitizing effect of GGS on TRAIL-induced apoptosis. (A) Hep3B cells were pretreated with 50 ng/ml of a DR5-specific blocking chimera antibody for 30 min followed by treatment with 30 μM GGS and 20 ng/ml TRAIL for 24 h. Cell viability was measured by the MTT assay. (B) Cell extracts were prepared for Western blot analysis of caspase-3 and PARP, and GAPDH was used as a loading control. (C) Hep3B and HepG2 cells were pre-treated with 20 μM of the pan-caspase inhibitor z-VAD-fmk for 30 min and further treated with 30 μM of GGS and 20 ng/ml TRAIL for 24 h to examine the effect of inhibiting caspases. Fragmented DNAs were extracted from treated cells and analyzed on a 1.5% agarose gel. (D) Flow cytometry was used to determine the DNA content. Cell morphology was examined under light microscopy (×400). Data are expressed as overall mean ± SE from three independent experiments. Statistical significance was determined by two-way ANOVA (*$P < 0.05$ vs. combined treatment with TRAIL and GGS).

increased in Hep3B cells treated with GGS (Fig. 2C). To further examine the DR5 transcriptional modulation by CHOP, we used the wild-type DR5-reponsive reporter construct pDR5/−605, which contains a region (−605/+3) of the DR5 promoter and a mutant version (pDR5/−605-mCHOP) of the promoter–reporter construct in which the potential CHOP-binding site of the DR5 promoter has been mutated. GGS treatment increased DR5 promoter activity in Hep3B cells transfected with the pDR5-605 plasmid but not in those transfected with pDR5-605-mCHOP (Fig. 2D), indicating that CHOP is responsible for GGS-induced DR5 upregulation in Hep3B cells.

3.3. GGS sensitizes HCC to TRAIL-induced apoptosis

Next, we assessed whether GGS sensitizes TRAIL-induced cell death. Treatment with 20 ng/ml TRAIL resulted in negligible death of Hep3B and HepG2 cells (<5%) at 24 h, suggesting that these cells are resistant to TRAIL-induced cytotoxicity. We examined the cytotoxic effects of GGS alone or in combination with TRAIL in these cell lines. GGS alone did not significantly induce growth inhibition. However, the combination of GGS and TRAIL strongly decreased cell viability when the concentration of TRAIL (20 ng/ml) was fixed (Fig. 3A). These results show that treatment with a combination of GGS and TRAIL effectively repressed cell growth in TRAIL-resistant HCC. Next, we investigated whether decreased cell viability by the combination treatment was due to induction of apoptotic signaling. We detected that treatment of Hep3B cells with a combination of 30 μM GGS and 20 ng/ml TRAIL for 24 h significantly increased the accumulation of sub-G_1 phase cells (Fig. 3B, first panel) and the population of cells stained by annexin-V (Fig. 3B, second panel), whereas treatment with GGS or TRAIL alone did not. When phase-contrast microscopy was used to examine changes in cell morphology, we also found that the cells treated with a combination of GGS and TRAIL displayed decreased cell numbers with some apoptotic shrinkage (Fig. 3B, third panel). Furthermore, Hep3B cells cultured with both GGS and TRAIL showed a marked change in condensed and destroyed chromatin in the nuclei (Fig. 3B, fourth panel). HCC has been demonstrated to be resistant to TRAIL because of insufficient activation of the extrinsic pathway and, more importantly, because of the blocking of death signaling at the mitochondrial level. Therefore, apoptotic events in the mitochondria were evaluated by measuring the mitochondrial membrane potential using $DiOC_6$. Marked reduction in the mitochondrial membrane potential was observed in cells treated with GGS and TRAIL (Fig. 3C). Additionally, this process was accompanied by the release of cytochrome *c* from the mitochondria into the cytosol (Fig. 3D). We also found that caspase-8, caspase-9, and caspase-3 activity increased significantly with the combined treatment (Fig. 3E). Similarly, cleavage forms of caspase-8, Bid, caspase-9, caspase-3, and PARP were observed after the combined treatment (Fig. 3F). We also found that the combined treatment increased bad expression, decreased cIAP-1 and XIAP expression, and sustained Bcl-2 and cIAP-2 expression (Fig. 3F). These data indicate that a combined treatment with GGS and TRAIL sensitized the cells to apoptosis and regulated the mitochondrial potential.

3.4. GGS sensitizes cells to TRAIL-induced apoptosis through DR5-dependent caspase activation

We found that GGS induced DR5 expression through a CHOP-dependent mechanism (Fig. 2). Recently, Akazawa et al. have

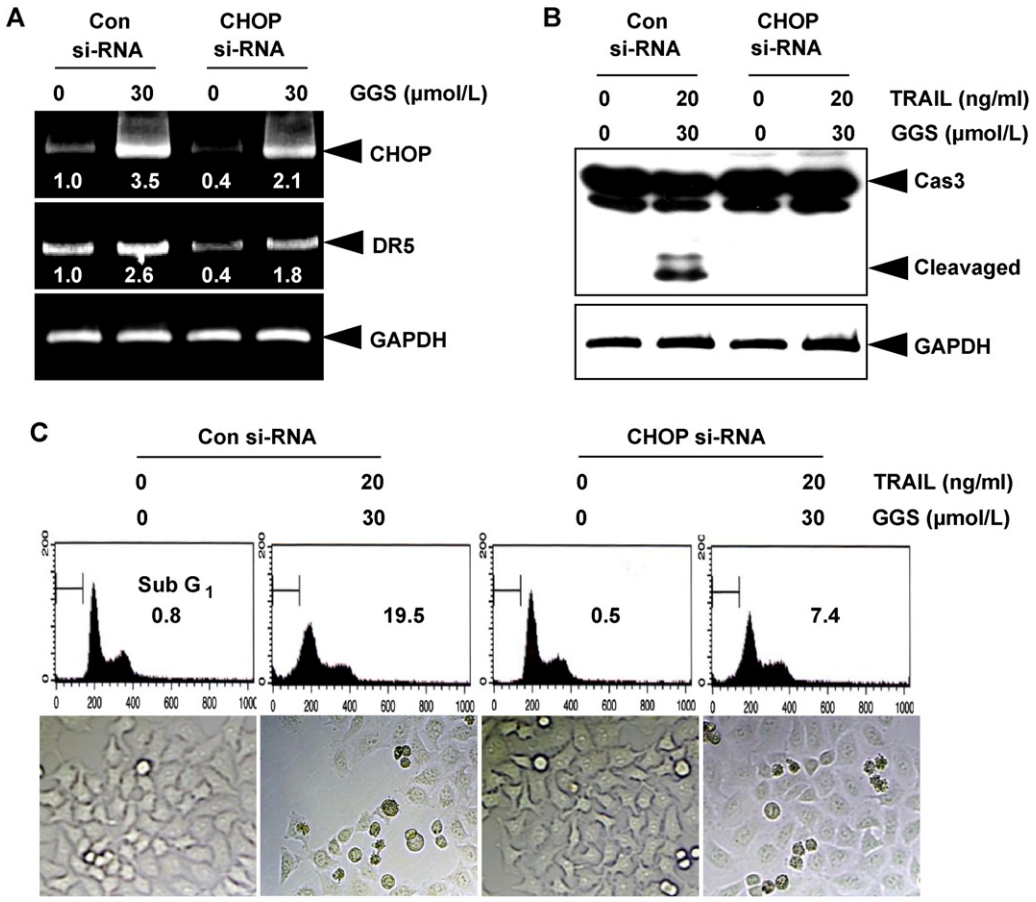

Fig. 5. CHOP mediates GGS-induced DR5 upregulation and apoptosis. (A) Hep3B cells were transfected with scrambled negative control RNA or siRNA duplexes against CHOP, incubated for 24 h, and then treated with 30 μM GGS alone for 24 h. RT-PCR of CHOP and DR5 was performed to confirm CHOP and DR5 downregulation by siRNA transfection. (B) Hep3B cells were transfected with CHOP siRNA. Twenty-four hours after the transfection, the cells were treated with the indicated concentrations of GGS/TRAIL for 24 h. Cell extracts were prepared for Western blot of caspase-3. GAPDH was used as a loading control. (C) Flow cytometry analysis was used to determine DNA content. Cell morphology was examined under light microscopy (×400).

shown that the DR5 signaling pathway is more prominently involved in TRAIL-induced apoptosis in malignant liver cell lines than DR4 [28]. To confirm the functional role of DR5 induced by GGS in the sensitization of TRAIL-induced apoptosis, we examined the effect of a DR5-specific blocking chimera antibody on GGS/TRAIL-induced apoptosis. The addition of a DR5 specific blocking antibody significantly blocked GGS/TRAIL-induced reduction of cell growth in Hep3B cells (Fig. 4A). Similarly, treatment with an anti-DR5 chimera antibody in Hep3B cells decreased the cleavage of caspase-3 and PARP induced by GGS/TRAIL (Fig. 4B). These results support the hypothesis that GGS-induced DR5 upregulation is critical for caspase activation and induction of TRAIL sensitivity in Hep3B cells. In this study, we also found that GGS did not affect DR4 surface expression at the transcriptional level (data not shown). We also tested the effects of a DR4-specific blocking chimeric antibody, which did not significantly block GGS/TRAIL-induced cell death in Hep3B cells (data not shown). Next, to determine the effect of caspase activation on GGS/TRAIL-induced apoptosis, we compared the effects of GGS/TRAIL on apoptosis induction in the absence and presence of z-VAD-fmk, a pan-caspase inhibitor. DNA fragmentation induced by GGS/TRAIL was completely blocked by pre-treatment with z-VAD-fmk (Fig. 4C). Pre-treatment with z-VAD-fmk also significantly blocked the accumulation of sub-G_1 phase cell populations and cell shrinkage (Fig. 4D). Taken together, these results suggest that GGS significantly enhanced TRAIL-induced apoptosis through the activation of caspases mediated by the DR5 signaling pathway.

3.5. CHOP mediates GGS-induced DR5 upregulation and sensitization to TRAIL

Recently, CHOP has been shown to be involved in thapsigargin- and tunicamycin-mediated DR5 upregulation, contributing to the sensitization of TRAIL-mediated apoptosis. We found that CHOP protein levels increased significantly with GGS treatment and preceded GGS-induced DR5 upregulation (Fig. 2A and B). We treated with CHOP siRNA in Hep3B cells to investigate whether CHOP is associated with GGS-mediated transcriptional activation of DR5. CHOP suppression by siRNA knockdown significantly inhibited GGS-induced DR5 upregulation, showing again that CHOP induction is required for GGS-induced DR5 upregulation (Fig. 5A). Moreover, we found that siRNA knockdown of CHOP blocked the activation of caspase-3, accumulation of the sub-G_1 cell population, and cell shrinkage induced by combined treatment with GGS/TRAIL (Fig. 5B and C). Nevertheless, transient knockdown of CHOP did not completely block cell death. Taken together, these results suggest that CHOP induction plays a critical role in both GGS-induced DR5 upregulation and GGS-facilitated TRAIL-induced apoptosis.

3.6. ROS mediate GGS-induced ER-stress and upregulation of CHOP and DR5

Hayashi et al. demonstrated that ROS induce ischemic neuronal cell death through induction of ER stress [29]. Indeed, recent reports have shown that ROS-mediated DR5 upregulation is critical for curcumin- or sulforaphane-sensitized TRAIL-induced apoptosis

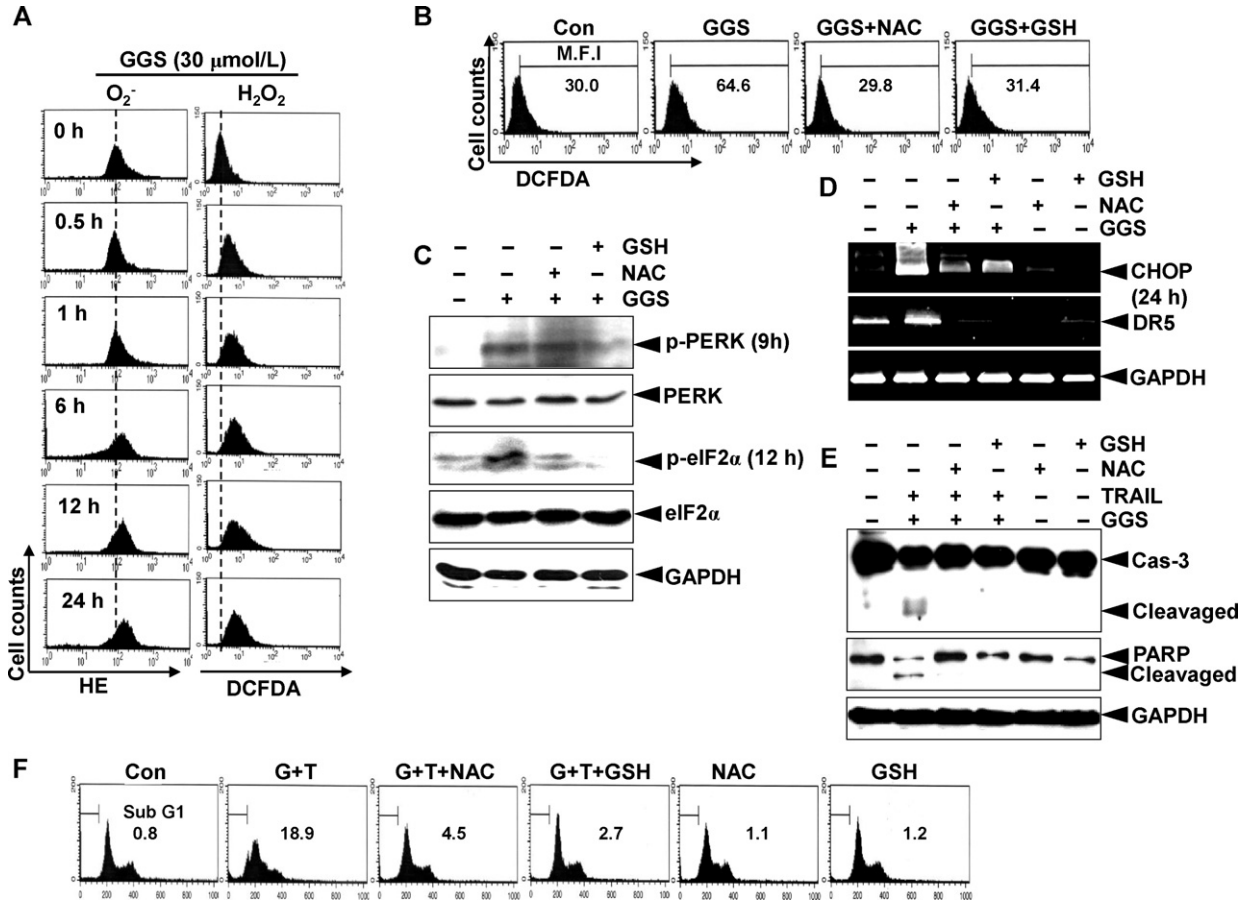

Fig. 6. ROS accumulation is a major target in GGS/TRAIL-induced apoptosis. (A) Hep3B cells were treated with 30 μM GGS at the indicated times and then stained with HE and DCFDA. (B) Hep3B cells were pretreated with 10 mM NAC or 5 mM GSH for 30 min and further treated with 30 μM GGS for 0.5 h. Redox status was measured using flow cytometry. (C and D) Hep3B cells were pretreated with 10 mM NAC and 5 mM GSH for 30 min and further treated with 30 μM GGS for the indicated times. Whole-cell protein lysates were prepared for detecting the indicated proteins by Western blot analysis (C). RT-PCR of CHOP, DR5, and GAPDH was performed (D). (E and F) Hep3B cells were pretreated with 10 mM NAC or 5 mM GSH for 30 min and further treated with 30 μM GGS and 20 ng/ml TRAIL for 24 h. Cell extracts were prepared for Western blot for caspase-3 and PARP. GAPDH was used as a loading control (E). Flow cytometry analysis was used to determine DNA content. Cell morphology was examined under light microscopy (×400) (F).

[16,30]. Therefore, we examined whether ROS generation could also be involved in GGS-induced ER stress and DR5 upregulation. HE and DCFDA-based fluorescence microscopy detection showed that treating Hep3B cells with GGS increased intracellular superoxide anion and hydrogen peroxide levels (Fig. 6A), which were prevented by NAC and GSH pretreatment (Fig. 6B). We also found that NAC and GSH pretreatment inhibited GGS-induced phosphorylation of PERK/eIF2α (Fig. 6C). Furthermore, we discovered that GGS-induced activation of CHOP/DR5 expression was completely blocked by NAC and GSH pretreatment (Fig. 6D). We next investigated whether ROS generation was directly associated with GGS/TRAIL-induced apoptosis. NAC and GSH pretreatment markedly blocked GGS/TRAIL-induced apoptosis and attenuated the cleavage of procaspase-3 and PARP (Fig. 6D and E). Taken together, these data clearly indicate that GGS/TRAIL-induced ER stress and apoptosis are mediated by ROS.

3.7. GGS-induced ER stress is associated with the induction of apoptosis

Because recent studies have suggested that the PERK/eIF2α signaling pathway plays an essential role in CHOP induction during ER stress [23,24], we tested whether CHOP induction is involved in mediating the GGS-induced activation of the PERK/eIF2α signaling pathway. We compared the effects of GGS on the levels of eIF2α and CHOP in the absence or presence of salubrinal, which selectively induces eIF2α phosphorylation. GGS increased the level of p-eIF2α in the absence of salubrinal, and this effect was substantially enhanced in the presence of salubrinal (Fig. 7A). Indeed, salubrinal significantly increased CHOP and DR5 mRNA levels induced by GGS, suggesting that GGS-initiated increases in the level of p-eIF2α are associated with CHOP and DR5 expression (Fig. 7B). Furthermore, salubrinal significantly augmented GGS/TRAIL-induced cleavage of caspase-3 and PARP (Fig. 7C). In agreement with this result, GGS/TRAIL-induced cell growth inhibition and an increase in sub-G_1 cell populations were also substantially enhanced by salubrinal (Fig. 7D and E). Thus, it seems that ER stress-mediated phosphorylation of eIF2α is involved in GGS/TRAIL-induced apoptosis.

4. Discussion

The safety of GGS makes it a useful candidate for complementary and preventive therapy [7]. Recently, GGS-induced anticancer activity was also demonstrated in various cell lines through NF-κB and STAT3 inhibition and activation of the JNK signaling pathway. Our present study demonstrated that GGS induced DR5 upregulation, resulting in the induction of apoptosis and an enhancement of TRAIL-induced apoptosis in HCC. As it has been well documented that CHOP is an important transcriptional factor regulating DR5 expression [31,32], we found that the regions containing the CHOP binding site were essential for GGS-mediated DR5 transactivation

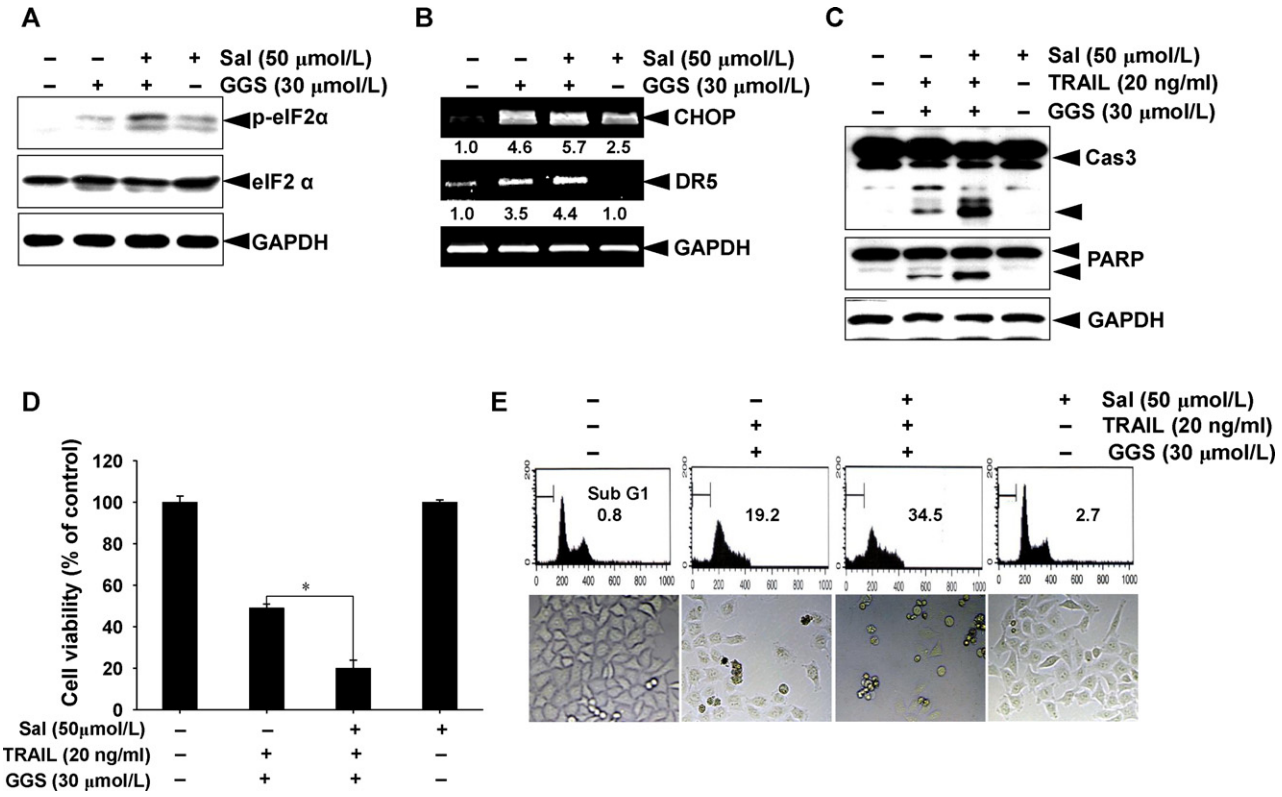

Fig. 7. GGS-induced CHOP/DR5 expression and TRAIL sensitization are mediated by eIF2α phosphorylation. (A) Hep3B cells were pre-treated with 50 μM salubrinal for 1 h and further treated with 30 μM GGS for 12 h to examine the effect of salubrinal (Sal) on GGS-induced eIF2α phosphorylation. eIF2α levels were determined by Western blot analysis. (B) Hep3B cells were pre-treated with 50 μM Sal for 1 h and further treated with 30 μM GGS for 24 h to examine the effect of Sal on GGS-induced CHOP/DR5 expression. CHOP and DR5 RNA levels were determined by RT-PCR. (C–E) To confirm the effect of salubrinal on the sensitization of cells to GGS in TRAIL-induced apoptosis, Hep3B cells were pre-treated with 50 μM Sal for 1 h and further treated with 30 μM GGS and 20 ng/ml TRAIL for 24 h. Western blot of caspase-3 and PARP was performed (C). Cell viability was determined by the MTT assay (D). Flow cytometry analysis was used to determine DNA content. Cell morphology was examined under light microscopy (×400) (E). Data are expressed as overall mean ± SE from three independent experiments. Statistical significance was determined by one-way ANOVA (*$P < 0.05$ vs. combined treatment with TRAIL and GGS).

using a mutation analysis of the DR5 5′-flanking region. Nevertheless, the regions why transient knockdown of CHOP did not completely restore cell viability are the possibility associated with other unknown molecular mechanisms or a little of incomplete knockdown of CHOP. Taken together, we concluded that CHOP induction participated in GGS-induced DR upregulation and TRAIL-induced apoptosis. We observed that CHOP induction by salubrinal treatment alone did not induce DR5 expression. Thus, it is possible that mechanisms other than CHOP may also be involved in mediating GGS-induced DR5 expression. Sp1 may also induce DR5 expression [33,34], although no reports have confirmed a Sp1 chromatin immunoprecipitation assay in the DR5 promoter region. In this study, we also found that GGS phosphorylation-dependently increased Sp1 DNA binding activity (data now shown). A future study should investigate whether Sp1 activation is involved in GGS-induced DR5 expression.

CHOP is a typical ER stress-regulated protein involved in ER stress-induced apoptosis. Thus, our GGS-induced CHOP expression finding suggests that GGS may trigger ER stress. We also found that GGS induced CHOP expression in an eIF2α phosphorylation-dependent manner. Thus, the PERK/eIF2α signaling pathway is thought to be the primary mechanism for CHOP and DR5 induction. However, other pathways (i.e., IRE1- and ATF6-mediated signaling pathways) also regulate CHOP expression during ER stress [35]. Thus, the mechanism underlying GGS-induced CHOP upregulation via the IRE1 pathway during ER stress needs further investigation.

Hayashi et al. demonstrated that ROS induces ischemic neuronal cell death through induction of ER stress [29]. Furthermore, it was reported that ROS generation is a major target for triggering and amplifying TRAIL-dependent apoptosis through DR5 expression [16,35]. In addition, Singh and his colleagues reported that GGS causes apoptotic cell death by ROS-dependent activation of JNK [12]. Therefore, we hypothesized that GGS sensitizes TRAIL-induced apoptosis by activating ROS-ER stress-CHOP signal cascades to induce DR5 expression. GGS-induced ROS generation was evident as early as 30 min and as late as 24 h after treatment with GGS, and pre-treatment with NAC or GSH, ROS inhibitors, significantly decreased GGS-induced ER stress and CHOP-dependent DR5 expression. Moreover, the apoptosis induced by GGS/TRAIL was also significantly attenuated in Hep3B cells by NAC or glutathione, suggesting that ROS generation is required to induce apoptosis. These results indicate that ROS act as upstream signaling molecules to initiate GGS-induced ER stress and CHOP/DR5 expression and are critical for sensitizing cells to TRAIL-induced apoptosis. Nevertheless, we need further study to elucidate the function of JNK as a key linker between ROS and ER-stress. In previous study, JNK activation is unaffected in apoptosis in IRE-$1^{-/-}$ fibroblasts [36]. The possibility is that ROS-induced activation of JNK sensitizes IRE for ER stress.

GGS-induced DR5 upregulation was associated with enhanced apoptotic signaling induced by TRAIL, as indicated by increased caspase-8 and Bid activation, reduction in the mitochondrial potential, activation of caspase-3, and cleavage of its substrate PARP. Recently, it was found that DR5 rather than DR4 predominantly contributes to TRAIL-induced lysosomal disruption and apoptosis in HCC Huh7 cells [35]. Although Huh7 cells express both TRAIL receptors, short hairpin RNA silencing of DR5 but not DR4 attenuated TRAIL-induced apoptosis. Consistently,

we found that a DR5/Fc chimera more effectively blocked GGS/TRAIL-induced apoptosis than a DR4/Fc chimera. These results suggest that GGS/TRAIL induces apoptosis through enhanced caspase-dependent apoptotic signaling by increased interaction between TRAIL and DR5.

Collectively, these results demonstrate that ER stress induced by GGS is similar to that observed for tunicamycin/thapsigargin [26,27]. In our study, we showed that a combined treatment of GGS and TRAIL effectively induced cell death not only in wild-type p53 (HepG2 cells) but also in deleted p53 (Hep3B cells) commonly through DR5 upregulation. In conclusion, GGS sensitized TRAIL-induced apoptosis by inducing ROS-dependent ER stress.

Conflicts of interest

No potential conflicts of interest were disclosed.

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